Mag. pharm. Thomas K. Unteregger

Development of Methods for the Continuous Purification of Biopharmaceuticals Using Electrophoresis and Monolithic Stationary Phases

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Ass.Prof. Dipl.-Ing. Dr.techn. Heidrun Gruber-Wölfler

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Zusammenfassung

Die pharmazeutische Industrie ist ständig auf der Suche nach Verbesserungsmöglichkeiten und Innovationen im Bereich der Arzneimittelherstellung zur Effizienzsteigerung und Kostenminimierung. Dazu werden immer mehr Batchprozesse in kontinuierliche Prozesse übergeführt. Bezogen auf Biopharmazeutika ist die Aufreinigung monoklonaler Antikörper ein entscheidender Produktionsschritt, der momentan von Protein A Affinitätschromatographie dominiert wird.

Diese Diplomarbeit soll dazu beitragen, eine kontinuierliche Reinigungsmöglichkeit für monoklonale Antikörper als Alternative zur derzeit üblichen Affinitätschromatographie zu entwickeln, die auf der Verwendung monolithischer stationärer Phasen bzw. auf dem Einsatz von Gelmatrizen, wie sie in der Elektrophorese gebräuchlich sind, basiert.

Im ersten Teil der Arbeit wurde mit Hilfe von Elektrophorese versucht, eine Gelmatrix für weiterführende Reinigungszwecke zu generieren. Im zweiten Abschnitt wurden – basierend auf Batchexperimenten – ein Normalphasen- und ein Imidazolin-Monolith auf ihre Bindungsaffinität zu einem monoklonalen Antikörper der Klasse IgG untersucht. Eine Bindung konnte in beiden Fällen nachgewiesen werden, wobei die Bindungsaffinität des Antikörpers zum Normalphasen-Monolithen höher war. Obwohl es nicht gelungen ist, den Antikörper im nativen Zustand von den beiden Monolithen zu eluieren, legt die vorhandene Bindungsaffinität nahe, die stationären Phasen zu modifizieren, um eine aktivitätserhaltende Elution zu ermöglichen.

Abstract

Pharmaceutical industry is always looking for improvements and innovations in the field of active pharmaceutical ingredient-manufacturing to increase efficiency and to minimize costs. Therefore, more and more batch processes are replaced by continuous processing. Regarding biopharmaceuticals, the purification of monoclonal antibodies is an essential production step that is dominated by protein A affinity chromatography.

This diploma thesis shall contribute to develop a new continuous purification process for monoclonal antibodies as an alternative to affinity chromatography. The new process should be based on monolithic stationary phases and on gel matrices, respectively, like they are used for electrophoresis.

In the first part of the thesis it was tried to generate a gel matrix for continuous purification purposes. In the second part the binding affinity of a monoclonal antibody of the class IgG to a normal phase- and an imidazoline-functionalized monolith was tested, based on batch trials. A binding affinity could be detected in both cases; however, the binding affinity of the antibody to the normal phase monolith was higher. Although it wasn’t possible to elute the antibody in a native state from the monoliths, the existing binding affinity suggests that the stationary phases should be modified to make an elution possible, where the activity of the antibody is preserved.

Acknowledgement

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Last but not least, I would like to thank my parents for their ongoing support.

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List of Abbreviations

#	number
(V/V)	volume concentration
~	approximately
°C	degree Celcius
µg	microgram
µl	microliter
2-PrOH	isopropyl alcohol
A	ampere
AA	acrylamide
AAa	acetic acid (anhydrous)
Ab	antibody
ACS	American Chemical Society
$AgNO_3$	silver nitrate
ACN	acetonitrile
API	active pharmaceutical ingredient
APS	ammonium persulfate
ATPE	aqueous two-phase extraction
BSA	bovine serum albumin
bzw.	beziehungsweise, respectively
C	Coomassie Staining
C&EN	Chemical & Engineering News
cf.	compare (from Latin *conferre*)
C_H	constant region of the antibody's heavy chain
CIPP	Capture / Intermediate Purification / Polishing
C_L	constant region of the antibody's light chain
CTAB	cetyltrimethylammonium bromide
Da	dalton (unified atomic mass unit)
DEA	diethylamine
dH_2O	distilled water
DMF	N,N-Dimethylformamide
DNA	desoxyribonucleic acid
E	eluent

EMBL	European Molecular Biology Laboratory
et al.	et alii (and others)
EtOH	ethanol
exp.	experiment
f.	following page
F_{ab}	antigen binding fragment of the antibody
F_c	crystallizable fragment of the antibody
FCS	fetal calf serum
ff.	following pages
Fig.	figure
FSB	final sample buffer
FWF	Fonds zur Förderung der wissenschaftlichen Forschung (Austrian Science Fund)
g	gram
GLB 2x	gel loading buffer 2x
GST	glutathione S-transferase
h	hour
H_2O	water
HAc	acetic acid
HAT	hypoxanthine-aminopterin-thymidine
H-chain	heavy chain of the antibody
His	histidine
HPLC	high performance liquid chromatography
I	imidazoline-functionalized monolith
i.e.	id est, that is
IEF	isoelectric focusing
Ig	immunoglobulin
IgX	immunoglobulin X (i.e. IgA, IgD, IgE, IgG, IgM)
IP	isoelectric point
IPTES	triethoxy-3-(2-imidazolin-1-yl)propylsilane
$I_{x\ mg}$	x mg imidazoline-functionalized monolith (i.e. 8mg, 12mg)
kDa	kilodalton
kg	kilogram
KSCN	potassium thiocyanate

L	ladder
l	liter
L-chain	light chain of the antibody
M	molar, molarity [mol/l], monolith
mA	milliampere
mAb	monoclonal antibody
mAbs	monoclonal antibodies
max.	maximal
MBP	maltose-binding protein
MeOH	methanol
mg	milligram
min	minute
ml	milliliter
mM	millimolar
Mn	number average molar mass
mol	mole
MS	mass spectrometry
MTMS	methyltrimethoxysilane
MW	molecular weight
N	normal phase monolith
n.a.	not available, no answer, not applicable
Na_2CO_3	sodium carbonate
$Na_2S_2O_3$	sodium thiosulfate
Na_2SO_4	sodium sulfate
Na-Ac	sodium acetate
NaCl	sodium chloride
nm	nanometer
$N_{x\ mg}$	x mg normal phase monolith (i.e. 8mg, 12mg)
P	plasma
p.	page
p.a.	pro analysi
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PEG	polyethylene glycol
pH	potential hydrogenii
Pkg	package
PP	polypropylene
RNA	ribonucleic acid
s	second
S	supernatant
SDS	sodium dodecyl sulfate
SiSt	Silver Staining
Tab.	table
TEMED	*N*,*N*,*N'*,*N'*-Tetramethylethylenediamine
TEOS	tetraethyl orthosilicate
TFA	trifluoroacetic acid
TKU	Thomas K. Unteregger
Tris	2-Amino-2-(hydroxymethyl)-propan-1,3-diol
TrisHCl	tris hydrochloride
TS	transferred supernatant(s)
TU	Technical University
V	volt
V_H	variable region of the antibody's heavy chain
V_L	variable region of the antibody's light chain
vs.	versus

1. Introduction

Monoclonal antibodies are used as APIs for the treatment of cancer, Crohn's disease, Bekhterev's disease, psoriasis, asthma and multiple sclerosis as well as for the prevention of transplant rejection. (Mutschler et. al., 2008) They make up one half of all the pharmaceutical products in development and clinical trials at the moment. Therefore, these drugs are of great interest in pharmaceutical industry. (Mountford et. al., 2014)
Since monoclonal antibodies are applied parenterally, high purification standards have to be fulfilled. (Aboul-Enein, 2010) Currently, protein A affinity chromatography is the most important purification method for this class of biomolecules. The disadvantages of protein A affinity chromatography, however, – like leakage of the ligand, high costs, discontinuity of the process, capacity limitations, selectivity restrictions and harsh elution conditions – leave room for improvement. (Mountford et. al., 2014; Mündges et. al., 2015)

This diploma thesis is part of a cooperation project between Graz University of Technology and University of Kaiserslautern entitled *Continuous Purification of (Bio)-Pharmaceuticals using Electro-Chromatography and Electrophoresis*. The project tries to overcome the mentioned problems above of protein A affinity chromatography by developing new stationary phases that should be finally implemented in a prototype for continuous annular electro-chromatography at University of Kaiserslautern (cf. *Fig. 1-1*). The results of this thesis shall help the project being accepted as a part of the FWF-funded program.

The first part of the thesis – *State of the Art* – provides theoretical facts that are essential for understanding the context of the thesis. This part includes chapters about the human blood and all its components, the immune system, antibodies in general and their purification strategies, electrophoresis and monoliths as stationary phases. The *Experimental* part of the thesis is divided into two sections: the first one concentrates on the development of a gel matrix as a basis for a new continuous purification process for monoclonal antibodies; in the second experimental part two monolithic stationary phases were tested concerning their ability of binding monoclonal antibodies of the class IgG. In the last part of the thesis – *Conclusions and Outlook* – the results of the experimental work are summed up and discussed again, including ideas for possible further proceeding.

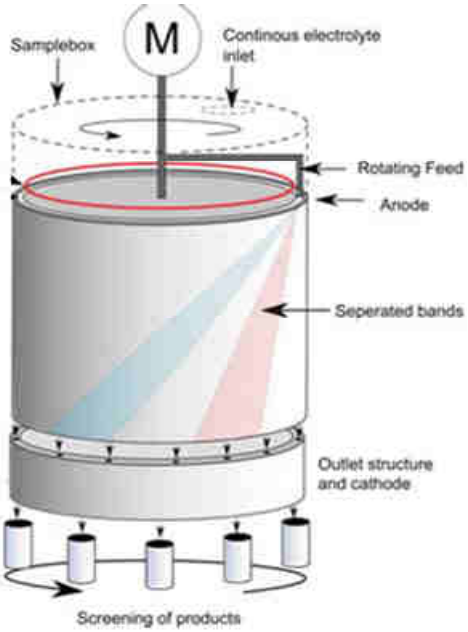

Fig. 1-1: scheme of the prototype for continuous annular electro-chromatography at University of Kaiserslautern (Braunbruck, 2015)

2. State of the Art

In this part all the theoretical facts will be provided that are essential for understanding the context of the diploma thesis.

2.1 The Human Blood

From a functional point of view, blood is a liquid tissue that is composed of cells and plasma. It serves as means of transport within the organism, as well as it is important for the homeostasis and the defense of pathogens. The volume and the composition of the blood are rather constant. (Mutschler et al., 2007)

The total amount of blood makes up about 7 – 8% of the body weight; for an adult this would be 4 – 6 l. Blood losses of more than 50% without immediate therapeutic measures are life-threatening. A healthy adult can compensate a volume loss of 10 – 15% without having significant disruptions in the cardiovascular system. Plasma proteins can be replaced within 3 – 5 days. The replacement of blood cells, however, takes much longer – after heavy losses it can take up to a month. (Mutschler et al., 2007)

2.1.1 Blood Plasma

Blood plasma is the non-cellular part of the blood with the appearance of a clear and yellow liquid. One liter plasma is composed of 0.9 l water, 9 g electrolytes, 65 – 80 g proteins and 20 g low-molecular organic substances. Blood plasma without clotting factors is called serum. 5 l of blood with a normal hematocrit contain 3 l plasma. (Mutschler et al., 2007)

Plasma proteins are a mixture of approximately 100 proteins. They can be separated in single fractions by means of different methods:

- salting out / precipitation
- adsorption chromatography
- ultracentrifugation
- electrophoresis / immunoelectrophoresis

(Mutschler et al., 2007)

With electrophoresis, plasma proteins can be divided into an albumin and a globulin fraction. The globulin fraction is subclassified into α_1-, α_2-, β- and γ-globulins. About 60%

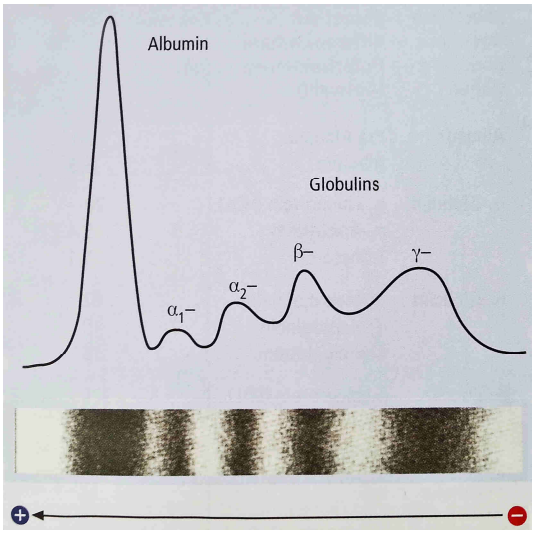

Fig. 2-1: electropherogram of the human serum (cf. Mutschler et. al., 2007)

of all plasma proteins are albumins. They have an elliptic molecular shape and they belong to the smallest plasma proteins. (Mutschler et al., 2007)

Gamma globulins are glycoproteins. Since they have important protective and defensive functions, they are also referred to as immunoglobulins (Ig). During electrophoresis γ-globulins are the slowest migrating plasma proteins. Via immunoelectrophoresis they can be classified into three large groups (IgG, IgA and IgM) and into two smaller ones (IgD and IgE). (Mutschler et al., 2007)

Fig. 2-1 shows the electropherogram of the human serum. In the lower part of the figure there is a scan of the stained paper strip after electrophoresis; in the upper part the related photometer curve is depicted. (Mutschler et al., 2007)

Plasma proteins in general are important for the maintenance of the colloid osmotic pressure. Furthermore, they have a buffer and a nutritive function; they also play a fundamental role in the immune response and the cascade of blood coagulation. Additionally, they serve as vehicles for hydrophobic substances like hormones, APIs, bilirubin and lipids, but also cations like Ca^{2+} can be bound. (Mutschler et al., 2007)

2.1.2 Red Blood Cells (Erythrocytes)

Erythrocytes have a diameter of 7.5 µm and a peripheral thickness of 2.5 µm. Due to their biconcave shape the thickness decreases to the center (cf. *Fig. 2-2*), which favors their formability during the passage of small capillaries. No nucleus is present; therefore, erythrocytes aren't able to synthesize DNA and RNA, respectively. Also mitochondria are missing. The cell metabolism takes place in form of glycolysis. (Mutschler et al., 2007)

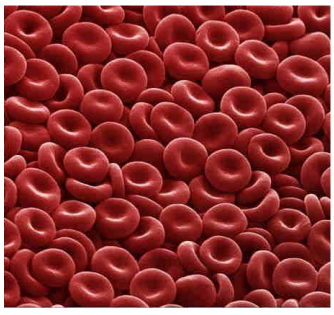

Fig. 2-2: erythrocytes (sciencedoing.blogspot.co.at)

All the important functions of the erythrocytes – like the transport of oxygen, contribution to the transport of carbon dioxide as well as an involvement in the regulation of the pH-value – are associated with hemoglobin, the pigment of the red blood cells. (Mutschler et al., 2007)

The volume fraction of erythrocytes in comparison to the total blood volume is called hematocrit. For men the hematocrit has an average value of 0.47, for women it is 0.42 – hence, erythrocytes account for nearly one half of the total blood volume. (Mutschler et al., 2007)

Erythropoiesis is the formation of red blood cells. Like for all the other blood cells it takes place in the red bone marrow. Erythropoietin is a renal hormone that stimulates erythropoiesis. (Mutschler et al., 2007)

Erythrocytes have a life cycle of 110 – 120 days. Every 24 h, 0.8% of all red blood cells are replaced by new ones. (Mutschler et al., 2007)

2.1.3 White Blood Cells (Leukocytes)

In contrast to erythrocytes, leukocytes have a nucleus. They play important roles in the immune system. In *Fig. 2-4* a scanning electron microscope image of a leukocyte can be seen. Leukocytes can be divided into granulocytes, lymphocytes and monocytes. Granulocytes again can be subdivided into neutrophils, eosinophils and basophils, depending on the shape of the cells and nuclei and on the colorability of the cytoplasmic granules (cf. *Fig. 2-3*). (Mutschler et al., 2007)

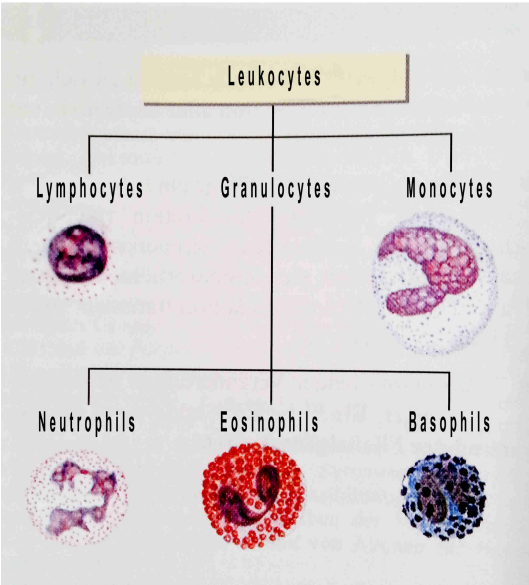

Fig. 2-3: leukocytes (cf. Mutschler et. al., 2007)

Only 5% of all the leukocytes are temporarily circulating in the blood, the rest is located in the bone marrow, in tissues and in organs, where they have specific functions. (Mutschler et al., 2007)

Granulocytes have polymorph nuclei. They play an important role in acute inflammation processes and in defending bacteria, viruses, protozoa and worms. Phagocytosis is their major task. (Mutschler et al., 2007)

90% of all the granulocytes that are circulating in the blood are neutrophils. They show a good amoeboid movement and they got small, poorly stainable granules in the cytoplasm. Neutrophils can phagocytize pathogens, tissue debris and foreign matters and are therefore important for the nonspecific defense. This species of granulocytes has a life cycle of only 4 – 5 days outside the bone marrow. (Mutschler et al., 2007)
Only 2-5% of the granulocytes in the blood are eosinophils. They got relatively big granules in the cytoplasm that can be stained intensively red with the acidic colorant eosin. Eosinophils are capable of amoeboid movement as well as they can phagocytize antigen-antibody complexes, dissimilar proteins and microorganisms. (Mutschler et al., 2007)
Basophils are the most infrequently occurring granulocytes in the circulating blood. Their granules become deeply blue-black when being stained with basic colorants. Basophils are secreting histamine, heparin and proteases. The mast cells of the tissues often can't be confined in their functionality from basophils. (Mutschler et al., 2007)

Like leukocytes, also lymphocytes show a heterogeneous cell population. B cells and T cells belong to the group of small lymphocytes; natural killer cells are referred to as large lymphocytes. Lymphocytes are important for specific defense mechanisms. Less than 1% of all lymphocytes are circulating in the bloodstream, the rest is located in secondary lymphatic organs. (Mutschler et al., 2007)

Monocytes are the largest blood cells. In tissues they are transforming into macrophages that are able to secrete more than a hundred substances. Important roles of macrophages are phagocytosis and specific defense mechanisms. (Mutschler et al., 2007)

2.1.4 Platelets (Thrombocytes)

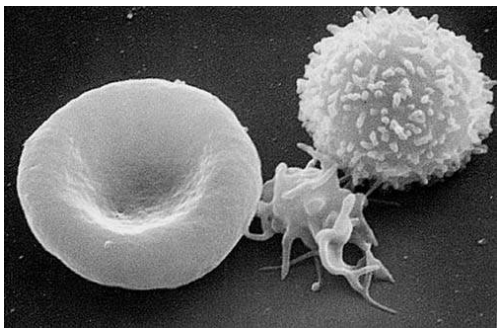

Fig. 2-4: from left to right: scanning electron microscope image of an erythrocyte, a thrombocyte and a leukocyte (pictures.doccheck.com)

Inactive thrombocytes are lenticular, anucleate disks with a life span of 8 – 12 days. Platelets have a diameter of 1.5 – 4 µm and a thickness of 0.5 – 4 µm. They are produced in the bone marrow and are very important for hemostasis, which is the technical term for the termination of bleeding. (Mutschler et al., 2007; Ammon et al., 2004)

2.2 Immunological Functions

In the narrower sense, resistance against infectious diseases is called immunity. We can differ between inborn and acquired immunity. Often, acquired immunity is also referred to as adaptive immunity. When adaptive immunity becomes part of the pathogenesis of a disease then it can even harm the organism. Autoimmune diseases and allergies are examples for that. (Kayser et al., 2005)

For defending potentially harmful substances or microorganisms the human body has unspecific and specific defense mechanisms where both humoral and cellular processes are involved. Unspecific mechanisms can render foreign bodies (antigens) harmless without preceding contact; specific defense mechanisms, however, are dependent on such a previous contact to the antigen, which leads to the production of antibodies. Those two immunological systems can work irrespective of each other, but a higher efficiency can be reached when they are cooperating. (Mutschler et al., 2008)

2.2.1 Unspecific Humoral Defense

The complement system, lysozyme, interferons and acute-phase proteins are responsible for this kind of immune defense. Some components can be assigned to several systems; the complement factors C3 and C4, for example, also belong to the group of acute-phase proteins. (Mutschler et al., 2008)

More than 20 activatable glycoproteins that are primarily synthesized in hepatocytes and macrophages build up the complement system. The single components of this system are indicated with a capital C. There are two ways how the complement system can be activated: the activation through antigen-antibody complexes is called *classical way*; the activation through structures on the surface of bacteria, viruses, fungi or protozoa is referred to as *alternative way*. Both ways end with the cascade C5 → C6 → C7 → C8 → C9. Most complement-proteins are precursors of enzymes that have to be activated by proteolysis. The biological achievements of the complement system are the defense of pathogens and the mediation of inflammation. (Mutschler et al., 2008)

Lysozyme is set free during the decay of phagocytic cells. It is able to cleave wall structures of particular bacteria via hydrolysis. (Mutschler et al., 2008)

Interferons belong to the group of immunomodulating cytokines. Cytokines are proteins or glycoproteins that are produced by the body with a regulatory effect on the immune system. Besides immunomodulating effects, interferons also have antiviral and

antiproliferative properties. According to their origin, they can be classified in α-, β- and γ-interferons. α-interferons are produced by different cells of the lymphatic system; β-interferons are generated by fibroblasts and epithelial cells and γ-interferon are built in lymphocytes. α- and β-interferons belong to the subgroup of type-I-interferons. They are primarily induced by viruses and parts of bacteria and fungi, they have their own receptor and they are preferentially antivirally effective. γ-interferon is also termed type-II-interferon; also this interferon type has its own receptor and it has mainly immunomodulating effects. (Mutschler et al., 2008)

When it comes to tissue injuries, acute-phase proteins are built. They are also referred to as *anti-inflammatory proteins*. Acute-phase proteins disintegrate lipids of destroyed cells and they act as proteases-inhibitors. (Mutschler et al., 2008)

2.2.2 Unspecific Cellular Defense

Phagocytes and natural killer cells make up the unspecific cellular defense. Neutrophils, eosinophils as well as monocytes and macrophages, respectively, belong to the group of phagocytes. This group is characterized by the abilities of amoeboid movement and phagocytosis. Neutrophils and eosinophils can be summed up to the subgroup of microphages. The blood cells with the best phagocytotic activity are monocytes. They are referred to as free macrophages; fixed macrophages are located outside the blood vessels in connective tissues and in many parenchymatous organs like the liver and the spleen. In addition to phagocytosis macrophages are antigen-presenting cells and therefore part of specific defense mechanisms, allergic reactions, transplant rejection and the elimination of tumor cells. Beyond that they are synthesizing different enzymes, some complement factors, interferons and interleukins. (Mutschler et al., 2008)

Natural killer cells are – as mentioned above – large lymphocytes. They are particularly specialized in the destruction of tumor cells and virus-infected cells. In contrast to the other lymphocytes they have no antigen-receptors. Thus, their function isn’t compulsory linked to antigen-antibody-reactions but anyway, the activity of natural killer cells is much higher when antibodies bind to their target cells. (Mutschler et al., 2008)

2.2.3 Specific Humoral Defense

Specific humoral defense mechanisms are triggered by antigen contact and mediated by B cells that are able to produce antibodies. (Mutschler et al., 2007)

For the organism antigens are foreign substances that lead to immunological defense measures in the blood and in tissues. Antigens are large molecules such as proteins, carbohydrates and sometimes even nucleic acids with a molecular weight of more than 3000 Da. When the body recognizes a resorbed or parenteral applied antigen he produces antibodies against it. Those antibodies are directed specifically against an antigen. Antibodies and antigens can bind reversibly to each other which results in a so called antigen-antibody-complex. (Mutschler et al., 2008) Antibodies will be discussed in more detail further down in the next chapter.

Antigenic determinants are those parts of the antigen that are important for their serological specificity; these substructures are the binding site for the particular antibody and they are normally located on the surface of the antigen molecule. Antigenic determinants are also referred to as epitopes. (Mutschler et al., 2008; Koolman et al., 2003)

Low molecular substances can obtain antigen properties when they bind to proteins or other macromolecules in the body. Those half-antigens are called haptens. (Mutschler et al., 2008)

Most antigens are stimulating both humoral and cellular defense mechanisms in the body. (Mutschler et al., 2008)

2.2.4 Specific Cellular Defense

T cells are the protagonist of the specific cellular defense. The origin of this lymphocyte population lies in the stem cells of the bone marrow; the thymus, however, is responsible for the maturing of the T cells – this is simultaneously the reason for their denomination. (Mutschler et al., 2007)

Antigen specific receptors on the surface of T cells are called T-cell-receptors. For every antigen there are specific T cells with the right structure of the receptor that meshes with the antigen. In contrast to B cells that recognize intact antigens, T cells only recognize fragmented antigens on the surface of antigen-presenting cells. After the first contact with the antigen a specific clone of the T cells starts to proliferate. Some of the newly formed daughter cells are long-living memory T cells. They have the ability to react fast and sometimes even fiercely when it comes to a repeated contact with the same antigen. (Mutschler et al., 2007)

A specific type of T cells is called T helper cells. They only recognize an antigen when other cells (antigen presenting cells) are presenting it to them. (Mutschler et al., 2007)

2.3 Antibodies

Antibodies are an important part of the humoral immune system. *Humoral* means that they are circulating passively in body liquids and cannot move actively to places where infections are present. Antibodies are able to bind antigens on the surface of pathogens to prevent somatic cells from being damaged (neutralization), they aggregate unicellular pathogens to make them better accessible for phagocytes (agglutination) and they activate the complement system to promote the innate immune response (opsonization). Furthermore, antibodies are very useful for diagnostic purposes. (Koolman et al., 2003)

There are five different isotypes of antibodies in the human plasma: IgA (with two subclasses), IgD, IgE, IgG (with four subclasses) and IgM. (Koolman et al., 2003; Löffler, 2005)

Fig. 2-5 shows the structure of a characteristic IgG molecule. Compared to the other immunoglobulins IgG is the highest concentrated antibody in the human serum – it can be regarded as the prototype of all the other immunoglobulins. (Löffler, 2005; Mutschler et al., 2007)

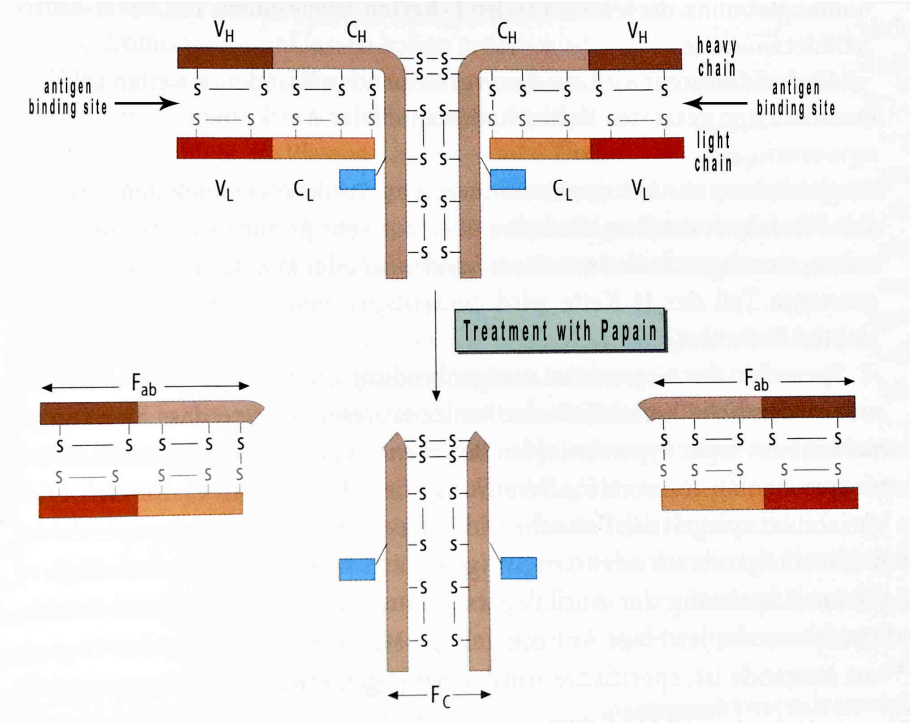

Fig. 2-5: structure of an IgG antibody (Löffler, 2005)

IgG is a symmetrically built, Y-shaped protein that is consisting of two identical light chains (L-chains) and two identical heavy chains (H-chains). Those chains are linked through non-covalent bonds and disulfide bridges. With the aid of the protease papain

antibodies can be split into three fragments: the two F_{ab}-fragments are able to bind antigens (F = fragment; $_{ab}$ = antigen binding); they are consisting of the L-chains and of pieces of the H-chains. The F_c fragment equals the remaining rest of the H-chains ($_c$ = crystallizable); all of the antibody's carbohydrates are part of this fragment. (Löffler, 2005; Koolman et al., 2003)

When comparing amino acid sequences of different antibodies it is obvious that some regions exhibit no or only little differences in their sequencing. Those regions are referred to as *invariable* or *constant* – in the case of the H-chains they are indicated with C_H, for the L-chains the abbreviation is C_L. (Löffler, 2005)

Especially in the area of the antigen binding site there is an exceeding variability of the amino acid sequence. Those regions are referred to as *variable* or even *hypervariable* – the designative abbreviations for the heavy and the light chains are V_H and V_L, respectively. This variability indicates that antibodies have to react in a very specific way with the different antigens what can only be reached in form of precise adaption of the antibody to the antigen. It is believed that the organism is able to produce specific antibodies against $10^7 – 10^8$ different antigens. (Löffler, 2005)

The subdivision of antibodies into the different isoforms (IgG, IgA, IgM, IgD and IgE) is based on the fact that there are five variants of the H-chains existing. They are indicated with the Greek letters α, δ, ε, γ and μ. There are also two variants of the light chains – κ and λ. Amongst other things *Tab. 2-1* gives an overview to which antibody classes the different variants of the H- and L-chains belong. (Löffler, 2005)

	IgG	IgA	IgM	IgD	IgE
molecular weight	150 kDa	160 kDa	950 kDa	170 kDa	190 kDa
heavy chains	γ	α	μ	δ	ε
light chains	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
amount in plasma [g/l]	8 – 18	0.9 – 4.5	0.6 – 2.8	0.003 – 0.4	1-14 x 10^{-4}
serum-Ig (%)	80	13	6	0.1	0.002
half-life (days)	23	6	5	3	2 – 3
placental passage	++	-	-	-	-
activation of the complement system	++	+	+++	-	-
bacterial inhibition (gram-negative)	+++	++ (with lysozyme)	+++ (with complement system)	-	-
viral inhibition	+++	+++	+	-	-
occurrence	serum	serum (monomers), epithelium, secretion (dimers), breast milk (dimers)	serum (pentamers), antigen receptors of B cells (monomers)	serum (monomers), antigen receptors of B cells (monomers)	serum (monomers), bound to basophils and mast cells

Tab. 2-1: characteristics, structural features and occurrence of human immunoglobulins (cf. Löffler, 2005; Mutschler, 2008)

IgA and IgM are potential multimers that are held together by disulfide bridges and by joining peptides. In *Fig. 2-6* those peptides are indicated with a capital J. (Koolman et al., 2003)

The central part of the antibodies – where papain attacks the molecule – is called hinge region *(cf. Fig. 2-6)*. This part is named like that because it works like a hinged joint what makes antibodies to very flexible proteins. (Koolman et al., 2003)

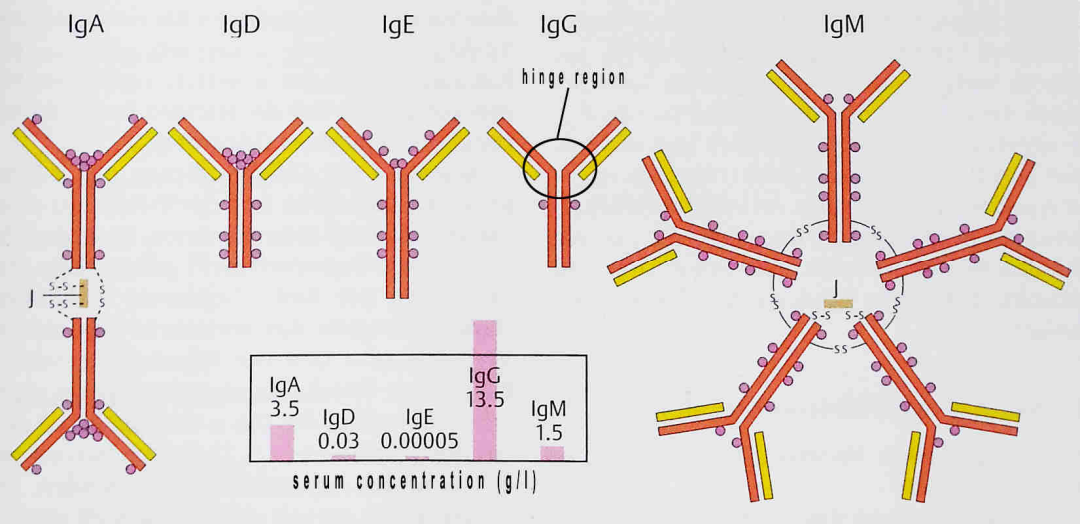

Fig. 2-6: immunoglobulin classes (cf. Koolman et al., 2003)

2.3.1 Antibody Production

In the context of antibody production B cells play a central role. B cells emerge from stem cells of the bone marrow. The bone marrow is also the place where B cells mature – so, the meaning of the B in the name of those cells becomes clear. B cells finally leave the bone marrow as mature, naive cells that are immunologically competent due to receptors on their surface that are able to recognize specific antigens. Those receptors are consisting of surface-immunoglobulins of the types IgD and IgM. In principle, the receptor equates the variable part of the same antibody that is secreted from the cell after antigen stimulation. B cells finally get to the spleen and the lymph nodes via the blood- and lymph stream. (Mutschler et al., 2007)

About 5 – 7 days after an antigen encountered immunologically competent B cells the antibody production starts. IgD and IgM are the first antibodies being secreted – therefore they are referred to as primary antibodies. T helper cells can provoke a shift to the production of the secondary antibodies IgG, IgA and IgE. (Mutschler et al., 2007) *Fig. 2-7* and *Fig. 2-8* represent the development and the clonal selection of B cells.

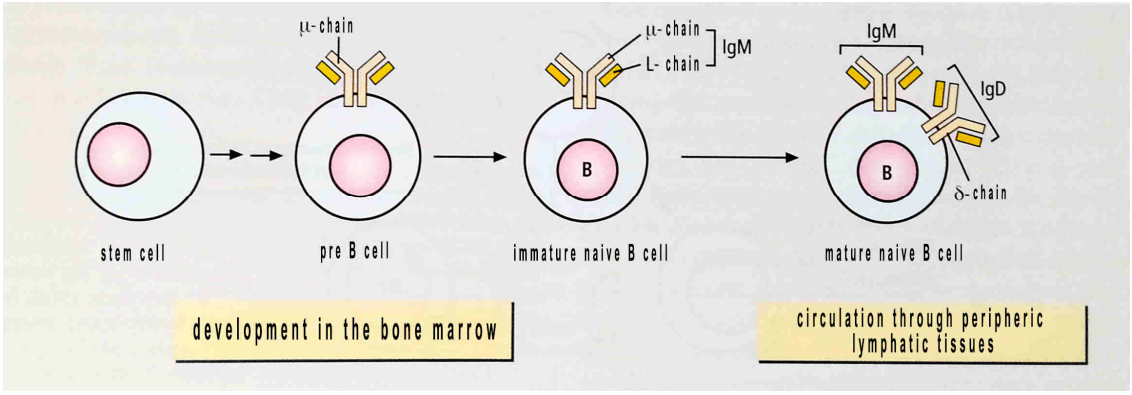

Fig. 2-7: development of B cells (cf. Mutschler et al., 2007)

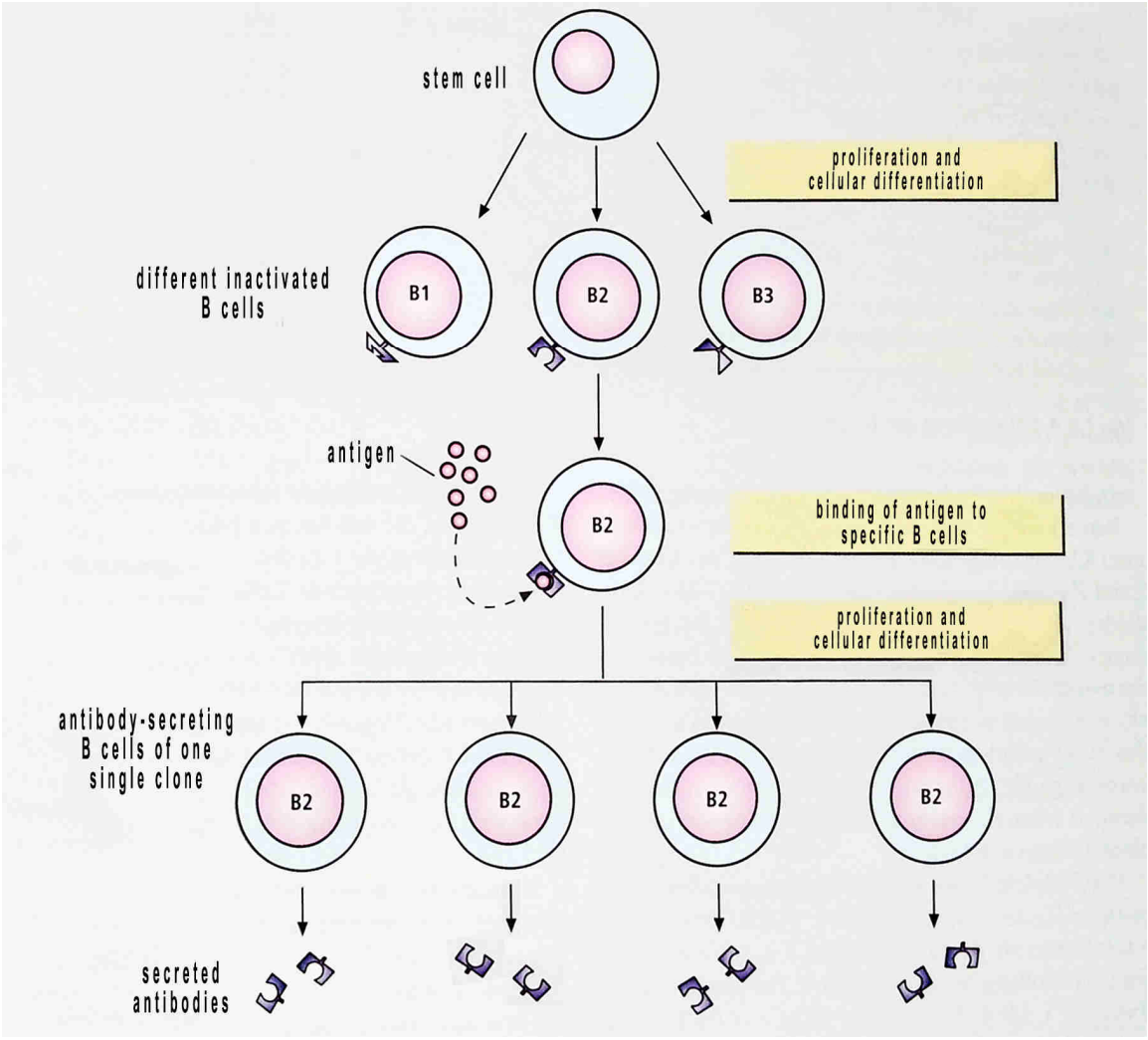

Fig. 2-8: clonal selection of B cells (cf. Mutschler et al., 2007)

2.3.2 Physiological Functions

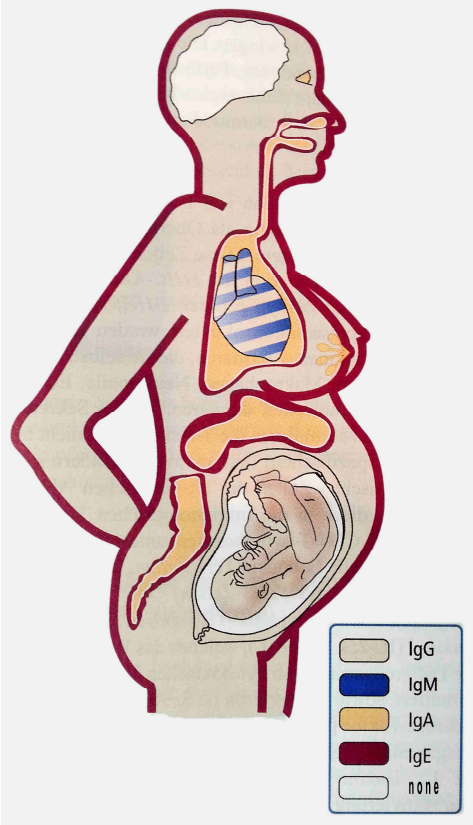

Fig. 2-9: distribution of the different immunoglobulins in the human body (Mutschler et al., 2008)

IgG makes up 80% of all immunoglobulins in the human serum. It can be divided into four subclasses (IgG_1 – IgG_4). During secondary responses to an antigen mainly IgG is built. It is the only immunoglobulin that is able to pass membranes. Therefore, IgG can get into the circulation of the unborn child through placental passage where it helps to eliminate microorganisms. For neonates it subsequently confers an important protection in the first months of their lives. (Mutschler et al., 2008)

IgA is specialized in defensive processes on mucosal surfaces of the organism. In the serum IgA mainly occurs in form of a monomer but it is the only antibody that can be secreted as a dimer. Its main purpose is to prevent pathogens (especially bacteria and viruses) and other materials from penetrating in mucous membranes. IgA can be found in breast milk so that neonates are participating on the mother's immunological protection through breastfeeding. (Mutschler et al., 2008)

IgM is the first secreted antibody when it comes to primary responses to an antigen. Its concentration decreases rapidly whilst the concentration of IgG still rises. IgM is the largest antibody; it can occur as a pentamer and it is much more effective than IgG. (Mutschler et al., 2008)

IgD is a relatively unexplored antibody. It is not only occurring in the serum. Like IgM, it is also bound to membranes of matured B cells. (Mutschler et al., 2008)

Also the physiological function of **IgE** is widely unknown. It is suggested that it plays a role in the immune response against parasite infestation, especially in the context of worm diseases. Mast cells and basophils have receptors for the F_c-fragment of IgE on their surface. The interaction of cell-bound IgE with a corresponding antigen can cause immediate hypersensitivities. (Mutschler et al., 2008) *Fig. 2-9* shows the distribution of the different immunoglobulins in the human body.

2.3.3 Monoclonal vs. Polyclonal Antibodies

Monoclonal antibodies (mAbs) are secreted from immune cells that are descending from one single antibody-producing mother cell – thus, they stem from a unique cell clone. mAbs are directed against one specific epitope of an immunogenic substance (antigen). Large antigens usually have several epitopes. This requires different mAbs that are secreted from different clones of B cells. Such a mixture of mAbs is referred to as polyclonal. (Koolman et al., 2003)

Fig. 2-10 shows how mAbs can be produced. As an initial step, lymphocytes from the spleen of an immunized mouse have to be isolated and fused with murine tumor cells (myeloma cells). This is necessary because the lifespan of antibody-secreting lymphocytes is restricted to a few weeks in cell cultures. The fusion with tumor cells results in cell hybrids, so-called hybridomas, which are potentially immortal. The fusion itself is a seldom event; its probability can be increased by the addition of PEG. (Koolman et al., 2003)

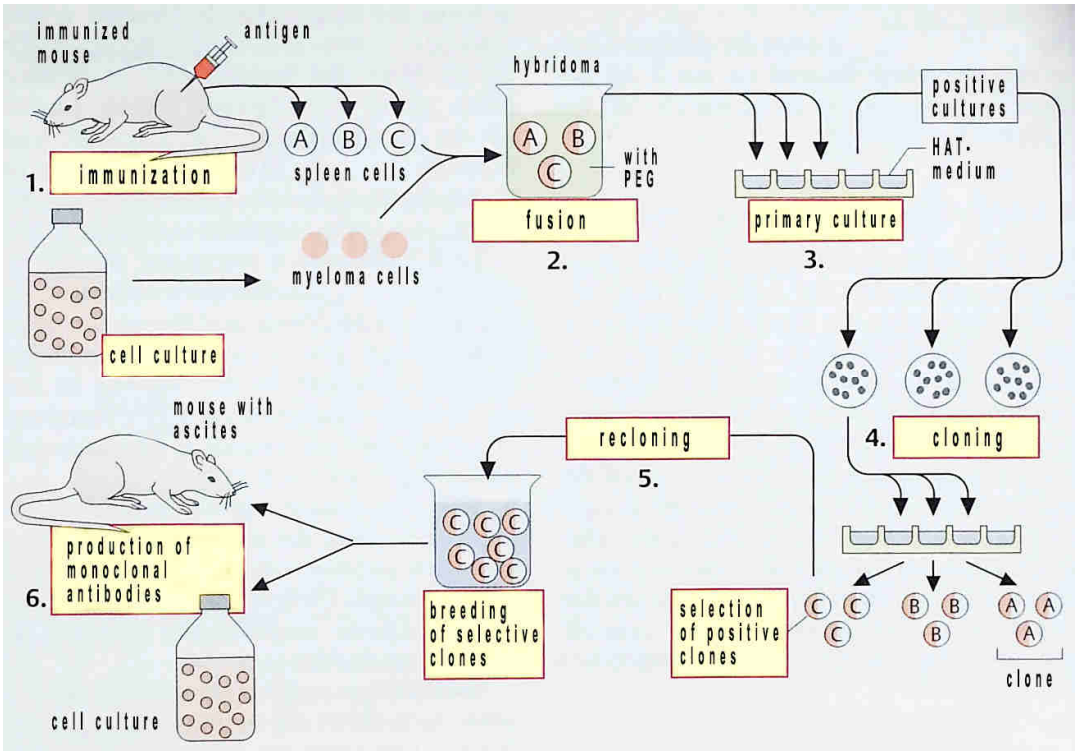

Fig. 2-10: production of monoclonal antibodies (cf. Koolman et al., 2003)

The next step comprises the incubation over a longer period in a primary culture with HAT-medium that is consisting of hypoxanthine, aminopterin and thymidine to selectively gain the successfully fused cells. Only hybridoma cells survive in this medium; myeloma cells and spleen cells die. (Koolman et al., 2003)

mAbs are only produced by some of the fused cells. To find those cells the hybridomas have to be singularized and proliferated by cloning. After the clones were tested for built mAb the positive cultures are selected by cloning again. This results in hybridomas that are producing mAbs. The production of the mAbs is finally carried out in vitro with the help of bioreactors or in vivo by generation of an ascites-liquid in a mouse. (Koolman et al., 2003) This procedure was awarded with the Nobel Prize in 1984. (Mutschler et al., 2007)

2.4 Purification Strategies

When purifying antibodies many different parameters like the purity required for the desired final application, the scale of purification, physico-chemical characteristics, production sources as well as the economy of the purification process have to be taken into account. (GE Healthcare, 2015)

Fig. 2-11 shows some important contaminations that can be expected when it comes to antibody purification, depending on the different production sources. (GE Healthcare, 2015)

	Molecular types	Quantity	Significant contaminants
Source: native			
Human serum	Polyclonal IgG, IgM, IgA, IgD, IgE	IgG 8 to 16 mg/ml IgM 0.5 to 2 mg/ml IgA 1 to 4 mg/ml IgE 10 to 400 ng/ml IgD up to 0.4 mg/ml	Albumin, transferrin, α_2-macroglobulin, and other serum proteins
Hybridoma: cell culture supernatant	Monoclonal	Up to 1 mg/ml	Phenol red, albumin, transferrin, bovine IgG, α_2-macroglobulin, other serum proteins, viruses
Hybridoma: cell culture supernatant, serum-free	Monoclonal	1 to 4 mg/ml	Albumin, transferrin (often added as supplements)
Ascites	Monoclonal	1 to 15 mg/ml	Lipids, albumin, transferrin, lipoproteins, endogenous IgG, other host proteins
Egg yolk	IgY	3 to 4 mg/ml	Lipids, lipoproteins, vitellin
Source: recombinant			
Extracellular protein expressed into supernatant	Monoclonal antibodies, tagged antibodies, antibody fusion proteins, Fab, or F(ab')$_2$ fragments	Depends upon expression system	Proteins from the host, e.g., *E. coli*, Chinese Hamster ovary (CHO) cells, general low level of contamination
Intracellular protein expression		Depends upon expression system	Proteins from the host, e.g., *E. coli*, phage

Fig. 2-11: antibody production sources and important contaminations (GE Healthcare, 2015)

Extraction, filtration, centrifugation, desalting and buffer exchange are important steps that are involved in many purification work flows. (GE Healthcare, 2015) In *Fig. 2-16* the workflow of a possible purification process is depicted.

Also the adding of specific tags to biomolecules during the upstream process can facilitate the purification in the course of the downstream processing. Histidine tags are very often used, mostly in the form of a $(His)_6$-sequence, but also glutathione S-transferase- and maltose-binding protein-tags are common practice (GST-, MBP-tags). The affinity of those tags to specific stationary phases can be used as a basis of the purification process, especially if the target molecule has no affinity to any known stationary phase. The tag itself can be removed in a further production step. (GE Healthcare, 2012; GE Healthcare, 2015)

2.4.1 Affinity Chromatography

The principle of affinity chromatography is a reversible interaction between the stationary phase and the analyte (cf. *Fig. 2-12*). This reversible interaction is based on covalent bonds between the separation matrix and the molecules of interest. Ligands can be coupled to the stationary matrix in order to enhance the separation efficiency. (GE Healthcare, 2007)

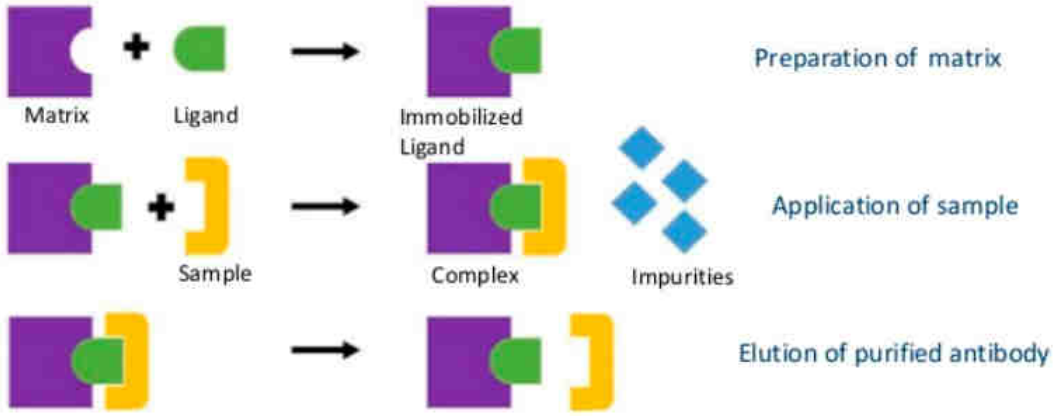

Fig. 2-12: principle of affinity chromatography (cf. image.slidesharecdn.com)

Protein A and G are bacterial proteins that show high affinity to the F_c-region of polyclonal and monoclonal antibodies of the class IgG. Protein A stems from *Staphylococcus aureus* and protein G stems from bacteria of the genus *Streptococcus*. These two proteins can be

coupled to Sepharose for chromatographic applications. (GE Healthcare, 2007) *Fig. 2-13* shows the binding affinity of protein A and G to different antibodies:

Species	Subclass	Protein G binding	Protein A binding
Human	IgA	–	variable
	IgD	–	–
	IgE	–	–
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	++++	–
	IgG_4	++++	++++
	IgM*	–	variable
Avian egg yolk	IgY†	–	–
Cow		++++	++
Dog		+	++
Goat		++	–
Guinea pig	IgG_1	++	++++
Hamster		++	+
Horse		++++	++
Koala		+	–
Llama		+	–
Monkey (rhesus)		++++	++++
Mouse	IgG_1	++++	+
	IgG_{2a}	++++	++++
	IgG_2	+++	+++
	IgG_3	+++	++
	IgM*	–	variable
Pig		+++	+++
Rabbit		+++	++++
Rat	IgG_1	+	–
	IgG_{2a}	++++	–
	IgG_{2b}	++	–
	IgG_3	++	+
Sheep		++	+/–

* *Purified using HiTrap IgM Purification HP columns.*
† *Purified using HiTrap IgY Purification HP columns.*
++++ = *strong binding.*
++ = *medium binding.*
– = *weak or no binding.*

Fig. 2-13: binding affinity of different antibodies to Protein A and G (GE Healthcare, 2015)

Free protein A and G can be used to get a first impression of the binding affinity to target molecules. When bound to a separation matrix, however, the binding affinity can be different. Rat IgG_1, for instance, does only bind to Protein A in combination with Sepharose, but it doesn't bind to free protein A. (GE Healthcare, 2015)

Protein G exhibits a better affinity to IgG than protein A. Additionally, the binding affinity to albumin is lower which results in better yields and purities. The elution conditions for

the target molecule, however, are – when working with protein A – less harsh compared to protein G. (GE Healthcare, 2015) *Fig. 2-14* gives an overview of the binding and elution conditions of different classes of IgG when protein A is used in combination with Sepharose for affinity chromatography.

Protein A has five regions with an affinity to the F_C-part of IgG. When protein A is coupled to Sepharose, at least two IgG-molecules can bind to one molecule of protein A. Besides IgG, also IgA and IgM have a binding affinity to protein A. (GE Healthcare, 2015)

Altogether it can be said that the selectivity, resolution and capacity of affinity chromatography are very high. Purity levels up to 99% can be reached and often, no other purification step is required. (GE Healthcare, 2015)

Species	Subclass	Protein A binding pH	Protein A elution pH
Human	IgG_1	6.0 to 7.0	3.5 to 4.5
	IgG_2	6.0 to 7.0	3.5 to 4.5
	IgG_3	8.0 to 9.0	≤ 7.0
	IgG_4	7.0 to 8.0	3.0 to 6.0
Cow	IgG_2	n.a.	2.0
Goat	IgG_2	n.a	5.8
Guinea pig	IgG_1	n.a.	4.8
	IgG_2	n.a	4.3
Mouse	IgG_1	8.0 to 9.0	4.5 to 6.0
	IgG_{2a}	7.0 to 8.0	3.5 to 5.5
	IgG_{2b}	Approx. 7.0	3.0 to 4.0
	IgG_3	Approx. 7.0	3.5 to 5.5
Rat	IgG_1	≥ 9.0	7.0 to 8.0
	IgG_{2a}	≥ 9.0	≤ 8.0
	IgG_{2b}	≥ 9.0	≤ 8.0
	IgG_3	8.0 to 9.0	3.0 to 4.0 (using 3 M potassium isothiocyanate)

Fig. 2-14: binding and elution conditions for different classes of IgG when using protein A + Sepharose for affinity chromatography (GE Healthcare, 2015)

2.4.2 The CIPP-Concept

If one step isn't enough to reach the required purity of the biomolecule, CIPP is a common purification strategy (cf. *Fig. 2-15*). CIPP is an acronym and stands for *Capture*, *Intermediate Purification* and *Polishing*. The *Capture* step encompasses – after preceding preparation, extraction and clarification – the isolation, concentration and stabilization of the targeted biomolecule. Potency and activity must not be lost during this step. The

Intermediate Purification step aims to remove bulk impurities like nucleic acids, viruses, endotoxins and other proteins. The *Polishing* step is used to reach the desired final high level purity by removing substances that are closely related to the product or trace impurities, respectively. The CIPP concept is used in industry for the purification of therapeutically used antibodies. (GE Healthcare, 2015)

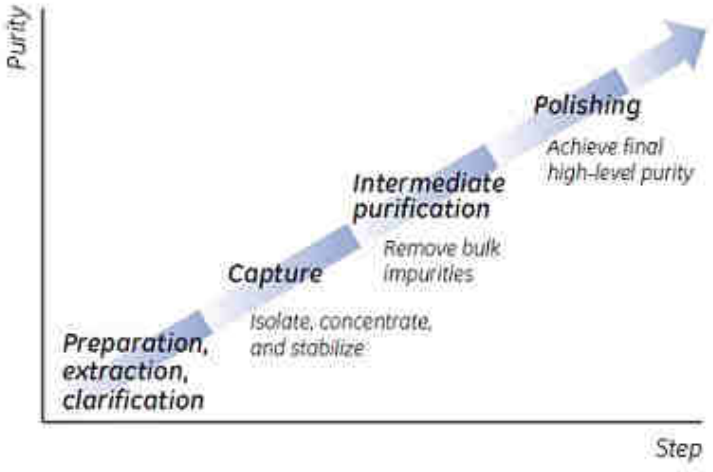

Fig. 2-15: CIPP concept (www.gelifesciences.com)

2.5 Alternative Approaches

Due to the disadvantages of protein A and G affinity chromatography – like mentioned in the introduction of the thesis – alternative approaches for purifying antibodies (and other biomolecules) are a research field of great interest in pharmaceutical industry. Below, a short overview of some alternative approaches shall be given:

2.5.1 Aqueous Two-Phase Extraction (ATPE)

Among others, the working group around Dr. Tim Zeiner at TU Dortmund University focuses on the development of an aqueous two-phase extraction system consisting of phosphate salt, polyethylene glycol (PEG) 2000 and water. In a first extraction step, the antibody accumulates in the PEG-rich top phase when NaCl is added to the system. After this step a back extraction step is used to accumulate the antibody in the phosphate-rich bottom phase – therefore, the concentration of NaCl has to be reduced. The third step encompasses the washing of the bottom phase with fresh top phase. During this washing step, the antibody remains in the bottom phase. So far, purities of 95% and yields up to 88% could be reached with this method for purifying IgG_1. (Mündges et al., 2015)

The figure below shows how ATPE can be integrated in a purification process:

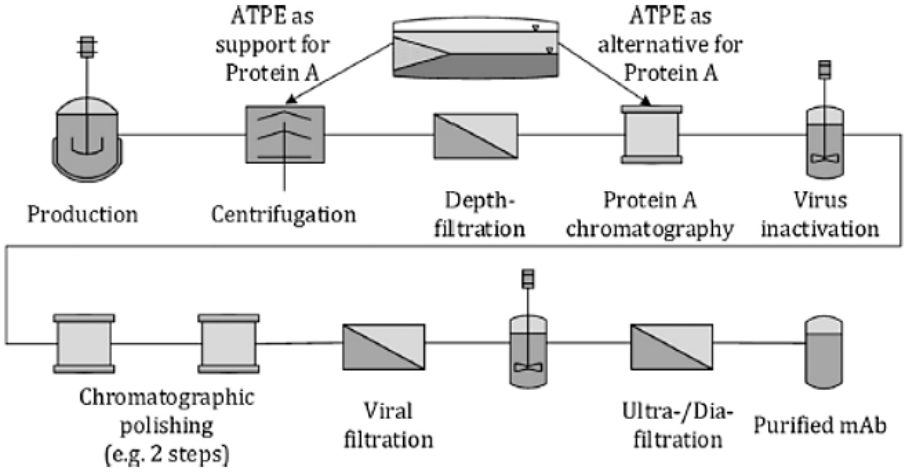

Fig. 2-16: ATPE integrated in a purification process (Mündges et. al., 2015)

2.5.2 Development of New Stationary Phases

When talking about stationary phases we have to distinguish between ligands and support matrices. Support matrices usually have an autonomous separation effect that can be enhanced or altered by ligands in the form of functional groups.

One support matrix that has drawn interest over the last decades are monoliths. (Barroso et. al., 2013) They have a macroporous structure that can be directly linked to the walls of the geometries where the separation process takes places (e.g. columns, capillaries) through in situ polymerization; thus, retaining frits aren't needed. Another advantage is that the back pressure of monolithic columns is up to three times lower compared to packed beds. (Allen et. al., 2003)

The separation effect of the monoliths can be positively influenced through a functionalization with ligands. In the course of this diploma thesis an imidazoline-functionalized silica gel based monolith was applied in addition to a normal phase silica gel based monolith.

2.6 Electrophoresis

The migration of charged particles in an electrical field with constant voltage or amperage is used for separating complex mixtures of biomolecules. Separation methods based on this principle are referred to as electrophoresis. (Jansohn et. al., 2012; Rücker et. al., 2008)

Electrophoretic techniques can be classified into free-flow or carrier-free electrophoresis, respectively, and carrier electrophoresis. When regarding carrier-free electrophoresis, the

electrophoretic migration takes place only in a buffer solution. Carrier electrophoresis is performed on papers or gels in a buffer solution. (Rücker et. al., 2008)

2.6.1 Gel Electrophoresis

The most common type of carrier electrophoresis is gel electrophoresis; usually it is carried out on polyacrylamide-, agarose-, starch- or cellulose acetate gels. Here, the separation of the single substances not only results from their charge, also the sieving effect of the gels because of differences in size and shape of the molecules that have to be separated has to be taken into account. Physical effects like convection and diffusion are prevented by the polymeric gel matrices. (Jansohn et. al., 2012)

The electrophoretic mobility of charged particles depends on the total net charge, size and shape of the molecule, the pore size of the carrier; the pH-value, temperature and ionic strength of the buffer and the strength of the electric field. (Jansohn et. al., 2012)

Gel electrophoresis can be performed continuously or discontinuously. Discontinuously means, that the carrier is composed inhomogeneously in matters of the gel composition. The technique using this principle is called disc electrophoresis. When an electrophoresis is performed continuously the gel matrix has a homogenous composition. (Jansohn et. al., 2012)

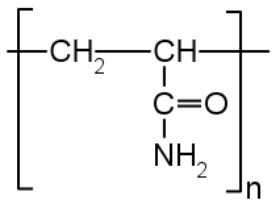

Fig. 2-17: chemical structure of polyacrylamide (wikipedia.org)

Polyacrylamide (cf. *Fig. 2-17*) has the best resolution of all the gel matrices listed above; therefore it is primarily used for analyzing proteins and DNA-fragments. Common acrylamide concentrations for preparing polyacrylamide gels lie in the range of 3 – 30% which accounts for a pore size of 0.5 – 0.2 nm.

Generally, large molecules are retained stronger than smaller ones when the acrylamide concentration is high. When using low concentrations of acrylamide the molecular sieve effect is little; in this case the separation is primarily based on different ratios of mass to charge. (Jansohn et. al., 2012)

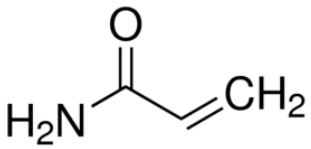

Fig. 2-18: chemical structure of acrylamide (wikipedia.org)

The three-dimensional structure of polyacrylamide is built after radical polymerization of the monomeric acrylamide (cf. *Fig. 2-17, Fig. 2-18*). The chemical reaction is catalyzed by

ammonium persulfate (APS; cf. *Fig. 2-19*) and tetramethylethylenediamine (TEMED, cf. *Fig. 2-20*). (Jansohn et. al., 2012)

DNA-molecules are negatively charged and therefore, they all migrate to the anode. Proteins, however, don't have a uniform charge and thus, they don't have a general migration direction. (Jansohn et. al., 2012)

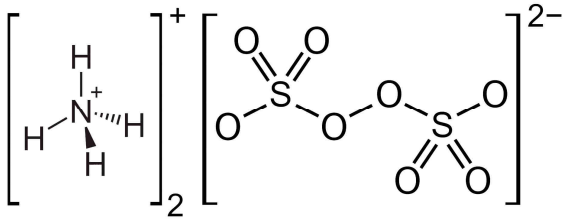

Fig. 2-19: chemical structure of APS (wikipedia.org)

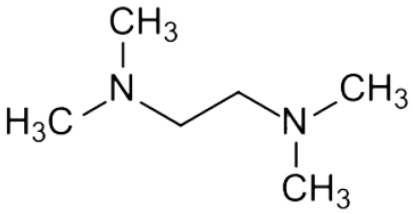

Fig. 2-20: chemical structure of TEMED (wikipedia.org)

When sodium dodecyl sulfate (SDS, cf. *Fig. 2-21*) is added to proteins, the intrinsic charge of the proteins is covered so effectively that anionic micelles with a constant net charge per mass unit are generated; those micelles are migrating all towards the anode. About 1.4 g of SDS bind to 1 g protein. In combination with heat denaturation to unfold the proteins a separation based on the protein mass can be reached. (Jansohn et. al., 2012) This method was firstly described by Ulrich K. Laemmli in 1970. (Laemmli, 1970)

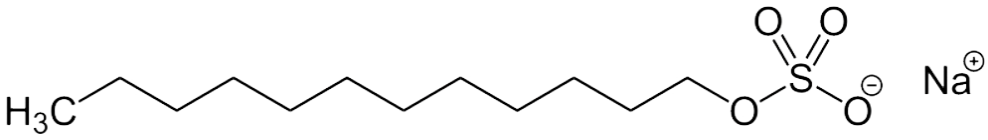

Fig. 2-21: chemical structure of SDS (wikipedia.org)

2.6.2 Standard Operation Procedure

When not using industrially manufactured gels that are available on the market, gels have to be casted in the lab before a gel electrophoresis can be performed. In the case of polyacrylamide the working steps are as follows: two glass plates – separated by a spacer – have to be cleaned with alcohol before they are put into the casting stand. The bottom of the plates should be sealed with a 2% agarose-solution to prevent the gel mixture from running out of the plates before the polymerization of the gel is finished.

To obtain better resolutions with sharper bands, gels for SDS-PAGE usually consist of two differently composed regions that are referred to as running- and stacking gel (cf. *Fig. 2-22*). The stacking gel has large polyacrylamide (PAA)-pores and its buffer is containing

chloride ions; those ions have a higher velocity of migration than the proteins. The electrophoresis buffer, however, is containing glycine ions with a lower velocity of migration than the proteins. As a consequence of that the proteins are accumulating between the ions with a low mobility (glycine ions) and the ions with a high mobility (chloride ions) depending on their velocity in the gradient of the field strength. This phenomenon is called *stacking effect* and it is leading to a concentration and pre-separation of the analytes which results in sharper bands on the gel. (Jansohn et. al., 2012)

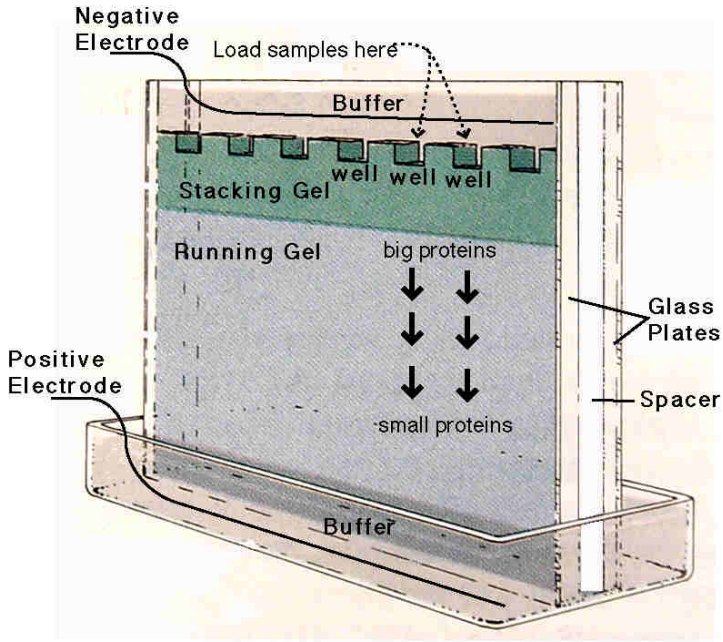

Fig. 2-22: scheme SDS-PAGE (ww2.chemistry.gatech.edu)

The running gel has smaller PAA-pores than the stacking gel as well as a higher salt concentration and a higher pH-value. When passing the border of the stacking- to the running gel the glycine ions can overrun the larger proteins. In the buffer system of the running gel the pile of proteins gets dissipated and the proteins get separated according to their molecular size. (Jansohn et. al., 2012)

At first, the running gel has to be casted. Therefore, the appropriate amounts of buffer, H_2O, APS and TEMED are pipetted into a vessel (cf. part *3. Experimental*). The solution is mixed before acrylamide is added. As soon as acrylamide is part of the mixture, the polymerization starts. The solution is pipetted into the spacing between the two glass plates; the formation of air bubbles should be prevented. The fill level of the running gel has to be adapted to the slots where the analytes will be applied. From the bottom of the slots to the beginning of the running gel the analytes should cover a distance of

approximately 1 – 2 cm. After the liquid mixture of the running gel got filled in the interspace of the glass plates, some isopropanol has to be added to the spacing to get a smooth surface of the running gel. The isopropanol will accumulate on top of the liquid because of its lower density. (Jansohn et. al., 2012)

After the polymerization of the running gel is finished, the isopropanol has to be removed with filter paper. Then, the stacking gel can be casted in the same way with the only difference that now the variant composition to the running gel has to be considered. When the mixture of the stacking gel got filled into the spacing of the glass plates, a comb has to be inserted to form the slots for applying the analytes. Again, a 2% agarose-solution can be used to seal the top of the glass plates at the left and the right end to prevent air from getting under the comb causing inhomogeneously shaped slots. Polymerized gels can be stored in the fridge wrapped in wet paper in a plastic bag. For native PAGE no stacking gel has to be casted, so the running gel mixture is filled to the top of the glass plates before the comb is inserted. The same counts for agarose gels, when the electrophoresis is performed vertically. (Jansohn et. al., 2012)

Now the electrophoresis can be performed. Therefore, the comb has to be removed from the gel, before the gel/glass plate-sandwich gets inserted into the electrophoresis chamber. After the chamber got filled up with the running buffer, the prepared solutions of the analytes can be applied to the slots. The composition of the analyte solutions is listed in part 3 of the thesis.

When the electrophoresis chamber got closed with the cover plate and plugged to the power supplier, the start button can be pressed after the desired settings of the power supplier were made. During the electrophoresis it has to be checked periodically that the fill level of the running buffer doesn’t deepen. When performing long runs, an ice bath for cooling has to be considered. The last steps after stopping the electrophoresis are the detection of the analytes on the gel with an adequate staining method and the ensuing computerized documentation via making a scan.

2.6.3 Detection Methods for Proteins

Proteins can be localized on gels as coloured complexes after reaction with dyes or silver salts. During the staining process proteins intertwine with the gel matrix; this isn’t desirable when proteins are purified preparatively via gel electrophoresis. (Jansohn et. al., 2012)

In the course of this diploma thesis coomassie- and silver staining were used as staining techniques: Coomassie brilliant blue R-250 (cf. *Fig. 2-23*) is a dye that binds unspecifically to nearly all proteins as a deep blue complex with a reddish tint (→R). The detection limit lies in the range of 0.1 – 2 µg protein per band on the gel. Coomassie brilliant blue G-250 (cf. *Fig. 2-24*) has the same sensitivity like R-250, but the blue of this dye is brighter and has a greenish tint (→G). The solubility of G-250, however, is worse; because of that it's usually used as a colloidal solution. The lower solubility of G-250 effectuates that it preferentially binds to the proteins instead of the gel matrix which leads to shorter destaining times. When thicker gels are used, though, the selectivity is reduced. (Jansohn et. al., 2012) Compared to Coomassie Brilliant Blue R250, Coomassie Brilliant Blue G250 has two additional methyl groups (cf. *Fig. 2-23*, *Fig. 2-24*)

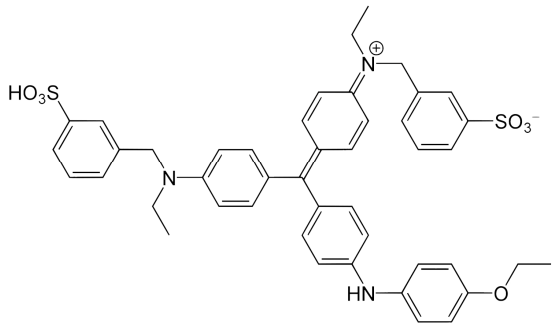

Fig. 2-23: chemical structure of Coomassie Brilliant Blue R250 (wikipedia.org)

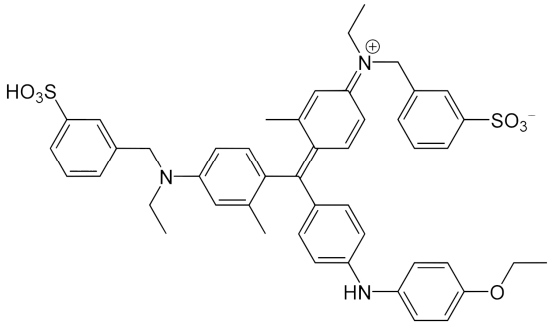

Fig. 2-24: chemical structure of Coomassie Brilliant Blue G250 (wikipedia.org)

When the electrophoresis run is finished, the gel is briefly rinsed with dH_2O before it comes into a tray with coomassie brilliant blue, methanol, distilled water and acetic acid for about half an hour; longer staining times may lead to better results. Then, the gel has to be rinsed with water again, before it is put into a destaining solution consisting of acetic acid, methanol and distilled water over night. After washing with dH_2O for about 10 min, the gel is ready for documentation. The recipe for preparing the staining and the destaining solutions, respectively, can be found in part 3 of the thesis. (EMBL, 2015)

Silver staining is 10 – 100-fold more sensitive than coomassie staining. Less than 1 ng protein per band can be detected with this method. During silver staining it comes to a complexation of silver ions with side chains of amino acids, especially with sulfhydryl- and carboxyl groups. (Jansohn et. al., 2012)

Although the sensitivity of silver staining is better, much more work steps are needed for this staining method: At first, the gel has to be rinsed with dH_2O before it comes into a tray

with methanol, water and acetic acid (50 ml MeOH / 22.5 ml dH_2O / 2.5 ml HAc) for 20 min. Then the gel is washed with 50% MeOH for 10 min, before it is washed with 50 ml dH_2O three times for 10 min. Afterwards the gel is put for 1 min in a 0.02% sodium thiosulfate solution (10 mg $Na_2S_2O_3$ in 50 ml dH_2O) to sensitize the gel. Then, the gel is washed in dH_2O two times for 1 min, before it is incubated with a cold 0.1% silver nitrate solution (50 mg $AgNO_3$ in 50 ml dH_2O) at 4 °C for 20 min. Then, the gel is washed in dH_2O again two times for 1 min. For the next step, the gel is developed in 0.04% formaldehyde in a 2% sodium carbonate solution (2 g Na_2CO_3 / 100 ml dH_2O / 40 µl formaldehyde). When the colour of the bands is satisfying, the development can be stopped with 5% acetic acid. After washing with dH_2O for about 10 min, the gel is ready for documentation. (EMBL, 2015)

2.7 Monoliths

Monoliths are an alternative to common stationary phases that are widely spread in chromatography for analytic and preparative purposes. While traditional stationary phases usually are particle shaped, monoliths exhibit a porous, one-piece structure with channels in the micrometer range (cf. *Fig. 2-25*, *Fig. 2-26*). (C&EN, 2006; Chromatographer, 2009)

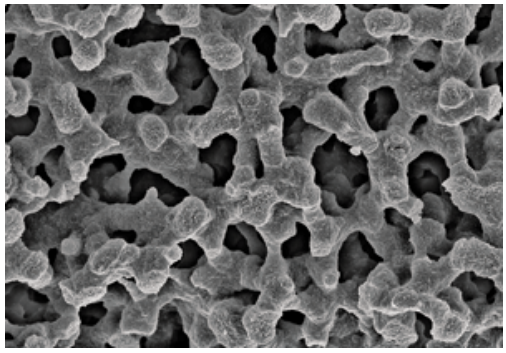

Fig. 2-25: porous structure of a monolith (C&EN, 2006)

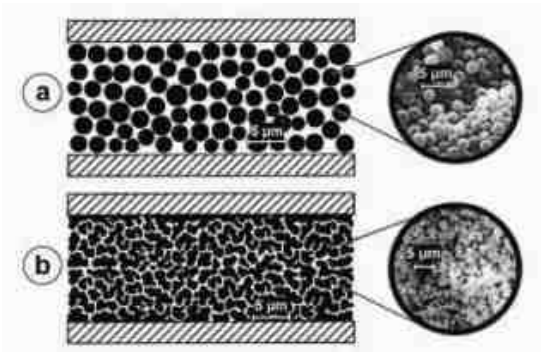

Fig. 2-26: a) particle shaped stationary phase, b) monolithic stationary phase in a column (Chromatographer, 2009)

The skeleton of monoliths can be organic or inorganic. Silica-based monoliths belong to the inorganic class. One way to produce them is the so-called sol-gel technology. Organic monoliths are polymer-based. Acrylamide, methacrylamide and styrene are often used as polymers for producing this class of monoliths. (Chromatographer, 2009)

The production of the monoliths used in the experimental part of this thesis is based on the sol-gel process (cf. *Fig. 2-27*). The silica compounds TMOS and MTMS that are depicted in the figure below have the function of monomeric precursors. (Kato et. al., 2005)

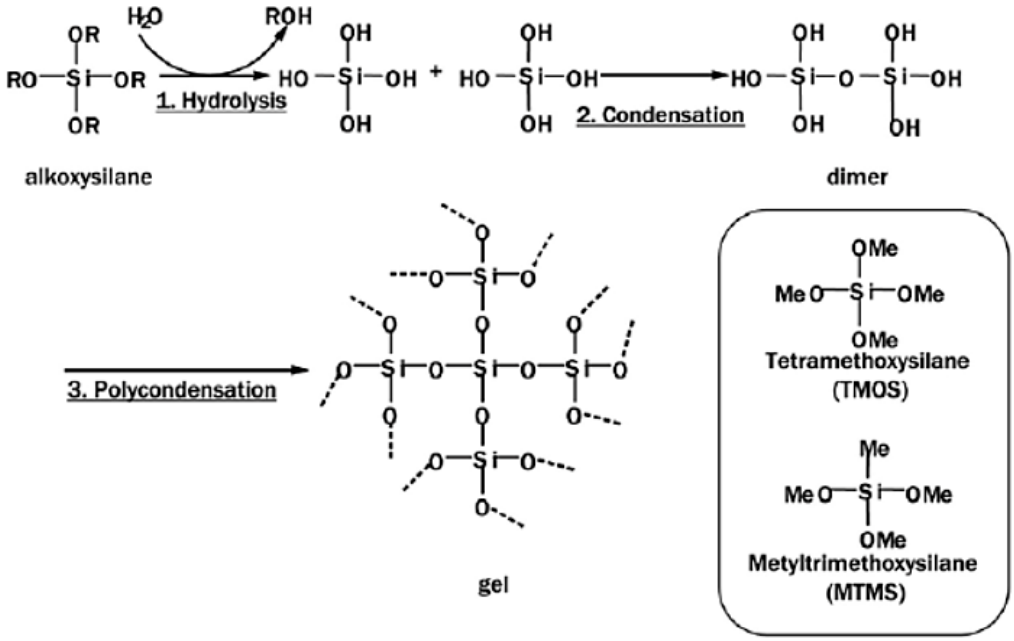

Fig. 2-27: monolith production via sol-gel reaction
(Kato et. al., 2005)

3. Experimental – Part 1

In this chapter the development of an optimal gel matrix as a basis for a new continuous purification process for monoclonal antibodies will be deduced in stages. All experiments were performed with of 10 x 10 cm.

3.1 Native Polyacrylamide Gel Electrophoresis

When performing a native PAGE, the proteins aren't denatured with SDS. Hence, the proteins can carry a net positive or a net negative charge. Because of that the electrophoresis were carried out both towards the anode and the cathode.

Furthermore, no protein ladder could be used since the protein ladder that was available in the lab is designed for SDS-PAGE and, therefore, denatured with SDS. Such ladders exhibit a differing mobility in comparison to native proteins. However, for reaching the goal of the experiments, a standardized ladder actually isn't required.

Additionally, also dyes like bromophenol blue were left aside when preparing the sample solutions to avoid a manipulation of the running behaviour of the analytes and not to endanger the native state of the protein. As a consequence of this it wasn't possible to monitor the proceeding of the electrophoresis with the naked eye which required an experimental and gradual approximation of the optimal runtime.

A rat monoclonal antibody, raised with IgG-depleted FCS, was the main analyte. The concentration of the antibody-stock-solution was 1.6 mg/ml. The supernatant of the cell culture where the antibody was raised dates back to 2009. Proteases and long-term storage may have reduced the amount and diversity of the ingredients due to decomposition.

Finally, the human plasma used for the following experiments stems from a male donor in his mid-twenties. Both supernatant and plasma served as impurities which are essential for the development of a purification process.

Standard operation procedure:

- Gel composition (amount for 3 gels, 1.0 mm):

final concentration [% PAA] in gel	**4**	**6**
30% AA [µl]	2.7	4
4x Gel Buffer pH 7.0 [ml]	5	5
H_2O [ml]	12.3	11
10% APS [µl]	75	75
TEMED [µl]	15	15
total amount [ml]	20.09	20.09

Tab. 3-1: native PAGE gel composition (Protaffin Biotechnologie AG)

- Gel Buffer 4x:
  250 mM TrisHCl
  pH 7.00

- Running Buffer 10x: (dilute to 1x before use)
  0.24 M TrisHCl
  1.92 M Glycine
  pH 7.00

- Gel Loading Buffer 2x:
  2x Gel Buffer
  20% Glycerol

- Staining Solution (Coomassie):
  1 g Coomassie Brilliant Blue R250
  500 ml MeOH
  400 ml dH_2O
  100 ml HAc

- Destaining Solution:
  500ml MeOH
  150ml HAc
  1350ml dH_2O

3.1.1 Trial 1 (native PAGE 6% towards anode – 1)

The aim of the first trials was to determine an optimally resolving gel concentration in combination with an adequate runtime. Based on the molecular weight of an IgG antibody of about 150 kDa, a low concentrated 6% PAA-gel was used for the first experiments. The parameters of trial 1 were defined as follows:
Runtime: 200 min
Voltage: 100V constant
Staining technique: Coomassie
The composition of the sample solutions can be taken from *Tab. 3-2*:

Slot	1	2	3	4
analyte	1.5 µg Ab (= 0.938 µl)	3 µg Ab (= 1.875 µl)	6 µg Ab (= 3.750 µl)	12.5 µl supernatant from cell culture
PBS	ad 10 µl	ad 10 µl	ad 10 µl	-
GLB 2x	10 µl	10 µl	10 µl	12.5 µl
total amount	20 µl	20 µl	20 µl	25 µl

Tab. 3-2: native PAGE 6% towards anode / cathode – 1 (TKU)

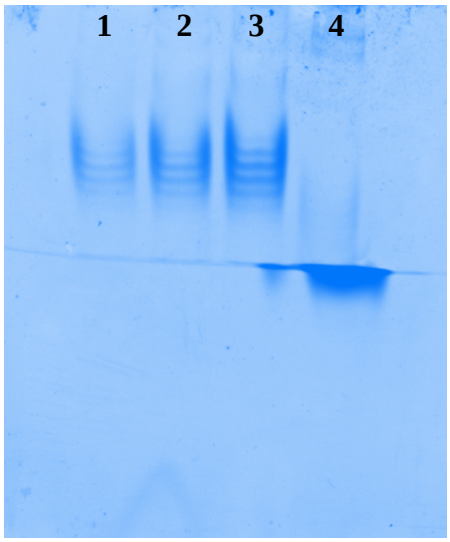

Fig. 3-1: native PAGE 6% towards anode -1 (TKU)

The scan of the stained gel (cf. *Fig 3-1*) shows that the run time was chosen too short. Thus, the analytes couldn't migrate further than to the middle of the gel.

The antibody split up into multiple bands (paths 1, 2, 3). Following the data sheet of the antibody-stock-solution, the protein was purified via protein G affinity chromatography, i.e. all of the bands should belong to the antibody itself and shouldn't represent any contamination. Maybe the multiple bands result from different isoforms of the antibody.

3.1.2 Trial 2 (native PAGE 6% towards cathode – 1)

The parameters and the composition of the sample solutions of this trial were the same as in trial 1. The only difference lies in the direction of the run (here towards cathode).

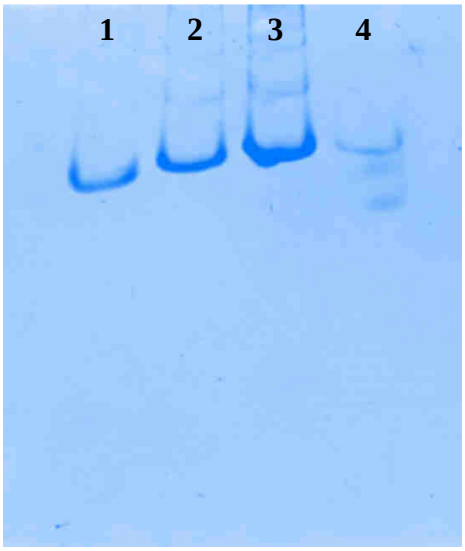

Fig. 3-2: native PAGE 6% towards cathode -1 (TKU)

In the paths of the two upper Ab-concentrations (2, 3), two weak bands are visible (cf. *Fig. 3-2*); maybe the antibody is contaminated.

Apart from that the antibody exhibits three thick and clearly separated single bands (1, 2, 3) in contrary to trial 1.

Additionally, there is no broad intensive band visible in path 4 of the supernatant like there was in trial 1 but we have one band on a level with the antibody bands of paths 1, 2 and 3. This could mean that there is some antibody in the

supernatant of the cell culture as well, but it could also mean that some sample spilled over from slot 3 while the sample solutions were applied.

3.1.3 Trial 3 (native PAGE 6% towards anode – 2)

Parameters for electrophoresis:

Runtime: ~ 300 min

Voltage: 100V constant

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 3-3*:

Slot	1	2	3	4	5	6	7
analyte	1.5 µg Ab (= 0.94 µl)	3 µg Ab (= 1.88 µl)	6 µg Ab (= 3.75 µl)	10 µl S	3 µg Ab + S ad 10 µl	10 µl P	3 µg Ab + P ad 10 µl
PBS	ad 10 µl	ad 10 µl	ad 10 µl	-	-	-	-
GLB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

Tab. 3-3: native PAGE 6% towards anode / cathode – 2 (TKU)

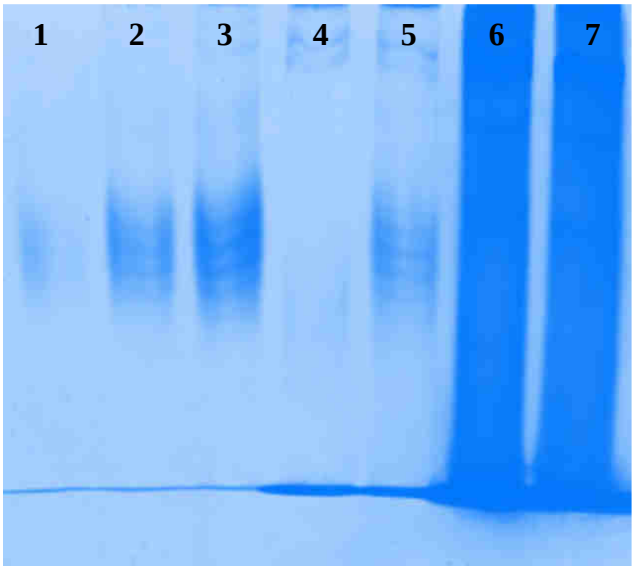

Fig. 3-3: native PAGE 6% towards anode – 2 (TKU)

The run was stopped due to an unexpected error of the power supplier caused by a minimal too high fill level (~2 mm) of the buffer in the electrophoresis chamber. It took some time until the error message was detected. During that time the run was stopped automatically by the device. Because of that the exact runtime can only be estimated.

Anyway, the pause in the run had no negative influence on the outcome of the experiment like it can be seen in *Fig. 3-3*. Again, there are multiple bands in the paths where the antibody was loaded (1, 2, 3, 5). Only in path 7 the antibody can't be seen because the concentration of the plasma was too high which resulted in two massive bands extending over the whole covered distance of the samples (6, 7). Like in trial 1, a continuous band is extending over the entire width of the gel. This band can't be seen when the electrophoresis is performed towards the cathode.

3.1.4 Trial 4 (native PAGE 6% towards cathode – 2)

Parameters for electrophoresis:

Runtime: 180 min

Voltage: 100V constant

Staining technique: Coomassie

Scheme of loaded samples: same as in trial 3, see *Tab. 3-3*

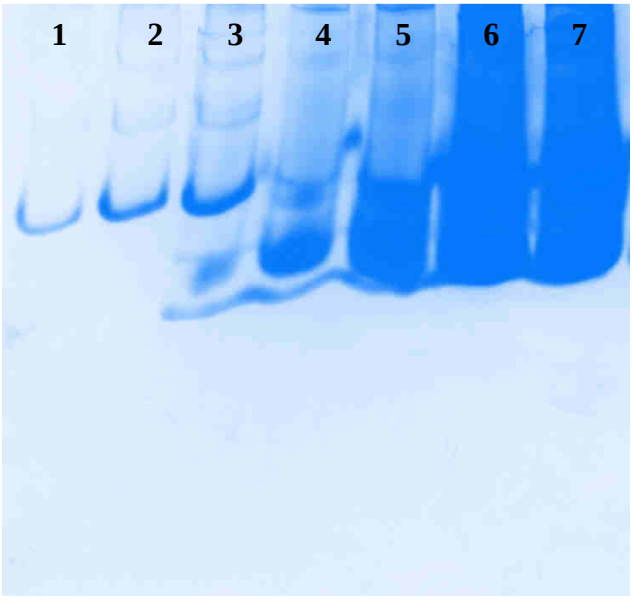

Fig. 3-4: native PAGE 6% towards cathode – 2 (TKU)

The run was stopped after 3 hours, because it was predictable that the plasma loading would be too high again. The samples for trials 3 and 4 were prepared simultaneously; therefore, the plasma concentration wasn't varied for this trial.

However, trial 4 was conducive to get a first impression of the plasma running behaviour when performing a native PAGE towards the cathode.

As anticipated, the plasma left two massive bands on the gel after the staining and destaining process. It seems that some sample spilled over from slot 4 to slot 3 while applying the sample solutions *(cf. Fig. 3-4)*.

3.1.5 Trial 5 (native PAGE 6% towards anode – 3)

Parameters for electrophoresis:

Runtime: 300 min

Voltage: 100V constant

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 3-4*:

Slot	1	2	3	4	5	6	7
analyte	1.5 µg Ab (= 0.94 µl)	3 µg Ab (= 1.88 µl)	6 µg Ab (= 3.75 µl)	5 µl S	3 µg Ab + 4.06 µl S	5 µl P	3 µg Ab + 4.06 µl P
PBS	ad 10 µl	ad 10 µl	ad 10 µl	5 µl	4.06 µl	5 µl	4.06 µl
GLB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

Tab. 3-4: native PAGE 6% towards anode / cathode – 3 (TKU)

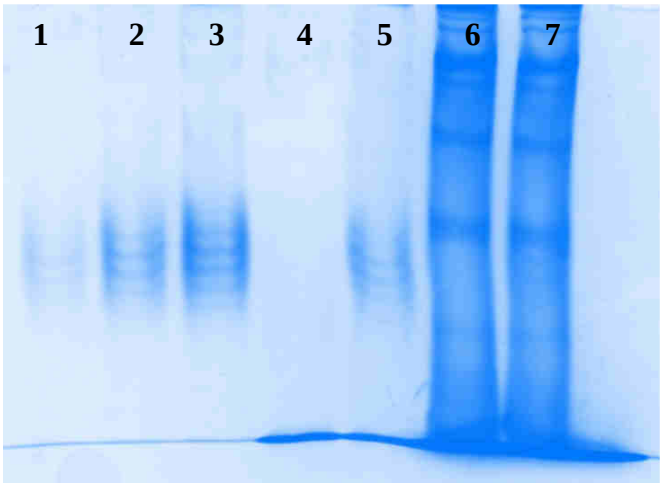

Fig. 3-5: native PAGE 6% towards anode – 3 (TKU)

The only difference from this trial to trial 3 is that this time only half of the amount of supernatant and plasma, respectively, were used in slots 4-7.

With these concentrations the plasma bands (6, 7) became more transparent, so that several sub-bands appeared (cf. *Tab. 3-5*). A further reduction of the plasma concentration would probably improve the result. In case of the supernatant, a higher concentration could be used again.

3.1.6 Trial 6 (native PAGE 6% towards cathode – 3)

The parameters and the composition of the sample solutions of this trial were the same as in trial 5.

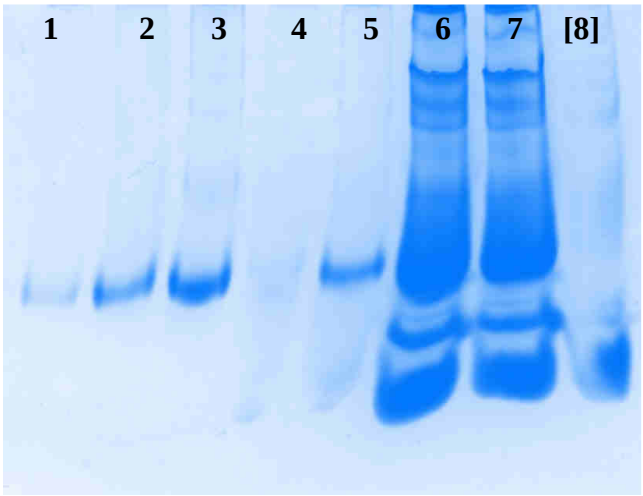

Fig. 3-6: native PAGE 6% towards cathode – 3 (TKU)

Now, also for the electrophoresis towards the cathode a runtime of 300 min was fixed for the first time; again with lower concentrations of the supernatant and the plasma, respectively, compared to trial 4.

A little bit of the plasma sample spilled over from slot 7 to slot 8 (cf. *Fig. 3-6*). A further reduction of the plasma concentration would probably improve the result.

3.1.7 Trial 7 (native PAGE 6% towards cathode – 4)

Parameters for electrophoresis:

Runtime: 360 min

Voltage: 100V constant

Staining technique: Coomassie

Slot	1	2	3	4	5	6	7
analyte	1.5 µg Ab (= 0.94 µl)	3 µg Ab (= 1.88 µl)	6 µg Ab (= 3.75 µl)	7.5 µl S	3 µg Ab + 6.09 µl S	2.5 µl P	3 µg Ab + 2.03 µl P
PBS	ad 10 µl	ad 10 µl	ad 10 µl	2.5 µl	2.03 µl	7.5 µl	6.09 µl
GLB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

Tab. 3-5: native PAGE 6% towards cathode – 4 (TKU)

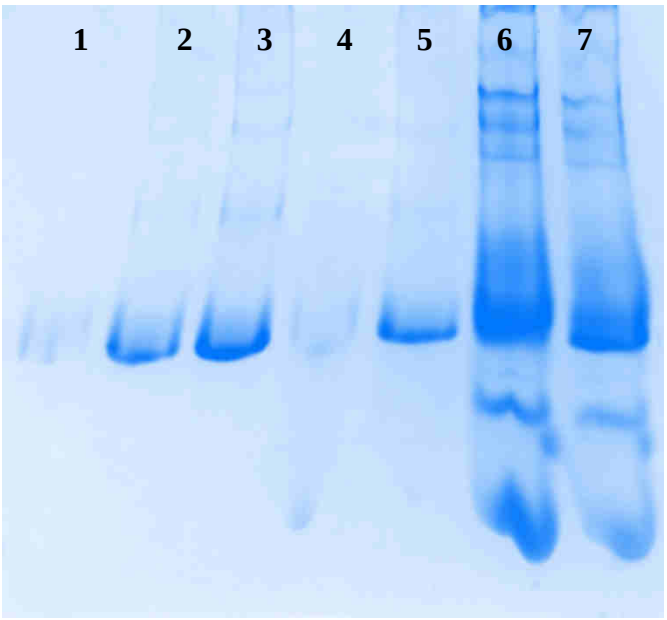

Fig. 3-7: native PAGE 6% towards cathode – 4 (TKU)

The composition of the sample solutions can be taken from *Tab. 3-5*; the corresponding gel to this trial is depicted in *Fig. 3-7*.

For this trial a runtime over 6 hours was tested. The concentration of the plasma in slots 6 and 7 was halved relating to trials 5 and 6. The concentration of the supernatant in slots 4 and 5, however, was chosen to be half as much again.

3.1.8 Trial 8 (native PAGE 4% towards anode)

Parameters for electrophoresis:

Runtime: 260 min

Voltage: 100V constant

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 3-6*:

Slot	1	2	3	4	5	6	7
analyte	1.5 µg Ab (= 0.94 µl)	3 µg Ab (= 1.88 µl)	6 µg Ab (= 3.75 µl)	7.5 µl S	3 µg Ab + 7.5 µl S	2.5 µl P	3 µg Ab + 2.5 µl P
PBS	ad 10 µl	ad 10 µl	ad 10 µl	2.5 µl	0.625 µl	7.5 µl	5.625 µl
GLB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

Tab. 3-6: native PAGE 4% towards anode / cathode (TKU)

By decreasing the PAA-gel concentration to 4% the runtime could be reduced for approximately 40 min in comparison to trial 5. However, such low concentrated PAA-gels are difficult to handle in the course of the staining/destaining process and during the

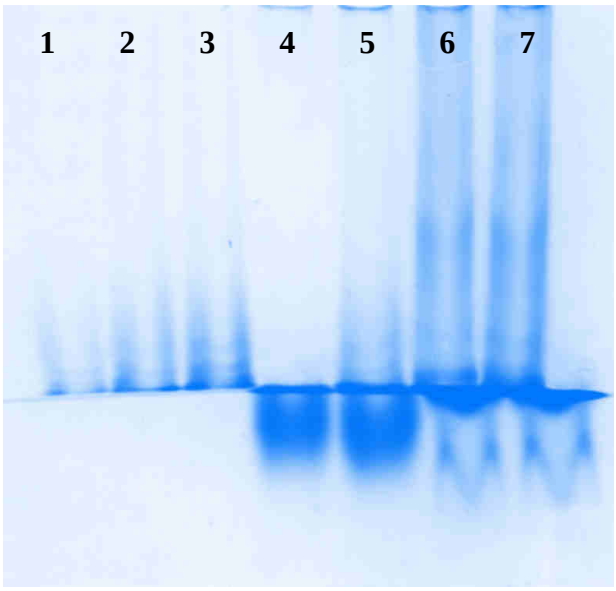

Fig. 3-8: native PAGE 4% towards anode (TKU)

working steps of the gel documentation, respectively. They tend to tear when being pulled over. Additionally, the separation capacity of the gel is reduced significantly what can be seen when regarding paths 1, 2 and 3 in *Fig. 3-8* in comparison to trial 5: the multiple bands in those paths, probably belonging to different isoforms of the antibody, became rather diffuse.

Furthermore, the continuous band that is extending over the entire width of the gel and that could be seen in all of the previous trials performed towards the anode now isn't located at the end of the migration distance of the analytes anymore; in fact, under this continuous band the other bands are proceeding in paths 4 – 7, in case of paths 6 and 7 the proceeded bands are even shifted to the right.

3.1.9 Trial 9 (native PAGE 4% towards cathode)

The parameters and the composition of the sample solutions of this trial were the same as in trial 8.

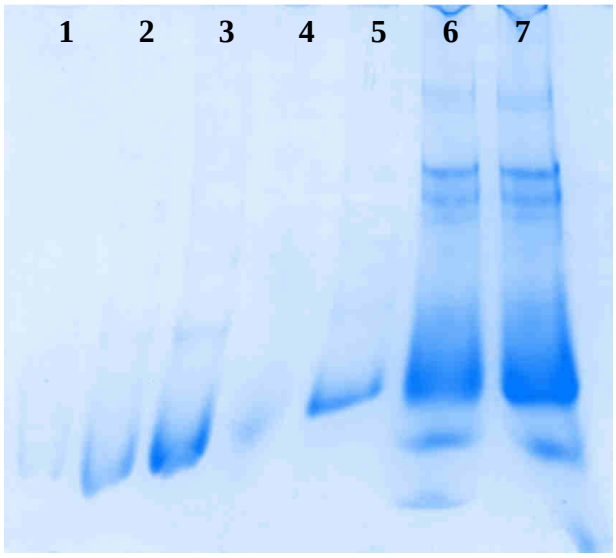

Fig. 3-9: native PAGE 4% towards cathode (TKU)

Like in trial 8, the runtime could be reduced in comparison to trials 6 and 7 when regarding the covered distance of the analytes in the gel. On the contrary to trial 8, this time the separation capacity isn't reduced compared to the experiments based on a higher-concentrated PAA-gel.

Merely, the bands don't lead straight through the gel (cf. *Fig. 3-9*), but this effect also occurred with higher concentrations of PAA, albeit less distinctive.

3.1.10 Possible Further Proceeding

Concerning native PAGE, the following proceeding would be possible: It could be tested, whether higher-concentrated PAA-gels (e.g. 10%) significantly affect the runtime in a negative way concerning the covered distance on the gel of the migrated analytes. An advantage of higher-concentrated PAA-gels would be that they are more stable and easier to handle when being pulled over.

Furthermore, different pH-values of the buffer solutions (gel-, running-, gel loading buffer) could be tested. Maybe the net charge of the mAb could be thusly affected in a positive way to receive sharper bands on the gel. By all means it has to be considered that harsh pH-values can denature proteins as well. Isoelectric focusing could be used to determine the exact isoelectric point of the provided mAb. Probably there is a relation between the optimal pH-value and the IP of the protein.

Finally, the developed native PAGE system could be adapted for protein standards as analytes. Those protein standards are available on the market as ready-to-use mixtures for HPLC-analysis.

However, it has to be considered that problems may occur when it comes to extracting the proteins from the PAA-gel matrix for recovery. Proteins that are cleaned via native PAGE could only be used for analytic purposes; on no account they would be suitable for therapeutic use.

3.2 SDS-PAGE

When using this method, all proteins are denatured by adding SDS to the sample solutions and by heating them up to 95 °C for 5 min. Because of the SDS, all proteins carry a net negative charge and therefore the experiments are all performed towards the anode. In comparison to native PAGE, SDS-PAGE leads to sharper bands of the migrated analytes on the stained gel and thus holds a greater potential for analytical purposes; for preparative work, however, SDS-PAGE isn't suitable. Anyhow, this method was used to receive a general impression about the differences concerning the running behaviour of the analytes in

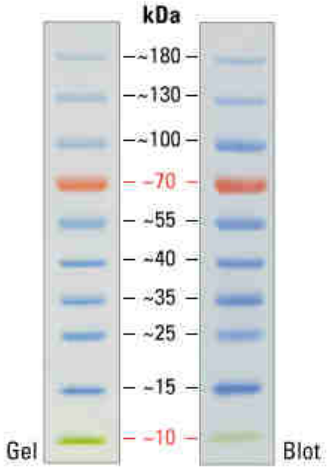

Fig. 3-10: band profile of the Page Ruler™ Prestained Protein Ladder for SDS-PAGE (www.thermofisher.com)

relation to the native PAGE method. This time, a prestained protein ladder was available. The band profile of this ladder can be taken from *Fig. 3-10*.

It has to be considered that some of the sample solutions may evaporate when it comes to heating – hence, it is advisable to prepare the sample solutions in abundance (i.e. 25 µl when 20 µl should be applied).

Standard operation procedure:

- Running gel (amount for 3 gels, 1mm):

final concentration [% PAA] in gel	**6**	**10**	**12**
30% AA [µl]	3	4.5	6
4x Gel Buffer, pH 8.8 [ml]	3.75	3.75	3.75
H_2O [ml]	8.25	6.75	5.25
10% APS [µl]	50	50	50
TEMED [µl]	10	10	10
total amount [ml]	15.06	15.06	15.06

Tab. 3-7: composition of the running gel for SDS-PAGE (Protaffin Biotechnologie AG)

- Stacking gel (amount for 3 gels, 1mm):
  (always the same concentration, independent of the composition of the running gel)

final concentration [% PAA] in gel	**3.9**
30% AA [µl]	650
4x Gel Buffer, pH 6.8 [ml]	1.25
H_2O [ml]	3.05
10% APS [µl]	25
TEMED [µl]	5
total amount [ml]	4.98

Tab. 3-8: composition of the stacking gel for SDS-PAGE (Protaffin Biotechnologie AG)

- Tris/Glycine Running Buffer 10x: (dilute to 1x before use)

  TrisHCl 29 g

  Glycine 144 g

  SDS 10 g

  dH_2O ad 1000 ml

The composition of the Gel Buffer can be taken from the previous chapter *Native Polyacrylamide Gel Electrophoresis*.

- Final Sample Buffer 2x:

Tris buffer, pH 6.8 (pre-cooled, 4°C)	10 ml
Glycerol	8 ml
SDS	1.6 g
β-Mercaptoethanol	2 ml
0.1% Bromophenolblue solution	2 ml
dH_2O	18 ml

3.2.1 Trial 10 (SDS-PAGE 6% Coomassie Staining)

Parameters for electrophoresis:

Runtime: 100 min

Voltage: 10 mA (constant) for 10 min, then 20 mA (constant) for 90 min

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 3-9*:

Slot	1	2	3	4	5	6	7
analyte	0.375 µg Ab (= 0.234 µl)	0.75 µg Ab (= 0.469 µl)	1.5 µg Ab (= 0.938 µl)	12.5 µl S	12.5 µl P	0.75 µg Ab + P ad 10 µl	1 µl L for CS 0.5 µl for SiSt
PBS	ad 10 µl	ad 10 µl	ad 10 µl	-	-	-	-
FSB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	-
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	1 µl for CS 0.5 µl for SiSt

Tab. 3-9: SDS-PAGE 6% Coomassie-/Silver Staining (TKU)

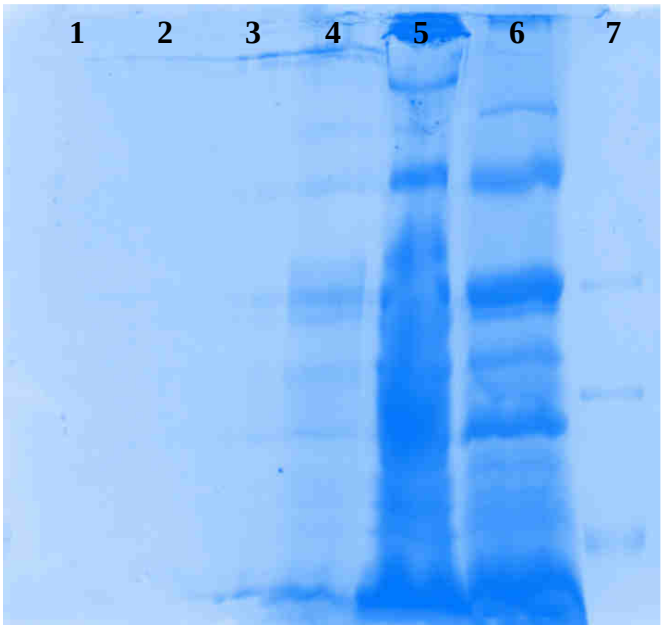

Fig. 3-11: SDS-PAGE 6% Coomassie Staining (TKU)

Only three out of ten ladder bands are visible on the Coomassie-stained gel (cf. *Fig. 3-11*).

The pure plasma was applied in a too high concentration (5). The antibody, however, is hardly visible. There is only a thin band in the upper region of the three Ab-paths (1, 2, 3). Maybe some of the antibody agglomerated during the denaturing process.

Altogether, this gel isn't meaningful; let's wait for the result of the silver staining trial.

3.2.2 Trial 11 (SDS-PAGE 6% Silver Staining)

The composition of the sample solutions can be taken from *Tab. 3-9*. The other parameters were as follows:

Runtime: 85 min

Voltage: 10 mA (constant) for 10 min, then 20 mA (constant) for 75 min

Staining technique: Silver Staining

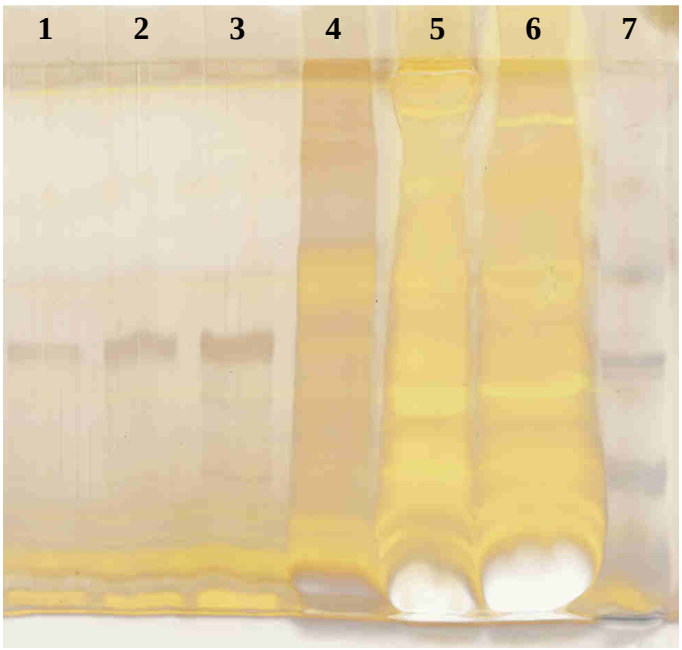

Fig. 3-12: SDS-PAGE 6% Silver Staining (TKU)

Silver Staining is a much more sensitive staining method than Coomassie Staining; this can be proved when taking a glance on the gel left-hand in *Fig. 3-12*. Although the same amount of analytes had been applied compared to trial 10, much more bands were visualized now.

Again, the ladder didn't run well on the gel, maybe it depends on the PAA-concentration, or maybe some other parameters like the running buffer bear the blame for that. It could be possible that the buffer was used too many times and hence the ions became exhausted. On all accounts, the plasma concentration was too high. There are many bands visible in the paths of the antibody (1, 2, 3). Some amount of the Ab really seems to be agglomerated, otherwise the bands in the upper region of paths 1, 2 and 3 can't be explained. It also seems that the antibody decayed into smaller fragments (see bands in paths 1, 2 and 3 at the bottom of the gel). In any case, some further experiments have to be conducted.

3.2.3 Trial 12 (SDS-PAGE 10% Coomassie Staining)

Parameters for electrophoresis:

Runtime: 75 min

Voltage: 5 mA (constant) for 10 min, then 10 mA (constant) for 65 min

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 3-10*:

Slot	1	2	3	4	5	6	7	8
analyte	0.375 µg Ab (= 0.234 µl)	0.75 µg Ab (= 0.469 µl)	1.5 µg Ab (= 0.938 µl)	5 µl S	0.75 µg Ab + 4.77 µl S	5 µl P	0.75 µg Ab + 4.77 µl P	1 µl L
PBS	ad 10 µl	ad 10 µl	ad 10 µl	5 µl	4.77 µl	5 µl	4.77 µl	-
FSB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	-
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	1 µl

Tab. 3-10: SDS-PAGE 10% Coomassie Staining (TKU)

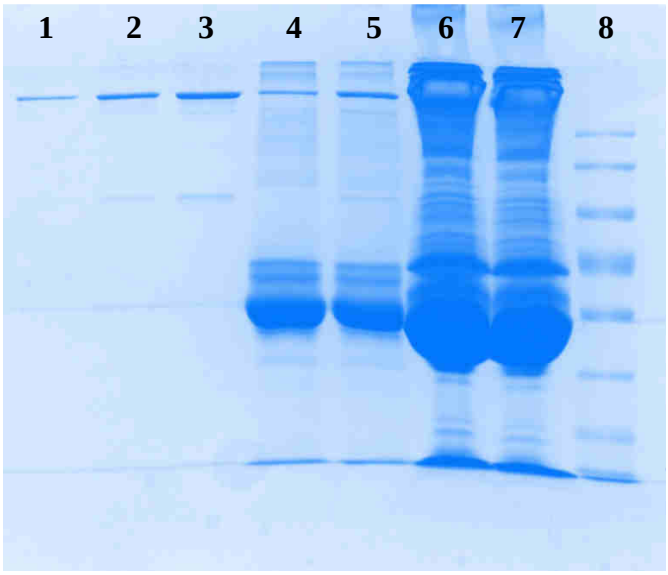

Fig. 3-13: SDS-PAGE 10% Coomassie Staining (TKU)

This time the SDS-PAGE worked far better. Only half as much of the supernatant and the plasma had been applied for this trial. The bands are sharper and even 9 bands of the ladder are visible (cf. *Fig. 3-13*).

Again, it seems that some amount of the antibody got agglomerated. Finally it has to be mentioned, that the plasma bands are still rather intensive so that the concentration of the plasma could be further reduced.

3.2.4 Trial 13 (SDS-PAGE 10% Silver Staining)

Parameters for electrophoresis:

Runtime: 75 min

Voltage: 5 mA (constant) for 10 min, then 10 mA (constant) for 65 min

Staining technique: Silver Staining

The composition of the sample solutions can be taken from *Tab. 3-11*:

Slot	1	2	3	4	5	6	7	8	9
analyte	0.375 µg Ab (= 0.234 µl)	0.75 µg Ab (= 0.469 µl)	1.5 µg Ab (= 0.938 µl)	5 µl S	0.75 µg Ab + 4.77 µl S	5 µl P	0.75 µg Ab + 4.77 µl P	0.5 µl L	1.5 µl L
PBS	ad 10 µl	ad 10 µl	ad 10 µl	5 µl	4.77 µl	5 µl	4.77 µl	-	-
FSB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	-	-
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	0.5 µl	1.5 µl

Tab. 3-11: SDS-PAGE 10% Silver Staining (TKU)

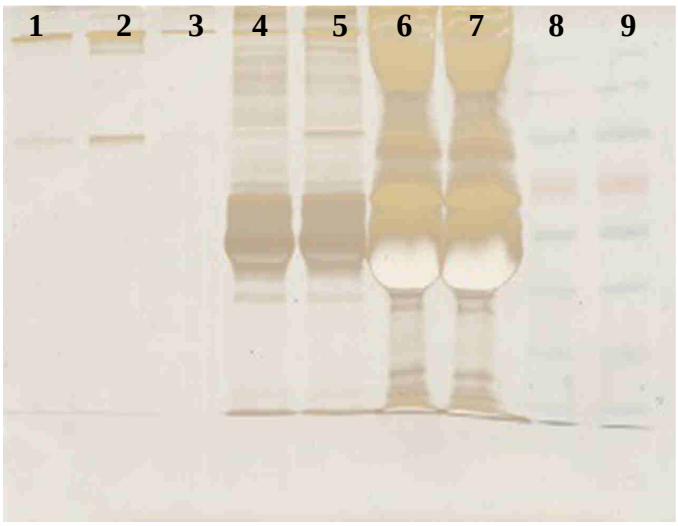

Fig. 3-14: SDS-PAGE 10% Silver Staining (TKU)

The stacking gel got detached during the staining process. Therefore, it is missing in *Fig. 3-14*. Two concentrations of the ladder had been applied to check whether a too low amount of the ladder was the reason why not all of the 10 bands could be made visible in the previous experiments; but again, only 9 bands of the ladder can be seen on the stained gel. The staining time, however, should have been longer in order to receive darker bands with a better contrast to the gel.

3.2.5 Trial 14 (SDS-PAGE 15% Coomassie Staining)

Runtime: 55 min

Voltage: 10 mA (constant) for 10 min, then 20 mA (constant) for 45 min

Staining technique: Coomassie

Slot	1	2	3	4	5	6	7
analyte	1.5 µg Ab (= 0.938 µl)	3 µg Ab (= 1.875 µl)	4 µl S	1.5 µg Ab + 4 µl S	2.5 µl P	1.5 µg Ab + 2.5 µl P	1 µl L for CS 0.5 µl for SiSt
PBS	ad 10 µl	ad 10 µl	6 µl	5.06 µl	7.5 µl	6.56 µl	-
FSB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	-
total amount	20 µl	20 µl	20 µl	20µl	20 µl	20 µl	1 µl for CS 0.5 µl for SiSt

Tab. 3-12: SDS-PAGE 15% Coomassie-/Silver Staining (TKU)

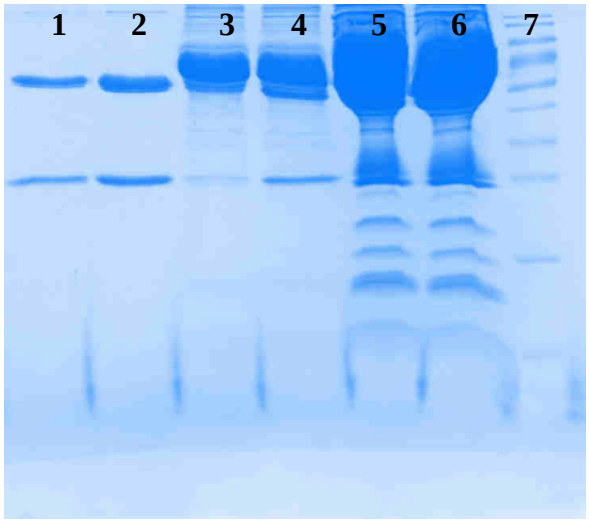

Fig. 3-15: SDS-PAGE 15% Coomassie Staining (TKU)

The composition of the sample solutions can be taken from *Tab. 3-12*. The stacking gel is missing in *Fig. 3-15*. This time all bands of the ladder could be made visible. Between all paths vertical bands can be seen in the lower half of the gel.

3.2.6 Trial 15 (SDS-PAGE 15% Silver Staining)

Parameters for electrophoresis:

Runtime: 55 min

Voltage: 10 mA (constant) for 10 min, then 20 mA (constant) for 45 min

Staining technique: Silver Staining

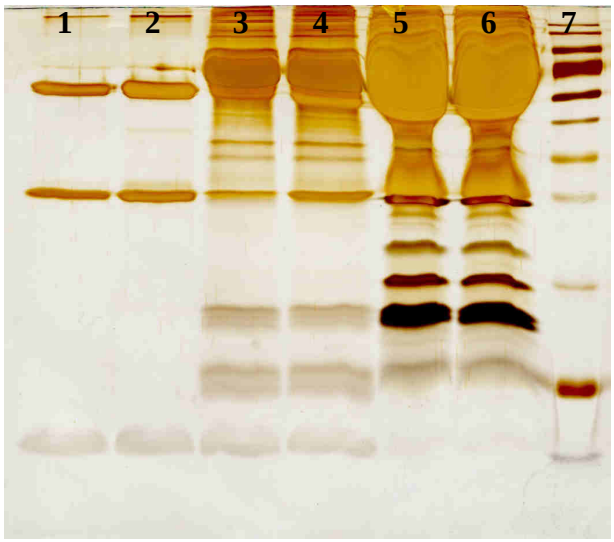

Fig. 3-16: SDS-PAGE 15% Silver Staining (TKU)

The composition of the sample solution was the same as in trial 14. The gel from this trial is depicted in *Fig. 3-16*.

Again, all bands of the ladder could be made visible. The band at the bottom of path 7 belongs to the dye front. The colour of the bands indicates the amount of analyte on the gel. Dark bands indicate a small amount; light brown bands indicate large amounts of the analyte.

3.2.7 Possible Further Proceeding:

The SDS- PAGE system could be used – in addition to native PAGE – for the characterization of the running behaviour of protein standards as analytes. Those protein standards are available on the market as ready-to-use mixtures for HPLC-analysis.

3.3 Native Agarose Gel Electrophoresis – 1

Due to the toxicity of acrylamide, agarose gels served as matrices for the separation process in the following trials.

The agarose gels were produced by heating specific amounts of agarose in an aqueous buffer solution. The gels were casted immediately when the solution was still very hot because agarose gel mixtures solidify very fast.

Agarose gels have large pores and are therefore primarily used for analytical and preparative purposes when working with nucleic acids. Usually, agarose gel electrophoresis is performed horizontally on thick gels – but, since the prototype with whom the continuous purification process shall be finally performed is designed for gels with a thickness of only 1 mm, it has to be tested whether such thin agarose gels are stable

enough making the approach with this gel matrix possible. On all accounts it has to be considered that agarose gels cannot be silver stained.

Standard operation procedure:

- Gel composition (amount for 3 gels, 1.0 mm):

final concentration [%] in gel	**1.2**	**1.4**	**1.6**	**2.0**
Agarose [g]	0.3	0.35	0.4	0.5
Gel Buffer A 1x [ml]	24.7	24.65	24.6	24.5
total amount [ml]	25	25	25	25

Tab. 3-13: agarose gel composition (TKU)

- Gel Buffer A / Running Buffer 10x: (dilute to 1x before use)
  0.24 M TrisHCl
  1.92 M Glycine
  pH 7.00

- Gel Buffer B 4x:
  250 mM TrisHCl
  pH 7.00

- Gel Loading Buffer 2x:
  2x Gel Buffer B
  20% Glycerol

3.3.1 Trial 16 (horizontal agarose gel electrophoresis 1.2%)

The composition of the sample solutions of this trial were the same as in trial 8 (cf. *Tab. 3-6*). Again, three different concentrations of the antibody were used; additionally, supernatant- and plasma solutions were applied – once, each of them pure and once, in separated slots, mixed with antibody. The casted gel was 7 mm thick. The other parameters were defined as follows:

Runtime: 60 min
Voltage: 100 V
Staining technique: Coomassie

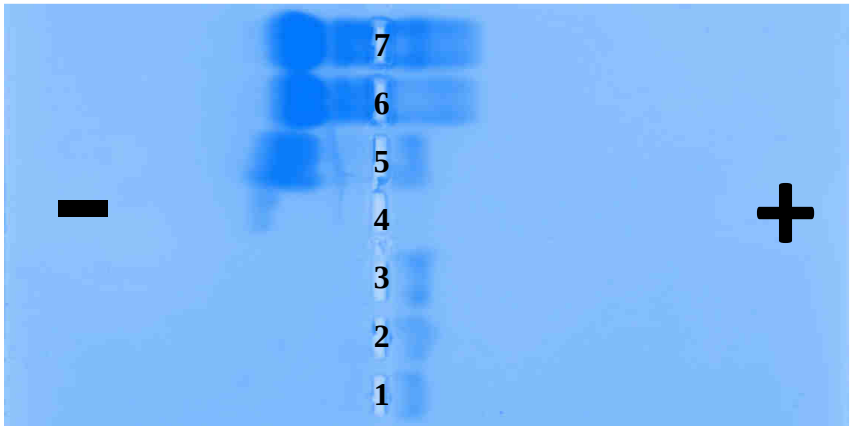

Fig. 3-17: horizontal agarose gel electrophoresis 1.2% (TKU)

The first agarose gel trial was performed 'traditionally' on a thick horizontal gel like it is usual for this type of electrophoresis. The sample solutions were applied approximately in the middle of the gel, so that the run was performed simultaneously both towards the anode and the cathode. This trial was used to get a first impression whether the analytes are suitable for an agarose gel matrix.

Slots 1, 2 and 3 were filled with antibody solutions in three different concentrations (1.5 µg, 3 µg, 6 µg). In *Fig. 3-17* it can be seen that the antibody only migrated towards the cathode (+).

Slot 4 was filled with 7.5 µl supernatant, in slot 5 a combination of supernatant and antibody was applied; in slot 6 there was pure plasma and slot 7 was filled with a mixture of plasma and antibody. It seems that some of the sample solution spilled over from slot 6 to slot 5; otherwise the intensive band on the anode side in slot 5 can't be explained, because this band isn't visible in slot 4, where the pure supernatant was applied.

Since the antibody only migrated towards the cathode a first purification could be reached by performing the run into the appropriate direction towards the positive pole.

3.3.2 Trial 17 (vertical agarose gel electrophoresis 1.2% towards anode - 1)

The next trials were used to test how an agarose gel electrophoresis works when the gel is only 1 mm thick. The parameters and the composition of the sample solutions of this trial were the same as in trial 16. The gel belonging to this trial is depicted in *Fig. 3-18*.

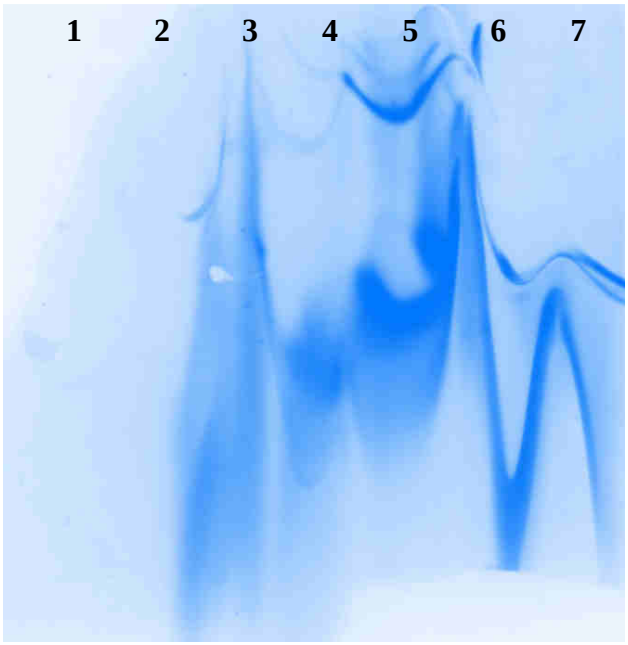

Fig. 3-18: vertical agarose gel electrophoresis 1.2% towards anode – 1 (TKU)

The first problems occurred when the sample solutions were applied because the slots were connected by thin arachnoid agarose membranes that hindered the sample solutions from sinking down in the slots; because of that the sample solutions spread to the neighbouring slots which resulted in blurred bands. In path three a bubble can be seen on the gel in form of a white spot. The left upper and the right lower edge of the gel are missing in *Fig. 3-18*; the gel was so thin, that those parts broke away although the gel was handled with caution. Like in trial 16, the antibody didn't migrate into the gel. The band in path 3 surely stems from the sample solution of path 4.

3.3.3 Trial 18 (vertical agarose gel electrophoresis 1.2% towards anode - 2)

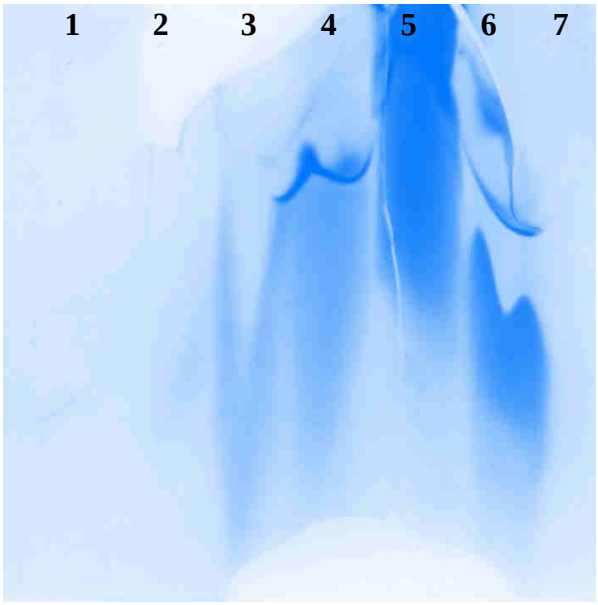

Fig. 3-19: vertical agarose gel electrophoresis 1.2% towards anode – 2 (TKU)

This trial was exactly the same like trial 17. Trials 17 and 18 were performed simultaneously. Actually, it was planned to perform this run towards the cathode, but the cables were plugged in the wrong way. So, both trials were performed towards the anode. Again, parts of the gel broke away and again the sample solutions spread to the neighbouring slots which resulted in blurred bands (cf. *Fig. 3-19*). The conclusion of trials 17 and 18 is that agarose gels with a concentration of only 1.2% are too thin and therefore prone to tearing.

3.3.4 Trial 19 (vertical agarose gel electrophoresis 2% towards anode)

Parameters for electrophoresis:

Runtime: 120 min / Voltage: 100 V / Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 3-14*:

Slot	1	2	3	4	5	6
analyte	3 µg Ab (= 1.88 µl)	6 µg Ab (= 3.75 µl)	7.5 µl S	3 µg Ab + 7.5 µl S	2.5 µl P	3 µg Ab + 2.5 µl P
PBS	ad 10 µl	ad 10 µl	2.5 µl	0.625 µl	7.5 µl	5.625 µl
GLB 2x	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

Tab. 3-14: vertical agarose gel electrophoresis 2% towards anode / cathode (TKU)

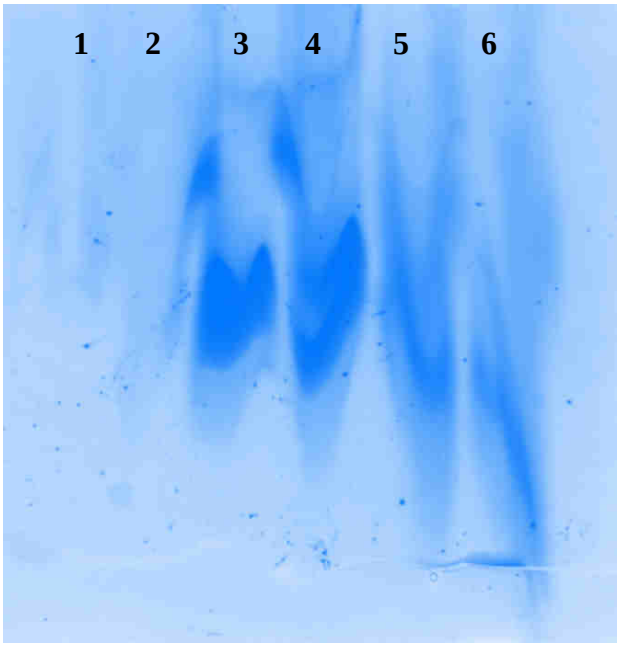

Fig. 3-20: vertical agarose gel electrophoresis 2% towards anode (TKU)

The concentration of agarose was raised to 2%, which lead to problems when the gel was casted because the agarose-buffer-solution gelatinized really fast; sometimes the solution didn't even reach the bottom of the glass plates before it solidified. It took serveral attempts until a usable gel could be created. In any case, the glass plates have to be cleaned with alcohol before the casting process to reduce bubble formation.

For this trial only two different concentrations of pure antibody solutions were applied. The thin arachnoid agarose membranes that connected the slots in the same way like it was described in the previous trials were removed by means of a pipette tip before the sample solutions were applied. It didn't succeed completely and hence some of the sample solutions again spread to the neighbouring slots which can be seen in *Fig. 3-20* by means of the blurred paths. The problem with the connected slots would be completely overcome when using the final prototype because then the feed would be applied continuously and therefore no slots would be needed at all. Although the agarose concentration was rather high the lower part of the gel broke away in course of the staining process. Agarose gels seem to be rather fracturable when casted very thin, e.g. with a thickness of only 1 mm.

3.3.5 Trial 20 (vertical agarose gel electrophoresis 2% towards cathode)

Fig. 3-21: vertical agarose gel electrophoresis 2% towards cathode (TKU)

The parameters and the composition of the sample solutions of this trial were the same as in trial 19. This time the thin arachnoid membranes could be removed from the slots. It seems that it needs some practice and a good eye to achieve this goal.

The separation itself worked well. All bands are clearly separated from each other (cf. *Fig. 3-21*) which means that none of the sample solution spilled over to the neighbouring slots. This time also the gel didn't get damaged during the single steps of the workflow.

3.3.6 Trial 21 (vertical agarose gel electrophoresis 1.6% towards anode)

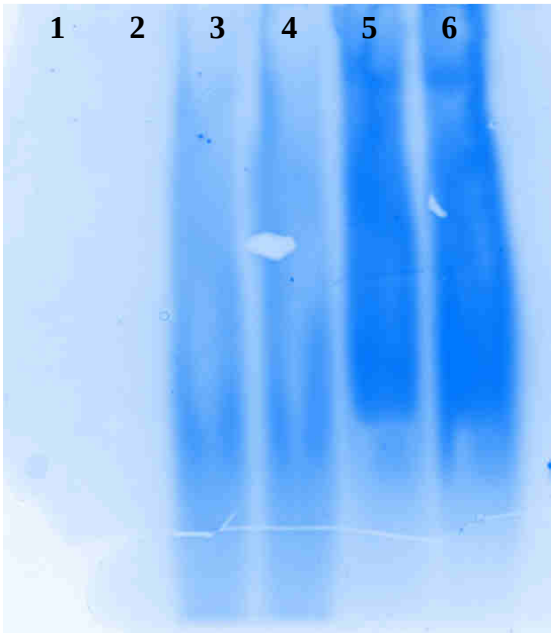

Fig. 3-22: vertical agarose gel electrophoresis 1.6% towards anode (TKU)

Parameters for electrophoresis:

Runtime: 107 min

Voltage: 100 V

Staining technique: Coomassie

Since the 2% gel was hard to cast, the agarose concentration was reduced to 1.6%. With this lowered concentration the robustness of the gel decreased too. Again, parts of the gel were torn away during the workflow. Also a bubble can be seen both in paths 4 and 6 in *Fig. 3-22*. The bands are clearly separated from each other.

3.3.7 Trial 22 (vertical agarose gel electrophoresis 1.6% towards cathode)

Fig. 3-23: vertical agarose gel electrophoresis 1.6% towards cathode (TKU)

A vertical crack in the gel can be seen between paths 2 and 3 in *Fig. 3-23*; beginning from the lower edge of the gel, rising upwards and drifting to the left.

There are two bubbles, each one in path 5 and 6, respectively. The separation process however worked pretty well.

Also with this concentration of agarose the casting of the gel turned out to be problematical as the solution was still too viscous.

3.3.8 Trial 23 (vertical agarose gel electrophoresis 1.4% towards anode)

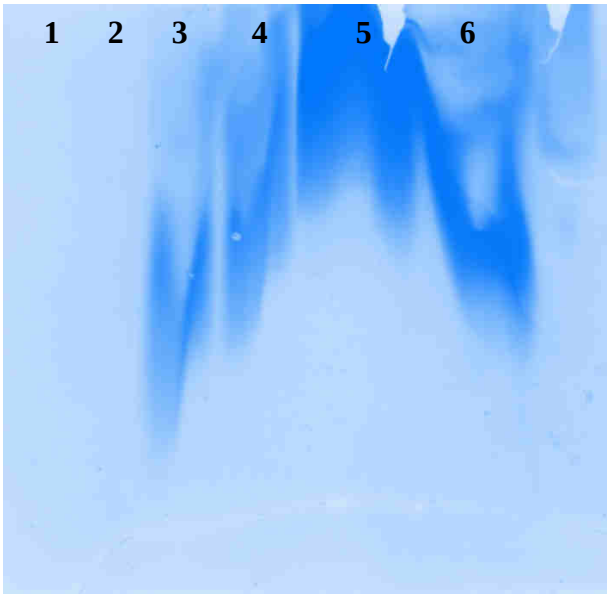

Fig. 3-24: vertical agarose gel electrophoresis 1.4% towards anode (TKU)

Parameters for electrophoresis:

Runtime: 90 min

Voltage: 100 V

Staining technique: Coomassie

The concentration of agarose was reduced again to 1.4%. This concentration was too low to obtain a stable gel. When removing the comb some teeth of the gel broke away and when the thin membranes inside the slots were removed some further teeth got damaged. Beginning from slot 5 the sample solutions spread over several slots resulting in two blurred and broad bands which can be seen in *Fig. 3-24*.

3.3.9 Trial 24 (vertical agarose gel electrophoresis 1.4% towards cathode)

Fig. 3-25: vertical agarose gel electrophoresis 1.4% towards cathode (TKU)

Parameters for electrophoresis:

Runtime: 150 min

Voltage: 100 V

Staining technique: Coomassie

The gel belonging to this trial is depicted in *Fig. 3-25*. All of the problems from the previous trials in this chapter occurred again.

As concluding remark it can be summarized that a separation process based on an agarose gel would be possible. The agarose gel itself, however, must be modified in some way, to make it more stable and better castable.

3.4 Native Agarose / PEG – Gel Electrophoresis

To obtain agarose gels with a higher robustness and a better castability PEG 400 was added to the agarose gel mixture.

Standard operation procedure:

- Gel composition (amount for 3 gels, 1.0 mm):

final concentration [% Agarose / % PEG] in gel	**1.5 / 10**	**1.8 / 10**
Agarose [g]	0.38	0.45
PEG 400 [ml]	2.5	2.5
Gel Buffer A 1x [ml]	22.12	22.05
total amount [ml]	25	25

Tab. 3-15: agarose / PEG 400 – gel composition (TKU)

- Gel Buffer A / Running Buffer 10x: (dilute to 1x before use)

  0.24 M TrisHCl

  1.92 M Glycine

  pH 7.00

The composition of the gel loading buffer can be taken from chapter 3.3.

3.4.1 Trial 25 (casting an 1.8% agarose / 10% PEG 400 gel – 1)

As a first approach a concentration of 1.8% agarose and 10% PEG 400 was tested to prove whether the viscosity of a highly concentrated agarose solution can be reduced by adding a low molecular PEG (MW 400) with a liquid state of matter.

The yellow marked parts in the figure below indicate gel-free areas. The hot solution solidified so fast during the casting process that it didn't even reach the ground of the casting stand. The gel ends under the upper level of the glass plates because the casting process was interrupted since the large gel-free parts at the bottom prevented any further use of the gel.

Fig. 3-26: casted 1.8% agarose / 10% PEG 400 gel – 1 (TKU)

3.4.2 Trial 26 (1.5% agarose / 10% PEG 400 gel electrophoresis towards cathode)

Parameters for electrophoresis:

Runtime: 180 min

Voltage: 100 V

Staining technique: Coomassie

To reduce the viscosity of the heated gel-solution the concentration of agarose was reduced to 1.5%. The figure below shows the casted gel, air bubbles are marked with yellow colour again.

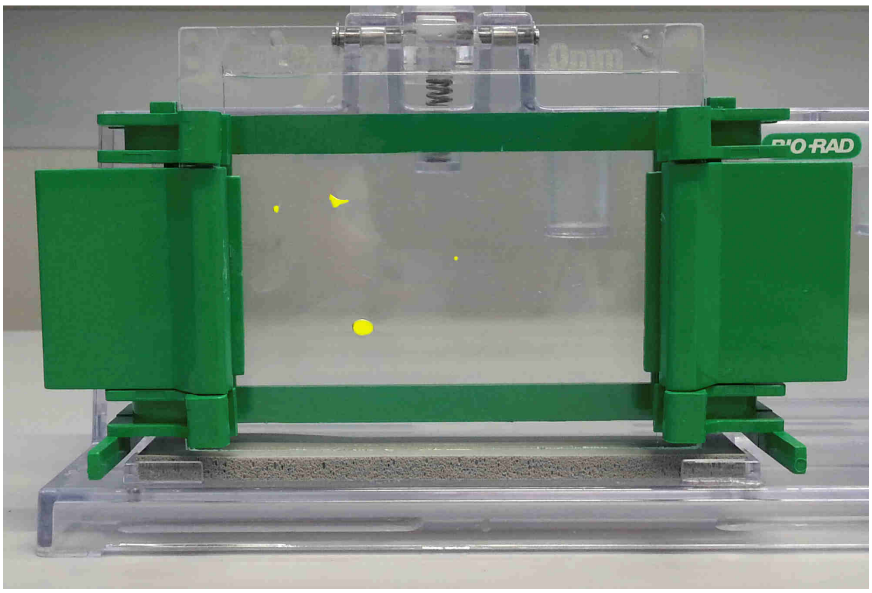

Fig. 3-27: casted 1.5% agarose / 10% PEG 400 gel (TKU)

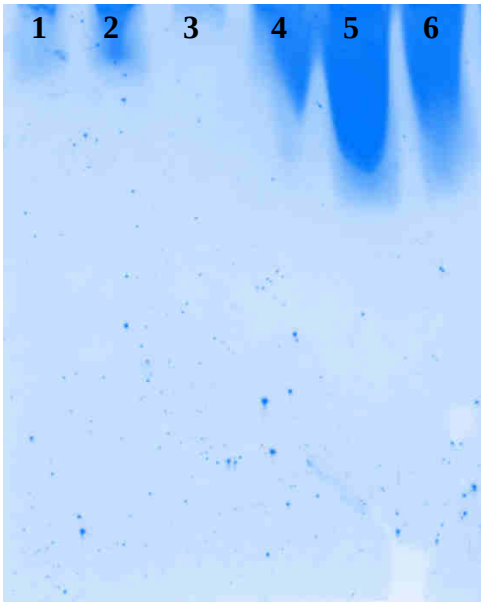

Fig. 3-28: agarose 1.5% / PEG 400 10% gel electrophoresis towards cathode (TKU)

This time it was possible to cast a useable gel but only one out of 4 casted gels was good enough to perform an electrophoresis; the other gels were too aerated.

The electrophoresis was performed towards the cathode because that's the pole to whom the antibody migrated in the trials that were performed with pure agarose.

The blue spots on the gel in *Fig. 3-28* could stem from PEG that was maybe poorly distributed in the solution. Altogether, this gel wasn't better to handle than pure agarose gels.

3.4.3 Trial 27 (casting an 1.8% agarose / 10% PEG 400 gel – 2)

The difference from this trial to trial 25 is that now pure water was used instead of Gel Buffer A 1x (cf. *Tab. 3-15*). By doing so the gel became castable (cf. *Fig. 3-26*, *Fig. 3-29*) and its elasticity was really good when it was removed from the glass plates. Apparently the ions of the buffer solution had a negative effect on the stability of the gel. In the following trials it will be tested whether a gel that was produced without any buffer

solution can be used for electrophoresis. If this approach won't work, the concentration of buffer solution in the gel will be reduced.

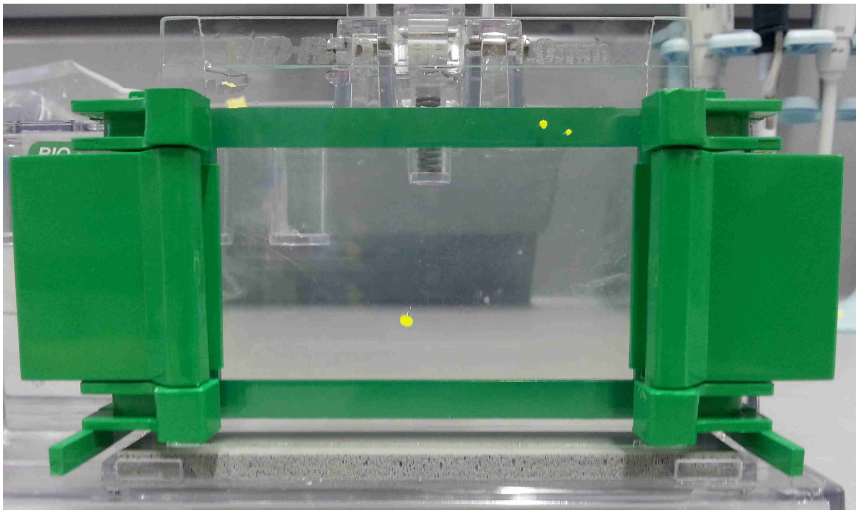

Fig. 3-29: casted 1.5% agarose / 10% PEG 400 gel – 2 (TKU)

3.4.4 Trials 28 - 31 (1.8% agarose / 10% PEG 400 gel electrophoresis towards anode / cathode; gels casted without buffer)

The gels of trials 28-31 were casted like it was described in trial 27, with pure water instead of Gel Buffer A 1x. The composition of the sample solutions can be taken from *Tab. 3-14;* the parameters of trials 28 and 29 were defined as follows:

Runtime: 115 min
Voltage: 100 V
Staining technique: Coomassie

For trials 30 and 31 the runtime was reduced to 60 min, all other parameters stayed the same. In *Fig. 3-30* all gels from those four trials are depicted in one single picture collage. None of those trials worked. The following errors occurred (in combination or alone): The analytes didn't migrate into the gel, the gels shrunk during the electrophoresis run (cf. *Fig. 3-31*) and the gels fell into pieces.

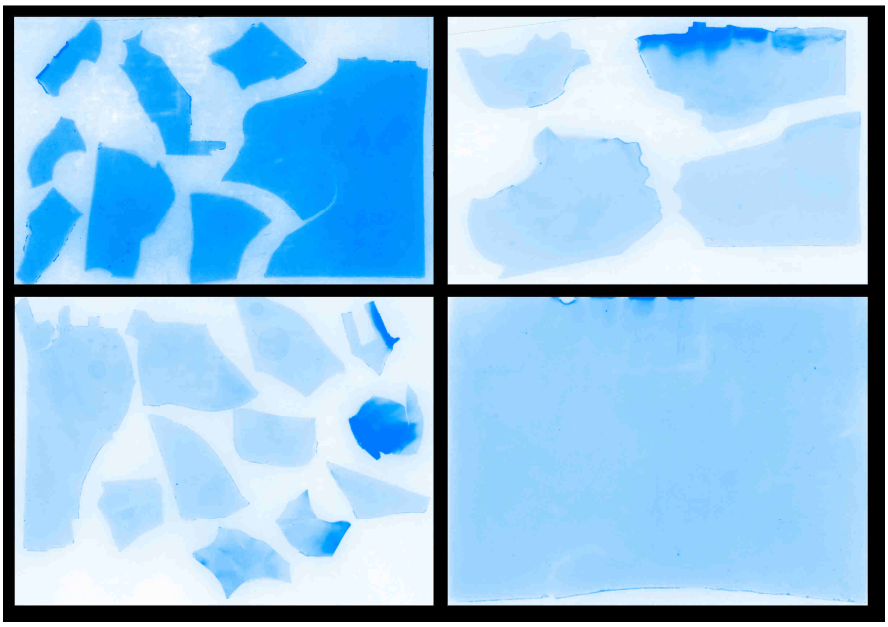

Fig. 3-30: trials 28-31; 1.8% agarose / 10% PEG 400 gel electrophoresis towards anode / cathode; gels casted without buffer (TKU)

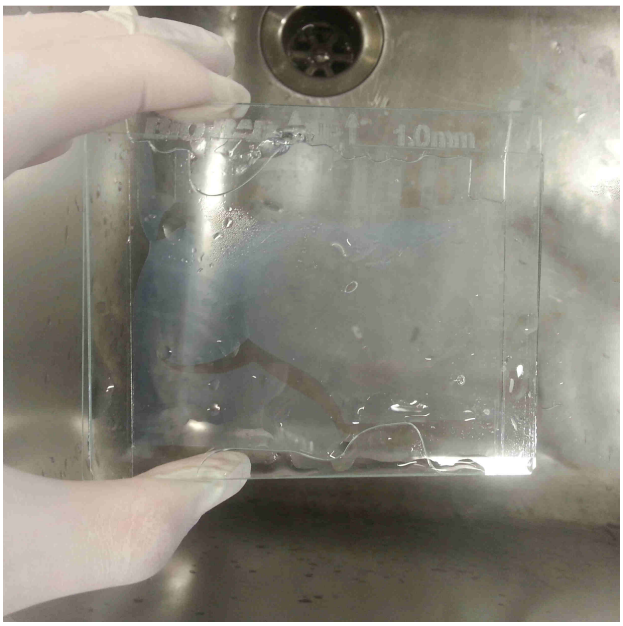

Fig. 3-31: shrunk gel after performed electrophoresis (TKU)

3.5 Native Agarose Gel Electrophoresis – 2

Since the approach with PEG as an additive to the gel mixture didn't improve the results of the experiments, only agarose was used again (like in the penultimate chapter) for the following trials. Initially, the gels were casted with pure water instead of Gel Buffer A 1x. In a second step the buffer concentration in the gel was halved in comparison to the other trials where buffer was a component of the gel mixture. The standard operation procedure can be taken from *3.3 Native Agarose Gel Electrophoresis – 1*.

3.5.1 Trial 32 (1.8% agarose towards anode; gel casted without buffer)

Parameters for electrophoresis:

Runtime: 65 min

Voltage: 100 V

Staining technique: Coomassie

Fig. 3-32: 1.8% agarose towards anode; gel casted without buffer (TKU)

The composition of the sample solutions can be taken from *Tab.3-14*. The gel belonging to this trial is depicted in *Fig. 3-32*.

The gel shrank to half of its size during the electrophoresis run. Furthermore, the analytes didn't migrate into the gel.

3.5.2 Trial 33 (1.8% agarose towards cathode; gel casted without buffer)

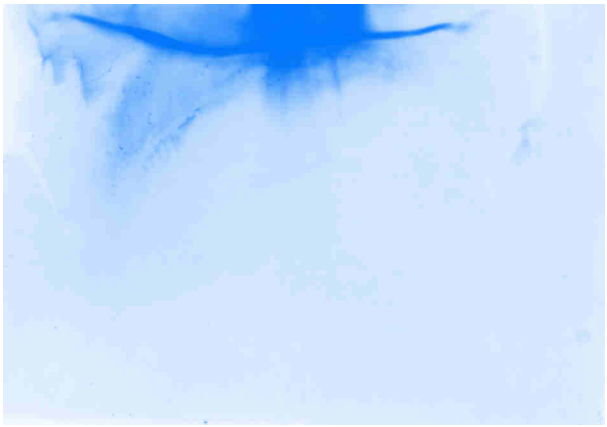

Fig. 3-33: 1.8% agarose towards cathode; gel casted without buffer (TKU)

The composition of the sample solutions can be taken from *Tab.3-14*; all other parameters can be taken from the previous trial.

Like in trial 32, the gel decreased in size during the runtime of the electrophoresis. This time the analytes migrated into the gel, but no proper bands can be seen on the gel in *Fig. 3-33*.

Summing up it can be said that gels that are casted without any buffer solution aren't appropriate for electrophoresis at all. It seems crucial for the separation process that some ions of the buffer solution are implemented in the gel matrix.

3.5.3 Trial 34 (1.8% agarose towards anode; gel casted with ½ x running buffer)

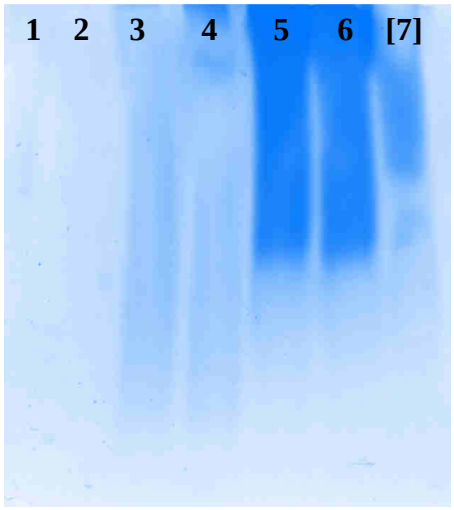

Fig. 3-34: 1.8% agarose towards anode; gel casted with ½ x running buffer (TKU)

Parameters for electrophoresis:

Runtime: 65 min / Voltage: 100 V / Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 3-14*. The corresponding gel is depicted in *Fig. 3-34*.

For this trial the concentration of buffer in the gel was halved compared to the other trials where the gels were casted with a buffer solution. Surprisingly, the reduction of the buffer concentration resulted in a gel that could be casted without any problems and also the stability of the gel was quite good. The concentration of ions in the gel was high enough, so that the analytes could migrate into the gel. Some sample solution spilled over from slot 6 to slot 7; because of that an additional band (7) can be seen on the gel.

3.5.4 Trial 35 (1.8% agarose towards cathode; gel casted with ½ x running buffer)

Fig. 3-35: 1.8% agarose towards cathode; gel casted with ½ x running buffer (TKU)

The composition of the sample solutions can be taken from *Tab. 3-14*; all other parameters can be taken from the previous trial. The corresponding gel is depicted in *Fig. 3-35*.

Again, the electrophoresis with the reduced buffer concentration in the gel worked well. In contrast to trial 34 where the electrophoresis was performed towards the anode the antibody bands are visible in this trial. This was predictable from other agarose-gel trials. Ideas for possible further proceeding will be discussed in the chapter *Conclusions and Outlook* of the thesis.

4. Experimental – Part 2

In the second experimental part of this thesis two monolithic stationary phases based on silica gel shall be tested concerning their ability of binding monoclonal antibodies of the class IgG. The same antibody as in the previous chapter had been used.

4.1 Production of the Monoliths

As an initial step the stationary monolithic phases were synthesized. In addition to a normal phase monolith, an imidazoline-functionalized monolith was produced. Since the production of those stationary phases is part of another diploma thesis carried out by Martin Griesbacher at the Institute for Process and Particle Engineering at Graz University of Technology simultaneously to this thesis, only a rough overview of the production process shall be given here.

The required amount of the substances needed for the production of the monoliths as well as their chemical function in the course of the synthesis is listed in *Tab. 4-1* and *Tab. 4-2*. *Tab. 4-1* is a modification of *Tab. 10.1* and *Tab. 10.2* from Braunbruck's dissertation. *Tab. 4-2* is relying on Griesbacher's diploma thesis.

Substance	Abbreviation	Amount	Function
Cetyltrimethylammonium bromide	CTAB	108 mg	porogen
Tetraethyl orthosilicate	TEOS	3.6 ml	crosslinker
N,*N*-Dimethylformamide	DMF	0.72 ml	drying controller
Distilled water	dH_2O	1.44 ml	hydrolysis reactant
Ethanol	EtOH	4.32 ml	solvent
Diethylamine	DEA	0.18 ml	catalyst

Tab. 4-1: composition of a normal phase monolith (Braunbruck, 2013)

Substance	Abbreviation	Amount	Function
Polyethylene glycol 6000	PEG 6000	500 mg	porogen
Tetraethyl orthosilicate	TEOS	2.5 ml	crosslinker
Triethoxy-3-(2-imidazolin-1-yl)propylsilane	IPTES	1.5 ml	ligand
Distilled water	dH_2O	1.5 ml	hydrolysis reactant
Isopropyl alcohol	2-PrOH	4.5 ml	solvent
Acetic acid (anhydrous)	AAa	0.05 ml	catalyst

Tab. 4-2: composition of a imidazoline-functionalized monolith (Griesbacher, 2016)

4.1.1 Production of the Normal Phase Monolith

CTAB was weighed in a 15 ml centrifugation tube. Then, the AAa, EtOH, dH_2O, TEOS and DMF were added to the tube successively in arbitrary order. The mixture was blended by means of a vortex mixer before it was put in a water bath for 30 min at 60 °C. After the mixture was cooled down to room temperature, DEA was added to the mixture and it was blended again with the vortexer before it was put in the drying oven for 18 h at 40 °C allowing the monolith to age. Next, the monolith was washed with dH_2O and then it was put again in the drying oven for 2 h at 40 °C overlaid with an aqueous 0.01 M ammonium hydroxide solution. Afterwards, the ammonium hydroxide solution was removed via filtration and the monolith was washed with 60% EtOH before it was dried in the drying oven over night at 40 °C. Finally, the monolith was pulverized with a mortar. (Griesbacher, 2016)

4.1.2 Production of the Imidazoline-Functionalized Monolith

First, the PEG was dissolved in 2-PrOH and dH_2O, using a 15 ml centrifugation tube as receptacle. Next, the AAa and TEOS were added and the mixture was blended by means of the vortexer, before it was heated to 60 °C for 30 min in a water bath. The mixture was cooled down to room temperature and it was blended again; then, IPTES was added and the mixture was put in the drying oven for 18 h at 40 °C allowing the monolith to age. All the other steps were the same as in *5.3.1 Production of the Normal Phase Monolith* (washing with dH_2O – processing/washing with ammonium hydroxide solution – washing with EtOH – drying – pulverization). (Griesbacher, 2016)

4.2 Swell Test

Before the stationary phases can be filled in a column for performing a chromatographic separation trial it has to be tested whether the monolithic substances are increasing their volume when being wetted. The swell test was performed at room temperature.

Therefore, 16 mg of the normal phase monolith (N) and 16 mg of the imidazoline-functionalized monolith (I) were filled in a 1.5 ml reaction tube. The filling level of the monoliths in the tubes was marked with a green line. Then, 300 µl PBS were added to each tube. After 18 hours the moisture expansion was checked. In *Fig. 4-1* and *Fig. 4-2* it can be seen that the volume of the monoliths didn’t increase.

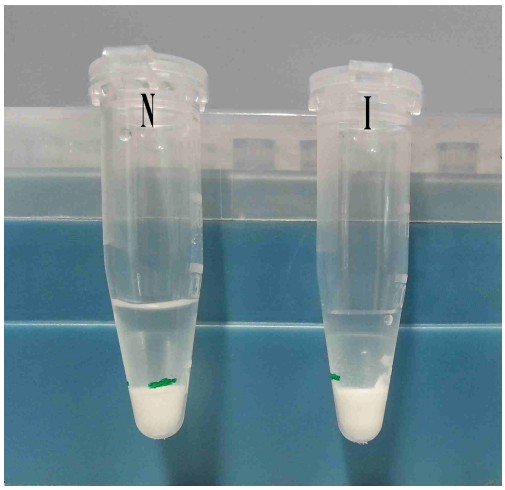

Fig. 4-1: monoliths after 18 h in PBS, before centrifugation (TKU)

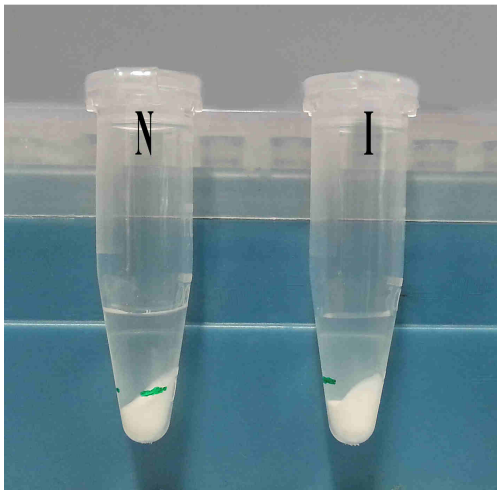

Fig. 4-2: monoliths after 18 h in PBS, after centrifugation (TKU)

4.3 Batch Experiments

The next important parameter that has to be tested is whether there is any binding affinity of the antibody to the monoliths. Therefore, batch experiments shall be performed, again using 1.5 ml reaction tubes. The experiments will be analyzed by SDS-PAGE.

4.3.1 Experiment 1 (antibody binding affinity – 1)

To get a first impression of the antibody binding affinity, two different amounts of the monoliths (8 and 12 mg) were each incubated with 6.25 µg of the antibody. PBS was added to get a final volume of 200 µl in one single reaction tube. After 30 min of incubation on a roll mixer the solid monolithic phase was separated from the liquid phase via centrifugation.

Then, 150 µl of the liquid supernatant were transferred in a new reaction tube so that now – in case of no binding affinity between the antibody and the monolith – 5 µg of the antibody would be in each of the reaction tubes with the transferred supernatants (TS). Only three quarters of the supernatant were transferred (150 µl of 200 µl); therefore, only three quarters of the initially applied antibody (5 µg of 6.25 µg) can maximally be in the transferred liquid, assuming a homogeneous distribution of the antibody in the liquid mixture. On the other hand – if the entire antibody got bound to the stationary phase – there should be no antibody in the reaction tubes with the TS.

SDS-PAGE was used for the detection of the antibody in the TS. For preparing the supernatants for the final electrophoresis, the liquid part of the TS was evaporated by means of a vacuum concentrator at 45 °C. Afterwards, 15 µl PBS and 15 µl FSB were

added to the residue, followed by a 5 min denaturation step at 95 °C. Finally, the SDS-PAGE was performed, based on the standard operation procedure like it is described in the first experimental part of the thesis (cf. *4.2 SDS-PAGE*).

The previously described work steps of the antibody binding affinity experiment are graphically summarized in *Fig. 4-3*. The *M* in the figure indicates the monolithic phase.

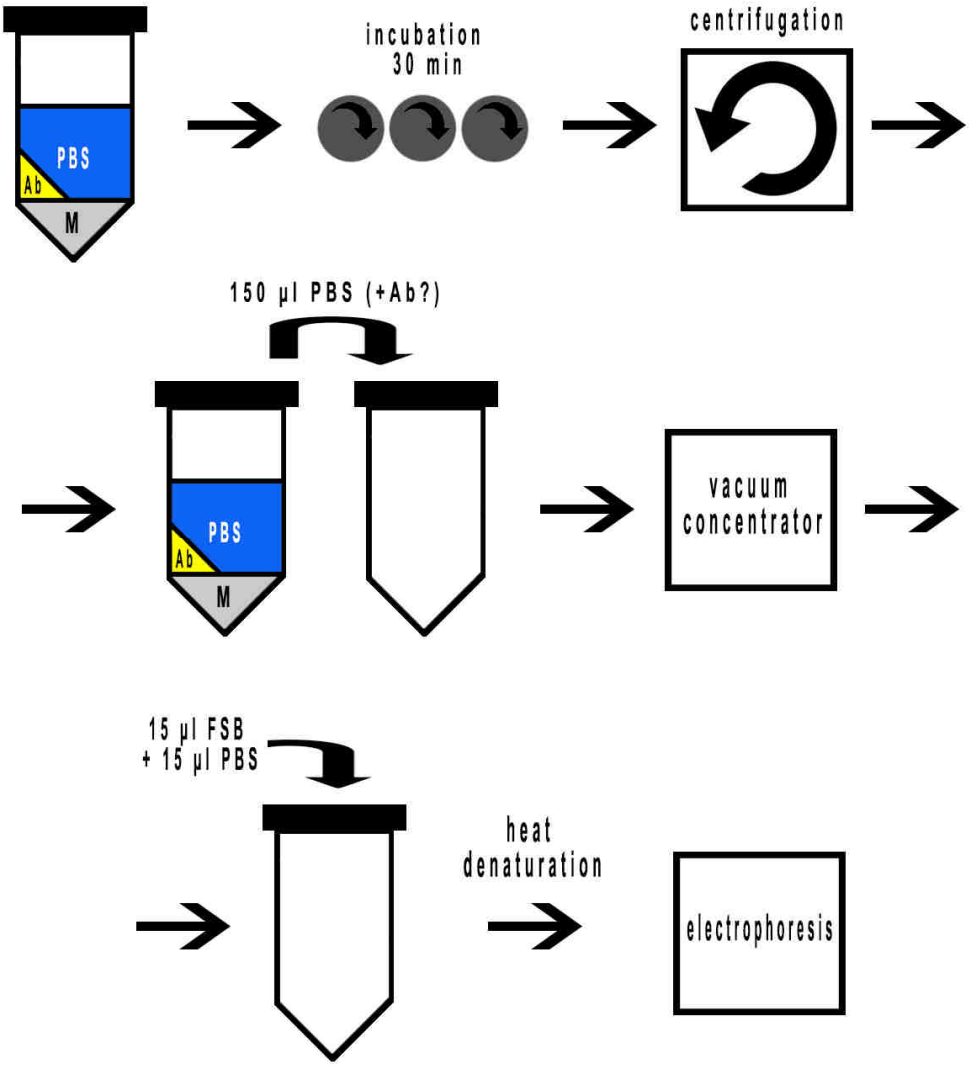

Fig. 4-3: scheme binding affinity batch experiment (TKU)

The composition of the sample solutions can be taken from *Tab. 4-3*.

Slot	1	2	3	4	5	6
analyte	5 µg Ab (= 3.125 µl)	max. 5 µg Ab (from I_{8mg}-batch)	max. 5 µg Ab (from N_{8mg}-batch)	max. 5 µg Ab (from I_{12mg}-batch)	max. 5 µg Ab (from N_{12mg}-batch)	1 µl L
PBS	ad 10 µl	15 µl	15 µl	15 µl	15 µl	-
FSB 2x	10 µl	15 µl	15 µl	15 µl	15 µl	-
total amount	20 µl	30 µl	30 µl	30 µl	30 µl	1 µl

Tab. 4-3: SDS-PAGE 12% controlling gel experiment 1 (TKU)

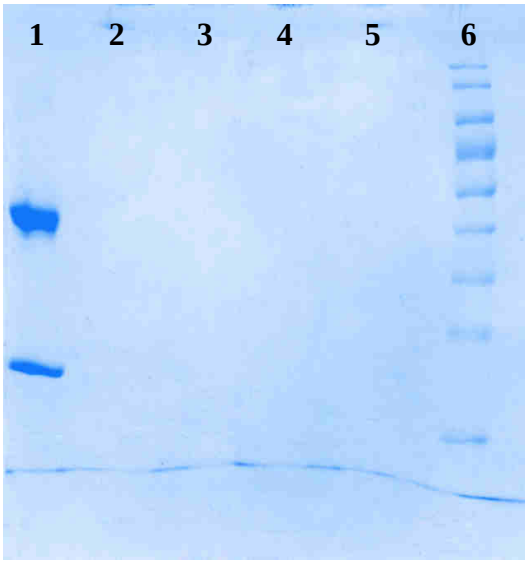

Fig. 4-4: SDS-PAGE 12% controlling gel experiment 1 (TKU)

Parameters for electrophoresis:
Runtime: 70 min
Voltage: 10 mA (constant) for 10 min, then 20 mA (constant) for 60 min
Staining technique: Coomassie

The corresponding gel is depicted in *Fig. 4-4*. No antibody could be detected in the TS which suggests, that the entire applied antibody was bound by the solid monolithic phase.

4.3.2 Experiment 2 (antibody binding capacity – 1)

This experiment was conducted to get an estimation of the antibody binding capacity of the monoliths. Therefore, the amount of the applied antibody was increased from 6.25 µg to 12.25 µg which lead to a maximal final antibody amount of 10 µg in the TS. This time, only 8 mg of the monoliths were used.

Fig. 4-5 gives an overview of the results of experiments 2 – 4. The final electrophoresis step of those trials was performed on one single gel; this gel is depicted in the following figure. The meaning of the Roman numbers in *Fig. 4-5* will be explained in the next paragraph.

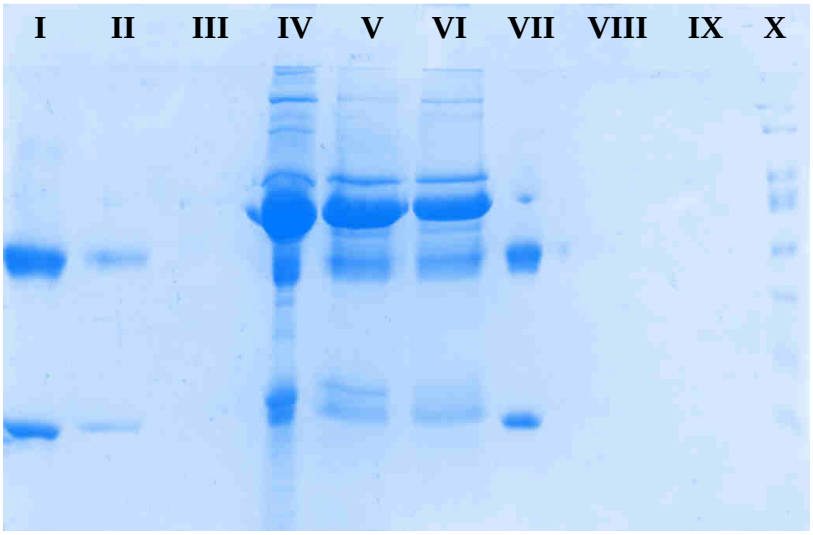

Fig. 4-5: SDS-PAGE 12% controlling gel overview experiments 2-4 (TKU)

For reasons of clear arrangement an extract of *Fig. 4-5* will be made for experiments 2 – 4 where only the paths that are important for the particular trial are shown in the figures. So – *Fig. 4-6*, *Fig. 4-7* and *Fig. 4-8* are a cut down version of *Fig. 4-5*. The black vertical line in *Fig. 4-6* and *Fig. 4-7* indicates that paths 3 and 4 on those gels didn't lie next to each other on the original gel (cf. *Fig. 4-5*). The Roman numbers indicate the paths on the original gel (cf. *Fig. 4-5*).

Slot	1	2	3	4
analyte	10 µg Ab (= 6.25 µl)	max. 10 µg Ab (from I_{8mg}-batch)	max. 10 µg Ab (from N_{8mg}-batch)	1 µl L
PBS	ad 10 µl	15 µl	15 µl	-
FSB 2x	10 µl	15 µl	15 µl	-
total amount	20 µl	30 µl	30 µl	1 µl

Tab. 4-4: SDS-PAGE 12% controlling gel experiment 2 (TKU)

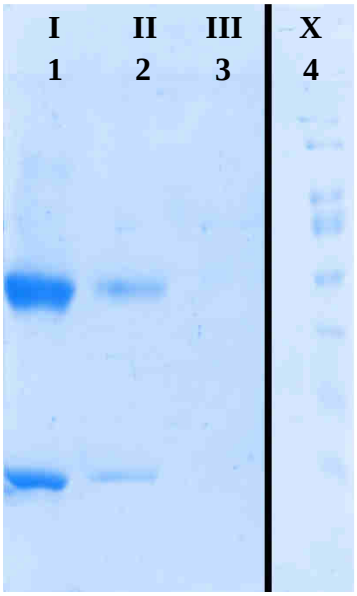

Fig. 4-6: SDS-PAGE 12% controlling gel experiment 2 (TKU)

The parameters of the electrophoresis were the same as in the previous experiment. The pipetting scheme for the gel left-hand can be taken from *Tab. 4-4*.

No antibody band is visible in path 3; this would suggest that the entire applied antibody bound to the stationary phase. In path 2 light antibody bands can be seen; this would suggest that only some of the applied antibody bound to the imidazoline-functionalized monolith, but it could also mean that some sample solution spilled over from slot 1 to slot 2. This trial has to be repeated (cf. exp. 6) to exclude that the analyte from path 1 is responsible for the bands that can be seen in path 2.

4.3.3 Experiment 3 (plasma binding affinity – 1)

In this experiment the binding affinity of human plasma to the monoliths will be tested.

Slot	1	2	3	4
analyte	2 µl P	max. 2 µl P (from I_{8mg}-batch)	max. 2 µl P (from N_{8mg}-batch)	1 µl L
PBS	ad 10 µl	15 µl	15 µl	-
FSB 2x	10 µl	15 µl	15 µl	-
total amount	20 µl	30 µl	30 µl	1 µl

Tab. 4-5: SDS-PAGE 12% controlling gel experiment 3 (TKU)

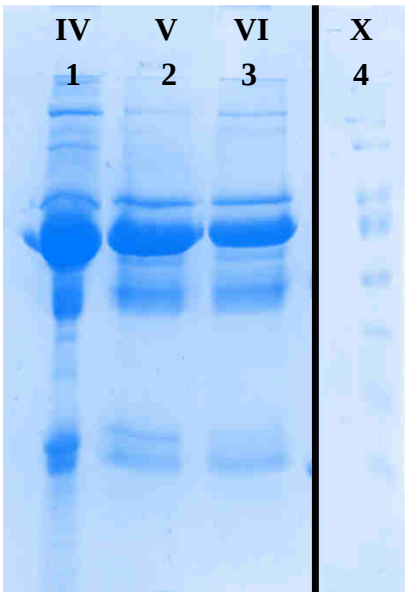

Fig. 4-7: SDS-PAGE 12% controlling gel experiment 3 (TKU)

The parameters of the electrophoresis were the same as in experiment 1. The pipetting scheme for the gel left-hand can be taken from *Tab. 4-5*.

Some plasma bands of path 1 vanished in paths 2 and 3; this suggests that some plasma proteins bound to the monoliths.

Some other bands lost intensity which could mean that not the whole amount of the corresponding proteins bound to the solid phases; but, when path 1 is compared to paths 2 and 3 it is obvious that path 1 is narrower than the other two paths. This could also be the reason for the decreased intensity of some of the bands in paths 2 and 3.

4.3.4 Experiment 4 (reduced incubation time)

Slot	1	2	3	4
analyte	5 µg Ab (= 3.125 µl)	max. 5 µg Ab (from I_{8mg}-batch)	max. 5 µg Ab (from N_{8mg}-batch)	1 µl L
PBS	ad 10 µl	15 µl	15 µl	-
FSB 2x	10 µl	15 µl	15 µl	-
total amount	20 µl	30 µl	30 µl	1 µl

Tab. 4-6: SDS-PAGE 12% controlling gel experiment 4 (TKU)

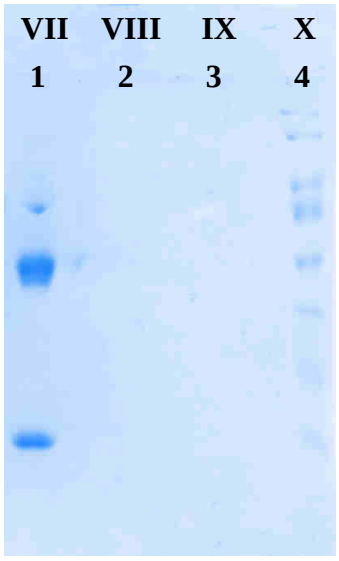

Fig. 4-8: SDS-PAGE 12% controlling gel experiment 4 (TKU)

This experiment was used to test whether a reduced incubation time of 15 instead of 30 min has any negative effect on the binding affinity of the antibody.

The parameters of the electrophoresis were the same as in experiment 1. The pipetting scheme for the gel left-hand can be taken from *Tab. 4-6*

No negative effect could be detected; no antibody bands can be seen in paths 2 and 3 which suggests that the whole antibody got bound by the solid monolithic phases.

4.3.5 Experiment 5 (elution test – 1)

This time it has to be tested whether the bound antibody can be eluted from the monoliths. As a first approach 1 x PBS with 1 M NaCl will serve as the eluent. The sodium ions are supposed to disassociate ionic interactions between the antibody and the monoliths. However, the exact binding mechanism is unknown. The elution test was performed like it is described in experiment 8 with the only exception that the washing step after the incubation was skipped. The washing step was introduced in experiment 8 by hindsight to avoid antibody contaminations from the incubation liquid. The composition of the sample solutions can be taken from *Tab. 4-7* and *Tab. 4-8*.

Slot	1	2	3	4	5
analyte	5 µg Ab (= 3.125 µl)	max. 10 µg Ab (from I_{8mg}-batch)	max. 10 µg Ab (from N_{8mg}-batch)	2 µl P	max. 2 µl P (from I_{8mg}-batch)
PBS	ad 10 µl	15 µl	15 µl	ad 10 µl	15 µl
FSB 2x	10 µl	15 µl	15 µl	10 µl	15 µl
total amount	20 µl	30 µl	30 µl	20 µl	30 µl

Tab. 4-7: SDS-PAGE 12% controlling gel experiment 5 [part 1] (TKU)

Slot	6	7	8	9	10
analyte	max. 2 µl P (from N_{8mg}-batch)	5 µg Ab (= 6.25 µl)	max. 5 µg Ab (from I_{8mg}-batch)	max. 5 µg Ab (from N_{8mg}-batch)	1 µl L
PBS	15 µl	ad 10 µl	15 µl	15 µl	-
FSB 2x	15 µl	10 µl	15 µl	15 µl	-
total amount	30 µl	20 µl	30 µl	30 µl	1 µl

Tab. 4-8: SDS-PAGE 12% controlling gel experiment 5 [part 2] (TKU)

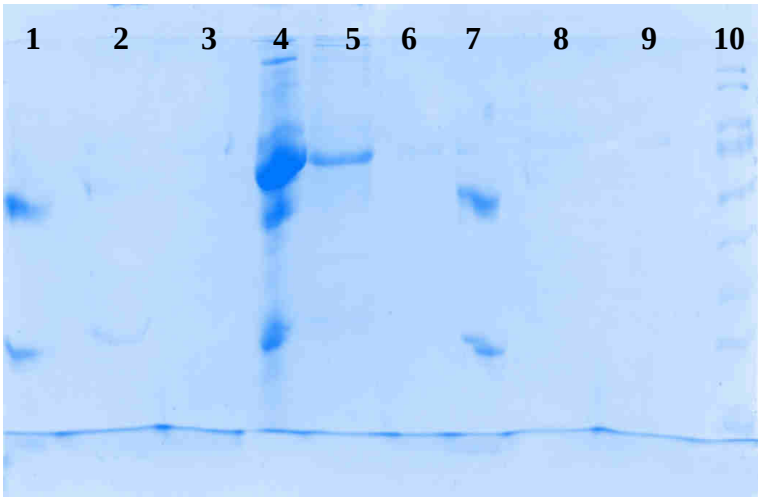

Fig. 4-9: SDS-PAGE 12% controlling gel experiment 5 (TKU)

The bands on the gel in *Fig. 4-9* aren't really sharp so maybe the ions of the eluent had a negative effect on the running behaviour of the analytes. No antibody could be eluted from

the monoliths. The light bands in path 2 could stem from path 1 in the case that some sample solution spilled over from path 1 to path 2 when the analytes were applied. The same counts for the band in path 5 – it could stem from the sample solution from path 4 too. Another elution test has to be performed. On all accounts, another eluent or a higher concentration of sodium chloride in PBS has to be chosen, because even if the elution in this experiment worked a bit, it didn't work well.

4.3.6 Experiment 6 (antibody binding capacity – 2)

Another antibody binding capacity experiment shall be performed. For this trial 12.25 µg, 25 µg and 50 µg of the antibody were used for incubating 8 mg of the monoliths which lead to a maximal final antibody amount of 10 µg, 20 µg and 40 µg in the TS. For the following experiments 10% PAA-gels will be used.

Slot	**1**	**2**	**3**	**4**	**5**
analyte	5 µg Ab (= 3.125 µl)	10 µg Ab (= 6.25 µl)	1 µl L	max. 10 µg Ab (from I_{8mg}-batch)	max. 10 µg Ab (from N_{8mg}-batch)
PBS	ad 10 µl	ad 10 µl	-	15 µl	15 µl
FSB 2x	10 µl	10 µl	-	15 µl	15 µl
total amount	20 µl	20 µl	1 µl	30 µl	30 µl

Tab. 4-9: SDS-PAGE 10% controlling gel experiment 6 [part 1] (TKU)

Slot	**6**	**7**	**8**	**9**
analyte	max. 20 µg Ab (from I_{8mg}-batch)	max. 20 µg Ab (from N_{8mg}-batch)	max. 40 µg Ab (from I_{8mg}-batch)	max. 40 µg Ab (from N_{8mg}-batch)
PBS	15 µl	15 µl	15 µl	15 µl
FSB 2x	15 µl	15 µl	15 µl	15 µl
total amount	30 µl	30 µl	30 µl	30 µl

Tab. 4-10: SDS-PAGE 10% controlling gel experiment 6 [part 2] (TKU)

The composition of the sample solutions can be taken from *Tab. 4-9* and *Tab. 4-10.*

Parameters for electrophoresis:

Runtime: 105 min

Voltage: 20 mA (constant) for 10 min, then 40 mA (constant) for 95 min [2 gels in one chamber]

Staining technique: Coomassie

In *Fig. 4-10* the result of this experiment can be seen. Paths 4 and 5 represent a repetition of experiment 2 (cf. *Fig. 4-6*). The result of experiment 2 could be affirmed with this trial. The antibody band in path 2 of *Fig. 4-6* could be reproduced, this time it can be excluded

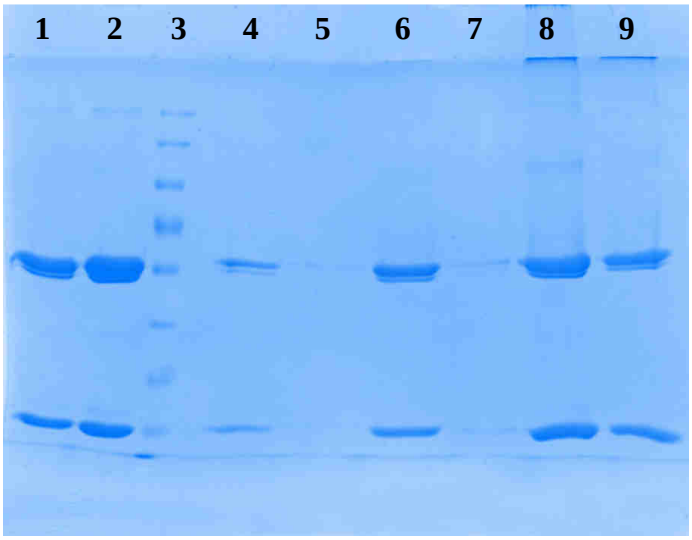

Fig. 4-10: SDS-PAGE 10% controlling gel experiment 6 (TKU)

that some sample solution spilled over from the neighboring antibody slot because the ladder path was inserted between paths 2 and 4 on the gel in *Fig. 4-10*. This time all the bands are much sharper than in the previous trials. Maybe there was something wrong with the last gel batch. Altogether it can be deduced that the normal phase monolith has a higher binding affinity to the antibody than the imidazoline-functionalized monolith.

4.3.7 Experiment 7 (elution test – 2)

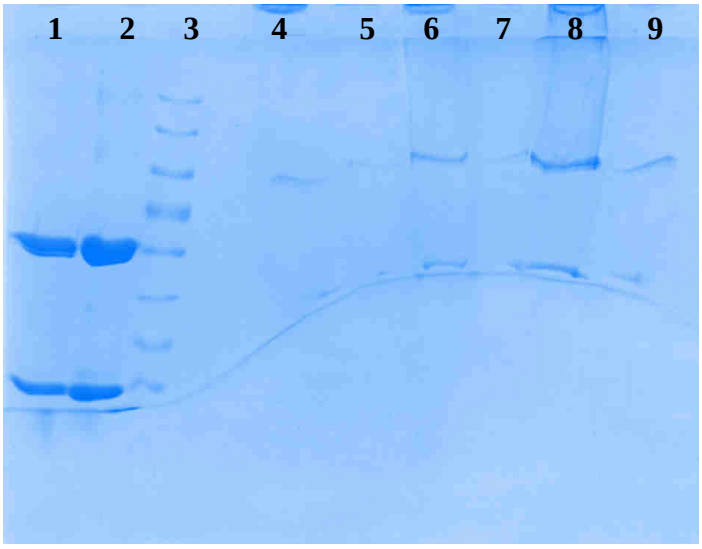

Fig. 4-11: SDS-PAGE 10% controlling gel experiment 7 (TKU)

This time 1 x PBS with 2 M NaCl served as the eluent. The pipetting scheme and all the other parameters for the electrophoresis can be taken from experiment 6. The elution test was again performed like it is described in experiment 8 with the only exception that the washing step after the incubation was skipped. The washing step was introduced in experiment 8 to avoid antibody contaminations from the incubation liquid; those contaminations could be responsible for the visible antibody bands in paths 4 – 9 in *Fig. 4-11*. Obviously, the ions in the eluent had a negative effect on the running behaviour of the analytes on the gel. The dye front isn't a straight horizontal line; it's taking the course of a curve, as soon as the ions were applied to the slots.

4.3.8 Experiment 8 (elution test – 3)

In this trial three different eluents were used:

- 1 x PBS with 2 M NaCl
- 8 M urea
- Na_2SO_4 in Tris pH 9.0 (Mountford et al., 2014)

First of all, 8 mg of the monoliths were each incubated with 37.5 µg of the antibody. PBS was added to get a final volume of 200 µl in one single reaction tube. After 30 min of incubation on a roll mixer the solid monolithic phase was separated from the liquid phase via centrifugation.

At this point, 150 µl of the liquid supernatant could have been transferred to a new reaction tube to continue with the antibody binding affinity test (cf. *experiment 1*). Since those experiments have already been conducted, as much of the liquid supernatant as possible was pipetted out of the reaction tubes with the monoliths to continue with the washing steps. The liquid supernatant was discarded.

For the first washing step 200 µl 1 x PBS were added to the reaction tubes with the monoliths and the tubes were again incubated for 30 min on a roll mixer. After a further centrifugation step, again as much of the liquid supernatant as possible was pipetted out of the tubes to finish the first washing step. Two further washing steps with 200 µl 1 x PBS were performed, but this time without incubating the tubes for 30 min on the roll mixer. Now, 200 µl of the eluents were added to the tubes with the monoliths. After an incubation of 30 min on the roll mixer, the tubes were left in the fridge over night. Then, 150 µl of the eluent were transferred in a new reaction tube so that now – in case of 100% binding affinity between the antibody and the monolith and in case of 100% elution rate – 10 µg of the antibody would be in each of the reaction tubes with the transferred supernatants (TS). Only three quarters of the supernatant were transferred (150 µl of 200 µl); therefore, only three quarters of the initially applied antibody (30 µg of 37.5 µg) can maximally be in the transferred liquid, assuming a homogeneous distribution of the antibody in the liquid mixture.

SDS-PAGE was used for the detection of the antibody in the TS. For preparing the supernatants for the final electrophoresis, the liquid part of the TS was evaporated by means of a vacuum concentrator at 45 °C. Afterwards, 15 µl PBS and 15 µl FSB were added to the residue, followed by a 5 min denaturation step at 95 °C. Finally, the SDS-

PAGE was performed, based on the standard operation procedure like it is described in the first experimental part of the thesis (cf. *4.2 SDS-PAGE*).

The previously described work steps of the elution test are graphically summarized in *Fig. 4-12*. The *M* in the figure indicates the monolithic phase, the *E* indicates the eluent.

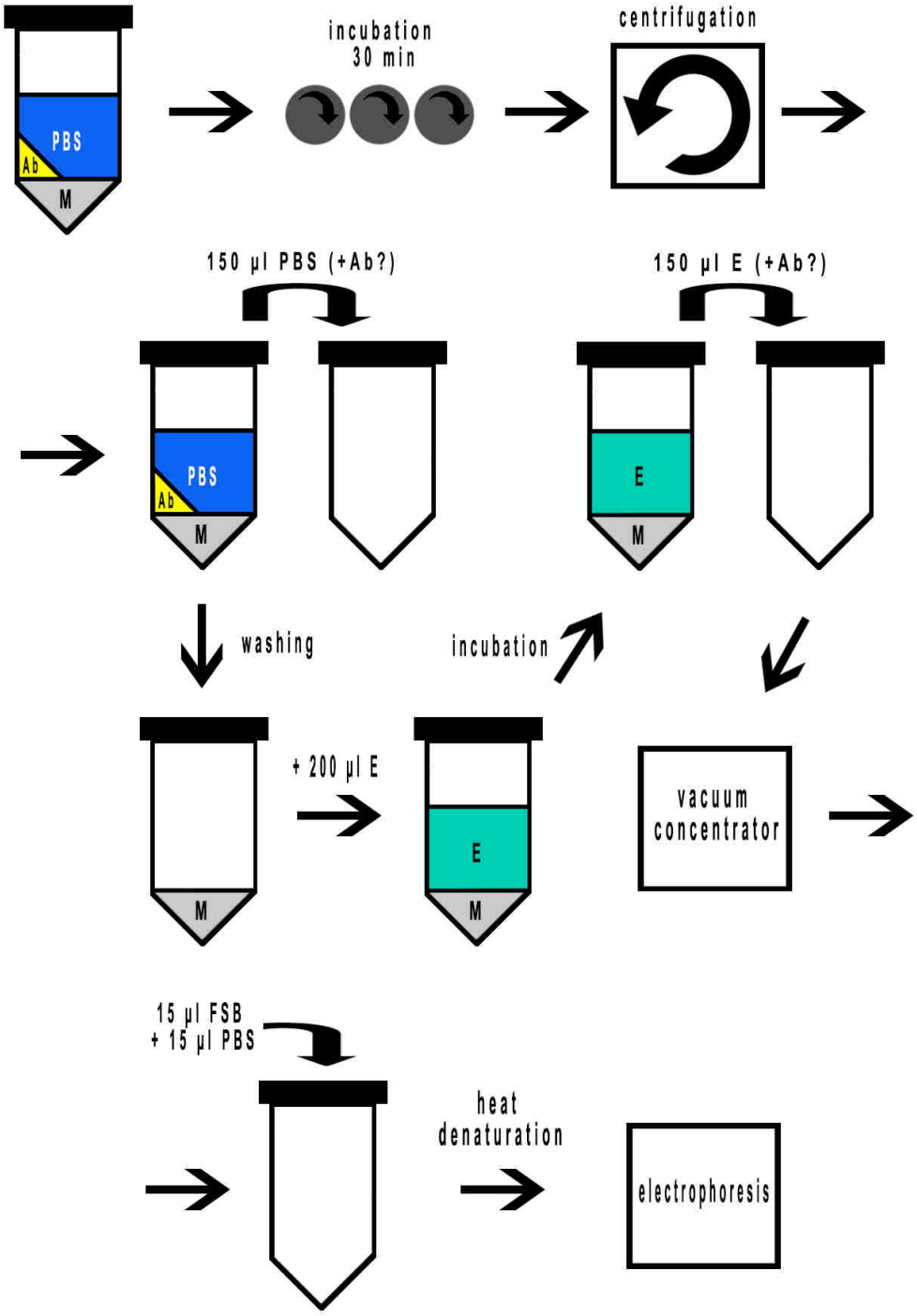

Fig. 4-12: scheme elution test (TKU)

The parameters of the electrophoresis are listed below:

Runtime: 110 min

Voltage: 20 mA (constant) for 10 min, then 40 mA (constant) for 100 min

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 4-11* and *Tab. 4-12*.

Slot	1	2	Slot	3	4
analyte	5 µg Ab (= 3.125 µl)	10 µg Ab (= 6.25 µl)	**analyte**	max. 30 µg Ab (from I_{8mg}-batch) 2 M NaCl/PBS	max. 30 µg Ab (from N_{8mg}-batch) 2 M NaCl/PBS
PBS	ad 10 µl	ad 10 µl	**eluent**	15 µl	15 µl
FSB 2x	10 µl	10 µl	**FSB 2x**	15 µl	15 µl
total amount	20 µl	20 µl	**total amount**	30 µl	30 µl

Tab. 4-11: SDS-PAGE 10% controlling gel experiment 6 [part 1] (TKU)

Slot	5	6	7	8	9
analyte	max. 30 µg Ab (from I_{8mg}-batch) urea	max. 30 µg Ab (from N_{8mg}-batch) urea	max. 30 µg Ab (from I_{8mg}-batch) Na_2SO_4/Tris pH 9	max. 30 µg Ab (from N_{8mg}-batch) Na_2SO_4/Tris pH 9	1 µl L
eluent	15 µl	15 µl	15 µl	15 µl	-
FSB 2x	15 µl	15 µl	15 µl	15 µl	-
total amount	30 µl	30 µl	30 µl	30 µl	1 µl

Tab. 4-12: SDS-PAGE 10% controlling gel experiment 6 [part 2] (TKU)

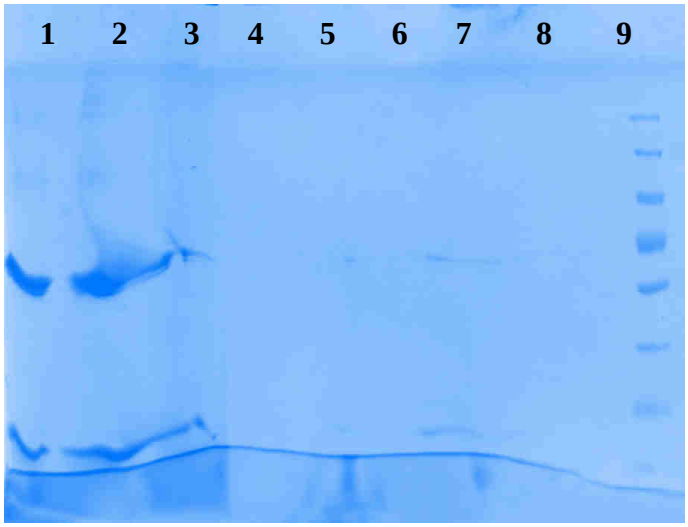

Fig. 4-13: SDS-PAGE 10% controlling gel experiment 8 (TKU)

In *Fig. 4-13* it can be seen that the eluents had a negative effect on the running behaviour of the analytes. However, the elution of the antibody didn't work. The antibody bands in path 3 surely spilled over from path 2 while the analytes were applied to the slots. Only in path 7 a slight antibody band is visible.

4.3.9 Experiment 9 (antibody binding affinity – 2)

This time, the antibody was eluted directly from the incubated monolithic phase with 1 x FSB. The *M* in *Fig. 4-14* again indicates the monolithic phase.

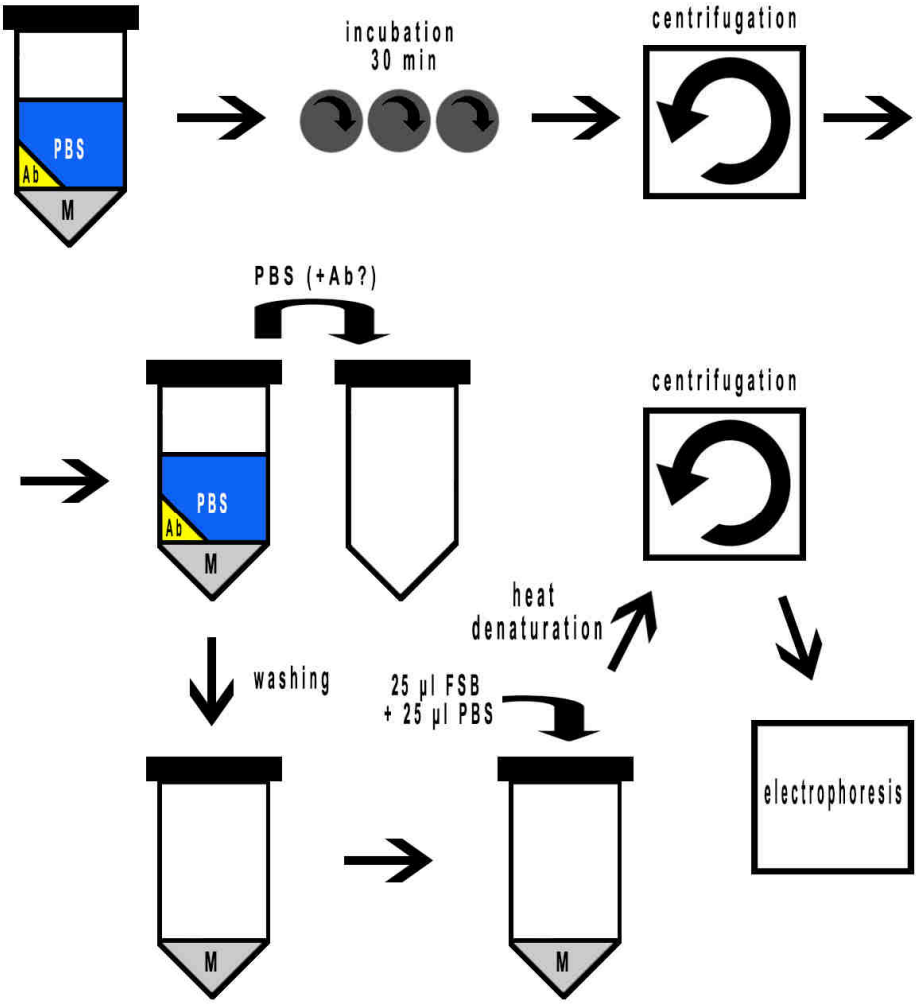

Fig. 4-14: scheme experiment 9 (TKU)

The composition of the sample solutions can be taken from *Tab. 4-13*. The parameters for the electrophoresis were: Runtime: 50 min / Voltage: 10 mA (constant) for 10 min, then 25 mA (constant) for 40 min / Staining technique: Coomassie

Slot	1	2	3	4	5	6
analyte	10 µg Ab (= 6.25 µl)	1 µl L	max. 10 µg Ab (from I_{8mg}-batch)	max. 10 µg Ab (from I_{8mg}-batch)	max. 10 µg Ab (from N_{8mg}-batch)	max. 10 µg Ab (from N_{8mg}-batch)
PBS	ad 10 µl	-	~ 10 µl	~ 10 µl	~ 10 µl	~ 10 µl
FSB 2x	10 µl	-	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	1 µl	20 µl	20 µl	20 µl	20 µl

Tab. 4-13: SDS-PAGE 10% controlling gel experiment 9 (TKU)

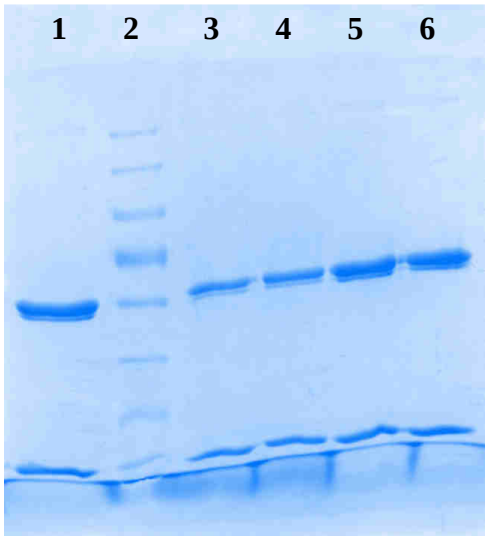

Fig. 4-15: SDS-PAGE 10% controlling gel experiment 9 (TKU)

With 1 x FSB serving as the eluent it was possible to elute the antibody from the monolithic phase which can be taken as a proof that the antibody really got bound to the monoliths. The eluted antibody, however, is denatured due to the effect of the FSB. This eluent couldn't be used in a purification process. In slots 3 and 4 and in slots 5 and 6 the same analyte was applied because the amount of analyte was simply too high for one single slot (cf. *Fig. 4-15*).

4.3.10 Experiment 10 (antibody binding affinity – 3 / plasma binding affinity – 2)

The same procedure as in the previous experiment was used but now also the binding affinity of plasma was tested. The data for performing the electrophoresis can be taken from experiment 9. The composition of the sample solutions can be taken from *Tab. 4-14*.

Slot	1	2	3	4	5	6	7
analyte	10 µg Ab (= 6.25 µl)	1.7 µl P	1 µl L	max. 10 µg Ab (from I_{8mg}-batch)	max. 10 µg Ab (from N_{8mg}-batch)	max. 1.7 µl P (from I_{8mg}-batch)	max. 10 µl P (from N_{8mg}-batch)
PBS	ad 10 µl	ad 10 µl	-	~ 10 µl	~ 10 µl	~ 10 µl	~ 10 µl
FSB 2x	10 µl	10 µl	-	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	20 µl	1 µl	20 µl	20 µl	20 µl	20 µl

Tab. 4-14: SDS-PAGE 10% controlling gel experiment 10 (TKU)

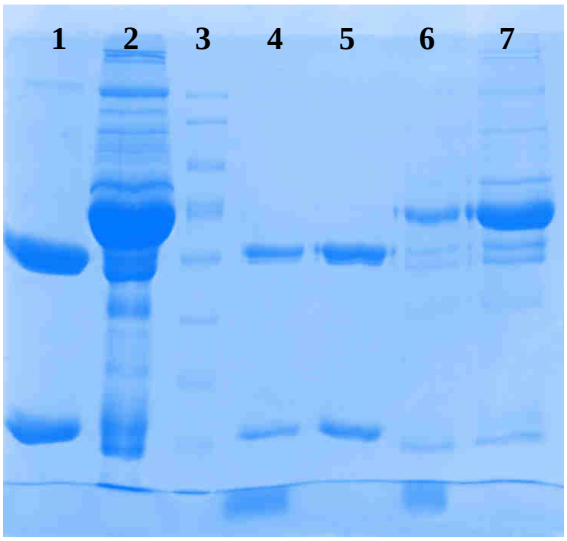

Fig. 4-16: SDS-PAGE 10% controlling gel experiment 10 (TKU)

The corresponding gel is depicted in *Fig. 4-16*. Paths 4 and 5 verify the result of experiment 9 that the antibody can be eluted from the incubated monoliths with FSB. Paths 6 and 7 indicate that some components of the plasma show a binding affinity to the monoliths too. The binding affinity of the plasma to the normal phase monolith is higher.

4.3.11 Experiment 11 (antibody binding affinity – 4 / plasma binding affinity – 3)

This time, the supernatant was tested for residues of the applied antibody and plasma, respectively, following the scheme that is depicted in *Fig. 4-17*. The *M* in the figure again indicates the monolithic phase.

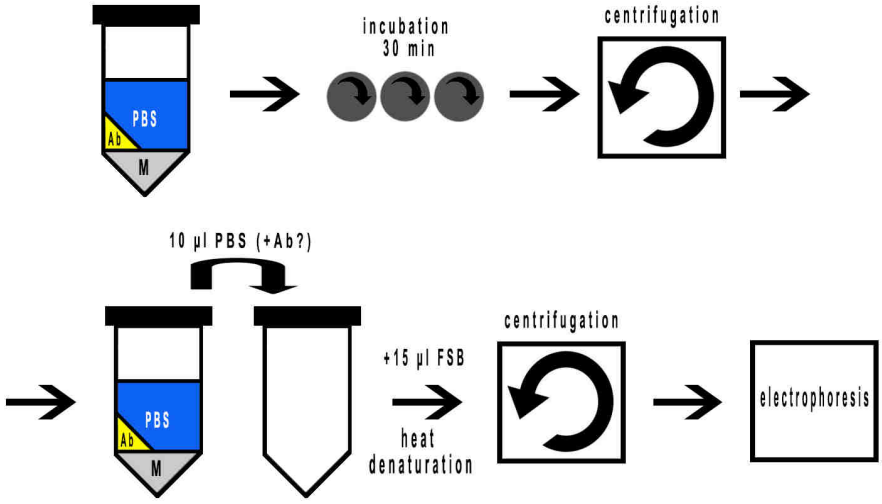

Fig. 4-17: scheme experiment 11 (TKU)

The parameters of the electrophoresis were the same as in experiment 9.

Slot	1	2	3	4	5	6	7
analyte	10 µg Ab (= 6.25 µl)	1.7 µl P	1 µl L	max. 10 µg Ab (from I_{8mg}-batch)	max. 10 µg Ab (from N_{8mg}-batch)	max. 1.7 µl P (from I_{8mg}-batch)	max. 10 µl P (from N_{8mg}-batch)
PBS	ad 10 µl	ad 10 µl	-	~ 10 µl	~ 10 µl	~ 10 µl	~ 10 µl
FSB 2x	10 µl	10 µl	-	10 µl	10 µl	10 µl	10 µl
total amount	20 µl	20 µl	1 µl	20 µl	20 µl	20 µl	20 µl

Tab. 4-15: SDS-PAGE 10% controlling gel experiment 11 (TKU)

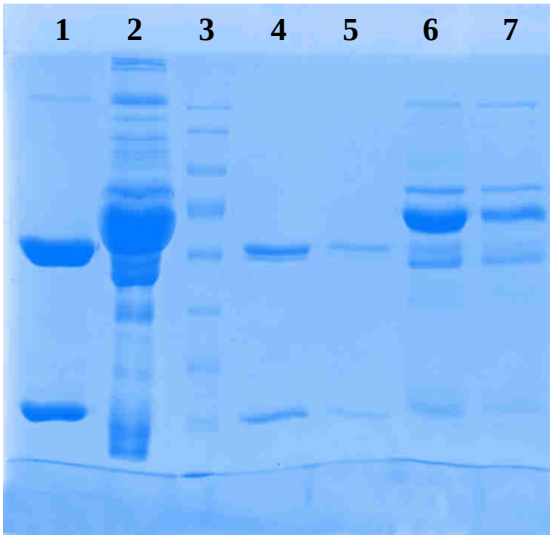

Fig. 4-18: SDS-PAGE 10% controlling gel experiment 11 (TKU)

The composition of the sample solutions can be taken from *Tab. 4-15*. This experiment was performed together with experiment 10, so the analyzed supernatant of this experiment should complement the result of the previous experiment. The results actually go well together: now, the bands from the I_{8mg}-batch are stronger (cf. *Fig. 4-18*) which supports the theory that the binding affinity of the normal phase monolith is higher.

4.3.12 Experiment 12 (elution test – 4)

The monoliths were incubated with 10 µg antibody to perform another elution test analogously to the procedure described in experiment 8 with the exceptions that now lower amounts of antibody and PBS as well as lower amounts of the eluents were used so that the vacuum concentration could be skipped. The eluent-supernatants were applied directly to the gels (cf. *Fig. 4-19*).

For this experiment the following eluents were used:

- Methanol 70% (V/V) + 0.1% TFA (cf. slots 3 & 4, *Fig. 4-19*)
- Acetonitrile 70% (V/V) + 0.1% TFA (cf. slots 5 & 6, *Fig. 4-19*)
- 50 mM sodium acetate adjusted with acetic acid to pH 4 (cf. slots 7 & 8, *Fig. 4-19*)
- Acetonitrile 90% (V/V) + 0.1% TFA (cf. slots 9 & 10, *Fig. 4-19*)

TFA was added to lower unspecific interactions between the monoliths and the antibody.

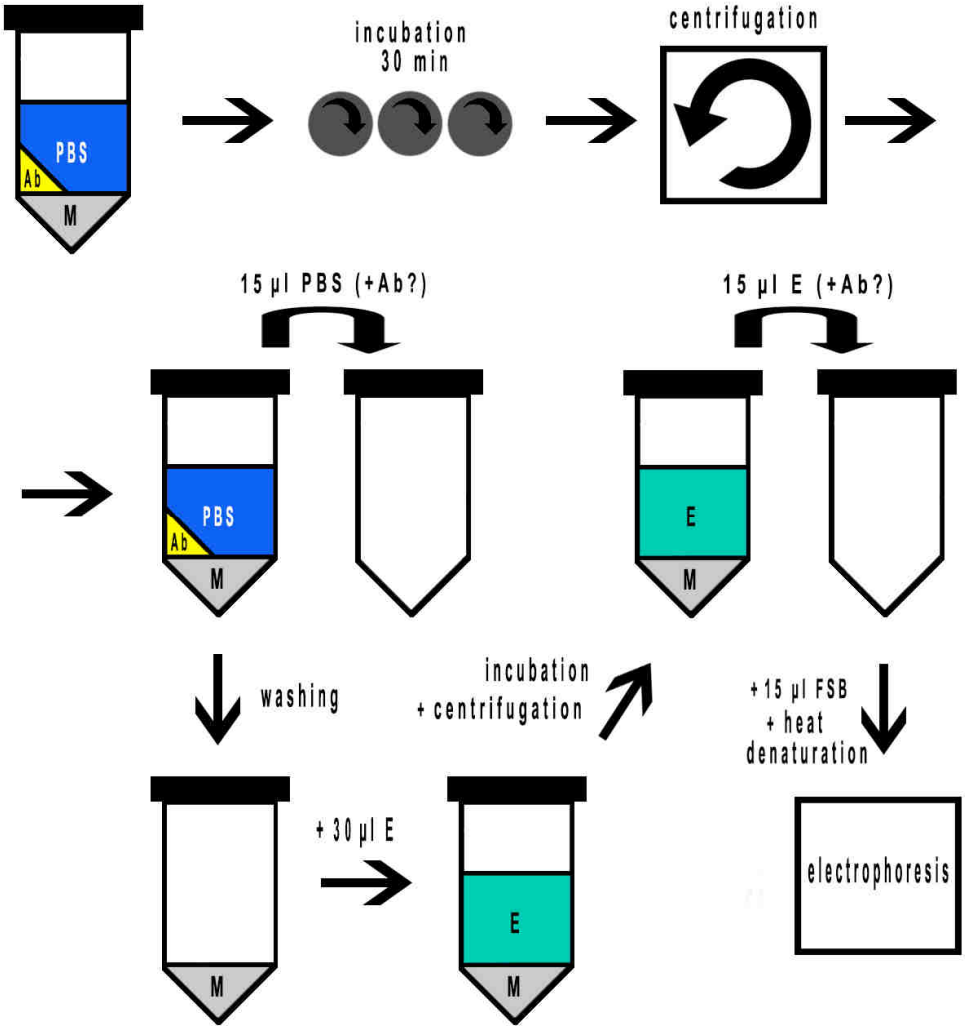

Fig. 4-19: scheme experiment 12 (TKU)

The parameters of the electrophoresis were:

Runtime: 55 min

Voltage: 20 mA (constant) for 10 min, then 40 mA (constant) for 45 min [2 gels in one chamber]

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 4-16* and *Tab. 4-17*.

Slot	1	2	Slot	3	4	5
analyte	10 µg Ab (= 6.25 µl)	1 µl L	**analyte**	max. 10 µg Ab (from I_{8mg}-batch) MeOH 70% + 0.1% TFA	max. 10 µg Ab (from N_{8mg}-batch) MeOH 70% + 0.1% TFA	max. 10 µg Ab (from I_{8mg}-batch) ACN 70% + 0.1% TFA
PBS	ad 10 µl	-	**eluent**	15 µl	15 µl	15 µl
FSB 2x	10 µl	-	**FSB 2x**	15 µl	15 µl	15 µl
total amount	20 µl	1 µl	**total amount**	30 µl	30 µl	30 µl

Tab. 4-16: SDS-PAGE 10% controlling gel experiment 12 [part 1] (TKU)

Slot	6	7	8	9	10
analyte	max. 10 µg Ab (from N_{8mg}-batch) ACN 70% + 0.1% TFA	max. 10 µg Ab (from I_{8mg}-batch) 50mM Na-Ac pH 4	max. 10 µg Ab (from N_{8mg}-batch) 50mM Na-Ac pH 4	max. 10 µg Ab (from I_{8mg}-batch) ACN 90% + 0.1% TFA	max. 10 µg Ab (from N_{8mg}-batch) ACN 90% + 0.1% TFA
eluent	15 µl	15 µl	15 µl	15 µl	15 µl
FSB 2x	15 µl	15 µl	15 µl	15 µl	15 µl
total amount	30 µl	30 µl	30 µl	30 µl	30 µl

Tab. 4-17: SDS-PAGE 10% controlling gel experiment 12 [part 2] (TKU)

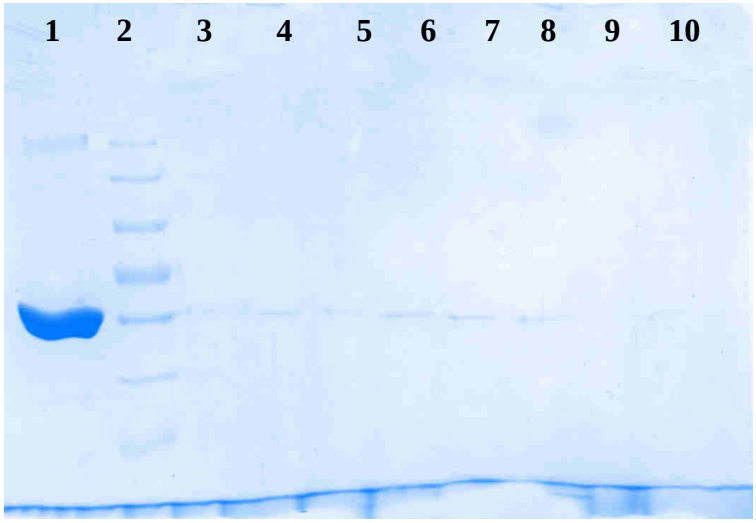

Fig. 4-20: SDS-PAGE 10% controlling gel experiment 12 (TKU)

In *Fig. 4-20* it can be seen that no antibody could be eluted from the incubated monolithic phases. The light bands in paths 3 – 8 probably represent contaminations from antibody that was displaced over the various work steps by transferring small amounts of the monoliths into a new reaction tube when only the liquid supernatant should had been transferred.

4.3.13 Experiment 13 (antibody binding affinity – 5)

The antibody was directly eluted from the incubated monolithic phase from experiment 12 with 1 x FSB after the different eluents of the previous experiment were applied (cf. *Fig. 4-14*). The parameters of the electrophoresis were the same as in the previous trial. The composition of the sample solutions can be taken from *Tab. 4-18* and *Tab. 4-19.*

Slot	1	2	3	4	5	6
analyte	10 µg Ab (= 6.25 µl)	1 µl L	max. 10 µg Ab (from I_{8mg}-batch) MeOH 70% + 0.1% TFA	max. 10 µg Ab (from N_{8mg}-batch) MeOH 70% + 0.1% TFA	max. 10 µg Ab (from I_{8mg}-batch) ACN 70% + 0.1% TFA	max. 10 µg Ab (from N_{8mg}-batch) ACN 70% + 0.1% TFA
PBS	ad 10 µl	-	15 µl	15 µl	15 µl	15 µl
FSB 2x	10 µl	-	15 µl	15 µl	15 µl	15 µl
total amount	20 µl	1 µl	30 µl	30 µl	30 µl	30 µl

Tab. 4-18: SDS-PAGE 10% controlling gel experiment 13 [part 1] (TKU)

Slot	7	8	9	10
analyte	max. 10 µg Ab (from I_{8mg}-batch) 50mM Na-Ac pH 4	max. 10 µg Ab (from N_{8mg}-batch) 50mM Na-Ac pH 4	max. 10 µg Ab (from I_{8mg}-batch) ACN 90% + 0.1% TFA	max. 10 µg Ab (from N_{8mg}-batch) ACN 90% + 0.1% TFA
PBS	15 µl	15 µl	15 µl	15 µl
FSB 2x	15 µl	15 µl	15 µl	15 µl
total amount	30 µl	30 µl	30 µl	30 µl

Tab. 4-19: SDS-PAGE 10% controlling gel experiment 13 [part 2] (TKU)

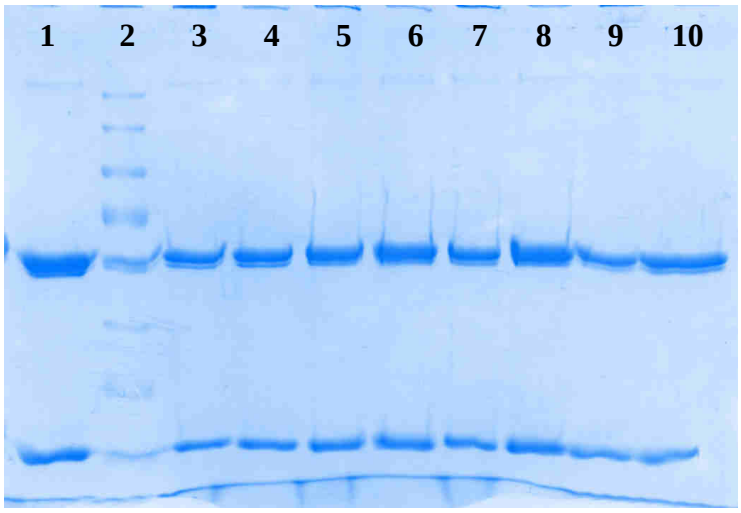

Fig. 4-21: SDS-PAGE 10% controlling gel experiment 13 (TKU)

The results of this experiment match the outcome of the previous experiment. With FSB the antibody could be eluted from the monolithic stationary phases (cf. *Fig. 4-21*).

4.3.14 Experiment 14 (antibody binding affinity – 6)

This time, the supernatant of experiment 12 was tested for residues of the applied antibody after incubation of the monoliths (cf. *Fig. 4-17*).

The numbers on the gel in *Fig. 4-21* are correlating with *Tab. 4-18* and *Tab. 4-19* of experiment 13. The parameters of the electrophoresis were the same as in experiment 12.

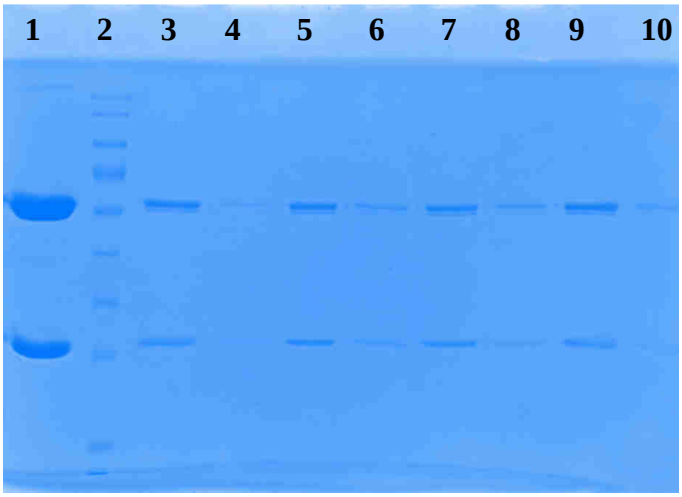

Fig. 4-22: SDS-PAGE 10% controlling gel experiment 14

Apparently, not the entire applied antibody got bound to the stationary phases because some antibody could be detected in the supernatants. The antibody bands in the paths of the imidazoline-functionalized monoliths are stronger which indicates the higher binding affinity of the normal phase monolith. The results of experiments 12 – 14, however, fit together.

4.3.15 Experiment 15 (elution test – 5 / antibody binding affinity – 7 / antibody binding affinity – 8)

This time, KSCN served as an eluent. Following the instructions of *Öztürk et al. (2008)*, a concentration of 0.1 M KSCN was used. Additionally, a pH-value of 4, but no buffer solution was mentioned in the paper, so 1 x PBS was used at pH 4.

Slots 5 and 8 were left blank to avoid contaminations of the analytes from the different test series, since all test series were analyzed on one single gel. In *Fig. 4-22*, paths 3 and 4 are representing the result of the elution test; on paths 6 and 7 the antibody was eluted directly from the incubated monolithic phase with 1 x FSB after the eluent was applied; on paths 9 and 10 the supernatant was tested for residues of the applied antibody and plasma, respectively, after incubation of the monoliths. The procedure for this experiment equals the procedure of experiments 12 – 14. The parameters of the electrophoresis were:

Runtime: 60 min

Voltage: 10 mA (constant) for 10 min, then 25 mA (constant) for 50 min

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 4-20* and *Tab. 4-21*.

Slot	1	2	3	4	5
analyte	10 µg Ab (= 6.25 µl)	1 µl L	max. 10 µg Ab (from I_{8mg}-batch) 0.1 M KSCN in 1 x PBS at pH 8 **eluent**	max. 10 µg Ab (from I_{8mg}-batch) 0.1 M KSCN in 1 x PBS at pH 8 **eluent**	-
PBS	ad 10 µl	-	15 µl	15 µl	-
FSB 2x	10 µl	-	15 µl	15 µl	-
total amount	20 µl	1 µl	30 µl	30 µl	-

Tab. 4-20: SDS-PAGE 10% controlling gel experiment 15 [part 1] (TKU)

Slot	6	7	8	9	10
analyte	max. 10 µg Ab (from I_{8mg}-batch) 0.1 M KSCN in 1 x PBS at pH 8 **monolith**	max. 10 µg Ab (from I_{8mg}-batch) 0.1 M KSCN in 1 x PBS at pH 8 **monolith**	-	max. 10 µg Ab (from I_{8mg}-batch) 0.1 M KSCN in 1 x PBS at pH 8 **incubation liquid**	max. 10 µg Ab (from I_{8mg}-batch) 0.1 M KSCN in 1 x PBS at pH 8 **incubation liquid**
PBS	15 µl	15 µl	-	15 µl	15 µl
FSB 2x	15 µl	15 µl	-	15 µl	15 µl
total amount	30 µl	30 µl	-	30 µl	30 µl

Tab. 4-21: SDS-PAGE 10% controlling gel experiment 15 [part 2] (TKU)

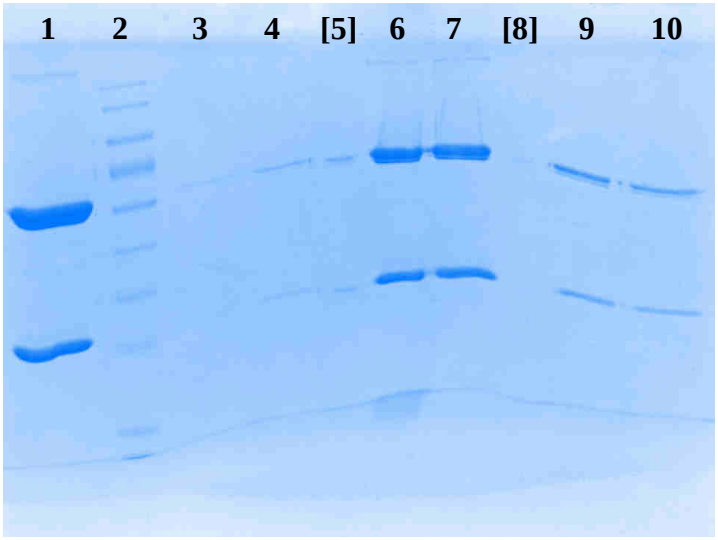

Fig. 4-23: SDS-PAGE 10% controlling gel experiment 15 (TKU)

Fig. 4-23 shows that also this approach of elution with 0.1 M KSCN in 1 x PBS at pH 4 didn't lead to the desired result. It can be seen that no significant amount of antibody could be eluted from the monoliths (cf. paths 3, 4). The light bands that are visible in those paths could stem from antibody that was carried off over the work steps. Paths 6 and 7 suggest that most of the applied antibody got bound to the monoliths. Paths 9 and 10 indicate that some of the antibody did not bind to the monolithic stationary phases.

4.3.16 Experiment 16 (elution test – 6)

The next three experiments equal experiments 12 – 14 with the difference that now BSA was added as a stabilizer for the antibody during the incubation because maybe the antibody got denatured by the stationary phase. This time the following eluents were used:

- Methanol 70% (V/V) + 0.1% TFA (cf. slots 5 & 6, *Fig. 4-23*)
- 50 mM sodium acetate adjusted with acetic acid to pH 4 (cf. slots 7 & 8, *Fig. 4-23*)
- Acetonitrile 70% (V/V) + 0.1% TFA (cf. slots 9 & 10, *Fig. 4-23*)

Parameters for electrophoresis:

Runtime: 60 min

Voltage: 300V [2 gels in one chamber]

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Tab. 4-22* and *Tab. 4-23*.

Slot	1	2	3	4	Slot	5
analyte	10 µg Ab (= 6.25 µl)	5 µg BSA	10 µg BSA	1 µl L	**analyte**	max. 10 µg Ab (from I_{8mg}-batch) MeOH 70% + 0.1% TFA + 1% BSA
PBS	ad 10 µl	ad 10 µl	ad 10 µl	-	**eluent**	15 µl
FSB 2x	10 µl	10 µl	10 µl	-	**FSB 2x**	15 µl
total amount	20 µl	20 µl	20 µl	1 µl	**total amount**	30 µl

Tab. 4-22: SDS-PAGE 10% controlling gel experiment 16 [part 1] (TKU)

Slot	6	7	8	9	10
analyte	max. 10 µg Ab (from N_{8mg}-batch) MeOH 70% + 0.1% TFA + 1% BSA	max. 10 µg Ab (from I_{8mg}-batch) 50mM Na-Ac pH 4 + 1% BSA	max. 10 µg Ab (from N_{8mg}-batch) 50mM Na-Ac pH 4 + 1% BSA	max. 10 µg Ab (from I_{8mg}-batch) ACN 70% + 0.1% TFA + 1% BSA	max. 10 µg Ab (from N_{8mg}-batch) ACN 70% + 0.1% TFA + 1% BSA
eluent	15 µl	15 µl	15 µl	15 µl	15 µl
FSB 2x	15 µl	15 µl	15 µl	15 µl	15 µl
total amount	30 µl	30 µl	30 µl	30 µl	30 µl

Tab. 4-23: SDS-PAGE 10% controlling gel experiment 16 [part 2] (TKU)

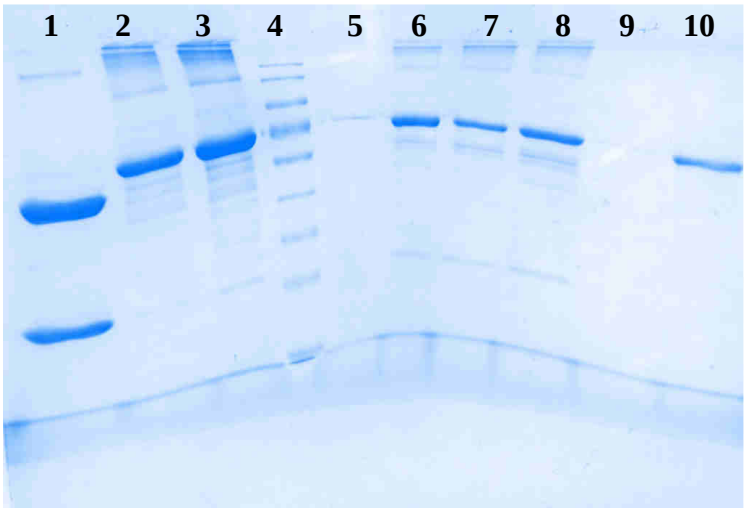

Fig. 4-24: SDS-PAGE 10% controlling gel experiment 16 (TKU)

Also this time the elution of the antibody didn't work. Amounts of BSA, however, could be eluted from the stationary phases. The eluents had a negative influence on the electrophoresis. The dye front isn't a straight horizontal line; it's taking the course of a curve.

4.3.17 Experiment 17 (antibody / BSA binding affinity – 1)

The antibody was eluted directly from the incubated monolithic phase from experiment 16 with 1 x FSB after the different eluents of the previous experiment were applied. The parameters of the electrophoresis were the same as in the previous trial. The composition of the sample solutions can be taken from *Tab. 4-24* and *Tab. 4-25*.

Slot	1	2	3	4	5	6
analyte	10 µg Ab (= 6.25 µl)	max. 10 µg BSA (from I_{8mg}-batch)	max. 10 µg BSA (from N_{8mg}-batch)	1 µl L	max. 10 µg Ab (from I_{8mg}-batch) MeOH 70% + 0.1% TFA + 1% BSA	max. 10 µg Ab (from N_{8mg}-batch) MeOH 70% + 0.1% TFA + 1% BSA
PBS	ad 10 µl	15 µl	15 µl	-	15 µl	15 µl
FSB 2x	10 µl	15 µl	15 µl	-	15 µl	15 µl
total amount	20 µl	30 µl	30 µl	1 µl	30 µl	30 µl

Tab. 4-24: SDS-PAGE 10% controlling gel experiment 17 [part 1] (TKU)

Slot	7	8	9	10
analyte	max. 10 µg Ab (from I_{8mg}-batch) 50mM Na-Ac pH 4 + 1% BSA	max. 10 µg Ab (from N_{8mg}-batch) 50mM Na-Ac pH 4 + 1% BSA	max. 10 µg Ab (from I_{8mg}-batch) ACN 70% + 0.1% TFA + 1% BSA	max. 10 µg Ab (from N_{8mg}-batch) ACN 70% + 0.1% TFA + 1% BSA
PBS	15 µl	15 µl	15 µl	15 µl
FSB 2x	15 µl	15 µl	15 µl	15 µl
total amount	30 µl	30 µl	30 µl	30 µl

Tab. 4-25: SDS-PAGE 10% controlling gel experiment 17 [part 2] (TKU)

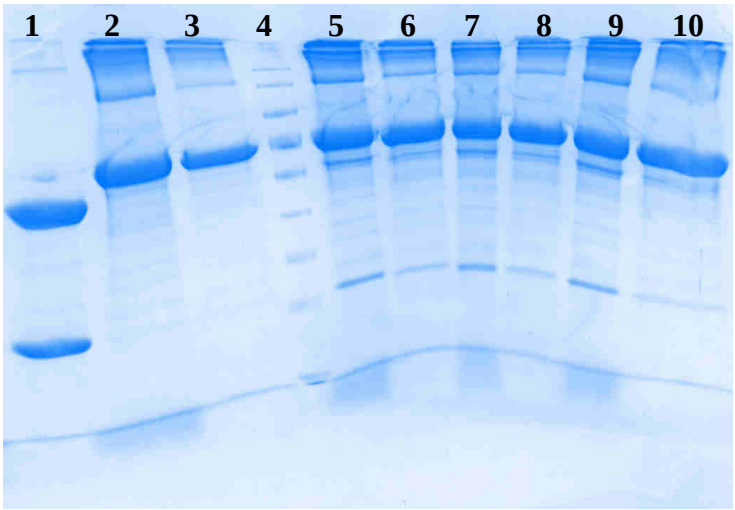

Fig. 4-25: SDS-PAGE 10% controlling gel experiment 17 (TKU)

The bands on the gel depicted in *Fig. 4-25* represent the compounds that were bound by the monolithic phases during incubation. The antibody bands are relatively light compared to the experiments where no BSA was used. It seems that the BSA concurred with the antibody referring to the binding affinity of the stationary phases.

4.3.18 Experiment 18 (antibody / BSA binding affinity – 2)

This time, the supernatant from experiment 16 was tested for residues of the applied antibody and BSA, respectively, after incubation of the monoliths.

The numbers on the gel in *Fig. 4-25* are correlating with *Tab. 4-24* and *Tab. 4-25* of experiment 17.

Parameters for electrophoresis:

Runtime: 50 min

Voltage: 10 mA (constant) for 10 min, then 25 mA (constant) for 40 min

Staining technique: Coomassie

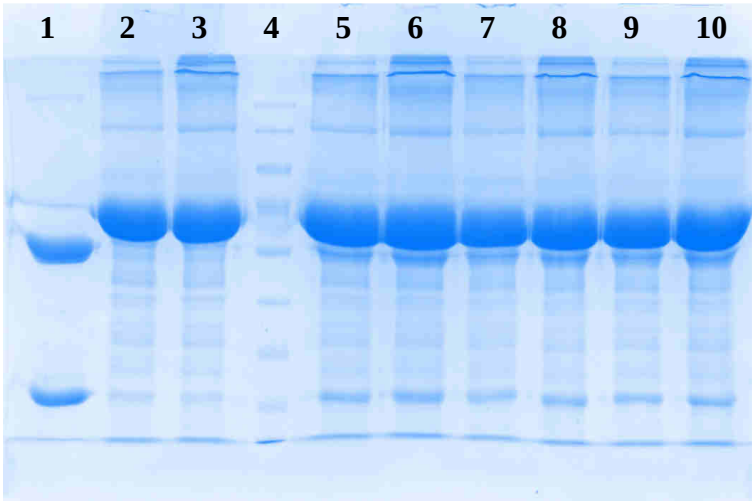

Fig. 4-26 shows that large amounts of BSA and antibody, respectively, did not interact with the stationary phases.

Therefore, many strong bands are visible on the gel left-hand.

Fig. 4-26: SDS-PAGE 10% controlling gel experiment 18 (TKU)

4.3.19 Experiment 19 (BSA binding affinity – 1)

This experiment equals experiment 17 with the only difference that now no antibody had been applied. Thus, the BSA was eluted directly from the incubated monolithic phase with 1 x FSB after the different eluents of experiment 16 were applied. Experiments 19 and 20 thus have the function of a blank test to get a better estimation which bands on the controlling gels of the previous three experiments belong to the BSA.

The parameters for the electrophoresis were:

Runtime: 75 min

Voltage: 20 mA (constant) for 10 min, then 40 mA (constant) for 65 min [2 gels in one chamber]

Staining technique: Coomassie

The composition of the sample solutions can be taken from *Fig. 4-26* and *Fig. 4-27*.

Slot	1	2	3	4	5	6
analyte	10 µg Ab (= 6.25 µl)	max. 10 µg BSA (from I_{8mg}-batch)	max. 10 µg BSA (from N_{8mg}-batch)	1 µl L	no antibody (from I_{8mg}-batch) MeOH 70% + 0.1% TFA + 1% BSA	no antibody (from N_{8mg}-batch) MeOH 70% + 0.1% TFA + 1% BSA
PBS	ad 10 µl	15 µl	15 µl	-	15 µl	15 µl
FSB 2x	10 µl	15 µl	15 µl	-	15 µl	15 µl
total amount	20 µl	30 µl	30 µl	1 µl	30 µl	30 µl

Tab. 4-26: SDS-PAGE 10% controlling gel experiment 19 [part 1] (TKU)

Slot	7	8	9	10
analyte	no antibody (I_{8mg}-batch) 50mM Na-Ac pH 4 + 1% BSA	no antibody (N_{8mg}-batch) 50mM Na-Ac pH 4 + 1% BSA	no antibody (I_{8mg}-batch) ACN 70% + 0.1% TFA + 1% BSA	no antibody (N_{8mg}-batch) ACN 70% + 0.1% TFA + 1% BSA
PBS	15 µl	15 µl	15 µl	15 µl
FSB 2x	15 µl	15 µl	15 µl	15 µl
total amount	30 µl	30 µl	30 µl	30 µl

Tab. 4-27: SDS-PAGE 10% controlling gel experiment 19 [part 2] (TKU)

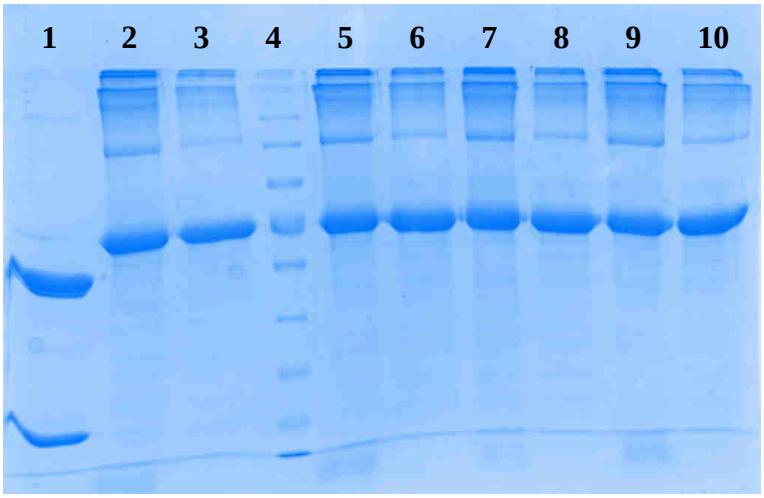

Fig. 4-27: SDS-PAGE 10% controlling gel experiment 19 (TKU)

Compared to *Fig. 4-25* the antibody bands are missing in *Fig. 4-27*.

4.3.20 Experiment 20 (BSA binding affinity – 2)

This experiment equals experiment 18 with the only difference that now no antibody had been applied. Thus, the supernatant was tested for residues of the applied BSA after incubation of the monoliths.

The tables and the parameters of the electrophoresis can be taken from experiment 19.

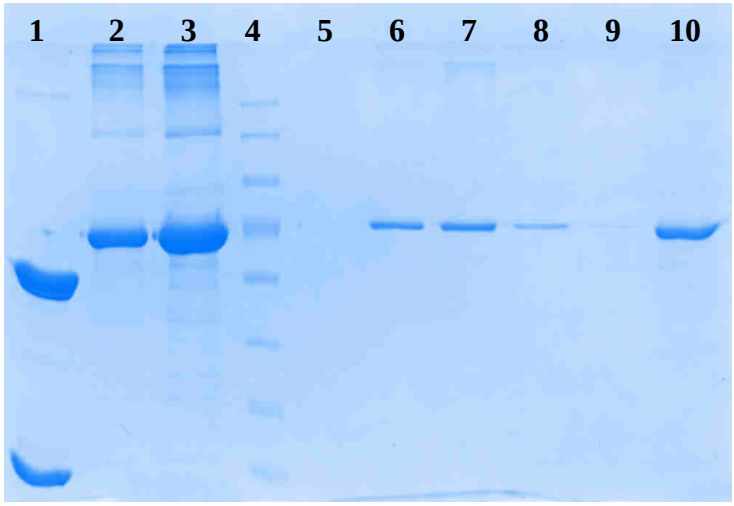

Fig. 4-28: SDS-PAGE 10% controlling gel experiment 20 (TKU)

When only BSA is applied to the stationary phases, the interaction between the analyte and the monoliths is much better (cf. *Fig. 4-28*). Compared to *Fig. 4-26* only small amounts of the analyte could be detected in the supernatant after incubation.

5. Conclusions and Outlook

In the course of this diploma thesis the suitability of different gel- and monolithic based stationary phases as a basis for a new continuous purification process for monoclonal antibodies was examined. The experimental work is divided into two parts: the first part focused on the development of an optimal gel matrix for continuous purification purposes; the second part concentrated on the synthesis and binding affinity of two monolithic stationary phases. All trials were conducted with a monoclonal antibody of the class IgG.

One important result of the first experimental part of the thesis is that buffer ions make agarose gels unstable. When the concentration of buffer in the gel was reduced, the castability and stability of the gel got influenced in such a positive way that it would make sense to perform some more trials with other buffer systems or with a modified buffer composition where only glycine or TrisHCl is used; also the pH-value of the buffer could be varied.
Proteins that are cleaned via native PAGE could only be used for analytical purposes due to the toxicity of acrylamide. Therefore, the native- and SDS-PAGE trials primarily served to get some basic information about electrophoresis in general as well as about the monoclonal antibody that was used for all the experiments in this thesis.

A positive result of the second experimental part of the thesis is the fact that the normal phase and the imidazoline-functionalized silica based monoliths both showed a binding affinity to the IgG-antibody. Unfortunately, the antibody couldn’t be eluted from the monoliths in a native state although different organic and inorganic solvents were used to elute the antibody by using hydrophobic and ionic interactions. At this point it has to be mentioned that the binding mechanism of the antibody to the monoliths is currently unknown. Also a low pH-value, the addition of 0.1% TFA to reduce the unspecific interactions between the antibody and the monoliths as well as the addition of BSA as a stabilizer to prevent a possible denaturation of the antibody by the stationary monolithic phases did not lead to the desired results. For further proceeding some other monoliths have to be synthesized; maybe monoliths with water inclusion would have a positive effect on the biomolecules. Furthermore, the normal phase and the imidazoline-functionalized monolith could be coated with protein A and G or with a mixture of the two bacterial proteins.

6. Working Materials

Chemicals

- 2-Mercaptoethanol (≥ 98%), 25 ml, Product# M3148-25ML, Lot#55396EMV, Sigma-Aldrich
- 2-Propanol (Rotisolv® HPLC), 2.5 l, Product# 7343.1, Lot# 956941, Carl Roth
- Acetonitrile (for HPLC), 2.5 l, Product# 83639.320, Lot# 15D091942, VWR Chemicals
- Albumin from bovine serum (minimum 98%, electrophoresis), 100 g, Product# A7906-100G, Lot# 038K0665, Sigma
- Ammonium hydroxide (33%), 2.5 l, Product# 6125, Lot# 0935701036, J.T.Baker
- Ammonium persulfate (≥ 98%), 25 g, Product# A3678-25G, Lot# 105K0700, Sigma
- Acetic acid (puriss., 99-100%), 2.5 l, Product# 27221-2.5L, Lot# SZBE2550V, Sigma-Aldrich
- Brilliant Blue R250 (for microscopy), 25 g, Product# 27816-25G, Lot# BCBG1219V, Sigma
- Bromophenol Blue sodium salt (for electrophoresis), 5 g, Product# B5525-5G, Lot# 069K1517, Sigma
- Diethylamine (≥ 99.5%), 250 ml, Product# 31730-250ML, Lot# BCBC2943V, Sigma-Aldrich
- Ethanol absolute (99.9%), 2.5 l, AAAH-5020-07025-201113, AustrAlco Österreichische Agrar-Alkohol Handelsges.m.b.H.
- Formaldehyde solution (min. 36.5% puriss.), 100 ml, Product# 33220, Lot# SZBA3430, Sigma-Aldrich
- Glycerol (≥ 99.5%), 1 l, Product# 191612-1L, Lot# 71596APV, Sigma-Aldrich
- Glycine (≥ 99%), 1 kg, Product# G7126-1KG, Lot# SZBE090AV, Sigma
- Hexadecyltrimethylammonium bromide (approx. 99%), 100 g, Product# H6269-100G, Lot# 117K0040, Sigma
- Human plasma, Lot# MN04051015, Institute of Pharmaceutical Sciences, University of Graz, working group of Univ.-Prof. Mag. Dr.rer.nat. Andreas Kungl
- Hydrochloric acid 1N (1 mol/l), 1 l, Product# K025.1, Lot# 1595113, Carl Roth

- Hydrochoric acid 25% (for analysis), 2.5 l, Product# 1.00316.2511, Lot# Z214516 044, Merck KGaA
- Methanol for HPLC LC-MS grade, 2.5 l, Product# 83638.320, Lot# 11Z3014, VWR
- N,N-Dimethylformamide (≥ 99.5%), 2.5 l, Product# 6251.2, Lot# 039102788, Carl Roth
- N,N,N',N'-Tetramethylethylenediamine (~ 99%), 25 ml, Product# T9281-25ML, Lot# BCBN1173V, Sigma
- Page Ruler™ Prestained Protein Ladder, 250 µl, Product# 26616, Lot# 00230161, Thermo Scientific
- Polyethylene glycol 400, 1 l, Product# 26602.290, Lot# 11B020500, VWR
- Poly(ethylene glycol) average Mn 6,000, 1 kg, Prod# 81260-1KG, Lot# BCBN8859V, Aldrich
- Potassium chloride (≥ 99.9995%), 25 g, Prod# 05257-25G-F, Lot# 0001424121, Fluka
- Potassium thiocanate (ACS reagent, ≥ 99.0 %), 500g, Prod# 81272, Lot# 82AD3070V, Sigma
- Rotiphorese® Gel 30 (37.5:1), 1 l, Prod# 3029.1, Lot# 204214417, Carl Roth
- Select agar, 250 g, Product# A5054-250G, Lot# 080M1575V, Sigma-Aldrich
- Silver nitrate (puriss. p.a.), 50 g, Product# 85228-50G, Lot# BCBC5599, Sigma-Aldrich
- Sodium carbonate (BioXtra, ≥ 99.0%), 500 g, Product# S7795-500G, Lot# BCBJ7599V, Sigma-Aldrich
- Sodium chloride p.a., 1 kg, Product# 1.06404.1000, Lot# K31900304 414, Merck
- Sodium dodecyl sulfate (≥ 98.5%), 100 g, Product# L3771-100G, Lot# SLBL1461V, Sigma
- Sodium hydroxide (≥ 99%), 1 kg, Product# 6771.1, Lot# 24677807, Carl Roth
- Sodium phosphate dibasic (cell culture tested), 1 kg, Product# S5136-1KG, Lot# 114K00741, Sigma
- Sodium phosphate monobasic monohydrate (ACS reagent, 98.0-102.0%), 1 kg, Product# S9638-1KG, Lot# 035K0178, Sigma-Aldrich
- Sodium sulfate (ACS reagent, ≥ 99%), 1 kg, Product# 239313-1KG, Lot# 138K6454 Sigma-Aldrich

- Sodium thiosulfate anhydrous (purum p.a.; ≥ 98%), 250 g, Product# 72049, Lot&Filling code 1309888 10207143, Fluka
- Tetraethyl orthosilicate (≥ 99%), 1 l, Product# 86578-1L, Lot# BCBG4027V, Aldrich Chemistry
- Triethoxy-3-(2-imidazolin-1-yl)propylsilane (≥ 97.0%), 50 ml, Product# 56760-50ML-F, Lot# BCBP9562V, Aldrich Chemistry
- Trifluoroacetic acid (99 %), 100 ml, Product# T6508-100ML, Lot# BCBF9404V, Sigma-Aldrich
- Trizma® base Primary Standard and Buffer (≥ 99.9%), 1 kg, Product# T1503-1KG, Lot# 000M5460, Sigma
- Urea (≥ 99.5%), 1 kg, Product# U1250-1KG, Lot# 119K1572, Sigma-Aldrich

Equipment

- Eppendorf Concentrator 5301, Serial# 0006057, Eppendorf AG
- FT-IR spectrometer Vertex 70, Bruker Austria GmbH
- GS-800 Calibrated Densitometer, Serial# WSD00712 A0061, System GS500, Bio-Rad Laboratories
- Mini-PROTEAN® Test Cell, Bio-Rad Laboratories
- Orion 3 Star pH Benchtop, snB14474, ThermoScientific
- PowerPac™ Basic, Serial# 041BR 42996, Bio-Rad Laboratories
- The Coulter Mixer, Cat# C5, Serial# 1257, Coulter Electronics Limited
- Universal Hood II, Serial# 721BR00412, Bio-Rad Laboratories

Software

- Image Lab™ Software, Bio-Rad Laboratories

Other Materials

- Cellstar® Tubes, 50ml, PP, graduated, conical bottom, blue screw cap, sterile, Cat# 227261, Lot# E14123NC, Greiner Bio-One GmbH
- Mini-Protean® Casting Stand, Pkg of 1, Product# 1653303, Bio-Rad Laboratories
- Mini-Protean® Comb, 10-well, 30 µl, Pkg of 50, Product# 4560013, Bio-Rad Laboratories
- Mini-Protean® System Glass Plates, Short Plates, 5 pack, Catalog# 1653308, Work Order# 60638, Bio-Rad Laboratories

- Mini-Protean® 3 System Glass Plates, Spacer Plates with 1.00 mm spacers, 5 pack, Catalog# 1653311, Work Order# 60679, Bio-Rad Laboratories
- Reaction Tubes, 1.5 ml, PP, graduated, attached cap, natural, Cat# 616201, Lot# E140935Y, Greiner Bio-One GmbH

7. List of Figures

8. List of Tables

9. References

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