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Immobilisation and evaluation of weak cation exchange ligands for immunoglobulin G purification

Verfasser

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1. Introduction

1.1. Preface

The aim of this diploma thesis is the investigation of a number of new mixed-modal hydrophobic weak cation exchange adsorbents (WCX) for the separation of antibodies through binding onto, and later elution from the functionalized surface of such an antibody binding adsorbents. The key issue is the development of a comparatively cheap as well as chemically stable WCX ligand with high affinity towards immunoglobulin G (IgG), which may be able to compete with affinity-based adsorbent Protein-A, which is presently the most used purification media for IgG.

The first part of this work is focused on the synthesis and immobilisation of promising weak cation exchange ligands onto a polymethacrylate based support material, the tentacle type Fractogel from Merck. An important goal in material preparation is to obtain high ligand densities and a high sanitation stability.

The subsequent evaluation of immobilized ligands includes antibody binding capacity measurements under static and dynamic conditions for stationary phases with different ligand densities, as well as, various buffer and sodium chloride concentrations.

The final step will be a comprehensive investigation of the most promising stationary phase with a real cell media supernatant. All collected flow-through fractions of a dynamic binding capacity study are to be analysed for their host cell protein (HCP) and DNA content.
An assessment of the most promising new stationary phases in terms of antibody enrichment and separation of impurities finalizes the evaluation process.

1.2. Structure and classification of antibodies

In mammal struggle against bacteria, viruses and other foreign particles the role of differentiating between “friend” and “foe” is the main task of antibodies.
Anitbodies, also known as immunoglobulins, are proteins which are built of four subunits. The approximately 450 to 550 amino acids (molecular mass: ~50 kDa) big subunit is called heavy chain, whereas the smaller one is called light chain, possessing around 200 amino acids (molecular mass: ~25 kDa)[1]. The antibody tetramer consists of 2 heavy chains and 2 light chains, which are connected via disulfide bridges, forming thereby a Y-shaped structure. In every immunoglobuin one light chain and one heavy chain are connected by disulfide bonds to form a symmetrical half of the tetramer. Disulfide bonds between the two heavy chains complete the structure.

Immunoglobulins are also divided into the five different classes IgA, IgD, IgE, IgG and IgM, which correspond to five different versions α, δ, ε, γ and μ of the heavy chain. Concerning the light chain there are only two different variations, κ and λ possible [1-4].
These different immunoglobulin classes are unequaly frequent in different body fluids where IgG is with ca.12 mg / mL the most abundant variant in blood-serum[4]. IgG with around 150 kDa is also the lightest of the immunoglobulins and can furthermore be divided into different IgG subclasses, IgG1, IgG2, IgG3 and IgG4, reflecting different γ-heavy chain variants[2].

Early investigation of antibodies showed that treating antibodies with a protease called papain results in the cleavage of the heavy chain at the so-called hinge region of an antibody, which provides different types of fragments.
The two fragments still capable of binding to its antigen are therefore called Fab, a short-cut for fragment-antibody-binding, whereas the remaining Fc fragment got its name from being easily crystallisable.
The Fab fragment consists of the light chain still connected via a disulfide bond with the amino terminal part of the heavy chain, whereas the Fc fragment contains the remaining carboxylic acid terminal parts of the two heavy chains.

Further investigation of the amino acid sequence revealed a module type assembly of these protein chains. The light chain consists of two modules, V_L and C_L, while the heavy chain is built out of 4 - 5 modules, V_H, C_H1, C_H2 and C_H3 for IgA, IgD and IgG, and additionally C_H4 for IgM and IgE (not shown in figure 1 below).

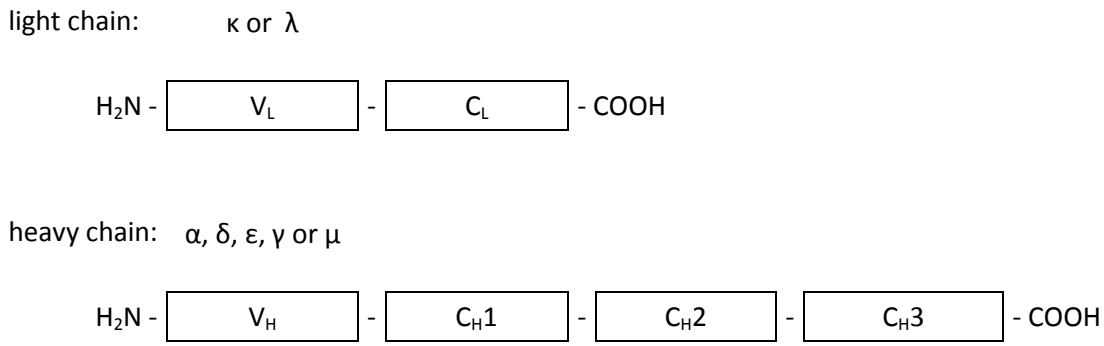


Figure 1: schematic composition of antibodies[4]

The names of these modules also reflect their tri-dimensional structure as well as their function. Hereby, V stands for “variable”, indicating that this module includes the antigen binding region (which is specific for each antibody), while C stands for “constant” and refers to its unvarying composition within a specific group of antibodies e.g. IgG.

The last variability in the structure of antibodies concerns the attachment of branched oligo-saccaride chains on sometimes multiple regions of the immunoglobulin, depending on the class as well as the organism from which they were expressed.
This biochemical modification is very common for surface-proteins as well as segregated proteins, and occurs at the so called post translational modification stage during the transportation of antibodies from the endoplasmic reticulum via the golgi apparatus to the surface of the cell[2] .

Antigen recognition is accomplished through three so called “hyper-variable regions”, which consists of 6 to 8 amino acids long loops on the surface of each variable module V_L or V_H, respectively. These antigen binding sites are also

called CDR´s, which stands for “complementary-determining regions” and their combination on both heavy and light chain is referred to as paratope[1].
The binding site on an antigen is known as epitope and as a matter of principle various parts of its surface can act as an interaction site for antibody binding. Therefore an antigen can possess multiple epitopes for many different antibodies.

Ultimately, antibodies can also be labelled as “polyclonal-antibodies” whether they are produced by several different clones of B lymphocyte cells or “monoclonal-antibodies” if they derive from one single cell culture line [1,2,5].

1.3. Production and purification of immunoglobulins

The antibody production process can be divided into two major steps. The first step, also referred to as “upstream processing”, covers the breeding and growing of cell cultures in bio-reactors, whereas the so called “downsteam processing” includes the subsequent purification of antibody from the harvested cell cultures broth [6-8,].
In-between these two steps, the host cell lysate requires an immediate stabilisation and protection from degradation of the target protein and goes hand in hand with inactivation or elimination of host cell proteases [9].
As with most separation and purification methods there is no one unique protocol that can be applied for every protein. Several possible routs may lead to the purified protein as end-product. However, a sequence of standardised purification steps with increasing selectivity for the target protein will most likely lead to the desired result [6,9].
Since the introduction of monoclonal antibodies [10], the producible concentration of immunoglobulin in cell culture feedstock increased continuously. Due to ongoing improvements in cell culture productivity and process efficiency, present immunoglobulin production yields of around 5 g/L will be increased soon to 8-10 g IgG/L [6,7,11].
These high antibody concentrations in cell culture solutions make high capacity purification methods necessary, at which affinity chromatography with Protein A resin with antibody binding capacities of over 50 g/L represents the state of the art in downstream processing of immunoglobulin G [6].

Nowadays, one crucial point that comes with Protein A chromatography, is the need to remove dissociated Protein A as a result from leaching of the separation column [12]. Therefore alternative affinity chromatographic ligands with similar IgG binding capacities are of great interest for small scale bench top purification as well as large scale industrial antibody production [6].

2. Experimental Section

2.1. Instrumentation

Dynamic binding capacity (DBC) studies were done on an Agilent 1200 HPLC-system composed of a binary pump, degasser, injector (not used), auto-sampler (not used) and multi wavelength UV/Vis detector from Agilent Technologies (Böblingen, Germany).
Additional equipment was a Gilson® Minipuls 3 peristaltic pump with 10 rollers and a 2 channels standard pump head from Gilson S.A.S., (Villiers-le-Bel, France), 6 port / 2 position switch valve and 13 port / 12 position switch valve from Agilent Technologies (Böblingen, Germany), pH/C-900 pH- and conductivity detector from GE Healthcare, (Vienna, Austria) and a SF-3120 automated fraction collector from Advantec MFS, Inc., (Dubin, USA).
Also for dynamic binding capacity measurement a “Super Compact” glass column system with10 mm inner diameter consisting of filling tube, thermo jacket, adapter sets, coupling elements, PEEK finger-tight fitting and 20 µm filter F membrane discs from Götec-Labortechnik GmbH, (Bickenbach, Germany) was employed.
Titrimetric determination of acidic ligands was done with a DL67 autotitrator with a 10 mL single burette drive and DG-111-SC Ag/AgCl pH-electrode from Mettler-Toledo GmbH (Giessen, Germany).

All IgG quantifications except “cell-feed” IgG were done on a SPECORD 50 UV/VIS spectrometer with WinASPECT software from Analytik Jena AG, (Jena, Germany) at a wavelength of 280 nm. 100-QS fused quartz cuvettes with 10 mm path length and 3500 µL capacity from Hellma Analytics, (Müllheim, Germany), as well as disposable UV semi-micro for 1500 µL or even UV micro cuvettes for

smaller sample volumes of 500 µL were ordered from Wagner & Munz GmbH, (Vienna, Austria).
Dialysis of cell culture feedstock was done in a 10 mL Spectra/Por Float-A-Lyzer tube with Biotech cellulose ester membrane form Spectrum Laboratories Inc., (Eindhoven, Netherlands)
IgG quantification of cell-feed samples and fractions was done on the same Agilent 1100 HPLC system using a 6-port / 2-position switching valve combined with a POROS® 20 µm Protein A ImmunoDetection® cartridge from Applied Biosystems,
(Vienna, Austria).

DNA-quantification was measured in ultramicro fluorescence cuvettes 105.251-QS with 3 x 3 mm path length and 45 µL measuring chamber volume, from Hellma Analytics, (Müllheim, Germany), with a LS 50B fluorescence spectrometer with FL WinLab software from PerkinElmer Life and Analytical Sciences, Inc., (Rodgau-Jügesheim, Germany).
Mini-PROTEAN 3 cell and PowerPac HC for gel electrophoresis were obtained from Bio-Rad Laboratories GmbH, (Vienna, Austria).

All ^{1}H and ^{13}C NMR measurements were done on a DRX400Mhz from Bruker by the NMR-group of the Institute of Organic Chemistry at the University of Vienna.
A Thermomixer® compact, a MiniSpin® centrifuge as well as micro test tubes (“Eppis”) and save-lock micro test tubes were purchased from Eppendorf AG, (Hamburg, Germany).
Scanning of gels was done with a HP Scanjet 4370 scanner from Hewlett-Packard Ges.m.b.H., (Vienna, Austria).
Also lots of standard labor equipment like fume hoods, rotary evaporator (rotavap), heating plates and various glassware etc. were used.

2.2. Chemicals

All chemicals from Aldrich and Fluka were ordered from Sigma-Aldrich Handels GmbH, (Vienna, Austria).
Chemicals from VWR were obtained from VWR International, (Vienna, Austria).
Fractogel® EMD Epoxy (M) with 40 – 90 µm particle size and 1000 µmol / g epoxide group density for dry gel was provided by Merck KGaA, (Darmstadt, Germany).
Electrophoresis kid and chemicals were purchased from Bio-Rad Laboratories Ges.m.b.H., (Vienna, Austria).
Beriglobin® polyclonal human IgG (160 mg / mL in 5 mL ampoule with 90% purity) were purchased from CSL Behring GmbH, (Vienna, Austria).
Gammanorm® polyclonal human IgG (165 mg / mL in 10 mL ampoule with 95% purity) were ordered from Octapharma AG, (Lachen, Switzerland) or (Vienna, Austria). Cell culture feedstock with IgG1, expressed by CHO cells was bought from Excellgene, (Monthey Valais, Switzerland)

Solvents :

Chemical :	CAS Nr.:	Supplier:
Methanol (MeOH) 98,5 % tech. grad	67-56-1	VWR
Water: deionized water (H_2O)		in-house
bidistilled water (bidest.)		in-house distilled
Dichloromethane (DCM)	75-09-2	Fluka
Ethyl acetate	141-78-6	various suppliers

Synthesis and Immobilisation:

Chemical :	CAS Nr.:	supplier:
Fractogel® EMD Epoxy (M) (40 – 90 µm)		Merck
Triethylamine (TEA) 99,5% puriss.	121-44-8	Fluka
3-Mercaptopropionic acid	107-96-0	Sigma-Aldrich
4-Aminobenzoic acid 99 %	150-13-0	Sigma-Aldrich
4-Mercaptobenzoic acid, tech., 90 %	1074-36-8	Sigma-Aldrich

Sulfanilic acid >99 %	121-57-3	Sigma-Aldrich
DL-Homocysteine thiolactone x HCl 99 %	6038-19-3	Fluka
N,N-Diisopropylethylamine (DIPEA)	7087-68-5	Fluka
3-Phenylpropionyl chlorid >98%	645-45-4	Fluka

Binding Capacity Studies:

Chemical :	CAS Nr.:	Supplier:
Gammanorm 165 mg/mL, 95 % IgG		Octapharma
Beriglobin 160 mg/mL, 90 % IgG		CSL Behring
Sodium chloride 99,5 %	7647-14-5	Fluka
Potassium dihydrogen phosphate 99,5 %	7778-77-0	Merck
di-Sodium hydrogen phosphate Dihydrate	10028-24-7	Fluka
Pluronic® F 68	9003-11-6	Sigma-Aldrich

SDS-PAGE:

Chemical :	CAS Nr.:	Supplier:
Sodium dodecyl sulphate (SDS) >99 %	151-21-3	Fluka
Ammoniumpersulfat (APS)	7727-54-0	Bio-Rad
Acrylamide/bis-Acrylamide (37,5 : 1) 30 % solution		Sigma-Aldrich
Acrylamide	79-06-1	
N,N´-Methylenebis(acrylamide)	110-26-9	
Tetramethylethylenediamine (TEMED)	110-18-9	Bio-Rad
Tris(hydroxymethyl)aminomethane (Tris)	77-86-1	Sigma-Aldrich
Tris-HCl running buffer 10x stocksolution		Bio-Rad
2-methyl-2-butanol (tertiary amyl alcohol)	75-85-4	Fluka
Protein marker for SDS-gels:		
Precision Plus Protein™ Unstained standards		Bio-Rad
Laemmli sample buffer, 30 mL		Bio-Rad

Chemicals use on various occasions:

Chemical :	CAS Nr.:	Supplier:
(Glacial) acetic acid	64-19-7	Sigma-Aldrich
Hydrochloric acid	7647-01-0	various suppliers
Sodium hydroxide	1313-73-2	various suppliers

0,1 M HCl was prepared with 8,04 mL concentrated hydrochloric acid adjusted to 1000 mL with bi-distilled water.

If not otherwise indicated, all chemicals used were of p.a. or HPLC-quality.

2.3. Synthesis and Immobilisation of selectors onto Fractogel® EMD Epoxy (M)

Fractogel® EMD Epoxy (M) with a particle size between 40 – 90 µm and pore size of around 800 Å is a polymethacrylate based resin produced with tentacle grafting technology from Merck KGaA. It was chosen as a support material for antibody purification media, because it is robust against cleaning-in-place (CIP), which is the sanitation procedure with 0.1-0.5 M sodium hydroxide solution. It can be versatile functionalised and it is available for different applications like ion-exchange, immobilized-metal-ion-affinity or size-exclusion-chromatography.

2.3.1. Gel A: Immobilisation of 3-mercaptopropionic acid

3-Mercaptopropionic acid was chosen as one of the first cationic exchange ligands in order to have a simple reference material as well as some sort of corner stone for amid bond based immobilisation procedures. Also some dynamic and static binding capacity studies were already made by group members. Therefore an established recipe for the immobilisation of the 3-mercaptopropionic acid, shown in the fig. 2 below, was succeeded and provided reproducible binding results [13].

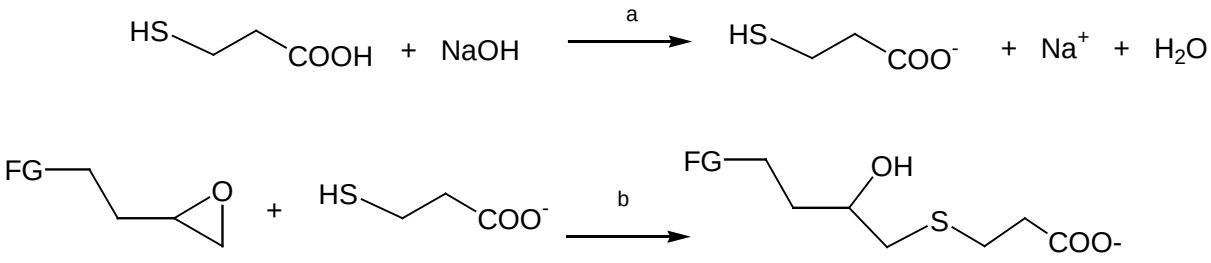


Figure 2: Conditions: (a) Neutralisation of 3-mercaptopropionic acid with NaOH (pH < 12) and dilute with water and MeOH (b) reflux under nitrogen atmosphere under mechanical stirring at 70°C, over night

Elemental analysis for the washed and dried modified Fractogel yielded a ligand density of 1234 µmol S / g gel.

2.3.2. Gel B: Immobilisation of 4-mercaptobenzoic acid

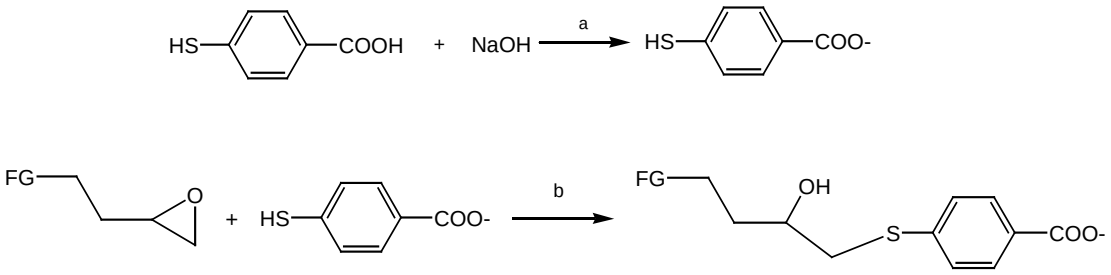


Figure 3: Conditions: (a) Neutralisation of 4-mercaptobenzoic acid with NaOH (pH < 12) and dilute with water (b) reflux under nitrogen atmosphere under mechanic stirring at 70°C, over night.

1,309 g (7,64 mmol) of 4-Mercaptobenzoic acid and 20 mL of water/methanol (50:50, (v/v)) were scaled into a 250 mL round bottom flask. Due to low solubility of 4-mercaptobenzoic acid, 1,6 mL (8 mmol) of 5 M NaOH were added. This mixture was refluxed for 10 minutes at 70°C under nitrogen atmosphere to form the corresponding sodium salt of the acid. In order to get a high-yield-immobilisation this solution was concentrated to ~10 mL on the rotary evaporator. A three-neck round bottom flask containing 1,5837 g Fractogel EMD Epoxy (M) (40 – 90 µm) and 5 mL methanol was flushed with nitrogen for 5 minutes before addition of the 4-mercapto sodium benzoate solution and 700 µL (5 mmol) of

triethylamin. This mixture was agitated with a mechanic stirrer and refluxed under nitrogen atmosphere at 70 °C over night. To remove residual reagent, the modified gel was washed several times with bi-distilled water, 0,1 M HCl solution, 0,5 M NaOH solution and methanol, respectively.
A ligand density of 950 µmol S / g Fractogel for a washed and dried equivalent was determined by elemental analysis.

2.3.3. Gel C: Immobilisation of 4-mercaptobenzoic acid with reduced ligand density

In order to find the most suitable immobilisation condition, preliminary testings were done with 100 mg of epoxide Fractogel in safe-lock Eppendorf micro tubes with different concentrations of 4-mercaptobenzoic acid. To every preparation an appropriate amount of 5 M NaOH as well as triethylamin was added and the pH-value was adjusted to be alkaline before each Eppendorf tube was filled up to the 1,5 mL mark with bi-distilled water. The samples were held at 65 °C and agitated over night on an Eppendorf thermomixer.
Employing an equimolar proportion of 0,1 mmol 4-mercaptobenzoic acid and 0,1 mmol epoxide groups on 100 mg Fractogel EMD an immobilisation rate of 58 % (576 µmol S / g Fractogel) could be achieved. An up-scaling of this small batch test-immobilisation for 1,5 g Fractogel, using 1,5 mmol of 4-mercaptobenzoic acid and prepared as prescribed above led to an immobilisation rate far above the desired rate of ~ 50 %. The elemental analysis for this preparation yielded a ligand density of 819 µmol S / g Fractogel.
Such deviation in ligand density can occur, when reaction conditions are to be up-scaled, since the reagent concentration has to be drastically changed due to the increase of the reaction batch.

2.3.4. Gel D: Immobilisation of 4-mercaptobenzoic acid with 50 % ligand density

As the upscaled immobilisation protocol yielded a higher ligand density than expected, a subsequent approach for a ligand density of 50 % on Fractogel gave the desired product.

In this reaction 0,155 g (0,905 mmol) of 4-mercaptobenzoic acid was coupled onto 1,5335 g (1,5335 mmol) of Fractogel EMD as previously described in 2.3.2 using the corresponding amounts of NaOH and TEA.

Elemental analysis showed a ligand density of 514 µmol S / g. After end-capping with 210 µl (3 mmol, ~4-fold molar excess) of β-mercaptoethanol under basic conditions as described in detail later, the ligand density of 561 µmol S / g Fractogel showed that residual epoxide groups were still present after ligand attachment.

2.3.5. Gel E: Immobilisation of 4-Aminobenzoic acid (para-aminobenzoic adic)

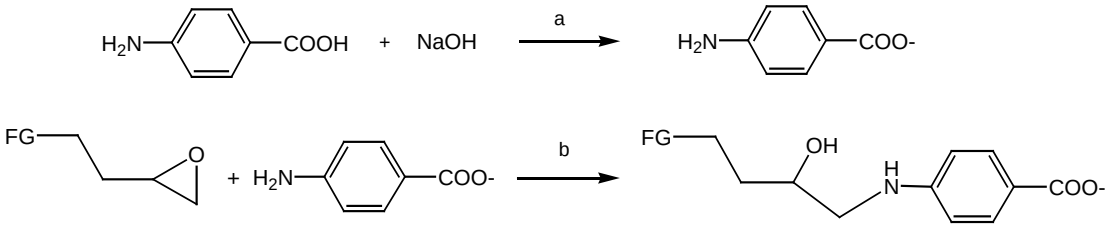


Figure 4: Conditions: (a) Neutralisation of 4-aminobenzoic acid with NaOH (pH < 12) and dilute with water (b) reflux under nitrogen atmosphere under mechanical stirring at 70°C, over night

For another immobilisation test, 60 mg (0,43 mmol, ~4-fold molar excess) 4-aminobenzoic acid, dissolved in 800 µL MeOH and 0,44 mmol of TEA was shaken with 100 mg Fractogel at 70 °C over night on an Eppendorf Thermoshaker. Elemental analysis showed very promising 869 µmol N / g ligand density, but another immobilisation test with 0,44 mmol NaOH instead of TEA provided only 563 µmol N / g Fractogel. This inconsistency can not be explained completely but maybe it has derived from residual TEA due to insufficient washing of the Fractogel, which has led to incorrect ligand densities.

For a bigger batch, 1,5252 g of Fractogel in 15 mL of MeOH were mixed with 850 µL (6,1 mol; ~4-fold molar excess) TEA and 849,7 mg (6,13 mol) of 4-aminobenzoic acid. The reaction slurry was mechanically stirred and refluxed over night at 70 °C under nitrogen and was then washed as previously described.
Elemental analysis of this immobilisation showed a ligand density of only 146 µmol N / g Fractogel which was the lowest observed yet.
This indicated that the epoxide Fractogel offers a lower reactivity for an amino-functionalized benzoic acid compared to a thiol functionalized variant and therefore no DBC or SBC studies were performed with Gel E.

2.3.6. Gel F: Immobilisation of 4-aminobenzene sulfonic acid (sulfanilic acid)

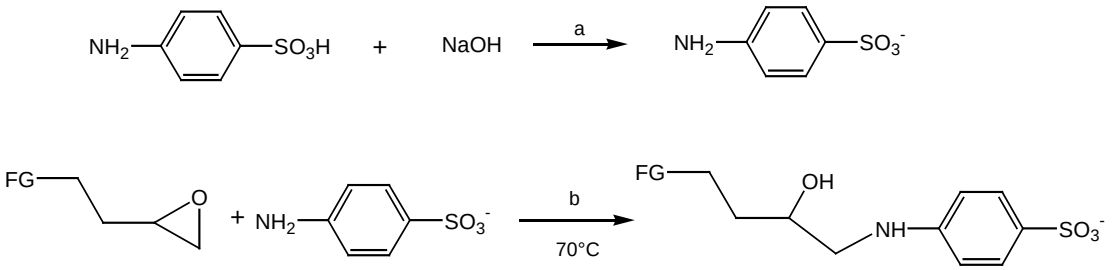


Figure 5: Conditions: (a) Neutralisation of 4-aminobenzene sulfonic adic with NaOH (pH < 12) and dilute with water (b) reflux under mechanical stirring at 70°C, over night

To find the best immobilisation condition for 4-aminobenzene sulfonic acid (sulfanilic acid) subsequent tests with 1, 2, 4 and 5-fold molar excess of sulfanilic acid were performed. Each sulfanilic acid equivalent was dissolved in an equimolar amount of 5 M NaOH and TEA before it was added to 100 mg of Fractogel and adjusted to 1 mL with H_2O. Sadly, according to elemental analysis, the immobilization yield for these tests were not higher than 246 µmol / g Fractogel.
In order to obtain the highest possible ligand density, 1,4622 g of Fractogel was suspended in a solution of MeOH and water at a ratio of 50:50 (v/v) and mixed with 2,558 g (14,622 mmmol, 10-fold molar excess) of 4-aminobenzene sulfonic acid. After addition of an appropriate amount of NaOH and TEA, the mixture was agitated at 70°C overnight as described above. This resulted in a ligand density

of 389 µmol S / g and 551 µmol N / g Fractogel, respectively. The excess of nitrogen over sulphur could account for residual triethylamine and therefore ligand density was referred to the sulphur content.
Since this ligand density was still a bit low, a similar approach with 20-fold molar excess of 4-aminobenzene sulfonic acid was prepared and this time the ligand density was 472 µmol S /g Fractogel or according to the nitrogen value, 480 µmol N / g Fractogel.

2.3.7. Gel G: Synthesis and immobilisation of 2-(3-phenylpropanamido)-4-sulfanylbutanoic acid (cinnamoyl-homocysteine)

Cinnamoyl-homocysteine was chosen as ligand molecule, because it exhibits an aromatic ring in combination with a carboxylic acid group.

2.3.7.1. Synthesis of N-(2-oxothiolan-3-yl)-3-phenylpropanamide

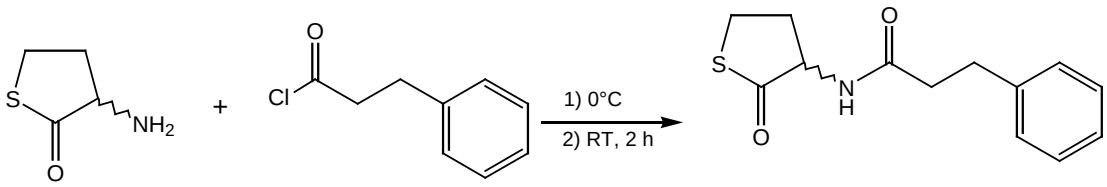


Figure 6: Conditions: Coupling of DL-homocystein thiolactone with 3-phenylpropyonyl chloride in the presence of DIPEA in DCM under reflux and magnetic stirring at RT, over night

1,58 g (10,3 mmol) of DL-homocysteine thiolactone hydrochloride and 3,58 mL (20,6 mmol) of N,N-diisopropylethylamine (DIPEA, Hünigs base) were dissolved in 6 mL dichlormethane and cooled to 0°C in an ice bath.
In a separate container, 1,56 ml (10,3 mmol) 3-phenylpropyonyl chloride was dissolved in 4 mL dichlormethane and cooled to 0 °C, before drop-wise addition to the homocysteine thiolactone solution. The reaction was stirred at room temperature over night. After a big part of the dichlormethane was removed on the rotavapor, the product was then extracted with ethyl acetate / citric acid (10 % w/v) from the concentrated solution. The organic phase was again washed with

10% citric acid solution and concentrated. The product was purified by flash chromatography (Yield: 1,74 g, 63,1 %) and its purity was confirmed by NMR.

TLC eluent: ethyl acetate : methanol / 2:1 (v/v)

Spectroscopic data: 1H NMR (CDCl3): δ 7,28 (m, 2H, aromatic), 7,20 (m, 3H, aromatic), 5,85 (broad s, 1H, -N**H**), 4,48 (m, 1H, -C**H**-NH), 3,33 (m, 1H, -S-C$\mathbf{H_a}H_b$), 3,23 (m, 1H, -S-$CH_a\mathbf{H_b}$), 2,97 (t, 2H, -C**H2**-CO), 2,91 (m, 1H, -S-CH_aH_b-C$\mathbf{H_c}H_d$-CH-NH), 2,55 (m, 2H, -CH_2-C$\mathbf{H_2}$-Ph), 1,80 (m, 1H, -S-CH_aH_b-$CH_c\mathbf{H_d}$-CH-NH).

2.3.7.2. Immobilisation of 2-(3-phenylpropanamido)-4-sulfanylbutanoic acid (cinnamoyl-homocysteine)

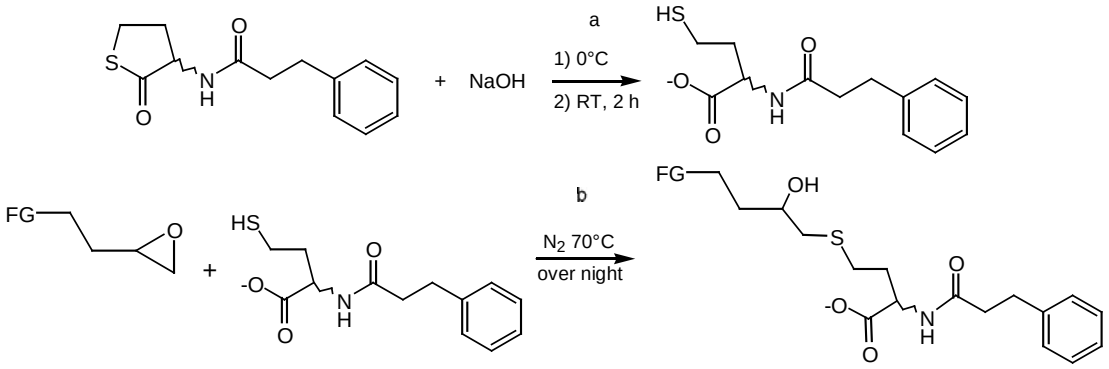


Figure 7: Conditions: (a) Ring opening of cinnamoyl-homocysteine with NaOH (b) Coupling onto Fractogel EMD in the presence of NaOH, reflux under nitrogen atmosphere using mechanic stirring at 70°C, over night

1,74 g of cinnamoyl-homocystein were dissolved in methanol and cooled in an ice bath to 0°C. To the cooled solution, 3,9 ml of 5 M NaOH (3-fold molar excess) was added dropwise. TLC showed that the reaction was completed after ~2 hours of agitation at room temperature with a magnetic stirrer. A small aliquot of that solution was taken beside for NMR and MS analysis. The rest was added to a suspension of 1,455 g Fractogel EMD in 4 mL MeOH. This mixture was then refluxed at 70 °C under nitrogen atmosphere and agitated with a mechanic stirrer, over night. Elemental analysis for the washed and dried gel showed a ligand density of 713 µmol S / g Fractogel.

2.3.8. Endcapping of residual epoxygroups

In order to prevent any non-specific interactions, the remaining residual epoxide groups had to be blocked or at least inactivated after ligand immobilization. The endcapping reaction was performed under conditions similar to the corresponding immobilisation reactions.

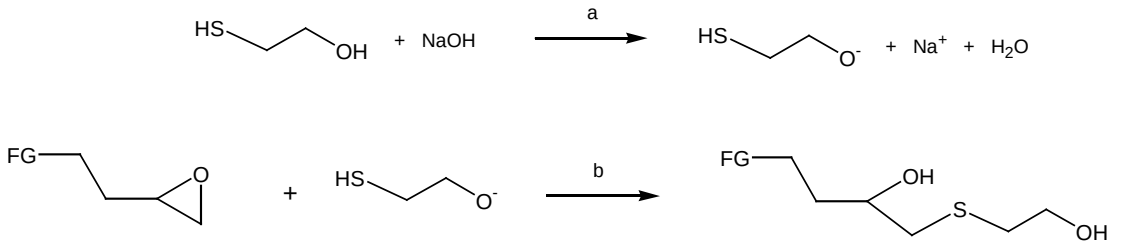


Figure 8: Conditions: Coupling of β-mercaptoethanol onto Fractogel EMD in presence of NaOH and TEA in Water / MeOH, reflux under nitrogen atmosphere under mechanical stirring at 70°C, over night

For each modified Fractogel the concentration of residual epoxide groups was calculated from the additional S-value of the elemental analysis of the β-mercaptoethanol endcapped adsorbent. The protocol is as follows: A 5-fold amount of β -mercaptoethanol was dissolved in an equimolar amount of NaOH (5 mol/L). This solution was then added to the suspension of the corresponding Fractogel in MeOH:H_2O / 1:1 (v/v) and equivalent amount of TEA. The reaction was mechanically stirred under nitrogen atmosphere at 70°C, over night. All Fractogels were washed as described above and ligand densities were determined via elemental analysis.

2.4. Ligand density measurement

The ligand density was determined via elemental analysis by the Micro Analytical Laboratory at the University of Vienna. Additionally the ligand densities were also verified through titration with 0,1 M NaOH (4 g NaOH pellets in 1 L bidest. water).
For the elemental analysis, an aliquot of ~100 µL of methanol washed gel was placed into an Eppendorf micro test tube with pierced aluminium foil cap and dried at 60°C over night. To remove residual solvent, the gel sample was

additionally vacuum dried at 60 °C, over night and submitted for elemental analysis.

For the titration, 100 to 250 mg of modified gel (depending on the expected ligand coverage) was washed and equilibrated in 0,1 M HCl for at least 30 minutes to ensure complete protonation of the acidic ligands. The gel sample was then washed several times with deionized water (and methanol if wettability was insufficient) until the pH-level was neutral. The washed slurry was transferred into a 100 mL beaker and filled up to the 60 mL mark with 0,5 M NaCl solution to ensure a sufficient conductivity. All titrations were performed with a Mettler DL67 autotitrator with mechanical stirrer and attached Ag/AgCl pH-elektrode (DG-111-SC). The titrator was programmed to add 2 mL of 0,1 M NaOH in 10 µL increments, every 30 – 300 seconds, depending on the drift of the pH-electrode, which should stay constant at ≤ 0,3 mV for 5 seconds. After the titration, the gel was transferred to a weighted glass funnel where it was subsequently washed with water and methanol. The gel sample was first dried over night at 60 °C and then under vacuum until a constant weight was reached. The ligand density was calculated from the consumption of 0,1 M NaOH at the equilibirium point (highest slope of the titrationcurve) divided by the mass of the vacuum dried Fractogel.

2.5. Static-Binding-Capacity (SBC)

The first survey of SBC measurements for the 4-mercaptobenzoic acid Fractogel at seven different pH-values (between pH 4,5 and 7,5). Figure 9 below shows the results of the DBC studies done by Dr. Heiner Graalfs at the Laboratory for Polymer Derivatization, Performance & Life Sciende Chemicals, Merck KGaA.

Thereby the optimum test condition for a maximum binding of IgG at various pH-values and sodium-chloride concentrations was identified.

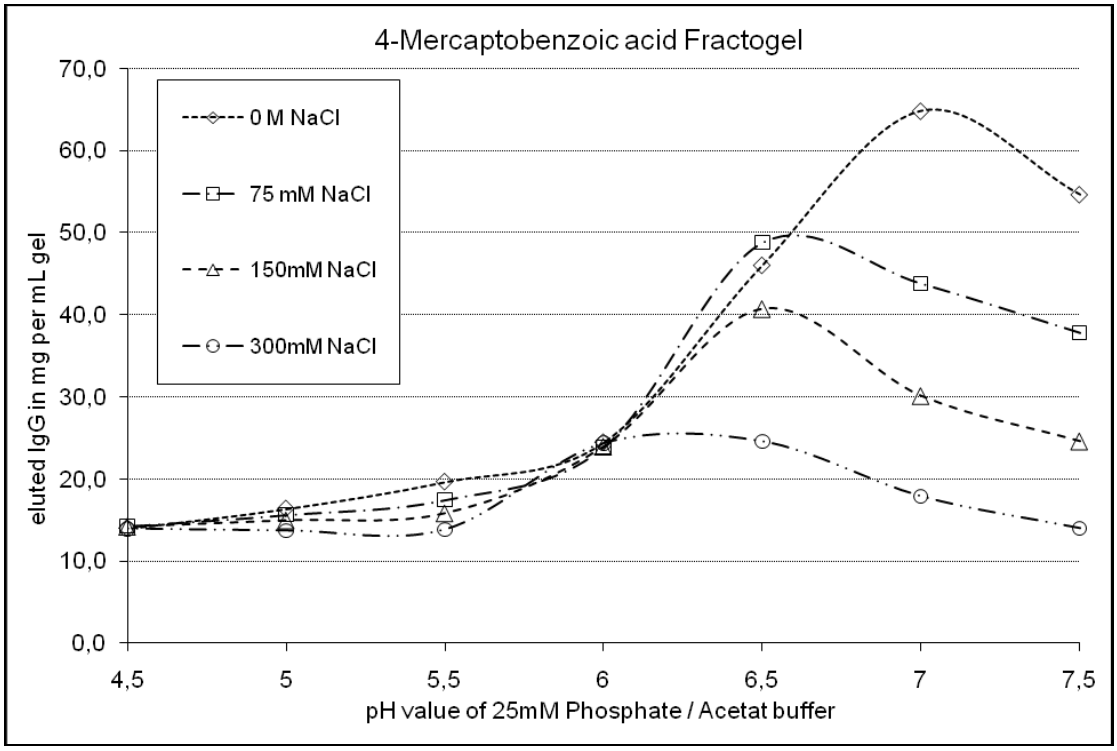


Figure 9: Recovered IgG in elution buffer in mg / mL Fractogel

Subsequent time depending SBC measurements for the 4-mercaptobenzoic acid Fractogel were performed with 5 mg/mL Gammanorm® IgG in PBS buffer. The ideal buffer conditions were taken from the diagram in Fig. 9, where a PBS buffer with 75 mM NaCl and 25 mM phosphate at pH 6,5 provided the maximum binding capacity for IgG.

Prior to all measurement the eligible gel was washed 1-2 times with 0,1 M HCl (to ensure protonation of the ligand) and at least two times with bi-distilled water until a neutral pH was achieved. The gel was briefly vacuum filtrated for 10 seconds and 30,0 mg of wet gel was scaled into Eppendorf reaction tubes.

To each Eppendorf tube, 1 mL of IgG solution was added and then shaken at 25 °C with 1400 rpm for 5, 15, 40, 60, 120 and 240 minutes on the Eppendorf thermomixer.

After the respective time of agitation, the gel was centrifuged for 60 seconds at 13.400 rpm and each supernatant was transferred into a fresh Eppi. The test gels were washed and shaken three times with 1 mL of PBS-washing-buffer (75mM NaCl and 25 mM Phosphate, pH 6,5) and then three times for 5 minutes with 1 mL of PBS-elution buffer (1 M NaCl and 25 mM phosphate buffer, pH 6,5). These wash and elution supernatants were also collected in Eppendorf test

tubes, after the samples were centrifuged for 60 seconds at 13.400 rpm. All samples were stored in the refrigerator at 4°C or in the freezer at 0°C, if not measured immediately. Quantification of IgG was done at 280 nm using UV-cuvettes on an UV/Vis Spectrometer. A calibration curve with external standards ranging from 0,1 to 1,1 mg/mL ($y = 1,3551x + 0,0812$ and $R^2 = 0,9994$) was used for the Gammanorm® IgG quantification in PBS buffer.

2.6. Dynamic-Binding-Capacity (DBC)

All tested gels were suspended in 1 M NaCl and allowed to swell for about 2 hours. Afterwards, the gels were transferred into 5 mL graduated cylinders and left to settle. The gel volume was adjusted to 1,1 mL, before it was transferred into the bio-chromatographic glass column with the lower variable piston with a 20 µm membrane filter disk already connected and tightened. The gel was allowed to sediment by gravity on top of the 20 µm filter disc inside the buffer filled column, in order to obtain an even surface. Then the second 20 µm filter disc was carefully placed above the gel. At last the second variable piston was inserted and the gel was purged with loading buffer, before it was compressed by 10 % to a volume of 1,0 mL which correspond to a 13 mm bed height.

The DBC measurements were done with an IgG concentration of 1 mg / mL (Beriglobin® IgG or Gammanorm® IgG) in 25 mM phosphate buffer with 75 mM and 150 mM NaCl at pH 6,5 or 7,4, respectively.

Also two DBC studies under more commercial (industrial) practice conditions with “mock feed solution” including 1 mg / mL Pluronic® F-68 with 150 mM NaCl at pH 6,5 as well as “dialyzed cell culture supernatant” (cell-feed) containing 1 mg / mL Pluronic® F-68 and 1 mg / mL NaN_3 with 75 mM NaCl at pH 6,5 were measured. It has to be noted, that Protein A affinity chromatography was carried out as stated in chapter 2.7.2 below and gave a initial IgG1 content of 32,9 µg / mL, which was raised by the addition of polyclonal IgG (Beriglobin) to the desired concentration of 1 mg / mL.

The peristaltic pump was used to load the IgG solution with 0,6 mL / min. onto the adsorbent in the column, until the UV/Vis- and conductivity- detector indicated a complete saturation of the gel with IgG by a plateau in the break-through curve.

All DBC studies were carried out until complete breakthrough of loaded IgG was achieved although the continuous increase in the so-called plateau comes with the risk of unspecific binding. This drawback had to be accepted in order to get comparable binding circumstances. For all tested gels, 100% breakthrough were reached after application of 200 – 320 mL of IgG solution, depending on pH value and NaCl concentration of the IgG feed solution.

Washing of the gel was done via HPLC pump with 80 mL PBS washing buffer with the same pH and salt concentration as the loading solution.The bound IgG was eluted with a PBS buffer solution containing 25 mM phosphate and 1 M NaCl at an adequate pH, which was applied as a one-step-gradient with a total volume of 100 mL elution buffer. Cleaning-in-place and regeneration was done with at 60 mL 0,5 M NaOH sanitizer solution followed by the washing of the gel with PBS washing buffer. During DBC measurements, 20 mL sample fractions were collected which correspond to a flow-through time of 33 minutes and 20 seconds per sample. For one whole run, a total volume of 500 mL were applied over a time period of 14 hours.

In the case of dialyzed cell culture feedstock, the collected fraction volume was reduced to 10 mL to refine the DBC measurement. Also elution was done as linear sodium-chloride gradient starting with 0 mM NaCl in 25 mM phosphate buffer and pH 6,5, which was raised to 1 M NaCl within 110 mL, followed by elution under isocratic salt conditions for 10 mL.

2.7. IgG quantification

IgG quantification for phosphate/acetate buffered solutions and “mock feed solution” that contained no other protein than IgG was done via UV/Vis absorption measurement at 280 nm.

IgG from dialyzed cell culture supernatant solutions containing a mix of proteins, DNA etc. had to be first isolated in order to remove all disturbing impurities. For the capture step an on-line HPLC method using an Agilent 1200 HPLC-system and a Protein-A ImmunoDetection® cartridge was used

2.7.1. UV/Vis absorption measurement

All measurements were performed on a Specord 50 UV/Vis spectrometer from Analytik Jena with 100-QS fused quartz cuvettes with 10 mm path length from Hellma Analytics. Separate calibration curves between 0,2 and 1,3 mg IgG / mL were generated for each buffer combination and each DBC and SBC run. Samples with an absorbance above 1,5 were diluted by as far as 1:5 with buffer solution to be in the linear range of the calibration curve. For sample volumes less than 1 mL disposal UV micro or semi-micro cuvettes were used for measurement and quantification of the standard solutions, respectively.

2.7.2. Quantification of IgG in cell culture feedstock with Protein A affinity chromatography

With additional proteins and DNA in the cell culture supernatant, antibody quantification could only be done after capturing of IgG onto a Protein-A affinity chromatographic column followed by elution and quantification of bound IgG.
The measurements were carried out by following an established routine[14] with a flow rate of 1 mL / minute, detection wavelength was set to 280 nm and the column temperature was adjusted to 25 °C. In order to avoid sample degradation, the sample tray was cooled to 10°C.
Each measurement sequence started with the injection of 100 µL of cell culture feed sample, followed by 15 minutes purging with application buffer containing 10 mM phosphate and 150 mM sodium chloride at pH 7,2. The bound IgG was then eluted with a 12 mM hydrochloric acid solution with pH 2 containing 150 mM sodium chloride for another 15 minutes, followed by re-equilibration of the Protein-A column for at least 6 minutes with the application buffer.
The measurements started with samples possessing the lowest IgG-concentration such as the washing-samples, followed by elution samples in reversed order and finishing with the cell culture supernatant application samples. In between sample injections with different sodium-chloride concentration as well as every 5-6 sample injection a blank sample was injected in order to obtain the

background noise value, which was subtracted from the sample peak as well as to detect a possible carry-over of IgG due to a column overload with IgG.

2.8. DNA quantification in cell supernatants:

Quantification of dsDNA in cell supernatant was done using the Quant-iT PicoGreen dsDNA reagents kit from Invitrogen [15].
The Quant-iT reagents kit contains PicoGreen as DNA binding reagent, a cyanine dye alternatively used instead of ethidium bromide, because it allows the trace determination of DNA in solution with increased intensity of fluorescence, highly favouring thereby double stranded DNA (dsDNA) over single stranded DNA (ssDNA) or RNA [16].

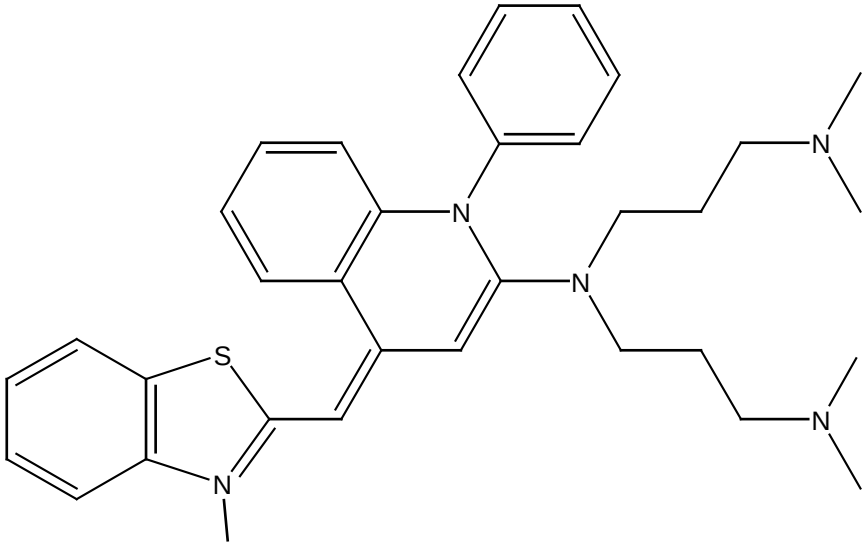


Figure 10: Structure of PicoGreen according to Zipper et al. [17]

The fluorescence measurements were carried out in ultra-micro cuvettes (45 µL cell volume) to reduce wastage of PicoGreen reagent, standard DNA for calibration as well as sample amount. Nonetheless calibration curves with linearity range of over 3 orders of magnitude were generated as shown in the fig.11 below.

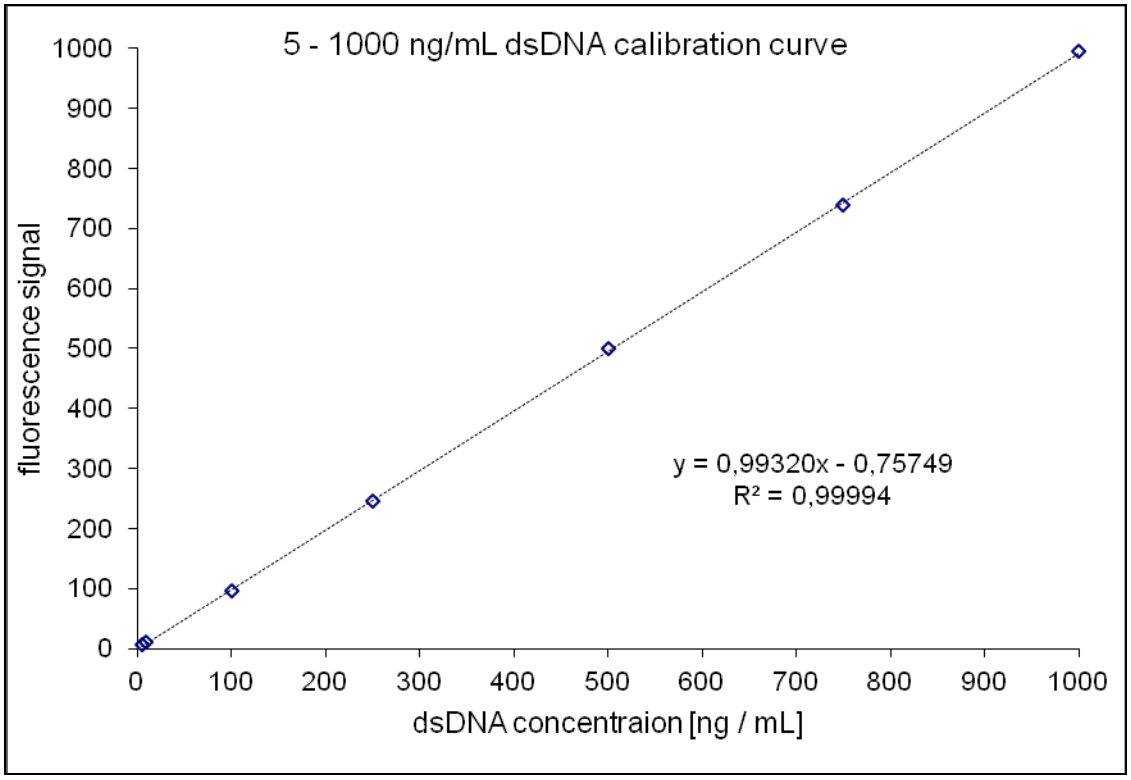


Fig. 11: Bacteriaphage λDNA calibration curve established with PicoGreen

2.8.1. Required Chemicals:

- PicoGreen dsDNA assay kit, (Invitrogen):
  - 25 mL 20xTE (Tris-EDTA) stock solution:
    200 mM TrisHCl, 20 mM EDTA pH=7,5
  - 1 mL dsDNA standard solution:
    100/mL Bacteriaphage λDNA in TE buffer
  - 1 mL PicoGreen dye stock solution in DMSO (light protection is advisable)
- HPLC-grad Ethanol (EtOH) or if not available Methanol (MeOH)
- Bi-distilled water: distilled “in-house”, sterile filtered through a 2 µm cellulose acetate membrane filter followed by ultrasonification for at least 10 minutes.

2.8.2. Preparation of reagents

Powder free examination gloves were worn at all stages from reagent preparation to measurement in order to avoid contamination of samples with DNA or DNase.
All solutions were prepared in DNA and DNase free Eppendorf Micro Test Tubes (1,5 mL), or "PCR tubes" (0,5 mL) for lower sample volumes. The diluted PicoGreen reagent solution was stored in a black DNA and DNase free Eppendorf test tube (if not available, it is possible to blacken an Eppendorf test tube with a black permanent marker and/or cover it with aluminium foil). Also the Eppendorf tube holder with test tubes containing the reagents, samples and mixed solutions was covered with aluminium foil to protect the reagents from light.

First, 3 mL of 1xTE buffer solution (10mM Tris-HCl, 1 mM EDTA, pH 7,5) were prepared by a 20-fold dilution of Tris-EDTA stock solution with sterile filtered bi-distilled water. The solution was prepared in two 1,5 mL Eppendorf test tubes and mixed thoroughly on the Eppendorf tube shaker for 30 seconds at room temperature.
Secondly, 150 µL of 2 µg/mL dsDNA solution were prepared through a 50-fold dilution by mixing 3 µL of the 100 µg/mL Bacteriaphage λ DNA in TE buffer with 147 µL of 1xTE buffer and were mixed on an Eppendorf tube shaker (1400 rpm, 30 seconds, room temperature).
With this 150 µl of 2 µg/mL λ-DNA solution a set of 8 diluted DNA standard solutions could be prepared, producing a 8-point straight calibration line. Note that for each new set of test samples , a new calibration curve was established. (However, in case of standard addition measurements, additional 60 µL of 2 µg/mL λ-DNA solution for each sample had to be taken into consideration.)
The PicoGreen reagent solution in 1xTE buffer was prepared in a black Eppendorf test tube by diluting 7,5 µL of PicoGreen stock solution (in DMSO) with 1492,5 µL TE buffer. After mixing for 30 sec., the PicoGreen reagent solution was placed in a test-tube rack and covered with aluminium foil for further protected from light.

The table below shows the required volumes of PicoGreen reagent, λ-DNA and 1xTE buffer in order to establish a 8 point standard calibration curve between 12,5 – 1000 ng dsDNA / µL

	TE buffer solution	λ-DNA solution	PicoGreen solution	End-volume	DNA conc. in endvolume
	40 µL		40 µL	80 µL	0 ng/mL (blank)
	39,5 µL	0,5 µL	40 µL	80 µL	12,5 ng/mL
	39 µL	1 µL	40 µL	80 µL	25 ng/mL
	38 µL	2 µL	40 µL	80 µL	50 ng/mL
	35 µL	5 µL	40 µL	80 µL	125 ng/mL
	30 µL	10 µL	40 µL	80 µL	250 ng/mL
	20 µL	20 µL	40 µL	80 µL	500 ng/mL
	10 µL	30 µL	40 µL	80 µL	750 ng/mL
		40 µL	40 µL	80 µL	1000 ng/mL
sum:	251,5 µL	108,5 µL	360 µL		

Table 1: Reagent volumes for a dsDNA standard curve: (12,5 – 1000 ng/mL DNA)

2.8.3. Measurement of the dsDNA calibration curve

All measurements were made on a Perkin Elmer LS 50B Luminescence spectrometer and FL WinLab software from PerkinElmer using an excitation wavelength of 480 nm and emission wavelength of 520 nm, according to the Quant-iT PicoGreen manual [15].

At the very first measurement with the Perkin Elmer LS 50B Luminescence spectrometer it was found that, with an integration time of 1 second, an excitation slit width of 10 nm and an emission slit width of 8 nm the spectrometer provided a fluorescence signal of approximately 800 – 900 units (out of maximal 999,999) for the highest dsDNA standard concentration measured.

Prior to each measurement the ultramicro fluorescence cuvette was washed carefully with bi-distilled water to avoid carry-over of DNA followed by EtOH (or MeOH) to remove residual water. The cuvette was then left to stand bottom up for about 5 minutes to dry.

Following the Quant-iT instruction the scheduled volumes of 1xTE buffer and DNA standard were mixed together in a small 500 µL Eppendorf test tube to give a preliminary volume of 40 µL as also shown in table 1 above. After adding 40 µL of PicoGreen solution and the DNA test solution was mixed thoroughly with a pipette, the test tube was placed in a light protected box and left for a pre-assigned reaction time of 3 to 4 minutes. The whole 80 µL were then transferred into the ultramicro-fluorescence cuvette and the fluorescence activity was determined.

An alternative measurement procedure was investigated, where the reaction mixture was prepared and mixed directly in the ultramicro-fluorescence cuvette. The comparison of the two procedures showed that both generated equivalent results.

2.8.4. Quantification of dsDNA from cell culture supernatant solutions

As pointed out in the user manual for the PicoGreen kit [15] and further described by Singer et al. [16], several compounds present in cell culture supernatant solutions can influence the sensitivity of the fluorescence assay.

With an estimated dsDNA concentration between ~1 – 25 µg/mL, it was decided to measure the samples in a 5 – 25-fold dilution. The following instruction shows the procedure for measuring 10 µL of sample solution in a final volume of 80 µL.

Furthermore, one challenge was the quantification of residual DNA in the presence of higher concentrations of immune-globulin-G antibody (IgG) and sodium chloride (NaCl). Both IgG and NaCl affected the PicoGreen fluorescence signal and therefore it is recommended to setup a calibration curve with similar IgG and NaCl concentrations.

In some cases the DNA concentration was verified via standard addition method. The table below shows an example of the required volumes of reagents to establish a standard addition curve with up to six measurement points.

Sample volume	TE buffer solution	λ-DNA solution	PicoGreen solution	End-volume	spiked DNA conc. in endvol.
10 µL	30 µL	0 µL	40 µL	80 µL	0 ng/mL
10 µL	25 µL	5 µL	40 µL	80 µL	125 ng/mL
10 µL	20 µL	10 µL	40 µL	80 µL	250 ng/mL
10 µL	15 µL	15 µL	40 µL	80 µL	375 ng/mL
10 µL	10 µL	20 µL	40 µL	80 µL	500 ng/mL
10 µL	0 µL	30 µL	40 µL	80 µL	750 ng/mL

Table 2: reagent volumes for dsDNA standard addition method

The sample measurements were carried out in the same way as previously described for DNA standard solutions by mixing 30 µL 1xTE-buffer and 10 µL sample with 40 µL PicoGreen solution in the same manner as described in chapter 2.8.3.
Both methods produced in most cases consistent findings for the majority of measurements. Some of the diverging results are stated in the results chapter below.

2.9. Gel electrophoresis

The SDS-PAGE was done with a Mini-PROTEAN 3 cell from Bio-Rad for a 8,0 cm x 7,3 cm gel using 0,75 mm spacer plates and a gel-volume of 4,2 ml [18]. If not mentioned different, gel electrophoresis was done as 2 parallel runs with 9 sample slots and 1 slot for the marker proteins on each gel.

2.9.1. Prearrangements for SDS-PAGE gels:

Filtered bidest water:

Bi-distilled water was filtered with an all-glass vacuum filtration unit through a cellulose acetate membrane filter with 0,22 µm mesh and ultrasonicated for at least 20 minutes. This “ultrapure” water was then used for buffer preparation and washing of the SDS-gels as well as the electrophoresis equipment.

Preparation of chemicals and buffers:

100 mg of sodium dodecylsulphate (SDS), as well as 100 mg ammonium persulfate (APS), were weigh into seperat Eppendorf micro tubes and each was mixed with1 mL of ultrapure water and ultrasonicated for at least 10 minutes.
A saturated solution of tertiary amyl alcohol in water was made by mixing the two solvents and storing the solution as a two-phase system. The bottom phase of this mix was then used as a topping barrier during polymerisation of the resolution gel.

Resolving gel buffer (1,5 M Tris-HCl, pH 8,8) and stacking gel buffer (0,5 M Tris-HCl, pH 6,8) were already prepared according to the instruction manual by a team member and were kept at 4°C in-between usage.
500 mL of Tris-HCl running buffer at pH 8,3 was prepared on a daily basis from a 10x stock solution new each day and was used at the most for a second SDS-PAGE run.

A stock solution of Laemmli sample buffer containing SDS, glycerol, Tris-HCl pH 6,8 and bromphenol blue as front marker was purchased from Bio-Rad Laboratories, Inc. and was used for sample preparations according to the instruction manual [19] as described in detail below.

2.9.2. Gelpreparation and casting:

For two gels a 10 mL monomer solution for the separation gel was prepared depending on the acrylamid / bisacrylamid concentration as shown is the list below. Also 5 mL of a 5 % acrylamid / bisacrylamid monomer solution for the stacking gel was prepared.

10 mL resolving gel:

percentage of monomer	filtered bidest. H2O	30 % solution acylamid / bisacrylamid	1,5 M tris-HCl pH 8,8 resolving buffer	10% w/v SDS
10%	4,1 mL	3,3 mL	2,5 mL	100 µL
9%	4,4 mL	3,0 mL	2,5 mL	100 µL
8%	4,7 mL	2,7 mL	2,5 mL	100 µL
7%	5,1 mL	2,3 mL	2,5 mL	100 µL
6%	5,4 mL	2,0 mL	2,5 mL	100 µL

5 mL stacking gel:

percentage of monomer	filtered bidest. H2O	30 % solution acylamid / bisacrylamid	0,5 M tris-HCl pH 6,8 stacking buffer	10% w/v SDS
5%	2,85 mL	0,85 mL	1,25 mL	50 µL

Table 3: Reagent volumes for the mixture of SDS-PAGE resolving gel and stacking gel, respectively

The two solutions were prepared in well-washed glass tubes and ultrasonicated for 15 minutes and let cooled back to room temperature before the addition of TEMED and ammonium persulfate and the preparation of the gel slides. After the glass plates were placed in the “gel cassette sandwich” and fixed on the “casting stand” the desired fill-height for the resolving gel was marked outside the glass plate. Polymerisation of the resolving gel was initialized by adding 100 µL of the 10 % w/v ammonium persulfate (APS) solution and 10 µL of tetramethyl-ethylenediamine (TEMED) solution to the premade monomer mix. The resulting solution was mixed shortly and then immediately transferred in between the glass plates with a “pasteur pipette”. After filling the chamber up to the desired height, tertiary amyl alcohol saturated water was placed on top of the gel to get an even gel surface. According to the manual, after 45 to 60 minutes of polymerisation time, the water solution on top of the gel was removed, rinsed with water and access water was carefully removed with the tip of a paper towel. Polymerisation of the stacking gel was started in the same way as before by adding 50 µL of 10 % w/v APS solution and 5 µL of TEMED to the stacking gel monomer solution.

This solution was well mixed and placed on top of the resolution gel up to the edge of the shorter glass plate. Right afterwards, a 10 toothed comb was carefully placed into the stacking solution without adding air bubbles. The gel was left to polymerize for at least 30 minutes, after which the comb was removed and the wells were washed with water.

The glass plates with the gels were taken out of the casting stand, residual gel from the edges of the plate was removed through washing with water, the gel plates were then placed into the "electrode assembly", as described in the instruction book and placed into the mini tank of the electrophoresis system.

After filling the inner and outer chamber with Tris-HCl running buffer pH 8,3 the electrophoresis system was ready for use.

2.9.3. Sample preparation and gel electrophoresis:

Sample preparation for denaturated SDS-PAGE was done by mixing 950 µL of laemmli sample buffer with 50 µL of β-mercaptoethanol. 100 µL of sample were mixed with 100 µL of this β-mercaptoethanol sample buffer. It is also possible for higher protein concentrations to mix 100 µL of sample with 200 µL of β-mercaptoethanol sample buffer.

For a native SDS-PAGE run, 100 µL of sample were mixed with 100 µL of sample buffer without addition of β-mercaptoethanol.

Each sample-buffer-mix was heated for 4 minutes at 95°C on a preheated Eppendorf thermo-mixer.

Between 2 and 10 µL sample-buffer-mix (depending on the protein concentration of the sample) were then placed into one of the wells of the SDS-PAGE gel as well as 5 µL of the molecular weight protein-marker mix (Precistion Plus Protein™, unstained standards) in the last well of each gel.

The picture below shows the molecular weight distribution between 10 – 250 kD for the protein marker from Bio-Rad Laboratories [20].

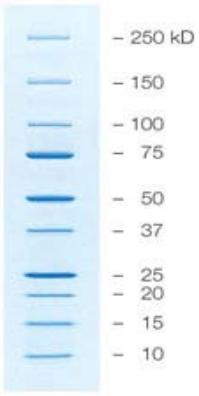


Figure 12: Molecular weight size contribution for Precision Plus Protein™ [20]

After all wells were filled the gel electrophoresis was started with 150 V as constant voltage. After about 30 – 40 minutes the gel electrophoresis was finished when the blue marker front reached the opposite part of the gel.

2.9.4. Silver staining of the proteins:

The detection of proteins was done via silver staining [21] with a ProteoSilver Plus silver stain kit from sigma. [22].
All solutions as listed below were made fresh from stock-solutions with filtered bi-distilled water and were used within two hours of preparation. Due to the relative high amounts of protein in the gel, the silver staining was done with the minor modification of just half the amount of sensitizer solution, silver solution and developer solution compared to the staining protocol from sigma. Shaking and mixing was done by hand and all solutions were decanted after appropriate incubation time and disposed adequately.

- Fixing solution: 50 mL ethanol + 10 mL acetic acid + 40 mL H_2O
- 30% ethanol solution: 30 mL ethanol + 70 mL H_2O
- Sensitizer solution: 0,5 mL ProteoSilver sensitizer + 49,5 mL H_2O
- Silver solution: 0,5 mL ProteoSilver silver solution + 49,5 mL H_2O
- Developer solution: 2,5 mL ProteoSilver developer 1 + 50 µL ProteoSilver developer 2 + 47,5 mL H_2O

Fixation of the gel was done in a glass beaker or crystallizing dish right after the gel electrophoresis was finished and the gel was washed by placing the gel in 100 mL fixing solution for at least 20 minutes. The gels were then washed with 100 mL of 30% ethanol solution and then with 200 mL of filtered bidest water for 10 minutes each. For enhanced binding of silver ions to the protein, the SDS-gel was suspended with 50 mL of sensitizer solution for 10 minutes and then washed twice with 200 mL of filtered bi-distilled water for 10 minutes each. After incubating the SDS-gel with 50 mL of silver solution for 10 minutes, the SDS-gel was shortly rinsed with 200 mL water. This final washing step is most delicate for the quality of the staining and must not exceed 30-60 seconds.

After decanting the washing solution, the SDS-gel was developed with 100 mL of developer solution for 5 minutes. The reaction was stopped by adding 5 mL of stop solution. Prior to scanning, the silver stained SDS-gel was washed with 200 mL of water for at least 15 minutes and then placed in between two transparent sheets. Arising air-bubbles were removed with caution and the gel was scanned with a HP Scanjet 4370 scanner from Hewlett-Packard and stored in 200 mL of pure water for further use or disposed adequate.

3. Results and discussion

3.1. Comparision of elemental analysis and acid/base titration with 1 M NaOH solution for ligand density determination

In order to determine the reliability of the acid/base titration method, test titrations of solutions containing glacial acetic acid or HCl using a 0,1 NaOH solution, showed a good accuracy. The result of this titration experiment can be seen in Table 4. Note that for the HCl solution constantly too high mol values were determined. The reproducibility of these titration results for HCl indicates that the inaccuracy lies in the concentration of the HCl solution rather than an error for the titration method itself.

glacial acidic acid:				0,1 M NaOH used	error of
volumn	mass	molar units		at equivalence point	measurement
in µL	in mg	in mmol		in mL	in %
90	9,441	0,1569		1,58	0,701
60	6,294	0,1046		1,04	-0,574
0,1 M HCl:				0,1 M NaOH used	error of
volumn		molar units		at equivalence point	measurement
in µL		in mmol		in mL	in %
1000		0,1		1,08	8,000
1000		0,1		1,08	8,000
1000		0,1		1,08	8,000
1000		0,1		1,07	7,000

Table 4: Titrations of glacial acidic acid and hydrochloric acid with 0,1 M NaOH

It has to be mentioned that the accurate consumption of 0,1 M NaOH was determined from a print-out of a detailed data list (not shown) and was not determined through graphic curve analysis of the figure-plots. These list print-outs from the titration automate also indicate even little equivalent points, which are shown as horizontal and vertical lines in the plot of the whole titration curve as shown in fig. 13 below.

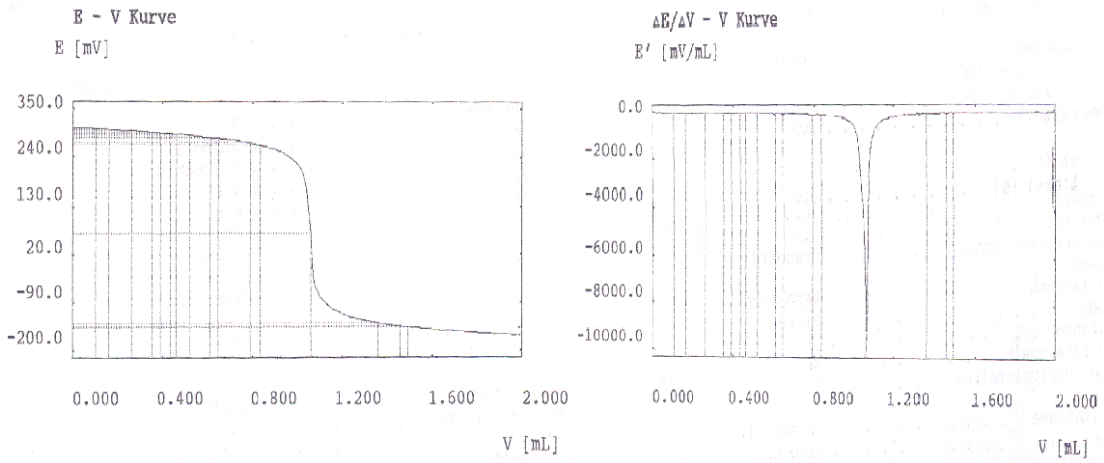


Figure 13: Titration curve for 1mL 0,1 M HCl using 0,1 M NaOH

The titration results for Fractogels with immobilized ligand showed sometimes deviating results compared to the ligand densities determined via elemental analysis. For example, three consecutive titrations of Gel B possessing a ligand density of 0,960 mmol/g according to elemental analysis results provided ligand densities between 1,074 and 1,103 mmol/g. With an epoxide-group density of 1,000 mmol/g for the support material this could only be explained by residual HCl from insufficient washing.

Gel D with a ligand density of 0,514 mmol/g on the other hand, gave a lower consumption volume of 0,1 M NaOH, which resulted in ligand densities between 0,227 and 0,270 mmol/g for the “non endcapped” gel as well as for the β-mercaptoethanol endcapped gel. Fig. 14 provides an example for such a titration with odd curve progression.

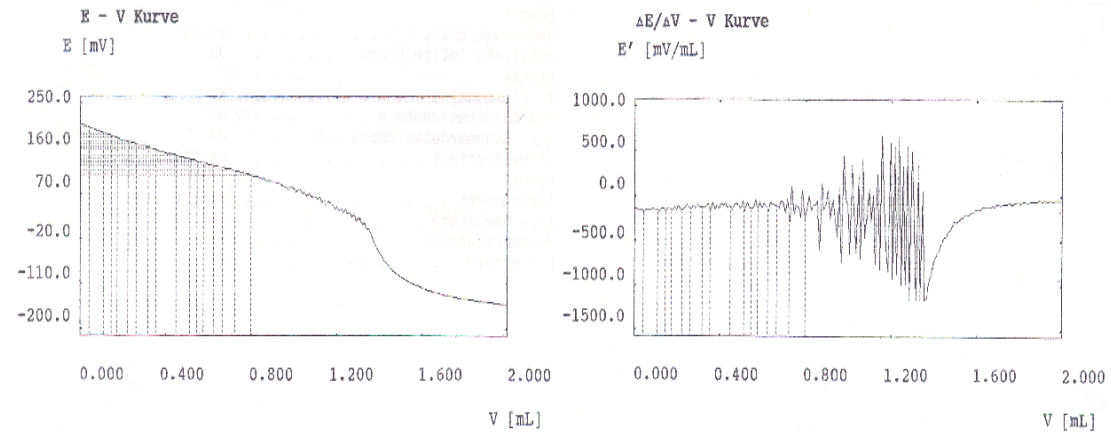


Figure 14: Titration curve for 572,0 mg Gel C titrated with 0,1 M NaOH

The cause for this uneven titration curve was assumed to lie in a too fast NaOH increment addition were the equilibrium for this acid-base titration was not reached yet. Therefore, the interval between additions of 0,1 M NaOH was adjusted to a maximum of 300 seconds, which however also extended the time for each titration drastically. Nevertheless similar ligand densities were observed, which could not be explained.

In case of Gel F the titration with NaOH yielded 0,443 mmol/g, which corresponds well with the 0,472 mmol/g ligand density obtained from elemental analysis. The titration curve for this material is shown in fig. 15.

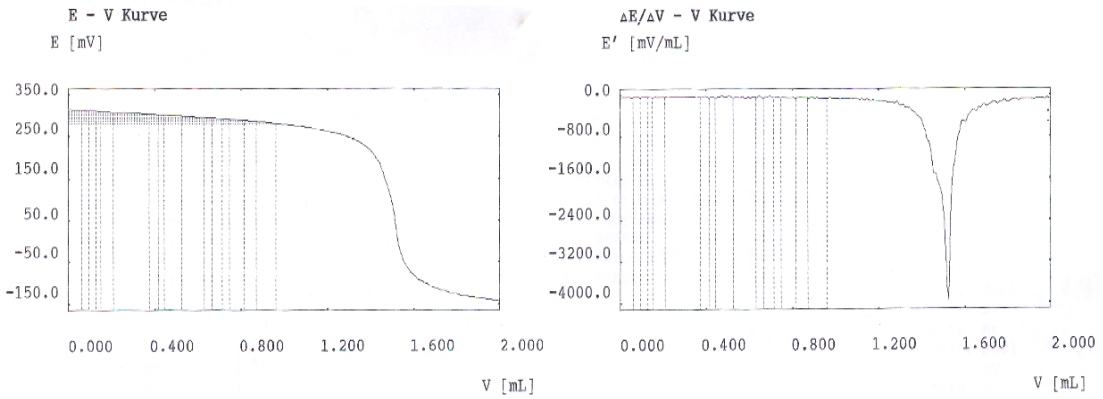


Figure 15: Titration curve for 345,4 mg Gel F titrated with 0,1 M NaOH

The last reaction with Fractogel with promising 0,713 mmol/g ligand density was the immobilisation of cinnamoyl-homocystein (Gel G). Unfortunately no evaluable titration curve for the verification of the ligand density via titration with 0,1 M NaOH could be generated.

3.2. Results for the immobilisation reaction of cinnamoyl-homocystein

Mass spectroscopical data from a sample of cinnamoyl-homocystein taken before immobilisation did not prove a reliable ring-opening reaction of the cinnamoyl-homocystein. When measured in positive ion mode, providing $[M+H]^+$ and $[M+Na]^+$ as shown in fig. 16 below, the MS spectra indicated a mixture of ~2/3 closed and ~1/3 open ring structure. Note that the $[M+Na]^+$ peak for the open ring structure seemed to be below the signal to noise ratio.

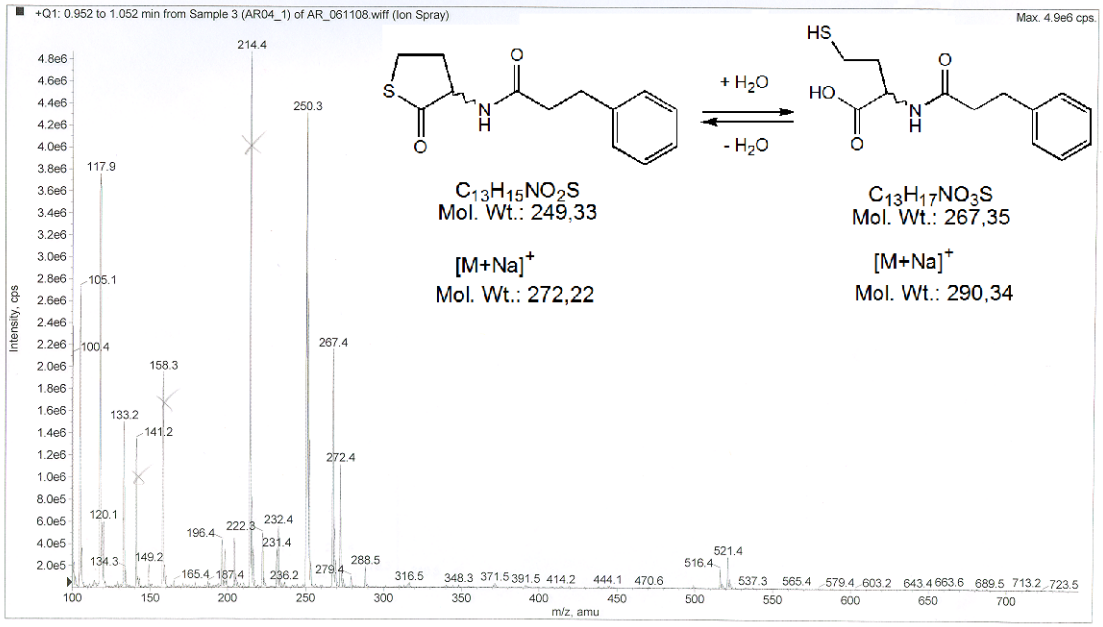


Figure 16: Ionspray-MS spectra of cinnamoyl-homocysteine with background peaks marked with X.

Nevertheless these H_2O addition and elimination reactions could have also taken place during the ionisation process and therefore any peak-rations most likely did not reflect the sample condition during the immobilisation reaction.

The thin-layer-chromatographic evaluation of the supernatant of the immobilisation reaction did indicate remaining cinnamoyl-homocystein, but NMR-spectra of a neutralized and cleaned fraction could not verify an intact structure for the molecule. According to this finding, a decomposition of the ligand-molecule during the immobilisation reaction seemed likely and therefore no further binding capacity studies with IgG for Gel G were made.

3.3. Kinetics of SBC studies for selected Fractogels

As previously mentioned, the static binding capacity study for Gel B with an reaction time of 2 hours was carried out by Dr. Heiner Graalfs and showed most promising results at pH levels around 6,5, combined with a high ligand density of 4-mercaptobenzoic acid Fractogel B.

Further investigation of this material, included a time-depending SBC study, which was carried out at reaction times between 5 minutes and 4 hours.

The fig. 16 below shows the kinetic study for Gel B using an IgG solution with of 5 mg / mL in 25 mM phosphate buffer with 75 mM NaCl at pH 6,5.

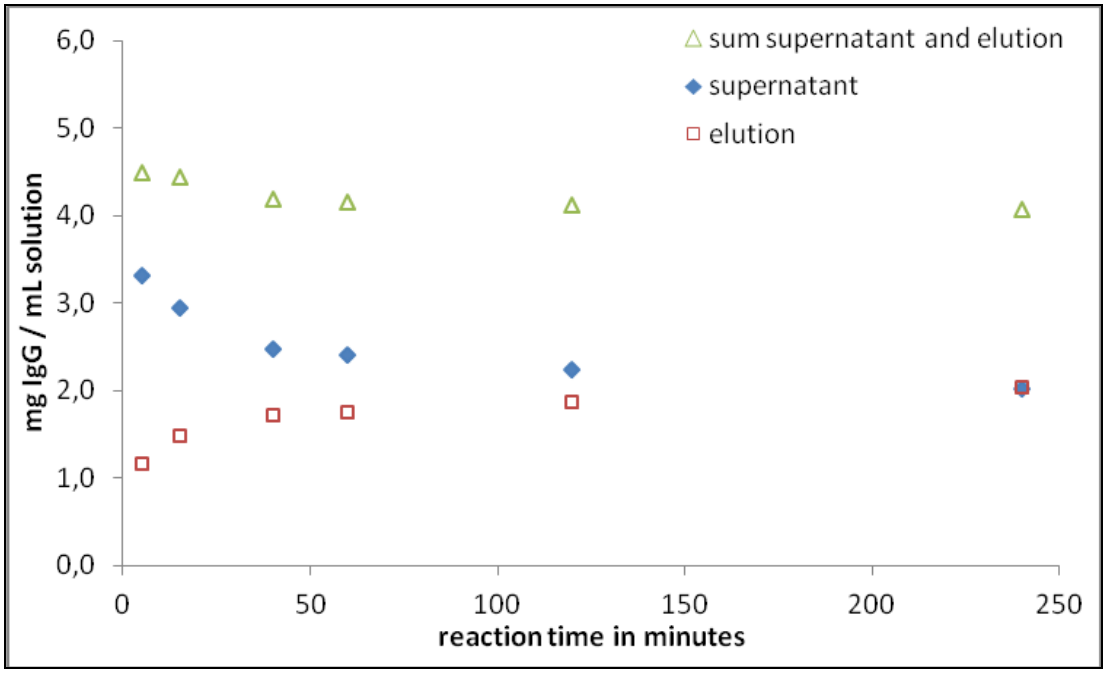


Figure 17: Time-dependant binding study for Gel B using a 5 mg/mL IgG solution

Each data point in fig. 17 resembles an average over triple measurements. It can be seen that for reaction times below 2 hours the binding sites on the gel are not completely saturated. This indicates a rather slow capture mechanism of IgG for Gel B.

The decreasing IgG concentration with increasing reaction time for the “sum of supernatant and elution” indicates an increased contribution of unspecific binding for longer reaction times; that is if one considers a constant efficiency for the washing steps prior to elution for all measurements.

For comparison the fig. 18 below shows a kinetic SBC study from Gel A carried out in 75 mM NaCl and 25 mM acetate/phosphate buffer at pH 5,5 with an IgG concentration of 8 mg/mL.

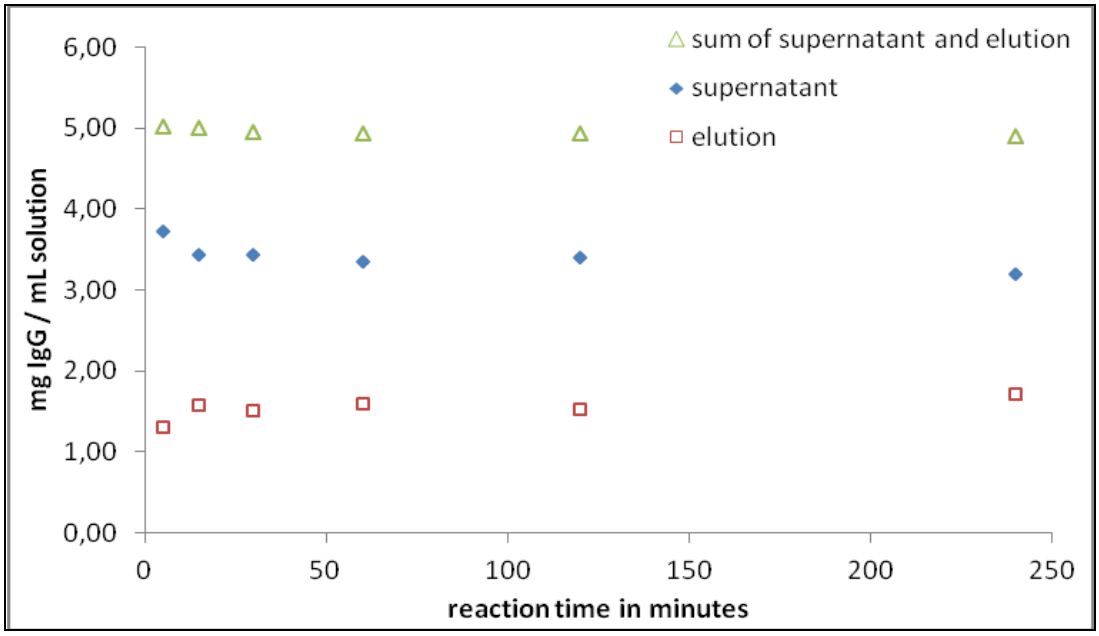


Figure 18: Time-dependant binding study for Gel A using a 8 mg/mL IgG solution

The most notable difference is that for Gel A a saturation of the binding sites for IgG is already reached after 15 minutes reaction time.

This could partly be explained by the higher IgG concentration but also points towards a much faster binding mechanism for Gel A.

3.4. Results of DBC studies for 4-mercaptobenzoic acid Fractogel with the highest ligand density (Gel B)

The very first DBC studies were measured with 1 mg/mL Beriglobin IgG and Gammanorm IgG, respectively, in 75 mM NaCl and 25 mM phosphate buffer at pH 6,5. The fig. 19 below shows the IgG concentration in mg/mL plotted against every collected flow-through fraction for both DBC runs.

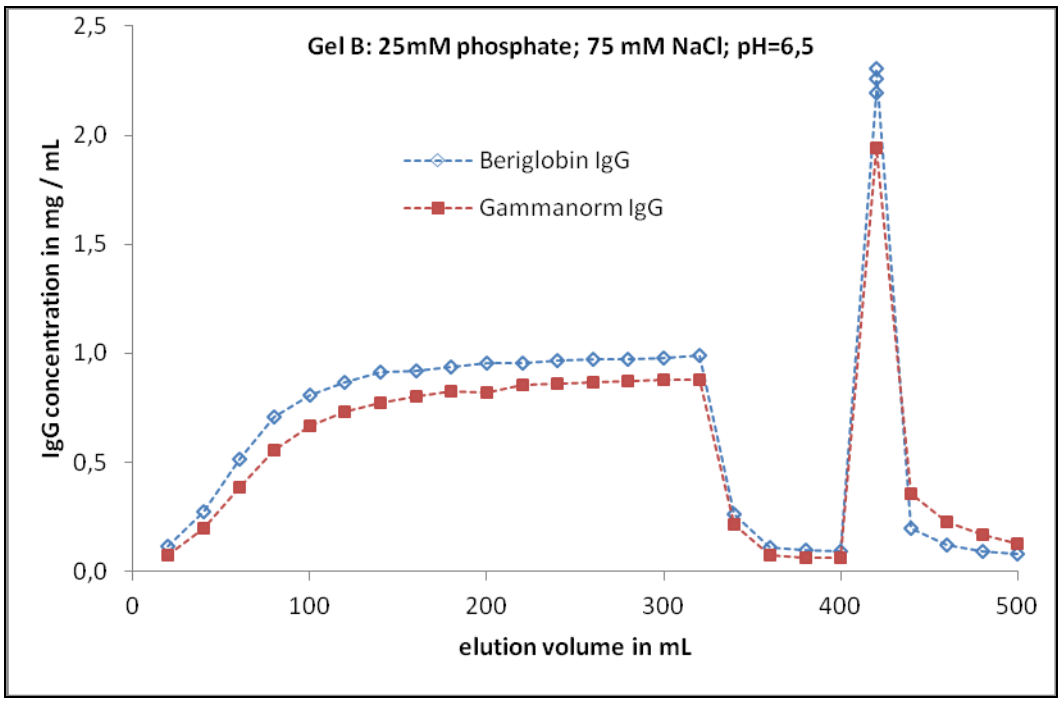


Figure 19: Comparison of binding behaviour for Gel B with two different commercial types of polyclonal IgG

The main reason for the deviating course of the two break-through curves lies in the slightly different IgG concentration of the two application feed solutions, 0,94 mg/mL for IgG Gammanorm and 1,02 mg/mL for IgG Beriglobin. In addition, this dissimilarity may also have led to different dynamic binding capacities for the two runs. This highlights that binding behaviour can be diverse regardless of a similar protein structure.

Nevertheless the total binding capacity after 100% saturation was 51,9 mg IgG/mL gel for Beriglobin and 52,6 mg IgG/mL gel for Gammanorm, as well as the calculated recovery of 83,7 % for Beriglobin and 85,2 for Gammanorm, was nearly the same for both runs. Therefore all further DBC measurements were carried out with Beriglobin IgG.

The next DBC measurements were carried out at higher sodium-chloride concentrations and at pH = 6,5 as well as at pH = 7,4 to mimic cell culture feed conditions. The elution capacity was decreasing with higher sodium-chloride concentration and higher pH value, which stands in accordance to findings from the SBC studies.

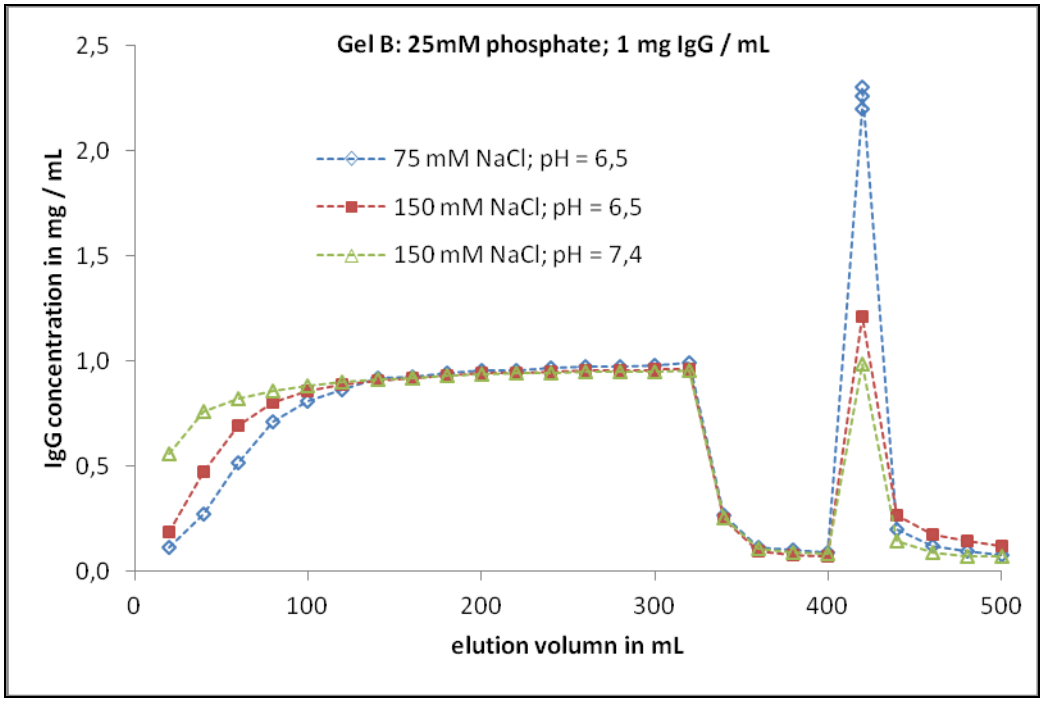


Figure 20: DBC measurements for Gel B at various pH values and sodium-chloride concentrations of the application feed.

DBC values at 100 % saturation, represent a balance between available binding sites and continuously delivered IgG in the feed. Therefore 100%-DBC values are not exactly the same as binding capacities obtained by static binding studies, which result from a partitioning equilibrium for an adjusted IgG concentration. Nevertheless the elution capacity for DBC stand in good accordance to those obtained by SBC measurements at similar test conditions (Table 5).

DBC measurements:	Beriglobin		SBC measurements:	Gammanorm
	mg IgG / mL gel			mg IgG / mL gel
75 mM NaCl; pH 6,5	51,9		75 mM NaCl; pH 6,5	48,8
150 mM NaCl, pH 6,5	39,2		150 mM NaCl, pH 6,5	40,7
150 mM NaCl, pH 7,4	25,3		150 mM NaCl, pH 7,5	24,6

Table 5: Comparison of DBC and SCB for similar NaCl concentrations and pH values

3.5. Comparision of DBC´s for 4-mercaptobenzoic acid Fractogels with different ligand density (Gel B, Gel C and Gel D)

Overall, three batches of 4-mercaptobenzoic acid Fractogels with ligand densities between 0,561 and 0,950 mmol/g gel were prepared and DBC studies were measured.
The results of elution capacity measurements for 1 mg IgG/mL in 75 mM NaCl and 25 mM phosphate buffer at pH 6,5 for the three gels are shown in Table 7, below.

	ligand density mmol / g gel	elution capacity mg IgG / mL gel
Gel B	0,960	51,9
Gel C	0,819	41,3
Gel D	0,561	25,2

Table. 7: Elution capacity for 4-mercaptobenzoic acid Fractogels with different ligand density at 75mM NaCl and 25 mM phosphate buffer at pH 6,5

The data set in Table 7 provides evidence for a somewhat linear correlation between ligand density and elution capacity. However, a linear regression with only 3 measurements is rather insignificant, since without the evaluation of gels with even lower ligand density this extrapolation is unreliable. On the other hand, gel preparations with very low ligand density could be inaccurate, due to the increasing risk of unspecific IgG binding onto the supporting material.

Further DBC measurements for Gel D under same conditions as used for Gel B, are shown in fig. 21 and illustrate similar binding behaviour for IgG with increasing NaCl concentration and pH value.

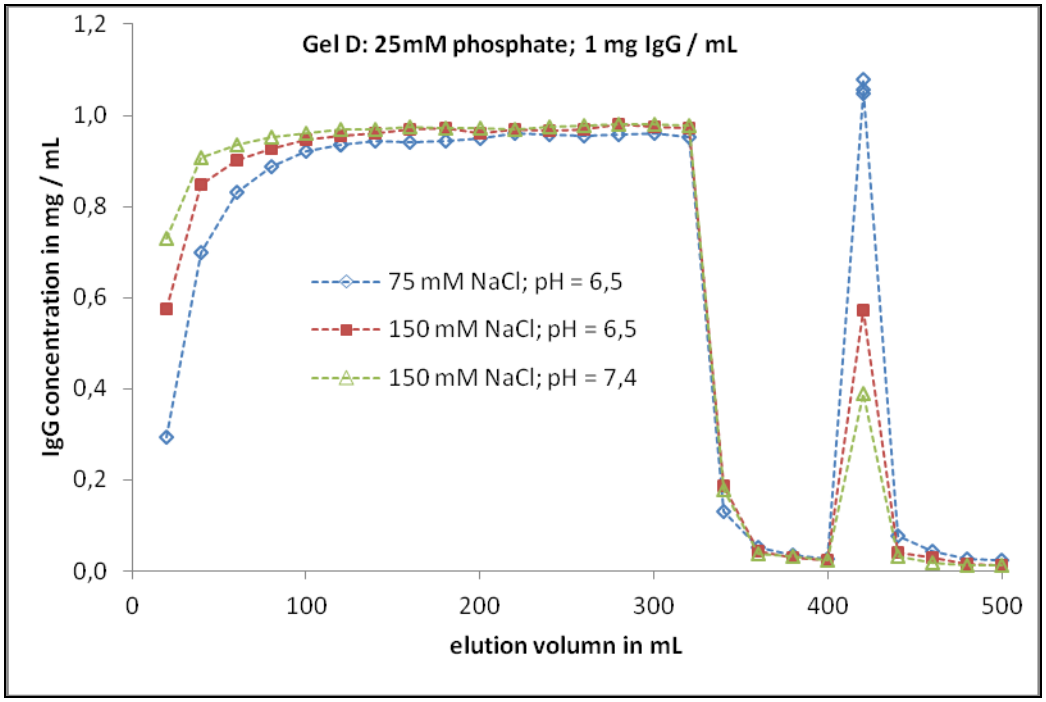


Figure 21: DBC measurements for Gel D at various pH levels and sodium-chloride concentrations.

A direct comparison of the measurements shown in fig. 22 illustrates the difference in elution capacity for the two 4-mercaptobenzoic acid Fractogels, Gel B and Gel D.

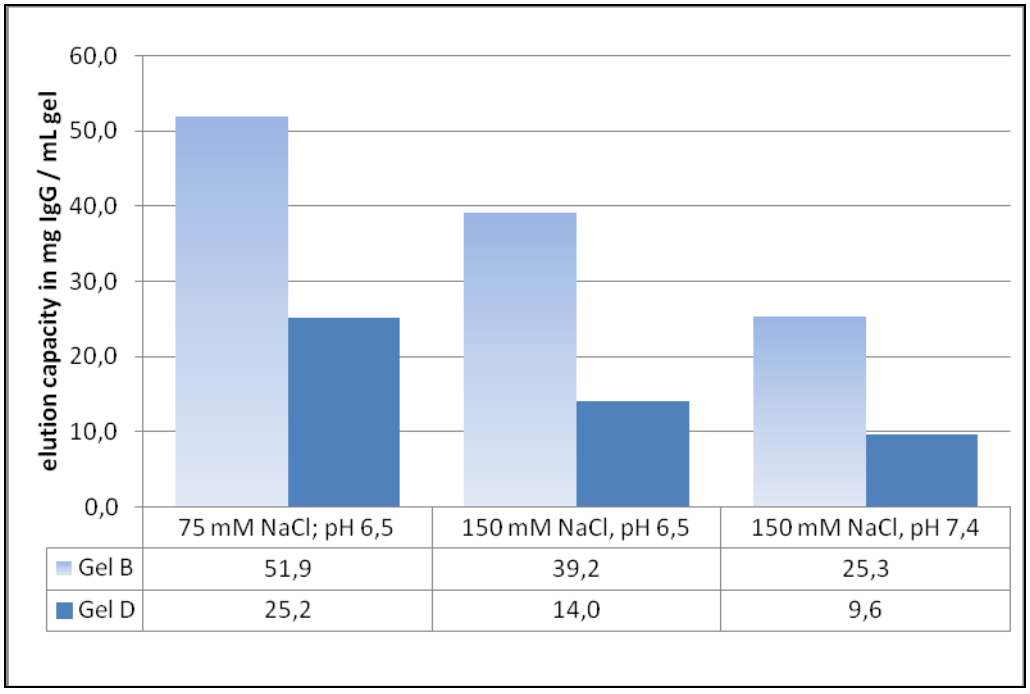


Figure 22: Comparison of the elution capacity for 4-mercaptobenzoic acid Fractogels with different ligand density.

While elemental analysis data yielded a ligand density ratio of 1 : 0,59 for the Gels B : D, their elution capacity ratio differ from that finding as shown in Tab. 7.

Buffer conditions:	elution capacity ratio:	
	Gel B :	Gel D
75 mM NaCl; pH 6,5	1 :	0,49
150 mM NaCl; pH 6,5	1 :	0,36
150 mM; NaCl, pH 7,5	1 :	0,38

Tab. 7: Elution capacity ratio for two 4-Mercaptobenzoic acid Fractogels with different ligand density

The fluctuations for the elution capacity ratio could not yet be explained, but may also result from measurement inaccuracy due to the fact that DBC studies are rather time-consuming and were only performed once for each buffer condition and therefore no statistical confidence interval could be determined.

3.6. DBC for 4-aminobenzene sulfonic acid Fractogel (Gel F)

Although, the two 4-aminobenzene sulfonic acid Fractogels were prepared with a 10-fold and 20-fold molar excess of ligand, they showed similar ligand density of 0,389 mmol/g and 0,472 mmol/g, respectively. DBC measurements were done only with Gel F_2 due to higher ligand density.

Fig. 23 shows the DBC measurement for 1 mg IgG/mL in 75 mM NaCl and 25 mM phosphate buffer at pH 6,5. This buffer composition was chosen for comparison reasons, since all other Fractogels were measured under this condition.

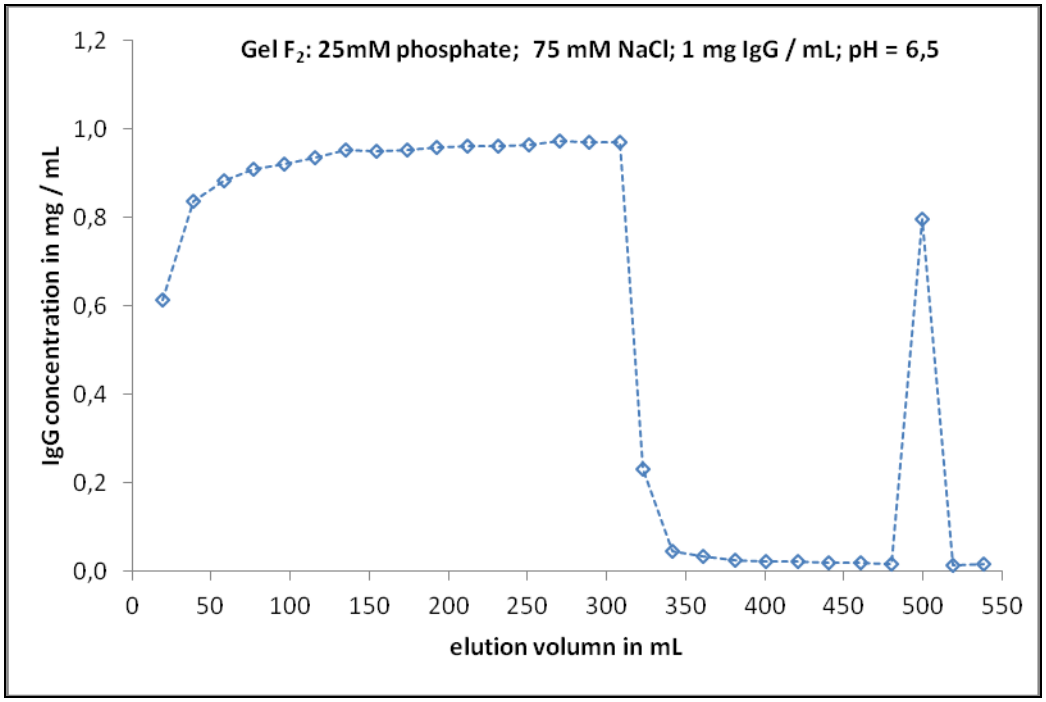


Figure 23: DBC measurements for Gel F_2: 25 mM phosphate buffer and 75 mM NaCl at pH 6,5

The low elution capacity of 17,8 mg IgG/mL gel and recovery of 61,11 % may result from the lower ligand density, which is near of the same order of magniture as Gel D. Also no SBC study was made for Gel F_2, so the buffer composition was probably not optimal for the 4-aminobenzene sulfonic acid ligand.

Also note that the slightly different DBC protocol with higher washing fraction volume prior to elution, which on the other hand should not have a great effect on the elution capacity of the gel.

3.7. DBC measurements for “mock feed solution" including 1 mg/mL Pluronic® F-68

Pluronic® F-68 is a non-ionic detergent, which is sometimes used as an additive to increase the water solubility of hydrophobic substances and to restrain foam formation.

Therefore as a first step towards a possible industrial application of 4-mercaptobenzoic acid Fractogel, a DBC measurement Pluronic® F-68 containing feed was performed in order to investigate the impact of Pluronic® F-68 on IgG

binding and IgG elution. Fig. 24 shows a comparison of DBC´s on Gel B for equal buffer conditions.

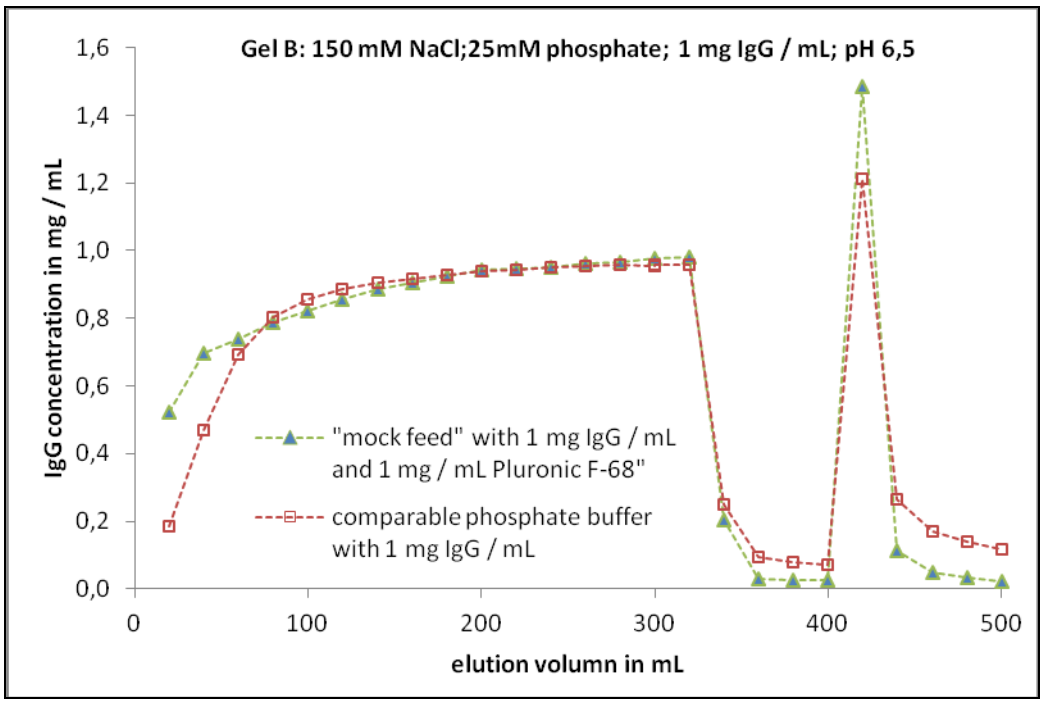


Figure 24: “mock feed” DBC study and comparison with a DBC measurement with smilar buffer composition for Gel B: 25 mM phosphate and 150 mM NaCl at pH 6,5.

Fig. 24 shows that in the presence of Pluronic® F-68 the IgG binding capacity of Gel B is slightly decreasing from 39,2 to 35,1 mg IgG/mL gel. Furthermore, also it shows that the IgG recovery was lowered to 80,1 %. This points toward a less favourable IgG binding, but until further measurements were done, this loss of binding capacity could also be within the statistical variability of the measurement.

Also note that the IgG concentration in the first elution fraction is slightly higher, which could indicate a faster displacement from the gel due to a better solubility of IgG.

3.8. DBC measurements of cell culture feedstock with Fractogel (Gel B)

As stated in the experimental section of this thesis, the elution of IgG was performed with a linear NaCl gradient, which led to a broadening of the elution peak.

Fig. 25 below shows the DBC measurement of a cell culture feedstock spiked with IgG sample with an overlay of the corresponding salt gradiet graph. This figure illustrates the connection between IgG concentration in the eluted fraction and the NaCl concentration of the applied elution buffer.

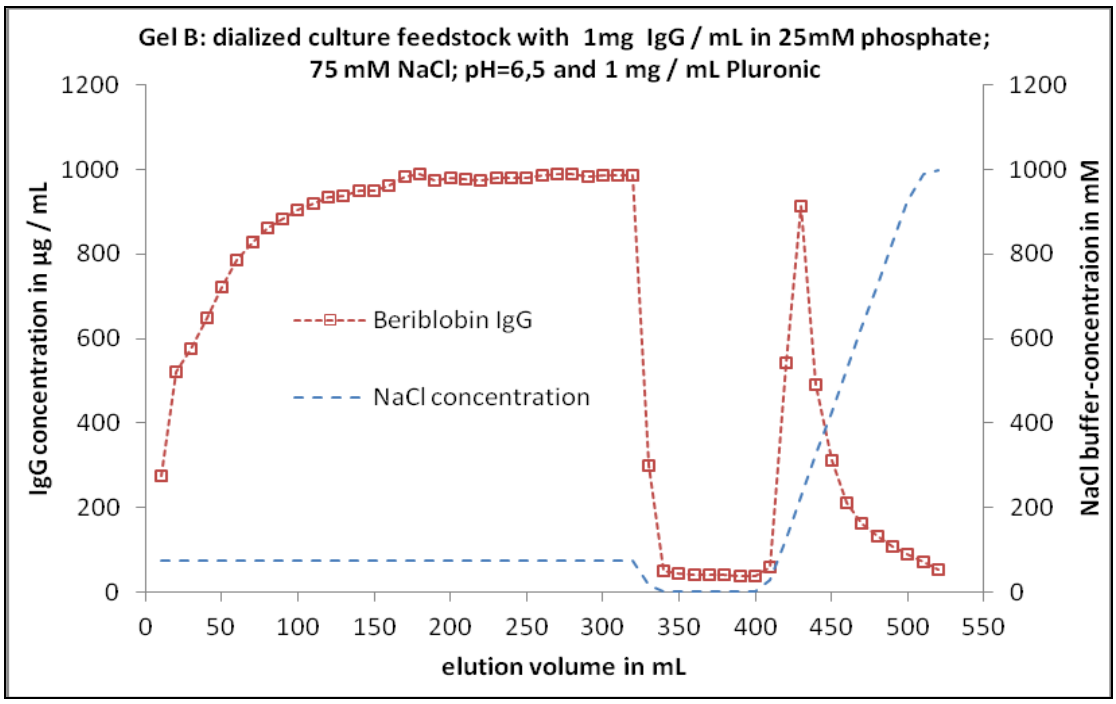


Figure 25: DBC measurement of a dialized cell culture feedstock with Gel B

The maximum for IgG elution is reached at a rather low salt concentration between 200 - 300 mM NaCl and therefore a much smaller peak-shape would have been obtained, if the NaCl concentration was raised undelayed. In regard to the following SDS-PAGE measurements of the collected fractions, a larger number of samples is rather helpful in order to get a more differential picture of eventual impurities. Also a large IgG concentration in the first elution sample could mask certain impurities.

3.9. Results for DNA quantification in cell supernatants

Measurements of two different batches of cell culture feedstock, A and B, yielded double stranded DNA (dsDNA) concentrations of 24,6 µg/mL for feed A and 2,24 µg/mL for feed B. These variations were caused by different production conditions such as different cell lines and possibly also influenced by the after-treatment of the cell supernatant, which the supplied refused to disclose. Nonetheless it can be expected that feed B was pre-purified since the DNA concentration is by a factor 10 lower than observed for feed B.

In terms of available quantity, cell culture feedstock B was chosen for further measurements and the salt concentration was predestined by the composition of the dialysis solution.
After dialysis and buffer salt adjustment to 75 mM NaCl and 25 mM phosphate at pH 6,5, a dsDNA concentration of 0,93 µg/mL for the application feed buffer was determined. Since IgG as well as high NaCl content is known to interfere with the Pico Green reagent [15], additional measurements with the standard addition method resulted in a slightly higher dsDNA concentration of 1,06 µg/mL for the dialysed feed B.

Finally, a DNA quantification for the gradient elution fractions G1 – G 11 from the DBC study with cell culture feedstock showed that the DNA concentration was decreasing to a level of maximal 0,15 µg/mL in fraction G3.
For the pooled elution fractions G1 – G11, the DNA concentration could even be reduced by 92,7 % to a concentration of 0,07 µg/mL.
Fig. 26 illustrates the correlation of IgG and DNA concentration for the DBC study with cell culture feed solution. IgG and DNA concentration are both expressed as % in reference to their original concentration of 1 mg IgG / mL and 0,93 µg DNA / mL in the cell culture feed solution.

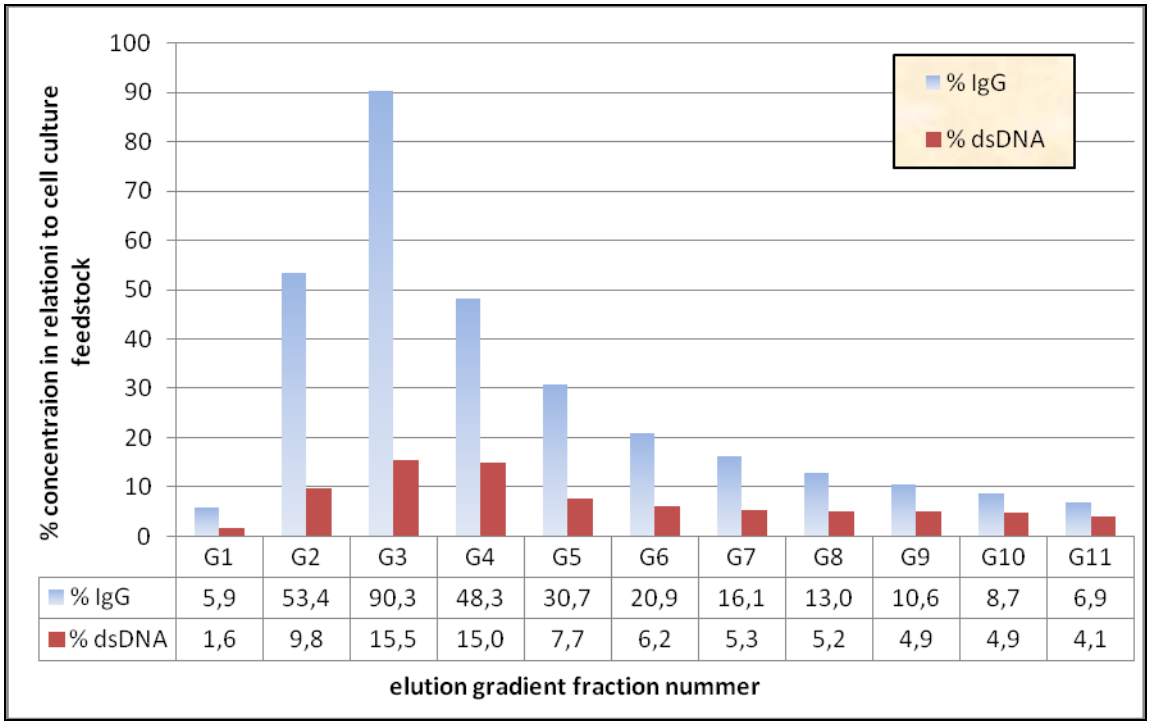


Figure 26: IgG and DNA content for elution fractions G1-G11

It is noticeable that the DNA concentration follows the curve progression of the IgG concentration. It must be mentioned that, according to the Quant-it PicoGreen instruction manual, a 0,1 % IgG concentration increases the observed fluorescence signal by 19 % whereas a 200 mM NaCl decreases the signal by 30 % [15].

The increase in DNA concentration, especially in fractions G2 – G5, very likely arises from such matrix-effects, but a partial binding of DNA besides the binding of IgG to the 4-Mercaptobenzoic acid Fractogel can not be excluded. It is therefore highly recommended to adapt the composition of the DNA-standard solutions to the IgG and NaCl concentration of each and every sample or to verify the obtained results additionally via standard addition method.

Due to this huge increase of required measurements a possible automatisation of DNA measurements is most desirable. Unfortunately, a micro-plate-reader adapter for such measurements was not available for the LS 50B fluorescence spectrometer, hence all DNA quantification and standard addition measurements had to be done manually with two ultramicro-fluorescence-cuvettes.

3.10. SDS-Page results for cell supernatants fractions

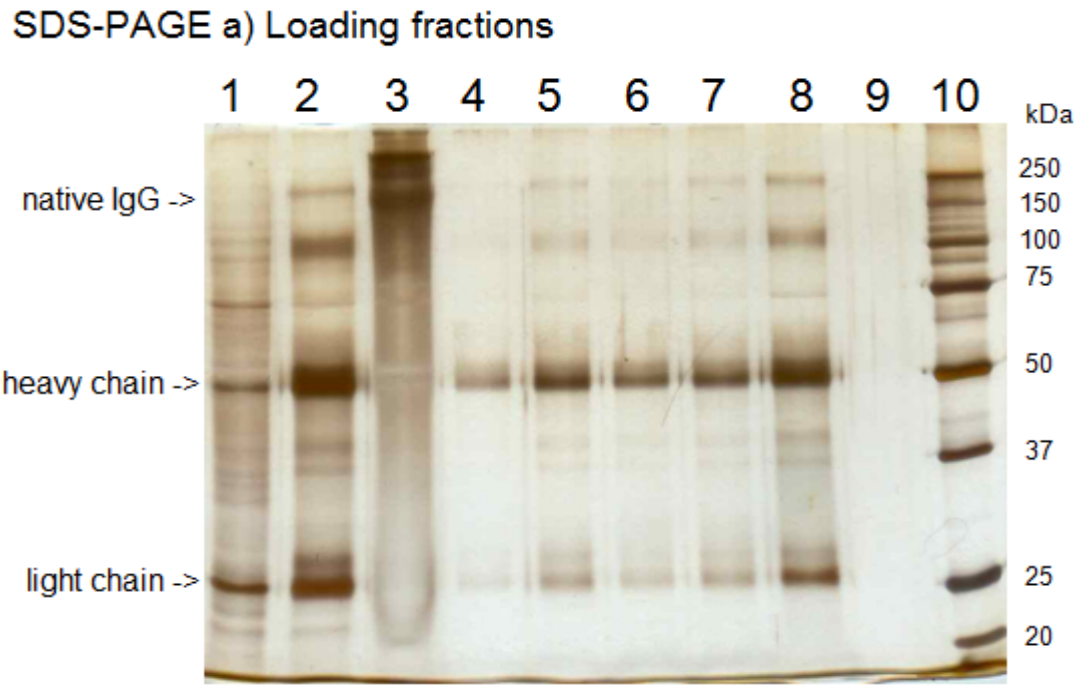
Since the main goal of this examination was to value the applicability of 4-mercaptobenzoic acid Fractogel for immunoglobulin enrichment, the final evaluation of the dynamic binding capacity study for Gel B using cell culture feed, included the analysis of selected DBC flow-through fractions using SDS-PAGE. The aim was to analyse a possible variation in the distribution of IgG and other bound proteins or feed impurities in general, in the different collected fractions of the DBC study.

Both SDS-Page gels, shown in Figures 27 and 28 were separated via 10% acylamid / bisacrylamid gels with TRIS-HCl buffer. Sample application volume was 3 µL and except for line 3 in gel a) all sample fractions were separated under reduced conditions as stated in chapter 2.9. This implies that disulfide bonds between each 150 kDa IgG tetramer were cleaved into two heavy chain fragments of 50 kDa size and two light chain fragments with 25 kDa size. Faint protein bands at ~150, 100 and 75 kDa may occur from an incomplete reduction of disulfide bonds. This results in the partial division of the IgG tetramer into various cleavage products such as the 100 kDa fragment, which resembles an assembly where the two heavy chains are still connected.

In both gels, the separation of the protein-marker mix in lane 10 served as an internal standard for a protein weight size calibration [20].

Also note that both analyzed SDS-Page gels were developed under rather intensive silver staining conditions in order to detect also low concentrations of feed impurities present in the respective sample fraction. The drawback of this intense staining was the overdeveloping of certain protein bands with higher protein content.

The first SDS-PAGE gel, shown in fig. 27, compares the composition of the applied feed solution with that of different loading fraction as well as the last column wash fraction.

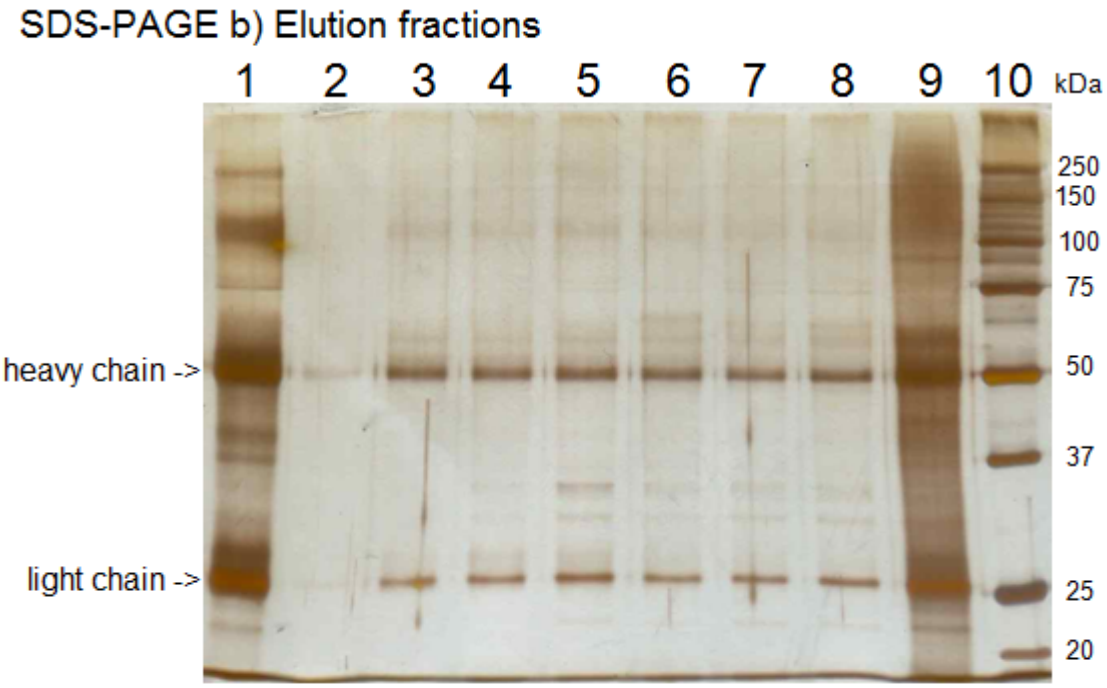


lane-nummber	description of the applied fraction
1	Dialysed cell culture feedstock (29,9 µg IgG / mL)
2	Dialysed cell culture feed spiked with berigolbin (1 mg IgG / mL)
3	Native SDS-PAGE of dialysed cell culture feed spiked with berigolbin
4	L1 Loading fraktion
5	L2 Loading fraktion
6	L3 Loading fraktion
7	L4 Loading fraktion
8	L17 Loading fraktion (last loading fraction)
9	W10 (last washing fraction)
10	proteinmarker

Figure 27: Silver stained SDS-PAGE gel a) including a sample description list

The lane 1-3 represent the protein distribution of the applied cell culture feed solution at various stages of preparation. Lane 1 visualizes the protein-content of the dialysed cell culture feedstock where the protein bands at 25 and 50 kDa confirmed a low initial concentration of 32,9 µg IgG1/mL compared to the cell culture feed spiked with polyclonal IgG in line 2, which possessed a significant higher IgG concentration of 1 mg/mL. Lane 3 yielded a bulky band at 150 kDa, since it resembles the same IgG-spiked cell culture feed sample from lane 2, but applied under native non-reduced conditions. Furthermore, lane 3 showed that the impurities in the sample mainly consist of rather high molecular weight impurities.

The lanes 4-7 represent the first four loading fractions and shows an early breakthrough of IgG already at the very first application fraction. The increasing IgG concentration and a low background of protein impurities proved that Gel B was not exclusively binding IgG. The protein composition of the final loading fraction 17 in lane 8 demonstrates a complete breakthrough of IgG as well as feed impurities, which is also reflected by its similarity compared with lane 2, the application feed solution. The sample in lane 9 represents the last washing fraction of the DBC run containing no detectable IgG or protein impurities.



lane-nummber	description of the applied fraction
1	Dialysed cell culture feed spiked with berigolbin (1 mg IgG/mL)
2	G1 Gradientenelution
3	G2 Gradientenelution
4	G3 Gradientenelution
5	G4 Gradientenelution
6	G5 Gradientenelution
7	G6 Gradientenelution
8	G7 Gradientenelution
9	0,5M NaOH CIP fraktion (brought to neutral pH with citric acid)
10	proteinmarker

Figure 28: Silver stained SDS-PAGE gel a) including a sample description list

The second SDS-PAGE gel, shown in fig. 28 illustrates the IgG elution fractions of the DBC run, where the lanes 2 – 8 stand for the first seven elution fraction G1 – G7. The last lane 9 displays the protein mixture of the first cleaning-in-place (CIP) fraction with 0,5 M NaOH as sanitizer solution.

In agreement with the IgG quantification via Protein-A affinity chromatography, the first elution fraction G1with an sodium-chloride concentration of ~30 mM NaCl contains a very little amounts of eluted IgG. The remaining elution fraction G2 – G7 seemed to carry a more or less equal amount of IgG as well as impurities, although the Protein-A affinity chromatography suggested a higher IgG concentration for the fractions G2 – G4 with a maximum of 0,914 mg IgG/mL in fraction G3. To conclude the evaluation of this SDS-PAGE run, the obtained purification factor for the pooled elution fractions was unsatisfying, since also noticeable amounts of feed impurities, especially host cell protein, were co-eluted with IgG. And although the major amount of impurities were washed from the 4-mercaptobenzoic acid Fractogel during the sanitizing step (CIP fraction) also a larger quantity of immunoglobulin G was lost.

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5. Appendix

5.1. List of tables

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Static and dynamic binding capacities of human immunoglobulin G on polymethacrylate based mixed-modal, thiophilic and hydrophobic cation exchangers

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ABSTRACT

The aim of this study was to investigate functional increments of ion exchange type ligands, which may improve the performance of mixed-modal ligands for antibody capture out of feed solutions with pH above 6.0 and containing sodium chloride concentrations of 150 mM and higher. For this purpose several functional groups such as sulfonyl, sulfanyl, amide, methoxy, short alkyl and aromatic moieties were tested in combination with a strong sulfonic acid and/or a weak carboxylic acid group. Therefore a series of ligands were synthesized and subsequently coupled onto epoxide activated Fractogel® EMD. In the first instance, all materials were tested by static binding capacity measurements (SBC) under test conditions, comprising a wide variety of different sodium chloride concentrations and differing pH values ranging from 4.5 to 7.5. From these preliminary experiment it was found that especially the aromatic groups improved the binding of human immunoglobulin G (h-IgG) under isotonic conditions, while other increments, e.g. thiophilic or amide groups, were not able to increase the capacity significantly. Taking the SBC results into account, the most promising materials were investigated under dynamic binding conditions (DBC) with a reduced selection of test conditions (pH 5.5, 6.5 and 7.4 at 75 and 150 mM NaCl). N-benzoyl-homocysteine (material **J**) and 3,5-dimethoxybenzoyl-homocysteine (material **K**) showed 100% DBCs of 37 mg/mL and 32 mg/mL in the presence of 75 mM NaCl and pH 6.5. Material **L** carrying mercaptobenzoic acid as a ligand and tested with the same solution provided a 100% DBC of 68 mg/mL. The influence of Pluronic F68 in a mock feed solution as well as in cell culture supernatant was investigated with the best performing bio-affinity type adsorbent, material **L**. For the real sample feed subsequent SDS-PAGE was conducted for the collected fractions.

1. Introduction

Although the importance of monoclonal antibodies (mAbs) in biopharmeutical industry is steadily growing, their large-scale manufacturing is still expensive [1] as downstream processing still accounts for up to 60% of the overall production costs [2]. The most prominent media in the purification of immunoglobulin G (IgG) are up to date Protein A resins, since they allow the direct and most-efficient capture of IgG from a cell culture supernatant [3]. Hence, Protein A media require a reduced number of pre-operational steps such as de-salting, buffer change and pH adjustments, which are not only laborious, but are most of all inconvenient. Their obvious drawbacks are, however, the chemical and enzymatic instability of the protein-type ligand, which could be significantly improved by protein engineering [4]. But although the availability and chemical stability of Protein A has been improved in recent years, it appears to be by a factor of 10 more expensive than alternative materials for antibody purification. Cleaning in place (CIP) and digestion by enzymes present in the cell culture feed may induce degradation and ligand leakage [5,6]. The reduced number of ligands present on the adsorbent surface leads subsequently to decreased binding capacities for the target protein. Furthermore, decoupled Protein A and degradation products thereof [7,8] have to be removed from the target mAb with additional polishing steps, which is rather counterproductive.

Since cation exchange type IgG capture media (CIEX) provide comparable IgG binding capacities to affinity-based adsorbents, but do not suffer from the same drawbacks, they resemble an interesting alternative to the latter [9–12]. The major advantage of CIEX media are their robustness against CIP and against autoclaving as well as their stability towards enzyme promoted ligand deterioration. Common cation exchangers perform best at buffer concentrations around 25 mM and at pH values of up to 0.5–1 pH units below or above the *p*I of the target protein [13,14]. The

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cell culture broth usually provides an isotonic environment with almost neutral pH and a salt concentration of about 150 mM sodium chloride. Under these conditions, the salt ions compete with the target protein for the ionic binding sites on the support material and decrease the binding capacity of the CIEX material. Furthermore, if an inappropriate pH is used, the charge densities on the ion exchange media may be reduced and on the surface of the target molecule the charge may change to an extent where their net polarity is reversed. In the worst case this will lead to a reduced or even no capture of IgG [15]. Consequently, the properties of the buffer system concerning conductivity and pH are of utmost importance in ion exchange chromatography and therefore additional unfavorable intermediate process steps often need to be implemented such as adjustment of pH and/or buffer dilution. The latter also includes an unfavorable increase of buffer volume and overall buffer consumption. Hence, it can be stated that the selectivity of CIEX-type adsorbents depend on the buffer conditions [16,17], which is an additional reason why the industrial application of strong (SCX) and weak (WCX) cation exchangers for mAb purification seem to be less popular although a considerable range of CIEX-type adsorbents are commercially available [18,19].

Examples for alternative process technologies, which are less dependent on buffer pH are thiophilic adsorption chromatography (TAC) and hydrophobic interaction chromatography (HIC) [20]. The IgG capture properties of thiophilic ligands are based on the attraction and interaction of spacer-chain embedded sulfanyl or sulfonyl groups towards antibodies. This was discovered in the year 1985 by Porath et al. [21]. The so-called electron donor–acceptor interaction properties of sulfur atoms within sulfur containing groups [22,23] are recognized as a responsible force for affinity and specificity of TAC adsorbents for antibody capture [24–26]. The major disadvantage of this adsorbent type is their need for high amount of structure forming (chaotropic) salts, without which no binding would take place. Both, sulfanyl and sulfonyl bonds are stated to exhibit thiophilic properties, but differ in their lipophilicity [22,23,27–29]. The beneficial influence of thiophilic spacer arms for IgG capture with the Mimetic Ligand™ A2P has been demonstrated using computational modeling studies as well as chromatographic performance evaluation [30,31].

Another method, often used for purification of antibodies under isotonic conditions, is hydrophobic interaction chromatography (HIC). Similar to thiophilic adsorbents, structure forming salts as, e.g. ammonium sulfate support the binding of antibodies to HIC media, but with a change in selectivity. HIC ligands consist of small aliphatic chains (C1–C6) or of one or more phenyl groups and are mainly used for the polishing of final MAb products [32,33]. Their selectivity towards IgG was found to be dependent on the chemical structure of the HIC ligands, namely whether they are aliphatic or aromatic in nature [24].

A further development along the line of MAb purification media development is the introduction of hydrophobic charge induction chromatographic (HCIC) ligands such as mercapto-ethyl-pyridine (MEP). Although this ligand type bears a basic group, it also contains an aromatic and therefore lipophilic moiety, if the pH is adjusted correctly. By increasing the pH-value, the charge of the aromatic heterocycle decreases and MEP ligands therefore behave similar to a HIC material. With decreasing pH, the ligand becomes positively charged and bound IgG is released [20,34,35].

The idea of combining cation exchange with thiophilic and/or hydrophobic moieties is not entirely new [23], but a more systematic investigation on the influence of structure variation is still missing. Commercially available adsorbents with similar mixed-modal CIEX-properties are, e.g. Capto™ MMC from GE Healthcare with N-benzoyl-homocysteine [36] and MBI-Hypercel™ with 2-mercapto-5-benzimidazolesulfonic acid from Pall Lifescience [37,38], as their interactive groups. Another interesting, but non-commercial material is 6-amino-4-hydroxy-2-naphthalene sulfonic acid [39]. These mixed-modal CIEX ligands (Fig. 1) exhibit untypical properties for cation exchangers. They bind IgG at higher pH values or higher salt concentrations compared to any common strong and weak cation exchange adsorbents.

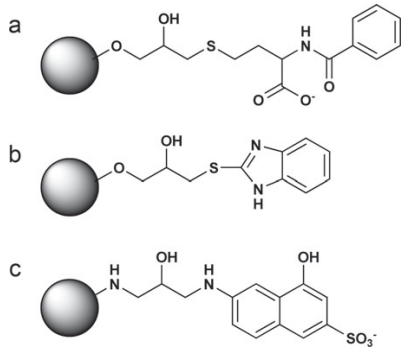

Fig. 1. Ligand structures of commercial adsorbents (a) Capto™ MMC, (b) MBI Hypercel™, and (c) 6-amino-4-hydroxy-2-naphthalene sulfonic acid.

The aim of this contribution lies in the creation of novel cation exchanger adsorbents with the capability to bind antibodies at near isotonic salt conditions and to investigate the influence of specific molecular scaffolds and functional groups on the overall binding properties. First preliminary investigations of their IgG capture performance were performed via static binding capacity (SBC) tests in dependence to the conductivity and pH-value of the test solutions containing standard polyclonal h-IgG. The best performing adsorbent was then characterized under near-real process conditions with a mock feed solution containing Pluronic F-68 in comparison to cell culture supernatant employing dynamic binding capacity (DBC) measurements.

2. Experimental

2.1. Reagents and chemicals

Pluronic F-68, sodium azide, sodium chloride, sodium hydroxide, sodium dodecyl sulfate, acetic acid, sodium dihydrogenphosphate, disodium hydrogenphosphate, 2-mercaptoethanol as well as the ProteoSilver™ Plus Silver Stain Kit (PROTSIL2) were purchased from Sigma–Aldrich (Vienna, Austria). Sodium acetate, acetic acid and pentyl alcohol was acquired from Merck (Darmstadt, Germany). 30% Acrylamide/bis solution, ammonium persulfate, precision plus protein standard (unstained), Laemmli sample buffer and N,N,N′,N′-tetra-methyl-ethylenediamine (TEMED) and 10× tris/glycine/SDS buffer were obtained from BIO-RAD (Vienna, Austria).

Fractogel® EMD Epoxy (M) with an epoxide group density of 1000 μmol/g dry gel, Fractogel® EMD SO3 (M) and Fractogel® EMD COO (M) had particle sizes of 40–90 μm and pore sizes of about 800 Å. All three Fractogel type media are cross-linked polymethacrylate resins modified with the tentacle-grafting technology from Merck KGaA (Darmstadt, Germany). Elemental analysis (sulfur content) was used for ligand density determination of Fractogel® EMD SO3 (440 μmol/g; C: 51.75%; H: 7.78%; N: 0.80%; S: 1.41%) and Fractogel® EMD COO (261 μmol/g; C: 52.34%; H: 7.37%; N: 0.37%; S: 0.069%). By titration with 1 M NaOH, 462 μmol/g and 510 μmol/g of ionic groups were determined for these two materials, respectively. The Capto™ MMC gel (average particle size: 75 μm, ionic capacity according to the manufacturer: 0.07–0.09 mmol/mL) was purchased from GE Healthcare (Vienna, Austria). Titration provided 504 μmol/g ionic acid groups and ele-

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mental analysis 509 μmol/g ligands (C: 47.34%; H: 6.6%; N: 0.83%; S: 1.63%).

Bi-distilled water was prepared in house, filtered through 22 μm cellulose acetate filter from Sartorius Stedim Biotech GmbH (Goettingen, Germany) and was ultrasonicated prior to use.

Polyclonal human immunoglobulin G (h-IgG) Gammanorm® (165 mg/mL with a purity of 95%) from Octapharma (Lachen, Switzerland) consisted of the IgG subclasses IgG_1 (59%), IgG_2 (36%), IgG_3 (4.9%) and IgG_4 (0.5%), respectively, and contained also a small amount of IgA (82.5 μg/mL). Polyclonal human IgG Beriglobin® (160 mg/mL with a purity of 95%) from CSL Behring (Vienna, Austria) comprised of IgG_1 (61%), IgG_2 (28%), IgG_3 (5%), IgG_4 (6%) and 1.7 mg/mL IgA. The cell culture supernatant was purchased from Excellgene (Monthey, Switzerland). It contained 39.6 μg/mL human monoclonal IgG1 (h-IgG1) expressed by a Chinese hamster ovary (CHO) cell line and 2.2 μg/mL DNA. It had a conductivity of 25.9 mS/cm (~218 mM NaCl) and a pH of 7.7 at 33.2 °C. After dialysis, the application feed contained 32.9 μg/mL h-IgG1 and 0.93 μg/mL DNA. It was adjusted to 1 mg/mL IgG using IgG (Beriglobin), 75 mM NaCl, 1 mg/mL Pluronic F-68, 1 mg/mL sodium azide and pH 6.5. The cell culture feed was dialyzed with Spectra/Por® Float-A-Lyzer® tubes (10 mL) from Spectrum Laboratories, Inc. (Eindhoven, Netherlands), which had a Biotech Cellulose Ester Membrane with a molecular weight cut-off (MWCO) of 3–5 kDa. Dialysis was performed twice using 2 L 10 mM PBS with 75 mM NaCl at pH 7.2.

2.2. Equipment

The dynamic binding capacity (DBC) measurements were performed using a Gilson® Minipuls 3 peristaltic pump (Villiers-le-Bel, France) for feed loading. This peristaltic pump was equipped with a standard pump head with 10 rollers and 2 channels. The feed flow was adjustable from 0.3 mL/min to 30 mL/min and the pump could withstand a back pressure of 5 bar. A calibrated polyvinylchloride (PVC) tubing with 1.02 mm ID was used. The low-pressure HPLC system consisting of a degasser, binary pump, auto injector (not used), 6-port/2-position switching valve (Agilent 1200 Series) and a multi wavelength detector UV/vis detector of the Agilent 1100 Series was from Agilent (Waldbrunn, Germany). Both pumps were connected over the 6-port 2-position switching valve to the bio-chromatographic column, which was connected to the MWD detector from Agilent, followed by the pH-conductivity device pH/C-900 from GE Healthcare (Vienna, Austria) and the automated sample collector Advantec SF-3120 (Dublin, USA).

For the dynamic binding capacity measurements, a C 10/10 column with two AC10 flow adapters from GE Healthcare (Vienna, Austria) was used. The 1 mL gel sample was packed at a bed-height of 1.3 cm between two Superformance Filter F membranes with 10 mm ID from Götec-Labortechnik GmbH (Mühltal, Germany).

The IgG content of all feed related samples was determined via Protein A high-performance liquid chromatography (Protein A HPLC) using a pre-packed Protein-A ImmunoDetection® sensor cartridge filled with 0.8 mL of POROS® 20 μm particles possessing pore sizes between 500 and 1000 nm from Applied Biosystems (Vienna, Austria). All other standard IgG samples were quantified photometrically on the UV/vis spectrometer Specord50 from Analytik Jena (Jena, Germany) at a wavelength of 280 nm and a path length of 10 mm. The DNA-content was measured with a Quant-iT PicoGreen dsDNA-kit from Invitrogen GmbH (Lofer, Austria) on the fluorescence spectrometer LS 50 B from PerkinElmer (Vienna, Austria). Hellma 105.251-QS ultra-micro fluorescence cuvettes with 3 mm × 3 mm light path and 45 μL cell volume were obtained through Wagner & Munz (Vienna, Austria). Data acquisition was performed with FL WinLab software from PerkinElmer (Vienna, Austria).

SDS-PAGE was performed on a Mini-Protean 3 device powered by a Mini-PROTEAN 3 Cell/PowerPac 300 System (220/240 V) of Bio-Rad (Vienna, Austria). The testing of different adsorbents via SBC test protocol 2 was performed fully automated, using the laboratory automation workstation Biomek® FX^P from Beckman Coulter Inc. (Krefeld, Germany) in combination with Multi Screen HTS filter plates with 0.65 μm for 96 well plates from Millipore (Schwalbach, Germany) and UV-Star Platte 96 K (Greiner Bio-One, Essen, Germany).

2.3. Sample analysis

2.3.1. Protein A-HPLC

The IgG content of the feed solutions was determined with the Protein A-HPLC method. The binding buffer was 10 mM phosphate buffer with 150 mM NaCl at pH 7.20, while the elution buffer was 12 mM HCl with 150 mM NaCl at a pH of approximately 2.5. The injection volume was 100 μL and the absorbance was measured at 280 nm [40].

2.3.2. DNA quantification

The quantification of dsDNA from cell culture supernatant as well as all related sample fractions was performed with the Quant-iTTM PicoGreen® dsDNA assay kit according to the technical instructions provided by Invitrogen (Lofer, Austria) [40,41].

2.3.3. SDS-gel electrophoresis

The 8% and 10%-Tris–HCl gels with a thickness of 0.75 mm and 10 sample wells were hand-cast according to the instruction manual provided by BioRad [42]. The protein samples were treated with Laemmli buffer [43] or with Laemmli buffer containing 5% 2-mercaptoethanol at a 1:1 ratio. In later case the samples were heated for 5 min at 90 °C to obtain information of the reduced protein molecules, before application of 5 μL of the sample-Laemmli mix to each sample wells.

Protein bands were stained with silver using the ProteoSilver™ Plus Silver Stain Kit (PROTSIL2) as recommended by the manufacturer [44]

2.4. Bio-chromatography

2.4.1. Kinetic binding study

For the equilibrium binding study three representative adsorbents (materials **A**, **E** and **L**) of the three adsorbent categories, strong thiophilic, weak aliphatic and weak aromatic cation exchanger were chosen. The test adsorbents were conditioned with 25 mM acetate/phosphate buffer with 75 mM NaCl at pH 5.5 for materials **A** and **E**, and with 75 mM NaCl at pH 6.5 for material **L**. The resins were suction dried via glass fritted funnel (pore size 3) under vacuum and aliquots of 3× 30 mg for each test adsorbent were transferred into 1.5 mL Eppendorf vials. The h-IgG (Gammanorm) solutions contained 8 mg/mL IgG for materials **A** and **E**, and 5 mg/mL IgG for material **L**. To each vial 1 mL of IgG solution was added and shaken for 5, 30, 60, 120 and 240 min at 25 °C and 1400 rpm on a thermomixer-compact from Eppendorf (Hamburg, Germany). After centrifugation at 10,000 rpm for 1 min with a mini-spin centrifuge from Eppendorf (Hamburg, Germany) the supernatant was removed. Each sample was washed for 5 min with 3× 1 mL application buffer, before elution of IgG with 3× 1 mL of application buffer with 1 M NaCl using an agitation time of 5 min. Note that for each wash and elution step, the sample vials were centrifuged at 1400 rpm. The supernatants for feed application and elution were measured at 280 nm. The solutions were diluted 1:4 if necessary. All values are the average of triple measurements. In order to state elution capacities in mg IgG per mL adsorbent, the correlation factors

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0.92 mL, 1.44 mL and 1.52 mL per 1 g adsorbent were determined for materials **A**, **E** and **L**.

2.4.2. Static binding capacity (SBC)

2.4.2.1. SBC test protocol 1. The SBC measurements were performed with standard polyclonal IgG solutions containing 5 mg/mL h-IgG, Gammanorm® in 25 mM PBS buffer prepared at seven different pH values (4.5, 5, 5.5, 6, 6.5. 7 and 7.5) and with 4 different NaCl concentrations (0, 75, 150 and 300 mM NaCl). To an aliquot of 10 μL of settled adsorbent, 200 μL of the IgG test solution (5 mg/mL) was added. The test slurry was agitated for 15 min, before the adsorbent was washed 3 times for 5 min with 1 mL binding buffer through shaking. Elution was induced by agitating the adsorbent 3 times for 5 min with elution buffer containing 1 M NaCl at the same pH as the binding buffer. The elution solutions were measured at 280 nm and diluted 1:4 if necessary. This protocol was performed manually.

2.4.2.2. SBC test protocol 2. Because of the insufficient buffering at low pH, the PBS buffer from test protocol 1 was exchanged for a 25 mM acetate/phosphate buffer. Also the IgG concentration was doubled and the incubation time of the adsorbent with antibody solution was increased to 2 h. The washing and elution steps were performed as previously described in SBC test protocol 1. This protocol was performed automated by the liquid handler Biomek® FX^P. Aliquots of 20 μL of the corresponding adsorbents (in suspension) were placed into the wells of the Multi Screen HTS filter plate, which was then positioned in the Biomek® FX^P device. All other steps were performed fully automated by the robotic workstation. After sedimentation of the adsorbent, the supernatant was removed by suction and 200 μL of the corresponding IgG test solutions (10 mg/mL buffer of the corresponding pH and sodium chloride concentration) were added. After 120 min of agitation the supernatants were removed by suction, transferred into a UV-Star Platte 96 K well plate and diluted by a 1:1 ratio. The IgG concentrations of the SBC-solutions were determined at a wavelength of 280 nm. The washing of the adsorbent was performed by adding 3× 200 μL acetate/phosphate buffer with a similar pH and sodium chloride concentration as the corresponding binding buffer and subsequent shaking for 5 min. The wash solutions were removed by suction. For IgG elution, 200 μL elution buffers with 1 M sodium chloride at pH 7.0 were added to the adsorbent. The elution solution was rinsed into a UV-Star Platte 96 K and measured at 280 nm. Afterwards the solution was diluted 1:1 and was re-measured.

In order to minimize the error inflicted by unspecific binding of IgG to the walls of the well plates, all "static binding capacities" presented in this study are in fact expressed via the elution capacities and resemble the average values of triple measurements. Their standard deviation was overall between 3% and 9%. Note that the actual binding capacities for the IgG capture steps are not shown.

2.4.3. Dynamic binding capacity (DBC)

All tested CIEX adsorbents were suspended in a 1 M NaCl solution and were allowed to settle in a graduated 5 mL volumetric flask to ensure an accurate determination of 1 mL of gel used for the measurement. The gel was inserted into the C 10/10 column on top of a 10 mm ID filter membrane discs placed on the AC10 flow adapters. The gel was allowed to settle by gravity, before the second filter disc and the second AC10 flow adapter were placed above the settled gel. The column was purged at a flow rate of 5 mL/min and the adapter was positioned to a final bed height of 13 mm.

The DBC measurements were performed with two standard IgG solutions prepared with IgG Gammanorm® and IgG Beriglobin® containing 1 mg/mL IgG in 25 mM phosphate buffer with 75 mM or 150 mM NaCl at pH values of 5.5, 6.5 and for the most promising adsorbent also at pH 7.4. As it is known from other studies that Pluronic can strongly affect the DBC results of MAb selective sorbents, Pluronic-F68 was included in the screening scheme. Therefore a further test was performed with a mock feed solution containing 1 mg/mL Pluronic F-68 and 150 mM NaCl at pH 6.5. In addition, material **L** was tested with a dialyzed cell culture supernatant, which was adjusted to pH 6.5 and 75 mM NaCl. It was spiked with IgG Beriglobin to increase the IgG concentration to 1 mg/mL, besides 1 mg/mL Pluronic F-68 and 1 mg/mL NaN_3. For all DBC measurements, if not stated otherwise, the polyclonal IgG, Beriglobin® was used at a concentration of 1 mg/mL in the corresponding application buffer.

After loading of the IgG test solution via peristaltic pump onto the column until 100% breakthrough of IgG was reached, the column was washed with the corresponding application buffer. The flow rate was 0.6 mL/min (57 cm/h), which is equivalent to a residence time of 1.3 min. The elution buffer had the same pH as the binding buffer, but its salt content was increased to 1 M NaCl. In the case of the dialyzed feed experiment, the wash solution consisted of 25 mM phosphate buffer and 0 mM NaCl. The elution of h-IgG was performed by applying firstly a linear salt gradient from 0 mM to 1 M NaCl within an elution volume of 110 mL, which was followed by an isocratic elution with 1 M NaCl for additional 10 mL. If not otherwise stated, all adsorbents were sanitized with 0.5 M NaOH. Note that sample fractions of 10 mL were collected.

2.5. Synthesis

Several ligands with different functional groups were synthesized and coupled onto the solid support, subsequently exhibiting different chemical properties. The synthesis of these ligands, their immobilization onto the support material and their physicochemical characterization are described in detail in electronic supplementary material. Note that ligand densities were determined via elemental analysis (EA) and acid/base titration (T) and are stated as μmol ligand per gram dry gel in Table 1. If not stated otherwise, ligand densities mentioned in this study refer to the results obtained by titration. A summary of the investigated mixed-modal CIEX-ligand structures are depicted in Fig. 2.

3. Results and discussion

3.1. Kinetic binding study

In order to determine the time-dependency of IgG capture for the various adsorbents discussed in this article, one of each of the three types of cation exchangers, strong thiophilic, weak aliphatic and weak aromatic (Fig. 2), was chosen as a representative for the group. The adsorbents are materials **A**, **E** and **L**. The equilibrium kinetics in Fig. 3 was determined via static binding capacity (SBC) tests, where each data point resembled the averaged elution capacity result of triple measurements with an average standard deviation of 8.3%.

A comparison of the three tested adsorbents reveals a fast IgG capture kinetic for materials **A** and **E**, where after 15 min saturation was accomplished and only minor changes in the elution capacities could be seen. Material **L** on the other hand shows a much slower binding kinetic compared to materials **A** and **E**. Partially, this result may have been caused by the slightly lower IgG concentration of 5 mg/mL used for the testing of material **L** instead of 8 mg/mL used for the former two adsorbents. However, the equilibration curve for material **L** indicates that this adsorbent binds IgG slightly slower, but possesses an overall higher binding capacity for IgG, since saturation was not yet reached after 40 min and even after 3 h the binding curve is still slightly ascending. Nevertheless, the imprecision of SBC measurements terminated after 120 min as it is the case for SBC test protocol 2 is low and can be considered being negligi-

Table 1
Overview of investigated mixed modal weak and strong cation exchange ligands together with their abbreviations (Abbr.) and ligand densities (LD) determined via elemental analysis (EA) and titration (T).

	Abbr.	Adsorbent	LD (EA) [μmol/g]	LD (T) [μmol/g]
Thiophilic sulfonic acid	**A**	3-Mercapto-1-propanesulfonic acid	1207	1023
	B1	3-Sulfonyl-1-propanesulfonic acid	955	637
	B2	3-Sulfonyl-1-propanesulfonic acid	1011	886
	C	2-(2-Sulfonylethoxy)-ethanesulfonic acid	840	564
	D	3,6-Dioxa-8-sulfonyl-1-octanesulfonic acid	790	457
Aliphatic HIC	**E**	3-Mercaptopropionic acid	1100	1043
	F	N-Acetyl-L-cysteine	1031	941
	G	N-Acetyl-D,L-homocysteine	843	727
	H	N-Acetyl-D,L-penicillamine	759	604
Aromatic HIC	**I**	N-Benzoyl-L-cysteine	709	641
	J	N-Benzoyl-D,L-homocysteine	887	843
	K	N-(3,5-Dimethoxybenzoyl)-D,L-homocysteine	909	738
	L	4-Mercaptobenzoic acid	950	950
Commercial	–	Fractogel® EMD SO_3	440	462
	–	Fractogel® EMD COO	261	510
	–	Capto™ MMC	509	504

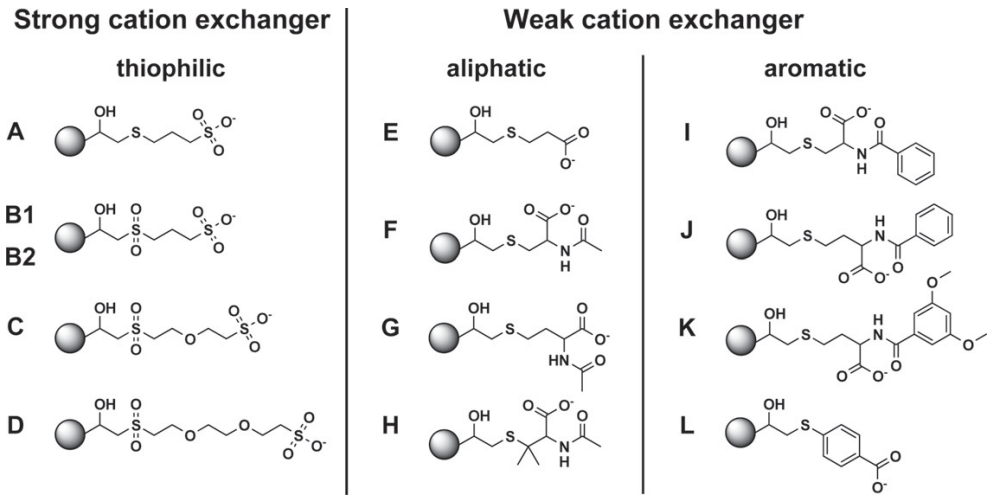

Fig. 2. Overview of investigated strong and weak cation exchanger adsorbents combined with a variety of different functional groups and different lipophilicities. Note that materials **B1** and **B2** are structurally identical, but were prepared with different synthesis protocols.

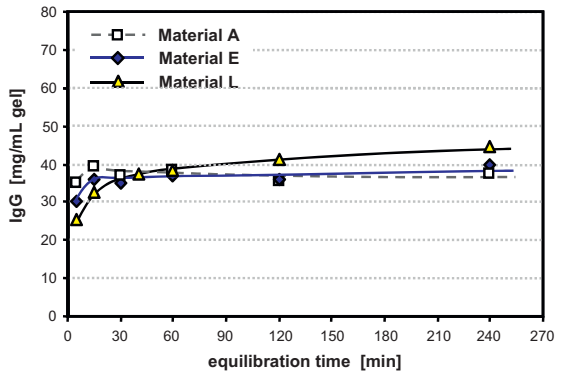

Fig. 3. Kinetic binding study for materials **A**, **E** and **L**, using 25 mM acetate/phosphate buffer with 75 mM NaCl adjusted to pH 5.5 for materials **A** and **E**, and adjusted to pH 6.5 for material **L**. 1 mL IgG test solution with 8 mg/mL IgG for materials **A** and **E**, and 5 mg/mL IgG for material **L** were added to 30 mg adsorbent and agitated for 5, 30, 60, 120 and 240 min at 25 °C. The data points are averaged elution capacities determined with triple measurements and calculated to mg IgG per mL adsorbent.

ble. In general, one has to differentiate between the fast primary binding, which exhibits in a strong binding of IgG to the adsorbent and a secondary binding that is rather slow and weak. Therefore, equilibration times above 2 h and static binding results determined by the difference between the IgG content in the supernatant and the original feed should be avoided. All SBC results presented in this article are solely the elution capacities.

3.2. Thiophilic cation exchange adsorbents with sulfonic acid group

The immobilization of sulfonic acid bearing ligands onto Fractogel® EMD Epoxy was conducted by coupling the dithiol functional ligands 1,3-propanedithiol, 2,2′-oxydiethanethiol, 3,6-dioxa-1,8-octanedithiol to the epoxide-activated support surface. The subsequent oxidation of the sulfanyl and the sulfhydryl groups to sulfonyl and sulfonic acid groups lead then to materials **B1**, **C** and **D** (Fig. 2 and Table 1). Alternatively, material **A** was prepared through direct immobilization of 3-mercapto-1-propanesulfonic acid onto the support. The subsequent oxidation of the sulfanyl group led to material **B2** (see electronic supplementary material, Fig. S2).

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These immobilization and oxidation reactions are straightforward and easy to accomplish providing sufficiently high ligand densities of 800–1000 μmol/g dry gel, respectively determined by elemental analysis (Table 1). Comparing the ligand densities obtained by elemental analysis (EA) with the titration results for the sulfhydryl determination using the 2,2′-dipyridyl disulfide (DPDS) method [45] (460–780 μmol/g) lead to rather strong deviations. However, the acid/base titration (T) results for the sulfonic acid group provided similar but lower values (460–640 μmol/g) as earlier obtained with the DPDS method. The reason for the strong differences of EA results and results obtained by DPDS method or titration lies in the possible cross-linking of surface bound ligands with epoxide groups in close proximity. This undesired reaction may occur during the preparation process despite the huge excess of dithiol-functional ligand used. The additional formation of disulfide bridges can only partially explain the imbalance between the DPDS results and the EA results, since disulfide bridges ought to break during the oxidation reaction, when the sulfur atoms are oxidized to sulfonic acid groups, and this imbalance still existed after the oxidation process. The slightly higher ligand densities obtained with elemental analysis compared to the acid/base titration results for materials **A** and **B2** as well as the higher ligand density of precursor material **A** compared to its oxidized analog, material **B2**, point clearly towards the formation of a small amount of disulfide bridges during the first reaction step.

Fig. 4 depicts the SBC results of materials **A**, **B1**, **B2**, **C** and **D** under all investigated pH values (pH 4–7.5) and salt conditions (0–300 mM NaCl) established with SBC test protocol 2. A comparison with the commercial strong cation exchanger Fractogel® EMD SO_3^- in Fig. 5 shows slightly lower yet comparable binding capacities for materials **A** and **B2**. The materials **B1**, **C** and **D** provide rather low IgG binding under all test conditions.

Note that material **B2** is structurally similar to material **B1**, with the difference that in case of **B1** the ligand was directly immobilized, while **B2** was prepared in a two step immobilization and oxidation protocol with material **A** as a precursor. The improved performance of material **B2** was clearly the result of the different preparation protocol, where no cross-linking of the ligand was possible combined with a slightly higher ligand density.

The main differences between materials **B1**, **C** and **D** are a slight decrease in ligand density, combined with an increase in the length of their spacer chains by one and two ethoxyl groups. Since no significant variation in IgG capture performance of these tree adsorbents was observed, it can be assumed that spacer length and slight variations in ligand densities in the range of ±100 μmol/g have little to no influence on the IgG capture performance of thiophilic sulfonic acid type adsorbents.

The difference in material performance observed for the precursor material **A,** which lack a sulfonyl group compared to material **B2**, leads to the conclusion that the incorporation of a sulfonyl group has a somewhat negative effect on IgG capture, when combined with a sulfonic acid head group.

Concerning IgG capture performance, all thiophilic sulfonic acid adsorbents have in common that they were not able to bind sufficient amounts of IgG under high pH values and high salt concentrations. In the absence of sodium chloride, material **A** shows a maximum binding capacity of 86 mg/mL at pH 5.0, while for material **B2** the maximum was of 72 mg/mL at the same pH. Fractogel® EMD SO_3 in Fig. 5 provided the best IgG capture performance with 118 mg/mL IgG at 0 mM NaCl and pH 5.0.

Note that the ligand densities of material **A** and **B2** were about 1023 μmol/g and 886 μmol/g, respectively, which was twice as high as that of the commercial sulfonic acid cation exchanger with a ligand density of around 440 μmol/g dry resin. The SBC results for Fractogel® EMD SO_3 and material **A** decrease strongly with increasing pH and reach 18 mg/mL and 16 mg/mL, respectively at a pH of

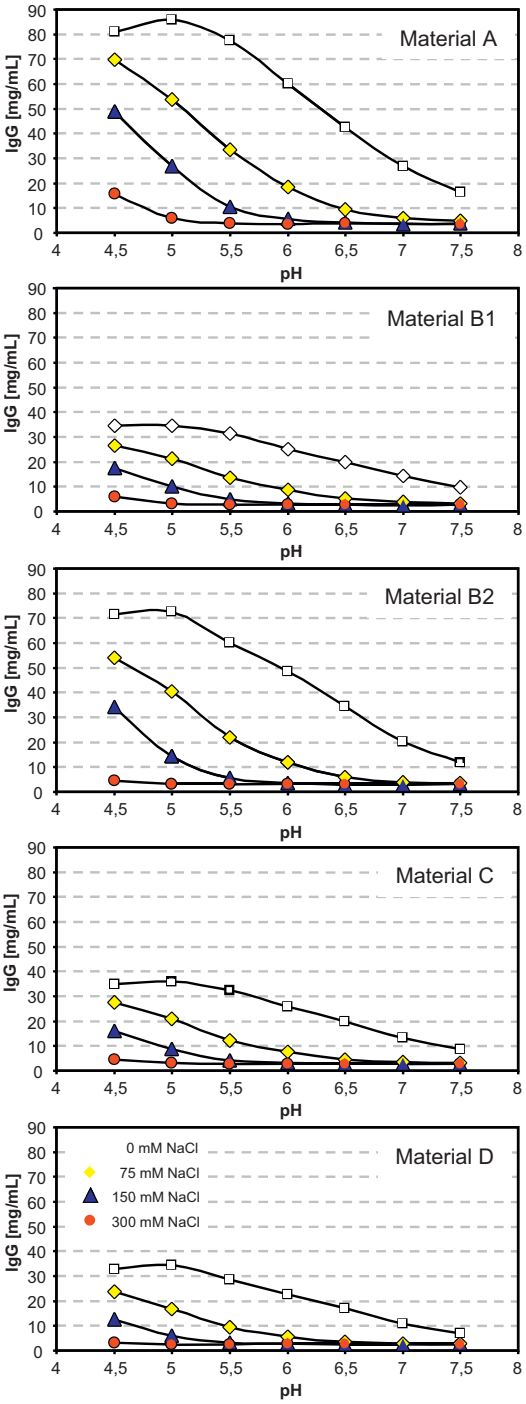

Fig. 4. Static binding capacity (SBC) results for materials **A**, **B1**, **B2**, **C** and **D** using polyclonal h-IgG are plotted against the pH-values (from pH 4.5 to 7.5) of test solutions containing varying amount of sodium chloride (0, 75, 150, 300 mM NaCl). SBC-tests were performed according to SBC test protocol 2.

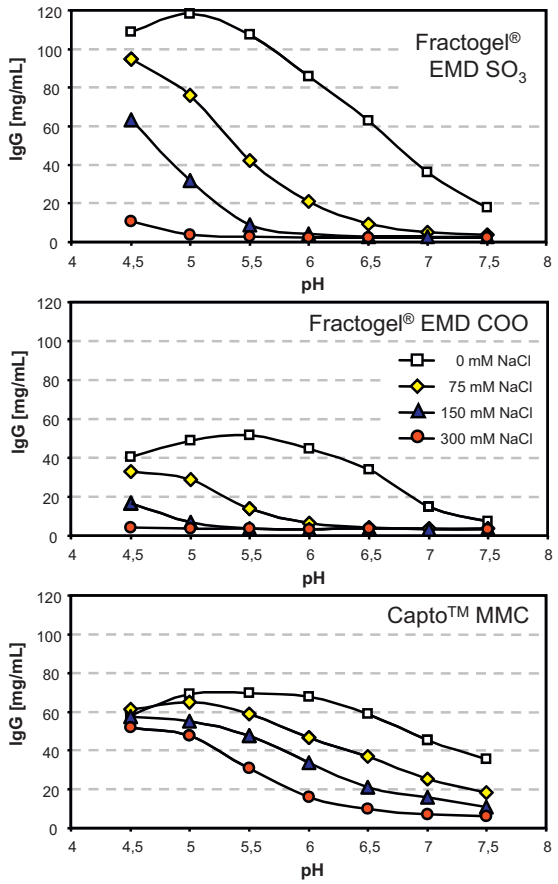

Fig. 5. SBC diagrams for the commercial cation exchanger adsorbents Fractogel® EMD SO3 and COO from Merck KGaA and Capto™ MMC from GE Healthcare tested with polyclonal h-IgG at different pH-values (from pH 4.5 to 7.5) and different sodium chloride concentrations (0, 75, 150 and 300 mM NaCl). SBC-tests were performed according to SBC test protocol 2.

7.5. On the other hand, a decrease of pH down to 4.5 provided only a slight decrease of elution capacities to 109 mg/mL and 81 mg/mL for the two investigated adsorbents at 0 mM NaCl. For sample solutions containing sodium chloride, the peak maximum shifted from 5.0 to 4.5. This led to elution capacities for Fractogel® EMD SO_3 and material **A** at 75 mM NaCl and pH 4.5 of 95 mg/mL and 70 mg/mL, respectively. In the presence of 150 mM NaCl and pH values of 5.5 and lower, both materials perform very similar, but in general, it can be concluded that Fractogel® EMD SO_3 performs slightly better compared to material **A,** independent of the pH value and salt content of the test solution.

Note that the use of thiophilic adsorbents always goes hand in hand with the addition of high amounts of structure forming salts, which follow the Hofmeister series [25]. In the present study no chaotropic additives were used, since they are rather undesired in large-scale downstream processing of mAbs. In case of sodium chloride, its structure forming effect can be considered being negligible.

3.3. Weak cation exchange adsorbent

Since the investigated sulfonic acid type adsorbents **A** and **B** did not seem to be much influenced by the change of the sulfanyl linkage to a sulfonyl group, all other adsorbents (**E** to **L**) were only prepared with a sulfanyl group. In any case, a conversion of the sulfanyl group to the corresponding sulfonyl group would not be possible with the oxidation protocol used in this study, since the carboxylic acid group would be oxidized to a per acid with the additional danger of ligand degradations. Nonetheless, it can be expected that a replacement of the sulfonic acid group by carboxylic acid ought to have a much stronger influence on material performance than a possible oxidation of the sulfanyl group as stated earlier.

In a first attempt, 3-mercaptopropionic acid was bound to the support material with a ligand density of 1043 μmol/g dry gel, which is denominated as material **E** (Fig. 2). As depicted in Figs. 5 and 6a, the static binding capacities of material **E** investigated at 0 mM and 75 mM NaCl using SBC test protocol 1 were all below the results obtained for the bench mark material Fractogel® EMD SO_3, which were established with SBC test protocol 2. As stated in Section 3.1, an equilibration time of 15 min was sufficient for material **E**. A significant deviation between SBC test protocol 1 and SBC test protocol 2 is not expected. For IgG test solutions with NaCl concentration of 150 mM and above, and with pH values below 6.0, binding capacities of material **E** were higher than those of Fractogel® EMD SO_3. For example at pH 5.5 and 150 mM NaCl binding capacities of 20 mg/mL for material **E** and 9 mg/mL for the bench mark material were obtained. Under higher pH conditions, both materials were not able to bind significant amounts of h-IgG.

A comparison with the weak cation exchanger Fractogel® EMD COO (ligand density: 509 μmol/g dry gel) measured with SBC test protocol 2 depicted in Fig. 5, exhibited for SBC tests with 0 mM NaCl generally lower elution capacities for material **E** (Fig. 4). Also at pH 4.5 and 75 mm NaCl, the commercial weak cation exchanger showed slightly higher capacities, namely 33 mg/mL compared to 22 mg/mL for material **E**. But for pH values between 4.5 and 5.5 at 150 mM NaCl as well as for pH 4.5 and 5.0 at 300 mM NaCl, material **E** performed much better than Fractogel® EMD COO. Material **E** was able to reach binding capacities of up to 15 mg/mL for test solutions containing 300 mM NaCl at pH 5.0, which was also the optimum pH for 150 mM NaCl solutions with a binding capacity of 26 mg/mL. For test solution containing 75 mM NaCl material **E** showed a maximum binding of 34 mg/mL IgG at pH 5.5. In case of test solutions without sodium chloride addition, a maximum elution capacity of 41 mg/mL at pH 6.0 was obtained. Note that for IgG solutions with pH values below or above the optimum pH value, a drop of binding capacities was observed. It is noticeable to mention that the earlier discussed thiophilic sulfonic acid ion exchangers showed peak maxima for test solutions containing 75 mM or more NaCl at pH 4.5.

Thus a question remains; why is Fractogel® EMD COO an insufficient IgG capture material under isotonic conditions, while another carboxylic acid carrying adsorbent is able to bind substantial amounts of IgG? Note that both adsorbents have the same underlying polymethacrylate support matrix, bearing a similar tentacle structures on their surface. It seems obvious that the different ligand attachment chemistries of the two adsorbents are responsible for their different IgG binding properties. Elemental analysis of the commercial weak cation exchanger provided evidence of the presence of nitrogen and the absence of sulfur, while material **E** possessed a sulfanyl linkage. It can therefore be assumed that the sulfanyl group is responsible for the enhanced binding properties of material **E**, although it is rather difficult to relate the different IgG capture performances decidedly to thiophilic properties or an altered overall lipophilicity.

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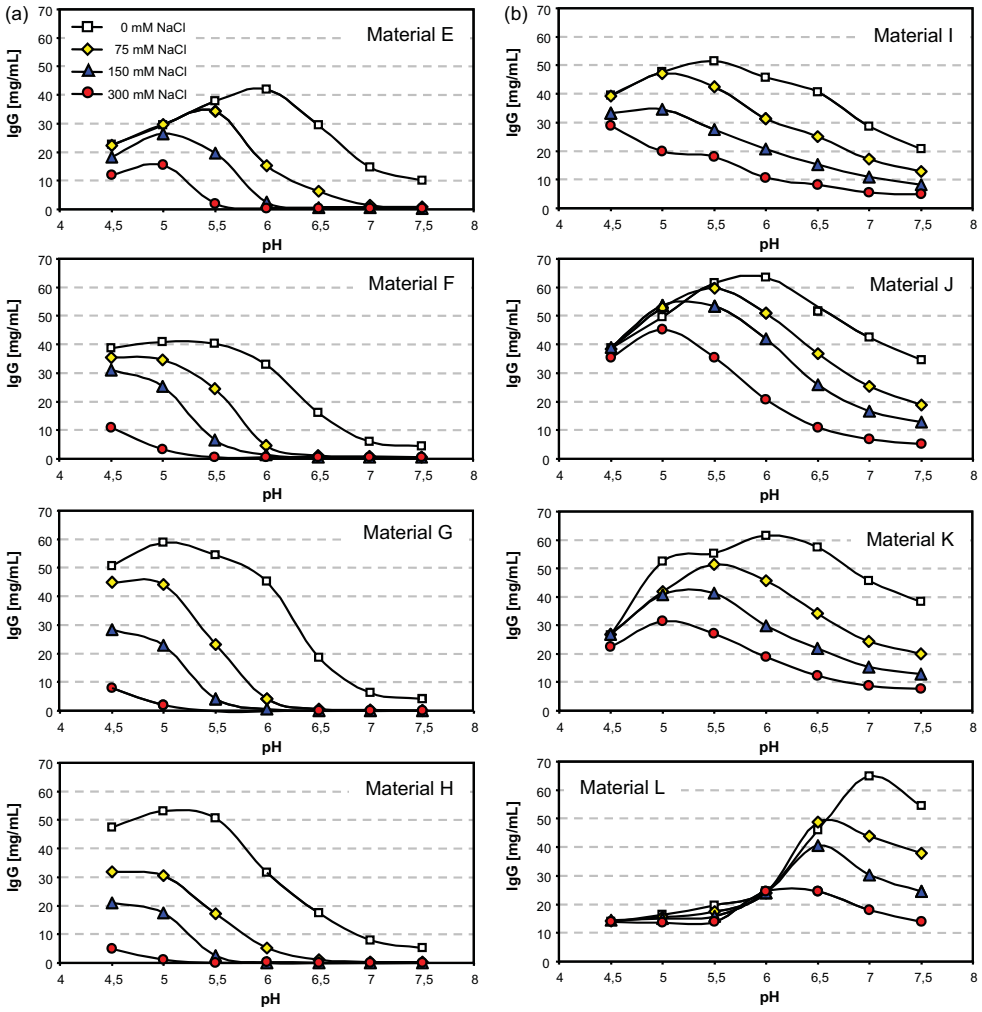

Fig. 6. SBC diagrams for the aliphatic HIC materials **E**, **F**, **G** and **H** were established with SBC test protocol 1, the aromatic HIC materials **I**, **J**, **K** and **L** were established with SBC test protocol 2. All adsorbents were tested with polyclonal h-IgG at different pH-values (pH 4.5–7.5) and different sodium chloride concentrations (0, 75, 150 and 300 mM NaCl).

3.4. Aliphatic hydrophobic interaction adsorbents

Based on the results obtained for 3-mercaptopropionic acid, further carboxylic acid bearing adsorbents with additional amide and aliphatic or aromatic moieties were prepared (Fig. 2).

All adsorbents were so-called "endcapped" with 2-mercaptoethanol to deactivate any residual epoxide groups. Note that the yields of the "endcapping" reactions judged by elemental analysis were for all adsorbents negligible. It can be expected that any residual epoxide groups present after ligand immobilization, ought to be unstable under the harsh immobilization conditions of pH >12 and may have reacted already to vicinal diol groups as a side reaction.

Cysteine and derivatives thereof were chosen as suitable ligands, because they exhibit a sulfhydryl group for ligand attachment and a carboxylic acid group accessible for capturing mAbs (see electronic supplementary material, Fig. S1). Furthermore several amino acids were already reported as potential antibody capture materials, especially histidine [46–48], but also tryptophane and phenylalanine [49]. An even further advanced material was the PAM (Protein A mimic) ligand [50], a tetrameric polypeptide with affinity for antibody capture at binding conditions of neutral pH and isotonic salt concentrations.

The first candidate N-acetyl-L-cysteine gained a maximum ligand density of 941 μmol/g and was abbreviated as material **F**. In comparison material **G**, carrying N-acetyl-D,L-homocysteine as a ligand, comes with an additional methylene group in the spacer chain. Material **H** carries N-acetyl-D,L-penicillamine as a ligand, which is similar to material **F** except for the two methyl groups attached to the carbon chain between the sulfanyl group and the carboxylic acid group.

Note that materials **F**, **G** and **H** were tested in batch experiments with SBC test protocol 1. Their results are depicted in Fig. 6a. While material **E** showed for measurements with decreasing salt content, a significant shift as well as increase of the binding capacity maximum with increasing pH for pH values above 4.5, e.g. pH 6 for 0 mM NaCl, this tendency was amiss or barely noticeable for material **F**, **G** and **H**. Only the curves obtained with test solutions containing 0 mM salt showed a similar, but much weaker trend. Another difference was the higher binding capacities for material **F**, **G** and **H** at low pH and 0, 75 and 150 mM NaCl compared to material **E**. With increasing pH, this behavior changes and material **E** exceeds the

n-acetylated materials. Only for the measurement with feed solutions containing 300 mM employing SBC test protocol 1, the IgG binding capacity of material **E** exceeds those of materials **F**, **G** and **H**. Above pH 5.5 none of these materials were able to capture substantial amounts of IgG in the presence of 300 mM NaCl.

Summing up these observations, the introduction of an amide group did not improve the IgG capture performance of investigated adsorbents under test conditions with pH values higher than 6.0 at 150 mM NaCl or higher. As can be seen in Table 2, the binding capacities for the materials discussed in this chapter were under the stated test condition within the range of strong cation exchangers as well as the investigated commercial ion exchangers Fractogel® EMD SO3 and Fractogel® EMD COO.

A comparison of the three cysteine related materials **F**, **G** and **H** exhibits only minor differences in performance. One of these deviations is the considerably higher capacity of material **G** compared to material **F** and **H**, although material **F** possess a higher ligand density. This effect is possibly based on the elongated spacer and the resulting increased lipophilicity of the ligand. The binding capacity curve for material **H** at 0 mM NaCl exceeds that of material **F** in Fig. 6a. For solutions with increased salt concentration, material **H** shows comparable or lower binding capacities compared to material **F**.

3.5. Aromatic hydrophobic interaction adsorbents

3.5.1. Static binding capacity study

The coupling of benzoyl-cysteine and benzoyl-homocysteine onto Fractogel led to the materials **I** and **J**. Furthermore 3,5-dimethoxybenzoyl-homocysteine was immobilized, which resulted in material **K** (Fig. 2 and Table 1). Fig. 6b shows the results of the SBC studies made by SBC test protocol 2. Besides a few exceptions, material **I** showed the lowest binding capacities of this series of aromatic cation exchangers, what can partially be assigned to the fact that it possesses the lowest ligand density of these 3 materials. The binding maximum for material **I** during the absence of sodium chloride was reached at pH 5.5 and was 52 mg/mL. Materials **J** and **K** exposed their maxima at pH 6.0 and showed binding capacities of 64 mg/mL and 61 mg/mL, respectively. The peak maximum of material **I** obtained by testing with test solution containing 75 mM sodium chloride was found again at an lower pH compared to materials **J** and **K**. Also the binding capacities measured were lower than those of materials **J** and **K**. In average, material **J** exhibits the highest capacities, followed closely by material **K** and material **I**. The source of these differences is the beneficial effect of a higher ligand density, since a higher number of ionic groups are able to bind more IgG due to an increased number of ionic interaction sites on the sorbent. The additional methoxy groups of material **K** did not significantly improve the performance of that material. For the desired binding condition at pH 6.5 and 150 mM NaCl, considerably high SBC values were found for materials **I**, **J** and **K**, namely 15.2, 26.0 and 21.8 mg/mL respectively (Table 2). Under this condition, material **E,** measured with SBC test protocol 1 exposed a binding capacity of only 0.53 mg/mL.

The commercial adsorbent Capto™ MMC gel from GE Healthcare has a very similar ligand structure compared to material **J** and provided therefore similar SBC results (Fig. 5), although the ligand density of Capto™ MMC is just about 500 μmol/g dry gel. Capto™ MMC is also a N-benzoyl-homocysteine based material, but with a different binding chemistry [51] carrying therefore a different spacer arm. The support material is Sepharose. During tests with feed solutions containing 0 mM NaCl and pH below 5.5, the binding capacities exceeded those of material **J**, but with increasing salt concentrations and increasing pH, material **J** performs better than Capto™ MMC by up to 8 mg/mL (Table 2).

The conclusion of these experiments is the positive influence of an aromatic moiety in close proximity to the carboxylic acid group, which favors IgG capture out of feed solutions containing more than 75 mM NaCl and possessing pH values above 5.5. Note that a close proximity relates to approximately 4 bonds between the aromatic and the carboxylic acid group. The subsequent next step was therefore, the coupling of 4-mercaptobenzoic acid onto epoxide activated Fractogel, which produces a sulfanyl bridge in the spacer chain. The resulting adsorbent was named material **L**.

Several ligands comparable to 4-mercaptobenzoic acid such as 4-aminobenzoic acid [52] and 2-mercaptonicotinic acid [53] have already been reported. They both possess a carboxylic acid group connected directly to an aromatic ring. The spacer chain of the nicotinic acid ligand contained a sulfanyl linkage at the same position as in material **L**. The aminobenzoic acid bearing material was coupled onto divinyl sulfone activated agarose and was tested only in the presence of 0.7 M $(NH_4)_2SO_4$, while the 2-mercaptonicotinic acid, which was also bound onto divinyl sulfone activated sepharose, was able to capture IgG out of human serum without the addition of lyotropic salts. Interestingly this ligand was not able to capture IgG in the absence of lyotropic salts, when it was connected via epichlorohydrin to the support material.

The ligand density of material **L** was about 950 μmol/g and the SBC-curves were surprisingly different compared to all the other investigated adsorbents (Fig. 6b). The ideal pH of the test solutions containing 75 mM and 150 mM NaCl was pH 6.5, and provided binding capacities of 49 mg/mL and 41 mg/mL, respectively, which represent a significant improvement compared to before mentioned ligands. Under these conditions material **J** showed binding capacities of 36.7 mg/mL and 26.0 mg/mL, respectively (Table 2). Interestingly, the binding capacities of material **L** at low pH dropped down to 14.2 and 14.4 mg/mL under above conditions. Note that under these conditions the benzoic acid is partly or even totally protonated and therefore possesses no ionic but very lipophilic character. On the other side, the target protein is highly (positively) charged and therefore the interactions are weak, and the affinity of the protein towards the ligand is low. As an outcome of all the SBC tests the three most promising materials **J**, **K** and **L** were chosen to be characterized in terms of their dynamic binding performance.

3.5.2. Dynamic binding capacity studies

For economic reasons and in order to save time, the previous SBC measurements served primarily for the elimination of the less promising ligands. The materials tested under dynamic conditions were the material **J**, **K, L** and the commercial adsorbent Capto™ MMC from GE Healthcare. For all dynamic binding capacity tests (DBC), adsorbent volumes of 1 mL at a bed height of 1.3 cm were used. In an additional measurement with material **L** using 2.5 mL adsorbent packed in the same column at a bed height of 3.2 cm, it was shown that both experimental settings provided comparable results for 100%-DBC and elution capacity (EC) (chapter 4 in supplementary information). The PBS buffer used, contained 75 or 150 mM sodium chloride with a pH of 5.5 or 6.5. In the case of material **L**, measurements at pH 5.5 were not performed. Instead DBC tests were conducted at pH 7.4 and 75 mM NaCl, which resemble conditions comparable to cell culture feed. The DBC results are summarized in Table 3. Material **J** and **K** provided very similar values for 100%-DBC and for the elution capacities (EC). These DBC results stood in good accordance with SBC results, although the latter were slightly higher than the dynamic test results. Material **J** showed an elution capacity of 31 mg/mL at pH 6.5 and 75 mM NaCl and material **K** showed an EC of 34 mg/mL. SBC measurements exhibited under similar conditions IgG binding capacities of 36.7 mg/mL and 34.3 mg/mL. This consistence between SBC and DBC measurements was found throughout all tests for material **J** and **K**. For SBC as well as DBC tests polyclonal h-IgG Beriglobin was used. A com-

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Table 2
Elution capacity (EC) results from static binding capacities (SBCs) tests for mixed modal cation exchanger adsorbents.

Thiophilic sulfonic acid[b]		Aliphatic HIC[a]		Aromatic HIC[b]	
Adsorbent	EC [mg/mL]	Adsorbent	EC [mg/mL]	Adsorbent	EC [mg/mL]
Fractogel EMD SO_3^-	2.98	Merck EMD COO^-[b]	3.52	Material **I**	15.2
Material **A**	4.12	Material **E**	0.53	Material **J**	26.0
Material **B1**	2.74	Material **F**	0.49	Material **K**	21.8
Material **B2**	2.95	Material **G**	0.00	Material **L**	40.7
Material **C**	2.78	Material **H**	0.06	Capto MMC	20.9
Material **D**	2.49				

[a] SBC test protocol 1 (manual) using PBS buffer at pH 6.5 and 150 mM NaCl.
[b] SBC test protocol 2 (automated) using PBS/acetate buffer at pH 6.5 and 150 mM NaCl.

Table 3
Summary of dynamic binding capacities at 10% and 100% breakthrough of IgG ($DBC_{10\%}$ and $DBC_{100\%}$) and elution capacities at $DBC_{100\%}$ ($EC_{100\%}$) for different feed solutions.

Application conditions			Material **J** (Benzoyl-homocysteine)		Material **K** (*3,5-Dimethoxybenzoyl-homocysteine*)		Material **L** (*Mercaptobenzoic acid*)	
pH	NaCl [mM]	Feed[a]	$DBC_{100\%}$ [mg/mL]	$EC_{100\%}$ [mg/mL]	$DBC_{100\%}$ [mg/mL]	$EC_{100\%}$ [mg/mL]	$DBC_{100\%}$ [mg/mL]	$EC_{100\%}$ [mg/mL]
5.5	75	A	55	53	46	47	–	–
		B	53	55	–	–	–	–
5.5	150	A	–	–	41	36	–	–
6.5	75	A	37	31	32	34	68	57
		B	–	–	–	–	68	57
		C	–	–	–	–	42	31
6.5	150	A	23	19	23	18	52	39
		B	15	18	–	–	–	–
		D	–	–	–	–	43	35
7.4	150	A	–	–	–	–	34	28

[a] *Feed solutions*: (A) IgG Beriglobin (1 mg/mL) in 25 mM PBS buffer. (B) IgG Gammanorm (1 mg/mL) in 25 mM PBS buffer. (C) Dialyzed cell culture feed with 10 mM PBS buffer with 75 mM NaCl, spiked with IgG Beriglobin to 1 mg/mL, Pluronic F-68 (1 mg/mL) and NaN_3 (1 mg/mL). (D) IgG Beriglobin in 150 mM NaCl spiked with Pluronic F-68 (1 mg/mL). *Test conditions*: 1 mL adsorbent at a flow rate of 0.6 mL/min and detection at 280 nm.

parison between two different types of polyclonal h-IgG, namely Beriglobin (feed A) and Gammanorm (feed B) was also done for the materials **J** and **L**. Table 3 summarizes the 100%-DBC and EC results investigated with four different test solutions, including a dialyzed cell culture feed (feed C) and a mock feed solution spiked with Pluronic-F68 (feed D).

Fig. 7 shows the IgG concentration plotted against the volume of applied feed. Material **L** exhibits practically same dynamic capture performance for feed A and feed B, which demonstrates that material **L** is not sensitive to slight variations in the composition of polyclonal h-IgG.

The dynamic test results for material **L** using feed A and B showed surprisingly high DBC values of about 68 mg/mL for 100% break-through of IgG and elution capacities of 57 mg/mL for test solutions containing pH 6.5 and 75 mM NaCl. In this case the dynamic measurements exceeded the results of the SBC measurements, which exhibited an elution capacity of 48.8 mg/mL.

In order to investigate the influence of the foam reducing agent Pluronic-F68 on the IgG capture performance of material **L**, a further DBC test with an IgG standard solution containing Pluronic F-68 (feed D) was conducted. The addition of Pluronic F-68 to the test solution led to a decrease of the elution capacity at 150 mM sodium chloride and pH 6.5 from 39 to 35 mg/mL. Additionally a DBC test was performed under isotonic conditions (pH: 7.4, NaCl: 150 mM) leading to an EC of 28 mg/mL and showed that substantial amounts of IgG could be captured also under such conditions. A final test of material **L** was done with a dialyzed cell culture supernatant containing typical impurities of a fermentation broth such as host cell proteins and DNA. Feed C was additionally spiked with Pluronic F-68 (1 mg/mL) and sodium azide (1 mg/mL), and was adjusted to pH 6.5 and 75 mM NaCl. The salt concentration resembles a 1:1 dilution of a feed solution with 150 mM NaCl and is in fact the ideal composition for antibody capture for material **L**. The elution of captured IgG was conducted with a sodium chloride gradient from 0 mM to 1 M NaCl.

For the entire DBC run, 10 mL fractions were collected and their antibody content was determined with the Protein A-HPLC method. For the application feed and a representative elution fraction also

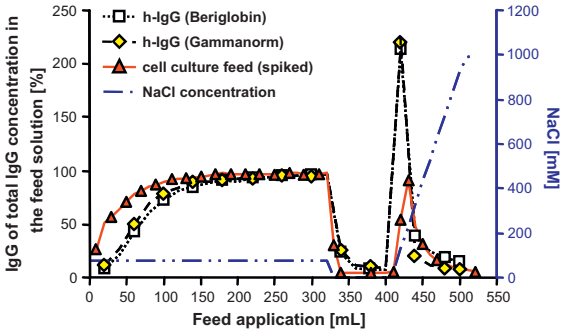

Fig. 7. Dynamic break-through curves of the aromatic HIC material **L** using polyclonal h-IgG Beriglobin (feed A: 1 mg/mL h-IgG in PBS with 75 mM NaCl at pH 6.5), polyclonal h-IgG Gammanorm (feed B: 1 mg/mL h-IgG in PBS with 75 mM NaCl at pH 6.5) and a dialyzed cell culture supernatant with monoclonal h-IgG1 (32 μg/mL) spiked with polyclonal IgG Gammanorm (feed C: 1 mg/mL h-IgG in PBS with 75 mM NaCl, 1 mg/mL Pluronic F-68 and 1 mg/mL NaN3 at pH 6.5). DBC-tests were performed at room temperature and at a flow-rate of 0.6 mL/min. For feed A and B the wash solutions had the composition of the binding buffer and the elution buffer contained 1 M NaCl at pH 6.5. The wash solution for the cell culture supernatant experiment consisted of a 25 mM phosphate buffer without salt and pH 6.5. The elution was performed by applying a linear salt gradient starting at 0 mM NaCl and ending at 1 M NaCl. Cleaning in place was conducted with 0.5 M NaOH solution for all 3 experiments.

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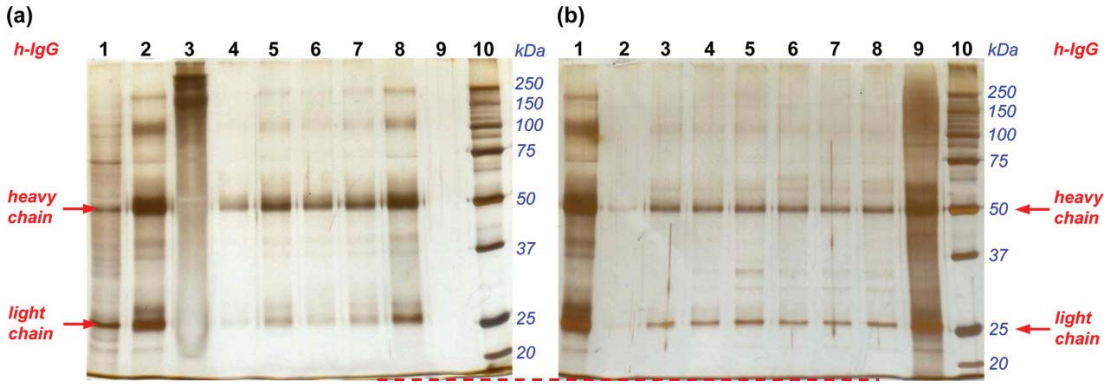

Fig. 8. Reduced and silver stained SDS-PAGE slab gels for collected DBC fractions (10 mL each) of material **L** tested with dialyzed cell culture supernatant (feed C). 10% Tris–HCl gel and 3 μL sample loads were used. (a) Lane 1: original dialyzed feed (32 μg h-IgG/mL); lane 2: dialyzed feed spiked with polyclonal h-IgG (feed C: 1 mg/mL h-IgG); lane 3: non-reduced feed C (spiked); lanes 4–7: loading fraction 1–4; lane 8: final loading fraction 17; lane 9: final wash fraction; lane 10: molecular weight marker. (b) Lane 1: feed C; lanes 2–8: elution fractions 1–7; lane 9: CIP (0.5 M NaOH); lane 10: molecular weight marker.

DNA-analysis was performed. These DNA tests exhibited that the removal of DNA was successful, which means that the aromatic ring did not interact with their purine or pyrimidine scaffolds. The amount of DNA dropped from 0.93 μg/mL to <0.15 μg/mL, which is below the limit of detection. Furthermore, the composition of some of the collected flow-through, wash and elution fractions was also visualized with SDS-PAGE using silver staining in Fig. 8.

3.5.3. SDS-PAGE

The SDS-PAGE gel in Fig. 8a shows in the lanes 1 and 2, the dialyzed feed before and after spiking with polyclonal IgG to a concentration of 1 mg/mL. Although the same sample volume was applied, the feed impurity composition of the spiked feed sample had shifted towards more distinct bands at approximately 28, 37, 40, 100 and 250 kDa. The non-reduced spiked feed in lane 3 suggests that most feed impurities were of high molecular weight of 75 kDa and higher. Furthermore, all three lanes were over-laid with a protein smear, which was absent in the flow-through fractions 1–4 (lanes 4–7). The first break-through of h-IgG was found in load fraction 1, while a slight break-through of the impurities 28, 37, 40, 100 and 250 kDa was observed for the following load samples. The final loading fraction 17 (lane 8) exhibited a significant beak-through of h-IgG and all feed impurities. The elution fractions 1 to 7 (lanes 2–8) in Fig. 8b showed no enrichment of h-IgG, which mirrors the results of the DBC diagram established by the quantification of h-IgG via the Protein A-HPLC method (Fig. 7). The most intensive bands of reduced h-IgG at 25 and 50 kD occurred in the elution fraction 3 (lane 4). The h-IgG content in this fraction was about 90% of what was present in the application feed. The elution fractions 2 and 4 (lane 3 and 5) contained only 54% and 48% h-IgG, respectively. The last elution fraction contained still 5% h-IgG, which indicates an insufficient elution. Only a small purification factor was obtained, since some impurities were co-eluted with h-IgG.

The absence of the protein smear and other feed impurities in the flow-through fractions 1–4 (lanes 4–7), their reduced presence in the elution fraction suggests and their most distinct presence in the cleaning-in-place (CIP) fraction in lane 9 suggests that material **L** is a mixed-modal adsorbent, which binds h-IgG and removes feed-impurities in a single purification step. The presence of the 25 and 50 kD bands indicates that h-IgG could not be effectively eluted with a shallow linear sodium chloride gradient. An elongated elution with 1 M sodium chloride would probably solve this problem, also an increased pH would be an option for improved elution conditions.

The elution peak maximum was reached after 30 mL of elution buffer had passed through the column, and the NaCl concentration at this point was around 200 mM. The shape of the IgG elution peak exhibited a tailing, which is not uncommon for polyclonal IgG, since it is rather complex and diverse in its composition. Cell culture supernatant with a higher concentration of mAb was unfortunately not available. The resulting elution capacity of 43 mg/mL was satisfying and further investigations on the field of aromatic acids will be conducted in the near future. In comparison to the cysteine and homocysteine based benzamido materials, the DBC results of material **L** were outstanding. At pH 6.5 and 150 mM sodium chloride the binding capacity of benzoyl homocysteine could be doubled. Also the commercial adsorbent Capto™ MMC, which possessed with 20 mg/mL 100% DBC almost the same binding capacity for feed A as material **J**, could be outperformed. A further advantage of 4-mercaptobenzoic acid is its simple structure and immobilization chemistry, which allows an easy large-scale production of the corresponding adsorbent. However, the question remains whether or not there is a thiophilic influence of the sulfur atom or not? Only a reference material where the sulfanyl linkage is exchanged (preferably by an oxo group) can answer this question. But what can be stated here is the fact, that the 4-mercaptobenzoic acid is able to bind IgG under physiological condition without the addition of structure forming salts.

4. Conclusion

During the creation, synthesis and characterization via SBC tests several findings crystallized out. First of all ligands based on dithiols coupled onto Fractogel turned out to provide unsatisfactory h-IgG capture results compared to the investigated commercial cation exchangers adsorbents. This seems to be the result of the bifunctionality of the CIEX-ligands in combination with the tentacle grafting of the support surface and their general tendency for disulfide cross-linking on this particular support. On the other hand, the direct coupling of 3-mercaptopropanesulfonic acid, which lack the possibility of disulfide bridge formation, provided comparable results to commercially available strong cation exchangers, e.g. Fractogel® EMD SO_3. However, their capture performance for h-IgG from solutions containing considerably high amount of sodium chloride was still insufficient. An improvement of the binding capacity could be observed, after replacement of the strong sulfonic acid group by a carboxylic acid group. Good results gave material **E**, the tentacle based gel bearing the mercaptopropionic acid ligand. The subsequent introduction of N-acetyl-cysteine and

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N-acetyl-homocysteine derivatives was not able to compete with this simple carboxylic acid compound, only the introduction of aromatic moieties brought an improvement of the IgG binding performance. Subsequently, these benzamido bearing carboxylic acid groups coupled onto Fractogel were able to bind antibodies with decent capacities at pH values higher than 6.0 and sodium chloride concentrations of 75 mM and higher, but there was still potential for improvement. Consequently a new ligand in the form of 4-mercaptobenzoic acid was introduced. It exhibited outstanding binding properties under conditions very near to neutral pH and at high sodium chloride concentrations. During DBC tests this material was able to bind up to 68 mg IgG per mL resin in the presence of 75 mM sodium chloride and a pH of 6.5, which is significantly higher than previous generations of mixed-modal materials, bearing, e.g. N-benzoyl-homocysteine based ligands. This high binding capacity makes this simple mixed-modal ion exchange based bioaffinity type material highly competitive to commercially available mixed-modal ligands for the capture of h-IgG from cell culture feed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.012.

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Supplementary Material

Performance evaluation of mixed-modal, thiophilic and hydrophobic cation exchanger adsorbents for the purification of human immunoglobulin G

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1. Reagents and equipment

L-Cystine, 1,3-propanedithiol, 2,2'-oxydiethanethiol, 3,6-dioxa-1,8-octane-dithiol, hydrogen peroxide (30%), 3-mercapto-1-propanesulfonic acid sodium salt, sodium hydroxide, N,N-diisopropylethylamine (DIPEA), triethylamine (TEA), 2,2'-dipyridyl disulfide (DPDS), hydrochloric acid (37% v/v), 3-mercaptopropionic acid, 4-mercaptobenzoic acid, 3,5-dimethoxybenzoyl chloride, N-acetyl-L-cysteine, N-acetyl-D,L-homocysteine thiolactone and D,L-homocysteine thiolactone hydrochloride were purchased from Sigma Aldrich (Vienna, Austria). benzoyl chloride was acquired from Merck (Darmstadt, Germany) and N-acetyl-D,L-penicillamine hemihydrate from Alfa Aesar (Karlsruhe, Germany). Tris (2-cyanoethyl) phosphine was a kind donation of CYTEC (New Jersey, USA).

If not otherwise stated, all solvents used for ligand synthesis were supplied by Sigma Aldrich (Vienna, Austria) and were of purest grade available, with exception of dichloromethane and methanol for flash chromatography, which were of technical grade. For flash chromatography silica gel 60 (0.040-0.063 mm particle size) from Merck (Darmstadt, Germany) was used.

Fractogel® EMD Epoxy (M), a cross-linked polymethacrylate resin modified with the tentacle-grafting technology and an epoxy group density of 1000 µmol/g dry gel, was provided by Merck KGaA (Darmstadt, Germany).

^{1}H NMR spectra were made with a Bruker DRX 400MHz spectrometer. The units for the chemical shift (δ) are given in parts per million (ppm). The mass spectrometric data were acquired on a PESciex API 365 triple quadrupole mass spectrometer from Applied Biosystems/MDS Sciex (Concord, Canada). The ion source was an electrospray device and, depending on the analyte, either positive or negative spectra or both were measured. The elemental analysis of sulfur containing materials were performed with an EA 1108 CHNS-O from Carlo Erba (Rodano, Italy), the determination of CHN was done with a "2400 Elemental Analyzer" from Perkin Elmer (Vienna, Austria). The titration of the ionic groups was performed on a Mettler Toledo autotitrator DL676 (single burette drive, 10 mL), equipped with an Ag/AgCl pH-electrode (DG-111-SC) (Giessen, Germany) with 0.1 M sodium hydroxide.

2. Synthesis of HIC-based cation exchange ligands

2.1 N-Benzoyl-D,L-homocysteine-thiolactone and N-(3,5-Dimethoxybenzoyl)-D,L-homocysteine-thiolactone

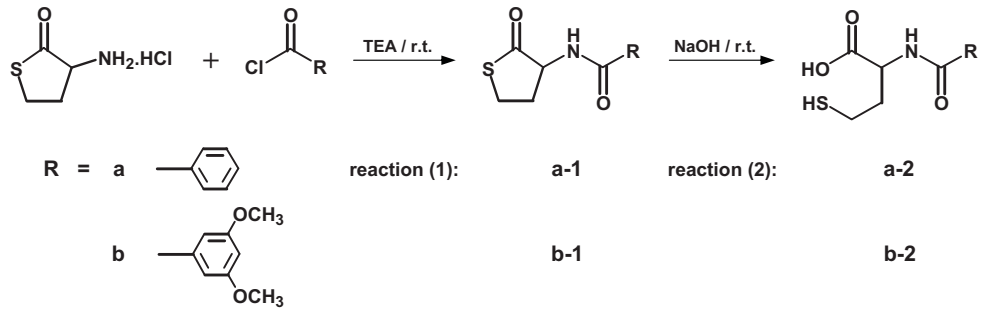

Figure S1: Reaction scheme for N-Benzoyl-D,L-homocysteine-thiolactone and N-(3,5-Dimethoxybenzoyl)-D,L-homocysteine-thiolactone

1.5 g D,L-Homocysteine-thiolactone hydrochloride (10 mmol) was dissolved in 16 mL dry dichloromethane with a two-fold excess of diisopropylethylamine (DIPEA) and cooled at 0°C in an ice bath. An equimolar amount of benzoyl chloride (a) or 3,5-dimethoxybenzoyl chloride (b) dissolved in dry dichloromethane was added dropwise to the reaction flask under constant stirring. The solution was kept under these conditions for 30 minutes and was then brought to room temperature, where it was allowed to stay another 18 hours until completion of the reaction was observed via TLC (**Fig. S1**). After evaporation of the solvent, the solid residue was re-dissolved in ethyl acetate and washed with an aqueous solution of citric acid (10% w/v). The organic phase was collected, the solvent evaporated and the solid white product was purified via flash-chromatography using silica gel 60 and dichloromethane/methanol (10:0.5).

(a-1) N-Benzoyl-D,L-homocysteine-thiolactone (J)

Reaction yield: 2g (90%)

MS (ESI, positive): 222.4 $[M+H]^+$, 104.7 $[M-116]^+$, 443.2 $[2M+H]^+$

^{1}H-NMR [400 MHz, CD_3OD]: δ = 7.86 (d, 2H), 7.57 (t, 1H), 7.48 (t, 2H), 4.93 - 4.87 (m, 1H), 3.53 - 3.45 (m, 1H), 3.38-3.33, (m, 1H), 2.70 - 2.62 (m, 1H), 2.41-2.29 (m, 1H)

(b-1) N-(3,5-Dimethoxybenzoyl)-D,L-homocysteine-thiolactone (K)

Reaction yield: 2.47 g (88%).

MS (ESI, positive): 282.2 $[M+H]^+$, 165.1 $[M-116]^+$,304.4 $[M+Na]^+$

1H-NMR [400 MHz, CD_3OD]: δ = 7.02 (d, 2H), 6.67 (t, 1H), 4.90-4.83 (m, 1H), 3.84 (s, 6H), 3.53-3.43 (m, 1H), 3.38-3.29 (m, 1H), 2.69-2.61 (m, 1H), 2.41-2.29 (m, 1H)

2.2. N-Benzoyl-D,L-homocsteine and N-(3,5-Dimethoxybenzoyl)-D,L-homocysteine

The opening of the thiolactone ring was performed under basic conditions. 10 mmol of N-benzoyl-D,L-homocysteine thiolactone (a-1) or N-(3,5-dimethoxybenzoyl)-D,L-homocysteine thiolactone (b-1) were dissolved in 6 mL of 5 M sodium hydroxide. In order to increase the solubility, 10 mL methanol was added (**Fig S1**). After 18 h of stirring under nitrogen, the reaction was stopped and the solvent was evaporated under reduced pressure. The remaining solid was dissolved in ethyl acetate and washed with 0.01 M HCl, providing a yield of 2.4 g (~99%).

(a-2) N-Benzoyl-D,L-homocsteine (J)

Reaction yield: 2,37 g (99%)

MS (ESI, positive): 240.2 $[M+H]^+$, 222.3 $[M-H_2O+H]^+$, 105.1 $[M-134]^+$

1H-NMR [CD_3OD]: δ = 7.88 (d, 2H), 7.56 (t, 1H), 4.86 – 4.80 (m, 1H), 2.72 – 2.58 (m, 2H), 2.28 – 2.13 (m, 2H)

(b-2) N-(3,5-Dimethoxybenzoyl)-D,L-homocysteine (K)

Reaction yield: 2.49 g (99%)

MS (ESI, positive): 300.4 $[M+H]^+$, 282.2 $[M-H_2O+H]^+$, 322.4 $[M+Na]^+$,

1H-NMR [400 MHz, CD_3OD]: δ = 7.04 (d, 2H), 6.62 (t, 1H), 4.84 - 4.78 (m, 1H), 3.86 (s, 6H), 2.73 – 2.55 (m, 2H), 2.28 - 2.11 (m, 2H)

2.3. N,N-Dibenzoyl-L-cystine

5 g L-Cystine (20.8 mmol) were solved in 50 mL 2 M sodium hydroxide and cooled down to 4°C. 45.7 mmol benzoyl chloride were added dropwise under stirring to the reaction mixture. After addition of benzoyl chloride, the solution was stirred for 2 h at room temperature, before adding hydrochloric acid (35% v/v) until the mixture reached a pH of 1. The reaction solution was then extracted 3 times with ethyl acetate. The organic phase were collected and evaporated under reduced pressure. Further purification via flash chromatography was performed with methanol/dichloromethane (50:1) on silica gel 60.

Reaction yield: 2.97 g (82%).

MS (ESI, positive): 449.2 $[M+H]^+$, 897.2 $[2M+H]^+$, 431 $[M-H_2O+H]^+$

MS (ESI, negative): 447 $[M-H]^-$, 895,3 $[2M-H]^-$

^{1}H-NMR [400 MHz, CD_3OD]: δ = 7.83 (d, 4H), 7.52 (t, 2H), 7.43 (t, 4H), 4.99 – 4.92 (m, 2H), 3.46 – 3.40 (m, 2H), 3.22 – 3.13 (q, 2H)

2.4. N-Benzoyl-L-cysteine (I)

N,N-Dibenzoyl-L-cystine (7.5 g, 17 mmol) was reduced according to Burns et al. [1] with tris (2-carboxyethyl) phosphine (TCEP) at pH 4.5. TCEP was prepared by refluxing tris (2-cyanoethyl) phosphine in concentrated HCL under nitrogen atmosphere for 2 h. The resulting solution was evaporated under reduced pressure. Afterwards N-benzoyl-L-cystine was mixed with a two fold excess of TCEP in 400mL of an ultra-sonicated ammonium acetate buffer system (25 mM) in methanol/water (1:1) at pH 4.5. Although the reduction was almost complete within one hour, the reaction was allowed to run

overnight under nitrogen atmosphere. The solvent was evaporated under reduced pressure and the remaining crude white solid was dissolved in 50 mM hydrochloric acid and extracted three times with ethyl acetate. After solvent removal using rotary evaporation, clean product were obtained. No further purification steps were necessary.

Reaction yield: 7.5 g (99%).
MS (ESI, positive): 226.3 $[M+H]^+$, 208.1 $[M-H_2O+H]^+$, 248.4 $[M+Na]^+$
^{1}H-NMR [400 MHz, CD_3OD]: δ = 7.87 (d, 2H), 7.55 (t, 1H), 7.47 (t, 2H), 4.81 – 4.75 (m, 1H), 3.15 – 3.09 (m, 1H), 3.04 – 2.96 (m, 1H)

3. Immobilization and modification of the ligands

Note that all ligands were immobilized onto Fractogel EMD Epoxy support material. The below listed ligands are abbreviated by letters A to L. The chemical structures of the corresponding adsorbents are listed in **Fig. 2** following the same abbreviations A to L as employed for the ligands.
The immobilization rate (ligand density) was determined via elemental analysis (EA) employing the sulfur and nitrogen content of the surface attached ligands. Alternatively, the sulfonic acid content and carboxylic acid content were determined via titration using 0.1 M NaOH (T). Throughout the article the ligand density based on the titration values are used as the effective ligand density. Alternatively the thiol content of the adsorbents were determined photometrically using the 2,2'-dipyridyl disulfide (DPDS) method [2].

3.1. Cation exchanger adsorbents with sulfanyl and sulfonyl-linkage

a) General procedure for ligand immobilization (sulfanyl-linkage):
To 20 mmol of the ligand (1,3-propanedithiol (2 mL), 2,2'-oxydiethanethiol (2.5 mL) or 3,6-dioxa-1,8-octane-dithiol (3.27 mL)) and 10 mmol of TEA (1.4 mL) were added to 1 g epoxy-activated support material (epoxide group density: 1 mmol/g) and heated to 70°C under nitrogen atmosphere for 18 h. The modified adsorbent was thoroughly washed with methanol.

Immobilization of 1,3-propanedithiol
Ligand density (EA): 1015 µmol/g dry gel; (C: 51.99%; H: 7.76%; N: 0.12%; S: 6.49%)
Thiol group density (DPDS): 783µmol/g dry gel

Immobilization of 2,2'-oxydiethanethiol
Ligand density (EA): 953 µmol/g dry gel; (C: 51.55%; H: 7.75%; N: 0.10%; S: 6.1%)
Thiol group density (DPDS): 660 µmol/g dry gel

Immobilization of 3,6-dioxa-1,8-octanedithiol
Ligand density (EA): 872 µmol/g dry gel; (C: 51.67%; H: 7.84%; N: 0.11%; S: 5.58%)
Thiol group density: 460 µmol/g dry gel

b) Oxidation of sulfanyl groups to sulfonyl groups (sulfonyl-linkage):

The oxidation of sulfanyl groups was performed with a freshly prepared performic acid solution, containing 28.5 mL formic acid with 5 mL H_2O_2 (30%). The performic acid solution was stirred for 2 h at room temperature. 3 g modified adsorbent were suspended in 20 mL methanol and 10 mL formic acid. The performic acid solution was added drop wise under gentle stirring and cooling with ice. Afterwards the solution was stirred for additional 4 hours at room temperature. The oxidized adsorbent was then extensively washed with distilled water and methanol [3,4].

c) Alternative procedure:

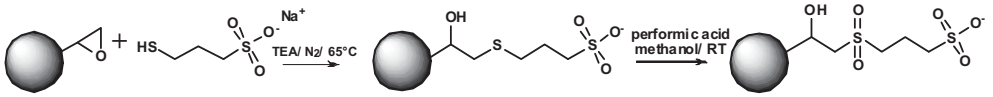

Figure S2: Coupling and oxidation of 3-mercapto-1-propanesulfonic acid

Material A (another production batch with a slightly lower ligand density) was oxidized according to the above mentioned general procedure leading to material B2, which possesses the same chemical structure as material B1.

3-Sulfonyl-1-propanesulfonic acid (B1)

Ligand density (EA): 955 µmol/g dry gel; (C: 48.16%; H: 7.23%; N: 0.16%; S: 6.12%)

Sulfonic acid density (T): 637 µmol/g dry gel);

3-Sulfonyl-1-propanesulfonic acid (B2)

Ligand density (EA): 1011 µmol/g dry gel; (C: 46.71%; H: 7.28%; N: 0.131%; S: 6.47%)

Sulfonic acid density (T): 886 µmol/g dry gel).

2-(2-Sulfonylethoxy)-ethanesulfonic acid (C)

Ligand density (EA): 840 µmol/g dry gel; (C: 46.29%; H: 6.95%; N: 0.511%; S: 5.38%)

Sulfonic acid density (T): 564 µmol/g dry gel

3,6-Dioxa-8-sulfonyl-1-octanesulfonic acid (D)

Ligand density (EA): 790 µmol/g dry gel, C: 48.72%; H: 7.36%; N: 0.36%; S: 5.06%)

Sulfonic acid density (T): 457 µmol/g dry gel

3.2. Cation exchange adsorbents with sulfanyl-linkage

Coupling of the carboxylic acid and sulfonic acid as well as cysteine and homocysteine based ligands.

a) General procedure for ligand immobilization:

A solution containing 10 mmol of 3-mercapto-1-propanesulfonic acid (A) in 15 mL methanol-water mixture (10:1), an equimolar amount of 5 M sodium hydroxide solution (10 mmol) and 10 mol-equivalents (relative to mol epoxide groups) of triethylamine (TEA) were mixed. This reaction solution was added to 1 g epoxy-activated support material (1000 µmol epoxy groups/dry g) and heated for 18 h at 65°C under nitrogen using a mechanical stirrer. The modified adsorbent was then washed with methanol, followed by subsequent wash-steps employing aqueous solutions of 50 mM citric acid, 0.5 M sodium hydroxide and distilled water until neutral pH was reached.

b) Deactivation of residual epoxide groups on the adsorbents:

Per gram adsorbent suspended in 15 mL methanol, 0.5 mL 2-mercaptoethanol was added. The reaction mixture was refluxed under nitrogen over night employing a mechanical stirrer. The adsorbent was washed with methanol until odorless.

Ligand density determination and acid content measurements were performed as previously described. The coverage of 2-mercaptoethanol was determined via elemental analysis. Note that if the reagent was not bound in stochiometrical quantities to the epoxide groups, the residual epoxy groups were endcapped by a ring-opening reaction using 2-mercaptoethanol. Note that this epoxide group endcapping was only performed on adsorbents with ligand densities below 950 µmol/g.

3-Mercapto-1-propanesulfonic acid (A)

In the case of 3-mercapto-1-propanesulfonic acid, which was purchased as a sodium salt, the free acid (A) was obtained through addition of the 5 M sodium hydroxide solution.

Ligand density (EA): 1207 µmol/g dry gel; (C: 49.27%; H: 7.27%; N: 0.13%; S: 7.73%)

Sulfonic acid density (T): 1023 µmol/g dry gel

3-Mercaptopropionic acid (E)

Ligand density (EA): 1100 µmol/g dry gel; (C: 52.22%; H: 7.7%; N: 0.15%; S: 3.34%)

Sulfonic acid density (T): 1043 µmol/g dry gel

N-Acetyl-L-cysteine (F)

Ligand density (EA): 1031 µmol/g dry gel; (C: 50.11%; H: 7.17%; N: 1.51%; S: 3.3%)

Carboxylic acid density (T): 941 µmol/g dry gel

N-Acetyl-D,L-homocysteine (G)

Ligand density (EA): 921 µmol/g dry gel; (C: 51.01%; H: 7.12%; N: 1.48%; S: 2.95%)

Carboxylic acid density (T): 835 µmol/g dry gel)

After endcapping with 2-mercaptoethanol:

Ligand density (EA): 843 µmol/g dry gel; (C: 51.01%; H: 7.58%; N: 1.402%; S: 2.7%)

Carboxylic acid density (T): 727 µmol/g dry gel

2-mercaptoethanol coverage: 0 µmol/g dry gel

N-Acetyl-D,L-penicillamine (H)

Ligand density (EA): 775 µmol/g dry gel; (C: 52.8%; H: 7.8%; N: 1.42%; S: 2.48%)

Carboxylic acid density (T): 703 µmol/g dry gel

After endcapping with 2-mercaptoethanol:

Ligand density (EA): 759 µmol/g dry gel; (C: 51.11%; H: 7.52%; N: 1.33%; S: 2.43%)

Carboxylic acid density (T): 604 µmol/g dry gel

2-Mercaptoethanol coverage: 0 µmol/g)

N-Benzoyl-L-cysteine (I)

Ligand density (EA): 715 µmol/g dry gel; (C: 54.44%; H: 7.28%; N: 1.38%; S: 2.29%)

Carboxylic acid Density (T): 634 µmol/g)

After endcapping with 2-mercaptoethanol:

Ligand density (EA): 709 µmol/g dry gel; (C: 53.73%; H: 7.2%; N: 1.38%; S: 2.27%)

Carboxylic acid density (T): 641 µmol/g

2-Mercaptoethanol coverage: 0 µmol/g dry gel

N-Benzoyl-D,L-homocysteine (J)

Ligand density (EA): 828 µmol/g dry gel; (C: 54.62%; H: 8.22%; N: 1.5%; S: 2.65%)

Carboxylic acid density (T): 820 µmol/g

After endcapping with 2-mercaptoethanol:

Ligand density (EA): 887 µmol/g dry gel; (C: 54.27%; H: 7.63%; N: 1.4%; S: 2.84%)

Carboxylic acid density (T): 843 µmol/g

2-Mercaptoethanol coverage: 50 µmol/g dry gel

N-(3,5-Dimethoxybenzoyl)-D,L-homocysteine (K)

Ligand density (EA): 930 µmol/g dry gel; (C: 54.22%; H: 7.34%; N: 1.47%; S: 2.99%)

Carboxylic acid density (T): 841 µmol/g

After endcapping with 2-mercaptoethanol:

Ligand density (EA): 909 µmol/g dry gel; (C: 54.23%; H: 7.33%; N: 1.43%; S: 2.91%)

Carboxylic acid density (T): 738 µmol/g

2-mercaptoethanol coverage: 0 µmol/g dry gel

4-Mercaptobenzoic acid (L)

Ligand density (EA): 950 µmol/g dry gel; (C: 51.62%; H: 7.11; N: <0.05%; S: 3.04%)

Carboxylic acid density (T): 950 µmol/g

4. Dynamic binding capacity test at increased bed height

An additional DBC test was performed with 2.5 mL of material **L** to determine its performance at an increased bed height compared to 1 mL as stated in the article resulting in a bed height of 3.2 cm. A test solution (feed B) containing 1mg/mL polyclonal h-IgG Gammanorm® in 25 mM phosphate buffer with 75 mM NaCl at pH 6.5 was applied onto the column until 100% breakthrough was reached. After washing with application buffer, the elution was performed with 25mM PBS-buffer with 1 M NaCl at pH 6.5 and a

flow rate of 0.6 mL/min, which equals a residence time of 3.25 min. Cleaning in place was conducted with 0.5 M NaOH solution Other parameters were equivalent to those described in the original article.

The result of this experiment was an elution capacity of 55 mg/mL. For 100% DBC a value of 68 mg/mL and for 10% DBC a value of 21 mg/mL were determined. These values stand in good accordance to the results obtained for the first experiment performed with an adsorbent volume of 1 mL, which exhibited an elution capacity of 57 mg/mL and 68 mg/mL for 100% DBC. Only the 10% DBC value differed with 11 mg/mL from the earlier experiment. The increase in 10% DBC is the result of the overall higher residence time of IgG molecules in the column and a shorter relative distribution area at the head of the column (**Fig. S3**).

For a better comparison of the two DBC curves established for the same adsorbent at different bed heights, these curves were normalized to the amount of applied feed. Due to the strongly differing application time for feed, wash and elution solution between the two experiments at different bed heights also their x-axis were displayed in two different scales to give credit to the 2.5 times higher bed height.

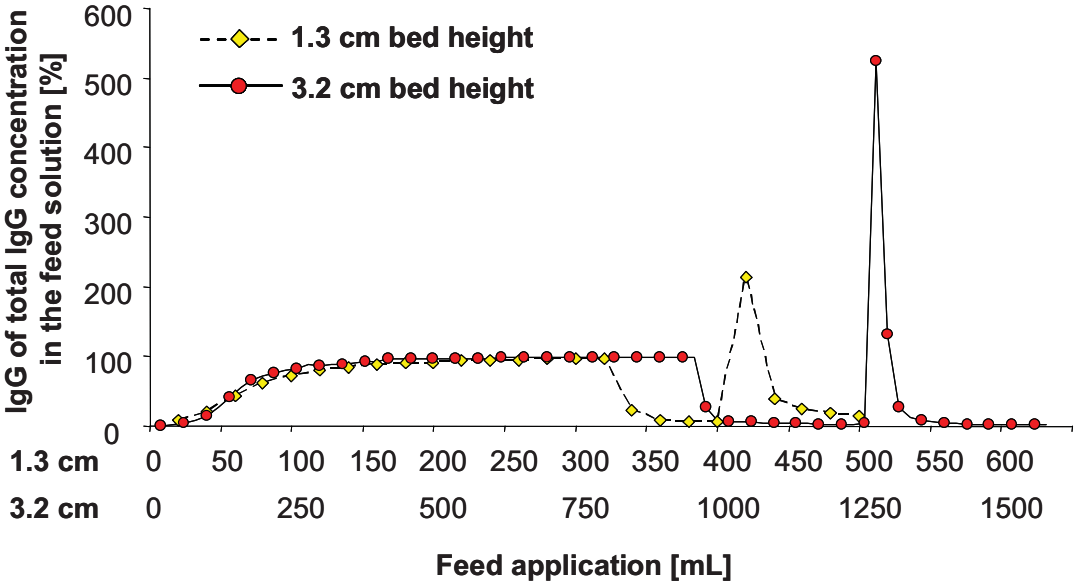

Figure S3: *Dynamic break-through curves for the aromatic HIC material* ***L*** *using polyclonal h-IgG (feed B) tested at 2 different bed heights (1.3 cm and 3.2 cm). For better comparison two different scales for the x axis were used in order to give credit to the different adsorbent volumes and therefore different amounts of application feed solutions applied. Nonetheless, the application time for feed, wash and elution solution was comparatively longer for the 3.2 cm bed height experiment compared to the DBC test at 1.3 cm bed height*

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Quantification of immunoglobulin G and characterization of process related impurities using coupled Protein A and size exclusion high performance liquid chromatography

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ABSTRACT

The present work describes two HPLC-UV methods for multi-protein quantification using (i) only a Protein A sensor cartridge (Protein A HPLC) and (ii) the same Protein A cartridge in combination with a size exclusion HPLC column (PSEC-HPLC). The possibility to simultaneously quantify immunoglobulin G (IgG) besides a non-binding protein such as bovine serum albumin (BSA) increases the applicability of Protein A HPLC. Its most pronounced feature is its independence of the buffer system, pH-value and salt content of the investigated sample solvent, which includes cell media. A comparison with the state-of-the-art, the photometrical Bradford method, shows that Protein A HPLC is as sensitive as Bradford, but that it comes with an extended linear range of 4 orders of magnitude, ranging from 0.15 [μg abs] to 1 [mg abs] absolute injected protein amount. The applicability of the PSEC-HPLC method is demonstrated for the analysis of real cell culture feed samples. While Protein A binds IgG, the SEC-column distributes the feed impurities by their molecular weight. The peak area ratios of IgG and the feed impurities of interest are then plotted against the collected sample fraction. These Protein A-Size-Exclusion-Chromatographic diagrams (PSEC-plot) combine the performance information of feed impurities and IgG in a single plot. Further it is shown that both methods are suitable for the performance evaluation of antibody purification media using static as well as dynamic binding experiments performed on DEAE-Fractogel and Capto Adhere. The investigated test samples were "mock" protein solutions with increasing complexity ranging from simple PBS buffer to serum free cell media and "real" cell culture feed solutions.

1. Introduction

In recent years, the demand for clinically applicable proteins, in particular human monoclonal antibodies, especially human or humanized immunoglobulin G (h-IgG) for medical purposes is increasing rapidly. Since decades, affinity materials based on Protein A or Protein G may it be in analytical scale [1–4], in semi-preparative lab-scale or process production scale [5,6] can be reckoned as state-of-the art for the purification of IgG [7]. The fact that Protein A was already immobilized onto a variety of different supports, e.g. sepharose [8], silica gel [9,10], monoliths [11–13], membranes [14] and magnetic beads [15] proof its dominance in the field of antibody purification.

The high cost of Protein A media, especially when employed in production scale processes and the increasing demand for pharmaceutically pure human monoclonal antibodies (HuMab), makes the development of newer less expensive antibody purification adsorbents such as bio-mimetica [16,17] and peptide-mimetica [18], or multi-step purification strategies without the use of Protein A adsorbents [19], highly interesting for researchers and industry alike.

Therefore, besides the development of new adsorbents for protein purification, also the implementation of simple, fast and thorough analytical screening methods for their characterization is of high importance. Hence it is of interest to not only measure the binding capacity of the adsorbent for IgG but also to be able to determine their cross-selectivity towards the presence of other non-target proteins under near-real condition.

Current methods to quantify proteins can be classified into three main groups, the photometrical, the electrophoretical and the chromatographic methods. The classic protein quantification techniques are surely the photometrical ones. While the direct quantification at 280 nm [20] is very simple, it requires fairly high protein amount to provide accurate results. Methods employing the formation of strongly absorbing protein-dye complexation products such as Bradford [21,22], Lowry [23], BCA [24], Cu(II)-neocuproine [25] or fluorescence based methods [26,27] show significantly increased sensitivity, enabling the detection of even

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trace amounts of proteins. However, these methods are also highly susceptible to interferences. Not only other proteins but also non-proteinageous sample components e.g. phenolic compounds [28,23] and polysaccharides [29,30] or even the buffer, pH-value and salt content of the sample [31] may lead to strong deviations, which necessitate own calibration curves for each and every sample matrix composition.

Also frequently used is the semi-quantitative detection of proteins, separated by their electrical charge differences on a SDS-Page slab gel, followed by visualization of the obtained protein bands by coomassie staining [32,33] or silver-staining [34–36,33]. Although this method does not provide distinct quantitative results, it gives the user a fast insight in the semi-quantitative molecular weight distribution of proteins for a fairly large number of samples. The use of sensitive staining methods e.g. silver-staining enables the detection of trace amounts of proteins down to the lower nanogram levels of 0.5–10 ng. More thorough quantitative information can be achieved in the combination with mass spectrometry [33].

Alternatively, chromatographic methods e.g. affinity chromatography [37,1,38], hydrophobic charge induction chromatography (HCIC) [39,40,38] and size-exclusion chromatography (SEC) [41–45] enable the separation as well as quantification of proteins from a more complex protein-mixture. Concerning complexity and diversity, real cell culture supernatants are among the most difficult samples to analyze and characterize. These multi-component mixtures comprise host-cell-proteins (HCP) [46–48], DNA [49,50,47,51], endotoxins [52,53], viruses [54,55] and others constituents [56].

Therefore the choice, which analytical method to employ depends strongly on the amount of information that one desires among other factors such as time, labor and grade of accuracy.

While size-exclusion-chromatography fractionates proteins by their molecular weight, affinity chromatography and ion-exchange chromatography capture only certain proteins depending on their physiological properties and charge distribution. The latter two are therefore more target specific compared to SEC, but may come with a slight loss on information. Protein A based affinity chromatography is for instance a simple, but highly effective method to isolate and quantify IgG from highly complex sample solutions.

Nonetheless, one has to consider that Protein A binds a number of different immunoglobulins, IgG1, IgG2 and IgG4, while IgG3 is not being bound [57–59]. Additionally, Protein A captures traces of IgA and IgM from different species. The diversity increases, when one considers the different isoforms of monoclonal antibodies, which differ in their disulfide connectivity, glycosylation, deamination, etc. [60–62]. This lead to the fact that even for Protein A-based affinity materials, the obtained quantitative results for IgG resembles a sum of data for a variety of different IgG-proteins, which only differ in their degree of variability and affinity towards the ligand protein.

The main advantage of analytical Protein A perfusion columns is the short analysis time of as low as 3 min per cycle for the determination of trace amounts of IgG from biological samples. Although a nice tool, their field of application is nonetheless limited by the fact that Protein A does only bind one species of protein, namely IgG.

Hence the aim of this study was to enhance the applicability of a simple analytical Protein A cartridge to simultaneously quantify up to two proteins, the Protein A binding protein IgG and a second flow-trough protein under near-real sample background conditions. Presently, performance evaluations of adsorbents suitable for antibody purification are conducted with one-protein solutions in standardized buffer systems using photometrical methods for protein quantification. The possibility to employ standard protein mixtures in serum-free cell media enables a more realistic screening of new adsorbents for antibody purification. A direct comparison of the Bradford method and Protein A HPLC will demonstrate the clear superiority of the latter.

A new instrumental set-up, implementing a size exclusion HPLC column in series after the Protein A cartridge (PSEC-method) enables not only the analysis of multi-protein mixture with more than two proteins. It also allows the characterization and comparison of different batches of real cell culture feed solutions using a PSEC-Plot. The value of the so-obtained information becomes clearer, when the same sample fractions used to set up a PSEC-Plot are visualized by simple size exclusion chromatograms and SDS-Page slab gels.

2. Experimental

2.1. Chemicals and standard solutions

Bovine serum albumin (BSA, Fraction V, 99%), ortho-phosphoric acid, phenol red, 10% Pluronic® F-68 solution, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium azide, sodium chloride, triethylamine, tris(hydroxymethyl)aminomethane and hydrochloric acid were purchased from Sigma–Aldrich (Vienna, Austria). HPLC-grade methanol was from VWR (Vienna, Austria) and HPLC-grade 2-propanol from Roth (Karlsruhe, Germany). Bovine gamma globulin (BGG; 2 mg/mL) from Pierce was purchased through THP Medical Products GmbH (Vienna, Austria), polyclonal human-immunoglobulin G, Gammanorm (h-IgG; 165 mg/mL) was from Octapharma (Germany). Bradford protein assay dye reagent was obtained from BIO-RAD (Vienna, Austria). EX-CELL^{TM}VPRO serum free cell medium (SFCM) without glutamine, containing 0.1% Pluronic® F68, 6 g/L glucose and 2.7 g/L sodium bicarbonate besides an undefined amount of hypoxanthine and thymidine was obtained from SAFC Biosciences Limited (Andover, UK). Cell supernatant containing human monoclonal antibody, h-IgG1 from CHO-cell expression system, with pH 7.5 and a conductivity of 17 mS/cm at 33 °C were from ExcellGene (Monthey, Valais, Switzerland). Note that a conductivity of 17 mS/cm corresponds to approximately 150 mM of sodium chloride. The exact properties of cell culture batches A to I are listed in Table S1 in the electronic supplementary material [63].

Bi-distilled water and all buffer solutions used in this study were distilled in-house and filtered through a 0.22 μm cellulose acetate membrane filter from Sartorius purchased through Wagner&Munz (Vienna, Austria) and ultrasonicated prior to use.

Stock solutions of polyclonal IgG (Gammanorm), BGG and BSA solution were prepared at a concentration of 10 mg/mL in the corresponding buffer and subsequently diluted to 1, 0.1 and 0.01 mg/mL in order to establish calibration curves with 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 50, 70, 100, 150, 200, 500, 700 and 1000 μg/mL. Sodium azide solutions in PBS and serum-free cell medium with 0.5, 1, 2 and 3 mg/mL were diluted from 10 mg/mL stock solutions. If not otherwise stated, a PBS buffer containing 10 mM phosphate and 150 mM sodium chloride with pH 7.20 and a TRIS buffer with 20 mM TRIS-HCl and pH 7.00 were used.

2.2. Instrumentation

2.2.1. HPLC-chromatography

All chromatographic measurements were performed on an Agilent 1100 series HPLC system equipped with a binary pump, column oven, temperature controlled sample tray, a multi-wavelength detector and a 2-position-6-port switching valve from Agilent (Vienna, Austria).

For the quantification of IgG a Protein-A ImmunoDetection® sensor cartridge from Applied Biosystems (Vienna, Austria) was

used. The rigid polymeric POROS beads in this pre-packed column posses large flow-through pores and smaller diffusion pores, which provide an accessible surface area of 1 m^2/cartridge.

The silica-based size exclusion columns TSKgel G3000SWXL with 7.8 mm ID × 30.0 cm length, 5 μm particle size and the guard column TSKgel SWXL with 6.0 mm ID × 4.0 cm length was from Tosoh Bioscience, Stuttgart, Germany.

2.2.2. Bio-chromatography

For column chromatography an L-6200A Intelligent Pump with a D-6000A Interface from Merck-Hitachi (Darmstadt, Germany) and a single wavelength, UV-975 Intelligent UV/VIS detector from Jasco, Biolab (Vienna, Austria) were used. Note that the preparative column was connected over a manual 2-position/6-port switching valve from Rheodyne to the purge-outlet of the HPLC-pump, while the high-pressure outlet of the HPLC-pump was sealed with a 10–32 peek plug. Feed application was performed with a Minipuls 3 peristaltic pump with a standard pump head with 10 rollers and 2 channels, accepting a feed-flow of 0.3 μL/min to 30 mL/min with a maximum back pressure of 5 bar and a polyvinylchloride (PVC) calibrated peristaltic tubing with 1.02 mm ID, from Gilson (Villiers-le-Bel, France).

For preparative bio-chromatography, a XK 16/20 column with 2 AK 16 adapters from GE-Healthcare (Vienna, Austria) and Superformance Filter F with 16 mm ID from Götec-Labortechnik GmbH (Mühltal, Germany) were used. The anion exchange adsorbent (2.5 mL) was placed between 2 Filter F discs and set to a bed height of 1 cm employing a compression factor of 20% [64–67]. Fractogel EMD DEAE (M) with a particle size of 40–90 μm and a diethyl aminoethyl (DEAE) group density of 460 μmol/g (dry) was from Merck KGaA (Darmstadt, Germany). Capto Adhere, a multimodal strong anion exchanger with N-benzyl-N-methyl ethanolamine ligands bound to highly cross-linked agarose beads, with a particle size of 75 μm and a ligand density of 0.09–0.12 mmol Cl/mL medium (730 μmol/g (dry) determined via elemental analysis of the nitrogen content) was from GE-Healthcare (Vienna, Austria).

2.2.3. Photometrical measurements

Photometrical measurements of proteins and phenol red were performed on the UV/VIS spectral-photometer SPECORD 50 from AnalyticJena AG (Jena, Germany). All Bradford measurements were performed with disposable semi-micro cuvettes from BioRad (Vienna, Austria), while for phenol red disposable cuvettes from Brandt, purchased through VWR (Vienna, Austria) were used. For the static binding tests a thermomixer-compact and a mini-spin centrifuge from Eppendorf (Hamburg, Germany) were used.

For DNA-quantification the PicoGreen dsDNA assay kit from Invitrogen (Lofer, Austria) was used in combination with the fluorescence spectrometer LS 50 B and FL WinLab software from Perkin Elmer (Vienna, Austria). Hellma 105.251-QS ultra-micro fluorescence cuvettes with 3 mm × 3 mm light path and 45 μL cell volume were obtained through Wagner & Munz (Vienna, Austria).

2.3. Chromatography

2.3.1. Protein A-HPLC

For the Protein A HPLC run an adsorption buffer (buffer A), containing 10 mM phosphate buffer with 150 mM sodium chloride at pH 7.2 and an elution buffer (buffer B) with 12 mM hydrochloric acid and 150 mM sodium chloride at pH 2 were used. The instrumental method was as follows: buffer A 5–15 min, buffer B 5–15 min and kept at buffer A for 6 min to re-equilibrate the Protein A cartridge for the next injection at a general flow-rate of 1 mL/min. A detection wavelength of 280 nm and sample injection volumes of 100 μL were used for all samples and standard solutions alike. The column temperature was kept constant at 25 °C and the sample tray was kept cooled at 10 °C to prevent an early fouling of feed samples. Note that all samples were set to a pH of approximately 7 after sample collection and stored at 4 °C if necessary [63].

2.3.2. Size exclusion HPLC combined with Protein A-HPLC

The instrumental set-up for the two columns, Protein A and SEC is shown in Fig. S1 [63]. In position-1, the Protein A-HPLC column is connected over the switching valve with the SEC-HPLC column. The flow-through peak, leaving the Protein A-HPLC column with buffer A enters the SEC-column, where these proteins are distributed by their molecular weight. Once the last sample peak leaves the SEC-column, the switching valve will change to position-2. Elution buffer B releases IgG from the Protein A column and direct it to the UV-detector. After the Protein A column has re-equilibrated with buffer A, the switching valve is set back to the starting position-1. Note that the SEC-column is at all times only in contact with buffer A [63].

2.4. Photometrical measurements

2.4.1. Bradford method for total protein analysis

For total protein quantification via Bradford standard solutions containing 1, 5, 10, 20, 30, 40 and 50 μg protein in 800 μL sample-buffer were prepared. 200 μL of Bradford dye reagent was added 30 min prior to the photometrical measurements. A blank sample with 800 μL of the corresponding buffer plus 200 μL Bradford reagent was used for background subtraction. Sample buffers tested were 20 mM TRIS-HCl at pH 7.0, pH 3.0 and pH 7.0 with 400 mM NaCl as well as 0.1 M HCl, PBS, SFCM and 2%-SFCM in TRIS, pH 7.0. Calibration curves were established for BSA, BGG and IgG. Note that the complex formation of dye and protein is time dependent. It is therefore advisable to re-measure the blank sample in regular intervals, especially if large numbers of sample are measured over a long period of time. This procedure increases the linearity range of all calibration curves established via Bradford.

2.4.2. UV absorbing additives

Phenol red stock solutions of 100 μg/mL in PBS and serum-free cell medium were subsequently diluted to 100, 75, 50, 25, 10, 5, 1 and 0.5 μg/mL. All samples were measured manually at 430 nm (0.5–50 μg/mL: $y = 0.0477x + 0.0185$; $R^2 = 0.9990$) as well as via Protein A-HPLC (0.5–100 μg/mL: $y = 62.9276x - 24.7251$; $R^2 = 0.99991$) in order to have a direct correlation between the phenol red content in the sample and the peak area, which has to be subtracted from the BSA containing flow-through peak.

The contribution of UV-absorbing additives in the IgG stock-solution (1–100 [μg abs] IgG: $y = 2.8131x - 2.1818$; $R^2 = 0.9994$) as well as of sodium azide (0.5, 1, 2 and 3 mg/mL: $y = 89.4102x + 10.0085$; $R^2 = 0.9995$) were determined with Protein A HPLC. These calibration values can be considered as being highly reproducible and therefore have not to be re-measured on a daily basis, but should be re-checked in regular intervals.

2.4.3. DNA-quantification

Quantification of DNA containing samples in PBS buffer were performed according to the protocol provided by Invitrogen using an excitation wavelength of 480 nm and a slit width of 10 nm as well as an emission wavelength of 520 nm plus a slit width of 8 nm. In order to save time as well as DNA-sample and reagent, the PicoGreen reagent solution was added directly into the ultra-micro cuvettes 2–4 min at the maximum before the measurement. Sample volumes were adjusted to 80 μL. Note that the diluted reagent was only used within 2 h after preparation, after which a signal reduction was observed, which may be due to a decomposition of reagent. External calibration curve ranging from 0.3 to 25 ng/mL dsDNA (with $y = 0.9288x + 0.6152$ and $R^2 = 0.9984$) and

0.01–0.5 μg/mL dsDNA (with $y = 1158.3x + 2.1132$ and $R^2 = 0.9999$) were used for DNA samples in PBS buffer.

For complex samples of DNA in cell culture broth, only a standard addition method, adding 0.25, 0.50 and 0.75 μg/mL ds-DNA to the sample solution was found to provide most reliable results ($y = 0.9536x + 591.8$ and $R^2 = 0.985$).

2.5. SDS-Page gel electrophoresis

A Mini-PROTEAN® 3 Cell with a Mini-PROTEAN 3 Cell/PowerPac 300 System (220/240 V) as well as all required chemicals including a Precision Plus Protein Standard (unstained) were obtained from Bio-Rad Laboratories Inc. (Vienna, Austria). All hand cast slab gels were prepared according to the general procedure for SDS-Page Laemmli buffer systems provided by Bio-Rad [68]. If not otherwise stated 10%-TRIS-HCl gels with a thickness of 0.75 mm and 10 sample wells were prepared. Analysis sample and Laemmli buffer (10% 2-mercaptoethanol) were mixed at a 1:1 ratio and reduced for 5 min at 90 °C before addition of 15 μL into each well. In case of the molecular weight marker, only 3 μL were applied.

Protein staining was performed with a ProteoSilver™ Plus Silver Stain Kit (PROT-SIL2) from Sigma (Vienna, Austria), according to the products technical information [69]. The only deviation from the protocol was that only 50 mL of the reagent solutions (sensitizer, silver and developer solution) were used. In order to obtain a clearer background, the developer solution was replaced by 50 mL bi-distilled water after the protein bands have almost, but not quite reached the desired intensity. The stop solution (5 mL 2% acetic acid in bi-distilled water) was added once the protein bands have fully reached their intended intensity.

2.6. Material characterization

2.6.1. Static binding capacity (SBC) experiments

For the static binding studies, the AIEX-adsorbents were conditioned with 0.1 M hydrochloric acid, rinsed with bi-distilled water until a neutral pH was reached and then filtered through a 2 mL pore 2 glass fritted funnel under vacuum for 30 s. Aliquots of 30 mg of wet AIEX-adsorbent were scaled into 1.5 mL Eppendorf vessels. Three different adsorption buffer systems were investigated, TRIS-HCl, PBS and serum free cell media containing 1 g/L Pluronic F68 (SFCM). For protein elution, 10 mM PBS-buffer with 400 mM NaCl, pH 7.2 was used. Three types of test samples were employed: (i) 10 mg/mL BSA in TRIS, PBS and SFCM, (ii) 10 mg/mL BSA plus 3 mg/mL IgG in TRIS, PBS and SFCM and (iii) buffer blank with SFCM. All gel-protein slurries were shaken at 25 °C and 1400 rpm before centrifugation at 10,000 rpm for 2 × 1 min and removal of the supernatant. The collected test fractions were quantified with the Protein A HPLC method.

(a) *Static binding capacity*: 1 mL of the corresponding protein samples were added to 30 mg suction dried gel. The gel-protein slurries were shaken for 2 h, before removal of the supernatant.
(b) *Wash-step (W)*: The remaining gel was washed for 5 min with 2 × 1 mL of bi-distilled water and 1 × 1 mL of the corresponding buffer. In case of cell media TRIS buffer was used.
(c) *Elution capacity (E)*: The final elution of bound proteins and feed components was performed with elution buffer employing a shacking time of 15 min at 25 °C.
(d) *Total binding capacity (TBC)*: The results for the three elution fractions and the final wash fraction were summarized, leaving the wash-steps with bi-distilled water out.

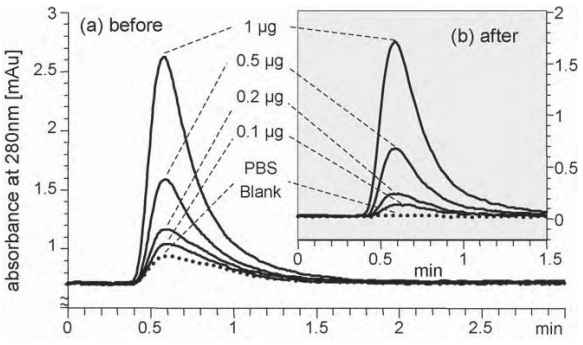

Fig. 1. Limit of detection for BSA in PBS-buffer using the Protein A HPLC method to quantify BSA besides IgG; (a) before subtraction of the PBS-blank and (b) after subtraction of the PBS-blank; (*"blank" in b was obtained through subtraction of 2 successive PBS-blanks*).

2.6.2. Dynamic binding capacity (DBC)

The dynamic binding capacity of a DEAE-Fractogel EMD material was performed with a HPLC-pump for column equilibration and rinsing (Fig. S2, position 1 [63]), and a peristaltic pump for feed application (Fig. S2, position 2). The DEAE-Fractogel column was rinsed with 30 mL 0.1 M HCl and with 10 mM PBS buffer, 75 mM NaCl (pH 7.2) until the adsorbent was fully equilibrated. Pump switching was performed manually with a 2-position-6-port switching valve. The cell supernatant was filtered through a 0.22 μm cellulose acetate filter from Sartorius (Goettingen, Germany) prior to use. The 10 mL aliquots of feed flow-through fractions were manually collected in reagent vials containing 10 mg of sodium azide, to prevent an early fouling of the feed samples. After a column wash-step with 10 mM PB without saline, the column was rinsed with 1 M sodium chloride until baseline was reached and was sanitized with 50 mL 0.5 M sodium hydroxide. All collected feed samples were quantified via Protein A-size-exclusion-chromatography.

3. Results and discussion

3.1. Method description

The general application of Protein A affinity materials is the binding of immunoglobulines IgG1, IgG2 and IgG4, while non-binding proteins including IgG3 and all other non-binding components present in cell supernatant solutions should pass unretained through the Protein A column. In general, the flow-through peak resembles a sum of all non-retained components in solution. However, once the exact amount of these components is known, it is possible to subtract the chromatographic peak areas of all known components and thereby determine the concentration of the non-binding target protein. Of course the limitation of this method lies in the magnitude of the background signal originating from the solution matrix in comparison to the amount of non-retained protein to be quantified. In the easiest case, if BSA is to be quantified besides IgG from a simple test mixture containing only bovine serum albumin and IgG in PBS, then only a small reproducible systems peak (Fig. 1, PBS blank) has to be subtracted. Note that also for IgG, the system generated peak was subtracted in order to obtain highly reproducible results. This general approach reduces the problem of unpredictable chromatographic artifacts, which were already mentioned by Compton in 1989.

The use of a protein test mixture containing only BSA and IgG may seem absurd, but one has to consider, that BSA is frequently

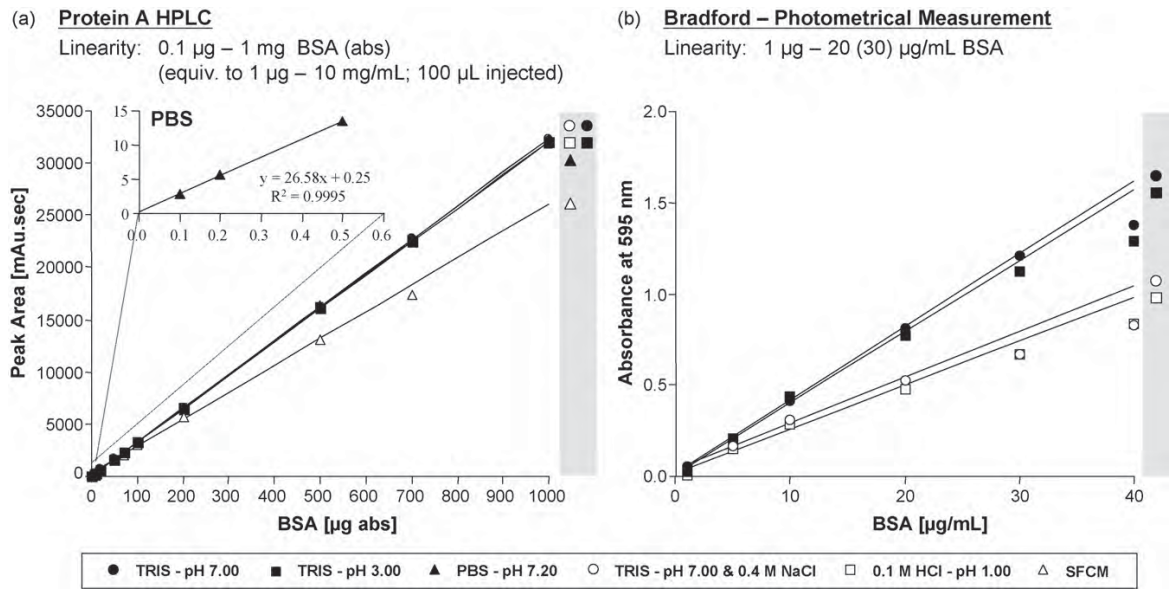

Fig. 2. Comparison of calibration curves for BSA dissolved in solutions with varying buffer system (TRIS, PBS and serum-free cell media (SFCM)), salt content (150 mM and 400 mM NaCl) and pH value (pH 7, pH 3 and pH 1) using (a) a Protein A-HPLC column and (b) Bradford (reagent blank was measured only at the beginning of each series).

used by material manufacturers as a single test protein to characterize and compare the binding properties of anion-exchange-type materials (AIEX). Note that BSA may not even be present in the antibody containing fermentation broth and therefore BSA is surely not an adequate representative to simulate the different feed impurities that are present in a real cell culture feed.

Fig. 1 shows the flow-through peaks of different amounts of BSA before and after subtraction of the peak area of the buffer blank. It can be seen in Fig. 1b that a complete subtraction of the background signal is possible and that BSA can be detected down to an absolute injection amount of 0.1 [µg abs] BSA. In order to obtain accurate and representable results, the corresponding buffer blank or in case of cell media (CM), an aliquot of CM ought to be injected after every 10–20 sample injection. The chromatogram of the inserted buffer injections also provide information about the general status of the column or the HPLC system, since the beginning of contamination is visualized by increasing system peak areas for the elution buffer, which can be as small as 9 [mAu s] and as high as 200 [mAu s] for a contaminated system.

It is also noteworthy to keep in mind that for the quantification of high BSA or IgG concentrations the adsorption time as well as the elution time for IgG has to be increased in order to prevent a carry over of BSA into the IgG elution peak or a carry over of non-eluted IgG into the IgG elution fraction of the following Protein A-HPLC run. Generally, a maximum adsorption time of 30 min for 1 mg BSA and an elution time of 15 min for 1 mg IgG are sufficient. Furthermore, it is advisable to include blank runs of the corresponding buffer after every 5–15 sample injection and after injection of samples with expectedly high protein content as well as before and after each series of measurements to ensure the accuracy of the results [1]. Also note that no difference was observed for calibration curves established by varying the injection volume or by varying the sample concentration, although the latter was used for all calibration curves discussed in this work. It is furthermore possible to establish calibration curves for BSA and IgG using dual standard protein mixtures, containing both the binding and the non-binding protein (data provided in Table S4 [63]). This reduces the analysis time for calibrant measurements by half. A comparison with calibration curves for BSA obtained with size exclusion chromatography shows no significant differences when Protein A HPLC or SEC-HPLC was used to quantify BSA (Table S4).

However, also for the simple stand-alone Protein A-HPLC method it is possible to quantify BSA in the presence of UV-absorbing additives such as sodium azide, phenol red or serum-free cell medium. The only important point to consider is that the exact amount of all back-ground sample components must be known plus their corresponding peak areas. The separately determined peak areas of the back-ground components have to be subtracted from the BSA containing flow-through peak, which make the establishment of own calibration curves for these compounds necessary.

3.2. Comparison of the Protein A HPLC method with the photometrical method from Bradford

Fig. 2 shows a comparison of calibration curves established with the Protein A method and the Bradford method for simple BSA solutions with different buffer system (TRIS, PBS, 0.1 M HCl and SFCM), pH-values (pH 7, pH 3 and pH 1) and sodium chloride content (0 mM and 400 mM). The Protein A HPLC method provides overlapping calibration curves for all investigated sample solutions ranging from 1 to 1000 µg BSA absolute injected [µg abs], with only serum-free cell media leading to a deviation for BSA amounts above 100 [µg abs]. A detailed list of the data set presented in Fig. 2a can be found in Table S2 in the electronic supplementary material [63].

The calibration curves established with the Bradford method in Fig. 2b show a stronger dependency towards changes in conductivity (0 mM and 400 mM NaCl) than changes in the pH value of the solution (pH 7 and pH 3). When using Bradford it is therefore necessary to establish own calibration curves for every buffer condition, which is not only laborious but also increases the analysis time tremendously (Fig. 5b).

Although both methods show the same lower linearity limit of 1 µg/mL, the upper linearity limit ends at about 30 µg/mL BSA for the Bradford method, while for the Protein A method an upper limit of up to 10 mg/mL for BSA and 5 mg/mL for IgG was determined. Nonetheless, even for the Protein A HPLC method it is advisable to dilute samples of high protein content to below 1 mg/mL in order

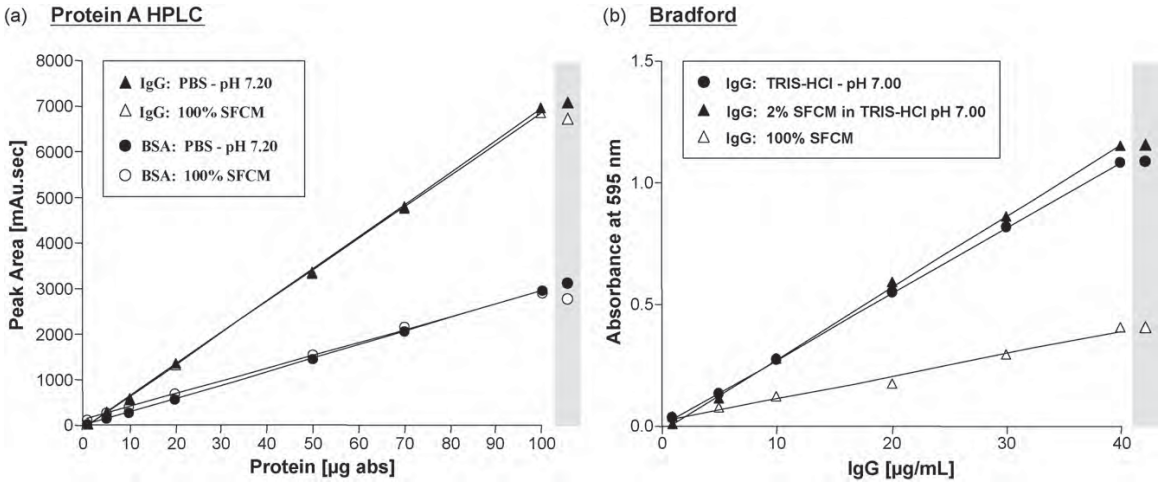

Fig. 3. Comparison of (a) calibration curves for BSA and IgG in PBS-buffer and serum free cell media (SFCM) using Protein A HPLC with (b) calibration curves for IgG in TRIS-buffer, TRIS with 2% SFCM and 100% SFCM using the Bradford method (reagent blank was measured before each sample measurement).

to reduce the HPLC run-times and to be able to use one calibration curve ranging from 0.1 to 100 [μg abs] (equiv. 1–1000 μg/mL) for all BSA containing samples independent of their buffer system, their pH-value and their salt content. Even for BSA and IgG in SFCM, which can be used as a mock-feed solution instead of cell culture feed for material screening, the same calibration curve (0.1–100 [μg abs]) can be used (Fig. 2a). The only limitations are the 10-fold increase of the limit of detection (LOD) of 1.5 [μg abs] and the limit of quantification (LOQ) of 5 [μg abs] for BSA in SFCM. These limitations are induced by the large background signal provided by the SFCM-blank.

Overall, the limit of detection and the limit of quantification was determined to be 0.15 [μg abs] and 0.5 [μg abs] for IgG and BSA in pure buffer systems such as PBS.

In comparison, Fig. 3b shows the calibration curve for IgG in TRIS buffer solutions with increasing amount of SFCM (0%, 2% and 100% SFCM) established with the Bradford method. For 2%-cell media in the sample solution, a calibration curve with a high linearity (R^2 = 0.9991) was obtained. With 100% cell media on the other hand only a correlation factor of 0.985 was achievable, even when measuring (and subtracting) the SFCM-reagent blank prior to each sample measurement. For the calibration curves of IgG and BSA in SFCM in Fig. 3a, established with the Protein A-HPLC method, no significant differences were observed for IgG as well as BSA, when SFCM was used instead of PBS buffer.

3.3. Analysis of artificial samples using Protein A-HPLC and the Bradford method

The classical way to analyze dual protein samples containing IgG and BSA, would be to quantify the total protein concentration via Bradford using bovine gamma globulin as the calibrate protein and to subtract therefrom the IgG-amount determined via Protein A-HPLC. This two-step approach will be compared with the simple Protein A-HPLC method.

Table 1 provides a summary of test results of various BSA and IgG test mixtures with different protein concentrations, different concentration ratios, different buffer solutions e.g. PBS and SFCM as well as with and without additives such as sodium azide and phenol red (PR). The use of SFCM and the addition of sodium azide and phenol red, increases subsequently the complexity of the background matrix and provide near-real mock feed solutions. Note that all three constituents can be present in real cell culture broth. While sodium azide prevents feed fouling, phenol red is sometimes added in order to detect pH changes during the fermentation process. Since they absorb partially in the UV range (220–280 nm), which

Table 1
Quantification of artificial test samples containing IgG and BSA besides the additives sodium azide and phenol red using Protein A-HPLC and the Bradford method.

	Buffer	Proteins		Additives[a]		Protein A-HPLC				BSA via Bradford[b]					
		IgG	BSA	NaN_3	PR	IgG		BSA		BGG-calib.		BSA-calib.		IgG-calib.	
		(μg/mL)	(μg/mL)	(μg/mL)	(mg/mL)	(μg/mL)	%	(μg/mL)	%	(μg/mL)	%	(μg/mL)	%	(μg/mL)	%
A	PBS	90	450	0.5	15.4	92.8	103	463.0	103	956.0	212	444.1	98.6	756.0	168
B	PBS	90	450	1	72.5	93.1	81	529.0	118	951.0	211	441.0	98.0	752.0	167
C	PBS	90	5	1	72.8	86.4	96	8.5	171	48.0	955	<0	–	20.3	406
D	PBS	5	90	1	–	4.7	94	90.8	101	149.7	166	82.2	91	138.2	154
E	PBS	90	5	1	–	89.2	99	4.8	96	12.3	247	<0	–	7.8	456
F	PBS	5	9	1	–	5.0	99	9.0	100	14.1	157	7.0	78	14.5	162
G	PBS	9	5	1	–	8.6	95	5.8	116	9.8	196	3.0	60	10.3	206
H	SFCM	90	50	1	–	88.3	98	53.4	107	206.3	413	88.0	176	61.2	122
I	SFCM	9	5	1	–	8.2	91	34.0	680	24.2	483	20.5	410	13.6	272

[a] For NaN_3 and phenol red (PR) own calibration curves were established.
[b] Total protein concentration were calculated using calibration curves for BGG, BSA and IgG; BSA concentrations were determined through subtraction of the corresponding IgG-concentration determined via Protein A-HPLC [42].

is characteristic for protein detection, it is essential to know their exact concentration in solution in order to subtract their contribution from the BSA containing flow-through peak.

The first results in Table 1 indicate that sodium azide and phenol red do not markedly disturb the quantification of BSA in PBS buffer, when BSA is present in a fairly high concentration, with only little amount of IgG and if BSA is also the calibrant (samples A, B and D). As soon as IgG is the dominant protein in solution, BSA is only weakly (sample F and G) or not at all detectable (sample C and E), depending on the amount of IgG and BSA in solution. The choice of protein for calibration is with Bradford critical. A comparison of results for samples A, B and C obtained with BGG, BSA and IgG for calibration shows a very strong deviation of results, which indicate that BGG provide highest false positive results followed by IgG. Especially samples with low BSA and high IgG concentration (sample E, G) in the presence of phenol red (sample C) or SFCM (sample H and I) are not accurately quantifiable with Bradford even when using BSA as the calibrant for the quantification of BSA.

In case of the Protein A-HPLC method, the only strong deviations were observed for samples with high phenol red content (sample B and C) and for samples with 5 μg/mL BSA (equiv. to 0.5 [μg abs]) in SFCM (sample I), which is below the stated LOD of 1.5 [μg abs] for BSA in SFCM.

3.4. Application of the Protein A HPLC method for material performance evaluation

The applicability of the Protein A-HPLC method for material performance evaluation was investigated with the commercial anion exchange material DEAE-Fractogel from Merck KGaA. In order to be able to compare the performance of DEAE-Fractogel for the capture of BSA under different buffer, salt and elution conditions, all tests were performed in batch mode via static binding capacity experiments.

This screening test was performed with the buffer systems PBS, TRIS and SFCM. Fig. 4 shows not only the SBC and the TBC (total binding capacity or elution capacity) results for both test proteins BSA and IgG, but provides additional information about the elution properties of the application buffers PBS and TRIS (Fraction W3). It is visible that the presence of 150 mM sodium chloride in PBS buffer leads to the elution of 20 μg/mg gel, while TRIS elutes half the amount of BSA. The protein eluting effect of PBS leads to a binding capacity of only 60 μg/mg gel for BSA, compared to 155 μg/mg gel for BSA in TRIS buffer. A comparison of the two test solutions containing only BSA (BSA single) and BSA spiked with IgG (BSA mix), shows that the presence of another protein reduces the binding capacity of the target protein by 6–8%. A comparison of SBC and TBC results shows the amount of BSA, which either bind only weakly or non-specifically to the adsorbent. BSA may elute during the wash steps W1 and W2 or remain on the support surface. A subtraction of the W1 and W2 shows that in case of PBS, 9.2 μg BSA per mg gel and for TRIS, 5.5 μg BSA per mg gel are not eluted from the anion exchange support (data provided in Table S7 [63]).

For SFCM two different strategies for data evaluation are provided and compared. In case of SFCM (A), the peak area of a SFCM blank injection was subtracted from the SBC-sample peak, while for SFCM (B), the peak area of the SBC-test run for SFCM was used. Since DEAE-Fractogel binds some constituents of SFCM, its concentration in the SBC supernatant is reduced. The subtraction of a smaller SFCM (test run) peak provides higher SBC results for BSA using the SFCM (B) approach. It can be expected that the SFCM (B) approach is more accurate than SFCM (A). A comparison of the W3 fractions of SFCM and TRIS, and a comparison of BSA (single) and BSA (mix) indicate that the constituents of the SFCM can influence the interaction between protein and adsorbent. The low W3 values for SFCM (B) show that less non-specific binding takes place. Surprisingly the TBC and SBC values for BSA (mix) in SFCM (B) are practically the same with 39 μg/mg gel.

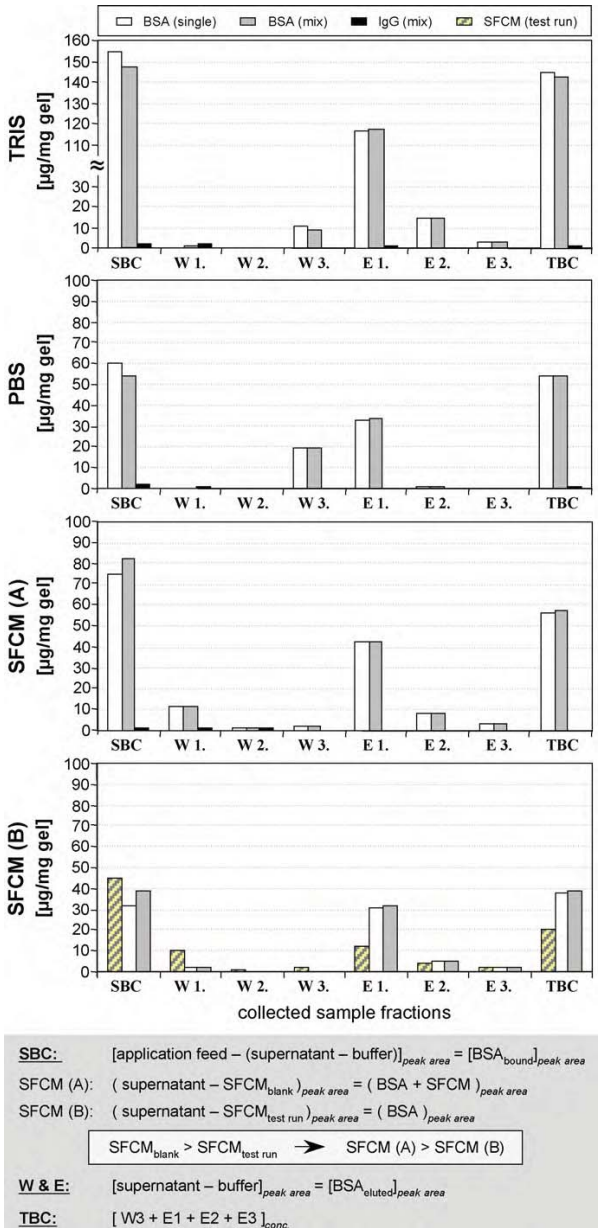

Fig. 4. Summary of results for static binding experiment with DEAE-Fractogel using single (single) and dual (mixed) protein solutions of BSA and IgG in 20 mM TRIS (pH 7.0), 10 mM PBS (150 mM NaCl, pH 7.2) and serum free cell media (SFCM). Analyzed supernatants: static binding capacity (SBC), wash (W1 and W2: bi-distilled water; W3: application buffer or for SFCM samples: TRIS), elution (E1–E3: PBS with 400 mM NaCl) and total binding capacity (TBC).

Naturally, the here described test protocol can be infinitely enlarged. Besides the influence of additives such as phenol red, sodium azide or Pluronic F67, also the efficiency of different sanitation solutions can be investigated with Protein A-HPLC.

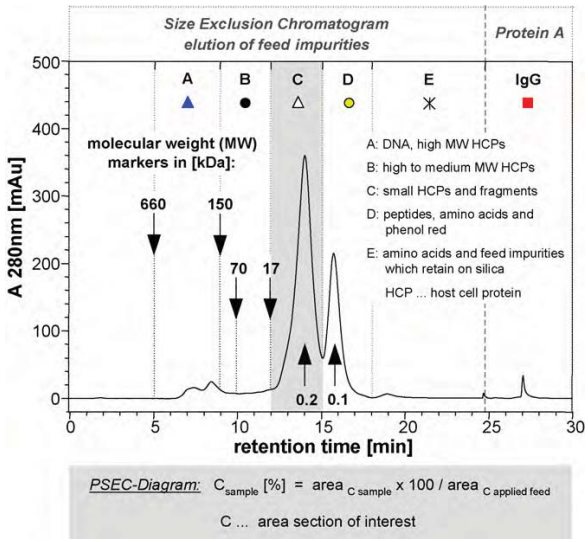

Fig. 5. Protein A-Size Exclusion Chromatogram (PSEC) of a cell culture supernatant containing 50 mg/L h-IgG1 is sliced into molecular weight areas of interest (A–E and IgG). Below is a simple formula describing the preparation of a PSEC-diagram from a set of sample fractions, using the MW-areas of the original application feed solution for comparison.

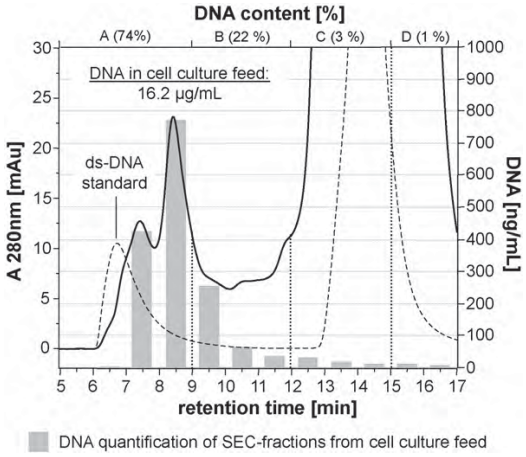

Fig. 6. DNA content of the corresponding MW-sections of the SE-chromatogram of cell culture feed determined via Quant-iT PicoGreen is shown in comparison to a ds-DNA standard of the same concentration.

3.5. Protein A combined with size exclusion chromatography

The application of Protein A HPLC, size exclusion HPLC for the quantification of IgG and the characterization of feed impurities from cell culture feed is known, but only as separate analytical methods [42]. The here described Protein A-size exclusion HPLC method (PSEC-method) fuses the two analytical methods into one. The use of one instrumental set-up and one application buffer saves not only time, but is also less laborious for the analyst.

Protein A-size exclusion chromatography (PSEC) can be divided into three steps. In the first instance IgG is being captured with the Protein A cartridge and in a second step all feed components, which did not bind to the Protein A adsorbent are being distributed by their molecular weight (MW) on the size-exclusion column. In the last stage, IgG is being eluted with the 12 mM HCl elution buffer (pH 2). Fig. 5 shows a PSEC-chromatogram of a cell culture feed where the SE-part of the chromatogram is being distributed into MW-areas (A–E), which are predefined by the retention times of a small set of MW-markers, namely thyroglobulin 660 kDa, IgG 150 kDa, myoglobuline 17 kDa, cytidine at 243 Da and p-amino-benzoic acid with 137 Da.

Generally speaking, small MW components such as phenol red will elute late from the SE column, while larger feed constituents such as DNA will practically flow-through the SEC column without being retained. The DNA content of the cell culture feed and the collected 1 mL fractions from a PSEC-run of 100 μL applied cell culture feed were determined via Quant-iT PicoGreen [70–72]. Since this fluorescent-spectrometric technique is very sensitive towards changes in the buffer system [73] and cell culture feed contains a huge back ground matrix besides only a trace amount of DNA, a standard addition method was chosen for the cell culture feed. The collected SEC fractions were quantified with a standard calibration curve using TRIS-EDTA buffer (TE) and PBS at a ratio of 50:30 (v/v). A DNA-content of 24.6 μg/mL in the cell culture feed was determined using an external calibration, while for the standard addition approach 22.3 μg/mL was found. For comparison, the latter amount of a standard ds-DNA solution was applied onto the SEC-column. Besides that 100 μL feed was applied and 1 mL fractions were collected and quantified, providing a DNA content of 16.2 μg/mL DNA in feed. Comparing the SE profiles of the standard DNA and the feed DNA in Fig. 6, it seems that the feed DNA contains more low MW DNA fragments ranging from 660 to 70 kDa, with a MW-maximum at approximately 300 kDa, while the standard ds-DNA flows practically unretained through the column.

Using the retention times of the MW-markers in Fig. 5 as a guidance, the SE-chromatogram was divided into 5 MW-sections, of which section A ranges from 660 to 150 kDa and contains 74% of the DNA from the feed, while section B ranges from 150 to 17 kDa and contains most of the larger proteins plus a small amount of 22% of DNA. Section C comprises small MW impurities from 17 to 150 kDa. In case of section D and especially area E, the here comprised impurities are expected to be smaller than 150 Da, but are on the contrary most likely higher MW-compounds, which are being retained on the silica-based surface of the SE-particles. Note that with increasing age and use of a silica-based SEC-column, not only the retention times of these higher MW-compounds will gradually increase, but also more feed impurities will be able to interact or even bind to the exposed silanol-groups of the silica surface.

3.5.1. Comparison of different cell culture feed batches using PSEC-HPLC

Since the composition of the constituents of cell culture feed samples changes from sample to sample, it is possible to visualize these changes by comparing the peak area ratios of the different SEC-areas relative to those of the original standard or application feed. The so obtained plot, the Protein A-SEC (PSEC) diagram can be used to characterize and compare different production batches of IgG containing cell culture broth (Fig. 7) or samples generated during performance evaluation of adsorbent for antibody purification using cell culture feed (Figs. 8 and 9).

In Fig. 7, the composition of 9 different batches of cell culture feed solutions from a CHO expression system for h-IgG1 production is being characterized with size exclusion HPLC, SDS-Page gel electrophoresis under reduced and non-reduced condition using silver staining and with a PSEC-plot. It is obvious that the SE-chromatograms of the different feed batches look more (B, C, D and G) or less (A, E, H and I) alike, providing only little information about the actual composition differences of these feed batches. On

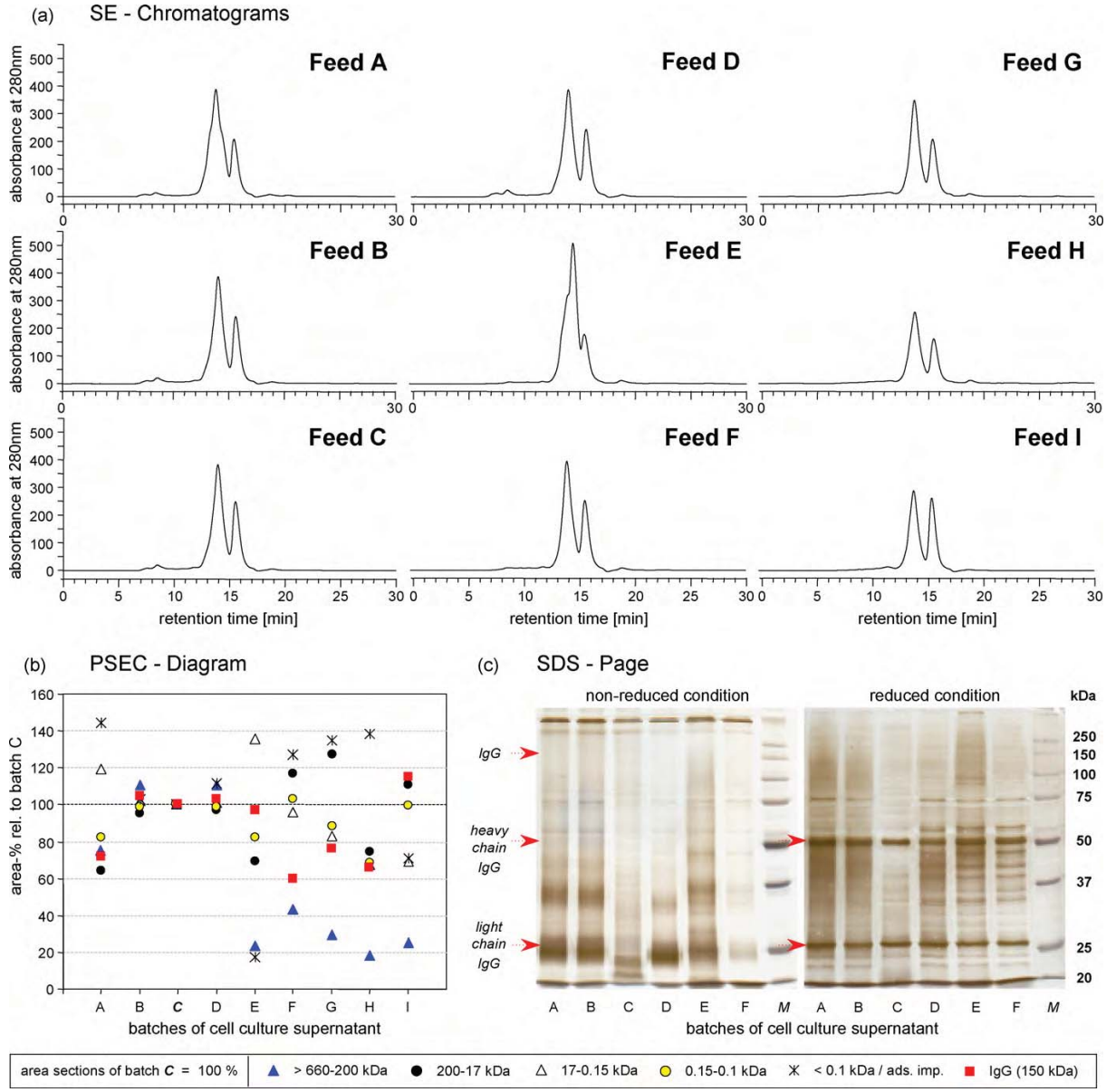

Fig. 7. Comparison of different cell culture feed batches (A–I) using (a) size-exclusion chromatography, (b) PSEC-diagram and (c) SDS-Page gel electrophoresis (gel: 10%-TRIS-HCl) for reduced and non-reduced feed samples with silver staining for protein visualization.

SDS-Page gels using non-reduced samples, the differences between these batches are also not so distinct, showing a similarity of batches A, B and D, batches C, E and F look very different to the former. Taking a look at the SDS-Page slab gel for the reduced samples feed A and B as well as feed D, E and F look alike. The PSEC-plot in Fig. 7b shows that the feed batches B, C and D are practically the same, which correspond to the fact that they are of the same production batch, but were delivered by Excellgene, separately bottled over a certain period of time. It is also visible that the feed impurity A (>660–200 kDa) content is lower for all the following batches E–I and that the IgG content of the final batch I is the highest of all investigated feed batches. The different results observed with SDS-Page and the PSEC-plot can be explained by the fact that they provide complementary results, while PSEC is focused on high MW constituents between 660 and 17 kDa, SDS-Page distributes primarily low-MW impurities between 100 and 20 kDa. In case of reduced samples, the SDS-Page slab gels shows a strong protein smear over the entire lane, which may cover up any distinct bands.

3.5.2. *Performance evaluation of anion-exchange materials using cell culture feed*

In Fig. 8 the dynamic break-through performance of a commercial DEAE-Fractogel material from Merck KGaA using cell culture feed is visualized in a PSEC-plot and on a SDS-Page slab gel using reduced conditions and silver staining. The PSEC-plot allows a comparison of the break-trough performance of feed impurities as well as IgG. It can be seen that DEAE-Fractogel completely removes high molecular weight impurities of 660–150 kDa from

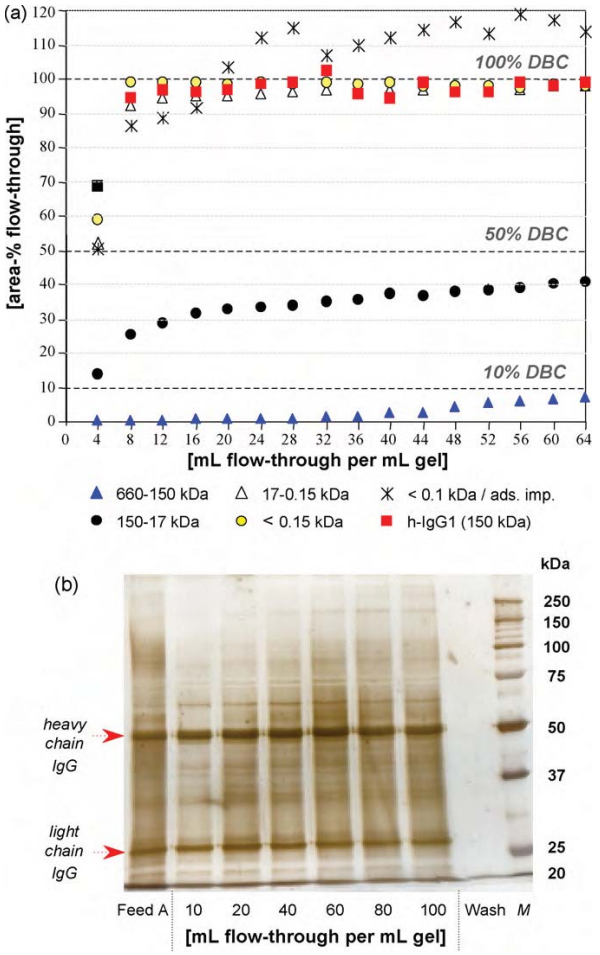

Fig. 8. Comparison of dynamic binding results for DEAE-Fractogel tested with cell culture feed using (a) PSEC-diagram and (b) SDS-Page gel electrophoresis (gel: 10%-TRIS-HCl).

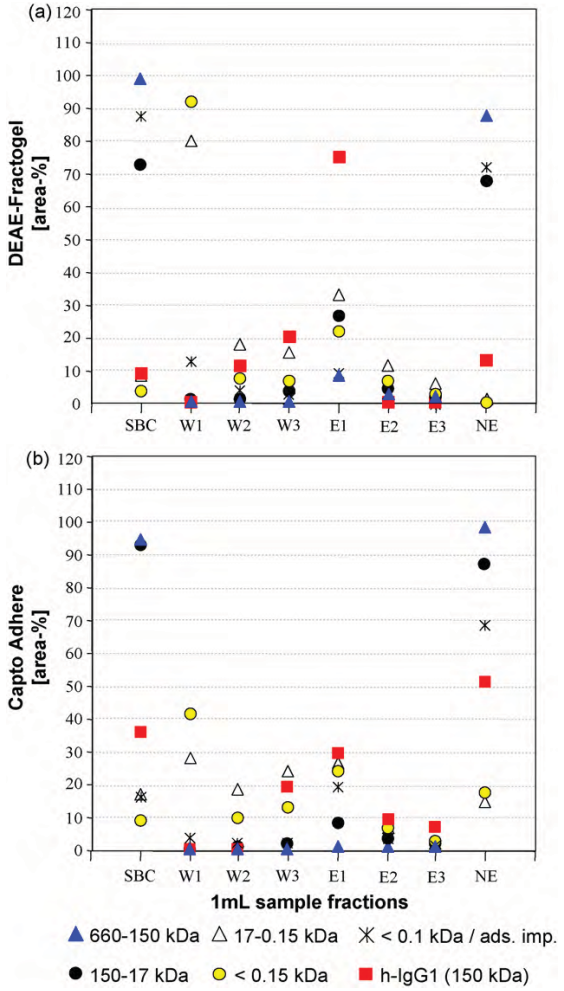

Fig. 9. Static binding (SBC), wash (W) and elution (E) profile of feed impurities and h-IgG1 from a cell culture feed tested on (a) DEAE-Fractogel and (b) CaptoAdhere; Wash solutions: PBS without NaCl (W1), with 75 mM (W2) and with 150 mM NaCl (W3); Elution solutions (E1–E3): PBS with 400 mM NaCl; Adsorbent: 25 mg (suction dried); static binding capacity (SBC = area% relative to application feed composition); wash, elution and non eluted fraction (NE = area% relative to bound impurities and h-IgG1).

the first 36 mL of feed per mL gel. In case of the molecular weight impurities in the range of 150–17 kDa, only a removal of 60–70% can be achieved. The remaining 30–40% resembles a multitude of impurities, which simply do not bind to this particular AIEX material. The lower MW-impurities do not bind much to DEAE-Fractogel, however, some impurities in the range of <0.1 kDa (impurity E), which may also resemble higher MW-impurities which slightly retain on the SEC column seem to weakly bind to the DEAE-Fractogel and be displaced once the adsorbent becomes saturated.

On SDS-Page the difference in the feed composition of the DBC flow-through fractions is seen in the increasing amount of high molecular weight impurities 100–250 kDa from the 10 mL to the 60 mL fraction. The first three fractions also show more distinct bands ranging from 30 to 40 kDa and less of the undefined smear that spreads over the entire lane. Sample fractions 60 mL, 80 mL and 100 mL do not show much difference anymore.

The performance of the same DEAE-Fractogel material for the capture of feed impurities from cell culture feed is compared to CaptoAdhere, using static binding capacity tests and PSEC-Plots to characterize these two adsorbents (Fig. 9). The SBC experiment is performed as previously described for standard protein solutions in Fig. 4, using 25 mg gel and 1 mL cell culture feed or 1 mL of the corresponding wash and elution solutions. Concerning the capture of high MW-impurities, it seems that Capto Adhere can bind slightly less DNA compared to DEAE-Fractogel, but more of the feed impurities 150–17 kDa. It is also shown that Capto Adhere binds about 38% IgG from the feed and only gradually releases 50% during the last wash step W3 with 150 mM NaCl and the elution steps E1, E2 and E3 with 400 mM NaCl. In case of DEAE-Fractogel, only 14% of the originally captured 9% of IgG does not elute from the adsorbent and 75% of bound IgG elute in the first elution fraction E1. Another interesting point is that DEAE-Fractogel can capture 87% of feed impurity E (<0.1 kDa plus ads. imp.), while Capto Adhere only binds 17% hereof under the here investigated test conditions.

The examples shown in Figs. 7–9 only represent a brief overview of the possible field of application for PSEC diagrams in the evaluation and comparison of new adsorbents designed for antibody purification. In follow-up publications we will use the here described methods to evaluate novel affinity-based weak

anion exchange type materials (AFFI-WAX), new triazine-based bio-mimetica B14 from Prometic Bioscience.Ltd and innovative endcapping strategies for azido-modified support materials for ligand immobilization via "Click Chemistry".

4. Conclusion

Up to date the major duty of analytical Protein A HPLC columns was to capture IgG from a highly complex mixture und doing so in as short a time as possible. Analysis times of a few minutes and high-through-put sample analysis made it an ideal tool for production monitoring.

The present study introduces a simple instrumental set-up that combines the capture of IgG with a Protein A HPLC column with the possibility to further distribute cell culture feed impurities with size-exclusion HPLC. The flexibility of this approach allows the user to decide whether he desires only a simple and fast IgG measurement or a slightly longer Protein A HPLC run for the simultaneous determination of the non-binding protein e.g. BSA besides IgG or if he needs the PSEC-HPLC method to analyze more complex biological samples. Although the latter may require fairly longer analysis time, it also provides the utmost information that can be gained by this simple instrumental set-up. The possibility to visualize the generated data in PSEC-plots enables the user to not only characterize and compare cell culture feed solutions of various production batches, but he may as well use the same method to characterize adsorbents for antibody purification using real cell culture supernatant for testing.

Moreover, the here described methods run fully automated, enabling a 24/7 non-stop sample analysis with column regeneration protocols included in the measurement sequence. In general, PSEC-HPLC is less laborious and provides more accurate and reliable quantitative results than protein determination via Bradford or SDS-Page can achieve.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.06.007.

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Supplementary Material

Quantification of immunoglobulin G and characterization of process related impurities using coupled Protein A and size exclusion high performance liquid chromatography

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1. HPLC-Method Description

Instrument: Agilent 1100 Series
Software: ChemStation for LC 3D system (Rev. B.01.03 [204])

The Agilent 1100 and 1200 systems are in actual fact not bio-compatible, since most instrumental parts as well as tubings are made of stainless steel. Therefore all steel tubings from the injection system down to the detector were replaced by tefzel tubings with slightly increased inner diameter to reduce the system back pressure. The screw threads of the outlet valves of both pumps (A and B) were sealed with teflon strips in order to prevent rusting. Note that the use of 12 mM hydrochloric acid, pH 2-3 as elution buffer for IgG removal from the Protein A cartridge is pretty harsh on the system. Since this elution buffer was recommended by the manufacturer Applied Biosystems, it was not replaced. However, in the long run for a 24 h – 7 d analysis scheme, the use of phosphoric acid instead of hydrochloric acid may be advisable. The use of citric acid is however not advisable, since it is prone to fouling and may lead to a strong bacterial contamination of the 100% aqueous HPLC-system.

1.1. Protein A HPLC Method

The here described Protein A HPLC method resembles the general “long” run with an adsorption time of 15 min and an elution time of 15 min, which was used for samples with high protein concentration and for samples with unknown protein and feed impurity content. For low sample concentration the adsorption time can be reduced to a minimum of 5 min and the elution time to 7 min. For samples with high protein content the injection volume should be reduced to 10-20 µL or the corresponding samples ought to be dilution by a factor of 5 to 10. The latter may be advantageous for samples which may precipitate at reduced temperatures, since the sample tray should be cooled in order to prevent early sample fouling.

Instrumental Set up

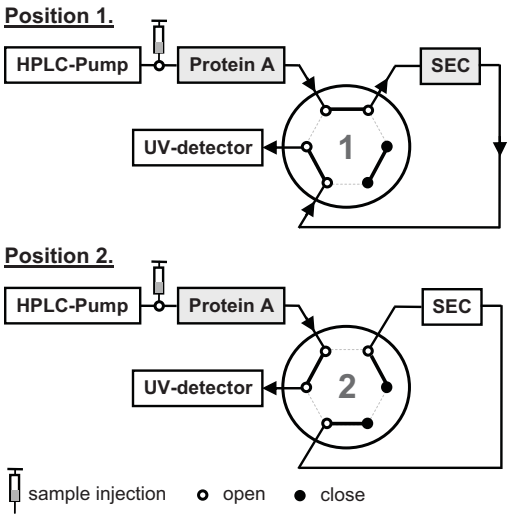

Figure S1. *Flow-chart showing the coupling of an analytical Protein A HPLC column with a SEC-column for the separate distribution of feed impurities on a SEC column after IgG capturing onto the Protein A column (position 1.) and the elution of IgG from the Protein A column (position 2.).*

Set up Pump:

Control: Flow: 1.000 mL/min
Stop time: 38.00 min
Post time: off

Solvent A: 10 mM PBS, 150 mM NaCl, pH 7.20
Solvent B: 12 mM HCl, 150 mM NaCl, pH 7.20

Pressure Limit: 0 – 150 bar

Time Table:

	Time	% B	Flow	Max. Pressure
1	0	0	1.000	150
2	15.00	0	1.000	150
3	15.01	100	1.000	150
4	32.00	100	1.000	150
5	32.01	0	1.000	150

Injection:

Injection with Needle Wash
Injection Volumn: 100 µL
Optimization: none

Column Thermostat: 25°C

Sample Thermostat: 10°C

MWD Signal:

Sample	BW	Reference	BW
280	10	550	20
260	*10*	*550*	*20*
220	*10*	*550*	*20*
432	*10*	*550*	*20*

Sample and Reference in [nm]
BW… band width [nm]

Set up Valve <2PS/6PT>

	Time	Position
1	0	Position 2
2	38.00	Position 2

Required Lamps:	UV and VIS
Peak Width (Response Time):	0.1 min (2s)
Slit:	4 min
Margin for neg. Absorbance:	100 mAu
Stop time:	as Pump
Autobalance:	Postrun

1.2. Protein A / SEC HPLC Method

If not otherwise stated the set-up for the Protein A / SEC HPLC method is the same as previously described for the Protein A HPLC method.

Set up Pump:

Control: Flow: 1.000 mL/min
Stop time: 55.00 min
Post time: off

Solvent A: 10 mM PBS, 150 mM NaCl, pH 7.20
Solvent B: 12 mM HCl, 150 mM NaCl, pH 7.20

Pressure Limit: 0 – 150 bar

Time Table:

	Time	% B	Flow	Max. Pressure
1	0	0	1.000	150
2	34.99	0	1.000	150
3	35.00	100	1.000	150
4	41.99	100	1.000	150
5	42.00	0	1.000	150
6	55.00	0	1.000	150

Set up Valve <2PS/6PT>

Position: Pos 1

Position 1: Protein A / SEC
Position 2: Protein A

Time Table:

	Time	Position
1	0	Position 1
2	34.50	Position 2
3	47.00	Position 1

1.3. Column Regeneration

1.3.1. Size-exclusion column (SEC)

Since the here employed SEC column (TSKgel G3000SWXL with 7.8 mm ID x 30.0 cm with the guard column TSKgel SWXL with 6.0 mm ID x 4.0 cm from Tosoh Bioscience, Stuttgart, Germany) has a silica lattice, the continuous use of such a column for the analysis of cell culture samples will rather sooner than later contaminate the column and change its separation performance. The below list of simple and not so simple wash procedures can be employed to extend the life-time of your column as well as increase the accuracy of your experimental results.

- In case of a slight contamination of the SEC column, which is shown in a slight peak broadening and peak tailing, 5-10 successive injections with 100 µL 20 mg/mL BSA in the application buffer with a 10 min column run time before the next injection are sufficient to fully regenerate the column performance as recommended by Tosoh. The solubilizing property of BSA was already mentioned by Compton, who suggested the use of BSA solutions to regenerate Protein A HPLC columns [1]. As a precaution this treatment was routinely performed after every 100-150 injection or after the column was not in use for a long period of time. Note that for PSEC runs, the Protein A-HPLC cartridge need not be disconnected and that a small wash-sequence of subsequent BSA injections can therefore be routinely included in a running analytical sequence.

- A frequently observed problem is the bacterial contamination of the tubings of the HPLC system and the columns in use. Such contaminations are visible by a high system peak for the elution sequence of the Protein A column. A useful remedy is the sequential injection (5-10) of 100 µL of 4 mg/mL sodium azide in PBS. Also this set of wash-injections can be performed on a regular basis prior to the BSA injections. If the tubings of the HPLC buffer supply are contaminated than a purging of the tubings with 4 mg/mL sodium azide in PBS can be performed. It would be advisable to rinse the tubings first before rinsing the column, in order not to contaminate the column any further.

- If these simple column regeneration protocols are not sufficient anymore, then a more extensive column wash procedure employing a minimum of 100 mL (8 column volumes, CV) of 0.5 M Na_2SO_4 in bidest. (pH 2.7) followed by 20 mL bidest and 100 mL 40% (v/v) methanol in bidest at a flow-rate of 0.5 to 1 mL/min is advisable. Note that the SEC-columns must be rinsed in the opposite flow-direction in order not to distribute the contaminants over the entire column and that in case of a strong contamination of the SEC-column higher volumes of the wash solutions and longer wash times may be needed [2]. Also note that a frequent use of this wash procedure leads to a loss in column performance, which is irreversible.

- For a SEC column, which was in constant use for protein analysis from a cell supernatant over more than a year, a full regeneration of the size-exclusion performance is very difficult to achieve, once a significant loss of resolution is observed. From a number of tested wash procedures which were originally designed for bonded silica reversed-phase column that were contaminated with protein residues [3], a simple column wash with 100% 2-propanol provided some improvement.

- Tosoh has also recommended chaotropic wash solutions of 0.1% SDS, 8 M urea or 6 M guanidine hydrochloride, but note that these compounds must be completely removed from the column, which may require elaborate rinsing [2]. Furthermore these reagents may be irreversibly adsorbed to the silica surface of the column packing [3] especially if the material has aged after a long period of use & regeneration, and exhibit therefore free silanol groups. The presence of free silanol-groups becomes obvious once high-molecular weight proteins are being retained on the SEC column, leading to SE-chromatograms with low peak height and strong peak tailing.

1.3.2. Protein A (PA ID sensor cartridge)

Although the Protein A sensor cartridge from Applied Biosystem is quite robust, a slight increase in column pressure over a short period of time should not be neglected. A thorough rinse as well as back-flush with elution buffer (12 mM HCl) for ½ to 1 hour every 2 weeks ensures that the column stays free of contaminants. It was furthermore observed that the life time of the Protein A cartridge was significantly increased when it was rinsed with 5-10 successive injections with 100 µL 20 mg/mL BSA and/or 4 mg/mL sodium azide in the application buffer, as previously mentioned for the Protein A - SEC column combination. Note that Compton had suggested a column wash with methanol after every 200 injections [1].
The last Protein A cartridge was used almost 24 hours for 5-7 days a week and that for an entire year, and the only observed difference was an increase in column pressure from 18 bar to 38 bar, without any significant changes in column performance. In comparison our first Protein A cartridge lasted only half a year and was discarded due to an irreversible column back pressure increase of over 100 bar.

2. Additional Information for the Experimental Section

2.1. Instrumental set-up for material performance evaluation

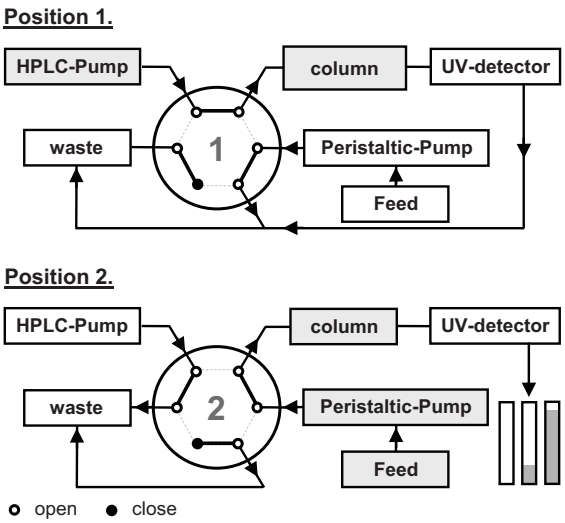

Figure S2. *Flow-chart for dynamic binding testing of adsorbents using cell culture feed. Column equilibration: position 1; Feed application: position 2; volume of collected sample fractions: 10 mL*

HPLC-system: Merck-Hitachi equipment with L-6200A Intelligent Pump with a D-6000A Interface, a single wavelength, UV-975 Intelligent UV/VIS detector from Jasco, Biolab and Minipuls 3 peristaltic pump from Gilson. Valve switching and sample collection was performed manually. Alternatively also an Agilent Instrument (Series 1100) with automated valve-switching and automated sample collector can be used. All data shown in this manuscript was performed with the Merck-Hitachi system.

2.2. Cell culture feed batches

Table S1. *Properties of buffer solutions and cell culture supernatant batches*

	NaCl	conductivity	pH-value	Temperature
	[mM]	[mS/cm]		[°C]
Buffer solutions:				
bi-dist. water	0	0.0	8.7	32.1
PB [a)]	0	1.7	7.4	32.3
PBS [a)]	75	9.8	7.2	32.0
PBS [a)]	150	18.9	7.2	32.0
PBS [a)]	300	34.8	7.1	32.2
Cell culture supernatants batches (ExcellGene)				
C	149 [b)]	18.3	7.4	33.6
D	140 [b)]	17.3	7.5	33.5
E	139 [b)]	17.2	7.5	33.5
F	139 [b)]	17.2	7.5	33.4
G	129 [b)]	16.0	7.5	33.3
H	132 [b)]	16.4	7.4	33.2
I	231 [b)]	27.4	7.6	33.2
J	218 [b)]	25.9	7.7	33.2
K	208 [b)]	24.8	7.2	33.5

[a)] 10 mM Phosphate buffer (PB) with increasing amount of sodium chloride (PBS)

[b)] estimation of salt content through correlation of the sodium chloride content and conductivity of standard PBS buffer solutions with increasing salt content and conductivity

($y = 0.1108\ x + 1.7580$; $R^2 = 0.9994$)

3. Additional Information for Results and Discussions

3.1. Comparison of Protein A HPLC and Bradford

Table S2 and **Table S3** show the corresponding data to the **Figure 2a** for Protein A HPLC and Figure 2b employing Bradford for protein quantification

Table S2 *Comparison of Protein A HPLC results (peak areas, limit of linearity LOL, calibration and correlation data) established with BSA solutions of varying BSA concentration, buffer systems, salt content and pH-values)*

Protein	**Buffer systems** [peak-areas [mAu sec]; absorbance at 280 nm (n = 1)]					
BSA [µg abs]	**TRIS-HCl pH 7**	**TRIS-HCl pH 3**	**TRIS-HCl pH 7, 0.4M NaCl**	**0.1M HCl pH 1**	**PBS pH 7.20**	**SFCM**
0.1	---	---	---	---	2.8 [b)]	---
0.2	---	---	---	---	5.7 [b)]	---
0.5	---	---	---	---	13.5 [b)]	---
1	30.9	25.2	30.5	30.6	28.5 [b)]	208.8
5	161.2	154.7	161.6	148.1	159.0	263.9
10	325.5	319.2	322.1	305.9	319.0	463.3
20	651.4	636.8	653.1	634.4	644.0	749.0
50	1638.3	1593.9	1632.6	1610.1	1645.3	1652.1
70	2262.2	2264.2	2282.2	2281.1	2273.2	2052.6
100	3232.4	3220.5	3248.8	3236.9	3275.0	2914.5
200	6499.6	6461.2	6571.2	6508.3	6511.1	5632.4
500	16233.9	16134.9	16228.6	16172.3	16317.6	13056.7
700	22744.2	22422.1	22702.9	22583.2	22719.9	17304.7
1000	32219.9	31944.4	32262.0	31733.3	32243.6	23097.6
LOL [a)] [µg abs]	> 1000	> 1000	> 1000	> 1000	> 1000	500
Calibr. curve [c)]	y=32.313x+16.137 R^2 = 0.99998	y=31.998x+17.203 R^2 = 0.99998	y=32.316x+23.952 R^2 = 0.99998	y=31.927x+40.627 R^2 = 0.99988	y=32.333x+23.766 R^2 = 0.99997	y=25.809x+257.76 R^2 = 0.9993

a) LOL = Limit of Linearity; [µg abs] = µg protein injected

b) calibration curve for BSA in PBS (0.1 - 1µg [abs]): y=28.459x+0.182, R^2 = 0.9990

c) calibration curve for 1 – 1000 [µg abs] BSA

Table S3 *Comparison of Bradford results (absorbance at 595 nm, limit of linearity LOL, calibration and correlation data) for BSA solutions in TRIS-HCl buffer containing varying amount of BSA (1-40 µg/mL) and salt (0M and 0.4M NaCl), and with varying pH-values (pH 7, 3 and 1))*

Protein	**Buffer systems** [absorbance at 595 nm (n = 3)]			
BSA [µg/mL]	**TRIS-HCl pH 7**	**TRIS-HCl pH 3**	**TRIS-HCl pH 7, 0.4M NaCl**	**0.1M HCl pH 1**
1	0.0559	0.0288	0.0393	0.0070
5	0.2102	0.2071	0.1710	0.1489
10	0.4153	0.4376	0.3127	0.2864
20	0.8160	0.7720	0.5267	0.4783
30	1.2103	1.1257	0.6699	0.6656
40	1.3749	1.2924	0.8249	0.8380
LOL [a] [µg/mL]	30	20	20	20
Calibration curve	y = 0.0401x + 0.0133 R^2 = 0.9999	y = 0.0390x + 0.0107 R^2 = 0.9930	y = 0.0253x + 0.0348 R^2 = 0.9897	y = 0.0242x + 0.0120 R^2 = 0.9796

For **Table S4** no corresponding Figures are provided.

Table S4 *Comparison of the calibration curves for BSA and IgG established separately with single standard solutions (IgG single and BSA single) and dual mixtures containing both, IgG and BSA (IgG combi and BSA combi) plus the calibration curve of BSA established with SEC-HPLC.*

Protein	**BSA**			**IgG**	
[µg abs] [a]	**single** [c]	**combi** [c]	**SEC** [d]	**single** [c]	**combi** [c]
0.1	3.0	3.5	2.8	4.2	2.6
0.5	13.1	15.2	17.2	19.3	27.0
1	27.7	33.9	38.4	45.0	60.1
5	159.8	161.8	215.7	323.4	348.7
10	321.1	333.8	422.0	676.6	711.9
50	1633.6	1666.5	1891.2	3629.0	3717.0
100	3312.8	3297.7	3658.1	7357.4	7434.2
150	4969.2	5045.1	5555.5	10947.4	11215.5
200	6646.4	6672.7	---	14764.8	14848.3
Calibration curve	y=33.2257x-8.6352 R^2 = 0.999988	y=33.4168x-3.6566 R^2 = 0.99994	y=36.8202x+17.9515 R^2 = 0.9998	y=73.7325x-35.8980 R^2 = 0.99995	y=74.5056x-13.8087 R^2 = 0.99998

a) [µg abs] = µg protein injected

b) calibration curve for BSA (combi): [BSA] $_{peak\ area}$ *– [IgG additives]* $_{peak\ area}$

c) Protein A HPLC method: single = protein solutions containing a single protein
combi = protein solutions containing both proteins, IgG and BSA

d) SEC = Size exclusion HPLC

Table S5 and **Table S6** show the corresponding data to the **Figure 3a** using Protein A HPLC and **Figure 3b** using Bradford for protein quantification

Table S5 *Quantification of BSA and IgG in PBS buffer and SFCM using Protein A HPLC*

Protein	**BSA**		**IgG**	
[µg abs] [a)]	**PBS**	**100% SFCM**	**PBS**	**100% SFCM**
1	26.0	121.1	39.9	42.6
5	144.5	276.5	290.6	273.8
10	287.2	420.7	580.3	603.2
20	582.6	710.1	1351.9	1329.9
50	1473.4	1556.6	3375.0	3339.7
70	2068.3	2155.6	4798.4	4769.0
100	2976.9	2902.4	6968.2	6879.2
Calibration curve	y=29.7707x-8.8990 R^2 = 0.99997	y=27.6212x+149.3405 R^2 = 0.99996	y=69.9762x-72.8425 R^2 = 0.9998	y=69.1703x-67.0854 R^2 = 0.99997

Table S6 *Quantification of IgG in the presence of increasing amount of SFCM (0%, 2% and 100%) in TRIS-HCl buffer (pH 7.0)*

Protein	**Buffer systems** [absorbance at 595 nm (n = 3)]		
IgG [µg/mL]	**TRIS-HCl pH7 0% SFCM**	**TRIS-HCl pH7 2% SFCM**	**100% SFCM**
1	0.0336	0.0095	0.0377
5	0.1316	0.1090	0.0773
10	0.2741	0.2794	0.1215
20	0.5559	0.5900	0.1750
30	0.8107	0.8603	0.2914
40	1.0836	1.1495	0.4053
Calibration curve	y = 0.0270x + 0.0042 R^2 = 0.9997	y = 0.0295x - 0.0209 R^2 = 0.9991	y = 0.0091x + 0.0236 R^2 = 0.9854

SFCM = serum-free cell media

Table S7 lists the numerical results, which are visualized in a diagram with bars in **Figure 3**.

Table S7. *Material performance evaluation of DEAE-Fractogel, employing different buffer systems and test conditions, using the Protein A HPLC method for protein quantification.*

			TRIS [µg/mg gel]	PBS [µg/mg gel]	SFCM [µg/mg gel]
SBC	**Blank run**	SFCM	---	---	45.35 [a]
	B	BSA	154.73	59.74	75.43 [b] / 31.69 [c]
	BI	BSA	147.75	53.77	82.59 / 38.51
		IgG	1.62	1.55	1.15
Wash 1. ***(bi-distilled water)***	**Blank run**	SFCM	---	---	10.06
	B	BSA	0.30	0.19	11.54 / 2.14
	BI	BSA	0.51	0.20	11.31 / 1.81
		IgG	2.08	1.44	1.34
Wash 2. ***(bi-distilled water)***	**Blank run**	SFCM	---	---	0.77
	B	BSA	0.26	0.11	0.75 / 0.31
	BI	BSA	0.33	0.13	0.87 / 0.40
		IgG	0.08	0.09	0.7
Wash 3. [d] ***(application buffer)***	**Blank run**	SFCM	---	---	2.36
	B	BSA	10.74	19.86	2.30 / 0.28
	BI	BSA	8.10	19.00	2.33 / 0.31
		IgG	0.11	0.15	0.09
Elution 1.	**Blank run**	SFCM	---	---	12.25
	B	BSA	117.11	32.57	42.47 / 31.00
	BI	BSA	117.31	33.33	42.98 / 31.41
		IgG	0.59	0.43	0.20
Elution 2.	**Blank run**	SFCM	---	---	3.65
	B	BSA	14.08	1.20	8.32 / 5.12
	BI	BSA	14.25	1.02	8.58 / 5.35
		IgG	0.10	0.08	0.10
Elution 3.	**Blank run**	SFCM	---	---	1.96
	B	BSA	3.07	0.29	3.13 / 1.71
	BI	BSA	3.24	0.29	3.01 / 1.59
		IgG	0.09	0.00	0.09
TBC	**Blank run**	SFCM	---	---	20.22
	B	BSA	145.00	53.92	56.22 / 38.11
	BI	BSA	142.90	53.64	56.90 / 38.66
		IgG	0.89	0.66	0.48

Abbreviations: static binding capacity (SBC), total binding capacity or elution capacity (TBC), serum-free cell medium (SFCM), bovine serum albumin (BSA; B), immunoglobulin G (IgG; I), solution with BSA and IgG (BI)

Buffer solutions: TRIS (20 mM TRIS-HCl, pH 7.20, 0 mM NaCl), PBS (10 mM phosphate buffer saline, pH 7.20, 150 mM NaCl), SFCM (commercial serum free cell media); elution buffer (PBS-buffer, 0.4 M NaCl)

[a] Constituents of SFCM were quantified using the same BSA calibration curve as for BSA containing samples

[b] Subtraction of the peak area of SFCM from a SFCM blank injection (= total bound fraction = bound BSA plus bound SFCM)

[c] Subtraction of the corresponding constituents from the SFCM material test (= bound BSA)

[d] Wash solution 3. for SFCM samples was TRIS-buffer.

References:

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[3] R.E. Majors, LC-GC Eur. 16 (2003) 404.

6. Summary

In the past 15 years Protein A has emerged as the most used ligand applied for affinity-based purification of immunoglobulin G [6]. The greatest advantage of Protein A affinity chromatography is, without a doubt, the possibility to isolate antibodies from a host cell lysate through a single purification step. The drawbacks of this state of the art method are the high cost of Protein A manufacturing as well as possible product contamination due to Protein A leakage.
Out of the many other possible methods for antibody purification, weak cation exchange adsorbents (WCX) could offer a cheap and robust alternative, or possibly even an attractive replacement for Protein A.

The first part of this work was focused on the immobilisation of aromatic ligands with weak cationic properties like carboxylic or sulfonic acid groups. Fractogel® EMD Epoxy (M) was chosen as support material because ligand molecules with thiol or amino group residues can easily be immobilized through a base catalyzed nucleophilic addition reaction onto the activated epoxide groups. From all tested molecules, 4-mercaptobenzoic acid proved to be an easy to handle ligand and provided possible immobilisation yields of up to 0,950 mmol/g from the available 1,000 mmol/g epoxide groups on Fractogel® EMD.

The analytical part of this diploma thesis was built around the IgG binding capacity measurements of the new created functionalized Fractogels.
4-Mercaptobenzoic acid Fractogel, as most promising candidate also yielded high antibody-binding-capacity of 52 mg IgG/g gel at nearly physiological conditions of 25 mM phosphate at pH 6,5 and a NaCl concentration of 75 mM.
Furthermore, binding capacity studies at same buffer conditions as stated above, for gels with reduced 4-mercaptobenzoic acid densities of 0,819 mmol/g and 0,514 mmol/g provided IgG binding capacities of 41 mg IgG/g gel and 25 mg IgG/g gel, respectively, which stood in good correlation with the corresponding ligand densities. This promising result would lead to the next possible optimisation step, namely the combination of 4-mercaptobenzoic acid with (one

or more) other functional moieties or (one or more) other ligands, to thereby obtain a pure “mixed-mode” stationary phases.

Continuing evaluations of the IgG binding capacity with cell culture feedstock revealed that the 4-mercaptobenzoic acid Fractogel was able to maintain a binding capacity of 31,4 mg IgG/g gel at a buffer composition of 25 mM phosphate, pH 6,5 and a NaCl concentration of 75 mM.

Also a decreasing DNA content for the collected elution fractions of the DBC study could be measured with the help of an especially adopted fluorescence measurement method. The biggest advantage of this adjusted method was the reduced wastage of fluorescence reagent. The measurements were carried out in ultra-micro cuvette with 45 µL measuring cell volume which required DNA sample volumes of only 10 µL.

Unfortunately, the 4-mercaptobenzoic acid ligand was not binding IgG exclusively and SDS-PAGE gels showed measurable amounts of host cell protein, which is not a big surprise, considering the simple structure of the ligand molecule.

Because of this insufficient selectivity towards IgG, a purification of immunoglobulin G directly from a cell culture feed solution, is therefore not the ideal application for the 4-mercaptobenzoic acid Fractogel.

7. Zusammenfassung

Über die letzen 15 Jahren hinweg hat sich Protein A als der am weitesten verbreitete Ligand in der affinitäts-basierenden Reinigungsmethode für Immunglobulin G etabliert [6]. Der große Vorteil der Protein A Affinitäts-chromatographie ist ohne Zweifel die Möglichkeit der Isolatioin von Antikörpern aus lysiertem Zellextrakt in einem einzigen Reinigungsschritt. Die Nachteile dieser „Stand-der-Technik"-Methode sind einerseits die hohen Herstellungs-kosten von Protein A, wie auch die mögliche Kontamination des gerreinigten Produktes durch Protein A Säulenbluten.
Aus der Vielzahl möglicher alternativer Methoden zur Antikörperreinigung könnten die sogenannten schwachen Kathionenaustauscher eine billige und zugleich robuste Alternative darstellen, bzw. die Protein A Affinitätschromatographie sogar ganz ersetzen.

Der erste Teil meiner Diplomarbeit beschäftigte sich mit der Immoblilisierung von aromatischen Liganden mit schwachen kationenaustauscher Eigenschaften wie zum Beispiel Carbonsäure- oder Sulfonsäuregruppen. Fractogel® EMD Epoxy (M) wurde als Trägermaterial ausgewählt da Ligandenmolküle mit Thiol- oder Aminogruppen leicht durch basisch katalysierte nucleophile Addition an die aktivierten Epoxydgruppen angehängt werden können. Von den getesteten Molekülen erwies sich 4-Mercaptobenzoesäure als einfach zu handhabender Ligand mit Immobilisierungsausbeuten von bis zu 0,950 mmol/g bezogen auf die zur Verfügung stehenden 1,000 mmol/g Epoxidgruppen von Fractogel® EMD.

Der analytische Teil dieser Diplomarbeit befaßte sich mit der IgG Bindungs-kapazitätsmessung dieser neue funktionalisierten Fractogele.
4-Mercaptobenzoesäure Fractogel, als vielversprechendster Kandidat, erreichte hohe Bindungskapazität von 52 mg IgG/g Gel, bei nahezu physiologischem Bedingungen von 25 mM Phosphat mit einem pH Wert von 6,5 und einer NaCl-Konzentration von 75 mM.
Zusätzlich zeigten die Bindungskapazitätsstudien unter den oben erwähnten Pufferbedingungen bei Gelen mit reduzierten Ligandendichten von 0,819 sowie

0,514 mmol/g Bindungskapazität von 41 bzw. 25 mg IgG/g gel und stehen somit in guter Korrelationen mit den gefundenden Ligandendichten.
Ein nächster möglicher Schritt wäre die Kombination von 4-Mercaptobenzoesäure mit (ein oder mehreren) anderen Molekülteilen oder Liganden zur Herstellung von sogenannten „mixed-mode“ stationären Phasen eröffnen.

Weiterführende Messungen von IgG Bindungskapazitätstudien mit Zellkultur-überstand zeigten daß 4-Mercaptobenzoesäure Fractogel auch bei Puffer-zusammensetzung von 25 mM Phosphat, einem pH Wert von 6,5 und einer NaCl Konzentration von 75 mM in der Lage war 31,4 mg IgG/g gel zu binden.
Außerdem konnte eine Verminderung der DNA Menge in den Elutionsfraktionen der dynamischen Bindungungskapazitätsstudie mithilfe einer angepassten fluoreszensspektroskopie Methode nachgewiesen werden. Der Hauptvorteil dieser angepaßten Methode war der reduzierte Verbrauch an Fluoreszenzreagenz. Die Messungen wurden in ultra-mikro fluoreszenz Küvetten mit 45 µL Messzellenvolumen durchgeführt wobei ein DNA-Probenvolumen von nur 10 µL benötigt wurde.
Unglücklicherweise bindet der 4-Mercaptobenzoesäureligand nicht ausschließlich IgG und SDS-PAGE Gele zeigten messbare Mengen von Wirtszellenprotein was aber, wegen der einfachen Struktur des Liganden, keine große Überraschung darstellt.
Aufgrund dieser ungenügenden Selektivität bezüglich IgG ist die Reinigung von Immunglobulin G direkt aus dem Zellkuturüberstand keine ideale Anwendung für das 4-Mercaptobenzoesäure Fraktogel.

8. Curriculum vitae

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1996 – 2011:	Studium der Chemie an der Universität Wien

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2002 – 2011:	Tutor bei verschiedenen chemischen Praktika für Biologie- und Chemiestudenten