Journal of Chromatography B, 878 (2010) 3382–3394

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Optimization of a ligand immobilization and azide group endcapping concept via "Click-Chemistry" for the preparation of adsorbents for antibody purification

Jeannie Horak*, Stefan Hofer, Wolfgang Lindner

Institute of Analytical Chemistry, University of Vienna, Waehringerstrasse 38, 1090-Vienna, Austria

ARTICLE INFO

Article history:
Received 11 August 2010
Accepted 23 October 2010
Available online 30 October 2010

Keywords:
Epoxide
Azide
Click reaction
Protein A
Size exclusion
Antibody purification
Protein aggregation
B14-ligand
Cell culture

ABSTRACT

This report describes and compares different strategies to deactivate (endcap) epoxide groups and azide groups on bio-chromatographic support surfaces, before and after ligand attachment. Adsorbents possessing epoxide groups were deactivated using acidic hydrolysis or were endcapped with 2-mercaptoethanol or 2-ethanolamine. The influence of surface-bound 2-ethanolamine was demonstrated for the triazine-type affinity adsorbent B14-2LP-FractoAIMs-1, which was tested in combination with the weak anion exchange material 3-aminoquinuclidine-FractoAIMs-3 (AQ-FA3). Azide groups were modified with 2-propargylalcohol using Click-Chemistry. Besides the conventional one-pot Click reaction, an alternative approach was introduced. This optimized Click protocol was employed (i) for the preparation of the weak anion exchange material AdQ-triazole-Fractogel (AdQ-TRZ-FG) and (ii) for the endcapping of residual azide groups with 3-propargyl alcohol. Using the new Click reaction protocol the ligand immobilization rate was doubled from 250 to 500 μmol/g dry adsorbent. Furthermore, the modified support surface was proven to be inert towards the binding of immunoglobulin G (IgG) as well as feed impurities. A thorough evaluation of modified surfaces and adsorbents was performed with dynamic binding experiments using cell culture supernatant containing monoclonal human immunoglobulin G (h-IgG-1). Besides SDS-Page, a recently introduced Protein A – size exclusion HPLC method (PSEC-HPLC) [1] was used to visualize the feed impurity composition and the IgG content of all collected sample fractions in simple PSEC-Plots. A surprising outcome of this study was the irreversible binding of IgG to azide modified surfaces. It was found that organic azide compounds, e.g. 1-azide-3-(2-propen-1-yloxy)-2-propanol (AGE-N3) promote antibody aggregation to a slightly higher extent than the inorganic sodium azide. The possibility that the Hofmeister Series of salt anions may be applicable to predict the properties of the corresponding organic compounds is discussed.

1. Introduction

In recent years, monoclonal antibodies, in particular human or humanized immunoglobulin G (IgGs) have emerged as one of the key products in biopharmaceutical industry. Monoclonal antibodies (Mabs) are expected to not only maintain their position in the market, but to even further expand and dominate the biopharmaceutical landscape in the years to come. The production of Mabs is indisputably a billion dollar business. There is a constant need to improve the process design for both, the use of improved cell lines with increased growth rates using optimized cell media compositions [2] or the possibility to introduce more efficient purification technologies in downstream processing. In turn, every new process developed must be critically analyzed to ensure financial viability of the process change.

In this context, inter alia, a problem remains, the loss of therapeutic activity caused by an association of proteins in their unfolded or partially unfolded state, which is especially critical if non-target proteins are incorporated [3]. The overall therapeutic activity of such conglomerates (complexes) is highly unpredictable and needless to say, their formation must be prevented. Another difficulty is that large aggregates are present in very small quantities of 0.3% or less. This makes their trace analytical detection rather difficult [4]. SDS-Page gel electrophoresis [5], field flow fractionation [5], turbidity [6] and size exclusion chromatography (SEC) combined with UV-detection, static light scattering detection (SLS) [7], dynamic light scattering detection (DLS) [4] and multi-angle laser light scattering (MALLS) detection [8,9] are frequently used techniques. The main factors for aggregate formation are the lowering of pH [10], heat [11,12] agitation [13], the addition or presence of certain preservatives or contaminants [14,13] and freezing as well as freeze-drying [15]. The lowering of the ion strength during ultra-/diafiltration in the last production steps seem to have a stronger effect on aggregate formation than sheer forces or a high

* Corresponding author. Tel.: +43 1 4277 52373; fax: +43 1 4277 9523.
E-mail address: Jeannie.Horak@univie.ac.at (J. Horak).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.10.025

local protein concentration [16]. With the increased productivity in upstream processing and a certain concentration dependency of aggregate formation, general interests are now shifted towards the production of tailor-made, aggregate-resistant antibodies to enable even higher production rates for Mabs [17].

Alternatively, it is possible to further improve the purification media to enable milder elution conditions, e.g. as it is the case for optimized Protein A media [18] or to modify the elution conditions, e.g. through addition of sodium chloride, ethylene glycol or urea [19].

Overall, the production costs for MABs as therapeutics become a key factor in downstream processing, resulting in the high demand to reduce these constraints. As a consequence the replacement of Protein A affinity materials with non-proteinageous, tailor-made affinity media for a specific drug or a group of drugs seem a promising alternative. Ligand library search based on "trial and error", and computer modeling studies are often used for small synthetic, bio-mimetic and peptide-mimetic affinity ligands [20–22]. Besides that, genetic engineering has proven to be a potential tool for the design of large protein-type ligands with pre-defined properties [23].

Although affinity ligands can be very selective in the capture of their target protein, the effect the underlying support media, the spacer chemistry and other support surface modifications may have on the overall material performance should not be neglected and should be reviewed critically. Extensive studies on the influence of spacer length and spacer chemistry have shown that a simple change in spacer-chemistry can come with a very profound change in the antibody capture properties of the A2P-ligand head group (a synthetic ligand designed for the capture of antibodies) [24–27]. Besides thiophilic and amino-spacer chains also a new immobilization concept, suitable for biological and bio-active affinity ligands [28,29] was employed, the so-called "Click-Chemistry". This copper (I) mediated 1,3-dipolar Huisgen cycloaddition reaction involves the coupling of an alkyne and an azide moiety, terminal on the ligand or the support surface. The result is the formation of a [1,2,3]-triazole linkage within the spacer chain [30]. Although advertised as easy going and straight-forward, still little is known about the precise mechanism of the coupling reaction [31–33]. A reaction scheme of a proposed mechanism is shown in Fig. 1. Recent studies show that Click-Chemistry is strongly dependant on the chemical properties of the reagents and the reactants used [34]. In case of solid phase reactions, the local environment surrounding the reacting groups, which includes the ligand itself may influence the yield of the Click-reaction strongly. Note that this immobilization concept was already applied for a variety of different chromatographic supports such as agarose [35,36], polymeric beads [37] and silica gel [38]. However, little to no information was provided concerning the possible reasons for the low immobilization rates.

The purpose of the presented study was to provide a better understanding about how and how strong the underlying surface modification can influence the performance of the final affinity adsorbent for the capture of h-IgG1. In order to unfold the contribution of a chosen endcapping strategy, it was necessary to investigate the modified support surfaces in the absence of the actual ligand. Only in the second step, an affinity-type ligand was introduced. It is important to mention that all modified support surfaces were tested with the same batch of cell culture feed to mimic near-real process conditions. This approach allowed a precise comparison of material performance. All test samples were measured with the in-house developed Protein A – size exclusion high performance liquid chromatographic (PSEC-HPLC) method [1] as well as SDS-Page gel electrophoresis using silver staining. In the final stage, possible reasons will be provided why azide-modified support surfaces bind IgG and how this problem was overcome using an improved Click Chemistry approach. The applicability of our optimized Click protocol was demonstrated for azide-group endcapping on support surfaces as well as affinity-ligand attachment.

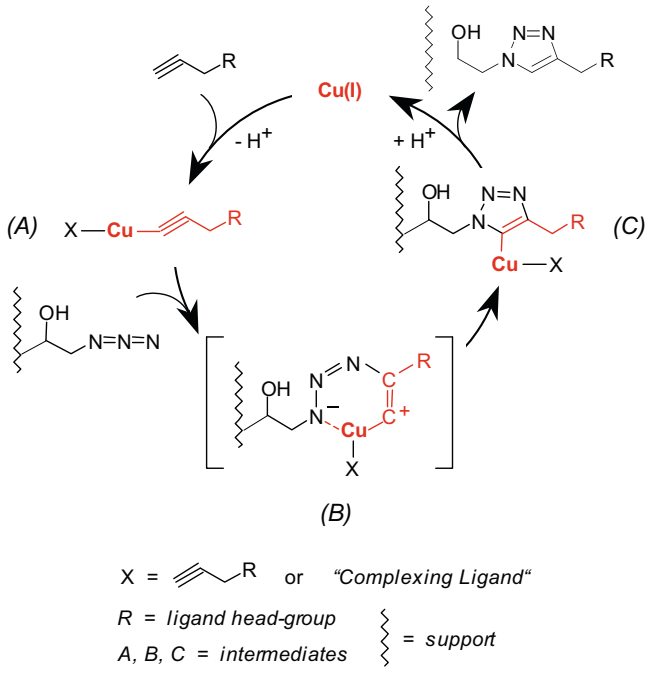

Fig. 1. Proposed mechanism for the Cu(I)-mediated 1,3-dipolar Huisgen cycloaddition reaction.

2. Experimental

2.1. Chemicals and materials

The chemicals 1-(2-propenyloxy)-2,3-epoxy propane (AGE), 1-azabicyclo [2.2.2] octan-3-amine dihydrochloride (AQ × 2 HCl), dicyclohexylcarbo diimide (DCC), ethylenediaminetetraacetic acid (EDTA), 4-pentynoic acid, 2-ethanolamine, 2-mercaptoethanol, 3-propargylalcohol, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium azide, sodium chloride (NaCl), sodium hydroxide, N-ethyl-diisopropylamine (DIPEA), triethylamine (TEA), citric acid (CA), hydrochloric acid and Amberlite IRA-402 were purchased from Sigma–Aldrich (Vienna, Austria).

Throughout this study only in-house bi-distilled water was used. Water, buffer solutions and cell culture feed were filtered through a 0.22 μm cellulose acetate membrane filter from Sartorius, purchased through Wagner&Munz (Vienna, Austria). Note that 10 mM phosphate buffer saline (PBS) solutions with increasing NaCl content of 0 mM, 75 mM, 150 mM and 300 mM will be denominated as PBS-O, PBS-75, PBS-150 and PBS-300 throughout this article.

Polyclonal human immunoglobulin G, Gammanorm (h-IgG, 165 mg/mL) was from Octapharma (Germany). Cell culture supernatant containing approximately 50 mg/L human monoclonal IgG1 (h-IgG1) from CHO-expression systems with a pH of 7.5 and a conductivity of 17 mS/cm at 33 °C was obtained from ExcellGene (Monthey, Valais, Switzerland). Isoelectric focusing (IEF) of Protein A purified monoclonal antibodies (MAb) from clarified cell culture broth was done in collaboration with Merck KGaA (Darmstadt, Germany). The IEF showed that the MAb consisted of an undeterminable number of variants with pIs between 7.5 and 9.3. Sample dialysis was performed with Spectra/Pore® CE Float-A-Lyzer® G2 with 10 mL volume size and a molecular-weight-cut-off (MWCO) of 3.5–5 kDa from Spectrum Europe B.V. (Breda, Netherlands) For further information see Fig. S2 [39].

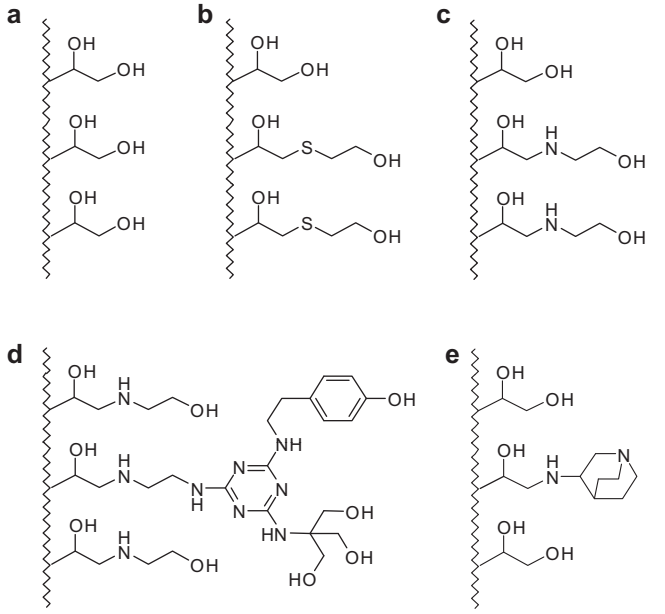

Fig. 2. Endcapping strategies for epoxide-activated Fractogel supports via (a) acid catalysed epoxide ring opening, (b) with 2-mercaptoethanol and (c) with 2-ethanolamine; (d) the IgG capture material B14-2LP-FA1, which comprises a triazine-core B14-ligand with a 1,3-diaminoethane spacer (2LP) bound to the non-commercial FractoAIMs-1 (FA1) support from Merck KGaA and is endcapped with 2-ethanolamine; (e) the weak anion exchanger 2-aminoquinuclidine-FractoAIMs-3 (AQ-FA3).

The Protein A HPLC Cartridge (PA ID Sensor Cartridge) from Applied Biosystems (Brunn am Gebirge, Austria) contained 0.8 mL of rigid polymeric POROS beads with an average particle size of 20 μm. It also possessed large flow-through pores and smaller diffusion pores with pore sizes ranging from 5000 to 10,000 Å. The overall accessible surface area was stated as 1 m^2/cartridge by the manufacturer. The silica-based TSKgel G3000SWXL column with 7.8 mm ID × 30.0 cm length (particle size: 5 μm) and the TSKgel SWXL guard column with 6.0 mm ID × 4.0 cm length were from Tosoh Bioscience (Stuttgart, Germany).

FractoAIMs®-1 (FA1) and FractoAIMs®-3 (FA3) were epoxy activated (~0.93 and 1.38 mmol/g (dry) gel), polymethacrylate-based, tentacle-grafted support media, designed by Merck KGaA (Darmstadt, Germany) for the EU-Project AIMs (Advanced Interactive Materials by Design). The spherical beads had a mean particle size of 40 μm with tuned pore-size and pore-size distribution. Note that FractoAIMs®-3 was obtained as a slurry in 2-propanol. The commercially available support, Fractogel® EMD Epoxy (M) from Merck KGaA (Darmstadt, Germany) had particle sizes of 40–90 μm, a pore size of about 80 nm and epoxide coverage of 1000 μmol/g dry gel.

The affinity sorbent B14-2LP-FA1 was equipped with a novel triazine-type B14-ligand that was linked with a 1,2-diaminoethane spacer to FractoAIMs-1 and was surface-endcapped with 2-ethanolamine. Note that B14-2LP-FA1 (Fig. 2d) is a non-commercial adsorbent that was provided by ProMetic BioSciences Ltd.™ (Cambridge, UK). Due to propriety rights owned by ProMetic BioSciences Ltd.™ the exact protocol for the preparation of B14-2LP-FA1 cannot be disclosed. The preparation of adsorbents with similar structure was described elsewhere [27].

2.2. Equipment and method description

2.2.1. Bio-chromatography

For dynamic binding experiments 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. All measurements were performed at 280 nm. Column switching was performed manually using a 2-position/6-port switching valve from Rheodyne. The application of biological sample was performed with a Minipuls 3 peristaltic pump and polyvinylchloride (PVC) calibrated peristaltic tubing with 1.02 mm ID, both from Gilson (Villiers-le-Bel, France).

For preparative bio-chromatography, XK 16/20 columns with two AK 16 adapters and C10/20 columns with two AC 10 adapters from GE-Healthcare (Vienna, Austria) were used. The former was used for anion-exchange type adsorbents, while affinity-type adsorbents were packed in the latter. Both column-types were employed in combination with Superformance Filter F with 16 mm ID or 10 mm ID from Götec-Labortechnik GmbH (Mühltal, Germany).

Further details concerning the experimental set-up and column packing can be found in our recently published article [1].

2.2.2. Online Protein A and size exclusion high performance liquid chromatography (PSEC-HPLC)

Following the previously described analytical method [1], all collected sample fractions were analyzed on an Agilent 1100 series LC system equipped with a binary pump, a multi-wavelength detector and a 2-position/6-port switching valve from Agilent (Vienna, Austria). The isolation and quantification of IgG was performed on a Protein A column, which was coupled in series with the SEC column in order to distribute the feed impurities by their molecular weight (MW) using 10 mM phosphate buffer with 150 mM NaCl, pH 7.20 (application buffer; PBS-150). The 2-position/6-port switching valve allowed the separate elution of IgG from the Protein A column using 12 mM HCl with 150 mM NaCl, pH 2–3 (elution buffer). If not otherwise stated, 100 μL sample volumes were applied and measurements were performed at 280 nm. The sample tray thermostat was set to 10 °C and the column thermostat was adjusted to 25 °C.

Establishment of PSEC-diagrams. In order to visualize and compare the experimental results of several PSEC-HPLC runs, the size-exclusion chromatograms of cell culture feed samples were sliced into molecular weight areas of interest; area sections A (660–150 kDa; ▲), B (150–17 kDa; ●), C (17–0.15 kDa; Δ), D (<0.15 kDa; ○) and E (<0.1 kDa plus adsorbed impurities (ads. imp.); ж). The Protein A section of the PSEC chromatogram provides the amount of bound or eluted h-IgG1 (150 kDa; ■). The percent peak area ratios of these sample chromatogram sections are calculated relative to the corresponding peak area sections of the application feed and plotted against subsequently collected material test fractions (10 mL). The amount of IgG and feed impurities in the application feed is thereby stated as 100% for each section. Since the SEC area sections are normalized by the amount of adsorbent used (1 mL for affinity adsorbents and 2.5 mL for anion exchange adsorbents), the feed-flow through a bio-chromatographic column is described as [mL feed per mL gel].

2.2.3. SDS-Page gel electrophoresis

The Mini-PROTEAN® 3 Cell with a Mini-PROTEAN 3 Cell/PowerPac 300 System (220/240 V) and 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 [40]. If not otherwise stated, 10%-Tris–HCl gels with 10 sample wells and 0.75 mm thickness were prepared. Samples were mixed in a 1:1 ratio with Laemmli buffer (10% 2-mercaptoethanol) and incubated for 5 min at 90 °C. In general, 15 μL sample and 3 μL molecular weight marker were applied. Protein staining was performed with the ProteoSilver™ Plus Silver Stain Kit (PROT-SIL2) from Sigma (Vienna, Austria) [1,41].

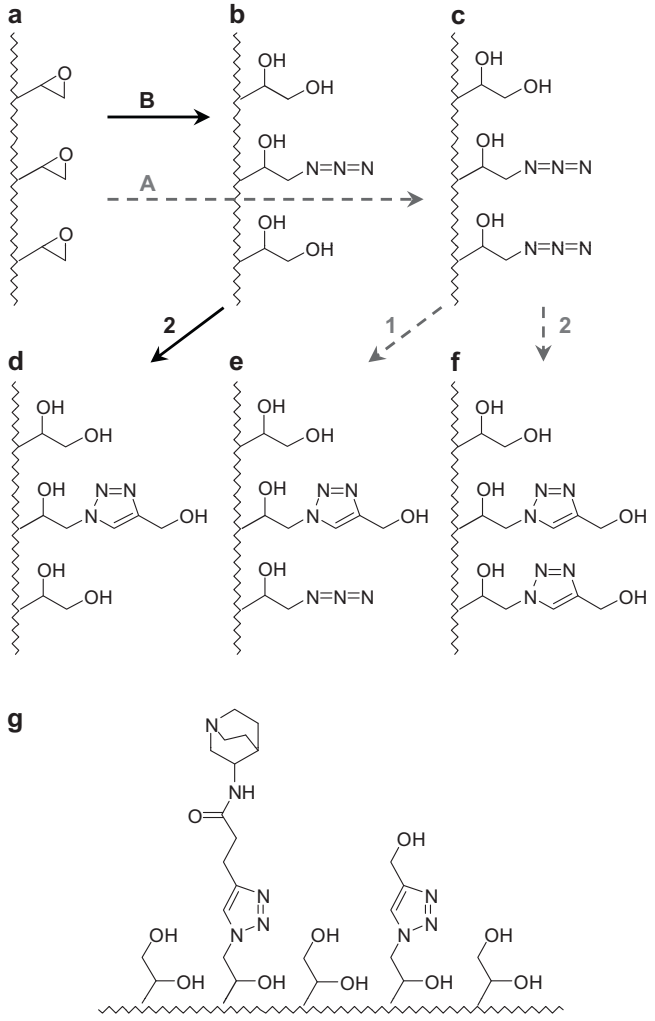

Fig. 3. Modification of (a) Epoxy-FractoAIMs-3 (FA3) with varying amount of sodium azide (A and B) leading to (b) FA3-N_3 (B, 750 μmol/g) and (c) FA3-N_3 (A, 1.38 mmol/g) and the endcapping of azide-groups with 3-propargylalcohol employing 2 different Click-reaction protocols (1 and 2), leading to the materials (d) FA3-TRZ-CH_2-OH (B2), (e) FA3-TRZ-CH_2-OH (A1) and (f) FA3-TRZ-CH_2-OH (A2). Material (g) AdQ-TRZ-Fractogel (B2; AdQ-TRZ: 500 μmol/g) was prepared on Azide-Fractogel (B, 676 μmol/g) employing Click protocol 2.

2.3. Preparation of materials

The synthesis of ligands, surface modifications of adsorbents and the preparation of affinity-type adsorbents (Figs. 2 and 3) are described in the electronic supplementary materials.

2.3.1. Click-protocol-1

To a suspension of 3 mL of FA3-N_3 (A) material (1 mol-equiv. azide groups: 1.38 mmol/g dry gel) in methanol, 4 mol-equiv. of 3-propargyl alcohol was added together with 3 mol-equiv. DIPEA and followed by the addition of 5–7 mol-equiv. CuI(I) in acetonitrile. The suspension was shaken on an orbital shaker for 3 days at room temperature. The final gel was washed successively with water, 10 mM HCl, 0.5 M NaOH, 0.1 M EDTA and methanol until the final material was completely white. FA3-triazole-CH_2-OH (A-1) was stored in 20% (v/v) aqueous methanolic solution until use (Fig. 3e).

Note that all solvents and solutions were ultrasonicated for several minutes before use and that the Click-reaction was performed under nitrogen to prevent an early oxidation of Cu(I) to Cu(II).

2.3.2. Click-protocol-2

Copper (I) was prepared through addition of sodium L-ascorbate (2 mol-equiv.) to an aqueous $CuSO_4$ solution (2 mol-equiv.), under streaming nitrogen. After addition of 3-propargyl alcohol (3 mol-equiv.) at room temperature, the bright yellow Cu(I)–propyne complex was mixed to the azide-modified support (azide groups: 1 mol-equiv.). The reaction slurry was covered with nitrogen and shaken for 3 days at room temperature on an orbital shaker. The addition of methanol and a few mL of brine enhanced the solubility of the alkyne–Cu(I) complex. Note that the Cu(I)–propyne alcohol complex precipitates, but will eventually dissolve as the Click reaction proceeds. The azide modified gel was washed with 10 mM HCl, 0.1 M EDTA, water and methanol until white in color (Fig. 3d and f).

2.4. Evaluation of material performance

2.4.1. Dynamic break-through experiments

For all dynamic binding capacity (DBC) experiments, the adsorbents were suspended in 1 M sodium chloride and left to settle in a graduated 5 mL volumetric cylinder. In general, 2.5 mL of test media were packed into 16 mm ID columns using Superformance filter discs to separate the gel from the column adapter. Only for the affinity material B14-2LP-FA1 a smaller gel amount of 1 mL adsorbent were used in combination with the 10 mm ID columns. After settling of the adsorbent by gravity, a bed height of 1 cm was adjusted employing a compression factor of 20%. The affinity adsorbent was equilibrated with application buffer (PBS-75, pH 7.20), while the anion exchange materials were rinsed with 0.1 M HCl prior to equilibration with the application buffer. In general, 10 mL sample volumes were collected. All citric acid (CA), hydrochloric acid (HCl) and cleaning-in-place (CIP) fractions were immediately neutralized with 5 M NaOH or 1 M CA solutions.

2.4.2. DBC for the endcapped epoxide and azide-activated adsorbents

After the dead volume (T0) of the column has elapsed, 10× 10 mL fractions of flow-through feed were collected. All columns were rinsed with 3× 10 mL of salt free PBS-0 buffer, followed by 1× 10 mL of PBS-75, PBS-150 and PBS-300, all at pH 7.2. IgG elution was performed with 2× 10 mL of 50 mM citric acid (CA, pH 3.50) and 1× 10 mL 0.1 M HCl solution. Before sanitation of the adsorbent (cleaning-in-place, CIP) with 2× 10 mL 0.5 M sodium hydroxide solution, the column was rinsed with PBS-0 until a neutral pH was reached.

2.4.3. DBC for B14-2LP-FA1/AQ-FA3 combination

After collecting 25 × 10 mL cell culture flow-through fractions, B14-2LP-FA1 was rinsed with PBS-0 until base line. IgG was eluted with 50 mM citric acid, pH 3.50. Before sanitation of the adsorbent with 0.5 M NaOH the column was thoroughly rinsed with PBS-0. The elution fractions were dialyzed for 24 h using CE Float-A-Lyzer® G2 membrane tubes and PBS-75 as dialysis buffer [39]. The pooled dialysis samples were applied onto the anion exchange adsorbent AQ-FA3 in flow-though mode without fraction collection. Bound feed impurities were eluted with PBS-300 from AQ-FA3.

2.4.4. DBC for AQ-FA3/B14-2LP-FA1 combination

The 200 mL cell culture feed were dialyzed twice for 24 h using PBS-75. The dialyzed feed was applied onto the anion exchange adsorbent AQ-FA3 in flow-though mode, before application onto B14-2LP-FA1. Fraction collection, IgG elution and column sanitation were performed as earlier mentioned.

2.4.5. Investigations on azide group promoted protein aggregation

The protein stock solutions A to G contained each 5 mg/mL IgG in PBS-75, pH 7.20. They were spiked with an increasing amount (1, 5, 10, 25, 50, 75 and 100 mg/mL) of the azide compounds, sodium azide or 1-azido-3-(2-propen-7-yloxy)-2-propanol (AGE-N_3). Note that the same molar quantities of sodium azide as well as AGE-N_3 were employed. All samples were shaken on a Thermomixer compact from Eppendorf (Vienna, Austria) at 25 °C and 1400 rpm for 3 h and 24 h, centrifuged and their supernatants measured with Protein A HPLC (injection volume: 10 μL).

Since shear forces as well as heat were reported to promote protein aggregation, three control samples without the addition of azide compounds, each containing only 5 mg/mL IgG were tested. One was stored motionless at 25 °C (control sample) and the two other samples were shaken at 25 °C (sample S) and 60 °C (sample H).

3. Results and discussion

3.1. Epoxide-activated support materials

In order to allow a fast and effective immobilization of affinity ligands, the adsorbent surface had to be sufficiently activated and hydrophilized. The polymethacrylate type support matrices used in this study have tentacle grafted surfaces. These tentacles increase the overall surface area and function as an extension of the actual spacer-chain. Since the epoxide groups on the tentacles as well as within the surface pores of the adsorbent are highly reactive, they have to be deactivated or endcapped after ligand immobilization. An example for the deactivation of reactive groups is shown in Fig. 2a for the acidic hydrolysis of epoxide groups. The endcapping of the epoxide group with 2-mercaptoethanol and 2-ethanolamine is visualized in Fig. 2b and c. Both concepts, the coupling of thiophilic ligands as well as amino-functional ligands onto solid supports are state of the art and therefore well discussed in literature [42–44]. A comprehensive study dealing with the influence of spacer-chain chemistry and support chemistry on the IgG capture performance of the bio-mimetic ligand A2P from a chromatographic as well as a theoretical thermodynamic viewpoint was recently published [27].

In order to unfold the contribution of these surface modifications to the overall performance of affinity adsorbents, it was necessary to investigate these surface modifications unimpaired by the affinity ligand. Only after that the affinity ligand B14 was introduced. The B14-2LP-FA1 adsorbent (Fig. 2d) with its 1,2-diaminoethane spacer arm plus a surface endcapping with 2-ethanolamine is admittedly an extreme example to demonstrate how strongly spacer-chemistry and surface-chemistry can influence material performance, but it also shows how such difficulties can be overcome during down-stream processing.

3.1.1. Endcapping strategies for epoxy-activated supports

For the control experiments Fig. 3 visualizes and compares the performance of the three most common end-capping strategies for epoxy activated support media, namely acidic hydrolysis (FA3-OH), the addition of 2-mercaptoethanol (FA3-S-$(CH_2)_2$-OH) and the addition of 2-ethanolamine (FA3-NH-$(CH_2)_2$-OH). The binding of IgG and feed impurities to the surface modified adsorbents during feed application (Fig. 4a) as well as their elution from the adsorbents during a number of wash, elution and sanitation steps (Fig. 4b) are displayed in Protein-A-Size-Exclusion-Chromatographic plots (PSEC-plots) [1].

These PSEC-plots show clearly that the simple incorporation of hydroxyl groups through acidic hydrolysis (FA3-OH) lead to a support surface, which is practically inert towards the binding of feed impurities as well as h-IgG1. Only a small negligible amount of feed impurity E binds to FA3-OH. Note that these SEC area section E impurities consist partially of low-MW-impurities below 0.1 kDa and partially of large MW-impurities that retain on the surface of the silica-based SEC-column (E: <0.1 kDa + ads. imp). The corresponding SDS-Page slab gel for FA3-OH in Fig. 4c shows that this support modification elute feed impurities with 25 kDa under salt-free condition (lane 2; W2) as well as when 75 mM NaCl (PBS-75; lane 3) is added. PBS buffer containing 150 mM NaCl (PBS-150; lane 4) removes the major part of the feed impurities with MW above 50 kDa. The elution of bound IgG (bands 25 kDa and 50 kDa) is seen in lane 4 and lane 5. The latter resembles the wash step with PBS-buffer containing 300 mM NaCl (PBS-300). The actual elution fractions CA1 and CA2 are completely clear. Only a slight smear of feed impurities and a small amount of IgG can be seen in the cleaning in place fraction S1 in lane 8.

The PSEC-plot for material FA3-S-$(CH_2)_2$-OH in Fig. 4 shows that this type of surface modification captures approximately 20% of feed impurities in the range of 150–17 kDa (area section B) during feed application. The corresponding elution diagram provide the information that also a small amount of feed impurity E has bound to the adsorbent and can easily be removed under salt-free condition (PBS-0; W1, W2 and W3) as well as with PBS-75 and PBS-150. The absence of impurity B in the elution diagram may indicate that it binds strongly to the adsorbent and cannot easily be eluted. Another possibility is that it has decomposed under the acidic elution condition, which would explain the protein smear over the entire lane 6 (C1) on the corresponding slab gel. It is also visible that FA3-S-$(CH_2)_2$-OH binds a small amount of IgG (lane 6). The elution of IgG is promoted by salt as well as by lowering of the pH (lane 6). IgG is found in the sample fractions PBS-75, PBS-150, PBS-300 and C1, with distinct bands for fractions PBS-150 and CA1.

Undoubtedly, adsorbent FA3-NH-$(CH_2)_2$-OH exhibits the strongest capture performance for feed impurities as well as for IgG. This surface modification binds about 80–90% of the high-MW feed impurities A (≥150 kDa), 40% of B (150–17 kDa) and 10% of E (<0.1 kDa plus ads. imp.), but also 10% of IgG (Fig. 4a). Taking a look at the protein composition of the elution fractions, it is clear from Fig. 4b that the few amino groups on the material surface can actually capture feed impurity A from 6 flow-through fractions (>600 area%) and feed impurity B from about 1.5 flow-through fractions. In accordance with the slab gel results, it is apparent that the largest portion of the bound feed impurities can only be removed from this adsorbent during sanitation (S1 and S2). It is also evident that a large amount of feed impurities elute together with IgG into the C1 fraction. The elution diagram shows that the salt wash steps PBS-75 and PBS-150 lead mainly to the elution of IgG and feed impurity E. The high MW impurities A and B start to elute with PBS-300. Since most IgG is found in fraction PBS-150 and CA1, but only little in PBS-300, it is most likely that IgG was bound by two different mechanisms to the adsorbents. The underlying hydroxyl groups, present due to acidic hydrolysis of residual epoxy groups after immobilization of 2-ethanolamine resemble the first discussed FA3-OH support. As previously stated, this surface modification tends to bind a small amount of IgG. This weakly bound IgG elutes as soon as salt is added to the wash solution. The 2-ethanolamine modification, on the other hand, binds IgG strongly and needs a pH switch towards lower pH (C1) or higher pH (S1 and S2) values in order to release IgG as well as feed impurities from its embrace.

Obviously adsorbent FA3-NH-$(CH_2)_2$-OH functions as an anion exchanger (AIEX), which makes any affinity-type adsorbent, that was endcapped with 2-ethanolamine or which possessed a spacer chain with embedded amino groups, a mixed-mode material with partial AIEX-properties. The most suitable endcapping strategy for

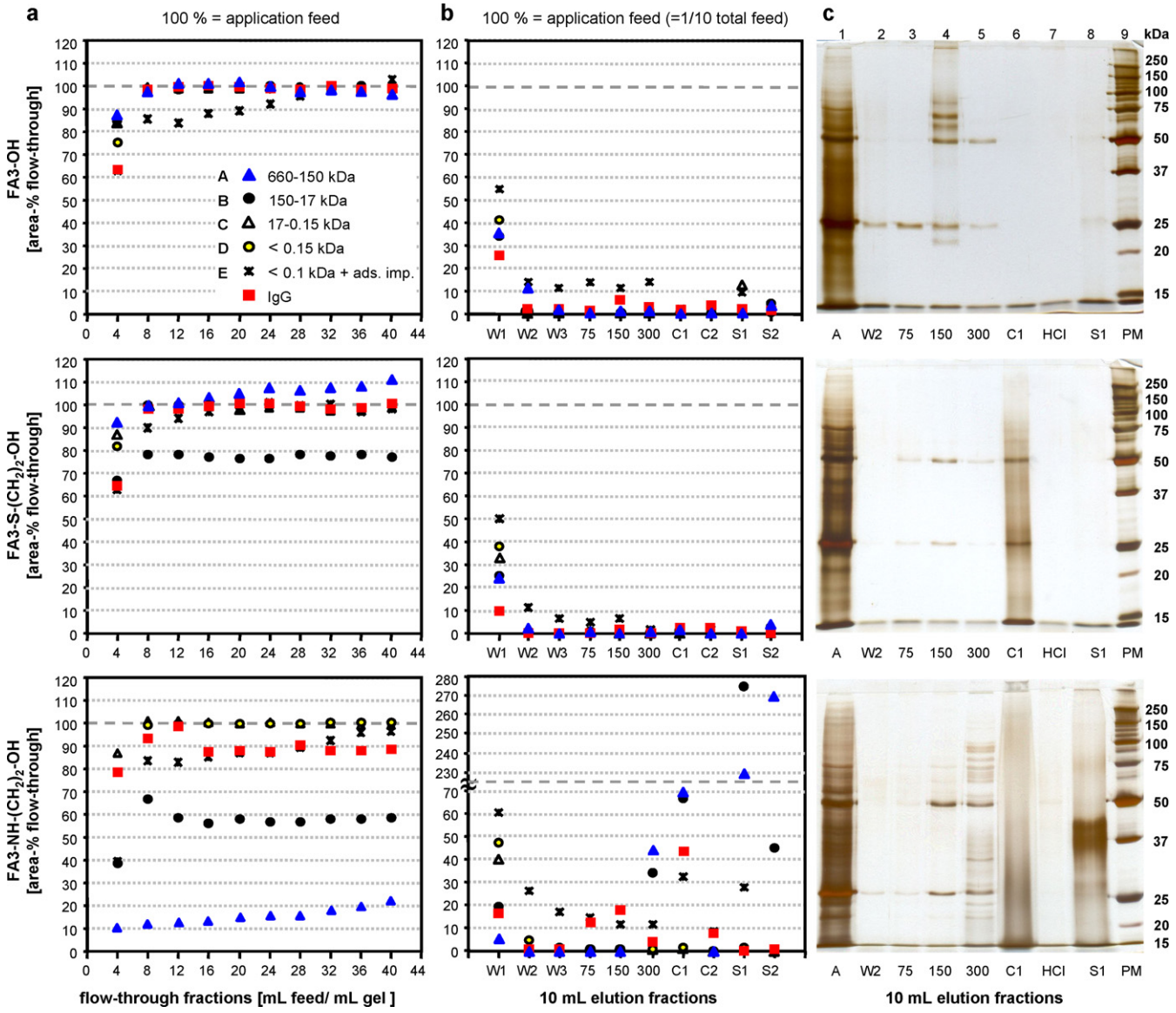

Fig. 4. Performance evaluation of FractoAIMs-3 Epoxy materials after acidic hydrolysis (FA3-OH) and after endcapping of epoxide groups with 2-mercaptoethanol (FA3-S-$(CH_2)_2$-OH) and 2-ethanolamine (FA3-NH-$(CH_2)_2$-OH), employing cell culture supernatant at pH 7.4 for testing. Sample composition were visualized with (a) PSEC-diagram for collected flow-through fractions, (b) PSEC-diagram for wash and elution fractions and (c) SDS-PAGE slab gels under reduced conditions; A: application feed, W2: wash solution (salt-free), 75: PBS with 75 mM NaCl, 150: PBS with 150 mM NaCl, 300: PBS with 300 mM NaCl, C1: 50 mM citric acid, pH 3.50, HCl: 0.1 M HCl, S1: 0.5 M NaOH; IgG light chain: 25 kDa, IgG heavy chain: 50 kDa.

epoxy-activated support materials was clearly the epoxy ring opening under acidic condition.

The reason for the sometimes observed deviation between chromatographically established PSEC-diagrams and SDS-Page results may lie on one hand on the large background signal of the neutralized citric acid (C) and sanitation (S) solutions (Fig. 4b), which were subtracted from the actual sample signal of the SEC run. On the other hand, silver staining is a very effective and sensitive staining technique, with which traces of proteins down to the lower nano-gram level can be visualized. Nonetheless, the color intensities of the silver-stained protein bands do not necessarily correlate with the actual concentration of proteins in solution. Therefore, the actual quantity of protein impurities in some of the slab gel lanes may just as well be too small to be chromatographically detectable.

3.1.2. Performance evaluation of B14-2LP-FA1

The following chapter describes two antibody purification strategies from cell culture supernatant. Both protocols include the affinity-type IgG-capture media B14-2LP-FA1 (Fig. 2d), the anion exchanger adsorbent AQ-FA3 (Fig. 2e) and a dialysis step prior to the AQ-FA3 clean-up. The main difference between the two protocols is the reversed order in which the two adsorbents are employed in the purification scheme.

Fig. 5a shows the SDS-Page gel and SEC results for the purification sequence using B14-2LP-FA1 in the first step, which is the IgG capture step. Lane 3 on the SDS-Page gel shows that the elution fraction (B14) contains predominantly IgG (short chain 25 kD and heavy chain 50 kDa) with only a slight smear of feed impurities across the lane. Distinct bands of feed impurities can be seen at 60, 90, 200 and 300 kDa, of which the 60 kDa band is the most pronounced one. The highly intense CIP lane of B14-2LP-FA1 in Fig. 5a proves that the underlying anion exchanger lattice of B14-2LP-FA1 leads to a very strong binding of feed impurities. It also proves that feed impurities that bind to B14-2LP-FA1, remain on the adsorbent and do not elute with IgG into the citric acid elution fraction (pH 3.50). Furthermore, Lane 6 (AX) shows that the

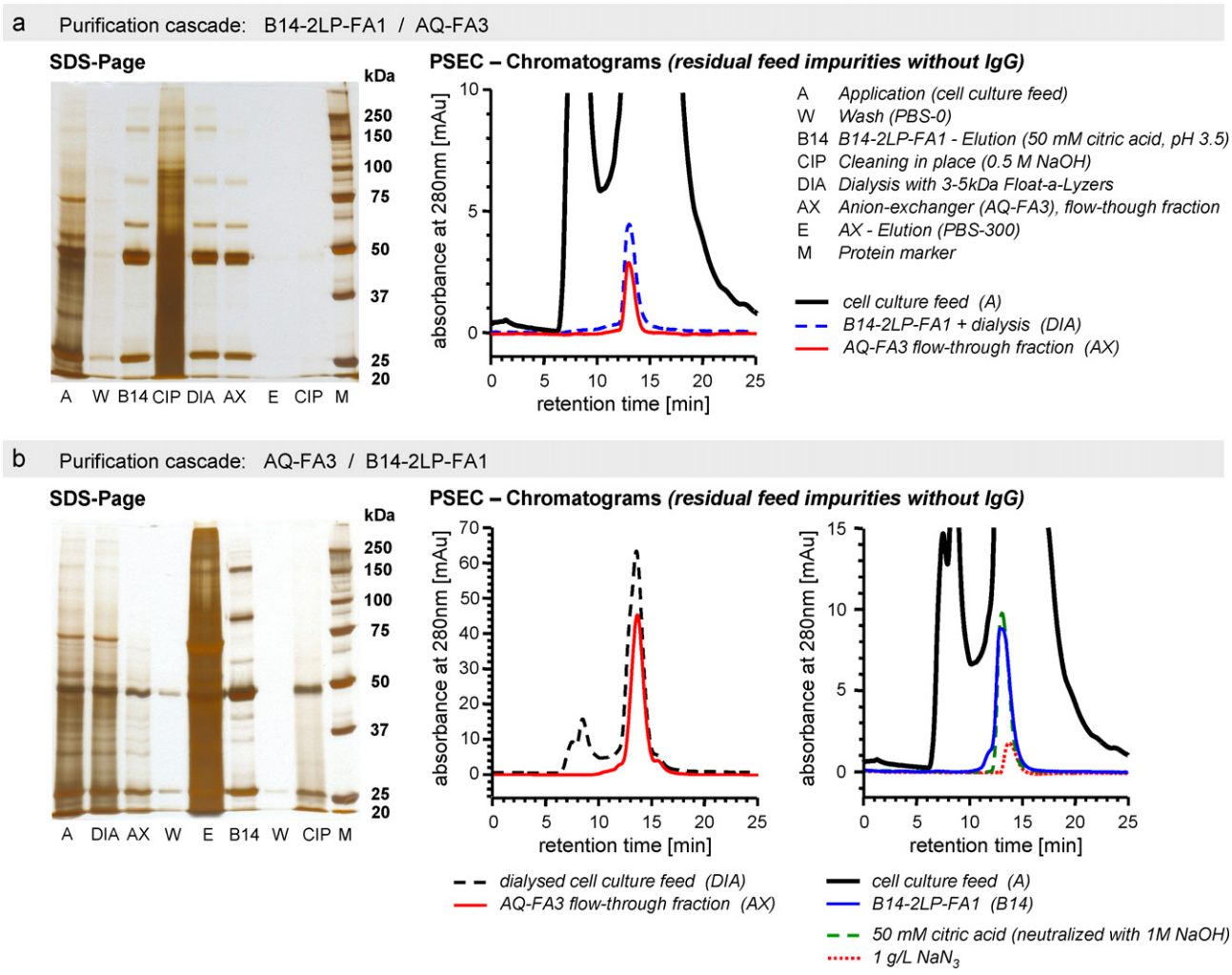

Fig. 5. Material performance evaluation via SDS-Page and PSEC-HPLC employing two different purification strategies for the isolation of monoclonal h-IgG1 from cell culture feed using the weak anion exchange adsorbent AQ-FA3 and the IgG capture media B14-2LP-FA1 in the order (a) B14-2LP-FA1/AQ-FA3 and in the reversed order (b) AQ-FA3/B14-2LP-FA1.

use of an anion exchange media in the final polishing step does not markedly improve the purity of the final IgG solution. Only the feed impurity band at 300 kDa and the slight smear across the lane can be removed. The impurity band 200 kDa is slightly reduced. However, although the impurities at 60 kDa and 90 kDa still remain in solution, the AQ-FA3 media could remove 19% of the feed impurities that have eluted from B14-2LP-FA1. The PSEC chromatogram of the elution fraction of B14-2LP-FA1 provides evidence that B14-

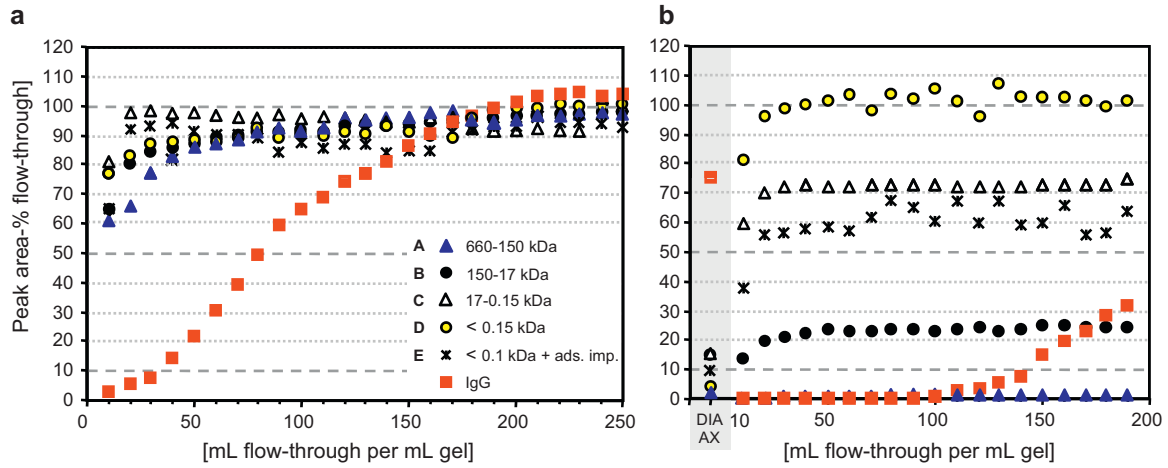

Fig. 6. PSEC-diagrams showing the dynamic break-through performance of B14-2LP-FA1 in the purification schemes (a) B14-2LP-FA1/AQ-FA3 and (b) AQ-FA3/B14-2LP-FA1, where DIA stands for dialysis (MWCO: 3–5 kDa) and AX for the pre-purification step using AQ-FA3.

2LP-FA1 can capture only 14% of the total amount of applied IgG, but can remove 99.88% of applied feed impurities.

It can be assumed that with the progressing capture of feed impurities, the binding capacity of B14-2LP-FA1 for IgG is gradually decreasing, due to the sterical hindrance exhibited by the large amount of impurities competing with the low concentration of IgG in the feed. In order to verify this hypothesis, B14-2LP-FA1 was tested with a "pre-purified" feed that was dialyzed and purified with AQ-FA3.

A comparison of the two SDS-Page gels in Fig. 5a and b shows that the two experiments lead to reciprocal results. As intended, the intense elution lane of AQ-FA3 (E) in Fig. 5b proves that AQ-FA3 can remove most of the feed impurities, before the feed enters the B14-2LP-FA1 column. The elution lane of B14-2LP-FA1 (B14) shows a slight smear across the lane with additional impurity bands at 30 kDa and 40 kDa. There are impurity bands at 60, 90 and 200 kDa, of which the latter two are most distinct. The presence of IgG (25 kDa and 50 kDa) in the CIP-fraction of B14-2LP-FA1 is most likely caused by a too short elution time due to a slight peak tailing. An evaluation of the PSEC-chromatograms for the AQ-FA3/B14-2LP-FA1 scheme lead to a total removal of 99.98% of feed impurities and a 5-fold increase in IgG-binding for the B14-2LP-FA1 adsorbent compared to the inverse purification scheme.

Fig. 6 shows the dynamic binding performance of B14-2LP-FA1, when employed in the first (Fig. 6a) and when applied in the second clean-up step (Fig. 6b). The left column in the PSEC-diagram in Fig. 6b visualizes the feed composition after dialysis and after clean-up with AQ-FA3. This feed composition was used as a reference for the corresponding PSEC-plot. Due to the lack of available pre-purified feed and the unexpected performance of B14-2LP-FA1, the feed application had to be terminated at 30%-DBC. A dynamic binding capacity of 3.7 g/L IgG at 30% column break-through was obtained. These two PSEC-plots show clearly that B14-2LP-FA1 has suffered the loss of IgG capture performance due to a seemingly unsuitable spacer chemistry and endcapping chemistry. But on the other hand the SDS-Page gels have shown that although B14-2LP-FA1 binds a large amount of feed impurities, which reduce its IgG binding capacity, these impurities do not elute with IgG. B14-2LP-FA1 can be classified as a mixed-mode adsorbent which isolates IgG and removes feed impurities in a single purification step.

3.2. Click-reaction for ligand immobilization on solid supports

3.2.1. Modified Click-reaction protocol

The so-called Click reaction is a cycloaddition reaction between azide and alkyne functional groups, leading to a [1,2,3]-triazole ring as the connecting link [30,45]. This reaction is known to be fast and straight forward; needing only a small catalytic amount of 1–5 mol% of copper (I) salt to promote the coupling reaction. Cu(I) can be directly employed using copper (I) iodide or using a $CuSO_4$/sodium ascorbate system, where Cu(I) is fashioned in aqueous solutions shortly before the coupling reaction is initiated [46].

The proposed mechanism for the Cu(I) mediated Click reaction is shown in Fig. 1. The formation of the intermediate A, the copper (I)–acetylide complex [33] and the intermediate C, the copper (I)–triazolide [32] are proven and documented. By this scheme Cu(I) exits this reaction cascade untempered and can enter the next reaction cycle as soon as the first intermediate has formed. However, the presence of oxygen in the solution may transform Cu(I) to its inactive Cu(II) counterpart, preventing its participation in the next cycle. Therefore, even for reactions employing CuI, the reducing agent sodium ascorbate has to be added to ensure that Cu(I) remains at all times in its reactive form.

Another difficulty that may occur is a possible complexation of the Cu(I) or Cu(II) ions by one of the reacting partners which includes the ligand head group and the solid support. The latter has occurred for the polymethacrylate-based supports FractoAIMs-3 and Fractogel. A possible remedy may be the addition of an "auxiliary or complexing ligand", which per se binds to Cu(I) but leaves one or more free sites to partake in the Click reaction. Such "complexing ligands" contain two or more sulfur or amino groups [47–49,32,50,37,51] that complexate with Cu(I) or Cu(II) [52]. Unfortunately, experiments with the "complexing ligand" bathophenanthroline disulfonate [49] had to be terminated due to irreversible binding of the reagent as well as the bathophenanthroline–Cu(I) complex to the Fractogel surface.

Another possibility to drive the cycloaddition reaction forward is to add more and more Cu(I) as the Click-reaction proceeds. However, this method leaves us with a large amount of surface-bound copper that has to be removed from the adsorbent after ligand immobilization. Plus, the surface bound copper provide a sterical hindrance, leading to much reduced immobilization rates.

The way out of this dilemma evolved in the implementation of a new procedure, which proved to be an applicable and simple alternative to the one-pot approach. Hereby, the alkyne modified ligand is combined and stirred with an equimolar amount of Cu(II) and sodium ascorbate until only the alkyne–copper complex, the so-called copper–acetylide is present in solution. This highly reactive intermediate is then added to the azide-modified polymethacrylate-type support under oxygen-free condition. With this new Click-procedure it is possible to double the ligand immobilization yield for AdQ-TRZ-Fractogel (B2) as well as produce fully azide group endcapped support surfaces after ligand immobilization (Fig. 3g).

Note that the complementary assembly, using alkyne-modified supports in combination with azide-terminal affinity ligands was not investigated, although literature had predicted that this reversed approach could provide much higher immobilization rates [37]. The choice to endow the ligand with the alkyne functionality was made deliberately. Cu(I) activated alkyne groups are very reactive and reportedly prone to unspecific binding of feed proteins [35]. Of course it may be possible to deactivate residual alkyne groups with azide-terminal endcapping reagents, but the reaction yield might not be sufficient and any remaining alkyne–Cu(I) complex is by far more reactive than idle azide-groups.

3.2.2. Endcapping strategies for azide group activated support surfaces

Fig. 7 shows the corresponding PSEC-diagrams and SDS-Page slab gels for the azide-modified FractoAIMs-3 support material (FA3-N_3) before and after azide-endcapping with 3-propargyl alcohol using two different endcapping protocols (Fig. 3; 1 and 2) for two different FA3-N_3 support materials (Fig. 3A and B). The two azide functional supports FA3-N_3 (A) and FA3-N_3 (B) possess azide-group densities of 1.38 mmol/g (A) and 750 μmol/g (B) dry gel. Note that the residual epoxide groups have been deactivated through acidic hydrolysis prior to any surface modification via Click-Chemistry. A description of the preparation of azide-group modified support media with tuned azide group density (Fig. S1) can be found in the electronic supplementary material.

Material FA3-N_3 (A) in Fig. 7a shows clearly that an azide-modified adsorbent without affinity ligand attached to its surface is on its own capable to capture a large amount of IgG. It binds about 86% of the applied h-IgG1 from the cell culture supernatant after 20% dynamic break through of IgG. Besides that FA3-N_3 (A) also binds mainly the feed impurities A (>150 kDa) and E (<0.1 kDa plus ads. imp.). Unexpectedly, the elution diagram in Fig. 7b and the SDS-gel in Fig. 7c of FA3-N_3 (A) show that only a small amount of "intact" IgG elute from the column. On the other hand, wash solutions W2, PBS-75 and PBS-150 (lanes 2–4) show an idle 25 kDa band, which may be light-chain IgG. This would indicate a degradation of IgG during clean-up. However, only the presence of both IgG polypep-

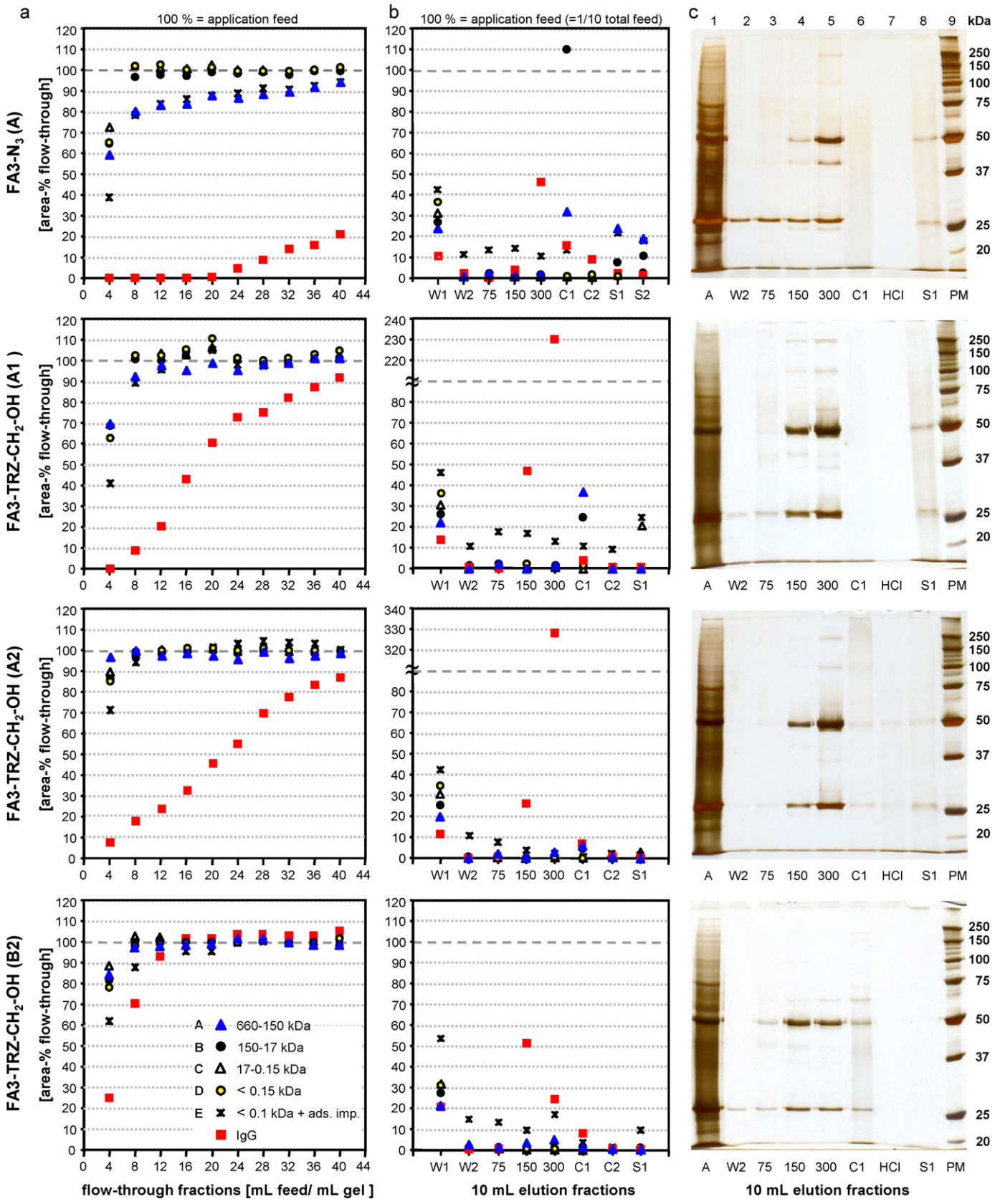

Fig. 7. Performance evaluation of Azido-FractoAIMs-3 support materials with and without azide group endcapping using 3-propargylalcohol, employing cell culture supernatant at pH 7.4 for testing. Sample composition were visualized with (a) PSEC-diagram for collected flow-through fractions, (b) PSEC-diagram for wash and elution fractions and (c) SDS-PAGE slab gel under reduced conditions; (A) application feed, W2: wash solution (salt-free), 75: PBS with 75 mM NaCl, 150: PBS with 150 mM NaCl, 300: PBS with 300 mM NaCl, C1: 50 mM citric acid, pH 3.50, HCl: 0.1 M HCl, S1: 0.5 M NaOH; IgG light chain: 25 kDa, IgG heavy chain: 50 kDa.

tide chains, the 25 kDa and the 50 kDa fragments within the same lane is a clear indicator for the presence of IgG. In the case that only one of the two characteristic peptide chains is present, this peptide may be a non-IgG related feed impurity or a degradation product of IgG. In the latter case, this IgG fragment may have been already present in the application feed or created during purification. In the latter case the "other" IgG fragment ought to posses other binding and elution properties.

To verify if an azide-modified support can actively break IgG molecules into their heavy and light chain, the former DBC-experiment was repeated with a standard IgG solution (100 mg/L human polyclonal IgG in PBS-75) in the absence of feed impurities. The absence of a lone 25 kDa lane in wash solution W2 plus the presence of both IgG fragments in the other wash and elution fractions prove that material FA3-N_3 (A) does not induce the fragmentation of IgG molecules (Fig. S3) [39]. Furthermore, it proves that the 25 kDa feed impurity was already present in the application feed.

The elution diagram of FA3-N_3 (A) in Fig. 7b shows that the elution of IgG is strongly salt dependant. IgG emerges strongest with PBS-300, while the low salt buffers PBS-75 or PBS-150 have almost no effect on IgG elution. A decreasing amount of IgG elutes with 50 mM citric acid, pH 3.50 (C1 and C2) and during sanitation (S1 and S2). On the SDS-Page gel, however only one distinct feed impurity band at about 40 kDa (lane 5) and a weak band at about 24 kDa are seen in the PBS-300 wash fraction. A slight smear of impurities over the entire lane is seen in the lanes PBS-75, PBS-150, PBS-300, C1 and S1. Possible reasons for the obviously low recovery of IgG will be discussed separately and in more detail in Section 3.2.4.

A comparison of the PSEC break-through diagrams for the two endcapped materials FA3-TRZ-CH_2-OH (A1) and FA3-TRZ-CH_2-OH (A2) in Fig. 7a show that both adsorbents are still endowed with the ability to capture IgG, but to a much smaller extent. The second endcapping strategy (B2) provides a support surface that binds little IgG and little to no feed impurities. Also in the PSEC elution diagrams in Fig. 7b the same tendency can be observed. While for FA3-TRZ-CH_2-OH (A1), feed impurity E can be found at about 10–20% in all collected fractions, impurity A and B are found with 37% and 25% only in the C1 elution fraction and impurity C only in S1. For material FA3-TRZ-CH_2-OH (A2) the elution PSEC diagram (Fig. 7b) shows only a slight elution of impurity E of about 3–12% in fraction W2 as well as in the three NaCl containing fractions. Only about 5% of feed impurity A is present in fraction C1. Concerning the elution behavior for IgG, both materials show the same tendency. With PBS-150 some of the bound IgG elute from the column, while the major amount elute with PBS-300. A comparison of SDS-gels show that FA3-TRZ-CH_2-OH (A2) does not bind feed impurities strongly, since the sanitation lane of the latter is clearer than that of FA3-TRZ-CH_2-OH (A1).

The main difference between the two surface modifications, FA3-TRZ-CH_2-OH (A2) and FA3-TRZ-CH_2-OH (B2) is the reduced azide group density of the latter. FA3-N_3 (B) possesses only 750 μmol/g azide groups, which is half the number of FA3-N_3 (A). Both adsorbents are endcapped with Click reaction protocol-2. The resulting endcapped support FA3-TRZ-CH_2-OH (B2) binds only little IgG, which mostly elutes with the salt wash step PBS-150, followed by PBS-300. Traces of IgG are found in the elution fraction C1. The sanitation solution is free of IgG and free of feed impurities. Overall it can be stated that a tuned number of azide groups, sufficient to achieve the desired ligand density, but not too densely set as to induce sterical hindrance during endcapping provides the best results, concerning IgG recovery and purity of the elution fractions.

3.2.3. Recovery of IgG for different endcapping strategies

A summary of bound and eluted IgG in Fig. 8, shows that 9% of applied IgG binds to FA3-NH-$(CH_2)_2$-OH during feed application,

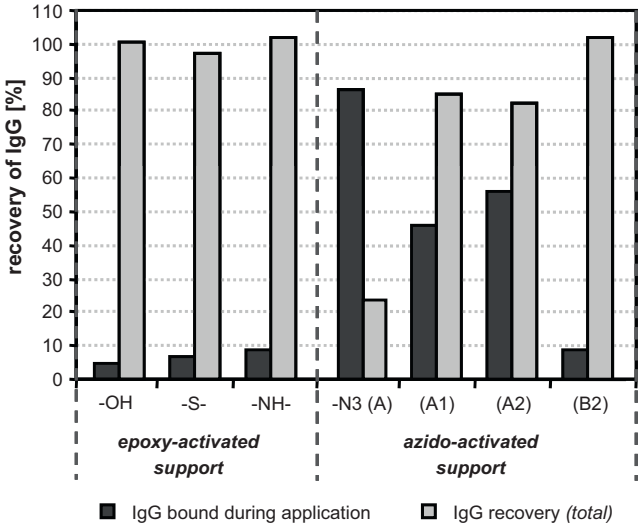

Fig. 8. Percentage of IgG, bound during application of cell culture supernatant containing h-IgG1 and the total recovery of IgG, which includes the wash, elution and sanitation steps for the epoxide-activated adsorbents FA3-OH (OH), FA3-S-$(CH_2)_2$-OH (–S–), FA3-NH-$(CH_2)_2$-OH (–NH–) and FA3-N_3 ($–N_3$) and the azide functionalized adsorbents FA3-TRZ-(CH_2)-OH (A1, A2 and B2).

while the simple surface modification FA3-OH captures only 5% IgG. For all three investigated surface modifications a total recovery of approximately 100% IgG can be obtained.

In case of the azide group activated supports and their endcapped versions, only material FA3-TRZ-CH_2-OH (B2) with the low azide group density using the Click protocol 2 provide comparable results, with 9% for the binding and 100% for the recovery of IgG. The non-endcapped support FA3-N_3 (A) with the high azide group density captures 86% of the applied IgG, but releases only 24% thereof. Comparing the two endcapped versions of FA3-N_3 (A), it is clear that FA3-TRZ-CH_2-OH (A1) binds approximately 10% less IgG, but only releases 3% more intact IgG compared to its counterpart. Nonetheless, Click protocol 2 is superior, because the resulting adsorbent FA3-TRZ-CH_2-OH (A2) binds less feed impurities, while capturing more IgG. Furthermore, it provides purer elution fractions as previously demonstrated in the PSEC diagrams and the SDS-Page gels in Fig. 7.

3.2.4. Azide group promoted aggregation of IgG

The big question that remains is what had happened to the IgG fraction, which was bound to FA3-N_3 (A), but was not even found in the final sanitation fractions? Is it still bound on the adsorbent or was it decomposed into smaller fragments?

In order to solve this dilemma, we will first take a look at the chemical properties of inorganic azide groups and then discus how organic surface bound azide groups may have interacted with IgG. In general, terminal azide groups are linear, polarized and possess an extended π-electron system that endows them with electron donor/acceptor properties with resonance structures quite similar to thiocyanate. From the smaller representative, the cyanate, we know that they adsorb antibodies via dipole–dipole interactions and can specifically interact with the sugar moieties within antibodies. But since this interaction is mostly used to immobilize antibodies onto surfaces for structural investigations such as scanning electron microscopy (SEM), little is unknown if these antibodies actually maintain their bioactivity after detachment [53,54].

The Hofmeister Series was created to describe the propensity of common salt anions and cations to increase the surface tension of a protein in solution and thus cause their precipitation. Nonetheless, we will suppose that surface bound anions

behave in a similar manner as their ionic analog. Consider that the position an anion partakes within the Hofmeister series corresponds to its degree of hydration in the following order: $SO_4^{2-} > HPO_4^{2-} > OH^- > F^- > HCOO^- > CH_3COO^- > Cl^- > Br^- > NO_3^- > I^- > SCN^- > ClO_4^-$ [55–57].

Furthermore, azide-anions posses similar free energy of hydration values [$-\Delta G_{hydr}$] and water absorbance properties [A_W] [58] as observed for SCN. Also an investigation on the electron-transfer properties of nucleophilic anions to 1-pyrenesulfonic acid radical cation (Py^+SA^-) in Nafion membranes provide similar attenuation factors [AF] for both, SCN and N3 for the difference in quenching within the membranes and in bulk solution [57].

[$-\Delta G_{hydr}$]: $SO_4 > Cl > Br > N_3 > SCN$ [58]
[A_W]: $SO_4 \gg Cl > Br > NO_2 > I > ClO_4 > SCN > N_3 > PO_4 > CrO_4$ [58]
[Cloud Point]: $H_2O > F > CH_3COO > Cl > Br > N_3 > NO_3$ [59]
[AF]: $SCN > N_3 > I > Cl$ [57]

Taking these reported properties of azide anions into account, it seems possible that they belong to the Group C of the Hofmeister Series, which comprises the non-hydrated anions [55]. It is also reported that chaotropic agents promote protein unfolding. They can promote conformational changes in the protein structure, which may induce the reduction or even complete loss of protein activity [60–62].

Considering that azide groups are poorly hydrated and rather hydrophobic in nature, it seems possible that they can alter or disrupt the water structure of proteins and enhance their own attraction for protein binding. This may explain the high binding capacity of IgG on azide-modified support media. If the surface bound azide groups incite protein unfolding and can actually promote the exposure of certain areas on the protein that are sensitive to inter-protein interactions then an excess of closely aligned azide groups may lead to a surface-induced aggregate formation of IgG. Such an aggregation would lead to high molecular weight (MW) entities of low concentration. Note that due to conformational changes of the antibody molecule, induced by a partial un-folding or association to higher MW conglomerates, a recognition and binding to Protein A may be hindered.

In order to verify this hypothesis, polyclonal h-IgG was incubated with sodium azide and with the organic azide compound, 1-azide-3-(2-propen-1-yloxy)-2-propanol (AGE-N_3) under various conditions.

Fig. 9a, shows clearly that IgG molecules are least sensitive to shear forces (S) provided by agitation than to heat (H), which is obviously the strongest aggregation inducer. Since all samples were agitated in the same manner, the value for sample (S) is practically the baseline value for all other test samples. It is also apparent that an increasing amount of sodium azide has no effect on IgG recovery as long as the incubation time is below 3 h. Once the contact time for IgG and sodium azide was increased to 24 h, the recovery rate for IgG decreases with increasing amount of sodium azide added to the solution. Surprisingly, this effect is more pronounced for the organic azide compound AGE-N_3 (Fig. 9b) than for sodium azide. Note that for AGE-N_3 the incubation time dependency is much reduced and differs only by 3–5%, with higher values for the long incubation times. Also less AGE-N_3 is needed to induce the same reduction of traceable IgG compared to sodium azide. While 100 g/L sodium azide (sample G) is necessary to provoke a 30% reduction of detectable IgG, only 25% of AGE-N_3 (sample D) is needed to obtain the same result. Another interesting feature is the visible appearance of the protein aggregates per se. The supernatants of the AGE-N_3 containing samples C and D are slightly milky and pinkish in color and their aggregates are thread-like in nature. In case of samples incubated with sodium azide, their precipitates are flocculent with a colorless clear to milky supernatant.

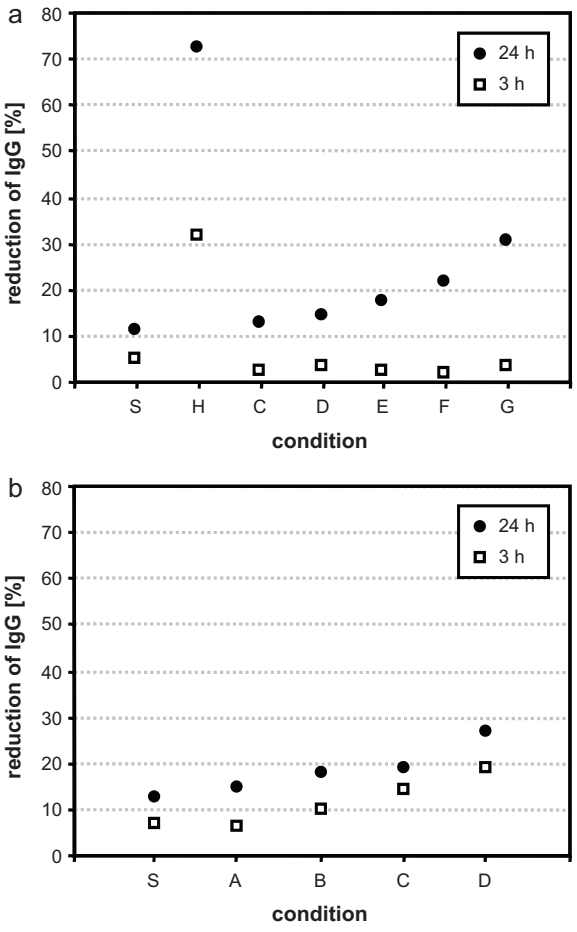

Fig. 9. Reduction of detectable IgG in solution after incubation of polyclonal h-IgG for 3 h and 24 h with solutions containing (a) NaN_3 and (b) AGE-N_3 and other aggregate forming conditions: S (azide-free, shear stress at 1400 rpm, 25 °C), H (azide-free, heat stress, 60 °C), and A to G resemble samples with addition of varying amount of NaN_3 or mol-equivalents of AGE-N_3 (1, 5, 10, 25, 50, 75, 100 g/L NaN_3) tested under otherwise same conditions at 25 °C and 1400 rpm. All samples were tested with Protein A HPLC.

The reason for the more pronounced protein aggregation effect of AGE-N_3 lies in its strong lipophilic nature, which makes it viable to interact stronger with hydrophobic patches on the surface of IgG molecules than its ionic counterpart. It can be assumed that sheer forces as well as the binding of IgG molecules to multiple attachment points on a support surface lead to a partial unfolding of their protein structure. The increased accessibility of hydrophobic areas on the protein will increase the interaction tendency between the IgG molecules as well as between IgG and the organic surface bound azide groups.

3.2.5. Performance of AdQ-triazole-Fractogel (AdQ-TRZ-FA3) with and without azide group endcapping

Click protocol 2 was used for the immobilization of the anion exchange ligand N-1-azabicyclo [2.2.2] oct-3-yl-4-pentynamide (AdQ-pentyne) onto an azide group modified Fractogel support as well as for the final endcapping of residual azide groups with 2-propargyl alcohol (Fig. 6g).

The reason for using Fractogel instead of the previously discussed FractoAIMs-3, is simply because the AdQ-pentyne ligand performs better in combination with Fractogel. This indicates that also the physical support properties such as pore size and pore size distribution play an important role in material performance. Since AdQ-TRZ-FA3 is a weak anion exchange material, the larger pore

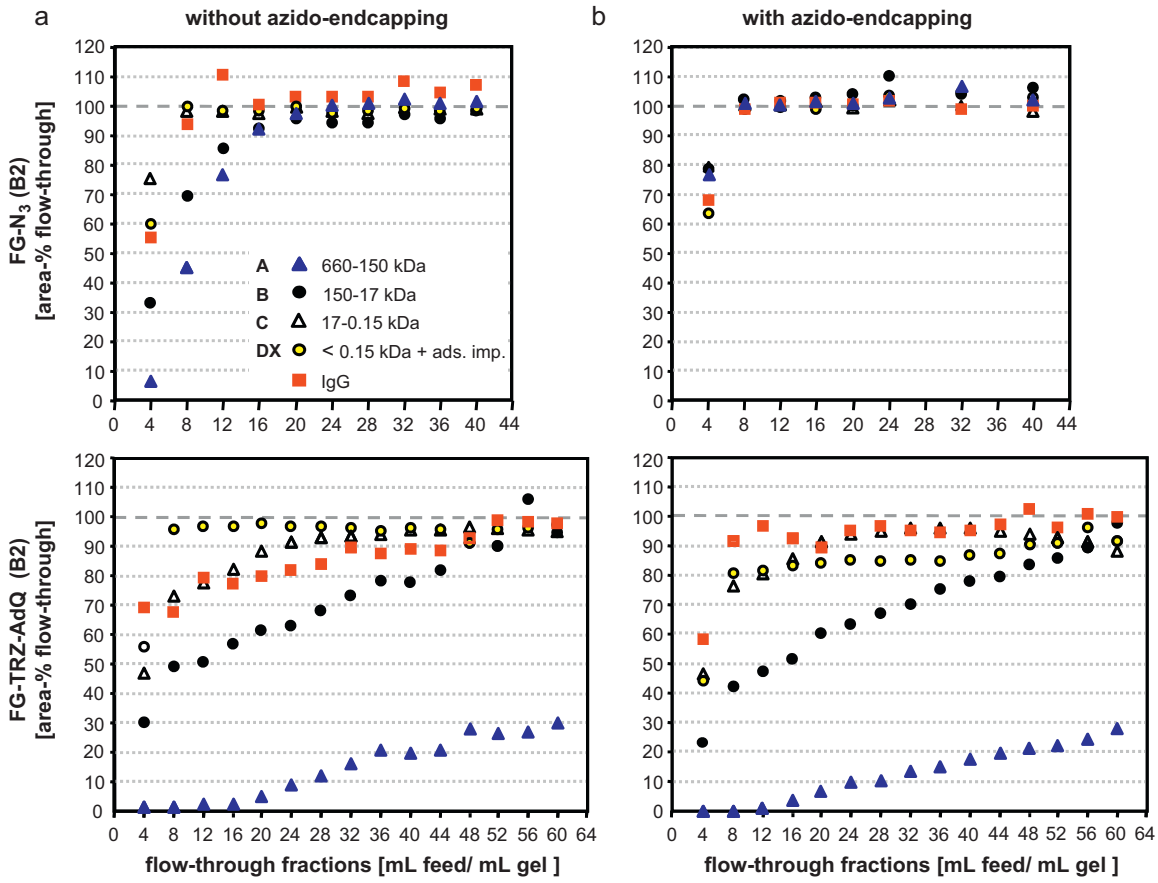

Fig. 10. PSEC-diagrams showing the dynamic break-through performance of (a) Azido-Fractogel material FG-N_3 (B2) and the modified adsorbent (b) FG-TRZ-AdQ (B2), both with and without azide group endcapping using the Azido-Fractogel with reduced azide group density (676 μmol/g) and Click-protocol 2 for the endcapping with 3-propargylalcohol.

size of Fractogel improves the binding performance of the adsorbents for the capture of feed impurities such as host cell proteins and DNA.

It is probable that the larger pore-size of Fractogel combined with the low azide group density of FG-N_3 (B2) facilitates the accessibility of azide groups for the Click-reaction. Note that with the conventional one-pot Click reaction (protocol 1) only a low AdQ-pentyne coverage of 250 μmol/g was obtained, while for protocol 2, 500 μmol/g could be achieved. Furthermore, the immobilization of the AdQ-pentyne ligand is very efficient, leaving only 176 μmol/g residual azide groups to be deactivated with 3-propargyl alcohol. The main difficulty observed during material preparation was the complexation of Cu(I) and Cu(I)–acetylide to the Fractogel support surface, which was significantly reduced with Click protocol 2.

The PSEC diagrams in Fig. 10 visualize the performance of FG-N_3 (B2) and AdQ-TRZ-FG (B2) before and after azide group endcapping using the same batch of cell culture feed as earlier employed for the preliminary investigations. While the non-endcapped Azido-Fractogel binds mainly high MW feed impurities A (660–150 kDa) and B (150–17 kDa) and little IgG, all feed impurities as well as IgG are not retained on the endcapped support. Comparing the two AdQ-TRZ-FG (B2) materials, it is apparent that the non-endcapped adsorbent binds more IgG and less of the low MW feed impurities than its azide group endcapped counterpart.

4. Conclusions

This study has show that any type of purification media will observe a natural loss in performance, once non-target molecules bind to the adsorbent. It does not appear to make a difference if they are small and large in number or large in molecular weight but present only in traces. In any case the undesired binding of non-target compounds induces also steric hindrance, blocking otherwise accessible binding sites for the target molecule.

The right choice of reagent or strategy for the endcapping or deactivation of the remaining reactive groups after ligand attachment can be crucial and difficult to make. For epoxide activated support surfaces a simple hydrolysis reaction, which leads to a fast and simple ring-opening seemed best suitable. The hydroxylated support surface is inert towards the capture of feed impurities as well as IgG. However, the introduction of 2-ethanolamine onto the surface for an affinity-type adsorbent such as B14-2LP-FA1 seems at first sight unwise. But at a second glance the so-modified adsorbent resembles a mixed-mode material, which combines the removal of feed impurities with the capture and release of practically pure Mab.

For azide-modified support surfaces, we have learned that even an optimized Click-reaction protocol for ligand attachment and azide group endcapping may not be sufficient to obtain inert support surfaces. Here we could show that a too close alignment of reactive groups can resemble for their neighboring groups a sterical hindrance in the final endcapping reaction after ligand immobilization. Another advantage of our modified Click approach, which is based on the off-line preparation of the copper (I)-acetylide complex lies in the tendency of polymethacrylate type support media to capture copper (I). Once bound to the surface, copper (I) is no more part of the reaction cycle and provide a profound hindrance for further immobilization reactions. Furthermore, we have discovered that inorganic as well as organic surface-bound azide groups do not only bind IgG but are also capable to induce aggregate for-

mation. However, with an optimized immobilization strategy for ligand attachment and surface endcapping via Click Chemistry, the modified adsorbent only exhibits the desired properties of the attached ligand without interference from the underlying support chemistry.

Acknowledgments

This study was performed within the EU-Project AIMs (Advanced Interactive Materials by Design; NMP3-CT-2004-500160), which is part of the 6th Framework Program of the European Union. The authors would like to thank ProMetic BioScience Ltd. for allowing us to evaluate their non-commercial affinity adsorbent, B14-2LP-FA1, Chris Sadler for making the B14 ligand and Sharon Williams for valuable discussions throughout the AIMs Project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.10.025.

References

[1] J. Horak, A. Ronacher, W. Lindner, J. Chromatogr. A 1217 (2010) 5092.
[2] P. Decaria, A. Smith, W. Whitford, BioProcess Int. 7 (2009) 44.
[3] J.P. Lopez-Bote, C. Langa, P. Lastres, C. Rius, A. Marquet, R. Ramos-Ruiz, C. Bernabeu, Scand. J. Immunol. 37 (1993) 593.
[4] K. Ahrer, A. Buchacher, G. Iberer, D. Josic, A. Jungbauer, J. Chromatogr. A 1009 (2003) 89.
[5] H.-C. Mahler, W. Friess, U. Grauschopf, S. Kiese, J. Pharm. Sci. 98 (2009) 2909.
[6] H.-C. Mahler, R. Mueller, W. Friess, A. Delille, S. Matheus, Eur. J. Pharm. Biopharm. 59 (2005) 407.
[7] K. Ahrer, A. Buchacher, G. Iberer, A. Jungbauer, J. Chromatogr. A 1043 (2004) 41.
[8] A. Hawe, W. Friess, M. Sutter, W. Jiskoot, Anal. Biochem. 378 (2008) 115.
[9] T. Wang, J.A. Lucey, J. Dairy Sci. 86 (2003) 3090.
[10] J.D. Lewis, R.T.C. Ju, A.I. Kim, S.L. Nail, J. Colloid Interface Sci. 196 (1997) 170.
[11] D.A. McCarthy, A.F. Drake, Mol. Immunol. 26 (1989) 875.
[12] I. Oreskes, D. Mandel, Anal. Biochem. 134 (1983) 199.
[13] R. Thirumangalathu, S. Krishnan, M.S. Ricci, D.N. Brems, T.W. Randolph, J.F. Carpenter, J. Pharm. Sci. 98 (2009) 3167.
[14] M. Garcia, M. Monge, G. Leon, S. Lizano, E. Segura, G. Solano, G. Rojas, J.M. Gutierrez, Biologicals 30 (2002) 143.
[15] J.-M. Sarciaux, S. Mansour, M.J. Hageman, S.L. Nail, J. Pharm. Sci. 88 (1999) 1354.
[16] K. Ahrer, A. Buchacher, G. Iberer, A. Jungbauer, J. Membr. Sci. 274 (2006) 108.
[17] L. Jespers, O. Schon, K. Famm, G. Winter, Nat. Biotechnol. 22 (2004) 1161.
[18] S. Gulich, M. Uhlen, S. Hober, J. Biotechnol. 76 (2000) 233.
[19] A.A. Shukla, P.J. Hinckley, P. Gupta, Y. Yigzaw, B. Hubbard, BioProcess Int. 3 (2005) 36.
[20] S. Kabir, Immunol. Invest. 31 (2002) 263.
[21] R. Li, V. Dowd, D.J. Stewart, S.J. Burton, C.R. Lowe, Nat. Biotechnol. 16 (1998) 190.
[22] A.C.A. Roque, M.A. Taipa, C.R. Lowe, J. Chromatogr. A 1064 (2005) 157.
[23] S. Hober, K. Nord, M. Linhult, J. Chromatogr. B 848 (2007) 40.
[24] V. Busini, D. Moiani, D. Moscatelli, L. Zamolo, C. Cavallotti, J. Phys. Chem. B 110 (2006) 23564.
[25] A.R. Newcombe, C. Cresswell, S. Davies, K. Watson, G. Harris, K. O'Donovan, R. Francis, J. Chromatogr. B 814 (2005) 209.
[26] L. Zamolo, V. Busini, D. Moiani, D. Moscatelli, C. Cavallotti, Biotechnol. Prog. 24 (2008) 527.
[27] L. Zamolo, M. Salvalaglio, C. Cavallotti, S. Hofer, J. Horak, W. Lindner, B. Galarza, C. Sadler, S. Williams, J. Phys. Chem. B 114 (2010) 9367.
[28] W.H. Binder, C. Kluger, Curr. Org. Chem. 10 (2006) 1791.
[29] W.H. Binder, R. Sachsenhofer, Macromol. Rapid Commun. 28 (2007) 15.
[30] H.C. Kolb, M.G. Finn, K.B. Sharpless, Angew. Chem. Int. Ed. 40 (2001) 2004.
[31] V. Aucagne, J. Berna, J.D. Crowley, S.M. Goldup, K.D. Haenni, D.A. Leigh, P.J. Lusby, V.E. Ronaldson, A.M.Z. Slawin, A. Viterisi, D.B. Walker, J. Am. Chem. Soc. 129 (2007) 11950.
[32] C. Nolte, P. Mayer, B.F. Straub, Angew. Chem. Int. Ed. 46 (2007) 2101.
[33] V.O. Rodionov, V.V. Fokin, M.G. Finn, Angew. Chem. Int. Ed. 44 (2005) 2210.
[34] Y. Angell, K. Burgess, Angew. Chem. Int. Ed. 46 (2007) 3649.
[35] B.P. Duckworth, J. Xu, T.A. Taton, A. Guo, M.D. Distefano, Bioconjugate Chem. 17 (2006) 967.
[36] S. Punna, E. Kaltgrad, M.G. Finn, Bioconjugate Chem. 16 (2005) 1536.
[37] M. Slater, M. Snauko, F. Svec, J.M.J. Frechet, Anal. Chem. 78 (2006) 4969.
[38] M. Ortega-Munoz, J. Lopez-Jaramillo, F. Hernandez-Mateo, F. Santoyo-Gonzalez, Adv. Synth. Catal. 348 (2006) 2410.
[39] Electronic Supplementary Material.
[40] B.-R.L. Instruction Manual for Mini-PROTEAN® 3 Cell, Inc., Available from: http://www.plant.uoguelph.ca/research/homepages/raizada/Equipment/RaizadaWeb%20Equipment%20PDFs/9B.%20miniprotean3%20cell%20manual.pdf.
[41] S.-A. Technical Bulletin for ProteoSilver™ Plus Silver Stain Kit (PROTSIL2), Inc., Available from: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/protsil2bul.Par.0001.File.tmp/protsil2bul.pdf.
[42] J. Hardouin, M. Duchateau, L. Canelle, C. Vlieghe, R. Joubert-Caron, M. Caron, J. Chromatogr. B 845 (2007) 226.
[43] J. Porath, T.W. Hutchens, Int. J. Quantum Chem. 14 (1987) 297.
[44] W. Schwartz, D. Judd, M. Wysocki, L. Guerrier, E. Birck-Wilson, E. Boschetti, J. Chromatogr. A 908 (2001) 251.
[45] W.D. Sharpless, P. Wu, T.V. Hansen, J.G. Lindberg, J. Chem. Educ. 82 (2005) 1833.
[46] K.M. Kacprzak, N.M. Maier, W. Lindner, Tetrahedron Lett. 47 (2006) 8721.
[47] T.R. Chan, R. Hilgraf, K.B. Sharpless, V.V. Fokin, Org. Lett. 6 (2004) 2853.
[48] W.G. Lewis, F.G. Magallon, V.V. Fokin, M.G. Finn, J. Am. Chem. Soc. 126 (2004) 9152.
[49] J.D. Megiatto Jr., D.I. Schuster, J. Am. Chem. Soc. 130 (2008) 12872.
[50] V.O. Rodionov, S.I. Presolski, D. Diaz Diaz, V.V. Fokin, M.G. Finn, J. Am. Chem. Soc. 129 (2007) 12705.
[51] A.E. Speers, G.C. Adam, B.F. Cravatt, J. Am. Chem. Soc. 125 (2003) 4686.
[52] E.A. Ambundo, Q. Yu, L.A. Ochrymowycz, D.B. Rorabacher, Inorg. Chem. 42 (2003) 5267.
[53] P.E. Mazeran, J.L. Loubet, C. Martelet, A. Theretz, Ultramicroscopy 60 (1995) 33.
[54] G. Zhou, L. Veron, A. Elaissari, T. Delair, C. Pichot, Polym. Int. 53 (2004) 603.
[55] E. Leontidis, Curr. Opin. Colloid Interface Sci. 7 (2002) 81.
[56] L.A. Munishkina, J. Henriques, V.N. Uversky, A.L. Fink, Biochemistry 43 (2004) 3289.
[57] T. Tachikawa, R. Ramaraj, M. Fujitsuka, T. Majima, J. Phys. Chem. B 109 (2005) 3381.
[58] P. Lo Nostro, L. Fratoni, B.W. Ninham, P. Baglioni, Biomacromolecules 3 (2002) 1217.
[59] M. Lagi, P. Lo Nostro, E. Fratini, B.W. Ninham, P. Baglioni, J. Phys. Chem. B 111 (2007) 589.
[60] S.-M. Choi, C.-Y. Ma, J. Agric. Food Chem. 53 (2005) 8046.
[61] S. Oscarsson, J. Chromatogr. B 666 (1995) 21.
[62] K.B. Song, S. Damodaran, Han'guk Saenghwa Hakhoechi 25 (1992) 393.