Journal of Chromatography B, 879 (2011) 1633–1640

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Influence of different spacer arms on Mimetic Ligand™ A2P and B14 membranes for human IgG purification

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ARTICLE INFO

Article history:
Received 15 February 2011
Accepted 31 March 2011
Available online 8 April 2011

Keywords:
Antibody purification
A2P
B14
Click-Chemistry
Azide-functionalized membrane
Affinity membranes

ABSTRACT

Microporous membranes are an attractive alternative to circumvent the typical drawbacks associated to bead-based chromatography. In particular, the present work intends to evaluate different affinity membranes for antibody capture, to be used as an alternative to Protein A resins. To this aim, two Mimetic Ligands™ A2P and B14, were coupled onto different epoxide and azide group activated membrane supports using different spacer arms and immobilization chemistries. The spacer chemistries investigated were 1,2-diaminoethane (2LP), 3,6-dioxa-1,8-octanedithiol (DES) and [1,2,3] triazole (TRZ). These new mimetic membrane materials were investigated by static and by dynamic binding capacity studies, using pure polyclonal human immunoglobulin G (IgG) solutions as well as a real cell culture supernatant containing monoclonal IgG_1. The best results were obtained by combining the new B14 ligand with a TRZ-spacer and an improved Epoxy 2 membrane support material. The new B14-TRZ-Epoxy 2 membrane adsorbent provided binding capacities of approximately 3.1 mg/mL, besides (i) a good selectivity towards IgG, (ii) high IgG recoveries of above 90%, (iii) a high Pluronic-F68 tolerance and (iv) no B14-ligand leakage under harsh cleaning-in-place conditions (0.6 M sodium hydroxide). Furthermore, foreseeable improvements in binding capacity will promote the implementation of membrane adsorbers in antibody manufacturing.

1. Introduction

The demand and the importance of biopharmaceuticals is constantly growing and antibiotics such as penicillin and cephalosporin, hormones (insulin), blood factors (Factor VIII, Factor IX) and interferons (α,β,γ) [1,2] belong to the most important drugs on the pharmaceutical market. In recent years, monoclonal antibodies (MAb) gained much importance. The reasons for their increasing dominance are their applicability against a multitude of diseases such as Alzheimer's syndrome and cancer [3] combined with a high approval rate by governmental institutions [4]. Presently, more than 18 antibodies have already been approved in the United States, and more than 150 antibody-based therapeutics are under development or undergoing clinical trials [5,6].

In addition, the production yields of MAbs have significantly improved and it is reasonable to expect titers in the order of 10 g/L in the near future [7]. Despite such improvements in upstream process development, the overall production costs are not reduced, since the real bottleneck in antibody manufacture lies in downstream processing, where purification costs may exceed 60% of the total production expenses [8]. The origin of that is directly related to stringent quality demands defined by purity, efficacy, stability, immunogenicity and several further aspects, which are clearly specified by regulatory agencies [9].

The most important step in downstream processing is the capture of MAbs from the cell culture supernatant, for which up to date Protein A-based resin adsorbents are the process platform of choice. Reason for their superior status is their high selectivity and high binding capacity for immunoglobulin G (IgG). Despite all benefits that Protein A adsorbents may offer, they also suffer from certain drawbacks such as high pressure drops, flow rate limitations and soft gel compression, which are typical for agarose-based separation techniques [10]. Furthermore, the rather high IgG molecular weight of 150 kDa entails additional restrictions due to the slow diffusion of the antibody molecules into the dead end pores of the adsorbent particles [11].

A promising alternative is represented by membrane adsorbers, which exhibit several advantages with respect to classical

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doi:10.1016/j.jchromb.2011.03.059

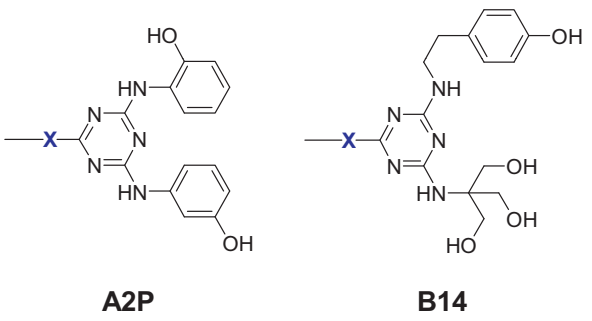

Fig. 1. Chemical structure of the Mimetic Ligands™ A2P and B14, which are linked with group X (X = NH or S) to the corresponding spacer chain.

packed-bed chromatography. Membranes have almost no pressure drop restrictions due to their superior flow properties and, in addition, diffusion mass transfer resistance is not important, since the main transport mechanism involved is convection [12].

For these reasons, membrane-based purification techniques are gaining increased interest in industrial applications. The use of anion exchange membranes in polishing steps of antibody manufacturing is well established, especially since they do not suffer from capacity limitations when operated in a flow through mode for trace impurity removal [13–15]. More recently, membrane adsorbers used in protein capture mode, such as cation exchange type membranes [16–18] and affinity based Protein-A membranes [10,19], were considered for IgG purification at production scale. Further improvements of membrane properties lead to a new Protein A based membrane from Sartorius Stedim Biotech, which possesses 12-times higher binding capacities for IgG compared to the former Sartobind Protein A affinity membrane [20].

However, Protein A is a large bacterial protein, which is rather sensitive towards harsh sanitation conditions and detached ligand has to be removed in additional polishing steps. Therefore, smaller and more stable ligands that can mimic the antibody binding region of Protein A represent an attractive alternative. Such synthetic ligands are inter alia the peptido-mimetic ligand D-PAM [21], the bio-mimetic triazine derived A2P ligand [22], and peptide sequences [23]. While D-PAM was created by screening of peptide libraries [24,25], A2P is the product of the combination of computational chemistry and a combinatorial ligand library approach, where the two phenolic substituents of the triazine core are designed to capture polyclonal IgG.

On the other hand, the newly developed B14-ligand from ProMetic BioSciences Ltd. was created to specifically capture human monoclonal IgG_1. B14 was found through a diverse and rationally designed combinatorial library approach, and possesses the same triazine-core as A2P, but with a different set of substituents (Fig. 1). A recent study describes the IgG capture performance of B14-ligand bound via triazole linkage (TRZ) [26,27] to a new polymethacrylate based adsorbent (FractoAIMs-3). It was shown that B14 is suitable for IgG_1 capture at low bed heights and high flow-rates from cell culture feed [28], which can be considered being ideal properties for a corresponding membrane adsorber. Furthermore, molecular dynamic simulations performed on different A2P-type affinity media showed that the bio-specific ligand is not the only factor which influences the antibody capture performance of affinity media [29,30]. It was found that spacer chain chemistry, immobilization chemistry and support chemistry may contribute in positive or negative ways. Therefore the present study includes a small variation of different ligand head groups (A2P and B14), spacer chains (2LP, DES and TRZ) and membrane supports, including two new cellulose-based membranes with improved pore structure and surface properties (Epoxy 2 and Epoxy 3). Membrane performance evaluation was performed with polyclonal IgG standard solutions as well as cell culture supernatant containing IgG_1, employing in the first instance static binding experiments and dynamic binding tests for the most promising membrane adsorbents.

2. Experimental

2.1. Materials and methods

2.1.1. Support media

Four different epoxide-activated regenerated cellulose membranes, namely Sartobind Epoxy™ and the new Epoxy 1, Epoxy 2 and Epoxy 3 membranes, were kindly provided by Sartorius Stedim Biotech GmbH (Goettingen, Germany). They differ in their physical and chemical parameters such as pore size, void volume, thickness and epoxide group density, as summarized in Table 1 and the latter in Table S1 of the electronic supplementary material. Sartobind Epoxy membranes are commercially available and have an average pore size of 0.45 μm and an epoxide group density between 2.0 and 2.2 μmol/cm^2, according to manufacturer's specifications. The Epoxy 1 and Epoxy 2 membranes have similar physical characteristics, but different epoxide group densities of 0.3–0.4 μmol/cm^2 for Epoxy 1 and ranging between 1.2 and 1.8 μmol/cm^2 for Epoxy 2 membranes. The Epoxy 3 material possesses not only the highest epoxide density, between 2.7 and 3.2 μmol/cm^2, but also the greatest thickness. Its large average pore size of more than 3 μm is mirrored by a very high permeability. For convenience, Sartobind Epoxy membranes will be denominated as Sartoepoxy throughout this paper.

2.1.2. Feed solutions

Polyclonal human immunoglobulin G, Gammanorm (165 mg/mL) from Octapharma (Stockholm, Sweden), was used as IgG source and diluted to the appropriate concentration with 0.1 M Phosphate Buffer Saline (PBS), pH 7.4. The cell culture supernatant employed in this study (ExcellGene, Monthey, Switzerland) contains monoclonal human IgG_1 at a concentration of about 0.11 mg/mL. 0.1 M PBS buffer (pH 7.4) was used as equilibration and washing buffer; 0.1 M glycine (pH 2.8), 0.05 M citric acid (pH 2.5) and 0.05 M acetic acid (pH 2.5) were used as elution buffers. Regeneration was accomplished using a 0.6 M NaOH solution.

2.1.3. Protein quantification

IgG concentration in pure protein solutions was determined with a UV-spectrophotometer Shimadzu UV-1601 (Milano, Italy)

Table 1
Summary of membrane properties.

Membrane	Thickness[a] [μm]	Pore size[b] [μm]	Porosity[a] [%]	Permeability[b] [mL/(min bar cm^2)]	BET surface area[a] [m^2/g]
Sartoepoxy	230	0.45	72.7	50–60	2.25
Epoxy 1	192	<0.45	54.5	35–45	4.01
Epoxy 2	192	<0.45	58.5	30–35	4.78
Epoxy 3	237	>3	72.2	400–600	0.93

[a] Data provided by the Membrane Technology group at the University of Twente, The Netherlands.
[b] Data provided by Sartorius Stedim Biotech, Germany.

at a wavelength of 280 nm. For complex solutions, IgG concentration was measured with a HPLC system Alliance 2695 with a dual wavelength UV-detector 2487 from Waters (Milano, Italy) using a Protein A affinity cartridge PA ID from Applied Biosystems (Monza, MI, Italy) [20]. The purity of the eluted fractions was determined with SDS PAGE under denaturing conditions using coomassie blue for protein staining. Electrophoresis was performed using the Criterion system from Bio-Rad Laboratories (Segrate, MI, Italy) [20].

2.2. Preparation and characterization of affinity membranes

The synthetic Mimetic Ligands™ were provided as A2P-monochloride and B14-monochloride by ProMetic Biosciences Ltd. (Cambridge, UK). All other synthesis related chemicals were purchased from Sigma Aldrich (Vienna, Austria). The synthesis protocols for the below mentioned ligand-spacer combinations were published by Zamolo et al. [30]. The membrane preparation protocols as well as the determination of accessible epoxide groups on a membrane surface via titration with thiosulfate and via elemental analysis after immobilization of 2-ethanolamine and 2-mercaptoethanol were summarized in Table S1 in the electronic supplementary materials. The chemical structures of investigated membrane adsorbers can be found in Fig. S1 in the electronic supplementary materials. Table 2 provides an overview of the various ligands, spacers and support combinations together with the corresponding ligand densities.

A short schematic overview is reported below regarding the different membrane protocols for the immobilization of A2P and B14, onto the different porous membrane supports, namely Sartoepoxy, Epoxy 1, Epoxy 2 and Epoxy 3:

(i) Direct ligand immobilization via 1,2-diaminoethane linkage (2LP), with final epoxide group deactivation using 2-ethanolamine;
(ii) Use of 3,6-dioxa-1,8-octanedithiol (DES) as spacer arm. The thiol-terminal A2P-DES ligand was linked via an epoxy-ring opening reaction onto the epoxy-activated membrane surface. The remaining epoxide groups were endcapped with 2-mercaptoethanol;
(iii) Use of [1,2,3]-triazole ring (TRZ) as spacer arm. The introduction of an alkyne group on the biomimetic ligands was performed through nucleophilic substitution of the chlorine atom of A2P-Cl and B14-Cl with 3-propargylamine, obtaining A2P-propyne and B14-propyne moieties. Ligand immobilization was performed via Click-Chemistry [27], employing a copper (I) mediated Huisgen 1,3-dipolar cycloaddition reaction to connect the alkyne modified affinity ligand to an azide-functionalized membrane support. No endcapping was performed for these membranes.
(iv) In order to evaluate possible non specific interactions between the target protein IgG and both spacer and surface, two additional test membranes were prepared, namely OH-$(CH_2)_2$-DES-Sartoepoxy and OH-CH_2-TRZ-Sartoepoxy. In case of OH-$(CH_2)_2$-DES-Sartoepoxy, 2-chloroethanol was attached to one of the two thiol-groups of 3,6-dioxa-1,8-octanedithiol prior to the immobilization of OH-$(CH_2)_2$-DES to Sartoepoxy. The OH-CH_2-TRZ-Sartoepoxy was prepared by coupling 3-propargylalcohol to azide modified Sartoepoxy membranes via Click-reaction with copper (I) as catalyst.

Note that in an earlier study Protein A Epoxy 1 showed good performance for IgG capture from cell culture supernatant [20]. Unfortunately, due to the low ligand densities of A2P and B14 ligands on Epoxy 1 membrane support, preliminary batch experiments showed rather low binding capacities for IgG. Nonetheless, in order to provide a thorough description and comparison of membrane performance, Epoxy 1 membranes were included in our discussion, although no experimental data were explicitly shown.

2.3. Membrane performance evaluation and characterization

Batch adsorption experiments were performed by soaking the membrane discs in IgG solutions of known concentration and kept under gentle agitation until equilibrium was reached. The protein concentration in the supernatant was determined at the beginning and at the end of each experiment by measuring the absorbance of the solution at 280 nm [20]. This protocol was followed also in preliminary experiments performed during the preparation of affinity membranes to determine their optimal ligand density.

Dynamic binding experiments were performed using an ÄKTA purifier FPLC system from GE Healthcare (Milano, Italy). Layered stacks of membranes were housed in a membrane unit of 2.5 cm diameter. Pure IgG standard solutions were used to characterize the membranes and to obtain dynamic isotherms. The effect of different operating conditions, including flow rate and feed concentration, was investigated. Membrane selectivity was determined in further experiments using cell culture supernatant.

3. Results and discussion

3.1. Mimetic affinity membrane preparation

The underlying concept of the synthetic Mimetic Ligands™ A2P and B14 relates to their property to bind to the Fc-region of IgG and hence they are possible alternatives to Protein A as affinity ligand. Both synthetic ligands are stable in sanitation solutions containing up to 1 M sodium hydroxide without the risk of ligand leakage or denaturation, which may not be the case for Protein A.

The benchmarks for this study were the A2P ligand, the 2LP spacer chain and the Sartoepoxy membrane supports [29,30]. The immobilization procedure was found to be strongly dependent on the chemical properties of the affinity ligand as well as the spacer chain and the support media. Therefore, it was necessary to perform extensive optimization experiments in order to achieve the desired ligand densities through the immobilization protocol. Naturally, an increase in ligand density would provide an increase in the IgG binding performance. However, since the mimetic ligand is much smaller than the target protein, IgG capacity will eventually reach a plateau with increasing A2P-TRZ densities, as shown for the A2P-TRZ-Sartoepoxy membrane in Table S2 in the electronic supplementary material. In case of the Epoxy 1 support membranes, no increase in ligand density was achievable and the low number of attached ligand naturally led to unsatisfactory IgG capture performance (data not shown). Epoxy 1 was therefore not further investigated.

Concerning the choice of linkage chemistry, the coupling of thiophilic affinity ligands onto epoxy-activated supports (i.e. agarose) is a known and well-established technique in biochemistry [19]. Their immobilization onto cellulose membranes is, however, more sensitive due to the difference in handling membrane sheets compared to resin-like media. It was observed that specially Epoxy 2 membranes are highly sensitive to shear forces, therefore a mechanical stirrer, which is frequently used for spherical beads, cannot be used. Shear-force induced surface degradation makes the use of magnetic stirring devices, for temperature-controlled reactions, and horizontal shakers, for reactions at room temperature, essential. A careful adjustment of the agitation speed is required in order to gently mix the reaction solution, but leave the membrane sheets practically stationary and floating in solution.

Table 2
Summary of investigated ligand-spacer-membrane combinations together with their ligand density.

Spacer[a]	Ligand	Membrane	Identification name	Ligand density [μmol/cm²]
2LP	A2P	Sartoepoxy	A2P-2LP-Sartoepoxy	0.25
	A2P	Epoxy 2	A2P-2LP-Epoxy 2	n/a
	A2P	Epoxy 3	A2P-2LP-Epoxy 3	n/a
	B14	Sartoepoxy	B14-2LP-Sartoepoxy	0.40
DES	A2P	Sartoepoxy	A2P-DES-Sartoepoxy	1.18
	OH-$(CH_2)_2$–	Sartoepoxy	OH-$(CH_2)_2$-DES-Sartoepoxy	n/a
TRZ	A2P	Sartoepoxy	A2P-TRZ-Sartoepoxy	0.73
	A2P	Epoxy 2	A2P-TRZ-Epoxy 2	0.22
	A2P	Epoxy 3	A2P-TRZ-Epoxy 3	0.48
	B14	Sartoepoxy	B14-TRZ-Sartoepoxy	0.70
	B14	Epoxy 2	B14-TRZ-Epoxy 2	0.26
	OH-CH_2–	Sartoepoxy	OH-CH_2-TRZ-Sartoepoxy	n/a

[a] spacer chain description: 1,2-diaminoethane (2LP), 3,6-dioxa-1,8-octanedithiol (DES) and [1,2,3] triazole (TRZ).

Employing this improved ligand immobilization procedure, an A2P-DES ligand density of 1.18 μmol/cm² could be obtained, which is double to triple higher than any ligand density for the other investigated membrane adsorbents. Although the static binding capacity of A2P-DES-Sartoepoxy materials was satisfactory, as shown in an earlier study [29], their elution recovery was rather low with the typical elution buffers used. Since this parameter indicates a loss of valuable IgG through irreversible binding, it was decided not to prepare nor investigate any B14-DES type membranes. Results for A2P-DES-Sartoepoxy are only shown in Table 2 and Fig. 2a strictly for comparison reasons.

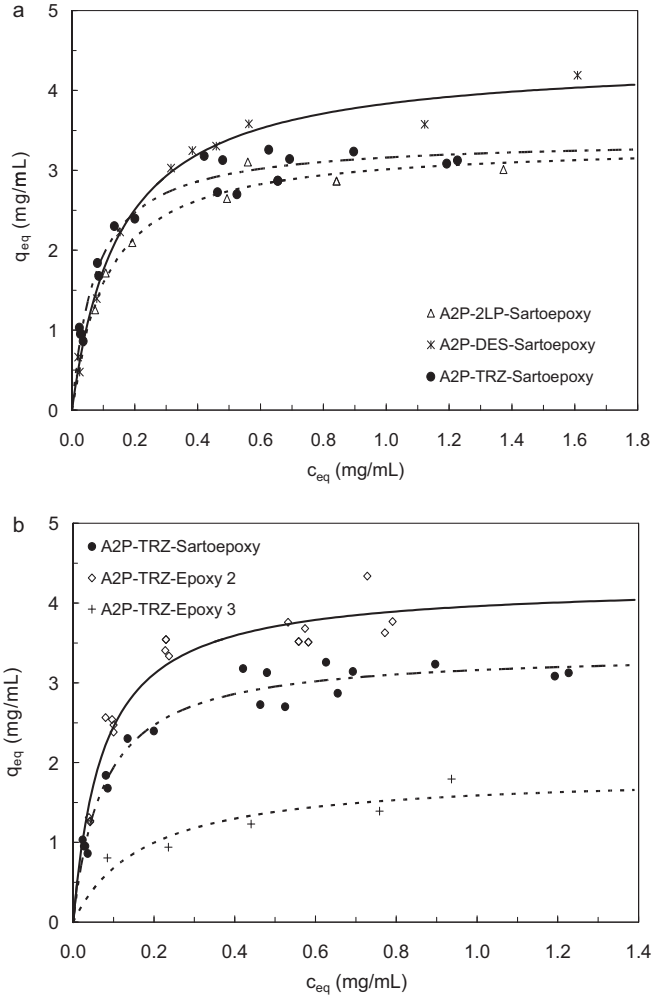

Fig. 2. Batch equilibrium adsorption isotherms using pure IgG solutions shows the effect of (a) spacer arm and (b) membrane support on the static binding capacity.

In case of the B14-2LP ligand-spacer combination, an earlier study for polymethacrylate beads had provided evidence that the amino groups incorporated in the spacer chain and the surface endcapping had lead to reduced IgG binding capacities due to secondary binding of feed impurities [26]. Comparable adsorbents with B14-TRZ ligand spacer combination lacked this drawback and even showed an improved IgG capture performance compared to commercial Protein A media, when operated at low bead height and fast flow rates [28].

The incorporation of a TRZ linkage involves the so-called "Click Chemistry" [27]. Unfortunately the Click reaction was in the present case not as straightforward as described in the literature for cycloaddition reactions of compounds in solution [31], therefore a modification of the reaction protocol was necessary. The usual amount of approximately 5 mol.% [32] of Cu(I) was not sufficient to catalyze the reaction to a satisfying extent and to obtain the desired ligand densities. Since copper (I) had to be added in large quantities (4 mol. equiv.) relative to the amount of available azide groups on the membrane surface, the overall problem that emerged was the final removal of copper (I) and (II) from the membrane surface. Another difficulty that arose was that copper (I) not only bound to the support surface but was also forming a complex with the amino and hydroxyl groups of the affinity-ligand. It is obvious that any remaining surface bound copper would reduce the IgG binding performance of the membrane adsorbent, since immobilized copper resembles a sterical hindrance for the target protein to reach the affinity ligand. A simple wash protocol using 50 mM citric acid and 0.5 M sodium hydroxide proved to be most effective for membrane adsorbents. An alternative approach employing a pre-activated copper (I)-acetylide complex was discussed elsewhere for anion exchange and B14-TRZ type adsorbents, but was not yet employed for membrane materials [27].

3.2. Batch characterization

The affinity membranes have been tested in batch experiments with pure polyclonal IgG solutions in order to investigate the effects that the ligand head group, the spacer arm and the immobilization chemistry, the membrane support and the number of active epoxy groups available for ligand coupling, may have on the overall material performance.

The experimental adsorption data were well described by the Langmuir model, which in equilibrium conditions can be expressed as:

$$q_{eq} = \frac{c_{eq} \cdot q_{max}}{c_{eq} + K_d}$$

where c_{eq} and q_{eq} are the equilibrium protein concentrations in the liquid and solid phase respectively, q_{max} represents the

Table 3
Langmuir equilibrium parameters and IgG recovery of the affinity membranes tested.

Membrane	q_{max} [mg/mL]	K_d [mg/mL]	Recovery [%]
A2P-2LP-Sartoepoxy	3.34	0.109	62
A2P-DES-Sartoepoxy	4.42	0.152	31
A2P-TRZ-Sartoepoxy	3.40	0.0752	51
A2P-2LP-Epoxy 2	3.03	0.151	n/a
A2P-TRZ-Epoxy 2	4.25	0.0735	59
A2P-2LP-Epoxy 3	1.99	0.0897	n/a
A2P-TRZ-Epoxy 3	1.87	0.175	35
B14-2LP-Sartoepoxy	3.45	1.91	n/a
B14-TRZ-Sartoepoxy	2.61	0.0540	91
B14-TRZ-Epoxy 2	3.07	0.152	93

maximum binding capacity and K_d is the Langmuir equilibrium dissociation constant.

The thermodynamic equilibrium parameters, obtained by applying the Langmuir model, together with the average values of IgG recovery are reported in Table 3.

3.2.1. Spacer arm and immobilization chemistry

The effect of the different spacer arms on IgG adsorption is shown in Fig. 2a, where the equilibrium isotherms for the A2P affinity membranes prepared using the Sartoepoxy matrix are reported. It can be noticed that A2P-DES-Sartoepoxy membranes have the highest maximum static binding capacity, namely 4.4 mg/mL, while A2P-2LP-Sartoepoxy and A2P-TRZ-Sartoepoxy have a comparable capacity of about 3.4 mg/mL. However, if we consider the elution step it can be noticed that all A2P membranes have rather poor performance and, among those, A2P-DES-Sartoepoxy is the membrane with the lowest recovery, 31% compared to 51% for A2P-TRZ-Sartoepoxy and 62% for A2P-2LP-Sartoepoxy [33]. The synergic combination of the A2P ligand with the DES spacer chemistry causes an enhancement of the affinity bond, which improves the binding capacity for IgG, but at the same time provides much reduced elution yields, which resemble a major drawback [34]; thus DES spacer was not further investigated. The change in spacer chemistry, from DES to TRZ for A2P bound to Sartoepoxy membranes, increases the IgG recovery by 20%. Although the IgG recovery increases by 30% when changing from DES to 2LP, caution must be paid before a general conclusion is drawn since these tests were performed with pure IgG solutions. In case of a real cell culture feed, IgG would compete for the A2P-ligand binding sites with feed impurities such as host cell proteins and DNA, as well as the anion exchange type moieties of the amino-functional 2LP spacer chain and the ethanolamine surface endcapping. It was proven for resin-type mimetic ligands with 2LP spacer chain that they can capture a significant amount of feed impurities, while their IgG binding performance was at the same time much reduced [26]. For the other two spacer variations, control experiments with support-spacer combinations without affinity ligands, namely Sartoepoxy-DES-$(CH_2)_2$-OH and Sartoepoxy-TRZ-CH_2-OH, were tested with pure IgG solution using static binding tests. They provided no significant evidence for non-specific binding of IgG towards either of the two spacer linkages. This result proofs that the strong binding of IgG onto A2P-DES-Sartoepoxy was exhibited by a synergetic effect between the DES spacer and the A2P-ligand. It furthermore shows that the TRZ-spacer per se does not influence IgG binding. However, for Azido-Sartoepoxy membranes without ligand and without endcapping with propargyl alcohol a maximum static binding capacity for IgG of 1.13 mg/mL was observed. This result correlates with recently published results for azide group modified resin type adsorbents [26]. It is therefore noteworthy to mention that the spacer arm and the support surface modification can significantly influence the IgG capture performance of an attached ligand-head group [26,28–30].

Overall, these results indicate that the Click-coupling procedure was the most promising approach compared to the other two alternatives. However, A2P-2LP-Epoxy 2, A2P-2LP-Epoxy 3 and B14-2LP-Sartoepoxy were prepared and investigated via static binding tests with pure IgG solution for the purpose of comparison.

3.2.2. Membrane support

From the results obtained in the previous section, only 2LP and TRZ affinity membranes were investigated with the improved Epoxy 2 and Epoxy 3 supports. The adsorption isotherms for the different A2P-TRZ affinity media employing pure IgG solutions under static binding condition are reported in Fig. 2b. A2P-TRZ-Epoxy 2 membranes have the highest binding capacity for IgG, followed by A2P-TRZ-Sartoepoxy and A2P-TRZ-Epoxy 3 membranes. It is apparent that Epoxy 3 membrane supports lead to a loss of separation performance. Interestingly, a similar trend is also observed for the 2LP modified membranes; however, the elution performance of these membranes was not reproducible and this problem became apparent for different production lots. A possible reason for this occurrence is the fact that epoxide groups can undergo an acid as well as base catalyzed ring-opening reactions in aqueous solution. A2P-2LP as well as ethanolamine are basic compounds. The number of residual epoxide groups after ligand attachment can vary from batch to batch and so does the number of the ethanolamine groups attached during epoxide group endcapping. As previously mentioned, these surface near amino groups are responsible for partial anion exchange type properties of adsorbents with 2LP spacer chain and ethanolamine endcapping. Their presence or absence may therefore have a very strong effect on material performance.

When considering the membrane structural parameters reported in Table 1, it is possible to derive more details on the separation performances of the different affinity membranes investigated. It is important to notice that the Epoxy 2 membrane support has the highest internal surface area per unit mass (4.78 m^2/g), but with 1.2–1.8 $\mu mol/cm^2$ the second lowest epoxide group density (Table S1). This reveals on one hand a poor, yet sufficient, epoxide functionalization of the available specific surface area of the membrane matrix, but on the other hand the reactive epoxide or azide groups are highly isolated, leaving enough space for ligand-protein interaction. In comparison the Epoxy 1 support possesses only a slightly smaller surface area of 4.01 m^2/g, but comes with a very low epoxide group density of 0.3–0.4 $\mu mol/cm^2$. For large and spacious ligands exhibiting a very strong affinity for IgG capture such as it is the case for Protein A, the Epoxy 1 support was classified as being the ideal membrane support [20]. For smaller mimetic ligands, however, the attachable ligand densities on Epoxy 1 was simply too low to provide reasonable IgG binding capacities. This was discovered for A2P-DES-Epoxy 1 as well as for A2P-TRZ-Epoxy 1, for which a static binding capacity of only 0.70 mg/mL was detected with an IgG concentration in the feed of 1.0 mg/mL.

However, affinity membranes prepared with the Epoxy 2 membrane support provide comparable or higher binding capacities with respect to the corresponding membranes prepared with the Sartoepoxy support (i.e. A2P-TRZ-Epoxy 2 has a higher binding capacity than A2P-TRZ-Sartoepoxy). Sartoepoxy has the third highest surface area with 2.25 m^2/g and the second highest epoxide group density with 2.0–2.2 $\mu mol/cm^2$. Both membrane types, Sartoepoxy and Epoxy 2 come with an easily accessible and interconnected microporous structure, a feature that allows the target protein to readily interact with the affinity binding sites.

The Epoxy 3 membrane on the other hand, has a very small surface area of only 0.93 m^2/g combined with the highest density of epoxide group, namely 2.7–3.2 $\mu mol/g$. The ligand coupling reaction for A2P-TRZ onto Epoxy 2 and Epoxy 3 supports provides

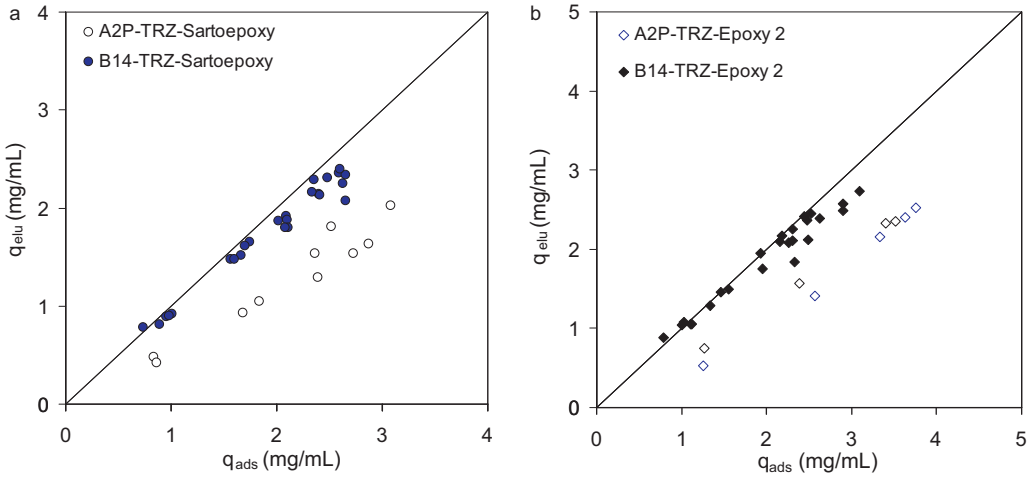

Fig. 3. Adsorption versus elution performance of A2P-TRZ and B14-TRZ affinity membranes in batch experiments using pure IgG solutions.

comparable low yield, which demonstrates that the more open membrane structure of Epoxy 2 facilitates IgG binding. Therefore it can be concluded that the Epoxy 2 matrix has a good potential for improvement. By increasing the epoxide group density, it may be possible to further increase the IgG binding capacity for Epoxy 2 type membrane adsorbents, making it competitive with chromatography beads at process scale.

3.2.3. *Affinity ligand comparison*

The new mimetic ligand B14 was immobilized only onto the two most promising membrane types, the standard Sartoepoxy and the Epoxy 2 membranes. Since A2P and B14 are designed with similar chemical and steric structure, it is reasonable to expect that the resulting interactions between the spacer arms investigated and the two ligands follow the same trend. Derived from preliminary tests, the Click reaction protocol can be considered the most promising coupling procedure, and will be hereafter applied for further experimentation and discussion.

When comparing affinity membranes that differ only by their immobilized ligand but not by the linking chemistry or the support, it was observed that A2P-TRZ-Epoxy 2 affinity membranes have a higher binding capacity for polyclonal IgG compared to the corresponding B14-TRZ Sartoepoxy membranes (Table 3). However, the recovery from A2P affinity membranes was consistently below 62% (Table 3), which is not satisfactory for industrial application purposes. On the other hand, IgG recovery from B14 affinity membranes was always greater than 90%, indicating that the membrane matrix does not influence the elution performance of mimetic A2P or B14 affinity membranes. Therefore it must be the chemical structure of the mimetic ligand, which induces low or high IgG recoveries as it can be observed from the data reported in Fig. 3. It is worth to note that all investigated membranes with TRZ-spacer chain were not azide group endcapped, but possess approximately the same number of residual azide groups on the membrane surface, namely 1.1 μmol/m^2 for TRZ-Sartoepoxy and 0.7 μmol/m^2 for TRZ-Epoxy 2. Although residual azide groups can reduce IgG recovery, the fact that their numbers are comparable for these two membrane types, makes their presence not important for comparison purposes.

3.3. *Dynamic characterization*

The behaviour of affinity membranes has been analyzed in dynamic experiments with both pure IgG solutions and a cell culture supernatant containing human monoclonal IgG_1, for an appropriate evaluation of their possible industrial application in the capture step of a monoclonal antibody production process. Due to the low recovery of A2P affinity membranes already observed in the batch experiments, a more extensive dynamic characterization was performed for B14 affinity membranes, for which the effects of flow rate and IgG concentration were also investigated. A2P-TRZ-Sartoepoxy membranes were tested only at one fixed value of IgG concentration, employing pure IgG solution at a constant flow rate of 2 mL/min.

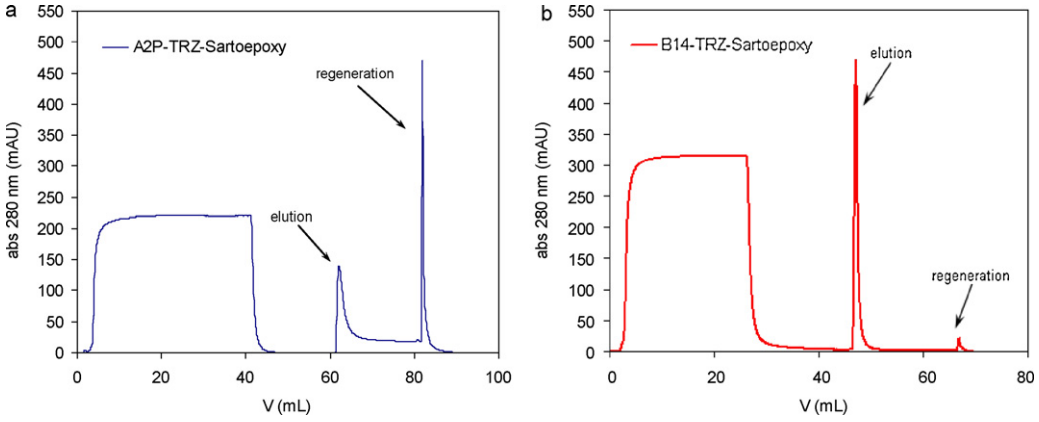

Fig. 4. Comparison between the IgG binding, elution and regeneration profiles for (a) A2P-TRZ-Sartoepoxy membranes, obtained with c_0 = 0.81 mg/mL in the feed, and for (b) B14-TRZ-Sartoepoxy membranes at c_0 = 1.07 mg/mL, for experiments performed at a constant flow rate of 2 mL/min in all chromatographic stages.

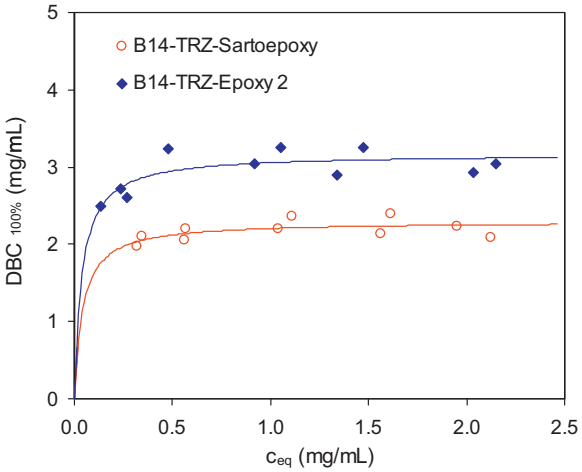

Fig. 5. Comparison between dynamic isotherms of B14-TRZ affinity membranes.

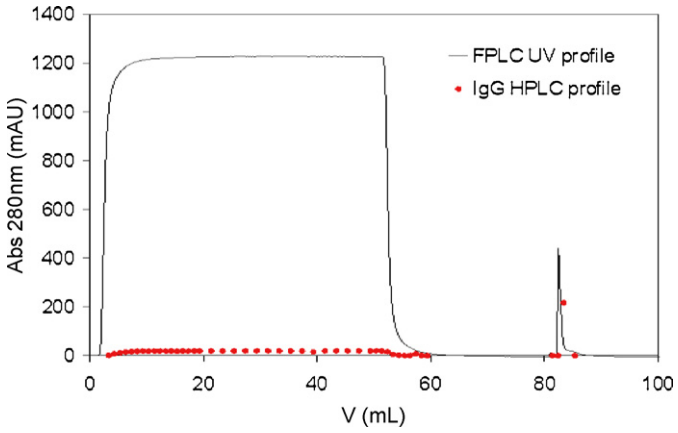

Fig. 6. Dynamic profiles obtained with the cell culture supernatant on B14-TRZ-Epoxy 2 membranes at a flow rate of 5 mL/min in all the chromatographic stages. FPLC UV signal (280 nm) overlays the IgG_1 concentration of the collected fractions (1 mL) determined by Protein A-HPLC analysis.

A comparison between A2P-TRZ-Sartoepoxy and B14-TRZ-Sartoepoxy membranes confirmed the results previously obtained in batch experiments, that is A2P-TRZ-Sartoepoxy membranes provide high dynamic binding capacities, but lower recoveries for IgG, as it is also evident from Fig. 4. The different area proportions of the elution and regeneration peaks can be seen as indicators of the binding strength towards IgG. In Fig. 4a, where a chromatographic cycle of A2P-TRZ-Sartoepoxy is presented, the regeneration peak has an area comparable with that of the elution peak. This indicates that after elution, a considerable amount of IgG still remains bound to the membrane adsorbent. A different behaviour was observed with the B14-TRZ-Sartoepoxy membrane, Fig. 4b, for which the regeneration peak is barely visible, indicating that almost all of the IgG adsorbed onto the affinity membranes is actually recovered in the elution step. Indeed, the average recovery for A2P-TRZ-Sartoepoxy membranes in dynamic experiments was 57%, while for B14-TRZ-Sartoepoxy membranes it was 92%, which correlates well with the batch experimental results.

All dynamic experiments were performed up to complete saturation in order to calculate the dynamic binding capacity at 100% breakthrough, $DBC_{100\%\ BT}$. The experimental values of $DBC_{100\%\ BT}$, obtained at different values of IgG concentration in the feed, were used to draw dynamic isotherms as it is shown in Fig. 5. The results were described using the Langmuir model, whose parameters are reported in Table 4. B14-TRZ-Epoxy 2 membranes are endowed with higher binding capacity for IgG than B14-TRZ-Sartoepoxy membranes, qualitatively confirming the results obtained in batch experiments.

Finally, the A2P-TRZ and B14-TRZ membranes were challenged with a cell culture supernatant possessing an IgG titer of 0.11 mg/mL. Fractions of 1 mL volume were collected and analyzed with HPLC using a Protein A HPLC column. An example of the UV profile for the cell culture supernatant and the concentration profile for IgG, obtained with Protein A HPLC analysis, are shown in Fig. 6. The dynamic binding capacities and the corresponding recoveries are reported in Table 5.

A2P membranes, which have higher binding capacity towards pure IgG compared to the corresponding B14 membranes (Table 3), showed a lower binding capacity, when tested with cell culture supernatant (Table 5). It is also important to remark that little to no IgG was recovered from A2P membranes when tested with cell culture feed. This is also illustrated by the weak IgG bands in the elution fraction of A2P-TRZ-Epoxy 2 on SDS-PAGE gels compared to the corresponding B14-TRZ membranes as shown in Fig. S2 from the electronic supplementary material. B14 modified supports showed a clear superior performance. Especially, the B14-TRZ-Epoxy 2 provided the highest binding capacity of 1.41 mg/mL, combined with an IgG recovery of 92%.

The theoretical binding capacity, evaluated from the dynamic isotherm for pure IgG solutions at a concentration of 0.11 mg/mL (IgG_1 titer in the supernatant), is 2.49 mg/mL for A2P-TRZ-Sartoepoxy, 2.55 mg/mL for A2P-TRZ-Epoxy 2, 1.67 mg/mL for B14-TRZ-Sartoepoxy and 2.37 mg/mL and B14-TRZ-Epoxy 2. However, the binding capacities obtained with the cell culture supernatant are sensibly lower than expected on the basis of pure IgG solutions, as it can be noticed from the data reported in Table 5. The reason for the strong deviation between results from pure IgG solutions and cell culture feed may lay in the known sensitivity of A2P towards Pluronic F-68 [30], which is mostly added as an anti-foaming agent to cell culture media. On the contrary, membranes carrying the new ligand B14, which was designed to be Pluronic F-68 tolerant, are able to capture and release more IgG from cell culture feed. Furthermore, one has to take into account that the cell culture feed used for these experiments contained only a small amount of IgG_1, while the feed related impurities are highly dominant (Fig. 6) and may compete with IgG for binding sites on the membrane adsorbent. Another possible explanation for that behaviour can be attributed to membrane fouling due to the complexity of the cell culture solution. This motivation is strengthened by the fact that the drop in binding capacity is less severe for the membranes prepared with the Epoxy 2 matrix, due to the more open structure than the Sartoepoxy membrane. The more probable solution, however, may lay in the lower number of residual azide groups on Epoxy 2 (0.7 μmol/cm^2) compared to Sartoepoxy

Table 4
Langmuir parameters and recovery of B14-TRZ affinity membranes determined with pure IgG solutions.

Membrane	$DBC_{100\%\ max}$ [mg/mL]	K_d [mg/mL]	Recovery [%]
B14-TRZ-Sartoepoxy	2.30	0.0411	92
B14-TRZ-Epoxy 2	3.17	0.0374	96

Table 5
$DBC_{100\%\ BT}$ and recovery of A2P-TRZ and B14-TRZ affinity membranes determined with cell culture supernatant with an IgG_1 titer of 0.11 mg/mL.

Membrane	$DBC_{100\%\ BT}$ [mg/mL]	Recovery [%]
A2P-TRZ-Sartoepoxy	0.49	7
A2P-TRZ-Epoxy 2	0.70	1
B14-TRZ-Sartoepoxy	0.74	35
B14-TRZ-Epoxy 2	1.41	92

membranes (1.0 μmol/cm^2). In a previous study, the influence of residual azide groups on the IgG capture and recovery performance of resin-type adsorbents was clearly shown [28]. It was discovered that organic azide groups are capable of disrupting the protein structure of IgG molecules, leading to an enhanced binding of IgG combined with a strongly reduced recovery from the adsorbent. The possibility of azide group endcapping after ligand attachment was not yet fully developed when this study was finalized, but resembles a possible next step in the performance optimization of B14-TRZ-Epoxy 2 membrane adsorbents.

4. Conclusions

New biomimetic affinity membranes with different ligand head groups, A2P and B14, various spacer-arms and immobilization chemistries, 2LP, DES and TRZ, and different membrane supports have been prepared and tested with pure IgG solutions to obtain indications on the feasibility of their application in an industrial antibody manufacturing process. Early results obtained with simple batch experiments did not only help to optimize the coupling reactions for the Click Chemistry protocol, they also lead to optimized ligand densities for the capture of IgG.

High binding capacities for IgG have been obtained with A2P affinity membranes, but the strong binding of IgG to the A2P ligand leads to rather low recoveries. This general tendency was observed for all A2P affinity membranes and represents an important factor, when considering a possible industrial application of A2P affinity membranes. However, this behaviour was not observed with membranes prepared with the new B14 ligand, where elution recoveries greater than 90% were obtained in all cases.

From all the experiments the following conclusions can be drawn: (i) B14-ligands perform better than A2P-ligands both in terms of elution yields and of Pluronic F-68 tolerance; (ii) the TRZ-spacer is clearly superior to the earlier investigated 2LP and DES-spacer chains; (iii) the new Epoxy 2 membrane support exhibits a better performance than the commercial Sartoepoxy membrane.

In addition, it is worth noticing that there is space for further optimization of the B14-TRZ-Epoxy 2 affinity membrane. It seems realistic that an increase of the epoxide group density on the internal porous surface of the new Epoxy 2 matrix will provide an enhancement of its binding performance for IgG. Furthermore, the preparation of mimetic membrane adsorbents with TRZ-spacer chain should include an azide group deactivation protocol. A surface endcapping will not only increase the IgG recovery rate, but will also provide a higher dynamic binding for IgG, since the new surface modification is practically inert towards the non-specific binding of feed related impurities as well as IgG. The attainment of these goals could produce affinity membranes that are more competitive with conventional packed-beads, assessing the use of membrane adsorbers for protein capture in downstream processing.

Acknowledgements

The authors greatly acknowledge the contribution of Michela Giovannangelo for her help in the experiments, Zandrie Borneman and the Membrane Technology Group at the University of Twente NL, for their data on membrane structural parameters, Rene Faber from Sartorius Stedim Biotech GmbH for the membrane support materials, Chris Sadler from ProMetic Biosciences Ltd. for his help with ligand preparation and immobilization. ProMetic BioSciences and Mimetic Ligands™ are trademarks of ProMetic BioSciences Ltd.

This work has been performed as part of the "Advanced Interactive Materials by Design" (AIMs) project, supported by the Sixth Research Framework Programme of the European Union (NMP3-CT-2004-500160).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.059.

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