A purification platform for antibodies and derived fragments using a *de novo* designed affinity adsorbent

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Abstract

Antibody technologies are the most representative success-case in the biopharmaceutical industry. Widely available purification technologies fail in providing a dedicated universal purification platform that can accommodate antibodies structural diversity, namely antibodies from non-human sources, as chicken IgY, and antigen-binding fragments. In this work, we took inspiration from natural and engineered antibody-binding ligands, to rationally design affinity adsorbents able to capture full-length antibodies and fragments. The one-pot Petasis and Ugi combinatorial reactions were sequentially employed to rapidly generate a library of putative solid-phase adsorbents. The best performing adsorbent yielded a single-step recovery, under mild conditions, of human and chicken whole antibodies, antigen-binding fragments and engineered single-domain antibodies from different complex feedstocks. Due to its simple preparation, the lead antibody adsorbent finds broad applicability as a universal purification platform to increase the availability of antibody technologies in research and development.

1 Introduction

Antibodies (monoclonal and polyclonal) are the dominant class of therapeutic and diagnostic molecules in the biopharma and biotech industry [1]. Antibody purification is dominated by protein A chromatography as an initial capture step of fully human IgG molecules and humanized monoclonal antibodies (mAbs), through binding to the constant domains (Fc region) of antibodies. However, protein A chromatography fails in providing a solution for the purification of fragments containing variable domains, and for full IgG molecules from non-mammal sources.

Engineered and designed antibodies, includes those containing variable antibody domains as antigen-binding fragments (Fab) and nanobodies (Nb), and have been increasingly attracting attention mainly due to their ease of manufacturing and tissue penetration while maintaining target specificity [2]. Avian IgY is also considered an interesting source of polyclonal antibodies, due to the high titres produced in egg yolk without repeated bleeding cycles as typically done in mammals [3].

There is currently no reported antibody purification platform that can address the isolation of such diverse, yet at the same time, similar, antibody molecular formats. Protein L chromatographic adsorbents can be considered a solution to purify light chain antibody fragments. However, protein L binds only kappa light chains, failing to purify fragments containing lambda light chains or heavy chains (e.g. Nb).

Designed low-molecular weight peptidomimetic ligands are an appealing option to develop tailor-made chromatographic adsorbents displaying affinity and selectivity towards target biological products [4]. Structurally, these ligands can be seen as intermediates between biological ligands, such as natural receptors (e.g. Protein A [5]) or peptides (e.g. PAM [6]), and synthetic molecules, as ion exchange or mixed-mode ligands. Peptidomimetic ligands combine the selectivity of natural receptors and the moderate affinity of engineered peptides, with superior chemical robustness, low-cost and scalability typical from synthetic ligands. Recently, our group introduced the Petasis-Ugi scaffold that originates from the successive one-pot multicomponent Petasis and Ugi reactions [7], offering higher chemical diversity (4 positions can be randomised) and possibility to fine-tune selectivity.

Here, we report for the first time an affinity chromatographic adsorbent that can simultaneously isolate both full-length and fragment antibodies containing only variable domains, hence acting as a purification platform for this class of biologicals. To achieve this, we exploited the sequential Petasis-Ugi multicomponent reactions to design a small but focused combinatorial library of adsorbents to bind a wide variety of these antibody structures.

We took inspiration from the key structural and functional components from natural (Protein A and Protein L) and engineered (triazine and Ugi peptidomimetic ligands) antibody affinity ligands used in chromatographic separations, to design the chemical substituents to include in the library. After performing a library screening for binding polyclonal human IgG, a lead adsorbent with an incredible propensity to act as a universal antibody purification platform was selected. Remarkably, it promoted the capture of polyclonal human and chicken IgY, preferable binding to antigen-binding fragments (human Fab versus Fc), and direct capture of nanobodies (Figure 1). In addition, the easiness of adsorbent synthesis characteristic of one-pot reactions, the estimated low-cost of the adsorbent (less than 1€/g), as well as the high robustness of synthetic scaffolds (typically withstand harsh CIP and SIP conditions [8]), and scaling-up potential (from 0.5g to 500g while still using benchtop equipment), turn it into a viable and useful tool for all life scientists developing antibodies for biotech and medical applications.

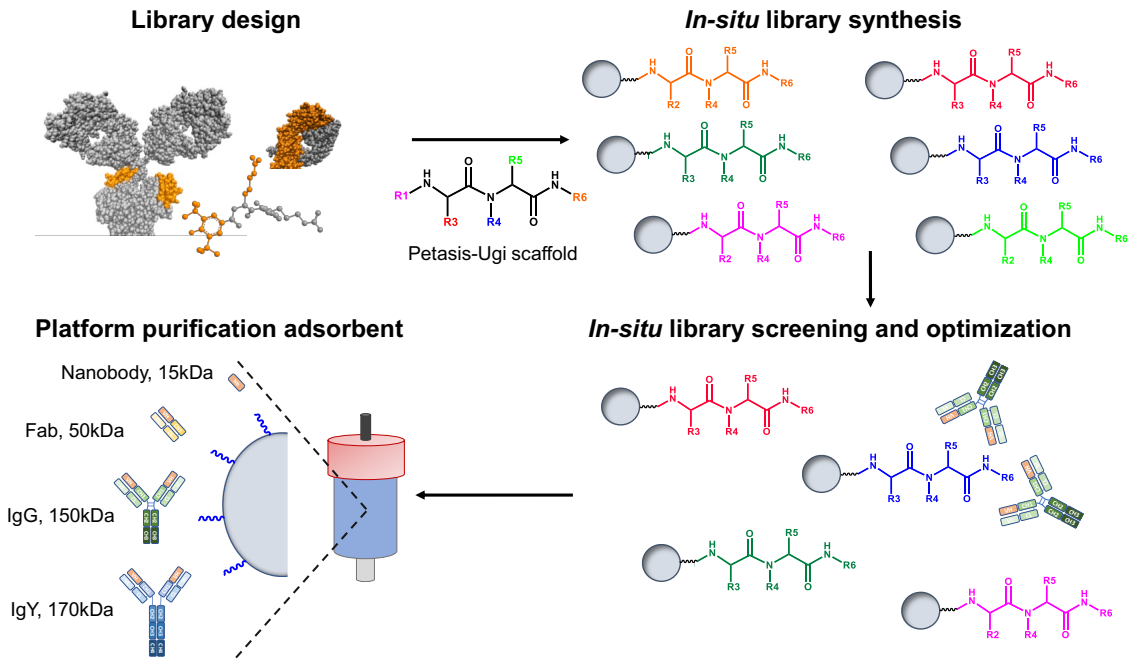


Figure 1 - Rational design and development of a platform purification adsorbent for antibodies and antibody fragments.

2 Materials and Methods

2.1 Materials

All used chemicals were at least 98% pure and solvents were pro-analysis. QuantiPro BCA, Pierce BCA, human plasma, cross-linked agarose (Sepharose™ CL-6B), and Fc solution were purchased from Sigma-Aldrich. Captiva 96-well filtration block, polypropylene columns (0.8cm x 6.5cm) and frits were purchased from Varian, and deep well plates riplate® sw 2 mL were acquired from Roth. The pure human immunoglobulin G (hIgG) solution Gammanorm (165 mg/mL), was supplied by Octapharma. The Fab solution was purchased to Rockland. The Trastuzumab, IgY and Nb cell culture supernatant were kindly provided by Prof. João Gonçalves, Dr. Mara Freire and Prof. Ulrich Rothbauer, respectively.

2.2 Library preparation

For the synthesis of the Petasis-Ugi combinatorial library, Sepharose CL-6B was epoxy-activated, aminated and the Petasis-Ugi reaction proceeded as described elsewhere [7]. For the lead ligand 21.5 μmol epoxy groups/g of moist agarose was obtained and an amine content, quantified by the Kaiser test, of 13.5 μmol NH_2/g of moist agarose. The combinatorial library comprised a total of 72 ligands, each ligand obtained from the combinations of boronic acids, amines and aldehydes.

2.3 Library screening for human IgG binding

The library ligands were regenerated using 0.1M NaOH in 30% isopropanol followed by distilled water (2 cycles of washes, 0.75mL/well) and then with 0.1M HCl followed by distilled water (2 cycles of washes, 0.75mL/well). The adsorbents were equilibrated using

binding buffer (15x 0.75mL/well). For the screening, a solution of pure IgG in PBS at pH 7.4 (0.5mg/mL) was prepared and 500µL was loaded to each well. The block was incubated for 1 hour at 25 ºC with agitation (50 rpm). The flowthrough and the washes were collected by centrifugation at 500 rpm during 30 seconds in 96-well transparent microplates. Equations 1-3 were used to assess the binding capacities, the percentage of binding and recovery:

$$\text{Binding capacity } \left({}^{mg_{target}}\!/_{g_{support}}\right) = \frac{\text{Amount target bound (mg)}}{\text{g support}} \quad (1)$$

$$\%\ \text{Binding} = \frac{\mu g\ \text{target bound}}{\mu g\ \text{target loaded}} \text{x } 100 \quad (2)$$

$$\%\ \text{Recovery} = \frac{\mu g\ \text{target eluted}}{\mu g\ \text{target bound}} \text{x } 100 \quad (3)$$

2.4 Purification of antibodies from different sources

For each test, 1 g of B1Al2A2 agarose was packed in gravity columns, in triplicate unless stated otherwise. Before the screenings the ligand was regenerated, followed by equilibration with the binding buffer, as described before. Commercial IgG solution, human plasma, egg yolk, Trastuzumab and the Nb cell culture supernatant were diluted in binding buffer to achieve a total protein concentration of 1 mg/mL and 500µL was added to the column. 5 CV of washes with binding buffer were performed, followed by elution in 5 CV. For the binding and elution buffer optimization the following buffers were used at pH 9: 20mM Tris (Buffer 1), 20mM Tris 0.5M sodium chloride (Buffer 2) and 20mM Tris 1M sodium chloride (Buffer 3); 20mM Sodium Phosphate 1M sodium chloride (Buffer E1); 20mM Sodium Phosphate, 1 M NaCl, 1% CHAPS; 20mM Sodium Phosphate, 1M Arginine (Buffer E3), 20mM Sodium Phosphate 0.5M imidazole (Buffer E4). All samples were collected in 1.5 mL microcentrifuge tubes for total protein concentration determination using BCA and quantiPro BCA assay reagent. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to evaluate the samples purity in a 12.5% acrylamide gel and ran at 100V. Images were acquired with Gel DocTM XR + System with Image LabTM software from Bio-Rad and analyzed with ImageJ software. The gels analyzed on ImageJ were first processed to a 32-bit image. Afterwards, each lane was selected and plotted in terms of band intensity given by its corresponding area. All protein peaks were selected, and the purity of target protein was calculated as the percentage of area of the corresponding bands in regards to the total area of the remaining proteins. The amount of target protein used for recovery calculations was determined considering the purity obtained by ImageJ software, and combined with BCA results regarding quantification of total protein.

2.5 Regeneration studies

The effect of the cleaning-in-place (CIP) step on the lifetime of the resin was assessed by performing a 24h incubation period of 1M NaOH with the resin. Pure IgG solutions were used for this tests and samples before and after CIP were compared for protein concentration calculations. To check the re-usability of the adsorbent, 5 consecutive

purification cycles with human plasma were performed. After each purification cycle, a CIP step with 1M NaOH was included.

2.6 Static partition equilibrium studies

Affinity constant and theoretical maximum capacity (Q_{max}) were estimated by static partition equilibrium studies. For this purpose, the adsorbent was equilibrated with 20mM Tris pH 9 and different concentrations of pure IgG (0 to 57.4 mg/mL) were incubated, in duplicate, with 0.25 g of B1Al2A2 functionalized agarose in a total volume of 250 µL. The solution was incubated overnight, at 25ºC at 150 rpm (orbital agitation). The supernatants were collected, and a BCA assay was performed to quantify the total protein. The data was fitted by the Langmuir isotherm according to the following equation:

$$q=\frac{Q_{max}K_aC}{1+K_aC} \quad (4)$$

Where q is the bound protein per mass of support (mg IgG/g resin) and C corresponds to the amount of unbound protein in equilibrium (in mg). The Q_{max} and K_a corresponds to binding capacity (in mg IgG/mL adsorbent) and association equilibrium constant (in M^{-1}), respectively.

2.7 Indirect flow cytometry – Anti-Her2

The anti-HER2 monoclonal antibodies activity before and after purification (in triplicates) was determined by flow cytometry. SKBR3 cells and HeLa cells (negative control) were harvested with dissociation buffer (0.6 nM EDTA in PBS), centrifuged at 300 x*g* for 5 min and ressuspended in ice cold PBS with 3% (w/v) BSA (Sigma) to a final concentration of 1 x 10^6 cells/ml. Cell suspension with 1 x 10^5 cells were incubated with 0.5 µg of anti-HER2 mAbs samples for 30 min at 4ºC, afterwards the cells were washed twice by centrifugation at 300 x*g* for 5 min and ressuspended in ice cold PBS with 3% (w/v) BSA. The detection was performed with addition of the secondary antibody Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody conjugated with FITC (Invitrogen), diluted to a final concentration of 1:400. The secondary antibody was incubated for 30 min at 4ºC in the dark, the cells were then washed by centrifugation as previously described and 10 µl of propidium iodide (10 µg/ml, Sigma) added to the stained cells. Assessment of antibody binding were performed using Guava easyCyte™ (Merck Millipore). Flow cytometry data was analysed with FlowJo software (TreeStar).

2.8 Biolayer Interferometry analysis

The dissociation constants of GFP-Nb purified by the Petasis-Ugi affinity adsorbent was determined on BLItz™ system (Pall ForteBio). Purified, biotinylated GFP was immobilized on Streptavidin (SA) dip and read biosensors (Pall ForteBio, cat. # 18-5020) using a concentration of 50 µM. For kinetic measurements three concentrations (120 nM, 250 nM, 1000 nM) of the GFP-Nb in diluent buffer (1 x PBS, 0.1% (w/V) BSA (Carl Roth, cat. # 8076), 0.1 % (w/v) TritonX-100 (Sigma-Aldrich, cat. # T8787)) was used. Each measurement was done in duplicates with an association time of 60 s followed by 120 s dissociation in diluent buffer. Kinetic constants were determined using BLItz software (BLItz Pro 1.2, Pall ForteBio) according to global fitting of data sets. To estimate the affinity of the GFP-Nb purified by the Petasis-Ugi affinity adsorbent the BLItz™ (ForteBio) system was used. Biotinylated GFP-Nb VB3 and VB6 were immobilized via their C-terminal His6-tag on Anti-Penta-HIS dip and read biosensors. Individual VB3 or VB6

coated biosensors were incubated with the soluble protein fraction comprising GFP-VIM or GFP. At least five readings at different GFP-VIM concentrations (9 – 370 nM) were used for VB3 and VB6 and a global fit was applied to calculate binding affinities. As negative control the highest concentration of GFP (370 nM) was used for the binding studies.

2.9 Circular Dichroism

Circular dichroism (CD) spectra were recorded on a JASCO J-1500 CD spectrometer (JASCO, Hiroshima, Japan). CD spectra were recorded in the range from 200 to 260 nm with quartz Suprasil® CD cuvettes (0.1 cm) at room temperature (ca. 25 ◦C). Each CD spectrum is the result of four accumulations originally recorded in degrees and converted to [θ], molar ellipticity, with the spectrometer software. The following acquisition parameters were used: data pitch, 0.5 nm; bandwidth, 1.0 nm; response, 4 s; and scan speed, 100 nm/min. CD spectra was then measured for sample solutions containing ca. 1mg/ml of IgY. Spectra of blank control samples, containing the buffer, were also measured and subtracted from the respective IgY CD spectra.

2.10 Molecular docking

The structure of the affinity ligand B1Al2A2 was prepared with Perkin Elmer Professional ChemDraw 17.1, and its stereoisomers created. The protein structures for the nanobody (PDB ID: 3K1K) and trastuzumab (PDB ID: 4HKZ)), were collected from the Protein Data Bank [9,10]. Both protein structures were then cleaned, and its energy minimized. Computational docking calculations with the affinity ligand and both nanobody and trastuzumab were performed with Autodock 4.2 [11]. The grid box comprised the whole protein structures and docking parameters ran as default for 100 runs and 25M energy evaluations. The best results for each docking run were considered for analysis. The CDR regions were determined using Paratome software [12].

3 Results

3.1 Rational design & screening of a combinatorial library of antibody ligands

The sequential Petasis-Ugi multicomponent reactions create a scaffold structure with up to five variable positions (Figure 2a) [7]. To rationally design the chemical substituents to include in the library, we detailed the key interactions established between antibodies and their natural ligands (proteins A and L), the chemical features of previously reported triazine and Ugi IgG-binding ligands, and the aminoacid residues present in reported antibody-binding peptides (Table S1 and S2). The chemical compounds were selected based on their structural resemblance with natural aminoacids that are known to be crucial for antibody binding or in those present at engineered synthetic affinity ligands with affinity to IgG (e.g. 22/8, 8/7, A3C1) [13–15].

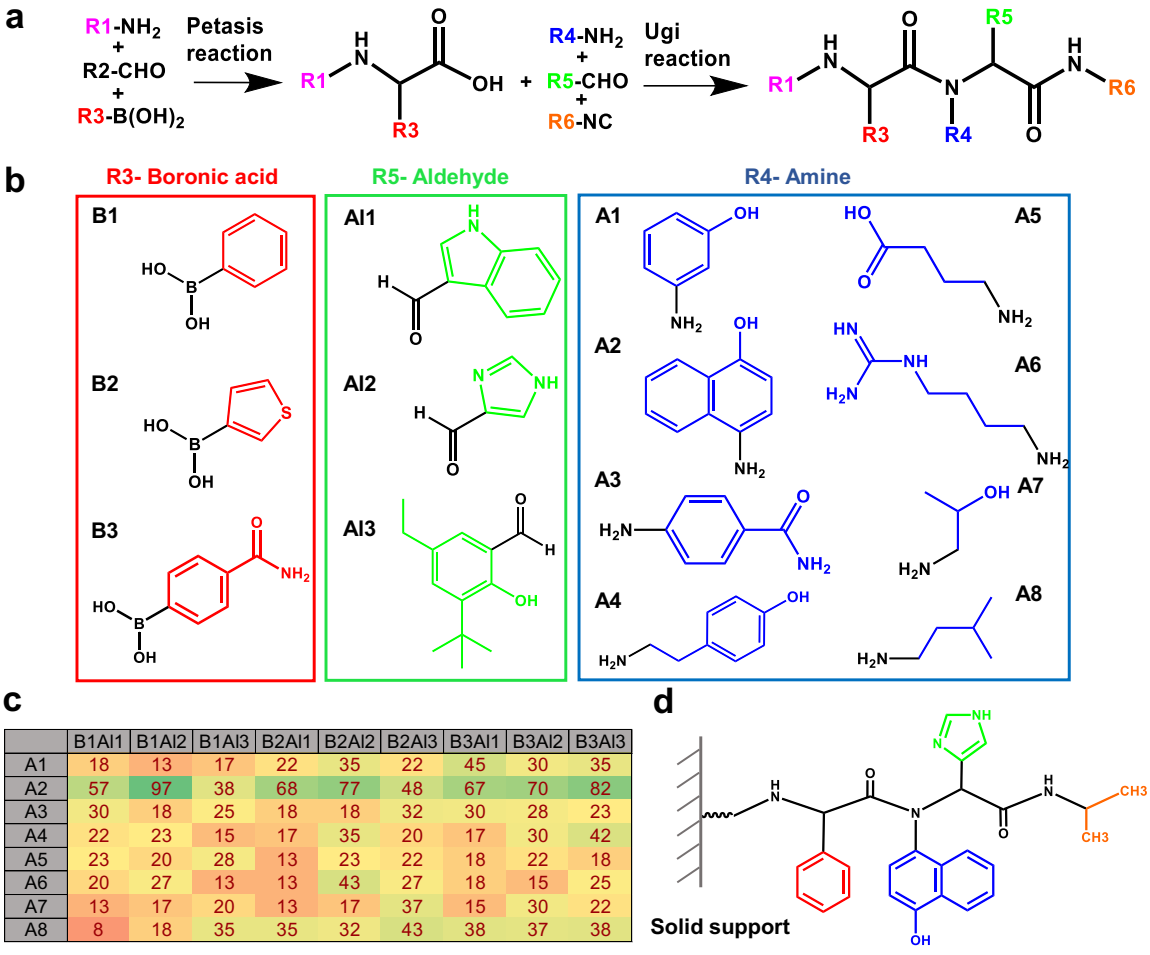
Using this information, and considering the requirements for solid-phase chemistry, we designed a Petasis-Ugi combinatorial library with 72 members and variation in the following positions (Figure 2b): the boronic acid component introduced during the Petasis reaction (R3 substituent), the amine and aldehyde components introduced in the Ugi reaction (R4 & R5 substituents). We left position R1 (amine component in Petasis reaction) as the anchoring point to the solid support (aminated cross-linked agarose beads) and position R6 (isocyanide component in Ugi reaction) kept constant.

After the solid-phase synthesis of the Petasis-Ugi combinatorial library, it was screened towards binding human polyclonal IgG at physiological conditions (Figure 2c). The

screening results revealed 7 ligands binding between 0.3-0.6 mg of IgG/g of support, which corresponds to more than 50% IgG binding (B1Al1A2, B1Al2A2, B2Al1A2, B2Al2A2, B3Al1A2, B3Al2A2 and B3Al3A2). There is a clear pattern of higher binding capacities in adsorbents containing the A2 compound, 4-amino-1-naphthol, which is found on other known antibody ligands namely triazine ligand 22/8 (protein A mimetic) and the Ugi ligand A3C1 (protein L mimetic). The adsorbent containing the ligand B1Al2A2, showed the highest percentage of binding (97%) and was selected as the lead ligand for further studies.

3.2 Adsorbent performance optimization

The structure of ligand B1Al2A2 has the potential to establish several interactions with a target (Figure 2d). Overall, it offers the possibility for the establishment of pi-type and hydrophobic interactions, as well as hydrogen bonding and mediation of charge interactions.



	B1Al1	B1Al2	B1Al3	B2Al1	B2Al2	B2Al3	B3Al1	B3Al2	B3Al3
A1	18	13	17	22	35	22	45	30	35
A2	57	97	38	68	77	48	67	70	82
A3	30	18	25	18	18	32	30	28	23
A4	22	23	15	17	35	20	17	30	42
A5	23	20	28	13	23	22	18	22	18
A6	20	27	13	13	43	27	18	15	25
A7	13	17	20	13	17	37	15	30	22
A8	8	18	35	35	32	43	38	37	38

Figure 2- a) Multicomponent Petasis and Ugi reactions are used sequentially to increase the diversity of the synthetic library. R1, R2, R3, R4, R5 and R6 stand for the aminated agarose, aldehyde, boronic acid, amine, aldehyde and isocyanide used in the reaction, respectively. b) Chemical structure of the components used to create the combinatorial library. Represented in red are the boronic acids (B), in green the aldehydes (Al) and in blue the amines (A). c) Results of the combinatorial library screening against human IgG under physiological conditions in PBS. Binding results after regeneration step with 0.1M NaOH in 30% isopropanol, expressed in terms of percentage of IgG bound. d)

Representation of the chemical structure of the adsorbent containing the lead ligand, B1Al2A2.

As a first step in understanding the binding and elution behaviour of the lead adsorbent, the conditions for binding and elution of human IgG were optimized. Polyclonal human IgG consists of a heterogeneous mixture of IgG classes, therefore it does not have an exact isoelectric point (pI) although a range can be assumed 6.5-9.5 [16]. The ligand B1Al2A2 has a theoretical pI of 8.3 but according to the distribution of its microspecies it maintains an overall neutral charge between pH 8-9.2 (Figure S1). Three binding conditions at pH 9 were tested as this pH ensures that most immunoglobulins will be charged while the ligand will be mostly neutral and charge repulsion is avoided (Figure S1).

The binding buffers tested were 20mM Tris (Buffer 1), 20mM Tris 0.5M sodium chloride (Buffer 2) and 20mM Tris 1M sodium chloride (Buffer 3). The increasing salt concentrations in the binding buffer will provide an understanding about the influence of hydrophobic and ionic interactions in antibody binding. The results showed that salt-free environment (Buffer 1) promoted the strongest interaction between the immunoglobulins and the ligand, achieving 0.79 mg of IgG/g support and 76% of binding (Figure S2). In these conditions, hydrophobic interactions are not emphasized and electrostatic interactions are less inhibited. When increasing the ionic strength in Buffer 2 and Buffer 3, the amount of IgG bound is lower (66% and 61% of binding, respectively). Buffer 1 was selected to proceed with the study of elution conditions.

Four different elution buffers were then tested, maintaining constant 20 mM sodium phosphate, which enabled higher ionic strength when compared to Tris buffer. Buffer E1 included a high salt concentration (1M NaCl), aiming the disruption of ionic-charged interactions (20mM Sodium Phosphate 1M sodium chloride); Buffer E2 included a high salt concentration (1 M NaCl) and a zwitterionic surfactant (1% CHAPS) known to break multicomponent protein-protein interactions; Buffer E3 included Arginine (1 M), an aminoacid known to facilitate the dissociation of the antibody−protein A complex and inhibit the aggregation of eluted antibodies [17]; Buffer E4 included imidazole (0.5 M) in an attempt to act as a competitor for the interactions established between the R5-imidazole component in the ligand and the target IgG. The results revealed that buffer E3 yielded the best results, recovering 89% of bound IgG (0.55 mg of IgG /g support) (Figure S3). Buffer E2 was the second-best option (recovering about 60% of bound protein). Arginine has thus a predominant influence in antibody recovery from the B1Al2A2 adsorbent. The success of arginine can be explained by the fact that arginine is charged at both N and C terminals as well as in the side chain (guanidinium group with a remarkable positive charge effect at the pH of operation). As such, arginine can establish a variety of interactions with antibodies, namely interacting with both positive and negative charged groups, aromatic and polar moieties [17]. The net charge of the affinity adsorbent can vary according to the pH, therefore showing platform-like features by moulding the operational parameters according to the target protein. Based on these results Buffer 1 and E3 buffers were selected for further purification studies, with small adjustments in pH of operation according to the biomolecule studied for purification.

3.3 Selectivity of the adsorbent towards antibody fragments

Finally, we set out to understand the selectivity of the adsorbent towards different antibody fragments, Fab and Fc. A mixture of Fc and IgG (1:1 ratio) was loaded onto the

adsorbent, but only IgG was observed in the elution fraction (Figure S4a). We further loaded pure Fab solution and pure Fc solutions onto the resin. Pure Fab recorded 86% recovery and 67% binding (0.65mg Fab/g adsorbent), whereas with pure Fc, only 38% was recovered and 13% of Fc (66µg Fc/ g adsorbent) was bound to the adsorbent (Figure 3a and S4b-c). These results indicate a preferential binding of the adsorbent for the Fab fragment.

3.4 Purification of antibodies from polyclonal sources from complex mixtures

The applicability of B1Al2A2 as an affinity adsorbent to capture polyclonal antibodies from crude samples was first assessed using human plasma. Plasma is a complex mixture and a valuable source of therapeutic proteins as albumin, coagulation factors and polyclonal immunoglobulins [18]. Using buffers 1 and E3 for binding and elution, respectively, it was possible to obtain a highly selective IgG capture step with overall 96% recovery at 89% purity, as determined by gel densitometry analysis (Figure 3b and S5a).

Another important parameter in affinity chromatographic adsorbents is the stability to regeneration and cleaning-in-place (CIP). The purification performance of B1Al2A2 adsorbent was assessed before and after incubation with 1M NaOH for 24h. The adsorbent withstood the harsh CIP conditions and maintained 90% recovery for IgG before and after the incubation, thus showing the robustness of the solid support (Figure S6). The re-usability of the adsorbent was tested in 5 consecutive purification cycles with human plasma with a CIP between each step (Figure S7). The results reveal a drop in recovery of IgG after the third cycle (from ~95% to 72%; and then to 50% in cycle 5). Despite this loss of performance, the selectivity towards IgG was unaffected throughout the 5 cycles, as seen in the eluted fractions (Figure S7b). Albumin remained mainly in the flowthrough fractions, hence showing a capability for re-utilization. It is likely that the regeneration conditions used would need further optimisation, as in this work standard conditions have been used.

We further assessed the possibility to capture IgY from chicken egg yolk. IgY is the major immunoglobulin in oviparous animals and it has been used as diagnostic and immunochemical reagent. Despite the phylogenetic difference between mammals and birds, IgY antibody is the functional equivalent to mammal IgG, with both molecules divided into heavy and light domains with constant and variable chains [3]. However, IgY has an approximate molecular weight of 170kDa, due to the additional constant domain in the heavy chain, and the hinge region is less flexible. The IgY used in this study is polyclonal with an isoelectric point within 5.7-7.6, hence, pH 7 was selected to improve the chances of interaction between antibody and ligand [19]. The results show a successful and highly selective purification of IgY at pH 7, as seen by SDS-PAGE (Figure S5b) with 93% purity and 42% recovery (Figure 3b). The reason for the low recovery obtained may lie in the physicochemical differences of IgY to other mammalian antibodies. The heavy chain of this immunoglobulin is based on the Fc domain of chicken IgY (Fcυ). Fcυ3- Fcυ4 domains have low identity (at amino acid level) with the mammalian domains of IgG. In addition, the Fcυ2 domain, believed to be the precursor of the hinge region of IgG, is much more rigid and can therefore limit mobility and accessibility of the affinity ligand to putative binding sites. An additional relevant feature is that common IgY purification procedures rely on precipitation methods and hydrophobic interactions driven by the contribution of aromatic residues. It is possible that the binding conditions for this protein mixture need to be optimized to increase the

recovery results. However, the obtained recovery for IgY purification without any optimization of the binding conditions is within range to other reported adsorbents for purification, such as MEP (59.5% recovery [20]), which are not as selective as B1Al2A2 (93% purity vs 90% in MEP), hence can be considered as a capture step for IgY.

The secondary structure of the pure fractions was assessed by circular dichroism (Figure S9), showing that the purification process does not compromise the structural integrity of the antibody and is comparable to other reports [21]. Interestingly, ovalbumin, a protein present in large quantities in egg yolk, is absent from the elution, thus emphasising the selectivity of the affinity adsorbent (Figure S5b). The successful purification of IgY suggests B1Al2A2 broader application to other classes of antibodies related to the structural domains characteristic of IgG.

3.5 Purification of monoclonal antibodies and nanobodies from complex mixtures and insight into binding mechanism

After the successful purification of IgG and IgY polyclonal antibodies from complex mixtures, the potential of B1Al2A2 adsorbent to purify monoclonal antibodies and nanobodies was sought. As a model protein, we used Trastuzumab, a monoclonal antibody targeting the HER2 receptor, which was produced in CHO cells. The conditions of operation were the same as previously defined, with a slight pH adjustment to 8 to have a positive net charge on the antibody (pI 8.7) [22]. Using these conditions, an efficient purification of Trastuzumab was obtained with 84% purity and 76% recovery of mAb (Figure 3a and 2c). Interestingly, purification of IgG from Human Plasma had better results (96% recovery), when compared to Trastuzumab from CHO cell culture (76% recovery) (Figure 3b). This decrease in performance can be explained from the differences in the characteristics of both mixtures. IgG from Human Plasma is polyclonal which comprises a much broader spectrum of antibodies isotypes than the case for Trastuzumab, which is a monoclonal antibody.

As previously verified, the net charge of the adsorbent at the pH of operation is neutral, thus showing that it is not necessary for the ligand and target protein to have opposite charges for binding to occur. The electrostatic/hydrophobic interactions and hydrogen bonds altogether contribute for target binding. However, when the cell culture supernatant was loaded into the resin at pH 9, no selectivity for the mAb was observed. Under these conditions the protein profile of the load, flowthrough and elution were the same (Figure S9a). Furthermore, when loading pH was set at 9.6, no proteins were visible in the elution (Figure S9b). These results demonstrate the mixed-mode nature of the adsorbent that simply by changing the pH of operation, the binding and recovery of the target protein can be finetuned. One of the critical quality parameters in downstream processing is the confirmation of functionality of the biomolecules after elution. The eluted Trastuzumab samples retained above 90% binding HER2 receptor, as shown in flow cytometry experiments, thus demonstrating that the purification step did not alter mAb's biological activity (Figure 3d).

The performance of B1Al2A2 adsorbent to capture and elute human full-length IgG and Fab fragments was demonstrated. We further assessed if the adsorbent could be suitable for the purification of small-engineered antibody fragments containing variable regions and antigen-binding properties. Nanobodies are derived from camelid antibodies and consist of the variable domain being 10 times smaller than full IgG. Due to the absence of post-translational modifications, nanobodies can be produced in bacterial

hosts. In this work we used an anti-GFP Nb produced in *E. Coli*. Following the same rational as before, binding pH was set at 8 based on the net charge of the biomolecule (pI 7.2) and the affinity adsorbent. According to our results, Nb could be successfully purified by the B1Al2A2 adsorbent, with 73% recovery and 87% purity in a single chromatographic step (Figure 3e). Moreover, the purified Nb retained binding towards its target GFP with a K_D of 6.35nM, as determined by BLI analysis, which is within range of the expected values as reported in [10] (Figure 3f).

The purification of this small engineered antibody which is analogous to the variable domain of the heavy chain of mammal antibodies, reinforces B1Al2A2 role in binding different antibody classes from distinct biological origins. Furthermore, this experiment suggested that the mechanism behind the interaction between B1Al2A2 and antibodies may be linked to the framework regions in the heavy chain of immunoglobulins, as it is the only common domain between Nb and the whole antibody. To better understand and obtain insight into the putative binding sites of B1Al2A2, a ligand-protein automated docking approach was performed. For this, Trastuzumab and Nb were selected to compare the binding site between a large full-length antibody and a small engineered fragment. The location of the predicted binding sites of B1Al2A2 are located outside the nanobody and trastuzumab CDR regions and within the framework regions which are maintained constant from antibody to antibody (Figure 3g and 3h). These results further corroborate the ligand's mechanism of binding to such different types of antibodies.

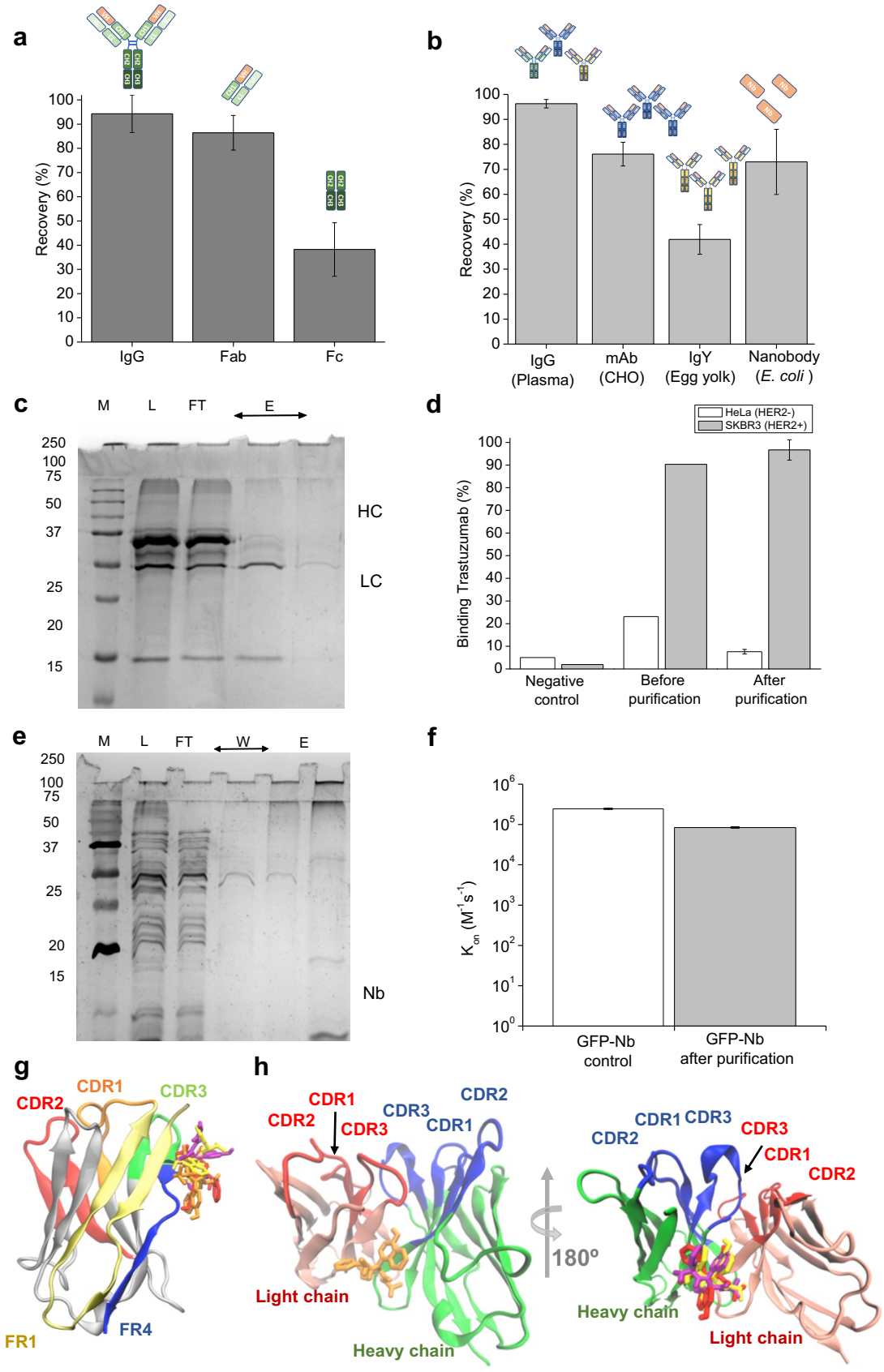


Figure 3 – Purification performance of the B1Al2A2 chromatographic adsorbent. a) Comparison of the purification performance for pure model proteins (IgG, Fab and Fc fragments), expressed in terms of recovery (n=2). b) Comparison of the purification of IgG, IgY and Nb from the respective complex mixture, expressed in terms of recovery

(n=3). c) Purification performance of B1Al2A2 using Trastuzumab from CHO cell culture supernatant. Coomassie blue stained SDS-PAGE of fractions collected during purification process and (d) confirmation of affinity using flow cytometry against HeLa cell line (does not express HER2 receptor) and SKBR3 cell line (expresses HER2 receptor). e) Purification of Nb from E. coli lysate. Coomassie blue stained SDS-PAGE of fractions collected during purification process and (f) confirmation of affinity determined by BLI against GFP (n=2). GFP-Nb control stands for the same Nb used in this study and its affinity results were determined in [10] using quartz crystal microbalance. Location of B1Al2A2 and nanobody (g) and B1Al2A2 and Trastuzumab (h) best docking poses in a 3-dimensional structure (Nanobody PDB ID: 3K1K; Trastuzumab PDB ID: 4HKZ). B1Al2A2 stereoisomers are colour coded (R,R- orange; R,S – yellow; S,R – red; S,S – purple) as well as the CDR, framework regions and light/heavy chains. M: Molecular weight marker; L: Load sample before purification; FT: Flowthrough fraction; W: Wash fraction; E: Elution fraction. Position of IgG heavy chain (HC, 50 kDa), light chain (LC, 25 kDa), and Nb (15 kDa) are indicated in the side of the gel.

3.6 Benchmarking study

The results show B1Al2A2 promising features as an affinity adsorbent for the purification of antibodies and derived fragments, as summarised in Table 1. We set out to compare how B1Al2A2 compares to other reported affinity adsorbents by doing a theoretical benchmarking, summarised in Table 2 and 3. In terms of its affinity constant, B1Al2A2's K_D for IgG is located at the higher range (1mM) when compared with other affinity adsorbents which explains the reversible binding of antibodies at mild elution conditions. Figure 3a and b demonstrate that the adsorbent performs best in the purification of full-length antibodies, therefore the K_D was determined only for this target product. The high purities obtained (>84%) for the different types of antibodies demonstrate the potential of the B1Al2A2 adsorbent to be used as a first capture step for antibodies. Furthermore, the recoveries obtained were comparable with other standard affinity supports (Table 3), thus reinforcing B1Al2A2 can be a reliable alternative for antibody purification.

Ligands reported to bind IgG, such as DAAG, 22/8 and AbSep, all reported high yields in purification of whole antibodies ranging from 85-99% [15,23,24]. However, their major drawback relies in the low pH used during elution. Furthermore, the use of these affinity ligands has only been reported to bind this class of antibodies. Ligands targeting the Fab fragment are less frequent. Nascimento *et al* reported a 12-mer peptide affinity ligand discovered in a phage display library which binds specifically kappa light chains, however, this faces the same issue as Protein L chromatography [25].

Table 1- Purification performance of B1Al2A2 for target proteins in terms of recovery, purity, biological activity and binding/ elution conditions. BLI- Biolayer Interferometry

		Target	Source	Recovery (%)	Purity (%)	Biological activity of purified protein	Binding condition	Elution condition
Pure Samples	Polyclonal	IgG (150kDa)	Human	94±8	-	-	20mM Tris pH 9	20mM NaP 1M L-arg pH 9
		Fab (50kDa)		86±7	-	-		
		Fc (50kDa)		38±11	-	-		
Complex mixtures	Polyclonal	Human IgG (150kDa)	Human Plasma	96±2	89	-	20mM Tris pH 9	20mM NaP 1M L-arg pH 9
		Avian IgY (170kDa)	Chicken egg yolk	42±6	93	-	20mM Tris pH 7	20mM NaP 1M L-arg pH 7
	Monoclonal	Trastuzumab (150kDa)	CHO Cell culture	76±5	84	97% HER2 binding (by flow cytometry)	20mM Tris pH 8	20mM NaP 1M L-arg pH 8
		Nanobody (15kDa)	*E. coli*	73±13	87	6.35nM K_d for GFP (by BLI)	20mM Tris pH 8	20mM NaP 1M L-arg pH 8

Some groups have recently developed a rational design strategy to confer Nb's binding to Protein A. JSR Life Sciences developed a methalacrylate-based resin, Amsphere A3 Protein A, which binds whole antibodies and was evolved to bind antibody fragments, including Nb, obtaining up to 98% recovery [26]. This resin can be considered as a purification platform for human antibodies and those derived from VH domain, however, as it is a modification of Protein A some of the disadvantages from its use remain, namely the use of low pH (3.5) and stability only under mild regeneration conditions (0.1M NaOH).

Other affinity ligands such as HWRGWV peptide, its derived peptoids (PL16), Protein A mimetic (PAM), Mercapto-ethyl-pyridine (MEP) and A2C7I1 have shown their transversal properties by purifying all human subclasses of antibodies as well as chicken IgY and llama IgG [20,27–31]. However, some of these affinity ligands are either non-selective or have only been reported in Fc-specific interactions, hence may not be applicable for isolation of antibody fragments containing only variable domains. One additional important feature of this affinity ligand is its easiness of production. Following the synthesis scheme outlined in this article, one could easily manufacture an in-house affinity adsorbent for different classes of antibodies at just 0.8€/g adsorbent. B1Al2A2 can therefore be considered as a cost-effective alternative purification platform to Mabselect Sure which costs around 35€/mL adsorbent (Sigma website). Despite the attempts to improve purification schemes, current technologies for purification of antibodies fail to have a broad application to different antibody molecular formats. The discovery of B1AL2A2 addresses the need for integration of novel affinity adsorbents that act as platform-like technologies. These can help shift the bottleneck of process design from the downstream processing, by designing more cost-effective production pipelines.

Table 2- Benchmarking B1Al2A2 performance compared to other affinity adsorbents. PAM- Protein A Mimetic

Ligand	Target	Binding capacity (mg/mL)	Affinity constant (K_D, M)	Elution condition	Ref.
Protein A (MabSelect Sure)	IgG	49.8	2.2×10^{-10}	10mM sodium formate, pH 3.6	[23] [a]
Protein G		18.9	2.2×10^{-7}	10mM sodium formate, pH 3.6	[23] [a]
Mabsorbent A2P (triazine based ligand)		35.1	3.9×10^{-7}	50mM sodium citrate, pH 3	[32][b]
DAAG (synthetic peptide)		49.6	3.8×10^{-7}	10mM sodium formate, 100mM NaCl, pH 3.6	[23] [a]
D_2AAG (synthetic peptide)		36.2	1.3×10^{-6}	10mM sodium formate, 100mM NaCl, pH 3.6	[23] [a]
22/8 (triazine based ligand)		152	7.1×10^{-6}	100mM glycine, pH 2.9	[8,15]
A2C11I1 (Ugi based ligand)		24.6	4.8×10^{-6}	100mM sodium phosphate, 30% ethylene glycol, pH 7	[33]
NKFRGKYK (peptide)		4.9	1.1×10^{-7}	100mM acetate, pH 4	[34] [c]
NARKFYKG (peptide)		5.0	1.5×10^{-7}	100mM acetate, pH 4	[34] [c]
AbSep (peptide)		78.0	5.3×10^{-6}	25mM phosphate, 1M NaCl, 20% PEG 600, pH 7	[24]
Cyclo[Link-M-WFRHY-K] (cyclic peptide)		19.7	7.6×10^{-6}	200mM acetate, pH 4	[35]
Protein L	Fab	-	6.5×10^{-10}	Glycine, pH 2	[36]
8/7 (triazine based ligand)		0.7	1.4×10^{-5}	100mM glycine, 50% ethylene glycol, pH 2	[13]
WIPNSEFEHERTK (peptide)		-	2.0×10^{-6}	20mM citric acid, pH 2.5	[25]
A3C1(Ugi based ligand)	hIgG, Fab	16.1 (Fab)	2.6×10^{-6}	100mM sodium bicarbonate, 0.1% CHAPS, pH 10	[14]
A2C7I1 (Ugi based ligand)		17.0 (hIgG)	5.3×10^{-5}	100mM sodium carbonate, 40% ethylene glycol, pH 10	[30]
MEP Hypercell (synthetic)	hIgG, IgY	33.3 (hIgG)	4.8×10^{-7}	100mM acetate, 50mM sodium chloride, pH 3	[23][a,][20,37,38]
Amsphere A3 Protein A	hIgG, Fab, Nb	33.5-49.6 (hIgG)	$0.39\text{-}3.9 \times 10^{-7}$	10mM Citrate, pH 3.5	[39]
HWRGWV (peptide)	Mammalian IgG, IgA, IgM	28.4 (hIgG)	1.0×10^{-5}	200mM phosphate, pH 4	[32][b,] [27]
PL-16 (HWRGWV peptoid)	Mammalian IgG, IgY, llama IgG	57.0 (hIgG)	5.4×10^{-7}	0.1-0.2M acetate buffer, pH 4	[28,40]
PAM	Mammalian IgG, IgA, IgM, IgE, IgY	25.0 (hIgG)	3.0×10^{-7}	100mM acetic acid, pH 3	[31]
B1Al2A2	hIgG, Fab, Nb, IgY	41.0 (hIgG)	1.0×10^{-4}	20mM phosphate, 1M arginine pH 7-9	-

[a,b,c] values retrieved from comparison in the same experimental conditions in the same paper

Table 3- Comparison of recovery and purities obtained for different chromatographic adsorbents for the purification of full-length antibodies and derivatives. n.a. – not applicable (refers to pure protein samples). * - Due to lack of standardized chromatographic method for IgY and Nb purification, MEP and Amsphere Protein A were selected.

Adsorbent	Target	Recovery (%)	Purity (%)	Reference
Protein A (MabSelect Sure)	IgG	>90	>90	[23]
Protein L (Capto L)	Fab	97.3	>90%	[41]
MEP *	IgY	59.5	90	[20]
Amsphere Protein A *	Nb	98	- n.a.	[26]
B1Al2A2 (This work)	IgG Fab IgY Nb	96±2 86±7 42±6 73±13	89 n.a. 93 87	-

4 Conclusions

Protein A chromatography, the standard chromatographic matrix used in the capture of antibodies, is effective in purifying whole antibodies, however it currently cannot address the purification of antibody fragments, such as Fab, Nb and ScFv, which are trending in the pharmaceutical industry [42,43].

We report the design and development of an affinity adsorbent (B1Al2A2) for the purification of antibodies from mammal and avian sources, and Fab-derived fragments using a novel Petasis-Ugi peptidomimetic scaffold. The Petasis-Ugi scaffold proposed is a valuable tool for generating rationally designed and inexpensive chromatographic adsorbents. The simplicity of the sequential 'one-pot' reactions enables its easy implementation in laboratories with the advantage of creating peptidomimetic molecules with added dimensional diversity by incorporating the two reactions sequentially. B1Al2A2 is capable of purifying IgG from human plasma, Trastuzumab from CHO culture cell supernatant, Nb from crude cell extract of *E. coli,* and avian IgY from egg yolk therefore establishing a platform for purification for a wide range of antibodies. The purity of the samples obtained from a single chromatographic step are high using only mild elution conditions with L-arginine and sodium phosphate at neutral pH. Through its mechanism of binding in the framework within the variable region of antibodies, we foresee a standardised use of B1Al2A2 as an affinity ligand platform for a first step of purification for antibodies and related fragments which has never been reported before.

Author contributions

ACAR designed the work. The experiments were conducted by MJBM, FT and AJMB. JG, UR, MF contributed for the manuscript. MJBM, ASP and ACAR prepared and revised the manuscript.

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Conflict of interests

The authors declare no conflict of interests.

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