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Evaluation of a novel agarose-based synthetic ligand adsorbent for the recovery of antibodies from ovine serum

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Abstract

This paper evaluates a prototype agarose-based affinity adsorbent utilizing a bound synthetic ligand designed to replace Protein A as an IgG-affinity capture resin and compares its purification characteristics with four commercially available matrices for the recovery of polyclonal antibodies from crude hyperimmune ovine serum. The novel adsorbent was found to show the highest dynamic capacity (29.2 mg/mL) of all matrices under evaluation—30% higher than the other commercial adsorbents evaluated. When using a post-load caprylic acid wash, IgG yields of over 85% and purities of over 90% were achieved consistently over multiple loading cycles. To evaluate bead diffusion, inverted confocal microscopy was used to visualise fluorescent antibody binding on to individual adsorbent beads in real time. The results indicate that the binding characteristics of the prototype adsorbent are similar to those obtained with Protein G Sepharose. This study indicates that the high-capacity prototype matrix is a feasible and potentially cost-effective alternative for the direct capture of antibodies from crude ovine serum and may therefore also be applicable to the purification of other complex industrial feedstocks such as transgenic milk or monoclonal antibodies expressed using recombinant technologies.

Keywords: Affinity chromatography; Antibody; Ovine; Polyclonal; Serum; Synthetic

1. Introduction

Antibody-based products are becoming an increasingly important class of therapy for the treatment of many clinical indications [1] and represent one of the largest categories of biotherapeutics currently under development [2,3]. The initial recovery step in many antibody-manufacturing processes involves either a fractionation step or chromatographic purification using ion exchange or affinity matrices [4]. Recombinant Protein A, for example, is an industrial standard that is commonly used to recover monoclonal antibodies from clarified cell culture supernatant [5,6]. Nevertheless, both Protein A and Protein G are highly expensive resins and require costly cleaning agents such as urea or guanidine hydrochloride. Furthermore, when purifying IgG from feedstocks such as crude ovine serum, the capture efficiency of Protein A and Protein G may be compromised by the viscosities and contaminants present in the feed [7] resulting in weak binding affinities [8] and a greater potential for product loss. Coupled to issues of resin shelf life and ligand leakage [9] as well as the need for robust column design to achieve economically viable levels of column re-use, such problems ultimately increase the financial and technical risks inherent to an antibody-manufacturing process employing Protein A or Protein G column capture.

These issues have driven the development of alternatives [10] and recently, it has been demonstrated that synthetic adsorbents based on low-molecular weight chemical ligands can be used successfully to capture antibodies from complex feedstocks (e.g. [11]). Synthetics are typically cheaper than recombinant Protein A or Protein G and permit the use of robust cleaning protocols which do not adversely affect binding capacity. This makes them highly attractive in the purification of crude

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Table 1
Adsorbents used in the study and details of their composition and size

Adsorbent	Manufacturer	Base matrix	Ligand	Particle size (μm)
Prototype	Millipore (Consett, Co Durham, U.K.)	Agarose	Bicyclic heteroaromatic (<500 Da)	20–100[a]
MEP Hypercel	Pall BioSepra (Cergy-Saint-Christophe, France)	Cellulose	4-Mercapto-ethyl-pyridine	80–100
ProSep G	Millipore (Consett, Co Durham, U.K.)	Controlled pore glass (CPG)	Recombinant Protein G	75–125[b]
MAbsorbent A2P	Prometic Biosciences (Cambridge, U.K.)	Agarose	Di-substituted phenolic derivative of tri-chlorotriazine	75–125
ProSep-vA Ultra	Millipore (Consett, Co Durham, U.K.)	Controlled pore glass (CPG)	Native Protein A	75–125

Please note that although at the time of writing the prototype adsorbent is not available commercially, samples may be requested from Millipore for evaluation following completion of materials transfer and control agreements.
[a] Determined by the method in Section 2.8.
[b] Assumed to be the same as ProSep A since both have a CPG base matrix.

feeds [12], such as for recovering polyclonal antibodies from hyperimmunised ovine serum [13] during the production of the FDA-approved rattlesnake antivenom, CroFab™ (Protherics U.K. Limited, Blaenwaun, Ffostrasol, Llandysul, Wales, U.K.). This paper evaluates a novel prototype agarose-based synthetic affinity adsorbent utilising a bicyclic heteroaromatic ligand of less than 500 Da (Millipore, Consett, Co Durham, U.K.) and compares the performance characteristics with four commercially available chromatographic resins (Table 1), as well as precipitation and centrifugation steps used currently for CroFab™ production [14,15] to determine the ability of the prototype to recover and purify IgG from crude hyperimmune ovine serum.

Process-scale chromatography requires a robust protocol that achieves consistently high yields and purities over repeated feed application cycles. To determine whether the prototype adsorbent was able to achieve these goals, the following investigations were conducted and form the subject of the remainder of the paper:

- Calculation of static and dynamic matrix capacities when challenged with purified polyclonal IgG.
- Determination of column re-use capacity when challenging the novel adsorbent with ovine serum.
- Evaluation of cleaning protocols for the novel adsorbent.
- Application of confocal microscopy to compare protein uptake patterns between the prototype and commercially available Protein G Sepharose (GE Healthcare, Buckinghamshire, U.K.).

2. Experimental

2.1. Purified polyclonal IgG

Polyclonal IgG was purified from ovine serum using a 10 mm Bioprocess™ system (GE Healthcare) within cGMP production facilities, Protherics U.K. Ltd. following the protocols described [7,11]. Frozen bags of ovine serum (Protherics Australasia Pty Ltd.) were thawed at room temperature and filtered using the following filter train: Kleenpak Nova Preflow 'UB' and Kleenpak Nova Supor EKV (Pall Europe Ltd.) to remove particulates and minor precipitates. The polyclonal IgG was purified using MEP Hypercel (Pall Europe Ltd.). A BPG 200™ chromatography column (20.0 cm diameter × 20.4 cm height, 6.54 L packed bed) was packed at 400 cm/h with H_2O and equilibrated with >3 CVs of 100 mM sodium phosphate pH 7.6. Clarified serum (5 L) was loaded onto the column at 140 cm/h and washed to baseline absorbance (∼3 CV) with 100 mM sodium phosphate pH 7.6. The bound polyclonal IgG was eluted at 80 cm/h using 100 mM citrate pH 2.5 (3.7 CV) and the IgG fraction (3 CV) collected in a single use Flexel® 3D Bag (Sartorius Stedim Biotech). A sample of eluted IgG was removed from the bulk and stored at 2–8 °C prior to use. The purified IgG had a purity of 98% as determined by non-reduced SDS-PAGE and scanning densitometry analysis as described in Section 2.7. The final IgG concentration was 30 mg/mL in 10 mM sodium phosphate pH 7.6, as determined by absorbance at A_{280} with a molar extinction coefficient of 225,000 M^{-1} cm^{-1}.

2.2. Static capacity measurements

The static antibody binding capacities of the five matrices were determined in order to provide an indication of the amount of IgG needed to achieve dynamic breakthrough from packed columns. 100 μL of each adsorbent was pipetted into Eppendorf tubes and spun down at 3000 rpm for 2 min, after which the supernatant was withdrawn and discarded. Each matrix was equilibrated by adding an excess (500 μL) of the relevant buffer (composition and concentration given in Table 2), agitated on a Minishaker (IKA Works, Wilmington, North Carolina, U.S.A.) at 2000 rpm for 10 min and spun down by centrifugation at 3000 rpm for 2 min. This was carried out in triplicate to leave the matrix fully immersed in equilibration buffer. To ensure that the matrix was fully saturated with protein, each tube was then overloaded with 500 μL of MEP Hypercel-purified IgG, produced as described in Section 2.1. Separate 100 μL aliquots of

Table 2
Buffer compositions

Adsorbent	Equilibration buffer	Washing buffer	Eluting buffer
Prototype	25 mM sodium phosphate/HCl pH 7.6	25 mM sodium phosphate/HCl pH 7.6	100 mM glycine/HCl pH 2.8
MEP Hypercel	100 mM sodium phosphate/HCl pH 7.8	150 mM mixed buffer (sodium phosphate, sodium chloride, caprylic acid) pH 7.6	100 mM citric acid monohydrate pH 2.3
ProSep G	0.01 M PBS pH 7.4	0.01 M PBS pH 7.4	100 mM glycine/HCl pH 2.8
MAbsorbent A2P	25 mM sodium phosphate/HCl pH 7.6	25 mM sodium phosphate/HCl pH 7.6	50 mM sodium citrate pH 4
ProSep-vA Ultra	0.01 M PBS pH 7.4	0.01 M PBS pH 7.4	100 mM glycine/HCl pH 2.8

All chemicals were obtained from Sigma–Aldrich Limited (Poole, Dorset, U.K.) All columns were equilibrated for five-column volumes; washing and elution steps were carried out to base line in the UV trace.

washed and equilibrated matrices were also incubated with ovine serum obtained from Protherics U.K. Limited (serum IgG concentration of 34 mg/mL). Prior to use, the serum was filtered using 0.2 μm Minisart syringe filters (Sartorius Limited, Surrey, U.K.) to remove particulates that could potentially interfere with IgG binding. Both sets of Eppendorf tubes were agitated on the Minishaker at 2000 rpm for 24 h. This duration was selected to provide conditions for maximal protein uptake. The tubes were spun down at 3000 rpm for 2 min and the supernatants were withdrawn and assayed for IgG concentration by gel analysis.

2.3. Dynamic capacity measurements

Dynamic capacities of the five adsorbents were determined at 10% breakthrough. The resins were packed into a C10/10 column (GE Healthcare) at 3.95 mL/min (300 cm/h) with five-column volumes of water and the beds were then operated with the buffers given in Table 2 using an ÄKTA Basic 100 chromatography system (GE Healthcare). The setup consists of a P900 pump, an INV907 injection valve, a PV908 outlet valve, a UV900 UV meter set to measure at 280 nm and a Frac-950 fractionator and is controlled by Unicorn software (version 4). For each packed bed, the operational flowrate was selected to achieve a residence time of 6 min, i.e. sufficient time to allow adequate diffusive penetration of the matrix particles by the load. Bed volumes and flow rates used in the dynamic capacity study are given in Table 3. Each bed was overloaded with 5 mL (150 mg) of 98% pure MEP Hypercel-purified IgG (Section 2.1) in order to create breakthrough curves. Dynamic capacities were then calculated as follows: the UV absorbance, A (arbitrary absorbance units: AU) of the flowthrough from the column was determined by the Beer–Lambert law:

$$A = \varepsilon c l \quad (1)$$

where ε is the extinction coefficient (225,000 M^{-1} cm^{-1}), c the flowthrough molar concentration of IgG at 10% breakthrough (3.0 mg/mL; 20 μM) and the path length l is 0.2 cm in the UV cell of the ÄKTA Basic 100. Based on this data it was determined that the 10% breakthrough point would occur at an absorbance of 900 mAU (280 nm). Capacities (DBC) were then determined from the following equation [16]:

$$\mathrm{DBC} = \frac{C_0 \times (V_\mathrm{L} - V_0)}{V_\mathrm{Bed}} \quad (2)$$

where C_0 is the IgG feed concentration (30 mg/mL), V_L the volume loaded up to 10% breakthrough at 900 mAU, V_0 the void volume of the beds (void fraction of 0.4 assumed for all matrices) and V_Bed is the packed bed (all volumes in millilitres). DBC results were averaged over three repeat runs (±4.5%).

2.4. Prototype re-use study

The re-use capacity of the prototype was determined over 10 cycles using the C10/10 column, again operating with a flow rate set to achieve a residence time of 6 min. Based on the results of the static and dynamic capacity measurements, 1 mL of 0.2 μm filtered ovine serum was applied to a 2.98 mL packed bed, washed through at 0.5 mL/min and the flowthrough was fractionated into 1 mL aliquots. The serum was washed through the column until the UV trace returned to baseline (<50 mAU), after which the IgG was eluted using 100 mM glycine/HCl pH 2.8 in a single eluted fraction. The flowthrough and elution por-

Table 3
Bed volumes and operational flowrates used in the dynamic capacity study

Adsorbent	Bed height (cm)	Bed volume (mL)	Flowrate (mL/min)	Flowrate (cm/h)
Prototype	4.2	3.3	0.55	42.0
MEP Hypercel	3.9	3.1	0.51	39.0
Prosep G	3.0	2.4	0.39	29.8
MAbsorbent A2P	5.0	3.9	0.66	50.4
ProSep-vA Ultra	5.3	4.2	0.69	52.7

Flowrates were used for all washing, eluting and re-equilibrating steps with each matrix. All flowrates were set so as to achieve residence times of 6 min for every bed. The diameter of all packed beds was 1 cm (C10/10 column).

tions were both analysed by SDS-PAGE and the 10-cycle run was repeated on a 3.14 mL bed using a mixed sodium phosphate/caprylic acid wash (same as the washing buffer for MEP Hypercel—see Table 2 for composition) prior to elution in an attempt to remove adsorbed albumin and other minor impurities to improve eluted IgG purity [7]. The washing flowrate was 0.52 mL/min. To determine the impact of repeated purification cycles, the dynamic capacity of the adsorbent was then determined again after 10 repeat runs by the method in Section 2.3.

2.5. Column cleaning

Suitable agents are needed to clean unwanted proteins from chromatography matrices—especially during the purification of crude materials. Although chemicals such as urea or guanidine hydrochloride are highly efficient for this purpose, their expense and disposal problems mean that 0.5 M NaOH and 0.1 M HCl were considered for this study instead. In both cases, five-column volumes of the solution were applied after elution, before re-equilibrating for five-column volumes and then reapplying the next feed sample.

2.6. Inverted confocal scanning laser microscopy

Inverted confocal laser microscopy was used to visualise the uptake of labelled IgG by individual beads of the prototype. Visualisation was achieved using the fluorescent Cy^{TM}3 dye (GE Healthcare, Buckinghamshire, U.K.) which was used to tag purified IgG prior to incubation with the matrix. As a control, the experiment was also repeated with Protein G Sepharose (GE Healthcare). The spherical beads of Protein G Sepharose were used instead of the ProSep G used in Sections 2.2 and 2.3 because the latter consists of angular beads owing to its glass base matrix, complicating its direct comparison with the spherical particles of the novel adsorbent.

The method described in [17] was employed to label the IgG. Two stocks of IgG were prepared by diluting a 30.0 mg/mL stock of purified IgG down to 2.0 mg/mL—one in 0.1 M sodium carbonate (buffer pH adjusted to 9.3) and the other in 0.01 M PBS pH 7.4. 1 mL of the sodium carbonate diluted IgG was then labelled fluorescently by addition to a vial of the Cy^{TM}3 dye and inverted gently for 40 min. Subsequently, the contents of the vial were added to 4 mL of the PBS-diluted IgG to produce a solution containing a total of 10 mg of IgG. To prepare the adsorbents, 100 μL of each resin was spun down at 3000 rpm for 2 min and washed three times using 1 mL of 0.01 M PBS. This resin volume was chosen to ensure that it would be completely saturated by the 10 mg IgG solution. After adding the PBS-washed matrix to the 10 mg of IgG, it was inverted gently and two 300 μL samples were withdrawn—one immediately after incubation and the other after 150 min (duration chosen to maximise uptake of IgG). Each 300 μL sample was spun down to recover the matrix and then washed three times with 1 mL of 0.01 M PBS to remove any unbound IgG and fluorophore. Each sample of matrix was then resuspended in 20 μL of PBS and analysed using an inverted confocal laser microscope (Leica Microsystems UK, Buckinghamshire, U.K.) at a 20-fold magnification using an excitation wavelength of 568 nm. The image of each bead was focused upon its central plane to ensure that the intensity of the monitored emissions accurately reflected IgG uptake along the entire cross-section of the bead. Intensities were measured using the Leica analysis software, which analysed images of the beads in order to determine fluorescence profiles. Intensity values for each bead were averaged over three scans and three beads were analysed at each time point to verify that similar intensity profiles were obtained.

Two further control experiments were also performed. First, the fluorescence signal from a sample of the novel adsorbent was monitored every 3 min over a 15-min period (the total duration taken to analyse a total of three beads at any time point) to determine whether there was any significant fluctuation over the course of the experiment as a result of photobleaching. Secondly, Sepharose CL 4B agarose beads without any ligand attached (GE Healthcare) were also analysed by the Cy^{TM}3 protocol to determine whether there was any non-specific binding of the fluorescently labelled IgG to the base matrix which would potentially interfere with the evaluation of the intensity profiles.

2.7. SDS-PAGE

Antibody concentrations were determined by non-reducing denaturing SDS-PAGE, using precast 4–20% Novex® Tris-Glycine gels (Invitrogen U.K., Paisley, U.K.). About 10 μL of each sample was mixed with an equal volume of 2 × SDS sample buffer (Invitrogen) and heated in boiling water for 2 min. Each well in the gel was loaded with 10 μL of the heated samples (approximate protein loadings of 5–10 μg per lane) and electrophoresed at 150 V for 90 min. Protein bands were visualised by staining with SafeStain (Invitrogen) for 1 h, after which the gel was destained with water for 1 h and then placed into fresh water overnight. The integrated optical densities (IOD) of each band were determined by scanning densitometry (LabWorks^{TM} version 4.5, Ultra-Violet Products Ltd., Cambridge, U.K.), and compared to calibrated IgG concentrations. IgG purities were calculated from the percentage band intensities as evaluated by densitometry analysis.

2.8. Matrix particle sizing

The size distribution of the prototype adsorbent particles were evaluated using a Mastersizer 2000 (detection range of 0.02–800 μm) equipped with a small volume sample dispersion unit (Malvern Instruments Ltd., Malvern, U.K.). The matrix was well mixed and added to the sample dispersion unit until an obscurance of 12% was reached. Sizes were evaluated with respect to the percentage of total particle volume. Measurements were repeated three times, with high levels of reproducibility observed between samples.

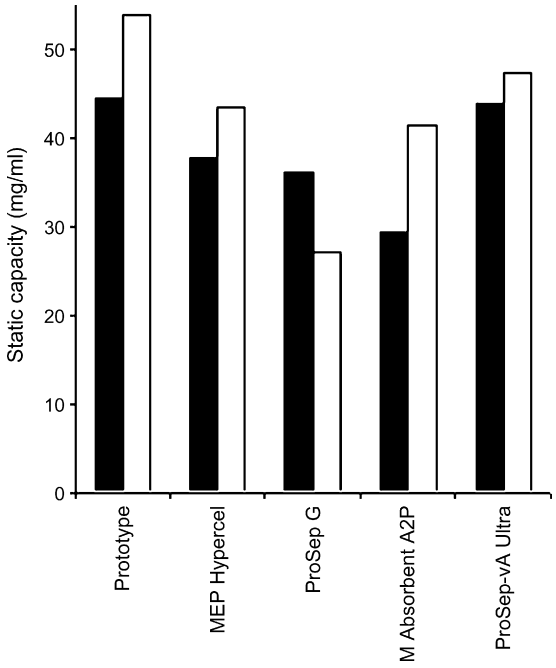

Fig. 1. Static capacities of the five adsorbents: (■) purified IgG; (□) serum.

3. Results and discussion

3.1. Static and dynamic capacity measurements

Fig. 1 shows the static IgG capacities of the five matrices and demonstrates that the prototype has the highest binding capacity when challenged with both purified IgG and ovine serum. Except for ProSep G, the capacities are all higher for ovine serum compared to the pure IgG, with the prototype having a capacity of 54 mg/mL—a value that is comparable to ProSep-vA Ultra and higher than MAbsorbent A2P, MEP Hypercel and ProSep G. Based on these results and to determine accurate dynamic IgG binding capacities, 150 mg of purified IgG (5 mL of 30 mg/mL) was applied to overload a packed bed of each matrix and breakthrough curves were generated. The volume of feed loaded up to 10% breakthrough at 900 mAU (see Section 2.3) was measured and inserted into Eq. (2) in order to calculate the dynamic binding capacities of the matrices. The results in Table 4 show that the prototype adsorbent has the highest capacity out of the five evaluated, with a dynamic binding capacity of 29.2 mg IgG/mL matrix under the conditions described—approximately 30% higher than the best commercial adsorbent evaluated.

Table 4
Calculated dynamic capacities of each adsorbent (the standard deviation (1 d.p.) is shown in parenthesis)

Adsorbent	Dynamic binding capacity (mg/mL)
Prototype	29.2 (±1.5)
MEP Hypercel	21.3 (±0.3)
ProSep G	18.7 (±0.4)
MAbsorbent A2P	16.3 (±1.1)
ProSep-vA Ultra	13.7 (±0.1)

3.2. Re-use study of the novel adsorbent

A packed column of the prototype was operated as described in Section 2.4 for a total of 10 cycles and the main flowthrough and elution peaks were fractionated and subjected to non-reduced SDS-PAGE analysis. The sample chromatogram in Fig. 2 was taken from the first run and shows that both the main flowthrough and elution peaks have small shoulders next to them (F1 and E1, respectively in Fig. 2). The flowthrough was collected in three 1 mL fractions, with the first consisting of the shoulder, whilst the eluate was collected in two separate fractions—the first containing the shoulder (E1) and the second containing the primary peak (E2). A gel of the flowthrough peaks from the third cycle is shown in Fig. 3, along with an elution shoulder. The flowthrough shoulder contains a small amount of both IgG and albumin, whereas the main flowthrough peaks and the shoulder of the elution peak mainly contain only albumin.

The 10 cycle re-use experiment was also repeated with a 3.14 mL column using a post-serum-load caprylic acid wash to determine whether this would improve the purity of the eluted IgG. Fig. 4 shows a gel of the primary elution peaks (i.e. peak E2 in Fig. 2) from both re-use studies. Yields of 85% or above were obtained consistently, both with and without the caprylic acid wash—comparable to the precipitation and centrifugation steps employed currently by Protherics for manufacture of CroFab™. Notably, after the fourth loading cycle when operating without the caprylic acid wash, the albumin-rich elution shoulder merged with the main IgG elution peak, reducing its purity and overall, purities ranged from 86 to 91% (cycles 1–4) and 79–84% (cycles 5–10). Addition of caprylic acid substantially removed residual albumin remaining on the column after serum application, resulting in high IgG purities of 92–98%, comparing well with the 91% value achieved using the precipitation and centrifugation steps employed at present [7]. The dynamic binding capacity of the caprylic acid-washed bed was then evaluated

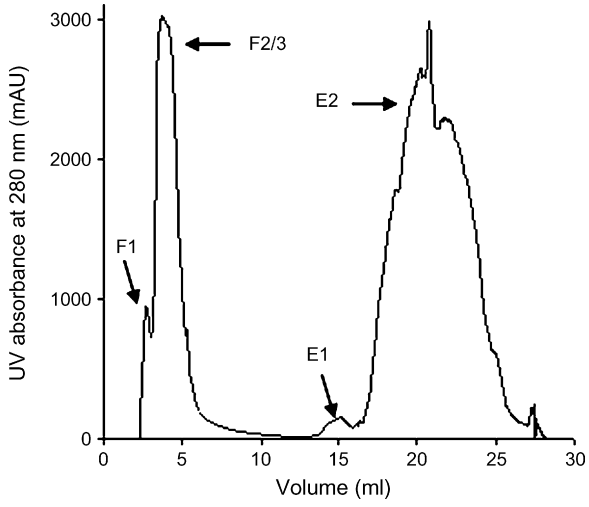

Fig. 2. A 280 nm chromatogram produced when challenging a packed bed of the prototype adsorbent with 0.2 μm filtered ovine serum. Labels refer to lanes in gel image in Fig. 3. F1 refers to the fraction taken consisting of the shoulder on the flowthrough, whilst F2 and F3 lie within the main flowthrough peak. E1 refers to the shoulder on the elution peak, whilst E2 is the primary elution peak.

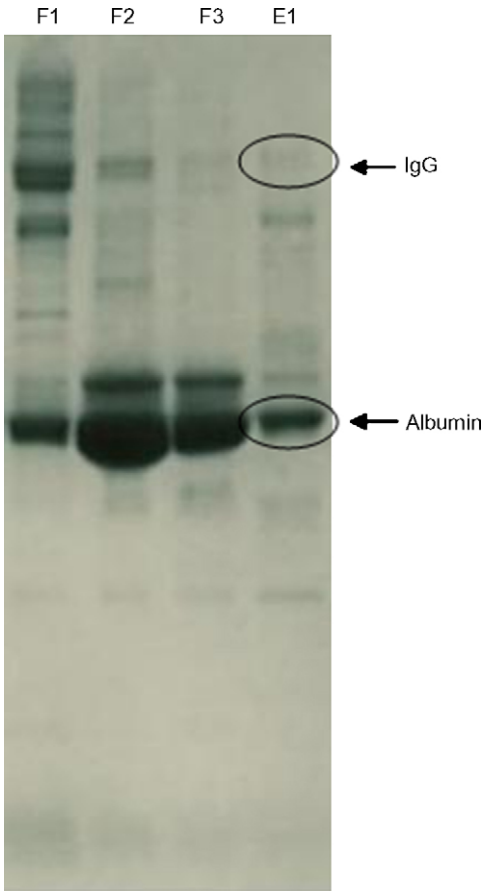

Fig. 3. SDS-PAGE gel showing the fractions identified in Fig. 2 (taken from the third cycle of the 10-cycle run). F1, shoulder of the main flowthrough peak; F2/F3, second and third fractions of the flowthrough peak; E1, shoulder of the main elution peak.

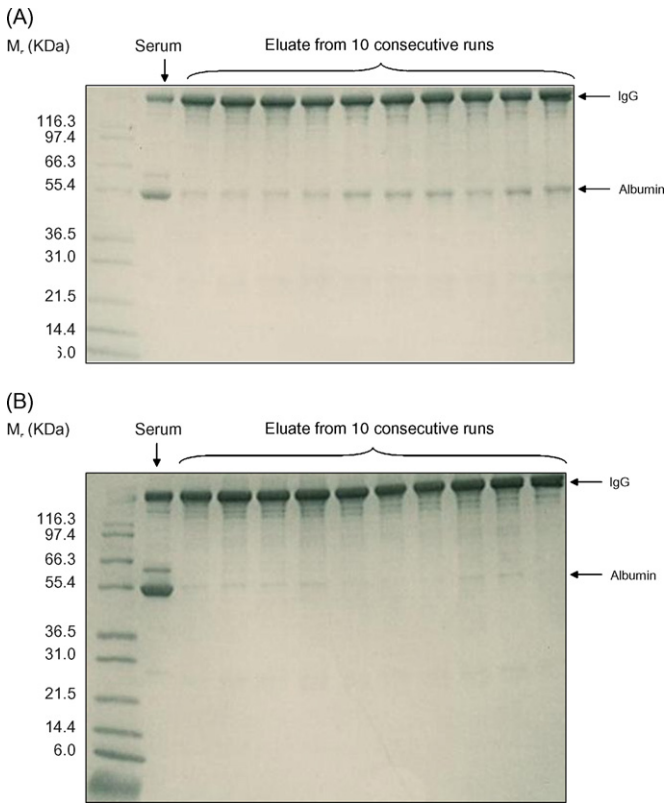

Fig. 4. SDS-PAGE gel of the eluates (primary peaks, i.e. E2 in Fig. 2) collected from 10 consecutive runs when a packed bed of the prototype was challenged with 1 mL of 0.2 μm filtered ovine serum: (A) without a caprylic acid wash; (B) with caprylic acid. Purity drops appreciably during runs 5–10 when operating without a caprylic acid wash.

again after 10 consecutive runs and an average of 27.2 mg/mL was obtained—close to the 29.2 mg/mL obtained in Section 3.1, suggesting minimal loss of binding capacity. It should be noted that when operating under the conditions described, the volume of the eluate was around 10–11 mL from each column run (10× the load volume; the authors propose that this is due to gradual elution of polyclonal isoforms from the column) suggesting that concentration would be needed before subsequent processing.

3.3. Column cleaning

Relatively harsh cleaning agents are desirable for process-scale chromatography when purifying crude materials in order to eliminate adsorbed protein and remove the potential risk of bioburden that could adversely affect subsequent column runs and product quality. Although chaotropic agents such as urea or guanidine hydrochloride are commonly used for cleaning Protein A columns and are highly efficient agents for removing unwanted contaminants, their high costs and the disposal problems associated with their use can make them unattractive for commercial operation [3]. A major advantage of the prototype adsorbent is that it utilises a conjugated chemical ligand and may therefore show tolerance to alternative cleaning solutions.

Given that sodium hydroxide is commonly used to clean non-protein ligands, initial experiments to evaluate the cleaning and sanitisation of the prototype adsorbent used five-column volumes of 0.5 M NaOH. A 3.14 mL packed bed of the prototype adsorbent was subjected to 1 mL of ovine serum, washed and eluted as described in Section 2.4 and then cleaned and sanitised with 0.5 M NaOH, prior to re-equilibration and application of another 1 mL of ovine feed. The chromatogram in Fig. 5a was then obtained, with no initial shoulder and only a small elution peak. The flowthrough was collected in 1 mL fractions and was shown by SDS-PAGE analysis to contain large amounts of IgG and serum proteins whilst the eluate lane only contained a faint band of IgG (Fig. 5b). The gel also revealed a significant amount of 'smearing' along the flowthrough lanes. Given that the only difference between this bed and those described in Section 3.2 was the application of a 0.5 M NaOH sanitisation step, the smearing may therefore represent leached ligand arising from NaOH wash that in turn results in a reduced column capacity. In this respect, the prototype behaves similarly to conventional Protein A resins in terms of its low tolerance of high pH conditions. It is important to note, however, that studies conducted at Millipore indicate that the matrix is stable against caustic reagents and hence the results observed with sodium hydroxide may also be due to residual NaOH present within the column.

Conversely, when five-column volumes of 0.1 M HCl were used to clean a 3.38 mL column of the prototype and the subse-

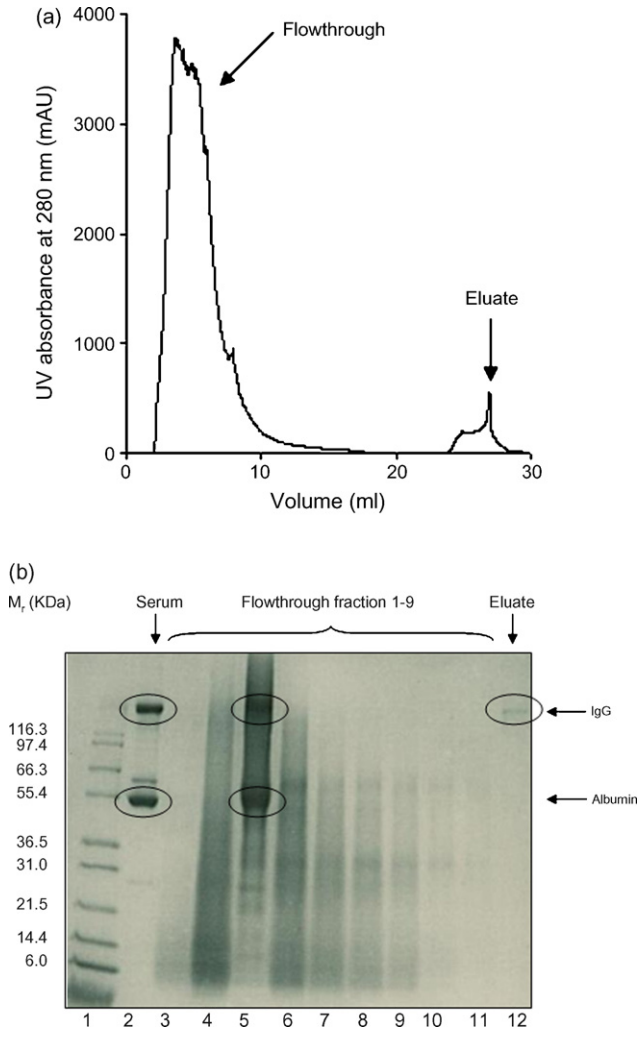

Fig. 5. (a) Chromatogram obtained when applying ovine serum after a five-column volume 0.5 M NaOH wash. Minimal IgG appears to bind to the column. (b) SDS-PAGE gel of the flowthrough and elution fractions after 0.5 M NaOH washing. Lanes: 1, marker; 2, ovine serum; 3–11, the nine flowthrough fractions; 12, eluate.

quent 1 mL ovine feed was applied, a similar chromatogram to Fig. 2 was obtained. Fractions of the flowthrough and the elution peaks were also shown by gel analysis to contain similar proportions of IgG and serum proteins to those obtained previously (Section 3.2, Figs. 3 and 4). Closer examination of the UV trace during the HCl wash showed small peaks appearing within one- to two-column volumes (data not shown), suggesting that there was very little build-up of adsorbed protein to the bed. The experiment was continued for repeated loading cycles, which demonstrated that consistently high yields (just under 90%) and purities (95%) were achieved over nine affinity cycles. Hence 0.1 M HCl appears to be a suitable agent for cleaning the prototype adsorbent when purifying polyclonal antibodies from serum.

3.4. Inverted confocal microscopy

Confocal microscopy was used to evaluate binding and subsequent diffusion of fluorescent IgG over time. Fig. 6 shows the

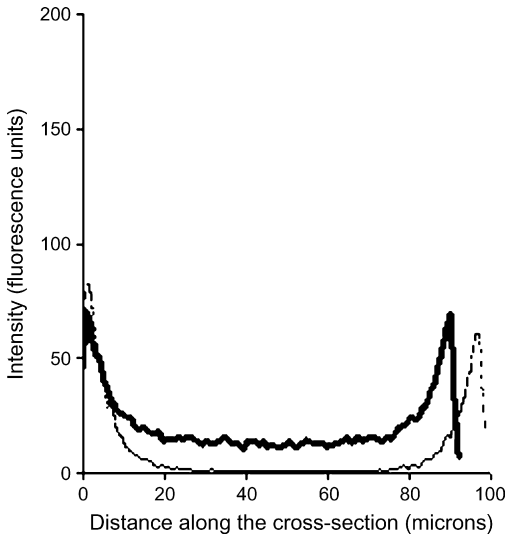

Fig. 6. Fluorescence intensity profile along the cross-section of the adsorbent particles immediately after matrix incubation: (▬▬) prototype adsorbent; (– – –) Protein G Sepharose.

fluorescence intensity profiles along the cross-sections of the beads of both the prototype and Protein G Sepharose immediately after matrix incubation, whilst Fig. 7 shows the equivalent profiles after 150 min. Immediately after incubation, the prototype shows similar intensities at the edge of the beads compared to the Protein G Sepharose. Conversely, the Protein G adsorbent shows no protein binding at all across most of the central 50% of the bead, whereas for the prototype there is an appreciable IgG concentration in the centre. After 150 min, both matrices exhibit similar profiles (Fig. 7). Despite deliberately over-saturating with protein, however, the IgG appears to concentrate primarily along the outer 20% of the beads in the case of both resins, leaving a central core that exhibits only a third of the intensity seen on the outside. The experiment was repeated with a 24 h incubation

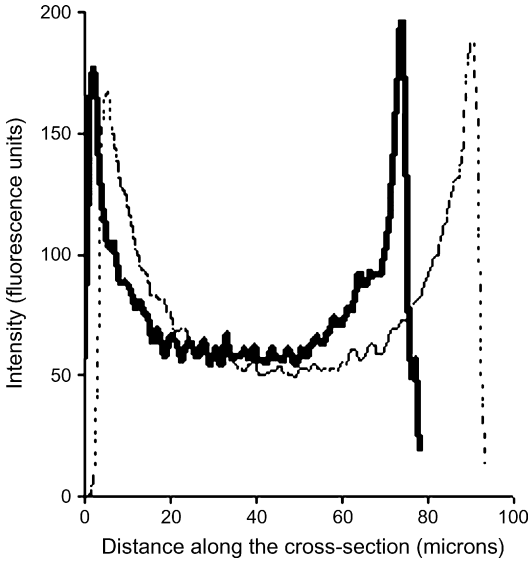

Fig. 7. Fluorescence intensity profile along the cross-section of the adsorbent particles after incubation for 150 min: (▬▬) prototype adsorbent; (– – –) Protein G Sepharose.

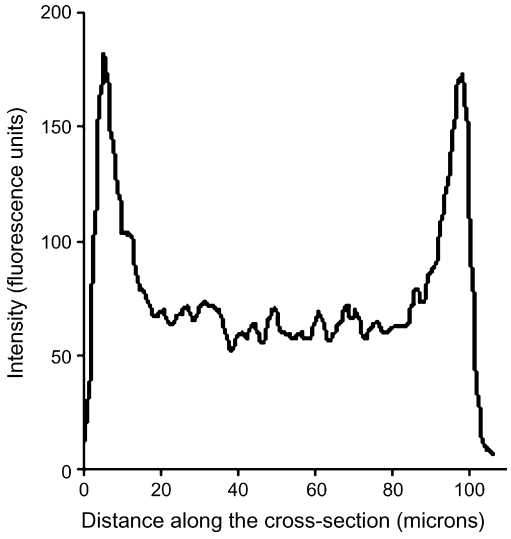

Fig. 8. Fluorescence intensity profile along the cross-section of a bead of the prototype adsorbent after incubation for 24 h.

of the prototype adsorbent. The results in Fig. 8 show that after this period of time, the intensity profile is similar to that obtained after 150 min, suggesting that equilibrium has been reached and no further IgG can bind. These data therefore imply that the IgG uptake characteristics of the novel adsorbent are similar to those of Protein G Sepharose and that the prototype may be a viable option for the commercial-scale capture and purification of polyclonal antibodies from crude industrial feedstocks such as ovine serum. Please note that due to commercial limitations at the time of writing, details of the bound synthetic chemical ligand (structure, charge, bead density and hydrophobicity) have not been disclosed and hence no further characterisation studies were undertaken.

In the control experiments, the intensity profile was shown to remain constant in the 15 min during which analysis of the prototype adsorbent occurred, implying that there was no photobleaching over the course of the experiments. It was also shown that there was no binding of the fluorescently labelled IgG to the Sepharose CL 4B agarose beads, implying that the fluorescence patterns seen with the prototype and Protein G Sepharose were due to the binding of IgG to the ligand and not due to non-specific binding of fluorophore. Intensity profiles were also comparable for each of the three beads analysed at each time point, suggesting that the data that formed the basis of the evaluation were a representative sample.

3.5. Comparison to other research in the field

The studies presented in this paper have sought to extend the growing body of research into alternatives to biospecific resins such as Protein A or Protein G for the purification of antibodies. Developments to date include:

- Engineering proteins and peptides to mimic antibody-binding activity.
- Creating pseudo-biospecific resins, such as those based on hydrophobic, thiophilic or hydroxyapatite ligands, or small molecule mimics developed using molecular modelling and combinatorial chemistry to replicate the interaction between the Phe132-Tyr-133 dipeptide in Protein A and antibody molecules.

Table 5
Capacities of engineered and pseudo-biospecific adsorbents used previously for antibody capture

Matrix	Feed material	IgG capacity (mg/mL)	Reference
Peptide (TG19318)	Rabbit serum	10–25	[22,23]
MAbsorbent A2P	Rabbit serum	10–15	[12]
MEP Hypercel	Bovine IgG	20	[24]

Examples of recent studies into the use of these media for antibody purification include the capture of human Fab from *E. coli* cell extracts using a mixed mode adsorbent operating in expanded bed mode [18] and the recovery of polyclonal IgG from a variety of feedstocks such as human plasma, ascites fluid and fetal calf serum using an artificial Protein A [19]. The results presented in the current paper show that the novel adsorbent compares well with these and other publications on synthetics. Data in the literature on yields achieved using pseudo-biospecific resins vary over a wide range, with values spanning from 4 to 99% [20], so the prototype appears to achieve recoveries at the top end of what is possible. Furthermore, the high purities obtained with the novel adsorbent are in line with those from other pseudo-biospecific resins (>90% in several cases [20]). The prototype is also similar to these matrices in that relatively inexpensive washing steps can be used prior to elution to remove adsorbed contaminants [7,21] and thus achieve purification factors equal to those of Protein A. Finally, the 29.2 mg/ml capacity of the novel adsorbent is relatively high compared to previous results (see Table 5).

3.6. Significance of the findings

Advances in fermentation technologies in recent years have resulted in significantly higher product titres that are now shifting the throughput bottleneck further downstream. From a commercial point of view, there is now a growing need to identify strategies that will allow recovery operations to handle higher concentration feeds. From this standpoint, the novel adsorbent offers a promising option for the direct capture and purification of antibodies from crude and viscous materials, achieving high yields and purities. This is aided by the relatively high binding capacity compared to commercially available alternatives and therefore compares favourably with the highly expensive Protein A and Protein G.

4. Conclusions

This paper has evaluated a prototype adsorbent for its ability to recover and purify polyclonal antibodies from crude hyperimmunised ovine serum. The adsorbent was shown to have a higher dynamic capacity for polyclonal IgG than a range of other commercially available matrices and with a post-load caprylic

acid wash, was shown to achieve consistently high yields and purities over 10 purification cycles with minimal drop in binding capacity. It was also demonstrated that 0.1 M hydrochloric acid could be applied to clean the column efficiently. Inverted confocal microscopy was used to visualise the antibody binding characteristics for individual beads of the prototype matrix, producing similar results to those achieved with a commercially available Protein G. These data indicate that the prototype adsorbent is a feasible option for the recovery of polyclonal antibodies from ovine serum and may therefore have wider applicability to other complex feedstocks of industrial relevance. The high binding capacities and eluted IgG purities observed suggest that this 'next generation' synthetic alternative to Protein A or G chromatography adsorbents may be suitable for the commercial scale purification of other products such as therapeutic monoclonal antibodies from mammalian cell culture or from feedstocks produced using transgenic technologies.

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References

[1] S. Flatman, I. Alam, J. Gerard, N. Mussa, J. Chromatogr. B 848 (2007) 79.
[2] A.C.A. Roque, C.R. Lowe, M.A. Taipa, Biotechnol. Prog. 20 (2004) 639.
[3] A.A. Shukla, B. Hubbard, T. Tressel, S. Guhan, D. Low, J. Chromatogr. B 848 (2007) 28.
[4] S. Farid, Adv. Biochem. Eng./Biotechnol. 101 (2006) 1.
[5] R.L. Fahrner, G.S. Blank, G.A. Zapata, J. Biotechnol. 75 (1999) 273.
[6] K. Swinnen, A. Krul, I. Van Goidsenhoven, N. Van Tichelt, A. Roosen, K. Van Houdt, J. Chromatogr. B 848 (2007) 97.
[7] A.R. Newcombe, C. Cresswell, S. Davies, K. Watson, G. Harris, K. O'Donovan, R. Francis, J. Chromatogr. B 814 (2005) 209.
[8] K. Huse, H.-J. Böhme, G.H. Scholz, J. Biochem. Biophys. Meth. 51 (2002) 217.
[9] H. Yavuz, S. Akgöl, R. Say, A. Denizli, Int. J. Biol. Macromol. 39 (2006) 303.
[10] S. Hober, K. Nord, M. Linhult, J. Chromatogr. B 848 (2007) 40.
[11] S. Chhatre, N.J. Titchener-Hooker, A.R. Newcombe, E. Keshavarz-Moore, Nat. Protoc. 2 (2007) 1763.
[12] H. Bak, O.R.T. Thomas, J. Chromatogr. B 848 (2007) 116.
[13] C. Newcombe, A.R. Newcombe, J. Chromatogr. B 848 (2007) 2.
[14] G. Neal, J. Christie, E. Keshavarz-Moore, P. Ayazi Shamlou, Biotechnol. Bioeng. 81 (2003) 149.
[15] G. Neal, R. Francis, P. Ayazi Shamlou, E. Keshavarz-Moore, Biotechnol. Appl. Biochem. 39 (2004) 241.
[16] Millipore Technical Brief: Determination of Dynamic Binding Capacity for ProSep®-vA Media, http://www.millipore.com, Literature Number TB1175EN00, 2005.
[17] P. Thillaivinayagalingam, K. O'Donovan, A.R. Newcombe, E. Keshavarz-Moore, J. Chromatogr. B 848 (2007) 88.
[18] M.B. Hansen, A. Lihme, M. Spitali, D.J. King, Bioseparation 8 (1999) 189.
[19] R. Li, V. Dowd, D.J. Stewart, S.J. Burton, C.R. Lowe, Nat. Biotechnol. 16 (1998) 190.
[20] A.C.A. Roque, C.S.O. Silva, M.A. Taipa, J. Chromatogr. A 1160 (2007) 44.
[21] E. Boschetti, TibTech 20 (8) (2002) 333.
[22] G. Fassina, A. Verdoliva, G. Palombo, M. Ruvo, G. Cassini, J. Mol. Recog. 11 (1998) 128.
[23] G. Fassina, M. Ruvo, G. Palombo, A. Verdoliva, M. Marino, J. Biochem. Biophys. Meth. 49 (2001) 481.
[24] V.B. Brochier, A. Schapman, P. Santambien, L. Britisch, Fast purification process optimization using mixed-mode chromatography sorbents in pre-packed mini-columns, J. Chromatogr. A, 2007, doi:10.1016/j.chroma.2007.08.086, in press.