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Characterisation of an industrial affinity process used in the manufacturing of digoxin-specific polyclonal Fab fragments☆

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Abstract

This paper describes the effect of several variables on the affinity process for the production of the FDA approved biotherapeutic product Digoxin Immune Fab (Ovine) (DigiFab™, Protherics Inc., TN, USA). The study considers the effects of column re-use on matrix capacity and on the subsequent recovery of the antibody product, and the impact of varying column loading on matrix performance. The methodology used could be equally applied to assess the feasibility of using an affinity matrix for commercial scale purification of alternative antibody derived biotherapeutics. The capacity and specific Fab recovery were calculated through 24 h equilibrium and mass balance studies. Results were assessed against data obtained through confocal scanning laser microscopy. Scale-down experiments produced specific Fab recoveries and purities that were comparable with those at production scale. The matrix capacity was found to be 45 ± 15 mg of Fab/ml of matrix. Through the use of fluorescent DigiFab and confocal scanning techniques, Fab uptake onto single affinity bead was evaluated. Average intensity values calculated for each sample provided direct real-time, measure of Fab binding and matrix capacity. The results suggest that the affinity matrix had a limited reuse life as a drop in recovery is observed following the completion of a small number of process cycles (30% after three runs). The findings support that which is seen at the current manufacturing scale, where the affinity column is used for a limited number of runs. Results from this study can be used as a basis for future optimisation of this purification process.

Keywords: Chromatography; Affinity purification; Antibody; Confocal scanning laser microscopy

1. Introduction

Antibodies and antibody derivatives are currently thought to constitute 20% of biopharmaceutical products in development [1] with annual revenue of $3–$4 billion [2]. It was estimated that in 2002, 470 antibody products were in development worldwide by some 200 companies [3]. Polyclonal antibodies are generated via hyper-immunisation in variety of host animals, including rabbit, goat, sheep and chicken [1]. Since animal serum contains a mixed population of antibodies against an entire range of epitopes, polyclonal antibodies are often considered more efficacious in some situations, especially for the treatment of acute illness and medical emergencies, as they can bind to multiple epitopes on the disease-causing agent [1]. However, hyperimmune serum will contain a significant amount of irrelevant and non specific immunoglobulins, hence the manufacturing challenge is to identify and separate specific Igs from this fraction [2]. This typically requires a custom affinity column to ensure sufficient removal of non specific antibodies or fragments. The use of custom affinity resins for commercial biopharmaceutical production presents a number of technical challenges. The target ligand must be commercially available and suitable for large scale conjugation to an activated base matrix. In addition, the custom affinity matrix must exhibit minimal non specific binding, show acceptable ligand stability and demonstrate low levels of ligand leakage to permit the necessary re-use of the affinity adsorbent. Furthermore, the capacity and re-use of a custom affinity column (over the duration of the affinity campaigns)

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plus the associated costs of matrix cleaning validation may have a significant impact on the commercial viability of a biotherapeutic purification process. For some affinity columns it may be more cost effective to treat each batch of affinity matrix as a disposable (single use or single campaign) consumable within the manufacturing process.

Affinity adsorbents account for greater than 35% of the total recovery raw material costs at large scale [4]. The ligands may also denature under harsh sanitization conditions and reduce dynamic capacity of the matrix. Custom ligands may be costly to develop and in-house coupling protocol is needed, it is therefore important to fully characterize the process step to produce the most efficient process and avoid costly mistakes.

Polyclonal therapeutic antigen binding fragments (Fabs) from cleaved antibodies have been used for many years to treat poisoning with digoxin, digitoxin, and a range of other structurally related compounds, including cardiotoxins from the oleander plant (*Nerium oleander*) [5] and toads (*Bufo* sp.) [6]. Digoxin is prescribed for treating a range of heart conditions but can cause life-threatening toxicity when blood levels of the drug rise above its narrow therapeutic range. Severe digitalis intoxication is preferentially treated by intravenous infusion of Fab fragments of digoxin-specific antibodies. At least two FDA approved biotherapeutic products are currently marketed for the treatment of digoxin toxicity, including Digoxin Immune Fab (Ovine) (DigiFab™, Protherics Inc., TN, USA). Digoxin-specific Fab is generally well tolerated and clinically effective in patients with potentially life-threatening digitalis toxicity [7].

In this paper, mass balances expressed in terms of total protein and antibody fragments, batch uptake kinetics and an imaging technique are employed in-order to optimise the existing industrial affinity process (Protherics UK Ltd.) used in the purification of DigiFab™ [8,9]. A ligand specific fluorescence technique utilising, confocal scanning microscopy [10–13] has been used to investigate the binding of fluorescent Fab to a single Fab specific affinity bead and to evaluate changes in binding capacity over multiple affinity runs.

2. Materials and methods

2.1. DigiFab manufacturing process outline

Sheep are immunized with a digoxin analogue, digoxin-dicarboxymethoxylamine (DDMA) and then serum collected. The immunoglobulin fraction is isolated from ovine serum by a series of precipitation and centrifugation steps. Isolated (total) IgG fractions were then digested with papain as previously described [14]. The digoxin-specific Fab is isolated by affinity chromatography. DDMA was used as the ligand for affinity process. Custom affinity columns (DDMA coupled to 6-amino Sepharose (Amersham Biosciences, Buckinghamshire, UK Ltd.)) are produced at Protherics UK Ltd. (Llandysul, Wales). Purified Fab was concentrated and diafiltered prior to lyophilisation. An outline of the DigiFab™ manufacturing process is shown in Fig. 1.

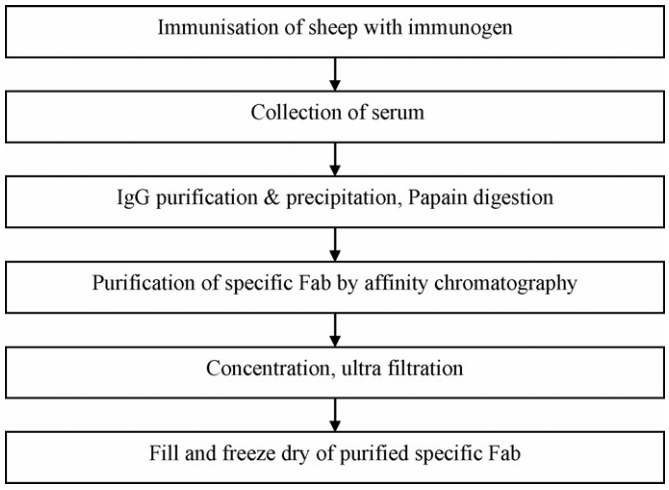

Fig. 1. DigiFab™ manufacturing process outline.

Table 1
Production and lab scale column set up

	Production	Scale down
Column height	5.50 cm	5.50 cm
Column diameter	20.00 cm	1.00 cm
Column volume	1.70 l	4.32 ml
Cross sectional area	314.16 cm^2	0.79 cm^2
Optimum loading range (total protein)	340–850 g	900–2250 mg
Load flow rate	10.00 l/h	0.40 ml/min
STB, acid wash and equilibration flow rate	13.00 l/h	0.54 ml/min
Elution flow rate	4.00 l/h	0.17 ml/min

Scale down was based on reduction of the column diameter by a factor of 20, while column length, pH of buffers and process time were kept constant. Manufacturing loading range is between 200 and 500 g/l of matrix.

2.2. DigiFab™ affinity purification

Production scale affinity chromatography for the purification of DigiFab™ was operated according to Table 1. The packed column was cleaned and sanitized using buffers listed in Table 2. Sanitisation of the affinity column was carried out using 20% (v/v) ethanol and 0.1 M sodium hydroxide. Ethanol and sodium hydroxide in this buffer means that it acts as a bactericidal agent and prevents microbial contamination of the column. During

Table 2
DigiFab™ affinity purification step

	Buffer	Number of CV[a]
Load[b]	Sheep serum load material	To desired volume
First wash (STB)	12 mM disodium tetraborate	3
Acid wash (PPL)	1% (v/v) propronic acid pH 3.7	14
Elution (ALP)	5% (v/v) acetonitrile and 3.6% (v/v) propionic acid pH 2.5–2.7	19
Equilibration (STB)	12 mM disodium tetraborate	4
Sanitisation	20% (v/v) ethanol and 0.1 M sodium hydroxide	4

[a] CV, column volume.
[b] Manufacturer's load range is 200–500 g/l of total protein.

the affinity run a sodium tetraborate (STB) wash was used for column equilibration and an acid wash was used to remove non specific antibodies or adsorbed impurities from the column prior to elution of specific Fab.

2.3. Scaled down chromatography studies

Scaled down chromatography runs were based on reduction of the column diameter by a factor of 20, while the flow rates, bed height, buffers and process time were kept constant [15,16]. The column was set up according to the information provided in Table 1. A frozen production sample (500 ml) of post digestion total Fab (in PBS) from previous a ultra filtration stage undertaken at Protherics UK Ltd. was thawed overnight and stored at 2–8 °C. The total protein concentration of load material was 57 mg/ml. Scaled down affinity chromatography was undertaken by packing a C10/10 column (Amersham Biosciences Ltd.) with 4.5 ml of DigiFab affinity matrix, 5.7 cm bed height × 1.0 cm diameter (custom manufactured matrix, Protherics UK Ltd.). The column was cleaned and sanitized, as described, prior to use and 1575 mg (load concentration of 350 mg/ml of matrix) of total protein was loaded (27.18 ml) onto the column. A sample of protein was filtered through a 0.2 μm syringe filter (Sartorious Ltd., Epsom, UK) to remove insoluble protein and to mimic current production conditions. The column was loaded at 30 cm/h (0.4 ml/min, residence time 11.3 min). After loading, the column was first washed with STB buffer for three column volumes (CV) at 40 cm/h (0.54 ml/min), then with acid wash for 14 CV at 40 cm/h. The bound protein (specific Fab) was eluted for 19 CV at 13 cm/h (0.17 ml/min) (Tables 1 and 2). The eluted fractions were pooled and the total protein concentration of antibody was determined by absorbance at 280 nm using an absorption coefficient (1.0 mg/ml) of 1.4. Fab purities were determined by Coomassie stained SDS-PAGE and scanning densitometry analysis. Results obtained regarding specific Fab yield, purity, papain and Fc concentration from this scale down run were compared with a manufacturing run (data from Protherics UK Ltd.).

2.4. Mass balance and recovery versus load calculations

A C10/10 column (Amersham Biosciences Ltd.) was packed with 4.32 ml (5.5 cm bed height, 1.0 cm diameter) of DigiFab affinity matrix. The column was cleaned and sanitized as described previously. Total protein concentration of load material was calculated to be 59 mg/ml (QC (Analytical) Laboratories, Protherics UK Ltd.). To evaluate matrix capacity, six affinity runs at 432, 864, 1296, 1728, 2160, and 3888 mg of total protein (7.27, 14.53, 21.8, 29.06, 36.33, and 65.39 ml) loading were carried out as mentioned above (see Section 3). Six consecutive runs on the same column were carried out to mimic current manufacturing conditions (currently the affinity column is used for a maximum of seven runs). The eluted fractions from each run were pooled and total protein recovered was determined. Mass balance on the first run (432 mg loading) and the fifth run (2160 mg loading) was determined. In this case all three fractions (STB wash, acid wash and elution) were collected and assayed for total protein concentration and specific Fab concentration.

2.5. 24 h equilibrium binding experiments

Eluted specific Fab from runs 2–5 (864–2260 mg loading) were pooled (protein concentration 2.0 mg/ml). The pH of pooled protein was adjusted to pH 6.48 at 21.5 °C using 1 M NaOH. The pH adjusted solution was then filtered using 0.2 μm filter (Sartorious Ltd.) and assayed for Fab concentration. The concentration after pH adjustment and filtration was 1.75 mg/ml. Centricon plus-20 centrifugal filter devices (Millipore Ltd., Tyne and Wear, UK) were used according to instructions supplied by Millipore to concentrate 350 ml of eluted protein at 1.75 mg/ml down to 2 ml at 287 mg/ml concentration. The protein solution was highly precipitated, hence it was diluted down to a more workable concentration of 40 mg/ml. Slide-A-Lyser dialysis cassette 10 kDa (Pierce Ltd., Rockford, UK) was used according to the manufacturer's instructions, to buffer exchange the concentrated protein in to PBS. The sample pH after overnight dialysis was pH 7.6. The final concentration of the sample after buffer exchange was 26 mg/ml.

Hundred microliters of DigiFab matrix (Protherics UK Ltd.) equilibrated in PBS was pipetted into a series of Eppendorf tubes. Samples of purified Fab were diluted in PBS accordingly to achieve concentrations ranging between 26 and 1 mg/ml. Hundred microliters of each sample was pipetted into separate Eppendorf tubes containing DigiFab affinity matrix and placed on an end over end stirrer. After 24 h the Eppendorf tubes were spun in a Beckman GS-6R centrifuge (Beckman Coulter UK Ltd., Buckinghamshire) at 1300 rpm for 2 min, to pack the matrix to the bottom of the tube. Eighty microliters of the mobile phase was removed and analyzed for total protein concentration. The data obtained from these 24 h equilibrium experiments were evaluated employing the Langmuir–Freundlich correlation given in Eq. (1) [17–20]:

$$C_s = \frac{Q_m C_m^n}{(1/K_a) + C_m^n} \quad (1)$$

where C_s represents the concentration of bound solute at equilibrium with the mobile phase having a concentration C_m, Q_m the maximum capacity of the matrix, K_a the association constant, and n represents Langmuir–Freundlich coefficient. The maximum capacity of the column was calculated by iteration using SigmaPlot (Systat Software UK Ltd., Hounslow, UK).

2.6. Confocal scanning laser microscopy

The fluorescence intensity profiles and the distribution of bound Fab onto the affinity adsorbent particles can be measured using confocal scanning laser microscopy. This permits the analysis of a two-dimensional optical section of the adsorbent particle. The fluorescent signal provides a direct measurement correlating the amount of protein uptake per matrix bead. This could then be used to estimate column capacity [13]. Hence, confocal scanning laser microscopy was used to monitor direct, real time binding of fluorescent DigiFab™ and to compare affinity

matrix performance after 1, 2, and 3 consecutive manufacturing batches. Total protein concentration of load material was calculated to be 59.46 mg/ml and 1296 mg (300 mg/ml) of total protein was loaded onto an affinity column as described above (Section 2.3, Tables 1 and 2). After one manufacturing run, the column was dismantled and the used matrix stored in 20% ethanol. This was repeated with affinity columns stored after two and three manufacturing runs and samples stored at 2–8 °C for further use.

Lyophilised vials of DigiFab™ (Protherics UK Ltd.) each containing 40 mg of Fab, were resuspended with 1 M sodium carbonate (pH 9.3 at room temperature). DigiFab™ was fluorescently labeled with Cy3 Dye (Amersham Bioscience Ltd.). One milliliter of DigiFab™ (2 mg/ml) was added to a vial of fluorophore (one vial contains 0.2–0.3 mg of dye) and left to incubate for 40 min (with occasional inversion) at room temperature. One vial of Cy3 dyes 1 mg of DigiFab™. One milliliter of each matrix (new, after 1, 2 and 3 consecutive runs) was centrifuged at 3000 rpm using microfuge 11 (Beckman Coulter Ltd.) for 2 min. One milliliter of PBS pH 7.4 was added to each matrix and centrifuged at 3000 rpm for 2 min. This process was repeated twice to wash the matrix and remove ethanol and the matrix gently resuspended in 200 μl PBS. Four test tubes were filled with 4 ml of 2 mg/ml unlabelled DigiFab™. To these, 1 ml of 2 mg/ml labeled DigiFab™ was added to give 10 mg of protein. To over saturate the affinity beads, 0.125 μl of each matrix was added to the protein sample according to the ratio of 1:40 (volume of matrix/volume of DigiFab™). This was left to incubate for 3 h (continual shaking). Three hundred microliters of this mixture was placed in an Eppendorf tube and spun down at 3000 rpm for 2 min. One milliliter of PBS was added to the matrix and centrifuged at 3000 rpm for 2 min. This was repeated twice to remove unbound Fab and free (unconjugated) fluorophore. Twelve microliters PBS was finally added to each matrix sample and left to equilibrate. Leica inverted confocal microscope (Leica Microsystems, Bucks Milton Keynes, UK Ltd.) was set up and operated according to manufacturer's instructions. A sample (12 μl) of each matrix (after 0, 1, 2, and 3 runs) was placed under the microscope (20× magnification) and 20 images of beads were taken (image size was set at 512 pixel). To ensure that the target bead was in the focal plane of the microscope, all images were focused at the centre of the bead. The beads were excited at 568 nm and emitted between 573 and 643 nm.The fluorescent signal of Fab bound to individual beads remained constant over the duration of the experiments and indicate that respective signal changes are due to variable levels of Fab binding and not due to photobleaching over the course of the experiments. Native sephahrose 4B matrix (with no ligand attached) (Amersham Bioscience Ltd.), non specific ovine polyclonal IgG and ovine Fab fragment (Stratech Scientific UK Ltd., Cambridgshire) were used as controls to show specific binding of Fab to affinity beads (data not shown). Since fluorescence intensity profiles show adsorption of Fab onto affinity surfaces, a change in intensity value corresponds to a change in the binding capacity of the column. The images were analyzed using ImageJ software (v1.33u, ImageJ, National Institutes of Health, MD, USA) and fluorescent intensities across the bead were calculated.

2.7. Total protein concentrations

Samples were diluted to 2 mg/ml (working range of spectrometer) and adsorption readings at 280 nm were observed using a Cecil CE 2041 spectrophotometer (Cecil Instruments UK Ltd., Cambridge). The total protein concentration was then determined using an adsorption coefficient at 280 nm of 1.4 (generic value for polycolonal antibody concentration, Quality Control Laboratories, Protherics UK Ltd.). Each sample were analysed three times and the maximum standard deviation was calculated to be ±1.5. Specific Fab concentrations and digoxin binding capacity was determined within the Quality Control Laboratories, Protherics UK Ltd., using a validated in-house Polarization Fluoroimmunoassay (PFIA) [21]. All specific Fab concentrations were calculated from three independent determinations and the maximum standard deviation was calculated to be ±4.5.

2.8. SDS-PAGE

Non reduced SDS-PAGE was carried out using Novex® Tris–glycine pre-cast 4–20% polyacrylamide gels (Invitrogen UK Ltd., Paisley) and a discontinuous Tris–glycine buffering system. All samples were prepared by mixing an equal volume with 2× SDS sample buffer (Invitrogen Ltd.) and heating in boiling water bath for 2 min. Gels were typically loaded with 5 μg protein per sample lane and electrophoresed at constant voltage of 150 V. Protein bands were visualized by staining for a minimum of 60 min with 0.1% (w/v) Coomassie Brilliant Blue R250 (Sigma–Aldrich UK Ltd., Gillingham, UK) and destained with 40% (w/v) methanol, 10% (w/v) glacial acetic acid. Band intensities were quantified by scanning densitometry analysis using ImageMaster Total Lab v2.01 (Amersham Biosciences Ltd.).

3. Results and discussion

3.1. Scaled down chromatography studies

Fig. 2 compares the purity of the eluted material from laboratory and production scale affinity chromatography runs. Eluted specific Fab (lanes 3, 4 and 5) from scaled down experiments showed banding patterns and intensities comparable to a DigiFab™ production reference (lane 6). A predominant band with an apparent molecular weight of 40–45 kDa was detected. Additional bands were also observed in the eluted product at 21.5 kDa which is inferred to be papain carried from previous digestion stage and a band at 31.0 kDa which is likely to be a Fc fragment [14]. Contaminating bands at 55.4 kDa (assumed to be ovine albumin) [1] and 10 kDa are observed in the load material and are removed by the affinity purification step. The total protein concentration of the eluted product was calculated to be 3.0 mg/ml (Table 3) which gave an overall specific Fab yield of 13%. Specifications for specific Fab yield defined by the manufacturers (Protherics UK Ltd.) when loading between 200 and 500 mg/ml are between 10 and 18%. Purity was

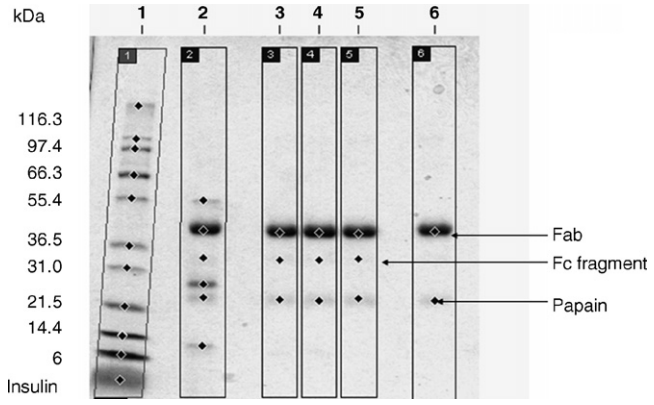

Fig. 2. Non reduced SDS-PAGE analysis of purified Fab, to compare scaled down runs with production run. Black dots represent bands detected by scanning densitometry analysis. Lane 1, molecular weight markers (Mark 12, Invitrogen UK). Lane 2, Affinity column load material (5 μg). Lanes 3, 4 and 5, scale down runs (5 μg load, purity 90%). Lane 6, production run (5 μg load, purity 84%). DigiFab product appears as expected with a predominant band at approximately 40–45 kDa in all samples. Minor impurities detected in the load material (lane 2) represent ovine albumin and digest fragments.

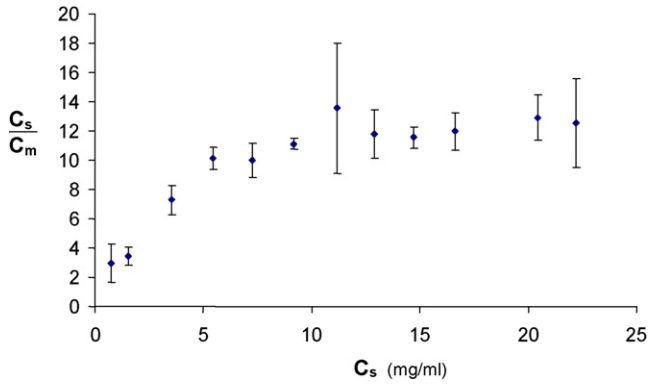

Fig. 3. Graph of C_s/C_m vs. C_s (Scatchard plot) where C_s is the concentration of bound solute in mg/ml and C_m is the concentration of solute in the mobile phase in mg/ml, produced a non linear plot indicating positive cooperativity. This data was fitted to Eq. (1) using SigmaPlot. SigmaPlot uses iteration to find the coefficients that give the best fit between the equation and the data. From this maximum capacity of the column was calculated to be 45 ± 15 mg/ml of matrix. Since volume of laboratory scale column was 4.32 ml, then total capacity of column was 193 ± 65 mg. Error bars shown represent standard deviation ($n = 6$).

calculated to be 90% and minor impurities were comparable with a production reference (Fig. 2). These results suggest that current production scale DigiFab™ affinity process can be successfully scaled down with eluted product purities comparable to samples manufactured at production scale. Therefore, the scale down experiments described were carried out as the basis for characterization of the affinity process.

3.2. *Performance characteristics of the current DigiFab™ affinity process*

C_s (concentration of bound solute in mg/ml) and C_m (concentration of solute in the mobile phase in mg/ml) values obtained from 24 h equilibrium experiment were plotted in terms of C_s/C_m versus C_s to produce a Scatchard plot (Fig. 3). A positive initial slope, characteristics of positive coopperativity [17–20] in the low protein region is observed (where the values of C_s/C_m increases with the increasing value of C_s for C_s less than 12 mg/ml). This implies stronger but slower binding. A plateau is observed in the region beyond 13 mg/ml protein concentration. This is likely due to the appearance at higher protein concentrations of an associated species with weaker binding affinity for the surface than the other species existing at lower concentrations [20]. Hence, beyond 13 mg/ml, greater proportions of Fab in the mobile phase are less active.

A nonlinear Scatchard plot (Fig. 3) indicates deviation from Langmuir behaviour and so a Langmuir–Freundlich (this can describe both concave up and concave down curves) model was used to calculate the maximum capacity of the column (Q_m), by fitting experimental values of C_s and C_m to Eq. (1) [17,18] using SigmaPlot (Systat Software Ltd.). SigmaPlot uses iteration to find the coefficients that give the best fit between the equation and the data [17]. The maximum capacity of the column (Q_m) was calculated to be 45 ± 15 mg/ml of matrix. This suggests the total capacity of the scale down column (column volume 4.32 ml) is 193 ± 65 mg. Therefore, loading above 300 mg/ml (Table 4) will exceed the saturation capacity of the column. The Langmuir–Freundlich coefficient (n) was calculated to be 1.87, and since this is greater than 1, this is another indication of positive cooperativity in binding and the heterogeneous nature of the adsorption process [17].

Mass balance data (Table 5a) shows that all proteins loaded are recovered in the flow-through and eluted fractions, indicating that all three buffers are performing as expected. STB provides favourable conditions for binding of specific Fab to the column, while acid wash is removing non specific Fab and minor impurities. Specific Fab (DigiFab™) is eluted from the adsorbent using a low pH elution buffer. Therefore, when operating the column, binding sites are not being blocked by proteins as residual protein is not left bound to the column.

At a loading concentration of 100 mg/ml total protein, 79 mg is the total specific Fab loaded. Of this 65 mg (Table 4) of specific

Table 3
Comparison between production (data from Protherics UK Ltd., DigiFab™ manufacturing run) and scale down runs

	Production	Scale down
Concentration of load material	57 g/l	57 mg/ml
Total protein concentration	3.0 mg/ml	3.0 mg/ml
Specific Fab yield[a]	12%	13%
Specific antibody concentration	1.8 mg/ml	1.1 mg/ml
Purity[b]	84%	90%
Papain concentration[c]	0.1% (w/w)	0.1% (w/w)
Fc concentration[d]	0.2% (w/w)	0.2% (w/w)

[a] Specific Fab yield dependent on current manufacturing batch (total Fab in elution/total protein loaded). Typical specific Fab titres: 10–18%.

[b] Purity (Fab purity (40–45 kDa) band) >80% calculated by non reduced SDS-PAGE and scanning densitometry analysis.

[c] Papain concentration (contamination from previous digestion stage) − product specification = 0.1% (w/w) (weight of papain/weight of total protein in elution).

[d] Fc concentration − product specification = 0.2% (w/w) (weight of Fc/weight of total protein in elution).

Table 4
Total protein and Fab loaded vs. recovered after 1–6 affinity runs (loading concentrations 100–900 mg/ml)

Total protein load (mg/ml)	In (total)		Out (total)		Specific Fab yield (%)	Total Fab recovered (%)	% Drop (assume maximum possible recovery for run 1)
	Protein (mg)	Fab (mg)	Protein (mg)	Fab (mg)			
100	432	79	76	65	15	82	100
200	864	157	136	116	13	74	90
300	1296	235	162	137	11	58	70
400	1728	314	187	159	9	51	61
500	2160	392	207	175	8	45	54
900	3888	706	239	203	5	29	35

All eluted samples have a purity value of 85%. From this total Fab out was calculated. It is assumed that, at 100 mg/ml loading the recoveries obtained are the maximum possible (since all eluted product had 85% purity value), hence drop in recoveries were calculated as % drop from run 1. Remaining proteins in each sample are classed as minor impurities. Total capacity of column is 193 ± 65 mg. Therefore, loading above 246 mg/ml will begin to over saturate the column. All six runs were carried out on a single scale down column to mimic current manufacturing process by Protherics UK Ltd., where an affinity column is used for a maximum of seven runs. Total Fab recovered was calculated as total Fab out/total Fab in. Where as specific Fab yield was calculated as total Fab out/total protein in. Data presented are calculated as a mean of three repeat determinations and maximum standard deviation was calculated to be ±1.5.

Table 5a
Total protein mass balance at 100 and 500 mg/ml load concentrations (432 and 2160 mg of total protein, respectively)

Total protein in (mg)	Protein out (mg)			Total protein out (%)
	STB	Acid	Elution	
432 (100 mg/ml)	355 83%	8 2%	76 18%	103
2160 (500 mg/ml)	1825 85%	248 12%	207 10%	107

Samples from the first and fifth run (from pervious experiment; Table 3 and Fig. 5) were collected and assayed accordingly. Total protein calculated using adsorption at 280 nm (using extension coefficient of 1.4, which is generic for polyclonal antibody). All protein loaded is recovered in various streams. Data presented are calculated as a mean of three repeat determinations and maximum standard deviation was calculated to be ±1.5.

Fab is recovered in the elution step, therefore 82% of total Fab loaded is recovered. Of this 65 mg of specific Fab, 41 mg (Table 5b) is active and 24 mg is non active Fab. A combination of using a fresh column and loading below capacity (Table 4) has resulted in high recoveries being observed.

At a loading concentration of 500 mg/ml total protein, 392 mg is the total specific Fab loaded. Of this 175 mg (Table 4) of specific Fab is recovered in the elution step, therefore 45% of total Fab loaded is recovered. The concentration of load material was above capacity and, in addition, the column had gone through five consecutive manufacturing runs so low recoveries were observed. Out of the total specific Fab loaded, 162 and 109 mg are active in the elution and acid wash streams, respectively (Table 5b). This means 121 mg is non active Fab in all three streams.

Table 4 shows that after two consecutive runs on the column, there is 11% drop in yield and by the third run, yield has dropped by 30%. Therefore, this column could only be used a limited number of times before recoveries have a commercial impact on the affinity process. This data is consistent with the current manufacturing process at large scale, with each affinity column used for a limited number of affinity cycles.

The first run at 100 mg/ml loading produced a specific Fab yield at the top end of the desired range of 10–18%. This is due to a fresh matrix being loaded below capacity, but as loading and number of runs on the column increases, specific Fab yield reduces (Fig. 4) and by 900 mg/ml loading specific Fab yield has dropped below the manufacturer's range to 5%. Since binding sites are not being blocked by unbound proteins (Table 5a), this reduction in specific Fab yield could be due to combination

Table 5b
Specific Fab mass balance at 100 and 500 mg/ml load concentrations (79.0 and 392 mg of total specific Fab, respectively)

Total specific Fab in (mg)	Specific Fab out (mg)			Total specific Fab out (%)
	STB	Acid	Elution	
79 (100 mg/ml)	0 0%	0 0%	41 53%	53
392 (500 mg/ml)	0 0%	109 28%	162 41%	69

Specific Fab concentration calculated using polarization fluoroamunoassay (PFIA), which assays for active Fab. Therefore, if both active and non active are taken into account then, all Fab loaded is recovered in various streams All specific Fab concentrations were calculated from three independent determinations and the maximum standard deviation was calculated to be ±4.5. Overall error in PFIA calculations (validated assay by Protherics UK Ltd) is ±10%.

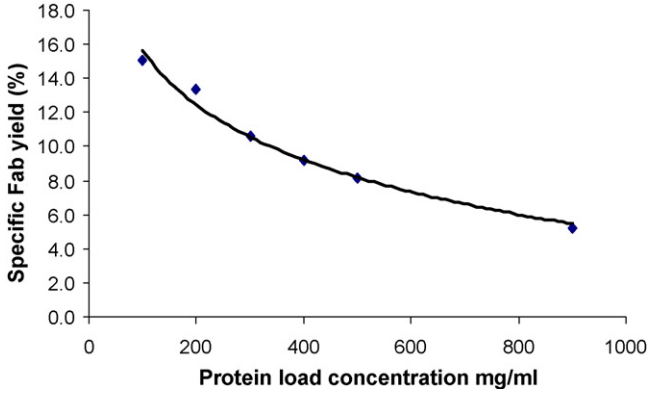

Fig. 4. Specific Fab yield (%) vs. load concentration. By 400 mg/ml loading specific Fab yield (%) is below manufacturers range of 10–18%. After three runs, column performance has dropped by 30%.

of loading above capacity, buffers impacting upon the affinity ligand or potential leaching of bound ligand.

3.3. Ligand leaching

Potential leaching of bound ligand was investigated using a biosensor (Biacore, Biacore international AB, Uppsala, Sweden) assay (manuscript in preparation). Fractions were collected from three blank affinity runs (no protein loaded) and assayed for leached DDMA eluting from the column. The results indicate minimal levels of leached ligand in all chromatographic fractions, with total levels of leached DDMA between 8 and 13 μg. As 29 mg of DDMA was coupled to the laboratory scale affinity column (data from Quality Control laboratories, Protherics UK Ltd.), the amount of ligand leaching off the column (0.05% total ligand per run) is likely to be insignificant over the number of affinity cycles evaluated for this study. Trace amounts of leached ligand in the purified Fab preparations are likely to be bound to Fab in solution and although the impact of DDMA toxicity has not been investigated as part of this report, the adsorbent described is currently used for production of an FDA approved biopharmaceutical DigiFab™, and the impact of ligand toxicity has been considered in detail as part of the biologics license application (BLA).

3.4. Use of alternative buffers

The effect of current buffers on column performance was investigated by running alternative buffers through the DigiFab™ scaled-down column. For this experiment STB was replaced by 25 mM phosphate buffer at pH 7.6, 50 mM citric acid adjusted to pH 3.6–3.9 was used as acid wash and eluted using 50 mM citric acid adjusted to pH 2.5–2.7. The absorbance profiles and recoveries of these runs mimicked those seen in previous laboratory scale DigiFab™ runs using existing production buffers and Fab appeared as expected by non reduced SDS-PAGE analysis, with banding patterns and intensities comparable to a DigiFab™ reference (Fig. 5). Total protein mass balance showed that all proteins loaded were recovered in the flow-throughs (data not shown); after each consecutive run, specific Fab yield reduces as observed when using existing buffers (Table 6). Therefore, after consecutive runs, the reductions in specific Fab yields seen (Table 4) are not due to buffers affecting the column matrix.

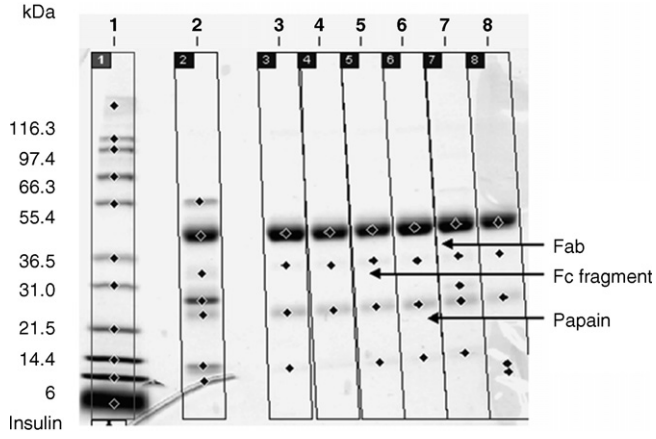

Fig. 5. Non reduced SDS-PAGE analysis of purified Fab collected from runs using alternative buffers. Black dots represent bands detected by scanning densitometry analysis. Lane 1, molecular weight markers (Mark 12, Invitrogen UK). Lane 2, affinity column load material (5 μg). Lanes 3 and 4 are scale down runs using alternative buffers at 200 mg/ml loading (5 μg load, purity 88%). Lanes 5 and 6 are runs at 300mg/ml loading (5 μg load, purity 87%). Lane 7, run at 400 mg/ml loading (5 μg load, purity 87%). Lane 8, run at 500 mg/ml loading (5 μg load, purity 85%). DigiFab product appears as expected with a predominant band at approximately 40–45 kDa in all samples. The purity of eluted Fab is equivalent to those seen when using current manufacturing buffers.

The results described in this report indicate that the reduction in column capacity over a limited number of affinity cycles is not due to leaching of bound ligand, non specific adsorption to the column or specific Fab that remains bound after elution. The results suggest that the reduction in column capacity occurs due to the physical characteristics of the chromatography adsorbent or ligand. The impacts of base matrix and cross linking are currently under investigation within this laboratory using three novel agarose absorbents, which have increased rigidity due to the presence of modified cross linking chemistries.

3.5. Confocal scanning laser microscopy

Confocal scanning laser microscopy was used to investigate real time, direct binding of DigiFab™ to affinity beads. It was hoped that this would provide additional data to assess matrix performance over consecutive affinity runs. No fluorescence signal was observed when labelled DigiFab™ was incubated with native sepharose 4B matrix (Amersham Bioscience Ltd.) or when fluorescently labelled non specific IgG or Fab (Stratech

Table 6
Fab loaded vs. recovered after 1–4 affinity runs (loading concentrations 200–500 mg/ml), using alternative buffers

Total protein load (mg/ml)	In (total) Fab (mg)	Out (total) Fab (mg)	Specific Fab yield (%)[a]	Total Fab recovered (%)[b]
200	157	133	13	85
300	235	176	11	75
400	314	193	9	63
500	392	187	8	48

Reduction in total Fab recovered and specific Fab yield after each consecutive run are equivalent to that seen when using current production buffers. Data presented are calculated as a mean of three repeat determinations and maximum standard deviation was calculated to be ±1.5.

[a] The specific Fab yield was calculated as a percentage of Fab out against total protein in.

[b] Total Fab recovered is a percentage of Fab out against total Fab in.

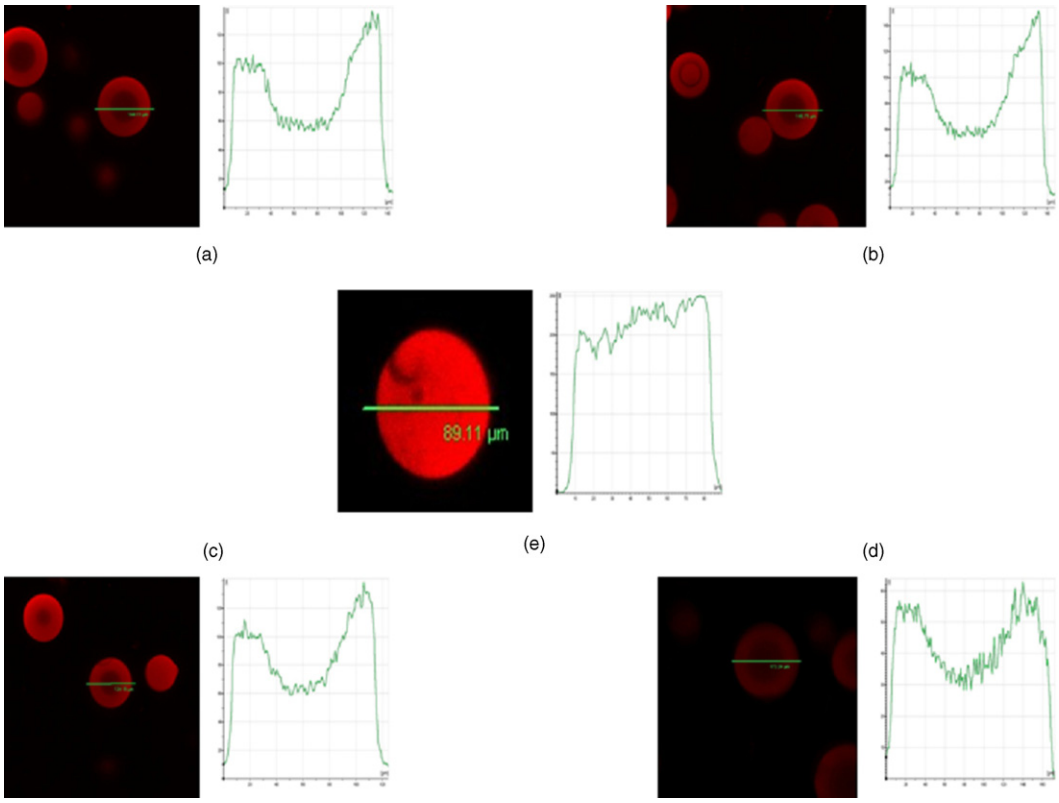

Fig. 6. Images and corresponding absorbance profiles (*x*-axis shows bead diameter in μm and *y*-axis shows fluorescence intensity). Four DigiFab^{TM} affinity matrix samples were analysed after 0, 1, 2, and 3 consecutive affinity runs were analysed using confocal scanning laser microscopy. All images were focused at the centre of the bead. The beads were excited at 568 nm and emitted between 573 and 643 nm. Repeat images taken at the end of the experiments indicate that the corresponding signal changes are not due to photobleaching over the course of the analysis. Controls using standard sepahrose 4B matrix and pure sheep IgG, Fab fragment produced blank images: (a) new matrix; (b) after one manufacturing run; (c) after two manufacturing runs; (d) after three manufacturing runs; (e) new matrix after 24 h incubation. According to the images, reduced intensities within the centre of the bead suggests that binding occurs predominantly to the outer layer of the bead.

Scientific Ltd.) were incubated with DDMA-coupled affinity beads (data not shown). Hence, this process is based on specific binding to DigiFab^{TM} matrix. Fluorescence intensity seen around the outer layer of the affinity bead (Fig. 6) is fluorescent Fab binding specifically to DigiFab^{TM} affinity ligand and not due to non specific binding of flurophore. Repeat images showed that, for the entire duration of the experiment (4 h), laser intensity remained constant, hence fluorescence changes are not due to photobleaching. Even though the column was over saturated, most of the Fab were bound to the outer layer only (Fig. 6). Protein was adsorbed to a dense outer layer in most cases and the fluorescence intensity profile shows that the inner part was only partly used for adsorption [10–13]. One interpretation of this result is that even though ligands are likely to be available in the inner core of the bead, the incubation time for the Fab was not sufficient for diffusion of Fab into the central core of the cross-linked agarose bead. To evaluate this further, a sample of new (unused) matrix was left to incubate with labelled Fab for 24 h and the resulting beads imaged. In this case all available surfaces were used for adsorption (Fig. 6e). The 3 h equilibration used in this experiment was similar to the current manufacturing process (during production the allowed equilibration time is approximately 200 min, depending on the load concentration), hence longer binding times are required to bind all Fab to the matrix.

Overall, average intensity calculated using Image J (v1.33u, National Institutes of Health) showed that intensity drops after each manufacturing run. Since mass balance data indicate that binding sites are not being blocked by adsorbed proteins, the reduction in fluorescence intensities are likely to be due to modification of the bound ligand. This change in intensity values after 1, 2 and 3 consecutive runs (Table 7) are consistent with the drop in specific Fab recoveries seen in Table 3. After three runs, intensity has dropped by 32% from first use. Therefore, it

Table 7
Comparison of average intensities after 1, 2 and 3 consecutive runs on the column

Matrix	Average intensity of 20 beads (λ) ± S.D.	% Intensity ± S.D.
New	6028 ± 1163	100
After 1 run	5653 ± 839	94 ± 5
After 2 run	5608 ± 345	93 ± 13
After 3 run	4070 ± 1061	68 ± 4.5

To obtain matrix for confocal analysis all affinity runs were carried out at 300 mg/ml loading. All matrices were then saturated in pure Fab and imaged. Twenty images per sample were taken and intensity values of them were determined using ImageJ software. Error shown represents standard deviation ($n = 20$). Intensity value represents Fab uptake on to a single affinity bead. After three affinity runs, bead intensities had dropped by 32%, consistent with a rapid reduction in column performance over three consecutive affinity runs.

can be concluded that this column could only be used a limited number of times.

4. Conclusions

Downstream processing of most protein biopharmaceuticals utilises a chromatographic purification step. This includes an affinity step often employed during latter stages of the purification process and it is critical to fully characterise the performance of the affinity column to prevent a process bottleneck and to develop a robust, optimised process. This paper employs mass balancing of protein and Fab fragments, capacity measurements using batch uptake kinetics and an imaging technique to investigate the impact of varying column loading and re-use on matrix performance. Confocal scanning microscopy was used to visualise the binding of an FDA approved biotherapeutic to a Fab specific affinity resin and the images were used to evaluate variations in binding capacity over multiple affinity cycles. The current manufacturing process was successfully scaled down by 20 fold and the maximum column capacity was calculated to be 193 ± 65 mg. Confocal images show Fab binding to the outer layer of the affinity bead, while its inner core is only partly used for adsorption. All results show that this column could only be used a limited number of times before reduced recoveries have a commercial impact on the affinity process, consistent with current large scale manufacturing operations. The results presented will be used in further investigations for future optimisation of the DigiFab^{TM} affinity process.

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References

[1] A.R. Newcombe, C. Cresswell, S. Davies, K. Watson, G. Harris, K. O'Donovan, R. Francis, J. Chromatogr. B 814 (2005) 209.
[2] J. Haurum, S. Bregenholt, IDrugs 8 (2005) 404.
[3] L. Gray, C-088V Dynamic antibody industry. Including polyclonals and monoclonals, Business communication company Inc., Norwalk, 2002, www.bccresearch.com/biotech/c088v.html.
[4] D.K. Follman, R.L. Fahrner, J. Chromatogr. A 1024 (2004) 79.
[5] R. Safadi, I. Levy, Y. Amitai, Y. Caraco, Arch. Intern. Med. 155 (1995) 2121.
[6] R.J. Flanagan, A.L. Jones, Drug Saf. 27 (2004) 1115.
[7] T.W. Smith, Am. J. Emerg. Med. 9 (1991) 1.
[8] Protherics UK Ltd., Blaenwaun, Ffostrasol, Llandysul, Ceredigion, Wales, UK, www.protherics.com.
[9] A.G. McMillin, W.E. Owen, T.L. Lambert, B.K. De, E.L. Frank, P.R. Bach, T.M. Annesley, W.L. Roberts, Clin. Chem. 48 (2002) 1580.
[10] A. Ljunglof, R. Hjorth, J. Chromatogr. A 743 (1996) 75.
[11] A. Ljunglof, J. Thommes, J. Chromatogr. A 813 (1998) 387.
[12] A. Ljunglof, M. Larsson, K.G. Knuuttila, J. Lindgren, J. Chromatogr. A 893 (2000) 235.
[13] A. Ljunglof, P. Bergvall, R. Bhikhabhai, R. Hjorth, J. Chromatogr. A 844 (1999) 129.
[14] C. Cresswell, A.R. Newcombe, S. Davies, I. Macpherson, P. Nelson, K. O'Donovan, R. Francis, Biotechnol. Appl. Biochem. 42 (2005) 163.
[15] S.M. Wheelwright, Protein Purification Design and Scale up of Downstream Processing, Hanser Publishers, New York, 1991.
[16] G. Sofer, L. Hagel, Handbook of Process Chromatography. A Guide to Optimisation, Scale-up and Validation, London Academic press, San Diego, 1997, pp. 100–106 and 289–298.
[17] S.Y. Suen, J. Chem. Technol. Biotechnol. 70 (1997) 278.
[18] L.C.L. Aquino, E.A. Miranda, I.S. Duarte, P.T.V. Rosa, S.M.A. Bueno, Braz. J. Chem. Eng. 20 (2003) 3.
[19] Q.M. Mao, M.T.W. Hearn, Biotechnol. Bioeng. 52 (1996) 204.
[20] R. Blanco, A. Arai, N. Grinberg, M. Yarmush, B.L. Karger, J. Chromatogr. 482 (1989) 1.
[21] D. Amitava, W. Alice, D. Pradip, Ther. Drug Monit. 21 (2) (1999) 251.