Characterisation of chromatography adsorbents for antibody bioprocessing

A thesis submitted to University College London for the degree of Master of Philosophy

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I, Bela Sheth, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature

Date

Dedicated to my husband, Michael

Acknowledgements

I would like to acknowledge the enormous contribution of my supervisor, Daniel Bracewell, in helping to develop this project.

For inviting me to visit Millipore (UK), I wish to thank my industrial supervisor, Jon Huddleston and the whole team at Durham.

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Abstract

Currently the purification of monoclonal antibodies for therapeutic purposes is reliant on protein A affinity chromatography. The rapid growth of this class of therapeutic and their high value makes the understanding of protein A chromatography an important target. There is a range of commercially available protein A chromatography media. The main differences between these media are the support matrix type, the pore size, the particle size, the amount of ligand attached to the matrix and the kind of protein A modification. The differences in these factors give rise to differences in compressibility, chemical and physical robustness, diffusion resistance and binding capacity of the adsorbents. The ideal media would have high specificity, high mass transfer and binding capacity, low non-specific adsorption and ligand leakage, incompressibility, resistance to alkaline condition for sanitization, chemical stability and cost effectiveness. Current resins offer a compromise, which balances what is achievable in respect of these features giving rise to an array of different solutions. Measurement of these parameters is often complex and agreed standards have yet to be determined. The objective of this study is to further develop understanding of these measurements for the assessment of the matrix performance.

This thesis employs a suite of techniques to characterise commercial and prototype adsorbents. The adsorbents that will be looked at are MabSelect (GE Healthcare), MabSelect Xtra (GE Healthcare), Prosep Ultra (Millipore), Protein A immobilised on 4CL Sepharose (GE Heatlhcare) in house and a prototype adsorbent with a Protein A mimic ligand (Millipore). Both down-scaled techniques of fixed bed chromatography, together with supporting analysis of equilibrium and dynamic behaviours are used. The latter will adopt standard and novel 'wet chemistry' approaches together with the increasingly adopted techniques of laser scanning confocal microscopy. Experiments are carried out using hIgG to study the static capacity, adsorption equilibrium and dynamic capacity of adsorbents. Other techniques will be used to study the kinetic uptake and desorption rates of adsorbents in different conditions. A novel approach using confocal microscopy is used to further understand the adsorption behaviour of individual beads of different sizes.

The main results that were drawn from these techniques are that MabSelect Xtra had the highest static capacity of 61.8mg/ml. It also showed the highest dynamic capacity at 2 mins, 4 mins and 8 mins residence time (0.66cm Omnifit column, bed height 6cm) when compared to other adsorbents. This is mainly due to the more porous nature of the MabSelect Xtra beads, which increased the surface area available for Protein A ligand immobilisation. From the adsorption equilibrium data the Kd values ranged from 181nM to 36nM. Such low values are expected by affinity adsorbents such as these. The uptake rate curves were similar for all the adsorbents. Hence the difference in particle size, pore size, the type of ligand or the material of the adsorbent itself did not have an effect on the uptake rate when carried out in a batch mode. A similar behaviour was shown for the desorption curves. The confocal analysis using a flow cell showed that all the adsorbents showed a shrinking core effect except for the prototype where the hIgG didn't penetrate into the bead and was only attached to the surface of the bead. It was found that the adsorption rate to the centre of each bead was linear. The different particle sizes within any particular type of matrix and also across different matrix did not result in different diffusion rates. From the adsorption curves produced it was seen that smaller beads reached saturation much faster than larger beads at any given time. This technique can have great benefits in understanding how individual beads of different adsorbents behave in different circumstances.

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1.1 Chromatography in Bioprocessing

The centre focus of bioprocessing is using living cells to make desired products, which is commonly carried out in a bioreactor. Downstream processing from this reactor gives concentrated and purified products. These products can be therapeutic proteins such as factor VIII and urokinase. It could also be diagnostic enzymes, insulin, monoclonal antibodies, dehydrogenase, antibiotics, proteases or amylases. In the recovery of biomolecules 5 stages can be distinguished. These can be seen in the figure below (Krijgsman, 1995).

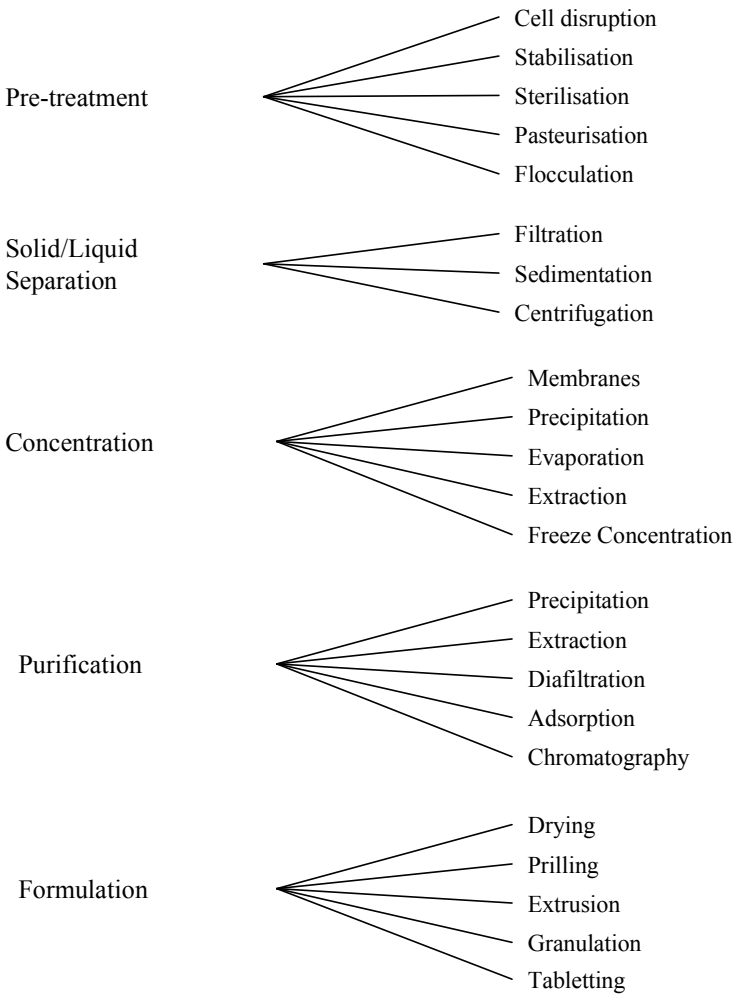


Fig 1.1 - Five major steps in the recovery of biomolecules

Just focusing on the antibody based therapies, the current global antibody pipeline, which comprises of more than a hundred antibodies in development, was ready to deliver 16 new products by 2008 (Swinnen et al., 2007). The table below shows some of the approved monoclonal antibodies present in the market (Shukla et al., 2007).

Trade name	Indication	Company	Year of approval
(a) Approved monoclonal antibodies			
Orthoclone OKT3	Acute kidney transplant rejection	Ortho Biotech	1986
ReoPro	Prevention of blood clot	Centocor	1994
Rituxan	Non-Hodgkin's lymphoma	Genentech/Biogen-IDEC	1997
Panorex	Colorectal cancer	GlaxoSmithKline	1995
Zenapax	Acute kidney transplant rejection	Hoffman-LaRoche	1997
Simulect	Prophylaxis of acute organ rejection in allogenic renal transplantation	Novartis	1998
Synagis	Respiratory synctial virus	Medimmune	1998
Remicade	Rheumatoid arthritis	Centocor	1998
Herceptin	Metastatic breast cancer	Genentech	1998
Mylotarg	Acute myelogenous lymphoma	Wyeth-Ayerst	2000
Campath-1H	B-cell chronic lymphocytic leukemia	Millenium/ILEX	2001
Zevalin	Non-Hodgkin's lymphoma	Biogen IDEC	2002
Humira	Rheumatoid arthritis	Abbott	2002
Bexxar	Non-Hodgkin's lymphoma	Corixa/GSK	2003
Xolair	Allergy	Genentech/Novartis	2003
Erbitux	Colon cancer	Imclone/BMS/Merck	2004
Avastin	Metastatic colon cancer	Genentech	2004
Raptiva	Psoriasis	Genentech/Xoma	2004
Tysabri	Multiple sclerosis	Biogen-Idec	2006
Vectibix	Metastatic colorectal cancer	Amgen	2006

Table 1.1 - Approved monoclonal antibodies available in the market

The graph below shows the projected annual production of monoclonal antibodies (Low et al., 2007).

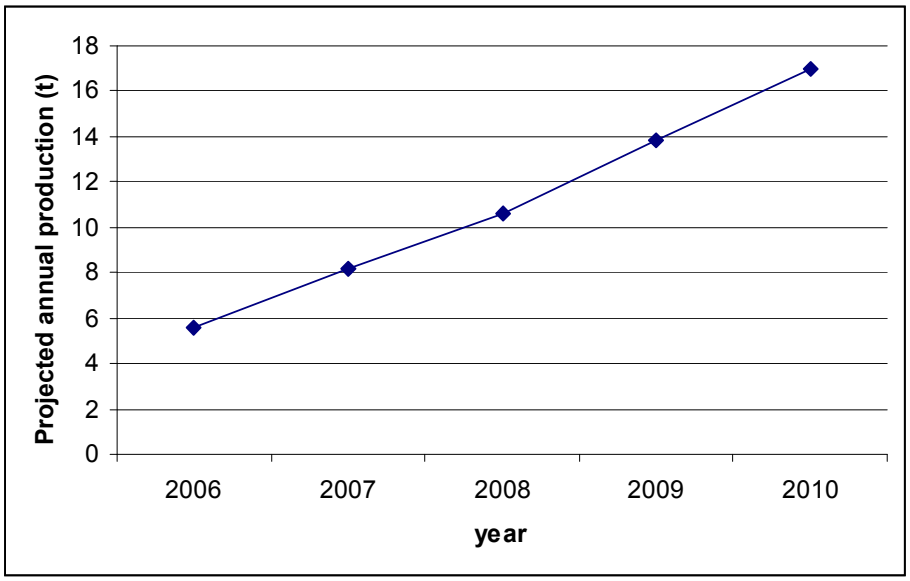


Fig 1.2 - Projected annual production of monoclonal antibodies. The numbers are in metric tonne.

In response to increasing market demands and pressure for cost reduction, the biotech industry has successfully increased bioreactor volumes up to 20,000 L (Swinnen et al., 2007). However process bottleneck has always been downstream and purification costs are outweighing cell culture costs.

This chapter mainly gives an introduction to chromatography focusing further on affinity chromatography that is used to purify monoclonal antibodies. Protein A chromatography is widely used to purify monoclonal antibodies mainly because of its high selectivity. To meet the demands with the increasing bioreactor volumes and cell culture expression levels an optimum use of expensive Protein A affinity resin (€6000–€9000/L resin) can have significant benefits (Swinnen et al., 2007).

Despite Protein A affinity chromatography being a predominant mAb capture step in bioprocessing it has several limitations. Firstly the high cost of the resins. Secondly the differences in the dynamic binding capacity at various flow rates and in the pressure-flow characteristics of various Protein A chromatographic media can result in wide variations in throughput (Shukla et al., 2007). A Protein A step also adds impurity in the process in the form of leached Protein A ligands. Another major limitation is the inability to increase large-scale column diameter to beyond 2m

without encountering significant issues with flow distribution and packing (Shukla et al., 2007). There is a ready market for the company that can produce a Protein A resin with a dynamic binding capacity of >50 g/L at an even lower residence time (Low et al., 2007). Some of the options that are currently being investigated include higher ligand densities on their resins, ligand orientation/accessibility, particle size, pore size and distribution, alternative protein A mimic synthetic ligands and more stable support matrices with increased mass transfer.

1.2 Affinity Chromatography

Affinity chromatography involves the use of ligands that attach to the media and that have binding affinity to specific molecules or a class of molecules.

Ligands can be bio-molecules, like protein ligands or can be synthetic molecules. Both types of ligand tend to have good specificity. But protein ligands have the disadvantage that they are expensive and mostly denature with the use of cleaning solutions, whereas synthetic ligands are less expensive and more stable.

The most commonly used protein ligand in production is the protein A ligand, which is specific to the IgG antibody (Section 1.6).

In affinity chromatography when the solution is introduced to the column the target protein is adsorbed while allowing contaminants (other proteins, lipids, carbohydrates, DNA, pigments, etc.) to pass through the column (Fig 1.3). The adsorbent itself is normally packed in a chromatography column; though the adsorption stage can be performed well by using the adsorbent as stirred slurry in batch binding mode. The next stage after adsorption is the wash stage, in which the adsorbent is washed to remove residual contaminants. The bound protein is then eluted in a pure form. Elution is normally achieved by changing the buffer or salt composition so that the protein can no longer interact with the immobilized ligand and is released. Affinity chromatography can be performed in a fixed bed or a fluidised bed.

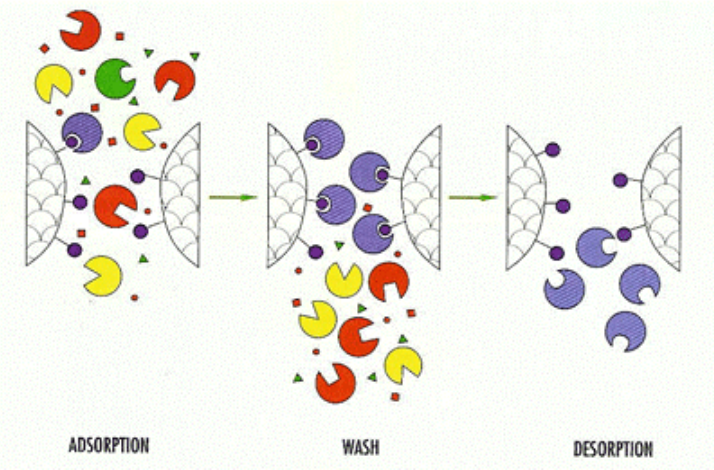


Fig 1.3 – Stages during the affinity chromatography process that includes adsorption, wash and desorption

1.3 Types of media

The support to which the ligands can be attached to can be of different materials.

1.3.1 Natural polymers

Natural polymers can be of agarose, cellulose or dextran. Their most beneficial point is that these materials have very low non-specific adsorption. This is because the polymer chains are very hydrophilic due to the presence of many hydroxyl groups and hence the proteins do not adhere to them.

Dextran and agarose have better flow properties than cellulose. Fibrous cellulose is extremely hard to pack (Jaunbauer et al., 2005). Its usefulness is limited by its fibrous and non-uniform character. The main disadvantage of dextran is its low degree of porosity. Agarose beads on the other hand are extremely soft and therefore cross-linked to increase the strength. However due to their loose structure it allows ready penetration by macromolecules with molecular weights in the order of several millions (Lowe et al., 1974). The uniform spherical shape of the particles in particular gives good flow properties.

1.3.2 Synthetic polymers

The three synthetic polymers of importance are hydrophobic vinyl polymers, polyacrylamide and polyvinylstyrene.

All of these are relatively hydrophobic and hence needs to be modified by coating the surface with a hydrophillic polymer to avoid low recovery (Jaunbauer et al., 2005). An advantage of the synthetic polymer-based media is their resistance to extreme chemical conditions, such as pH.

The main disadvantage with polystyrene adsorbents is their relatively low porosity. Polyacrylamide beads on the other hand are superior to many polymeric supports due to their polyethylene backbone, which increases chemical stability. They tend to have a more uniform physical state and porosity, permitting the penetration of macromolecules with molecular weights of upto 500,000 (Lowe et al., 1974). Another advantage is that they posses many modifiable groups which enables the covalent attachment of a variety of ligands.

1.3.3 Inorganic media

The commercially available inorganic media are made of ceramic, silicate or glass. The silicate media are coated with several other materials. The most common glass media used consists of irregularly shaped controlled porous glass (CPG) particles. These adsorbents are rigid and can work at high flow rates. Due to their controlled pore size they tend to generate sharp exclusion limits (Lowe et al., 1974).

1.4 Modes of Chromatography

1.4.1 Elution

Elution is carried out by first introducing a small sample of the mixture on to the column. It is then eluted with a mobile phase, which has a lesser affinity to the stationary phase than the sample components. The components then move along the column depending on their relative affinity for the stationary phase but at a slower rate than the eluent. Hence the components can be completely separated with a zone of mobile phase between them. This mode of chromatography is commonly used for analytical purposes (Braithwaite et al., 1985).

1.4.2 Frontal Analysis

Frontal analysis is executed by continuously adding the sample onto the column. The component with the least affinity for the stationary phase will pass along the

column while the component with the greater affinity will get adsorbed to the stationary phase. Eventually this component will pass along the column, when the capacity limit of the stationary phase is exceeded. This mode of chromatography is used to achieve breakthrough curves (Section 1.8.2).

1.4.3 Displacement

Displacement is carried out by first introducing the sample mixture onto the column. Elution then occurs when a displacing solvent is passed through the column, which has a greater affinity for the stationary phase than the sample components.

1.5 Antibody

Antibodies are protein molecules that play a crucial role in the immune system, which defends the body against toxins that enter the bloodstream and counters the disease threat posed by invading microbes and viruses. Antibodies are made in white blood cells, B-lymphocytes. Each B-lymphocyte makes copies of its own unique antibody, which are then displayed on its outer surface. Immunoglobin G is the major antibody in serum. It has a Y-shaped structure composed of four protein subunits which comprises of two identical light chains and two identical heavy chains as shown in Fig 1.4.

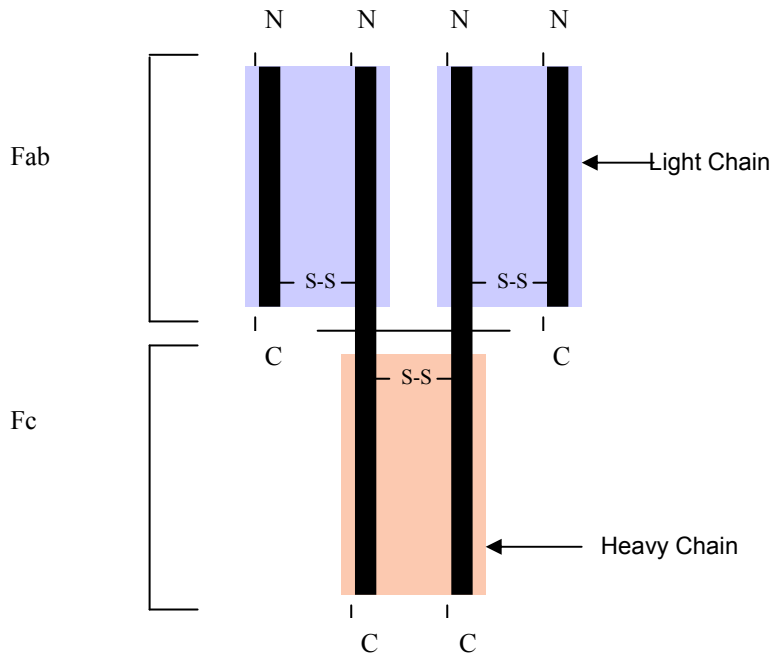


Fig 1.4 – Structure of IgG molecule (L. Stryer 1995)

Monoclonal antibodies can be used therapeutically, to protect against diseases; they can also help to diagnose a wide variety of illnesses, and can detect the presence of drugs, viral and bacterial products, and other unusual or abnormal substances in the blood. Their specificity makes monoclonal antibody technology so valuable. Given such a diversity of uses for these disease-fighting substances, their production in pure quantities has long been the focus of scientific investigation. The monoclonal antibody (mAb) market has grown rapidly in recent years, reaching sales of $14bn in 2005, an increase of 36.5% from 2004 sales of $10.3bn.

Monoclonal antibodies are homogenous because they are synthesised by a population of identical cells (a clone). Each such population is descended from a single hybridoma cell formed by fusing an antibody producing cell with a tumour cell that has the capacity for unlimited proliferation. Hence it possesses a structure that can only bind to one epitopal group on one antigen. Polyclonal antibodies, in contrast with monoclonal ones are products of many different populations of antibodies-producing cells and hence differ somewhat in their precise specificity and affinity for the antigen. Hence a polyclonal antibody mixture has typically most or all of the antibodies acting against all epitopal groups for antigens on the molecule.

1.6 Protein A affinity chromatography

1.6.1 Protein A

Protein A affinity chromatography is widely applied in the commercial production of IgG. Protein A, which has a molecular weight of 42,000, is a cell wall protein from Staphlococcus *aureas* with affinity for the Fc region of IgG. The amino-terminal region contains five highly homologous IgG-binding domains (Fig 1.5). All five IgG-binding domains of SpA bind to IgG via the Fc region (Jansson et al., 1993). Binding of the antibody is normally done at pH 7 and elution at pH 2-3 (Jungbauer et al., 2005). The two binding domains at the C-terminal are non-immunoglobulin binding regions and are thought to be responsible for the binding of Protein A to the bacterial cell wall.

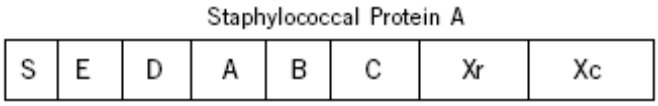


Fig 1.5 - Schematic drawing of regions encoded by the gene for Staphylococcal protein A. S is the signal sequence. E, D, A, B and C are the immunoglobulin binding regions. Xr and Xc are C-terminal located, non-immunoglobulin binding regions (Uhlen et al., 1984).

1.6.2 Commercially available Protein A media

There are various commercially available protein A chromatography media. The main differences between these media are the support matrix type, protein A ligand modification, the pore size and the particle size. The differences in these factors give rise to differences in compressibility, chemical and physical robustness, diffusion resistance and binding capacity of the adsorbents (McCue et al., 2003). Products of different manufacturers were researched to arrive at this table.

Adsorbent	Manufacturer	Matrix type	Ligand type	Particle/Pore size	Static/dynamic capacity	Adsorbent special features
nProtein A Sepharose 4 Fast Flow	GE Healthcare, UK	Crosslinked agarose – 4%	Native protein A	Particle size – 90μm	Static capacity – 35mg/ml	2 times total IgG capacity than Protein A Sepharose CL-4B. Replaces former Protein A Sepharose 4 Fast Flow
rProteinA Sepharose Fast Flow	GE Healthcare, UK	Crosslinked agarose – 4%	Recombinant Protein A. C-terminal Cysteine attached to rProtein A	Particle size – 60-165μm	Static Capacity – 50mg/ml	Much higher dynamic capacity. Thioether coupling providing single point attachment of the protein A. Oriented coupling enhances the binding of IgG
rmp Protein A Sepharose Fast Flow	GE Healthcare, UK	Crosslinked agarose – 4%	Recombinant Protein A. Multipoint ligand attachment	Particle size – 60-165μm	Static capacity – 35mg/ml	Low ligand leakage. Increase chemical stability due to multipoint ligand attachment
Protein A Sepharose CL-4B	GE Healthcare, UK	Crosslinked agarose – 4%	Native protein A	Particle size – 90μm	Static capacity – 20mg/ml	NG
HiTrap rProtein AFF	GE Healthcare, UK	Crosslinked agarose – 4%	Recombinant Protein A C-terminal Cysteine attached to rProtein A	Particle size – 90μm	Static Capacity – 50mg/ml	Pre packed Column – 1ml and 5ml
Streamline rProtein A	GE Healthcare, UK	Crosslinked agarose – 4%. Modified with inert metal alloy core for providing required high density	Recombinant Protein A. C-terminal Cysteine attached to rProtein A	Particle size – 80-165μm	Static Capacity – 50mg/ml	Expanded bed adsorption. Capture IgG directly from unclarified feed stock.
MabSelect	GE Healthcare, UK	Crosslinked agarose	NG	Particle size – 85μm	Dynamic capacity – 30mg/ml	Large feed volumes – more than 10000L

Adsorbent	Manufacturer	Matrix type	Ligand type	Particle/Pore size	Static/dynamic capacity	Adsorbent special features
MabSelect Sure	GE Healthcare, UK	Crosslinked agarose	Alkali stabilised Protein A	Particle size – 85μm	Dynamic capacity – 30mg/ml	Increase chemical stability during sanitization
MabSelect Xtra	GE Healthcare, UK	Crosslinked agarose	NG	Particle size – 75μm	Dynamic capacity – 40mg/ml	High dynamic capacity as adsorbent more porous than other MabSelect family adsorbents
IPA-300	RepliGen Corp., Waltham, MA, USA	Crosslinked agarose	NG	NG	NG	NG
IPA - 400	RepliGen Corp., Waltham, MA, USA	Crosslinked agarose	NG	NG	NG	NG
IPA - 500	RepliGen Corp., Waltham, MA, USA	Crosslinked agarose	NG	Particle size – 90μm	NG	Modified to double binding capacity
Affi-Gel Protein A Gel	Bio-Rad, Hercules, CA, USA	Crosslinked agarose	Native protein A	NG	Static Capacity- 20mg/ml	NG
Affi-Prep Protein A	Bio-Rad, Hercules, CA, USA	Polymeric – Macroporous matrix	NG	NG	Static Capacity- 16-23 mg/ml	Used for medium to high pressure
MAbsorbent A2P	Affinity Chromatography Ltd. (Prometic Biosciences), Freeport, Isle of Man, UK	Crosslinked agarose – 6%	Synthetic ligand (aromatic triazine derivative)- mimic recombinant and native Protein A.	Particle size - ~100μm	Static Capacity – 50mg/ml	Use of Synthetic ligand – less expensive than Protein A ligands. Only used for humanized or human antibody.
MAbsorbent A1P	Affinity Chromatography Ltd. (Prometic Biosciences), Freeport, Isle of Man, UK	Crosslinked agarose – 6%	Synthetic ligand (aromatic triazine derivative)- mimic recombinant and native Protein A.	Particle size - ~100μm	Static Capacity – 50mg/ml	Use of Synthetic ligand – less expensive than Protein A ligands. Only used for murine antibody.
Protein A Ceramic HyperD F	Pall corporation 2200 Northern Boulevard East Hills, USA	Polyacrylamide gel in ceramic macrobead	Recombinant Protein A	Particle size - 50μm	Dynamic Capacity – 30mg/ml	Rigid. Less back pressure issues.

Adsorbent	Manufacturer	Matrix type	Ligand type	Particle/Pore size	Static/dynamic capacity	Adsorbent special features
Poros A 50 High Capacity	PerSeptive Biosystems, Framingham, MA, USA	Polystyrene divenylbenzene	NG	Particle size – 50μm Pore size – 50 to 1000nm	NG	Discrete classes of pores. Large ”through pores” allow convection flow through particles, quickly carrying molecules to small ”diffusive pores” inside. Used large scale for low pressure application
Poros A 20	PerSeptive Biosystems, Framingham, MA, USA	Polystyrene divenylbenzene	NG	Particle size – 20μm Pore size – 50 to 1000nm		Discrete classes of pores. Large ”through pores” allow convection flow through particles, quickly carrying molecules to small ”diffusive pores” inside. Used samller scale for high resolution.
UltraLink Immobilized Protein A Plus	Pierce, Rockford, IL, USA	Polymeric	Native protein A	Particle size–50 to 80μm Pore size-100nm	Static Capacity – 30 mg/ml	Rigid – Used for high pressure Higher capacity than UltraLink Immobilized Protein A
UltraLink Immobilized Protein A	Pierce, Rockford, IL, USA	Polymeric	Native protein A	Particle size – 50 to 80μm Pore size – 100nm	Static Capacity – 16 mg/ml	Rigid – Used for high pressure
Agarose Protein A	Pierce, Rockford, IL, USA	Crosslinked agarose – 6%	NG	NG	Static Capacity – 12-19 mg/ml	NG
Agarose Protein A Plus	Pierce, Rockford, IL, USA	Crosslinked agarose – 6%	NG	NG	Static Capacity – 34 mg/ml	Higher capacity than Agarose Protein A
Prosep-vA Ultra	Miilipore Bioprocessing, Consett, UK	Porous glass	Native Protein A	Particle size - ~100μm Pore size – 70nm	Static Capacity – 56 mg/ml	Rigid, incompressible, low back pressure and operate at high flow rates.
Prosep-vA High Capacity	Miilipore Bioprocessing, Consett, UK	Porous glass	Native Protein A	Particle size - ~100μm Pore size – 100nm	Static Capacity – 40 mg/ml	Rigid, incompressible, low back pressure and operate at high flow rates.
Protein A Cellthru 300	Sterogen Bioseparations, n.a. 200–300 n.a. Carlsbad, CA, USA	Agarose content – 4%	Recombinant Protein A	Particle size – 200 to 300μm	Static capacity – 35mg/ml Dynamic capacity – 28mg/ml	Used for unclarified, viscous feed stream

Adsorbent	Manufacturer	Matrix type	Ligand type	Particle/Pore size	Static/dynamic capacity	Adsorbent special features
Protein A Ultraflow	Sterogen Bioseparations, n.a. 200–300 n.a. Carlsbad, CA, USA	Agarose content – 4%	Recombinant Protein A	Particle size – 40 to 160μm	Static capacity – 50mg/ml Dynamic capacity – 40mg/ml	NG

Table 1.2 – Commercially available native, recombinant and mimic Protein A affinity chromatography media

1.7 Factors effecting performance of adsorbents

The ideal features of a chromatographic media would be high specificity, high mass transfer, high binding capacity, low non-specific adsorption, incompressibility, resistance to alkaline condition for sanitization, chemically stability, low ligand leakage and cost effectiveness (Jungbauer et al., 2005). There are various factors that affect these features.

1.7.1 Pore size

Smaller pores mean an increase in the surface area available for protein A immobilisation. Previous studies have shown that Prosep vA-Ultra (pore size 70nm) has a higher static capacity than Prosep vA-High capacity (pore size100nm) due to the fact that it has got smaller pores (McCue et al., 2003). Smaller pores also means a much stronger structure and hence the media being less compressible.
But the disadvantage would be an increase in the diffusion resistance. Hence the time taken to reach the static capacity would be slower for adsorbents with smaller pores when compared to adsorbents with larger pores (McCue et al., 2003). On the other hand larger pores would mean less diffusion resistance but a decrease in protein A immobilisation and a more compressible structure (Hahn et al., 2005).

1.7.2 Particle size

Smaller particle size would mean better mass transfer and better access to internal bead volume. This is because pore diffusivity depends on the square of the particle diameter (Hahn et al., 2003). But there would be a higher pressure drop across a packed bed due to the smaller particle size. On the other hand larger particles would mean lower pressure drop across a packed bed, but the mass transfer and efficiency would be poorer. This was also shown in previous studies. Poros 50 (particle size 45-50μm) showed a higher dynamic capacity than prototype Poros LP (particle size 55-60μm). But Poros LP had a lower pressure drop (higher permeability) at higher flow rates when compared to Poros 50 (Fahrner et al., 1999).

1.7.3 Support material

The material used for the matrix can affect the performance. The different kinds of support materials used were given in Table 1.2. The main factor that is affected by the support material is the compressibility of the media. Previous studies have shown that agarose adsorbents are more compressible than porous glass adsorbents (Stickel et al., 2001). Pressure-flow profiles of different adsorbents showed that non-linear profiles are characteristics of compressible adsorbents. Linear pressure - flow curves, on the other hand, as that of Prosep-rA adsorbent are characteristics of incompressible adsorbents. Hence depending on the matrix type adsorbents can be used at higher flow rates without being compressed.

1.7.4 Protein A modification

Ligands are always exposed to the harsh conditions of operational cycle especially to the intermediate cleaning and sanitization procedures. The way the Protein A ligand is modified makes a difference in the performance of the adsorbent. Many companies tend to use the native form of Protein A. But many companies also tend to use recombinant Protein A with specific modifications. Three examples are described below.

Firstly, In the case of rProteinA Sepharose Fast Flow (GE Healthcare) the recombinant protein A has been engineered to include a C-terminal cysteine. The coupling conditions are controlled to favour a thioether coupling providing single point attachment of the protein A (Fig 1.6). The oriented coupling enhances the binding of IgG thus increasing the dynamic capacity of the adsorbent.

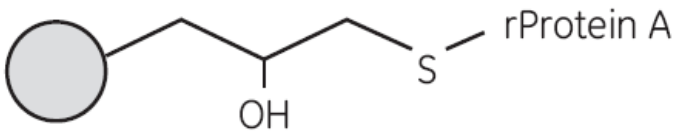


Fig 1.6 – C-terminal cysteine favours oriented thioether coupling (GE Healthcare)

Secondly, in the case of rmp Protein A Sepharose Fast Flow (GE Healthcare) a recombinant protein A is used with a molecular weight of 44 600. It contains five antigen-binding domains, which allow multiple attachment points to the Sepharose

Fast Flow support. The attachment is carried out through reductive animation, which creates chemically stable amide bonds. This results in less ligand detaching from the support during elution and cleaning, and hence less Protein A leakage.
Lastly, in the case of MabSelect Sure (GE Healthcare) the recombinant protein used is modified such that the amino acids sensitive to alkali were identified and substituted with more stable ones. This made the adsorbent more stable to alkali and hence more stable during cleaning and sanitization.
There are also several Protein A mimics available in the market which are synthetic ligands and hence less expensive than the native form. One example is the MAbsorbent A2P by Prometic Biosciences.

1.7.5 Column Packing

To obtain an optimal separation, sharp symmetrical chromatographic peaks must be obtained. A theoretical plate model is used to understand the processes going on inside the column and understand the column efficiency. The plate model supposes that the chromatographic column contains a large number of separate layers, called theoretical plates. The efficiency of the column can be expressed in terms of theoretical plates in a column N (the more plates the better), or by stating the plate height, the Height Equivalent to a Theoretical Plate (HETP - the smaller the better).

The HETP is calculated by the following formula: HETP = L / N where L is the length of the column. A more realistic understanding of the processes at work inside a column is using the Van Deemter Equation. The resulting band shape of a chromatographic peak is affected by the rate of elution. It is also affected by the different paths available to solute molecules as they travel between particles of stationary phase. If we consider the various mechanisms, which contribute to band broadening, we arrive at the Van Deemter equation for plate height (Deemter et al., 1956);

$$HETP = A + B / u + C u$$

Here u is the average velocity of the mobile phase and A, B and C are factors which contribute to band broadening. The constant A refers to eddy diffusion, B refers to longitudinal diffusion and C refers to resistance to mass transfer. Below is a typical

Van Deemter Plot. Such plots are of considerable use in determining the optimum mobile phase flow rate.

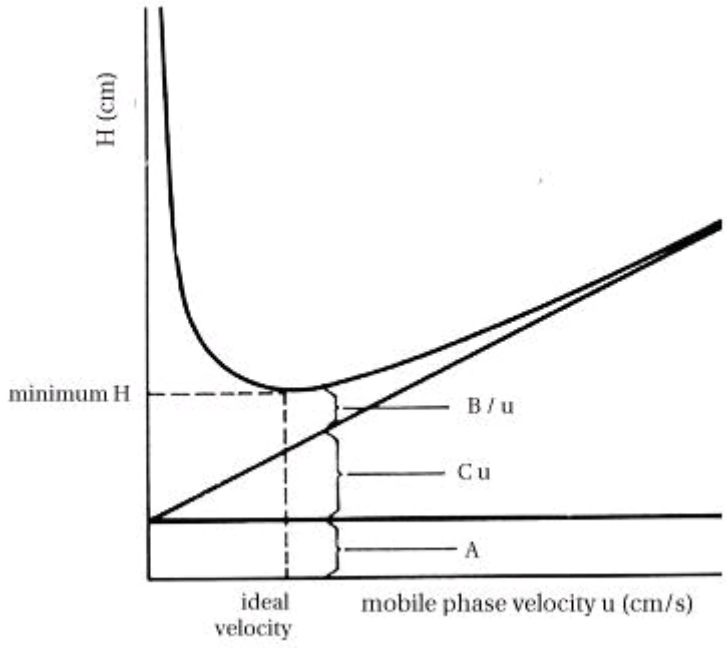


Fig 1.7 – A typical Van Deemter Plot (Deemter et al., 1956)

1.8 Performance measurements

There are various tests that can be carried out to evaluate the performance of the adsorbents. It is generally not possible to reconcile all the ideal features and a compromise would need to be made. It is then up to the manufacturer to decide which criteria are more important in the manufacturing of the adsorbents.

1.8.1 Adsorption isotherms

When the antibody solution is in contact with the adsorbent an equilibrium is achieved between the antibodies in the solution and the antibodies bound to the adsorbent.

Any concave adsorbent isotherm is favourable which means strong adsorption occurs even in dilute solution. Any convex adsorbent isotherm on the other hand is said to be unfavourable. Fig 1.8 shows the different types of adsorption isotherms.

- *Linear isotherm* – Linear isotherms are not very common in bioseparation (Belter et al., 1988) and are given by $q^* = KdC^*$, where q^* is the amount of solute adsorbed per amount of adsorbent, C^* is the solute concentration in solution and Kd is the equilibrium dissociation constant.

- *Freundlich isotherm* – This isotherm is normally used to describe adsorption of a wide variety of antibiotics, steroids and hormones (Belter et al., 1988) and is given by $q^* = KdC^{*n}$, where n is a constant.

- *Langmuir isotherm* – This isotherm is often used to describe adsorption data for proteins. This is given by;

  $$q^* = (Q_m C^*)/(Kd+C^*)$$

  Where Q_m is the maximum amount of solute that can be adsorbed per amount of adsorbent. The Kd value gives information on the selectivity of the affinity system. Kd value of ~ 5×10^{-7}M is typical for a strong, highly selective affinity system (Hahn et al., 2005).

The Langmuir isotherm is derived from kinetic mechanism. It is based on the following hypotheses:

- The surface of the adsorbent is uniform; hence all the adsorption sites are uniform.
- Adsorbed molecules do not interact.
- All adsorption occurs through the same mechanism.
- Only a monolayer of adsorbed molecules is formed. They do not deposit on other adsorbed molecules.

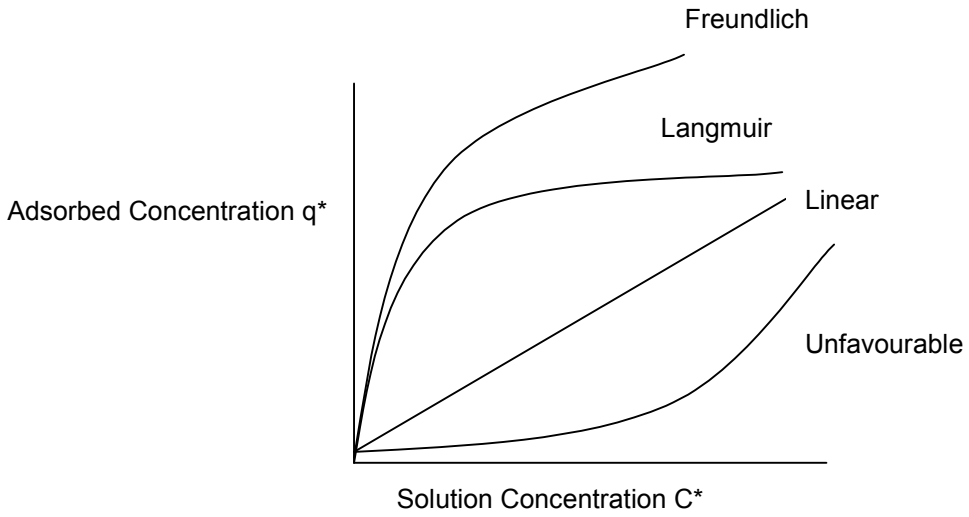


Fig 1.8 - Types of adsorption isotherms, which are Freundlich, Langmuir, Linear and Unfavourable

1.8.2 Breakthrough curves (Dynamic Capacity)

Breakthrough curves give an indication of when the column is completely saturated. When this happens, the concentration of target protein going in equals to the concentration of target protein coming out. It gives an indication to when to stop the loading depending on how much bed remains unused and how much of the product is lost (Fig 1.9). Excessive loading would give rise to loss of some product, whereas insufficient loading would leave the column under utilized (Bailey et al., 1986).

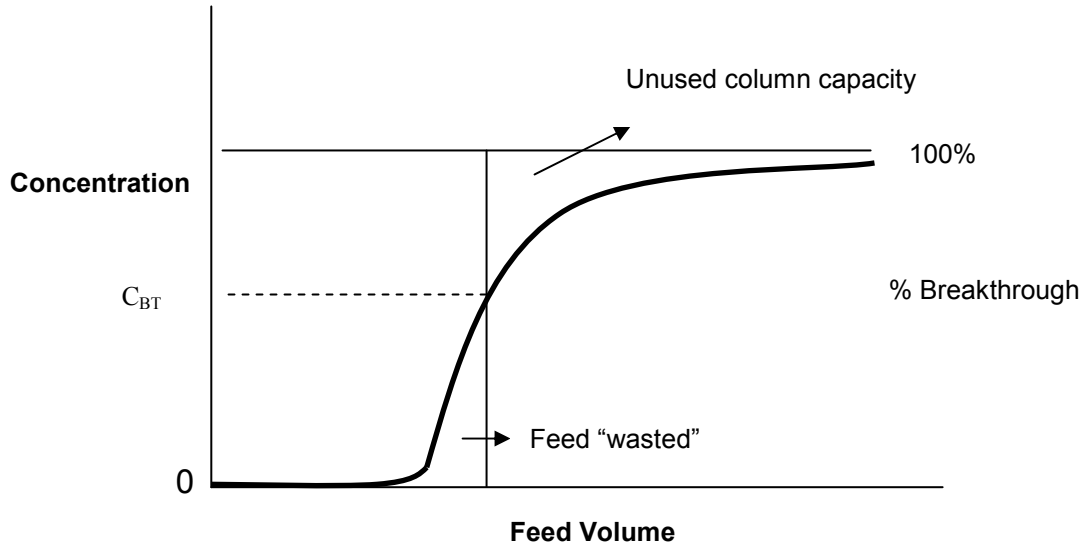


Fig 1.9 – Breakthrough curve

The gradient of the curve gives an indication of how sepecific the binding is. For example, steeper the curve the more specific the binding.

Breakthrough curves are normally used to measure the dynamic capacity of a media. The column is loaded with protein solution at a specific concentration and flow rate. The loading is stopped at a specific percentage breakthrough and the protein is eluted to get the dynamic capacity.

Dynamic capacity can be increased by either decreasing the flow rate and hence increasing the residence time in the column or by increasing the column height, which also increases the residence time in the column (Hahn et al., 2003, Hahn et al., 2005).

Increasing the flow rate normally decreases the dynamic capacity. This is mainly due to the fact that using a faster flow rate decresaes the rate of mass transfer of the protein to the interior adsorption sites of the matrix. In other words, less protein would bind to the adsorbents. The extent of the decrease in the mass transfer rate depends on the particle size and the pore size of the adsorbents. When comparing different types of media, adsorbent that tend to show good mass transfer properties will have a higher dynamic capacity at higher flow rates. However some media which have poorer mass transfer properties might have a higher equilibrium binding capacity overall and will tend to show a higher dynamic capacity at longer residence time (Hahn et al., 2003).

In order to decrease the breakthrough time a load with higher concentration can be used. The distribution of protein adsorbed in the resin is determined by the

intraparticle diffusion rate of the protein into the matrix, which depends on the concentration gradient of the protein and the resin porosity (Pan et al., 2005). When the feed concentration increases, it results in the solute shooting into the interior adsorption sites of the matrix, which leads to the higher adsorption capacity at larger initial feed concentration.

1.8.3 Batch adsorption

Batch adsorption mostly involves the mixing of the adsorbent with the protein solution. Leaving it for an extended amount of time (24hours) would give an indication of the static capacity of the adsorbent. Whereas drawing samples at specific intervals and measuring the concentration would give the uptake kinetics for the adsorbent. One of the accurate ways to carry this out would be to recirculate the stream through a spectrometer (Chang et al., 1998, McCue et al., 2003). But, again, this method causes a time delay and might not be suitable for media that reaches its static capacity in the first few minutes of mixing. Hence other methods like replacing the external loop with a dip probe can be considered (Tscheliessnig et al., 2005). Confocal microscopy can also be very useful in understanding the kinetic uptakes of the adsorbent (Section 1.9). Batch adsorption kinetics can give a very good indication of the mass transfer properties and static capacity of adsorbents.

1.8.4 Shallow beds

This is commonly performed by using a micro-column with approximately 5–20 μl of adsorbent (Lewus et al., 1998, Hahn et al., 2005). The IgG solution is passed through the column at a specific flow rate. Desorption is then carried out to get the mass of IgG adsorbed to the media. The kinetic uptake can then be constructed by varying the contact time.

1.8.5 Desorption

There are a few papers available on adsorption uptake kinetics, but hardly any on desorption of IgG when using Protein A ligand. The rate at which desorption takes place for different adsorbents might give a lot of information about the media in terms of mass transfer. Batch desorption kinetics can be performed in a similar way to batch adsorption. When desorption is carried out in dynamic mode it gives an

idea of the amount of IgG that might still be bound to the adsorbent. Whereas the peak of the eluent can show how concentrated the eluent might be. This is a factor that needs to be taken into consideration as a very high concentration might lead to aggregation of the antibody and hence it might be favourable to have broader elution peaks.

1.8.6 Protein A leakage

In order to ensure product safety it is necessary to have a minimum amount of protein A leakage in the eluent. Protein A leakage is mostly measured by using Protein A ELISA to get the protein A concentration in the eluent (Hahn et al., 2006).

1.8.7 Purity

In order to understand the selectivity of the adsorbent, using just pure IgG solution is not sufficient, adding impurities to the feed will give a better understanding. The entire chromatographic cycle can be carried out by adding impurities to the protein solution for example 2.5% Fetal calf serum (Hahn et al., 2003) or cell culture supernatant (Hahn et al., 2006). The eluent can then be tested by SDS /Page to measure its purity.

1.8.8 Life Cycle Studies

Having an indication of how many cycles an adsorbent can perform without it affecting its performance can be very important for economic reasons. This can be done by performing repetitive purification cycles, which includes loading, washing, elution, regeneration and CIP steps. Factors like protein A leakage, host cell protein in the eluent and dynamic capacity can then be studied. The type of protein ligand used, has a great influence on the amount of protein A leakage. Previous studies have shown that MabSelect Sure showed the least amount of protein A leakage when compared to MabSelect Xtra and Prosep vA-Ultra due to its alkaline stabilized protein A ligand (Hahn et al., 2006). The capacity can be studied by measuring the IgG concentration in the eluent or also by using laser scanning confocal microscopy, which can give an indication of adsorbent fouling.

1.9 Confocal Microscopy

Dynamic capacity and kinetic adsorption have been studied by using packed column or batch adsorption experiments as mentioned in section 1.8.2 and 1.8.3. Confocal microscopy, on the other hand, can be used to analyse the adsorption of IgG on a single particle.

In confocal microscopy the specimen is fluorescently labelled to be detected by the confocal scanning microscope. The depth discriminating property of the confocal microscopy can be used to carry out virtual optical sectioning of a specimen.

Adsorption and kinetic properties of the media can be studied by attaining images of a single particle by incubating the adsorbent for different time intervals in fluorescently labelled IgG solution (Ljunglof et al., 1996). In order to get images in a more dynamic condition; flow cells (Fig 1.10) can be used (Dziennik et al., 2003). Here the particles are packed behind a coverslip and the adsorbent is kept in place with the help of a frit and the fluorescently labelled IgG feed solution is passed through the adsorbent with the help of a pump.

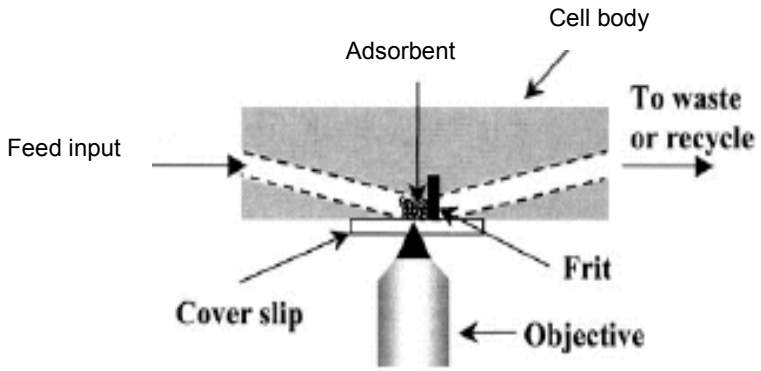


Fig 1.10 – Flow cell set up used to view labelled individual beads under a confocal microscope

These images give an indication of where the IgG molecules tend to bind on a single particle at different time intervals, whether on the surface or more in the interior of the porous particles. A similar procedure can be used to get dynamic desorption images.

Confocal microscopy can also be used to get an idea of adsorbent fouling by taking samples of adsorbent from different chromatographic cycles and incubating them in fluorescently labelled IgG and viewing the particles under a confocal microscope (Siu et al., 2006, Thillaivinayagalingam et al., 2007). Confocal microscopy can also

be used to see where the ligand is attached to the adsorbent by fluorescently labelling the ligands itself.

1.10 Conclusion

There are several factors to consider when designing a protein A affinity chromatography adsorbent. Different elements would have an effect on different performance measurements. Several methodologies tend to help manufacturers to characterise these adsorbents. It is usually the case that not all the desired characteristics of the adsorbents can be achieved and a trade off needs to be made.

Selectivity of the adsorbent can be of importance as it can eliminate further downstream processing steps, hence reducing costs. Higher binding capacity and better mass transfer properties are important especially due to the fact that feedstock expression levels are increasing and the affinity step needs to be able to purify a large amount of feed. These factors are important especially to companies where the affinity chromatography is a bottleneck in the bioprocess. On the other hand, the production rate might be of great importance too. Some adsorbents might have a higher dynamic capacity but can only perform at lower flow velocities due to compressibility issues, whereas other adsorbents might have a lower dynamic capacity but can withstand higher flow rates without getting compressed. Hence more cycles would be required to process a given amount of antibodies, but as each cycle is run quickly the overall production rate might increase. Then the factor to consider would be whether the adsorbent's performance is affected by the increase in the number of cycles.

Hence by having a methodology that can be used to study various characteristics and the performance of different adsorbents can help companies to design adsorbents that would be beneficial to manufacturing companies, by taking the economical factor into consideration too. The next chapter will look into some methods that can be used to characterise affinity adsorbents.

1.11 Target of the thesis

This thesis will mainly focus on designing a set of techniques to understand the performance of different affinity chromatography adsorbents. The different

adsorbents that are considered in this thesis are MabSelect (GE Healthcare), MabSelect Xtra (GE Healthcare), Prosep Ultra (Millipore), Protein A immobilised on 4CL Sepharose (GE Heatlhcare) in house and a prototype adsorbent with a Protein A mimic ligand (Millipore). These adsorbents are considered specifically as they have different particle size, porosity, support material and the type of ligand. The major methods used in this thesis looks at the static capacity, adsorption equilibrium data, dynamic capacity, uptake kinetics and desorption kinetics of different adsorbents. A more novel technology using confocal microscopy is also designed using a flow cell, which gives a good indication of the visual characteristics of an adsorbent, diffusion rate to the centre of the bead and the adsorption rates at an individual bead level. It can be of great benefits to have a set of methods that can be used to characterise adsorbents especially to manufactures of novel adsorbents.

Chapter 2 – Performance measurement of affinity chromatography adsorbents

2.1 Abstract

Affinity chromatography plays a big role in the purification of monoclonal antibodies. To make the process more cost effective and to be able to handle the large bioreactor volumes produced, manufacturers of chromatography media are looking for robust methodologies to characterise the performance of these media. This chapter looks at designing and analysing a range of different performance measurement methods. Any new adsorbent in production can be subjected to these methods to understand their characteristics and compare them with other commercially available adsorbents.

Four different affinity adsorbents are looked at in this chapter, which are MabSelect (GE Healthcare), MabSelect Xtra (GE Healthcare), Prosep Ultra (Millipore, Consett) and a prototype adsorbent immobilised with a synthetic ligand (Protein A mimic). All these four adsorbents have distinct qualities in terms of pore size, particle size, ligand type and the material the adsorbent is made from.

In this chapter, 6 different methods were designed and analysed. The static capacity experiment showed that MabSelect Xtra has the highest static capacity of 61.8mg/ml. The adsorption equilibrium data showed very low Kd values as expected by such affinity adsorbents. MabSelect Xtra and Prosep Ultra showed steeper equilibrium curves possibly indicating that these media have higher affinity to hIgG at lower hIgG concentration. Dynamic capacity carried out in a 0.66cm Omnifit column with a bed height of 6cm at 2, 4 and 8 min residence time showed MabSelect Xtra to have the highest dynamic capacity of 37.1mg/ml at 2 min residence time. By designing a method using the AKTA^{TM} Basic, studies of uptake kinetics using 1mg/ml hIgG were carried out, which showed all the four media to have very similar hIgG uptake rates. The same experiment was carried out using a higher concentration of hIgG (3mg/ml) on MabSelect and MabSelect Xtra. The uptake rate was much faster, however it was similar for both the adsorbents. Similarly desorption rate was carried out on the four media at room temperature

and at 4^0C to slow the process down. Again all the four media showed similar characteristics at both the temperatures.

2.2 Introduction

This chapter aims to study the performance of a few commercially available Protein A media along with a prototype. These adsorbents are MabSelect (GE Healthcare), MabSelect Xtra (GE Healthcare) and Prosep Ultra (Millipore, Consett). MabSelect consists of highly cross-linked agarose beads with a mean particle size of 85μm. MabSelect Xtra also consists of cross-linked agarose beads but it has a more open structure than that of MabSelect and hence greater porosity. The mean particle size is 75μm, smaller than MabSelect (Hahn et al., 2005). Prosep Ultra consists of irregularly shaped porous glass particles of ~100μm and a pore size of 70nm. The prototype consists of cross-linked agarose beads, but with a different percentage of agarose used when compared with MabSelect and MabSelect Xtra. The particle size is smaller with a mean of 51μm and the ligand used is a chemical ligand as opposed to Protein A ligand. This chapter gives an indication of a methodology that can be used to characterise chromatographic adsorbents. It will study the performance of different media, hence relating performance of the media to the support matrix type, particle size and pore size.

2.3 Static capacity

2.3.1 Materials for determining the static capacity

Chromatography media

MabSelect, MabSelectXtra media were from GE Healthcare (UK) and Prosep Ultra and a prototype media (CIGL) were from Millipore (Consett, UK).

Chemicals

Phosphate buffered saline (PBS) pH 7.4 was from Sigma-Aldrich (Gillingham, Dorset), which is biotechnology performance certified (product – P5368). Human polyclonal IgG was also from Sigma-Aldrich in the form of a 99% lyophilised powder (product G4386).

2.3.2 Method for determining the static capacity

The static capacity for MabSelect, MabSelect Xtra, Propsep Ultra and the prototype (CIGL) were determined by batch experiment. The method below needs to be repeated for each adsorbent. 55 ml of hIgG with a concentration of 1 mg/ml of hIgG in PBS pH 7.4 was made in a beaker and the solution was sterile filtered using 0.22µm syringe filters (Sartorius). Three, 20ml falcon tubes were labelled A, B and C and the fourth falcon tube was labelled as the adsorbent. 1 ml (2.3cm) of adsorbent was measured in a 5ml extraction column (Bioconnections, Leeds) by suction drying it with a syringe. The adsorbent was washed with 4CV of PBS. The adsorbent was put into the falcon tube labelled as the adsorbent with the help of a syringe. The extraction column was washed with 4ml PBS and the same 4ml was added to the falcon tube with the adsorbent. The adsorbent falcon tube was placed on a stirrer. When the beads were in uniform suspension, 500µL of the suspension was taken using a 1ml pipette and added to each of the three 20ml falcon tubes labelled A, B and C (hence making triplicates for each sample). 14ml of 1mg/ml hIgG solution was added into each of the three falcon tubes, A, B and C. The three falcon tubes were put on a rotator for 6 hours. It was taken off the rotator and the beads were left to settle. Each sample was analysed in a spectrometer by measuring the UV absorbance in a quartz cuvette at 280nm and the data was recorded, using PBS as blank. The average of the 3 samples was used to calculate the static capacity.

The static capacity Qs was calculated using the formula below:

Qs = {(1mg/ml)*14ml hIgG solution – [(Protein concentration in solution left after run/measured feed concentration)*1mg/ml](14.5ml total volume)}/0.1ml beads

The concentration was measured using the formula below:
Concentration (mg/ml) = A (mAu)/1.38 (Ext.coeff)

2.3.3 Results

The table below shows the average static capacity of the three samples for each of the adsorbents. Appendix A shows the static capacity for all the samples.

Adsorbent	Static capacity (mg/ml)
MabSelect	57.6
MabSelect Xtra	61.8
Prosep Ultra	56.4
Prototype (CIGL)	59.3

Table 2.1 - Static capacity for MabSelect, MabSelectXtra, Prosep Ultra and CIGL. MabSelect Xtra shows the highest static capacity of 61.8mg/ml. Average of 3 samples was taken.

2.4 Adsorption equilibrium data used to determine maximum capacity and dissociation constant

2.4.1 Materials for adsorption equilibrium experiments

Chromatography media

MabSelect, MabSelectXtra media were from GE Healthcare (UK) and Prosep Ultra and a prototype media (CIGL) were from Millipore (Consett, UK).

Chemicals

Phosphate buffered saline (PBS) pH 7.4 was from Sigma-Aldrich (Gillingham, Dorset), which is biotechnology performance certified (product – P5368). Human polyclonal IgG was also from Sigma-Aldrich in the form of a 99% lyophilised powder (product G4386).

2.4.2 Method for adsorption equilibrium experiments

Adsorption equilibrium data were determined by batch experiment for MabSelect, MabSelect Xtra, Propsep Ultra and the prototype (CIGL). The method below needs to be repeated for each adsorbent. 80ml of 0.5mg/ml of hIgG in PBS pH 7.4 was made in a beaker and the solution was sterile filtered using 0.22μm syringe filter (Sartorius). Six, 20ml falcon tubes were labelled A, B, C, D, E and F and the seventh 20ml falcon tube was labelled as the adsorbent.

1 ml (2.3cm) of adsorbent was measured in a 5ml extraction column (Bioconnections, Leeds) by suction drying it with a syringe. The adsorbent was washed with 4CV of PBS. The adsorbent was put into the falcon tube labelled as the adsorbent with the help of a syringe. The extraction column was washed with 4ml PBS and the same 4ml was added to the falcon tube with the adsorbent. The adsorbent falcon tube was placed on a stirrer. When the beads were in uniform suspension, 500μL of the suspension was taken using a 1ml pipette and added to each of the 20ml falcon tubes labelled A, B, C, D, E and F (hence making 6 samples). 4ml, 8ml, 10ml, 12ml, 16ml and 20ml of 0.5mg/ml hIgG solution was added into falcon tubes labelled A, B, C, D, E and F respectively. The final volume of liquid was made up to 20ml with PBS. The tubes were put on a rotator for 6 hours. It was taken off the rotator and the beads were left to settle. Each sample was analysed in a spectrometer by measuring the UV absorbance in a quartz cuvette at 280nm and the data was recorded, using PBS as blank.

The equilibrium concentration in bulk liquid (C*) was calculated using the formula below:

C* mg/ml = Feed concentration * Protein concentration in solution left after run/measured feed concentration

The equilibrium concentration on adsorbent (q*) was calculated using the formula below:

q* mg/ml = (0.5mg/ml) * volume of hIgG solution – [(Feed concentration) * (Protein concentration in solution left after run/measured feed concentration)](20.5ml total volume)

This gave results for 0.1ml of media and hence the data was converted for 1ml of media, which was then used to plot the graphs.

Kd (M) was calculated using the following formula:
Kd (M) = Kd (mg/ml)/ 150000 (Approximate RMM of IgG)

2.4.3 Results

Two different ways were used to determine the maximum capacity and the dissociation constant. In the first way, the data collected from the experiment was linearised and the linearised form of the Langmuir isotherm (Hanes plot) was used to get the maximum capacity and the dissociation constant, where 1/slope gives the maximum capacity and y intercept equals to Kd/Q_{max}.
In the second way the experimental data was fitted using Langmuir isotherm and the maximum capacity and dissociation constant were determined using the Solver function in Excel, without linearising the isotherm.

The data collected was for human polyclonal IgG, which included the IgG_3 that doesn't bind to protein A (MabSelect, MabSelect Xtra, Prosep Ultra). Hence the data was corrected to take this factor into account. To get the corrected values, 5.5% (Hahn et al., 2005) from the initial feed concentration was subtracted to give the following results. This correction only applies to MabSelect, MabSelect Xtra and Prosep Ultra as CIGL tends to bind IgG_3.

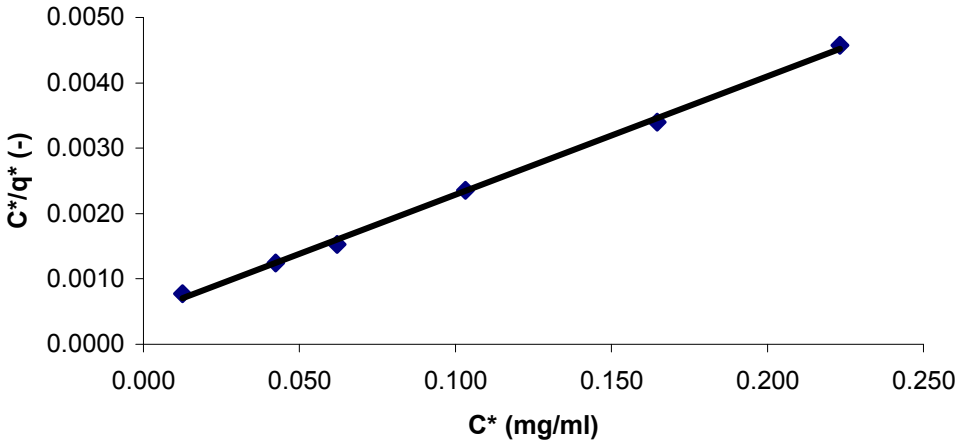


Fig 2.1 – Hanes Plot – MabSelect. Linearised form of Langmuir isotherm for Mabselect. 1/slope gives the maximum capacity and y intercept equals to $Kd/Q_{max.}$ The equation for the line is y=0.01813x + 0.00047 with an R^2 value of 0.99832. Here $Q_{max} = 55.1$ and Kd = 0.026mg/ml

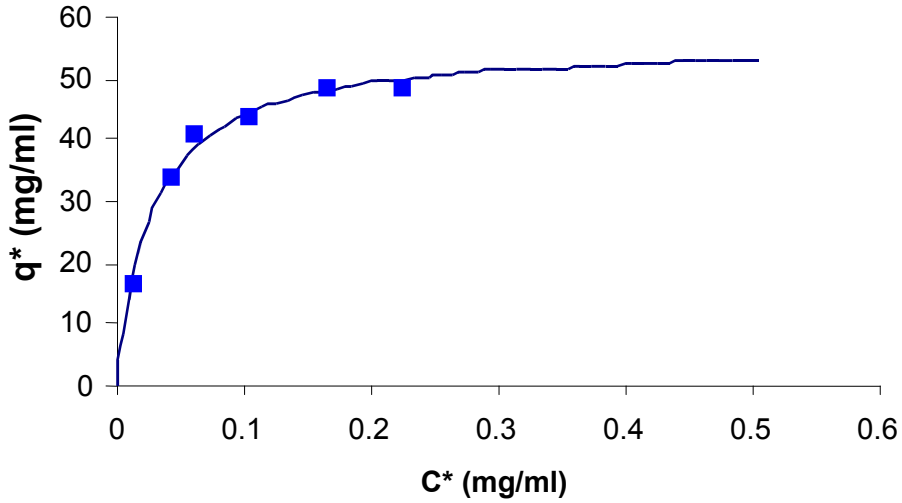


Fig 2.2 – Adsorption Equilibrium – MabSelect. Experimental data for MabSelect fitted using Langmuir isotherm and the maximum capacity and dissociation constant were determined using the Solver function in Excel.

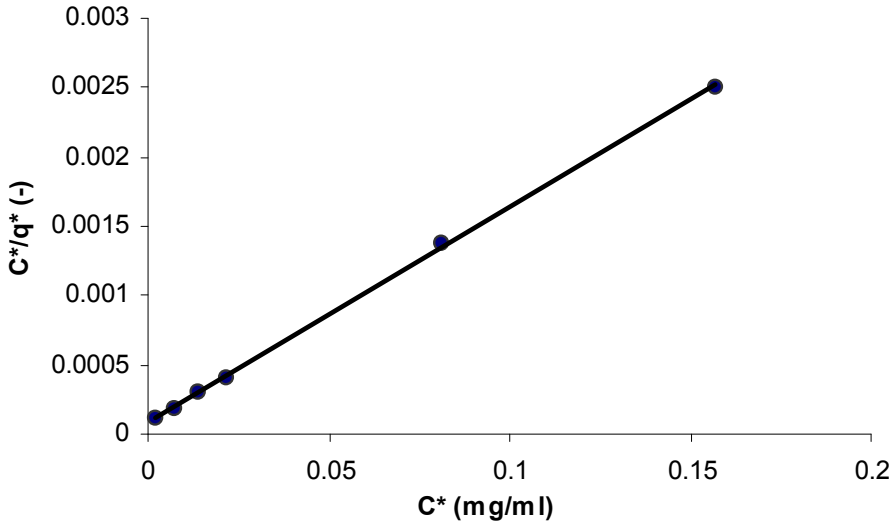


Fig 2.3 – Hanes Plot – MabSelect Xtra. Linearised form of Langmuir isotherm for Mabselect Xtra. 1/slope gives the maximum capacity and y intercept equals to $Kd/Q_{max.}$ The equation for the line is y=0.01554x + 0.00009 with an R^2 value of 0.99975. Here $Q_{max} = 64.4$ and Kd = 0.006mg/ml

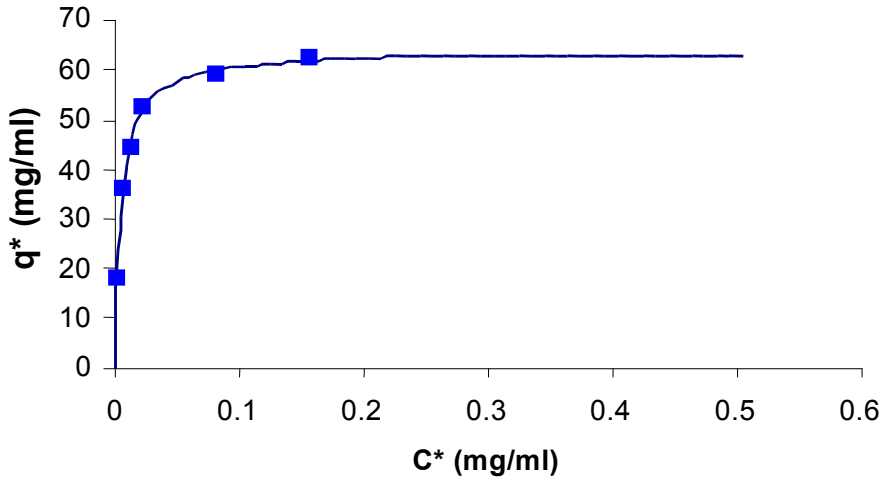


Fig 2.4 – Adsorption Equilibrium – MabSelect Xtra. Experimental data for MabSelect Xtra fitted using Langmuir isotherm and the maximum capacity and dissociation constant were determined using the Solver function in Excel

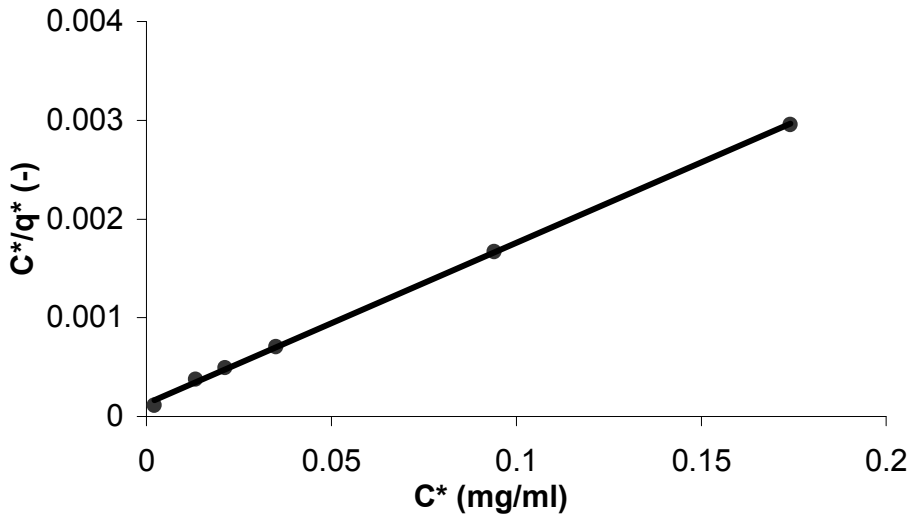


Fig 2.5 – Hanes Plot – Prosep Ultra. Linearised form of Langmuir isotherm for Prosep Ultra. 1/slope gives the maximum capacity and y intercept equals to $Kd/Q_{max.}$ The equation for the line is y=0.01630x + 0.00013 with an R^2 value of 0.99927. Here $Q_{max} =$ 61.3 and Kd = 0.008mg/ml

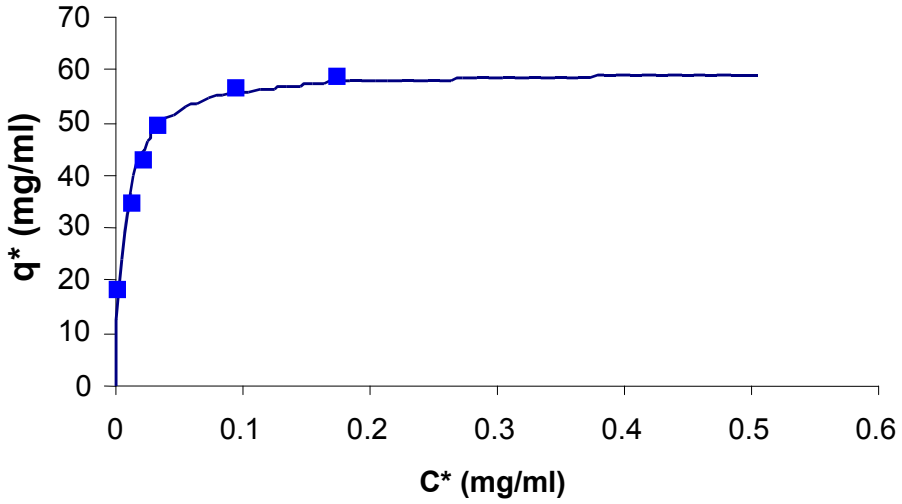


Fig 2.6 – Adsorption Equilibrium – Prosep Ultra. Experimental data for Prosep Ultra fitted using Langmuir isotherm and the maximum capacity and dissociation constant were determined using the Solver function in Excel

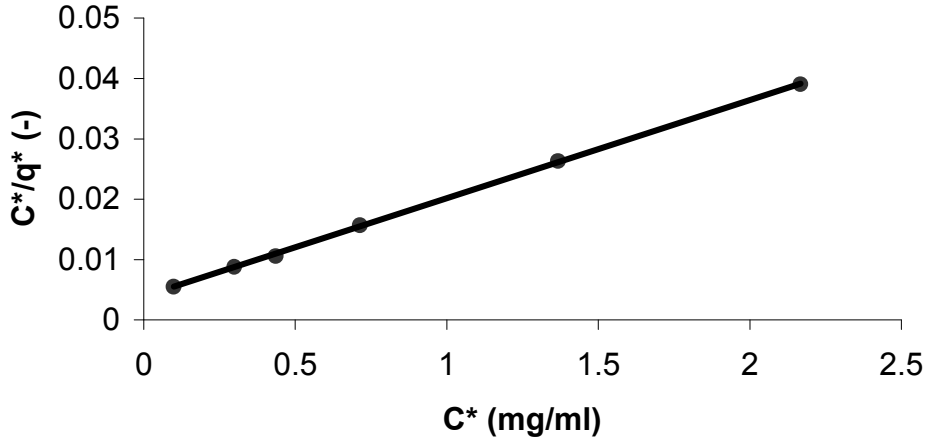


Fig 2.7 – Hanes Plot – CIGL. Linearised form of Langmuir isotherm for CIGL. 1/slope gives the maximum capacity and y intercept equals to $Kd/Q_{max.}$ The equation for the line is $y=0.0163x + 0.0039$ with an R^2 value of 0.9997. Here $Q_{max} = 61.5$ and Kd = 0.024mg/ml

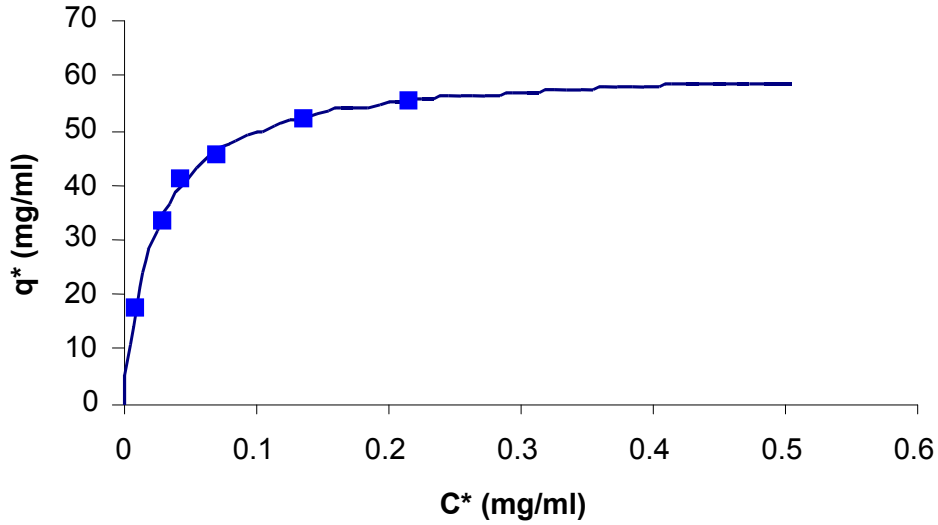


Fig 2.8 – Adsorption Equilibrium – CIGL. Experimental data for CIGL fitted using Langmuir isotherm and the maximum capacity and dissociation constant were determined using the Solver function in Excel

Adsorbent	Q_{max} (mg/ml)	Kd (mg/ml)	Kd (nM)
MabSelect	55.1	0.026	173
MabSelect Xtra	64.4	0.006	38.6
Prosep Ultra	61.3	0.008	53.2
CIGL	61.5	0.024	160

Table 2.2 - A summary of maximum capacity and dissociation constant determined using Hanes Plot for MabSelect, MabSelect Xtra, Prosep Ultra and CIGL.

Adsorbent	Q_{max} (mg/ml)	Kd (mg/ml)	Kd (nM)
MabSelect	55.9	0.027	181
MabSelect Xtra	63.4	0.005	35.8
Prosep Ultra	60	0.007	49.9
CIGL	61.3	0.023	156

Table 2.3 - A summary of maximum capacity and dissociation constant determined using Solver function in Excel for MabSelect, MabSelect Xtra, Prosep Ultra and CIGL.

2.5. Dynamic capacity

2.5.1 Material for determining dynamic capacity

Chromatography media

MabSelect, MabSelectXtra media were from GE Healthcare (UK) and Prosep Ultra and a prototype media (CIGL) were from Millipore (Consett, UK).

Chemicals

Phosphate buffered saline (PBS) pH 7.4 was from Sigma-Aldrich (Gillingham, Dorset), which is biotechnology performance certified (product – P5368). Human polyclonal IgG was also from Sigma-Aldrich in the form of a 99% lyophilised powder (product G4386). Glycine was also from Sigma-Aldrich.

2.5.2 Method for determining dynamic capacity

The adsorbents were packed into a 0.66cm Omnifit column to a bed height of 6cm. Frontal analysis runs were performed using AKTA^{TM} Basic and antibody concentrations were monitored on a spectrometer by UV absorbance at 280nm. The packed column was equilibrated with PBS pH 7.4. The column was loaded with 1mg/ml hIgG in PBS. The loading flow rate was varied between 1– 0.25 ml/min to give residence times between 2-8mins respectively. The loading was stopped at 20% breakthrough to take into account 10% IgG_3 (value decided internally by Millipore, UK) that doesn't bind to Protien A and hence give a 10% breakthrough value (value decided internally by Millipore, UK). The column was washed with PBS pH 7.4. The bound hIgG was eluted using 0.1M glycine pH 2 and collected. The sample was analysed by measuring the UV absorbance on a spectrometer at 280nm to determine the hIgG concentration. The dynamic capacity of the adsorbents was calculated.

Experimental data can be seen in Appendix A

2.5.3 Results

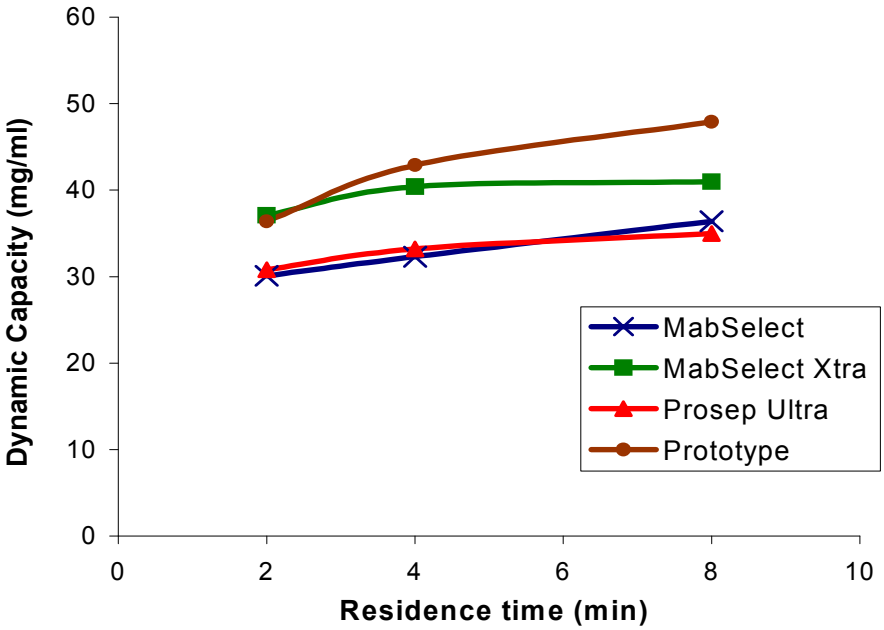


Fig 2.9 – Dynamic capacity determined at 2min, 4min and 8min residence time for MabSelect, MabSelect Xtra, Prosep Ultra and CIGL. 0.66cm Omnifit column with a bed height of 6cm was used. Loading was stopped at 20% breakthrough.

Adsorbent	**Dynamic Binding Capacity (mg/ml)**		
	2mins	**4mins**	**8mins**
MabSelect	30.1	32.3	36.4
MabSelect Xtra	37.1	40.4	41
Prosep Ultra	30.8	33.2	35
CIGL	36.4	42.9	47.9

Table 2.4 - Summary of dynamic capacity determined at 2min, 4min and 8min residence time for MabSelect, MabSelect Xtra, Prosep Ultra and CIGL. An Omnifit column with a 6cm bed height, appr. 2ml adsorbent was used.

CIGL shows an exceptionally high dynamic capacity at 8min residence time. This is due to the fact that IgG_3 tends to bind to CIGL and hence the loading should have been stopped at 10% breakthrough instead of 20% for this media for 2min, 4min

and 8min residence time. Due to lack of time this experiment wasn't repeated for CIGL at 10% breakthrough.

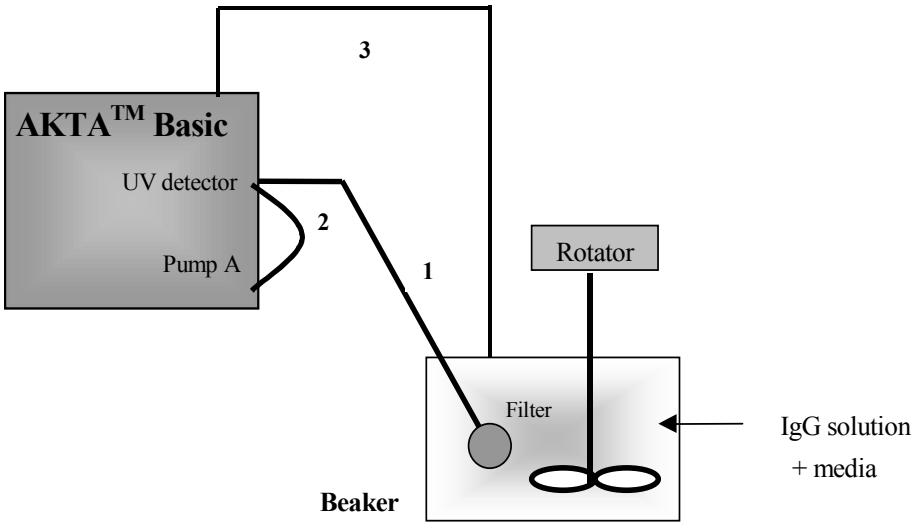
2.6 Batch uptake rate

2.6.1. Method to determine uptake rate

In this experiment phosphate buffered saline (PBS) pH 7.4 was from Sigma-Aldrich (Gillingham, Dorset), which is biotechnology performance certified (product – P5368). Human polyclonal IgG was also from Sigma-Aldrich in the form of a 99% lyophilised powder (product G4386).

Response time

Antibody uptake kinetics was determined by continuously recirculating a small stream of hIgG solution through the AKTA^{TM} Basic. The hIgG solution is mixed with the adsorbent in a vessel with the help of an impeller (Fig 2.10).



Tube 1 – Connected from filter to outlet of UV detector
Tube 2 – Connected from inlet of UV detector to Pump A of AKTA^{TM} Basic.
Tube 3 – Waste loop

Fig 2.10- Uptake rate equipment setup used to determine the uptake kinetics for different media. It consists of a stream of hIgG solution recirculating through the AKTA^{TM} Basic from a beaker consisting of homogenous mixture of hIgG and media solution.

At any point in time as a small stream of solution is leaving the system it is not in contact with the adsorbent for a certain amount of period. This gives a difference in the hIgG concentration between the stream of solution drawn and that in the beaker. As a result it takes a certain amount of time before the system stabilises and shows a constant UV 280nm reading. This in other words is the response time of the system. In order to get the response time the following method was carried out.

First the system was equilibrated with PBS. A solution of hIgG was prepared in a 250ml beaker. Stream of hIgG solution was drawn from the beaker through a filter membrane by using AKTA^{TM} Basic. The point at which the flow was started equals to time 0 in Fig 2.11. This was the start time, which was specified manually when the flow was started. The time taken to give a constant UV 280nm reading equals to the response time of the system. The aim is to keep the response time to a minimum, as this would give more accurate results when carrying out the uptake kinetic experiment. Two sets of experiments were carried out at a flow rate of 4ml/min and 6ml/min.

The response time was estimated from the graph below (Fig 2.11). Two flow rates were used of 4ml/min and 6ml/min. The hIgG solution is detected quicker when using a flow rate of 6ml/min but they both tend to show a response time of ~20-25sec. Higher flow rates couldn't be used as the media in the solution tend to stick to the filter. Hence a flow rate of 4ml/min was used to carry out the experiment.

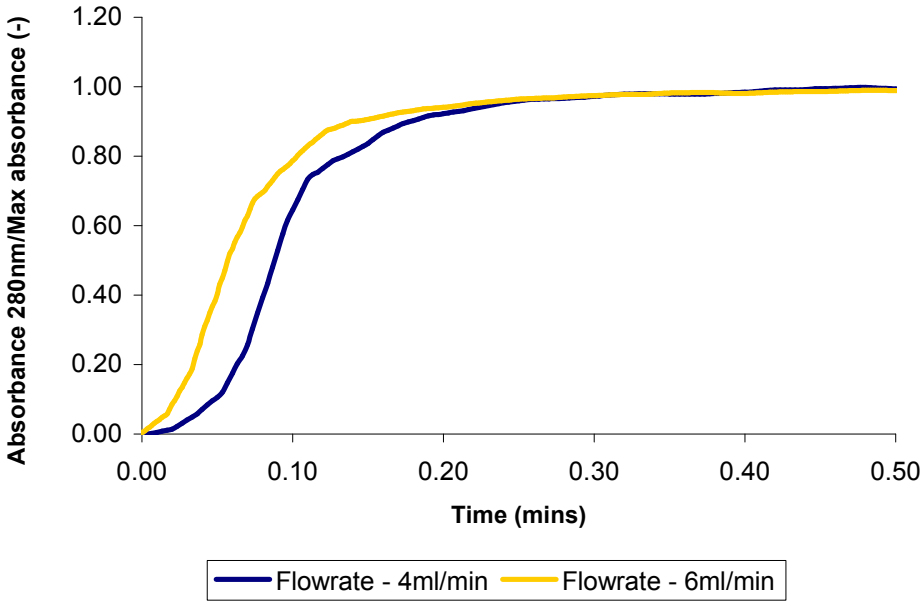


Fig 2.11 – Response time here is defined as the amount of time before the system stabilises and shows a constant UV 280nm reading. This equilibration time (response time) is recorded at 4ml/min and 6ml/min flow rate which shows to be ~20-25sec.

Uptake Rate

Antibody uptake kinetics was determined by preparing 100ml of 1mg/ml of hIgG solution in PBS. The solution was agitated with a marine impeller (2cm diameter, 2 blades) at 145rpm. The hIgG concentration was monitored by continuously recirculating a small stream of solution through the AKTA^{TM} Basic. The response time was ~20-25sec at a flow rate of 4ml/min. 1ml of media was measured with the help of an extraction column (Bioconnections Ltd, Leeds). This was suction dried and added to the solution. Time 0 on the graph is when the suction dried media was poured into the hIgG solution, which was specified manually. The setup was left to run for 2-3 hours. This same procedure was repeated by preparing 100ml of 3mg/ml of hIgG solution in PBS.

Characterisation of mixing and reproducibility of results

Two sets of experiments were carried out with CIGL to get reproducible results. One of them was carried out at 145rpm and the other at 200rpm.The difference in the mixing speed is not very large but the fact that reproducible results are achieved can prove that the mixing is uniform and the uptake rate is not affected by the change in mixing speed. Extremely high mixing speed could be used but this might damage the adsorbent. A very high speed was not needed to be tested, as from the graph below both the results produced are very similar, thus proving that a mixing speed above 145rpm doesn't affect the results. Below 125rpm the beads were not uniformly mixed visually. Moving of the impeller position was needed occasionally at 145rpm and 200rpm due to the beads settling down in certain areas of the beaker. This experiment was carried out without the use of any baffles. All the experiments were carried out at 145rpm, except for Prosep Ultra, which needed mixing at 200rpm as visually the beads tend to settle down at 145rpm. In Fig 2.12 Q_{max} equals to the maximum capacity of the adsorbents achieved at the end of the experiment (120mins).

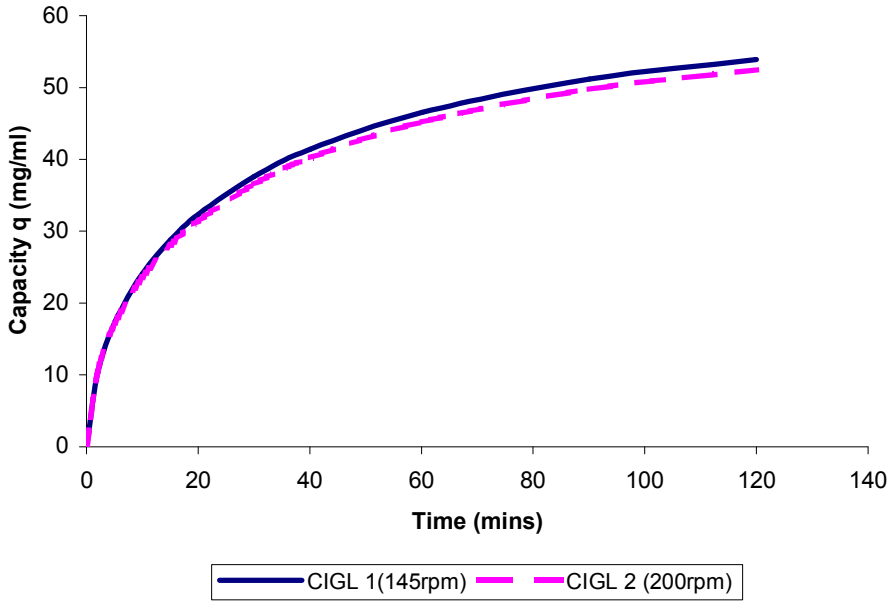


Fig 2.12 - Uptake kinetic curves for two samples of CIGL where the impeller speed of 145rpm and 200rpm was used to check mixing characterisation and reproducibility.

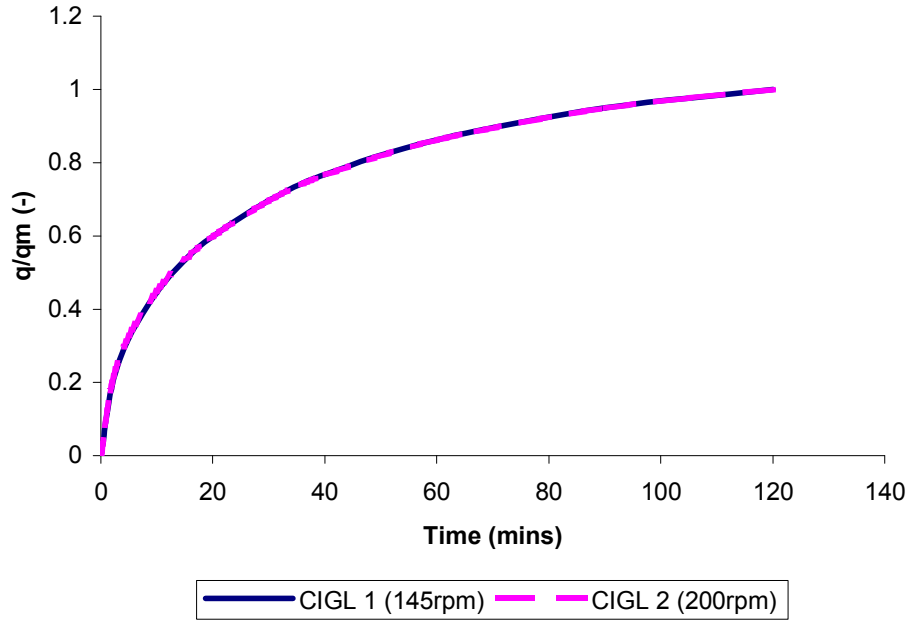


Fig 2.13 - Uptake kinetic curves for two samples of CIGL where maximum capacity at the end of adsorption for each sample equals to 1. The two graphs showed to be reproducible.

2.6.2 Results

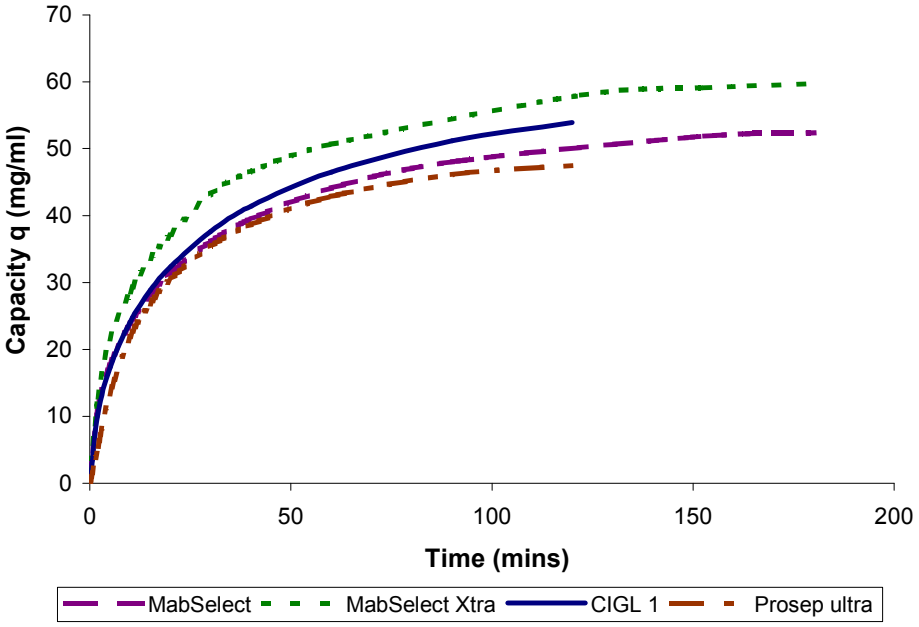


Fig 2.14 – Uptake kinetic curves for MabSelect, MabSelect Xtra, Prosep Ultra and CIGL. Impeller speed of 145 rpm was used except for Prosep Ultra where 200rpm was used. Maximum capacity determined at 120 mins.

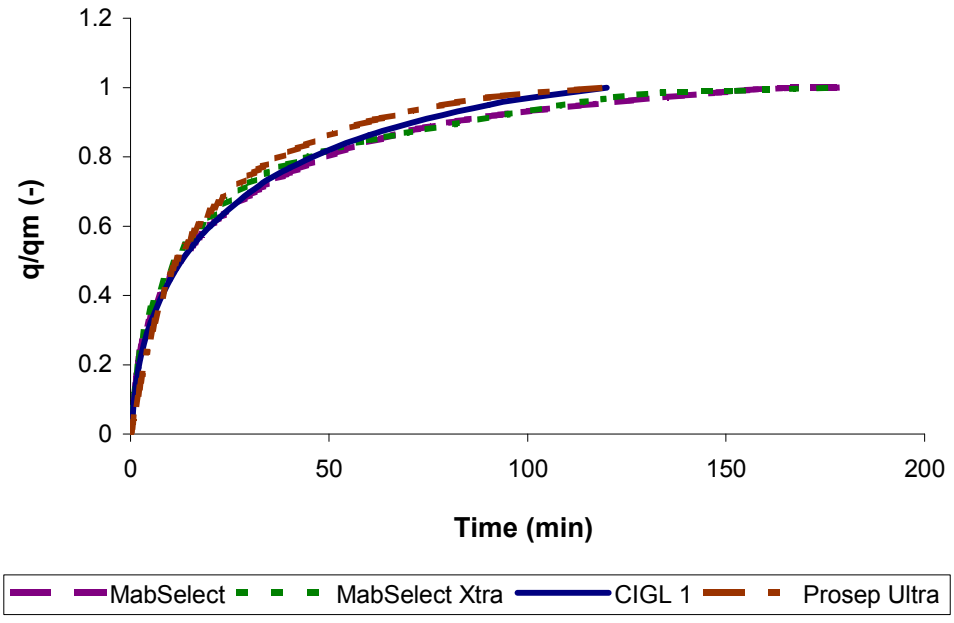


Fig 2.15 – Uptake kinetic curves for MabSelect, MabSelect Xtra, Prosep Ultra and CIGL where maximum capacity at the end of adsorption for each media equals to 1. The uptake rate shows to be very similar for all four media.

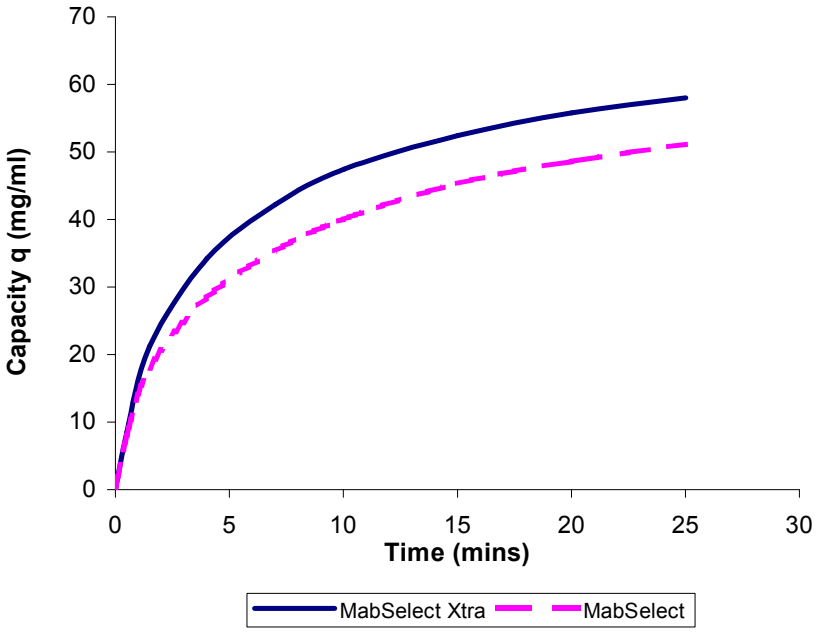


Fig 2.16 – Uptake kinetic curves for MabSelect and MabSelect Xtra using higher concentration of hIgG (3mg/ml) and an impeller speed of 145rpm. The uptake rate shows to be very similar for the two media.

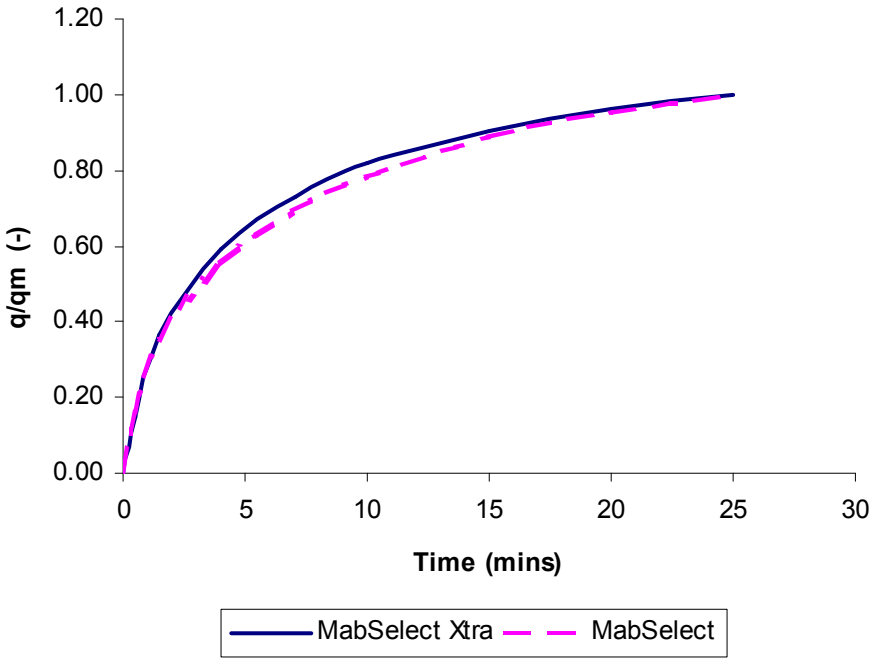


Fig 2.17 – Normalised version of the uptake kinetic curves for MabSelect and MabSelect Xtra using higher concentration of hIgG (3mg/ml) and an impeller speed of 145rpm. The uptake rate shows to be very similar for the two media.

2.7 Desorption Kinetics

2.7.1 Method to analyse desorption rate

In this experiment phosphate buffered saline (PBS) pH 7.4 was from Sigma-Aldrich (Gillingham, Dorset), which is biotechnology performance certified (product – P5368). Glycine was also from Sigma-Aldrich.

After the completion of the uptake the media was left to settle. The media was collected in an extraction column (Bioconnections Ltd, Leeds) and washed with PBS. It was suspended in 1ml of PBS in the extraction column. It was then transferred to 100ml of 0.1M glycine pH 2. Time 0 on the graph is when the suspended media was poured into the glycine solution, which was specified manually. The solution was agitated with a marine impeller at 145rpm. The hIGg concentration was monitored by continuously recirculating a small stream of solution through the AKTA^{TM} Basic. The setup was left to run until a steady absorbance reading was achieved. The data was used to get the desorption kinetics.

Characterisation of mixing and reproducibility of results

Two sets of experiments were carried out with CIGL to get reproducible results. One of them was carried out at 145rpm and the other at 200rpm for the same reason as mentioned in section 2.4. From the graph below both the results produced are very similar, thus proving that the mixing speed doesn't affect the results.

The end point of the experiment was taken as zero, hence assuming that the amount of antibody released is equal to the amount that was bound to the adsorbent initially. This was done because the transfer of the suspended adsorbent from the extraction column to the elution buffer caused some loss of the media.

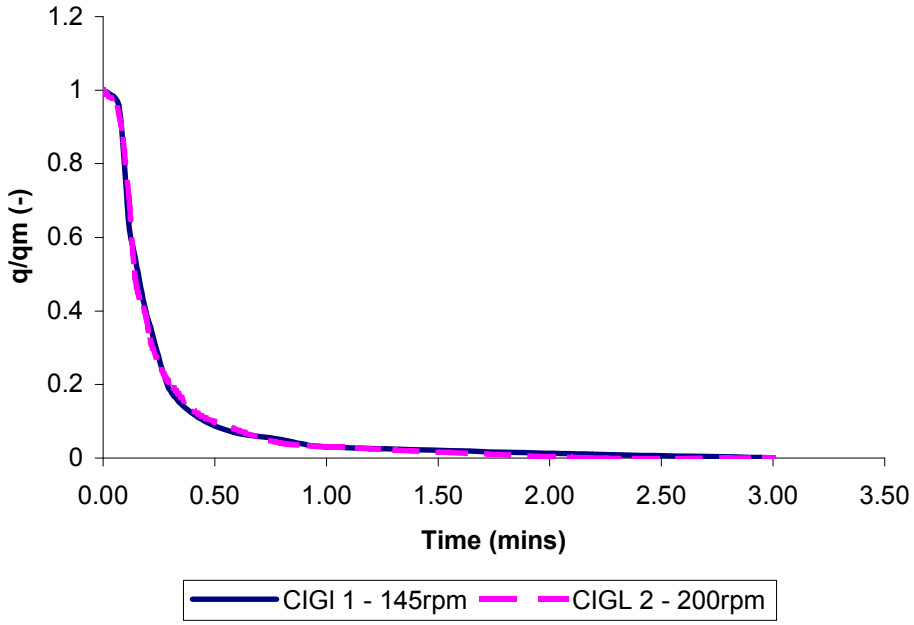


Fig 2.18 – Desorption kinetic curves for two samples of CIGL where the impeller speed of 145rpm and 200rpm was used to check mixing characterisation and reproducibility

2.7.2 Results

Fig 2.19 shows the desorption curves for all the four media. From the graph it is difficult to say if the slight differences are due to the type of adsorbents or due to experimental inaccuracies.

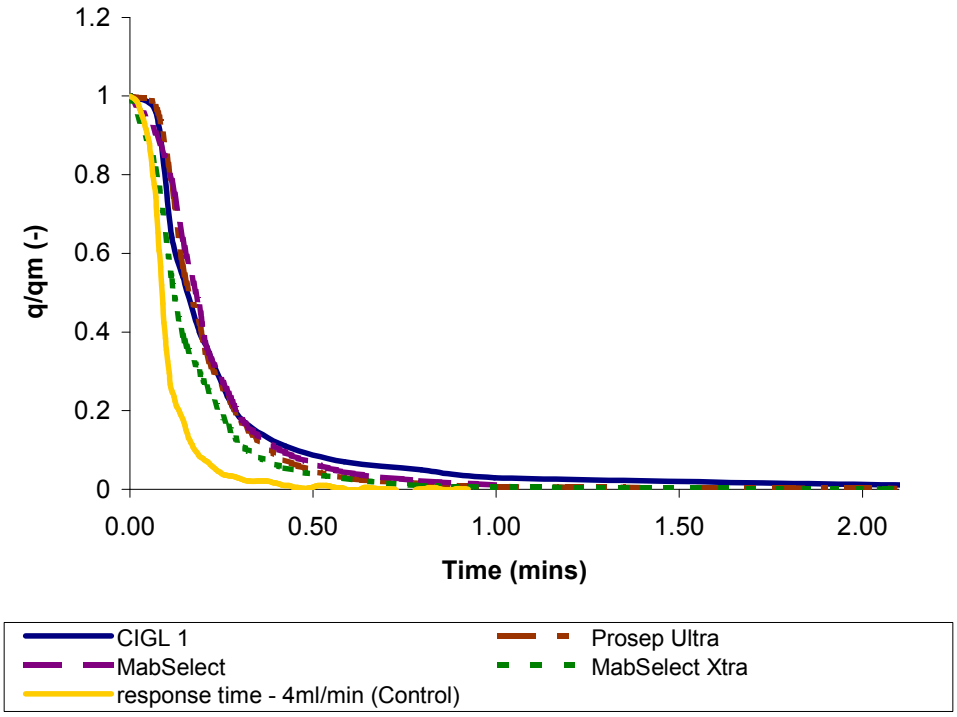


Fig 2.19 – Desorption kinetic curves for MabSelect, MabSelect Xtra, Prosep Ultra and CIGL. Impeller speed of 145 rpm was used except for Prosep Ultra where 200rpm was used. Desorption rate shows to be very similar for all four media.

2.8 Desorption at 4^{o}C

2.8.1 Method to analyse desorption kinetics at 4^{o}C

In this experiment phosphate buffered saline (PBS) pH 7.4 was from Sigma-Aldrich (Gillingham, Dorset), which is biotechnology performance certified (product – P5368). Glycine was also from Sigma-Aldrich.

After the completion of the uptake experiment the media was left to settle. The media was collected in an extraction column (Bioconnections Ltd, Leeds) and washed with PBS. It was suspended in 1ml of PBS in the extraction column. It was then transferred to 100ml of 0.1M glycine pH 2. This glycine solution in a beaker was placed with ice around it to maintain the temperature at 4^{o}C, which was measured by a temperature probe. Time 0 on the graph is when the suspended media was poured into the glycine solution, which was specified manually. The solution was agitated with a marine impeller at 145rpm. The hIGg concentration was monitored by continuously recirculating a small stream of solution through the

AKTA^{TM} Basic. The setup was left to run until a steady absorbance reading was achieved. The data was used to get the desorption kinetics.

2.8.2 Results

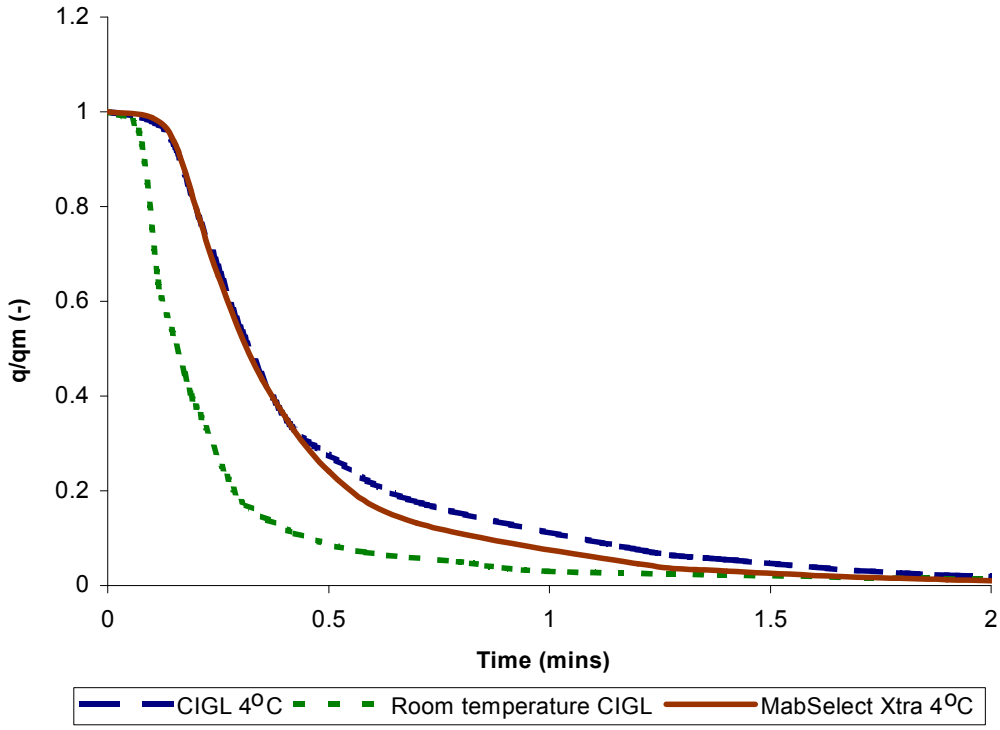


Fig 2.20 – Desorption carried out on CIGL and MabSelect Xtra at 4^{o}C to slow down the rate of desorption. Similar results showed for the two media at 4^{o}C. Impeller speed of 145 rpm was used

2.9 Discussion

From the static capacity data MabSelect Xtra has the highest static capacity of 61.8mg/ml. This was expected due to the more porous nature of the bead, which increased the surface area available for Protein A ligand immobilisation. MabSelect and Prosep Ultra showed similar static capacity data as proposed by the manufacturers. The prototype in fact showed relatively good static capacity of 59.2mg/ml.

The adsorption isotherm data from the Hanes Plot and by using the Solver function from Excel were very similar. Both the methods to analyse the data seem to be accurate. The Q_{max} values estimated from the adsorption isotherms were very similar to that achieved from the static capacity experiments. The Kd values range from 181nM to 36nM. Such low values are expected by affinity adsorbents such as these. The adsorption isotherms were slightly steeper for MabSelect Xtra and Prosep Ultra when compared to MabSelect and the prototype. This probably means that MabSlect Xtra and Prosep Ultra have higher affinity to hIgG at lower hIgG concentration. It must be noted that 5.5% was subtracted from the initial feed concentration. This percentage is the estimated amount of IgG_3 that doesn't bind to MabSelect, MabSelect Xtra and Prosep Ultra. To make this method further accurate it is recommended to use monoclonal IgG without IgG_3.

Results from the dynamic capacity experiment showed MabSelect Xtra to have a greater dynamic capacity than the other three adsorbents. This is again due to the more porous nature of the beads and also due to the smaller size of the beads. The dynamic capacity for all four media increased with increase in the residence time. However the increase in the dynamic capacity for MabSelect Xtra from 4min residence time to 8min was much less when compared to other adsorbents. This could be due to an increase in resistance due to stoichiometric reasons. It must be noted here that the prototype showed exceptionally high dynamic capacity. This is due to the fact that IgG_3 binds to CIGL and hence the loading should have been stopped at 10% breakthrough instead of 20%. Hence CIGL cannot be used in this case for direct comparison.

A reliable system was designed to understand the uptake kinetics of different adsorbents. The system produced reproducible results for CIGL, proving the system to be accurate and reliable. From the results all the four adsorbents had very similar uptake rates. Hence the difference in particle size, pore size, the type of ligand or the material of the adsorbent itself did not have an effect on the uptake rate when carried out in a batch mode. The same experiment was carried out on MabSelect and MabSelect Xtra at a higher hIgG concentration. The time taken to approach saturation was much faster as expected and hence the uptake rate was much faster. This is due to the increase in diffusion rate due to a higher concentration gradient.

Desorption experiments again showed reproducibility when carried out on two samples of CIGL at two different impeller speeds. The actual desorption of hIgG from the adsorbent was achieved very quickly within a few minutes. Once again the desorption rate was very similar for all the four adsorbents.

In order to slow down the desorption process to get a closer look at any differences between the adsorbents, the process was carried out at 4^{o}C for CIGL and MabSelect Xtra. This definitely slowed down the process when compared to the desorption experiment carried out at room temperature. However the desoprtion rate was again very similar for CIGL and MabSelect Xtra.

2.10 Conclusion

The methods designed and carried out in this chapter demonstrates different ways to characterise adsorbents in different situations. In a batch mode the static capacity gives the maximum amount of hIgG that can bind to different adsorbents. The adsorption equilibrium curve gives a strong indication of the affinity of the adsorbent towards hIgG and the predicted maximum capacity. The uptake kinetics and desorption kinetics can help someone understand the rate at which different adsorbents can bind and release hIgG in a batch mode at different hIgG concentrations. The dynamic capacity gives an indication of how different adsorbents would perform when packed in a column. Clearly it can be seen that some methods show a clear distinction between different adsorbents whereas some show very similar results. This provides a set of methods to compare novel adsorbents against commercially available adsorbents and help manufactures understand which performance factors of an adsorbent need further analysis.
Batch methods can be very easy to perform and can give useful information on different adsorbent kinetics. The method is also less expensive than using a column and less time consuming. There are no issues involved with column packing and there is consistency in the data, as opposed to fixed bed methods where column packing can play a major role in the kind of data generated. There are also no minor technical issues such as air bubbles being introduced in the column. The data can also be comparable with different batch methods, where as comparing data generated from fixed bed experiments can be an issue, especially when different bed heights and column diameters has been used. Batch method used in this thesis to find kinetic adsorption data is even more reliable because the stream

of hIgG is being re-circulated and at no time are any samples been taken out of the system for analysis.

The next chapter looks into another more novel technology that uses laser scanning confocal microscopy. This method gives a better indication of the performance of matrices at a single bead level and also gives a more visual characterisation of the adsorbents.

Chapter 3 - Confocal analysis using flow cell

3.1 Abstract

The use of laser scanning confocal microscopy has proved to have several advantages. Its is a non-destructive process where individual beads can be analysed over time. Quantitative and qualitative analysis can then be carried out on the images produced. This chapter looks into the use of a flow cell placed under a confocal microscope where a channel of the flow cell is packed with different matrices and a continuous flow of 2mg/ml hIgG is passed through the channel with a flow rate of 0.1ml/min. The channels are 1cm in length and have a diameter of 0.1cm. It can pack ~8μl of media. The microscope is set up such that xy images of beads are taken every two minutes through the centre of various sized beads.

The following chapter studies four different matrices, which are MabSelect, MabSelect Xtra, Protein A immobilised on CL4 Sepahrose and a prototype media (CIGL). From the images a good understanding of the binding process could be derived visually for all the matrices. They all showed a shrinking core effect except for the prototype where the hIgG didn't penetrate into the bead and was only attached to the surface of the bead. Further quantitative analysis was carried out on these images. From the intensity profiles of the beads diffusion rates and uptake curves for individual beads could be calculated. It was found that the adsorption rate to the centre of each bead was linear. The different particle sizes within any particular type of matrix and also across different matrix did not result in different diffusion rates. The diffusion rates here were found to be approximately constant at around 0.72 min^{-1}. These matrices are designed to perform effectively at high concentrations. It could be possible that the difference in the diffusion rate might be visible at much lower hIgG concentrations. From the adsorption curves produced it can clearly be seen that smaller beads reached saturation much faster than larger beads. From this study we can conclude that this methodology can prove to be very useful in understanding the visual binding effects of different matrices and also understanding diffusion rates and adsorption rates at an individual bead level especially in chromatography media where heterogeneity in bead size exists.

3.2 Introduction

Chromatography is all but ubiquitous in the purification of biological molecules. The performance of chromatographic separations is usually evaluated using breakthrough curves, adsorption isotherms or static binding capacities. However, these methods do not provide a complete understanding of the process because of the complexity involved in this type of operation. At the level of individual beads it is difficult to infer from these macroscopic methods the spatial and temporaly nature of the binding within the bead. This maybe of particular relevance if the beads are heterogeneous in nature i.e. have significant size distribution, as is the case with most chromatographic media used in process scale operations. Microtome sectioning of beads has been used in the past to study the adsorption process within chromatographic media. Beads were sectioned using a microtome, and fluorescently labelled proteins then visualised (Subramanian et al., 1994). This has obvious disadvantages; it is a destructive technique and the method requires significant time to prepare samples for imaging, making many events difficult to study in this manner. More recently, the use of laser scanning confocal microscopy (CSLM) was introduced to circumvent these issues and due to its ability to show an optical section through the depth of beads (Ljunglof et al., 1996). CLSM, which allows quantitative measurement of the product and/or contaminants as they are adsorbed on to the beads, is an invaluable tool for a direct assessment of binding events at the bead level. The technique has been used to study the mechanism of transport and adsorption of various protein molecules.

CLSM can also be used to calculate the uptake curve for different chromatography matrices. By relating the relative fluorescence intensity obtained at different times to the value at equilibrium, the degree of saturation versus time could be calculated. This would then be used to calculate the solid phase concentration at different time intervals (Ljunglof et al., 1998). CLSM has been used to assess matrix performance over consecutive affinity runs (Thillaivinayagalingam et al., 2007) and to study protein adsorption to chromatographic matrices (Protein A to IgG Sepharose 6 Fast Flow) (Ljunglof et al., 1996). This method has also investigated the fouling in chromatography and examined the effectiveness of CIP protocols (C. Siu et al., 2006). CSLM has also been used in the past to understand the adsorption of plasmid DNA to individual adsorbent particles (Ljunglof et al., 1999). The

mechanism of transport of the protein molecules into the pores of the adsorbent has also been studied using this technique (Subramanian et al., 2005). The effect of pH and conductivity during ion-exchange adsorption was evaluated using CLSM and the mechanism of adsorption under varying conditions has been discussed in the paper by Harinarayan et al. (2006).

Several experiments have been reported using a batch mode where the media and the protein conjugated with dye have been stirred continuously and samples withdrawn at regular intervals to be analysed under confocal microscope. The main drawback of using finite bath method of adsorption is that the same bead is not evaluated over a period of time, instead different beads are examined at different times. Kasche et al. (2003) used a technique where the beads were fixed in the wedge between the microscopic slide and a coverslide. The solution containing the adsorbate was then flowed through these beads and the adsorption process examined. A further improvement to this method is the use of flow cell by Schroeder et al. (2006) and micro-column by Hubbuch et al. (2002). IEX adsorption process has been carried out, by imaging particles under confocal microscopy using a flow cell (Dziennik et al., 2003). This has the biggest advantage as contacting and imaging are carried out simultaneously as opposed to sequentially hence removing the time lag. Secondly the same bead can be evaluated over a certain period of time which gives a more realistic picture compared to batch mode studies. The rate of adsorption and diffusion coefficient of various protein molecules were studied by this technique and compared with the conventional method of measurements. This direct method of measurement of diffusion coefficient was further used to model the adsorption process to a greater accuracy.

The increased use of monoclonal antibodies (MAbs) in medicine has generated a greater interest in the efficient and economical purification strategies. The selective binding of IgG to protein A is used to capture them directly by affinity chromatography principles. A variety of adsorbents with protein A as ligands are available commercially for the purification of IgG. The performances of 15 different adsorbents were compared by Hant et al. (2003) in terms of dynamic binding capacities and rate constants. Porous agarose based media were found to exhibit higher dynamic binding capacity However, the higher mass transfer with non-agarose based media noticed was attributed to the smaller particle diameter of these adsorbents. These adsorbents were compared by indirect measurements and not by CLSM. These indirect measurements do not allow the study of effect of

particle size of the adsorbents, inherent in the commercial adsorbents, while CLSM can be very useful in this context.

The porosity of the adsorbent beads depends on the matrix type and the size of the beads. The transport of protein molecules through the beads depends on the porosity and the size of the adsorbate. The effect of bead size on the adsorption process has not been studied using CLSM. However, using the results from various literatures, Kasche et al. (2003) deduce that the concentration of the adsorbed molecules in the first 10-30 % of the radius of the adsorbent increases for both macroporous and polymeric network adsorbents (radius of adsorbent in the range of 20-100 micrometers). Higher percentage values apply for smaller sized adsorbent, which indicates that the penetration of the product is higher for smaller sized particles. A significant scatter in the uptake profile of lysozyme (on to Sepharose SP) by CLSM studies (direct measurement) was observed when comparing with indirect measurement (fluid phase concentration measurement) (Ljunglof et al., 1998). This was proposed to be the difference in the particle size of the beads, which differed, by 5 micrometer even after a visual selection of similar sized beads.

This chapter will be looking at hIgG adsorption on different affinity matrix using CSLM and a flow cell. The adsorption profile has been used to understand the difference in the adsorption of hIgG to beads of different sizes that belongs to the same matrix.

3.3 Materials and Method

3.3.1. Materials

Chromatography media

MabSelect and MabSelectXtra media were from GE Healthcare (UK). The prototype media was from Millipore (Consett, UK). The media where Protein A is immobilised on 4CL Sepharose (GE Healthcare, UK) was produced in-house at Millipore.

Chemicals

Chemical for buffer preparation was from Sigma-Aldrich (Gillingham, Dorset). Phosphate buffered saline pH 7.4 is biotechnology performance certified (product – P5368). Sodium carbonate was also from Sigma-Aldrich. Human polyclonal IgG was also from Sigma-Aldrich in the form of a 99% lyophilised powder (product G4386). Cy3 fluorescent dye was purchased from GE Healthcare (UK). Sephadex G-50 used to remove unconjugated Cy3 dye was purchased from Sigma-Aldrich (Gillingham, Dorset).

3.3.2 Equipment

Leica inverted confocal microscope was set up. An objective of 20X was used. Image size was set at 512x512 pixels. The beads were excited at 568nm and emitted between 573-643nm. Leica software was used for analysis.

A flow cell manufactured in-house at University College London was used to pack the chromatography beads (Figure 3.1). It is a pyrex block with four channels drilled through it. The channels are 1cm in length and have a diameter of 0.1cm. It can pack ~8μl of media. Frits were placed just outside the channel to keep the beads in place. A coverslip is attached on the face of the block with the help of an adhesive (Araldite) to seal the channel.

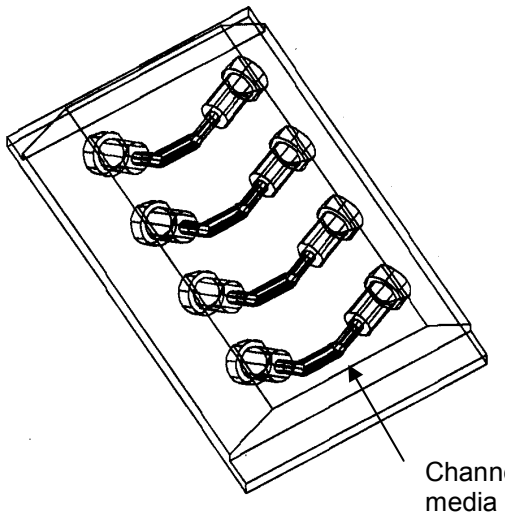


Fig 3.1- Flow cell with a channel of diameter 0.1cm and a length of 1cm was used to pack ~8μl of media, which could be placed under an inverted confocal microscope.

3.4. Method

3.4.1 Sample preparation

1ml of hIgG (5mg/ml) was made in 0.1M sodium carbonate (pH 9.3 at room temperature) which was fluorescently, labelled with Cy3. The hIgG solution was added to a vial of fluorophore (1 vial contains 0.2-0.3mg of dye) and was left to incubate for 40 min at room temperature with occasional inversion. 1 vial of Cy3 dyes 1mg of hIgG.

The sample was passed through a gel filtration column to remove unconjugated Cy3 dye. Column XD 16 (GE Healthcare) was packed with Sephadex G-50 under gravity. The labelled protein, the first band to elute was collected by visual inspection. The sample was then made to 10ml with PBS. The concentration was made to 2mg/ml by adding 15mg of hIgG. The dilution of labelled protein with unlabelled protein is necessary to avoid total internal filtering of the confocal laser light by the fluorescent dye.

3.4.2 Equipment setting

The flow cell was packed with the chromatography media. A syringe pump was connected to the flow cell with the help of tubings and HPLC connectors. The media was washed with 0.5ml of PBS at a flow rate of 0.1ml/min.

The flow cell was placed under the inverted confocal microscope. The Z series and the stage of the microscope were adjusted such that visually images of a few beads could be taken from the centre and an area with different size beads could be selected. The labelled hIgG solution was passed through the column with a syringe pump at 0.1ml/min (Figure 3.2). The microscope was programmed such that xy images were taken every 2mins for 40mins. The fluorescent intensity across the beads at every time interval could then be studied using the Leica software (Figure 3.3).

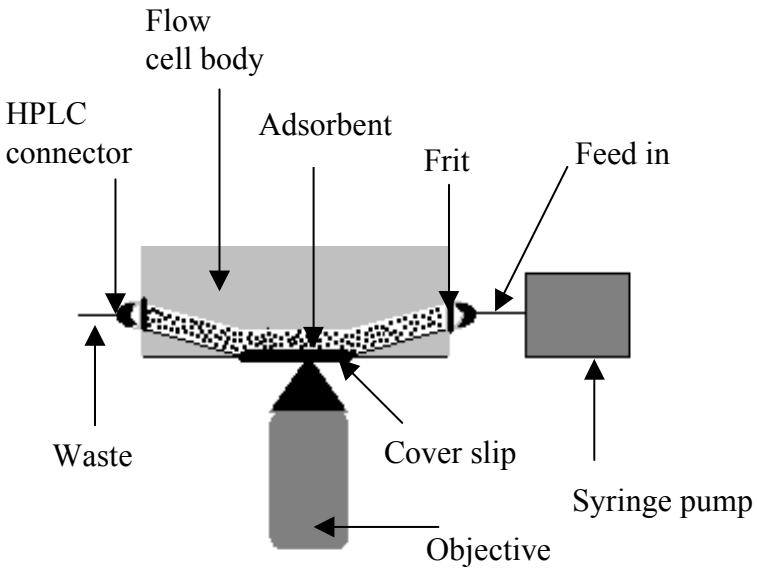


Fig 3.2 - Flow cell setting under confocal microscope. 2mg/ml hIgG was passed through the channel at 0.1ml/min flowrate with a syringe pump.

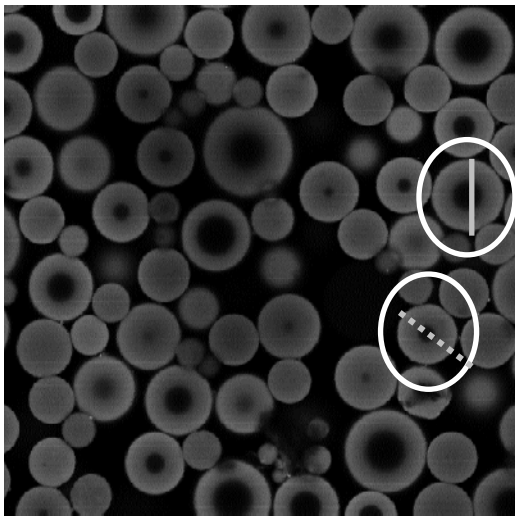
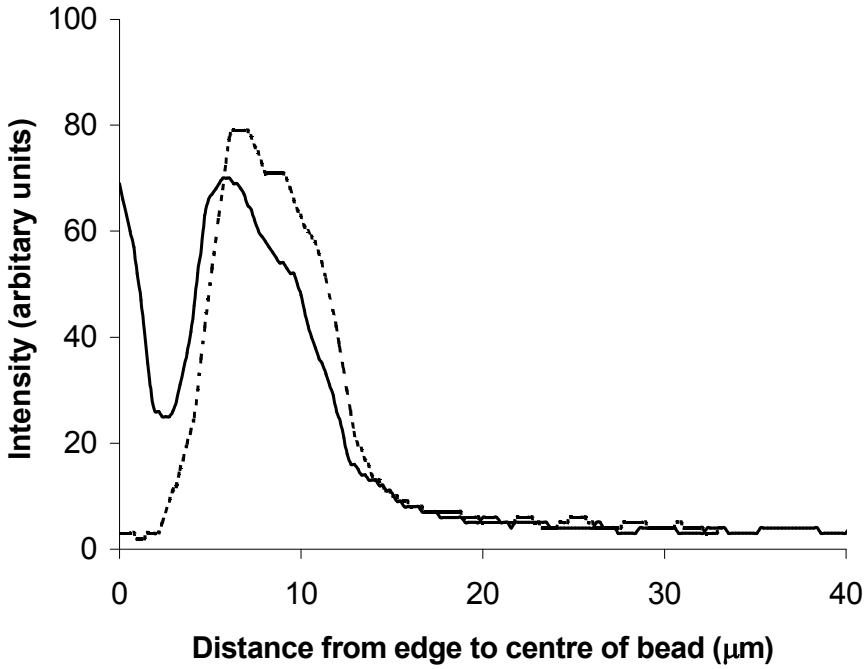


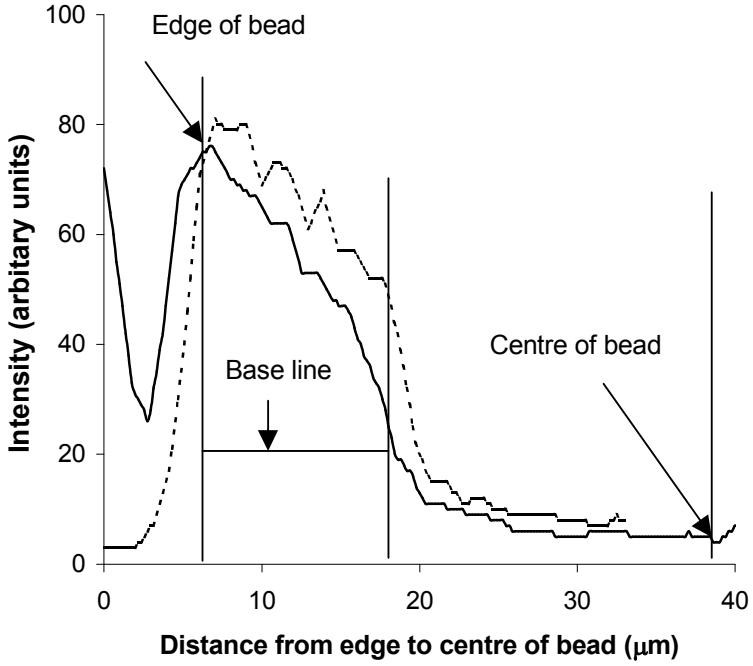
Fig 3.3 - Image of beads seen under the microscope at 30 mins. The images of the chosen beads are from the centre and of different sizes (50μm and 70μm). The line across the bead helps produce the intensity profile across that section as seen in figure 3.4

3.4.3 Analysis

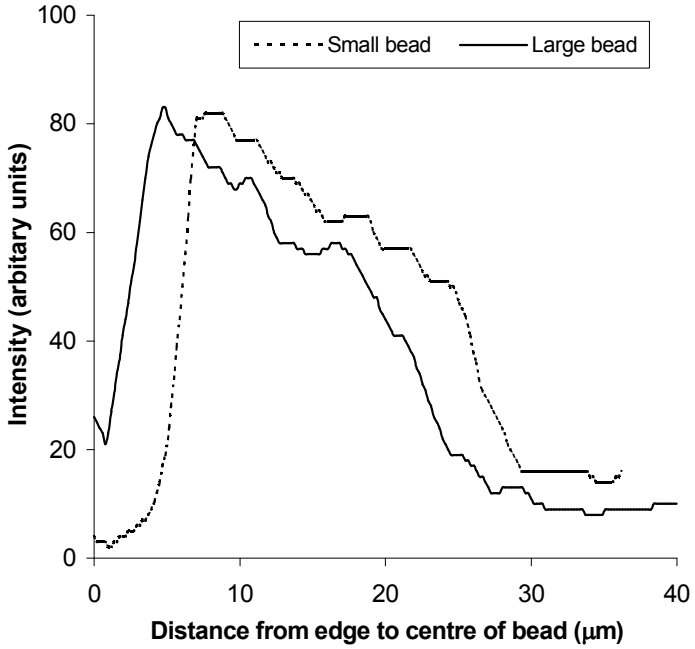
The intensity profile across each bead for each time interval is produced by the Leica software. The distance penetrated by the labelled hIgG to the centre of the bead for each time interval can be estimated from the profile by choosing a certain intensity baseline (Figure 3.4). This was chosen in the following way. The average maximum intensity was calculated from the intensity value shown on the outside surface of the bead. This was subtracted from the intensity reading of the background (where there are no beads). 10% of this value was added to the background intensity and this was taken as the baseline.


100
80
60
40
20
0
Intensity (arbitary units)
0
10
20
30
40
Distance from edge to centre of bead (μm)

A


100
80
60
40
20
0
Intensity (arbitary units)
Edge of bead
Base line
Centre of bead
0
10
20
30
40
Distance from edge to centre of bead (μm)

B



C

Fig 3.4 - Intensity profile across a small bead (~50μm) and a large bead (~70μm) at A; 10mins, B; 20mins and C; 30 mins time intervals. The distance travelled by hIgG to the centre of the bead with time can be estimated from the profile

3.5 Results and Discussion

3.5.1 Visual images

The images obtained using confocal studies at the end of 10, 20 and 30 minutes for the various adsorbent media is presented in Figure 3.5. Only one bead for each matrix is selected and shown here. These specific beads are chosen such that the images of these sections are taken from the centre of the bead. MabSelect and MabSelect Xtra tend to show a typical shrinking core effect. Whereas for the prototype it can be seen that hIgG doesn't tend to penetrate inside the bead and most of it just binds to the outer surface of the bead. The adsorbent where Protein A is immobilised on 4CL Sepharose also shows a shrinking core effect, but the areas where the beads are in contact with each other doesn't tend to allow hIgG to penetrate the inside of the bead until at a later stage in time. Hence this method gives a clear visual characterisation of hIgG binding to different chromatography matrices (Figure 3.5).

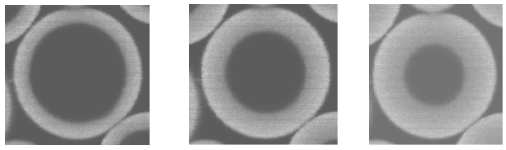
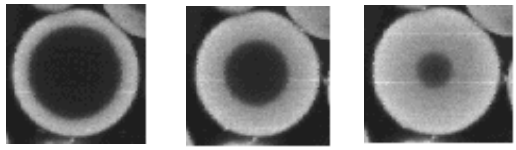
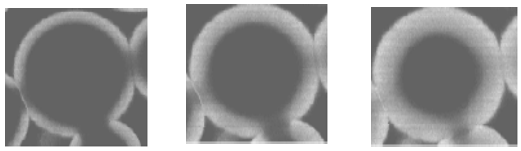
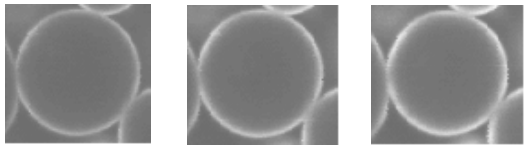
Adsorbent	Time (mins)		
	10	20	30
MabSelect™ Average Particle size - 85µm 4% agarose			
MabSelect Xtra™ Average Particle size - 75µm 4% agarose			
ProteinA on 4CL Sepharose Average Particle size - 100µm 4% agarose			
Prototype Average Particle size - 60µm 6% agarose			

Fig 3.5 - Visual Images of Mabselect, MabSelect Xtra, Prototype (Protein A Mimic ligand) and Protein A immobilised on 4CL Sepharose media taken at 10, 20 and 30 mins in a flow cell. 2mg/ml hIgG was passed through the channel at 0.1ml/min flowrate.

3.5.2 Diffusion rate

Analysis of data obtained from the intensity profile graphs is used to obtain the diffusion rates shown in Figure 3.6. The analysis was carried out on 2 samples of MabSelect, 1 sample of MabSelect Xtra and Protein A immobilised on 4CL Sepharose. The experiment was carried on different days and using separately prepared labelled protein samples each time. Hence the slight difference in packing of the column, microscope specifications and labelled protein sample concentration could contribute to the difference in the diffusion rate for different matrices. From Figure 3.6 it can also be clearly shown that the adsorption rate to the centre of each

bead is linear. The results of diffusion rates obtained using confocal studies for various matrices and difference particle sizes within the same matrix is presented in Table 3.1. At lower feed concentrations (0.1-0.5 mg/ml) there is a dependency between affinity and diffusivity (Hahn et al., 2005). But at higher concentration (3mg/ml) all the media with different particle size had similar isotherm, thus affinity is not dependent on diffusivity at this concentration. This means particle size difference which could lead to higher diffusivity is not really beneficial in terms of higher mass transfer if the feed concentration is increased beyond a certain level.

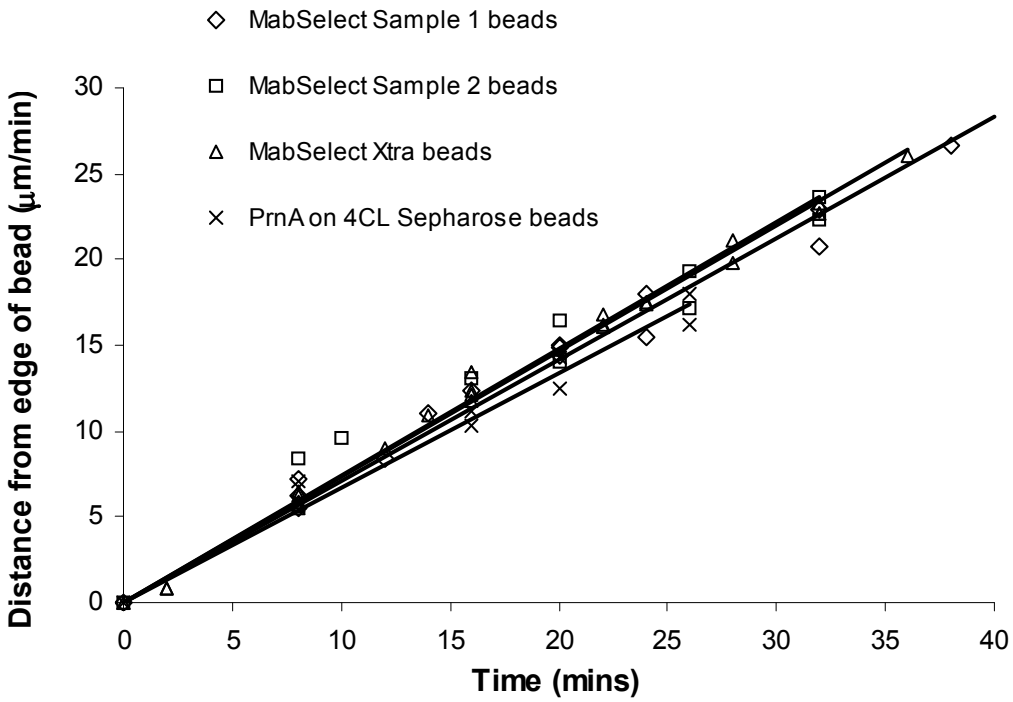


Fig 3.6 - Adsorption rate for different size beads for two different samples of MabSelect, 1 sample of MabSelect Xtra and Protein A immobilised on 4CL Sepharose

Chromatography media	Bead size (μm)	Gradient (min^{-1})
MabSelect Xtra	75	0.74
Manufacture size - 75μm	70	0.72
	66	0.74
	55	0.73
	53	0.74
MabSelect Sample 1	103	0.72
Manufacture size - 85μm	89	0.73
	86	0.67
MabSelect Sample 2	72	0.71
	82	0.77
ProteinA on 4CL	124	0.66
Sepharose	107	0.68
Manufacture size - 100μm		

Table 3.1 - Diffusion rate for different size beads and for different media (R^2 value of 0.977)

In the current study, the diffusion rates as seen from Table 3.1 seem to be same among different bead sizes and also bead types. MabSelect and MabSelect Xtra had similar diffusion rates. The different particle sizes within any particular type of matrix and also across different matrix did not result in different diffusion rates. The diffusion rates were found to be approximately constant at around 0.72 min^{-1}.

3.5.3 Adsorption rate

By integration of intensity profiles the average volumetric intensity of the beads was calculated. From this data uptake curves for different size beads of MabSelect Xtra was plotted (Figure 3.7). It can clearly be seen that smaller beads reach saturation much faster than larger beads indicating a higher adsorption rate as the proportion of the bead that has absorbed the hIgG is higher for smaller beads than larger bead at a given time.

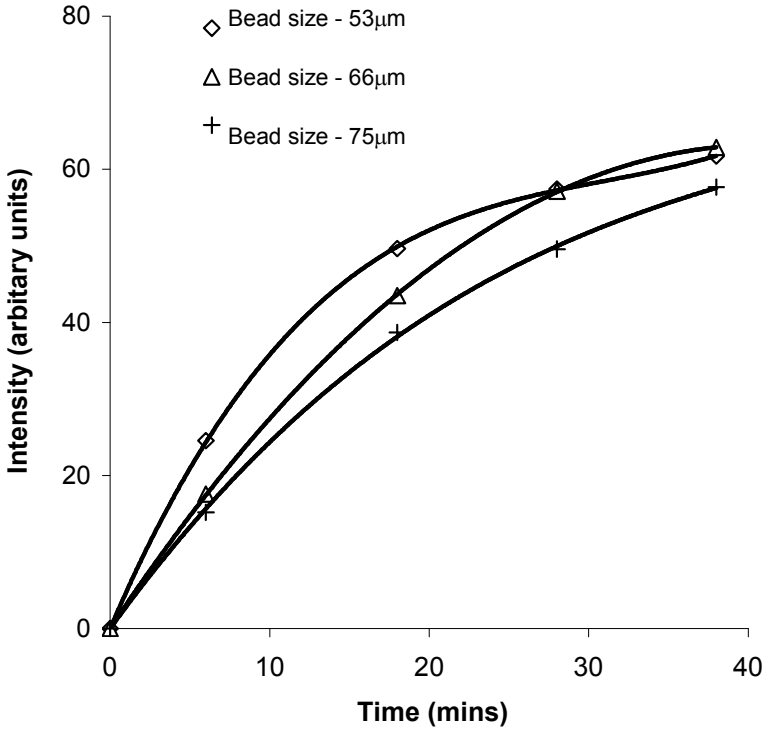


Fig 3.7 - Uptake curves for different size beads of MabSelect Xtra plotted from data calculated from intensity profiles

The adsorption rate as seen from Figure 3.7 is higher for smaller sized bead compared to larger size bead. Perhaps constant diffusion rate means the front reaches same length of the pore at any particular time for different sized beads.
From our studies we have demonstrated that particle size of the media needs to be considered even if diffusion rates are similar. Modelling of chromatography operations to predict the performance assumes a single particle size. The accuracy of the modelling can be improved by considering the heterogeneity of the media. The adsorption performance of individual beads can be measured quantitatively by confocal microscopy thus allowing creating an accurate model.

3.6 Conclusion

Although using confocal microscopy to study adsorbents is a complex procedure it can give very detailed information on different adsorbents. The methods looked at in chapter 2 were very informative, however it did not give information on individual beads. The uptake rate kinetics in chapter 2 showed similar uptake rates for all the

adsorbents, however using confocal microscopy showed a difference in the uptake rates of different sized beads. Similarly in Chapter 2 the static capacity of the prototype (CIGL) adsorbent was higher than MabSelect and Prosep Ultra, however after carrying out the confocal experiment the visual images showed that hIgG only tends to bind to the surface of the CIGL beads. This probably implies that the prototype has excellent stoichiometric properties and needs further analysis. Hence the use of confocal microscopy and the flow cell creates further questions on the characteristics of different adsorbents, which helps make the analysis more robust.

Chapter 4 - Future Work

4.1 Introduction

So far this thesis looked into a few experimental methods that have proved to be very accurate and reliable such as static capacity, adsorption isotherms, dynamic capacity and uptake kinetics. These methods gave a good indication of the performance of the different kind of matrices. This thesis also looked into a more novel approach of using confocal microscopy using a flow cell to get a further understanding of the different kind of matrices. However there are various other areas that needs to be explored further. This chapter looks at some of these areas where further work is necessary to achieve a toolbox of methods that can be used to understand and evaluate the performance of different matrices.

4.2 Ligand density

It can be of great importance to understand how much protein A ligand is present in 1ml of media. Certain media might show a high static capacity but have relatively low protein A ligand density and the high static capacity might be due to another factor. In order to understand if this is the case, and compare adsorbents with each other a robust technique to calculate the ligand density can be very useful.

One such method that can be carried out and tested further uses BCA (Bicinchoninic acid). A standard curve is produced using different concentrations of Protein A and adding BCA to it and measuring the OD values at 562nm. Similarly BCA can be added to a known mass of adsorbent and the concentration of Protein A can be calculated using the standard curve produced.

4.3 Use of Optical Biosensor Technology

SPR (surface plasmon resonance) technology enables real-time detection and monitoring of biomolecular events and provides quantitative information. SPR arises when light is reflected under certain conditions from a conducting film at the interface between 2 media of different refractive index. Here the ligand is immobilised on the sensor surface, or indirectly via an immobilised capturing

molecule and the solution with the desired product to be captured (e.g. IgG) is passed over it. The data derived can be used to understand how specific the binding is between two molecules. It can also be used to understand the kinetics (rate of association and dissociation) and affinity. The data tends to give information on the thickness, density and mass of the desired product attached, which can help understand stoichiometry information.

Chapter 5 – Thesis Conclusion

5.1 Summary of the thesis

The work in this thesis was focused on designing reliable methods that can be used to characterise different kinds of adsorbents. The batch methods used to analyse different properties of adsorbents such as static capacity, adsorption equilibrium data and kinetic uptake and desorption rate are easy to perform, can be reproduced easily, can be performed at a low cost and there are no issues related to packing columns. The batch uptake kinetic method was well designed and was accurate as a small stream of hIgG solution was analysed continuously and at no point were any samples taken out of the system for analysis. The results showed that MabSelect Xtra had the highest static capacity. All the adsorbents had very low Kd values as expected by affinity adsorbents such as these. The use of Hanes Plot and the Solver function in Excel to calculate the Kd and Q_{max} values showed to be accurate methods. All the media showed similar kinetic and desorption rates. Dynamic capacity gives further information on how adsorbents would perform when packed in a column. A small 0.66cm Omnifit column with a bed height of only 6cm was used to minimise the time of experiment and cost. Data was collected at 2min, 4min and 8 mins residence time. The results showed MabSelect Xtra to have the highest dynamic capacity. The relatively high static and dynamic capacity for MabSelect Xtra is due to the more porous nature of the beads, which increased the surface area available for Protein A ligand immobilisation and also due to the smaller size of the beads.

Another more novel method using a flow cell, labelled hIgG and a laser scanning confocal microscopy was designed to the see the adsorption properties of different media at individual bead level. A channel of the flow cell was packed with different media (~8μl) and placed under the microscope. Continuous flow of 2mg/ml hIgG was passed through the channel with a flow rate of 0.1ml/min. This method uses a minute amount of media, has shown to be accurate for the experiments carried out in this thesis, gives information on individual beads visually and also gives uptake rates of individual beads of different sizes of the same media. It was found that the adsorption rate to the centre of the bead for all the matrices was linear and smaller beads reached saturation much faster than larger beads at any given time. Visually all the beads showed the shrinking core effect except for the prototype media. With this method, images of the beads can be seen continuously with time and again no samples need to be taken out of the system for analyses.

Manufacturers of affinity adsorbents need to take into account several factors while designing and producing a specific matrix. Some of these factors are purity (selectivity), capacity, recovery, binding and elution, alkali resistance, adsorbents scale-up, stability, ligand loss (leakage) and safety (toxicity). Many manufactures are showing increasing interest in designing synthetic ligands for affinity chromatography. Synthetic ligands are less expensive than biological ligands, synthesis of these ligands are simpler and they are more resistant to sterilisation. There is a need to design methods that can be used to analyse the above performance characteristics of affinity adsorbents. Some of these methods can be used to analyse caustic stability of adsorbents, dynamic capacity using MAb feedstock, pressure flow characteristics and life cycle studies. All these methods put together can form a toolbox of methods to characterise different adsorbents.

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Appendix

Appendix A - Static Capacity

MabSelect

Feed Concentration (mg/ml) 0.878

OD reading	Concentration (mg/ml)	Static capacity (mg/ml)
0.692	0.501	57.2
0.693	0.502	57.1
0.682	0.494	58.4
Average Static Capacity (mg/ml)		**57.6**

MabSelect Xtra

Feed Concentration (mg/ml) 0.946

OD reading	Concentration (mg/ml)	Static capacity (mg/ml)
0.7	0.507	62.3
0.706	0.512	61.6
0.705	0.511	61.7
Average Static Capacity (mg/ml)		**61.8**

Prosep Ultra

Feed Concentration (mg/ml) 0.896

OD reading	Concentration (mg/ml)	Static capacity (mg/ml)
0.71	0.5145	56.7
0.711	0.5152	56.6
0.718	0.5203	55.8
Average Static Capacity (mg/ml)		**56.4**

CIGL

Feed Concentration (mg/ml) 0.986

OD reading	Concentration (mg/ml)	Static capacity (mg/ml)
0.757	0.549	59.3
0.757	0.549	59.3
0.758	0.549	59.2
Average Static Capacity (mg/ml)		**59.3**

Appendix B – Dynamic Capacity

MabSelect

2mins residence time, 1ml/min flowrate

Plates/meter = 1674
HETP = 0.05972
As = 1.51

Feed concentration = 0.989 with OD reading of 273mAu
20% breakthrough is at 54.6mAu
concentration = 9.4mg/ml
Volume = 6.4ml
mass = 60.2mg
Dynamic capacity = 30.1mg/ml

4mins residence time, 0.5ml/min flowrate

Plates/meter = 1548
HETP = 0.05988
As = 1.71

Feed concentration = 0.989 with OD reading of 273mAu
20% breakthrough is at 54.6mAu
concentration = 9.93mg/ml
Volume = 6.5ml
mass = 64.5mg
Dynamic capacity = 32.3mg/ml

8mins residence time, 0.25ml/min flowrate

Plates/meter = 1520
HETP = 0.05980
As = 1.7

Feed concentration = 1.123 with OD reading of 310mAu
20% breakthrough is at 62mAu
concentration = 10.1mg/ml
Volume = 7.2ml
mass = 72.7mg
Dynamic capacity = 36.4mg/ml

MabSelect Xtra

2mins residence time, 1ml/min flowrate

Plates/meter = 2381
HETP = 0.04200
As = 0.84

Feed concentration = 1.025 with OD reading of 283mAu

20% breakthrough is at 56.6mAu
concentration = 13.5mg/ml
Volume = 5.5ml
mass = 74.25mg
Dynamic capacity = 37.1mg/ml

4mins residence time, 0.5ml/min flowrate

Plates/meter = 2167
HETP = 0.04628
As = 0.84

Feed concentration = 1.105 with OD reading of 305mAu
20% breakthrough is at 61mAu
concentration = 13.93mg/ml
Volume = 5.8ml
mass = 80.79mg
Dynamic capacity = 40.4mg/ml

8mins residence time, 0.25ml/min flowrate

Plates/meter = 2081
HETP = 0.04805
As = 0.85

Feed concentration = 1.08 with OD reading of 298.6mAu
20% breakthrough is at 59.72mAu
concentration = 12.06mg/ml
Volume = 6.8ml
mass = 82mg
Dynamic capacity = 41mg/ml

Prosep Ultra

2mins residence time, 1ml/min flowrate

Plates/meter = 1146
HETP = 0.08729
As = 1.86

Feed concentration = 1.01 with OD reading of 280mAu
20% breakthrough is at 56mAu
concentration = 8.8mg/ml
Volume = 7ml
mass = 61.6mg
Dynamic capacity = 30.8mg/ml

4mins residence time, 0.5ml/min flowrate

Plates/meter = 1051
HETP = 0.09514
As = 2.27

Feed concentration = 1.01 with OD reading of 280mAu
20% breakthrough is at 56mAu
concentration = 9.35mg/ml
Volume = 7.1ml
mass = 66.4mg
Dynamic capacity = 33.2mg/ml

8mins residence time, 0.25ml/min flowrate

Plates/meter = 1072
HETP = 0.09332
As = 2.44

Feed concentration = 1.01 with OD reading of 280mAu
20% breakthrough is at 56mAu
concentration = 10.77mg/ml
Volume = 6.5ml
mass = 70mg
Dynamic capacity = 35mg/ml

CIGL

2mins residence time, 1ml/min flowrate

Plates/meter = 2041
HETP = 0.04901
As = 1.6

Feed concentration = 1.07 with OD reading of 295mAu
20% breakthrough is at 59mAu
concentration = 8.67mg/ml
Volume = 8.4ml
mass = 72.8mg
Dynamic capacity = 36.4mg/ml

4mins residence time, 0.5ml/min flowrate

Plates/meter = 2148
HETP = 0.04656
As = 1.02

Feed concentration = 1.07 with OD reading of 295mAu
20% breakthrough is at 59mAu
concentration = 11mg/ml
Volume = 7.8ml
mass = 85.8mg
Dynamic capacity = 42.9mg/ml

8mins residence time, 0.25ml/min flowrate

Plates/meter = 1992
HETP = 0.05019
As = 1.66

Feed concentration = 1.07 with OD reading of 295mAu

20% breakthrough is at 59mAu
concentration = 13.68mg/ml
Volume = 7ml
mass = 95.77mg
Dynamic capacity = 47.9mg/ml