NON-POROUS PSEUDOAFFINITY SUPPORTS FOR THE RECOVERY OF ANTIBODIES

by

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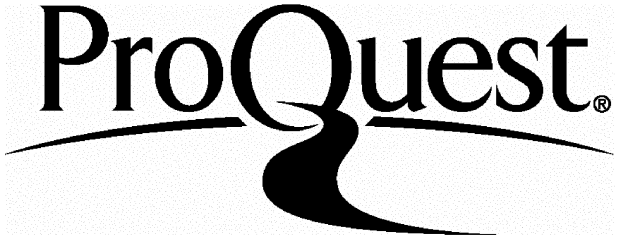
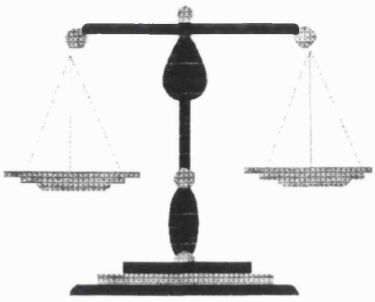


To Nicky

Balance is the Key



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ABSTRACT

The major costs of production of monoclonal antibodies (MAb's) are incurred during downstream processing. Techniques which utilise the specificity of an affinity interaction have the potential to reduce the number of unit operations to a minimum and thus enable savings to be made in the overall costs of production.

The preparation and detailed characterisation of two types of immobilised metal affinity adsorbent for the recovery of MAb's from chinese hamster ovary (CHO) culture is described in this thesis.

Non-porous metal chelating perfluorocarbon supports were prepared by coating particulate perfluorocarbon materials with polyvinyl alcohol which was subsequently activated and derivatised with iminodiacetic acid (IDA) functions. The preparation conditions were systematically optimised to produce stable coated and highly derivatised metal chelate adsorbents (2-5μmoles Zn^{2+}/mL). Supports charged with zinc ions selectively bound MAb and non-specific adsorption to uncharged supports was not observed. The specific binding capacity of the best chelating perfluorocarbon adsorbent (chelating PTFEP; 9mg/mL) is close to that of a porous metal chelate adsorbent (chelating Streamline™; 13mg/mL). The chelating perfluorocarbons are very stable to treatment with a wide variety of chemicals, and this combined with their non-porous nature make them attractive alternatives to conventional affinity adsorbents.

Non-porous magnetic chelators were produced from iron oxide particles made by the controlled alkali precipitation of iron salts. The iron oxide cores were silanised and subsequently coated with polyglutaraldehyde, epoxy activated and coupled with IDA. These supports are densely derivatised with IDA (30-60μmoles Me^{2+}/mL) and exhibit high specific binding capacities for the test MAb's. Although Ni^{2+} charged supports were much more effective than Zn^{2+} charged supports for the recovery of MAb from PBS (~40mg/mL c.f. ~21mg/mL), this advantage was lost when the adsorbents were employed in real process liquors. The presence of suspended cells appeared to have no effect on performance but some component(s) in the CHO medium selectively desorped (~50%) of nickel from charged supports. Nickel and zinc charged magnetic chelators were used to recover MAb directly from CHO fermentation's. MAb's bound to zinc and nickel charged adsorbents could be recovered at high yield and purity (~99%) and no detrimental effects of medium components or suspended material were observed.

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Glossary

APTES	Aminopropyl triethoxysilane
CHO	Chinese Hamster Ovary
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylene Diamine Triacetic Acid
FPLC	Fast Protein Liquid Chromatography
HGMS	High Gradient Magnetic Separation
HPLAC	High Performance Liquid Affinity Chromatography
HPLC	High Performance Liquid Chromatography
IDA	Iminodiacetic Acid
IgG	Immunoglobulin G
IMAC	Immobilised Metal Affinity Chromatography
LDH	Lactate dehydrogenase
MAb	Monoclonal Antibody
MSFB	Magnetically Stabilised Fluidised Bed
PBS	Phosphate Buffered Saline
PERCAS	Perfluorocarbon Emulsion Reactor for Continuous Affinity Separation
PF8TES	Perfluorooctyl triethoxysilane
PTFE	Polytetrafluoroethylene
PTFEP	Polytetrafluoroethylene propylene
PVA	Polyvinyl alcohol
VSM	Vibrating Sample Magnetometry

1. INTRODUCTION

This chapter discusses some of the considerations pertinent to the purification of biotechnological products such as monoclonal antibodies. A brief look at the economic considerations of production puts into context some of the issues involved in the fashioning of the optimal purification protocol. Approaches to reduce the number of unit operations in the downstream process are discussed and in particular the principles of affinity adsorption and its potential applications are examined. Finally the requirements for the selection of an affinity adsorbent to recover monoclonal antibodies from cell culture are considered.

1.1 Production of monoclonal antibodies

The production of monoclonal antibodies and other biotechnological products is conventionally separated into two components, upstream and downstream processes. The upstream process is defined as the production of the desired product by cell culture or fermentation. This includes the use of genetic engineering to introduce the required recombinant DNA into a relevant host organism and the subsequent expression of the DNA by the host in a fermentor or other culture system. The downstream process is defined as a series of steps which when followed result in the recovery of purified product from the upstream. Downstream processing is composed of a series of unit operations designed to produce a quantity of the product suitable for the defined end use. A unit operation refers to a distinct chemical or physical process for example filtration, centrifugation, or chromatography. Both downstream and upstream processes impinge on and have an effect on each other (Cartwright, 1987; Kelley and Hatton, 1991).

1.1.1 Economic considerations for the production of monoclonal antibodies

Economic considerations are crucial throughout the development and operation of all the processes required to produce monoclonal antibodies and indeed other recombinant proteins. The industrial production of a product has to be profitable in order for a return on the investment to be gained. Profit can be measured in a number

of ways including unit production cost, cost of sales, return on investment (Datar *et al.*, 1993). One way to maximise profit is therefore to minimise costs. A useful approximation of the cost of producing a particular product (Equation 1.1) illustrates the relationship between process costs and product yield as adapted from Sofer and Nystrom (1989).

Equation 1.1 Ctot = Process Costs / Product Yield

Ctot is the total production cost. Process costs includes a wide range of variable costs such as capital, overhead, labour, materials, validation etc. Product yield is the yield of the product in kilograms. There are therefore two ways to bring down production costs, reduce the cost of the process and, or increase the product yield.

A production cost estimate for the production of a monoclonal antibody is $5000 per g product (Lebherz, 1987) and it is estimated that a large percentage of this total cost is incurred by the downstream process. Dwyer (1984) estimated the burden to be as high as 90%. This is probably an overestimate as other processes such as validation, assay development and product formulation also contribute heavily. Whatever the actual percentage burden it is still likely to be significant and therefore cutting the costs involved in the downstream process is an appealing way to cut total costs.

Process costs can be split into three arbitrary groups: material and utility expenses, labour costs and capital costs. These three areas can be affected by judicious choice of process unit operations, the efficiency of these operations, size and scale of equipment and the degree of automation.

One significant material cost is likely to be the cost of separation media, which is discussed in more detail later. Labour costs are dependent on the batch size and on production time (Wheelwright, 1991). A large batch size and short production time is the ideal to be aimed for. Automation of processes is another major way to reduce labour costs (Kenney and Chase, 1987).

The capital costs for any production process are to a certain extent dependent on the number and scale of the individual unit operations making up the process. While it is

often not possible to reduce the number of unit operations upstream, the scope for reducing the number of downstream operations is quite considerable (1.1.2). A minimised number of steps therefore reduces process costs and is probably the most effective and therefore important way in which to reduce costs.

The other way to reduce the total production cost is to increase product yield. Yield is defined as the percentage of the product actually recovered by a process. This can be achieved by increasing the efficiency of upstream processes such as fermentation or cell culture and/or increasing the efficiency of the downstream processing.

The main ways to directly influence costs of downstream processes by reducing process cost and/or increasing product yield are summarised below:

Reduce losses of yield:-

Improve recovery/efficiency of individual unit operations

Reduce number of unit operations:-

Direct recovery from bioreactor

Combine purification steps

Replace initial recovery steps such as centrifugation etc.

Reduce costs of separation media:-

Increase life time of separation media

Reduce quantity of separation media

Automate purification step

Therefore the ideal purification unit operation, in terms of cost, is an automated, single step, highly selective purification with a large capacity for the protein to be purified and an ability to work in feed solutions such as fermentation broth. The attainment of this ideal system will be the subject of much of the discussion in this introduction.

1.1.2 Considerations for the large scale purification of monoclonal antibodies

In general there are three stages that are considered when purifying large quantities of monoclonal antibody, feed pretreatment, initial purification and final purification (Schmidt, 1989).

Prior to chromatographic purification the feed stock is often pre-treated so that the resulting feed stream is free of particulate matter and fouling solids. There are a large number of methods used to carry out this pretreatment; centrifugation (Naveh and Siegel, 1991), ultrafiltration (Birch *et al.*, 1987), and depth filtration (Lee, 1989) to name but a few. All these methods can be considered as high capacity, low selectivity techniques. Capacity is a measure of the amount of antibody that can be recovered from a given volume of feed stock and selectivity is a measure of the degree of separation of the antibody from contaminants. The advantages of pretreatment techniques are a reduction in volume, allowing further unit operations to be scaled down, and a reduction in the number and concentration of some of the contaminants, particularly particulate matter. The disadvantage of such methods is a reduction in the yield of the purification, a percentage of the antibody inevitably being lost during the pretreatment process.

Typically pretreatment is followed by the use of ion exchange or protein A chromatography and subsequently final purification steps (Schmidt, 1989). Techniques which have both high capacity and high selectivity could potentially be used to replace pretreatment unit operations and/or final purification resulting in an increase in yield and reduction of costs.

1.1.3 Comparison of methods for the purification of monoclonal antibodies.

There are a variety of techniques used to purify monoclonal antibodies. As discussed previously the considerations for large scale one step purification are high capacity, high specificity, robustness, ability to work in 'dirty' feedstreams and cost. Capacity, resolution and the ability to work in 'dirty' feedstreams are to a certain extent dependent upon the mode of operation (1.2.2).. Some of the more common purification methods are summarized in Table 1.1.

Gel filtration or size exclusion chromatography partitions molecules between the liquid and solid phase on the basis of size. Large molecules cannot penetrate the pores of the gel and therefore pass quickly through the column. The flow rate can be tailored to effect separations of molecules similar but not identical in size (Low, 1988). The overriding limitation of gel filtration is that it is confined to a packed bed operation and is therefore unsuitable for use in particulate containing feedstreams (1.2.2.2). Instead it is most often used as a final polishing step to remove low levels of contaminants and to exchange buffers for product formulation (Lillheoj and Malik, 1989).

Hydroxyapatite is a form of calcium phosphate and as such has weak anionic and weak cationic functional groups. Monoclonal antibodies are bound to hydroxyapatite more strongly than the majority of contaminating proteins when adsorbed in low ionic strength phosphate buffers at neutral pH, elution is achieved by increasing the ionic strength (Juarez-Salinas *et al.*, 1987). The recent development of commercial high performance variants of hydroxyapatitate products has allowed the rapid development for a variety of applications including preparative HPLC for purification of antibody processed from 250 mg of total protein per cycle (Brooks and Stevens, 1985). However a study comparing anion exchange, protein A affinity and hydroxyapatite separations concluded that hydroxyapatite was the least efficient in terms of yield and purity (Manil *et al.*, 1986).

Ion exchange chromatography binds monoclonal antibodies on the basis of their charge (Lillehoj and Malik, 1989). Anion exchangers tend however to bind contaminants such as albumin, thereby reducing the capacity and the lifetime of the adsorbent (Posillico *et al.*, 1987). Cation exchangers have been shown to be effective for a wide variety of monoclonal antibodies (Malm, 1987) and are therefore believed to be better suited. Ion exchange adsorbents have a high capacity (30mg protein per mL adsorbent) and allow high throughput rates. Ion exchange chromatography is selective to a certain degree, has a concentrating effect and it can be used to isolate proteins in one step (Scopes, 1988). The main disadvantages of ion exchange is the lack of complete specificity, and dilution is often required to lower the ionic strength of feedstock which may be a problem in large scale applications. In addition Roe (1987) showed that the

performance of an ion exchange resin in crude broth was considerably reduced. The majority of large scale applications involve a pretreatment step such as ultrafiltration to remove particulates and reduce the volume prior to dilution with appropriate buffers.

Table 1.1 The suitability of common separation media for one step purification

Unit Operation	Basis for separation	Capacity	Resolution	Cost of Media	Ability to work in 'dirty' feedstreams
Affinity	Bio-specificity	High	Excellent	Relatively Expensive	Depends on mode of operation
Ion Exchange	Net charge	High	Relatively high	Relatively inexpensive	Depends on mode of operation
Gel filtration	Size	Moderate	Moderate	Cheap	Unable
Hydroxyapatite	Unknown	Moderate	Moderate	Relatively inexpensive	No applications reported

For example Scott *et al.* (1987) used an initial ultrafiltration step followed by cation exchange to adsorb antibody and subsequent anion exchange to adsorb contaminants to yield antibody of better than 90% purity. The lack of complete specificity means that ion-exchange has limitations in the purification of antibodies but can have a place in purification schemes with more specific recoveries. Kenney and Chase (1987) demonstrated the applicability of a large scale anion exchange unit operation as the final clean up of affinity purified antibody destined for therapeutic use.

Affinity separation is a purification technique the basis of separation of which is the biological specificity of many macromolecules (1.2.1). The main advantage of affinity

over other separations such as ion exchange or hydrophobic interaction is the great specificity and therefore excellent resolution. Bonnerjea *et al.* (1986) show that affinity techniques are nearly an order of magnitude more effective than other purification techniques, the average affinity step achieved a purification of just over 100 fold compared to less than 12 fold for other techniques. Another advantage of affinity separations is the ability to purify product from very dilute solutions (Knight, 1989).

However despite approximately 20 years of development, largely by empirical techniques affinity as a separation tool has a number of practical limitations for the large scale purification of proteins. The main limitations of affinity separations at a large scale are due to the design of the affinity adsorbent itself (1.3). Both the ligand and support material used have not been fully optimised for large scale purification's and therefore their development and widespread use have been slow (Hill and Hirchenstein, 1983; Quadri, 1985).

The designer of affinity adsorbents for large scale purification needs to keep several considerations in mind. Many of the following points continue to limit the usefulness and widespread applicability of affinity adsorption techniques and are discussed in more detail later.

- Stability of adsorbent (ligand and support) to chemical and biological degradation.
- Susceptibility of adsorbent to fouling and non-specific adsorption.
- Suitability of adsorbent for use in alternative mode of operation to packed bed.
- Cost of adsorbent.
- Toxicitiy of any leachates

A more detailed discussion of affinity separations and attempts to address these limitations follows (1.2 and 1.3)

1.2 Affinity Adsorption.

1.2.1 Principles of affinity interactions.

1.2.1.1 Bio-specificity of proteins.

Proteins are biopolymers and consist of amino acids linked together by peptide bonds. They possess a wealth of information in the sequence and properties of the amino acid residues and the well defined three dimensional structure which changes in response to the environment. A general account of proteins can be found in most good biochemistry books such as Stryer (1988). In essence the structure and biospecificity of a protein such as a monoclonal antibody is a function of the four levels of structure which give the antibody its conformation or three dimensional shape. The conformation of a protein defines its function such as it's ability to interact with other molecules for a specific purpose or as a structural entity (Stryer, 1988).

The capability of a protein to recognize a molecule, can be qualitatively described in terms of specificity (Katchalski-Katzar, 1983). The shape of the protein and complementary molecule needs to be compatible so that any interaction is a closely knit one, specificity in this context is therefore defined as recognising and maintaining favourable interactions between the ligand and specific protein. Examples of such interactions include those between an enzyme and substrate, antibody and antigen, hormones and receptors, as well as a host of others which are observed in biochemistry.

1.2.1.2 The affinity interaction

The interaction of a protein (P) and ligand (L) to form a protein ligand complex (PL) can be described as a reversible reaction (Lowe and Dean, 1974) which can therefore be expressed as an equation (Equation 1.2.). The forward reaction i.e. the formation of PL is called adsorption and the reverse is termed desorption.

Equation 1.2 $P + L \rightleftharpoons PL$

The degree of affinity of the ligand for the molecule is important in the design, preparation and performance of the affinity adsorbent. The affinity is described by the dissociation constant Kd and is defined by Equation 1.3.

Equation 1.3 $Kd = [P][L] / [PL]$

The reciprocal of Kd is a measure of the affinity of the ligand for the protein, the association constant. It also defines the strength of the interaction. An interaction with a low Kd value therefore has a high affinity constant. As a general rule Kd values in the range of 10^{-4} to 10^{-8}M are normally required for most affinity purification techniques (Janson, 1984). Above this range not only is adsorption and therefore recovery poor, but non-specific interactions increase as the association constants of other proteins in the solution become proportionally greater. Conversely below 10^{-8} M the strength of the interaction is so strong that it becomes difficult to disrupt the protein-ligand association. Recovery of the bound protein will therefore be poor and the adsorbent will have a reduced capacity on re-use. In addition the protein may be damaged in the elution process and lose its biological function.

1.2.1.3 Ligand concentration.

Generally the higher the ligand concentration the greater the capacity of the adsorbent. In particular for efficient adsorption the concentration of accessible and active immobilised ligand is an important factor. For a low affinity system (Kd $>10^{-4}$M) a ligand concentration of about 10^{-2}M is required assuming no impairment of affinity is caused by the immobilisation (Lowe and Dean, 1974).

The concentration of ligand is a fundamental parameter which defines the strength of protein ligand interaction as can be seen from Equation 1.4, a rearranged form of Equation 1.3.

Equation 1.4 $[L] / Kd = [PL] / [P]$

It can be seen that the ratio of ligand to the dissociation constant equals the ratio of protein ligand complex to the concentration of protein in solution, which can be renamed the distribution coefficient. This implies that in order for substantial

adsorption, say 95 %, of the protein from solution the value of Kd needs to be several orders of magnitude lower than the concentration of ligand. Thus, for example an □ ligand at a concentration of 10mg/mL should have a Kd value of less than 10^{-5}M for substantial adsorption to take place (Harris and Angal, 1989).

As the concentration of immobilised ligand is increased the strength of binding of adsorbate also increases (Lowe and Dean, 1974). Presumably the reason for the importance of ligand concentration is because the equilibrium between bound protein and free protein is altered in favour of free protein when the concentration of ligand is decreased.

The number of ligands capable of binding with the protein, and therefore the capacity is dependent upon the surface area of the matrix. A matrix with a large surface area can not only immobilise more ligand per unit volume of support but the kinetics of the interaction between the adsorbent and specific protein to be separated are optimal. To this end the design of the support to which the affinity ligand is attached is very important (1.3.3).

The optimum ligand concentration is one reached by compromising of the increased capacity at high ligand densities and the reduction in resolution and wastage of ligand at excessively high ligand concentrations. One further consideration is that the use of non-porous or pellicular supports reduce the problems of decreased accessibility and therefore allow the use of relatively high ligand densities (Hearn and Davies, 1990).

1.2.1.4 Throughput rate

Although flow rate or throughput rate implies a column operation the important characteristic to note is that a high flow rate implies a low contacting time between the ligand and protein. Contacting time is of equal relevance to other types of affinity separation.

Flow rate or contacting time depends on the rate of formation of the protein-ligand complex, which is dependent upon the Kd value. If the time taken to reach equilibrium is long then low flow rates need to be used. For despite less protein molecules colliding with immobilised ligands the complementary pair are held closer together for

longer which is essential if adsorption is to be carried out. For complete adsorption the slowest flow rate acceptable needs to be used. For economic reasons high flow rates and therefore high throughputs are required. So determining the optimum flow rate is a matter of compromise between recovery of the product and the economics of the process.

1.2.2 Modes of operation for the purification of monoclonal antibodies.

A traditional purification process involves a number of unit operations which progressively enrich the required product by eliminating contaminating proteins. In contrast a single step purification involves the selective adsorption of the product thus eliminating the major part of the contaminants in one step. The main reason to aim for a single step purification is the lower costs involved in this approach. Jones (1991) compared a typical purification method involving precipitation, ion exchange, dialysis, gel filtration, hydrophobic interaction and three ion exchange polishing steps with a single affinity chromatography purification. As each unit operation is not 100% efficient yield losses occur at each stage. The loss of yield is accumulative so that the final yield of the multistep purification was 9% whereas the one step purification had a yield of 71% and in addition the product was purified to a greater degree. Yield losses translate into an increase in production costs (Equation 1.1). Bearing in mind that multistep purification's are the commonest approach in industry it is no wonder therefore that 50-80% of the costs of production of therapeutic products are incurred by the downstream processing (VanBrunt, 1988).

Affinity adsorption is probably the only technique capable of performing an economically viable and efficient one step purification because of the specific nature of the affinity interaction (1.2.1). The challenge however is to perform an affinity adsorption in the presence of large quantities of gross contaminants such as cells, cell debris, particulate matter, colloidal material, lipids and other fouling substances as would be encountered at such an early stage in the downstream process.

Table 1.2 Modes of operation for the affinity adsorption of monoclonal antibodies.

Mode of operation	Resolution	Suitability for primary recovery	Considerations
Batch adsorption	+++	+++++	Recovery critically dependent on kinetics of adsorption (Kd) Recovery of adsorbent after contacting (Loss of yield)
Packed bed adsorption	+++++	+	Fouling of column prevents application in feedstock containing particulate matter
Expanded bed adsorption	++++	++++	Throughputs limited by adsorbent design and feedstock composition
Fluidised bed adsorption	+++	++++	Throughputs limited by adsorbent design.
Magnetically stabilised fluidised bed adsorption	+++	+++	Specialist media required. Poorer resolution than fluidised bed
Magnetic affinity adsorption	+++	+++++	Recovery requires special media.
Affinity membrane adsorption	+++	+++	Fouling of membranes

There are a number of ways in which the affinity interaction can be exploited in order to purify products. The choice of mode of operation depends upon such characteristics as the ability to scale up, economics and the nature of the feedstock. Certain modes of operation are unsuitable for use in the early stages of downstream processing. In addition the efficiency of a mode of operation is dependent on the nature of the solid phase on which the ligand is immobilised. The lack of suitable solid phases has meant that many of the modes of operation discussed below have not achieved their full potential to date. The choice of ligand is also critical as in order to work and be reused. In the harsh environment to be found at early stages of the downstream process, the ligand must be robust.

Table 1.2 illustrates a variety of modes of operation and gives an indication of their suitability for use at large scale and applicability as a method for direct recovery or one step purification.

1.2.2.1 Batch adsorption.

A simple practical approach is described by Chase (1988). The adsorbent is added to the feed stream containing the protein, normally contacting is carried out in a stirred tank of some description. After contacting the adsorbent is recovered by filtration or centrifugation and is then packed into a column in which the protein is eluted and recovered.

To obtain good yields the partition coefficient (α), defined as the fraction of soluble protein adsorbed at any one time, needs to be close to unity. A partition coefficient of 1 means that all the protein in solution is adsorbed. The value of α is critical as it reflects the value of dissociation constant (Kd), which has a bearing on the efficiency of adsorption. In order to achieve an 80-90% yield of protein, α values must be greater than 0.98 (Scopes, 1988). If α is sufficiently high then batch adsorption holds great advantages over other separation techniques.

Scale up of batch adsorption is relatively simple, requiring a method to contact the adsorbent with feed stream and recover the adsorbent. Large volumes of feedstock can be processed by increasing the amount of adsorbent. The amount of adsorbent

used depends also upon the relationship between the concentration of the product and the affinity of the adsorbent. A low concentration of product compared to the affinity means binding will be poor. If high recovery is required then the concentration should be much greater than the value of Kd in order for a significant percentage of the capacity of the adsorbent to be used (Chase, 1988). It may therefore be necessary to concentrate the feedstream before carrying out a batch adsorption in order to make the relationship favourable. This introduces another unit operation with the associated penalties of loss of yield and cost.

The time taken to complete a batch adsorption is dependent upon the contacting time required to adsorb sufficient protein and this is dependent upon the affinity of the adsorbent for the protein. An adsorbent with high affinity, low dissociation constant, will require a shorter contacting time than a system with low affinity. In addition affinity adsorption requires longer contacting times, in comparison to less specific adsorption techniques, due to the orientation specific nature of the affinity interaction. For example the position of histidine residues on the target protein will influence the kinetics in an IMAC separation. For porous supports contacting time is also dependent on the diffusion of protein through pores. Non-porous supports, as there is no pore diffusion, should allow much quicker binding.

One method to increase the usefulness of batch adsorption particularly for cases where absorption is slow or incomplete is continuous affinity-recycle extraction (CARE) (Gordon and Cooney, 1990). The adsorbent is contacted with the feedstream, the specific protein binds to the ligand and other contaminants pass through a filter which excludes the passage of the adsorbent. Adsorbent is continually removed to a desorbing stage in which elution buffer is added to the adsorbent. The protein is eluted and passes through a filter where it is recovered and the adsorbent is then recycled back into the adsorbing stage. In this way the purification can be carried out continuously. Pungor *et al.* (1987) demonstrated that the recovery of *B*-galactosidase from unpretreated homogenized cells was 70%, compared to 90% for treated system, and that cell debris in the eluant stream was removed.

A potential problem of batch adsorption is the efficient and speedy recovery of the adsorbent-protein complex. As centrifugation or filtration are commonly used there must be a large difference in size and density between the adsorbent and cell debris etc. One approach is to use large adsorbent particles. Wang and Sobnosky (1985) developed an adsorbent consisting of adsorbent particles immobilised within a hydrogel made of calcium aglinate and K-carrageenan. The large size of the resulting adsorbent meant that it could be recovered from suspension culture without disrupting the culture. In addition the hydrogel excluded very large macromolecules and cell debris thus reducing fouling caused by these components. The main disadvantage of such an approach is that the effective resistance to diffusion is increased thus slowing the adsorption process.

The main advantage therefore of batch adsorption is its applicability at an early stage of downstream processing even to the point of direct recovery from the fermentor vessel (Rofler *et al.*, 1984; Freeman *et al.*, 1993). The problem of recovering the adsorbent-protein complex however remains a major stumbling block to its widespread use, although the use of magnetic collection technology is promising (1.2.2.6).

1.2.2.2 Packed Bed adsorption.

In packed bed adsorption the adsorbent is packed into a column through which the protein in solution is passed. In contrast to batch adsorption the theory of column adsorption is more complex and difficult to describe mathematically. There are several models to explain the behaviour of macromolecules such as proteins, in an affinity column quantitatively (Yang and Tsao, 1982; Jungbauer, 1993).

Qualitatively the protein can be thought of as occupying three states in a column (Lowe and Dean, 1974). It can be adsorbed reversibly onto the ligand, it is this non covalent interaction which is the basis of the separation. Alternatively it can be trapped within the support particle, if the support is porous, but not adsorbed to the ligand. Or it can be in the free liquid phase between the particles of the matrix. The protein will randomly occupy these three states, the percentage occupying any one state at any time depends on the value of the dissociation constant (Equation 1.3).

Because of the column design the protein has numerous chances to interact with the immobilised ligand thus affinity chromatography has excellent powers of resolution explaining its widespread use in a wide variety of applications (Lee, 1987; Skera *et al.*, 1991; Kaul and Mattiasson, 1992).

The main disadvantage of packed bed adsorption is the susceptibility of the column to fouling and blocking from cells, cell debris, lipids and other fouling materials. The adsorbent, usually porous, is prone to fouling and the column itself can become blocked particularly at the inlet. This causes an increase in back pressure which can damage the adsorbent and limits throughput rates and decreases the efficiency of the separation. For this reason packed bed separations are not suitable for use in 'dirty' feed streams encountered at the early stages of downstream processing and require pretreatment steps in order to be a viable separation method.

1.2.2.3 Fluidised bed adsorption.

An ordinary fluidised bed is formed by passing feedstream or buffer up through a packed bed column. At low flow rates the adsorbent particles lie on one another and on the bottom plate of the column. Increasing the flow causes the particles to move apart and at a particular flow rate, termed the fluidisation velocity, the adsorbent particles become suspended within the fluid stream. At a sufficiently high flow rate, termed the terminal velocity, the adsorbent particles will be transported out of the column. At intermediate flow rates between these two points the bed as a whole remains motionless relative to the walls of the column but the individual particles move within the bed. The bed at this point is said to be fluidised (Gaillot *et al.*, 1990). The fluidisation and terminal velocity of a solid depends upon its settling velocity which is a function of its size and density. Settling velocity (V cm/sec) is defined by the Equation 1.5 (Sinnot, 1983). Where g is the gravitational force cm/sec^2, a is the radius of particles in cm, n is the coefficient of viscosity g/cm-sec, $\rho 1$ is the density of the particle and $\rho 2$ is the density of the suspending medium (g/cm^3).

Equation 1.5 $V=(2ga^2 (\rho 1-\rho 2)) / 9n$

The terminal velocities of the feed stream components must be lower than that of the adsorbent so that the cell debris etc. passes through the column whilst the adsorbent is retained. In addition the terminal velocity of the adsorbent must be sufficiently high to allow the use of acceptable throughput rates and reasonable cycle times.

The design of adsorbent is critical and has been the focus of much research effort (Burns and Lyddiatt, 1995). One method to increase the terminal velocity of an adsorbent and thus allow higher throughput rates is to increase the density of the adsorbent. Densified cellulose adsorbents (Gilchrist and Lyddiatt, 1995), densified agarose adsorbents (Chang and Chase 1994) and perfluorocarbon affinity emulsions (McCreath *et al.*, 1992) have all been developed for use in fluidised beds.

The main advantage of fluidised bed separations is their applicability for the purification of products from feed streams containing particulate matter and fouling materials thus reducing the number of unit operations. The voidage between adsorbent particles allows cells and cell debris to pass through, providing the terminal velocity of the adsorbent is higher than that of the particulate matter (Draeger and Chase, 1991). In addition the pressure drop across a fluidised bed is much less than that across a packed bed of comparable volume, this means that higher throughputs can be used, within the confines of the adsorbents terminal velocity, with a subsequent decrease in cycle time and increase in economic savings.

The main disadvantage is the reduction in performance compared to an idealised packed bed. As the adsorbent particles are being mixed within the fluid in effect the adsorbent is contacting the feedstream in a manner analogous to batch adsorption rather than in the chromatographic fashion of a packed bed and therefore resolution is poorer (Sadana and Raju, 1990). Cycle times are shorter than those for batch adsorption as the separation takes place within the same vessel. Two other disadvantages are that firstly large amounts of solvent are required unless the feed stream is at the correct physiological state for adsorption and secondly extensive dilution can occur during desorption unless the flow is reversed and elution is carried out under packed bed conditions.

As a result of the poor chromatographic performance of fluidised bed adsorption, in order to exploit the full capacity, it is necessary to recycle the feedstock this will increase the cycle time but will prevent yield losses (Somers *et al.*, 1989). Fluidised bed adsorption has potential for use as a continuous purification method (Gordon *et al.*, 1990). McCreath *et al.* (1992) demonstrated the semi-continuous purification of albumin using non-porous perfluorocarbon emulsions, the yield was 87% and the albumin was recovered at 91% purity.

1.2.2.4 Expanded bed adsorption.

The main disadvantage of fluidised bed adsorption (1.2.2.3) is that back mixing reduces the performance of the separation. By preventing the back mixing it should be possible to recreate more closely the conditions present in a packed bed, i.e. where the adsorbent beads are stationary and flow through the column approximates plug flow, and takes advantage of chromatographic behaviour. There are several methods to minimize back mixing and 'stabilise' the bed. Firstly the use of baffles within the column will prevent much of the back mixing, however this is not appropriate for batch protocols as it best suits continuous countercurrent operations. The use of magnetically susceptible adsorbents and the application of a uniform magnetic field will also stabilise the bed, so called magnetically stabilised fluidised beds (1.2.2.5).

The alternative method is to use a flow rate which results in the adsorbent particles being fluidised but does not result in gross movements within the bed, the resulting bed is said to be stabilised and is termed an expanded bed. As there is little movement of the adsorbent particles the flow approximates plug flow, similar to that experienced within a packed bed, as can be seen by visual inspection and residence time distributions (DeLuca *et al.*, 1994). The operation is carried out in a column similar in design to a conventional column used in packed bed operation with two major differences. Firstly the flow adapter at the bottom of the column must ensure an even distribution of flow over the cross section of the bed thus eliminating channeling and other flow irregularities which would destabilise the bed and disrupt the performance of the separation DeLuca *et al.* (1994). Secondly the top adapter needs to be movable,

the sequence of operations of an expanded bed absorption illustrates this need (Chase, 1994).

The expansion characteristics of the bed are described by Richardson and Zaki (1954) in Equation 1.6 where U is the superficial velocity of liquid flow (m/s), U_t is the terminal velocity of an isolated particle such as an adsorbent bead (m/s) and ε is the voidage of the bed. The Richardson and Zaki constant ^{n} describes the flow within the bed, a value of 4.8 corresponding to laminar flow.

Equation 1.6 $U = U_t \varepsilon^n$

The expansion characteristics are a strong function therefore of the physical properties of the adsorbent and feed stream. U_t is defined by the diameter and density of the particles. It is the difference between values of U_t for the adsorbent and feedstream which define the flow rates that can be used to produce a stable bed and allow the removal of particulate matter from the feed stream. Maximising the differences between the terminal velocity of the support and the feedstream is important to maximise the throughputs used.

Conventional adsorbents are unsuitable for use in commercially viable expanded bed operation as the difference in terminal velocities of the adsorbent and feed stream components is too small for the use of acceptable throughput rates (Chase and Draeger, 1992). New adsorbents have been designed that have greater density and/or size allowing higher throughputs to be used (Gilchrist and Lyddiatt, 1995; Chang and Chase, 1994). Streamline™ manufactured by Pharmacia is an agarose based adsorbent with a large mean size and size distribution (100-300μm) and a quartz core which increases the density to approximately 1.2Kg/m^3. Streamline™ derivatised with ion exchange ligands has been used to purify a secreted recombinant protein from *E.coli* in a single operation with yields exceeding 90% (Chang and Chase, 1994). Crude fermentor broth was added directly to an expanded bed column which resulted in the removal of 99.99% of cells and a sixteen fold reduction in the volume of the process stream. Thommes *et al.* (1994) demonstrated the purification of IgG from hybridoma culture with recoveries of approximately 80% and the elimination of all cells and cell

debris. Both examples amply illustrate the power of expanded bed absorption and its applicability as a single step purification method.

A limitation of expanded bed operation is the compromise between the degree of bed expansion and throughput. Expansion of the bed greater than twice the settled bed height results in an increase in the voidage between particles, which decreases the efficiency of adsorption, with the product to be purified leaving the bed without contacting any of the adsorbent. Increasing the viscosity of the feedstock, the presence of cells, lipids etc., causes an increase in bed height at a given flow rate (DeLuca *et al.*, 1994). Another limitation of expanded bed adsorption is the time that is required to stabilise the bed after a change in conditions such as changing the flow rates or altering the viscosity of the applied solution (De Luca *et al.*, 1994). The practical outcome of this behaviour is that when altering conditions the operator must wait a period of time in order for the bed to be fully stabilised. This obviously has implications for productivity and cycle times (Thommes *et al.*, 1994).

1.2.2.5 Magnetically stabilised fluidised beds.

As mentioned previously (1.2.2.3) fluidised beds give poor performance because of the large degree of back mixing. Stabilising the adsorbent beads should improve the performance of the separation. If the fluidised adsorbent particles are magnetically susceptible and a uniform field is applied across the bed then magnetic dipoles are induced in the adsorbent causing the magnetic adsorbent to be spatially fixed, and thus stabilising the bed. The beds are renamed magnetically stabilised fluidised beds or MSFB (Rosenweig, 1979).

Rosenweig *et al.* (1981) demonstrated that MSFB's exhibited plug flow across a wide range of field strengths and fluid velocities, similar to that encountered in expanded beds. The application of a magnetic field in effect causes the magnetic adsorbent particles to line up parallel to the direction of the magnetic field lines producing chains of adsorbent beads. Rosenweig *et al.* (1981) established that in order to maintain a constant fluidised bed height increasing the magnetic field strength meant that the flow rate had to be increased correspondingly. This in effect means that it is possible to use

high throughputs and still maintain a bed which is stabilised for optimal performance. This control over bed expansion is the major advantage of MSFB over fluidised and expanded bed modes of operation.

One limitation of MSFB as a mode of operation is the lack of a suitable magnetic support (1.3.3.4). Various porous or pellicular supports are available such as agarose, in which magnetite has been adsorbed (Nixon *et al.*, 1991), and magnogel, a mixture of calcium alginate and magnetite (Burns and Graves 1985). In fact it is possible to use non-magnetic supports, Chetty and Burns (1991) fluidised nickel particles and a conventional porous adsorbent Macrosorb and stabilised the bed magnetically, as long as approximately 20% of the adsorbent is magnetically susceptible.

The size of the adsorbent is particularly crucial for MSFB. In packed beds, with small adsorbent particles, mass transfer in the solid (adsorbent) phase is the most important factor for efficiency and performance. In contrast mass transfer in the liquid phase is more important for fluidised beds, because of the voidage within the bed. Decreasing the size of the adsorbent increases the degree of bed expansion so that efficiency becomes worse and although MSFB can control the level of bed expansion visual observations indicate that channeling is more dramatic for small adsorbents (Nixon *et al.*, 1991).

A major limitation of MSFB is that it appears to be less efficient than a comparable fluidised bed. Nixon and co. workers (1991) hypothesised that this surprising observation is a function of bed structure. The adsorbent particles in MSFB being more ordered than in a fluidised bed resulting in poorer contacting between the adsorbent and product.

MSFB can be adapted for use as a continuous separation process (Burns and Grave, 1985). Chetty and Burns (1991) demonstrated the continuous recovery of lysozyme from a mixture containing myoglobin. Other applications of MSFB have included the use of MSFB as a bioreactor for plant cell culture (Bramble *et al.*, 1990) and as an immobilised enzyme reactor for the production of glucose (Kang *et al.*, 1995). The

applications of MSFB for use in protein separations have however been limited and no solution to the problems highlighted by Nixon *et al.* (1991) have been forthcoming.

1.2.2.6 Magnetic affinity separation.

Differences in the magnetic properties of materials can be used as a basis for separation. Biological systems with intrinsic differences in magnetic properties are relatively few, one example is red blood cells which contain high concentrations of haemoglobin, a fact which can be used to separate red blood cells from whole blood (Melville *et al.*, 1982). In order to exploit magnetic separation technology in other biological systems it is therefore necessary to render a protein magnetic by the attachment of a magnetically responsive element.

The simplest way of making proteins or cells magnetic is to add a magnetic solid such as magnetite to the solution. The protein adsorbs non-specifically to the solid conferring magnetic properties upon the protein. This process is totally unselective but is useful in removing total cell mass from solution (Dunlop *et al.*, 1984). Introducing a magnetic particle which binds specifically to the required product can be achieved by immobilising a ligand specific to the protein to be purified onto a support which exhibits magnetic properties.

Magnetic affinity separations is comprised of several stages 1) adsorption, 2) magnetic recovery of product-adsorbent complex, 3) wash to remove contaminants, 4) elution of product and 5) recovery of adsorbent for reuse. The adsorption stage is normally carried out in a batch mode fashion by contacting the magnetic adsorbent with the process stream in order to allow specific adsorption of the protein (1.2.2.1). The magnetically susceptible adsorbent is recovered by the application of an external magnetic field which causes the adsorbent to be retained allowing contaminating material to be removed.

The collection of magnetic supports can be carried out at a small scale using small hand held permanent magnets. Permanent magnets have been used to effect recovery of lactate dehydrogenase from porcine muscle homogenate (Ennis and Wisdom, 1991). Magnetised agarose was recovered after contacting with 80mL of crude homogenate,

by immersing a small permanent magnet (inside a resealable waterproof bag) into the feedstock. The magnet was then removed from the bag and the recovered adsorbent was packed into a column where elution of lactate dehydrogenase was carried out.

However larger scale separations are more difficult to achieve. Magnetic field strength is described by an inverse square law which states that the greater the distance a magnetically susceptible particle is from the source the lower the force acting upon it (Duffin, 1990). This means that in order to effect collection of magnetic particles from large volumes of solution the strength of the field has to be increased to compensate for the decay of the magnetic field density. The practical limit to the strength of permanent magnetic fields is 10,000 to 100,000gauss and the production of high field densities is technically demanding (Whitesides *et al.*, 1983).

Another consideration is the relationship between the applied magnetic field and the magnetic force acting on the magnetic particle described by Equation 1.7 (Menz *et al.*, 1986).

Equation 1.7 $Fx = V\ Xv\ H\ dH/dx$

Fx is the force acting on the particle in direction x, V is the volume of the particle and Xv is the magnetic susceptibility per unit volume. The applied magnetic field H causes the alignment of the particle, parallel to the applied field, and its movement towards the point of highest magnetic field gradient (dH/dx). This has a number of implications, firstly the smaller the volume of the particle the greater the magnetic field strength required for capture and secondly increasing the magnetic field strength will result in a greater force acting upon a particle of given volume and magnetic susceptibility.

There are a number of different kinds of industrial scale magnetic separator which attempt to get around the problems of producing high magnetic field gradients. Rotating drum magnetic separators consist of a drum fitted internally with magnets to produce a high intensity field at the drums circumference. The drum is rotated about a horizontal axis so that the lower part of the drum is immersed in the fluid stream. The magnetic particles are attracted to the drum circumference and the drum is rotated so that the adsorbent can be removed (Munro *et al.*, 1981). The separators are also more

efficient when processing fluids with low viscosity's at low flow rates, and thus are unsuitable for use as an early downstream processing unit operation.

The higher the density of the magnetic field the more efficient the separation and therefore its applicability as a magnetic separator within a downstream process (Equation 1.7). In an attempt to increase the intensity of an electromagnetic field the method for high gradient magnetic separation (HGMS) was discovered (Bitter, 1936). The basic concept is a bed filled with stainless steel wool surrounded by electromagnetic coils. When the power is applied to the electromagnet it induces a magnetic field in the steel wool. The small fibres of the steel wool distort the applied magnetic field and generate strong local magnetic field gradients, the sum of the external magnetic field and the magnetic field induced in the steel wire (Whitesides *et al.*, 1983). This structure is the most economical and effective way of magnetising a working volume up to field strengths of 2 Tesla (Oder, 1976).

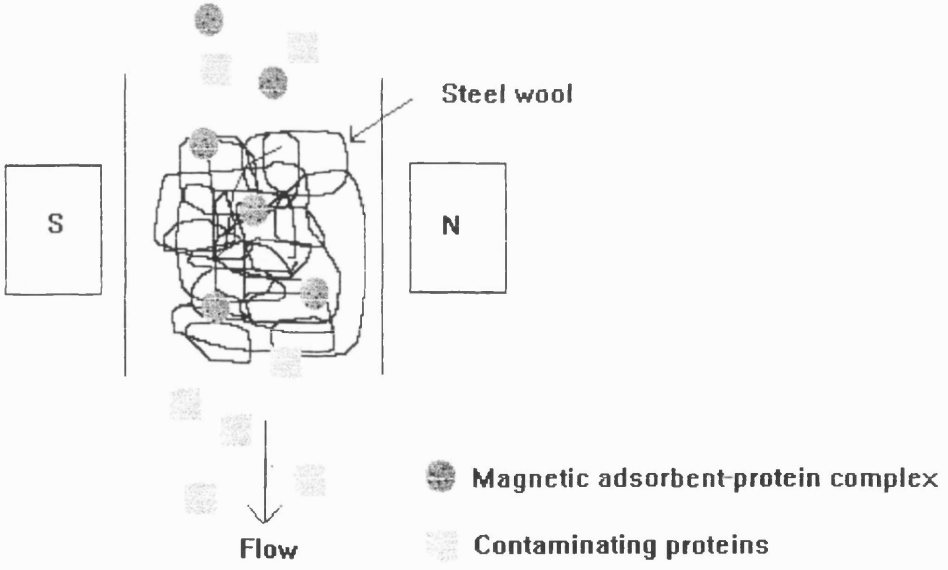
There are several models attempting to explain the behaviour of HGMS devices, (Watson, 1973 and 1975). The conclusions of these models, which have been verified experimentally (Luborsky and Drummond, 1975), are that in order to maintain the performance of the separation increasing the flow rate requires a concurrent increase in the intensity of the applied magnetic field. The filter performance is superior when the applied magnetic field and fluid flow are parallel compared to when they are perpendicular, horizontal orientation causes build up of cell debris.

The local magnetic gradients generated by the steel wool fibres attract and retain magnetic particles in the solution whilst the external field is being applied. A particle will be captured when the ratio of the magnetic force acting on the particle exceeds the sum of the hydrodynamic drag force and the net weight of the particle (Oder, 1976). The chances of a captured magnetic particle being swept away after having been attracted by the capture surface of the steel wool is strongly dependent upon the flow rate used through the bed and the strength of the magnetic field (Oder, 1976).

After some time the steel wool surface will have reached its practical capacity for retaining the magnetic material, and breakthrough of the magnetic material will occur.

At this time processing is terminated, the magnetic field is removed and the bed washed through to collect the magnetic particles. In order to increase the resolution of the separation the bed can be washed to remove any particulate matter trapped within the steel wool whilst the magnetic field is still present. In the case of affinity adsorption the magnetic adsorbent can be packed into a conventional column and the adsorbed protein eluted in the conventional manner. Alternatively the separation can be carried out entirely in the HGMS filter unit by maintaining the magnetic field and firstly washing the adsorbent to remove contaminants and then pass an elution buffer through the bed to cause elution of the adsorbed protein. The protein can then be collected in the eluent volume and on removing the magnetic field the adsorbent particles can be collected and recycled to the adsorption stage.

Figure 1.1 Model of High Gradient Magnetic Separation



The nature of the matrix used to generate the high field gradients is particularly important. Ferromagnetic stainless steel wool is strongly magnetic and has a large surface area to volume ratio so that efficiency at high throughputs should be good. If particulate matter does begin to clog the wool then it can be removed and washed and

sterilised and then replaced relatively easily. One disadvantage of using steel wool as a matrix is the fact that it will retain some magnetic properties after the removal of the external field, this can make recovery of the adsorbent particles difficult. Paramagnetic materials are less efficient in terms of capacity but recovery of the adsorbent after use is likely to be more efficient (Oder, 1976).

HGMS can be adapted as a continuous separation method with the use of a carousel separator (Marston, 1975), the steel wool matrix rotates through zones of adsorption, washing, and recovery (Kelland and Maxwell, 1975). Alternatively it should be possible to operate HGMS in a semi continuous fashion by the use of a number of HGMS canister's.

The ideal magnetic adsorbent material is small (0.1-1μm) and weakly magnetic, for a number of pertinent reasons (1.3.3.4). The high field densities generated by HGMS would allow the recovery of such material more efficiently than the other types of magnetic separator. One of the principle advantages of magnetic separation is the ability to be used in feed streams containing particulate matter, lipids and colloidal material (Munro, 1976). A comparative study of magnetic and non-magnetic techniques illustrates the rapidity and efficiency of using such supports (Hirschbein and Whitesides, 1982).

Ennis and Wisdom (1991) demonstrated the applicability of magnetic separation, on a small scale, in the early stages of processing by purifying lactate dehydrogenase (LDH) directly from porcine muscle homogenate. A conventional purification involving centrifugation and Reactive Red 120 dye ligand chromatography resulted in 50% losses of LDH compared to the magnetic separation yield of approximately 100%. The purification of LDH was also more efficiently carried out by the magnetic extraction, 15 fold purification compared to 5 fold. The major loss of LDH, in a conventional process, is incurred during the centrifugation of the homogenate (40%) prior to chromatography. With the ability of magnetic extraction techniques to be applied to debris laden feed stocks this source of loss can be eliminated (Halling and Dunnill, 1980). The main reason HGMS has not been developed as a unit operation

for the purification of proteins has been the availability of a suitable adsorbent (1.3.3.4).

1.2.2.7 Miscellaneous affinity separations.

Membranes have traditionally been used to separate molecules on the basis of size. It is possible to immobilise affinity ligands to the surface of membranes in order to combine the specificity of affinity interactions with the excellent transport properties of thin film membranes (Heath and Belfort, 1987; Kaul and Mattiasson, 1992). Zale *et al.* (1990) used protein A immobilised to a membrane to isolate murine monoclonal antibodies from clarified cell culture supernatant. Compared with an agarose column of comparable size the yield was two orders of magnitude higher. They also claim the process is up to 100 times faster then conventional affinity chromatography as carried out in an expanded bed. Although fouling is a problem with affinity membranes there are examples of their use at an early stage in downstream processing eliminating the need for concentration and partial purification steps (Spalding, 1991).

An adaptation of the affinity membrane system is the use of affinity cross-flow filtration (ACFF). This is a combination of the specificity of an affinity interaction and the efficiency of cross flow ultra filtration (Herak and Merrill, 1990; Labrou and Clonis, 1994). Weiner *et al.* (1994) immobilised protein A onto microparticles for the isolation of a specific IgG from clarified hybridoma culture. The microparticles were prepared from bacterial cell wall fragments and contacted batchwise with feedstream in a stirred tank. The microparticles were retained upstream of a ultrafiltration membrane, collected, and the IgG eluted from the support. The design of a suitable support to which the affinity ligand can be immobilised and the susceptibility for fouling of membranes, are the major reasons for the lack of large scale applications. Krause *et al.*, (1991) for example, demonstrated that due to fouling recovery of product was approximately halved.Affinity partitioning is a technique in which the selectivity of aqueous two-phase systems, (Huddleston *et al.*, 1991), is improved by coupling a specific ligand to either of the two polymers used. The ligand is attached to the polymer in which the protein to be purified partitions to conventionally. The

specificity of the ligand-product interaction increases the the partition coefficient, the ratio of the protein to be purified in the two phases (Joelsson and Johansson, 1987).

Affinity precipitation is a method by which the selectivity of precipitation as a downstream operation is improved. There are two main approaches to effect affinity precipitation, the first utilises the affinity interaction to make complexes to large to be soluble (Van Dam *et al.*, 1989). The second is to induce precipitation of the affinity complex by the alteration of conditions or addition of a group not directly involved in the affinity interaction (Kaul and Mattiasson 1992; Morris *et al.*, 1993). Despite the fact that neither the adsorption stage or precipitation stage have been optimised successfully to date the technique is a promising one to introduce the resolving power of affinity separation at an early stage in the purification of proteins.

1.3 The Affinity Adsorbent

1.3.1 Immobilisation of ligand.

The immobilisation of the ligand onto the matrix support is a crucial stage in the preparation of an affinity adsorbent. The term adsorbent is defined as the combination of a matrix with a specific ligand. Immobilisation will affect the capacity of the adsorbent, the concentration of accessible and sterically unhindered ligands, the stability of coupling bond, and the lifetime of the adsorbent.

The immobilization of the ligand onto the adsorbent is normally carried out in two stages: activation of the matrix followed by coupling of the ligand. Activation is the procedure by which chemical groups upon the matrix are chemically manipulated so that complementary groups on the ligand can be reacted to form a chemical bond. Activation is accomplished by introducing an electrophilic group onto the matrix which can then react with nucleophiles on the ligand. Available active groups include hydroxyl groups, carboxylic groups, amino groups and sulfhydryl groups (Wheelwright, 1991). Activation can increase the final ligand concentration, which is particularly important for low affinity systems, by increasing the number of groups, such as hydroxyl groups, able to combine with affinity ligands (Porath and Sundberg, 1974).

The activation method used will in some respects determine the performance of the adsorbent in that it determines the type of coupling bond between the matrix and ligand. Often the activation and coupling techniques are synonymous and are therefore given the same name. Table 1.3 illustrates some of the commonly used coupling or activation reactions used. The comparative stability of the bonds is listed in descending order, secondary amine bonds being the most stable whilst isourea bonds are the least stable. The suitability of the coupling method depends on the nature of the ligand and the support and the particular application for the affinity separation.

Table 1.3 Common activation and coupling methods

Activation method	Activated group	Group used in binding	Bond type (Stability)
Bisoxirane	Hydroxyl	Amine, thiol	Secondary amine (++++)
Divinylsulfone	Hydroxyl	Amine	Secondary amine (++++)
2-Fluro-3-methyl pyridinium tosylate	Hydroxyl	Amine, thiol	Secondary amine (++++)
Carbodiimide	Carboxyl	Amine	Amide (+++)
Carbonyl diimidazole	Hydroxyl	Amine	Urethane (++)
Cyanogen bromide	Hydroxyl	Amine	Isourea (+)

Adapted from Lowe and Dean (1974).

The stability of the bond between ligand and support determines the lifetime of the adsorbent. The conditions the adsorbent is subjected to during elution, regeneration and sterilisation in particular, may well disrupt weak bonds between ligand and matrix causing leaching of the ligand from the support. A weakly bound ligand if leached from the matrix causes a reduction in the lifetime and the capacity of the adsorbent. The presence of ligand in the purified product also presents regulatory problems as it will contaminate the product. The leaching of protein A, for example, requires the use of additional purification steps to remove it from the product. The extra expense of a stable coupling chemistry must therefore balanced against the expense of 'clean up' purification steps and the reduction in the lifetime of the adsorbent.

Cyanogen bromide (CNBr) reacts with hydroxyl groups in agarose and other polysaccharide matrices to form a reactive group that further reacts with primary amines upon the ligand (Axen *et al.*, 1968). The N-substituted isourea bond that is formed is charged at physiological pH's and therefore imparts anionic exchange properties upon the support, a potential source of non-specific interactions. The isourea derivative is susceptible to nucleophilic attack and slow hydrolysis at extremes of pH and temperatures above 4°C resulting in leakage of the ligand. Despite these major disadvantages, including toxicity of the reactants, for historical reasons CNBr activation is probably the widest used procedure.

An alternative method is epoxidation, bis-oxiranes (bis-epoxides) react with hydroxyl or amino groups at alkaline pH to yield derivatives with a reactive long chain hydrophilic oxirane group. The oxirane can be reacted with nucleophiles (e.g. proteins) under alkaline conditions to produce affinity adsorbents (Sundberg and Porath, 1974). Compared to CNBr activation there are lower numbers of hydrophobic and ionic groups thus reducing non-specific interactions. The oxirane acts as a spacer arm therefore it is not necessary to insert one as is the case in CNBr activation's. Oxirane coupled ligands are also extremely stable, the ether bond being particularly stable (Hermanson *et al.*, 1992).

Another role of the activation method is often the introduction of a spacer arm e.g. epoxidation. The purpose of a spacer arm is to distance the ligand from the support to reduce steric hindrances. For example the matrix may alter the conformation of the ligand and or the protein if they are too close. A spacer arm is a short length of a hydrophilic hydrocarbon which reduces the effects of steric hindrances resulting in a subsequent increase in the strength of the affinity interaction and an increase in capacity of the adsorbent (Cuatrecasas *et al.*, 1968).

After ligand immobilisation there will be residual sites on the activated matrix which will be potential sources of non-specific binding. It is therefore important to block or cap these unused activation sites. Small short hydrophilic compounds without secondary functionality's are the best choices of blocking agents as they will not be a source of non-specific binding themselves. Common capping agents include glycine, ethanolamine, tris and cysteine (Hermanson *et al.*, 1992). The alternative is to leave the adsorbent in ligand coupling buffer to hydrolyse the remaining activated sites.

1.3.2 Selection of Affinity ligand.

Affinity adsorption is based upon the biospecific interactions between a substance and another complementary molecule (1.2.1). There are two broad classifications of ligand, biospecific and pseudobiospecific. Biospecific ligands mimic natural biological affinities and are therefore very specific. Examples of this type of ligand are immobilised enzymes, hormones and receptors, antigens and antibodies. Pseudobiospecific or group specific ligands have a much broader specificity and are capable of interacting with a variety of molecules sharing a common motif in their conformational structure. Thus some knowledge of the protein to be purified its structure or biological properties is invaluable in choosing a ligand, the alternative is a trial and error approach.

At the present time it is difficult to establish rules of thumb for the choosing of the best ligand for the purification of a particular protein. There are however a number of factors that need to be considered when choosing a ligand which is suitable for a particular purification.

Specificity

Flexibility

Strength of interaction

Stability of ligand and protein to be purified

Cost

Biospecific ligands have very specific affinities for the complementary molecule, for example the Kd value for a typical antibody-antigen interaction is in the range 10^{-8} to 10^{-12}M (Van Oss *et al.*, 1986). A strong interaction (low Kd value) means that product loss is likely to be low and it should be possible to carry out a high yielding single step purification from very dilute solutions. However strong affinities often require extreme conditions in order for the product to be desorbed from the ligand. This may reduce the lifetime of the ligand and support and also result in loss of product due to denaturation (Herion and Bollen, 1983). If milder elution conditions are used to prevent denaturation then the desorption will be incomplete and losses in product yield and the reduction of the adsorbent capacity in subsequent purification's which may be just as severe (Yarmush *et al.*, 1992).

In contrast pseudobiospecific ligands have much broader specificity's, often referred to as general ligands, and as a consequence have a lower affinity than biospecific ligands (10^{-4}-10^{-7} M). The weaker interaction may result in poorer recovery of the product, a reduction in capacity and increase in non-specific adsorption of unwanted contaminants. However the ability to purify a variety of products using the same ligand may be an advantage in terms of reducing the costs of development of the process and validation. The main disadvantage with ligands that can purify a variety of biomolecules is the reduction in specificity, but if the adsorption and desorption conditions can be used to fine tune the specificity then this problem can be reduced.

Thus a trade off between the specificity of the ligand and its flexibility needs to be made for industrial applications (Lowe and Dean, 1974).

The stability of the ligand, and indeed the protein to be purified, will determine the extremes of conditions the adsorbent can withstand during elution, regeneration and sanitisation. In addition it will ordain the suitability of the ligand for use in purification's from process streams containing a large cocktail of contaminants. In particular the presence of proteases will seriously reduce the efficiency of the separation and the lifetime of adsorbents with proteinaceous ligands.

The cost of the ligand per se and its immobilisation onto the support are other serious considerations. Biological ligands need to be produced and purified from biological sources which adds to the cost. The lifetime of the ligand will also influence the cost. Stable ligands that remain functional for a long time and do not significantly leach from the adsorbent will be more cost effective. Ligand leakage is dependent to a certain extent upon the immobilisation method used (1.3.1).

As a consequence of the above, pseudobiospecific ligands are likely to be favoured for applications in 'dirty' process streams i.e. as a single step purification process. This is due to their robustness, cheapness in comparison to biospecific ligands and their flexibility. Biospecific ligands are likely therefore to be restricted to applications where the greater selectivity and therefore resolution is a more appropriate design consideration, for example the purification of high value therapeutic proteins (Hill and Hirtenstein, 1983; Clonis, 1987) or applications where a pseudobiospecific ligand can't be found for the specific protein of interest.

There follows a discussion of some of the key ligands used in the purification of monoclonal antibodies.

1.3.2.1 Protein A

Protein A is a single polypeptide chain of molecular mass 42KD from the cell wall of several strains of *Staphylococcus aureus*. It interacts with the Fc or constant region of immunoglobulins of mammalian species. Because it does not bind to the variable antigenic determinant region but the constant region, protein A can be classed as a

pseudo-immuno-affinity ligand. It contains four binding sites with a dissociation constant of approximately 10^{-8} M (Hermanson *et al.*, 1992). Although the main specificity of protein A is for IgG's other immunoglobulins such as IgA and IgM bind to a certain extent (Langone, 1982).

Protein A is usually immobilised through its amine groups. By controlling the amount of protein A added to the immobilisation reaction it is possible to generate supports with capacities ranging from 12-15mg human IgG per mL of gel, as an average capacity, to 40mg human IgG per mL of gel and above (Hermanson *et al.*, 1992). Protein A is relatively stable but leaching of protein A from affinity adsorbents even at low levels (2-10ng protein A per mg of antibody) causes problems because of it's toxicity (Naveh and Siegel, 1991).

Protein A is an expensive ligand, for example on average protein A gels are an order of magnitude more expensive than gels immobilised with chelating groups or dyes (Pharmacia Biotech, 1994). The effect of this on the production costs of a therapeutic antibody is illustrated by the difference between an ion exchange separation ($53 per g of product) and $217 per g for a protein A separation (Sadana and Beelaram, 1994).

Protein A has been used in a number of large scale applications. An example of this is the 140 fold purification of antimelanoma monoclonal antibody 9.2.27. pretreated by ultrafiltration using a 500mL protein A-Sepharose column (Lee, 1987). However the levels of endotoxins, DNA, minor protein contaminants, serum immunoglobulins and of leached protein A were too high and required further purification operations. The use of protein A as the very first purification step is not widely recommended because of the presence of fouling solids and proteases which would reduce the capacity of the adsorbent (Scott *et al.*, 1987). Despite these disadvantages protein A Sepharose columns have been used for the commercial production of antibody for over 100 cycles from clarified and concentrated supernatant without a significant reduction in capacity (Kenney and Chase, 1987).

An alternative to protein A is a related protein called protein G. Protein G is a cell wall protein of group G *Streptococci* (Bjorck and Kronvall, 1984). Like protein A,

protein G binds to the Fc region of a wide variety of IgG's. The specificity's of the two ligands are different so there is considerable interest in using protein G when protein A is unsuitable (Lee *et al.*, 1992). Protein G enjoys similar advantages and disadvantages, namely cost and stability, to protein A.

1.3.2.2 Avid AL.

Avid AL™ (Bioprobe International, Tustin CA) is a synthetic low molecular weight ligand prepared by the reaction of fluropyridine and mercaptoethanol with a solid support such as Sepharose. The resulting structure is believed to act as an electron acceptor, and interacts specifically with IgG's, which are believed to behave as electron donors (Ngo and Khatter, 1992).

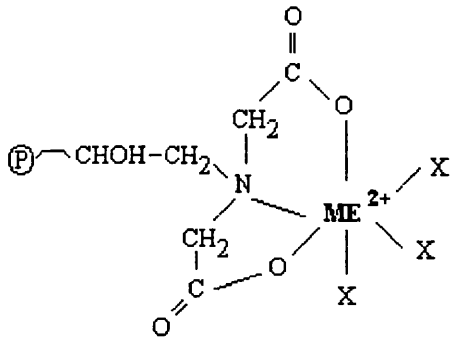
In comparison to protein A and protein G, Avid AL™ has a wider specificity, binding many species that the proteinaceous ligands do not. In addition elution can often be carried out under neutral conditions which will help to protect the antibody from adverse effects. Because it is synthetic it is more stable under conditions known to inactivate protein A and protein G (Ngo and Khatter, 1992). Avid AL™ has been used to purify IgG's directly from PBS/diluted serum (Khatter *et al.*, 1992). However compared to protein A the binding strength is on the low side (Kd 17μM) (Ngo and Khatter, 1992).

1.3.2.3 Metal Chelate Ligands.

Some proteins show an affinity for metal ions, a phenomenon that has been exploited as a basis for affinity separations (Porath and Belew, 1983; Arnold, 1991). Most work has been carried out using iminodiacetic acid (IDA) a metal ion chelator and the first row transition metals such as nickel (Ni^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), and iron (Fe^{2+}). IDA is a tridentate chelator meaning that the nitrogen atom and two carboxylate oxygen's bind to the metal ion (Figure 1.2.). There are four coordination sites which bind the metal ion at least one of which is occupied by buffer salt or water molecules. These can be readily displaced by a protein containing a strong electron donor such as histidine at slightly alkaline pH. Other chelators such as tris(carboxymethyl) ethylenediamine (TED) and nitrilotriacetic acid (NTA) bind metal ions (Me^{2+}) more

strongly than IDA. This is useful in applications where a biomolecule has such high affinity that the Me^{2+} ions are scavenged from the adsorbent (Arnold, 1991).

Figure 1.2 Structure of iminodiacetic acid



The metal chelator is immobilised onto a solid support, metal ions are in turn immobilised by 'charging' the column with concentrated (50mM) metal salt solution. The concentration of ions that can be immobilised is proportional to the amount of immobilised chelating ligand. Excess ions are removed by washing before the adsorption stage. Adsorption is usually maximal at higher pH's, salt is often added to reduce non-specific ion exchange effects, whilst elution is carried out by lowering the pH to protonate the donor groups of the adsorbed protein. Alternatively lewis acids such as metal ions which compete for the adsorption sites of the protein, or a lewis base such as imidazole which competes for the chelated metal ion can be added (Arnold, 1991). Stepwise or gradient elution can be used to increase the specificity of the technique by separating proteins on the basis of the strength of their interactions with the chelated metal ions.

The main functional group in proteins capable of binding with metal ions are amino acid residues with electron donating groups. Hemdan and Porath (1985) demonstrated the relative binding of free amino acids to immobilised Ni^{2+} ions. Cysteine, histidine and argininine were retained most strongly, an order of magnitude more than other amino acids, whereas glutamic and aspartic acids were not retained at all. Hemdan and Porath (1985) also showed that increasing ionic strength further enhanced retention and that sodium chloride was the most effective salt. In practice the presence of

histidine dominates protein binding, other amino acids and functional groups have a very much smaller contribution to the affinity of a protein for a chelated metal ligand (Arnold, 1991). The imidazole ring of the histidine residue is believed to form a coordination bond with the metal ion increasing the degree and strength of the interaction. This view is supported by the observation that retention is highly dependent upon the pH of the buffer and the pKa of imidazole (Sulkowski, 1985).

The number of available histidine residues and their accessibility on the proteins surface are important factors. For IDA-Cu^{2+} and IDA-Ni^{2+} at neutral pH, accessible histidines are the primary adsorption sites (Hemdan *et al.*, 1989). The interaction is dependent upon the number and position of residues relative to one another. The presence of multiple histidines appears to complicate the equilibrium binding one would expect. Todd *et al.*, (1994) describe the resulting multisite binding as comprising of a weak and strong binding interactions. A protein containing two proximal, surface accessible histidines can bind in one of two ways to immobilised metal ions. If they bind one at a time i.e. independently then the affinity is comparable to that of a protein containing a single histidine residue. If on the other hand the density of the immobilised Me^{2+} ion is such that the two histidine residues can bind simultaneously to two distinct ligand sites then the affinity is much higher. It is therefore possible by controlling ligand density, charging the adsorbent with different concentrations of metal salt, to resolve proteins with different numbers of histidine residues (Todd *et al.*, 1994).

The affinity of immobilised metal ions towards a biomolecule is also dependent upon the metal ion chosen. Immobilised Cu^{2+} has an approximately fifteen times greater affinity for imidazole than Ni^{2+}, which in turn has three times greater affinity than Zn^{2+} and Co^{2+} (Arnold, 1991). If the affinity towards the product is too great, then harsh elution conditions need to be used in order to recover the product, conditions which may cause denaturation of the product. Another factor to consider is the toxicity of the metal. Copper in particular is known to be toxic and therefore its presence in the final product would not be desired.

The stability and inexpensive nature of metal chelating ligands, which are low molecular weight compounds, is a great advantage. Regeneration is simple, metal ions

are stripped from the adsorbent which is simply recharged before being recycled. A possible disadvantage is the lack of specificity however this can be tailored by selecting an appropriate metal and ligand density, and appropriate adsorption and elution conditions. In addition the use of molecular biology techniques to produce polyhistidine fusion tails is a rapidly growing application for metal chelate separations and greatly increases the resolving power (Hochuli, 1988; Sassenfeld, 1990; Nygren *et al.*, 1994).

1.3.3 Selection of matrix.

Affinity adsorption can be divided into two distinct steps, the mass transfer of the specific protein from the sample to the immobilised ligand and the interaction between the ligand and specific protein. The support matrix will affect both these phenomena. The type and scale of the separation will define to some extent the requirements the support needs to meet. For example a process scale separation will require a support with a large capacity and one able to withstand high flow rates. In contrast in an analytical separation high capacity and maximum flow rates will not be major considerations.

The ideal material for a support or matrix would have the combination of the following characteristics (Table 1.4). The relative importance of each of the characteristics depends on the purification protocol used and the scale (Narayanan and Crane, 1990).

In general there are two types of support; non-porous and porous. Porous supports are the most commonly used, they consist of a network of pores in which the ligand is immobilised and therefore through which the protein must diffuse in order to interact specifically to effect a separation. In contrast the ligand in a non-porous support is immobilised upon the external surface of the support.

Table 1.4 Characteristics of the ideal support material

Insoluble: to prevent contamination and aid separation
Minimal non-specific adsorption: hydrophilic, no charged groups
Large interfacial surface area: large capacity
If porous: highly permeable
If non-porous: small diameter - large surface area to volume ratio.
Easily derivatisable:
Non-compressible to withstand high throughput rates
Chemically stable: particularly to regeneration and elution.
Sterilisable
Easy to regenerate: column lifetime
Inexpensive: dependent on all of above.

The interaction of protein and immobilised affinity ligand does not occur instantaneously. There are a number of steps that occur as summarised by the step model of adsorption (Chase, 1988). The first is mass transfer of the bulk liquid to the outer surface of the particles, which is opposed by film diffusion resistance. When a fluid flows past a solid surface there is a stagnant layer of fluid that covers the surface and even though this layer is usually thin it resists movement of solute molecules across it. The second step, occurring only in porous supports, is solute diffusion into the pores which is opposed by pore diffusion resistance. Thirdly the chemical interaction at the binding site of the ligand, opposed by surface reaction resistance. Of the three resistance's to mass transfer, pore diffusion resistance is rate limiting in porous supports, for non-porous supports the rate limiting step will be film diffusion

resistance because of the absence of pores. The comparative lack of diffusional limitations is a significant advantage of non-porous supports over porous ones (Halling and Dunill, 1980).

The time taken for the mass transfer of the protein from the mobile phase to the surface of the ligand needs to be considered in terms of the throughput rate. If the mass transfer rate is slow then the flow rate or contacting time needs to be adjusted so that the protein has sufficient time to interact with the ligand otherwise the protein will not be adsorbed and it will appear in the void volume. The mass transfer rate depends on the nature of the support, particle size, permeability, molecular weight of the ligand and the protein to be adsorbed (Katoh, 1987).

The nature of the ligand-protein interaction is susceptible to changes in the microenvironment. The ligand immobilised upon the support is surrounded by a microenvironment that is in part defined by the nature of the support. The effect of the matrix upon this microenvironment is crucial to the efficiency of the separation (Turkova, 1983). Ligands directly immobilised onto supports are not as effective as those immobilised via a spacer molecule, this is due to steric effects imposed by the physiochemical nature of the matrix (Lowe and Dean, 1974). Hydrophilic conditions are usually necessary to maintain the conformation of the protein to be purified and so in order to mimic the affinity interaction in free solution the matrix needs to be hydrophilic (Hammond and Scawen, 1989).

In order to be capable of resolving proteins effectively the amount of non-specific adsorption should be minimal. Non-specific adsorption is the adsorption of molecules, other than the required protein to the support, spacer arm or to the ligand itself. Generally this means no hydrophobic or highly charged areas on the surfaces of the support (Narayanan and Crane, 1990). This is very difficult to achieve without processing of some kind or careful design as proteins are polyelectrolytic, they have many different charged groups, and are therefore very likely to bind non-specifically.

To accomplish an affinity separation usefully on a large scale the separation process must have a large capacity for the protein to be separated. Capacity is commonly

defined as the total amount of protein (mg) per unit volume of the support. The adsorption capacity to a certain extent is predicted by the type of ligand and its affinity for the protein (1.2.1). But the number and accessibility of ligand binding sites depends on the size and shape of the support and the linkage of ligand to the support. Small porous particles have a larger surface area to volume ratio than bigger particles and therefore a greater number of accessible adsorption sites which increases resolution (Wheelwright, 1991). Horstman demonstrated that the capacity of Sepharose based gels increased with decreasing particle size (Horstman *et al.*, 1986). A limitation of non-porous adsorbents is their reduced capacity compared to conventional porous supports due to the smaller surface area (Kobos *et al.*, 1989). Increasing the surface area to volume ratio by reducing the diameter of the support particle will increase the capacity of the adsorbent. A 0.1-1μm particle has a comparable surface area to a porous macroparticle of about 100μm (Groman and Wilchek, 1987).

The mechanical strength of the support determines the maximum flow rate or throughput rate that can be applied, in that it determines the maximum pressure the support can withstand without collapsing or compressing. Mechanical strength is also necessary to prevent damage caused by abrasion of particles due to high throughput rates and handling during column packing and re-packing. One of the major limitations of porous supports is their compressibility under elevated pressures. When a porous support is compressed the pores are partially closed thereby preventing diffusion of the protein into the support reducing the capacity of the support and the yield of the separation. One way to increase the mechanical strength of porous supports is cross linking of the network of pores, however there is sometimes, but not always, a trade off with permeability and a reduction in mass transfer rate (Lowe and Dean, 1974).

Chemical stability is a crucial property for any matrix. An unstable matrix will contribute to ligand leaching and therefore reduction in yield of the purification over time. Chemical stability is particularly crucial during elution, cleaning and sanitisation when the support is likely to be exposed to the harshest conditions. Any effects here will almost certainly reduce the capability of the support for regeneration.

Regeneration is the process by which after elution of the protein the support is made ready for another cycle of purification.

The choice of affinity matrix depends on many of the factors discussed above, other aspects such as cost and scale will also be very important. There is a certain amount of compromise to be made to achieve the ideal affinity support so no one support material can be classed as ideal but some are better than others. The advantages and disadvantages of some of the more important supports are discussed below.

1.3.3.1 Conventional porous supports.

Conventional supports can be arbitrarily divided into two groups, biopolymer supports and synthetic supports. Some supports are combinations of the two types. Described below are examples of the more important types of porous supports.

Biopolymer supports are based upon polysaccharide structures and include such examples as agarose, cellulose, and dextran. Historically biopolymeric supports were the first to be used as chromatographic matrices for bioprocesses and they continue to be widely used. Agarose is a macroporus support with a large number of hydroxyl groups on the polysaccharide backbone, which confer a fair degree of hydrophilicity and are also easily manipulated for ligand immobilisation (Harris and Angal, 1989). Agarose is normally crosslinked in order to increase stability and physical strength. Pharmacia produce at least two versions of crosslinked agarose, CL-Sepharose and Fast Flow Sepharose. These can operate at low to medium pressures but are still prone to compression under high pressures. Superose is a highly crosslinked agarose gel matrix of 10μm particles and can operate at the high pressures (3-10MPa) of HPLC (Hammond and Scawen, 1989). Recently the Sepharose based supports have been adapted for use in expanded bed adsorption (1.2.2.4). Streamline™ comprises of a large Sepharose type bead (100-300μm) which has a quartz core incorporated within it to increase the density, an important feature for expanded bed operations (Chang and Chase, 1994).

There are a number of inorganic supports that have been used as adsorbents in downstream processing (Green and Wase, 1986). The commonest are silica based

supports. Silica is widely used in HPLC applications such as reversed phase chromatography as they are mechanically stable and therefore able to withstand high pressures (Dean *et al.*, 1985). The main disadvantages associated with the use of silica in affinity purification are the high degrees of nonspecific adsorption to the matrix and the stability of the matrix. Coating with silane reduces nonspecific adsorption and increases the stability in alkaline pH. The main application for silica supports is in HPLC or high performance liquid affinity chromatography (HPLAC) (Ohlson *et al.*, 1989).

Synthetic supports are produced by the polymerisation of monomers. Co-polymerisation can be carried out by combining synthetic monomers with natural matrix materials such as agarose or other synthetic polymers. Cross linking reagents are widely used to form the polymer, there are a large number of commercially available matrices (Hermanson *et al.*, 1992). Typically synthetic matrices have better chemical and physical stability than natural matrices, pressures of 2-3bar can be tolerated and the best of the supports can stand the sorts of pressures encountered in HPLC. This strength allows increased linear flow rates thus greater throughputs with consequent reduction in time and production costs. Thus they are particularly suited to the rigours of process scale production. Examples of such supports include Polyacrylamide (Hjerten and Mosbach, 1962), Trisacryl (Allary *et al.*, 1991) and Azlactone beads (Hermanson *et al.*, 1992). The advantages of this type of synthetic support over biopolymer supports is that they are resistant to microbial attack, more stable at pH extremes and generally more chemically stable. Physical stability is dependent upon the processing conditions but is generally fair.

The main disadvantage with all the supports discussed above is the fact that they are porous. This means that they are susceptible to fouling and are therefore unsuitable for use at an early stage in the downstream process and in addition diffusion limitations affect performance.

1.3.3.2 Perfusion supports

The problem of pore diffusion restriction inside the matrix particle is probably one of the main problems in improving chromatographic peformance in a packed bed. One way to overcome this is to use particles which have throughpores, large pores which allow convective flow into the particle supplementing the diffusion of the protein. So called perfusion chromatography (Afeyan, 1990) reduces the resistance to molecular movement, and also allows convection, which is faster than diffusion, to take place. A recent development has been the production of a polystyrene/divinylbenzene copolymer which is coated with polyhydroxylic polymers or polyethyleneimine, depending upon the application, to form a bead like matrix with conventional small pores (50-100nm) and larger through pores (600-800nm). These large through pores allow convective flow of the liquid phase thus reducing the diffusional resistance to mass transfer. Because of this the liquid phase can be transported much quicker through the matrix thus allowing higher flow rates to be used in a column operation without a loss of capacity or resolution.

To date perfusion chromatography has been restricted to HPLC applications partly because of the small size of beads available and also because the properties are better exploited by the high pressures and increased flow rates produced by HPLC technology (Hermanson *et al.*, 1992). Initially the supports were ion-exchange ones for HPLC or reversed phase HPLC, but immobilised protein A and iminodiacetic acid and specific immunoglobulin supports have been prepared by various activation methods (Hermanson *et al.*, 1992). Lehman *et al.* (1993) used perfusion supports (POROS™) to purify recombinant tick anticoagulant peptide. The use of perfusion cation exchange chromatography and perfusion reversed phase HPLC reduced the process time by one-half and increased the yield obtained from 32% to 47%. Katoh *et al.* (1994) demonstrated that at flow rates of 500cm/hour POROS™ was comparable to an equivalent HPLC adsorbent in terms of time to break through. A new perfusion support has recently been developed, Superporous agarose (Gustavsson and Larsson, 1996).

1.3.3.3 Perfluorocarbon supports

One of the drawbacks of many commercial matrix materials is their fragility under conditions such as pressure, pH or temperature used in the separation process and particularly in the regeneration and clean up processes (Knight, 1989). Perfluorocarbons consist entirely of carbon and fluorine atoms but do have a superficial resemblance to hydrocarbon based molecules. Perfluorocarbons are chemically and biologically inert as well as being thermally stable, they have a high density (1.8-2.1g/mL) and are insoluble in organic and aqueous solvents (Stewart *et al.*, 1990). These properties would suggest an almost ideal material for use as a matrix (Table 1.4).

Perfluorocarbons are very hydrophobic, a potential source of nonspecific absorption, and as a consequence are non-wettable in aqueous solvents, making ligand immobilisation difficult. Their hydrophobic nature has been utilised in HPLC applications (Williams *et al.*, 1986). Until recently the hydrophobic nature of perfluorocarbons has precluded their application in affinity separations. The use of fluorosurfactants to 'wet' the fluorocarbon surface enabled the adsorption of ligands. Fluorosurfactants are analogous to hydrocarbon surfactants in that they contain a polar head and a perfluoroalkyl/aryl tail. The perfluoroalkyl tail consists of a carbon chain with the normal hydrogen atoms replaced with fluorine atoms. The perfluoroalkyl tails can be adsorbed to fluorocarbon surfaces, in effect causing wetting of the surface (Bee *et al.*, 1983).

Hato *et al.* (1986) used fluorosurfactants to 'wet' a fluorocarbon surface to which an enzyme was adsorbed and crosslinked with glutaraldehyde for stability. Kobos *et al.* (1988) reacted the fluorosurfactant (perfluorooctyl) propanoyl imidazolide with urease and then adsorbed the perfluroalkylated enzyme (pf-enzyme) onto the fluorocarbon surface. The disadvantage with this approach is that the surface of the perfluorocarbon support in effect remains hydrophobic and therefore is a potential source of non-specific interactions. By washing the support with a neutral fluorosurfactant it is possible to render the support more hydrophilic (Kobos *et al.*, 1989). Another disadvantage with direct perfluoroalkylated adsorption of ligand onto a fluorocarbon

surface is retention of ligand activity, particularly for proteinaceous ligands, as the ligand is reacting directly with the fluorosurfactant. It is possible to control the degree to which the ligand is inactivated by controlling the ratio of fluorosurfactant to ligand (Kobos *et al.*, 1989). Leakage of ligand is dependent upon the number of perfluoroalkyl chains per ligand molecule. Stewart *et al.* (1989) demonstrated that leaching of ligand was a particular problem with directly adsorbed perfluroalkylated dye ligands.

An alternative method is to coat the perfluorocarbon surface with a neutral hydrophilic layer onto which ligands can be immobilised. Stewart *et al.* (1990) reacted polyvinyl alcohol (PVA) with a fluorosurfactant, perfluorooctanoyl chloride. The resulting perfluoroalkylated-PVA was then adsorbed onto the fluorocarbon support. The exact nature of the interaction between the perfluorocarbon support and perfluoralkylated polymer is unclear. It may be due to strong, essentially irreversible (Kobos *et al.*, 1988), hydrophobic interactions between the support and strongly hydrophobic perfluoroalkyl groups or a more specific (fluorophilic) interaction between fluorine atoms (Kobos *et al.*, 1989). The high avidity of perfluoroalkylated biomolecules for fluorocarbon surfaces means that a variety of types of flurocarbon supports can be used, including solid particles, membranes, films and fibres and liquid perfluorocarbon droplets. This versatility is attractive for a range of applications including affinity separations (Kobos *et al.*, 1989).

The PVA coat confers hydrophilicity to the perfluorocarbon and makes it relatively easy to immobilise a wide range of ligands, via the hydroxyl groups, using standard immobilisation methods (1.3.1). To date most applications have used group specific dyes (Stewart *et al.*, 1990) but there is a great potential for immobilising other ligands such as protein A, metal chelating groups etc. via the derivatised hydroxyl groups of the PVA coat (Pitfield 1992).

Stewart and co-workers (1990) prepared a pefluorocarbon pseudoaffinity dye adsorbent which had a dynamic capacity for albumin of 15.7mg per mL of support which compares favourably with the capacities of other dye adsorbents with the same dye (5-18mg/ml). The limiting factor to increase the capacity of the adsorbent is the

surface area, the perfluorocarbon support used by Stewart *et al.* (1990), Perflex®, has a surface area of only 6-8m^2/g. The low capacity of the adsorbents may well be outweighed by the reduction in non-specific absorption, due to the hydrophilicity of the PVA coat and to the remarkable stability of the adsorbent. Stewart *et al.* (1990) demonstrated that a dye-PVA-perfluorocarbon adsorbent showed leakage of dye under only the most extreme conditions i.e. 24 hour exposure to 6M sodium hydroxide or 5M hydrochloric acid, and under extended tests exposure to conditions such as molar sodium hydroxide, hydrochloric acid, urea over nine months did not have any discernible effect.

One innovative application of perfluorocarbon-PVA chemistry has been the preparation of liquid perfluorocarbon affinity supports (McCreath *et al.*, 1992). Liquid perfluorocarbons are compressible under high pressures and are therefore not suitable for packed bed operation. They are however potentially very useful for expanded bed or fluidised bed operations.

McCreath *et al.* (1992) coated perfluorodecalin, a liquid perfluorocarbon, via flurosurfactant action with a dye ligand -PVA conjugate. The resulting affinity emulsion drops (20μm mean diameter) were crosslinked, in order to increase stability during pumping operations, with glutaraldehyde to form floccules (20-60drops/floccule). The larger size (125μm.) actually makes the supports more suitable for fluidised operations and in fact increased the capacity towards human serum albumin, 0.37mg/mL for discrete droplets compared to 1.81mg/mL for the floccules (McCreath *et al.*, 1993).

The ability to transport liquid perfluorocarbons and their high density has been exploited by workers in Cambridge (McCreath *et al.*, 1993; 1994; Owen *et al.*, 1994) in the development a perfluorocarbon emulsion reactor for continuous affinity separation (PERCAS). The PERCAS system consists of four stages i.e. adsorption, washing, elution and reequilibration. The non-porous nature of the affinity emulsion allows rapid binding kinetics and the density of the affinity floccules enables rapid collection. The stability of the adsorbent and the continuous nature of the separation means that it is possible to treat large volumes of feedstock with a relatively small

amount of adsorbent. McCreath *et al.* (1994) demonstrated that the productivity of the PERCAS system was approximately 2.25 times higher than a expanded bed operation for the purification of glucose-6-phosphate dehydrogenase from homogenised bakers yeast under the same process conditions.

In conclusion the main advantages of perfluorocarbon supports over porous supports are their stability and therefore reusability, in addition their non-porous nature means they are less susceptible to fouling and therefore are potentially useful in purification's from 'dirty' feed streams. The lower capacity, due to smaller surface area, may well be outweighed by the above advantages.

1.3.3.4 Magnetic Supports

Knowledge of the basic magnetic properties of particular materials and their behaviour in a magnetic field is fundamental to understanding and exploiting magnetic separation (1.2.2.6). There are three different categories of magnetism dependent on their response to an externally applied magnetic field (Duffin, 1990).

Most materials such as water, sugars, lipids and many proteins when placed in a magnetic field are relatively unaffected. They are termed diamagnetic or magnetically unresponsive. Paramagnetic materials such as free radicals, oxygen and proteins containing metal ions are attracted towards the region of highest field strength, the strength of the paramagnetic force is comparable to the force of gravity in magnitude (Whitesides *et al.*, 1983). Paramagnetic materials are of limited use for magnetic separations as the strength of the magnetic force is comparatively weak (100-500 times weaker) compared to that experienced by ferromagnetic materials. Ferromagnetic materials include iron, cobalt, nickel and some of their compounds, of particular importance due to its wide availability is magnetite Fe_3O_4.

The magnetic force acting upon the particle as induced by the application of an external field is calculated from the equation given above (Equation 1.7). Equation 1.7 shows that the magnetic nature of the adsorbent whether it is paramagnetic or ferromagnetic i.e. its magnetic susceptibility, the size of the adsorbent, and the strength and gradient of the field will affect the force acting upon the adsorbent. In order for

efficient recovery the force F_X must be sufficiently high to retain and recover the magnetic adsorbent in the separation device (1.2.2.6). Therefore large, strongly ferromagnetic particles are more efficiently recovered than small paramagnetic or diamagnetic particles by a given magnetic field of fixed strength and gradient. Another consideration is the support particle needs to be sufficiently magnetically responsive for efficient collection yet it must lack any magnetic properties during the adsorption step. This is necessary to prevent agglomeration of the support particles which reduces the accessibility of the immobilised ligands and therefore reduces the capacity of the adsorbent. Agglomeration is a potential problem for small non-porous supports in particular as they will tend to behave as porous supports, as a consequence losing much of their advantage over porous supports.

Ferromagnetic materials become magnetic in weak applied fields from 500 to 5000gauss and are therefore termed as having high magnetic susceptibility. After the removal of the external field ferromagnetic materials exhibit residual magnetism, this is called magnetic hysteresis or magnetic memory (Duffin, 1990). Of particular interest is that very small particles <300Å of normally ferromagnetic materials are too small to posses normal magnetic domains but have high magnetic susceptibility and saturation and do not possess magnetic memories (Cullity, 1972). These materials are termed superparamagnetic and the absence of a magnetic memory means when the external field is turned off they lose all magnetic properties making collection and therefore separation easier. The development of useful magnetic separations has been made possible by the use of superparamagnetic supports.

Equation 1.7 states that the volume of the particle influences the force induced by the applied magnetic field. Large supports require lower magnetic field strengths for recovery than smaller supports. There are a number of disadvantages associated with using large supports for batch adsorption namely low surface area for non-porous supports, increased diffusional limitations for porous supports and rapid settling of adsorbents (1.3.3). The use of HGMS (1.2.2.6) allows the rapid and efficient recovery of small magnetically susceptible particles. So such particles are ideally suited for magnetic affinity separations.

Most applications of magnetic adsorbents for purification of proteins use magnetic porous supports such as magongel (Dean *et al.*, 1985). Magnogel consists of agarose crosslinked with epichlorohydrin with magnetite incorporated in the interior of the gel beads. The presence of magnetic particles does not significantly reduce the effective porosity compared to conventional porous beads however the capacity is reduced by as much as 20-50% (Mosbach and Andersson 1977; Ennis and Wisdom 1991). Goetz *et al.* (1991) developed a magnetic pellicular support, an impermeable core of magnetic material coated with a thin porous layer of silica. The support was used in magnetically stabilised fluidised beds as a bioreactor for the conversion of sucrose into component monosacharrides by immobilised invertase. The surface area was as expected lower than that of conventional fully porous adsorbents but the behaviour of the adsorbent was comparable (Halling and Dunnill, 1980).

Non-porous supports can be prepared in a variety of ways. Munro *et al.* (1975) directly adsorbed chymotrypsin and β-galactosidase to the surface of nickel particles and then crosslinked the enzymes with glutaraldehyde to produce a multi-layer enzyme envelope. However the nickel core was found to be unstable in acidic pH and slowly dissolved releasing metal ions which irreversibly inhibited the enzymes. Van Leemputten and Horisberger (1974) immobilised trypsin to magnetite particles (0.3-0.7μm) in the same manner with similar results. Direct adsorption is also likely to be an inefficient method of ligand immobilisation because of steric hindrances imposed by the magnetic core. The reduction of corrosion of the magnetic core is a particular challenge for the synthesis of non-porous magnetic adsorbents.

In an effort to prevent the inactivation of chymotrypsin by nickel ions, Munro *et al.* (1975) coated nickel powder with a thin layer of bovine serum albumin (BSA) before immobilising the enzyme. This slowed down the corrosion of the nickel and the release of nickel ions and protected the immobilised chymotrypsin from loss of activity to a limited extent because of the porosity of the BSA layer. Munro *et al.* (1977) demonstrated the advantages of coating with organic polymers which further reduced the levels of nickel ions being released. Pieters and Bardelletti (1992) used

polyethyleneimine to coat magnetite particles followed by the coupling of adipic hydrazide as a spacer arm for the immobilisation of glucose oxidase and glucoamylase.

An alternative method to immobilise ligands on non-porous supports can be achieved by silanisation. Robinson *et al.* (1973) used aminopropyltriethoxysilane (APTES) to immobilise enzymes directly to magnetite, and Halling and Dunnill (1979) used the same technique to immobilise chymotrypsin to a nickel-nickel oxide support. However the silane bond between silane and metal surface was relatively unstable and the leakage of enzyme from these supports was significant. Halling and Dunnill (1979) greatly improved the stability of the silane layer by coating silanised support with glutaraldehyde to form a polyglutaraldehyde coat. The polyglutaraldehyde coat stabilises the silane layer, by joining together many silane linkages, and the free aldehyde groups allows immobilisation of a wide variety of ligands.

The advantages of porous and to a lesser extent pellicular supports are firstly as they are based upon conventional supports the coupling chemistries are well established and easy to carry out. The size of the beads is not critical for the recovery by magnetic filtration and finally magnetic agglomeration if it occurs is believed to be a minor problem. The convenience of using porous supports because of their widespread use as matrix supports is however outweighed by the advantages of non-porous supports.

The main advantages of non-porous supports are:

- Reduction in mass transfer limitations due to non-porous nature.
- Reduced susceptibility to fouling.
- Reduction in effects of attrition.

The small size and high density of non-porous magnetic supports make external mass transfer better than conventional porous supports, mass transfer rates increase with decreasing particle size (Halling and Dunnill, 1980).

Munro *et al.* (1977) compared the fouling of non-porous and porous supports by stirring them in chilled whole milk for 48h. There was a slight fall in the activity of chymotrypsin immobilised to a non-porous nickel support whilst a cellulose support was seriously affected. Halling and Dunnill (1980) studied the effect of fouling on

ferrite-lactate catalysts and concluded that only a small fall in activity could be attributed to fouling and when fouling did occur it was easily removed by stirring in buffer or detergent. In contrast it is much harder to remove deposited fouling material from the pores of porous supports. Non-porous adsorbents should therefore behave better in feedstreams containing fouling solids, thus allowing the selective recovery of proteins from liquors containing suspended solids and thereby reducing the need for pretreatment unit operations that are normally required before a chromatographic separation. Several examples of this are available in the literature, the purification of L-asparginase and *B*-galactosidase from *E.coli* cell homogenates, and the recovery of alcohol dehydrogenase from crude liver homogenate (Mosbach and Andersson, 1977).

Although no conclusive studies on the effects of attrition have been carried out, many non-porous supports are prepared by quite vigorous mechanical methods such as ball milling of the magnetic particle to produce the correct uniform size (Halling and Dunnill, 1979). Therefore it seems unlikely that the conditions in stirred tank reactors are likely to cause significant attrition. Munro *et al.* (1977) observed some attrition of rock magnetite and ferrite although this was believed to be caused by the fact that the magnetic particles were loose aggregates of smaller particles. In contrast conventional supports are prone to substantial attrition, although this is less important for smaller supports (Regan *et al.*, 1974).

The ideal non-porous support should have superparamagnetic properties. There are two commercially available non-porous magnetic supports, BioMag® (Advanced Magnetics Inc.) and Dynabeads (Dynal). BioMag® is a super paramagnetic iron oxide core coated to provide primary amino groups or carboxyl groups that are activated prior to ligand immobilisation. A wide variety of ligands have been immobilised such as Biotin, albumin, antisera, Protein A etc. (Groman 1985). Dynabeads are uniform porous polystyrene beads into which superparamagnetic particles of magnetite are deposited. The pores are then filled in and coated with polymeric compounds to reduce the surface area from $100m^2/g$ to $5m^2/g$ and provide a surface for immobilisation of the required ligand (Haukanes and Kvam, 1993). The small size of Biomag® (0.5 to 1.5μm) means that it has a much higher surface area than Dynabeads

(2.8 to 4.5μm). Therefore the capacity for the ligand and specific protein is much higher, 200mg/g compared to 2-50mg/g. In a study by Trickett *et al.* (1990) BioMag® particles were concluded to be slightly better than Dynabeads for the immunomagnetic purging of acute lymphoblastic leukaemia cells from bone marrow to be transplanted. Other applications of the beads are reviewed by Vaccaro (1990) and Haukanes and Kvam (1993).

1.4 Aims

There is a large potential to increase the efficiency and the yield of downstream processes, and thereby reduce costs, by introducing an affinity separation at an early stage in the process. Traditional affinity adsorbents and modes of operation require pretreatment of the feedstream to reduce the levels of particulate matter and gross contaminants.

The aim of this project is therefore to examine alternative adsorbents and modes of operation for their ability to replace conventional downstream unit operations.

It should be noted that non-porous supports have great potential for operating in fouling feedstreams as they are less prone to fouling and easier to clean (Munro et al., 1977; Halling and Dunnill, 1979). Pseudoaffinity ligands, such as metal chelates (1.3.2.3), are also suitable for use in such an environment because of their greater stability than biological affinity ligands (1.3.2)

This specific aim of this project is therefore to design and test non-porous pseudoaffinity supports for the recovery of monoclonal antibodies from cell culture.

Two distinct types of support will be examined. The first is a chelating perfluorocarbon support. The design of this support and its characterisation are detailed in Chapter 2. The second type of support is termed a chelating magnetic support or magnetic chelator (Chapter 3). The application of these non-porous chelating adsorbents for the recovery of monoclonal antibodies is discussed in the final chapter (Chapter 4)

2. CHELATING PERFLUOROCARBONS

2.1 Introduction

Solid perfluorocarbon polymers such as polytetrafluoroethylene (PTFE) are characterised by their high density and physical strength, and being incompressible this allows the use of high throughputs and pressures. In addition perfluorocarbons show marked chemical and biological inertness and complete insolubility in aqueous and organic solvents, allowing extreme conditions for cleaning and sterilisation to be employed. Until recently the chemical inertness and hydrophobicity of powdered perfluorocarbon aggregates has prevented their use in affinity chromatography, where hydrophilicity and ease of derivatisation are two essential requirements for an affinity matrix (Groman and Wilchek, 1987). However, developments in a number of laboratories (Stewart *et al.*, 1989; 1990; Kobos *et al.*, 1989) have shown that the surface of perfluorocarbons can be modified in order to present these features and thus make perfluorocarbons an ideal material for use as a base matrix.

This chapter details the preparation and characterisation of chelating perfluorocarbon supports. Perflurocarbon base matrices were coated with polyvinyl alcohol and subsequently activated and coupled to a chelating ligand (IDA). The optimum methods for the preparation of these supports were determined, and the binding characteristics of a metal binding monoclonal antibody (MAb1) investigated. Finally applications for the use of such pseudoaffinity adsorbents are discussed.

2.2 Experimental

2.2.1 Materials

The monoclonal antibody MAb1 was received from the Wellcome Foundation Ltd. (Beckenham, Kent, UK).

Particulate perfluoropolymer base materials were obtained from three sources. MP1500 manufactured by E.I. Du Pont was kindly donated by K. & K. Greef Ltd. (London, UK). Polytetrafluoroethylene (PTFE wax) powder of low molecular weight was obtained from Goodfellow Cambridge Ltd. (Cambridge, UK) and poly(tetrafluoroethylene propylene) (PTFEP) chromatographic grade, a co-polymer of tetrafluoroethylene and propylene, was purchased from Polysciences Inc. (Northampton, UK).

Iminodiacetic acid (IDA) and 1,4 Butane dioldiglycidyl ether were procured from Sigma (Poole, UK). Polyvinyl alcohols (all 80-89% hydrolysed) of molecular weights - 9-10K, 13-23K, 31-50K and 85-146K - were obtained from Aldrich (Dorset, UK) while polyvinyl alcohol with an average molecular weight of 77K (88% hydroysed) was obtained from ICN Biochemicals (Oxfordshire, UK). Perfluoroctanoyl chloride was purchased from Fluorochem (Old Glossop, UK)

Chelating Fast Flow Sepharose™ was purchased from Pharmacia Bioprocess Technology AB (Uppsala, Sweden) whilst chelating Streamline™ was a gift from the same company.

The HPLC assay utilised a Beckman HPLC system (Beckman Instruments, High Wycombe, UK) with a HiPAC Protein A column (ChromatoChem Inc. Montana USA), kindly donated by Wellcome Research Laboratories (Beckenham, Kent, UK).

Chemicals used in the preparation of HPLC buffers were purchased as HPLC grade where possible. All other chemicals employed were of AnalaR grade and obtained from Sigma (Poole, UK) and BDH Chemicals (Poole, UK).

2.2.2 Preparation of metal chelating perfluorocarbon supports

2.2.2.1 Coating of perfluorocarbon matrices

The procedure used to coat the perfluoropolymers with a neutral, hydrophilic coat of polyvinyl alcohol (PVA) was adapted from methods used by Stewart *et al.* (1990) to coat and derivatise perfluorocarbon supports with biomimetic dye ligands.

Aqueous solutions of polyvinyl alcohols (10-20mg/mL of various molecular weights) were prepared by dissolving in water at 50°C. Perfluorooctanoyl chloride (160μL, 15mmoles) was then added to 40mL of PVA solution and mixed on a tumbling shaker for 20min. Subsequently 10mL aliquots of the PVA/perfluorooctanoyl chloride mixture were added to approximately 2g portions of the perfluoropolymers. The reaction mixtures were tumbled overnight at room temperature on a rotary shaker (ReaX2, Heidolph, Germany) and the PVA-coated supports were recovered by vacuum-filtration on glass fibre paper (GF/A Whatman). The PVA-modified supports were washed sequentially in the following solutions- 5 times with 50mL distilled water; twice with 50mL of 50% (v/v) acetone; once with 50mL 100% (v/v) acetone; once with 50mL 50% (v/v) acetone; once with 50 mL (30% v/v) acetone-water; and finally 5 times with 50mL distilled water. Care was taken not to expose the support to the air in order to prevent drying out.

2.2.2.2 Epoxide activation of polyvinyl alcohol coated perfluorocarbon matrices

The routes and methods employed in functionalisation of PVA coated supports were adapted from procedures developed by Sundberg and Porath (1974). PVA-coated supports were recovered either by low speed centrifugation (500rpm 3min) in a Beckman GS6 benchtop centrifuge (Beckman Instruments, High Wycombe, UK) or vacuum-filtration, and ~1g quantities of moist support placed in 50mL tubes. Aliquots (10mL) of solution containing 0.1-0.6M NaOH; 10-60% (v/v) 1,4 Butane dioldiglycidyl ether; and 1mg/mL of sodium borohydride were added to the moist support and the reaction mixtures contacted for various times (7, 15, 24h) on a rotary shaker at room temperature. The epoxy-activated perfluorocarbon supports were

recovered by vacuum-filtration on GF/A filters and washed 2-3 times with 50mL volumes of distilled water before immediate coupling with IDA

2.2.2.3 Coupling of iminodiacetic acid

Approximately 1g (moist weight) portions of the epoxy activated supports were resuspended in 2mL of distilled water in a 25mL conical flask, to which was added 2mL of a solution containing of 1-30% (w/v) IDA in 2M Na_2CO_3 and 140μg/mL $NaBH_4$. Coupling was allowed to proceed at 60°C for 15h in a shaking water bath (Clifton Digital Shaker bath, Nickel Electro Ltd. Weston-SuperMare, England).

The IDA-coupled perfluorocarbon supports were washed by filtration under vacuum with 5 times 100mL volumes of distilled water and excess unreacted epoxide groups blocked by resuspension in 1M ethanolamine at 4°C for 2-3 days.

2.2.2.4 Charging of chelating supports

Chelating supports were charged with metal ions just before use. 'Blocked' supports were washed from any contaminating solution with water on a vacuum filtration rig with GF/A Whatman filters, 3-4 times with 50mL volumes of water. A fixed volume (50mL) of 50mM $ZnCl_2$ or other metal salt solution was added to the support and left to drain slowly under gravity with intermittent stirring (15min). The charged supports were then washed 5 times with 100mL volumes of water and finally resuspended in phosphate buffered saline (PBS) pH7.2 ready for use. Controls or 'uncharged' supports were prepared by washing in 50mL of 0.1M EDTA in order to ensure no metal ions were bound, and subsequently washed with 5 times 100mL water before being resuspended in PBS.

2.2.3 Characterisation of chelating perfluorocarbon supports

2.2.3.1 Pseudoaffinity adsorption studies of a monoclonal antibody by metal chelating adsorbents.

A range of concentrations of the antibody (0.5-6.0mg/mL) were made up in pre weighed 1.5mL eppendorfs in 1mL of PBS pH 7.2. An approximately 1:1 slurry (w/w) of filter dried Zinc charged support with PBS pH 7.2 was prepared. A volume (200μL) of the 1:1 slurry were added to each of the eppendorfs. The support/protein mixtures were routinely incubated in sealed eppendorfs on a vibrax shaker (1000rpm) at 4°C for 24-36h to allow equilibrium to occur between the adsorbent and liquid phase (Chase and Draeger, 1992). At the end of the incubation the supports were separated by pulse centrifugation and the supernatants analysed for residual antibody content. The amount of bound antibody was calculated by difference and adsorption isotherms plotted.

2.2.3.2 Determination of stability of chelating perfluorocarbon supports

Portions of charged and washed chelating perfluorocarbon supports (250mg moist weight) were placed into preweighed eppendorfs to which was added 1mL of 0.1M EDTA. The supports were mixed on a vibrating shaker at 1000rpm for approximately 10 minutes before being recovered by centrifugation. The supernatant was removed and analysed for metal content in order to determine the ligand density (2.2.4.2).

The support was washed several times in water and then contacted with 1mL aliquots of various solutions (1M NaOH, 1M HCl, acetone etc.) and left at room temperature for one month. After this time the supports were recovered by centrifugation washed several times in water before being charged with 1mL of 50mM metal salt solution. The newly charged supports were washed in 1mL aliquots of water and the metal ions stripped from the support by resuspension in 1mL of 0.1M EDTA, which was analysed for metal content (2.2.3). The loss of IDA functions can be inferred from the decrease in immobilised ligand density after exposure to particular solutions.

2.2.4 Analytical methods

2.2.4.1 Determination of the degree of adsorption of polyvinyl alcohol

Polyvinyl alcohol (PVA) in free solution was measured spectrophotometrically at 670nm using an Iodine reagent based method (Garvey *et al.*, 1974). A PVA solution (1mg/mL) was made up in water. Appropriate dilution's were made and 0.5mL aliquots of Garvey's reagent were added to 1mL volumes of PVA solutions. The resulting mixture was mixed gently and left for twenty minutes before the absorbance was read at 670nm. The presence of PVA is indicated by the formation of a green-blue colour. A standard curve was thus generated from which the concentration of PVA could be extrapolated within the range 0.1-1.0 absorbance units.

Garvey's reagent

- 0.45% w/v Potassium iodide
- 0.225% w/v Iodine
- 3.6% w/v Boric acid

In water

The degree of adsorption of polyvinyl alcohol onto perfluoropolymer particles from solution was determined by measuring the concentration of PVA in free solution before and after contacting with perfluorocarbon. The amount of bound or adsorbed PVA was then calculated by difference and expressed as mg PVA/g support.

2.2.4.2 Immobilised Ligand concentration

The density of immobilised IDA was determined by inference from the measurements of immobilised metal ions (Me^{2+}) concentrations since it has been shown by Hochuli (1988) and others that there is a 1:1 stoichiometry for the binding of Me^{2+} ions to immobilised IDA when Me^{2+} has been supplied in excess concentrations.

Approximately 250mg (moist weight) portions of charged support were placed into pre-weighed eppendorfs into which 1mL of distilled water was pipetted. The tube contents were shaken on a vibrating shaker (IKA VXR, Staufen, Germany) (1000rpm,

5min), and supports recovered by low speed centrifugation in a Beckman 12 microcentrifuge (Beckman Instruments, High Wycombe, UK) (1000rpm, 30s). The supernatants were removed and retained for analysis by atomic absorption spectrophotometry to establish effectiveness of wash step. The supports were then resuspended in 1mL of 0.1M EDTA, 1M NaCl and mixed on a vibrating shaker (1000rpm) for approximately 20 minutes before recovering supports by centrifugation and analysing the metal content of the supernatants by atomic absorption spectrophotometry. Zinc was determined at 213.9nm and Nickel at 232.4nm, using single element lamps operated at 10mA currents with an air-acetylene flame in a Perkin-Elmer AA 3100 Atomic Absorption instrument (Perkin Elmer, Seer Green, UK.). The amount of support used in tests were determined at the end of an experiment by drying samples in a Savant Speedvac vacuum desiccator (International Equipment Co., Luton, UK). Ligand densities were then expressed as μmoles metal ion per g of dried support.

2.2.4.3 Determination of protein concentration

Protein concentration was determined using Coomassie® Plus Protein assay reagent (Pierce and Warriner Ltd. Cheshire, UK.). The basis for the assay being a shift in absorbance from 465nm to 595nm, when the active ingredient Coomassie® Blue G-250, binds to proteins.

The protocol followed was that supplied with the reagent for use with microtiter plates (Redinbaugh and Campbell, 1985). Calibration curves were prepared from stock solutions of Bovine serum albumin (2mg/mL). Duplicates were prepared for each sample and the absorbance read at 595nm on a Dynatech MR7000 plate reader (Billingshurst, W.Sussex, UK).

2.2.4.4 Determination of antibody concentration

In experiments where the only protein added was the monoclonal antibody, the concentration was routinely determined by measuring the optical density at 280nm in quartz cuvettes. A 1mg/mL solution of pure MAb1 has an absorbance of 1.32 at

280nm, from which a standard curve can be plotted (A.J. Sheppard, pers. communication).

Where the antibody was present within a background of other proteins and substances likely to interfere with the spectophotometric analysis a HPLC based assay was used. A HiPAC® Super Protein A silica 0.83mL column (ChromatoChem Inc. Montana USA) was used in conjunction with a Beckman HPLC system (Beckman Instruments, High Wycombe, UK) comprising of a 166 detector, 507 autosampler and 126 solvent delivery module controlled by Beckman Gold® software.

The standard run involved equilibration and absorption in 0.2M Citrate buffer pH6.0 followed by sequential washes in 0.2M Citrate 0.15M NaCl buffer pH5.4 and water. Elution was achieved with 2% acetic acid 0.15M NaCl, the eluant being monitored at 280nm. The column was calibrated by applying known concentrations of the monoclonal antibody MAb1. The Beckman Gold® software provided calibration data and subsequently extrapolated this to unknown samples.

Table 2.1 Properties of particulate perfluorocarbon materials

Information	Perflex®[a]	MP1500	PTFEP	PTFE wax
Manufacturer	E.I. Du Pont	E.I. Du Pont	Polyscience	Goodfellows
Material	Polytetrafluoro-ethylene	Polytetrafluoro-ethylene	Poly (tetrafluoro ethylene propylene) copolymer	Polytetrafluoro-ethylene (low MW)
Particle size range (μm)	na	10-35	na	6-9
Mean Particle Size (μm)	35	20	10	7.5
Specific surface area (m^2/g)	6-8	11	na	5
Density (g/cm^3)	1.8-2.1	2.2	2.12-2.18	2.2
Settling velocity (cm/sec)[b]	0.08	0.026	0.0065	0.0042

[a] Material used by Stewart *et al.*, (1989)
[b] Values calculated using Stoke's equation (Equation 1.5)
na = Manufacturers data not available

2.3 Results

2.3.1 Selection of base material

In previous work with solid perfluorocarbons at the University of Cambridge (Stewart *et al.*, 1988; 1989) a sintered particulate polytetrafluoroethylene of mean particle size 35μm and a surface area of 6-8m^2/g was used. Unfortunately this material, tradename Perflex®, is no longer being manufactured by E.I. Du Pont and so alternatives had to be found.

Three different perfluorocarbon base particles representing a range of sizes and physical properties (Table 2.1) were selected as starting materials for the preparation of metal chelating affinity supports. All the perfluoropolymers have similar densities of approximately 2.2g/cm^3 and therefore size is the main difference, an especially important factor in determining settling velocity.

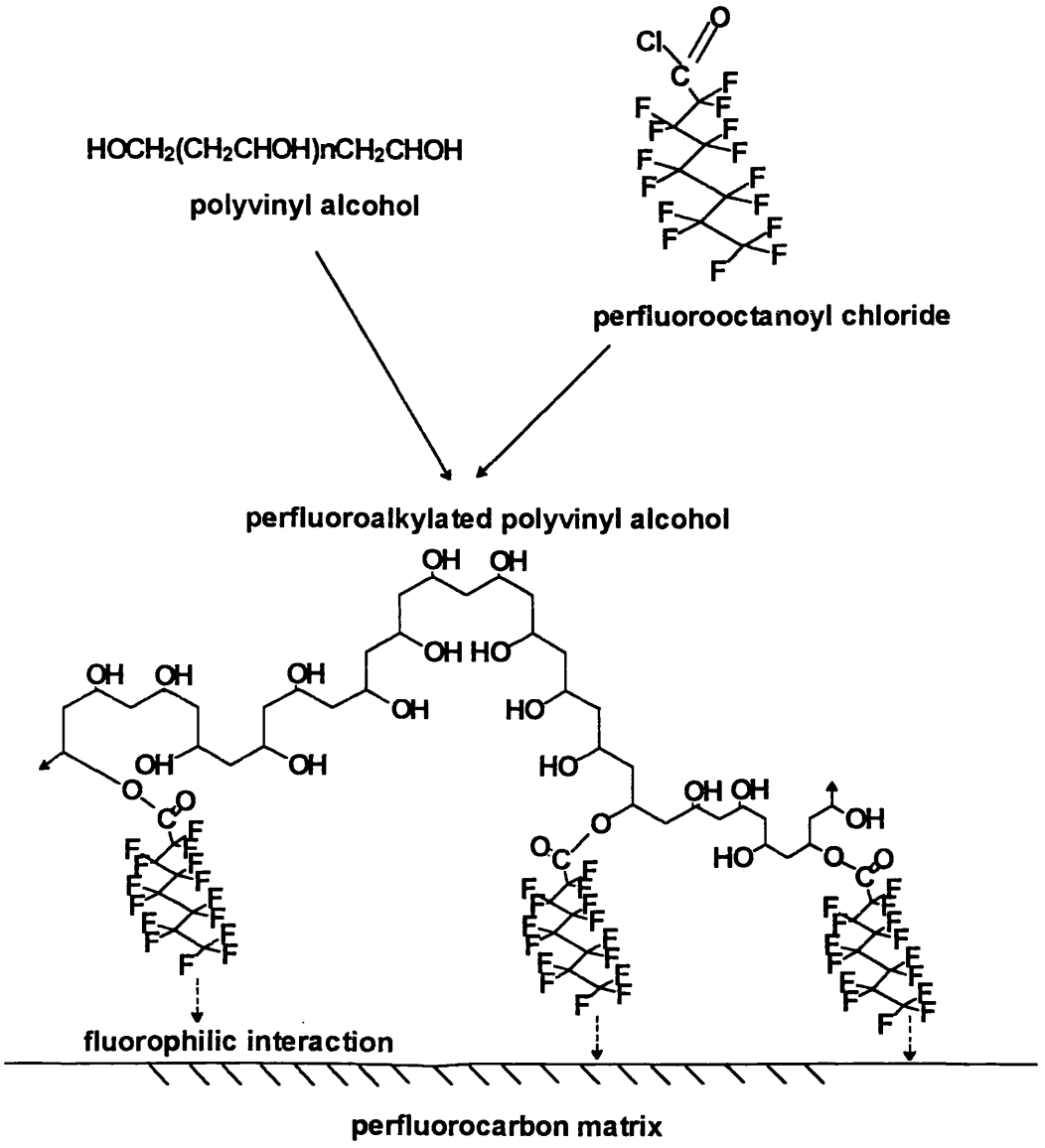
Preliminary tests identified that the 20μm MP1500 particle supplied by DuPont was the easiest to handle and most closely resembled the discontinued Perflex®. As the base materials are very similar in terms of their chemical and physical nature this particle was chosen for the preparation of prototype metal chelating perfluorocarbon supports.

2.3.2 Preparation route

Figure 2.1 illustrates the method employed to coat the hydrophobic perfluorocarbon base matrix with a hydrophilic polyvinyl alcohol (PVA) layer. PVA in aqueous solution is esterified with perfluoroctanoyl chloride, and is securely adsorbed on the perfluorocarbon support by multiple 'fluorophilic' interactions (Kobos *et al.*, 1989; Stewart *et al.*, 1990).

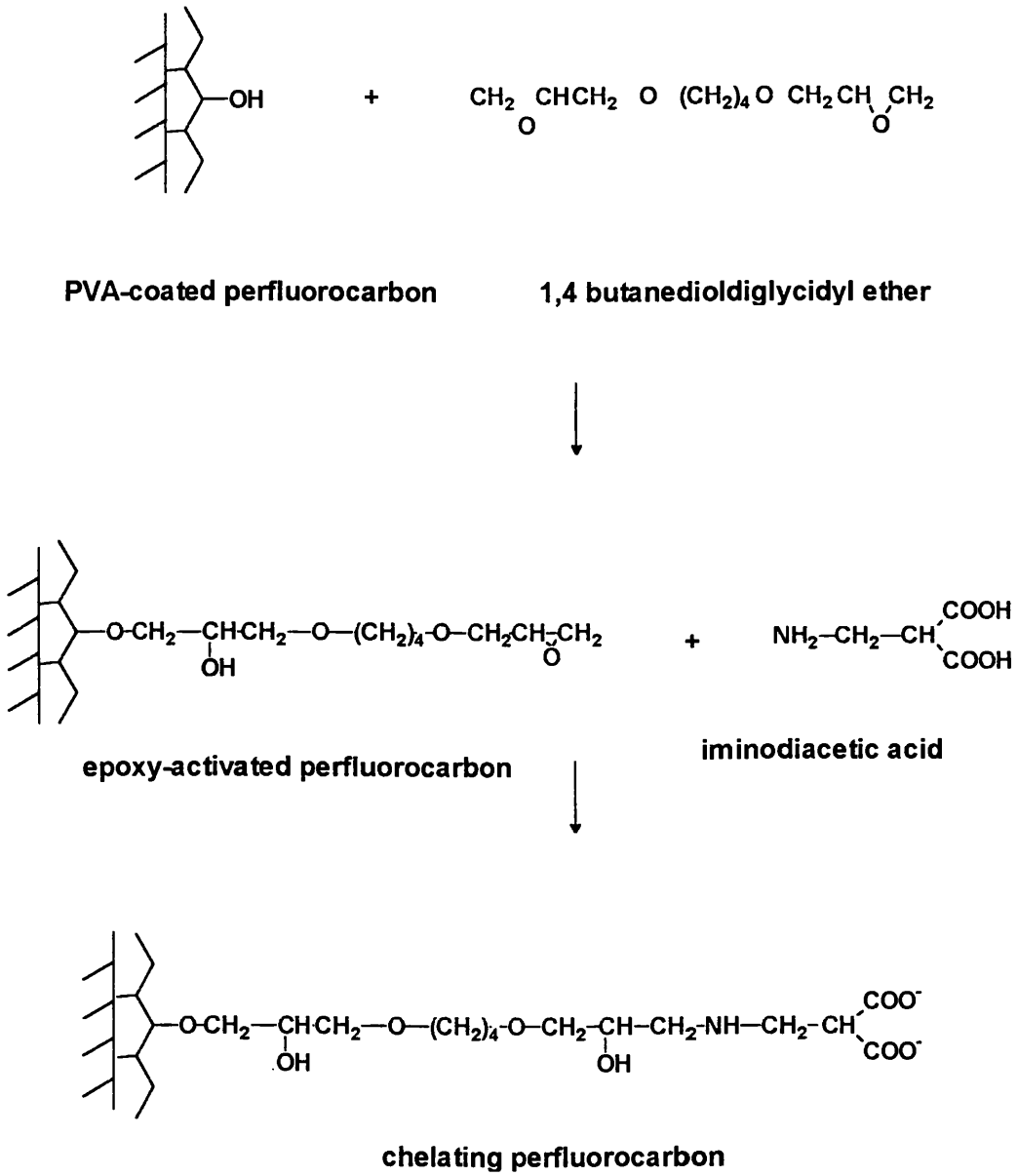
The strategy employed for the substitution of iminodiacetic acid (IDA) functions onto a PVA-coated perfluorocarbon particle is shown in Figure 2.2. The reaction route involves activating the PVA coat around the perfluorocarbon particle with an epoxide spacer and coupling the IDA to the adsorbed epoxy activated surface.

Figure 2.1 Scheme for the preparation of polyvinyl alcohol coated perfluorocarbons



Adapted from Stewart et al. (1990)

Figure 2.2 Scheme for the preparation of chelating perfluorocarbon adsorbents



2.3.3 Optimisation of ligand density

Given that clear relationships exist between the surface hydroxyl density, intermediate epoxide density, ligand density, and protein binding capacity (Sundberg and Porath, 1974; O'Brien *et al.*, 1996), a series of optimisation experiments was undertaken.

2.3.3.1 Adsorption of polyvinyl alcohol to perfluorocarbons

Early studies demonstrated that the mode of mixing, the size and shape of container, and the ratio of liquid/solid phases were crucial to the success of the PVA coating step. Complete coverage of perfluorocarbon surfaces and secure anchorage of the PVA coat are essential for an effective bioaffinity adsorbent. Exposed areas of the strongly hydrophobic base matrix nucleate particle aggregation, cause loss of particles adhering to surfaces of containers and also give rise to unacceptable levels of non-specific binding. The best results were obtained by vigorously tumbling mixtures containing at least 2mL (usually 5mL) of perfluoroalkylated PVA solution (15-20mg/mL) per g of particulate perfluorocarbon, in containers of ~2.5 times the volume of the liquid phase. These conditions consistently yielded stable modified perfluorocarbon supports, homogeneously coated with perfluoroalkylated-PVA. The PVA-coated support particles are easily distinguished from uncoated or partially coated preparations. The starting materials are opaque white non-wettable powders. The PVA-coated particles, on the other hand, are wettable and appear waxy and translucent. Furthermore effective coating i.e. the presence of PVA on the modified surface of perfluorocarbon particles could be demonstrated by staining with iodine reagent as described earlier (2.2.4.1). Coated-particles are stained various colours depending on the levels of adsorbed PVA, ranging from brown through green to blue (at the highest levels of adsorbed PVA). Uncoated perfluorocarbons failed to take up any stain and remained white.

2.3.3.2 Effect of free PVA concentration on the surface coverage of MP1500 perfluorocarbon support with PVA (9-10K).

Portions (2g) of MP1500 perfluoropolymer were mixed with 10mL aliquots of various concentrations of perfluoroalkylated-polyvinyl alcohol solution and the reaction mixtures were contacted overnight at room temperature. The supports were subsequently recovered and the supernatants assayed for residual PVA using the iodometric assay described in analysis section. Surface coverage was expressed as mg PVA adsorbed per g of perfluoropolymer.

Figure 2.3a shows the degree of adsorption of perfluoroalkylated polyvinyl alcohol (9-10K) to MP1500 perfluorocarbon support over a range of input concentrations (0-20mg/mL). Maximal surface coverage (30-36mg PVA per g of support) could be achieved using PVA concentrations between 10-20mg/mL. More concentrated PVA solutions than this are difficult to prepare because of the low solubility of the polymer. Figure 2.3b is an adsorption isotherm for the adsorption of 9-10K perfluoroalkylated PVA to MP1500, capacity (mg/g) versus C* the equilibrium concentration. A theoretical maximum capacity of 49.5mg PVA per g of MP1500 can be calculated when extrapolating to infinite PVA concentration. Figure 2.4 shows the relationship between ligand density of metal chelating perfluoropolymers and the percentage of support covered by PVA. No further increase in ligand density was observed above ~80% coverage relating to ~30mg PVA per g support using the derivatisation and coupling conditions employed (30% epoxide; 15% IDA). The calculated number of Zn^{2+}-IDA ligands bound per molecule of PVA is 48 for MP1500 with a PVA surface coverage of ~15%. Increasing the packing density of PVA reduces the number of sites accessible for derivatisation through steric hindrance, and at maximum coverage ~15 Zn^{2+}-IDA ligands are attached to each PVA molecule.

Figure 2.3a Adsorption of 9-10K PVA to MP1500

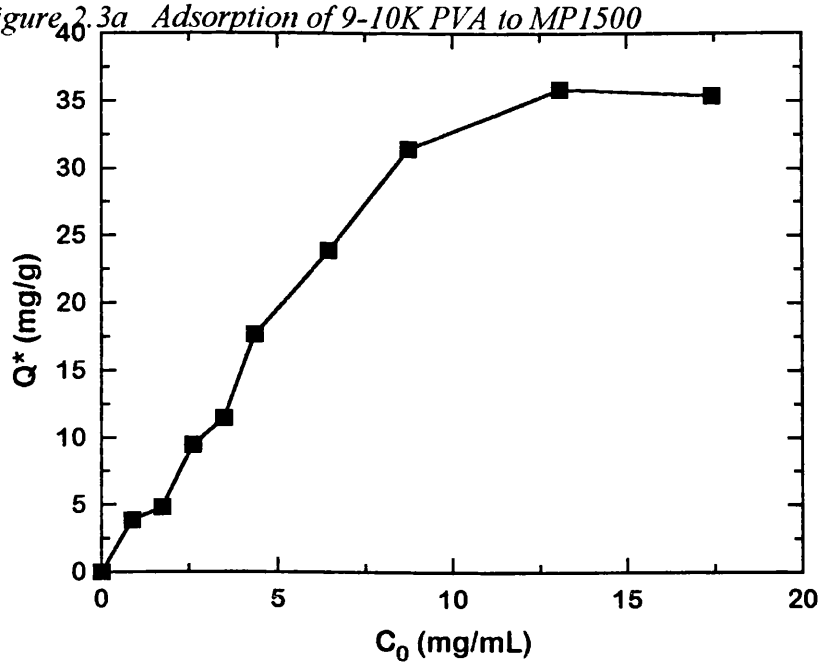
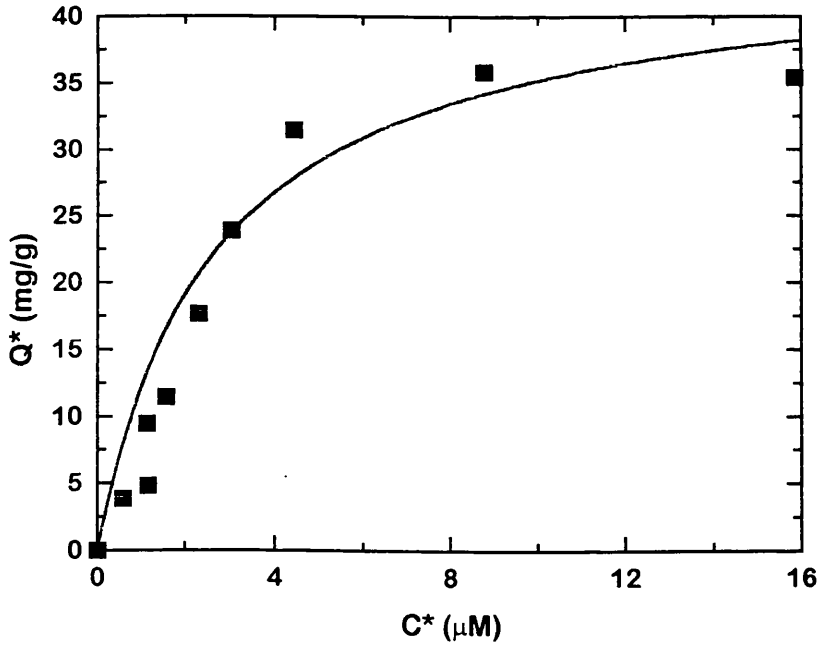


Figure 2.3b Isotherm for adsorption of 9-10K PVA to MP1500



MP1500 material was mixed with various concentrations of 9-10K perfluoroalkylated-PVA for 15h at room temperature. Uptake (Q) of PVA was calculated as the difference between initial and equilibrium concentrations per g support.*

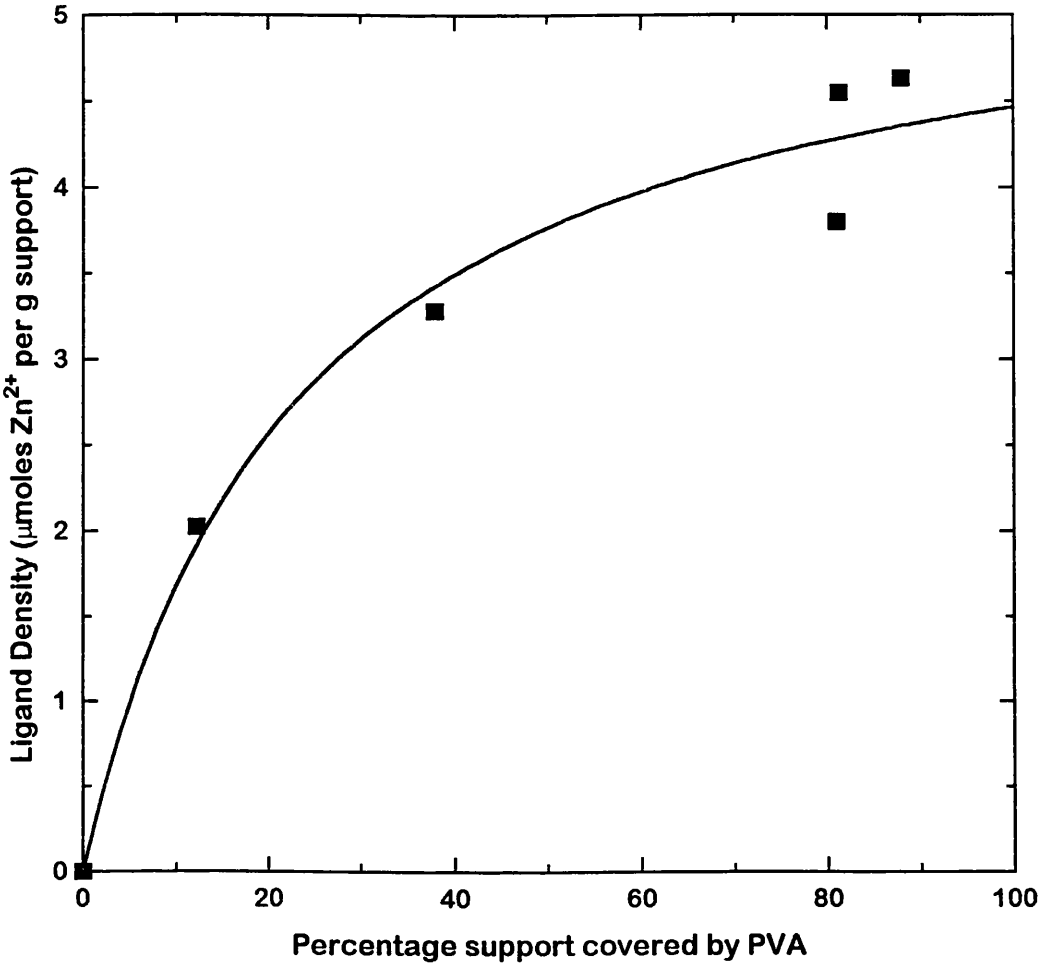


Figure 2.4 Relationship between immobilised ligand density (μmoles/g) and percentage coverage of PVA on MP1500

MP1500 was PVA coated at various concentrations of 9-10K PVA and subsequently epoxide activated and coupled with IDA at optimum conditions.

2.3.3.3 Effect of molecular weight of polyvinyl alcohol upon PVA adsorption to perfluorocarbon base materials

Garvey *et al.* (1974) investigated the effect of increasing the chain length (molecular weight) of PVA's on the adsorption to polystyrene particles. Increasing the chain length was accompanied by increases in both the levels of adsorbed PVA and the tightness of binding. The same trend was observed when perfluoroalklyated PVA's were adsorbed onto MP1500 perfluorocarbon particles. Maximum levels of adsorbed PVA on the MP1500 particles were again reached at input concentrations of 15-20mg/mL. Increasing the chain length of PVA resulted in higher levels of adsorbed PVA (36.5mg PVA 9-10K /g c.f. 64mg PVA 77K /g). This effect was also observed for the other perfluorocarbon base materials as summarised in Table 2.2.

Table 2.2 Amounts of PVA adsorbed onto particulate perfluorocarbons

Particulate perfluorocarbon	Adsorbed PVA (mg/g)			
	9-10 K	13-23 K	31-50 K	77 K
MP1500	36.5	38.2	43.0	64
PTFEP	39.5	40.9	45.8	nd
PTFE wax	nd	18.4	nd	nd

nd = not determined

2.3.3.4 Effect of molecular weight of polyvinyl alcohol upon ligand density of chelating perfluorocarbon supports.

MP1500 PVA-coated supports coated were activated and coupled with IDA. Figure 2.5 shows how the ligand densities vary with the change in mean molecular weight of the PVA polymer coat. Maximum ligand densities were obtained on supports coated with the smallest molecular weight PVA (9-10K) even though these supports possessed the lowest amounts of surface bound PVA (Table 2.2). All other PVA-coated supports had ligand densities in the range 3.6-3.9μmoles/g. The higher densities achieved with the 9-10K PVA may be attributed to a higher proportion of primary hydroxyl moeities per unit chain length. However it is also likely that the packing of the PVA molecules upon the surface of the perfluorocarbon particles has an important effect upon the final ligand density. If perfluoroalkylated-PVA molecules are densely packed then the number of sites accessible for derivatisation by the epoxide activation agent are reduced due to steric hindrances. Furthermore steric hindrance is likely to be worse on supports coated with long polymer chains. The effectiveness of the derivatisation step has obvious implications upon the final ligand density. Measurements of ligand densities on partially coated and fully coated particles indicates that steric hindrance due to close packing is indeed evident (Figure 2.6). As successive polymer chains are packed next to each other on the perfluorocarbon surface the density of ligand per mg of PVA coat falls. This clearly has important implications on the resulting ligand densities and may also influence the stability of the support. Preliminary observations suggest that supports coated with higher molecular weight PVA' s are more stable. They appear more uniform in size and physical characteristics and less 'fines' are observed.

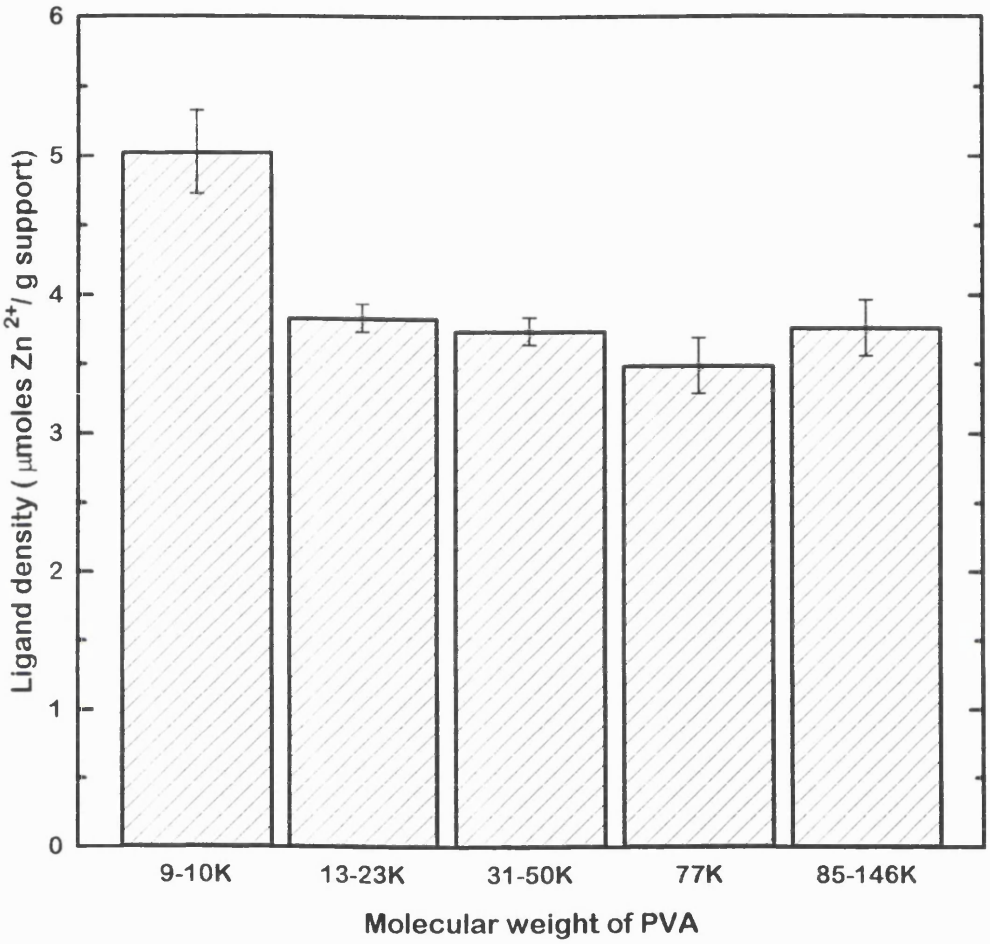


Figure 2.5 Effect of molecular weight of adsorbed PVA on the ligand density of chelating MP1500 particles.

Adsorption of perfluoroalkylated PVA's was performed at saturating conditions (15-20mg/mL). Supports were activated with 30% 1,4-butane diol diglycidyl ether for 15h and coupled with 15% IDA. The error bars represent a standard deviation of 3-6% (n=4).

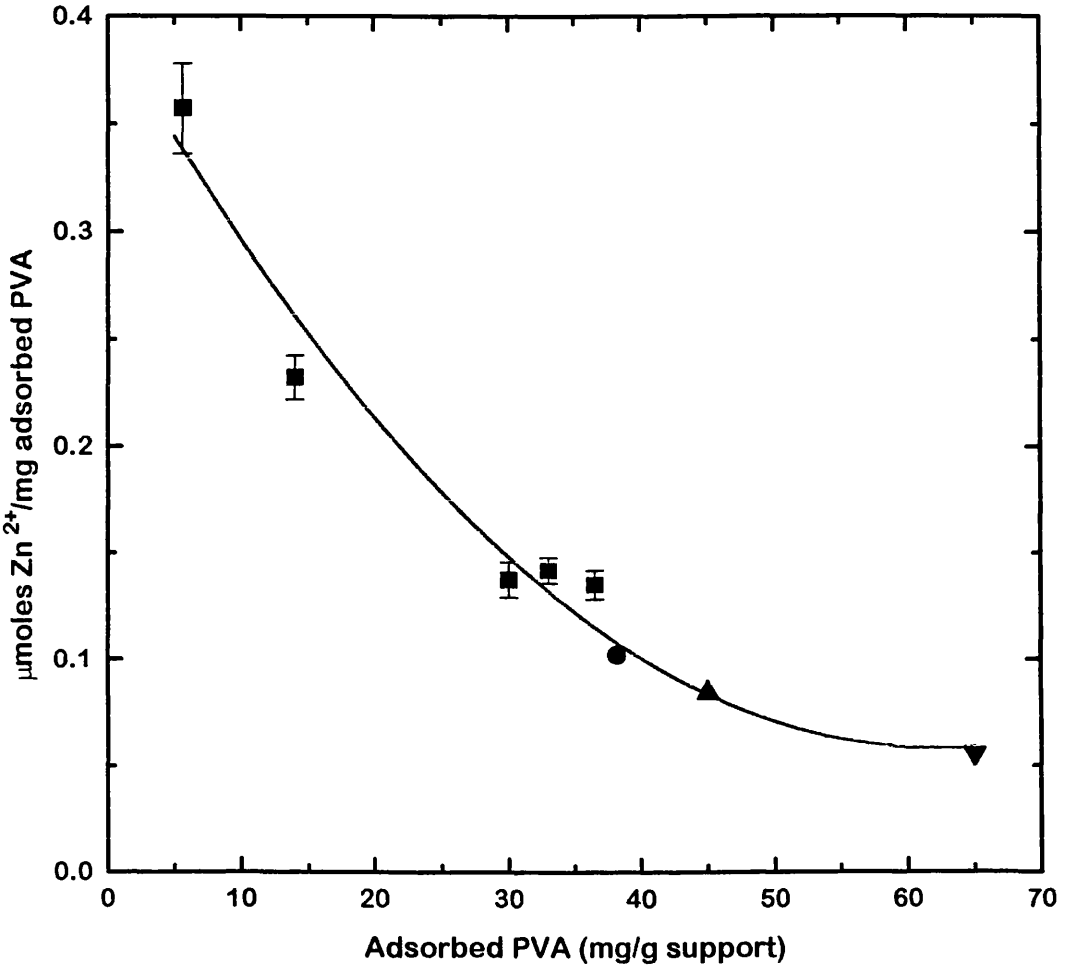


Figure 2.6 Relationship between amount of adsorbed PVA and its accessibility for derivatisation with IDA.

Supports with known amounts of adsorbed PVA (■ 9-10K; ● 13-23K; ▲ 31-50K; ▼ 77K) were activated and coupled with IDA under optimal conditions. The ligand densities were determined as described in Analysis.

2.3.3.5 Effects of 1-4 butanedioldiglycidyl ether concentration upon ligand density of metal chelating perfluorocarbon supports

1,4 butanedioldiglycidyl ether, a common epoxide activation agent reacts with hydroxyl groups upon a support, in this case the hydroxyl groups of the PVA coat, to give a spacer arm to which ligands can be coupled under alkaline conditions (Sundberg and Porath, 1974). PVA-coated MP1500 was directly epoxide activated under reducing alkaline conditions using a range of 1,4 butane dioldiglycidyl ether concentrations for 7h. The epoxy-activated supports were then coupled using 15% IDA and excess epoxide groups blocked with ethanolamine before charging the surface chelate groups with Zn^{2+} ions. The ligand densities, μmoles Zn^{2+} per g of support, were then measured as described above (2.2.4.2).

Figure 2.7 shows the effect of 1,4-butane dioldiglycidyl ether concentration on the ultimate ligand density achieved after coupling to surface oxirane groups. Maximum ligand densities (5μmoles Zn^{2+}/g) were obtained with 30% 1,4-butane dioldiglycidyl ether. Further discussion of the relationship between epoxide concentration and ligand density follows (2.3.3.6).

Strong evidence for the presence of surface attached IDA was demonstrated by washing Zn^{2+} charged supports with 0.1M acetate buffer 1M NaCl pH 3.8. The ligand densities dropped markedly as a result of the breaking up of the Zn^{2+} IDA complex, which is unstable below a pH of ~4-4.5, liberating Zn^{2+} ions into the bulk phase. Essentially complete desorption of the immobilised Zn^{2+} ions from charged supports occured at pH 3.8 and also in the presence of competition from agents such as imidazole and EDTA. In contrast washing Zn^{2+} charged supports with solutions of near neutral pH had little or on effect upon the immobilised zinc density.

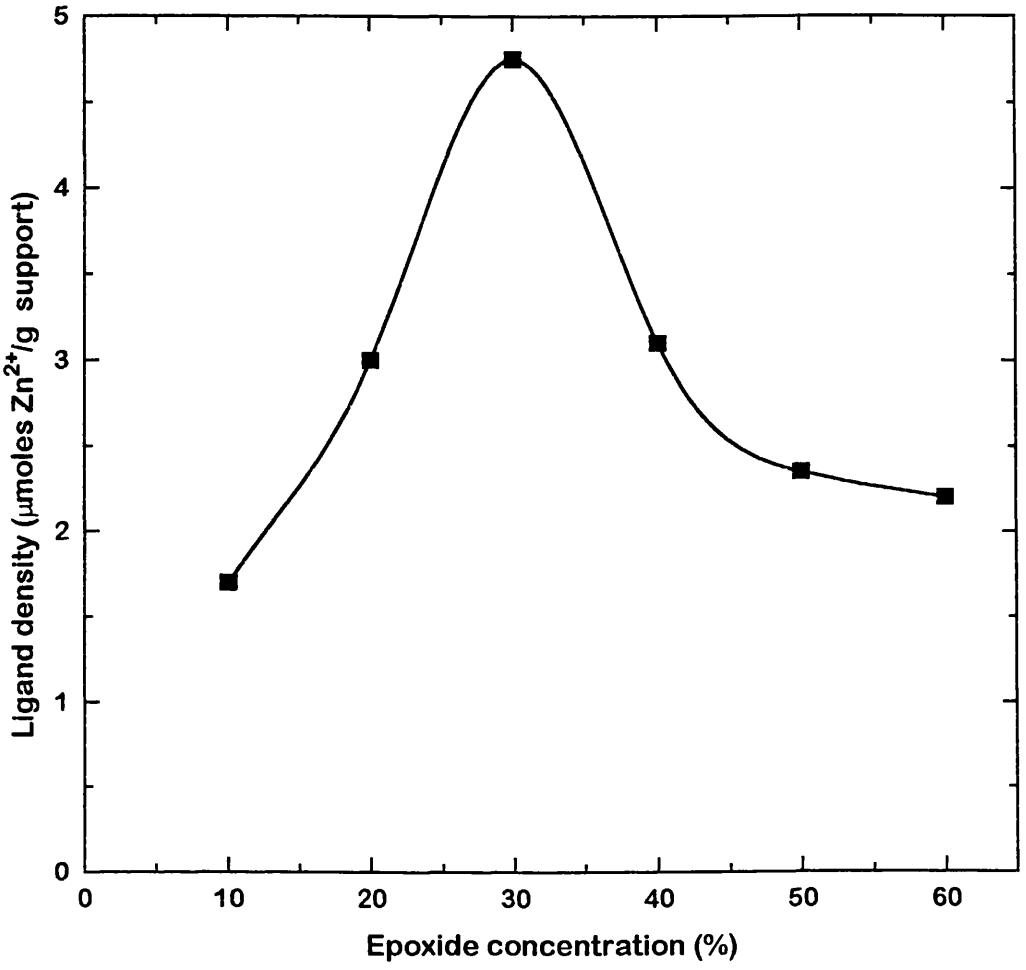


Figure 2.7 The effect of epoxide concentration (% 1,4-butane diol diglycidyl ether) upon the ligand density of chelating MP1500 particles.

Supports were epoxide activated at various epoxide concentrations (10-60%) for 7h before coupling with 15% IDA.

2.3.3.6 Effects of 1,4-butane dioldiglycidyl ether concentration and epoxidation time upon ligand density of metal chelating-perfluorocarbon supports

The concerted effects of time of the activation and concentration of 1,4-butane dioldiglycidyl ether are illustrated in Figure 2.8 which shows profiles of ligand density against time of activation, over a range of epoxide concentrations (10-60% v/v). At the lowest epoxide concentration (10%) only low ligand densities, 2μmoles of Zn^{2+}/g of support, could be achieved. Doubling the concentration to 20% had a marked effect upon ligand density which reached 3μmoles Zn^{2+}/g after 7h, and continued to rise over the 24h period to attain maximal levels of 5μmoles Zn^{2+}/g. The same ligand density could be achieved in considerably less time (15h) by raising the epoxide concentration yet further to 30%. However at this epoxide concentration extending the time of activation to 24h resulted in a significant reduction in ligand density.

The decline of ligand density is also observed with higher concentrations (40-60%) of epoxide at 15h. This decline reflects the onset of cross-linking of neighbouring bi-functional epoxide groups, which effectively reduces the number of sites available for coupling to IDA. The bell shaped curve of Figure 2.7 evidently reflects the increased contribution of the gelation side reaction with increasing epoxide concentration resulting in lower measured ligand densities. Cross-linking is a very serious problem at the highest concentrations and longest activation times employed where the effects are clearly visible to the naked eye. Inter-particle cross-linking is so extensive that the support becomes a globular mass effectively useless as an affinity support. At 7h no visible signs of 'gelation' are apparent over the entire range of concentrations investigated (10-60%). After 15h 'gelation' is evident only at the highest concentration (50% and 60%) but at 24h gelation is observed with epoxide concentrations from 30-60%.

Maximum levels of immobilised ligand (~5μmoles/g) were therefore achieved using either 20% epoxide for 15h or 30% epoxide for 7h. Metal chelating supports prepared under both conditions exhibited no apparent differences in their 'protein binding' or 'handling' characteristics.

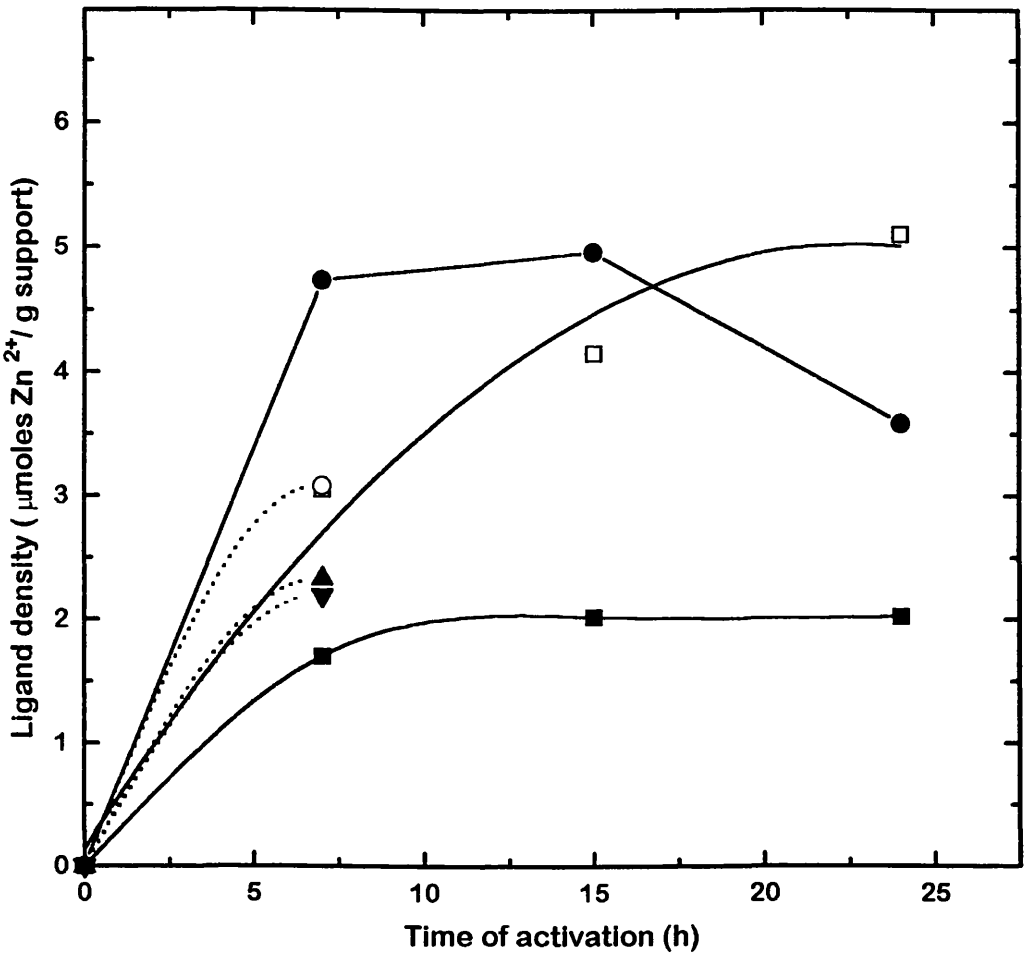


Figure 2.8 Concerted effects of time of activation and concentration of epoxide (% 1,4-butane diol diglycidyl ether) upon the ligand density of chelating MP1500 particles.

Supports were epoxide activated at various epoxide concentrations and for different times (−■− 10% epoxide; −□− 20% epoxide; −●− 30% epoxide; ○ 40% epoxide; ▲ 50% epoxide; ▼ 60% epoxide) before coupling with 15% IDA. Each data point is the average of two experiments. Ligand density measurements were not carried out on chelating MP1500 activated at 40-60% epoxide after 7h due to the gelation of the samples.

2.3.3.7 Effect of sodium hydroxide concentration during epoxide activation upon ligand density of metal-chelating MP1500 support.

The effects of sodium hydroxide concentration during epoxidation on the ligand density of metal chelating supports is presented in Figure 2.9. PVA-coated supports were activated using 30% 1,4-butane diol diglycidyl ether over a range of concentrations of sodium hydroxide. Epoxy-activated supports were subsequently coupled using 15% IDA, and charged with Zn^{2+} (2.2.3).

The coupling efficiency reaches maximum levels at NaOH concentrations above 0.3M. Beyond this point theres is no change in ligand density, which remains at 5μmoles/g. Routinely a final molarity of 0.4M NaOH was used for subsequent experiments.

2.3.4 Coupling of ligand to epoxy-activated perfluorocarbons

To investigate the effect of IDA concentration upon the ligand density of the affinity supports epoxidation was carried out using optimal conditions of 30% 1,4-butane diol diglycidyl ether and 0.4M NaOH for 7h. The epoxide activated supports were then coupled with IDA at various concentrations for 15h and charged with Zn^{2+} ions.

Figure 2.10 illustrates the effect of the initial IDA concentration upon the final ligand density. Concentrations in excess of 15% IDA are necessary to achieve the highest levels of bound ligand. In routine experiments 15% IDA for 15h were the coupling conditions employed.

2.3.5 Transferring optimum conditions to other perfluoropolymers

The optimum conditions for the preparation of metal chelating perfluorocarbon supports were subsequently transferred to the surface modification of the smaller perfluorocarbon particles and their ligand densities measured after coupling. Ligand densities of 10-12μmoles/g were consistently achieved for PTFE wax (6-9μm) and PTFEP (10μm) particles.

The PTFE wax support possessed difficult handling characteristics. In agitated solutions it readily formed a stable foam and particle recovery by centrifugation or filtration proved difficult. Another worry about using the PTFE wax support at a

process scale is that the particle is readily compressible and therefore not ideal for chromatographic operations involving high pressures and flow rates. Despite the small particle size of the PTFEP material, it did not present the same handling problems as the PTFE wax. Although some work was carried out using the PTFE wax further work concentrated on comparing the MP1500 20μm and PTFEP 10μm particles.

Experiments confirmed that the conditions optimised for the preparation of chelating MP1500 were also optimal for the preparation of chelating PTFEP. Input concentrations of 15-20mg/mL perfluoroalkylated PVA were routinely used for preparation of PVA-coated PTFEP. Epoxidation conditions of 30% epoxide, 0.4M NaOH for 7h and ligand coupling conditions of 15% IDA for 15h consistently gave chelating PTFEP affinity supports with ligand densities of 10-11μmoles Zn^{2+} /g.

One interesting observation was that the amounts of different perfluoroalkylated-PVA's adsorbed onto the surface of PTFEP particles were very similar to those on MP1500, suggesting roughly equal surface areas for the two supports (Table 2.2). The capacity of the PTFE wax for the 13-23K polymer was less than that adsorbed by the MP1500 and PTFEP support, and this finding is also consistent with the manufacturers figures for surface area per g (see Tables 2.1 & 2.2).

However in contrast to the results obtained with MP1500 (Figure 2.7) no distinct optimum epoxide concentration was observed with PTFEP particle, which remained constant (10.3-11.9μmoles Zn^{2+}/g) between 10 and 40% epoxide (Figure 2.11). However the gelation phenomenon described earlier for MP1500 supports was also observed at concentrations above 50% over a 7h time period. After epoxide activation the particle size could be observed to be increased suggesting some degree of inter-particle linking. The higher the concentration of epoxide, the more pronounced the aggregation. This would suggest that the smaller size of the PTFEP means that inter-particle cross-linking is more widespread. However 30% epoxidation for 7h, the optimum conditions for chelating MP1500 preparation, did not result in any discernable 'gelation'

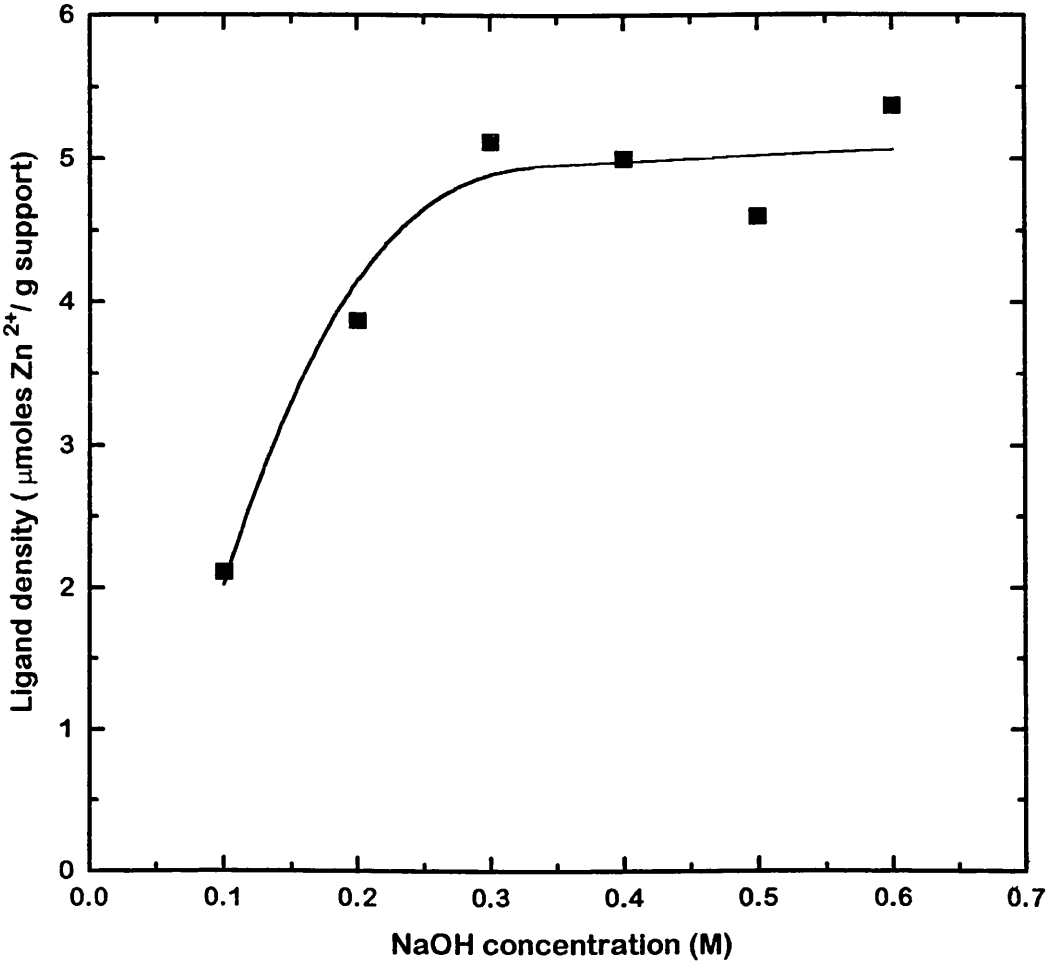


Figure 2.9 Effect of sodium hydroxide concentration during epoxide activation on the ligand density of chelating MP1500 particles.

Supports were activated with 30% 1,4-butane diol diglycidyl ether for 7h and coupled with 15% IDA. The data points represent the averages of two experiments.

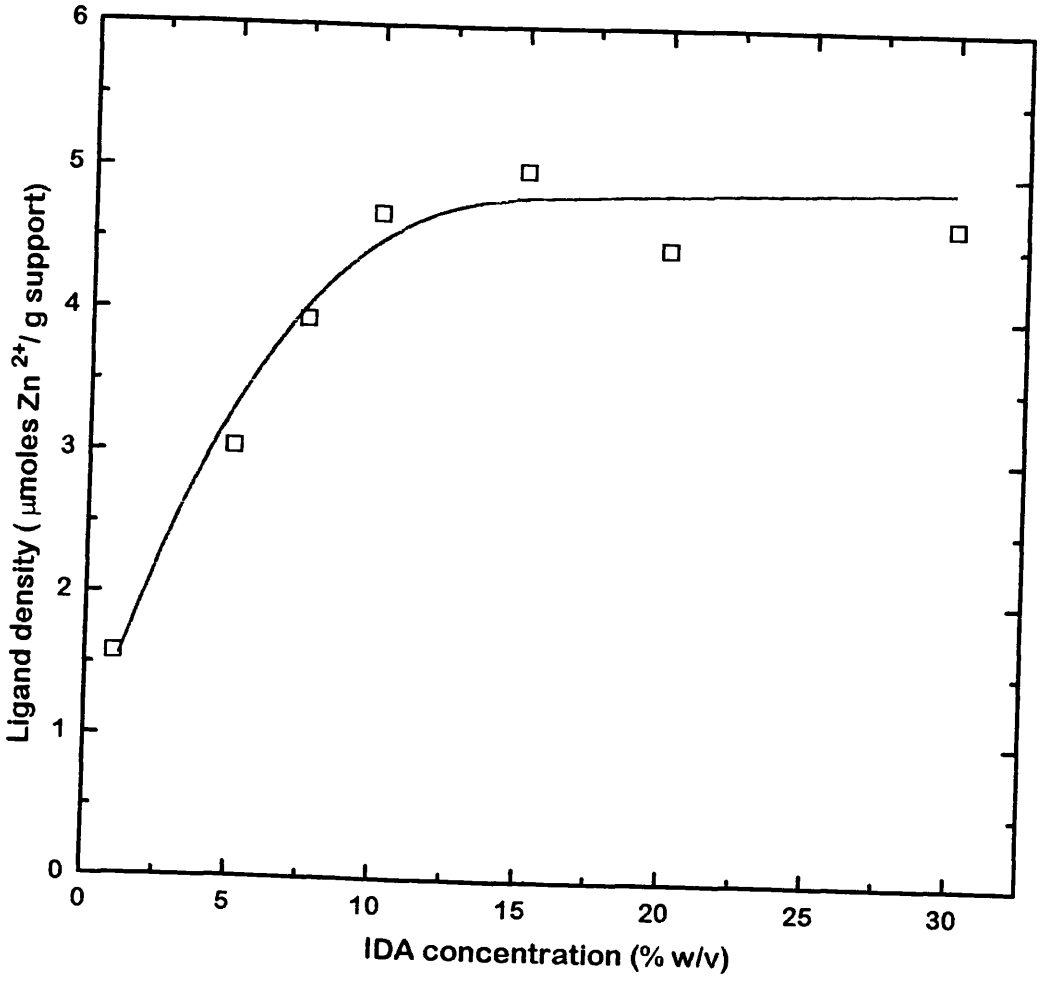


Figure 2.10 Effect of IDA concentration on the ligand density of chelating MP1500 particles.

Supports were activated with 30% 1,4-butane diol diglycidyl ether for 7h and coupled with IDA at various concentrations. Each data point is the average of three experiments.

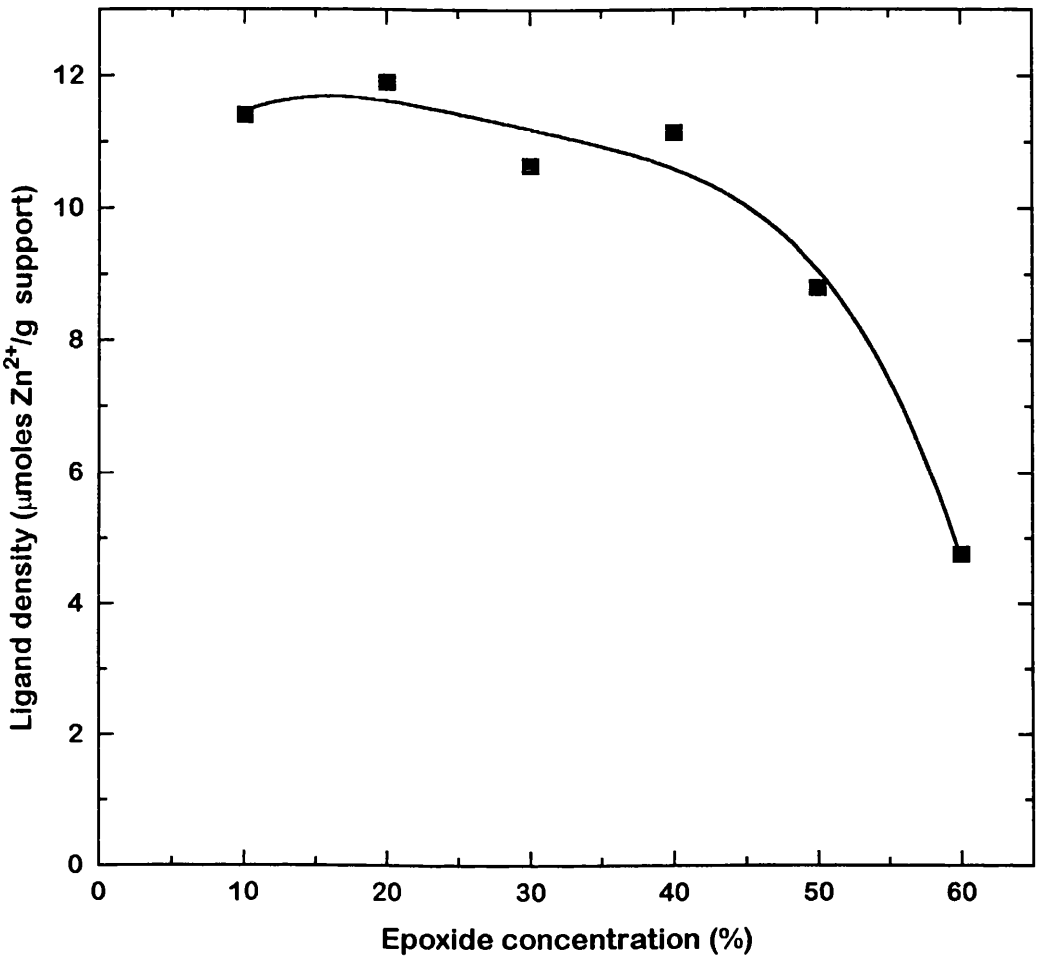


Figure 2.11 Effect of epoxide concentration (% 1,4-butane diol diglycidyl ether) upon ligand density of chelating PTFEP.

PVA coated supports were epoxide activated at various epoxide concentrations (10-60%) for 7h before coupling with 15% IDA.

2.3.6 Pseudoaffinity adsorption of a monoclonal antibody to chelating perfluorocarbon supports and commercial agarose based supports.

The different perfluorocarbon materials were coated with 9-10K PVA polymer, grafted with IDA and charged with Zn^{2+} ions using the optimum conditions discussed above (2.3.5) before testing in batch adsorption experiments (2.2.3.1) The test protein used was an monoclonal antibody known to bind commercial Zn^{2+}-IDA gels, MAb1 (A.J. Sheppard, pers. communication). Adsorption isotherms were measured at near neutrality at which surface histidines are unprotonated and free to co-ordinate with bound metal ions (Porath and Belew, 1983; Todd *et al.*, 1994). Equilibrium adsorption isotherms for MAb1 on metal chelating perfluorocarbon supports are shown in Figure 2.12. Figure 2.12a is a plot of binding capacity at equilibrium, Q*, versus the input MAb concentration, C0. Plots of this kind are useful for rapidly estimating the working capacities at a given MAb input concentration. Equilibrium adsorption isotherms are shown in Figure 2.12b. Q*, the amount of protein bound per g of support at equilibrium is plotted against C*, the liquid phase or 'free' antibody concentration at equilibrium. The solid lines represent the fit of 'best fit' hyperbolas (Langmuir isotherm model) to the data, where Q* is the amount of protein adsorbed per g of support at equilibrium and C* is the liquid-phase antibody concentration at equilibrium. The Langmuir model (Langmuir, 1916) is fully described by two constants, a dissociation constant, Kd and the maximum capacity for adsorbed antibody, Qmax (Equation 2.1).

Equation 2.1 $Q = (Qmax \times Kd \times C) / (1+Kd \times C)$

The Langmuir constants derived from the data in Figures 2.12b and values for working capacity at 0.5mg/mL antibody Figure 2.12b are presented in Table 2.3. Scatchard (1949) plots confirmed that the binding of MAb1 to Zn^{2+}-charged perfluorocarbon supports was adequately described by the simple Langmuir model (Figure 2.13).

Figure 2.12a Adsorption of MAb1 to chelating perfluorocarbons

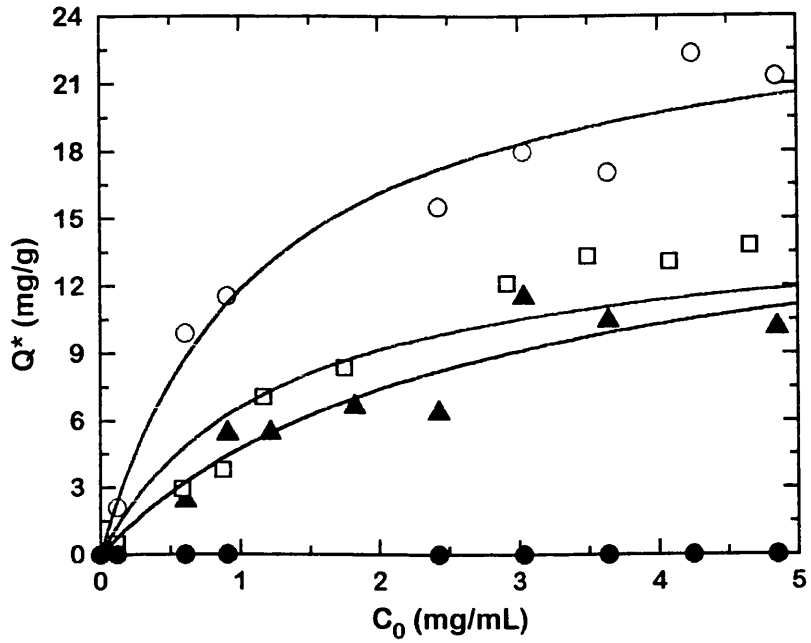
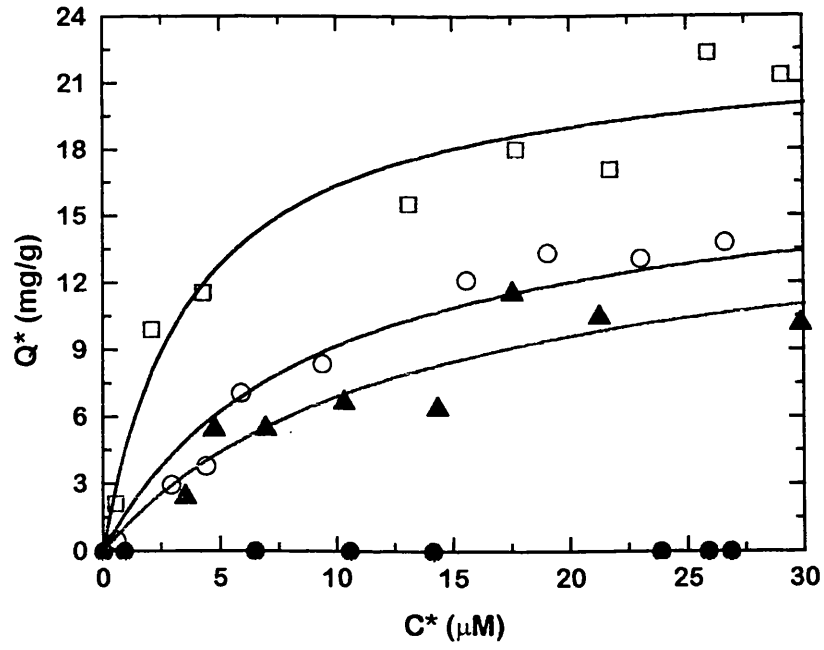


Figure 2.12b Adsorption isotherm for binding of MAb1 to chelating perfluorocarbons



Zn^{2+} charged chelating perfluorocarbons □ PTFEP; ○ MP1500; ▲ PTFE wax and ● uncharged MP1500 were employed in the adsorption of MAb1 from PBS pH7.2

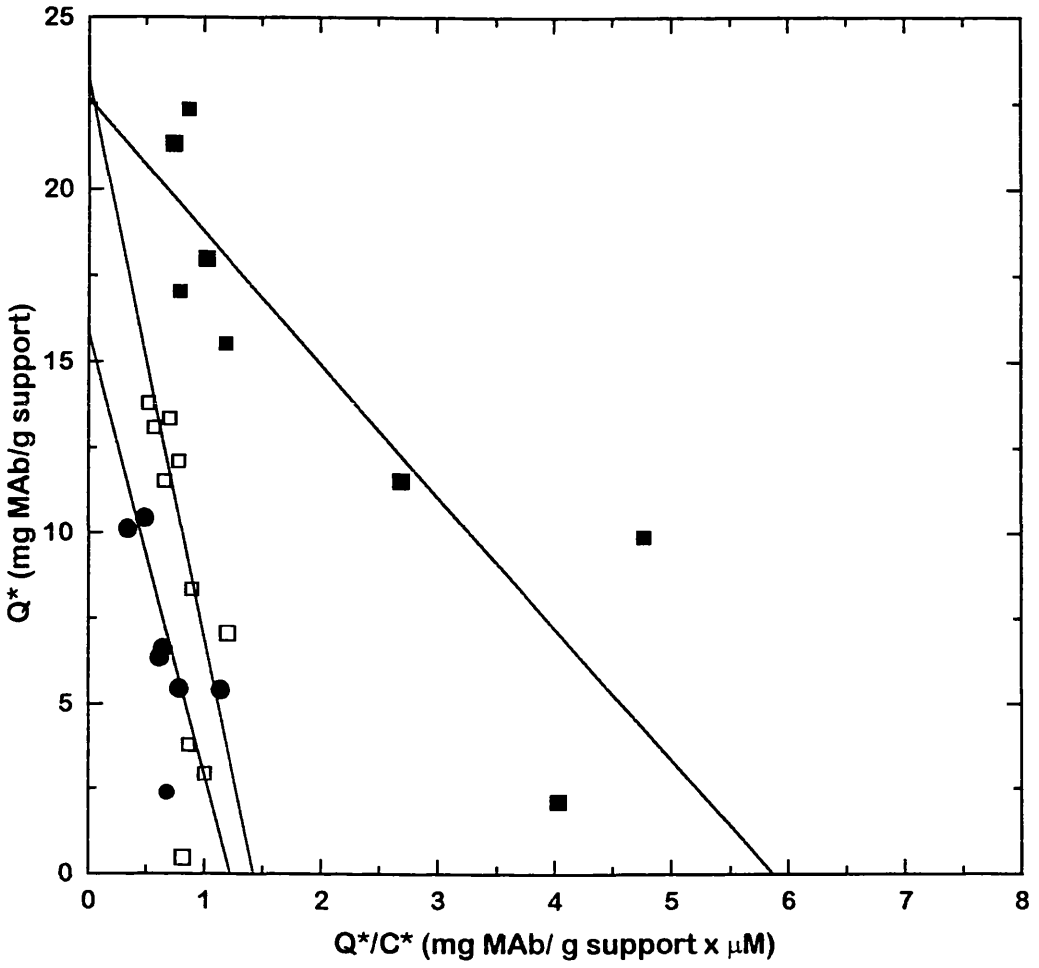


Figure 2.13 Scatchard Plots for Zn^{2+}-chelating perfluorocarbons.

The lines represent the fit of Langmuir binding constants (derived from Best fit hyperbolas) in equilibrium plots of Q vs. C*. The intercepts on the y-axes are the Qmax values and on the x-axes, the initial slopes (Qmax / Kd)*

■ *PTFEP;* □ *MP1500;* ● *PTFE wax*

Table 2.3 Binding parameters of Zn^{2+}-chelating perfluorocarbons for MAb1

Parameter	MP1500	PTFEP	PTFE wax
Qmax (mg/g)[a]	17.5	22.6	15.8
Kd (μM)[a]	9.1	3.8	13.0
Initial slope (Qmax/Kd)[a]	1.9	6.0	1.2
Working capacity at 0.5mg/mL MAb1 (mg/g)[b]	4.2	7.7	2.8

[a] Langmuir parameters derived from data in Figure 2.12b

[b] Interpolated from Figure 2.12a

All three of the non-porous Zn^{2+}-chelating perfluorocarbon supports prepared, bound the MAb1. As expected the capacities for non-porous supports of these dimensions were relatively low. Specific binding capacities are evidently related to particle size and surface area available. The maximum capacities and lowest Kd were obtained with the 10μm PTFEP based support (Qmax = 22.6mg/g; Kd = 3.8μM). No non-specific binding of MAb1 to any of the uncharged chelating perfluorocarbon adsorbents was observed, confirming that binding to Zn^{2+}-charged particles was specfically mediated via immobilised Zn^{2+} ions.

The chelating PTFE wax support, in addition to earlier observations on the difficulty of handling this material (2.3.5), possessed the lowest binding capacity (15.8mg/g) despite its small particle size. This material was deemed unsuitable and has not been studied further.

Adsorption of MAb1 to PTFEP was stronger than that to MP1500 (Dissociation constants, Kd of ~$4x10^{-6}$M for PTFEP and ~$9x10^{-6}$M for MP1500). The tightness of binding is reflected by the initial slope of the isotherm (i.e. Qmax/Kd). Thus the binding to PTFEP support is ~2 times stronger. This increased strength in the binding of MAb1 to chelating PTFEP is reflected in the values of working capacity (7.7mg/g for PTFEP c.f 4.2mg/g for MP1500).

In Figure 2.14 equilibrium adsorption isotherms for MAb1 to two types of chelating agarose based adsorbents, chelating Sepharose Fast Flow and chelating Streamline, are shown. Capacities on these porous hydrogel supports are expressed as mg/g of dried gel. The capacities for the Streamline support were slightly lower (162mg/g) than those of the 90μm fast flow matrix (230 mg/g). This finding is consistent with the presence of a solid quartz core reducing the effective capacity and the findings of others (Chang and Chase, 1994) showing reduced capacities for the binding of BSA to DEAE derivatised Streamline compared to the Fast Flow matrix. The dissociation constants measured for the commercial supports were lower than those recorded for the chelating perfluorocarbons though not markedly so, all lying in the μmolar range. Somewhat unexpected was the difference in Kd for the interaction of MAb1 with the two supports (~1 x 10^{-6}M and ~2 x 10^{-6}M for Fast flow and Streamline respectively) and the initial slope of the isotherm for MAb1 binding to the fast flow matrix was roughly three times that of the Streamline support. Again this finding has also been made by Chang and Chase (1994) who observed Kd value of 0.4 x 10^{-6}M for DEAE-Fast Flow Sepharose and 2 x 10^{-6}M for DEAE-Streamline matrix towards BSA. The reasons for this apparent reduction in binding affinity remains unclear at present.

2.3.7 Stability of chelating supports to adverse conditions

An experiment was carried out to investigate the stability of chelating MP1500 and chelating PTFEP (2.2.3.2). The results shown in Figure 2.15 show that over the period which the test was carried out (one month) there were no significant decreases in ligand density. Significant decrease was taken to be a decrease of greater than 10%, this being the approximate error involved in the measurement of ligand density. Indeed some measurements of ligand density after the test was complete show an increase in ligand density which obviously can not be correct and is explained by the errors involved in measurement.

Figure 2.14a Adsorption of MAb1 to porous supports

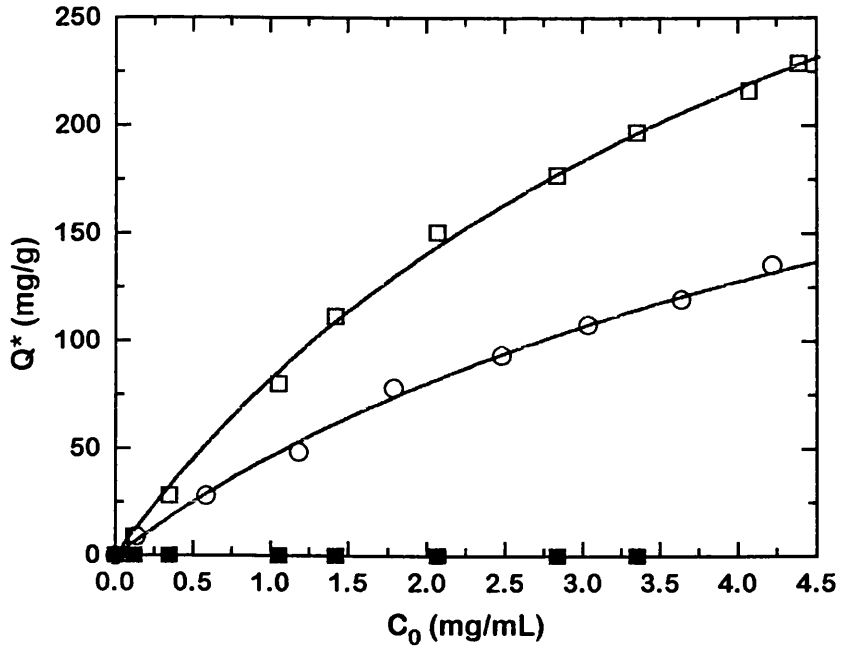
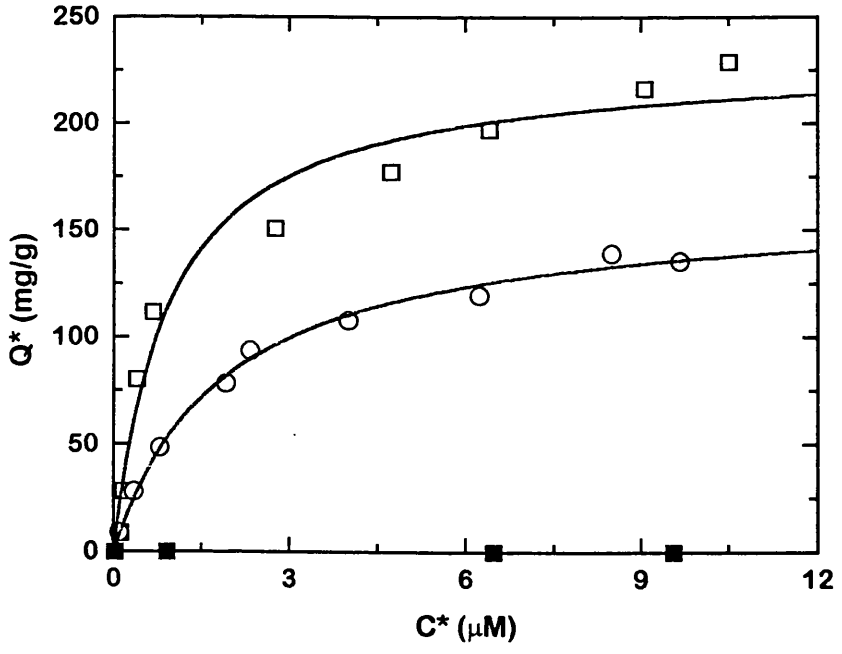


Figure 2.14b Adsorption isotherm for binding of MAb1 to porous supports



Zinc charged chelating Fast Flow Sepharose □; chelating Streamline O and uncharged supports ■ were contacted with various concentrations of MAb1 in PBS pH7.2

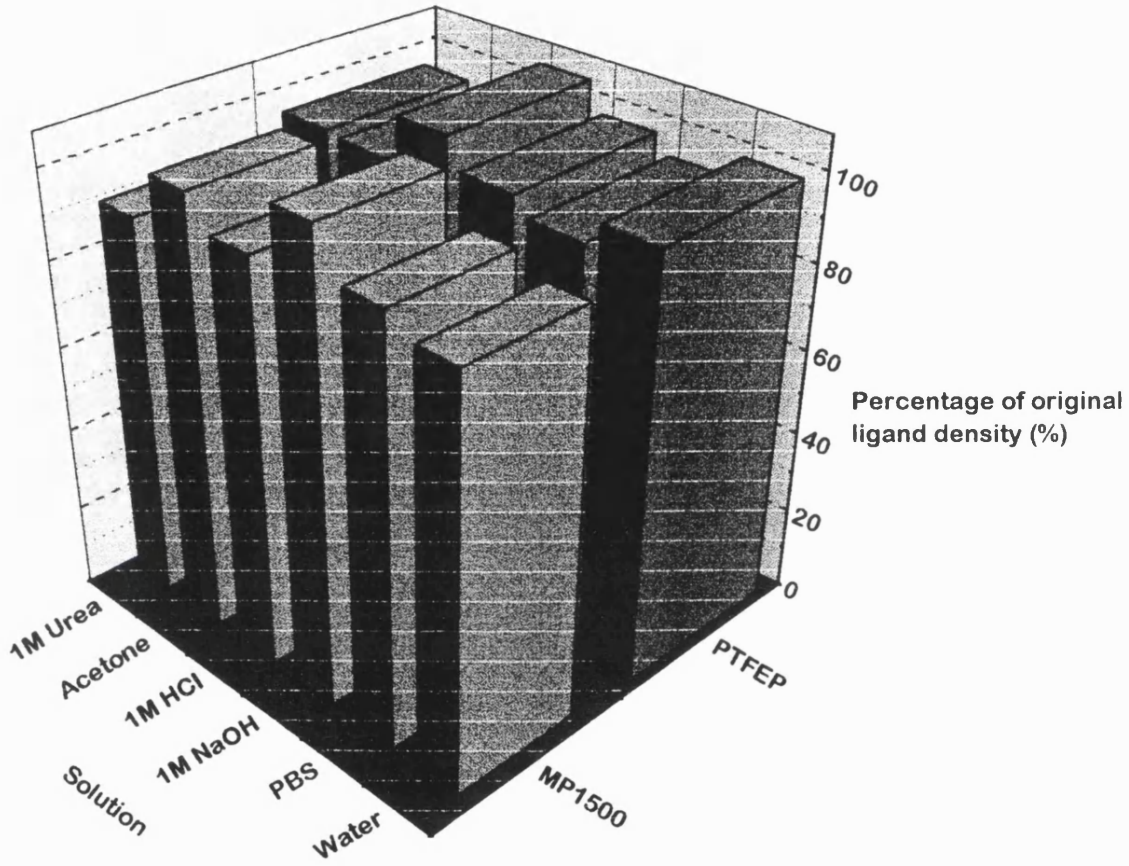


Figure 2.15 Stability of chelating perfluorocarbon supports

Chelating perfluorocarbon supports were contacted with various solutions for one month. Changes in ligand density are expressed as a percentage of the original ligand density

2.4 Discussion

2.4.1 Preparation of metal chelating perfluorocarbons

Successful coating of perfluorocarbon base matrices with polyvinyl alcohol (PVA) and the introduction of chelating groups is clearly demonstrated by the iodometric staining of the PVA coat, the selective desorption of Zn^{2+} with acetate at pH 3.8 and the selective binding of MAb1 to supports only in the presence of added Zn^{2+} ions.

The optimum conditions for the preparation of metal chelating perfluorocarbons were elucidated and when applied consistently yielded preparations with similar ligand densities and binding characteristics.

It appears that it is possible to directly adsorb PVA to the surface of perfluorocarbons (Pitfield, 1992). The perfluorocarbon support is prewashed in a polar organic solvent such as acetone and PVA in aqueous solution is then adsorbed. The resulting PVA coat is then crosslinked with terephthaldehyde before subsequent activation and ligand coupling steps (Pitfield 1992). MP1500 was coated with PVA by first washing the PTFE powder in acetone and then contacting with 20mg/mL PVA (9-10K). Subsequently these supports were epoxide activated and coupled with IDA. The resulting ligand densities were identical to those of chelating MP1500 prepared by perfluoroalkylation of PVA. The crosslinking of the PVA coat with terephthaldehyde was not considered necessary as epoxide activation results in crosslinking of the PVA coat. This method of PVA coating would therefore appear to be an alternative to the method used in this thesis.

One potential cause for concern during the preparation of these metal chelating supports was the evidence that epoxidation when carried out at high concentrations of epoxide (1-4 butanedioldiglycidyl ether) over extended time periods caused inter-particle crosslinking. If this crosslinking is allowed to continue unchecked the resulting preparation, because of the decreased surface area per unit weight of support due to 'gelation', is effectively useless as a pseudoaffinity support. If the optimum epoxide concentrations and time periods are strictly adhered to (30% epoxide, 7h; 20% epoxide, 15h) then 'gelation' should not be observed and will present no problem. A

certain degree of cross linking is likely to be desirable in order to stabilise the PVA coat.

There are alternative chemistries in the literature which could be employed to introduce chelating groups onto the PVA coat which would not be subject to the competing cross linking side reaction experienced during epoxidation. One approach may be to react a molecule such as mercaptoethylamine with the hydoxyl containing surface of PVA resulting in an amine bearing surface coupled through a stable thioether bond (Dean *et al.*, 1985). Subsequent treatment of the particles with bromoacetic acid should yield a support densely covered with IDA functions.

2.4.2 Binding characteristics of chelating perfluorocarbon supports

Chelating MP1500 and chelating PTFEP supports exhibited suprisingly high capacities for non-porous supports of such dimensions, indicating highly folded surfaces, particularly the MP1500 particle. Although the maximum capacities for the two supports are very similar the interaction strengths are very different. The ligand density on the PTFEP support is twice that of MP1500. Todd *et al.*, (1994) and O'Brien *et al.*, (1994) have recently demonstrated the importance of high ligand density in order to achieve multi site interactions with Cu^{2+}-IDA adsorbents, resulting in dramatic enhancement of apparent binding strengths. The difference in binding strength between the PTFEP and the MP1500 supports may therefore reflect the difference in ligand densities and the ability of the PTFEP but not MP1500 support to support multi-site and therefore stronger binding.

The effective surface areas of MP1500 and PTFEP appear to be approximately the same based upon their capacities for PVA (Table 2.2) and binding capacities (Table 2.3). The manufacturers of MP1500 quote a surface area of $11 m^2/g$ (Table 2.1) therefore one could assume that the surface area of PTFEP is approximately the same or a little more. Given that MP1500 has approximately the same surface area for capture of target protein as the smaller PTFEP particle then if the ligand density on the MP1500 particles could be raised sufficiently the MP1500 particles would be especially attractive.

Table 2.4 Comparison of Zn^{2+}-chelating supports for MAb1

	Perfluorocarbon based supports			Agarose based supports	
	chelating MP1500	chelating PTFEP	chelating PTFE wax	chelating Sepharose FF	chelating Streamline
Average particle Size (μm)	20	10	7	90	200
Density (gcm^{-3})	2.2	2.12-2.18	2.2	1.13	1.18
Ligand Density (μmoles Zn^{2+}/g)	4-5	10-11	10-12	30-60	<50
Kd (μM)	9.1	3.82	7.79	0.93	1.89
Qmax (μmoles/g)	0.11	0.15	0.09	1.53	1.09
Packing density (g/mL)	0.34-0.44	0.40	0.30-0.50	0.06	0.08
Capacity mg antibody per unit volume packed bed (mg/mL)	9.1	7.0	6.3	13.8	12.9

It is unlikely however that any significant increase in ligand density on PVA-coated MP1500 particles could be achieved using the epoxide activation route. We calculated that 14-15 molecules of IDA are bound per molecule of PVA (9-10K molecular weight) adsorbed onto MP1500 particles. The number of hydroxyls esterified with perfluorooctanyl tails is unknown but the total number of available hydroxyl groups on a polymer chain of this length is 198.

A comparison of binding characteristics and physical properties of non-porous chelating perfluorocarbons and 'conventional' porous supports is presented in Table 2.4. Table 2.4 demonstrates that the chelating perfluorocarbon supports are comparable with porous supports in terms of such parameters as capacity, Kd etc. However due to the low surface area of the chelating perfluorocarbons even when the capacity is expressed as MAb per unit volume of packed bed, a more realistic comparison, the values are still lower than those obtained for the chelating porous adsorbents.

Despite the advantages of chelating perfluorocarbon supports, namely stability, rapid binding kinetics and reduction in the effects of fouling, the lower binding performance is likely to have major implications for commercial application.

2.4.3 Selection of mode of operation for the application of metal chelating perfluorocarbon supports.

As discussed previously (1.2.2) the mode of operation employed to affect a purification is of prime importance when considering a one step purification or direct recovery of a monoclonal antibody. The presence of cells, cell debris and other fouling solids and components means that traditional column or packed bed operations are not an option for process scale purifications. Indeed packing chelating MP1500 in an XK16/20 (Pharmacia) column under low flow rates (1mL/min) resulted in a serious increase in backpressure resulting in the abandonment of work to investigate the operation of the chelating perfluorocarbons in packed bed mode.

Other modes of operation that these chelating perfluorocarbons would be suitable for are batch adsorption, fluidised bed absorption or expanded bed absorption.

For both expanded bed absorption and fluidised bed absorption to be successful at a process scale the adsorbent should be sufficiently dense or large for throughputs in the range 100-300cm/h to be used. In an expanded bed operation the attainment of a commercially viable throughput is a major consideration and current limitation of the technique (1.2.2.4). Ideally throughputs higher than 300cm/h would be used if the adsorbent could maintain a stable bed at such a flow rate and bed expansion could be maintained at twice settled height. For an effective fluidised bed operation the operational throughput is dependent upon the fluidisation velocity of the adsorbent and the speed and strength of the interaction between target protein and immobilised ligand. In both operations the density and size of the adsorbent will define to a certain extent the maximum throughput used.

McCreath *et al.* (1995) demonstrated the expanded bed operation of dye-perfluoropolymer supports, the base perfluorocarbon material in question Perflex® or FEP has a mean particle size of 35μm and settling velocity of 0.08cm/sec (Table 2.1). In expanded bed experiments McCreath *et al.* (1995) demonstrated that a stable expanded bed could be achieved at a throughput of 134cm/h. Dye-FEP has a higher settling velocity than either chelating-MP1500 (0.026cm/sec) or chelating PTFEP (0.0065cm/sec) this would suggest that either of these materials would form a stable expanded bed at throughputs less than 134cm/h. Throughputs of 100cm/h were observed to produce an unstable expanded bed of MP1500. This suggests that the chelating perfluorocarbons developed here would be unsuitable for expanded bed operation.

There is some potential for fluidised bed operation but the low fluidisation velocity of these particles does suggest that throughputs through such a fluidised bed would be on the low side and therefore of limited use as a process option. The choice between chelating MP1500 and chelating PTFEP for this application would be a compromise on the higher fluidisation velocity of chelating MP1500 and the higher ligand density of chelating PTFEP.

The final option for the application of these chelating perfluorocarbons is in batch absorption. As the adsorbents are non-porous then the kinetics of the interaction between ligand and protein are likely to be fast as there are non diffusional limitations. The other advantages of chelating perfluorocarbons, resistance to fouling, reusability, stability and cost mean that there is some potential for their deployment as batch adsorbents.

The size of the chelating perfluorocarbons is approximately the same as that of CHO cells (18μm Jordan *et al.*, 1996; 13-14μm Boraston *et al.*, 1996) therefore filtration or the use of a basket centrifuge would not be an efficient means of recovering adsorbents in the presence of cells. The higher density of the chelating perfluorocarbons compared to CHO cells means that their Stoke's settling velocities are approximately 10-30 times higher. This in theory would mean that recovery could be carried out in a gravity settling tank (Lavanchy *et al.*, 1964). However the use of some kind of centrifugation technique would be highly recommended in order to decrease the processing time. Two possibilities are tubular bowl centrifuge and scroll decanter centrifuge. The higher solids capacity of the scroll decanter centrifuge and the ability to perform continuos recovery would be considerable advantages over tubular bowl centrifuges (Sinnot, 1983). Only low centrifugal forces should be necessary which is an advantage in reducing possible effects of support damage during centrifugation. The effect of centrifugation on CHO cells would also have to be considered.

2.5 Conclusions

The non-porous chelating perfluorocarbon supports specifically bind MAb1 and are comparable in terms of binding capacity and binding strength with conventional porous adsorbents in batch mode (1.2.2.1). The efficient recovery of 'loaded' supports should be possible by centrifugation, but will require experimental confirmation.

The main disadvantage of the chelating perfluorocarbon supports is the relatively low specific binding capacity for MAb. A large amount of support would be required to recover the MAb from large scale CHO culture. The economic viability of this would depend on the final commercial price of the adsorbent and the effect of the benefits such as stability on the lifetime of the adsorbent.

Increasing the surface area of the perfluorocarbon base matrix should result in higher binding capacities. The synthesis of macroporous perfluoropolymer adsorbents by combining perfluorocarbon and a polymer such as methacrlyate would be one way in which to achieve higher specific surface areas (McCreath, pers. communication; Svec and Frechet, 1996). The resulting support could be coated with PVA and derivatised as described above (2.2.2). It is likely that fouling could be a potential problem but the stable nature of the perfluorocarbon material would mean that strenuous cleaning conditions could be applied without affecting the reusability of the adsorbent. In addition this approach would probably result in larger supports (100μm) which will make recovery from suspension easier.

An alternative approach is to reduce the size of the adsorbents and thereby increase the surface area. However reducing the size would have the undesirable effect of reducing the settling velocity, thus making recovery from batch adsorption more difficult. By making the adsorbent magnetically susceptible then after contacting the adsorbent with the feedstream in a batch mode it will be possible to recover the support magnetically (1.2.2.6).

It is this approach which is discussed in Chapter 3 and constitutes the rest of this thesis.

3. MAGNETIC CHELATORS

3.1 Introduction

As a direct consequence of their dimensions the non-porous perfluorocarbon supports developed in Chapter 2 suffer two major drawbacks despite their attractive features such as low non-specific binding and stability. These are low effective binding capacity per unit volume and very low settling velocities. Collectively these factors seriously limit the effective use of non-porous perfluorocarbon supports for the direct recovery of protein products from crude process liquors.

In these laboratories submicron non-porous magnetic adsorbents derivatised with metal chelates (O'Brien, 1996; O'Brien *et al.*, 1996; submitted) and with biomimetic dyes (Pannu *et al.*, in preparation) have been prepared and tested for the selective recovery of a variety of proteins (recombinant T4 lysosyme, cytochromes c, haemoglobin, alcohol dehydrogenase and lactate dehydrogenase). Magnetic separation is especially attractive at large scale and is currently being investigated in our laboratories (Pannu *et al.*, in preparation; Zulqarnain *et al.*, in preparation) for the recovery of 'loaded' magnetic dye linked supports from crude yeast homogenates. At scale high gradient magnetic separation (HGMS) is probably the only practical means for recovering support particles of submicron dimensions from feedstocks containing biological particulates of similar dimensions.

This chapter discusses the preparation and characterisation of coated non-porous magnetic support particles derivatised with iminodiacetic acid and illustrates how they can be employed to selectively recover monoclonal antibodies direct from CHO fermentation broth containing suspended cells. A commercially available amine-terminated iron oxide particle of 0.5-1.5μm dimensions, BioMag® 4100B (Perseptive Diagnostics, Mass., USA) was chosen as the starting material for the preparation of magnetic chelator supports. In later studies a similar starting material was prepared by following the patented protocol describing the preparation of BioMag® (Josephson, 1987).

3.2 Experimental

3.2.1 Materials

MAb1 and MAb2 monoclonal antibodies were received from the Wellcome Research Foundation Ltd. (Beckenham, Kent, UK.). For experiments carried out in the presence of Chinese hamster ovary (CHO) cells, fermentation's were provided by the Wellcome Research Foundation Ltd. (Beckenham, Kent, UK).

BioMag® 4100B, superparamagnetic amine-terminated iron oxide particles (PerSeptive Diagnostics, Cambridge, Mass., USA) were supplied by Metachem Diagnostics (Piddington, Northants, UK).

The following materials were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK):- aminopropyltriethoxysilane (APTES), glutaraldehyde (grade I), 1,4-butanediol diglycidyl ether and iminodiacetic acid (IDA).

All other chemicals employed, unless specified, were AnalaR grade and obtained from Sigma and BDH chemical companies (Poole Dorset, UK).

3.2.2 Magnetic particle separation

At all stages in the preparation and in the use of magnetic particles, recovery was effected by separation using permanent magnet devices. For the preparation of the superparamagnetic amine-terminated iron oxide starting material, made according to the patent instructions (Josephson, 1987), vessels containing the material were usually placed on top of ordinary laboratory magnetic stirrers which produce low magnetic fields of 100-200 Oersteds. Alternatively when rapid particle separation was required beakers containing particles were placed on top of homemade devices consisting of a ceramic magnets producing 2500 - 3000 Oersteds, fixed to a wooden board. For all small scale procedures and in all binding experiments magnetic particles were recovered quickly using tube racks (2–50mL vial sizes) fitted with neodymium–iron–boron bar magnets producing fields of ~1000 Oersteds (PerSeptive Diagnostics, Cambridge, Mass., USA).

3.2.3 Preparation and characterisation of magnetic chelators

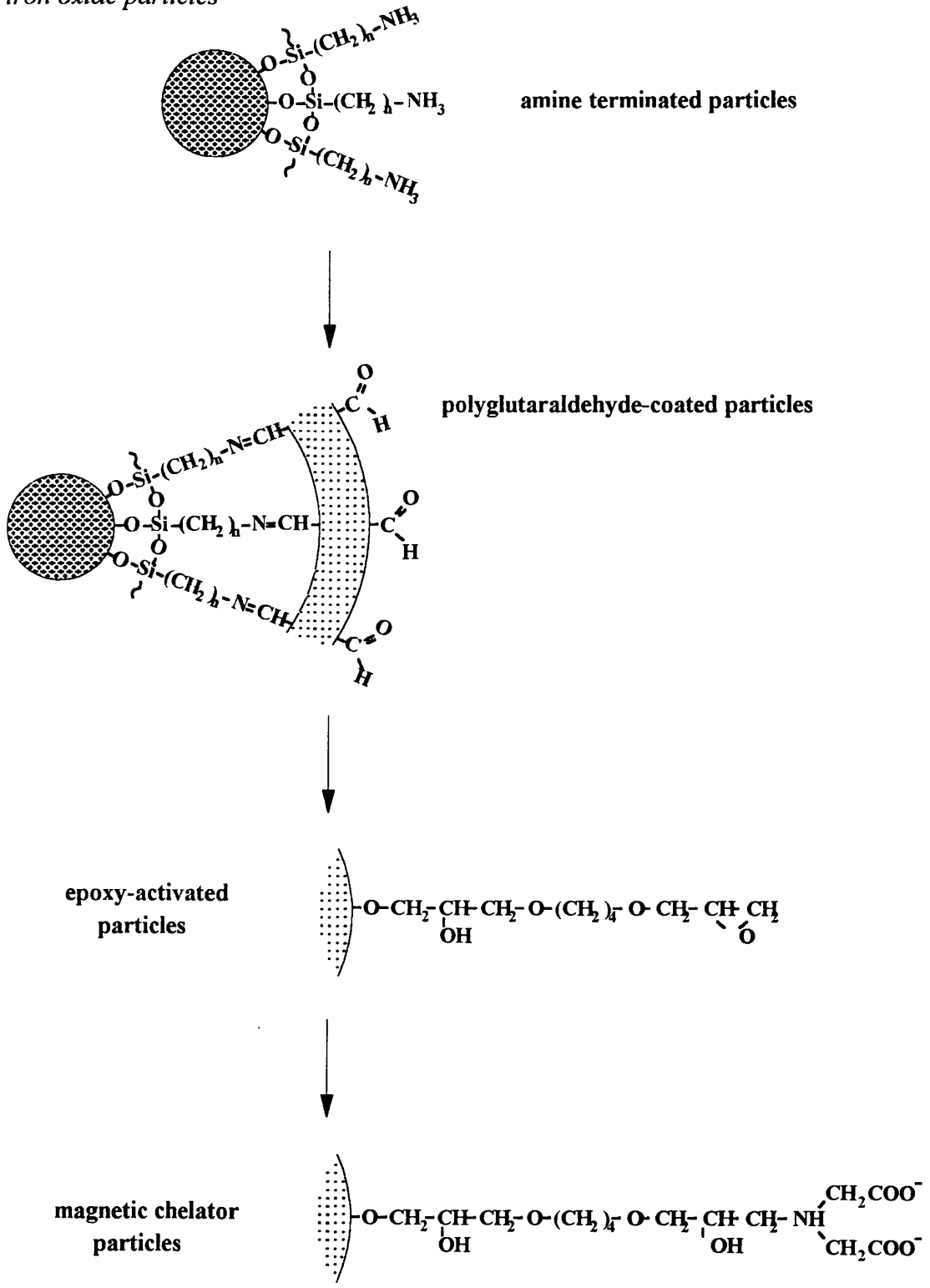
The preparation of metal chelating magnetic adsorbents or magnetic chelators as they have been termed by O'Brien *et al.* (1996) is shown schematically in Figure 3.1 and is described in detail below.

3.2.3.1 Preparation of amine terminated (SIN) iron oxide particles

Fine iron oxide core particles were prepared by precipitation in strong alkali according to the patented procedure (Josephson, 1987). All operations were carried out at room temperature unless stated otherwise. One hundred and fifty mL of 1M $FeCl_2$ was mixed with an equal volume of 0.5M $FeCl_3$ (Final concentration 0.5M $FeCl_2$ and 0.25M $FeCl_3$) filtered through 0.45μm glass fibre filters (Whatman GF/A). The filters are blinded relatively rapidly and fresh filters were used after filtration of ~100mL of solution. The filtered iron chloride solution (300mL) and 300mL of 5M NaOH (pre-equilibrated at 60°C) were then poured simultaneously into a 1L beaker containing 150mL of filtered distilled water. The mixture was stirred vigorously (~500rpm) during addition of the alkali and iron salt solution; using an overhead mixer fitted with a rushton turbine (Citenco, Herts, UK). A black precipitate formed almost immediately. Mixing was continued for a further five minutes and the particles were allowed to settle out of suspension overnight. Approximately 15g of particles were formed at this stage.

The liquid phase above the particle slurry was removed by decanting and the particles were washed ~5 times by cycles of 'resuspension' and 'settling'* with ~800mL volumes of distilled water until the pH of the liquid phase reached neutrality. The settling of particles between washes was greatly accelerated by placing the beakers containing magnetic suspensions on top of laboratory magnetic stirrers which produce magnetic fields of ~150 Oersteds. At this stage the particle slurry was washed twice with 800mL of 0.2M NaCl, as above*, and then resuspended to 1L volume with methanol (AnalaR grade). Following two further washes with methanol the final concentration of water in the methanol particle suspension was ~1% (v/v).

Figure 3.1 Scheme for the preparation of magnetic chelators from amine terminated iron oxide particles



Kindly drawn by Irini Theodossiou

After settling of the particles ~750mL of the liquid top phase was removed leaving ~15g of particles in ~250mL of methanol.

SIN type particles were prepared by silanisation with aminopropyltriethoxy silane (APTES). All silanisation steps and glycerol dehydration steps were performed in a fume cupboard. Ten mL of APTES was added to the iron oxide particle suspension and was mixed for 3 min at 3000 rpm using a Kinematica PT-6000 homogeniser with a 40mm polytron head (Polytron, Switzerland). Subsequently 5mL of glacial acetic acid was added and the homogenisation speed instantly raised to 11600rpm. After 10min the mixing speed was reduced to 4650rpm and mixing was allowed to continue for a further 2h.

Just before the 2h elapsed, 200mL of glycerol was poured into the beaker containing silane coated metal oxide particles and mixing continued for a few minutes until a homogeneous suspension was obtained. The blended solution was poured into a round bottomed flask (500mL) placed on an electric heating mantle. A constant stream of nitrogen was passed into the flask which was heated slowly to 150°C with gentle stirring provided from above using a glass chain-linked stirring rod driven by a Cintenco FHP motor (Cintenco, Borehamwood, UK). A condenser was fitted to collect methanol and silane vapours driven off by the heat. Heating was stopped when the particle volume was reduced to ~200mL and no further condensate was collected.

The flask and its contents were allowed to cool to room temperature. Silane coated particles were washed free of glycerol by 3 cycles of resuspension and settling with 2L portions of distilled water.

3.2.3.2 Polyglutaraldehyde coating of amine-terminated iron oxide particles

Commercially obtained amine-terminated iron oxide particles (3.2.1) and SIN type particles prepared 'in house' (3.2.3.1) were coated with polyglutaraldehyde using a simplified version of the method employed by Halling and Dunnill (1979) and O'Brien *et al.* (1996).

Amine-terminated particles at a final concentration of ~5g/L were mixed with glutaraldehdye at a final concentration of 2% (v/v) in a fume cupboard. The pH was

rapidly adjusted to 11 and was maintained at this level for 1h by manual addition of 1M NaOH. Mixing at ~500rpm was achieved using a Cintenco motor fitted with a stainless steel paddle. Polyglutaraldehyde-coated particles were recovered by accelerated settling using laboratory magnetic stirrers and were washed extensively with copious quantities of water until glutaraldehyde could not be detected in the washes, using the Tollens test (3.2.4.1).

3.2.3.3 Epoxide activation of polyglutaraldehyde coated iron oxide particles

Polyglutaraldehyde coated supports were activated with 1,4 Butane dioldiglycidyl ether according to the method described by O'Brien *et al.* (1996).

Suspensions of polyglutaraldehyde coated particles (25mg/mL) were epoxide activated using final reactant concentrations of 40% 1,4 Butane dioldiglycidyl ether, 0.1M NaOH and 1mg/mL of sodium borohydride, and mixed by tumbling for 15h (REA X2; Heidolph, Germany). After activation the particles were washed 8 times in a volume of distilled water.

3.2.3.4 Iminodiacetic acid coupling of epoxide activated magnetic supports

IDA coupling was achieved using 30% IDA in 2M Na_2CO_3 and 0.6mg/mL $NaBH_4$. The epoxide activated particles (25mg/mL) were incubated at 60°C for 24h in a shaking waterbath (Clifton Digital Shaker bath, Nickel Electro Ltd. Weston-Super-Mare, UK). After coupling the particles were separated using a low magnetic field and the bulk phase removed. The particle slurry was washed 4 times in a volume of distilled water and finally resuspended in 1M ethanolamine and kept at 4°C for 48h. The magnetic chelator particles were then washed three times in distilled water and stored at 4°C.

3.2.3.5 Charging of magnetic chelators

Magnetic chelator supports were charged with metal ions before use. Supports were washed 3-4 times with 50mL aliquots of water and recovered on a magnetic rack. A fixed volume (50mL) of 50mM $ZnCl_2$ or 50mM $NiCl_2$ solution in water were added to the supports and mixed on a tumbling shaker for 15min. The charged supports were

then washed with 5x 50mL portions of water and twice with phosphate buffered saline (PBS) pH7.2 before use. Controls or 'uncharged' supports were prepared by treating with 50mL of 0.1M EDTA (to ensure no metal ions were bound) prior to washing with water and equilibrating with PBS.

3.2.3.6 Pseudoaffinity adsorption/desorption studies with monoclonal antibodies MAb1 and MAb2.

Routinely magnetic chelator supports, usually at 2 or 4mg/mL particle concentration but in some experiments ranging from 0.4 to 15mg/mL, were incubated at 25°C on a vibrating shaker (IKA Labortechnik, Staufen, Germany) or a Luckham R100 shaker for volumes over 2mL. Incubation of the supports was carried out for various times from (2min-15h) with various concentrations of the antibodies (0.1-6.0mg/mL) in PBS pH 7.2 or CHO culture medium. At the end of each test the supports were magnetically separated and the supernatants analysed for all or some of the following; antibody concentration, protein concentration and metal ion concentration (3.2.4). The amounts of bound antibody were calculated by difference.

In some studies magnetic supports 'loaded' with antibody were employed in desorption studies. Routinely supports were washed 3-4 times in PBS pH 7.2 and subsequently twice with PBS containing 12mM EDTA.

3.2.3.7 Pseudoaffinity absorption/desorption studies with MAb2 in CHO fermentation broth containing viable CHO cells.

Studies to investigate the adsorption/desorption of MAb2 in CHO fermentation broth containing viable CHO cells were routinely carried out using zinc charged, nickel charged and uncharged magnetic chelators at particle concentrations of 0.2-4.5mg/mL. Two types of CHO fermentation were investigated. Simple batch fermentation's, harvested after 3-4 days, with a MAb2 titre of 50-100μg/mL and fed batch fermentation's, 3 feeds of fresh media components over 7 days, with MAb2 titres ~4 fold higher (200-500μg/mL). CHO cell viability was normally maintained above ~70%

but the time of harvest and the length of time before processing meant that cell viability in experiments was generally lower (~50-20%).

Magnetic chelators were added to sterile cell culture pots containing 50-300mL of culture broth. Incubation at room temperature was carried out on a Luckham R100 shaker. Samples (5-15mL) were removed every 5-10min over a period of 70min. Magnetic chelators were recovered magnetically and the supernatants analysed for antibody and protein content in addition cell viability was measured by the dye exclusion method (3.2.4.7). Uptake of MAb2 was calculated by the difference between initial MAb2 concentration (C_0) and supernatant concentration (C).

Recovery of MAb2 from magnetic supports 'loaded' with antibody was routinely achieved by washing supports 3-6 times in PBS pH 7.2 (15-50mL) and subsequently twice with PBS containing 12mM EDTA.

3.2.4 Analytical methods

3.2.4.1 Assay for the presence of glutaraldehyde

The presence of aldehyde groups was determined using the Tollens test (Harwood and Moody, 1990). Tollens reagent was prepared by adding 30% ammonia solution dropwise to 3mL of $AgNO_3$ (60mg/mL) with stirring until the brown precipitate formed initially dissolved to yield a slightly cloudy grey/white solution. Three mL of KOH (70mg/mL) was then added followed by enough concentrated ammonia solution to make the solution almost clear again.

Aliquots (0.5mL) of freshly prepared Tollens reagent were added to an equal volume of unknown sample. The formation of a black precipitate or a silver mirror on the walls of the test tube, either immediately or on warming the tube in a beaker of hot water, constituted a positive test for the presence of aldehyde

3.2.4.2 Determination of weight of magnetic chelator particles

The amounts of supports used in tests was determined at the end of experiments by drying samples in a Savant Speedvac vacuum desiccator (International Equipment Co., Luton, UK) and weighing on an analytical balance. To back up dry weight

measurements, samples were also dissolved in 1mL of conc. HCl in eppendorf vials. The concentration of iron was then determined at 324.8nm using a single element lamp operated at 16mA with a Perkin-Elmer AA 3100 Atomic absorption instrument using an air-acetylene flame. Using known weights of supports dissolved in 1mL of conc. HCl a calibration curve relating particle weight to Fe content was constructed. Measurement of the soluble iron content by atomic absorption spectrophotometry enabled accurate determination of low amounts of support to be made.

3.2.4.3 Immobilised ligand concentration

One mL aliquots containing ~10mg of charged support particles were pipetted into eppendorf vials and washed 2-3 times by cycles of magnetic recovery and resuspension in distilled water and mixing on a vibrating shaker (IKA VXR, Staufen, Germany) (1000rpm, 5min). The supernatants were removed and retained for analysis by atomic absorption spectrophotometry to establish effectiveness of each wash step. The supports were then resuspended in 1mL of 0.1M EDTA and mixed on a vibrating shaker (1000rpm) for approximately 20min before recovering supports magnetically and analysing the metal content of the supernatants by atomic absorption spectrophotometry. Zinc was determined at 213.9nm and nickel at 232.4nm, using single element lamps operated at 10mA with an air-acetylene flame in a Perkin-Elmer AA 3100 Atomic Absorption instrument (Perkin Elmer, Seer Green, UK.). Prior calibration with known concentrations of metal ions allowed accurate determination of metal content to be made. Ligand densities were then expressed as μmoles metal ion per g of dried support. Dry weight of the supports being determined as described above (3.2.4.2).

3.2.4.4 Determination of protein concentration

Protein content was determined using Coomassie® Plus Protein assay reagent (Pierce and Warriner Ltd., Cheshire, UK). The reagent is based on the Bradford assay (Bradford, 1976). The protocol followed was that supplied with the reagent for use with microtiter plates (Biorad, New York, USA). Calibration curves were prepared from stock solutions of Bovine serum albumin (2mg/mL). Duplicates were prepared

for each sample and the absorbance read at 595nm on a Dynatech MR7000 plate reader (Billingshurst, W.Sussex, UK).

3.2.4.5 Determination of antibody concentration

Antibody content was determined by a protein A HPLC assay (2.2.4.4). Alternatively a chelating HiTrap (1mL) column was used (Pharmacia Biotech, St. Albans, UK). The protocol used was an adaptation of that provided with the column. The column was washed with 5mL water and charged with 1mL of 0.1M $ZnSO_4$. Residual zinc was removed by washing in 5mL of water before equilibration in 5mL PBS pH7.2. The sample was then loaded and the column washed with 5mL PBS pH7.2. Elution was effected by 5mL PBS 12mM EDTA. The eluted fraction was assayed for protein as described above (3.2.4.4)

3.2.4.6 Particle size analysis

Particle size measurements were kindly performed by Kamran Zulqarnain using a laser particle analyser.

3.2.4.7 Measurement of Cell viability

Cell viability was measured by the dye exclusion method (Petersen *et al.*, 1988) using erythrosin B (Sigma, Dorset, UK). One hundred microliter portions of samples were added to 700μL aliquots of 0.04% erythrosin B in PBS. The number of live, viable cells (cells that did not take up any dye) was quantified, using a light microscope and haemocytometer and was expressed as a percentage of the total cell count.

3.3 Results

3.3.1 Chemical and physical characterisation of magnetic chelator supports and starting materials.

Magnetic chelator supports and amine-terminated starting materials were subjected to numerous tests prior to their use in batch binding experiments with the MAb's.

Figure 3.2 shows particle size distributions for the homemade SIN-type and BioMag® 4100B amine terminated materials and the magnetic chelators prepared from these materials. The particle distribution for amine terminated SIN type particles and for BioMag® 4100B is approximately the same 0.5μm-1.5μm.

Furthermore in simple tests with laboratory magnetic racks no differences between the commercial and homemade supports were observed. However the SIN type particles were darker in colour (black-brown) than the BioMag® 4100B (brown with slight reddish tint), and the free exposed amine densities of the SIN-type material were roughly half those of the commercial amine terminated supports (data not shown).

Following polyglutaraldehyde coating, epoxidation and IDA coupling the particle sizes for both types of metal chelating supports (i.e. those prepared from SIN type and BioMag® 4100B materials) were slightly higher (closed symbols) and there is some evidence for particle agglomeration resulting in a second peak at ~2-3μm.

The IDA ligand densities measured using Zn^{2+} and Ni^{2+} ions were approximately 90-100μmoles/g for the magnetic chelators prepared from homemade SIN type particles ('In house' magnetic chelators) compared to ~200μmoles/g for magnetic chelators prepared from BioMag® 4100B (commercial or BioMag magnetic chelators). The colour and behaviour of both types of magnetic chelator remained similar to that of the respective starting materials. Physical properties of the starting materials and magnetic chelators are summarised in Table 3.1.

Although the particle sizes of the two magnetic chelators are similar the difference in amine and ligand densities would suggest a difference in surface area (4.6).

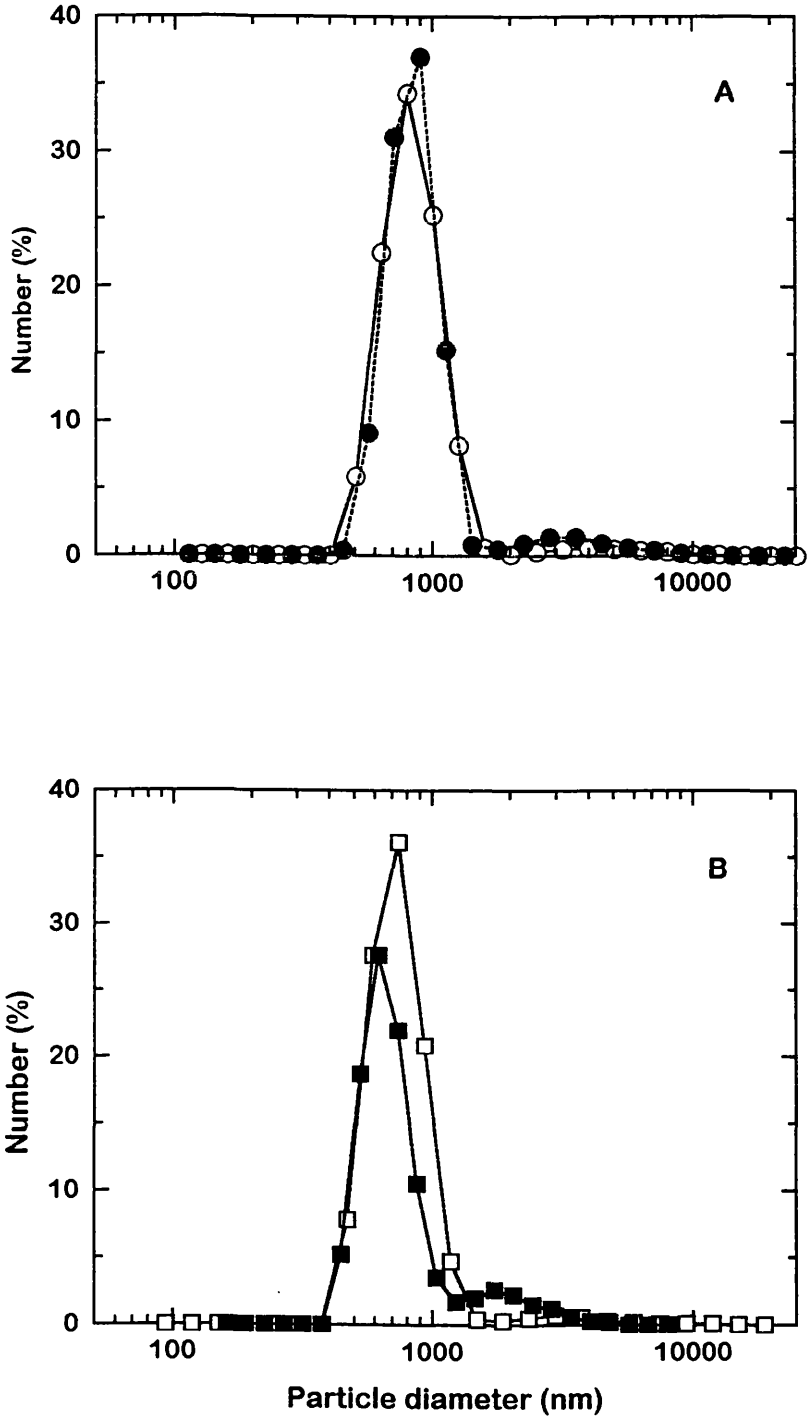


Figure 3.2 Particle size analysis of starting materials and magnetic chelators

Panel A- BioMag (○ BioMag® 4100B amine terminated material ; ● BioMag magnetic chelators)

Panel B- 'In house' (□ SIN type amine terminated material ; ■ 'In house' magnetic chelators)

Table 3.1 Physical properties of starting materials and magnetic chelators

Information	BioMag® 4100B	'In house' SIN type
Particle size range (μm)	0.5-1.5	0.5-1.5
Mean Particle Size (μm)	0.8	0.8
Specific surface area (m^2/g)	>100[a]	nd
Packing density (g/mL)	0.25-0.35	0.25-0.35
Ligand density (μmoles/g) Zn^{2+} / Ni^{2+}	200 / 200	90 / 100

[a] Manufacters data

nd not determined

O'Brien (1996) demonstrated that the majority (95.8%) of functional amine groups i.e. silane groups, were coated and blocked by the polyglutaraldehyde coat. The inclusion of sodium borohydride in the epoxidation step is believed to convert the aldehyde functions to hydroxyl functions. The final epoxide density of supports epoxide activated without sodium borohydride was significantly lower than in the presence of this reducing agent (S.M. O'Brien pers. communication). A sharp decrease in the number of epoxide groups after coupling of IDA (O.R.T. Thomas pers. communication) illustrates that the coupling step was effective, remaining epoxide groups were blocked by the addition of ethanolamine. This evidence together with the observation of specific metal ion mediated affinity towards MAb's suggests that the preparation scheme (Figure 3.1) is an accurate description of the surface chemistry of the magnetic chelators.

3.3.2 General features of the binding of MAb1 to BioMag magnetic chelator particles.

In initial studies presented here BioMag magnetic chelators prepared using the commercially available amine-terminated iron oxide particles (BioMag® 4100B) as the starting material were employed in batch adsorption studies with MAb1.

Equilibrium adsorption experiments were carried out using a range of monoclonal antibody input concentrations and zinc and nickel charged BioMag magnetic chelators at a fixed concentration of ~2mg/mL (arbitrarily chosen on the basis of previous studies by O'Brien *et al.*, 1996; submitted). The results of these studies are presented in Figure 3.3. The data derived from these experiments was plotted in 2 ways. Figure 3.3a gives plots of binding capacity at equilibrium, Q*, versus the input MAb concentration, C_0. Plots of this kind are useful for rapidly estimating the working capacities at a given MAb input concentration. Equilibrium adsorption isotherms are shown in Figure 3.3b. Q*, the amount of protein bound per g of support at equilibrium is plotted against C*, the liquid phase or 'free' antibody concentration at equilibrium. The data were fitted using the simple Langmuir model (Equation- 2.1) using the Microcal® Origin V3.5 'Best fit hyperbola' program.

Langmuir constants Qmax and Kd derived from the Q* vs. C* plots in Figure 3.3b and working capacities at 100 and 500μg/mL MAb obtained from Q* vs. C_0 plots in Figure 3.3a are summarised in Table 3.2.

Both zinc and nickel charged magnetic chelators bound MAb1. No non-specific binding of MAb1 was observed for the uncharged supports. This observation agrees well with previous studies using Zn^{2+} charged chelating perfluorocarbons and confirms that the binding to metal charged supports is mediated by immobilised metal ions. The maximum binding capacity figures for the nickel charged support (121.1mg/g) were nearly twice that of the zinc charged particles (69.5mg/g) and the Kd values were much lower (~10^{-7}M c.f. $2x10^{-6}$M). Furthermore the initial slope (Qmax/Kd) of the adsorption isotherm, describing the binding of MAb1 to nickel charged supports, was nearly 40 times greater.

This has important implications for the anticipated operational performance which is reflected in the differences in working capacity values for the zinc and nickel charged adsorbents.

The concentration of MAb typically found in CHO culture broth at the end of batch and fed-batch fermentation's are <100μg/mL and 500μg/mL respectively. At a MAb titre of 500μg/mL therefore the working capacity (Qw) for nickel charged supports are ~3 times higher (105 c.f. 35mg/g) and rise to nearly six times greater (58 c.f. 10mg/g) at an MAb input concentration of 100μg/mL.

Table 3.2 Summary of binding features of MAb1 for BioMag magnetic chelators

Parameter	Zn^{2+} charged	Ni^{2+} charged
Qmax (mg/g)	69.5	121.1
Kd (μM) [a]	1.96	0.09
Initial slope (Qmax/Kd)[a]	3.54×10^{1}	1.34×10^{3}
Working capacity at 500μg/mL MAb (mg/g) [b]	35	103
Working capacity at 100μg/mL MAb (mg/g) [b]	10	58

[a] derived from Langmuir adsorption isotherm model Figure 3.3b

[b] interpolated from Figure 3.3a

Figure 3.3a Adsorption of MAb1 to BioMag magnetic chelators

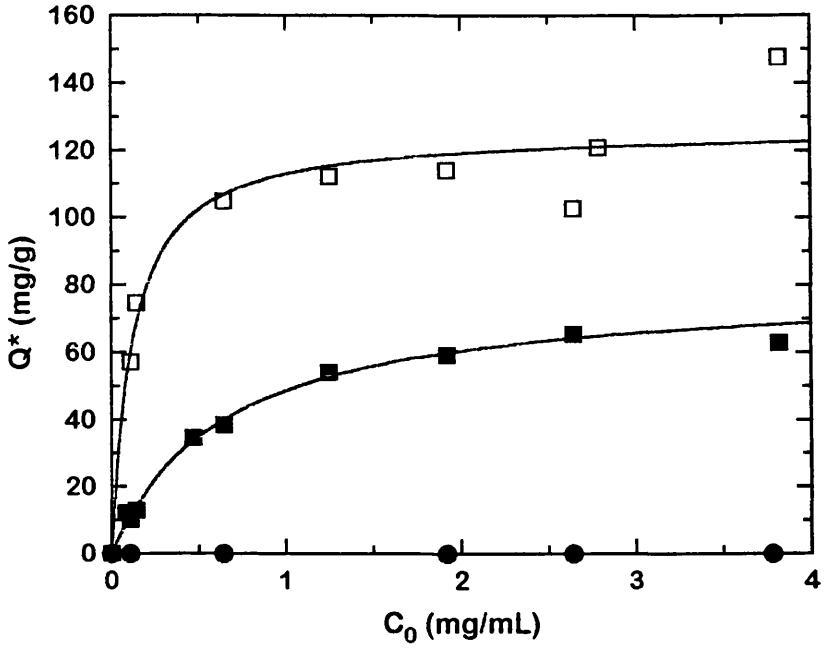
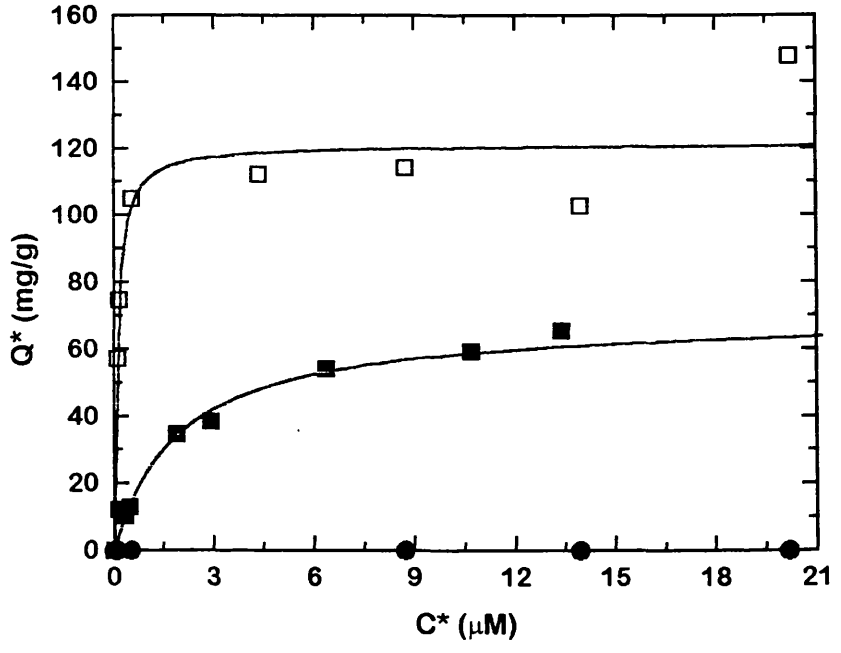


Figure 3.3b Adsorption isotherm for binding of MAb1 to BioMag magnetic chelators



Zinc ■ and Nickel □ charged and uncharged ● BioMag magnetic chelator particles were employed at 2mg/mL for the adsorption of various concentrations of MAb1 from PBS pH7.2

A series of studies were conducted to examine first, the kinetics of binding and second the concentration of support particles that would be required to achieve a high level of MAb adsorption from the bulk phase.

Figure 3.4 shows typical plots of equilibrium binding experiments (C^*/C_0 vs. time) for nickel and zinc charged BioMag magnetic chelators. For both supports equilibrium binding was achieved very rapidly in 5mins or less. Macromolecules binding to non-porous particles do not face the problems typically encountered with porous supports (e.g. transmission through pores and internal diffusion limitations) and so these rapid binding kinetics were not unexpected.

In agreement with the isotherm data, nickel charged supports were more effective than the same concentration (4mg/mL) of zinc charged particles, adsorbing ~40% of the input MAb compared to <25% for zinc charged particles.

The effective binding capacities at equilibrium (plotted in the insert to Figure 3.4) indicates working capacities for zinc and nickel charged particles under the conditions of these tests (4mg/mL particles; 500μg/mL MAb1) of 55.7±4.2mg/g (n=6) and 30.1±6.4mg/g (n=6) respectively (based on average data collected from 5-60min).

The results of batch binding experiments in which the support particle concentrations were varied from 0.5mg/mL to 13mg/mL are shown in Figure 3.5.

Clearly Ni^{2+} charged particles are significantly more effective than those charged with Zn^{2+} ions. For example the particle concentration required to bind 50% of the monoclonal antibody was ~4mg/mL for nickel charged supports compared to ~9mg/mL for zinc-IDA linked particles. This corresponds to working capacities of ~73mg and 33mg of MAb1 per gram of Ni^{2+} and Zn^{2+} charged adsorbents respectively. For true equilibrium binding, the amount of MAb1 bound should principally depend on the free MAb concentration in the liquid bulk phase. The insert in Figure 3.5 shows how the equilibrium binding capacities vary with change in support concentration.

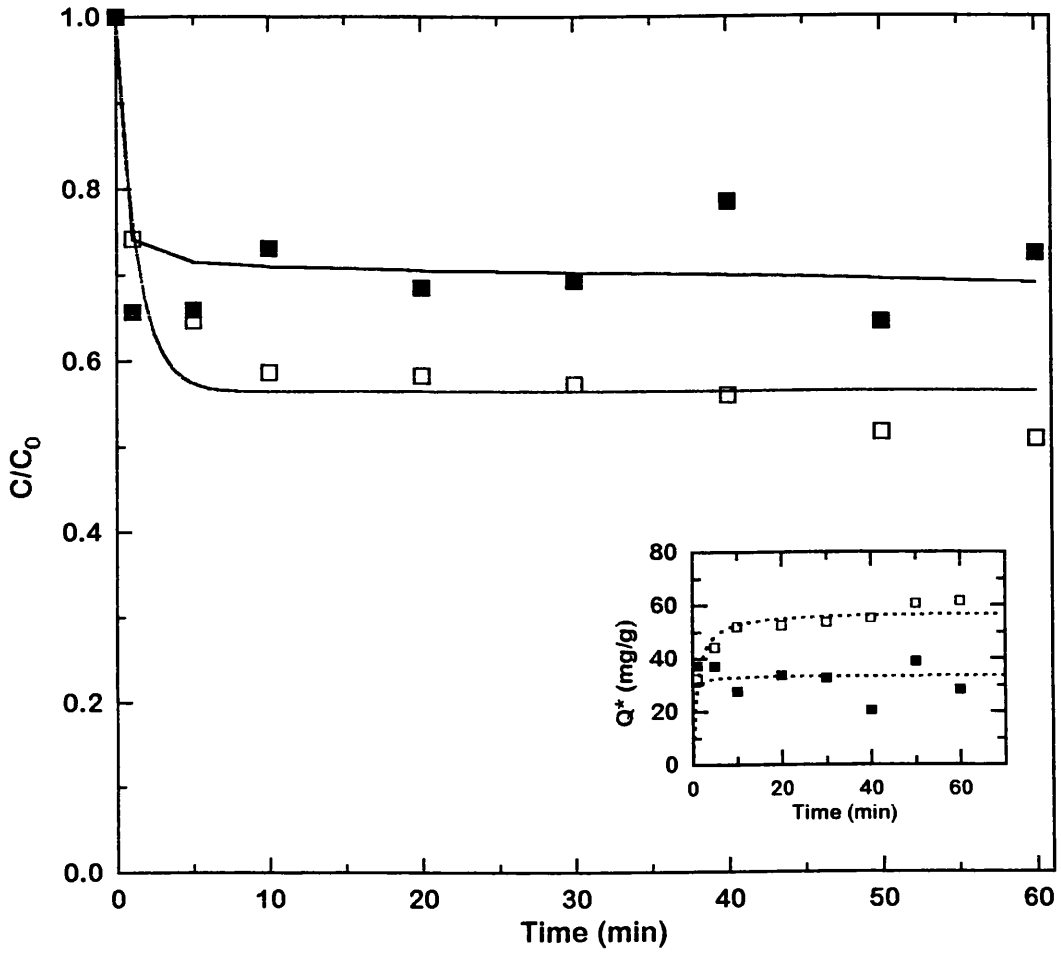


Figure 3.4 Equilibrium binding of MAb1 by zinc and nickel charged BioMag magnetic chelator particles.

MAb1 (500μg/mL) in PBS pH7.2 was contacted with Zinc ■ and Nickel □ charged BioMag particles at a particle concentration of 4mg/mL.

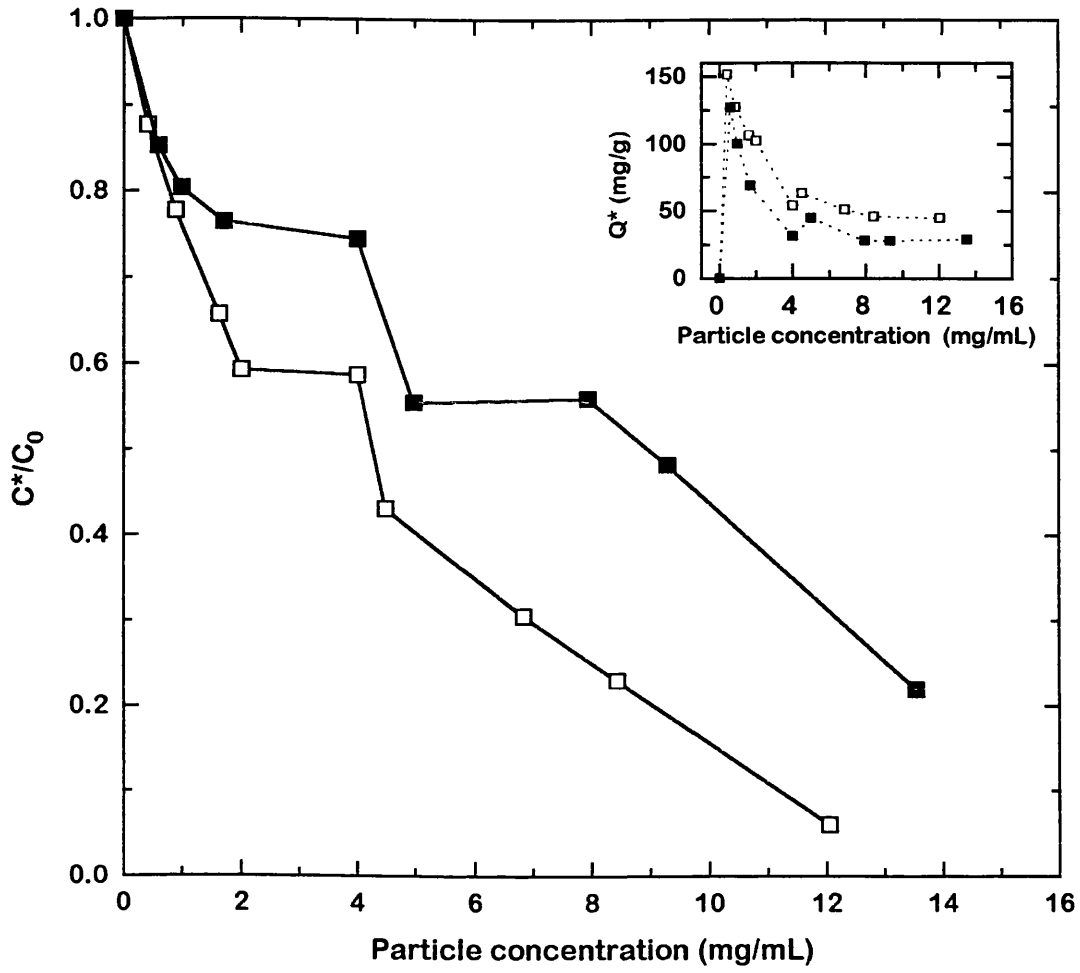


Figure 3.5 Effect of concentration of metal charged magnetic chelator upon the uptake of MAb1

MAb1 (500μg/mL) in PBS pH7.2 was contacted with Zinc ■ and Nickel □ charged BioMag magnetic chelators at various concentration (0.4-14mg/mL). The particles were contacted for one hour before C MAb1 concentrations were measured*

As expected the amounts of MAb1 adsorbed per unit weight of support were highest (~150mg/g and ~125mg/g for Ni^{2+} and Zn^{2+} charged supports) at very low particle concentrations and decreased significantly from these values however as the particle concentration was raised above 4mg/mL and the decline in working capacity of both zinc and nickel charged particles was less marked.

MAb1 could be recovered quantitatively from 'loaded' supports by selective desorption with EDTA. The results of typical binding/ desorption experiments for MAb1 with Ni^{2+} and Zn^{2+} charged BioMag magnetic chelators are presented in Table 3.3. Three washes with PBS failed to dislodge adsorbed MAb and only two washes with PBS 12mM EDTA were required to recover approximately 99% of the bound MAb. Furthermore ~85% was eluted with one wash of the EDTA solution and a further ~14% in the second.

Table 3.3 Recovery of MAb1 from BioMag magnetic chelators.

	MAb1 content (μg)		% recovery	
	Ni^{2+}	Zn^{2+}	Ni^{2+}	Zn^{2+}
Wash 1 PBS pH7.2	nd	nd		
Wash 2 PBS pH7.2	nd	nd		
Wash 3 PBS pH7.2	nd	nd		
Wash 4 PBS pH7.2 12mM EDTA	125	69	85.6	84.2
Wash 5 PBS pH7.2 12mM EDTA	20	12	13.7	14.6
Wash 6 PBS pH7.2 12mM EDTA	nd	nd	nd	nd

Zn^{2+} and Ni^{2+} charged BioMag magnetic chelators 'loaded' with MAb1 were employed in batch desorption studies. MAb1 content of the washes was analysed by Bradford assay (3.2.4.4). Experimental error is estimated as approximately 10%.

3.3.3 General features of the binding of MAb2 to magnetic chelator particles.

Following these promising studies with MAb1, attention was switched to another IgG1, MAb2, since the continued supply of pure MAb1 and CHO fermentation's producing this MAb could not be guaranteed.

Pseudoaffinity adsorption of MAb2 to zinc and nickel charged magnetic chelators was investigated as described above (3.2.3.6). The results are plotted in Figure 3.6a and Figure 3.6b, data derived from these figures is presented in Table 3.4. As for MAb1, no adsorption of MAb2 to uncharged supports was noted. Not surprisingly experimentally determined Qmax and Kd values; describing the adsorption of MAb2 to zinc and nickel charged supports were very similar to those obtained with MAb1.

Table 3.4 Summary of binding features of MAb2 for BioMag magnetic chelators.

Parameter	Zn^{2+} charged	Ni^{2+} charged
Qmax (mg/g)[a]	68.8	131.3
Kd (μM)[a]	1.39	0.17
Initial slope (Qmax/Kd)[a]	4.9×10^{1}	7.7×10^{2}
Working capacity at 500μg/mL MAb (mg/g)[b]	44	104
Working capacity at 100μg/mL MAb (mg/g)[b]	15	55

[a] derived from Langmuir adsorption isotherm model Figure 3.6b
[b] interpolated from Figure 3.6a

Figure 3.6a Adsorption of MAb2 to BioMag magnetic chelators

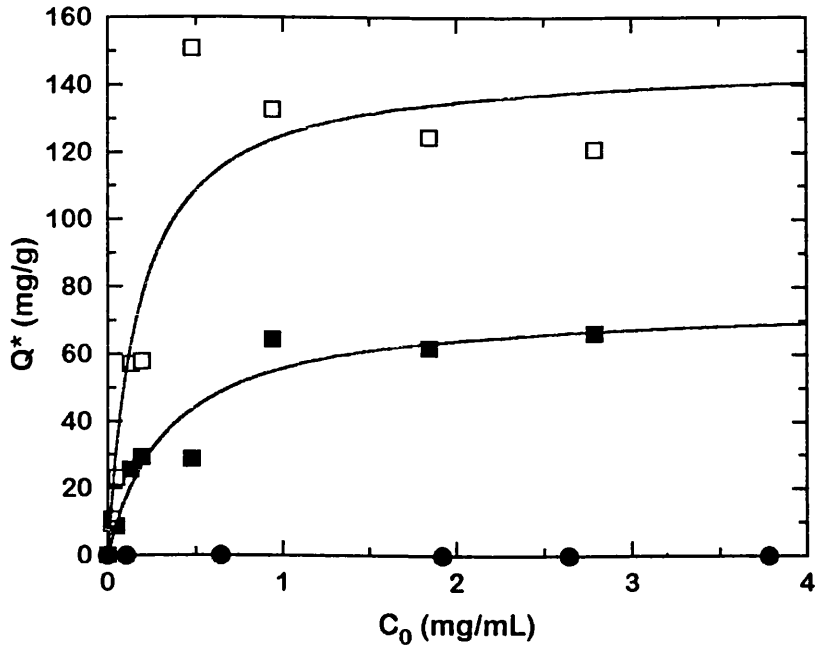
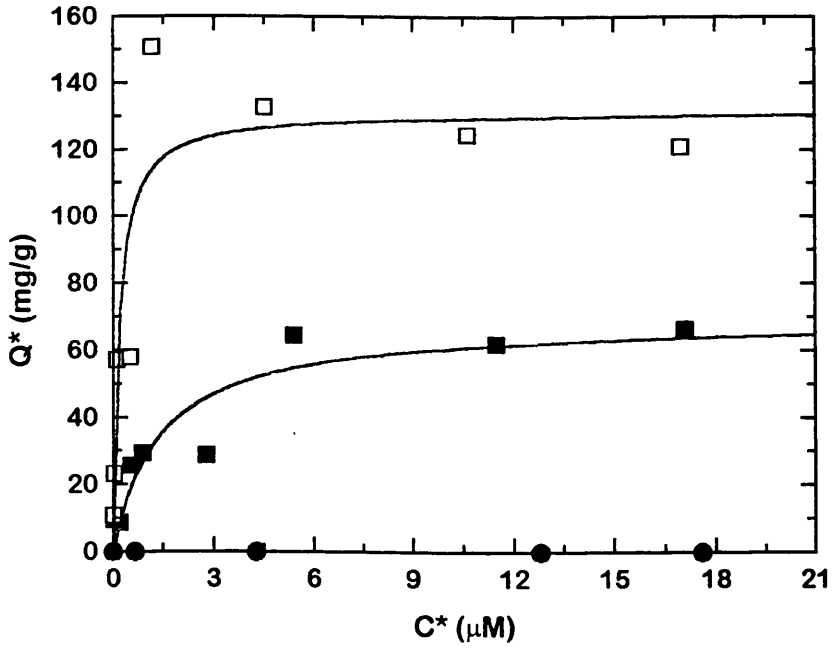


Figure 3.6b Adsorption isotherm for binding of MAb2 to BioMag magnetic chelators



Zinc ■ and Nickel □ charged and uncharged ● BioMag magnetic chelator particles were employed at 2mg/mL for the adsorption of various concentrations of MAb2 from PBS pH7.2

In the studies described thus far, the recovery of monoclonal antibodies using magnetic supports has been carried out using phosphate buffered saline as the suspending medium. Table 3.5 summarises the results of experiments in which the performance of magnetic chelators was investigated in more complex environments: MAb2 'spiked' into sterile CHO culture medium at concentrations of 100μg/mL and 500μg/mL and batch fermentation's (MAb2 titre of 79.2μg/mL) containing viable CHO cells. BioMag magnetic chelator particles were then challenged with these solutions. Control experiments using PBS as a suspending buffer were also carried out and the results of these are included for comparison.

In all cases the adsorption kinetics meant that equilibrium binding was reached quickly (within 5min) as was to be expected from previous studies with MAb1 (3.3.2). Figure 3.7 shows a typical profile of the uptake of MAb2 from crude CHO culture containing viable cells.

In the absence of MAb2 negligible amounts of CHO culture medium proteins were adsorbed onto Zn^{2+}, Ni^{2+} or even uncharged magnetic chelator supports. Comparison of the amounts of MAb2 bound by zinc charged supports in PBS and CHO Medium, at both MAb input concentrations, indicate no drop in binding capacity. The same cannot be said for nickel charged supports where there is a drop in working capacities (Qw) on switching from PBS to medium. At input concentrations of 100μg/mL and 500μg/mL the working capacities drop from 58mg/g to 16.5mg/g and from 109 to 45mg/g respectively.

The working capacities of zinc charged BioMag magnetic chelators challenged with crude CHO culture broth containing cells were very similar to those of the same support with either PBS or CHO culture medium. The amounts of MAb2 bound by nickel supports presented with crude CHO broth were similar to those obtained with supports challenged with CHO culture medium containing spiked MAb2 at comparable levels. Taken collectively these results suggest that the presence of suspended cells has little effect on the adsorption of MAb2 but that some component of CHO culture medium adversely affect the nickel charged supports.

Table 3.5 Effect of suspending medium on the working capacities (Qw) of charged BioMag magnetic chelator supports.

Solution	MAb1 (μg/mL)	Soluble protein (μg/mL)	Qw (mg/g) Zn^{2+} charged		Qw (mg/g) Ni^{2+} charged	
			Antibody	Protein	Antibody	Protein
PBS	100	nd	10 ± 1.6	nd	58 ± 6.4;	nd
PBS	500	nd	34± 5.3	nd	109 ± 12.1	nd
Media	0	741	0	<2	0	<2
Media	100	810	10.5 ± 2.1	nd	16.5 ± 4.3	
Media	500	1273	35 ± 3	nd	44.5 ± 4	
Crude	79	1366	10 ± 2.4	12	13 ± 1.5	16

Zn^{2-} and Ni^{2+} charged BioMag magnetic chelator particles (1.5-2mg/mL) were employed in batch binding experiments. All values are given as mean ± sample standard deviation (n=6). MAb2 was 'spiked' into sterile CHO culture media at 100 and 500μg/mL. Crude batch fermentations containing viable CHO cells and MAb2 (79.2μg/mL). Specific MAb uptake was measured by HPLC and soluble protein uptake was measured by Bradford assay nd not determined

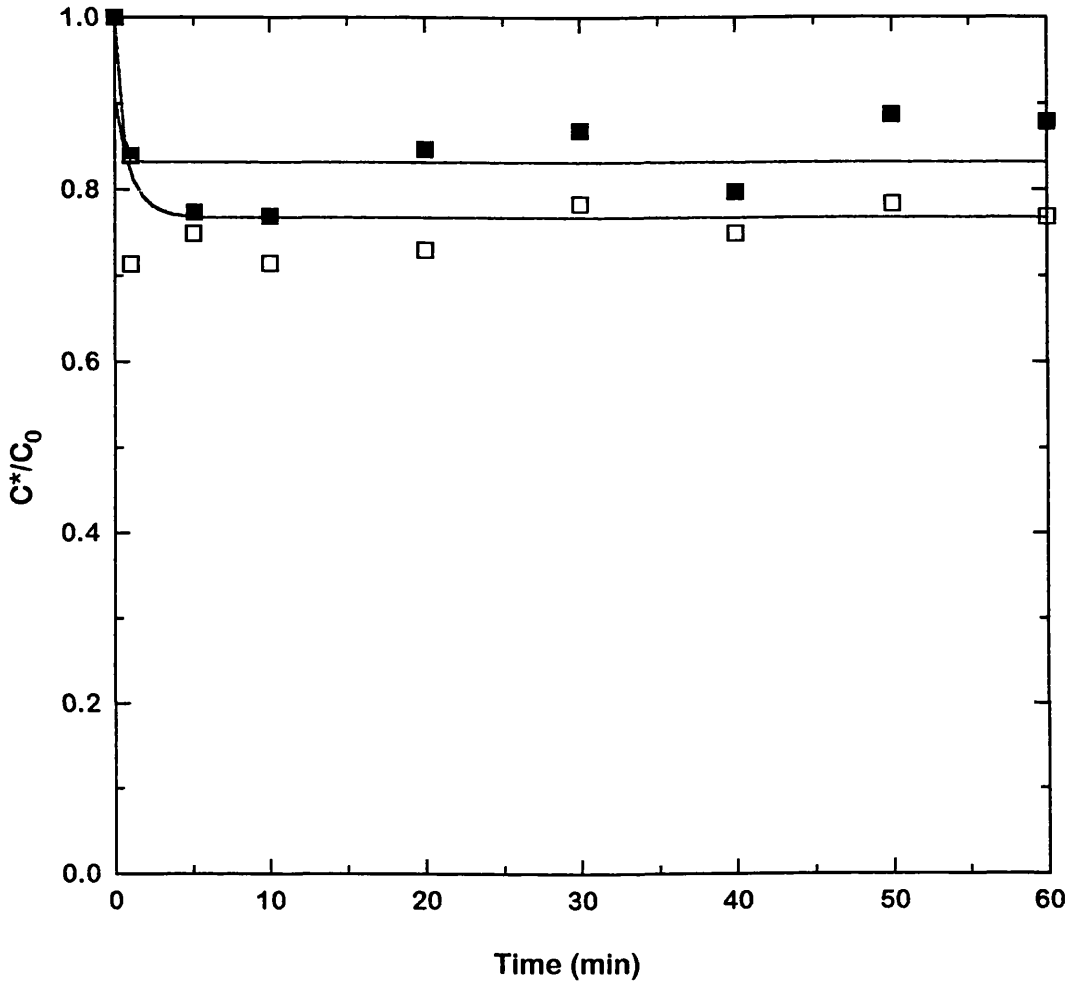


Figure 3.7 Uptake of MAb2 from CHO culture by BioMag magnetic chelators

Zinc ■ and Nickel □ charged BioMag magnetic chelators (1.5mg/mL) were contacted with crude CHO culture broth containing cells. The MAb2 titre was 79.2μg/mL.

Measurements of ligand density of the magnetic supports before and after binding tests suggested that nickel ions were selectively desorbed from the magnetic chelators by some component or components of the CHO medium. The ligand density of nickel charged supports dropped from 200μmoles/g to 100μmoles/g at the end of the test and was accompanied by nickel appearing in the medium. Zinc charged supports were less affected with ligand densities dropping from 200μmoles/g to 186μmoles/g.

Desorption studies of MAb2 from 'loaded' BioMag magnetic chelators challenged with crude CHO culture containing cells are summarised in Table 3.6. Two washes with PBS removed some loosely bound or entrapped protein (~17% for zinc and ~21% for nickel charged) but no MAb2 was detected. Approximately 99% of the bound MAb2 was recovered from either Zn^{2+} or Ni^{2+} charged supports. With an estimated purification factor of 17. The purity of the MAb was calculated on the basis of protein content and was not analysed by SDS-PAGE.

Table 3.6 Recovery of MAb2 from 'loaded' BioMag chelators challenged with crude CHO culture

	% of bound recovered			
	Zinc charged		Nickel charged	
	MAb2	Protein	MAb2	Protein
Wash 1 PBS pH7.2	nd	11.9	nd	14.8
Wash 2 PBS pH7.2	nd	5.2	nd	5.4
Wash 3 PBS pH7.2	nd	nd	nd	nd
Wash 4 PBS pH7.2	nd	nd	nd	nd
Wash 5 PBS pH7.2 12mM EDTA	86.7	73.1	75.9	61.2
Wash 6 PBS pH7.2 12mM EDTA	12.4	9.5	23.5	18.5
Wash 7 PBS pH7.2 12mM EDTA	nd	nd	nd	nd

BioMag magnetic chelators (1.5mg/mL final concentration) were incubated with crude CHO culture containing cells (MAb2 titre: 79.2μg/mL) for 60min. Supports were magnetically recovered and washed in the above solutions.

3.3.4 Pseudoaffinity adsorption/desorption studies of MAb2 with 'In house' magnetic chelators

All of the experiments described above (3.3.2; 3.3.3) have been carried out with magnetic chelator supports prepared using the commercially available amine terminated starting material, BioMag® 4100B. Due to the high cost per g of this support material and given the lack of alternative base particles with the necessary characteristics for an effective magnetic affinity adsorbent, the production of 15g batches of SIN-type particles was undertaken, according to the patent which describes the preparation of BioMag® 4100B (Josephson, 1987).

Magnetic chelators based on the amine terminated SIN particle were prepared and examined in the same way as described for magnetic chelators based on the BioMag® particle. The results of adsorption isotherm experiments with these 'in house' supports and MAb1 and MAb2 are presented in Figure 3.8a and 3.8b and the important binding parameters are summarised in Table 3.7. For these supports the values of Qmax were lower than those of BioMag chelators by a factor of ~1.3 for zinc and ~1.5 for nickel charged adsorbents. However similar trends in the binding behaviour of MAb's 1 and 2 to 'In house' magnetic chelators were observed as with the BioMag chelators (3.2.3.6). Again the binding of MAb's to Ni^{2+} charged supports in PBS is superior in terms of both Qmax and Kd values than Zn^{2+} charged supports. No binding of the MAb to uncharged supports was observed.

Table 3.7 Binding parameters of Zn^{2+} and Ni^{2+} charged 'In house' magnetic chelator supports for MAb1 and MAb2

Parameter	Zn^{2+} MAb1	Zn^{2+} MAb2	Ni^{2+} MAb1	Ni^{2+} MAb2
Qmax (mg/g)[a]	51.6	52.8	78.5	84.9
Kd (μM)[a]	0.68	0.47	0.12	0.27
Initial slope (Qmax / Kd)	75.8	112.3	654.2	314.4
Working capacity at 500μg/mL (mg/g)[b]	43	43	69	73
Working capacity at 100μg/mL (mg/g)[b]	26	23	35	42

[a] derived from Langmuir adsorption isotherm model Figure 3.8b
[b] interpolated from Figure 3.8a

Figure 3.8a Adsorption of MAb1 and MAb2 to 'In house' magnetic chelators

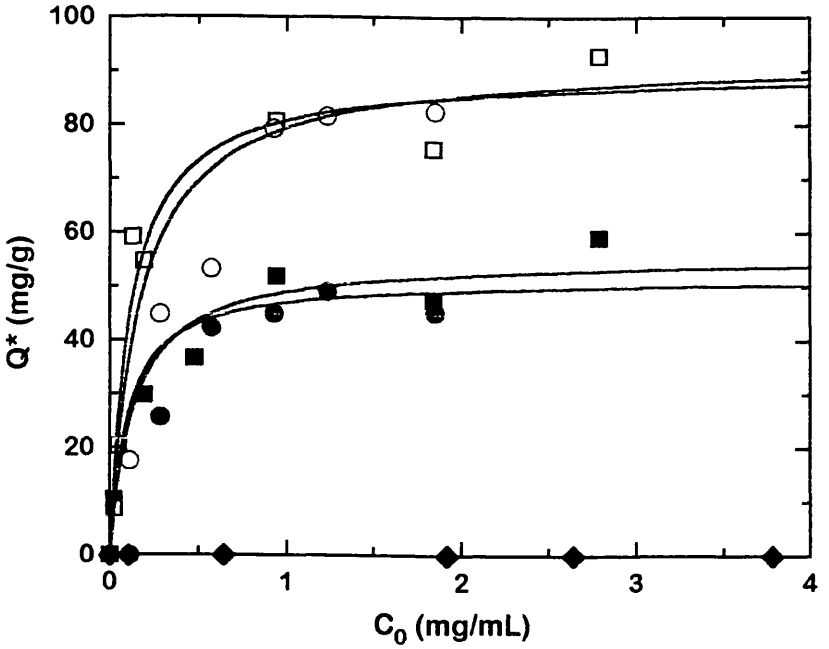
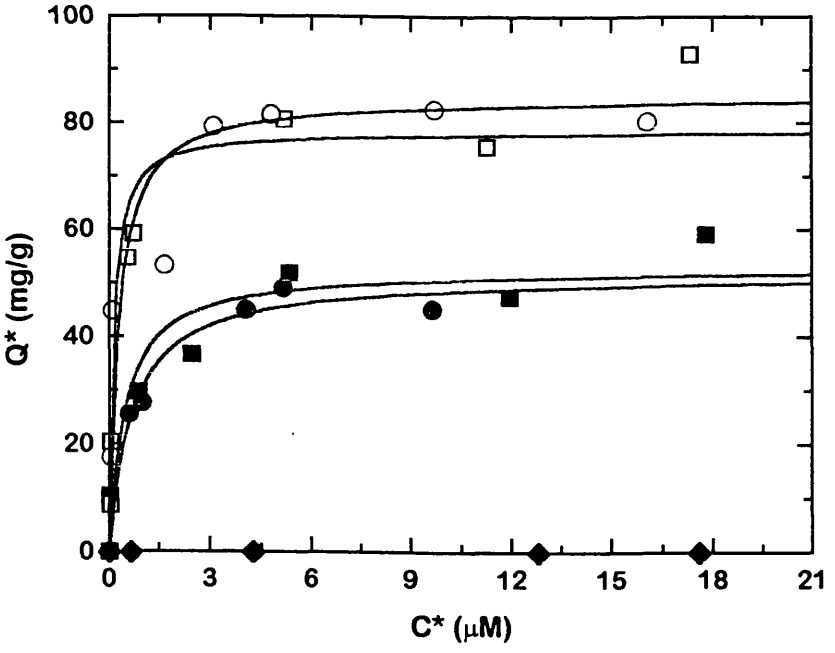


Figure 3.8b Adsorption isotherm for binding of MAb1 and MAb2 to 'In house' magnetic chelators



'In house' magnetic chelators were employed at a final concentration of 2mg/mL.

MAb1 in PBS pH7.2 was incubated with zinc ■ and nickel □ charged 'inhouse' particles.

Zinc ● and Nickel ○ charged 'In house' adsorbents were contacted with MAb2 in PBS pH7.2.

Uncharged supports ◆ were also employed.

Having confirmed that the 'In house' SIN-type magnetic chelators behaved in a similar way to the BioMag magnetic chelators, 'in house' magnetic chelators were applied directly to the recovery of MAb2 from a crude high titre (428μg/mL) fed batch fermentation broth containing suspended cells. Various concentrations (0.3-4.5mg/mL) of 'In house' magnetic chelators were added to crude CHO broth containing cells and were incubated for various times from 5min to 15h. The results of these experiments are summarised in Table 3.8.

As expected from previous studies, equilibrium binding was achieved very rapidly, within 5min (data not shown).

Table 3.8 Effect of concentration of 'In house' magnetic chelators on uptake of MAb2 from crude CHO broth.

	Working capacity- Qw (mg/g)		
Particle concentration (mg/mL)	Uncharged	Zinc charged	Nickel charged
0.3	0	77.5 ±7.6	96.1 ± 11.1
1.0	nd	31.7 ± 7.5	nd
1.5	nd	33.1 ± 8.3	nd
4.5	2 ± 1	32.0 ± 5.6	37.9 ± 5.5

'In house' magnetic chelators at various concentrations were contacted with crude CHO culture (MAb2 titre: 428μg/mL) containing cells. Uptake was measured as the difference between C_0 and C* MAb2 content. All values given as mean ± standard deviation (n=6) nd- not determined

Uncharged supports failed to bind the MAb and nonspecific binding of proteins other than MAb2 to uncharged and zinc charged supports was at insignificant levels. The highest working capacities (77.5 and 96.1mg/g for zinc and nickel charged supports) were noted at the lowest particle concentration (0.3mg/mL). However the Qw's of zinc charged supports at the higher particle concentrations examined were similar ~32-33mg/g. At the highest particle concentration employed (4.5mg/mL) 34% and 39% of the MAb2 were adsorbed from the crude CHO broth by zinc and nickel charged particles respectively. Moreover excellent recovery (~99%) of the MAb was achieved following sequential washes with PBS to remove loosely adsorbed and entrained proteins and elution with PBS 12mM EDTA (Table 3.9). An estimate of purity given that the protein content is equivalent to the MAb2 content is ~99%, a purification factor of ~3 for the recovery from high titre fermentations.

Table 3.9 Recovery of MAb2 from 'In house' magnetic chelators challenged with crude CHO broth.

	% of bound recovered			
	Zinc charged		Nickel charged	
	MAb2	Protein	MAb2	Protein
Wash 1 PBS pH7.2	nd	22.6	nd	11.5
Wash 2 PBS pH7.2	nd	6.1	nd	10.6
Wash 3 PBS pH7.2	nd	nd	nd	nd
Wash 4 PBS pH7.2	nd	nd	nd	nd
Wash 5 PBS pH7.2 12mM EDTA	85.1	62.1	86.2	70.5
Wash 6 PBS pH7.2 12mM EDTA	14.0	9.2	12.9	7.3

'In house' magnetic chelators were employed at a final concentration of 1.5mg/mL MAb2 titre and contacted with CHO fermentation (428μg/mL MAb2) for 60min. Supports were magnetically recovered and washed in the above solutions

Atomic absorption measurements demonstrated that during these batch binding experiments the ligand densities of nickel and zinc charged supports fell from 100 to 40μmoles/g and from 90 to 85μmoles for Ni^{2+} and Zn^{2+} charged supports respectively. These results confirm earlier observations of the preferential desorption of Ni^{2+} ions from magnetic chelators by a component/s of the CHO medium (3.3.2; 3.3.3).

3.3.5 The effect of magnetic chelator particles upon the viability of CHO cells.

The effects of contacting CHO cells in culture broth with magnetic particles on the viability of the cells was also examined. Zinc charged SIN-type chelator particles were incubated with crude CHO culture from a fed batch fermentation producing MAb2 for various times and the results of these tests are presented in Figure 3.9. The cell viability were initially very low ~10% and the measurements of cell viability were erratic. However compared to controls with no support added the viability would appear to be unaffected by the presence of magnetic chelator particles (insert to Figure 3.9).

This observation is supported by other experiments (data not shown) for nickel charged and uncharged 'In house' supports and for BioMag based magnetic chelators.

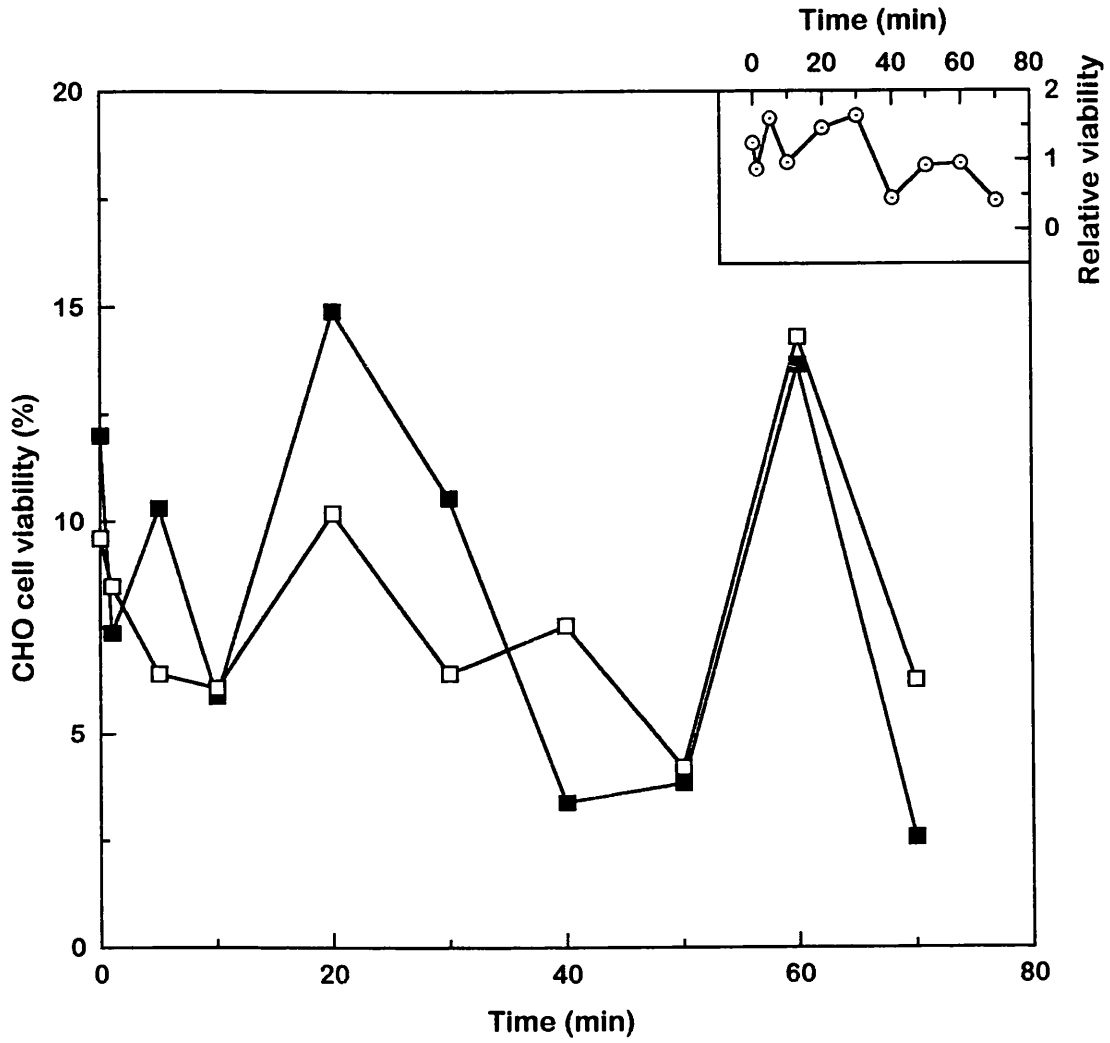


Figure 3.9 Effect of magnetic particles upon viability of CHO cells

Zn^{2+} charged 'In house' magnetic chelators at 1mg/mL −■− *and a Control* −□− *with no added magnetic chelator (400mL crude CHO culture containing viable cells) were incubated on a Luckham R100 shaker at room temperature. Measurements of cell viability were carried out in duplicate*

3.4 Discussion

3.4.1 Characteristics of magnetic chelators

A considerable amount of work has been undertaken in these laboratories to optimise the ligand densities of chelating magnetic supports using commercial BioMag preparations (O'Brien *et al.*, 1994; 1996; O'Brien 1996). These optimum conditions were routinely used to coat and derivatise the commercial magnetic chelators and 'in house' magnetic chelators described here. For the BioMag magnetic chelators ligand densities of 200μmoles Ni^{2+} or Zn^{2+}/g support were routinely achieved. Despite using the same conditions for preparation the 'in house' magnetic chelators had lower ligand densities (90-100μmoles Me^{2+}/g support). The particle size of the two starting materials and the final magnetic chelators are little different but perhaps results can be explained by differences in the iron oxide crystal size (4.6)

The ligand densities of the chelating magnetic supports are higher than those recorded by O'Brien (1996). This is due to differences in the solutions used for charging the supports. O'Brien (1996) charged the supports with metal salt solutions in 0.1M acetate buffer 1M NaCl pH 3.8 and then washed them in the same buffer to remove loosely bound metal ions. In fact if Ni^{2+} or Zn^{2+} charged supports are washed with 0.1M acetate buffer 1M NaCl pH 3.8. virtually complete desorption of the bound metal occurs. In contrast Cu^{2+} which has a much greater affinity for IDA than either Ni^{2+} or Zn^{2+}, remains bound under these conditions.

3.4.2 Binding of monoclonal antibodies by metal charged magnetic chelators

The binding of MAb1 and MAb2 with BioMag magnetic chelator and 'in house' magnetic chelator is summarised in Table 3.10. It can be seen that the binding of the two antibodies is very similar in terms of capacity and strength of interaction. This is not an unexpected result as the antibodies are both humanised IgG1's and the basis for binding is likely to be the same (4.1.1).

The capacity of the 'In house' magnetic chelators for both antibodies is less than that of the BioMag magnetic chelators. However the Kd values of Zn^{2+}charged 'in house' supports are lower than the commercial preparation (4.1.3).

The molecular dimensions of IgG are 150Å (Berry *et al.*, 1991; Tsoka, 1996). Assuming that loose cubic packing of the IgG is adopted and there is no change in the molecular footprint of the IgG a maximum binding capacity of 130mg MAb/g of support corresponds to a surface area/g of $118m^2/g$. The similarity between this figure and the effective surface area of the BioMag magnetic chelators as quoted by the manufacturers ($<100m^2/g$) indicates that fairly tight packing of the MAb on the support surface is achieved. O'Brien *et al.* (submitted) estimated monolayer surface coverage of magnetic chelator particles as $\sim120m^2/g$ using cytochromes *c* as probes. In contrast the same calculation for the 'In house' magnetic chelators yields a value of $\sim75m^2/g$.

The binding of the MAb's to Me^{2+} charged magnetic chelators is adequately described by the Langmuir model (Langmuir, 1916) and was not improved further by using the biLangmuir model (Todd *et al.*, 1994). Furthermore from Scatchard plots (not shown) the mode of binding did not appear to deviate from Langmuir binding. The ligand spacing would suggest that there is potential for the interaction of MAb with more than one immobilised metal ion. Ligand spacing has been calculated as 9Å for BioMag magnetic chelator and 12Å for 'In house' magnetic chelators. Compared to 150Å as a dimension for a MAb (Tsoka, 1996), clearly the MAb sits over a large number of potential binding sites. However the $HisX_3HisXHis$ binding motif of the MAb's, believed to be responsible for binding (4.1), will be able to interact with 1 or possibly a maximum of 2 bound metal ions simultaneously.

Table 3.10 Comparison of chelating magnetic supports for MAb1 and MAb2

Support:	BioMag magnetic chelator				'In house' magnetic chelator			
Monoclonal antibody:	MAb1	MAb2	MAb1	MAb2	MAb1	MAb2	MAb1	MAb2
Metal:	Zn^{2+}	Zn^{2+}	Ni^{2+}	Ni^{2+}	Zn^{2+}	Zn^{2+}	Ni^{2+}	Ni^{2+}
Qmax (mg/g)	69.5	68.8	121.1	131.3	51.6	52.8	84.9	78.5
Kd (μM)	1.96	1.39	0.09	0.17	0.68	0.47	0.12	0.27
Initial slope (Qmax/Kd)	3.54×10^1	4.9×10^1	1.34×10^3	7.7×10^2	7.5×10^2	1.1×10^3	6.5×10^3	3.14×10^2
Working capacity (mg/g) 500 / 100 μg/mL MAb	35 / 10	44 / 15	103 / 58	104 / 55	43 / 26	43 / 23	69 / 35	73 / 42

3.4.3 High Gradient Magnetic separation of magnetic supports

The strength of the externally applied field, the throughput rates used, the size and nature of the matrix within the canister will all affect performance of a HGMS operation (Watson and Hocking, 1975). Preliminary studies were carried out to investigate some of the above mentioned factors. Breakthrough curves were measured under varying conditions of field strength and flow rate. The volume to breakthrough indicates to a certain extent the efficiency of the operation. A typical breakthrough plot (Figure 3.10) demonstrates the effect of magnetic field upon breakthrough. The higher the applied magnetic field the longer breakthrough is delayed and the higher the capacity for the support (0T- 10mg/mL; 0.5T- 104mg/mL; 1.3T- 177mg/mL). However it proved difficult to remove the support from the ferromagnetic matrix without resorting to mechanical agitation. The higher the field used the greater the residual magnetic properties exhibited, by the HGMS matrix in particular. It therefore appears necessary to compromise between high field strength, and therefore capacity, and recovery of magnetic particles after separation. Alternatively paramagnetic matrices could be used. Compared to ferromagnetic matrices, the capacity of a paramagnetic matrix for magnetic particles is lower, but the lack of residual magnetic properties means that recovery is much more efficient (Pannu, in preparation).

Current work in our laboratories (Pannu, in preparation) has noted that the higher the throughputs used in the loading of magnetic dye supports the higher the magnetic field needs to be in order for efficiency of recovery not to be compromised, at low field strengths (0.3T) the optimal linear throughput rate is ~800cm/hr. LDH from yeast homogenate was recovered by HGMS recovery of dye chelators and subsequent washing to remove unbound material. Elution of LDH from dye magnetic supports was achieved by the incorporation of a closed loop containing elution buffer which was circulated through the canister with the field switched off in order to improve mixing. The field was then reapplied and supports magnetically recovered allowing the eluted LDH to be collected.

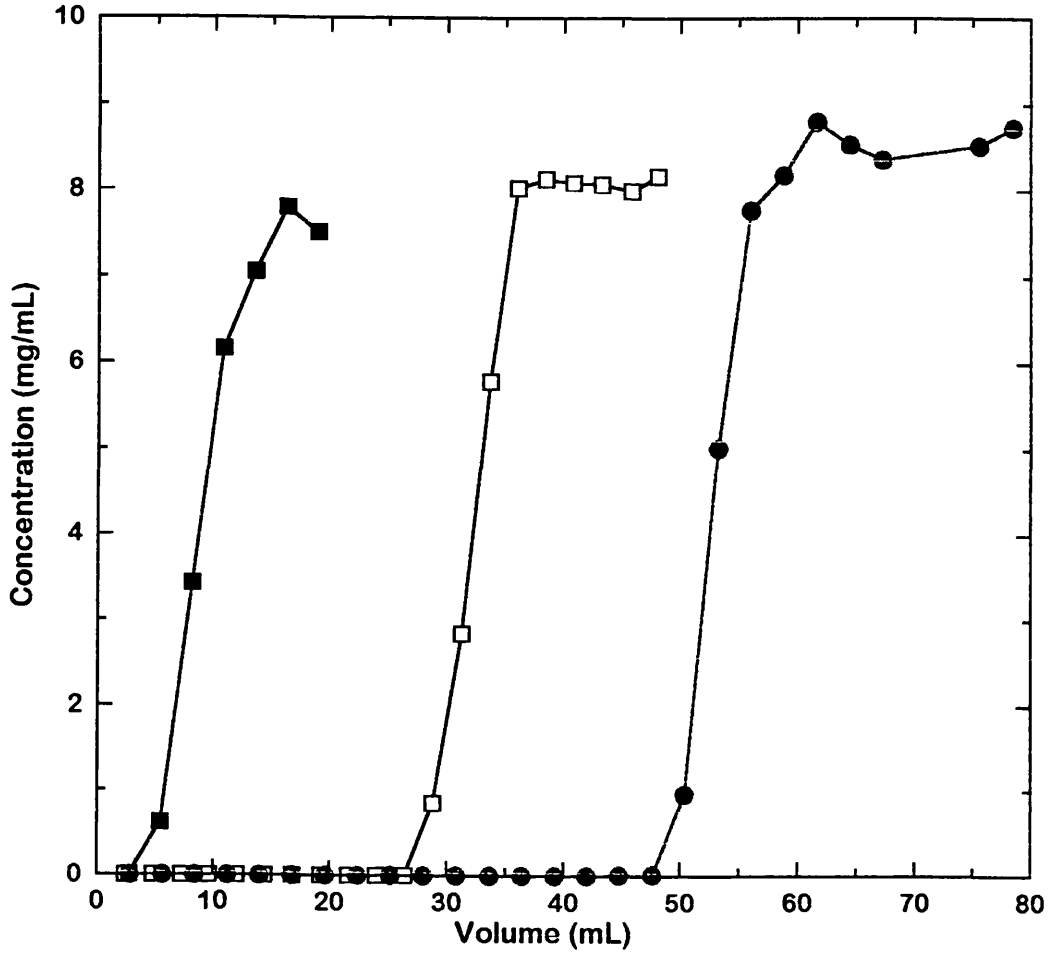


Figure 3.10 Effect of magnetic field strength on breakthrough profile for HGMS canister.

Amine terminated SIN type magnetic support in water (9-11mg/mL) were loaded at a flow rate of 2.9mL/min onto the HGMS cannister (1.1cm i.d IBF (Sepracor) column packed with ferromagnetic stainless steel wool #434 coated with polyvinyl alcohol to protect against corrosion and nonspecific adsorption- Theodossiou, 1993) at a variety of magnetic field strengths: 0T ■ ; 0.5T □ and 1.3T ●. *A variable field bipole electromagnet (Tesla Engineering, Storrington, UK.) controlled by a 20V D.C. transformer (Drake Transformers, Wickford, UK) was used to generate a magnetic field. Concentration of support at the cannister outlet was determined by measurement of optical density at 500nm and cross-reference to a calibration curve.*

Dye chelators were then recovered by flushing of the HGMS with no externally applied field at high flow rates. Ongoing studies aim to optimise the HGMS operation for process scale use (Zulqarnain *et al.*, in preparation)

A tentative calculation of antibody capacity of a HGMS canister based on the capacity of the HGMS canister for the magnetically susceptible supports (0.4g/mL) and the capacity of the support for MAb2 (50mg/g) gives a value of 20mg MAb/mL of HGMS canister. For one step process scale recovery of MAb from CHO culture (MAb titre of 500μg/mL in 1000L.) this would require the use of a 25L HGMS canister to recover 10Kg of support. The scale up of HGMS operations should be relatively easy as HGMS is used in the industrial processing of clay (Whitesides *et al.*, 1983).

Throughputs estimated at 800cm/hr for loading would mean that recovery could be achieved in one working day given appropriate design of the HGMS canister. Alternatively the batch processing of smaller canisters, the space between the poles of the electromagnet used in these studies can fit a vessel with a maximum volume of 3.4L, could be adopted.

3.5 Conclusions

From the adsorption isotherms for Zn^{2+} and Ni^{2+} charged magnetic chelators, (both 'In house' and BioMag) it would appear that Ni^{2+} charged supports offers significant advantages over the Zn^{2+} charged particles in terms of tightness of binding and working capacity. However this does not appear to be the case in the 'real system'. When MAb2 is recovered from CHO medium or from CHO fermentation's containing cells the performance of Ni^{2+} charged supports is lower than one would expect based on its binding in PBS. In contrast the performance of Zn^{2+} charged supports (in terms of working capacity) is apparently unaffected by the 'real system' compared to that in PBS pH 7.2 (Table 3.3). The reason for this 'leveling' on the Qw of the zinc and nickel charged supports may be that Ni^{2+} ions and not Zn^{2+} are selectively desorbed by some components. The added presence of cells does not appear to play a significant role

The high ligand densities of the magnetic chelators may account for the absence of nonspecific binding in crude CHO cultures. O'Brien (1996) observed that increasing the ligand density above 30μmoles Cu^{2+}/g eradicated non specific binding of proteins in *E.coli* homogenate. Possible reasons for this may be that interactions with the base matrix are minimised because of the high levels of substitution with IDA (calculated ligand spacing values of ~12Å and ~9Å for 'In house' and BioMag adsorbents respectively).

The use of these magnetic chelators for the recovery of MAb2 has been demonstrated. The application of HGMS in conjunction with the batch adsorption from crude CHO culture has great potential for the direct or primary recovery of monoclonal antibodies at a process scale.

The final chapter in this thesis is a discussion of the two types of support developed in this thesis, their application for the recovery of monoclonal antibodies from cell culture and some considerations for future work to realise the potential of these supports.

4. GENERAL DISCUSSION

This chapter is a general discussion of the chelating supports developed in Chapters 2 and 3. The application of these supports for the recovery of monoclonal antibodies is considered and areas for future work are briefly examined.

4.1 Use of IMAC for the purification of monoclonal antibodies.

The application of IMAC for the purification of monoclonal antibodies is not widely reported in the literature. Porath and Olin (1983) noted that IgG fractions of human serum bound to Ni^{2+} charged supports and Ill *et al* (1993) observed that IgG_1 antibody bound with relatively high affinity to Ni^{2+} charged IMAC columns. Using proteolytic digestion and genetic engineering techniques Hale and Beidler (1994) demonstrated that the portion of the IgG molecule responsible for binding to immobilised metal ions is near the C terminus of the CH3 region. The amino acid sequence of a variety of IgG's from different species and subclasses revealed a native histidine (**H**) rich region at this point (Table 4.1).

Table 4.1 Comparison of C-terminal sequences of IgG heavy chains from various species.

Residue number	Sequence	Species/Subclass
312	HEALHNHYTQKSLSLSPGK	Human γ1
308	HEALHNHYTQKSLSLSLGK	Human γ4
306	HEALHNHHTQKNLSRSPGK	Mouse γ3
305	HEALHNHVTQKSLSLSLGK	Rabbit γ

Table adapted from Hale and Beidler (1994). The residue numbers represent the position of the first amino acid of each peptide in the heavy chain. The last residue of each peptide represents the C-terminal amino acid of the heavy chain.

Common to all the sequences shown in Table 4.1 is a motif thought to be a likely site for IMAC interaction (**$HisX_3HisXHis$**). The $HisX_3His$ motif (where His is a histidine residue and X is any other amino acid) has been shown to be a particularly powerful motif for IMAC interactions, particularly if it forms part of an α helix (Suh, 1991; Todd *et al.*, 1991).

Both MAb's used in this thesis are IgG1's. The amino acid sequence of MAb1 reveals this same metal binding motif (Crowe *et al.*, 1992) at the C-terminus of the heavy chain and it is likely that this region is also present in MAb2 (A.J. Sheppard personal communication)

The fact that a wide variety of IgG's, including the humanised MAb's used in this study have this native metal binding site, has important implications for the generic nature of IMAC for the purification of monoclonal antibodies.

4.2 Leaching of metal from supports

It has been noted that leaching of the metals from metal charged magnetic chelators occurs in the presence of media components and that the fall in nickel density is significantly greater than the fall in zinc density.

The selective removal of nickel from metal charged chelating supports in batch mode was graphically demonstrated when chelating perfluorocarbons were examined for the recovery of MAb2 from CHO culture. Chelating MP1500 when charged with Ni^{2+} turns from a white to a green colour, indicating the presence of surface attached nickel ions. On addition to CHO culture feedstock containing CHO cells the supports quickly turned from green to white verifying the removal of nickel. Furthermore the working capacity dropped from 4.2mg/g (in PBS) to 0.15mg/g (in CHO broth). Ni^{2+} charged perfluorocarbon particles have much lower surface areas and ligand densities (10-20 fold) than Ni^{2+} charged magnetic chelators and therefore the effects of CHO culture media components are unsurprisingly greater, in terms of MAb capacity.

Andersson and co-workers have reported that certain albumins can selectively scavenge certain metal ions from IDA via high affinity sites (Andersson *et al.*, 1991).

However this is not the case for IgG's, as experiments carried out in PBS do not show any selective scavenging by the MAb's, leaving the possibility of media components selectively removing nickel in preference to zinc. There are no serum albumins in the CHO medium used in these studies (A.J. Sheppard pers. communication) and in any case the affinity of albumins for zinc is of the same order if not higher than their affinity for nickel (Andersson *et al.*, 1991).

The composition of CHO culture medium is highly complex with well over 100 different chemical constituents. Although the media recipe is proprietary information it is known that there are low levels of EDTA and histidine as well as other components such as amino acids that have some capability for metal ion chelation. The affinity between nickel and IDA according to the literature is higher than that for zinc and IDA (Arnold, 1991), but what is not known is the affinity of media components such as EDTA for Zn^{2+} and Ni^{2+}. If a component has a higher affinity for nickel than that exisiting between Ni^{2+} and IDA then selective removal can be envisaged. The complex nature of the CHO culture medium would make determining which compound is responsible difficult. In addition several media components could be acting synergistically thus complicating elucidation.

Evidence that the disruption in binding occurs in the presence of medium without cells would suggest that CHO cells do not interfere with the binding of MAb to the chelating supports. In support of this, O'Brien (1996) noted that the presence of *E.coli* cells had no effect upon the binding or a recombinant T4 lysozyme to Cu^{2+} charged magnetic supports. However components of the culture medium seriously affected the use of these adsorbents. Cu^{2+} ions were selectively desorbed and the level of binding of T4 lysozyme was much reduced.

In conclusion the presence of small molecules in the medium appears to have a dramatic effect on the binding of MAb's to Ni^{2+} charged supports. Experiments to determine the effects of individual components and the effect of dilution of CHO broth with PBS would need to be carried out in order to capitalise on the superior binding performance of nickel charged adsorbents observed in PBS.

Table 4.2 Comparison of Zn^{2+} chelating non porous adsorbents and 'conventional' porous adsorbents for the recovery of MAb1

	Magnetic chelators		Chelating perfluorocarbons		Chelating Sepharoses'	
	Biomag	In house	PTFEP	MP1500	Fast Flow	Streamline
Qmax (mg/g)	69.5	51.6	22.6	17.5	230	162
Kd (μM)	1.96	0.47	3.8	9.1	0.94	1.89
Packing Density[1]	0.3	0.3	0.4	0.4	0.06	0.08
Capacity mg antibody per unit volume packed bed (mg/mL)	20.8	15.5	9.1	7.0	13.8	12.9
Working capacity (mg/mL) at 500 / 100 μg/mL MAb	10.5 / 3	12.9 / 7.8	3.1 / 0.8	1.68 / 0.4	2.7 / 0.6	1.92 / 0.4

[1] Packing density of the chelating perfluorocarbons and chelating Sepharose's was determined by packing a 1mL HiTrap column (Pharmacia) with the support and subsequently determining the dry weight of the packed adsorbent. Packing density of the magnetic chelators is based on the capacity of a HGMS cannister (Pannu *et al.*, in preparation)

4.3 The relationship between cell viability and purification of MAb's

An understanding of the relationship between cell viability and purification of MAb's by pseudoaffinity adsorption is important to gain as ideally purification would be carried out in the presence of cells, thereby removing the need for clarification steps such as depth filtration. Direct recovery of MAb's from cell culture would only be possible if cells did not interfere with MAb adsorption to affinity supports. There is no evidence that the presence of cells affects the purification of MAb by magnetic chelators (Table 3.5). In addition preliminary studies indicate that there appears to be little effect upon the viability of CHO cells in the presence of magnetic chelators (3.3.5). These observations would need to be tested more rigorously at different viability's of CHO cells and concentrations of magnetic chelators in order for firmer conclusions to be reached. In addition indirect measurements of cell viability such as DNA or LDH release would supplement the information obtained from the dye viability method (3.2.4.7). The dye viability assay is a measure of membrane function rather than viability per se and does not take into account cells which are lysed (Petersen *et al.*, 1988). Indeed whether a cell is counted as 'dead' or is ignored as a cell fragment is subjective.

Cell viability of the CHO cells is maintained above 70% during fermentation because the presence of large amounts of dead and lysed cells is known to cause fouling of depth filtration membranes and chromatography columns (A.J. Sheppard personal communication). However this is less likely to be a problem for magnetic separations using the non-porous magnetic chelators and HGMS. In these laboratories no fouling of magnetic chelator and magnetic dye supports or HGMS matrix material has been observed when using crude yeast homogenate (Pannu, in preparation) and *E.coli* homogenates (O'Brien, 1996). In addition the majority of the proteases released from lysed CHO cells are active under acidic conditions and there is no evidence that disruption of the cells causes antibody degradation in this system (A.J. Sheppard personal communication)

4.4 Comparison of non-porous chelating adsorbents to conventional porous adsorbents

For the purposes of this discussion conventional supports are taken to be porous adsorbents commonly used in laboratory and process applications. Two 'conventional' supports were studied, chelating Fast Flow Sepharose commonly used in FPLC applications and chelating Streamline a purpose designed support for use in expanded bed operations.

A comparison of the binding of MAb1 to Zn^{2+} charged porous and non-porous chelating adsorbents is given in Table 4.2. The values for dissociation constant (Kd) can be compared directly between supports when binding the same test protein, in this case MAb1. Kd values describing the interaction between MAb1 and Zn^{2+} chelating supports are all within the 10^{-6}M range necessary for effective binding (Chase, 1988). The values for the magnetic chelators in particular compare very favourably with the porous supports.

Figure 4.1 demonstrates the relationship between ligand density and affinity for MAb1 as described by Kd. The ligand density is expressed as μmoles Me^{2+}/m^2 surface area in order to more effectively compare porous and non-porous adsorbents. The higher the ligand density the lower the value of Kd. Nickel charged supports are as expected more effective than their zinc charged counterparts.

The values for ligand density and binding capacity, in this thesis, have been determined and expressed per g dry weight. Whilst this is a very accurate way of determining such values the conventional method of expressing capacity is per mL. This is a somewhat confusing term unless it is specified that the volume (mL) is settled volume of a support and buffer solution (50:50), as is usually the case, or more informatively volume of packed bed or reactor volume. This last value implies that the adsorbent is packed in a column and ligand density or binding capacity is measured within the column.

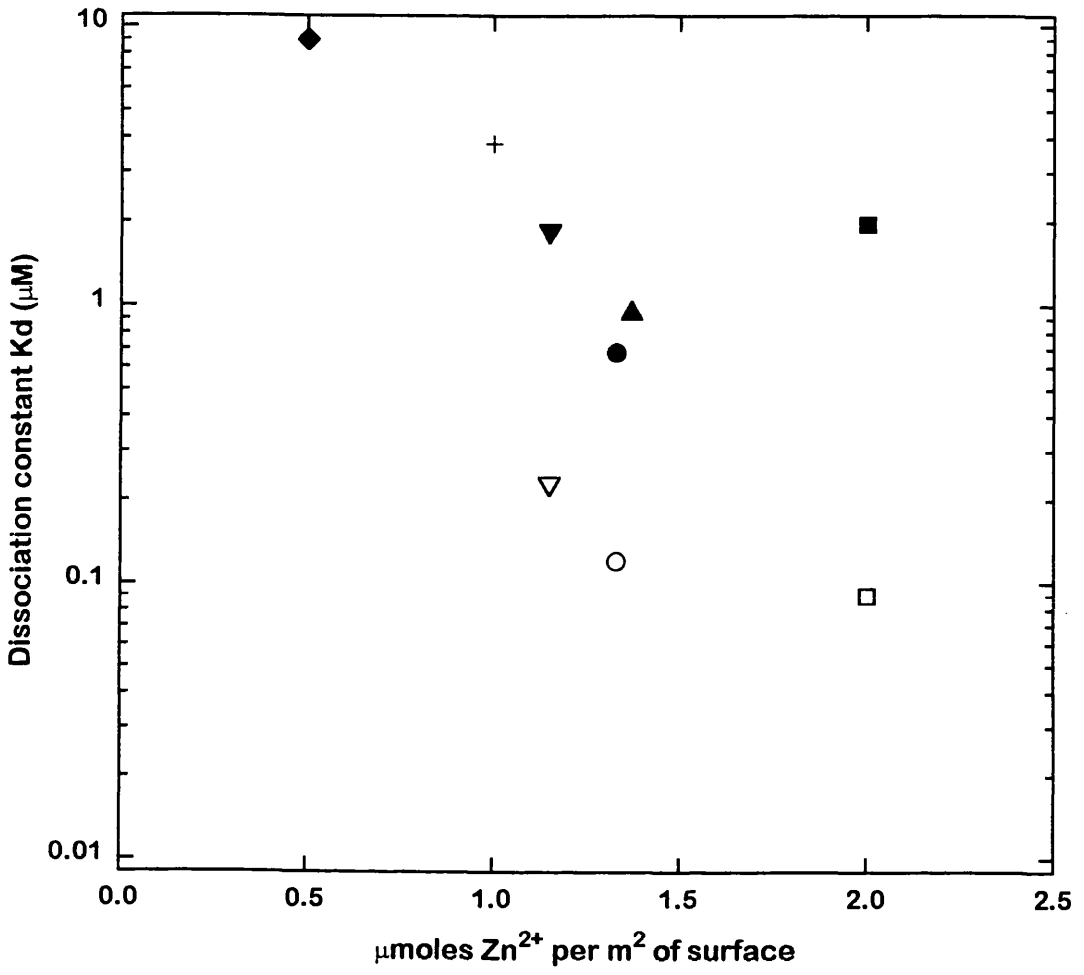


Figure 4.1 Relationship between dissociation constant (Kd) and ligand density per m^2 surface area for chelating adsorbents binding to MAb1.

♦ Zn^{2+}- chelating MP1500; + Zn^{2+}-chelating PTFEP; ▼ Zn^{2+}-chelating Streamline; ∇ Ni^{2+}-chelating Streamline; ▲ Zn^{2+}-chelating Fast Flow Sepharose; ■ Zn^{2+}-chelating BioMag magnetic chelator; □ Ni^{2+}-chelating BioMag magnetic chelator; ● Zn^{2+}-chelating 'In house' magnetic chelator; O Ni^{2+}-chelating 'In house' magnetic chelator.

The higher density of the non-porous support materials permits a higher loading of adsorbent in a packed column on a weight per unit volume basis. Calculating the binding capacity per unit volume of packed bed, or indeed of a reactor such as HGMS canister is done by consideration of the packing density of the adsorbent. The packing density is the weight of support per unit volume of the reactor (g/mL).

The capacity of Zn^{2+} magnetic chelators for MAb1 is significantly better than for the other supports. In particular the capacity of BioMag magnetic chelators is about 1.5 times higher than the porous adsorbents (~21mg/mL cf. ~14mg/mL respectively). Of particular interest for the process application of chelating adsorbents are the values for working capacity. At a MAb1 concentration of 500μg/mL or 100μg/mL the Qw of Zn^{2+} charged magnetic chelators are ~3-5 times greater than either the chelating perfluorocarbons or chelating Sepharoses'. The chelating perfluorocarbon adsorbents although less effective than the magnetic chelators do compare favorably with the chelating porous supports (chelating PTFEP; 3mg/mL and chelating Streamline 2.7mg/mL).

One potential advantage of chelating non-porous supports over porous supports is their resistance to fouling. Although fouling has not been directly investigated in this thesis there are a number of indirect observations that would suggest fouling is unlikely to be a major problem for the application of these non-porous supports. Firstly adsorption of MAb to chelating non-porous supports is determined by the presence of immobilised metal ions. As a consequence of this there is very little non-specific adsorption. This is supported by the finding that there is negligible uptake of media components by the magnetic chelators, and the recovery of relatively pure MAb from 'loaded' supports. In addition the effect of CHO cells on the uptake of MAb appears to be negligible. Munro *et al.* (1977) and Halling and Dunnill (1980) reported that fouling of non-porous supports is significantly less than that experienced with porous supports and when fouling does occur that it is easier to remove.

One other advantage associated with the non-porous nature of the supports developed here is rapid equilibrium binding. In order for an MAb to bind to porous supports it must physically enter the pores where the ligands are immobilised. This process is

hampered by pore diffusion resistance, which effectively increases the time taken to reach equilibrium. For example equilibrium binding to non-porous magnetic chelators is rapidly achieved within 5min compared to 20min for chelating Fast Flow Sepharose (data not shown).

The stability of chelating perfluorocarbons to adverse conditions is particularly impressive (2.3.7; Stewart *et al.*, 1990). It was not felt necessary to directly compare the chelating perfluorocarbons with chelating Sepharoses' as the stability of the latter is well documented (Hermanson *et al.*, 1992; Pharmacia Biotech, 1994). The stability of the magnetic chelator particles is known to be less than ideal (O'Brien, 1996). The repeated action of chelating agents and acidic buffers causes the corrosion of the iron oxide core. Some protection is gained by coating with silane and polyglutaraldehyde but pinholes in these coats will allow small molecules to corrode the core. In effect the magnetic core will shrink with each use and eventually the loss of its magnetic properties can be envisaged. Though this will presumably occur long after the support has lost its coat structure and therefore usefulness as an affinity adsorbent. This has obvious implications for the long term reuse of the magnetic chelators. This said the chemistry involved in the preparation of the supports, epoxide activation and IDA coupling, is relatively stable. 'In house' magnetic chelators were regenerated with 0.1M NaOH which had no effect on subsequent ligand density or binding capacity in crude CHO fermentation feedstock.

In conclusion the non-porous supports developed and characterised here outperform or are comparable to the conventional porous supports in terms of binding characteristics to MAb's. Combined with the inherent advantages of being non-porous these supports are eminently suitable for the direct recovery or one step purification of MAb.

4.5 Application of chelating non-porous supports for the recovery of monoclonal antibodies

The supports developed in this thesis are most suited to be used in batch mode. The batchwise adsorption of the MAb in a stirred tank reactor followed by recovery of the adsorbent-MAb complex by centrifugation for the chelating perfluorocarbons and magnetic recovery (HGMS) for the magnetic chelators. For efficient batch adsorption the affinity of the adsorbents for the MAb's is critical. The Kd values, a measure of affinity, for the chelating perfluorocarbons and magnetic chelators are in the 10^{-6}-10^{-7}M range which is acceptable for batch adsorption (Chase, 1988). In addition the target concentration of MAb to be purified will influence the efficiency of adsorption. One way to anticipate the efficiency of a batch adsorption is the ratio of input concentration C_0 to Kd (C_0/Kd). A low MAb concentration compared to the value of Kd will result in poor binding and necessitate the use of large amounts of adsorbent to recover significant amounts of the MAb. The titre of MAb from the CHO fermentation's investigated here is approximately 500μg/mL for fed batch and ~100μg/mL for simple batch fermentation's (A.J. Sheppard personal communication). The chelating perfluorocarbons have high Kd values and at target concentrations of 500μg/mL equivalent to 3.3μM antibody C_0/Kd is 0.87 for chelating PTFEP and 0.36 for chelating MP1500. For chelating PTFE wax the ratio is 0.25, a value too low for significant uptake. The magnetic chelators perform much better for Ni^{2+} charged BioMag magnetic chelator C_0/Kd >20 which should result in significant adsorption of MAb. For Zn^{2+} charged magnetic chelators as one would expect the ratio is less favourable but in all cases is greater than 1. At a target concentration of 100μg/mL the C_0/Kd ratios are much less ~7 and ~1 for Ni^{2+} and Zn^{2+} charged BioMag magnetic chelator respectively and 0.07 for chelating PTFEP charged with zinc ions.

Decreasing the value of Kd would be one way to improve the efficiency of adsorption. Experiments comparing equilibrium binding performance of Zn^{2+} charged chelating Fast Flow Sepharose at 25°C and at 4°C showed a decrease in Kd values by a factor of approximately 1.5. A decrease in Kd of this order (or greater) should be possible to achieve if binding of the MAb's to the other chelating supports were carried out at

4°C. However processing at 4°C could present some problems such as increased fouling, e.g. lipid material precipitating onto supports, and in addition special equipment would be required to maintain the temperature. An alternative way to decrease the value of Kd would be the use of other ligands, such as protein A, with greater affinity for MAb's or the use of affinity fusion tails (Sassenfeld and Brewer, 1984; Hochuli, 1988; Essen and Skera, 1993).

Another option to increase the C_0/Kd ratio is to increase the MAb concentration by prior concentration of the MAb. However this would introduce a unit operation with the associated penalties of cost and loss of yield.

It may not be desirable to alter the C_0/Kd ratio by the approaches discussed above i.e. lower Kd or increase C_0. The use of large amounts of support, which would depend on the final cost of these magnetic adsorbents if they were made commercially, would increase the quantity of MAb recovered in a given batch operation. Alternatively semi-continuos recovery of MAb is likely to improve the overall product yield from fermentation. A suitable semi-continuous recovery using magnetic chelators could consist of the following steps. The adsorbent is added to a vessel containing the feedstock where MAb is bound to the adsorbent, the kinetics of which will be fast due to their non-porous nature. Feedstock is continuously removed and the adsorbent recovered by HGMS. The feedstock if it contains significant quantities of the MAb can at this point be recycled to the contacting vessel. The adsorbent is washed to remove cells and contaminating proteins and the MAb eluted. The adsorbent would then be regenerated by recharging with metal ion and recycled to the contacting vessel. It may well be possible to effect recovery directly from the fermentor by such an approach.

4.6 Comparison of magnetic properties and particle characteristics of BioMag and 'In house' magnetic chelators.

The reasons for the difference in binding characteristics of the 'In house' magnetic chelators prepared from the SIN type material and those prepared from BioMag® 4100B were initially unclear. The particle size distributions are virtually the same (Figure 3.2) and yet the ligand densities and the binding capacities of the two MAb's for the 'In house' magnetic chelators is roughly half that of BioMag magnetic chelators. Slight differences in colour of the two adsorbents were noted but no other differences in particle characteristics were apparent. One clue was that the calculated monolayer surface coverage of MAb at infinite concentration for the 'in house' magnetic chelators suggested that these supports had a lower specific surface area than BioMag magnetic chelators (~75 c.f. ~120 m^2/g).

Further evidence for differences between the particles was revealed by analysis of the magnetic properties. The analysis was carried out in collaboration with Quentin Pankhurst at the Physics department here at UCL. A brief summary of the findings is given here.

The iron salt ratio (Fe^{2+} : Fe^{3+}) used in the alkali precipitation of iron oxide (3.2.3.1) for the preparation of SIN type amine terminated particles, and subsequently used for the preparation of 'in house' magnetic chelators, is 2:1. In the patent describing the preparation of BioMag® 4100B (Josephson, 1987) it is claimed that the ratio can be changed from 1:4 to 4:1 without substantially changing the characteristics of the particles. However, using techniques such as vibrating sample magnetometry (VSM) and Mössbauer spectroscopy, Zulqarnain *et al.* (in preparation) have shown that altering the ratio of Fe^{2+} : Fe^{3+} crucially effects the magnetic properties and surface area of the resulting iron oxide particles.

Figure 4.2 shows the results of vibrating sample magnetometry (VSM) analysis of commercial BioMag® amine terminated particles, the SIN type (2:1) amine terminated particles used in this thesis and amine terminated particles prepared from a 1:2 (Fe^{2+} : Fe^{3+}) ratio.

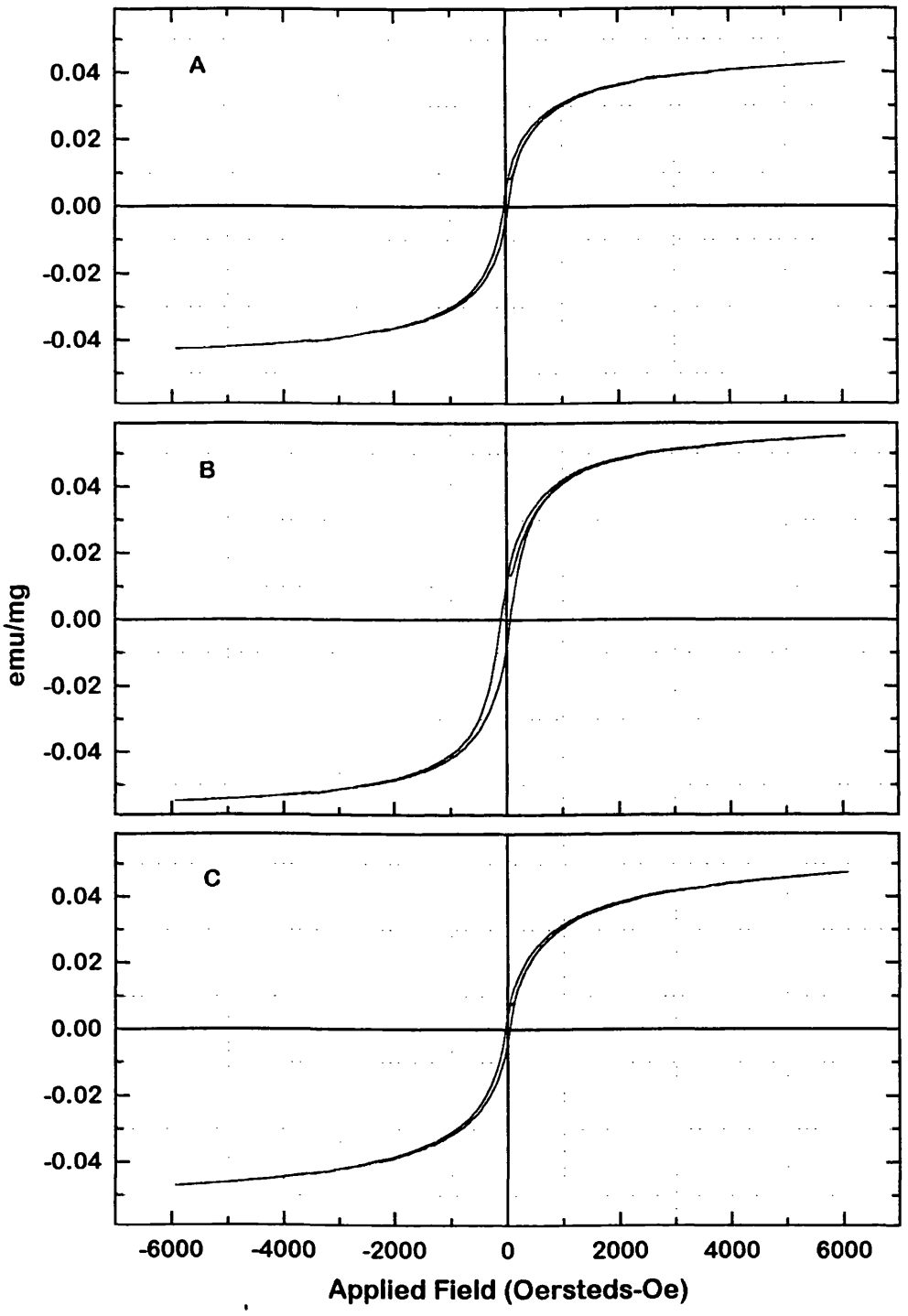


Figure 4.2 VSM data for amine terminated starting materials

Panel A; BioMag® 4100B *Panel B; 2:1 Fe^{2+} : Fe^{3+}(SIN type)* *Panel C; 1:2 Fe^{2+} : Fe^{3+}*

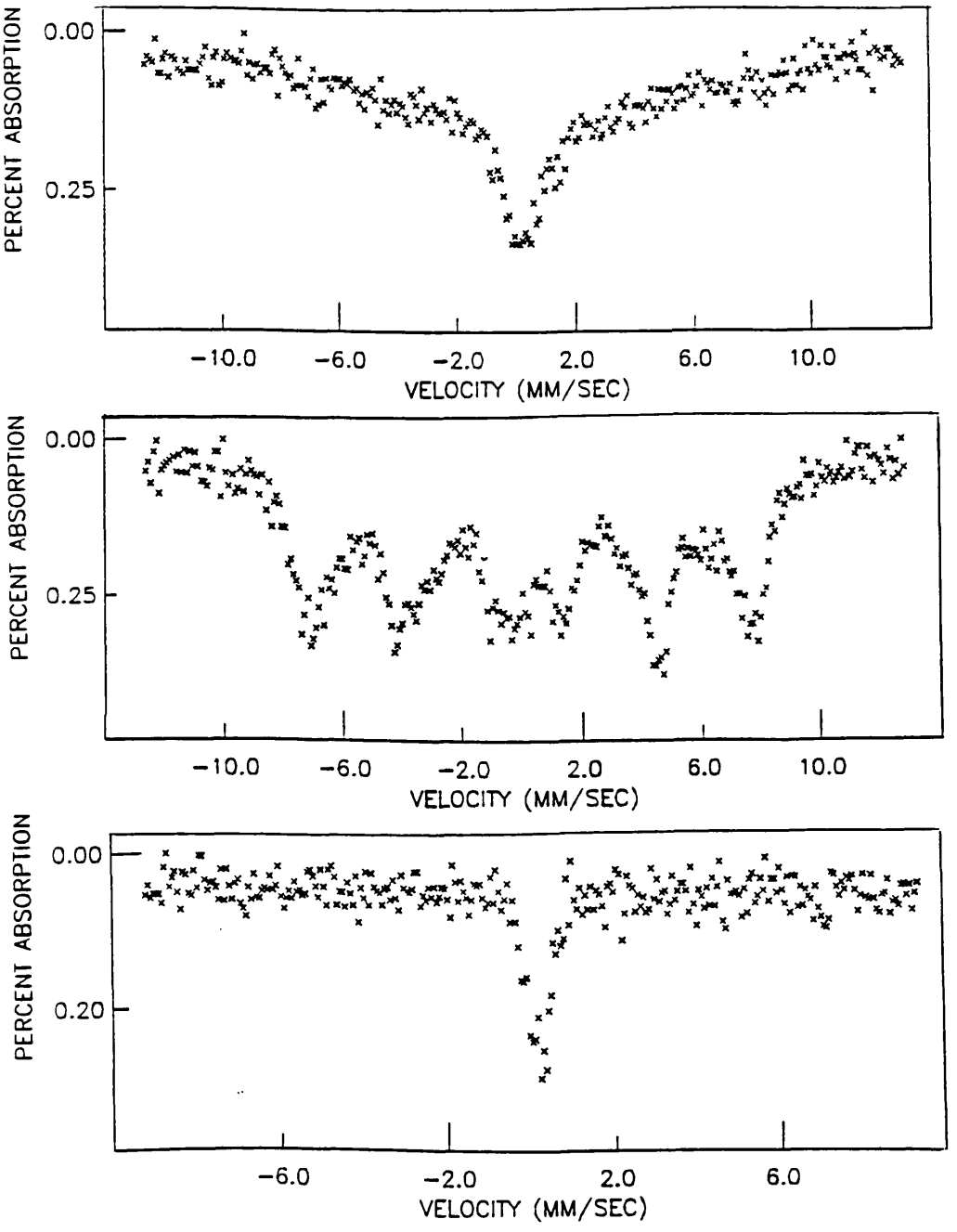


Figure 4.3 Mössbauer data for amine terminated starting materials

Panel A; BioMag® 4100B *Panel B; 2:1 Fe^{2+} : Fe^{3+}(SIN type)* *Panel C; 1:2 Fe^{2+} : Fe^{3+}*

VSM is a useful technique for analysing the magnetic behaviour of macroscopic samples containing many fine particles. The characteristic hysteresis loops generated from VSM analysis qualitatively describe the behaviour of magnetically susceptible particles in an applied magnetic field. In addition it is possible to obtain from these plots a number of values which describe the behaviour quantitatively. The saturation magnetisation (M_s) is the magnetisation at infinite field, the remnant magnetisation (M_r) is the magnetisation at zero field and finally coercivity (H_c) is the applied field at which the magnetisation falls to zero. A perfectly superparamagnetic material should have a completely closed loop and values of M_r and H_c of 0. The more ferromagnetic a material is the more open the loop and the higher the values of M_r and H_c (Duffin, 1990)

The curves for BioMag® 4100B and 1:2 amine terminated iron oxide particles (Panel A and Panel C) are very similar. M_r and H_c values are virtually identical, H_c values of ~40Oe for both particles and M_r values of 0.00457emu/mg for BioMag® 4100B and 0.00487emu/mg for 1:2. However in Panel B the loop is more open and values for M_r (0.0115emu/mg) and H_c (99.8Oe) for 2:1 amine terminated particles are higher indicating that the 1:2 material is more ferromagnetic than the other samples. These observations give indirect evidence for differences in crystal size. The larger a magnetic crystal the more likelihood that on removal of the externally applied field it will retain some magnetic properties i.e. it will behave ferromagnetically. As crystal size decreases this ability is lost and the coercivity (H_c) drops, as a result of the increasing effect of thermal interactions on the crystal magnetisation, and eventually falls to zero. It is likely therefore that the crystal size of the 2:1 particles is higher than that of the 1:2 or BioMag® 4100B particles.

Figure 4.3 illustrates the analysis of amine terminated iron oxide materials by Mössbauer spectroscopy. Mössbauer spectroscopy is a useful technique for determining not only the magnetic nature of a sample but also can be used to identify the magnetic material (Pankhurst *et al.*, 1994). In Figure 4.3 the spectra, measured at room temperature, for BioMag® 4100B and 1:2 (Panels A and C respectively) are virtually identical and typical of weakly interacting superparamagnetic materials

(Pankhurst and Pollard, 1993). In contrast the 6 line spectra shown in Panel B is characteristic of superferromagnetically ordered material. Magnetic interactions between crystals are significant indicating that the particles retain some magnetic properties after induction of magnetic moment. By lowering the temperature at which the analysis is carried out it is possible to measure the ground state of the sample and thereby identify the mineral form of the iron oxide. The particles contain a mixed population of iron oxides including maghemite (γFe_2O_3) and magnetite (Fe_3O_4). The indications are (Zulqarnain *et al.*, in preparation) that the BioMag 4100B and 1:2 material are richer in maghemite and that the 2:1 material consists largely of magnetite. This is supported by the observation that the 2:1 material is blacker, characteristic colour of magnetite, than the other two materials.

Detailed analysis of low temperature Mössbauer spectroscopy supports the observation that the crystals of BioMag 4100B and 1:2 material are smaller than those of the 2:1 material (Q.A. Pankhurst personal communication).

Taken collectively all the evidence (VSM, Mössbauer spectroscopy, ligand density, binding characteristics and particle size analysis) point to differences in crystal size but not in particle size of the magnetic chelators i.e. smaller crystals for BioMag 4100B and 1:2 material than the 2:1 material. A particle of roughly the same dimensions but consisting of smaller crystals would be expected not only to have different magnetic properties but also different specific surface areas. For example a 1μm particle consisting of 300Å crystals will have a greater surface area than one consisting of 500Å crystals, the surface of the particle being more heavily crenated. Crystals smaller than 300Å can not sustain a magnetic moment in the absence of a magnetic field (Pankhurst and Pollard, 1993).

The precipitation conditions therefore critically effect the crystal size of the resulting iron oxide which influences the nature of the magnetic properties of the particles and their surface area. The magnetic properties of BioMag and material prepared 'in house' from a 1:2 Fe^{2+} : Fe^{3} ratio are ideal for the purposes of a non-porous magnetic affinity support. In addition the higher surface area, and therefore higher ligand density and binding capacity, make these two materials the ideal choice for a starting

material for the preparation of non-porous affinity adsorbents. Magnetic affinity adsorbents prepared from 1:2 Fe^{2+} : Fe^{3} ratio Iron oxide are currently being investigated in these laboratories.

4.7 The ideal magnetic affinity adsorbent

The 'ideal' magnetic affinity support would have many of the properties of the magnetic adsorbents developed here (Chapter3) e.g. high ligand density and specific binding capacity, and superparamagnetic properties. However the stability of these supports is known to be less than ideal, leaching of iron from the magnetic core being the main problem. The polyglutaraldehyde coat and silane layer provide some protection but corrosion of the magnetic core is a major consideration for extended use (O'Brien, 1996). In contrast the stability of the perfluorocarbon supports is excellent due to the inherent properties of the perfluorocarbon base matrix (Chapter2). Work has been carried out to investigate the coating of magnetic cores with perfluoropolymers to yield stable corrosion resistant supports.

In our laboratories Theodossiou (1993) successfully prepared corrosion resistant dye-linked PVA coated magnetic adsorbents by coating metal oxide cores with perfluorosilanes such as perfluorooctyl triethoxysilane (PF8TES) to create a pseudo-perfluorocarbon coat. The methods used by Theodossiou (1993) were adapted for the perfluorosilanisation of 'in house' SIN type material. Perfluorosilane coated SIN type iron oxide was successfully prepared and coated with PVA, but in common with the supports developed by Theodossiou (1993) ligand densities were disappointingly low. During preparation the perfluorosilane coated particles are cured prior to coating with PVA and this step is responsible for a significant loss in surface area due to particle aggregation. Various methods to reduce the size of the cured particles were tried but in the main were unsuccessful. Hand grinding, with a pestle and mortar, and a mechanical grinder both failed to reduce the particle size to anything like the 0.5-1.5μm size of 'in house' particles prepared by the glycerol dehydration method (3.2.3.1). A more rigourous investigation of grinding methods may result in an

approach to achieve the very small particle size necessary for an effective magnetic affinity adsorbent.

Preliminary experiments were carried out to investigate the PVA coating of perfluorosilanised iron oxide SIN type particles, prepared according to the methods developed by Theodossiou (1993). Two methods of PVA adsorption were investigated i.e. the direct adsorption of PVA and the adsorption of perfluoromodified PVA. Direct adsorption of PVA was carried out by wetting the particles with acetone before contacting with 15mg/mL (9-10K) PVA. Alternatively PVA (9-10K; 15mg/mL) was perfluoromodified with perfluorooctanoyl chloride (2.2.2.1). PVA adsorption appeared to occur in both instances (data not shown) and the resulting PVA coated particles were epoxy activated and coupled to IDA as described above (3.2.3.3; 3.2.3.4). The particle size and therefore surface area of the resulting PVA coated particles meant that ligand densities were very low (0.30μmoles Cu^{2+}/g: direct adsorption of PVA; and 0.35μmoles Cu^{2+}/g: perfluoromodified PVA). Corrosion resistance of the magnetic chelators coated with perfluoroalkylated PVA (perfluoromodified PVA) appeared to be slightly better than those directly adsorbed with PVA (results not shown). This suggests that it is desirable to reinforce the pseudo-perfluorocarbon layer by the use of perfluoroalkylating agents rather than rely on direct adsorption of the PVA polymer. However these results would need to be confirmed, for a firm conclusion to be drawn.

In order to get away from the problems of particle aggregation during the perfluorosilanisation procedure used by Theodossiou (1993). The silane deposition method described in 3.2.3.1 was adapted by Pannu (in preparation). Fine particles coated with perfluorosilane were prepared however the silane modified iron oxide particles were so hydrophobic as to cause significant aggregation during the silane deposition and glycerol dehydration steps. Furthermore recovery of the perfluorosilane modified particles from glycerol solution proved extremely difficult. Reducing the hydrophobicity of the perfluorosilanised iron oxide particles by lowering the concentration of PF8TES used may be one way in which to eliminate the need for

grinding. This would have the effect of reducing the extent of perfluorosilanisation, which may or may not affect the corrosion resistance of the final affinity support.

An alternative to the perfluorosilane coating and subsequent creation of a pseudo-perfluorocarbon layer is the direct coating of magnetic cores with perfluorocarbons. There are a number of technical difficulties with spraying or coating very small material such as the iron oxide cores, particularly preventing aggregation during the coating process. One way around these potential problems may be the adaptation of the method used by Lee *et al.* (1996) to prepare ultrafine magnetic particles by alkali precipitation of iron salts into aqueous polyvinyl alcohol solution. Lee *et al.* (1996) used PVA to prevent coagulation of the precipitated magnetite, however they observed that the magnetite particles were completely coated by a physically absorbed PVA layer. It is possible to dissolve certain fluoropolymers such as Teflon AF1600 (DuPont, UK) in perfluorinated solvents such as Fluoroinert® and there are liquid perfluorocarbons such as perfluorodecalin. It may therefore be possible to precipitate iron salts (1:2 Fe^{2+}:Fe^{3+}) into a fluoropolymer solution and thereby coat the magnetic particles with perfluorocarbon. The magnetic-perfluorocarbon particles could then be PVA coated and coupled to affinity ligands such as IDA to produce extremely stable magnetic adsorbents with high surface area.

The coating of the magnetic support with PVA would allow the coupling of a wide variety of ligands. This thesis has shown that it is possible to couple IDA to PVA coated supports by epoxide activation. But the use of alternative ligands would be an option. In particular protein A or protein A mimics could be immobilised by the use of conventional techniques (Hermanson *et al.*, 1992). Avid AL (Ngo and Khatter, 1992) and Histidine (El-Kak *et al.*, 1992) are alternative ligands known to bind to IgG's, althougth the reported interaction strengths are on the low side. Finally the use of biomimetic dyes which have been designed for enhanced specificity towards antibodies may prove a promising approach (Lowe *et al.*, 1992; K Jones personal communication).

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