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SYNTHESIS AND STUDY OF CHELATING POLYMERS AND THEIR APPLICATION TO PROTEIN AND METAL SEPARATION FROM AQUEOUS SOLUTIONS USING NOVEL METAL AFFINITY INTERACTION TECHNIQUES

By

Javier Enrique García-Barrón

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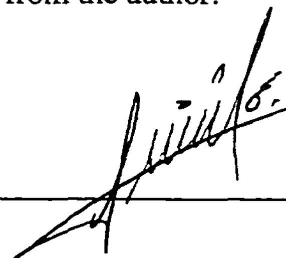
SIGNED: 

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To my beautiful wife Armida and tender daughter Maria Esther with love.

To my mother Maria Esther for being my inspiration.

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ABSTRACT

The main objective of this research work was the development, synthesis, and study of polymeric chelating derivatives. These derivatives were characterized in terms of their specific metal affinity interaction with biomolecues and metal ions. These engineered materials were used to test their feasibility as tools for separation of proteins and heavy metal ions from aqueous solutions using different affinity separation techniques.

Linear and branched polymers were synthesized to create a variety of materials. Among the linear polymers synthesized was the chelated monomethoxy poly(ethylene) glycol (PEG-IDA). This derivative was used in metal affinity partitioning and metal affinity electrophoresis for fast protein-metal interaction analysis. Also a linear heterobifunctional poly(ethylene) glycol (Biotin - PEG - IDA) was synthesized and used as a tool to develop a modified enzyme-linked immuno sorbent assay (ELISA). A multi-armed high molecular weight chelating poly(ethylene) glycol (Star PEG-IDA) was prepared to enhance the separation of protein mixture in gel permeation chromatography. Iminodiacetic poly(ethyleneimine) (PEI-IDA) was prepared and used as a soluble chelating polymer in complexation-ultrafiltration studies for heavy metal ion removal from aqueous solutions. Similar PEIs were also used as casting polymers for the synthesis of affinity adsorbents useful in chromatographic applications. Either as a soluble macromolecule or as a casting polymer for the preparation of adsorbents, PEI chelated derivatives were used for ultratrace metal ion preconcentration and metal ion separations.

All polymeric materials prepared were characterized using analytical techniques which include elementary analysis, atomic absorption, UV and IR spectroscopy, high performance liquid chromatography and several colorimetric assays for the determination of end groups and product purity.

Metal affinity separation techniques studied with the aforementioned derivatives included: affinity partitioning, affinity electrophoresis and affinity size exclusion for protein purification; affinity complexation-ultrafiltration and metal ion affinity chromatography for removal of heavy metal. Efficient separation of protein mixtures were achieved based on selective affinity by some of the chelated polymers here described and extremely high metal adsorption capacities were found for some of the PEI-based adsorbents prepared.

Even though, some of these techniques are still in developmental stages, the results are very promising and encouraging for biotechnical and environmental applications.

CHAPTER 1

INTRODUCTION

1.1 RESEARCH MOTIVATION

With the growth of biotechnology, new and cleaner separation processes with low environmental impact are needed. These two requirements have become a major concern for controlling the development of chemical engineering processes.

Since most bioreactor product streams are dilute, and the design products are in general diluted in high volumes of aqueous media, the concentration driving force available for separation is small at the outset of a particular process. The problems of separating biological products are numerous, and the separation of complex molecules from multicomponent mixtures at low concentrations are challenging. Factors such as shear-dependent biological products, environments that are sensitive to chemical and physical changes, temperature, pH, and concentration throughout the process have a deep influence on the choice of separation processes, and upon the design of individual separation operations.

The development of techniques and methods for the separation and purification of biological molecules, such as proteins and peptides, has been an important prerequisite for many of the advancements made in science and biotechnology. The research and development focused in the area of applied biotechnology has concentrated in part, in the identification and expression of new proteins, and in innovations in drug delivery systems. The production and end use of such specialist low-volume products has only involved a minor engineering dimension for extraction and purification of these products on a small scale. Many such products, are successfully manufactured using techniques which are

based on bench-scale preparative processes. The small quantities of products involved, and their high value, provide little incentive for the application and development of new separation processes, or even for the improvement of existing techniques. However, there has been an increasing industrial awareness of the importance and potential for improving process productivity and efficiency by enhancing the process engineering of downstream operations.

The evident shift towards cleaner processes has also become a factor controlling the development of chemical engineering processes. This shift demands a very significant involvement of separations and recycled processes in order to ensure cleaner discharges, more efficient use of raw materials, and energy to ensure recycle of products whenever possible. The waste water treatment industry, a large user of biochemical engineering processes, is also under pressure to meet higher standards of discharge, by properly engineered treatment and separation processes, to reduce the level of contaminants for instance, of metal ions in effluents.

The understanding of the basis of selectivity and affinity phenomena at the molecular level, has made a significant impact upon biochemical separation processes. The ability to selectively engineer molecular structures using biochemical processes can allow the needs of separation and recovery to be used iteratively in the design of an overall process for separation of a particular contaminant. It is also possible to design and produce molecules whose structure is tailored to a given set of criteria. This is a particularly valuable development when applied to production of surfaces or molecules with specific affinity characteristics. The subject of molecular recognition is seen as being of fundamental importance to the understanding and choice of separation of existing purification techniques and the development of processes for the recovery of biomolecules and molecules such as metal ions.

1.2 DISSERTATION ORGANIZATION

This work represents an effort towards the development of engineered molecular structures tailored to satisfy a set of affinity and selectivity criteria to be used in the design of an overall process for purification and separation of molecules such as proteins or metal ions.

Chapter 2 comprises a literature review of the use of different polymers and techniques used in bioseparation and metal purification processes.

The experimental work presented here was divided into four sections. The first section, chapter 3, is a summary of the synthesis of all chelating polymeric materials prepared in this work. A more detailed explanation of the preparation and application of the polymers is given in each corresponding chapter. The second section, which is composed of chapter 4 through 6, focuses on the preparation of several polymeric materials and their application to protein purification studies. Chapter 4 presents the synthesis of chelated monomethoxy-PEGs and their application for fast protein-metal interaction analysis using affinity electrophoresis. The preparation of a multi-chelated PEG and its use in metal ion affinity size exclusion chromatography as a protein purification tool is discussed in chapter 5. In chapter 6 a new enzyme-linked immuno sorbent assay (ELISA) for characterization of specific antibodies is presented. Here, a heterobifunctional polyethylene glycol molecule was synthesized to be used as a tool for the development of this ELISA method in collaboration with Professor Vijayalakshmi (*Université de Technologie de Compiegne*, France).

The third section of this dissertation, which includes chapters 7 and 8, is devoted to metal purification studies. Chapter 7 describes the synthesis of a hyperbranched chelating polymer and its use in preconcentration and extraction of metal ions from aqueous

solutions. In chapter 8, the modification of solid supports for the preparation of chromatographic metal affinity adsorbents is discussed.

The final section comprises chapter 9 and 10. Chapter 9 presents an improved method for cleavage of protecting organic groups as an intermediate step in the synthesis of heterobifunctional PEG derivatives. Chapter 10 describes a protocol for the preparation and use of chelating ligands in aqueous two-phase systems (ATPS) for protein purification. This chapter has been written in such way that can be used as a guide in ATPS. Chapter 11 contains a summary and general conclusions on all the previous chapters.

This dissertation has been written in such way that each chapter could be understood independently of the rest of the dissertation. Certain chapters are based on papers submitted for publication as follows:

Chapter 6: Nedonchelle, E., Leduc, C., García, J. E., Guzmán, R.Z., and Vijayalakshmi, M.A., "Production of Neo-metalloenzymes by De Novo Biosynthesis : new ELISA method for their characterization. Accepted for publication in *Ann. N.Y. Acad. Sci.* (1998).

Chapter 8: Porath, J., Garcia, J.E. and Guzman, R.Z. " Polymolecular Molding or Casting and its Application to the Synthesis of Metal Affinity Adsorbents. Submitted to Science. (1999).

Chapter 9 : García, J. E., and Guzmán, R.Z., " Catalytic Transfer Hydrogenation of Bifunctional Poly(ethylene glycol) Derivatives Using Palladium-Poly(ethyleneimine) Catalyst . Accepted for publication in *J. Org. Chem.* (1997).

Chapter 10 : García, J. E., and Guzmán, R.Z., "Metal Affinity Protein Partitioning" in Methods in Biotechnology : Aqueous Two-Phase Systems. Bioremediation Protocols. *Humana Press Inc.* (in Press, 1999).

CHAPTER 2

LITERATURE REVIEW

2.1 POLYMERIC MATERIALS AND THEIR APPLICATION IN BIOTECHNOLOGY

2.1.1 Poly (ethylene) Glycol

Poly (ethylene) glycol (PEG) is a highly water soluble polymer with excellent mechanical and thermal stability (Maxfield *et al.*, 1975). PEG is of low toxicity when administered orally and only large quantities may cause adverse reactions (Smyth *et al.*, 1955). Due to its hydrophilic and hydrophobic properties, PEG has been used as a chemical modifier to modified the physicochemical properties and to obtain information on protein structure. The main objective of the chemical modification is to produce proteins that can be used as therapeutic agents in environments where native proteins are unable to interact.

According to Ajisaka *et al.* (1980), Boccu *et al.* (1982), and Beauchamp *et al.* (1983) among others, PEG-modified proteins can increase their plasma half-life, the more PEG attached per protein molecule the greater the extension of half-life. Also reduced immunogenicity and antigenicity has been observed as a result of PEG modification (Ritcher *et al.*, 1983) which is particularly important since immunological responses limit the clinical use of proteins (Delgado *et al.*, 1992). An increased protein solubility is expected with PEG because PEG is highly water-soluble due to hydrogen bonding of two to three water molecules per ethyleneoxide molecule (Maxfield *et al.*, 1975). PEG modification reduces also protein denaturation on exposure to heating or interfaces, in

water the PEG chains are solvated and may reduce the molecular motion that are intrinsic to denaturation (Baillargeon *et al.* , 1988).

PEG-protein modification represents a significant advance in the pharmaceutical industry. Also, the application of PEG-modifications in biotechnology is very promising, the conserved biological activity of PEG-proteins in organic solvents may allow the synthesis of compounds with a 3D structure determined by the conformation of the active site of the enzyme which is unlikely to be obtained by classical chemical synthesis (Delgado *et al.*, 1992). PEG-proteins has had a role in biotechnological applications such as aqueous two-phase systems and affinity electrophoresis as described below.

2.1.2 Heterobifuctional Poly(ethylene) Glycol

Synthesis of heterobifunctional poly (ethylene) glycol derivatives (X-PEG-Y) and especially the purification of these products are somewhat difficult to accomplish. In fact, despite its relevance in biotechnology, only a few synthetic approaches have been reported in the literature (Ehteshami, 1996; Zalipsky and Barany, 1986; Zalipsky and Barany, 1990). Heterobifunctional PEGs are linear PEG chains having two different end groups. A properly chosen combination of terminal end groups (X and Y), leads to a more selective attachment to two different molecules. There is an increasing interest in using these PEGs derivatives to construct heterobifunctional PEGs having a targeting moiety such as a receptor-specific-protein or carbohydrate on one terminal end and a therapeutic such as DNA for gene delivery, an oligonucleotide for antisense therapy, or a small molecule drug on the other end.

Heterobifunctional PEGs derivatives are being applied in the pharmaceutical industry and are of growing interest as tools in the area of drug delivery. Heterobifunctional maleimido PEG N-succinimidyl carboxylate was used by Paige *et al.*

(1995) to link recombinant human granulocyte colony stimulating factor (rhG-CSF) to human serum albumin (HSA) which resulted in extended circulation half-life and increased serum stability relative to rhG-CSF. Pardridge *et al.* (1999) introduced a novel approach using biotin-PEG-NHS to facilitate delivery of drugs to the brain. Biotin-PEG-NHS was linked to I-labeled epidermal growth factor (EGF) to OX 26, a monoclonal antibody to brain capillary endothelial transferrin receptors. Zalipsky *et al.* (1997) prepared a heterobifunctional PEG having a tBoc-protected hydrazide at one end and a N-succinimidylcarbonate at the other. This allows selective linkage to the phospholipid, distearoylphosphatidylethanolamine (DSPE) and the tBoc ester is then activated as a hydrazide. The hydrazide-activated PEG DSPE can replace PEG DSPE in a liposome formulation. The method offers the possibility of targeted delivery of therapeutics held in the liposome vesicle. The system also offers the possibility of long presentation of small peptides normally short-lived in circulation.

2.1.3 Branched Poly(ethyleneimine) Polymers

Poly(ethyleimine) polymers with complexing properties have gained considerable importance in terms of their potential applications in waste water treatment for the removal of metal ions (Chaufer *et al.*, 1998). PEI chelating polymers consist of a polymer backbone and a grafted chelating group. This chelating group may be incorporated in the monomer structure or added to the polymer backbone by a polymer analogous reaction (Bartulin *et al.* , 1986). The formation of chelates by polymers has been used for concentration, separation and extraction of metal ions (Geckeler *et al.*, 1980 and Efendiev *et al.*, 1981).

PEI polymers as metal ion complexing agents have also been important in solvent extraction chemistry (Frechet, 1981). Principles that are understood in this process can also be applied to solid-liquid extraction processes.

Chelating adsorbents are frequently used in industry and laboratory in order to trap metal ions for different purposes: removal of metal ions from contaminated water, preconcentrate metal ions and separate analytes from interfering contaminants prior to their determination by an instrumental method. The metal binding capacity and the metal binding strength are important characteristics of a chelator. A high capacity is usually an advantage, whereas the requirements for strong metal binding may vary depending on the analytical task.

Poly (ethylene) imine (PEI), a polymeric amine, has been used in many studies as a complexing agent (Strathmann, *et al.*, 1978 and Geckeler *et al.*, 1980) as well as a versatile source of chelating derivatives (Christensen, *et al.*, 1982 and Bhattacharya *et al.*, 1980) for removing metal ions from aqueous solutions by means of complexation-ultrafiltration. The branched structure of PEI was demonstrated leading to better chelating properties than a linear structure sterically unfavorable for complex formation (Kobayashi *et al.*, 1987). PEI also offers high content of functional groups, high water solubility and chemical stability which make it suitable for complexation studies.

2.1.4 Polymeric Adsorbents

The principle of bioaffinity chromatography, using polymeric supports modified with affinity ligands covalently bound to the solid matrix, has been known for many years. Campbell *et al.* (1951) were the first to use this principle in 1951 for the isolation of antibodies on a column of cellulose with a covalently attached antigen. A significant contribution in the development of bioaffinity chromatography was the method of affinity

attachment to activated agarose introduced by Porath and coworkers (Porath *et al.*, 1967; Axen *et al.*, 1967; Axen and Ernback, 1971). Agarose and dextran (natural polysaccharides) are among the most commonly used supports in affinity chromatography procedures. Agarose is composed of water soluble polysaccharide chains that are aggregated into beads. Chelating ligands can be easily coupled to the agarose or dextran matrices after epoxy activation. In 1968, Cuatrecasas *et al.* employed agarose supports in bioaffinity chromatography for the isolation of nuclease, chymotrypsin and carboxypeptidase. Cuatrecasas used the term affinity chromatography for the first time for this type of isolation, however other terms like " bioaffinity chromatography " (Porath, 1973) or " biospecific adsorption "
(O'Carra, 1974) have been used to described this phenomena.

Suitable compounds to be used as affinity ligands for the isolation of biomolecules must bind such products specifically and reversibly. Iminodiacetic acid (IDA), a chelating agent, is one of the most commonly used ligands in affinity chromatography. Its use gave raise to the development of what is now known as immobilized metal ion affinity chromatography (IMAC). The two carboxyl groups and the one nitrogen atom in this compound are able to donate electrons to a metal ion, forming a polydentate chelate. This complex is stable and the immobilized metal is able to create additional stable chelates with side groups of certain amino acids (Figure 2.1). The selection of metal to immobilize in the chelator is important for increasing the affinity towards a particular biomolecule. The most commonly used metal ions are Cu(II), Ni(II), Co(II) and Zn(II). The retention of the target molecule in a column depends on the number and type of amino acid side chains that can interact with the immobilized metal. If IDA is used as the chelating ligand, immobilized Cu(II) and Zn(II) will leave one coordinating site for proteins, while Ni(II) will leave three sites.

Several affinity techniques have been applied in biotechnology for separation and purification of biomolecules and in environmental clean ups. Figure 2.2 shows the techniques developed and use in affinity chromatography. Some of these techniques are described next.

2.2 METAL AFFINITY SEPARATION TECHNIQUES

2.2.1 Immobilized Metal Affinity Chromatography

Immobilized metal ion affinity chromatography (IMAC) was introduced by Porath *et al.* in 1975, who used immobilized metal ions chelated to insoluble chromatographic matrices to specifically fractionate serum proteins. IMAC is an electron donor-acceptor mechanism. In IMAC, the metal ion immobilized through a chelating agent to an insoluble matrix acts as an electron acceptor and the imidazole nitrogens of the accessible histidines of the surface of a protein act as the electron donors (Vijayalakshmi, 1989). It is known that there is a direct relationship between the number of unprotonated accessible histidine residues and the strength of protein binding to the polymer-supported metal ion (Sulkowski, 1987; Birkenmeier *et al.*, 1991; Wuenschell *et al.*, 1990). IMAC has been widely used for the purification of biomolecules. In IMAC, a metal chelate ligand is first covalently attached to the solid support and the column is then loaded with the selected metal ion. Chelates are much more stable than a metal complex due to the loss in free energy when a ring is formed. After equilibration of the column with an appropriate buffer, a protein solution is passed through the matrix. Elution of the bound protein from the matrix is easy, either by adjusting the pH, altering the ionic strength or introducing a competitive agent that can displace the bound amino acid groups.

There are several advantages to IMAC over other affinity chromatography methods. Metal chelates in IMAC columns are stable over a range of conditions including changes in pH and temperature. This quality makes IMAC a versatile separation technique for biological products. IMAC columns usually have a high binding capacity. The chelating ligand can be coupled to chromatographic matrices at high densities allowing the immobilization of large quantities of metal for coordination with amino acids of incoming proteins. The type of metal-protein interaction involved in IMAC reduces the potential of protein denaturation enabling the maintenance of biological activity (Arnold, 1991). The chelating ligands for IMAC are also relatively inexpensive in comparison to biospecific affinity chromatography. Among the most used ligands in IMAC are iminodiacetic acid (IDA) and tris-carboxymethylated ethylene diamine (TED). The IDA ligand is tridentate and can form a double five-membered ring chelate with hexacoordinate metal ions, while TED (tris-carboxymethylated ethylene diamine) is pentadentate and capable of forming four five-membered rings. These ligands work better with transition metal ions, which have several possible coordination sites and act as electron acceptors in the presence of this type of derivatives.

One disadvantage in IMAC is the possibility of metal leakage from the column. However, the metal in the protein can be extracted if the solution is additionally passed through a chelate column lacking immobilized metal ions. Another disadvantage is that IMAC is not biospecific, but the technique can be modified to increase specificity by selection of the appropriate ligands and ligand densities, metal ions, and buffer conditions to target the desired molecule.

2.2.2 Aqueous Two-Phase Systems

Partitioning of proteins in aqueous two-phase systems (ATPS) has become an established and well-known method for the separation and purification of biological materials (Walter *et al.*, 1985; Albertsson, 1986). In order to increase the selectivity and effectiveness of the separation, a ligand which partitions favorably into one of the phases and with affinity for the target molecule is introduced into the system. Thus, once binding occurs, the desired material preferentially distributes into the polymer-ligand rich phase. In general, the affinity ligand is attached covalently to one of the phase forming polymers, which ensures its primary distribution to one phase of the system. In affinity partitioning, ligands attached to poly(ethylene glycol) (PEG) have been widely described and used extensively in the separation and purification of proteins and cells, derivatives include PEG-linked dyes (Koppershlager *et al.*, 1981), PEG-linked antibodies (Sharp *et al.*, 1986) and PEG-linked long chained fatty acids (Shanbhag *et al.*, 1979). Similar derivatives for dextran (Dx) and procedures to prepare new affinity ligands are described by Harris (1985) and Harris and Yalpani (1985).

Resembling the IMAC separation scheme, metal affinity partitioning (MAP) has been under development as an alternative affinity approach to increase the selectivity of protein separation by incorporating chelated transition metal ions covalently bound to PEG as affinity ligands (Wuenschell *et al.*, 1990; Chung *et al.*, 1991; Birkenmeier *et al.*, 1991) and cell separation (Goubran-Botros *et al.*, 1991; Walter *et al.*, 1993; Laboureau *et al.*, 1996).

Typical two-phase systems for protein separations include aqueous solutions of PEG and dextran or PEG and a salt such as sodium sulfate or sodium carbonate. When a metal-chelated-PEG is added to the system containing the protein mixture, proteins with

affinity for the chelated metal interact and partition preferentially in the PEG-rich phase. In these systems, the partition coefficients (K) of proteins which contain surface accessible amino acids, particularly histidine residues, can significantly increase with the addition of a relatively small amount of the metal-chelated polymer derivative.

As in IMAC, the most commonly used chelating-PEG derivative by far has been IDA-PEG complexed with first row transition metal ions such as Cu(II), Co(II), Zn(II) and Ni(II), particularly with copper. One advantage found in MAP is the fact that, unlike many affinity interactions which are disrupted in high concentrations of salts, metal ion coordination in aqueous solutions is promoted by phase-forming salts. Therefore, the partitioning of proteins with Cu(II)IDA-PEG appears to be more effective in PEG-salt compared with PEG-Dx systems (Suh *et al.*, 1990; Otto *et al.*, 1993).

Metal affinity protein partitioning has become a very useful and versatile method for protein fractionation and characterization when used with the appropriate chemistry. By choosing an appropriate chelating-polymer and metal ion in a given aqueous two-phase system, a high partition coefficient (K) for the protein of interest or degree of purification can be achieved.

2.2.3 Affinity Gel Electrophoresis

Immobilized metal ion gel affinity electrophoresis (IMAGE) is another technique derived from the affinity concept introduced by IMAC. Electrophoresis is a technique which separates charged molecules according to differences of electric charge and molecular structure. When two molecules have the same charge but different mobilities, the faster moving substance will overrun and migrate ahead of the slower one. In affinity electrophoresis a charged molecule migrates through a matrix containing specific ligands under the influence of an electric field. The mobility of the molecule relative to its mobility

in a gel containing no affinity ligand reflects interactions with the immobilized ligand (Schossler *et al.*, 1985). The concept of affinity electrophoresis was introduced by Bog-Hansen (1973) and used by Horejsi and Kocourek (1974). Every electrophoretic process that studies biospecific interactions belongs to this category of affinity electrophoresis. Early this decade, Vijayalakshmi and coworkers combined the principle of electrophoretic separation and the interaction of a biomolecule with a ligand immobilized within the electrophoresis gel support to develop a new affinity technique called immobilized metal ion affinity gel electrophoresis (IMAGE). This affinity technique is a useful analytical tool for the quantitative determination of binding constants between biomolecules and chelated metal ions, for the efficient design of preparative-scale purification of proteins and enzymes using chromatographic of phase partition techniques, for the detection of protein unfolding and for the eventual study of structure/function relationships of modified proteins (Goubran-Botros *et al.*, 1992).

As a direct extension of the generic IMA principle, metal affinity electrophoresis (IMAGE) is an electron donor-acceptor mechanism. The metal ion immobilized through a chelating agent to an electrophoretic gel acts as an electron acceptor and the imidazole nitrogens of the accessible histidines of the surface of a protein act as the electron donors. This protein-metal interaction should be maintained regardless of the applied electrical field. Ligand immobilization by covalent attachment to the solid support enhances both separation and resolution of the analyte. Accurate and precise binding constants are obtained when the ligand is firmly immobilized within the support matrix. When metal-affinity electrophoresis is used as a preparative procedure, covalent immobilization of the chelating ligand reduces the possibility of leakage of metal ion complexes into the purified protein sample.

Among potential applications for metal-affinity electrophoresis are: studying interactions between proteins and metal ion complexes and determining apparent binding

constants (Birkenmeier, *et al.*, 1991), determining the content and distribution of metal-binding sites in peptides and on the surfaces of native proteins, probing metal-binding centers in denatured proteins (Wuenschell *et al.*, 1990), studying protein folding and processing by following changes in the accessibility of metal-coordinating residues, separating proteins that are very similar in mass charge (i.e., isoenzymes, mutants) but differ their metal-binding characteristics (Holmes *et al.*, 1992), detecting metal-binding centers of fusion peptides engineered into proteins to simplify purification or regulate activity and to predict the outcome of metal-affinity chromatography separations with very small samples and determining binding stoichiometries of biosystems.

2.2.4 Affinity Ultrafiltration

Affinity ultrafiltration is a relative new separation technique that uses the affinity concept combined with ultrafiltration. The combination of chelating interactions with a filtration separation process yields metal affinity ultrafiltration. This technique was developed in tl.e 80's (Mattiason *et al.*, 1986; Luong *et al.*, 1987) The principle of the process, as in affinity chromatography, lies in the ability of the ligand to form an affinity complex with the analyte to be purified. The complex will be held by a membrane in an ultrafiltration process, allowing impurities to pass through the filter unretained. The ligand used is generally a high molecular weight molecule with a minimum ten fold difference in molecular weight from the ultrafiltration membrane cut off to ensure good retention. In addition, it is desirable for the ligand to have a high capacity for the analyte that is being retained.

Affinity ultrafiltration has been used as an alternative to affinity chromatography for the purification of biomolecules. When a chelating agent is used as the ligand, the system

provides the same type of specific interaction of IMAC. This method offers some potential, involves no phase change, no chemical addition and has low energy requirements (Luong *et al.*, 1992). In comparison with affinity chromatography, affinity ultrafiltration has the ability to process unclarified and viscous liquids, and can be applied to the direct purification of biomolecules from spent media, which can cut processing cost significantly. It is also better suited for scaling up and continuos operation.

As in any other affinity separation technique, operating conditions and choice of ligand are very important. Some of the desired characteristics in a ligand are resistance to pH changes, shear force and temperature. In addition, the ligand should have a strong affinity for the target molecule, provide specific binding sites and be suitable for recycling.

Among the disadvantages of this technique is the non-specific adsorption of the biomaterials to the affinity ligand. This problem can be overcome by tailoring affinity ligands. Another limitation is the lack of appropriate membranes that provide a narrow distribution of molecular weight permeability, high flux and long operating life. A 10 fold difference in molecular weight is required in between the ligand and the biomolecule in order to have good separation. When the biomolecule to be purified has several binding sites, crosslinking may occur with the affinity ligand. An insoluble precipitate may form and increase the potential for fouling of the membrane. In comparison to affinity chromatography, a higher ligand-biomolecule binding constant
is required so that the complex is not easily dissociated during washing.

Some criteria for the selection of a membrane include: high permeability for the solvent, sharp MW cut off, good mechanical durability, high fouling resistance, easy maintenance and long life Luong *et al.* (1987).

Metal affinity ultrafiltration has been used also as a method for preconcentration and extraction of metal ions using several PEI derivatives (Chaufer and Deratani 1998). There has been increased research in this separation technique due to its ability to selectively remove metal ions at high flow rates. This is a promising technique that is still in development for waste treatment.

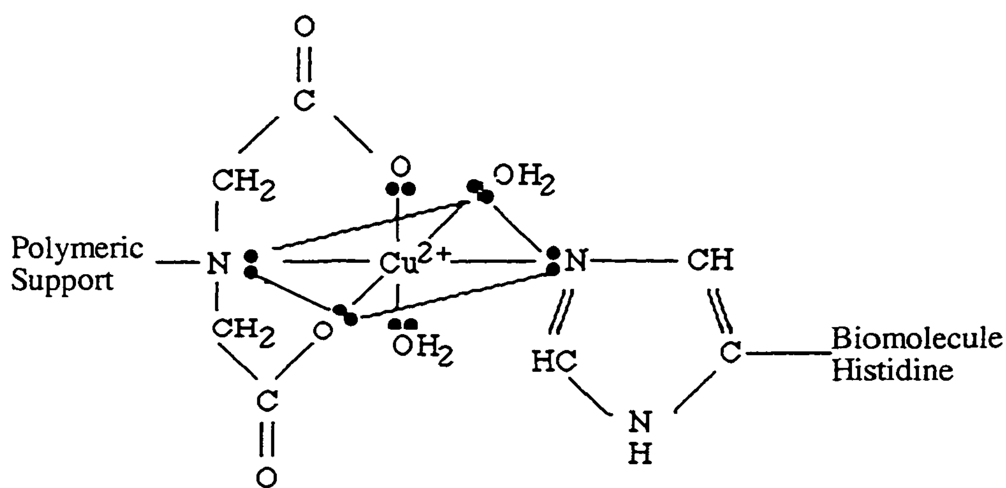


Figure 2.1 IDA-Metal-Protein Affinity Complex. IDA chelates the metal ion, the histidine of the biomolecule is bound to the immobilized metal. Heavy transition metal ions behave as electron acceptors providing several coordination sites.

(Wong et al., 1991)

Immobilized Metal affinity Techniques

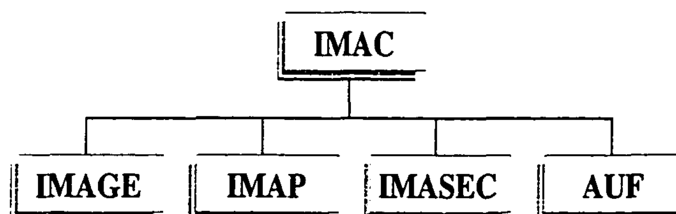


Figure 2.2 Immobilized Metal Affinity Techniques. IMAC and its related techniques can be useful in a broad range of applications.

CHAPTER 3

SYNTHESIS AND CHARACTERIZATION OF POLYMERIC CHELATING DERIVATIVES

3.1 HETEROBIFUNCTIONAL POLY(ETHYLENE) GLYCOLS

Synthesis of heterobifunctional poly (ethylene) glycol derivatives (X-PEG-Y) and especially the purification of these products are somewhat difficult to accomplish. In fact, despite its relevance in biotechnology, only a few synthetic approaches have been reported in the literature (Ehteshami, 1996; Zalipsky and Barany, 1986; Zalipsky and Barany, 1990). Heterobifunctional PEGs are linear PEG chains having two different end groups. A proper combination of terminal end groups (X and Y), leads to a more selective attachment to two different molecules.

Biotin - PEG - [IDA (Zn(II)] was prepared and characterized in our laboratory according to our own protocols (Ehteshami, 1996). A detailed description of the application of this Bifunctional PEG derivative for the screening of [IDA-Zn(II)] specific antibodies is presented in chapter 6.

Catalytic Transfer Hydrogenation of Heterobifunctional PEG Derivatives Using Palladium Poly (ethyleneimine) Catalyst.

The key and limiting step in the development of Bifunctional derivatives is the ability to synthesize and purify monoprotected functionalized polymers. Here an improved method for the removal of the organic protecting group (Z) from the heterobiprotected PEG (Boc-PEG-Z) is described.

Catalytic Removal of the Benzyl Protective Group Z

Boc-PEG-Z and Boc-PEG-Boc (PEG's derivatives MW 1900) were prepared and characterized in our laboratory according to our own protocols (Ehteshami, 1996).

Palladium-poly(ethyleneimine) in methanol was poured into a 2 ml chromatography column. 2 gr. (~ 1 mmole) of the mixture, consisting of Boc-PEG-Boc, and Boc-PEG-Z was dissolved in a 4.4% formic acid-methanol solution at about 5 mg/ml. The solution was allowed to flow slowly through the column (2 ml/min) in a continuous recycled loop for a period of 4 hr under a nitrogen atmosphere. The rate of catalyzed hydrogenolysis of the Boc-PEG-Z and Boc-PEG-Boc mixture was monitored by measuring the rate of deblocking of the amino group in the resulting monoprotected derivative Boc-PEG-NH_2 using the 2,4,6-trinitrobenzenesulfonic acid method (TNBS test) (Habeeb, 1966). An additional bed volume of formic acid-methanol was passed through the column to ensure complete removal of sample from the column. The eluent was evaporated under reduced pressure. To prevent breakdown of the Boc-PEG-NH_2 and Boc-PEG-Boc, the mixture once evaporated and crystallized, was diluted with water, partially evaporated at reduced

pressure, and then extracted into ethyl acetate (Coleman and Royer, 1980). After evaporation of the solvent a white solid material was obtained.

Preparation of Heterobifunctional Biotin - PEG - IDA

The heterobifunctional PEG biotin-PEG-IDA (BPI) was synthesized according to a modified method of Ehteshami *et al.* (1996). Briefly, diamino-PEG with a molecular weight of 2000 Daltons was used as the starting material to prepared a heterobifunctional PEG of the form Boc - PEG -NH_2 where Boc is a blocking group. Imidiacetic acid (IDA) was coupled to the amino group, followed by cleavage of the Boc protecting group with 15 % TFA to expose the second amino group which was then used for biotinylation using *N*-hydroxysuccinimide biotin. Briefly, 1 mol of amino - PEG - IDA was dissolved in buffer phosphate pH 8.5. Immediately prior to use, 1.1 mmoles of NHS Biotin were dissolved in DMSO (1 mg in 75 μl) and the mixture was added to the heterobifunctional PEG solution. After incubation for 30 minutes at room temperature, the excess NHS-biotin was removed with a Centricon-30 microconcentrator. The final product Biotin - PEG - IDA was characterized using the Avidin-Biotin test (Green, 1963) according to the hyperchroic effect when biotin binds to one of the active sites of Avidin. This heterobifunctional Biotin - PEG - IDA was used as a tool for the screening of the hapten [IDA Zn(II)] specific antibodies as described in chapter 6.

Preparation of Heterobifunctional ConA - PEG - IDA

The best characterized lectin is concanavalin A (ConA) which exists at physiological pH as a tetramer of identical subunits of 26,000 Daltons. Each monomer has one saccharide binding site with specificity for a D-mannosyl or a D-glucosyl moieties. (Sharon and Lis, 1972). The lectin concanavalin A (ConA) was coupled to soluble monomethoxy and heterobifunctional poly (ethylene) glycol according to our own protocols. The resulting modified lectin could be used in an insulin delivery system which utilizes a glycosylated insulin. In this system, the glycosylated insulin which is bioactive could be displaced from the modified ConA by glucose in response to the amount of glucose present. Glucose competes for binding sites on the lectin. This system offers the potential advantage of releasing insulin in amounts controlled by the concentration of glucose present. The presence of the polymer on the lectin surface would physically prevent cells and enzymes from attacking the drug. A brief description of the modification of ConA with PEG derivatives and characterization of the conjugate is presented below.

ConA Activation and Modification

Briefly, 20 mg concanavalin A (20% pure) were dissolved in 10 ml of a 50 mM buffer phosphate solution at pH 8.0 , 1 mM calcium and manganese ions and 0.15 mM NaCl. To 3.5 ml of ConA solution, 450 mg of glucose (as the saccharide binding site blocking agent) and 4.6 μl of a 50% glutaraldehyde (as the activation agent) solution were added. The solution was incubated for 20 minutes and the excess of glutaraldehyde was removed by chromatography using a PD10 column equilibrated with 3 bed volumes of 50 mM tris buffer. ConA was then eluted with 3.5 ml of a 50 mM NaAC buffer pH 5.0. The activated lectin was reacted with 2 μmoles of the bifunctional amino-PEG-IDA for 24 hr.

The mixture was purified by dialysis using a 25K MW cut off membrane against tris buffer pH 8.0. The progress of coupling was followed by the rate of disappearance of free amino groups as measured by the TNBS test (Habeeb, 1966). After lyophilization, a yellow material was collected.

Affinity changes based on the protein activity of the modified and unmodified lectin were measured using a polysaccharide light scattering assay (Sigma). In column adsorption experiments, the introduction of PEG chains on the surface of ConA (up to 12 chains per subunit according to TNBS) prevented this protein from been adsorbed on G type gel columns. The unmodified ConA was readily adsorbed.

Determination of Concanavalin Bioactivity

A polysaccharide light scattering assay was routinely employed for the determination of ConA binding properties. (Richardson and Behke, 1976; Sigma's protocol). Typically, 2 ml of ConA were incubated for 10 min at 25 °C. At the end of this period, 20 μl of a 10 mg/ml glycogen solution were added and the mixture was gently agitated. The optical density was measured at 420 nm during 5 min. Activity is defined as the absorbance reached during this period. Table 3.1 shows the results of the activity assay of unmodified and modified ConA. The bioactivity of ConA is conserved.

System	*Activity (AU at 420 nm)*
Native Concanavalin A	0.65
ConA - PEG (5000 MW)	0.55
ConA - PEG - IDA (2000 MW)	0.41

Table 3.1 Results of ConA conjugates activity assay.

Preparation of Heterobifunctional Fluorescein - PEG - IDA

The heterobifunctional PEG Fluorescein-PEG-IDA (FPI) was synthesized in a similar fashion as the Biotin - PEG -IDA (BPI) derivative. The amino group of heterobifunctional amino - PEG - IDA was used for labeling using *N*-hydroxysuccinimide fluorescein. Briefly, 1 mol of amino - PEG - IDA was dissolved in buffer phosphate pH 8.5. Immediately prior to use, 1.1 mmoles of NHS Fluorescein were dissolved in DMSO (1 mg in 75 μl) and the mixture was added to the heterobifunctional PEG. After incubation in the dark for 30 minutes at room temperature, the excess NHS-fluorescein was removed with a Centricon-30 microconcentrator.

3.2 MONOMETHOXY POLY(ETHYLENE) GLYCOL

Polyethylene glycol-iminodiacetic acid (PEG-IDA) loaded with transition metal ions (Cu^{2+}, Ni^{2+} and Zn^{2+}) was used to prepare several active biogels for electrophoretic analysis of biomolecules. A more detailed explanation is given in chapter 4.

Preparation of Chelated Monomethoxy Poly(ethylene) Glycol
[M]-PEG-(IDA Me^{2+})

Methoxy polyethylene glycol amino (M)-PEG - NH_2 was synthesized according to Cordes (1985). Briefly, 5 g of (M)-PEG - OH were melted at 70 ° C and water was removed under vacuum. After addition of 1 ml of distilled thionyl chloride ($SOCl_2$), the mixture was rotated for 24 h at 65 ° C under a nitrogen atmosphere to exclude moisture. After removing the excess of thionyl chloride by evaporation under vacuum, the residue was dissolved in 250 ml of absolute ethanol and precipitated at 4° C. The dried (M)-PEG - Cl was dissolved in 150 ml of water and 150 ml of ammonium solution (28%) were added. The solution was placed in a sealed plastic tube and left for 100 h at 55 ° C in a dry oven. Lyophilization of the solvent yielded (M)-PEG - (NH2) .

Carboxymethylation of (M)-PEG-amino was performed by reacting 5 gr. of (M)-PEG - (NH2) with a 50 molar excess of bromoacetic acid over the amino content. The mixture was kept at pH 9 and stirred for 24 hr to yield (M)-PEG - (IDA).

(M)-PEG - (IDA) was metallized with transition metal ions [Cu(II), Ni(II) and Zn(II)] by dissolving 5 gr. of the ligand in 100 ml of buffer acetate pH 4.0 containing the metal sulfate salt in a 50 molar excess over the PEG ligands. The solution was stirred overnight and (M) -PEG- (IDA-Me^{2+}) was extracted in chloroform. The metal bound to the chelator was determined by atomic absorption using appropriate standards.

3.3 STAR POLY(ETHYLENE) GLYCOL

Star PEG a high MW multi-PEG chelator was used to investigate its affinity interaction with specific proteins. Synthesis and application of Star PEG-(IDA-Cu^{2+})$_{64}$ in metal ion size exclusion chromatography is presented in chapter 5.

Preparation of Chelated Star Poly(ethylene) Glycol Star PEG -(IDA-Cu^{2+})$_{64}$

Amino star PEG - $(NH_2)_{64}$ was synthesized according to our own protocols: 1 g of PEG star was melted at 70 °C and water was removed under vacuum. After addition of 1 ml of distilled thionyl chloride ($SOCl_2$), the mixture was rotated for 24 h at 65 ° C under a nitrogen atmosphere to exclude moisture. After removing the excess of thionyl chloride by evaporation under vacuum, the residue was dissolved in 150 ml of absolute ethanol and precipitated at 4 ° C. The dried star PEG - $(Cl)_{64}$ was dissolved in 150 ml of water and 150 ml of ammonium solution (28%) were added. The solution was placed in a sealed plastic tube and left for 100 h at 55 °C in a dry oven. After amination, the PEG star was filtrated in an ultrafiltration cell to eliminate the excess of ammonium. Lyophilization of the solvent yielded 0.81 g of PEG - (NH2) 64.

Carboxymethylation of star PEG-amino was performed by reacting 1 gr. of Star PEG - $(NH_2)_{64}$ with a 50 molar excess of bromoacetic acid over the amino content. The mixture was kept at pH 9 and stirred for 24 hr. Unreacted bromoacetic acid was removed by ultrafiltration and the solution lyophilized to obtained 0.8 gr. of Star PEG - $(IDA)_{64}$.

Star PEG-$(IDA)_{64}$ was loaded with copper ions by dissolving 0.5 gr. of the ligand in 10 ml of buffer acetate pH 4.0 containing cupric sulfate salt in a 50 molar excess over the PEG ligands. The solution was stirred overnight and the free copper was removed by ultrafiltration until no copper was detected by light scattering. The solution was lyophilized yielding 0.45 gr. of Star PEG - (IDA-Cu^{2+})$_{64}$

The Extent of amination of star PEG - $(NH_2)_{64}$ was tested by light scattering using the TNBS test (Habeeb, 1966) indicating complete amination. After carboxymethylation of amino star PEG, the TNBS tested negative indicating no amino groups left in the polymer. Metal loading was analyzed by atomic absorption and the copper capacity of star PEG -

$(NH_2)_{64}$ was found to be 60 mmoles of Cu^{2+} per mmole of polymer corresponding to 94% of metal loading.

3.4 HYPERBRANCHED POLY(ETHYLENEIMINE) (PEI) DERIVATIVES

Preparation of Soluble Chelated Poly (ethyleneimine) (PEI)

Carboxymethylation of Poly (ethyleneimine) was performed by reacting the desired amount of PEI solution with a 50 molar excess of bromoacetic acid over the primary amino content. Primary amino groups in the PEI solution were measured using the TNBS method described by Habeeb (1966). The mixture was kept at pH 9 and stirred for 24 hr. Unreacted bromoacetic acid was removed by ultrafiltration. The carboxymethylated PEI (CMPEI) solution was tested with TNBS to ensure complete chelation. The application of soluble chelated PEI in preconcentration and removal of metal from aqueous solutions is discussed in chapter 7.

Preparation of Agarose-based PEI Supports

Preparation of Novarose-PEI Adsorbents (CP complex)

10 g of suction dry epoxy-activated Novarose were washed twice with 25 ml of 0.1 M carbonate buffer at pH 11 on a glass filter, suction dried and weighed. For the preparation of the adsorbent a PEI/Novarose ratio ~ 0.5 (W/W) and a PEI concentration in the range of 25% to 35% (W/W) are recommended. Thus, 5 g of PEI (25,000 or 750,000 molecular weight) were added to 10 ml of 0.1M sodium carbonate buffer pH 11 in a 100 ml screwcapped plastic bottle and the viscous solution homogenized on a mixer. The pH of

the solution was measured and if necessary adjusted with 6M NaOH or 5M HCl. The Novarose was added to the bottle which was then placed on a shaker at room temperature. After 24 hr the non-bound PEI was removed from the adsorbent on a glass-filter by washing with deionized water until the effluent was neutral. The adsorbent was kept in 20% (V/V) ethanol in a refrigerator.

Preparation of Novarose-PEI_2 Adsorbents (PC_n complex)

Sixty grams of Novarose-PEI gel (CP-complex) were placed in a one liter conical flask together with 40 ml epichlorohydrin, 40 ml isopropanol, 40 ml H_2O and 60 grams of K_2CO_3 ($KHCO_3$ is produced progressively during the reaction time). The suspension was shaken over night (or a specified time 16 hours for example). The suspension was then diluted to double the volume with water (to dissolve salts) and the gel was collected in a glass-filter funnel and thoroughly washed with distilled water, ethanol (to get rid of the excess epichlorohydrin) and finally distilled water. The activated Novarose-PEI was suspended in an aqueous solution of PEI to produce Novarose-PEI_1- PEI_2 (PC_n complex). We may use the same PEI or a PEI of different molecular weight. As in the first coupling reaction, a very high concentration of the PEI will result in a high capacity. After some reaction time there will still be epoxy groups present. These groups may be reacted with TREN to further increase metal adsorption capacity. A progressive spontaneous hydrolysis of the epoxy groups is likely to occur. This limits the time we should select. Six hours exposure in case of PEI 2000 after which TREN is added to the suspension and the reaction time may be extended over 24 hours (or much more if this should be convenient).

Several Novarose-based gels were prepared from this starting materials and were used for metal binding studies. Results are presented in chapter 8.

CHAPTER 4

PEG-Chelated Modified Gels for Electrophoretic Protein Analysis and Characterization

4.1 ABSTRACT

In this work, we describe alternative materials for electrophoretic analysis of biomolecules. Agarose gels usually used in electrophoretic protein analysis have been modified with functional polymer-chelators. Polyethylene glycol-iminodiacetic acid (PEG-IDA) loaded with transition metal ions (Cu^{2+} , Ni^{2+} and Zn^{2+}) was used in appropriate concentrations with agarose to prepare several active biogels. In this approach, biomolecules are separated and characterized according to their electrophoretic mobilities and also to their specific affinities for the different chelated metals in biochemical systems (Goubran-Botros *et al.*, 1992).

Immobilized metal ion affinity electrophoresis technique in one dimension was applied to the separation and characterization of several model proteins. Dissociation constants for protein-chelator complexes were calculated from the relative mobilities measured in a series of gels formed with different concentrations of metal.

4.2 INTRODUCTION

Electrophoresis is a technique which separates charged molecules according to differences of electric charge and molecular structure. When two molecules have the same charge but different mobilities, the faster moving substance will overrun and migrate ahead

of the slower one. In affinity electrophoresis a charged molecule migrates through a matrix containing specific ligands under the influence of an electric field. The mobility of the molecule relative to its mobility in a gel containing no affinity ligand reflects interactions with the immobilized ligand (Schossler *et al.*, 1985). The concept of affinity electrophoresis was introduced by Bog-Hansen (1973), and used by Horejsi and Kocourek (1974). Every electrophoretic process that studies biospecific interactions belongs to this category of affinity electrophoresis. Early this decade, Vijayalakshmi and co-workers combined the principle of electrophoretic separation and the interaction of a biomolecule with a ligand immobilized within the electrophoresis gel support to develop a new affinity technique called immobilized metal ion affinity gel electrophoresis (IMAGE). IMAGE is a useful analytical tool for the quantitative determination of binding constants between biomolecules and chelated metal ions, for the efficient design of preparative-scale purification of proteins and enzymes using chromatographic of phase partition techniques, for the detection of protein unfolding and for the eventual study of structure/function relationships of modified proteins (Goubran-Botros *et al.*, 1992).

IMAGE as in IMAC, is an electron donor-acceptor mechanism. The metal ion immobilized through a chelating agent to an insoluble gel acts as an electron acceptor and the imidazole nitrogens of the accessible histidines of the surface of a protein act as the electron donors (Vijayalakshmi, 1989). It is known that there is a direct relationship between the number of unprotonated accessible histidine residues and the strength of protein binding to the polymer-supported metal ion (Sulkowski, 1987; Birkenmeir *et al.*, 1991; Wuenschell *et al.*, 1990). Therefore, if metal affinity electrophoresis (IMAE) is a direct extension of the generic IMA principle, this relationship should be maintained regardless of the applied electrical field. Ligand immobilization by covalent attachment to the support enhances both separation and resolution of the analyte. Accurate and precise binding constants are obtained when the ligand is firmly immobilized within the support

matrix. When metal-affinity electrophoresis is used as a preparative procedure, covalent immobilization of the chelating ligand reduces the likelihood that the metal ion complexes will leach into the purified protein sample.

Among potential applications for metal-affinity electrophoresis are: studying interactions between proteins and metal ion complexes and determining apparent binding constants, determining the content and distribution of metal-binding sites in peptides and on the surfaces of native proteins, probing metal-binding centers in denatured proteins, studying protein folding and processing by following changes in the accessibility of metal-coordinating residues, separating proteins that are very similar in mass charge (i.e., isoenzymes, mutants), but differ their metal-binding characteristics, detecting metal-binding centers of fusion peptides engineered into proteins to simplify purification or regulate activity, predicting the outcome of metal-affinity chromatography separations with very small samples and determining binding stoichiometries of biosystems.

In this chapter, following the description of Goubran-Botros *et al.* (1992), we present studies where biomolecules are separated and characterized according to their electrophoretic mobilities and also to their specific affinities for the different chelated metals.

4.3 MATERIALS AND METHODS

Materials

Agarose (type VII), methoxy polyethyleneglycol 5000, coomasie brilliant blue G250, cytochrome C (horse heart), cytochrome C (bovine heart) and ribonuclease A and B (from bovine pancreas) were purchased from Sigma (St. Louis, MO). Gel-bond film was obtained from Pharmacia (Uppsala, Sweden). All other chemicals utilized were of analytical or reagent grade.

Methods

Synthesis of Methoxy-Poly(ethyleneglycol) Chelating Ligand (M)-PEG-(IDA Me^{2+})

Methoxy polyethylene glycol amino (M)-PEG-NH_2 was synthesized according to Cordes (1985). Briefly, 5 g of (M)-PEG - OH were melted at 70 ° C and water was removed under vacuum. After addition of 1 ml of distilled thionyl chloride ($SOCl_2$), the mixture was rotated for 24 h at 65 ° C under a nitrogen atmosphere to exclude moisture. After removing the excess of thionyl chloride by evaporation under vacuum, the residue was dissolved in 250 ml of absolute ethanol and precipitated at 4 ° C. The dried (M)-PEG-Cl was dissolved in 150 ml of water and 150 ml of ammonium solution (28%) were added. The solution was placed in a sealed plastic tube and left for 100 h at 55 ° C in a dry oven. Lyophilization of the solvent yielded (M)-PEG-(NH_2).

Carboxymethylation of (M)-PEG-amino was performed by reacting 5 gr. of (M)-PEG-(NH_2) with a 50 molar excess of bromoacetic acid over the amino content. The mixture was kept at pH 9 and stirred for 24 hr to yield (M)-PEG-(IDA).

(M)-PEG-(IDA) was metallized with transition metal ions by dissolving 5 gr. of the ligand in 100 ml of buffer acetate pH 4.0 containing the metal sulfate salt in a 50 molar excess over the PEG ligands. The solution was stirred overnight and (M)-PEG- (IDA-Me^{2+}) was extracted in chloroform. The metal bound to the chelator was determined by atomic absorption using appropriate standards.

Preparation of Ligand-Incorporated Electrophoretic Gels

The soluble polymer (M)-PEG-(IDA-Me^{2+}), containing the affinity ligand IDA-Me(II) in the concentration range of 0 to 1.5% (w/v) of PEG 5000-IDA-Me(II) representing metal concentrations in the range of 0 to 3.0 mM, was incorporated into the 2% melted agarose (type VII) solution at 70 °C. The ligand containing mixture was then spread on the hydrophilic side of the gel bond for gel casting.

Electrophoretic Conditions

The amount of protein to be injected should not saturate all interaction sites and should permit the formation and dissociation of the ligand-protein complex. Also, the protein content of the sample should be sufficient to be detected by Coomassie blue staining but should also be as small as possible to form a fine band in agarose gels. With Coomassie blue staining, samples containing 5 μg of protein in 20 μl volume were introduced into the wells.

Electrophoresis experiments were run for 4 h in the conventional way using a LHB 2117 multiphor II electrophoresis unit connected to a power supply model 1000/500 from BioRad under the following electrophoretic conditions: electric field of 5 V/cm of electrophoretic plate, current intensity 15 mA, electrophoretic pH 7.2 buffered with Tris-acetate 0.1 M. Protein staining was done with 0.5% (w/v) Coomassie brilliant blue G-250 in methanol:acetic acid:water (4:1:5, v/v) for 10 minutes and destaining with methanol:acetic acid:water (4:1:5, v/v) for 5 h.

To determine the specific effects of metal-protein interactions on electrophoretic mobility, gels containing the metal ion complex were compared to a control gel lacking the metal-containing monomer.

Determining Dissociation Constants

The mobility decrease of a protein caused by gel immobilized metal is a function of the metal concentration. Its dissociation constant for the interaction between the protein and the immobilized metal ion complex, can be calculated by metal-affinity electrophoresis performed using a series of gels containing Me(II) concentrations from 0 to 1.5 mM. At concentrations greater than 1.5 mM, the proteins mobility is very low and accurate measurement of differential migration is difficult. The equilibrium for protein-ligand complex dissociation can be expressed as follows

$$P + C \leftrightarrow PC \qquad [1]$$

at equilibrium

$$K_d = \frac{[P][C]}{[PC]} \qquad [2]$$

where [P] and [C] are the concentrations of free protein and metal, [PC] is the concentration of protein-metal complex. K_d is the dissociation constant of the complex. When the protein is applied to the agarose gel containing a given concentration of immobilized metal ion, part of the protein interacts with the metal resulting in a decreased mobility. The extent of the mobility depends on the concentration of the metal and the following equation can be written (Takeo, 1984)

$$\frac{d_o}{d} = \frac{[P]_t}{[P]} \qquad [3]$$

where d_o is the migration distance of the protein in the absence of metal and d is that obtained in the presence of metal, $[P]_t$ is the total protein concentration and [P] is the free protein. Since $[P]_t = [P] + [PC]$ equation [3] becomes

$$\frac{d_o}{d} = 1 + \frac{[C]}{K_d} \qquad [4]$$

If all the affinity ligand is immobilized, as it is in this system, Equation [4] can be expressed as

$$\frac{do - d}{d} = \frac{[C]}{K_d} \qquad [5]$$

The dissociation constant (K_d) from affinity electrophoresis is obtained from the reciprocal of the slope of a plot of the relative retardation (d_o - d)/d versus the metal concentration (Takeo, 1984), where d_o is the mobility of proteins in the control gel, d is the mobility of proteins in the metal-affinity gel, C is the total concentration of metal, K_d dissociation constant of the protein-immobilized metal complex.

4.4 RESULTS AND DISCUSSION

Metal affinity gel electrophoresis was used to study several model proteins. The differential mobility distance (d_0-d)/d as a function of immobilized ligand [M]-PEG-IDA (Me $^{2+}$) mM concentration was plotted for each protein with different transition metals (Cu^{2+}, N^{i2+} and Zn^{2+}). The dissociation constants were then calculated from the reciprocal of slope of the plots (figures 4.2 to 4.10) according to Bog-Hansen Takeo equation (figure 4.1). Table 4.1 summarizes the dissociation constants obtained for Cytochrome C from horse heart , cytochrome C from bovine heart, ribonuclease A and B at pH 7.2. The range of pH values at which the systems can be run will depend on the relative migration of the free protein and the protein-ligand complex at the given pH.

Tris-acetate, 0.1 M, pH 7.2, was selected because the optimum operating pH in IMA-Elec is near neutral pH (Goubran-Botros *et al.*, 1992). In general, the dissociation constant for the proteins was lower in the case of immobilized [M]-PEG-IDA (Cu $^{2+}$), that is, higher

association constant. As expected, the affinity of proteins for the chelated metal increases with the number of d electrons in the metal and declines with zinc. This is in agreement with the Irving-Williams series for immobilized metals in polymer matrices (Albertsson, 1958). According to Sulkowski (1987), copper is able to recognize even a single exposed histidine or even dispersed histidines on the surface of a protein, whereas zinc recognizes only groups of histidines with His-$(x)_{2,3}$-His or histidines linked by an α-helix.

It is known that protein-metal affinity interactions are based on the number of accessible histidines (Arnold, 1991). Immobilized metal ion affinity electrophoresis agrees well with other metal affinity techniques such as IMAC and IMAP . Cytochrome C from horse heart and bovine heart have 1 and 2 exposed histidines respectively and their dissociation constants were found to be of the same order of magnitude. For Rnase, the dissociation constant for Rnase B was in general higher than Rnase A. Ribonuclease A and B both have 4 accessible histidines. However, ribonuclease B is glycosylated at Asn 34 and ribonuclease A is non glycosilated. According to Goubran-Botros *et al.* (1992), glycosylation can influence at least through long range forces and by steric hindrance the microenvironment of histidines 119 and 48. Therefore Rnase B behaves as a protein with only 2 exposed histidines.

It is important to emphasize that Takeo's equation has some quantitative limitations and that some assumptions need to be specified in order to be valid. This assumptions include : complete immobilization of the ligand within the gel, that all molecules of the immobilized ligand are equally accessible to interaction with the protein, homogeneous microdistribution of the ligand, negligible influence of the electric field on K_d and that the nature of the gel has a negligible influence on K_d.

The values here obtained for K_d in all the experiments, should therefore be considered as apparent constants (a more complete set of experimental data is required to eliminate the uncertainty of the results). An average of 10-20% error was found in all the determinations of K_d. It is clear that the actual thermodynamic constants must differ from the apparent constants due to all the assumptions listed above.

Even though several assumptions have been made , the estimated dissociation constants agree well with the values report elsewhere. However, the values of K_d obtained by IMAGE should be taken carefully and those assumptions need to be considered. The values of the dissociation constants here calculated are then to be taken as comparative values for a fast determination of the strength of a protein-ligand interaction.

4.5 CONCLUSIONS

In immobilized metal ion affinity electrophoresis, the basic concept of protein recognition is conserved. According to the electrophoretic dissociation constants calculated for proteins in the presence of different metal ions, the strength of the affinity interactions follows the order Cu^{2+} >Ni^{2+} >Zn^{2+} . The affinity of proteins for the immobilized metal increases with the number of accessible histidines on their surface. Immobilized metal ion affinity electrophoresis can be used as a technique to characterize the surface of proteins.

The K_d values obtained by gel electrophoresis are estimated values since they are influenced by the presence of electric fields. However, under the appropriate experimental conditions, metal affinity electrophoresis offers a rapid analytical technique for protein screening.

Affinity Electrophoresis

Bog-Hansen-Takeo Equation

$$\frac{d}{d_o - d} = \frac{K_i}{C_i}\left[1 + \frac{C}{K}\right]$$

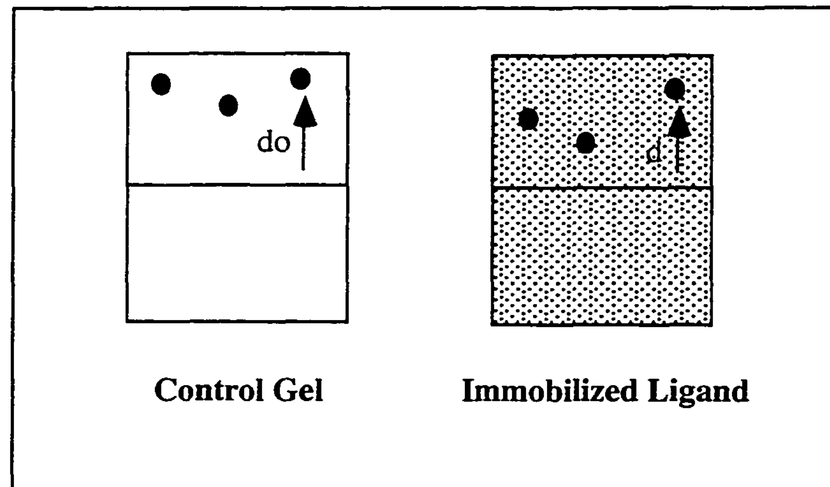


Figure 4.1 Migration distances of proteins measured on vertical electrophoretic gels. The dissociation constant (K_d) of a protein-immobilized ligand complex was calculated from the reciprocal of the slope of a plot of the relative retardation (d_o-d) /d versus the ligand concentration according to the Bog-Hansen-Takeo equation (1984).

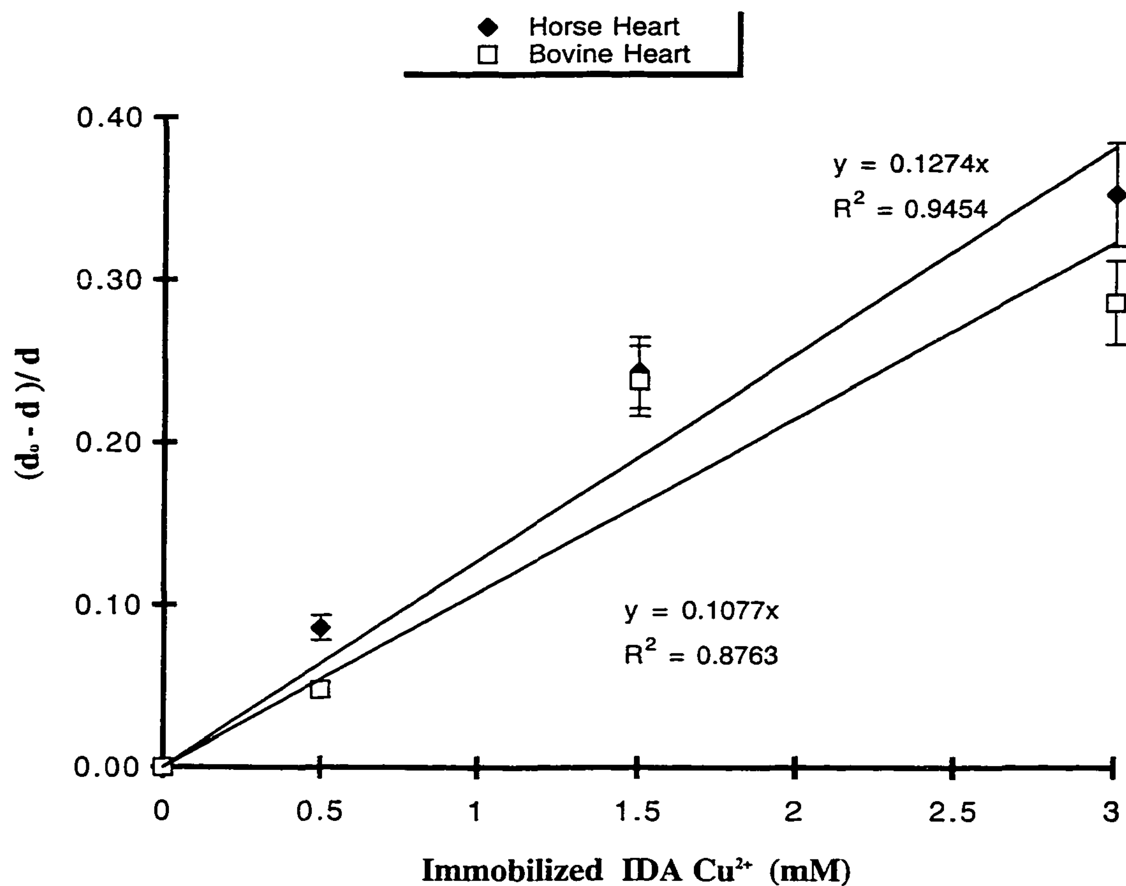


Figure 4.2 Differential mobility of proteins as a function of IDA Cu^{2+} concentration expressed in mM. Electrophoretic buffer, 20 mM Tris-acetate (pH 7.2). Cytochrome C form horse heart, K_d= 7.85 mM; Cytochrome C from bovine heart, K_d= 9.29 mM.

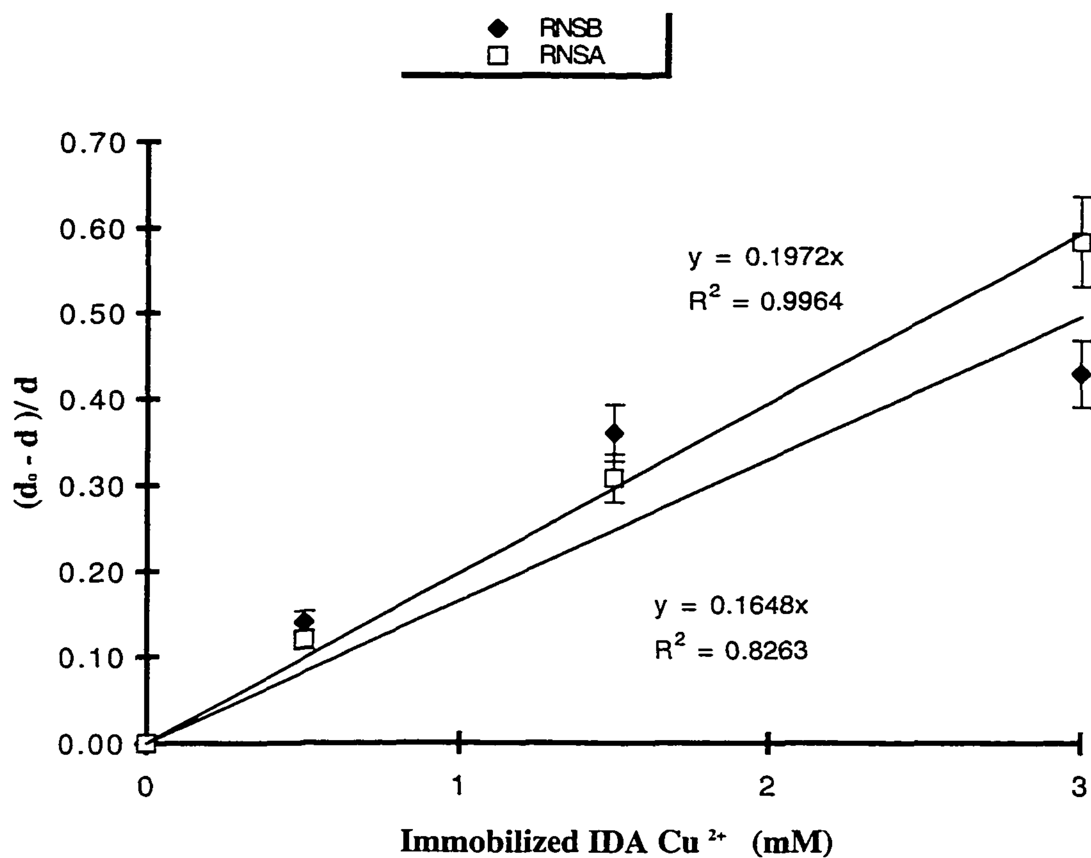


Figure 4.3 Differential mobility of proteins as a function of IDA Cu^{2+} concentration expressed in mM. Electrophoretic buffer, 20 mM Tris-acetate (pH 7.2). Ribonuclease A, K_d= 5.07 mM ; Ribonuclease B, K_d= 6.07 mM.

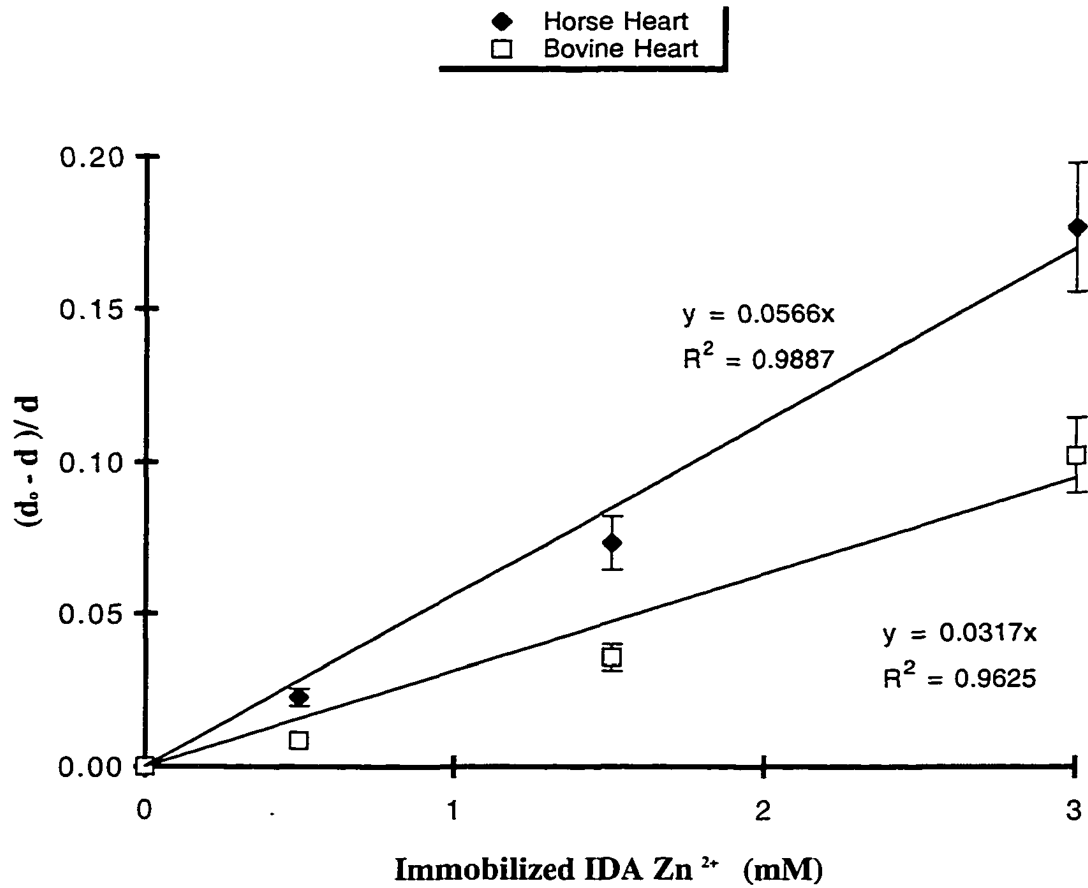


Figure 4.4 Differential mobility of proteins as a function of IDA Zn^{2+} concentration expressed in mM. Electrophoretic buffer, 20 mM Tris-acetate (pH 7.2). Cytochrome C form horse heart, K_d= 17.67 mM; Cytochrome C from bovine heart, K_d= 31.55 mM.

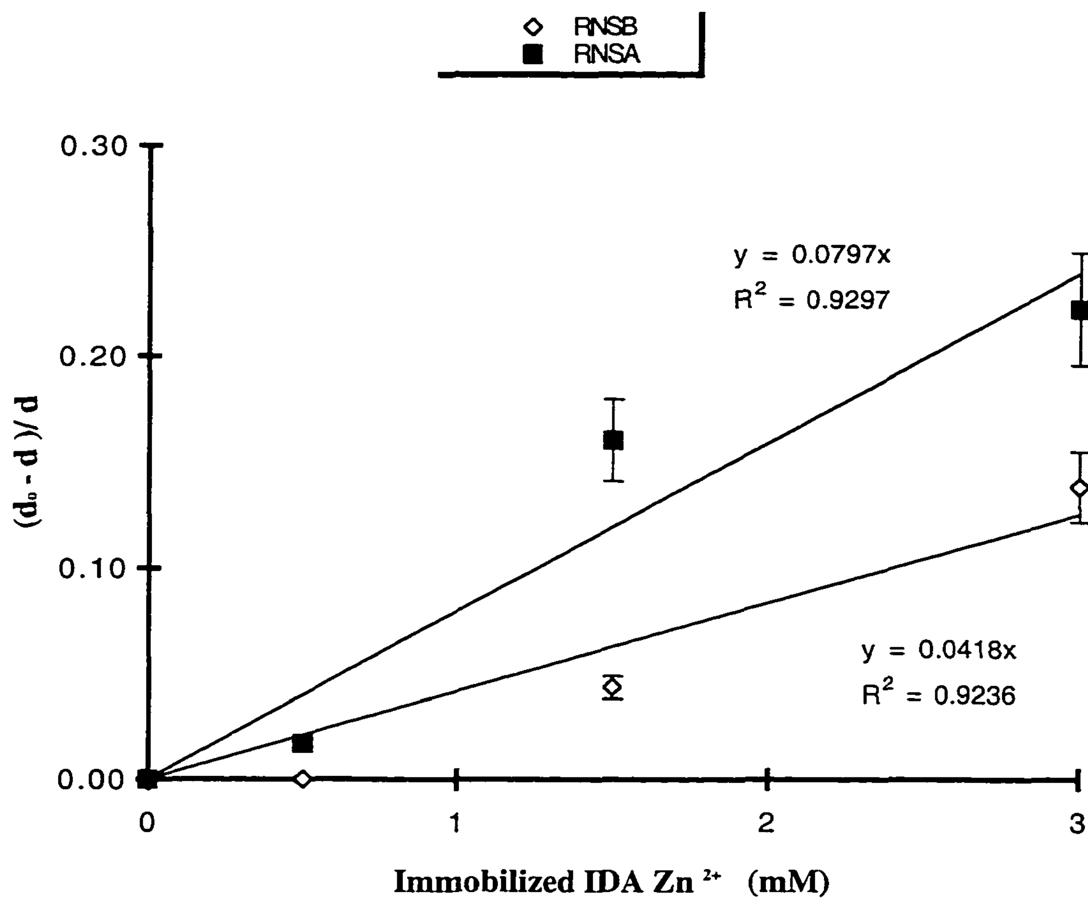


Figure 4.5 Differential mobility of proteins as a function of IDA Zn^{2+} concentration expressed in mM. Electrophoretic buffer, 20 mM Tris-acetate (pH 7.2). Ribonuclease A, K_d= 12.55 mM; Ribonuclease B, K_d= 23.92 mM.

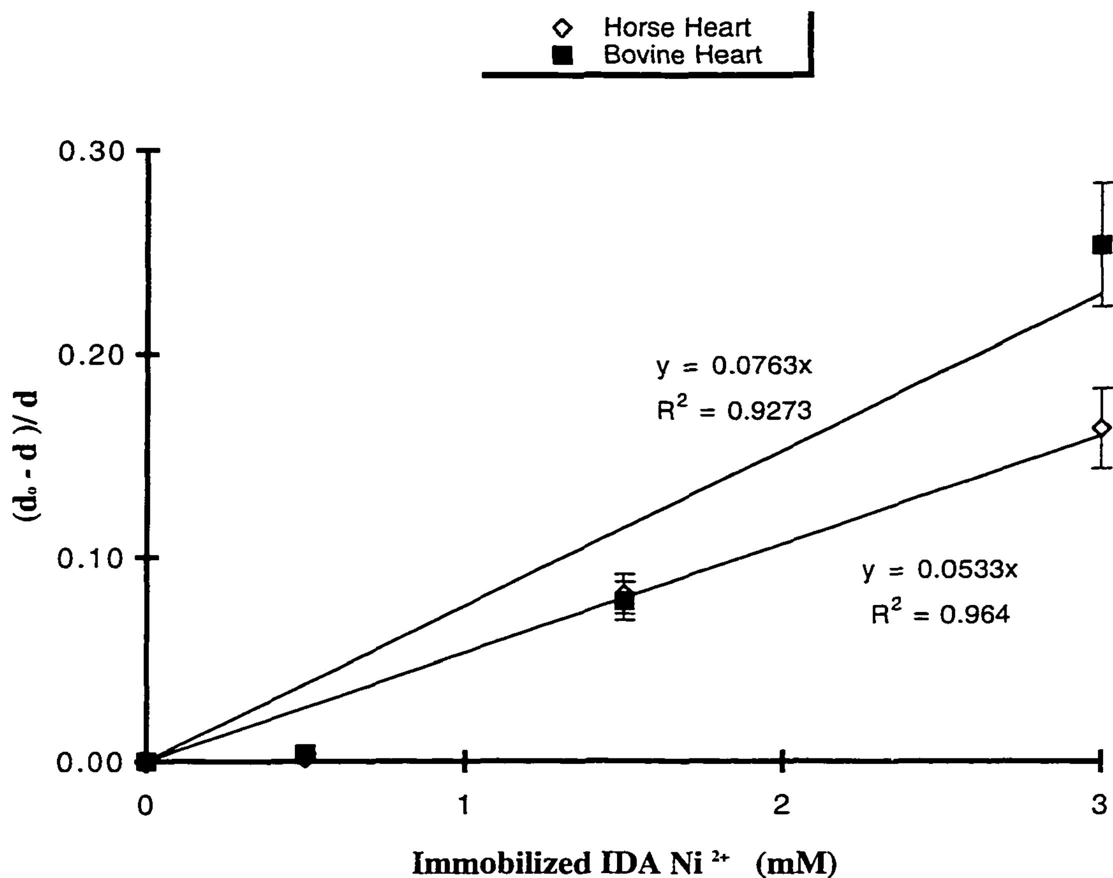


Figure 4.6 Differential mobility of proteins as a function of IDA Ni^{2+} concentration expressed in mM. Electrophoretic buffer, 20 mM Tris-acetate (pH 7.2). Cytochrome C form horse heart, K_d= 18.76 mM; Cytochrome C from bovine, K_d=13.11 mM.

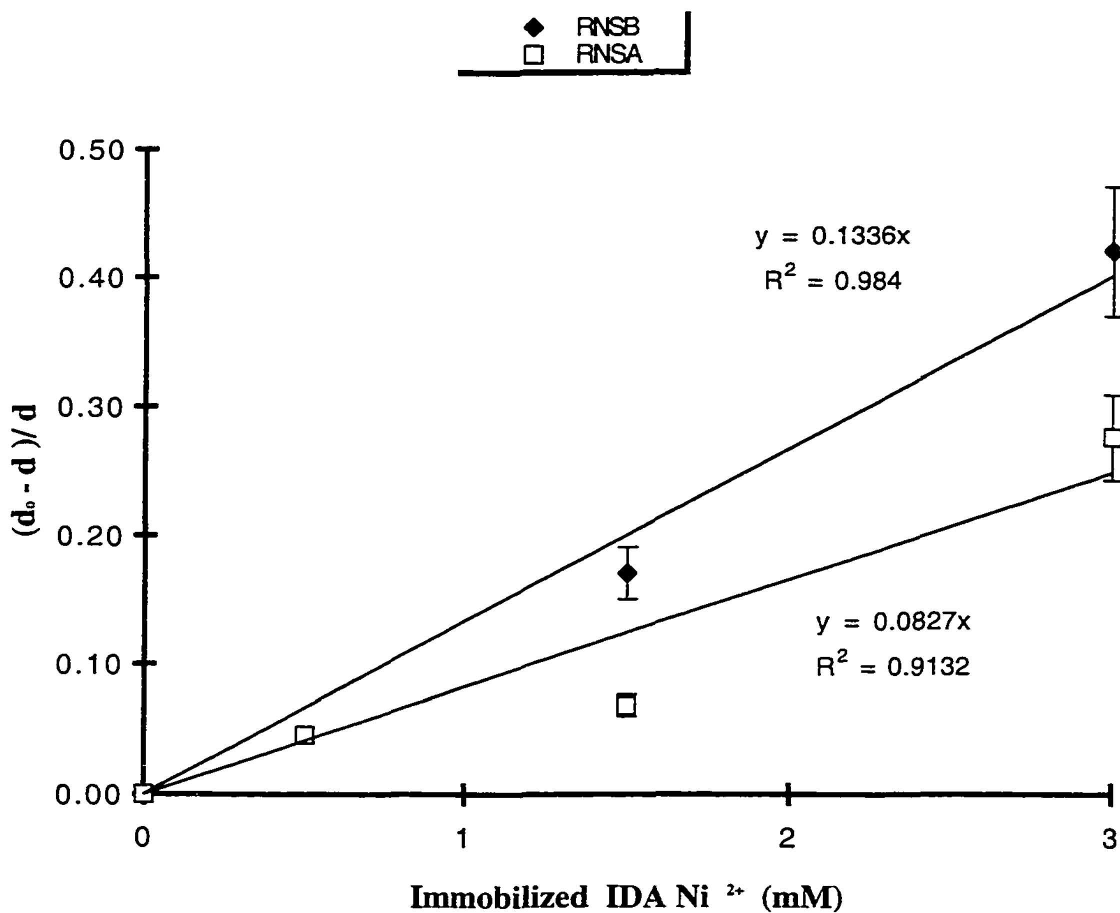


Figure 4.7 Differential mobility of proteins as a function of IDA Ni^{2+} concentration expressed in mM. Electrophoretic buffer, 20 mM Tris-acetate (pH 7.2). Ribonuclease A, K_d=12.09 mM ; Ribonuclease B, K_d=7.49 mM.

Protein	*Cu(II) IDA*	*Ni(II) IDA*	*Zn(II) IDA*
Cytochrome C (Horse Heart)	7.85	17.67	18.76
Cytochrome C (Bovine Heart)	9.29	31.55	13.11
Ribonuclease A	5.07	12.55	12.06
Ribonuclease B	6.07	23.92	7.49

Table 4.1 Dissociation constants K_d (mM) for model proteins and gel-immobilized Me (II) IDA.

CHAPTER 5

Metal Ion Affinity Size Exclusion Chromatography

5.1 ABSTRACT

Metal affinity size exclusion chromatography (MASEC) is a purification method that we are developing to enhance the separation of biomolecule mixtures using gel permeation chromatography. MASEC is based on the combination of the metal affinity principle and gel permeation. In this work, star PEG-$(OH)_{64}$ with a molecular weight of 700,000 Da was used to prepare a multi-armed chelator bearing iminodiacetic acid groups (IDA). The resulting PEG macroligand, loaded with copper, was allowed to interact in a batch mode with mixtures of hemoglobin, cytochrome C and ovalbumin. Due to its preferential affinity binding for hemoglobin, the ligand served as a carrier for this protein in gel permeation chromatography. Also due to its high molecular size, chelating star PEG acted as a protein-complementary molecule of bound hemoglobin modifying its retention time in the gel permeation column, allowing an efficient protein fractionation. Here we present the experimental results of the fractionation of mixtures of bovine hemoglobin with ovalbumin and horse heart cytochrome C by MASEC. Mathematical modeling to predict the differential chromatographic separation of this protein mixture is also presented.

5.2 INTRODUCTION

Downstream processing is of growing importance in biotechnology. There is a need for large-scale purification technology to take care of the initial purification steps. However, there is a greater demand for high resolving methods to purify the products.

With the production of substances using genetically modified microorganisms, the demands placed on purification technology have increased. Therefore, the commercialization of biotechnology depends on the ability to purify biomolecules such as peptides, proteins and enzymes. These biomolecules are usually sensitive to pH, shear stress, temperature, and solvents commonly used in purification techniques.

In recent years, several studies in purification based on ligand-biomolecule interactions have been conducted to provide more effective purification and recovery procedures for very dilute and fragile products. These studies include affinity cross-flow filtration (Luong *et al.*, 1987) and affinity ultrafiltration (Mattiasson *et al.*, 1984). Purification and recovery is then one area demanding innovative process development.

The present work describes the results of an alternative mode of affinity interaction that has a potential to develop as a feasible protein separation method.

5.3 MATERIALS AND METHODS

Materials

Novarose-SE 1000/17 column was acquired from inovata AB (Bromma, Sweden). Bovine hemoglobin, human hemoglobin, horse hemoglobin, porcine hemoglobin, whale myoglobin, horse myoglobin, ovalbumin, cytochrome C from horse heart, thionyl chloride ($SOCl_2$) and picrylsulfonic acid (2,4,6-trinitrobenzenesulfonic acid, TNBS) were acquired from Sigma (St. Louis, MO).

Star polyethylene glycol with hydroxyl ends and an average molecular weight of 700,000 Daltons was purchased from Shear Waters Polymers, Inc. (Huntsville, Alabama). All other chemicals utilized were of analytical or reagent grade.

Equipment

Size exclusion chromatography was performed in a HPLC ISCO system composed of a 2360 model gradient programmer, a 2350 model pump and a V^4 absorbance detector unit operating at 280 or 405 nm. A novarose-SE 1000/17 column was used for all analytical determinations performed using chemresearch chromatographic data management/system controller version 2.4 provided by ISCO. In all experiments, the amount of protein adsorbed by star PEG chelator and unbound protein were calculated by measuring the area under the peaks obtained in each chromatogram. Samples were buffered with 0.02 M tris and 0.1 M NaCl buffer pH 7.5. SEC was carried out at a flow rate of 0.5 ml/min.

Methods

Experimental

Preparation of Amino Star-PEG - $(NH_2)_{64}$

Amino star PEG - $(NH_2)_{64}$ was synthesized as follows: 1 g of PEG star was melted at 70 °C and water was removed under vacuum. After addition of 1 ml of distilled thionyl chloride ($SOCl_2$), the mixture was rotated for 24 h at 65 ° C under a nitrogen atmosphere to exclude moisture. After removing the excess of thionyl chloride by

evaporation under vacuum, the residue was dissolved in 150 ml of absolute ethanol and precipitated at 4 °C. The dried star PEG - $(Cl)_{64}$ was dissolved in 150 ml of water and 150 ml of ammonium solution (28%) were added. The solution was placed in a sealed plastic tube and left for 100 h at 55 °C in a dry oven. After amination, the PEG star was filtrated in an ultrafiltration cell to eliminate the excess of ammonium. Lyophilization of the solvent yielded 0.81 g of PEG - $(NH_2)_{64}$.

Preparation of Chelating Star PEG - $(IDA)_{64}$

Carboxymethylation of star PEG-amino was performed by reacting 1 gr. of Star PEG - $(NH_2)_{64}$ with a 50 molar excess of bromoacetic acid over the amino content. The mixture was kept at pH 9 and stirred for 24 hr. Unreacted bromoacetic acid was removed by ultrafiltration and the solution lyophilized to obtained 0.8 gr. of Star PEG - $(IDA)_{64}$.

Metal Loading of Star PEG - $(IDA)_{64}$

Star PEG-$(IDA)_{64}$ was loaded with copper ions by dissolving 0.5 gr. of the ligand in 10 ml of buffer acetate pH 4.0 containing cupric sulfate salt in a 50 molar excess over the PEG ligands. The solution was stirred overnight and the free copper was removed by ultrafiltration until no copper was detected by light scattering. The solution was lyophilized yielding 0.45 gr. of Star PEG -$(IDA\text{-}Cu^{2+})_{64}$

Measurement of Amino Content and Copper Capacity

The Extent of amination of star PEG - $(NH_2)_{64}$ was tested by light scattering using the TNBS test (Habeeb, 1966) indicating complete amination. After carboxymethylation of amino star PEG, the TNBS tested negative indicating no amino groups left in the polymer. Metal loading was analyzed by atomic absorption and the copper capacity of star PEG - $(NH_2)_{64}$ was found to be 60 μmoles of Cu^{2+} per μmole of polymer corresponding to 94% of metal loading.

Determination of PEG -(IDA Cu^{2+})$_{64}$-Protein Adsorption Isotherms

To estimate the capacity of PEG -(IDA Cu^{2+}) $_{64}$ for proteins, several protein-star chelator samples were prepared in a batch mode using 5 mg/ml of PEG -(IDA Cu^{2+}) $_{64}$ and different protein concentrations in the range of 0 to 12 mg/ml. Samples were gently shaken and incubated for a period of 1 hr to ensure maximum protein binding. Samples were then analyzed by SEC.

Analysis and Purification of Proteins

Protein purification and separation of protein mixtures was performed by mixing the sample containing the target molecule with PEG -(IDA Cu^{2+}) $_{64}$ in a batch mode. After incubation for 1 hr, the mixture was analyzed by SEC. In all cases, the protein concentration was 0.5 mg/ml whereas 5 mg/ml of PEG -(IDA Cu^{2+}) $_{64}$ was used.

SEC Mathematical Modeling

One-Dimensional Lumped Parameter Model Prediction of Chromatographic Protein Separations

In this section, the objective is to model size exclusion chromatography (SEC) using one-dimensional lumped-parameter model of differential chromatography to predict chromatography protein separation following the model of Gibbs and Lightfoot model (1986) and that of Lightfoot et al. (1991). The assumptions to this model are:

- Linear chromatography for an adsorptive system
- Differential chromatography (pulse input)
- Size exclusion is treated as an special case ($K_d = 0$)
- We use an asymptotic long-time solution (valid for long columns)
- Neglect non-uniformities within the bed

  (Homogeneous packing and flow distribution)
- Isothermal and isocratic

Governing Equations

- Convective transport of solute by the mobile phase in a one dimensional, pseudo-continuum model

$$\varepsilon_b\left(\frac{\partial C_f}{\partial t}+v\frac{\partial C_f}{\partial Z}-E\frac{\partial^2 C_f}{\partial Z^2}\right)=-(1-\varepsilon_b)\frac{\partial C_b}{\partial t} \qquad [1]$$

where :

C_f and C_b = Conc. of a solute in the mobile and stationary phases respectively (mg/ml)

v = interstitial velocity based on the area $A_o\ \varepsilon_b$ (cm/min)

ε_b = interstitial void fraction of the packed bed

E = convective axial dispersion coefficient which accounts for axial molecular diffusion (cm^2/min)

t = time (min)

z = axial coordinate

- The solute transport inside a porous sorbent particle is described by :

$$\frac{\partial w_s}{\partial t} = \frac{D_p}{r^2} \frac{\partial \left(r^2 \frac{\partial w_p}{\partial r} \right)}{\partial r} - F(w_p, w_s) \qquad \text{for} \qquad 0 \le r \le R \qquad [2]$$

$$\frac{\partial w_p}{\partial t} = F(w_p, w_s) \qquad \text{for} \qquad 0 \le r \le R \qquad [3]$$

$$\frac{\partial wp}{\partial r} = 0 \qquad \text{for} \qquad r = 0 \qquad [4]$$

w_p = local concentrations of solute present in the pore fluid

w_s = local concentrations of solute adsorbed on the internal surfaces

(based on a unit volume of stationary phase) (mg/ml)

- The local adsorption kinetics is expressed as :

$$F(w_p, w_s) = K_a \left(w_p - \frac{w_s}{K_d} \right) \qquad [5]$$

where :

K_a = forward rate constant of adsorption (1/min)

K_d = equilibrium constant (the ratio of the mass of adsorbed of solute to that of unadsorbed solute within a particle at equilibrium)

- The stationary phase concentration of solute, C_b, is given by :

$$C_b \equiv \left(\frac{3}{4\pi R^3}\right)\int_0^R (w_p + w_s)4\pi r^2 dr \qquad [6]$$

matching the conditions of flux continuity at the particle surface

$$-D_p \frac{\partial w_p}{\partial r} = K_c\left(\frac{1}{\varepsilon *_p} w_p - C_f\right) \quad \text{for} \qquad r = R \qquad [7]$$

where :

K_c = fluid phase mass transfer coefficient (cm/min)

$\varepsilon*_p$ = solute-dependent inclusion porosity.

- The initial conditions for the concentrations in the fluid and stationary phases are given by :

$$C_f(z, t = 0) = \frac{m_o}{A_o \varepsilon_b} \delta(z) \quad [8]$$

$$C_b(z, t = 0) = 0 \quad [9]$$

where :

A_o = column cross-sectional area (cm^2)

m_o = mass of solute initially injected (mg)

ε_b = interstitial void fraction

$\delta(\ )$ = Dirac delta function.

Solution to the model

- long columns in a sense that the boundary conditions have little effect on the peak shape
- the long-column form considers that the concentration field decays rapidly at long distances

$$\lim_{z \to \pm \alpha} C_f = \lim_{z \to \pm \alpha} C_b = 0 \quad [10]$$

- The long-time solution of a linear chromatography is a Gaussian function of the axial coordinate z in the column (Reis et al., 1979) given by :

$$C_f(z,t) = \frac{m_o u}{A_o \varepsilon_b \sqrt{2\pi H z_o}} \exp\left[\frac{-(z - z_o)^2}{2Hz_o}\right] \qquad [11]$$

where :

$$z_o \equiv uvt \qquad [12]$$

$$u \equiv \frac{\varepsilon_b}{\varepsilon_b + (1-\varepsilon_b)\varepsilon_p{}^*(1+K_d)} \qquad [13]$$

v = interstitial fluid velocity (cm/min)

u = fraction of solute in the moving fluid phase at long times

$\mathbf{K_d}$ = equilibrium constant for adsorption ($K_d = 0$ for SEC)

$\boldsymbol{\varepsilon_p}$* = species-dependent inclusion porosity

5.4 RESULTS AND DISCUSSION

It was stated earlier in this chapter that the retention time of a bound molecule in a SEC column can be modified when using a macroligand as a carrier. The principle behind this technique is to use a gel permeation column that will exclude some molecules in a mixture of similar size and delay the molecule of interest that interacts with a macromolecular chelator that is large enough to be retained in the gel. In this study, PEG -(IDA Cu^{2+}) 64 with a molecular weight of 700,000 Daltons was prepared and used as the interactive macromolecule. Table 5.1 shows the ability of chelating PEG -(IDA Cu^{2+}) 64 to interact with several proteins. It is clear that the protein-chelator interaction follows the basic principle of metal affinity interactions introduced by Porath and co-workers in 1975. Protein adsorption is governed by the number or density of accessible histidines on the surface of the protein (Vijayalakshmi, 1989). Of all the proteins tested, hemoglobin , with an average of 20 accessible histidines on its surface, was adsorbed almost completely. Ovalbumin and Cytochrome C, with 3 and 2 histidines residues respectively , were only partially removed.

Bovine hemoglobin was selected as the model protein to study the adsorption kinetics of PEG -(IDA Cu^{2+}) 64 . Bovine hemoglobin was chosen for this study due to its high affinity interaction with chelators of this kind. Protein adsorption is relatively fast with a rate constant $K = 82\ M^{-1}/S$. Figure 5.4 shows that the initial amount of protein is depleted almost 100% in less than one hour. Figure 5.5 shows the adsorption isotherm and capacity of PEG -(IDA Cu^{2+}) 64 for bovine hemoglobin. The capacity of metal chelated star PEG for hemoglobin was found to be 0.56 mg of protein per mg of polymer or equivalently 10 moles of bovine hemoglobin per mole of polymer. The copper capacity of the chelated star PEG was calculated as 60 moles of copper per mole of polymer. This represents 94 % of metal loading since the star PEG used in this study had 64 chelated

arms. The amount of protein adsorbed was low as expected. This result may be due to steric hindrance since hemoglobin is a big molecule preventing the protein from interacting with the polymer. Hemoglobin has 24 accessible histidines acting as metal binding sites, therefore, protein binding is not likely a one to one protein-chelator interaction. The adsorption isotherm obtained is typically of the Langmuir type, as for many other types of reactions where the solutes are bound tightly, usually as a result of multipoint interactions between the adsorption sites and the solutes. According to Langmuir's model, the association constant of hemoglobin with PEG -(IDA Cu^{2+}) 64 was Ka = 3.4 X 10^5 M^{-1}. This value is a quantitative measure of the stability of the complex and the affinity of the protein for the chelated polymer.

Figures 5.6 through 5.11 show the selective separation of mixtures of proteins based on the differential affinity of proteins for PEG -(IDA Cu^{2+}) 64. A mixture of bovine hemoglobin and ovalbumin (figure 5.8) or bovine hemoglobin and cytochrome C (figure 5.9) could not be separated by gel permeation alone due to their similar retention time. When PEG -(IDA Cu^{2+}) 64 was added to the mixtures, and incubated for 1 hour, hemoglobin was selectively adsorbed by the polymer, allowing the effective separation and purification of ovalbumin and cytochrome C (figures 5.10 and 5.11).

Figures 5.12 and 5.13 show the predicted protein separation as compare to the experimental results. The one dimensional differential chromatography model used simulated very accurately the separation of the protein mixtures. This model was fitted using only the species-dependent inclusion porosity (ε^*_p) as the one-lumped parameter. The efficiency plate (H) used for the calculations was 0.04 cm.

5.5 CONCLUSIONS

Immobilized metal ion affinity size exclusion chromatography is another technique derived from the immobilized metal ion affinity chromatography principle. The chelator-protein affinity interaction is also controlled by the number of accessible histidine residues on the protein surface that can interact by coordination bonds with immobilized transition metal chelates. With the introduction of modified star polyethylene glycol PEG-(IDA-Cu^{2+})$_{64}$ into size exclusion chromatography, a combined effect has been accomplished. Affinity ligand interactions and size exclusion chromatography have been grouped to successfully enhance protein extraction. Star polyethylene glycol PEG-(IDA-Cu)$_{64}$, a 700,000 Da MW polymer, contains 64 arms that work as flexible spacers able to grab proteins as any other chelator of its type. Due to its relatively high molecular weight, the protein retention times in gel permeation column can be displaced to early elution times allowing an alternative method for protein separation.

Finally, a one dimensional differential chromatographic mathematical model has been proposed that was able to predict very accurately protein separation.

Functional Star-Shaped PEG - (OH $)_n$

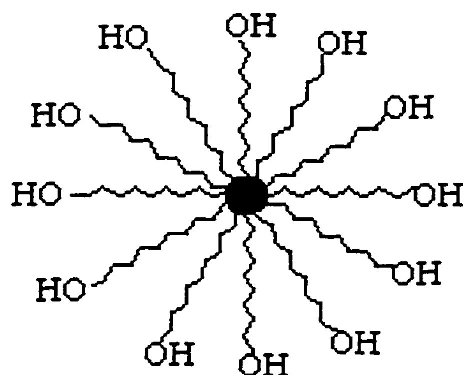


Figure 5.1 Star PEGs are multi-armed PEGs made by polymerization of ethylene oxide from a cross-linked divinyl benzene core. The large number of arms, as compared to usual linear or branched PEGs permits formation of strong hydrogels and ready immobilization on surfaces. Star PEGs hydroxyl groups can be modified and activated to be coupled to a variety of ligands, including proteins.

(Gnanou, *et al.*, 1988; Merril, 1992).

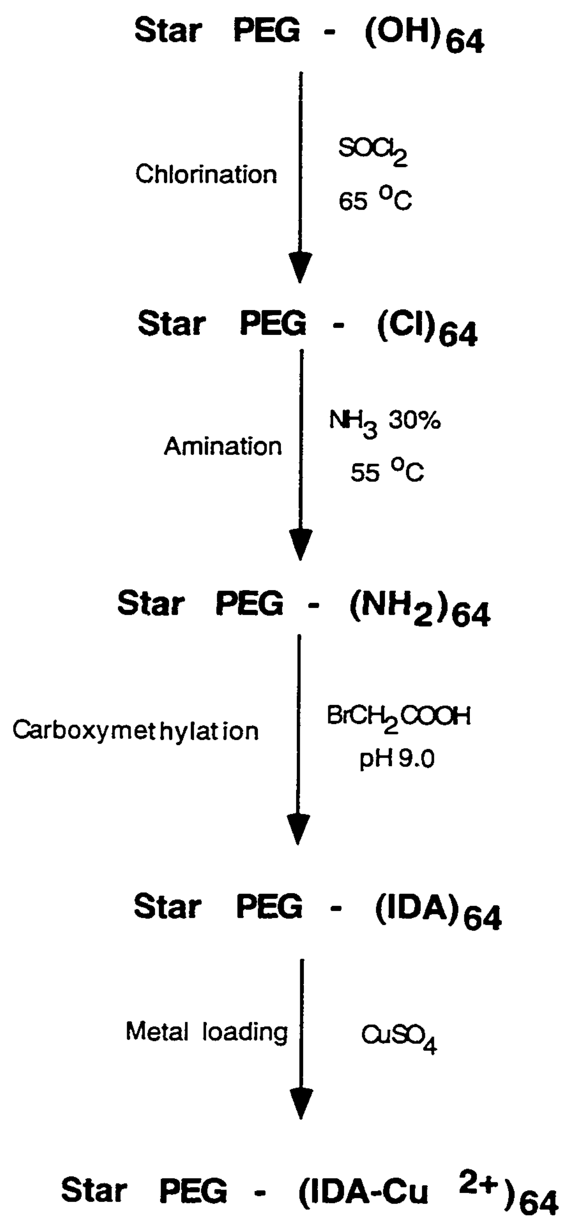


Figure 5.2 Synthetic route of Star PEG - (IDA-Cu)$_{64}$

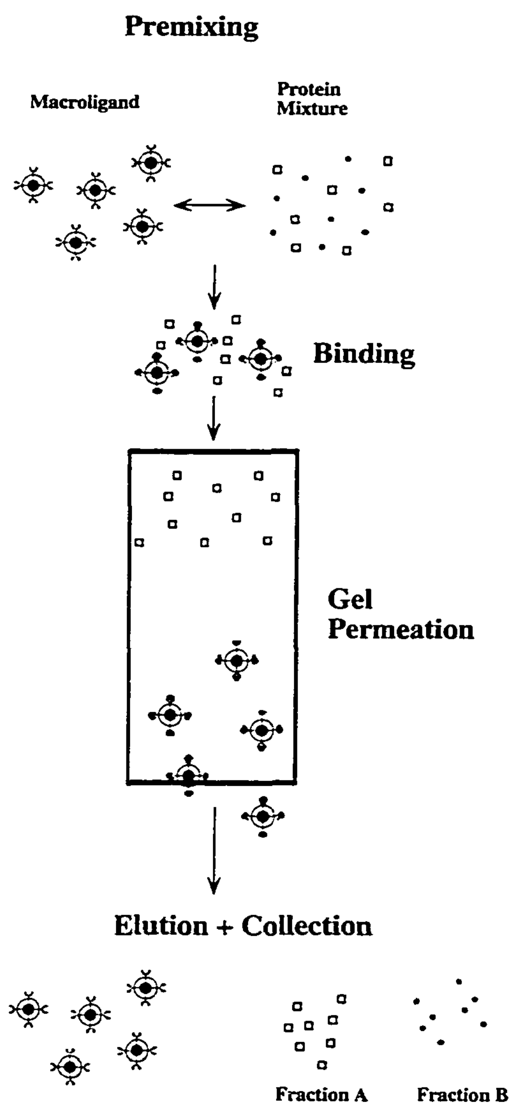


Figure 5.3 Idealized concept of Metal Affinity Size Exclusion Chromatography (MASEC)

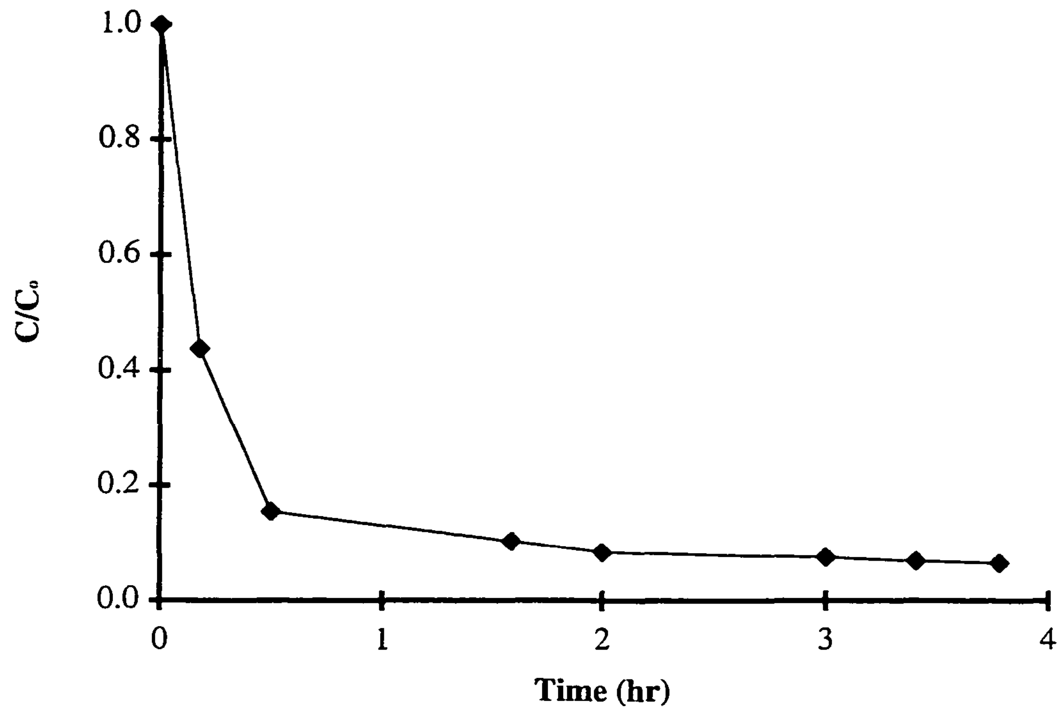


Figure 5.4 Adsorption rate of 0.5 mg/ml bovine hemoglobin in 5 mg/ml star PEG-(IDA Cu^{2+}) 64. Hemoglobin was almost completely adsorbed in 1 hr. Adsorption was found to best fit a second order reaction rate with K = 82 M^{-1}/ S.

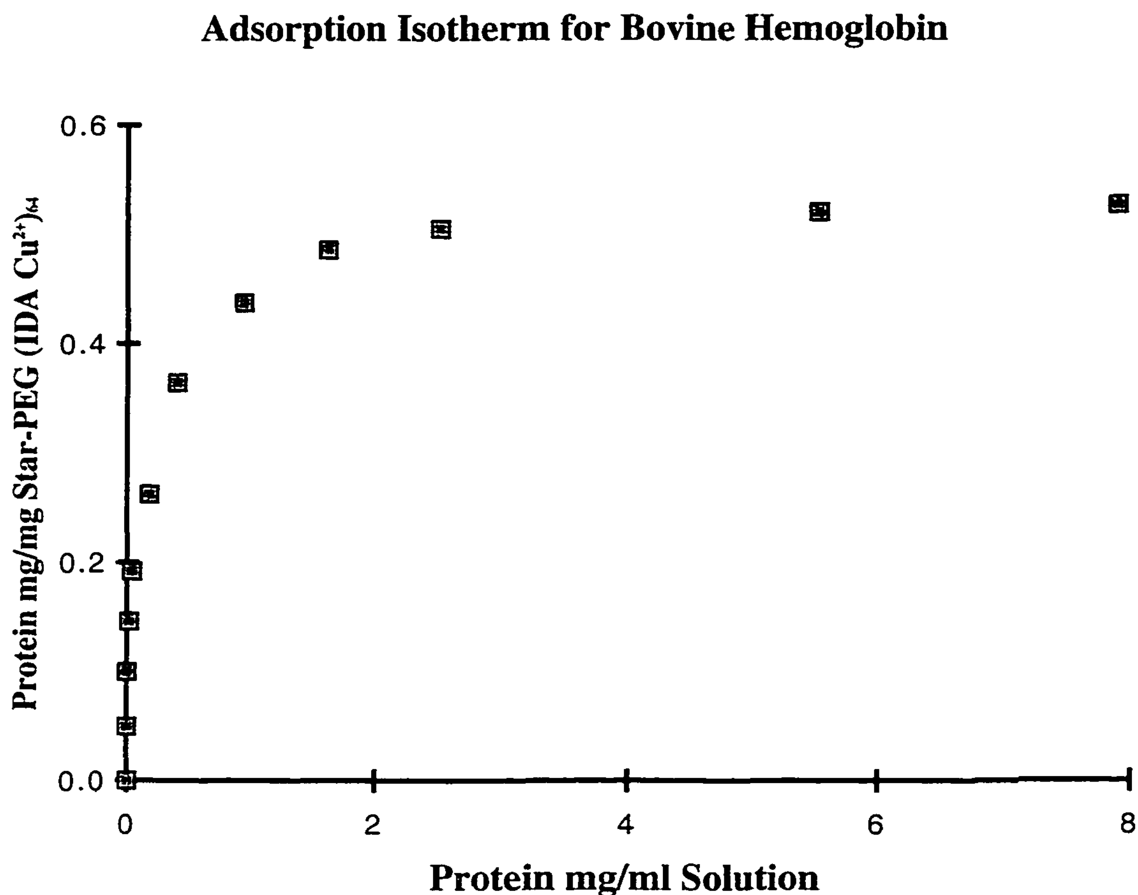


Figure 5.5 Adsorption isotherm for bovine Hemoglobin using 5 mg/ml of star PEG - (IDA Cu^{2+})$_{64}$. The amount of protein adsorbed depends upon the initial concentration of the protein to which the chelator is exposed until a saturation level is reached. This is the level at which the chelating capacity of star PEG - (IDA Cu^{2+})$_{64}$ can be measured with certainty. According to the Langmuir model, the dissociation constant for this reaction was found to be K_d = 2.9 µmols/l.

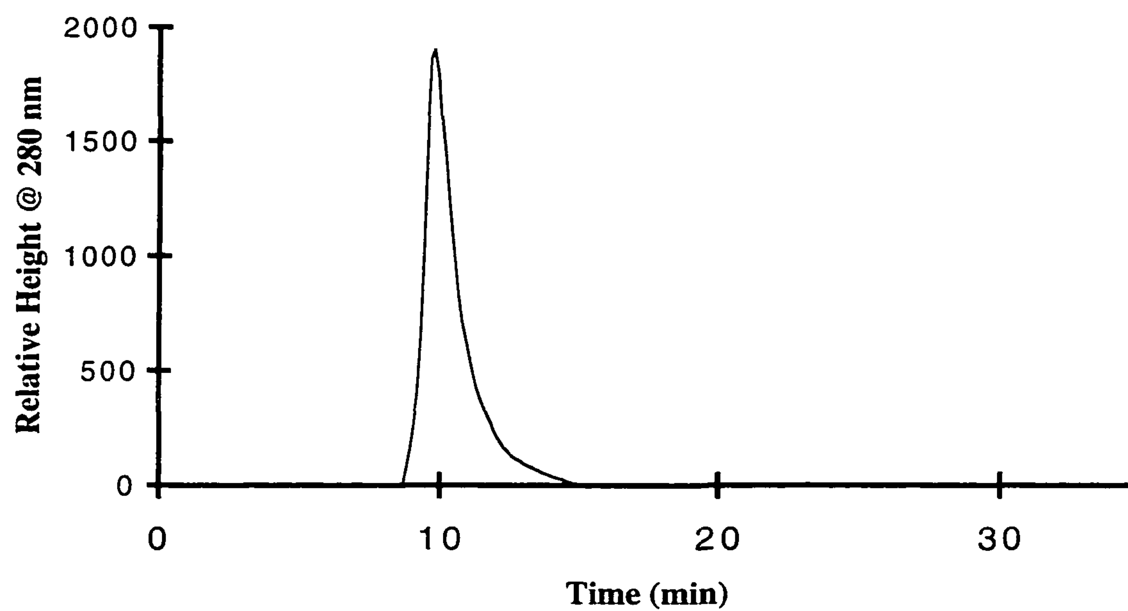


Figure 5.6 SEC of 5.0 mg/ml PEG - (IDA Cu^{2+})$_{64}$ on Novarose SE 1000/17. PEG - (IDA Cu^{2+})$_{64}$ Tr = 11.5 minutes.

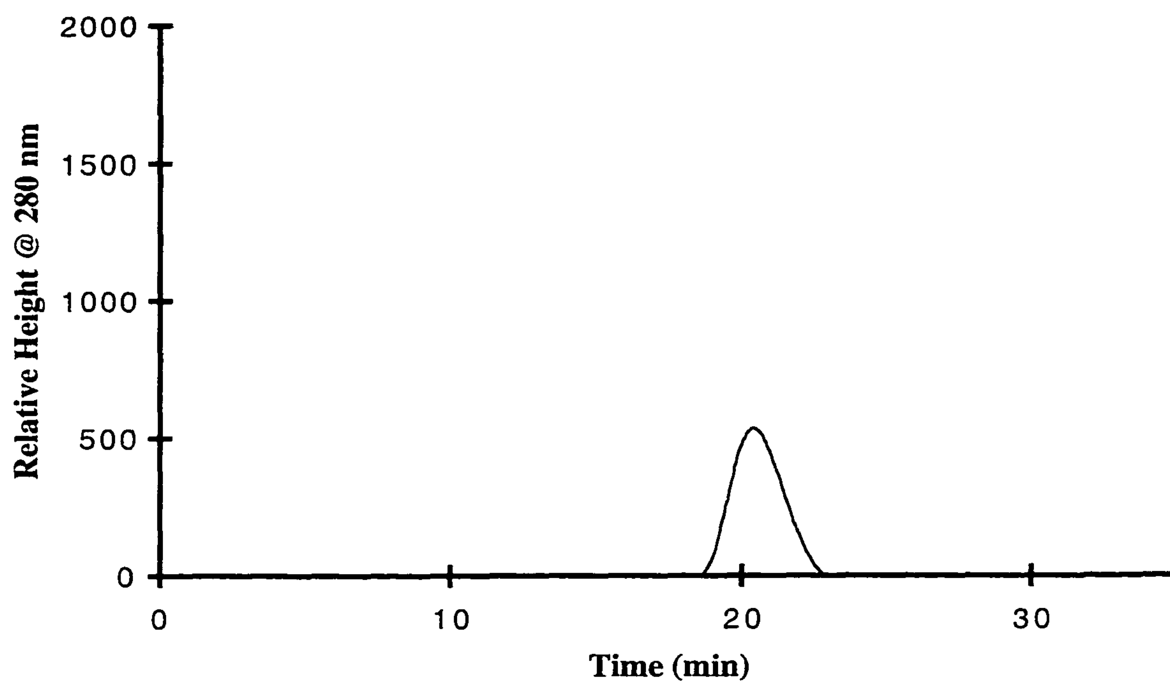


Figure 5.7 SEC of 0.5 mg/ml bovine hemoglobin on Novarose SE 1000/17. Bovine hemoglobin Tr = 22 minutes.

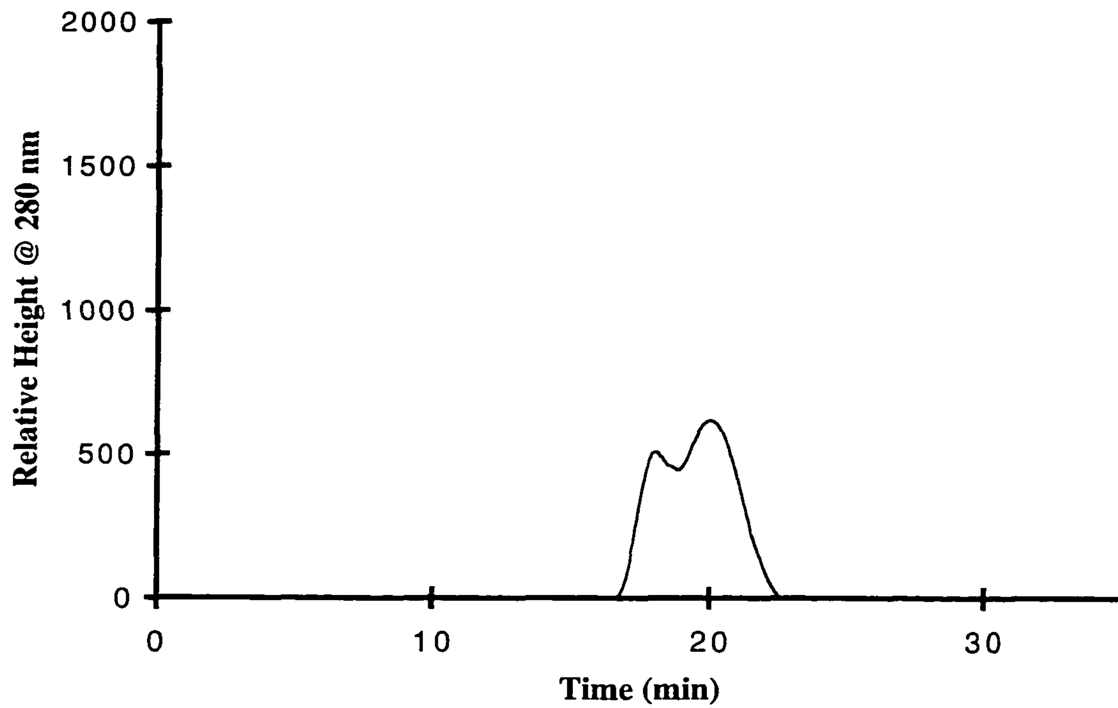


Figure 5.8 SEC of 0.5 mg/ml bovine hemoglobin and 0.5 mg/ml ovalbumin on Novarose SE 1000/17. Bovine hemoglobin Tr = 22 min., ovalbumin Tr = 18 minutes.

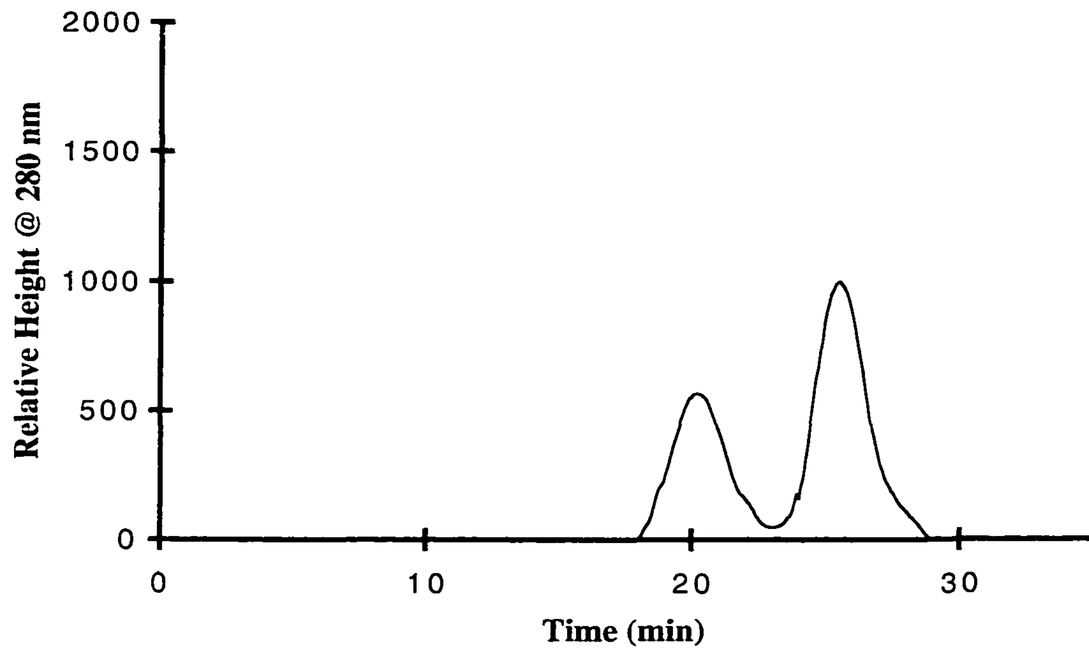


Figure 5.9 SEC of 0.5 mg/ml bovine hemoglobin, 0.5 mg/ml horse heart cytochrome C on Novarose SE 1000/17. Bovine hemoglobin Tr = 22 min., cytochrome C Tr = 26 minutes.

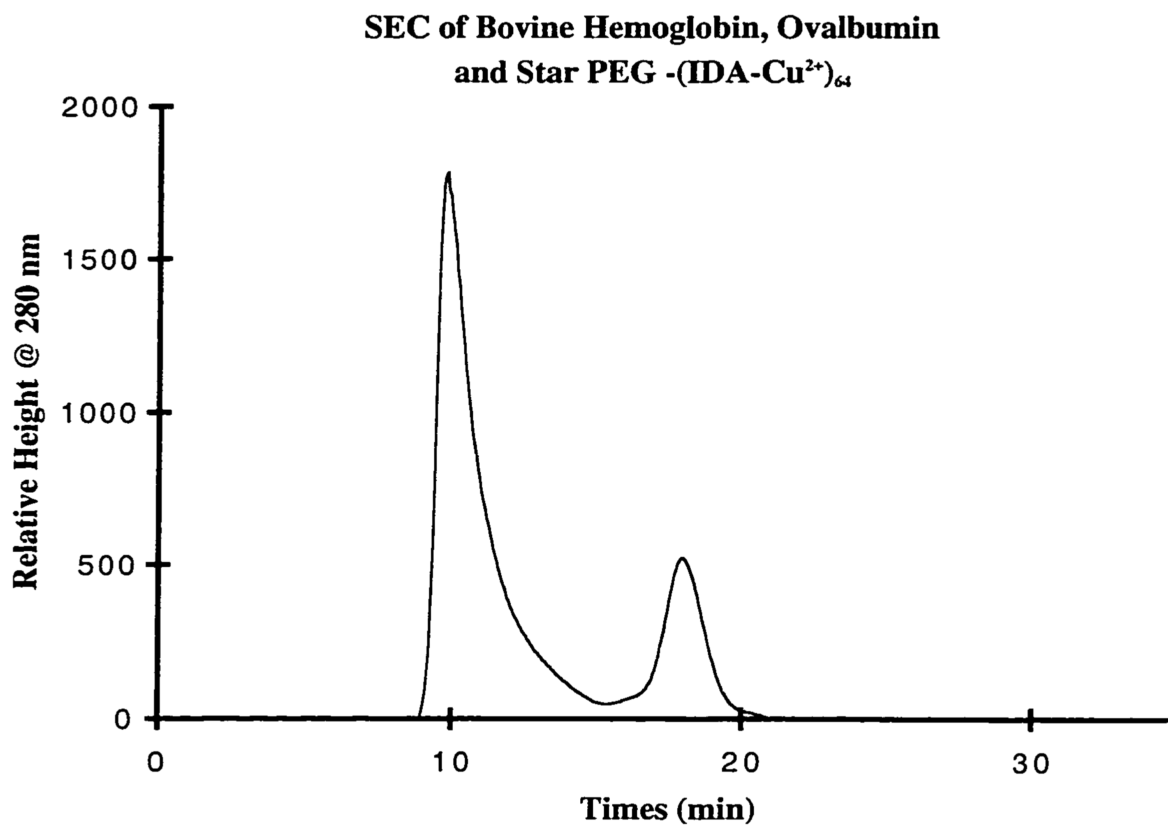


Figure 5.10 SEC of 0.5 mg/ml bovine hemoglobin, 0.5 mg/ml ovalbumin and 5.0 mg/ml PEG - (IDA Cu^{2+})$_{64}$ on Novarose SE 1000/17. Hemoglobin was separated from ovalbumin based on selective affinity towards PEG - (IDA Cu^{2+})$_{64}$. Protein chelator complex Tr = 11 minutes. Ovalbumin Tr = 18 minutes.

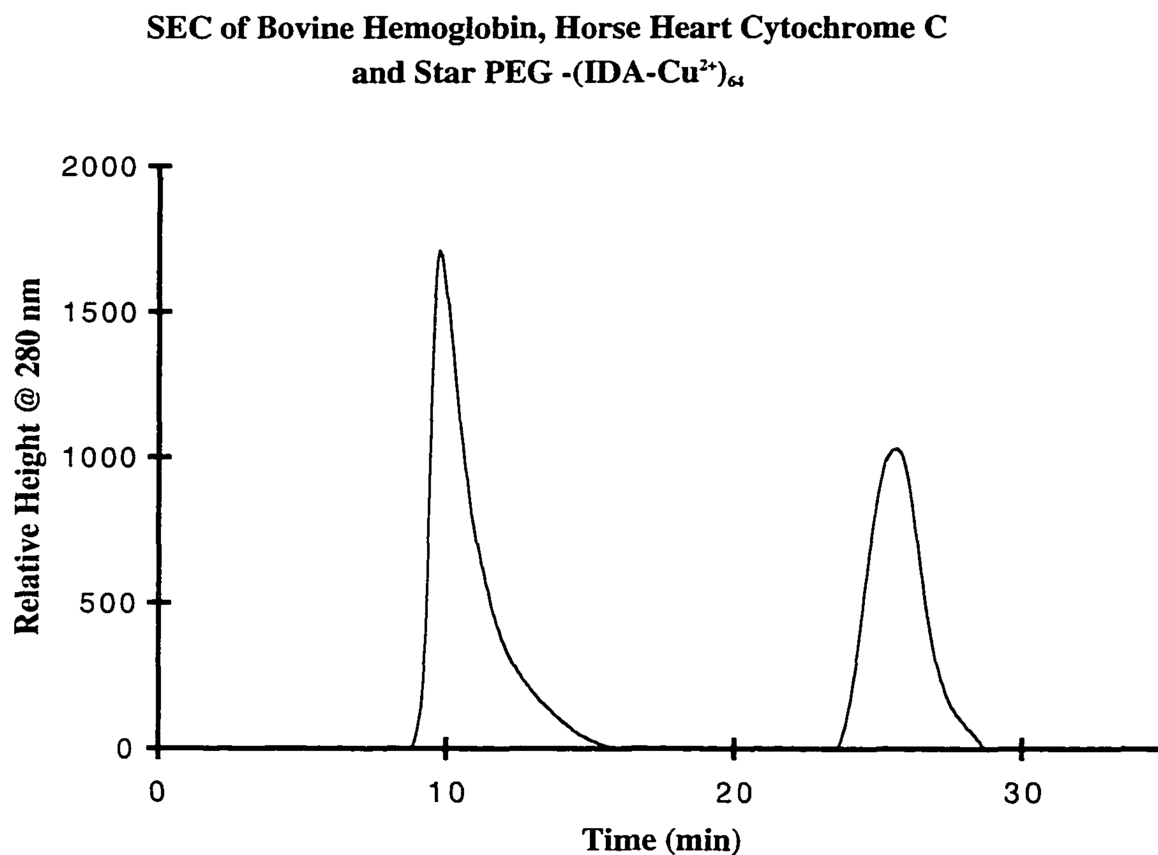


Figure 5.11 SEC of 0.5 mg/ml bovine hemoglobin, 0.5 mg/ml horse heart cytochrome C and 5.0 mg/ml PEG - (IDA Cu^{2+})$_{64}$ on Novarose SE 1000/17. Hemoglobin was separated from cytochrome C based on selective affinity towards PEG - (IDA Cu^{2+})$_{64}$. Protein chelator complex Tr = 11 min. Cytochrome C Tr = 26 minutes.

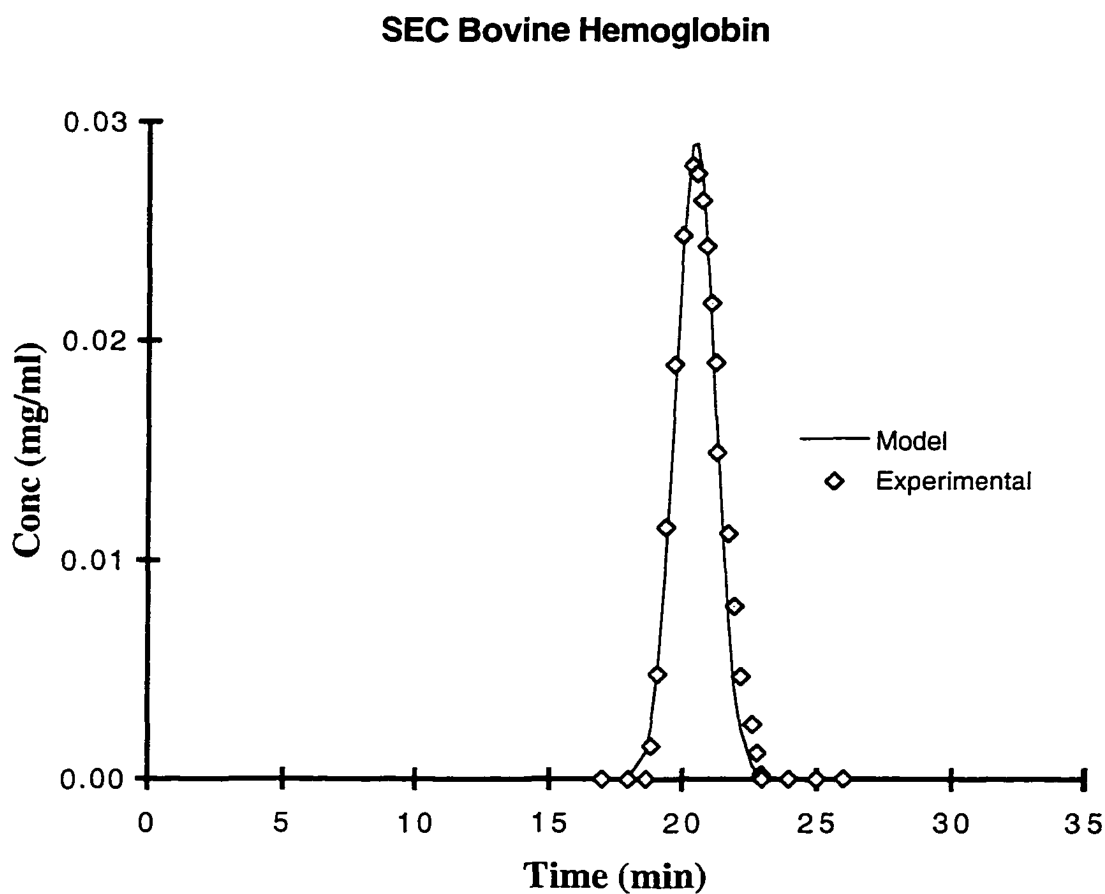


Figure 5.12 Comparison of an effluent chromatogram profile of bovine hemoglobin with a best fit (solid line) Gaussian expression given by equation 11. 0.03 mg sample of hemoglobin was injected at 0.5 ml/min in a 300 x 8 mm I.D. column packed with Novarose 1000/17 gel from Inovata. (λ = 280 nm, ε_p= 0.5, ε^*_p= 0.5, H= 0.04 cm).

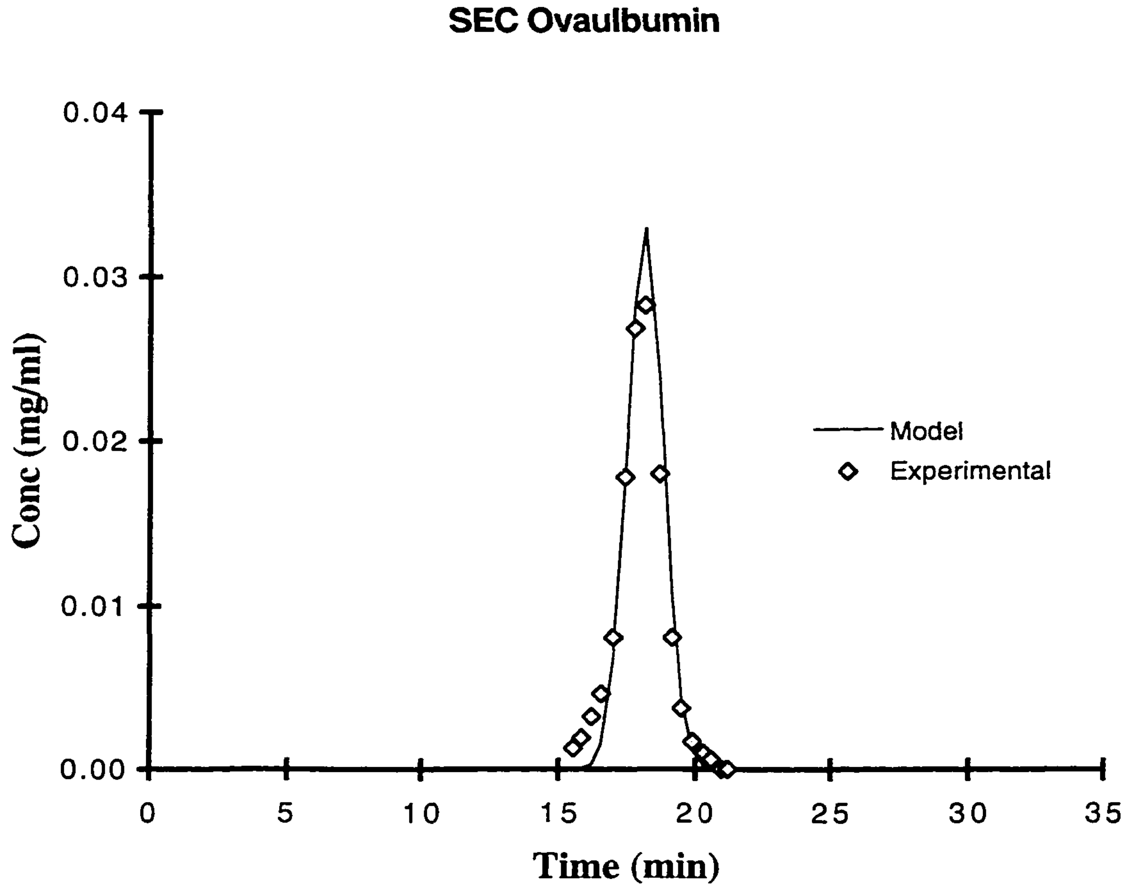


Figure 5.13 Comparison of an effluent chromatogram profile of ovalbumin with a best fit (solid line) Gaussian expression given by equation 11. 0.03 mg sample of ovalbumin was injected at 0.5 ml/min in a 300 x 8 mm I.D. column packed with Novarose 1000/17 gel from Inovata. (λ = 280 nm, ε_p= 0.5, ε^*_p= 0.33, H= 0.04 cm).

PROTEIN	*HISTIDINES*	*% PROTEIN EXTRACTED*
Human Hemoglobin	24	~100.0
Horse Hemoglobin	24	~100.0
Bovine Hemoglobin	20	~100.0
Whale Myoglobin	5	34.0
Horse Myoglobin	4	27.0
Ovalbumin	3	22.0
Cytochrome C Horse Heart	2	2.50

Table 5.1 Fraction of protein adsorbed by 5 mg/ml of star PEG- IDA $(Cu)_{64}$. In all cases, the initial protein concentration was 0.5 mg/ml. As stated by Vijayalakshmi in 1989, the interaction of proteins with metal chelators is governed mainly by the number or density of accessible histidines.

CHAPTER 6

PRODUCTION OF "NEOMETALLOENZYMES" BY *DE NOVO* BIOSYNTHESIS

6.1 ABSTRACT

Several approaches known for producing " neometalloenzymes " are classified into two categories: protein engineering using antibodies as starting materials and " de novo " biosynthesis of metal binding antibodies with potential catalytic metal-binding structure. This latter approach was chosen in this study. Because of the absolute need for the unequivocal screening of the hapten [IDA-Zn(II)] specific antibodies, a new ELISA method was developed using a biheaded polyethylene glycol with biotin on one end and the hapten in the other end. This new ELISA method was validated with rabbit sera since polyclonal anti-zinc-iminodiacetate [IDA-Zn(II)] antibodies are produced in rabbits.

6.2 INTRODUCTION

Enzymes and antibodies belong to two different classes of proteins. Enzymes are known for their catalytic properties, while antibodies are known for their high specificity of antigen binding.

The association of these properties can, in principle generate an antibody with catalytic activity, an " abzyme " . This concept was first announced by Pauling in 1946 and was further explained by Jencks in 1969. According to him, as enzymes operate by binding and stabilizing the transition state of the reaction catalyzed, any antibody that can selectively bind to the transition state molecule of the catalytic reaction should exhibit

catalytic activity. This was demonstrated by the group of Lerner (1986) and that of Schultz simultaneously in 1986, by eliciting antibodies against the transition state analogues of the enzymes.

Although this approach is very widely used, other alternative approaches are studied, particularly for obtaining "abzymes" where cofactors (e.g., metal ions) are needed. These approaches included engineering antibodies and Fv or Fab fragments expressed in *E. coli* for metal-binding sites (Skerra and Pluckthum, 1988). Engineering of metal-binding sites was based on the structural features established in metalloproteins, particularly zinc enzymes (Valee and Auld, 1990). Thus, Iverson *et al.* (1990) and Roberts *et al.* (1990) introduced zinc coordination sites in the light chain of an antibody. Later, Wade *et al.* (1993) studied further and established their structures.

The structural comparison between the metalloenzymes skeleton and the CDRs of the antibody light chains allowed the identification of potentially catalytic sites, the most interesting being a β-sheet structure in the CDRs. Partial mutation of four amino acid residues in these regions led to metal-binding sites with equivalent affinities, exhibited by native metalloenzymes. Nevertheless, the substrate binding and the substrate catalysis by these neometalloproteins were yet a challenge.

Another attempt to simulate carbonic anhydrase-like activity was to construct a "minibody" by introducing three histidine residues in a β-sheet structure of the heavy chain of an antibody (Pessi *et al.*, 1993). Once again, this "minibody" did not show catalytic efficiency. The attempts to change the structural zinc-binding sites in zinc-finger proteins by peptide deletion into catalytic zinc-binding sites as in metalloenzymes, by Merckle *et al.* (1991) resulted in a tetrahedric zinc-binding site with water as the fourth ligand. Despite this structural identity with the catalytic zinc-binding sites, no catalytic activity could be observed with these engineered zinc-finger structures.

These above-cited approaches have demonstrated the feasibility for the obtention of "neometalloproteins", some of them with esterase activity. Nevertheless, the obtention of "neometalloenzymes" for the amide bond cleavage still remains generally a challenge.

However, since the seminal contribution of Iverson and Lerner in 1989, the approach of using metal chelates alone or complexed with the "peptide substrate" as antigens for producing "neometalloenzymes" with specific proteolytic activities has shown some promise (Barbas *et al.*, 1993).

Along the same lines, the antibodies against metal chelates, originally aimed at radio imaging (Reardan *et al.*, 1985; Le Doussal *et al.*, 1990; Phelps *et al.*, 1990) have been studied for their potential zinc-binding capacity and hence show zinc carboxypeptidase-like activities. Boden *et al.* (1995) and Rouvier *et al.* (1997) studied the zinc-binding site's structure in the anti-DTPA-zinc antibody and indicated the potential for zinc carboxypeptidase activity.

A close scrutiny of the zinc-binding sites in the zinc enzymes along with the accumulated knowledge and basic ground rules established in the zinc-iminodiacetate [IDA-Zn(II)] recognition by proteins (Sulkowski, 1987; Berna *et al.*, 1997) prompted us to explore the possibilities of using IDA-Zn(II) as the hapten to elicit antibodies with corresponding paratopes with similar structures, as in zinc enzymes.

However, before looking for the " catalytic antibody ", many practical questions are to be addressed. Thus, in this work, we will report our data on these practical aspects of eliciting and characterizing such antibodies, particularly a new ELISA method that allowed the study of the IDA-Zn(II) (hapten) specific binding to the hapten specific site and avoided any cross-reactivity and nonspecific binding.

6.3 MATERIALS AND METHODS

Preparation of BSA-IDA-Zn(II) Conjugated Used for Immunization of Rabbits

The iminodiacetate (IDA) was coupled to bovine serum albumin (BSA) using epoxy activation of BSA.

Two conjugates, one low substitution and the other high substitution with hapten, were prepared by activating 100 mg BSA in 10 ml of 0.1 M sodium carbonate buffer at pH 9.5, using 0.005 mmol and 0.9 mmol, respectively, of 1,4-butanediol diglycidylether. The reaction was carried out at 37 °C for 4 hours under constant stirring. Then, 4 ml of each activated BSA was added respectively to 0.08 mmol and 0.72 mmol of IDA dissolved in 1.2 ml of 0.2 M bicarbonate buffer at pH 9. The coupling reaction was carried out at 37 °C overnight.

The BSA-IDA conjugates thus prepared were desalted on a PD10 column and characterized by MALDI-TOF mass spectrometry at the Department of Microbiology, University of Gent, Belgium, by B Devreese under the Direction of J. Van Beeumen.

Thus, two types of BSA-IDA conjugates, one with low substitution with a BSA-IDA molar ratio of 1:10 and the second high substituted with a 1:50 BSA:IDA molar ratio, were obtained. The BSA-IDA conjugates thus obtained are schematically represented in Figure 6.1.

Immunization

The BSA-IDA conjugates were saturated with zinc by mixing a 1 ml solution of 3.5 mg of the conjugates with 1.5 ml of 10 mM zinc sulfate solution for 1 hour at 37°C, except in the case of studying the time course of zinc chelation. After desalting the remaining

excess of Zn, the resulting 2 ml solution of 1 mg/ml of each conjugate was mixed well with 2 ml of Freund adjuvant (complete for the first injection, incomplete for the other) and injected into two rabbits with four subcutaneous injections. In the case of mice, only the high substituted conjugate (BSA:IDA = 1:50) was used after saturation with Zn (as described above). One hundred μg of the antigen well mixed with the adjuvant was injected by three intraperitoneal injections. Sera samples were taken 10 days after immunization. Three consecutive challenges with the antigen, each at 3-week intervals, were administered and sera were analyzed.

Preparation of Biotin-PEG-IDA Conjugate

The biotin-PEG-IDA (BPI) conjugate was synthesized according to a modified method of Ehteshami *et al.*, (1996). Briefly, diamino-PEG with a molecular weight of 2000 Daltons was used as the starting material to prepared a heterobifunctional PEG of the form X - PEG -Y where X is a blocking group (t-BOC) and Y represents a primary amino group. Imidiacetic acid (IDA) was coupled to the amino group, followed by deblocking the amino group which was then used for biotinylation using *N*-hydroxysuccinimide biotin. The final product was Biotin - PEG - IDA.

Enzyme-Linked immuno sorbent assay (ELISA)

Antigen (hapten with nonprotein carrier) immobilization: Avidin DX(Biosys) was diluted with coating buffer (15 mM Na_2CO_3-35 mM $NaHCO_3$, pH 9.5) to 10 μg/ml and then coated with 0.1 ml of the solution for 2 h at 37 °C. Any residual binding capacity of the plates was blocked with 3% gelatin in pH 7 PBS for 90 min. at 37 °C.

The hapten carrier conjugate (Zn-IDA-PEG-Biotin) was obtained by mixing 100 μM BPI in 10 ml of 100 mM zinc sulfate solution for 2 h at 37 °C, and 0.1 ml was directly added to the wells and incubated for 90 min. at 37 °C.

ELISA assay : Sera from rabbits and mice were diluted in pH 7 PBS and 0.1 ml/well was incubated for 1 h at 37 °C. The detection antibody, goat anti-rabbit or anti-mouse labeled with horseradish peroxidase, was diluted 1/4000 in 10 mM phosphate buffer (pH 6.4)-0.15 M NaCl-0.1% Tween 20 and incubated for 1 h at 37 °C. Each incubation step was terminated by washing the microtiter plates with pH 7 PBS containing 0.1% Tween 20.

Finally, 0.1 Ml/well of substrate solution for peroxidase (3 mM *o* - phenylenediamine dihydrochloride, 50 mM citric acid, 100 mM Na_2HPO_4, 16 μl H_2O_2 9%, pH 5) was added and the reaction was stopped after 30-min. incubation at room temperature in the dark with 0.1 ml HCl/2N. The absorption was measured at 490 nm.

6.4 RESULTS AND DISCUSSION

Optimization of the Hapten-Protein Carrier Conjugate for Immunization

Two series of conjugates, one with low IDA substitution (BSA:IDA = 1:10) and the second with high IDA content (BSA:IDA = 1:50), were prepared by covalent coupling of IDA to BSA using 1,4-butanediol diglycidyl ether as the epoxy coupling reagent, as described under materials and methods. In both cases, the conjugate was treated with a large excess of zinc ions before injecting into the animals in order to ensure a proper molar ratio of IDA to zinc and to account for any eventual zinc scavenging in the *in vivo* system.

The data indicated that the conjugate with the higher hapten to BSA molar ratio (BSA:IDA = 1:50) gave better specific immune responses. Furthermore, the antigen in the absence of zinc, that is, BSA-IDA, resulted in some response in ELISA. However, the Zn-IDA-BSA conjugate gave a higher ELISA specific response when tested with Zn-IDA as the total hapten conjugated to the nonprotein carrier with the ELISA system (data not shown).

Optimization of Parameters for the ELISA Test with the "Biheaded PEG Carrier"

This method of using avidin-biotin complexation to present the hapten IDA-Zn(II) on the ELISA plate is somewhat different from conventional ELISA techniques. The important issues to be addressed specifically to our antibody screening are (I) ensuring the coordinated metal's presence in the PEG conjugate and (II) differential recognition of the hapten with and without zinc by the immunized sera. All optimization studies were carried out with sera from rabbits immunized with the BSA-IDA-Zn(II) conjugates.

Influence of the BPI-Zinc Salt Solution Equilibration Time on the ELISA Responses

The aim of the experiment was to screen those antibodies specifically recognizing the complete hapten, namely, the chelated metal (IDA-Zn(II), with the exclusion of those recognizing only the chelating group (IDA). Thus, the hapten presentation in its intact composition becomes a major issue. The chelation of zinc to PEG-conjugated IDA may depend upon the molar concentration ratios of IDA to Zn^{2+} as well as upon the time course of stable formation.

Hence, we studied the ELISA response on both nonimmunized and immunized sera of rabbits as a function of time of BPI-Zn salt equilibration, using saturating (high excess molar ratio) zinc sulfate solution.

The data shown in Figure 6.2 show that at more than 100 minutes of contact time between BPI and 100 mM $ZnSO_4$ solution, prior to fixing this hapten on the ELISA plate, through avidin-biotin complexation, maximum responses were recorded for the immunized sera; in contrast, the nonimmunized sera did not show any variation and the responses were almost at the baseline level.

Verification of Hapten Conjugate Immobilization onto the ELISA Plates

Hapten immobilization on the ELISA plates is a "sine qua non" condition for the assay method. In our case, the hapten is presented to the antibody-containing sera as described in Figure 6.3.

In order to verify the efficient hapten PEG conjugate immobilization, we used an avidin-coated ELISA plate conjugated with biotinylated peroxidase and determined the efficiency of increasing concentrations of BPI (0 to 800 nM) to displace this biotinylated enzyme from the plate.

The data shown in Figure 6.4 indicate the following: at as low as 25 nM BPI, the peroxidase activity on the plate decreased drastically; and at 200 nM BPI concentration, total displacement of the initially coupled biotinylated peroxidase was observed.

Thus, with the concentration of 100 μM BPI used in our ELISA assays, we ensure an efficient coupling of the hapten PEG conjugate to the plate via the avidin groups. This in turn ensures the correct presentation of the hapten (Figure 6.3) to the antibody to be screened.

Other Parameters

In any conventional ELISA assay, gelatin is used to block the excess groups on the ELISA plates in order to suppress any nonspecific binding of the enzyme-linked second antibody, which will result in a high background noise.

In the range of gelatin concentration tested (0-3%), we found that the background noise was totally suppressed at a 3% concentration of gelatin (data not shown), resulting in an increase hapten specific response in the case of the immunized serum (Tussen, 1985).

The optimum ELISA assay conditions, determined with rabbit sera, were: 10 μg/ml avidin coating, 100 μM biotin-PEG-IDA-Zn, 3% gelatin for blocking excess groups, assay at pH 7.0.

The most important step for the screening of small-size hapten specific monoclonal antibodies is to develop a reliable and sensitive ELISA method, which demands the right choice of hapten presentation in the ELISA plates. The use of nonprotein carriers such as dextran has been studied in order to ensure a noncross-reaction system for hapten specific antibody detection.

Nevertheless, the biotin-PEG-hapten system described here has many advantages over the dextran-hapten conjugates as the former forms 1:1 molar ratio conjugates and their immobilization via avidin on the ELISA plates ensures the most fitting spatial orientation of the hapten for antibody-specific association. In the case of dextran-hapten conjugates, the carrier to hapten ratio cannot be precisely determined and this in turn may result in the presentation of the hapten at different orientations. Furthermore, the coupling of metal-chelate hapten [IDA-ZN(II)] results in at least partial insolubilization of the dextran, making the carrier conjugate impossible to be coated on the ELISA plates.

6.5 CONCLUSIONS

We have demonstrated the feasibility of a heterobifunctional polyethylene glycol derivative as a nonprotein carrier in immunochemical (ELISA) tests for metal-chelate antibodies. This approach is of great interest to eliminate the cross-reactivity with the hapten carrier and to differentiate between specific and nonspecific recognition.

This nonprotein carrier concept combined with the avidin-biotin system could be envisaged as a universal method for the screening of antibodies specific to any small-size hapten.

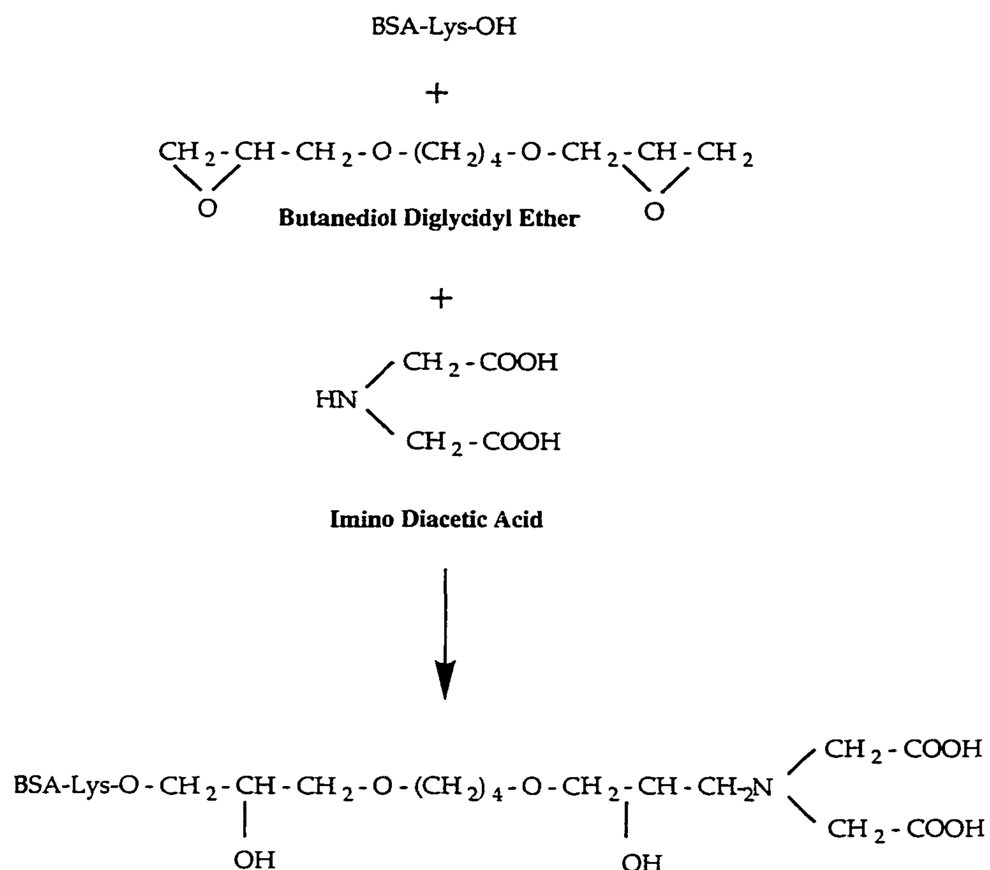


Figure 6.1 BSA-epoxy-IDA conjugation. The iminodiacetate (IDA) was coupled to bovine serum albumin (BSA) using epoxy activation of BSA.

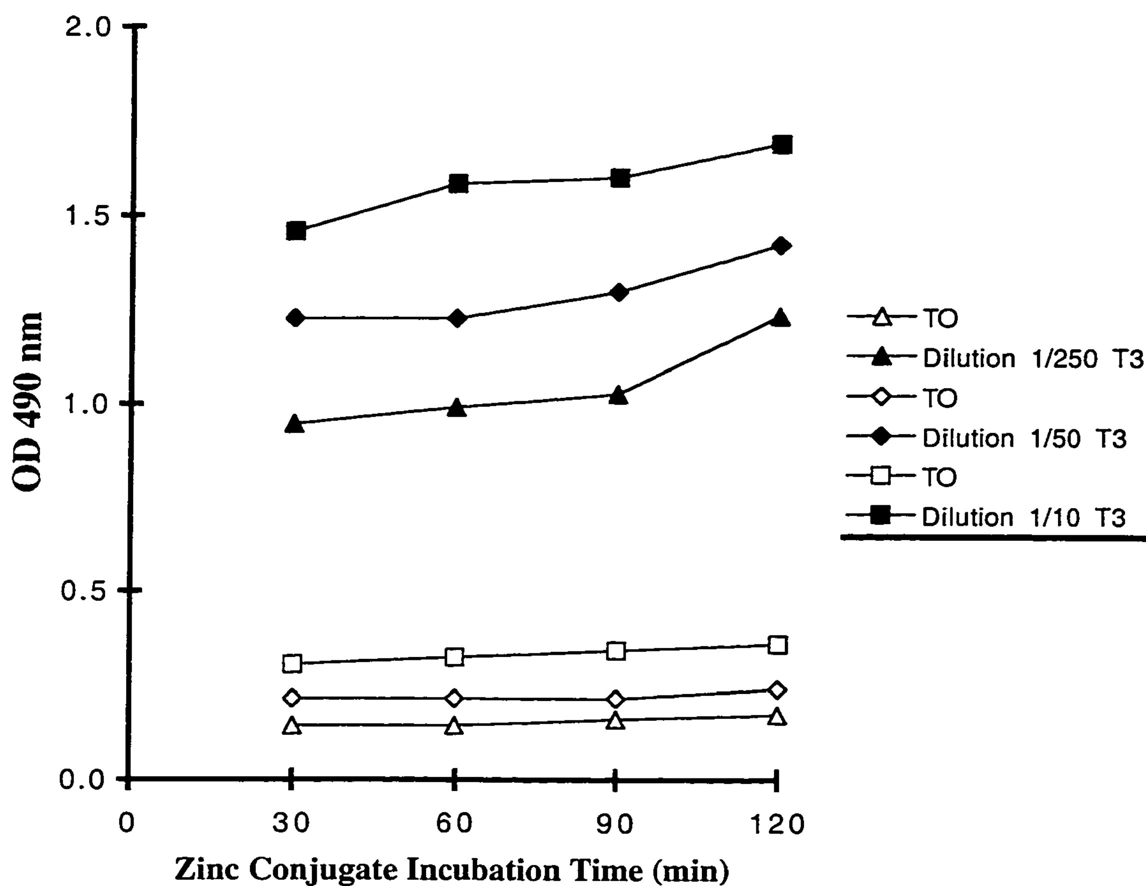


Figure 6.2 Response of the immunized rabbit antibody as a function of the Zn(II) incubation time for preparing the total hapten (BPI-Zinc complex) for the ELISA test. The immunized (T3) and nonimmunized (TO) sera responses were observed for three dilutions (1/10, 1/50 and 1/250) following the protocol presented in the text.

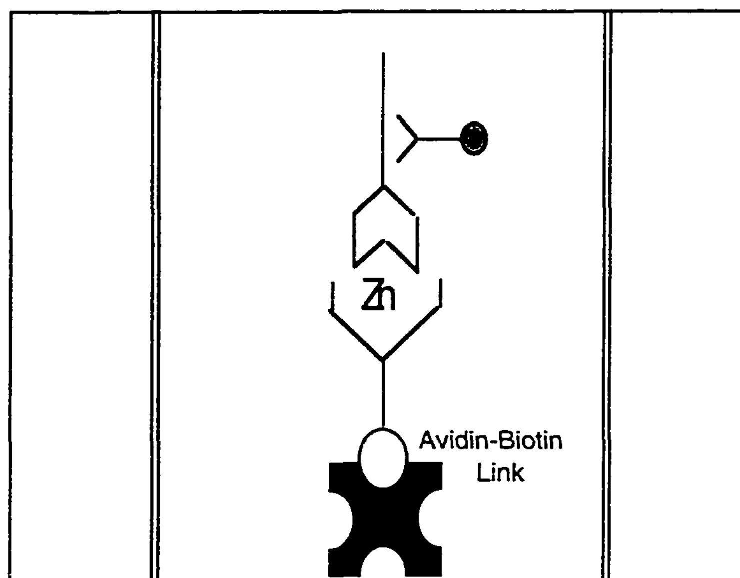


Figure 6.3 The principle of the enzyme-linked immuno sorbent assay (ELISA) The biotin-PEG-IDAZn(II) is immobilized on the microtiter plates by the avidin-biotin link. After incubation with diluted sera, the specific recognition was revealed by a labeled antibody.

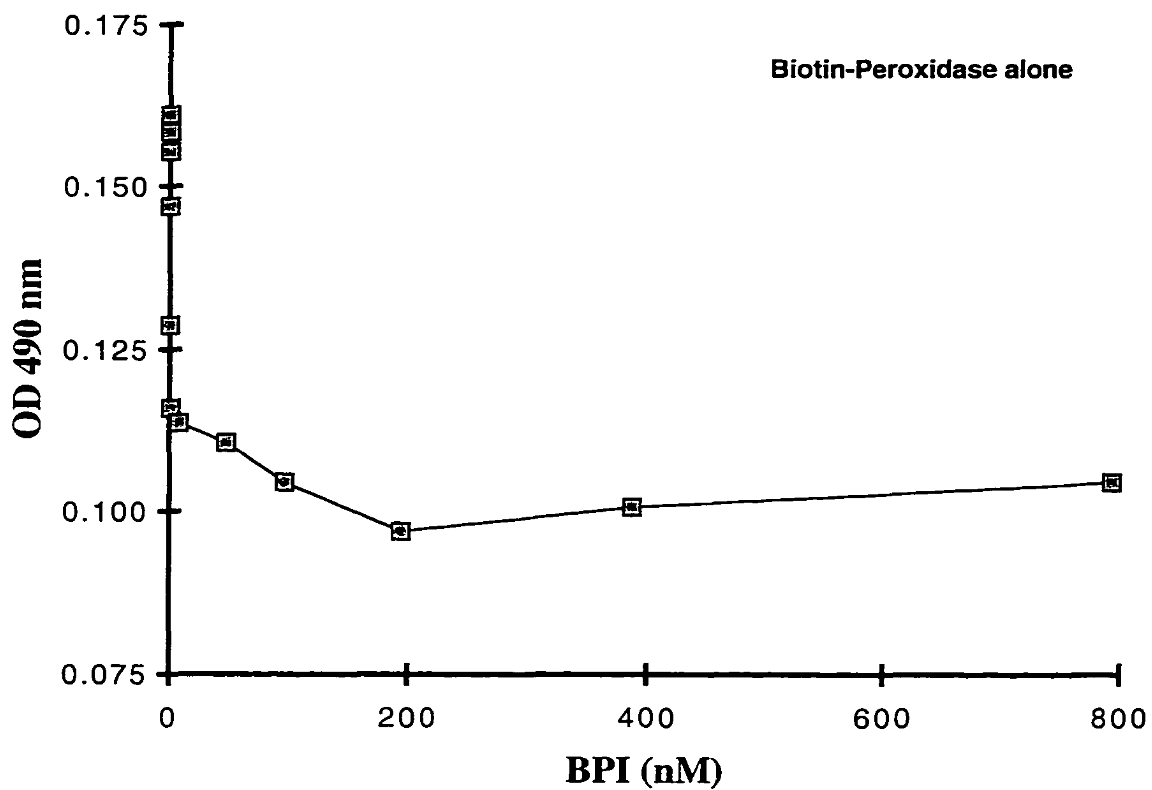


Figure 6.4 Competition between biotin-PEG-IDA (BPI) and biotin-peroxidase. Displacement of different concentrations of BPI (from 6 pM to 50 μM) was measured.

CHAPTER 7

SYNTHESIS OF CHELATED POLY (ETHYLENEIMINE) DERIVATIVES AND THIER APPLICATION IN METAL ION REMOVAL FROM AQUEOUS SOLUTIONS

7.1 ABSTRACT

Chelation of metal ions by high MW water-soluble macroligands and retention of the resulting complex by ultrafiltration leads to a method of concentration and extraction of metal ions from aqueous solutions.

This chapter describes the modification of a soluble polymer, poly (ethyleneimine) (PEI), to enhance its binding capacity for metal ions. PEI with an average molecular weight of 750,000 was carboxymethylated with bromoacetic acid to create a chelating polymer bearing imino diacetic acid (IDA) groups. The efficiency of this chelating polymer in affinity ultrafiltration was tested by extracting metal ions such as Cu^{2+}, Ni^{2+}, Co^{2+} and Fe^{3+} from aqueous solutions. The capacity of carboxymethylated PEI 750,000 MW for the transition metal ions tested at pH 5.0 was found to be close to 0.4 g of metal per g of polymer. Adsorption isotherms were constructed and correlated by Langmuir's model to calculate equilibrium parameters. Since PEI-metal complexes are colored complexes, a colorimetric method to determine complexation and binding constants was also used. Metal capacity and binding strength for a particular metal-chelator complex depend on the complex coordination number. UV spectroscopy was used to characterize coordination chemistry for PEI's macromolecular chelates.

This chapter presents the developmental stages of this affinity technique as it applies to the treatment of metal containing waste. Results of metal removal and binding studies are discussed.

7.2 INTRODUCTION

Chelators are frequently used in analytical chemistry to preconcentrate metal ions and to separate the analytes from interfering concomitants prior to their determination by an instrumental method. Poly (ethyleneimine) (PEI) is a polymer with a high content of amine-nitrogen and thus a chelating ligand (Malamas *et al.*, 1984). PEI is formed by polymerization of ethyleneimine (Aziridine) which leads to a high molecular weight polymer. PEI is amorphous and highly soluble in water. The polymer has the repeating unit C_2H_5N and is also highly branched for every 3-7.5 nitrogen in a linear unit (Dick *et al.*, 1970). A compact network of the PEI structure has been deduced from the observed dependence of the intrinsic viscosity on molecular weight and the small changes in the reduced viscosity with increased charge density on the polymer at low ionic strength (Bloys, 1978). A schematic representation of PEI structure is shown in figure 7.1. In PEI, branching leads to a certain complexity in the behavior of the protonated forms of the polymer due to the presence of primary, secondary and tertiary amines in the approximate ratio of 1:1:1 (Steinmann *et al.*, 1994). The multiplicity of amine functions in PEI provides ideal ligand for complexation with heavy metal ions. However, complexes of PEI with metal ions are in most cases water soluble. For the purpose of removal of heavy metal ions from aqueous solutions, it is desirable to prepare chelating agents whose metal complexes are readily separated from the aqueous phase. Some chelating ligands can be covalently bound to a macromolecule, such as PEI, and then used to extract metal ions from aqueous solutions by means of filtration. These complexes exhibit great stability, good water solubility, and a wide spectrum of chelated metals (Schwarzenbach *et al.*, 1946). Fully reacted acid polymer is insoluble in water but becomes water-soluble as sodium salt.

The combination of chelating interactions with a filtration separation process yielded affinity ultrafiltration. This technique was developed in the early 1980's and was the

natural extension of the pseudo affinity ultrafiltration used to purify metal ions from aqueous solutions (Mattiason *et al.*, 1984). The principle of the process lies in the ability of the ligand to form an affinity complex with the analyte to be purified. The complex will be held by a membrane in an ultrafiltration process, allowing impurities to pass through the filter unretained. The ligand used is generally a high molecular weight molecule with a minimum ten fold difference in molecular weight from the ultrafiltration membrane cut off to ensure good retention. In addition, it is desirable for the ligand to have a high capacity for the analyte that is being retained. There has been increased research in this separation technique due to its ability to selectively remove metal ions at high flow rates (Chaufer and Deratani, 1988).

The preparation of a chelating polymer and its use as a metal binding macromolecule is reported in this chapter.

7.3 MATERIALS AND METHODS

Materials

Poly (ethyleneimine) (PEI) with an average molecular weight of 750,000 was purchased from Aldrich (Milwaukee, WI). Ferric Chloride (III), sulfate salts of copper (II) and nickel (II), bromoacetic acid and picrylsulfonic acid (2,4,6-trinitrobenzenesulfonic acid, TNBS) were acquired from Sigma (St. Louis, MO). All other chemicals utilized were of analytical or reagent grade.

Equipment

Ultrafiltration experiments for polymer purification and metal binding measurements were carried out using a 10 ml stirred cell model 8010 with a YM10 membrane or centricon concentrators (centricon 30M) both obtained from Amicon (Beverly, MA).

Methods

Modification of Poly (ethyleneimine) (PEI)

Carboxymethylation of Poly (ethyleneimine) was performed by reacting the desired amount of PEI solution with a 50 molar excess of bromoacetic acid over the primary amino content. Primary amino groups in the PEI solution were measured using the TNBS method described by Habeeb (1966). The mixture was kept at pH 9 and stirred for 24 hr. Unreacted bromoacetic acid was removed by ultrafiltration. The carboxymethylated PEI (CMPEI) solution was tested with TNBS to ensure complete chelation.

Determination of Adsorption Isotherms

Adsorption isotherm were constructed using batch samples at pH 5.0 in sodium acetate 0.5 M. Several samples were prepared by adding 1.5 mg g of PEI or CMPEI and 6 ml of buffer containing the appropriate amount of Cu^{2+}, Ni^{2+} and Fe^{3+} ions to have a final ratio in the range of 0 to 10 mg of metal per mg of polymer. Samples were gently mix and left in an shaker overnight to reach equilibrium. After centrifugation the supernatant was tested for metal content using a Shimadzu UV spectrophotometer. The amount of

metal ion absorbed by the polymer was then calculated based on the initial amount of metal ion added to the solution.

Determination of Metal Binding Capacity and Binding Constants

Langmuir's Method

Assuming that the immobilized binding site, B, on the adsorbent has an affinity for an adsorbate, A, the interaction can be described by a relationship of the form:

$$A + B \underset{K_2}{\overset{K_1}{\leftrightarrow}} AB \qquad [1]$$

where AB is the bound complex, K_1 and K_2 are the forward and reverse rate constants respectively, and their ration (K_2/K_1) is the dissociation constant, K_d.

Several investigators have shown that the equilibrium relationship between free and bound adsorbate can be describe by a Langmuir-type isotherm (Graves *et al.*, 1974; Wankat *et al.*, 1974; Eveleigh *et al.*, 1977). Chase *et al.* (1984) described a mass-transfer rate equation that is consistent with the Langmuir's isotherm. The mass transfer of the adsorbate to the immobilized phase can be described by:

$$\frac{dq}{dt} = K_1 C(q_m - q) - K_2 q \qquad [2]$$

where C is the concentration of the free adsorbate, q is the concentration of bound adsorbate, and q_m is the maximum concentration of bound adsorbate. At equilibrium, equation [2] can be reduced to the adsorption isotherm:

$$q^* = \frac{q_m C^*}{Kd + C^*} \quad [3]$$

where the superscript (*) denotes an equilibrium value.

Equation [3] can be written in a linear form as follows:

$$\frac{C^*}{q} = \frac{K_d}{q_m} + \frac{C^*}{q_m} \quad [4]$$

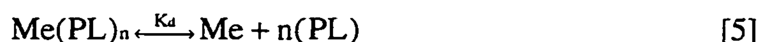
Equation [4] was used to determine values for the dissociation constant, K_d, and maximum binding capacity of the adsorbent, q_m.

UV-Spectrophotometric Method

UV spectroscopy is a characterization method that can be used for the investigation of colored macromolecular chelates. Studies on the stoichiometric composition of PEI metal complexes were carried out using the molar ratio method (Mukherji *et al.,* 1958). The dissociation constant can be spectro-photometrically determined. Yoe and Jones (1944) found that for a very stable complex a plot of optical density against molar ratio of component A to component B, with A constant, rose from the origin as a straight line and broke sharply to constant density at the molar ratio of the components in the complex.

However, a complex that undergoes appreciable dissociation in solution gave a continuos curve which became approximately parallel to the molar ratio axis when an excess of the variable component was added. Here, the extinction values for the appropriate wavelength were plotted versus the molar ration of PEI-nitrogen to metal ion. Extinction values were obtained by measuring the absorbance of a series of samples with constant PEI content (0.47 mg) and different metal concentrations to give 1: 1 to 9: 1 nitrogen to metal molar ratio. Determination of dissociation constants and complex compositions is described below.

The dissociation of metal complexes can be expressed as follows:



$$Me(PL)_n \overset{K_d}{\longleftrightarrow} Me + n(PL) \qquad [5]$$

where Me is the metal, PL is the chelating unit of polymer ligand (usually 1/3 of total repeating units in the polymer), and n is the number of PL units coordinated to the metallic ion. The equilibrium constant K_d can be expressed by the following equation:

$$K_d = \frac{[Me][PL]^n}{[Me(PL)_n]} \qquad [6]$$

Using the equilibrium data from isotherms obtained as described above, the K_d values were determined using the appropriate coordination number which depends on the metal ion and type of chelator.

Equation [6] can also be written as follows:

$$K_d = \frac{\alpha(\alpha nC)^n}{1-\alpha} \qquad [7]$$

where C is the initial concentration of the complex and α is the complex dissociation constant.

The quantitative relation for the calculation of the dissociation constants from the extinction values is defined as follows:

$$\alpha = \frac{E_m - E_c}{E_m} \qquad [8]$$

where E_m is the maximum extinction for the complex and E_c is the extinction for a specific coordination number.

7.4 RESULTS AND DISCUSSION

Figure 7.1 shows the average structure of PEI. The high content of amine-nitrogen in poly (ethyleneimine) (PEI) makes this polymer a highly water-soluble chelating ligand. Due to the presence of primary, secondary and tertiary amines in the approximate ratio 1:1:1, the polymer can show a complex behavior of its protonated forms. PEI is a hyperbranched polymer with a repeating unit C_2H_5N with about one branch for every 3-7.5 nitrogen in a linear unit which provides good properties for membrane retention. The high content of amino groups in the PEI structure allows suitable polymer modification. When

PEI is carboxymethylated, a new chelating polymer is obtained with reduced solubility which may be desirable for macromolecule isolation as contrary to PEI. Carboxymethylated complexes exhibit great stability, only good water solubility and chelates several metals ions (Schwarzenbach *et al.*, 1946).

In this work, poly (ethyleneimine) was reacted with bromoacetic acid to create carboxymethylated PEI (CMPEI) a chelating polymer bearing imino diacetic acid (IDA) groups. The chelating properties of PEI and CMPEI were studied and stability constants calculated for Cu (II), Ni (II), Co (II) and Fe (III).

Table 7.1 shows the metal capacities and binding constants for PEI and CMPEI calculated based on the equilibrium data obtained according to the procedure described in materials and methods. Since transition metal ions have several possible coordination sites, they may act as electron acceptors in the presence of chelating polymers such as PEI and CMPEI. Therefore, Me-PEI or Me-CMPEI complexes may show different coordination chemistry. Figures 7.10, 7.11 and 7.12 show the most probable coordination chemistry for copper, nickel and iron calculated using the molar ratio method. It can be seen that with PEI, copper can form complexes with different coordination numbers depending on the polymer concentration. Figure 7.11 shows that nickel can only form PEI complexes with coordination numbers of four or six whereas iron may complex PEI with coordination numbers of 3 or 6. Complexation studies with CMPEI were not performed however, a coordination number of 4 is probable due to the presence of IDA groups. Therefore binding constants for the resulting PEI/CMPEI metal complexes were determined on the basis of content of chelating groups in the polymer and on the premises of a coordination number of 4 which leads to metal complexes of the form $Me(PL)_4$ for Me-PEI and $Me(PL)_2$ for Me-CMPEI. Based on a coordination number of 4, PEI and CMPEI would have a theoretical metal capacity of 1.94 and 7.88 mmoles of metal per gram of polymer respectively. According to table 7.1 and 7.2 the copper capacity obtained from the isotherms (figures 7.4

and 7.6) were 0.6 for PEI and 0.55 for CMPEI which exceeded the theoretical capacity. It can be seen, however, that the adsorption isotherms in figures 7.4 and 7.6 reached a plateau at low "saturation levels". The plateau seems to represent the saturation of chelating units in the polymers. For PEI-Cu this plateau is at 0.23 g/g and 0.20 g/g for CMPEI-Cu . These values represent very closely the theoretical metal capacities expected solely from chelating interactions. The excessive high metal capacity reported in table 7.1 for copper can be explained in terms of further complexation or electronic interaction due to the high amino content and polymer branching. It is not surprising that metal ions can be trapped in the polymer network. This can be interpreted as a consequence of the presence of intercatenarian zones in the polymer network as shown in figure 7.13. Therefore, macrocyclic ring systems can be formed and they may vary in size and number of coordinating ring atoms. The exceptionally high affinity for some metal ions could be therefore caused by macrocyclic complexation possibly reinforced by steric inclusion effects specially in the case of PEI.

The presence of IDA groups in CMPEI limit the interaction of one metal ion with two nitrogen repeating units (four coordination sites), whereas PEI is likely to have 4 nitrogen repeating units interacting with only one metal ion. The net result for PEI is the formation of metal complexes with higher stability as evidenced by stronger binding constants (table 7.1) but lower metal capacity as compared to CMPEI. PEI is a positively charged polyelectrolyte with a pKa = 8.49 (Kobayashi *et al.*, 1987) and the retention of protonated PEI decreases markedly with an increase of the ionic strength of the solution. Metal transfer as its macromolecular complex can be significant. Adsorption results can be explained by the passage of a part of the polymeric complex through the membrane rather than by a decrease of the PEI metal capacity. The highly branched structure of PEI may account for the fact that its retention is more affected by the presence of salts since for a given MW the more branched polymer has a smaller hydrodynamic volume.

Table 7.2 shows the complex association constants for PEI and CMPEI with the different metals. These constants were calculated using the Langmuir's model which assumes a 1: 1 metal complex association (MePL) which leads to considerably weaker metal interactions. For this reason Langmuir binding constants were found to be orders of magnitude lower than the calculated constants for higher complex stoichiometry. In figures 7.4, 7.6 and 7.8 we see that the adsorption isotherms for copper and iron, deviate from the Langmuir isotherm. Adsorption seems to proceed in several stages specially in the case of iron (figure 7.8). Deviations from Langmuir's model may be due to the presence of sites of different activity (several nitrogen centers) and to a non-stoichiometric binding, that is more than one nitrogen center interacting with one metal ion. It is then expected to have a higher complex stability than the ones predicted by Langmuir (Tables 7.1 and 7.2).

The data for PEI complexes is in agreement with the Irving-Williams series for selectivity and complex stability: Cu(II) > Ni(II). PEI showed a high stability of the copper chelate which was calculated of two orders of magnitude higher than PEI-Ni complex. CMPEI could be an iron-selective complex ($K_a = 1.4 \times 10^7\ M^{-2}$). The polymer ligand containing carboxyl groups exhibited high selectivity for Iron Fe(III) > Cu(II) ~ Ni(II) ~ Co(II). In general, our results seem to agree well with earlier findings reported in the literature (Geckeler *et al.*, 1980). Chapter 8 describes solid phase studies for CMPEI and PEI that are in agreement with the data collected in this study.

7.5 CONCLUSIONS

Removal of metal ions from dilute solutions is readily performed by complexation with macromolecules and retention by ultrafiltration. This preconcentration technique appears as a promising process for metal removal with the advantage of being in homogeneous phase which may avoid diffusion problems.

Poly (ethyleneimine) demonstrated to be an efficient chelating polymer for the complexation-ultrafiltration process. Whether selectivity or capacity are required, PEI can be easily modified to create polymer chelators bearing specific chelating groups. Metal recovery can be done by appropriate elution methods and the polymer ligands can be reused.

Application of this complexation-ultrafiltration technique to metal-waste water treatment is under development. Here we have presented the developmental stages of this affinity technique as it applies to the treatment of metal containing waste.

POLYETHYLENEIMINE (PEI)

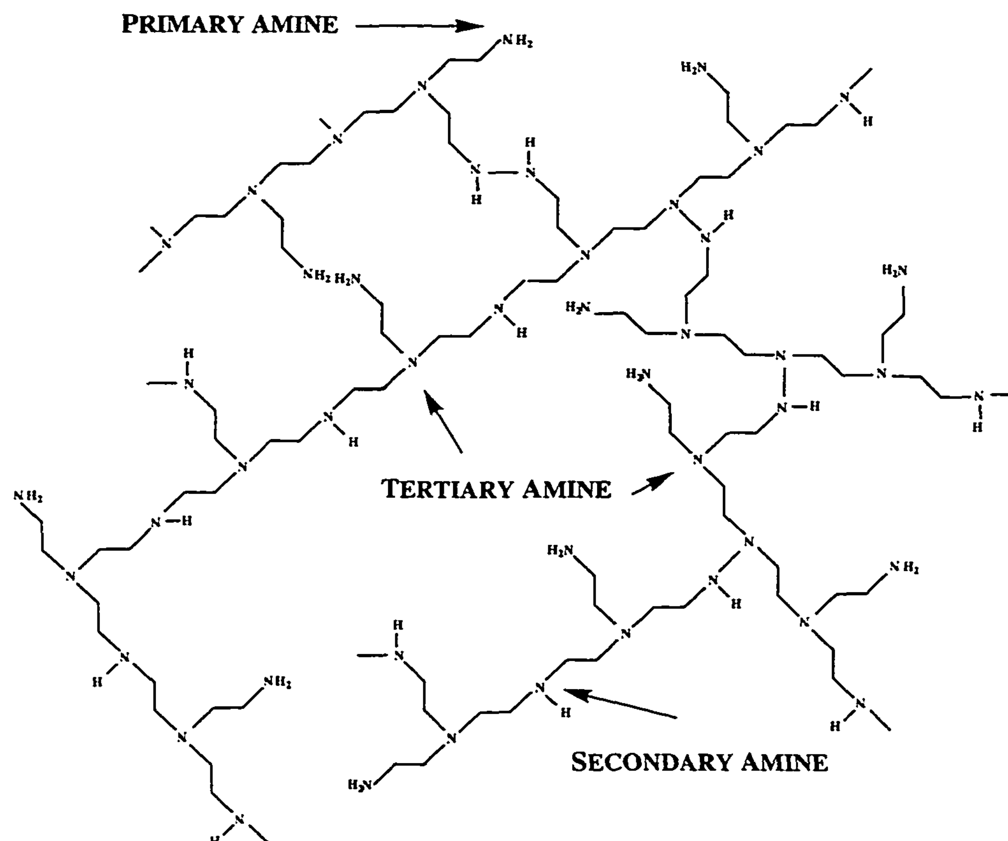


Figure 7.1 Schematic representation of the average structure of PEI. Branching leads to a certain complexity in the behavior of the protonated forms of PEI due to the presence of primary, secondary and tertiary amino groups in the approximate ratio 1:1:1 (Steinmann *et al., 1994).*

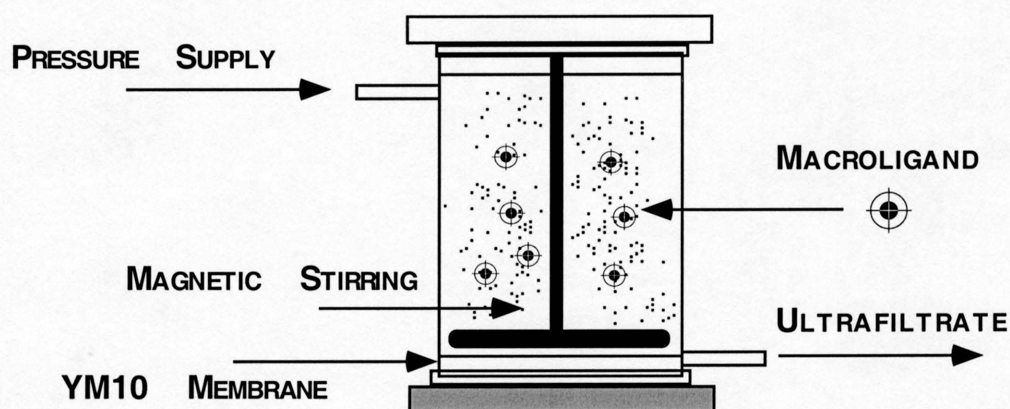


Figure 7.2 Conventional Ultrafiltration System. Stirred cells used with ultrafilters provide high flow rates with concentrated solutions. Gentle magnetic stirring controls concentration polarization with minimal exposure to shear denaturation.

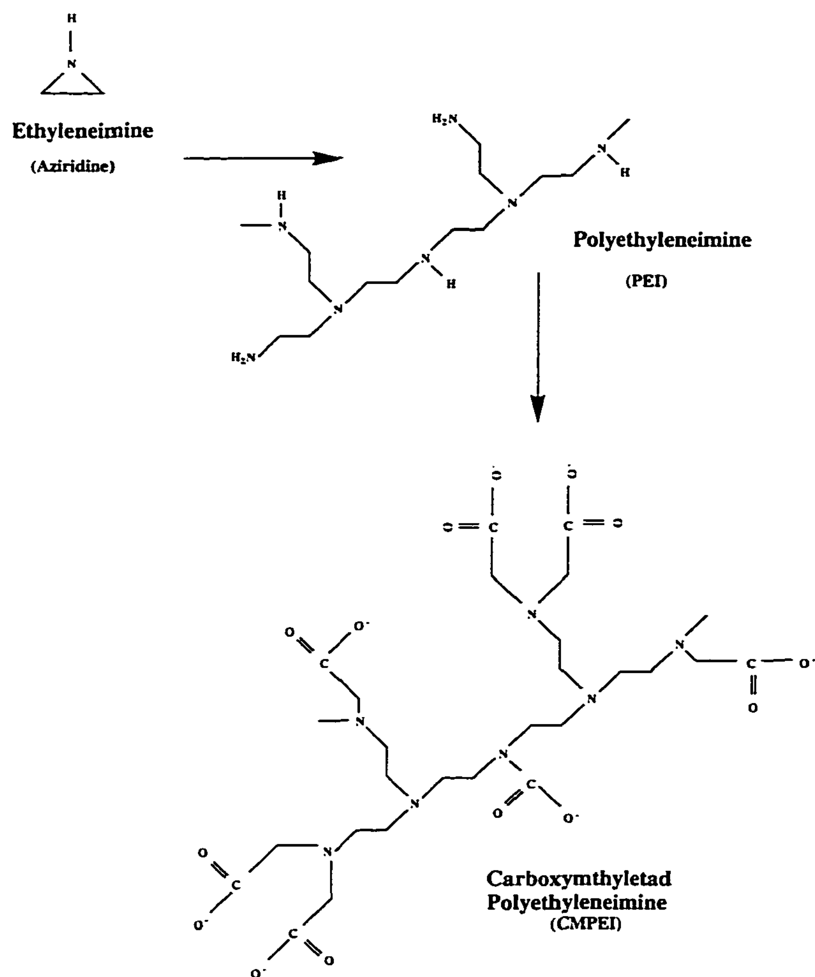


Figure 7.3 Synthesis of Carboxymethylated Poly (ethyleneimine) (CMPEI). The exceptionally binding capacity of CMPEI for some metals ions may be the result of macrocyclic complexation, possibly reinforced by sterically-caused inclusion effects.

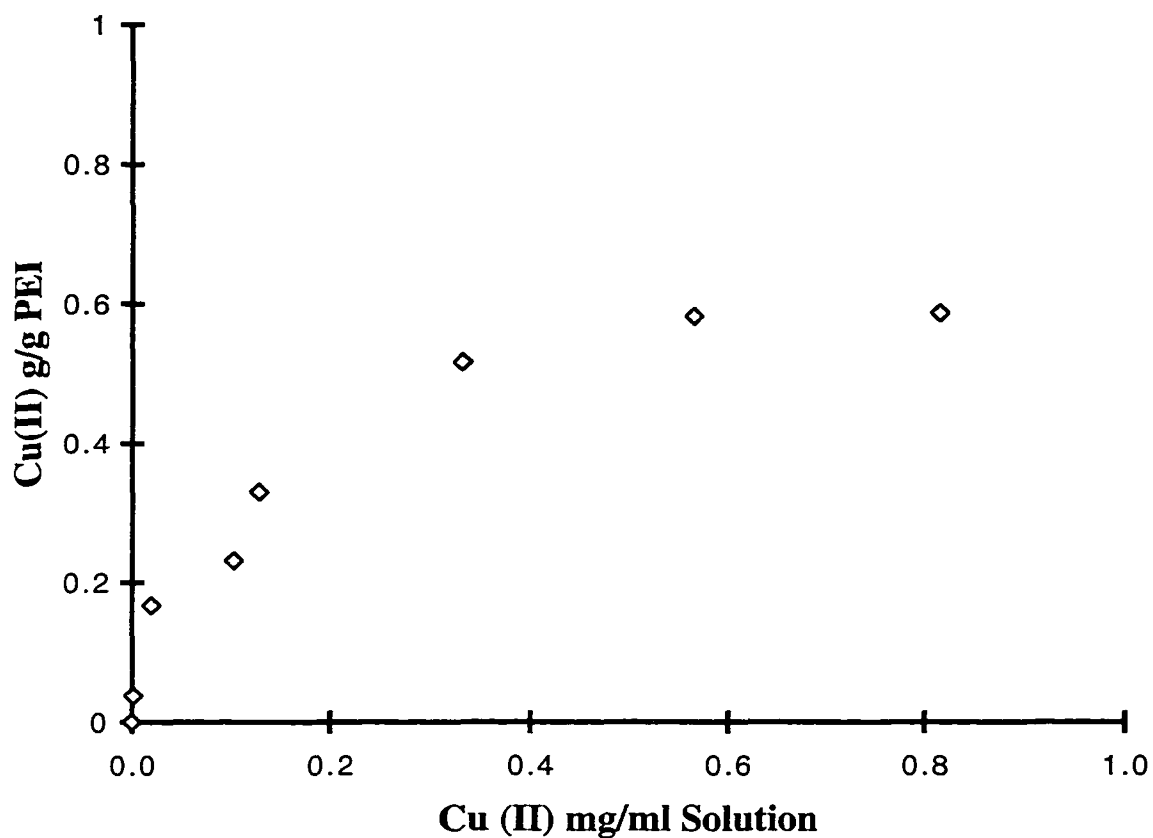


Figure 7.4 Adsorption isotherm for Cu(II) at pH 5.0 in buffer NaAc 0.1 M using Poly (ethyleneimine) with an average MW of 750,000 Daltons. Based on a metal coordination number of 4, the PEI- Cu(II) binding constant was found to be $K_a = 2 \times 10^{17}\ M^{-4}$

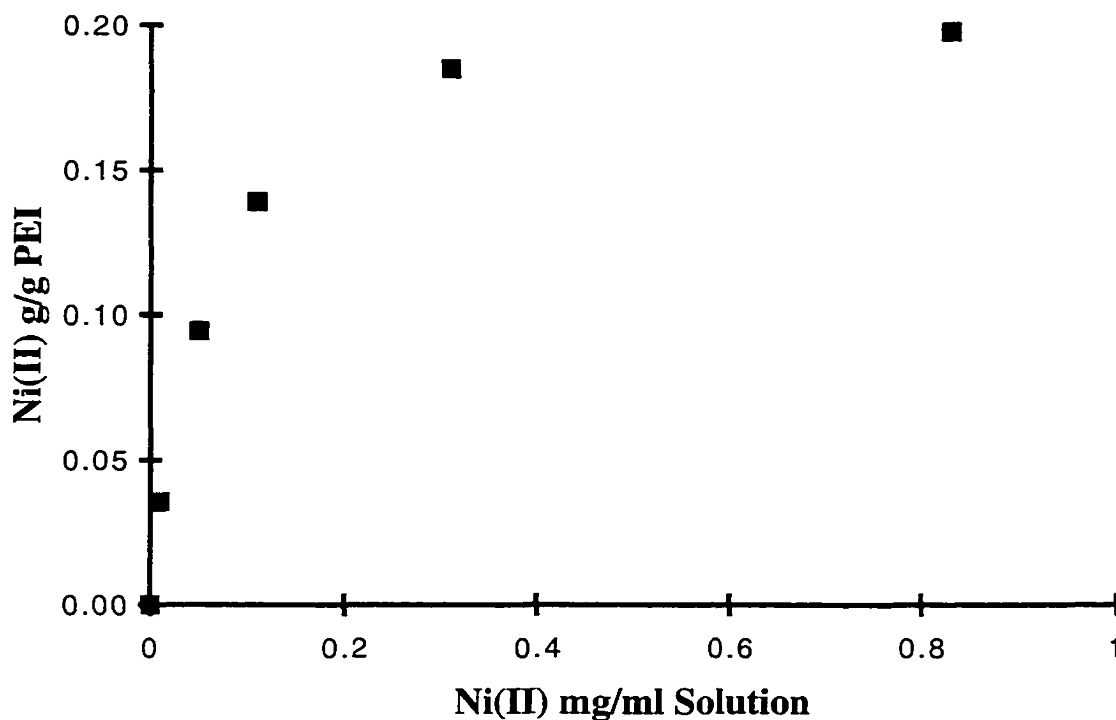


Figure 7.5 Adsorption isotherm for Ni(II) at pH 5.0 in buffer NaAc 0.1 M using Poly (ethyleneimine) with an average MW of 750,000 Daltons. Based on a metal coordination number of 4, the PEI- Ni(II) binding constant was found to be $K_a = 5.6 \times 10^{13}\ M^{-4}$

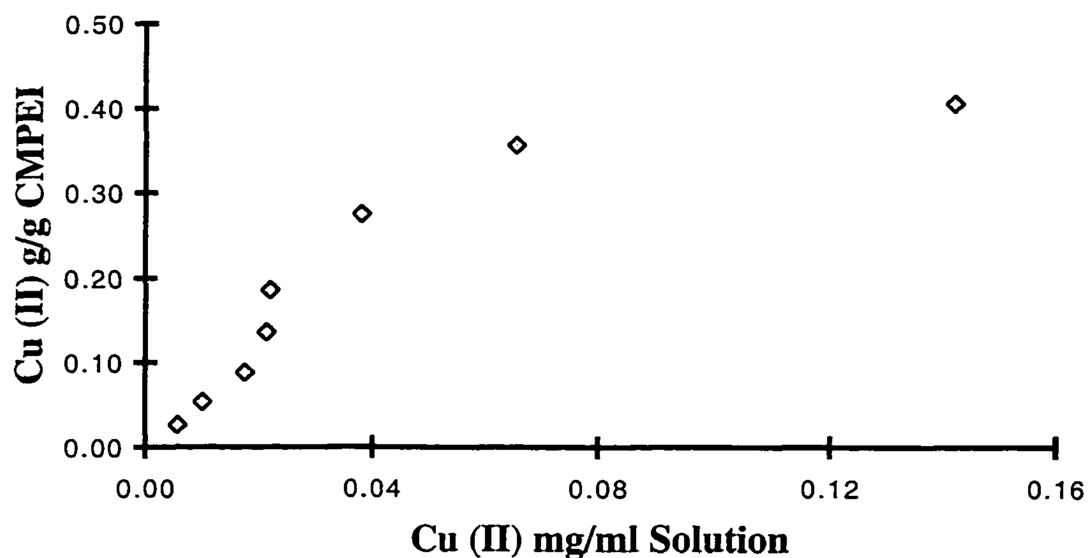


Figure 7.6 Adsorption isotherm for Cu(II) at pH 5.0 in buffer NaAc 0.1 M using Carboxymethylated-Poly (ethyleneimine) with an average MW of 750,000 Daltons. Based on a metal coordination number of 4, the CMPEI- Cu(II) binding constant was found to be $K_a = 1.8 \times 10^{6}\ M^{-2}$

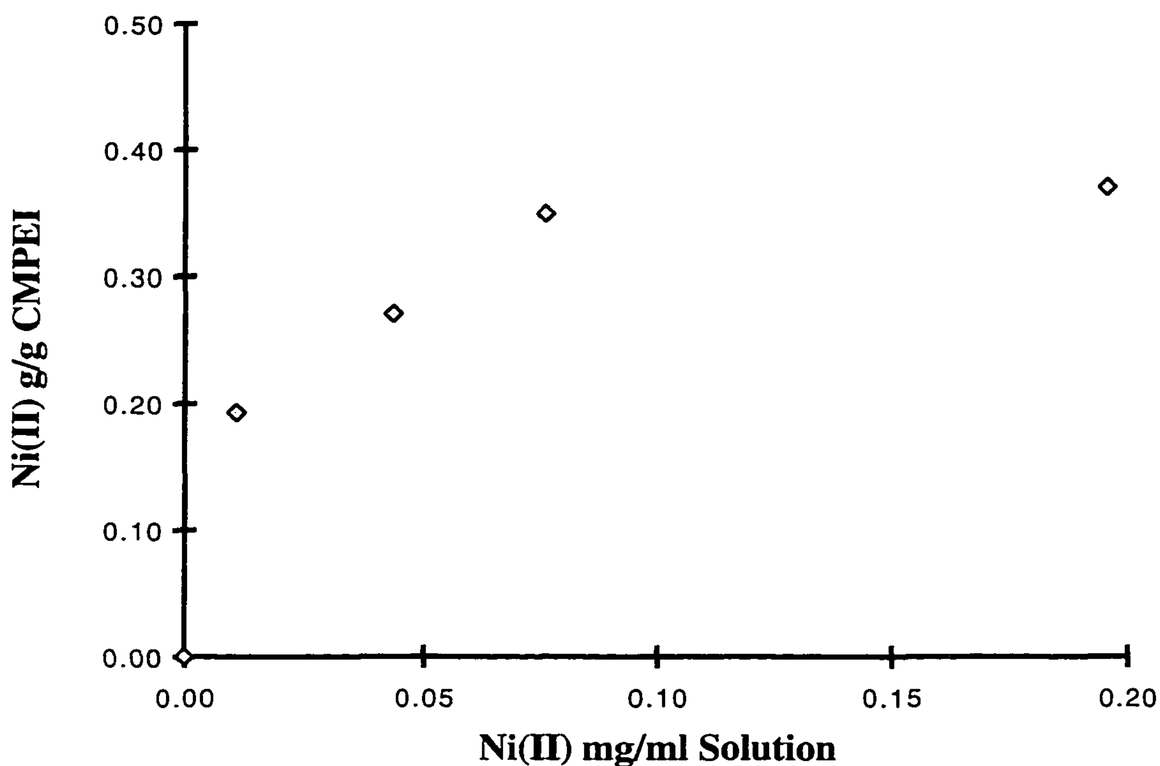


Figure 7.7 Adsorption isotherm for Ni(II) at pH 5.0 in buffer NaAc 0.1 M using Carboxymethylated-Poly (ethyleneimine) with an average MW of 750,000 Daltons. Based on a metal coordination number of 4, the CMPEI- Ni(II) binding constant was found to be $K_a = 9.5 \times 10^6 M^{-2}$

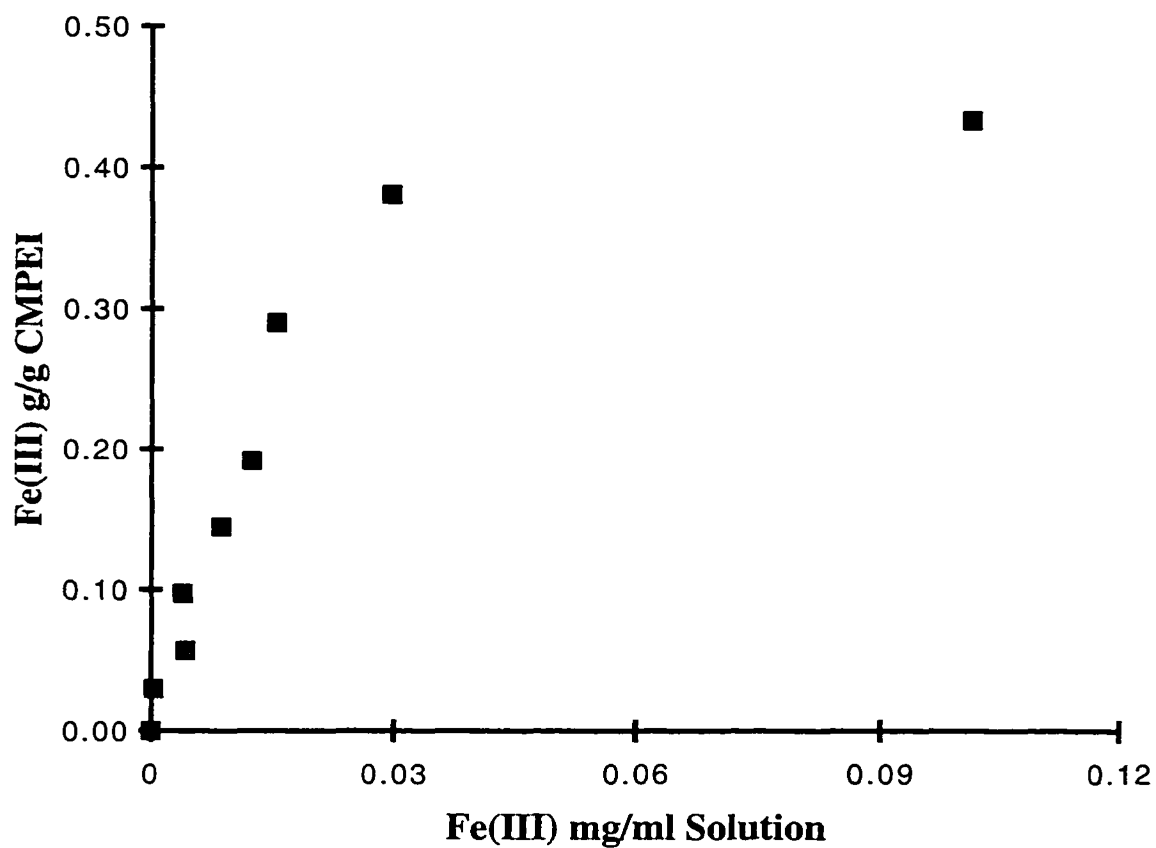


Figure 7.8 Adsorption isotherm for Fe(III) at pH 5.0 in buffer NaAc 0.1 M using Carboxymethylated-Poly (ethyleneimine) with an average MW of 750,000 Daltons. Based on a metal coordination number of 4, the CMPEI- Co(II) binding constant was found to be $K_a = 9.1 \times 10^{6}\ M^{-2}$

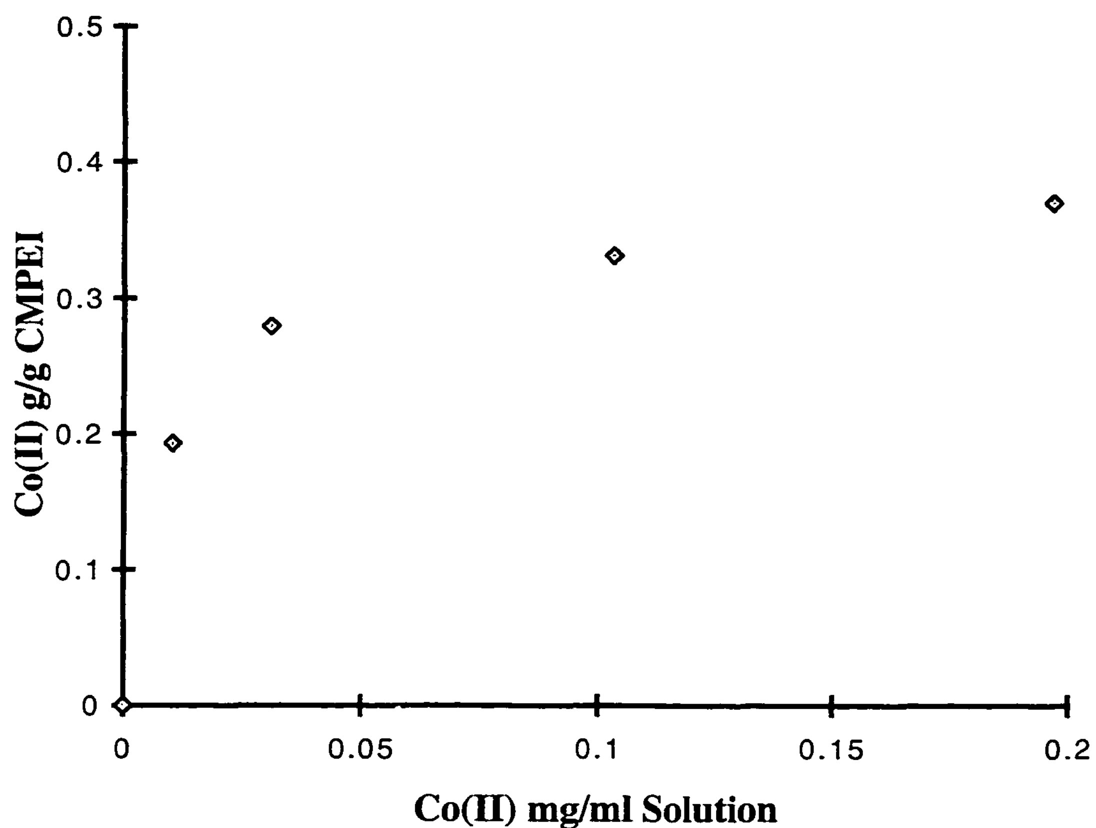


Figure 7.9 Adsorption isotherm for Co(II) at pH 5.0 in buffer NaAc 0.1 M using Carboxymethylated-Poly (ethyleneimine) with an average MW of 750,000 Daltons. Based on a metal coordination number of 4, the CMPEI- Fe(III) binding constant was found to be $K_a = 1.4 \times 10^{7}\ M^{-2}$

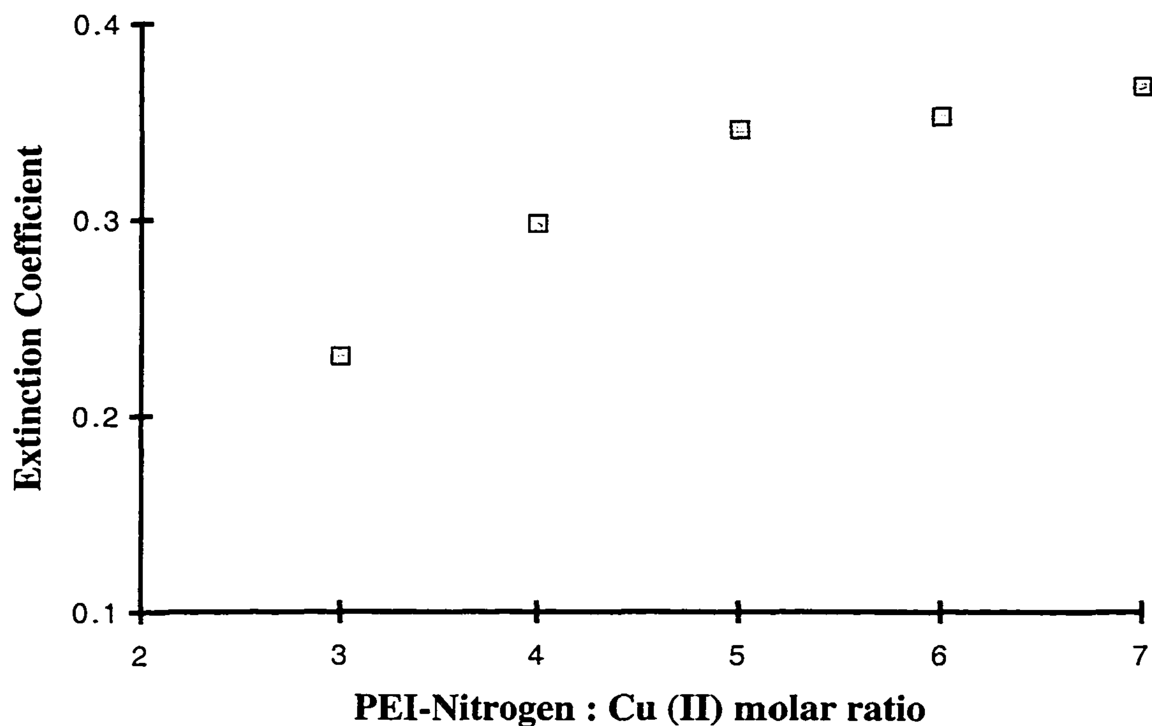


Figure 7.10 Extinction curve of the PEI-Cu(II) complex for the determination of the complex coordination number and calculation of the dissociation constant according to the molar ratio method (Mukherji *et al.*, 1958). Extinction is plotted versus the ratio of PEI nitrogen to copper. For a coordination number of 4, the dissociation constant for PEI-Cu(II) was found to be $K_d = 5 \times 10^{-18}$

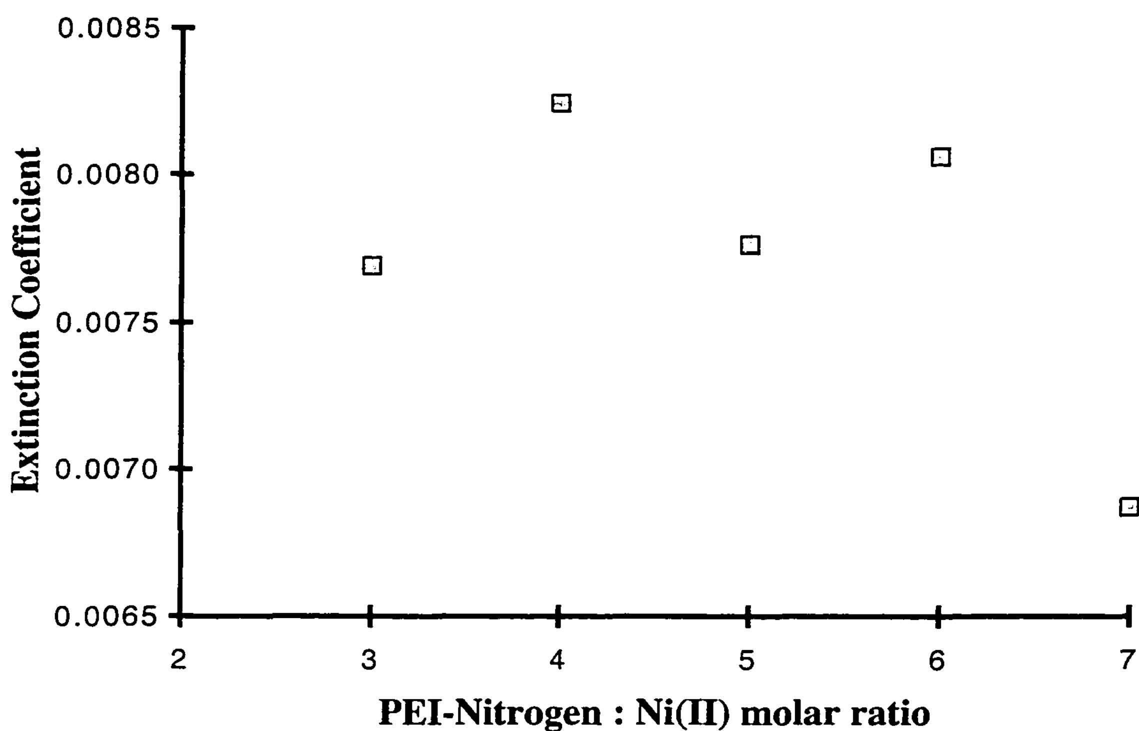


Figure 7.11 Extinction curve of the PEI-Ni(II) complex for the determination of the complex coordination number by the molar ratio method (Mukherji *et al.*, 1958). Extinction is plotted versus the ratio of PEI nitrogen to nickel. Determination of the dissociation constant according to the molar ratio method is not possible since the curve did not show a constant maximal extinction but rather two peaks that indicate only the formation of complexes with coordination numbers of four and six.

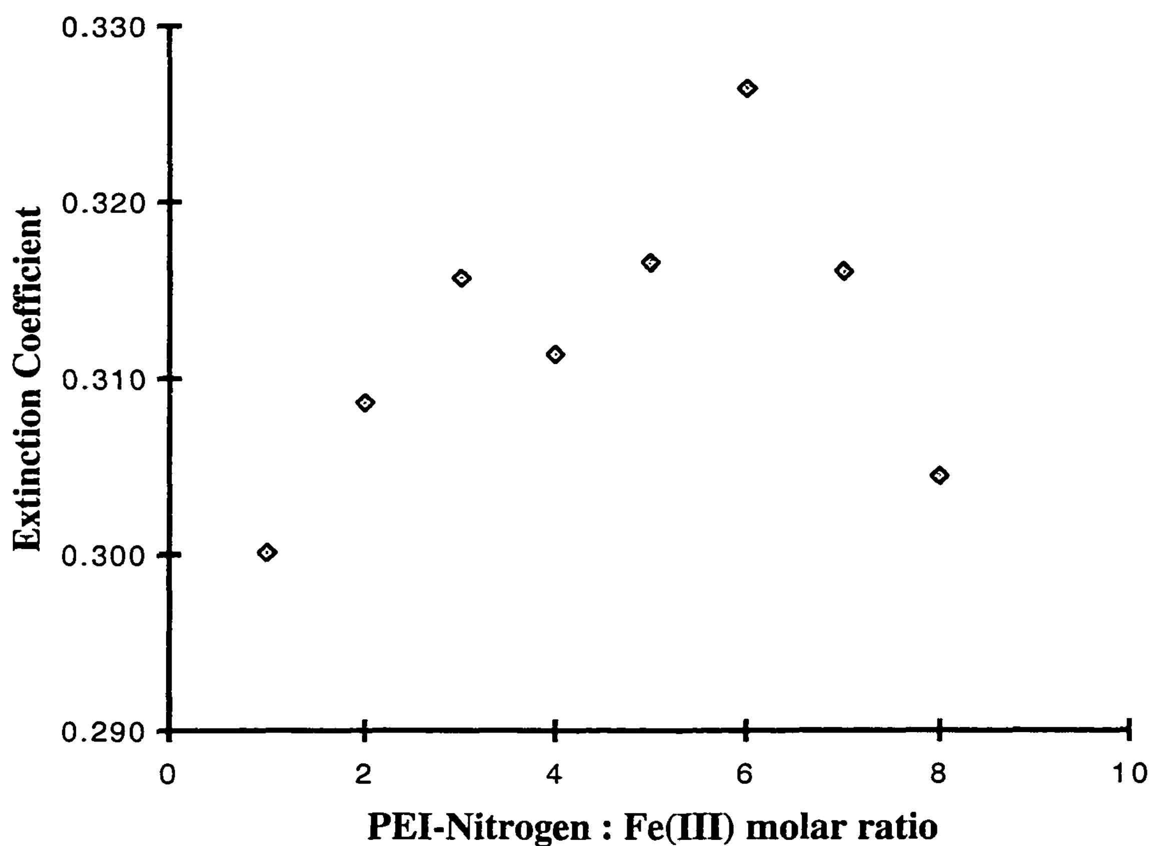


Figure 7.12 Extinction curve of the PEI-Fe(III) complex for the determination of the complex coordination number by the molar ratio method (Mukherji *et al.*, 1958). Extinction is plotted versus the ratio of PEI nitrogen to iron. Determination of the dissociation constant according to the molar ratio method is not possible since the curve did not show a constant maximal extinction but rather two peaks that indicate only the formation of complexes with coordination numbers of three and six.

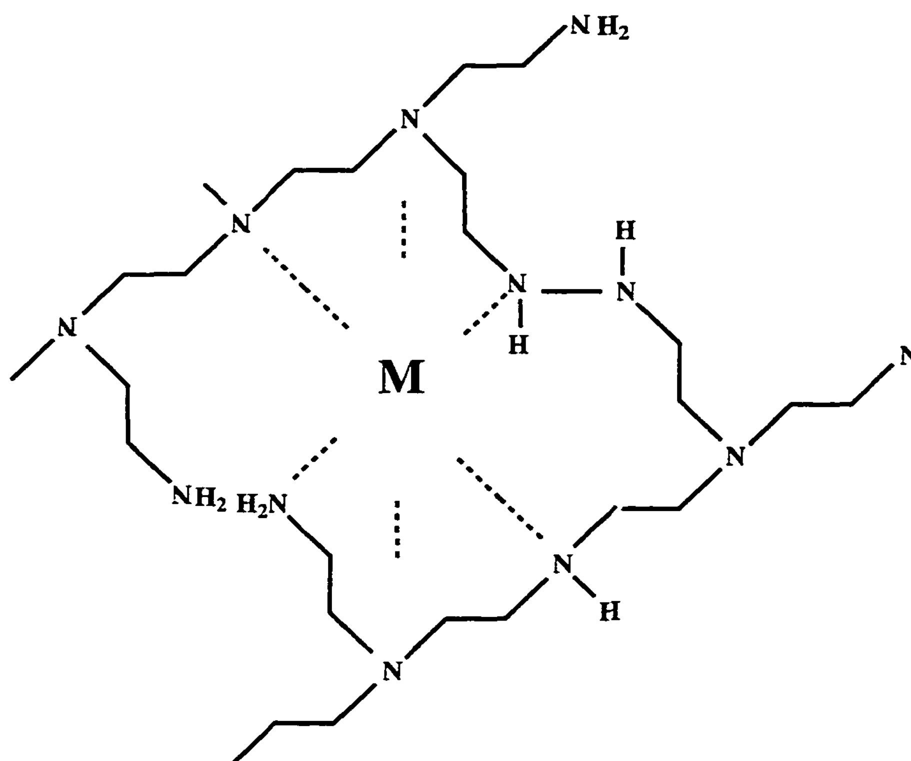


Figure 7.13 Poly (ethyleneimine) (PEI) showing "intracatenarian" metal cross-links. The exceptionally binding association constant of PEI for some metals ions may be caused to macrocyclic complexation, possibly reinforced by steric effects.

Macroligand-Metal Complex	*Binding Constant* K_a	*Experimental Metal Capacity (g/g polymer)*	*Theoretical Metal Capacity (g/g polymer)*
PEI-Cu(II)	$2.0 \times 10^{17}\ M^{-4}$	0.6	0.12
PEI-Ni(II)	$5.6 \times 10^{13}\ M^{-4}$	0.21	0.11
CMPEI-Cu(II)	$1.8 \times 10^{6}\ M^{-2}$	0.55	0.24
CMPEI-Ni(II)	$9.5 \times 10^{6}\ M^{-2}$	0.39	0.22
CMPEI-Co(II)	$9.1 \times 10^{6}\ M^{-2}$	0.39	0.22
CMPEI-Fe(III)	$1.4 \times 10^{7}\ M^{-2}$	0.51	0.21

Table 7.1 Comparison of metal capacities and binding constants determined for poly (ethyleneimine) and carboxymethylated poly (ethyleneimine). Calculations were made on the basis of content of chelating functional groups and on the premises of a coordination number of 4.

Macroligand-Metal Complex	*Binding Constant K_a (M^{-1})*	*Metal Capacity (g/g polymer)*
PEI-Cu(II)	7.4×10^3	0.6
PEI-Ni(II)	1.1×10^3	0.21
CMPEI-Cu(II)	1.3×10^3	0.55
CMPEI-Ni(II)	4.1×10^3	0.39
CMPEI-Co(II)	4.7×10^3	0.39
CMPEI-Fe(II)	7.2×10^3	0.51

Table 7.2 Adsorption parameters calculated according to the Langmuir's model for Cu(II), Ni(II), Co(II) and Fe(III) at pH 5.0 in buffer NaAc 0.1 M using Carboxymethylated-Poly (ethyleneimine) and Poly (ethyleneimine) with an average MW of 750,000 Daltons. Modification of PEI increased metal binding capacity but binding strength decreased.

CHAPTER 8

Polymolecular Molding or Casting and its Application to the Synthesis of Metal Affinity Adsorbents

8.1 ABSTRACT

Polymer molding is a method for synthesis of porous products of preformed shape. The concept is exemplified with agarose beads as "molds" and polyethyleneimines as castings. The agarose component of a strongly and extremely rigid cross-linked polyethylene-agarose copolymer complex, formed by the molding, may be selectively degraded to form a molded product or "memory gel" (M-gel). It consists essentially of cross-linked polyethyleneimine. The M-gel is chemically highly resistant, hydrophilic or amphiphilic and very rigid. Both the copolymer complex and the M-gel are anion exchangers and also metal chelate polymers of very high metal ion adsorption capacities. They are useful for ultratrace metal ion preconcentration and metal ion separation and may also serve as starting materials for synthesis of affinity adsorbents.

8.2 INTRODUCTION

There are increasing demands for microporous materials: zeolites and zeolite like products for nanotechnology (Chiu *et al.*, 1999) and organic hydrogels as well as hydrophilized silica for various applications in life sciences (Turkova *et al.*, 1993).

Both kinds of material may also be used in environmental cleaning-up processes.

Agarose has become the most frequently used support in affinity chromatography, gel electrophoresis and also in nucleic acid sequencing.

Agarose beads are cross-linked to increase their rigidity and heat stability (Porath *et al.*, 1971) in order to meet many quality demands in chromatographic applications. Still more rigid gels are now in request for high performance liquid chromatography. This chapter describes the synthesis of hydrogels of extreme rigidity and very high metal adsorption capacities. We use a technique that may be regarded as a kind of molding or casting: "polymolecular or polymer molding" (Meyers *et al.*, 1977). In general terms this concept may be delineated as follows:

A soluble polymer, C, is used as "casting" substance and a porous polymer, P, as the "mold". C and P are linked by covalent bonding (Fig. 8.1). In the first step polymer P acts as a template. Next, reactive groups are introduced to form an initial CP-complex (Fig. 8.1A), then follows exposure to more C, but this time with CP-complex as template for grafting to form a denser C copolymer component interlaced with P (Fig. 8.1B). Very compact, covalently-linked-adsorbate complexes, PC_n , are formed (Fig. 8.1C) by iterative activation, cross-linking and interpolymer bridging ("coupling") in which polymer C progressively is filling up the microvoids and channels of the porous network of P (Fig. 8.1D). Figure 8.1E shows the final degradation product. It should be observed that P may have any physical shape such as a particle, fiber, membrane or a sponge.

In CP_n, or CP-complex, there will be regions of high and low density of the C-component, and the high density regions are located around molecular fibers or cords making up the P-network (Fig. 8.2A). Presumably, the polymer C density of the CP-complex decreases gradient-wise from the position of the chain or fiber to the center of the micropores (Fig. 8.2B). Now by disintegrating P selectively by chemical degradation, the residual polymer C consists of a solid molecular framework that in shape is similar to that

of the mold P (Fig. 8.2B). Not only will the molecular structure reflect the geometry of the original scaffolding of P, but it will also retain its macroscopic size and shape. Since the cross-linked polymer C (X-poly C) product is a macroscopic replica of the original matrix, the molded gel conveys a "memory" of P and it may be called a "memory gel" (MX-poly C). If a soft gel such as cross-linked dextran (Sephadex) is used as P the outcome of the molding will be a softer product than if cross-linked agarose is used as the P-component. This reflects differences in the spatial structures of the polymer networks. In this work, we have used small and large molecular size polyethyleneimines as the C-component for grafting on Novarose (cross-linked agarose) supports.

8.3 MATERIALS AND METHODS

Materials

Novarose products were obtained from INOVATA (Bromma, Sweden). Polyethyleneimines (PEI) of different average molecular weights, were purchased from Aldrich (Milwaukee, WI). All other chemicals utilized were of analytical or reagent grade.

Equipment

Frontal analysis for metal ion binding capacity measurements was carried out using a LKB system from Pharmacia (Uppsala, Sweden) consisting of a HR 5/2 column (5 x 25 mm) connected to a P-1 pump, UV-1 optical unit and to a 2195 pH/ion monitor.

Methods

Preparation of Novarose-PEI Adsorbents (CP complex)

10 g of suction dry epoxy-activated Novarose were washed twice with 25 ml of 0.1 M carbonate buffer at pH 11 on a glass filter, suction dried and weighed. For the preparation of the adsorbent a PEI/Novarose ratio ~ 0.5 (W/W) and a PEI concentration in the range of 25% to 35% (W/W) are recommended. Thus, 5 g of PEI (25,000 or 750,000 molecular weight) were added to 10 ml of 0.1M sodium carbonate buffer pH 11 in a 100 ml screwcapped plastic bottle and the viscous solution homogenized on a mixer. The pH of the solution was measured and if necessary adjusted with 6M NaOH or 5M HCl. The Novarose was added to the bottle which was then placed on a shaker at room temperature. After 24 hr the non-bound PEI was removed from the adsorbent on a glass-filter by washing with deionized water until the effluent was neutral. The adsorbent was kept in 20% (V/V) ethanol in a refrigerator.

Preparation of Novarose-PEI_2 Adsorbents (PC_n complex)

Sixty grams of Novarose-PEI gel (CP-complex) were placed in a one liter conical flask together with 40 ml epichlorohydrin, 40 ml isopropanol, 40 ml H_2O and 60 grams of K_2CO_3 ($KHCO_3$ is produced progressively during the reaction time). The suspension was shaken over night (or a specified time 16 hours for example). The suspension was then diluted to double the volume with water (to dissolve salts) and the gel was collected in a glass-filter funnel and thoroughly washed with distilled water, ethanol (to get rid of the excess epichlorohydrin) and finally distilled water. The activated Novarose-PEI was suspended in an aqueous solution of PEI to produce Novarose-PEI_1- PEI_2 (PC_n complex).

We may use the same PEI or a PEI of different molecular weight. As in the first coupling reaction, a very high concentration of the PEI will result in a high capacity. After some reaction time there will still be epoxy groups present. These groups may be reacted with TREN to further increase metal adsorption capacity. A progressive spontaneous hydrolysis of the epoxy groups is likely to occur. This limits the time we should select. Six hours exposure in case of PEI 2000 after which TREN is added to the suspension and the reaction time may be extended over 24 hours (or much more if this should be convenient).

Crosslinking of PC_n Adsorbents (X-P complex)

Cross-linked adsorbent (X-PEI_1-agarose) was synthesized as follows: One hundred grams dry-suctioned Novarose Act ^{High} 1000/40 were suspended in 100 ml distilled water to which 20 ml of tetraethylene pentamine were added. Reaction was allowed to proceed for 70 hours. The gel was washed with water and then saturated with copper sulfate. After another washing with water followed by 0.1 M Na_2CO_3, 10 grams of this PEI_1-agarose was suspended in 10 ml of distilled water and 10 ml of butanol-bisglycidylether was added. The suspension was shaken for 60 minutes at room temperature, the gel was collected on a glass filter and washed with ethanol and water. The cross-linked gel (X-PEI_1-agarose), was immediately converted to X-PEI_2-agarose by a 24 hr treatment at room temperature with 50% aqueous solution of 2.7g PEI (Average MWr, ca 750,000). The gel was saturated with copper and the exhaustibly dried product was analyzed: Cu: 6.72%, N: 6.24%

Glutaraldehyde may be used as a cross-linking and coupling reagent for production of X-PEI_n-agarose but the products tend to become deeply colored. Control of the reactions are especially cumbersome due to progressive polymerization of the reagent.

Degradation of cross-linked Adsorbents

80g of X-PEI_2 – agarose gel were treated for 24 hours with 200 ml 50% H_2SO_4. The gel was washed with large volumes of distilled water until neutral pH. The gel was suspended in 100 ml of saturated solution of $NaIO_4$ (room temperature). After 20 hours the brown gel was collected, thoroughly washed with distilled water, resuspended and treated with 4g of $NaBH_4$ in small portions to give 42g of yellowish gel.

Measurement of Primary Amino Groups

Primary amino group content of the prepared gel adsorbents was measured using a modified solid phase TNBS method described by Antoni *et al.*, (1983). 0.5 ml of the gel to be analyzed was placed in a 15 ml conical tube. This sample was taken by first washing the gel thoroughly with deionized water. The gel was resuspended in roughly twice its volume with deionized water and a portion of the slurry was transferred to a pre-weighed 15 ml conical tube. The conical tube was centrifuged and the supernatant was removed. The tube was reweighed and a mass of deionized water was added equivalent to the mass of the gel contained in the tube. The gel was suspended in the additional deionized water and 100 μl of the suspension was quickly removed with a pipet and dispensed into an eppendorf tube. This tube was then centrifuged and the supernatant removed, leaving a fairly accurately measured 50 μg of gel.

To the 50 μg of gel, 5 ml of 0.1 M borax ($Na_2B_4O_7$) and 5 ml of 20 mM TNBS were added. A reference was prepared in a 15 ml conical tube by mixing 5 ml of 0.1 M borax and 5 ml of 20 mM TNBS. The samples were placed on a water bath-shaker at 37 °C

for 2 hr. The tubes were then removed, and centrifuged. 0.5 ml of the supernatant were transferred to a test tube containing 9 ml of 0.1 M borax and 0.5 ml of 30 mM glycine (sample). Another 0.5 ml of the supernatant was transferred to a test tube containing 9 ml of 0.1 M borax and 0.5 ml of deionized water (blank). The reaction in the test tubes was allowed to proceed for 25 minutes at room temperature. The absorbances of the samples were measured at a wavelength of 335 nm against the blanks. The amino group content of the gels was determined by the absorbance difference between the reference and the test gels using a calibration curve prepared for the TNP-derivative of glycine obtained.

Measurement of Metal Capacity

The capacity of the prepared gel was calculated by frontal analysis. All experiments were carried out using an LKB HPLC system. The prepared adsorbent was packed in a 3.4 x 0.5 cm I.D. column as described by the manufacturer. Before metal loading, the gel was equilibrated with 5 volumes of a 0.1M sodium acetate buffer pH 5. Frontal analysis was then performed according to standard procedures (Tiselius, 1940; Kasai *et al.*, 1986; Kyprianou and Yon, 1982). The equilibrated column was continuously fed with a 20 mM copper sulfate solution at a flowrate of 1 ml/min. The absorbance of the effluent was monitored at 280 nm and recorded using a chromatography data acquisition system (SMAD). The solution was fed until the absorbance of the effluent reached a constant plateau level equal to that of the absorbance of the solution being fed to the column. The elution volume was determined from the median bisector of the front of the elution profile.

Alternatively, the metal capacity of the gels can be estimated if a quick reference value is needed as follows: The column is packed and the flow rate adjusted to 1.0 ml/min. The column is equilibrated with 10 volumes of deionized water and equilibrated with 5 volumes of buffer. A selected volume of 20 mM copper sulfate solution is loaded based on

the anticipated copper capacity of the adsorbent. The column is then washed with 10 column volumes of deionized water resulting in a distinct colored immobile front. The capacity is determined by measuring the height of the front resulting from the known concentration of metal solution. For a column diameter of 0.5 cm, 1 cm of gel height is equivalent to 0.2 ml of gel.

Determination of Adsorption Isotherms

The adsorption isotherms were determined using a modified batch mode described by Steinmann *et al.* (1994). The isotherms were constructed using batch samples at pH 5.0 in sodium acetate 0.1 M. Several samples were prepared by adding 0.20 g of gel, and 40 ml of buffer containing the appropriate amount of metal ions. The samples were gently mixed and left in an shaker overnight to reach equilibrium. The samples were centrifuged and the supernatant tested for metal content by UV. The amount of metal ion absorbed in the gel was then calculated based on the initial amount of metal ion added to the solution.

8.4 RESULTS AND DISCUSSION

Primary Amino Content

The analysis of amino groups in solid phase requires a quantitative measurement of the TNBS remaining in solution after it has reacted with the polymer in the gel. The TNBS still present in solution is determined by means of reaction with an excess of glycine. The analysis at various concentrations of TNP derivative is shown in Fig 8.4 . The molar

absorption coefficient of the trinitrophenyl derivative of glycine calculated from this plot and utilized for the calculations was $\varepsilon = 8000$.

To complete the reaction of the primary amines in the gel, the amount of TNBS used, must be the lowest possible but high enough to ensure proper analytical results when reacted with glycine. Table 8.1 shows the primary amino content calculated for different Novarose adsorbents. High molecular weight PEI yielded more primary amino groups on the gel. However, when a second layer of PEI was added, crosslinking occurred and primary amines decreased. The structure of PEI reveals the presence of primary, secondary and tertiary amines in a 1:1:1 ratio. Therefore, the total nitrogen bound to the gels is 4 times the primary amines measured. Elementary analysis showed that the average nitrogen content in the gels is about 6.24%.

Metal Capacity

Figures 8.5 and 8.6 show the frontal analysis of several Novarose gels. The shape of the elution front for most of the gels is sigmoidal. This may be due to a not very homogeneous local distribution of the metal ion through out the column and distribution of the polymer itself in the gel. As illustrated in figure 8.2, there are regions of high and low density of polyethyleneimine (C component). These regions should be located around the fibers of the agarose matrix decreasing gradient wise from the position of the chain to the center of the pore. When the amount of polymer in the gel was increased, as in the case of Novarose PEI_2, diffusion and permeation rates decreased as shown by frontal analysis (steric limitations). After digestion of the gels, the agarose matrix was degraded and the diffusive limitations slightly decreased. Table 8.2 shows the dynamic binding capacities measured for several adsorbents. Although the dynamic binding capacity is best determine

by frontal analysis, this value is influenced by several factors such as flow rate, molecular size, pore size and polymer concentration. However, dynamic binding may be preferable to batch studies since it represents actual operating conditions.

Figures 8.7 through 8.11 show the adsorption isotherms of cu(II), Ni(II) and Fe(III) with different adsorbents. As expected batch analysis showed higher capacities due to the increased exposure time. Copper analysis shows that four nitrogens atoms are coordinated to one copper ion. The degree of cross-linking and the concentration of the ethyleneamine units strongly affect the strength and selectivity in adsorption of transition element ions. At the upper extreme of the reachable PEI-density some portions of the metal ions are irreversibly adsorbed and can not be eluted even at high concentrations of strong acids. Table 8.3 shows the capacities of Novarose adsorbents for transition metal ions. The exceptionally strong affinity may be caused by metal capture within intercatenarian polymacrocyclic structures in the cross-linked PEI network. This may be the case of Fe(III) in CMPEI adsorbents. Ionic deposition is also a possible condition responsible of high binding. All the facts mentioned offer some problems but also much promise for further development. Metal ions were collected in a very narrow zone, indicating a concentration factor of the order of 10^6. It may be interesting to note that Steinmann *et at.* (1994) found IDA-agarose to accumulate metal ions 50 times faster than the commercial chelating resin Chelex 100. The hydrogels derived from agarose and discussed here also seem to have similar excellent permeation and diffusion properties.

The agarose gel structure (Rees *et al.*, 1977) is remarkable among hydrogels with respect to its behavior towards solvents. Starting out from an agar bed in water, a series of sequential solvent displacements were carried out in order of decreasing solvent polarity followed by displacements in reverse. Solvent change can be made starting from water to ethanol and further to acetone, acetonitrile, pyridine and ending with hydrocarbons.

Making the solvent changes in reverse order we return the gel to its original state. The bed volume does not change much throughout these operations. In other words, agarose gels are not only hydrophilic but also close to "ideally amphiphilic" in their solvent behavior. Similar volume stability characterize PEI-agarose and MX-PEI. Presumably, also for those products the gel solvent behavior reflects unusual gel structure stability, a fact that has important implications. Figure 8.12 shows that gels in beaded form can be packed in beds that allow extremely high flow rates under pressure. Linear flow rates as high as 30 meters per hour have been reached in PEI-agarose beds and 6 meters per hour in MX-PEI beds without bed compacting. Solid phase synthesis in gel media, imbibed in many suitable solvents, should be possible. Some interesting pressure/flow rate relationships were observed in test columns of similar size and packing. Flow rate increased more gradually in the PEI-agarose beds but reached higher values than was the case for the degradation products PEI-AF and MX-PEI (Fig. 8.12). On the other hand, in the last mentioned beds a steep rise to the limiting value occurred so at lower pressures higher flow rates were achieved than in the agarose bed. Beside these changes we also observed by frontal analysis that permeability and diffusion rates increase when the agarose component is degraded.

8.5 CONCLUSIONS

In this study we have demonstrated molecular molding with epoxy-activated agarose beads as the mold P and branched polyethyleneimine, PEI of different molecular weight, as the C component. P can easily be degraded under conditions where cross-linked PEI is stable. By selecting PEI of different molecular size, chemisorption and coupling efficiency may be improved to give a PEI-agarose complex of high polyamine content. The final product, that we have denoted as MX-PEI, consists of cross-linked PEI with short residual

segments left over after the exhaustive degradation of the agarose component. It consists essentially of cross-linked polyethyleneimine.

The conditions for synthesis of the adsorbents have not yet been optimized so as to obtain the most desired properties with respect to rigidity, permeation and diffusion. Functionalization of PEI is easier than it is for agarose. By molecular molding and subsequent agarose degradation PEI in the form MX-PEI has become available as a suitable matrix for synthesis of a variety of adsorbents, and for binding or inclusion of catalysts such as metallic palladium.

Preliminary functionalization studies of PEI-agarose has been initiated. Introduction of picolyl groups increases capacity and strength of adsorption. Carboxymethylation and Mannich condensation of 8-hydroxyquinoline, for example, broaden the selectivity in direction to hard metal ions. From these observations we conclude that a new methodology has been introduced for synthesis of rigid, very high capacity adsorbents. However, extensive cross-linking produces extremely rigid particles at the expense of capacity. Much developmental work will therefore be necessary to find the necessary balance of the mechanical and chemical properties for each desired field of application. It has been shown in preliminary experiments that PEI agarose and its chelating derivatives are extremely efficient adsorbents for preconcentration of metal ions from very dilute solutions and they can also be used for metal ion separation by displacement chromatography.

The PEI-adsorbents can be autoclaved and they can be converted into leakage-proof products which make them potentially useful for biological and medical applications (e.g. for detoxification). The excellent adsorption properties can be explored for decontamination of metals from industrial and environmental waters and there is potential for their use in solution mining and hydrometallurgy. For applications in these last mentioned areas less expensive raw materials for the mold partner in the synthesis will be necessary. Since the molecular molding concept should be applicable also to other polysaccharides, such as

cellulose, the indicated adsorption techniques can eventually, be extended to very large-scale industrial applications.

The idea of incorporating one polymer A in a polymer B and then disintegrating polymer B to obtain a product consisting of A is apparently not new but to the best of our knowledge the kind of polymer molding concept here presented seems to be original. A recent comprehensive study of agarose structure has been made by Anders (1995).

Reactions between hydroxyls and oxirane reagent requires strong alkalinity (pH 12 or more) whereas amino groups are activated at pH 9 or lower. Systematic kinetics studies of these reactions should promote further development of polymeric adsorbents.

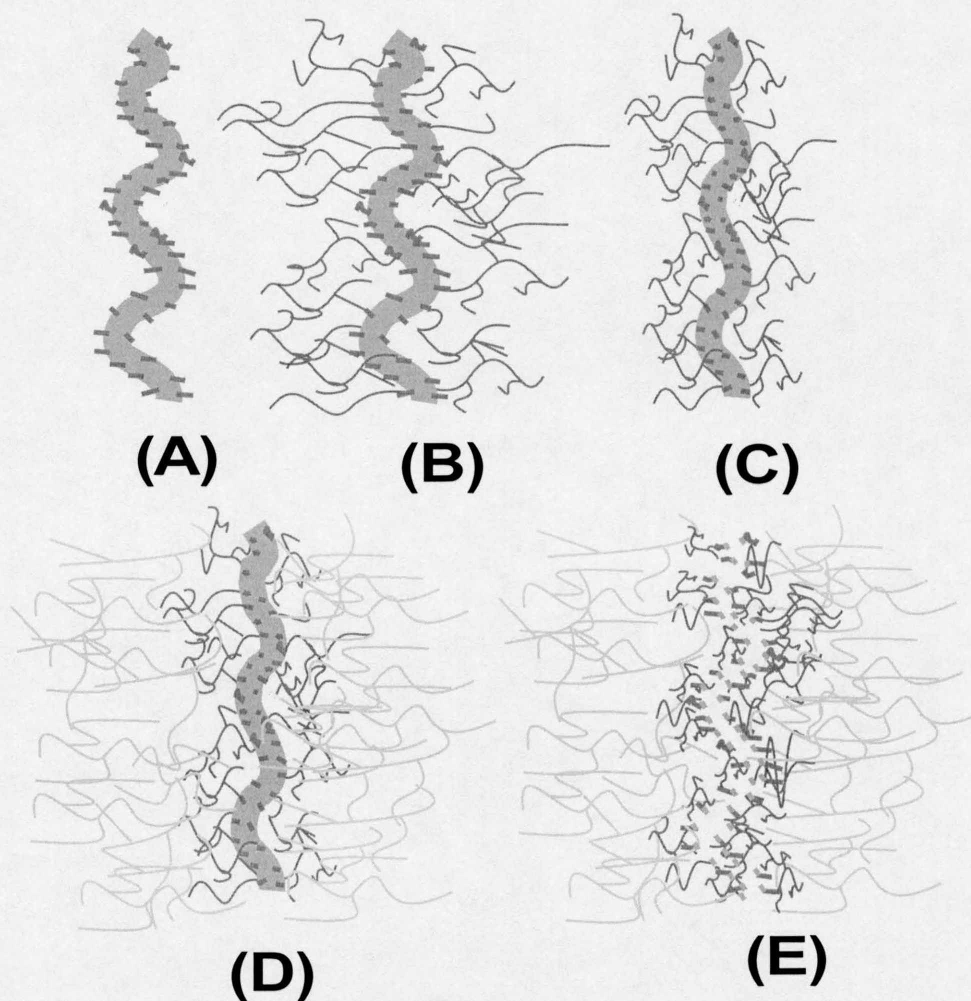


Figure 8.1 Schematic representation of the molding principle exemplified with agarose polyethylene imine. Agarose is used as mold and polyethyleneimine as casting. A) PEI-agarose B) PEI_2-agarose after short exposure C) PEI_2-agarose after long exposure leads to lining up and interchain cross-linking or bridging D) PEI_3-agarose with long PEI molecules that are not externally cross-linked E) Agarose disintegrated by hydrolysis leaving degraded galactose residues attached to the internally cross-linked and bridged PEI. (MX-PEI-AF or MX-PEI with agarose fragments).

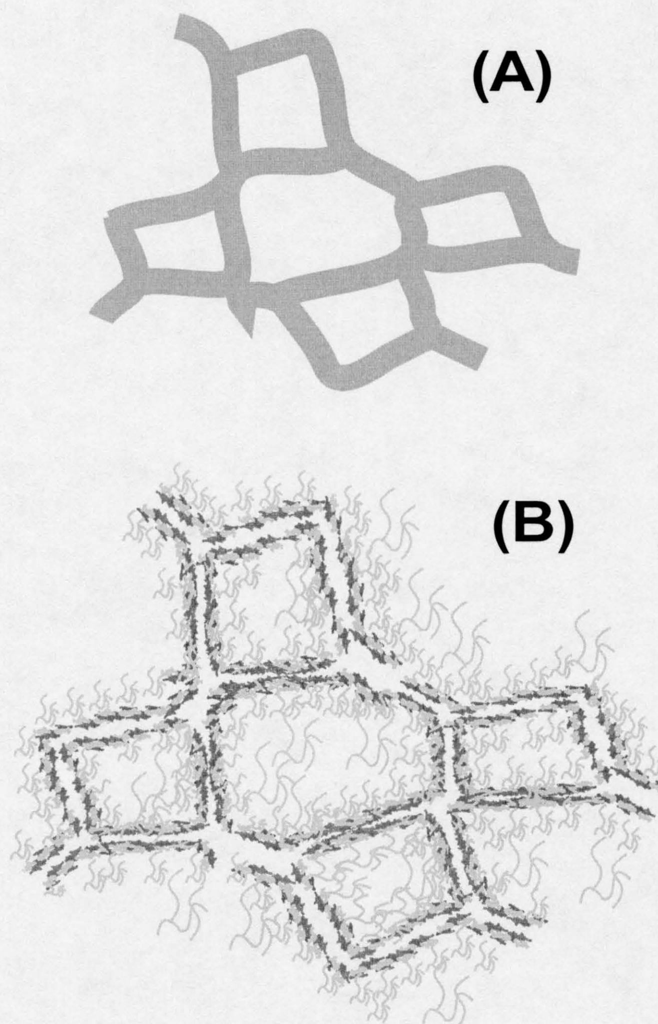


Figure 8.2 Idealized extension of network structures showing schematically pores or cavities of the original matrix and the final molded product. The internal regions of the cavities in B are less density populated by PEI chains. Size exclusion is expected to take place in addition to adsorption. The reduction in size limit for molecular exclusion when converting A to B is also expected to depend on the spatial interrelation between cavity dimensions of P and the space and density distribution of polymer C within the cavity. Presumably removal of the agarose results in some relaxing of the rigidity of the scaffolding gel network.

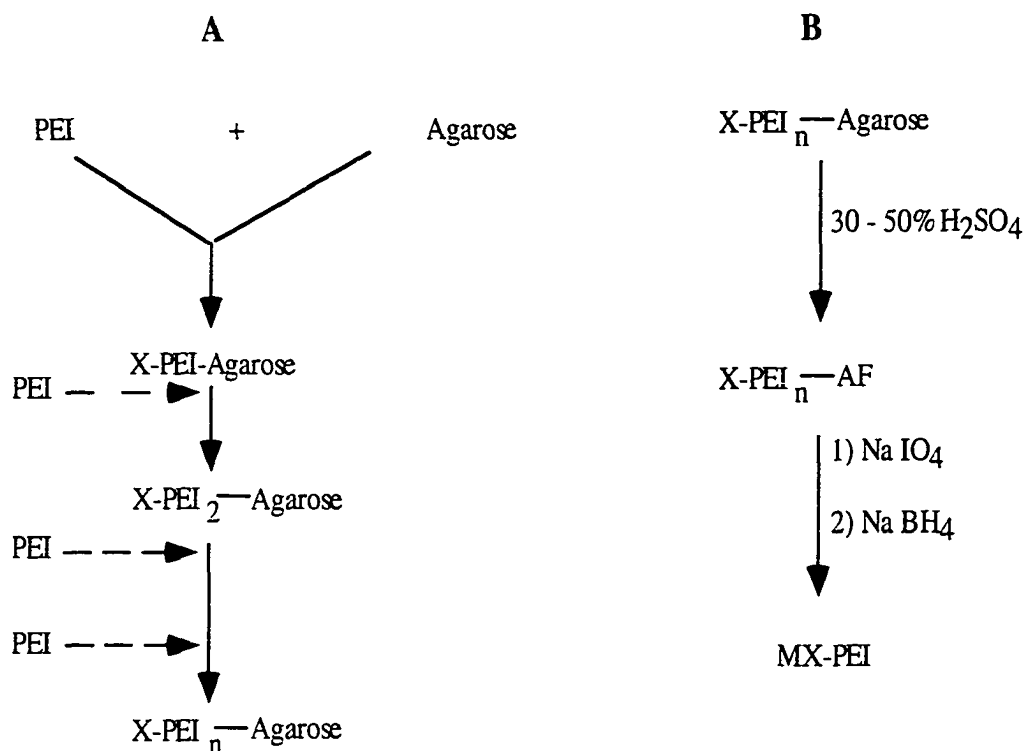


Figure 8.3 Synthesis of "memory" gel MX-PEI. This final product consists of cross-linked PEI with short residual segments left after the degradation of the agarose matrix. It is essentially cross-linked polyethyleneimine. (A) Illustrates the n-consecutive steps in the synthesis of the polyethyleneimine-agarose complex. (B) Degradation sequence from the complex to the cross-linked polyethyleneimine. (AF) signifies agarose-fragments, presumably consisting of galactose residues. (MX-PEI) signifies molded cross-linked PEI or memory of the shaped and structure of the original mold such as of agarose particles.

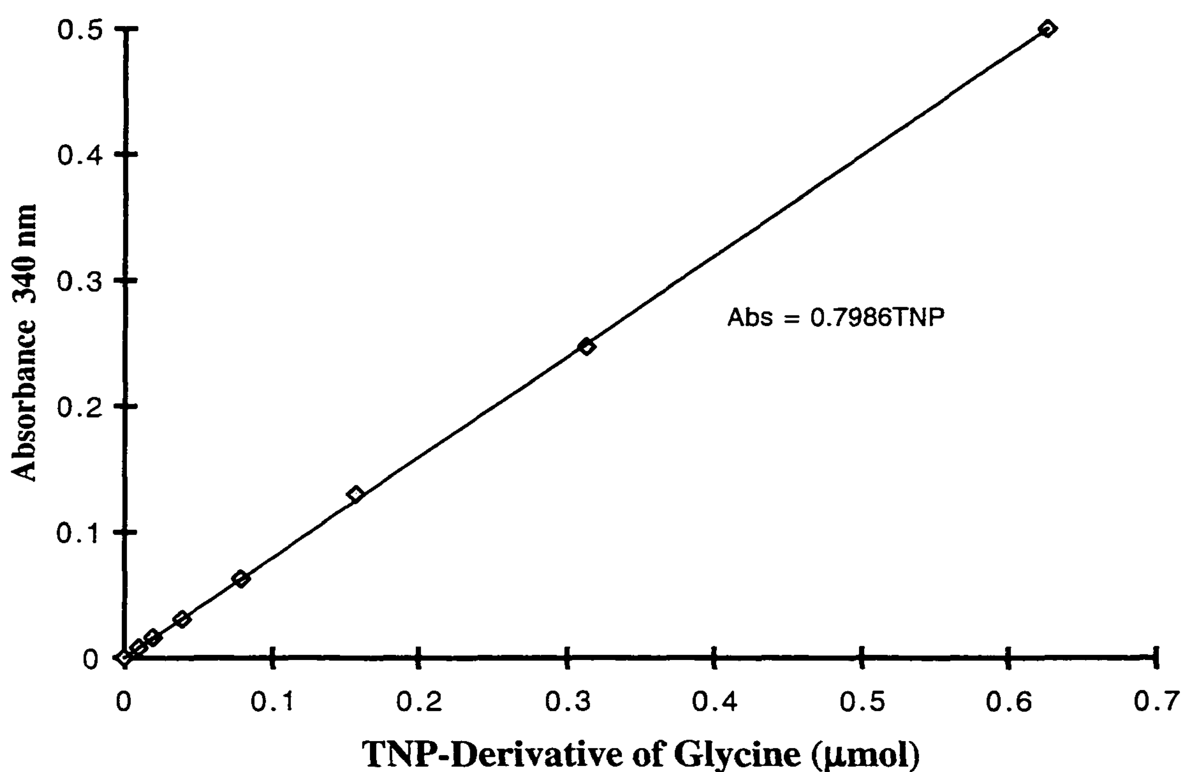


Figure 8.4 Colorimetric analysis at various concentrations of TNP derivative of glycine. The calibration curve of the reaction product of TNBS with glycine is linear. The molar absorption coefficient of the trinitrophenyl derivative calculated from this plot was $\varepsilon = 8{,}000$. Samples were prepared as follows: 0.5 ml of Glycine (30mM), 0.5 ml of TNBS sample and 9 ml of Borax 0.1 M.

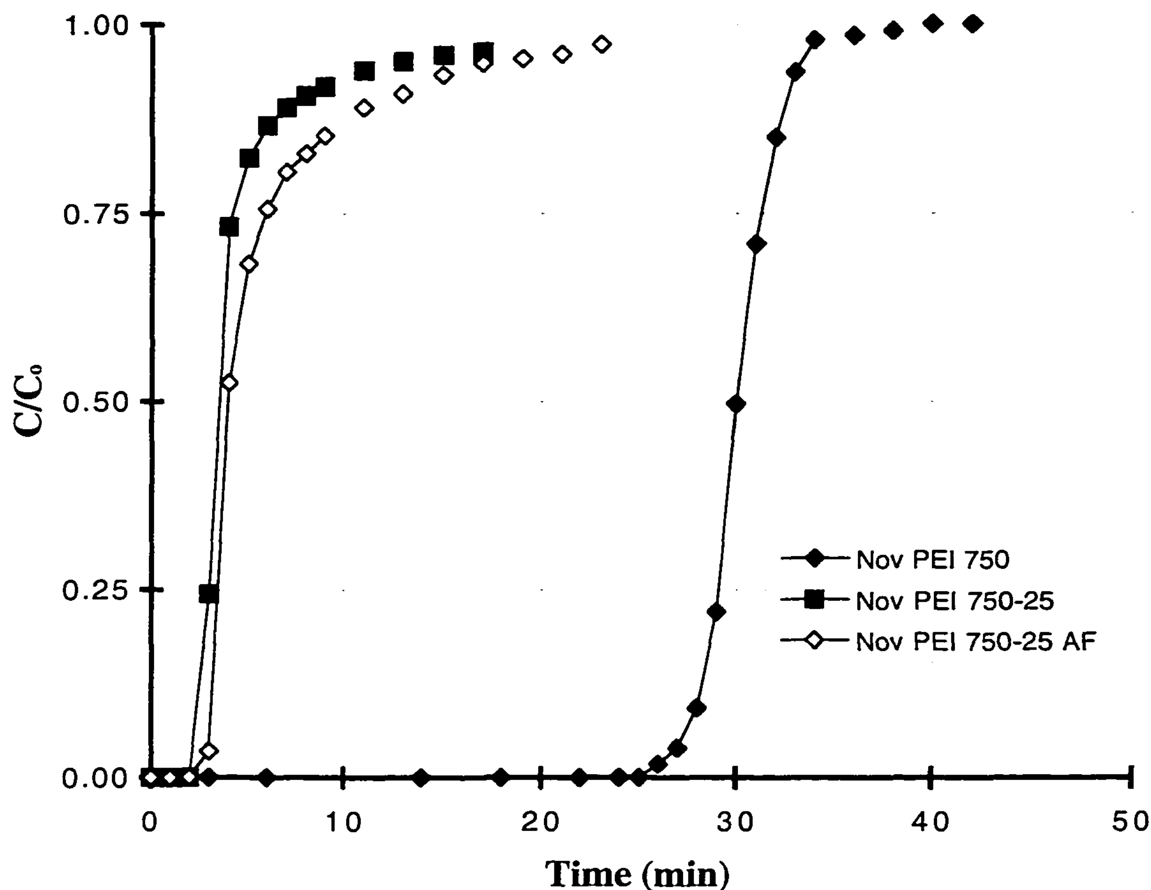


Figure 8.5 Frontal analysis of Novarose Act.^{High} PEI 750 adsorbents. Columns of similar size and packing were used in all the experiments. The bed volume was 0.55 ml , the flowrate 1 ml/min and C_o = 20 mM. PEI-agarose beds showed higher dynamic copper capacity. Permeability and diffusion rates decreased when a second layer of PEI was added the agarose matrix as evidenced by lower capacity. When the agarose component was degraded diffusion limitations were partially eliminated.

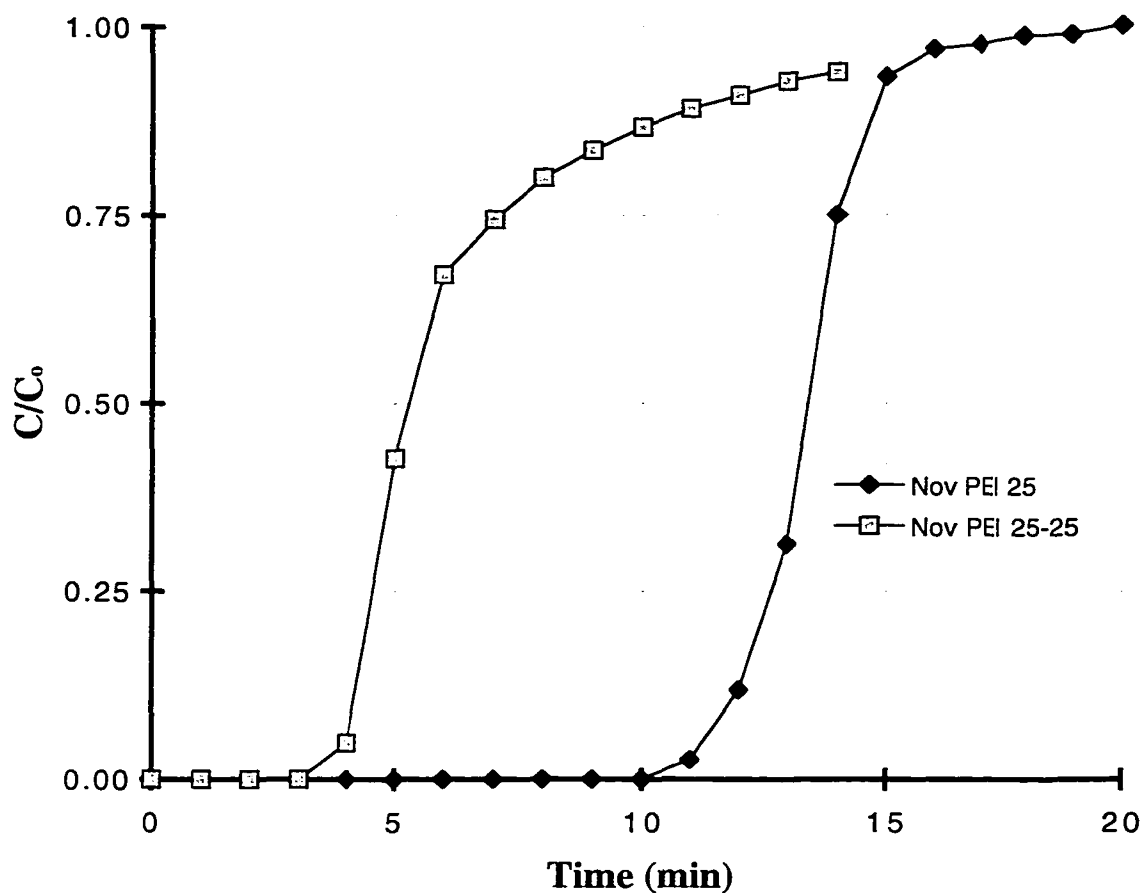


Figure 8.6 Frontal analysis of Novarose Act.^{High} PEI 25 adsorbents. Columns of similar size and packing were used in all the experiments. The bed volume was 0.55 ml, the flowrate 1 ml/min and C_0 = 20 mM. PEI-agarose beds showed higher dynamic copper capacity. Permeability and diffusion rates decreased when a second layer of PEI was added the agarose matrix as evidenced by lower capacity.

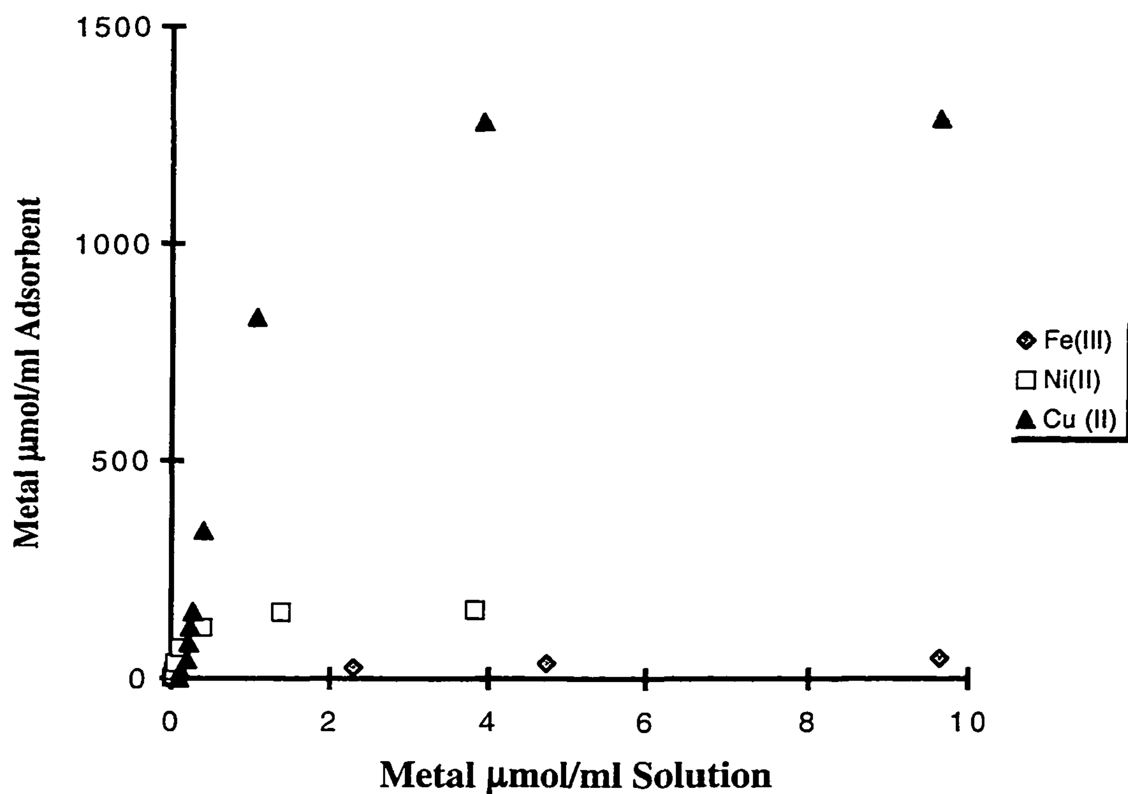


Figure 8.7 Adsorption isotherm for Cu(II), Ni(II) and Fe(III) at pH 5.0 in buffer NaAc 0.1 M on Novarose Act. High 100/40 modified with polyethyleneimine 750,000. The isotherm was determined after 24 hr in a batch mode using 0.2 g of gel and metal concentration in the range from 0 to 20 mM.

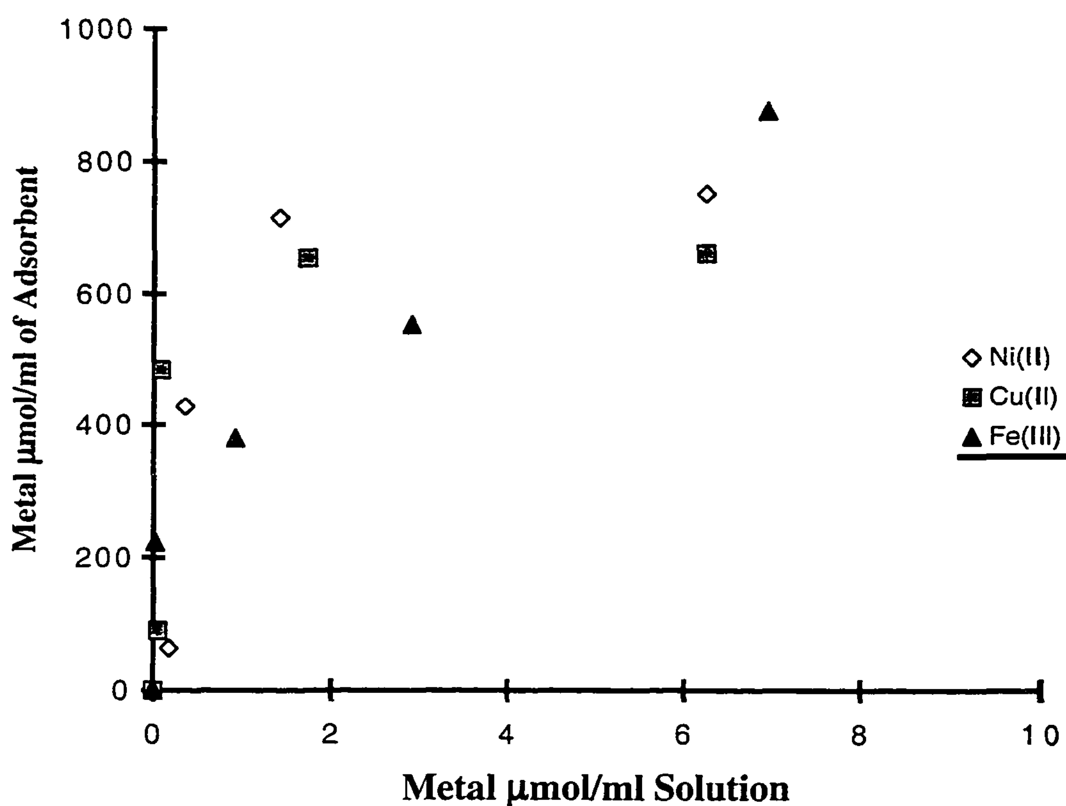


Figure 8.8 Adsorption isotherm for Cu(II), Ni(II) and Fe(III) at pH 5.0 in buffer NaAc 0.1 M on Novarose Act. High 100/40 modified with carboxymethylated polyethyleneimine 750,000. The isotherm was determined after 24 hr in a batch mode using 0.2 g of gel and metal concentration in the range from 0 to 20 mM.

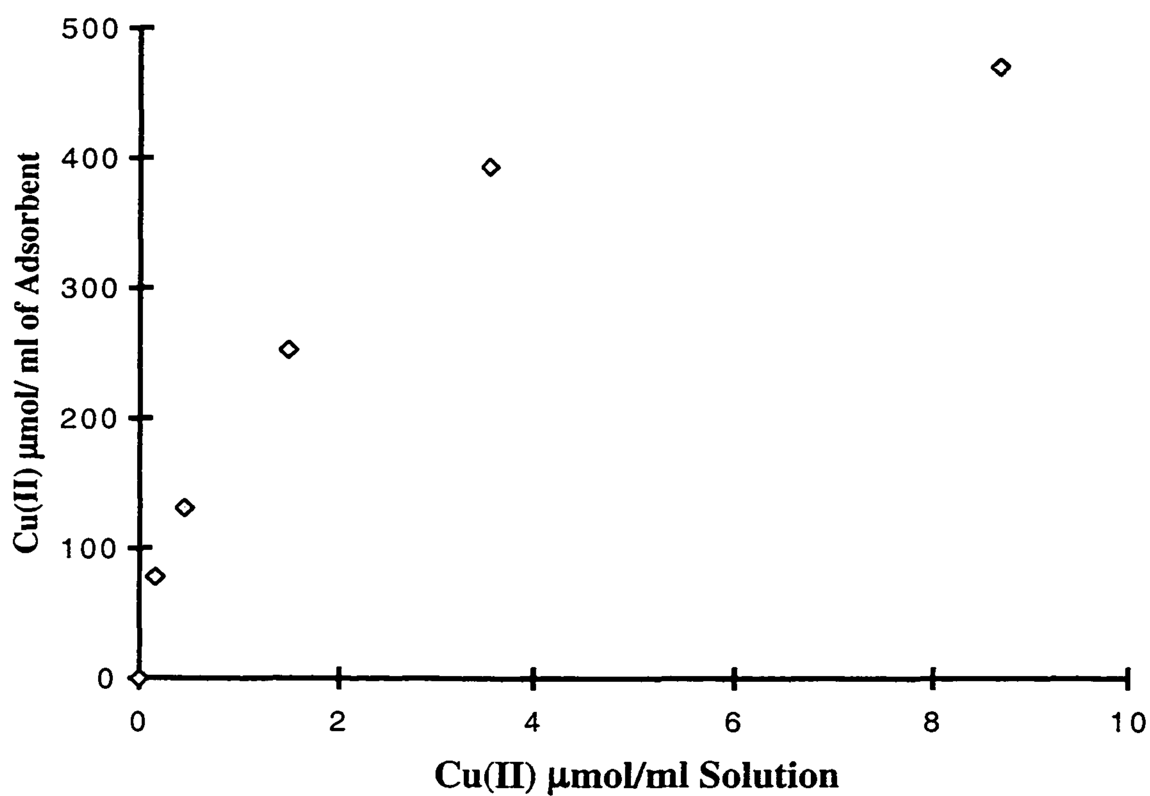


Figure 8.9 Adsorption isotherm for Cu(II) at pH 5.0 in buffer NaAc 0.1 M on Novarose Act. High 100/40 modified with polyethyleneimine 25,000. The adsorbent was then cross-linked with butanol-bisglycidyl ether. The isotherm was determined after 24 hr in a batch mode using 0.2 g of gel and metal concentration in the range from 0 to 20 mM.

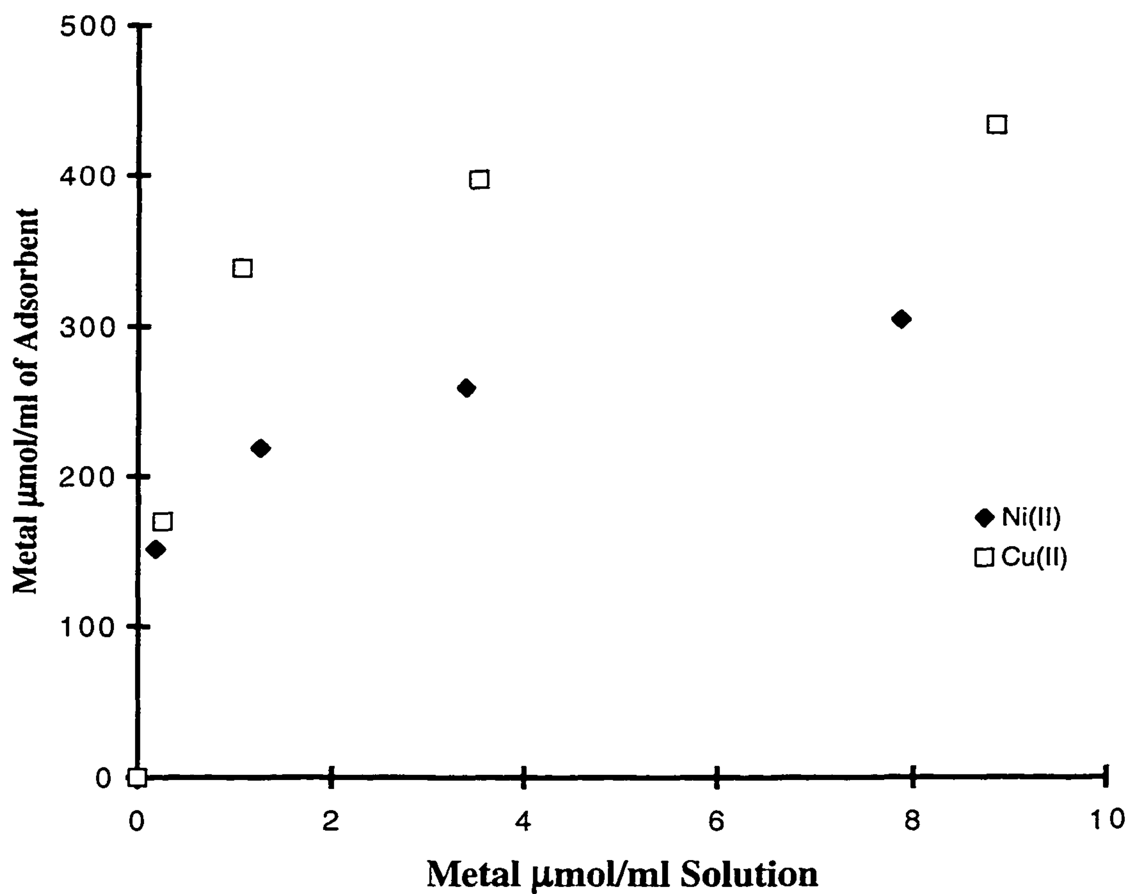


Figure 8.10 Adsorption isotherm for Cu(II) and Ni(II) at pH 5.0 in buffer NaAc 0.1 M on Novarose Act. High 100/40 modified with PEI-AF 25,000. The adsorbent was degraded with H_2SO_4 to eliminate the agarose component. The isotherm was determined after 24 hr in a batch mode using 0.2 g of gel and metal concentration in the range from 0 to 20 mM.

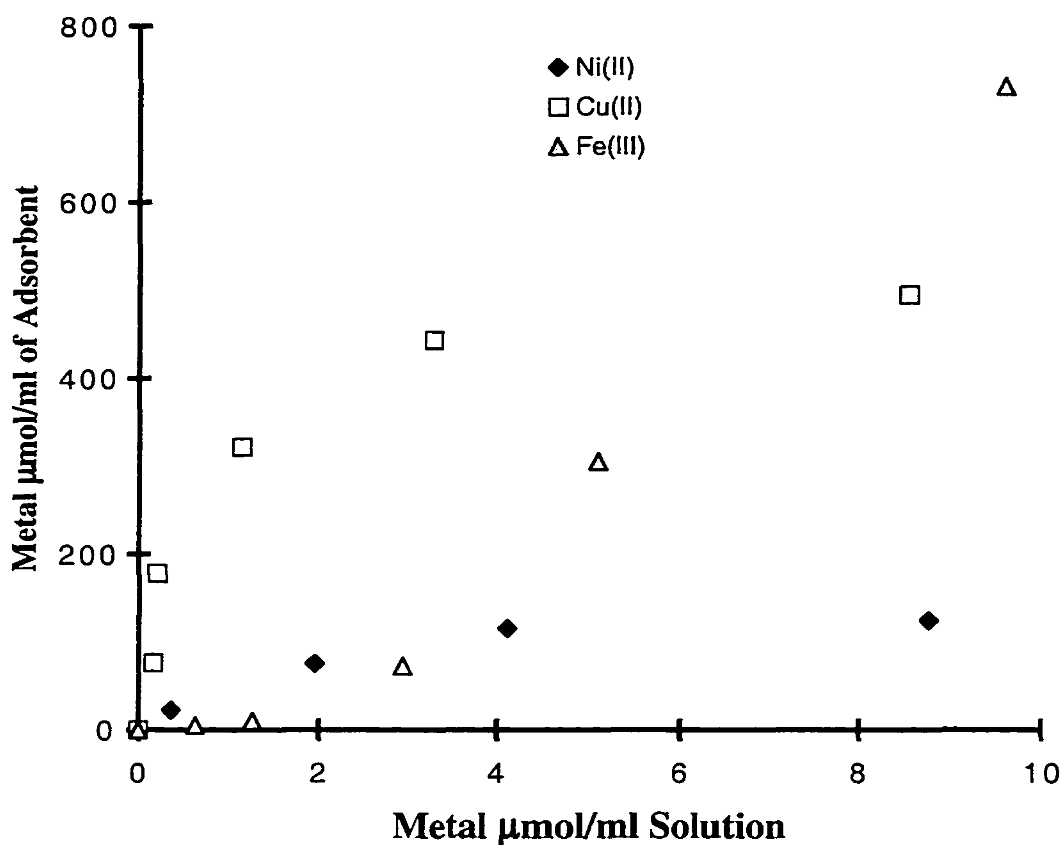


Figure 8.11 Adsorption isotherm for Cu(II), Ni(II) and Fe(III) at pH 5.0 in buffer NaAc 0.1 M. The adsorbent was Novarose Act. High 100/40 modified with a double layer of polyethyleneimine 25,000. The isotherm was determined after 24 hr in a batch mode using 0.2 g of gel and metal concentration in the range from 0 to 20 mM.

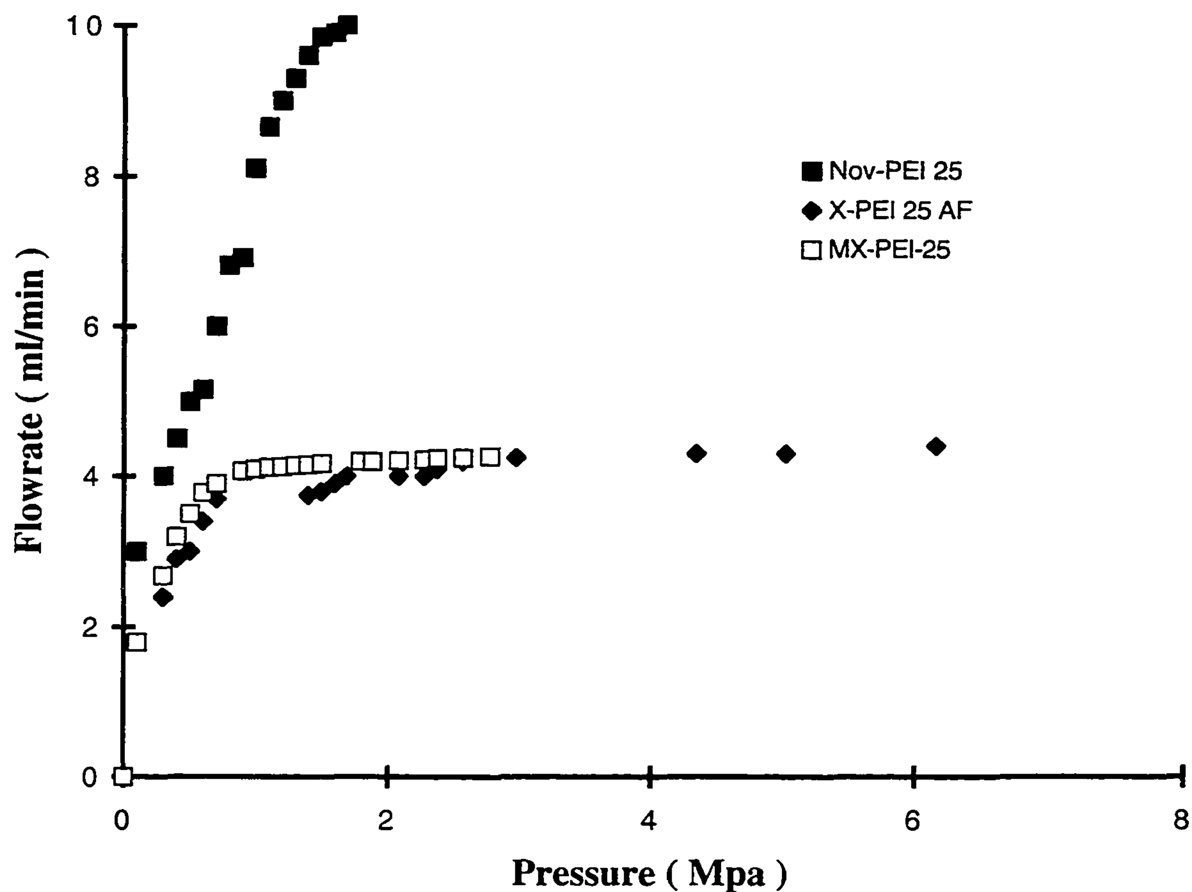


Figure 8.12 Pressure-flowrate studies on Novarose Act High PEI 25,000 adsorbents. Flow rate increased more gradually in the PEI-agarose beds and reached higher values than with the degradation products PEI-AF and MX-PEI. In the latter beds a steep rise to the limiting value occurred so that at lower flowrates higher pressures were achieved than in the agarose bed.

Gel Novarose Act. ^{High}	*Primary Amino groups (μmol/ml gel)*
Nov 100/40 PEI 25	880
Nov 100/40 PEI 25 - 25	601
Nov 10/40 PEI 750	580
Nov 100/40 PEI 750	985
Nov 10000/40 PEI 750	480
Nov 100/40 PEI 750 - 25	594

Table 8.1 Novarose Act. ^{High} primary amino content characterization. Primary amino group content of the prepared gel adsorbents was measured using a modified solid phase TNBS method described by Antoni *et al.*, (1983). High molecular weight PEI yielded more primary amino groups on the gel. However, when a second layer of PEI was added, crosslinking/bridging occurred and primary amines decreased.

Gel	*Cu^{2+} Capacity (μmol/ml gel)*
Novarose Act ^{High} 100/40 PEI 25	500
Novarose Act ^{High} 100/40 PEI 25-25	200
Novarose Act ^{High} 100/40 PEI 750-25	150
Novarose Act ^{High} 100/40 PEI750-25 AF	130
Novarose Act ^{High} 100/40 CMPEI 750	1200
Novarose Act ^{High} 10/40 PEI 750	550
Novarose Act ^{High} 100/40 PEI 750	1000
Novarose Act ^{High} 1000/40 PEI 750	1000
Novarose Act ^{High} 10000/40 PEI 750	400

Table 8.2 Novarose Act. ^{High} dynamic copper capacity measurements by frontal analysis. The column volume was 0.55 ml and the flowrate 1 ml/min. The dynamic binding capacity is best determined by frontal analysis, however this value is influenced by several factors such as flow rate, molecular size, pore size and polymer concentration. Permeability and diffusion rates decreased when a second PEI layer was added to the agarose matrix (Novarose-PEI_2) as observed by a drop in dynamic copper capacity . Diffusion limitations can be decreased when the agarose component is degraded (PEI_2-AF).

ADSORBENT	*CU(II)* *μmol/ml*	*Ni(II)* *μmol/ml*	*Fe(III)* *μmol/ml*
Novarose Act ^{High} PEI 750	1200	160	50
Novarose Act ^{High} X-PEI 750	500	N/A	N/A
Novarose Act ^{High} CM PEI 750	650	750	Asymptotic
Novarose Act ^{High} PEI 25	500	350	N/A
Novarose Act ^{High} PEI 25 FA	450	300	N/A
Novarose Act ^{High} 100/40 PEI 25-25	500	120	Asymptotic

Table 8.3 Metal capacity calculated for different Novarose Act^{High} 100/40 PEI adsorbents in batch after 24 hr. The degree of cross-linking and the concentration of the ethyleneamine units strongly affect the strength, capacity and selectivity in adsorption of transition metal ions. At the upper extreme of the reachable PEI-density some portions of the metal ions are irreversibly adsorbed and can not be eluted even at high concentrations of strong acids. The exceptionally strong affinity and high capacities may be caused by metal capture within intercatenarian polymacrocyclic structures in the cross-linked PEI network especially for Fe(III).

CHAPTER 9

CATALYTIC HYDROGENATION OF BIFUNCTIONAL POLY(ETHYLENE)GLYCOL DERIVATIVES USING PALLADIUM-POLY(ETHYLENEIMINE) CATALYST.

9.1 ABSTRACT

Palladium-Poly(ethylenimine) (Pd-PEI) supported in silica gel provides high catalytic activity and stability in the hydrogenolysis of the protective carbobenzoxy group (Z). This palladium catalyst has been used here to cleavage the Z protective group from a relatively large heterobiprotected soluble poly(ethylene)glycol derivative, Boc-PEG-Z, and to obtain a corresponding monoprotected compound, Boc-PEG-NH_2. Here Boc is the t-butyloxycarbonyl protective group. The monoprotected poly(ethylene)glycol derivative has been used for the preparation of heterobifunctional molecules with structure X-PEG-Y, where X and Y are two different functional moieties. Under the specific reaction and extraction conditions of the product, the hydrogenolysis does not affect the Boc group when removing the Z moiety. In the present work the rate of the hydrogenation of Pd-PEI catalyst is compared with that of palladium black, one of the most common used palladium catalysts used in this type of hydrogenation.

9.2 INTRODUCTION

Synthesis of heterobifunctional derivatives (X-PEG-Y) and especially the purification of these products are somewhat difficult to accomplish. In fact, despite its relevance in biotechnology, only a few approaches have been reported in the literature (Ehteshami, 1996; Zalipsky and Barany, 1986; Zalipsky and Barany, 1990). The key and limiting step in the development of bifunctional derivatives is the ability to synthesize and purify monoprotected functionalized polymers. Different strategies are being studied in our laboratory in order to improve the methods to produce several bifunctional derivatives. One of these strategies includes the cleavage of the Z protecting group from Boc-PEG-Z derivatives by catalytic transfer hydrogenation with Palladium black as a catalyst (Ehteshami, 1996). Once the Z group is removed a functional amino group of the form Boc-PEG-NH_2 is obtained. However this approach provides a slow rate of the amino group deblocking and low yields. In this report, palladium-poly(ethylenimine) has been tested as an alternative catalyst. Poly(ethylenimine) has good mechanical and chemical stability and has been used as a support for metals (Coleman and Royer, 1980; ElAmin *et al.*, 1979). Palladium-poly(ethylenimine) exhibits high catalytic activity and stability in the hydrogenolysis of the carbobenzoxy group (Z) and has been shown to be useful in the removal of this protective group from molecules such as small peptides and amino acids (ElAmin et al., 1979). This catalyst can be easily regenerated for further use with no loss of catalytic activity. In this work, a continuous recycled loop system through a Pd-PEI catalytic column appears to improve dramatically the rate of hydrogenation with high yields of the desired soluble monoprotected poly(ethylene)glycol derivatives.

9.3 MATERIALS AND METHODS

Materials

Boc-PEG-Z and Boc-PEG-Boc (PEG's derivatives MW 1900) were prepared and characterized in our laboratory according to our own protocols (Ehteshami, 1996). Palladium-poly(ethylenimine) (Pd-PEI) beads (20-40 mesh), palladium black, Chloroform, methyl alcohol, and formic acid, all reagent grade, were obtained from Aldrich (Milwaukee, WI).

Methods

Catalytic Removal of the Benzyl protective Group Z

Palladium-poly(ethylenimine) in methanol was poured into a 2 ml chromatography column. 2 gr (~ 1 mmole) of the mixture, consisting of Boc-PEG-Boc, and Boc-PEG-Z was dissolved in a 4.4% formic acid-methanol solution at about 5 mg/ml. The solution was allowed to flow slowly through the column (2 ml/min) in a continuous recycled loop for a period of 4 hr under a nitrogen atmosphere. The rate of catalyzed hydrogenolysis of the Boc-PEG-Z and Boc-PEG-Boc mixture was monitored by measuring the rate of deblocking of the amino group in the resulting monoprotected derivative Boc-PEG-NH_2 using the 2,4,6-trinitrobenzenesulfonic acid method (TNBS test) (Habeeb, 1966). An additional bed volume of formic acid-methanol was passed through the column to ensure complete removal of sample from the column. The eluent was evaporated under reduced pressure. To prevent breakdown of the Boc-PEG-NH_2 and Boc-PEG-Boc, the mixture

once evaporated and crystallized, was diluted with water, partially evaporated at reduced pressure, and then extracted into ethyl acetate (Coleman and Royer, 1980). After evaporation of the solvent a white solid material was obtained. A similar experiment was performed with the same mixture on palladium black as a catalyst, to compare the efficiency of the two systems.

9.4 RESULTS AND DISCUSSIONS

The rate of catalytic cleavage of the carbobenzoxy group (Z) of the biprotected poly(ethylene)glycol derivative Boc-PEG-Z with Pd-PEI was more effective when compared with the rate observed with Pd-black. Figure 9.1 shows the results of both catalytic systems. The deblocking with Pd-PEI was almost complete (>95%) in 1 hr, while Pd-black provided less than 50% deblocking. As evidenced by IR analysis, in all the experiments with Pd-PEI and Pd-black the second protective group (Boc) was not removed from the derivatives. The Boc group was removed selectively with TFA in subsequent bifunctional preparations. The utility and relevance of the Pd-PEI catalytic hydrogenation in this work is their effectiveness in removing protective groups when they are coupled to relatively large linear polymers (e.g.,PEG's). These results compared favorably with literature reports where Pd-PEI has been used to remove the same type of protective group from small peptides and amino acids (ElAmin et al., 1979). The great activity of Pd-PEI catalyst compared to Pd-black could be possibly due to the distribution of Pd on the large surface area available with PEI. The high rate has been also explained in terms of the possible attraction of the formic acid to the PEI matrix (Coleman and Royer, 1980).

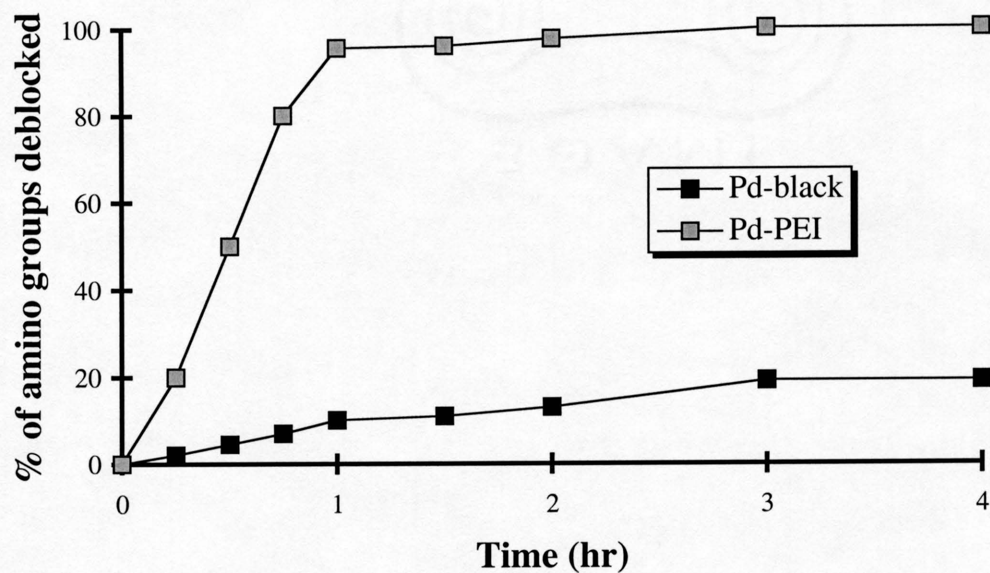


Figure 9.1 Rate of hydrogenation of the biprotected poly(ethylene)glycol derivative Boc-PEG-Z. The % of deblocked amino groups was measured during the experiment by taking samples of the solution from the continuous loop through the column at time intervals of 25 min.

CHAPTER 10

METAL AFFINITY PROTEIN PARTITIONING

10.1 INTRODUCTION

The partitioning in aqueous two-phase systems (ATPS) has become an established and well-known method for the separation and purification of biological materials (Albertsson, 1986; Walter *et al.*, 1985; Diamond and Hsu, 1992). In order to increase the selectivity and effectiveness of the separation, a ligand which partitions favorably into one of the phases and with affinity for the material of interest is introduced into the system. Thus, once binding occurs, the desired material preferentially distributes into the polymer-ligand rich phase. In general, the affinity ligand is attached covalently to one of the phase forming polymers, which ensures its primary distribution to one phase of the system. In affinity partitioning, ligands attached to poly(ethylene glycol) (PEG) have been widely described and used extensively in the separation and purification of proteins and cells, derivatives include PEG-linked dyes (Koppershlager *et al.*, 1981), PEG-linked antibodies (Sharp *et al.*, 1986) and PEG-linked long chained fatty acids (Shanbhag and Johansson, 1979). Similar derivatives for dextran (Dx) and procedures to prepare new affinity ligands are described by Harris (1985) and Harris and Yalpani (1985).

Metal affinity partitioning (MAP), in a similar fashion, has been developed as an alternative approach to increase the selectivity of separation by incorporating chelated transition metal ions covalently bound to PEG as affinity ligands (Wuenschell *et al.*, 1990; Chung *et al.*, 1991; *Birkenmeier et al.*, 1991). In this method the affinity interaction

depends on the chelated metal ions with particular accessible amino acids (metal-coordinating ligands) on the protein surface.

This partitioning method evolved from the concept of immobilized metal ion affinity chromatography (IMAC), a technique first introduced by Porath *et al.*, (1975) who used immobilized metal ions chelated to insoluble chromatographic matrices to specifically fractionate serum proteins. Resembling the IMAC separation scheme, metal ion chelators have been attached to water soluble polymers such as PEG and used as specific ligands for separation of proteins (Wuenschell *et al.*, 1990; Chung *et al.*, 1991; *Birkenmeier et al.*, 1991) and cells (Goubran-Botros *et al.*, 1991; Walter and Widen, 1993; Laboureau *et al.*, 1996). Typical two-phase systems for protein separations include aqueous solutions of PEG and dextran or PEG and a salt such as sodium sulfate or sodium carbonate. When a metal-chelated-PEG is added to the system containing the protein mixture, proteins with affinity for the chelated metal interact and partition preferentially in the PEG-rich phase. In these systems, the partition coefficients (K) of proteins which contain surface accessible amino acids, particularly histidine residues, can significantly increase with the addition of a relatively small amount (1-2%) of the metal-chelated polymer derivative.

Some of the chelating-PEG derivatives used in protein partitioning include iminodiacetic acid (IDA)-PEG, the L- and D-isomers of aspartic acid (Asp)-PEG, the L- and D-isomers of methionine (Met)-PEG (Wuenschell *et al.*, 1991), as well as derivatives of tris-carboxymethylated ethylene diamine (TED), and carboxymethylated (tris(2-aminoethyl)amine) (Cm-TREN) (Aguinaga-Diaz *et al.*, 1996). The binding properties of these chelating-PEG ligands are based on the chelating effect that some of their individual atoms display by acting as electron donors. Oxygen and nitrogen atoms in these molecules form coordination bonds with the metal ion in solution producing a metal chelate. Chelates are much more stable than a metal complex due to the loss in free energy when a ring is

formed. The IDA ligand is tridentate and can form a double five-membered ring chelate with hexacoordinate metal ions, while TED (tris-carboxymethylated ethylene diamine) is pentadentate and capable of forming four five-membered rings. These ligands work better with transition metal ions, which have several possible coordination sites and act as electron acceptors in the presence of this type of derivatives.

The most commonly used chelating-PEG derivative by far has been IDA-PEG complexed with first row transition metal ions such as Cu(II), Co(II), Zn(II) and Ni(II), particularly with copper. Other metal ions for example, Fe(III) which has affinity for phosphoryl groups, has been studied in the partitioning of phosphorylated proteins in PEG-Dx two-phase systems (Chung *et al.*, 1991). A significant observation in MAP is the fact that, unlike many affinity interactions which are disrupted in high concentrations of salts, metal ion coordination in aqueous solutions is promoted by phase-forming salts. As a consequence, the partitioning of proteins with Cu(II)IDA-PEG appears to be more effective in PEG-salt compared with PEG-Dx systems (Suh *et al.*, 1990; Otto and *Birkenmeier*, 1993). Some of the most typical PEG-salt systems are prepared using sodium, magnesium and ammonium sulfate, potassium phosphate and sodium carbonate salts.

Metal affinity protein partitioning has become a very useful and versatile method for protein fractionation and characterization. However, certain requirements have to be considered in order to take full advantage of this affinity technique. First, an appropriate chemistry must be developed for the synthesis of soluble chelating-polymers. Second, one has to consider the relevance of the protein surface topography on their recognition by metal chelates. It is now well established that accessible histidine residues serve as predominant metal binding sites. Even in the case of a protein from different species (e.g., isoenzymes) binding to the chelating-derivatives is a function of their primary structure with respect to the content and distribution of histidine residues (Otto and Birkemeier,

1993). In addition, the microenvironment of the electron donors on the surface protein may result in a variable mechanism of protein recognition by the metal ligand (Porath *et al.*, 1975). By choosing an appropriate chelating-polymer and metal ion in a given aqueous two-phase system, a high partition coefficient (K) for the protein of interest or degree of purification can be achieved. The methodology described here is focused on the metal affinity protein partitioning in PEG-salt systems.

10.2 MATERIALS AND METHODS

Materials

1. Poly (ethylene glycol) 8000 (OH-PEG-OH), PEG for synthesis of chelating derivatives (see Note 1) and methoxy poly (ethylene glycol) 5000 (M-PEG-OH) were obtained from Sigma (St. Louis, MO).

2. Anhydrous sodium sulfate, sodium and potassium phosphate monobasic and dibasic and sodium acetate were purchase from J.T. Baker Chemicals.

3. Buffer solutions. 0.1 M sodium phosphate buffer, pH 8.0. Sodium acetate 50 mM buffer, pH 4.0

4. Proteins. Model proteins used to characterize two-phase partitioning systems with effective chelating-polymers can be purchased from Sigma. Typically to prepare standard proteins solutions, dissolve 1 mg of protein in 1 g of 0.1M sodium phosphate buffer at various pH values. Examples of proteins used in affinity metal partitioning are listed in Table 10.1.

5. Reagents for synthesis of chelating derivatives (IDA-PEG). All reagents should be of analytical grade. Thionyl chloride, bromoacetic acid, ammonium hydroxide, ethylene diamine, iminodiacetic acid, absolute ethanol (Aldrich, Milwaukee; WI). Prepare ammonia solutions of 25%.

6. Metal ions. Sulfate salts of Cu(II), Ni(II), Co(II) and Zn(II) and Fe(III) from $FeCl_3$ (Sigma, St. Louis; MO).

Methods

Preparation of Chelating-PEG Derivatives, IDA-PEG and TED-PEG

1. Preparation of poly (ethylene glycol) chloride (PEG-Cl). Melt 60 g of methoxy (M)-PEG-OH 5000 (see Note 1) at 65 °C. The residual water contained in the MPEG can be removed under vacuum. Add 3 ml of distilled thionyl chloride and rotate the sample for 6 h at 65 ° C under a nitrogen atmosphere to exclude moisture (see Note 2). After removing the excess of thionyl chloride by evaporation using a rotary evaporator, dissolve the residue in 3 l of absolute ethanol and bring the mixture at 4 ° C in a refrigerator. Under these conditions the PEG-Cl is much less soluble in ethanol and will simply precipitate (crystallize) out of the solution. After filtration to separate the solid (PEG-Cl) from the liquid, place the PEG-Cl to dry in an oven at low temperature. The yield is usually high, from 90 to 95%.
2. Preparation of PEG-amino derivative. Dissolve 50 g of dried PEG-chloride in 150 ml of water. Add 150 ml of ammonia solution (25%). Place the solution in a sealed plastic tube and leave it reacting for 100 h at 55°C in an oven. After evaporation of the solvent and residual ammonia using a rotary evaporator (usually the temperature needed to remove the solvent and reactants is 40-55 °C) the amino-PEG derivative is obtained in high yield.
3. Carboxymethylation, preparation of derivative PEG-IDA (see Note 3). Dissolve 15 g of the amino-PEG in 100 ml of water. Carboxymethylation is carried out by adding 15 g of bromoacetic acid and adjusting the pH to 8.5 (see Note 4). Allow the reaction proceed for 12 h at room temperature. Then, add 100 ml of water and extract the PEG-IDA three times with 300 ml of chloroform. The chloroform in the PEG-rich

chloroform phase can be removed by distillation or evaporation using a rotary evaporator (usually the temperature needed to remove it completely is 30-40 °C). The characterization of IDA-PEG is usually done determining the nitrogen content by elemental analysis. The yield is usually found from 75 to 85%.

4. Preparation of tris-carboxymethylated ethylene diamine-poly(ethylene glycol) PEG-TED. The synthesis of the derivative M-PEG-TED or PEG-TED is practically similar to the above procedure, the compound M-PEG-Cl is reacted with ethylene diamine (NH_2-CH_2-CH_2-NH_2) instead of ammonium hydroxide for amination. The group PEG-NH-CH_2-CH_2-NH_2 is similarly carboxylated with bromoacetic acid to give the final compound PEG-TED (see Note 5)
5. Preparation of metal-chelator-PEG. Metal loading of the ligand is performed by dissolving 6 g of the ligand in 30 ml of 50 mM sodium acetate buffer pH 4.0 containing the desired metal ion as sulfate salt in 15 to 20 molar excess over the ligand (see Note 4). Stir the solution for 1 h and extract the metal chelator five times with chloroform. Remove the chloroform by evaporation. The metal content in the aqueous solution (metal not bound to chelator) is usually determined by atomic absorption using appropriate standards. Figure 10.1 presents the general scheme of the reaction steps to prepare Cu(II)-IDA-PEG(5000) (see Note 3).

Preparation of Affinity Two-Phase Systems

1. Preparation of PEG-salt two-phase systems. The two-phase systems are prepared on a weight basis from stock solutions of PEG 8000 (OH-PEG-OH) (40%, w/w), and sodium sulfate (16%, w/w). Two-phase systems (4.0 g total weight) are made by

combining 1.4 g of the PEG stock solution, 2.0 g of the salt solution, and 0.6 g of a 0.5 mg/ml mixture of protein solution in the desired buffer. For affinity partitioning using PEG-chelate-metal derivatives, replace a portion of the PEG 8000 with the metal chelator as needed (e.g., usually 1-3% w/w of total PEG is replaced for example, by the chelator Cu(II)-IDA-PEG(5000)). The final compositions of the polymer and the salt in this case are 14% PEG and 8% sodium sulfate (see Notes 6, 7).

2. PEG-Dx systems. A two-phase systems can be prepared on a weight basis from stock solutions of PEG 5000 or PEG 8000. A two-phase system (4.0 g total weight) consisting of PEG 5000 (40%, w/w), and Dx T500 (13.5%, w/w) is made by combining 0.70 g of the PEG stock solution, 1.30 g of the Dx solution, and 2.0 g of 1 mg/ml protein solution in the desired buffer. For the metal containing systems, replace a portion of the PEG 5000 with the metal chelator (e.g., Cu(II)-IDA-PEG(5000)) as needed, as in the PEG-salt case. The final compositions of both polymers in this case are 7.0% PEG and 4.4% Dx (see Notes 6, 7).

3. Once the systems have been prepared, invert them 50 times at room temperature and allow them to reach equilibrium for 15-30 minutes (see Note 8). Use a syringe with a large thin needle (in order to avoid contamination as much as possible) to withdraw samples from top and bottom phases, mixed them with a known volume of distilled water and analyze them for protein content (see Note 9) .

Determination of Protein Partition Coefficients

1. All protein partitioning experiments in these systems are usually carried out at room temperature. The partition coefficient (K_o) for each protein is calculated as the ratio of the concentration in the top and bottom phase (see Note 9). If measured by light scattering (at 409 or 280 nm), K_o is defined as the ratio of the absorbance of the protein in the top phase to the absorbance of the protein in the bottom phase. The effect of the metal-chelator-PEG in the partition of any protein is expressed as Δlog K which is given by $\Delta \log K = [\ln K_{aff} - \ln K_o]$, where K_{aff} and K_o are the partition coefficient of the species in the presence and absence of the metal-chelator PEG in the system respectively (see Note 10).

Recovery of Proteins Under Study

1. After the partition experiment, the phase containing the complex protein-metal-chelate-PEG (top phase) is separated from the bottom phase. The protein of interest is unbound from the complex by changes in pH or by adding competing ligands or chemical agents (such as EDTA) to dissociate the protein metal chelate complex (see Note 11). Once dissociation occurs, to the polymer phase containing the desired protein add high concentrations of salt, enough to form a new two-phase system with the residual PEG phase (e.g., to form a system consisting of PEG (40%, w/w), and salt (16%, w/w). The salt could be the same salt used in the initial two-phase formation (sodium sulfate) or another one (e.g., potassium phosphate), always maintaining a low pH (less than 5). Under these conditions, due to the absence of affinity binding groups for the protein

in the system and to the high ionic strength, the protein of interest will partition preferentially to the salt rich phase.

10.3 NOTES

1. Monometoxy poly(ethylene glycol) 5000 is one of the few PEG's of this type available commercially. To avoid protein precipitation during extraction, it is necessary to use metal affinity ligands that are essentially monofunctional (M-PEG-OH) or by derivatizing bifunctional PEG (OH-PEG-OH) with a very small amount of IDA.
2. Thionyl chloride and chloroform are highly toxic chemicals. It is necessary to handle these compounds with care under a hood.
3. Alternatively, PEG-IDA can be synthesized by reacting 5 g of the derivative PEG-Cl with 2.1 g of iminodiacetic acid in 50 ml of water adding 2 g of potassium carbonate. The solution is refluxed for 48 hr. Then 5 to 8 g of sodium sulfate are added to the hot reaction mixture, which after cooled down separates into two phases. The PEG phase (top phase) is retained and diluted to 50 to 100 ml. The PEG solution is dialyzed against 1% sodium bicarbonate, and finally extensively against water for 48 to 60 hr. After lyophilization the final product PEG-IDA is obtained (Wuenschell *et al.*, 1990).
4. Check that carboxymethylation and metal loading reactions are carried out at the proper pH at all times so as to maintain the reactivity of the reactants.
5. Figure 10.2 below shows schematically the structure of several chelating-PEG ligands used in affinity partitioning of proteins.
6. Affinity chelating ligands that are required in small final concentrations can be prepared at high enough concentrated stocks, and added in small aliquots (1-3% of the total PEG phase volume) to the complete phase system, with little effect on the phase

composition. In this way a series of systems with constant polymer concentration and different ligand concentrations can be easily prepared. On the other hand, if large quantities of the chelating-PEG ligand are needed, the derivative is substituted on a weight basis for PEG during the preparation of the specific phase system (Johansson *et al.*, 1983).

7. The effectiveness of partitioning in aqueous phase systems as a separation technique lies in its versatility and its sensitivity to a wide range of features of the partitioned material. Therefore, appropriate phase systems must be chosen. The selection of a useful phase system involves some experimentation. It is important to observe all the operating conditions, during the formation of the two-phase systems. Detailed description of phase diagrams with different polymer-polymer and polymer-salts systems are given in the literature (Zaslavsky, 1995). In metal affinity separations the change in protein partitioning coefficients due to the chelating ligand increases with increasing polymer-chelate concentration to a saturation value. Some of the most relevant parameters to consider include pH, temperature, polymer base molecular weight and concentration, etc. In PEG-salt systems it has been observed particularly that the higher the molecular weight of the polymer, the lower its concentration required for phase separation. The procedures described here for the formation of PEG-Salt and PEG-Dx two-phase systems correspond to commonly used two-phase systems.
8. To speed up phase separation, the systems can be centrifuged at 1200-1600 x g during 5-15 minutes to ensure that phase equilibrium has been reached.
9. Several analytical techniques for protein analysis can be applied to quantify the protein content in each phase. Protein concentrations should be calculated whenever possible, spectrophotometrically. Alternatively protein concentrations can be determined by the biuret method, which is unaffected by the presence of PEG. When using other

analytical methods because the polymers may affect certain chemical assays it is convenient to measure the effect of the phases and ligands on the intended assay. In other cases K will be calculated as the ratio of the concentration or radioactivity or any biological activity, e.g., enzyme activity of immuno-assay (ELISA) of a species in the upper phase and the lower phase.

10. Partitioning of histidine rich proteins with PEG-IDA-Cu(II) can be greatly enhanced in PEG-salt compared to PEG-Dx systems. In order to assess the effect of the chelating-PEG (say IDA-PEG (5000)) in the partitioning of the selected proteins, different amounts (e.g., 1%, 2%, etc.) of the polymer forming phase (say PEG (8000)) is substituted with the chelating-polymer. Control partition experiments are carried out using simply the unmodified polymer PEG (5000) instead of the chelating-polymer. Comparison of partitioning results for several model proteins are shown in Table 10.2.

11. At pH values above 5, the binding of proteins to Cu(II)-IDA-PEG is strengthened in the presence of salts. At lower pH values salts seem to suppress the binding. Interactions between the chelate metal ion and histidine-containing proteins can be reversed by Lewis acids (H^+), which compete for binding to the protein, alternatively the protein can be displaced by a Lewis base (imidazole, N-acetylhistidine), which competes for coordination to the chelated metal ion. In other cases a strong chelating agent such as EDTA is used to break up the ternary protein-metal ion-IDA complex.

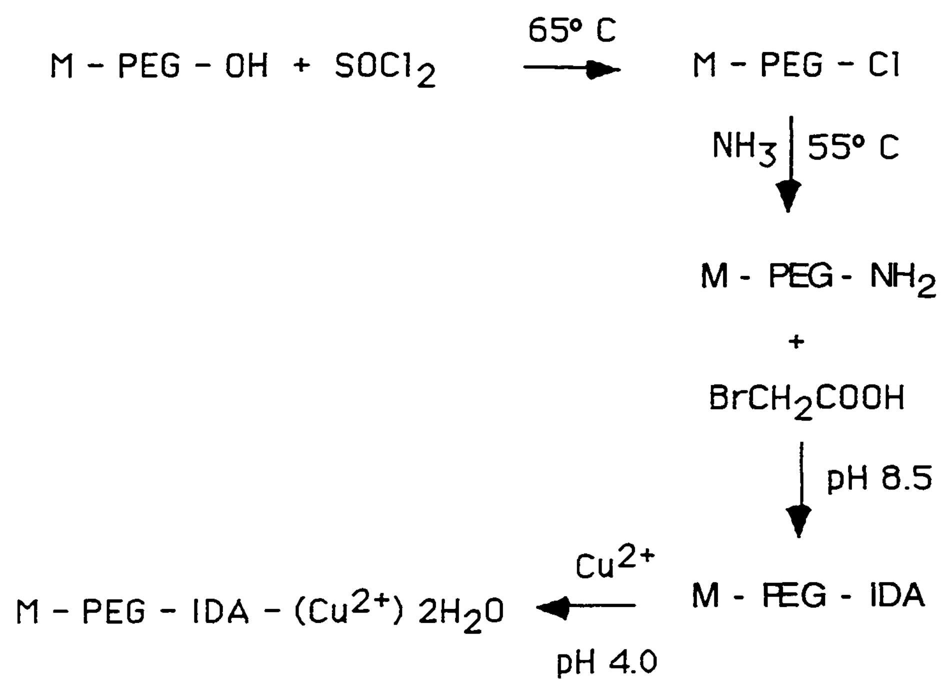
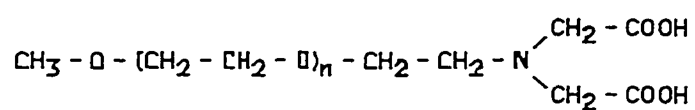
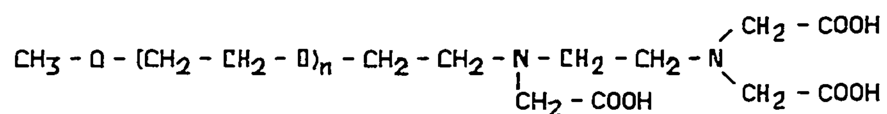


Figure 10.1 Synthesis of chelated monomethoxy poly (ethylene) glycol metallized with copper.

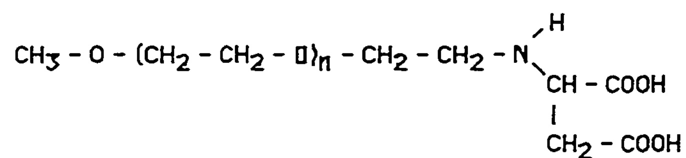
a) PEG -IDA



b) PEG -TED



c) PEG - (L-Asp)



d) PEG - (Cm-TREN)

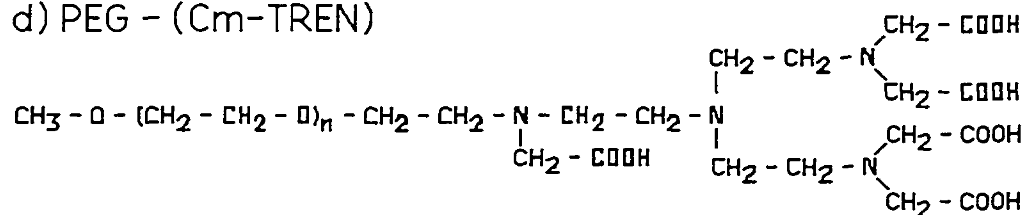


Figure 10.2 Common chelators used in aqueous two-phase systems.

Protein	**Surface histidines**
Human hemoglobin	24
Bovine hemoglobin	20
Whale myoglobin	5
Horse myoglobin	4

Table 10.1 Suitable proteins for characterization of metal affinity partitioning.

Protein	PEG-Salt ln (K/Ko)	PEG-Dextran ln (K/Ko)	Surface Histidines
Horse myoglobin	5.29	0.6	4
Whale myoglobin	7.29	0.7	5
Bovine hemoglobin	7.92	3.3	20
Human hemoglobin	7.67	3.6	24

Table 10.2 Metal Affinity Partitioning of Proteins Using PEG-IDA-Cu(II) in PEG-Salt and PEG-Dextran Aqueous Two-Phase Systems. In the absence (K_o) and presence (K) of PEG-IDA-Cu(II) derivatives. Composition of PEG-salt two-phase systems: 8.0% sodium sulfate, 14% PEG (8000) (with and without substitution of 1.4% PEG(5000)-IDA-Cu(II)), and 10 mM sodium phosphate, pH 7.8 (17). Composition of PEG-dextran two-phase systems: 4.4% dextran T500, 7% PEG 8000 [with and without substitution of 1% PEG(8000)-IDA-Cu(II)], 0.1 M NaCl, and 10 mM sodium phosphate, pH 8.0 (Wuenschell *et al.*, 1990).

CHAPTER 11

SUMMARY AND CONCLUSIONS

Several chelating polymeric derivatives have been synthesized and characterized. Among the soluble materials studied are linear, mono and heterobifunctional chelating poly (ethylene) glycols, multi-chelating poly(ethylene) glycol, and hyperbranched poly(ethyleneimine). Also, different poly (ethyleneimine) solid supports were prepared.

The feasibility and applicability of the chelating derivatives as tools for separation of proteins and heavy metal ions using several affinity purification techniques have been tested. Although some of these separation methods are still in development, in general the results are very encouraging for biotechnical and environmental applications.

The following are general conclusions that can be drawn from the research work presented in this dissertation.

1. New and improved synthetic strategies for soluble and insoluble chelating polymers has been achieved.
2. The synthesized chelating polymeric materials can be applied in the pharmaceutical industry as drug delivery tools. Also these materials can be used as chemical modifiers in biotechnology to alter the physicochemical properties and to obtain information on structural features of proteins.
3. Chelating polymers are also useful in the environmental field for ultratrace heavy metal ion preconcentration and separation from metal containing waste.

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