DOKUZ EYLUL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PREPARATION AND CHARACTERIZATION OF CIBACRON BLUE F3GA ATTACHED POLY(ACRYLAMIDE-ALLYLGLYCIDYL ETHER) CRYOGELS FOR ALBUMIN PURIFICATION FROM HUMAN PLASMA

by
Nazan DEMİRYAS

June, 2006
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Nazan Demiryas

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We have read the thesis entitled **"PREPARATION AND CHARACTERIZATION OF CIBACRON BLUE F3GA ATTACHED POLY(ACRYLAMIDE-ALLYLGLYCIDYL ETHER) CRYOGELS FOR ALBUMIN PURIFICATION FROM HUMAN PLASMA"** completed by **NAZAN DEMİRYAS** under supervision of **ASSIST. PROF. DR. NALAN TÜZMEN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

Poly(acrylamide-allyl glycidyl ether) [poly(AAm-AGE)] cryogel was prepared by bulk polymerization which proceeds in aqueous solution of monomers frozen inside a glass column (cryo-polymerization). After thawing, the monolithic cryogel contains a continuous polymeric matrix having interconnected pores of 10-100 μm size. Cibacron Blue F3GA was immobilized by covalent binding onto poly(AAm-AGE) cryogel via epoxy groups. Poly(AAm-AGE) cryogel was characterized by swelling studies, FTIR, scanning electron microscopy and elemental analysis. The equilibrium swelling degree of the poly(AAm-AGE) monolithic cryogel was 6.84 g H_2O/g cryogel. Poly(AAm-AGE) cryogel containing 68.9 μmol Cibacron Blue F3GA/g were used in the adsorption/desorption of human serum albumin (HSA) from aqueous solutions and human plasma. The non-specific adsorption of HSA was very low (0.2 mg/g). The maximum amount of HSA adsorption from aqueous solution in phosphate buffer was 27 mg/g at pH 5.0. Higher HSA adsorption value was obtained from human plasma (up to 74.2 mg/g) with a purity of 92%. Desorption of HSA from Cibacron Blue F3GA attached poly(AAm-AGE) cryogel was obtained using 0.1 M Tris/HCl buffer containing 0.5 M NaCl. It was observed that HSA could be repeatedly adsorbed and desorbed with poly(AAm-AGE) cryogel without significant loss in the adsorption capacity.

Key words: Dye-affinity Chromatography, Cryogel, Protein Purification, Albumin

İNSAN PLAZMASINDAN ALBUMİN SAFLAŞTIRILMASI İÇİN CİBACRON BLUE F3GA TAKILI POLİ(AKRİLAMİD-ALLİL GLİSİDİL ETER) KRİYOJELLERİN HAZIRLANMASI VE KARAKTERİZASYONU

ÖZ

Poli(akrilamid-allil glisidil eter) [poli(AAm-AGE)] kriyojeli monomerlerin sulu çözeltilerinin cam bir kolon içerisinde dondurulmasıyla gerçekleştirilen yığın polimerizasyonu ile hazırlandı. Eridikden sonra, monolitik kriyojel, 10-100μm boyutunda gözeneklere sahip sürekli polimerik bir matriks oluşturdu. Cibacron Blue F3GA, epoksi grupları üzerinden poli(AAm-AGE) kriyojeline kovalent olarak immobilize edildi. Poli(AAm-AGE) kriyojeli, şişme testi, FTIR, taramalı elektron mikroskobu (SEM) ve elemental analiz ile karakterize edildi. Poli(AAm-AGE) monolitik kriyojelinin denge şişme oranı 6,84 g H_2O / g kriyojel olarak bulundu. 68,9 μmol Cibacron Blue F3GA/ g kriyojel içeren poli(AAm-AGE) kriyojeli, sulu çözelti ve insan plazmasından insan serum albumin (HSA) adsorpsiyon-desorpsiyon çalışmalarında kullanıldı. HSA' in spesifik olmayan adsorpsiyonu 0,2 mg/g olarak bulundu. Sulu çözeltiden maksimum HSA adsorpsiyon miktarı, pH 5'de 27 mg/g olarak bulundu. En yüksek HSA adsorpsiyonu, %92 saflıkla plazmadan sağlandı (74,2 mg/g). Cibacron Blue F3GA takılı poli(AAm-AGE) kriyojelinden HSA'nin desorpsiyonu 0,5 M NaCl içeren 0,1 M Tris/HCl tamponu kullanılarak gerçekleştirildi. Adsorpsiyon kapasitesinde herhangi bir azalma olmaksızın, poli(AAm-AGE) kriyojeli ile HSA'nin tekrarlanabilir bir şekilde adsorlanıp desorplandığı gözlendi.

Anahtar Kelimeler: Boya-Afinite kromatografisi, Kriyojel, Protein Saflaştırma, Albümin

CONTENTS

Page

CHAPTER ONE

INTRODUCTION

1.1 Polymer Monoliths

Monoliths, also called continuous stationary phases, are important tools for bioseparation, biotransformation, and synthesis. Although the first experiments were reported in the late 1960s and early 1970s, their commercial breakthrough could not be achieved until the last decade. These materials are characterized by low mass transfer resistance. Thus all applications involving large molecules exhibit in principle better performance compared to conventional beaded media. Monoliths are also used in the area of catalysis and combustion. These honeycomb-like structures do not possess many features typical of monoliths for bioseparation and biotransformation. According to the common definition, monoliths are structures which are cast in one piece. Monoliths for combustion and catalysis have paralel channels without interconnection and channel diameters up to several millimeters. The channels of monoliths used for bioseparation are highly interconnected and partially constricted with a size usually less than 5 μm. Examples of monoliths for bioseparation and biotransformation are polymethacrylate block polymers (CIM disksm, CIM tubesm, Swiftm columns) produced directly by free radical polymerization induced thermally (Svec, 1999; Josic, 2001a; Josic, 2001b) or by radiation (Grasselli, Smolko, Hargittai, & Safrany 2001), polymethacrylate based monoliths with templated pores (Chirica, 2001; Hahn, 2001), monoliths prepared from compressed polyacrylamide gels (Hjerten, 1988; Hjerten, 1992) silica columns manufactured as single blocks by a sol-gel process (Minakuchi, 1996; Ishizuka, 1998) silica xerogels (Fields, 1996), emulsion-derived polymeric foams such as polyHIPEm (Mercier, Deleuze, & Mondain-Monval, 2000), monoliths prepared via metathesis polymerization (Jungbauer & Hahn, 2004) continuous urea-formaldehyde resins (Sun, Chai, 2002) monoliths prepared from carbon microspheres (Liang, 2003; Yamamoto, 2002) graphitized carbon monoliths (Liang, Dai & Guiochon, 2003) monoliths cast from cellulose (Noel, Sandeson & Spark,

2002) and superporous agarose (Gustavson & Larsson 2001), cryogels from polyacrylamide (Arvidsson, 2002; Kumar, 2003) and poly(vinyl alcohol), polyacrylamide-coated ceramics (Jungbauer & Hahn, 2004), rolled woven fabrics (Yang, 1992; Hamaker, 1999), and adsorptive membranes of various types (Jungbauer & Hahn, 2004). As long as they are used as a single sheet, membranes can be considered as monoliths with an extreme geometry in which the dimension in the axial direction is very short. Membranes are prepared as very thin layers and therefore scale up is not possible while preserving this monolithic structure. However, they can be stacked to provide an additional volume and consequently also more capacity. Currently four companies are manufacturing monolithic columns for application in bioseparation. An overview of their products is given in Table 1.1.

Table 1.1 Overview pf commercially available monoliths forn applications in bioseparation and bioconversion.

Trade name	Manufacturer	Material	Channel diameter	Examples of formats
CIM	BIA Separations	Polymethacrylate	1500 nm	Disks: 3.0 × 12 mm ID Tubes: 45 × 15 mm ID 110 × 34 mm ID 150 × 105 mm ID
UNO	Bio-Rad	Polyacrylamide	1000 nm	Columns: 35 × 7 mm ID 53 × 12 mm ID 68 × 15 mm ID 58 mL, 196 mL, 2540 mL [98]
SWIFT	Isco	Polymethacrylate	1500 nm	Rods: 50 × 4.6 mm ID 100 × 10 mm ID
Ultimate	LC-Packings	Polystyene divenylben-zene	Not available	Columns: 50 × 0.1 mm ID 50 × 0.2 mm ID
Chromolith	Merck	Silica	Macropores: 2 μm Mesopores: 13 nm	Rods: 100 × 4.6 mm ID 100 × 25 mm ID

Monoliths are frequently used for capillary electrochromatography; the most recent applications have been reviewed (Siouffi, 2003). Most of the recent progress concerns characterization of monoliths regarding mass transfer and hydrodynamic properties, the application of cryogels for particle separation and immobilization of cells, affinity monoliths. (Jungbauer & Hahn, 2004).

1.2 Porous Monoliths

Porous monoliths are a new category of materials developed the last decade. These materials are prepared using a simple molding process carried out within the confines of a closed mold. Polymerization of a mixture that typically contains monomers, free-radical initiator, and porogenic solvent affords macroporous materials with large through-pores that enable flow-through applications. The versatility of the preparation technique is demonstrated by its use with hydrophobic, hydrophilic, ionizable, and zwitterionic monomers. The porous properties of the monolith can be controlled over a broad range. These, in turn, determine the hydrodynamic properties of the devices that contain the molded material is dominated very much by convection, and the monolithic devices perform well even at very high flow rates. The applications of monolithic materials are demonstrated on the chromatographic separation of biological compounds and synthetic polymers, electrochromatography, gas chromatography, enzyme immobilization, molecular recognition, and in advanced detection systems. Grifting of the pore walls with selected polymers leads to materials with completely changed surface chemistries (Lozinsky, et al., 2003).

1.3 Macroporous Polymers

Macroporous polymers emerged in the late 1950s as a result of the search for polymeric matrices suitable for the manufacture of ion-exchange resins with better osmotic shock resistance and faster kinetics. In contrast to the polymers that require solvent swelling to become porous, macroporous polymers are characterized by a permanent porous structure formed during their preparation that persists even in the dry state. Their internal structure consists of numerous interconnected cavities (pores) of different sizes, and their structure rigidity is secured through extensive crosslinking. To achieve the desired porosity, the polymerization mixture should contain both a crosslinking monomer and an inert agent, the porogen. Solvating or

non-solvating solvents for the polymer that is formed, but also other soluble non-crosslinked polymers, or even mixtures of such polymers and solvents can serve as porogens. Although the vast majority of current macroporous beads are based on styrene-divinylbenzene copolymers, other monomers including acrylates, methacrylatees, vinylpyridines, vinylpyrrolidone, and vinyl acetate have also been utilized. Macroporous polymers are finding numerous applications as both commodity and specialty materials. While the former category includes ion-exchangers and adsorbents, supports for solid phase synthesis, polymeric reagents, polymer-supported catalysts, and chromatographic packingss fit well into the latter (Lozinsky, et al., 2003).

1.4 Mass Transfer and Hydrodynamic Properties

Mass transfer properties and hydrodynamic properties of monoliths two most important terms for the physical characterization of monolithic materials are hydraulic permeability and dispersion. For hydraulic permeability an apparent particle diameter can be defined. This number is equivalent to the particle diameter of a column filled with particles that has the same bed porosity. Pressure vs.flow profiles are generated and approximated with the Konecny-Carman equation.

$$\frac{\Delta p}{L} = \frac{150\,\mu}{d_{equ}^2} \cdot \frac{(1-\varepsilon_b)^2}{\varepsilon_b^3}$$

where Δp is the pressure drop, L is the length of the column, μ is the dynamic viscosity, and ε_b is the porosity of the monolith relevant for the macroscopic flow. Silica monoliths with a bimodal pore size distribution have been characterized (Leinweber, Lubda, Cabrera, & Tallarek, 2002). These monoliths consist of macropores, where the macroscopic flow occurs, and intraskeleton mesopores reachable only by diffusional transport, and have several characteristic length scales. Leinweber defined a characteristic length for both hydraulic permeability and dispersion for the monolith and correspondingly an equivalent particle diameter. Since the equivalent particle diameter is rather small, long columns packed with these particles could not be operated due to a large back pressure. Mesopores in the

skeletons provide for a high surface area but the mass transfer in these pores is controlled by diffusion. This eliminates one of the intrinsic advantages of monoliths, the convection-controlled mass transfer. (Tallarek, & Leinweber, 2002) also defined a characteristic diffusional length for monoliths with intraskeleton mesopores. The concept of "augmented diffusivity" affected by convection in beads with large pores has been introduced (Rodrigues, Lopes, Lu, Loureiro, & Dias, 1992). Regarding dispersion, monoliths with intraskeleton mesopores behave similarly to these beds (Siouffi, 2003). In contrast, monoliths without mesopores exhibit completely different mass transfer characteristics. For example, the peak broadening is affected only by the hydrodynamic dispersion, the socalled A term in the van Deemter equation. Jungbauer and Hahn compared a typical bead and a monolithic column and found that the reduced height equivalent to a theoretical plate (HETP) does not change with reduced velocity for monoliths (Figure 1.1). Attempts have also been made to model both flow and mass transfer properties of monoliths in order to find the effect of the monolithic structure on them. The pores can be modeled as lattices with certain connectivity or as a pore network. The networks can be described as random, semi-ordered random but macroscopically uniform, random with spatial correlation, and heterogeneous. The connectivity and the optimal distance of the nodes and their orientation are not yet fully understood (Jungbauer & Hahn, 2004).

In an attempt to take advantage of the enhanced mass-transfer properties in stationary phases by combining both the micropellicular and the monolithic configuration, porogen mixtures that favor the formation of macro-pores and suppress the formation of micro-pores were used. 1-Decanol and tetrahydrofuran were utilized as macro- and meso-porogen, respectively, for the preparation of poly(styrene/divinylbenzene)-based monoliths, resulting in a stationary phase configuration that can be adequately described as a micropellicular monolith.

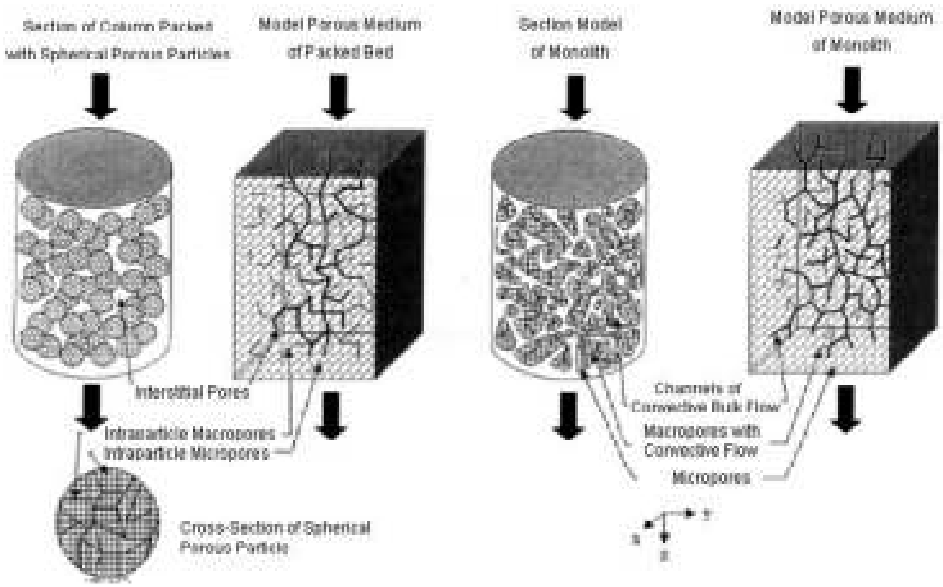

Figure 1.1 Schematic representation of network modeling of packed- and monolithic column.

Figure 1.1 reflects an ‘‘actual’’ model porous medium structure taking into account pore length and pore connectivity variability. For the realistic representation of the porous structure in the column, the pore size distribution of the network (model porous medium) should be similar to the pore size distributions encountered in the interstitial pores and in the pores of the particles. In a column being in operation in practice, the interstitial and intraparticle pores are correlated (Hollewand & Gladden, 1992), and thus, cannot be assigned to the network randomly. Therefore, in order to generate a realistic model porous medium (network) representation of the packed column, the interstitial pores are assigned in a semirandom manner to a percolating cluster which transects the lattice. The assignment of the intraparticle pores in the network is completely random. It should be noted here that there could exist certain porous chromatographic particles in which the macropores are not randomly distributed relative to the micropores (non-random bidisperse pore network); in such a case, our approach in constructing the intraparticle pore network is slightly modified by employing in the assignment of the intraparticle pores the procedure reported. In the case of a monolith (continuous bed), the interstitial pores in Figure 1.2 correspond to the large pores of the monolith while the intraparticle pores in Figure 1.2 correspond to the small and medium in size pores of the monolith. The details of the construction of the lattice network and the computational methods employed in this work are presented in the reports of Meyers (Meyers, 1998).

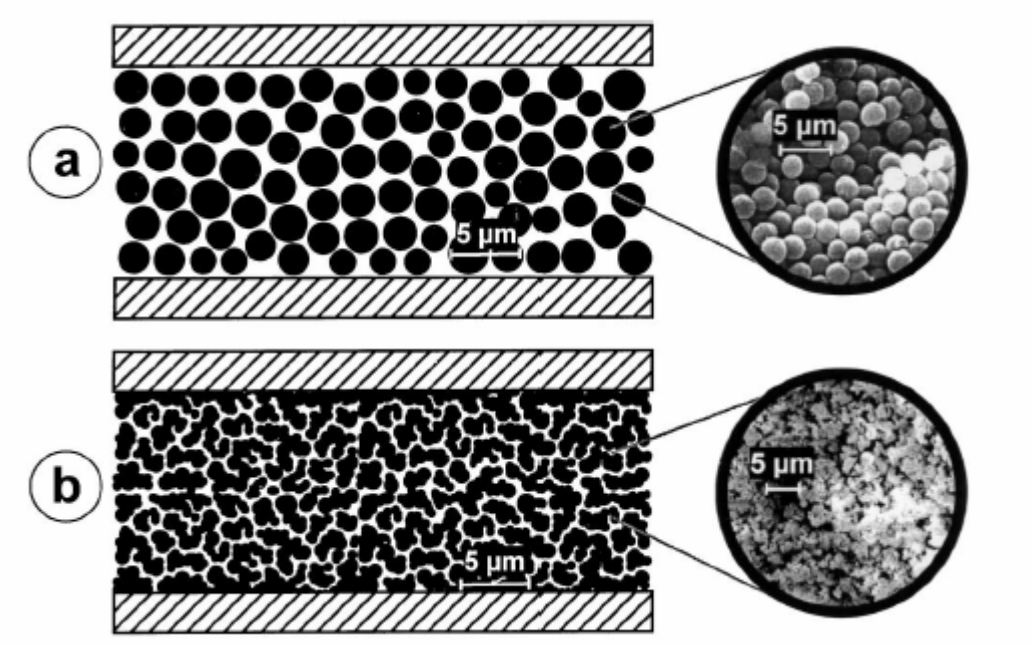

Figure 1.2 Illustration of the structural differences between packed- and monolithic column

Figure 1.2 illustrates the structural differences between a column packed with micropellicular particles (Figure 1.2 a) and one packed with a micropellicular monolith (Figure 1.2 b). In the conventional column, the chromatographic bed consists of tightly packed, micropellicular particles (Figure 1.2 a). Intra-particular mass-transfer resistances are confined to the very thin outer shell of the particles and, therefore, drastically reduced. The micropellicular, monolithic column bed is made of one single piece of a porous solid with relatively large channels for convective flow (Figure 1.2 b). Mathematical modeling has shown that increased pore connectivity, which represents the number of pores connected at a node of the porous network, plays a key role in improving the transport of the adsorbate molecules in the monolithic stationary phase configuration (Meyers, 1998). Moreover, mass transfer in a micropellicular monolith is further enhanced due to the absence of micro-pores. Comparing the electron micrographs in Figure 1.2 a and b, one can see that the number of channels penetrating the chromatographic bed is much higher in the monolith than in the column packed with micropellicular particles. In addition, the diameter of the channels is reduced in the monolithic structure, allowing a considerable decrease in the path-lengths required for mass transfer between the mobile and stationary phase.

1.5 Disadvantages and Limitations

Although the monolithic stationary phases possess a number of unique properties compared to traditional stationary phases, some disadvantages and limitations are inevitable. For the polymeric monolithic stationary phases, most of them are known to swell in organic solvents. This frequently leads to a lack of stability. Furthermore, the preparation of polymeric monoliths usually leads to micropores, which negatively affect the efficiency and peak symmetry of the column. Therefore, it is not easy to obtain high efficiency for small molecules. The low column capacity may be another significant disadvantage for their applications, which may be attributed to their low specific surface area compared to traditional packings. Although some attempts have been made to increase the specific surface area, most of the resulting polymeric monoliths in previous reports only showed relatively low specific surface areas (Xie, 1998; Svec, 1999; Peters, 1999; Mayer, 2000). The reason was mainly due to the high proportion of large pores in the total pore volume. Although several new methods have been developed, the preparation of preparative-scale polymeric monoliths was still difficult (Peters, 1997; Podgornik, 2000). The silica monoliths can provide either micrometer-size through-pores or high specific surface areas and can be well suited for both small molecules and biopolymers, but as with other silica packings, they cannot be used under too high and low pH.

Monolithic columns with wall-supported continuous porous beds have shown great potential as alternatives to packed columns in CEC because of their inherent advantages such as the absence of end-frits and the stability of the column bed. However, these columns also have their disadvantages and limitations. The disadvantage for polymer monolithic column is similar to that in HPLC, swelling of organic polymer in solvent and inadequate stability of silica-based monolithic column under extreme pH conditions. For particle-fixed monolithic columns, some disadvantage is obvious, such as damage of the surface of the reversed-phase silica for particle-sintered monolithic columns (Asiaie, Huang, Faman, & Horvath, 1998), reduced retentive characteristics for some particle-entrapped monolithic columns

(Chirica & Remcho, 1999) and poor efficiency for particle-loaded monolithic columns (Dulay, 1998; Ratnayake, 2000).

1.6 Cryogel

Polymeric materials combined under the name ‘gels’ are the systems ‘polymer – immobilized (solvate) solvent’, in which macromolecules connected via non-fluctuating bonds form a 3D-network (i.e. via the bonds that, to a large extent, remain unchanged with time). The gel morphology (homo- or heterophase) is determined by the method of gel preparation, and the nature of the bonds is determined by the chemical structure of the polymers. The role of the solvent immobilized within a 3D-polymer network in gels is crucial because the solvent does not allow the formation of a compact polymer mass, preventing the collapse of the system. Gels are physical objects that can withstand considerable reversible deformation without flowing or destruction. According to the nature of intermolecular bonds in the junctions of polymer network, gels can be divided into two groups: chemical and physical gels. A more detailed classification of gels, which reflects the particular process resulting in gel-formation, is presented in Table 1.2.

Table 1.2 Classification of polymeric gels and formation processes.

Polymeric gels			
Type	**Physicochemical causes of gel-formation**	**Examples**	**Comments**
Chemotropic gels	Intermolecular chemical bonds resulting in 3D covalent network	– Polyacrylamide gels; – Ion-exchange resins based on polystyrene or polyacrylate matrices; – Cross-linked dextran gels known as Sephadexes, etc.	This is a large group of gels; the gel-formation occurs during branched polymerization of monomeric precursors, or during covalent cross-linking of polymeric precursors. The gels are widely used in biotechnology.
Ionotropic gels	Ion-exchange reactions giving rise to stable intermolecular ionic (salt) bonds	– Gels based on polyelectrolyte complexes like alginate-polylysine or chitosan-polyphosphate mixed matrices, etc.	These gels are stable in media of definite composition but could easily be dissolved by changing, for example, the pH or ionic composition in outer liquid medium. The gels are used as carriers of immobilized (encapsulated) microbial, plant and animal cells.
Chelatotropic gels	Chelating reactions giving rise to stable intermolecular coordination bonds	Gels formed on addition of multivalent strongly coordinating metal ions (e.g., Cu(II) or Co(II)) to chitosan solutions or Cr(III) ions to carboxymethyl cellulose solution or alginate gels cross-linked by, for example, calcium ions.	Ca-alginategels are used for cell immobilization, other chelatropic gels have not yet found biotechnological applications.
Solvotropic (or solvatotropic) gels	Gelation due to the changes of solvent composition	The gels formed as a result of so-called coacervation phenomenon; such type of gelation is an intermediate stage in the processes of wet-formation of films and fibers, for example, from cellulose nitrates or cellulose acetates.	Gels are formed, when a non-solvent is added to the polymer solution thus reducing the polymer affinity to the medium and promoting non-covalent polymer–polymer interactions. No documented biotechnological application.
Thermotropic gels	Gelation caused by heating of an initial polymer system	– Gels of hydrophobically modified hydroxyethyl cellulose; – Ovalbumin and egg white gels.	Intermolecular hydrophobic interactions have a significant role in the gel-formation.
Psychrotropic (from ψυχρος – psychria – chill) gels	Gelation caused by chilling (no freezing) of initial polymer system	– Gelatine gels; – Starch gels; – Agarose and agar–agar gels; – Carrageenan gels.	Psychrotropic gels are well known; they are related to the physical ones. The gels are widely used in biotechnology as solid media for cell cultivation, as chromatographic materials or as electrophoresis matrices.
Cryotropic (from κρυος – kryos – frost, ice) gels (**cryogels**)	Gelation induced by freezing of an initial system.	These gels constitute the topic of the present review.	

Cryogels are gel matrices that are formed in moderately frozen solutions of monomeric or polymeric precursors. Cryogels typically have interconnected macropores (or supermacropores), allowing unhindered diffusion of solutes of practically any size, as well as mass transport of nano- and even microparticles. The unique structure of cryogels, in combination with their osmotic, chemical and mechanical stability, makes them attractive matrices for chromatography of biological nanoparticles (plasmids, viruses, cell organelles) and even whole cells. At present, polymeric gels have applications in many different areas of biotechnology including use as chromatographic materials, carriers for the immobilization of molecules and cells, matrices for electrophoresis and immunodiffusion, and as a gel basis for solid cultural media. A variety of problems associated with using polymer gels, as well as the broad range of biological objects encountered, lead to new, often contradictory, requirements for the gels. These requirements stimulate the development and commercialization of new gel materials for biological applications. One of the new types of polymer gels with considerable potential in biotechnology is ‘cryogels’ (from the Greek krios (kryos) meaning frost or ice). Cryogels are formed

as a result of cryogenic treatment (freezing, storage in the frozen state for a definite time and defrosting) of low- or highmolecular- weight precursors, as well as colloid systems–all capable of gelling. Cryogels were first reported, 40 years ago and their properties, which are rather unusual for polymer gels, soon attracted attention. The biomedical and biotechnological potential of these materials has now been recognized (Lozisky et al., 2003).

Given that the biotechnological applications of cryogels are discussed, they deserve a more detailed consideration, especially regarding the properties that distinguish them from other gel types. The formation of cryogels is schematically presented in Figure 1.3, in which the features of cryotropic gelation processes are also listed. Cryotropic gelation produces polymeric materials with essentially different morphology compared with gels obtained in non-frozen systems. Cryogels could be of any chemical type–covalent, ionic or non-covalent. Obviously, only the precursors of heat induced (thermotropic) gels cannot be used for the preparation of cryogels. With some exceptions, freeze-dried polymeric materials soaked in solvent (in which the polymer swells without dissolution) can be considered as materials with macro and microstructure similar to that of cryogels. The solvent freezing followed by the sublimation of solvent crystals (ice in case of aqueous systems) forms a system of interconnected pores in the polymeric material. However, no gel formation takes place perse in unfrozen liquid microphase (Figure 1.3 , unfrozen liquid microphase, UFLMP). Freeze-dried materials can be produced only as relatively thin objects, for example, films, plates or small beads. The production of freeze-dried cylinders or thick blocks is impractical from a technical point of view. On the contrary, cryogels can be formed in any desirable shape, for example, blocks, cylinders, tubes, granules and disks. Moreover, the production of cryogels is simpler than production of freze-dried materials because solvent removal under reduced pressure is not necessary. A system of large interconnected pores is a main characteristic feature of cryogels; some cryogels possess spongy morphology. The pore system in such sponge-like gels ensures unhindered convectional transport of solutes within the cryogels, contrary to diffusion of solutes in traditional homophase gels. The size of macropores within cryogels varies from tens or even hundreds to only a few micrometers–micrographs in Figure 1.3 illustrate these cases. The

interconnected system of large pores makes various cryogels promising materials for the production of new chromatographic matrices tailormade for the separation of biological nano- and microparticles (plasmids, viruses, cell organelles and even intact cells), and also for the implementation as carriers for immobilization of molecules and cells (Lozisky et al., 2003).

The main characteristic features of the cryotropic gelation processes ;

1)The reaction mixture containing gel-forming agents is frozen at temperatures a few degrees centigrade below the solvent crystallization point. The frozen system, despite looking as a single solid block, remains essentially heterogeneous and contains so-called unfrozen liquidmicrophase (UFLMP) along with the crystals of the frozen solvent.

2)Gel-forming reagents are concentrated in UFLMP, that is, cryoconcentration takes place. As UFLMP presents only a small portion of total initial volume, the concentration of gel precursors increases dramatically promoting the gel-formation. In fact, owing to cryoconcentration, the gel formation in such frozen systems proceeds sometimes faster than in liquid medium, when using the same initial concentration of precursors.

3)The crystals of frozen solvent perform as a pore-forming agent. When melted, they leave voids, macropores filled with the solvent. The surface tension between solvent and gel phase rounds the shape of the pores, making pore surface smoother. When freezing, the solvent crystals grow till they meet the facets of other crystals, so after thawing a system of interconnected pores arises inside the gel. The dimensions and shape of the pores depend on many factors, the most important are the concentration of precursors and the regimes of cryogenic treatment.

4)The polymer phase of the cryogel has, in turn, micropores formed in between the polymer chains. Thus, cryogels have both heterophase and heteroporous structure (Figure 1.3).

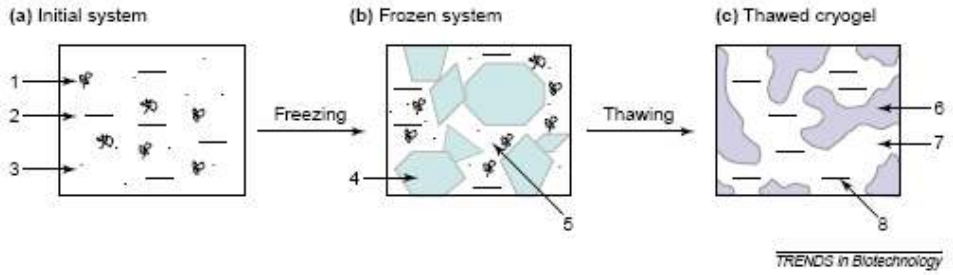

Figure 1.3 1, macromolecules in a solution; 2, solvent; 3, low-molecular solutes; 4, polycrystals of frozen solvent; 5, unfrozen liquid microphase; 6, polymeric framework of a cryogel; 7, macropores; 8, solvent. (Lozinsky et al., 2003)

One of the important parameters affecting the performance of porous hydrophilic materials is pore structure. There is no general technique to measure pore size and pore size distribution of the porous hydrophilic gels in their intact wet state. The porosity and pore size distribution of a porous rigid material in a dry state is usually determined by mercury intrusion porosimetry. Indirect methods, like inverse size exclusion chromatography (ISEC), were used for the structural characterization of monolithic porous sorbents. The porous structure in the pAAm-cryogels is controlled by the freezing temperature, the concentration of monomers in the initial reaction mixture and the type and content of a crosslinker. Varying the content of monomers in the initial reaction mixture resulted in pAAm-cryogels with different properties and hence different sizes of pores and different thicknesses of pore walls (Table 1.3). One could try to discriminate three parameters affecting the ultimate performance of pAAm cryogels, namely pore size (and pore size distribution), the thickness of pore walls and the density of pore walls expressed as the concentration. Pore size controls the flow resistance of the pAAm-cryogels, together with the thickness of pore walls, these parameters define the total pore volume of the pAAm-cryogel sample. The thickness of pore walls along with density of pore walls will determine the macroscopic mechanical properties of the pAAm cryogel. While the pore size and pore wall density will affect the accessibility of the ligands chemically bound to the polymer backbone. The water flow resistance of the pAAm-cryogels (estimated as the water flow rate through the columns filled with monolithic pAAm-cryogels at a certain hydrostatic pressure) gives an integral characteristic of the pore structure of the material. The water flow resistance of 15-pAAm-cryogel increased

almost an order of magnitude as compared to 6-pAAm-cryogels. Concomitantly, the swelling degree of the pAAm-cryogels decreased significantly while the portion of polymer-bound water (determined from adsorption of water vapor by dry pAAm-cryogel samples) increased with increasing monomer concentration in the reaction feed (Table 1.3). For traditional homogeneous gels prepared from the same monomer feed at room temperature, the swelling degree also decreased with increasing monomer concentration in the feed (Table 1.3). The swelling degree of the 10-pAAm cryogel (prepared from 10% monomer concentration) was practically the same as swelling degree of the gels prepared at 20^{o}C from 18% monomer concentration (Table 1.3) due to the cryoconcentration of the dissolved reagents in small nonfrozen regions where the polymerization reaction proceeded. It should be noted that the interconnected structure of pores in cryogels was reflected in their extremely fast swelling when dry samples were brought in contact with water. The cryogels were swollen within a few seconds with a swelling rate decreasing as the monomer concentration in the feed increased. Again, the behavior was consistent with the assumption of smaller pores and thicker walls in cryogels produced from feeds with higher monomer concentration. Frozen water in 18-pAAm-cryogel started melting at a lower temperature and was melting in a wider temperature range than in 6-pAAm- and 10-pAAm-cryogels, respectively. The amount of non-freezable water decreased with increasing monomer concentration in the feed (Table 1.4). That is in accordance with a previous assumption that the increase in monomer concentration in the feed resulted in the pAAmcryogels with higher density of pore walls. The higher the concentration of polymer chains in the pore walls, the less water is bound to the polymer via hydrogen bonding. As less water is incorporated in a three-dimensional gel matrix, more water becomes freezable. Another reason could be the increased amount of non-frozen (supercooled) water in the smaller pores in pAAm-cryogels produced from feeds with higher monomer concentration. (Plieva et al., 2005)

Table 1.3 Properties of pAAm-cryojels produced at -12°C from feeds with different monomer concentration

Monomer concentration[a] (%)	Notation	Swelling degree/g H_2O per g dried polymer (for gels)	Swelling degree/g H_2O per g dried polymer (for cryogels)	Polymer bound water (% of total water in cryogel)[b]	Water flow resistance of cryogels h^{-1c}
6	6-pAAm	14.7	5.5	4.0	870
10	10-pAAm	9.2	4.5	7.2	370
15	15-pAAm	5.9	3.9	11.3	81
18	18-pAAm	4.4	3.5	14.0	61
22	22-pAAm	3.9	3.2	17.3	20

[a] Vinyl (AAm + AGE)/divinyl (cross-linker) ratio was 15 : 1 (mol/mol). [b] The amount of polymer bound water was determined from water vapor adsorption by dry cryogels (for details see Experimental) and presented as percentage of total water in cryogel. [c] Water flow resistance of pAAm-cryogels was measured as a flow rate through pAAm-cryogel columns at constant hydrostatic pressure equal to 1 m water column.

Table 1.4 Water in different state in pAAm-cryogels calculated from DSC measurements

Cryogel sample	Total water (%)	Freezable water[a] (% of total water)	Non-freezable water (% of total water)
6-pAAm	97.3	93.7	6.3
10-pAAm	95.0	96.1	3.9
18-pAAm	92.2	98.1	1.9

[a] Freezable water includes free water and weakly bound water.

The freezing rate is one of the crucial parameters during the preparation of the monolithic cryogels that influence the porous properties of the monolithic cryogels. The lower the freezing rate (or the higher the freezing temperature), the larger the size of the growing ice crystals and, as a result, the larger the pore size of the cryogels prepared. However, at high freezing temperature there is a risk that the solution to be frozen will be in a supercooled state. Thus, the temperature should be low enough to assure freezing of the reaction mixture. The faster the freezing (or the lower the freezing temperature), the more nucleation is promoted, and the greater the number of crystals of smaller size. Low freezing temperature resulted in the preparation of supermacroporous cryogels with increased hydrodynamic resistance due to the formation of a less porous structure with bimodal pore size distribution. The total pore volume of the monoliths prepared at two different temperatures (–128^{o} C and –188^{o}C) was practically the same (90%) but with changed partition between the large pores (estimated as amount of water that can be squeezed out from the cryogel mechanically) and small pores (estimated as the amount of water kept

inside the cryogel by capillary forces). Despite the increased hydrodynamic resistance of the monoliths prepared at low temperature, the flow of mobile phase through these monoliths remained predominantly convective due to the large size of the pores (tens of μm) indicated by SEM photos. The flow resistance through the monolithic columns (estimated as water flow rate through the monolithic cryogel column at constant hydrostatic pressure equal to 1 m of water column) increased when the monoliths were prepared at low temperature, –188^{o}C.

Normally, the gel-fraction yield (the portion of monomers polymerized to form a gel) for the cryogels lies in the range of 70–80%. The remaining monomers (around 20%) are not polymerized at all or are polymerized to form soluble polymers or oligomers and are removed in the washing step. To be sure that the washed pAAm-cryogel matrix does not contain any residual monomers (mainly AAm), experiments on extraction of free monomers from the cryogel matrix were performed (Plieva, Andersson, Galaev, & Mattiasson, 2004).

For biomedical and biotechnological applications porous gels are usually modified with a ligand capable of selective interactions with some biological target, e.g., ligands (ionexchange, hydrophobic, affinity) in chromatographic matrices to ensure selective binding of proteins or plasmid DNA; anchoring peptides or proteins in scaffolds for tissue engineering to ensure attachment and proliferation of cells; catalytically active enzymes in biocatalysts etc. (Plieva et al., 2005)

1.7 Affinity Chromatography

1.7.1 General Aspect of Bioaffinity Chromatography

Affinity sorption is already a well established method for identification, purification and separation of complex biomolecules. This may be achieved by a number of traditional techniques such as gel permeation chromatography, high performance liquid chromatography, chromatofocusing, electrophoresis, centrifugation, etc., in that the process relies on the differences in the physical

properties (e.g., size, charge and hydrophobicity) of molecules to be treated. In contrast, affinity sorption techniques exploit the unique property of extremely specific biological recognition (Scopes, 1982; Gribnan, 1982; Chaiken, 1983). This is due to the complementarity of surface geometry and special arrangement of the ligand and the binding site of the biomolecule. All biological processes depend on specific interactions between molecules. These interactions might occur between a protein and low molecular weight substances (e.g., between substrates or regulatory compounds and enzymes; between bioformative molecules-hormones, transmittors, etc., and receptors, and so on), but biospecific interactions occur even more often between two or several biopolymers, particularly proteins. Affinity chromatography enables the separation of almost any biomolecule on the basis of its biological function or individual chemical structure. Examples can be found from all areas of structural and physiological biochemistry, such as in multimolecular assemblies, effector-receptor interactions, DNA-protein interactions, and antigen-antibody binding.

Affinity chromatography owes its name to the exploitation of these various biological affinities for adsorption to a solid phase (Wilcheck, 1984; Jonson, 1998). One of the members of the pair in the interaction, the ligand, is immobilized on the solid phase, whereas the other, the counterligand (most often a protein), is adsorbed from the extract that is passing through the column. Examples of such affinity systems are listed in Table 1.5.

Affinity sorption requires that the compound to be isolated is capable of reversibly binding (i.e., sorption-elution) to a sorbent which consists of a complementary substance (i.e., the so-called ligand) immobilized on a suitable insoluble support, i.e., the so-called carrier.

Table 1.5 Examples of Biological Interactions Used in Affinity Chromatography.

Ligand	Counter ligand
Antibody	Antigen, virus, cell
Inhibitor	Enzyme (ligands are often substrate analogs or cofactor analogs)
Lectin	Polysaccharide, glycoprotein, cell surface receptor, membrane protein, cell
Nucleic acid	Nucleic acid binding protein (enzyme or histone)
Hormone, vitamin	Receptor, carrier protein
Sugar	Lectin, enzyme, or other sugar-binding protein

The term affinity chromatography has been given quite different connotations by different authors. Sometimes it is very broad, including all kinds of adsorption chromatographies based on nontraditional ligands, in the extreme all chromatographies except ion exchange. Often it is meant to include immobilized metal ion affinity chromatography (IMAC), covalent chromatography, hydrophobic interaction chromatography, and so on. In other cases it refers only to ligands based on biologically functional pairs, such as enzyme-inhibitor complexes. The term not only to include functional pairs but also the so-called biomimetic ligands, particularly dyes whose binding apparently often occurs to active sites of functional enzymes although the dye molecules themselves of course do not exist in the functional context of the cell. Thus chromatography based on the formation of specific complexes such as enzyme-substrate, enzyme-inhibitor, etc., i.e on biological recognition, is termed bioaffinity or biospecific chromatography and the respective interaction-biospecific adsorption or bioaffinity (Porath, 1973). The original term “affinity chromatography” acquired a broader meaning also including hydrophobic chromatography, covalent chromatography, metal-chelate chromatography, chromatography on synthetic ligands, etc., i.e chromatography procedures based on different, less specific types of interaction. The broad scope of the various applications of affinity has generated the development of subspecialty

techniques, many of which are now recognized by their own nomenclature. Table 1.6 summarizes some of these techniques. As can be seen from Table 1.6, some of these subcategories have become accepted useful techniques (Wilcheck & Miron, 1999).

Table 1.6 Subcategories of affinity chromatography.

Affinity Chromatography	
	1. Hydrophobic Chromatography
	2. Immunoaffinity Chromatography
	3. Covalent AC
	4. Metal-Chelate AC
	5. Moleculaar Imprinting Affinity
	6. Membrane-Based AC
	7. Affinity Tails Chromatography
	8. Lectin Affinity
	9. Dye-Ligand AC
	10. Reseptor Affinity
	11. Weak AC
	12. Perfusion AC
	13. Thiophilic Chromatography
	14. High Performance AC
	15. Affinity Density Pertubation
	16. Library-Derived Affinity
	17. Affinity Partitioning
	18. Affinity Electrophoresis
	19. Affinity Capillary Electrophoresis
	20. Centrifuged AC
	21. Affinity Repulsion Chromatography

Schematic represantation of bioaffinity is shown in Figure 1.4. Affinity chromatography demonstrated in this figure is based on the simple principle that every biomolecule usually recognize another natural or artificial molecule. A wide variety of ligands may be covalently attached to an inert support matrix, and subsequently packed into a chromatographic column.

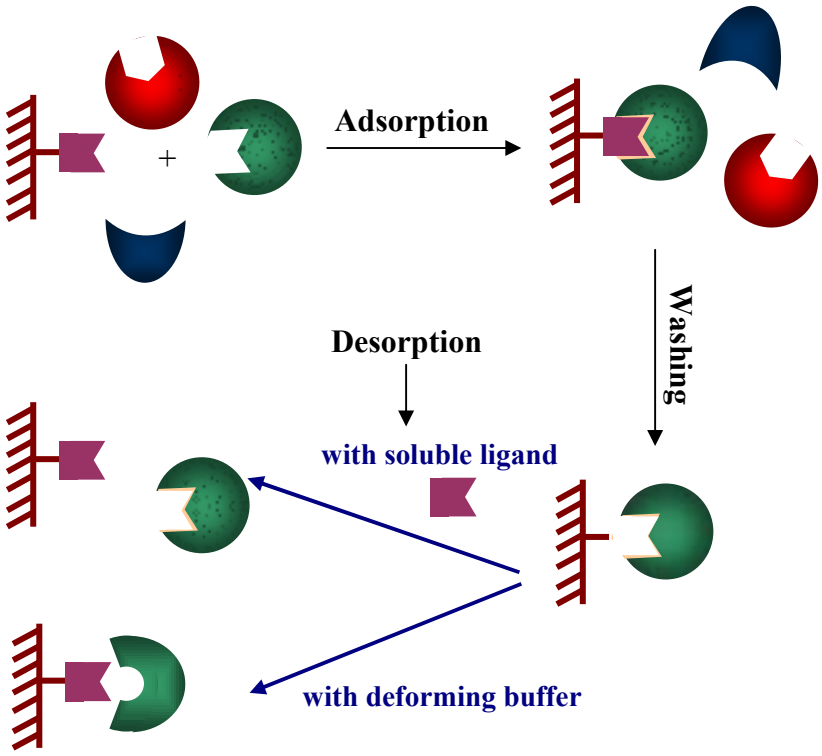

Figure 1.4 Schematic representation of the main steps in affinity chromatoraphy.

In such a system, only the protein molecules which selectively bind to the immobilized ligand will be retained on the column. Washing the column with a suitable buffer will flush out all unbound molecules. There are several techniques permit to desorb the product to be purified from the immobilized ligand.

Because affinity chromatography proper relies on the functional properties, active and inactive forms can often be separated. This is however, not unique to affinity methods. Covalent chromatography can do the same thing when the activity depends on a functional thiol group in the protein. By affinity elution, ion-exchange chromatography is also able to separate according to functional properties. These are, however, exceptions to what is a rule for the affinity methods.

Affinity chromatography has proved to be of great value also in the fractionation of nucleic acids, where complementary base sequences can be used as ligands, and

in the separation of cells, where cell surface receptors are the basis of the affinity. Its main use has, however, been in the context of protein purification.

A field that has been so successful that it is often treated separately is affinity based on antigen-antibody interactions, called immunosorption. Sometimes this is the only available route to the purification of a protein and is especially attractive when there is a suitable monoclonal antibody at hand.

Very often the use of affinity chromatography requires that the investigator synthesizes the adsorbent. The methods for doing this, which are described later, are well worked out and are also easily adopted for those not skilled in synthetic organic chemistry. To further simplify the task, activated gel matrices ready for the reaction with a ligand are commerically available. The immobilization of a ligand can, in the best cases, be a very simple affair. In addition, immobilizations are just as easy for proteins as for small molecules.

A property that needs special consideration is the association strength between ligand and counterligand. If it is too weak there will be no adsorption, whereas if it is too strong it will be difficult to elute the protein adsorbed. It is always important to find conditions, such as pH, salt concentration, or inclusion of, for example, detergent or other substances, that promote the dissociation of the complex without destroying the active protein at the same time. It is often here that the major difficulties with affinity methods are encountered. Ligands can be extremely selective, but they may also be only group specific. The latter type includes glycoprotein-lectin interactions, several dye-enzyme interactions, and interactions with immobilized cofactors. However, these interactions have also proved to be extremely helpful in solving many separation problems. Good examples are ligands that are group selective against immunoglobulins (e.g., staphylococcal protein A or streptococcal protein G) (Janson & Ryden, 1998).

1.7.2 Dye-Ligand Affinity Chromatography

In affinity chromatography a molecule having specific recognition capability (‘’ligand’’ or ‘’binder’’) is immobilized on a suitable insoluble support (‘’matrix’’ or ‘’carrier’’), which is usually a polymeric material in bead or membrane form. The molecule to be isolated (‘’analyte’’ or ‘’target’’) is selectively captured (‘’adsorbed’’) by the complementary ligand immobilized on the matrix by simply passing the solution containing the target through the chromatographic column under favorable conditions. The target molecules are then eluted (‘’desorbed’’) by using proper elutants under conditions favoring desorption, by adjusting the pH, ionic strength or temperature, using specific solvents or competitive free ligands, so that the interaction between the ligand and target is broken and the target molecules are obtained in a purified form. Since its first introduction (Cuatrecasas, Wilchek, & Anfinsen, 1968), thousands of different molecules (enzymes, antibodies, hormones, vitamins, receptors, many variety of other proteins and glycoproteins, RNA, DNA, etc.), even bacteria, viruses, and cells have been separated or purified by affinity chromatography (Deutscher, 1990; Matejtschuk, 1997; Scouten, 1981; Turkova, 1993) A wide variety of functional molecules, including enzymes, coenzymes, cofactors, antibodies, amino acids, oligopeptides, proteins, nucleic acids, and oligonucleotides may be used as ligands in the design of novel sorbents. These ligands are extremely specific in most cases. However, they are expensive, due to high cost of production and/or extensive purification steps. In the process of the preparation of specific sorbents, it is difficult to immobilize certain ligands on the supporting matrix with retention of their original biological activity. Precautions are also required in their use (at sorption and elution steps) and storage. Dye-ligands have been considered as one of the important alternatives to natural counterparts for specific affinity chromatography to circumvent many of their drawbacks, mentioned above (Denizli & Pişkin, 2001).

1.7.2.1 Reactive Dye Structure and Chemistry

Dye-ligands are able to bind most types of proteins, especially enzymes, in some cases in a remarkably specific manner. They are commercially available, inexpensive, and can easily be immobilized, especially on matrices bearing hydroxyl groups (Denizli & Pişkin, 2001), stable against biological and chemical attack, storage adsorbent without loss of activity, reusable: cleaning and sterilization, high capacity (Boyer, & Hsu, 1992). Although dyes are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many proteins by mimicking the structure of the substrates, cofactors, or binding agents for those proteins (Denizli & Pişkin, 2001).

A number of textile dyes, known as reactive dyes, have been used for protein purification in dye-ligand affinity systems, since they bind a variety of proteins in a selective and reversible manner. Most of the reactive dyes used in dye-affinity systems consist of a chromophore (either azo dyes, anthraquinone, or phathalocyanine), linked to a reactive group often a mono- or dichlorotriazine ring. They also have sulfonic acid groups to provide the desired solubility of the molecule in aqueous media. These groups are negatively charged at all pH values. Some dyes contain carboxyl, amino, chloride, or metal complexing groups; most contain nitrogen both in or outside on aromatic ring (Denizli & Pişkin, 2001).

These dyes are prepared by the reaction of cyanuric chloric (Figure 1.5 a) with an amino-containing dye, thereby producing a dichlorotriazinyl dye, which corresponds to the Procion MX range of dyes produced by ICI (Figure 1.5 b). The triazine ring in these dyes contain two labile chlorine atoms which makes dyes of this type highly reactive. Subsequent reaction of these dyes with an amine or alcohol causes the replacement of one of the triazinyl chlorine atoms and produces a less reactive monochlorotriazine dye which corresponds to ICI's Procion H range and Ciba-Geigy's Cibacron range (Figure 1.5 c) (Boyer, & Hsu, 1992). The only difference betwen Cibacron and Procion H series are the position of sulfonate group on the aniline ring, which is in *ortho*-position on Cibacron, but in *meta*- or *para*-position in Procion H series (Denizli & Pişkin, 2001).

Two dichlorotriazinyl molecules can be coupled with a bifunctional molecule (e.g., Diaminobenzene) to form bifunctional triazinyl dyes. An example is Procion H-E from ICI is shown in Figure 1.5 d. Some other examples of triazinyl dyes are monofluoro-triazinyl (Cibacron, Ciba-Geigy), trichloropyrimidnyl (Drimarene, Sandoz), and difluorochloropyrimidnyl (Lavafix, Bayer and Drimarene, Sandoz), which are shown in Figure 1.5 e–g, respectively. Note that, when the chloride atoms on the triazinyl ring are replaced with other groups, the reactivity of the dye is reduced, substantially. Dye-molecules having more chloride (or fluoride) atoms can easily react with the nucleophilic groups. The structure of several typical triazinyl dyes are shown in Figure 1.5. Note that, when the chloride atoms on the triazinyl ring are replaced with other groups, the reactivity of the dye is reduced, substantially. Dye-molecules having more chloride (or fluoride) atoms can easily react with the nucleophilic groups on the matrix at the ligand-immobilization step (Denizli & Pişkin, 2001).

An important strategy is to tailor-make, or redesign the dye structure to improve the specificity of textile dyes for target proteins. This new type of ligand is called Abiomimetic dyeB. It carries all the advantages of the parent (unmodified) dye including high specificity. This concept was first applied (Clonis, Stead, & Lowe, 1987) early in the 1980s and then

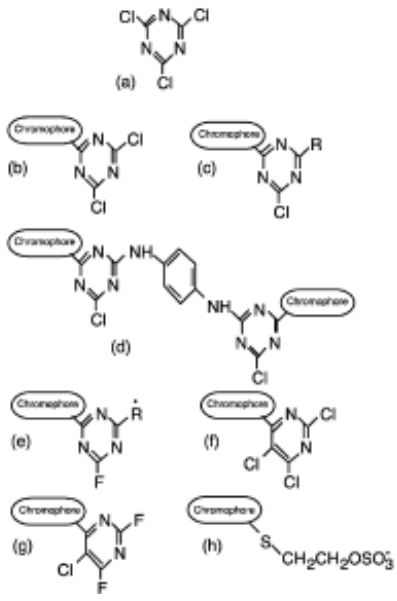

Figure 1.5 Structure of some of the reactive dye molecules; (a) cyanuric chloride; (b) Procion MX series (ICI); (c) Cibacron (Ciba-Geigy) and Procion H (ICI); (d) Procion H-E (ICI); (e) Monofluorotriazinyl, Cibacron, Ciba-Geigy; (f) Trichloropyrimidnyl, Drimarene, Sandoz; (g) Difluorochloro-pyrimidnyl, Levafix, Bayer and Drimarene, Sandoz; (h) Sulfatoethyl sulfone, Hoechst.

successfully used by them and also by others for specific enzyme recovery, as recently reviewed (Clonis et al., 2000). The first biomimetic dye was prepared by linking benzamidine to the reactive chlorotriazine ring via a diaminomethylbenzene group. It was used for the specific separation of trypsin from chymotrypsin (Denizli & Pişkin, 2001). Dye-ligands having two recognition moieties on the triazine ring were designed to isolate kallikrein from a crude pancreatic extract (Clonis, Goldfinch, & Lowe, 1981). By using biomimetic Cibacron Blue dye (phosphonated via a *p*-aminobenzyl ring), it was possible to purify alkaline phosphates from calf intestinal extract 280–330-fold in one chromatographic step after specific elution with inorganic phosphate (Burton, & Lowe, 1993). A similar biomimetic dye, prepared by using a diaminohexane spacer, was used to purify the same enzyme from the same source 120- to 140-fold (Clonis, & Lowe, 1991). A similar success was reported for the biomimetic-dye-affinity separation of alcohol dehydrogenase from horse liver by using Cibacron Blue 3GA bearing sulfonate, carboxylate, phosphonate, alcoholic, amido and trimethylammonium groups as terminal-ring substitutes (Lowe, Burton, Pearson, Clonis, Stead, 1986). Developments in

computational technology, especially in contemporary molecular modeling and bioinformatics, greatly improved the design of new series of biomimetic dye ligands. It was earlier recognized that anthraquinone-moiety-containing aromatic sulfonated dyes, such as Cibacron Blue 3GA, Procion Blue H-B and MX-R and Vilmafix Blue A-R tend to bind preferentially to the nucleotide-binding site of several proteins and mimic the binding of naturally occurring anionic coenzymes (e.g., NADH, FAD). Anthraquinone dichlorotriazine dyes (such as VBAR) also act as affinity labels of MDH (Labrou & Clonis, 1995) and LDH (Denizli & Pişkin, 2001). A three-dimensional structural model of LDH as a guide, appropriate structure changes of the dye molecules have allowed a biomimetic design of the ligand to improve the purification of L-lactate dehydrogenase (Labrou, Eliopoulos, & Clonis, 1999). The terminal biomimetic moiety bears a carboxyl group or a ketoacid structure linked to the triazine ring, thus mimicking natural ligands of L-malate dehydrogenase and these dyes have shown high specificity in the affinity purification of this enzyme (Labrou, Eliopoulos, & Clonis, 1996). Ketoacid-group recognizing enzymes (i.e., formate dehydogenase, oxaloacetate decarboxylase and oxalate oxidase) were purified by using biomimetic ligands (mercaptopyruvic-, m-amino- benzoic-, and amino-ethyloxamic-biomimetic dyes) (Kotsira, 1997; Labrou, 1995; Labrou, 1999). Molecular modeling has recently been applied for the design of triazine non-dye ligands for Protein A (Li, Dowd, Stewart, Burton, & Lowe, 1998), and human IgG (Teng, Sproule, Husain, & Lowe, 2000).

1.7.2.2 Immobilization of Reactive Dyes on Chromatographic Matrices

The most commonly used matrices for dye-ligand chromatography are gel filtration media including cross-linked agarose, cross-linked dextran, and beaded cellulose. Cross-linked agarose appears to be the best ‘’general purpose’’ matrix, due to its structural stability, flow properties, low incidence of non spesific adsorption, and open pore structure which allows high protein binding capacity. The dye-matrices are typically prepared with the dye immobilized directly, rather than indirectly via a spacer arm, because of significantly advantages in ligand leakage, capacity, and simplicity of immobilization (Denizli & Pişkin, 2001).

There are many methods for immobilization of ligand molecules onto the support matrix. First of all immobilization should be attempted through the least critical region (not from the active site) of the ligand molecule, to ensure minimal interference on the specific interaction between the immobilized ligand and the target molecules. Note that chemicals and experimental conditions applied may cause deleteriotion of the ligand molecules (means lost of their activity or functionality) during activation or coupling steps, therefore should carefully be selected. The active sites of biological molecules are often located deep within the three-dimensional structure of the molecule, which may cause an important steric hindrance between complementary ligand and target molecules. In these circumstances spacer arms, usually short alkyl chains, are frequently imposed between the matrix and the ligand to ensure their accessibility to the target (Denizli & Pişkin, 2001).

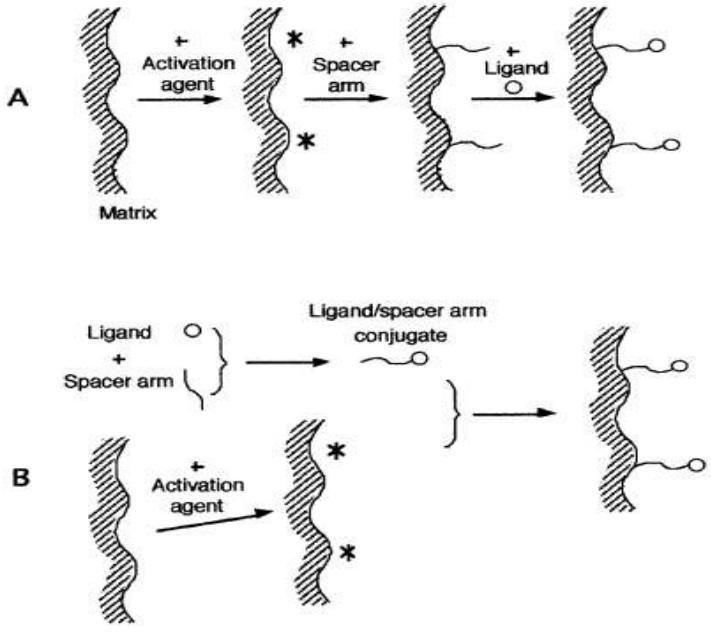

Figure 1.6 Strategies for coupling of ligand to the support matrix; (A) coupling through spacer arms; (B) coupling through spacer arm-ligand conjugates.

Many of the reactive dyes are immobilized onto matrix by direct reactions between the reactive groups (mainly hydroxyl groups) on the matrix and the dye molecules (hrough chloride or fluoride atoms) on triazinyl groups. Nonreactive dyes can be coupled to the matrix by the usual activation procedures, and the subject has been extensively reviewed (Denizli & Pişkin, 2001). Direct coupling of reactive triazinyl dyes to the matrices bearing hydroxyl groups is a simple, inexpensive and safe method (Baird, 1976; Burton, 1988; Clonis 1986; Clonis 1987; Hey 1981,). Coupling is achieved at alkaline conditions by nucleophilic substitution of hydroxyl groups with the reactive chlorine on the dye molecules. Nucleophiles are generated by the high pH, which promotes the ionisation of the matrix hydroxyl groups. Note that high pH (usually above 12) may cause hydrolysis of the chlorotriazines in the aqueous media, therefore very high pH values should be avoided. To couple the reactive dyes to the matrix hydroxyl groups, the matrix is incubated within the aqueous medium containing about 0.2% dye at pH 10–11 (adjusted with 0.1 M NaHCO , 1% Na CO or 0.1 M NaOH). A salt (NaCl, 2%) is also 3 2 3 included in the incubation medium, which salts-out and adsorbed of the dye molecules on the matrix surface, and therefore allows favourable hydrolysis and immobilization. Coupling can be achieved at room temperature (20–30^{o}C) at pH: 10–12 in about 2–3 days with monochlorotriazinyl dyes (e.g., Cibacron and Procion H series). After immobilization or use of these sorbents, in order to remove any uncovalently interacting dye (after dye immobilization) and all strongly bound protein molecules (after interaction with protein molecules), sorbents are treated with first water and then one of the followings: 1–2 M salt, 6 M urea in 0.5 M NaOH, 8 M urea, dimethylsulphoxide, 1–10 mg/cm^3 BSA, ethylene glycol, 20% ethanol in water. The dye-immobilized adsorbents should be stored in a dilute buffer solution at pH 8–9, with a bacteriostat-containing solution (e.g., 25% ethanol and 0.02% sodium azide) (Denizli & Pişkin, 2001).

1.7.2.3 Interactions between dye-ligands and proteins

The binding site of a protein is a unique stereochemical arrangement of ionic, polar, and hydrophobic groups in its three-dimensional structure, and where the polypeptide chains probably exhibit greatest flexibility. The dye-ligand molecules

participate in non-covalent interaction with the protein to achieve tight and specific binding. It has been shown in many kinetic studies that triazinyl dyes interact with an enzyme in a way involving the binding site (the substrate or coenzyme binding site, or the ‘’active site’’) for a natural biological ligand (NADH, NADPH, NAD^+, $NADP^+$, GTP, IMP, ATP, HMG-CoA, folate, etc.) of that enzyme so that this natural ligand cannot bind (Denizli & Pişkin, 2001). Many form of inhibition, including competitive, non-competitive, and mixed inhibition have been observed in these interactions. Triazine dyes, polysulphonated aromatic chromophores, mimic the naturally occuring heterocycles such as nucleotide mono-, di-, and triphosphates, NAD, NADH, flavins, acethyl-CoA and folic acid and inactivate typical nucleotide-dependent enzymes with different efficacy (Kaminska, Dzieciol, Koscielak, & Triazine, 1999). Thus, they can be used as affinity ligands for glycosyltransferases. Several spectrophotometric techniques including UV visible, FTIR, NMR, ESR, and circular dichroism, have been utilized to explain dye protein interactions, the existence of competitive ligands (e.g., substrates and coenzymes) and perturbing solutes (e.g., salts and organic solvents) (Federici, 1985; Lascu, 1984; Skotland, 1981; Subramanian, 1984). These studies have revealed that confirmation of both the dye and enzyme is important, and the interactions might be a mixture of electrostatic and hydrophobic forces, and also at discrete sites rather than in an indiscriminate fashion. Interactions of the parent dyes (especially Cibacron Blue F3G-A) and their analogs with several oxidoreductases, phosphokinases, and ATPases have been investigated (Denizli & Pişkin, 2001). These studies have shown that both the anthraquinone and the adjacent benzene sulfonate rings on these dyes are important in binding to the enzymes. They do bind to the enzyme molecules at a similar position and in a way similar to the AMP moiety of the coenzyme. Molecular models have shown a rough resemblance between Cibacron Blue F3G-A and NAD^+, but the most important similarities are with the planar ring structure and the negative charge groups. It has been shown by X-ray crystallography that this blue dye binds to liver alcohol dehydrogenase at an NAD^+ site, with correspondences of the adenine and ribose rings but not the nicotinamide. Thus, it was proposed that the dye is an analog of ADP-ribose, and it interacts with the ‘’nucleotide fold’’ found in AMP, IMP, ATP, NAD^+, $NADP^+$, and CTP binding sites of the corresponding enzymes. Cibacron Blue F3G-A have been an ideal dye-ligand for especially nucleotide-

binding proteins. The monochlorotriazinyl dyes (e.g., Cibacron Blue F3G-A, Procion Blue H-B) are usually not sufficiently reactive to inactivate irreversibly. But there are some exceptions (Witt, & Roskoski, 1980). It was also observed that divalent metal ions (e.g., Zn^{2+}, Mg^{2+}, Ca^{2+}) may increase considerably the inhibition of enzymes with these dyes by binding onto both the substrate and coenzyme binding sites (Hughes, Lowe, & Sherwood, 1982). Dichlorotriazinyl dyes (e.g., Procion Blue MX-R) have a greater reactivity, and exhibit irreversible inactivation of the enzymes (e.g., alcohol dehydrogenase) at the coenzyme-binding site. The interaction between the dye ligand and proteins can be concluded as follows: Dye molecules mimic natural ligands, and bind some protein molecules very specifically at their active points. However, under same conditions all proteins can be adsorbed onto dye-ligand affinity sorbents, which means that these ligands provide numerous opportunities for other interactions with other parts of the proteins. Most proteins are bound nonspecifically by complex combination of electrostatic, hydrophobic, hydrogen bonding, and charge-transfer interactions, all of which are possible considering the structural nature of the dyes (Denizli & Pişkin, 2001).

1.8 Human Serum Albumin (HSA)

Human serum albumin (HSA) is the most abundant protein in the circulatory system (Norbert, 1976). It consists of a single, non-glycosylated, polypeptide chain containing 585 amino acid residues and has many physiological functions which contribute significantly to colloid osmotic blood pressure and aid in the transport, distribution and metabolism of many endogeneous and exogeneous substances including bile acids, bilirubin, long-chain fatty acids, amino acids (notably tryptophan, thyrosine and cysteine), steroids (progesterone, testosterone, aldosterone, cortisol), metal ions such as copper, zinc, calcium and magnesium and numerous pharmaceuticals (Carter, & Ho, 1994). HSA commonly used for therapeutic purposes such as shock, heavy loss of blood etc., requires relatively high purity for medical use. Research on HSA separation has attracted considerable attention for its great potential in blood protein manufacture. HSA is at present commonly isolated from human plasma by Cohn's classical blood fractionation procedure (Cohn et al., 1946). Cohn's method concerns precipitation of proteins using ethanol with varying pH, ionic

strength and temperature. But this technique, which is the oldest method of industrial fractionation of blood proteins, is not highly specific and can give partially denaturated proteins (Kassab, Yavuz, Odabası, & Denizli, 2000).

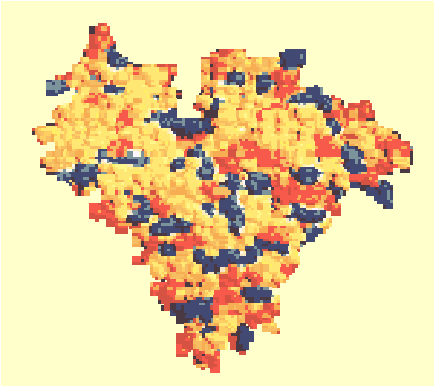

Figure 1.7 Three dimensional structure of HSA

CHAPTER TWO
EXPERIMENTAL METHODS AND MATERIALS

2.1 Materials

Acrylamide (AAm, more than 99.9% pure, electrophoresis reagent), allyl glycidyl ether (AGE, 99%), N,N'-methylene-bis(acrylamide) (MBAAm) and amonium persulfate (APS) were supplied by Sigma (St Louis, USA). N,N,N',N'-tetramethylene diamine (TEMED) was obtained from Fluka A.G. (Buchs, Switzerland). Cibacron Blue F3GA was obtained from Polyscience (Warrington, USA) and used without further purification. Human serum albumin (HSA, 98% pure by gel electrophoresis, fatty acid free, 67 kDa) was purchased from Aldrich (Munich, Germany). All other chemicals were of the highest purity commercially available and were used without further purification. Coomassie Blue for the Bradford Protein assay was from BioRad (Richmond, CA, USA). All water used in the experiments was purified using a All water used in the adsorption experiments was purified using a Millipore S.A.S 67120 Molsheim-France facility whose quality management system is approved by an accredited registering body to the ISO 9001. Laboratory glassware was kept overnight in a 5% nitric acid solution. Before use the glassware was rinsed with deionised water and dried in a dust-free environment.

2.2 Production of poly(AAm-AGE) cryogelic cryogel

Production of poly(AAm-AGE) cryogelic cryogel was performed using the Arvidsson et al's procedure (Arvidsson et al., 2002). AGE was selected in order to insert reactive epoxy groups in the cryogel. Briefly, monomers (10 ml of AAm, 1 ml of AGE) were dissolved in deionized water and the mixture was degassed under vacuum for about 5 min to eliminate soluble oxygen. Total concentration of monomers was 6% (w/v). The cryogel was produced by free radical polymerization initiated by TEMED (120 µl) and APS (100 mg). After adding APS (1% (w/v) of the total

monomers) the solution was cooled in an ice bath for 2-3 min. TEMED (1% (w/v) of the total monomers) was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into plastic syringe (5 ml, id. 0.8 cm) with closed outled at the bottom. The polymerization solution in the syringe was frozen at - 12^{o}C for 24 h and then thawed at room temperature. After washing with 200 ml of water, the cryogel was stored in buffer containing 0.02% sodium azide at 4^{o}C until use.

2.3 Cibacron Blue F3GA Immobilization

Cibacron Blue F3GA immobilization studies were carried out in a recirculating system equipped with a water jacket for temperature control. The cryogel was washed with 30 ml of water. Then, 100 ml of Cibacron Blue F3GA solution (5 mg/ml) containing NaOH (5 g) was pumped through the plastic column under recirculation at 80^{o}C for 2 h. Under these experimental conditions, a chemical reaction took place between the group of the Cibacron Blue F3GA containing chloride and the epoxide group of the poly(AAm-AGE) cryogel. The adsorption was followed by monitoring the decrease in UV absorbance at 630 nm. After incubation, the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel was washed with distilled water and methanol until all the physically adsorbed Cibacron Blue F3GA were removed. The modified cryogel was then stored at 4^{o}C with 0.02% sodium azide to prevent microbial contamination.

2.4 Characterization of cryogelic cryogel

The swelling degree of the cryogel (S) was determined as follows: cryogel sample was washed on porous filter until washing was clear. Then it was sucked dry and then transferred to pre-weighed vial and weighed ($m_{wet\ gel}$). After drying to constant mass in the oven at 60^{o}C, the mass of dried sample was determined ($m_{dry\ gel}$). The swelling degree was calculated as:

$$S = (m_{wet\ gel} - m_{dry\ gel}) / m_{dry\ gel} \quad (1)$$

The morphology of a cross section of the dried cryogel was investigated by scanning electron microscope. The sample was fixed in 2.5% gluaraldehyde in 0.15 M sodium cacodylate buffer overnight, post-fixed in 1% osmium tetroxide for 1 h. Then the sample was dehydrated stepwise in ethanol and transferred to a critical point drier temperated to + 10^{o}C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to + 40^{o}C and the pressure to ca. 100 bar. Liquid CO_2 was transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage. Release of the pressure at a constant temperature of + 40^{o}C resulted in dried cryogel sample. Finally, it was coated with gold-palladium (40: 60) and examined using a JEOL JSM 5600 scanning electron microscope (Tokyo, Japan).

FTIR spectra of the Cibacron Blue F3GA, the poly(AAm-AGE) cryogel and Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel were obtained by using a FTIR spectro-photometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogel (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a tablet, and the spectrum was then recorded.

2.5 Chromatographic Procedures

2.5.1 HSA-Adsorption from Aqueous Solutions

The HSA adsorption studies were carried out in a recirculating system equipped with a water jacket for temperature control. The cryogel was washed with 30 ml of water and then equilibrated with 25 mM phosphate buffer containing 0.1 M NaCl (pH 7.4). Then, the prepared HSA solution (50 ml of the aqueous HSA solution) was pumped through the column under recirculation for 2 h. The adsorption was followed by monitoring the decrease in UV absorbance at 280 nm. Effects of HSA concentration, pH of the medium and ionic strength on the adsorption capacity were studied. The flow rate of the solution was changed in the range of 0.2-2.0 ml/min. To observe the effects of the initial concentration of HSA on adsorption, it was changed

between 0.1-3.0 mg/mL. To determine the effect of pH on the adsorption, pH of the solution was changed between 4.0 and 8.0. To observe the effects of ionic strength, NaCl solution was used at ionic strength values of 0.01 and 0.6.

2.5.2 Desorption and Repeated Use

In all cases adsorbed HSA molecules were desorbed using 0.1 M Tris/HCl buffer containing 0.5 M NaCl. In a typical desorption experiment, 50 ml of the desorption agent was pumped through the cryogelic column at a flow rate of 0.2 ml/min for 1 h. The final HSA concentration in the desorption medium was spectroscopically determined. When desorption was achieved, the cryogel was cleaned with 1 M NaOH and then re-equilibrated with 25 mM phosphate buffer containing 0.1 M NaCl (pH 7.4). The desorption ratio was calculated from the amount of HSA adsorbed on the cryogel and the final HSA concentration in the desorption medium. In order to test the repeated use of poly(AAm-AGE) cryogel, HSA adsorption-desorption cycle was repeated for ten times using the same cryogelic column. In order to regenerate and sterilize, after the desorption, the cryogel was washed with 1 M NaOH solution.

2.5.3 HSA-Adsorption from Human Plasma

Human blood is collected from thoroughly controlled voluntary blood donors. Each unit separately controlled and found negative for HBS antigen and HIV I, II and hepatitis C antibodies. No preservatives are added to the samples. Human blood was collected into EDTA-containing vacutainers and red blood cells were separated from plasma by centrifugation at 4000 g for 30 min at room temperature, then filtered (3 μm Sartorius filter) and frozen at -20^{o}C. Before use, the plasma was thawed for 1 h at 37^{o}C. Before application, the viscous sample was diluted with 25 mM phosphate buffer containing 0.1 M NaCl (pH 7.4). Dilution ratios were 1/2 and 1/10. 50 ml of the human plasma with a HSA content of 37.7 mg/ml was pumped through the cryogel column at a flow rate of 0.2 ml/min for 1 h. Human serum albumin concentration was determined by using Ciba Corning Albumin Reagent (Ciba Corning Diagnostics Ltd,

Halstead, Essex, England; Catalog Ref. No: 229241) which based on bromocresol green (BCG) dye method (Tietz, 1986). In order to show dye specificity, adsorption of other blood proteins (i.e., fibrinogen and γ-globulins) was also monitored. Total protein concentration was measured by using the total protein reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, England; Catalog Ref. No: 712076) at 540 nm which based on Biuret reaction (Tietz, 1986). Chronometric determination of fibrinogen according to the Clauss method on plasma was performed by using Fibrinogene-Kit (Ref No: 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France) (Clauss, 1957). g-globulin concentration was determined from the difference.

The purity of HSA was assayed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separating gel (9 x 7.5 cm) and 6% stacking gels were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid-methanol-water (1:5:5, v/v/v) and destained in ethanol-acetic acid-water (1:4:6, v/v/v). Electrophoresis was run for 2 h with a voltage of 110 V. Lysozyme and human serum albumin were used as standards.

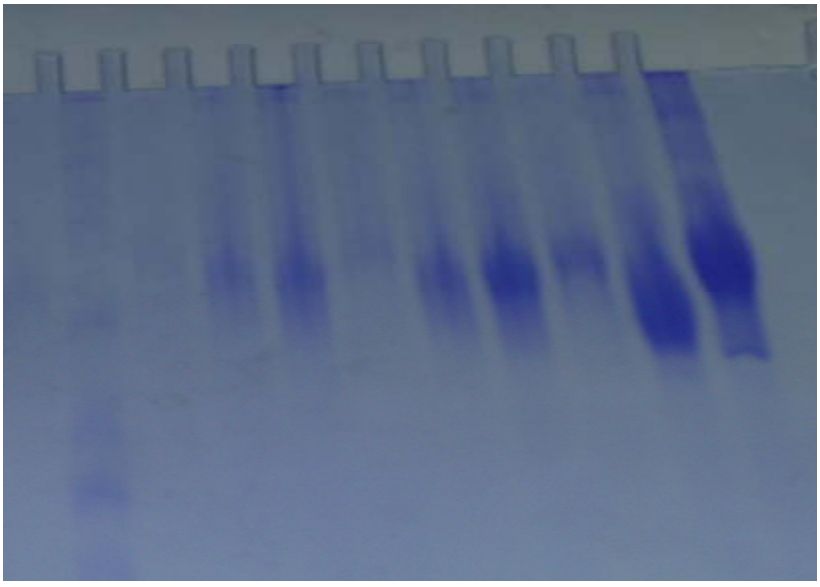

Figure 2.1 SDS-PAGE of HSA

CHAPTER THREE

RESULTS AND DISCUSSIONS

3.1 Characterization of cryogel

A supermacroporous monolithic cryogel was produced by copolymerization in the frozen state of monomers, acrylamide (AAm) and allyl glycidyl ether (AGE) with N,N'-methylene-bis(acrylamide) (MBAAm) as a cross-linker in the presence of amonium persulfate (APS)/ N,N,N',N'-tetramethylene diamine (TEMED) as initiator/activator pair. The functional epoxy groups on the surface of the pores in monolithic cryogels allowed their modification with the ligand, Cibacron Blue F3GA. The scanning electron micrograph of the internal structure of the monolithic cryogel is shown in Figure 3.1. Poly(AAm-AGE) cryogel produced in such a way have non-porous and thin polymer walls, large continuous inter-connected pores (10-100 μm in diameter, supermacro-porous) that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the protein molecules, allowing them to pass easily. The general shape of HSA can be viewed as three tennis balls in a can or cylinder. The Stokes radius is 3.9 nm. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible. The equilibrium swelling degree of the poly(AAm-AGE) monolithic cryogel was 6.84 g H_2O/g cryogel. Poly(AAm-AGE) monolithic cryogel is opaque, sponge like and elastic. This cryogel can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogel was submerged in water, it soaked in water and within 1-2 s restored its original size and shape.

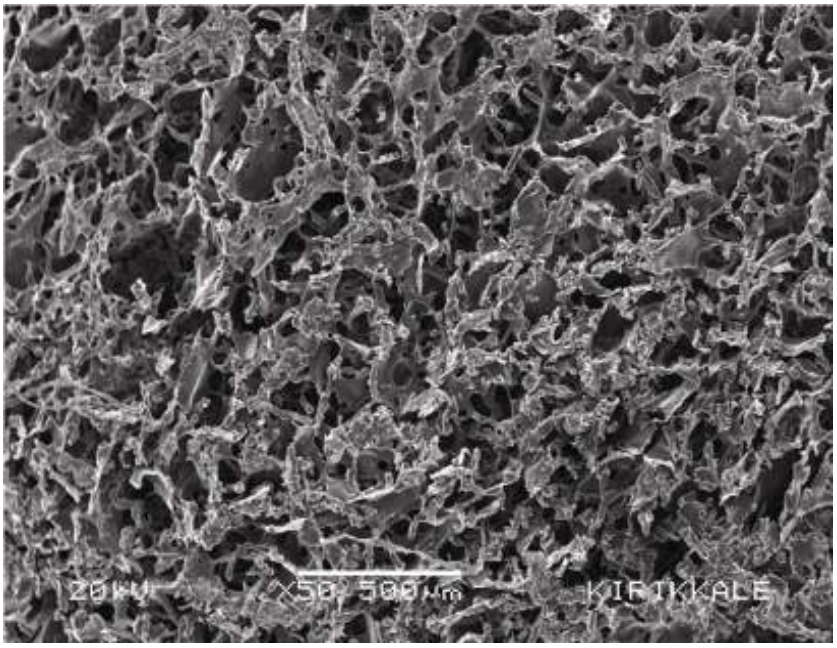

Figure 3. 1 Scanning electron micrograph of the inner part of the supermacroporous poly(AAm-AGE) monolithic cryogel matrix.

The schematic diagram for the immobilization of Cibacron Blue F3GA molecules through the poly(AAm-AGE) cryogel was given in Figure 3.2. Cibacron Blue F3GA was used as the biomimetic dye-ligand for specific binding of HSA. Cibacron Blue F3GA has been found to interact with many proteins that possess a dinucleotide-binding domain (Champluvier, & Kula, 1992). However, other proteins such as interferon (Jankowski, 1976; Karakoç, 2004) and human serum albumin (Leatherbarrow, 1980; Uzun, 2004) which may not possess a dinucleotide-domain have also been shown to bind to Cibacron Blue F3GA. The interaction between the dye and protein can be as follows: Dye molecules mimic natural ligands, and bind some proteins very specifically at their active points. However, under same conditions all proteins can be adsorbed onto dye-ligand affinity adsorbents, which means that these ligands provide numerous opportunities for other interactions with other parts of the proteins. Most proteins are bound nonspecifically by complex combination of electrostatic, hydrophobic, hydrogen bonding, and charge-transfer interactions, all of which are possible considering the structural nature of the dyes. It is reported that Cibacron Blue F3GA has no side effect on biochemical systems (Hanggi, & Carr,

1985). However, all commercial reactive dyes (including Cibacron Blue F3GA) contain various impurities which may affect their biochemical and related use. Reactive dyes have been purified by a number of chromatographic procedures such as thin layer chromatography, high performance liquid chromatography and column chromatography on Silica gel or sephadex. However it is suggested that purification of reactive dyes is necessary only when free dyes are used (Boyer, & Hsu, 1993). In cases where attached dyes are used, purification of the dye before immobilization is not likely to be necessary, because few of the contaminants will be attached on the support matrix, and proper washing of the matrix should remove adsorbed contaminants (Lowe & Wiseman 1984).

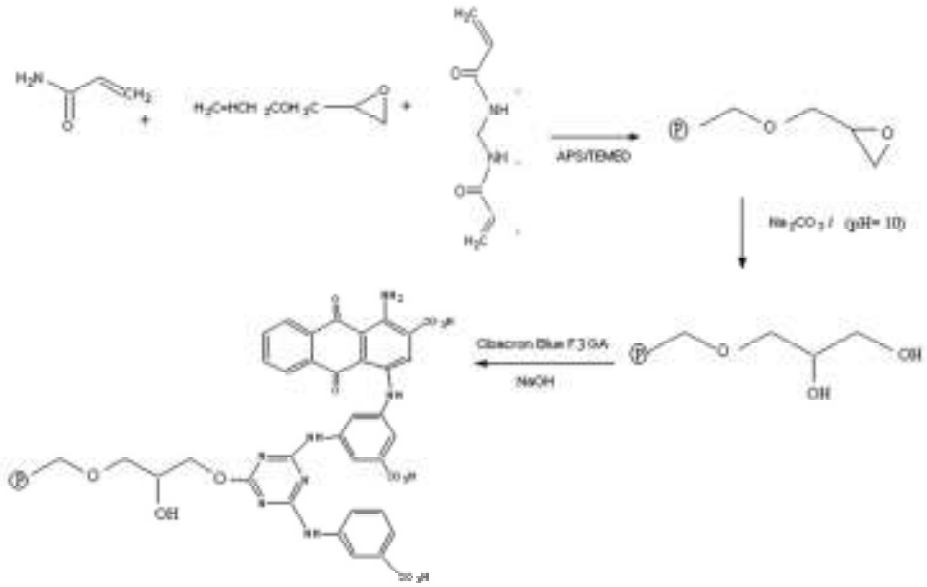

Figure 3.2 Schematic diagram for the immobilization of dye molecules through the poly(AAm-AGE) cryogel.

Cibacron Blue F3GA is covalently attached on poly(AAm-AGE) cryogel, via the reaction between the chloride groups of the reactive dyes and the epoxide groups of the AGE**.** The FTIR bands observed around 1160 cm^{-1} was assigned to symmetric stretching of S=O, as also pointed out on the chemical structure of the Cibacron Blue F3GA (Figure 3.3). The band observed at 3500 cm^{-1} was assigned to the -OH functional group. After Cibacron Blue F3GA attachment, the intensity of the -OH band increases due to NH stretching. The split of the band at 3300-3500 cm^{-1}

indicates also SO_3H and NH_2 groups. These bands show the attachment of Cibacron Blue F3GA within the poly(AAm-AGE) cryogel. The visual observations (the colour of the cryogel) ensured attachment of dye molecules**.** The dye loading was 68.9 μmol/g.

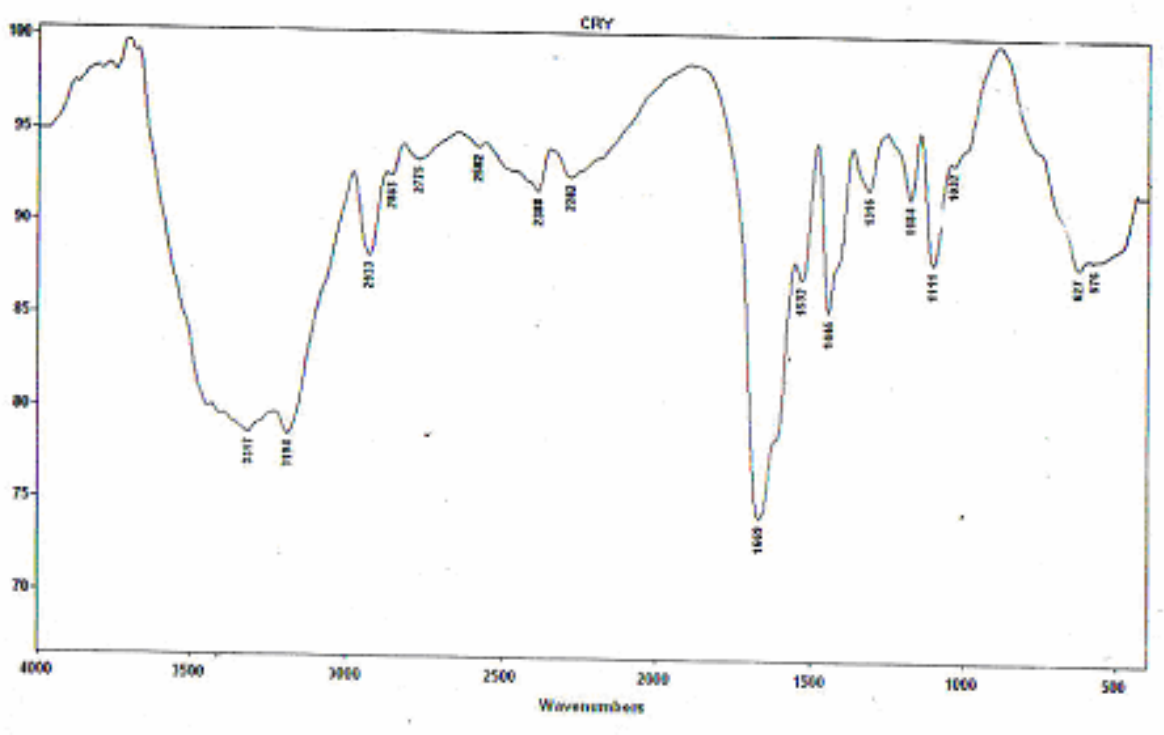

Figure 3.3 FTIR spectrum of Cibacron Blue F3GA attached poly(AAm-AGE).

3.2 HSA Adsorption from Aqueous Solutions

3.2.1 Effects of pH

Figure 3.4 shows the effect of pH on the adsorption of HSA onto Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel. The maximum adsorption of HSA was observed at pH 5.0, which is the isoelectric point of HSA. With the increase of pH above and below the pH 5.0, the HSA adsorption capacity decreased. The decrease in the HSA adsorption capacity can be attributed to electrostatic repulsion effects between the identically charged groups. At the isoelectric points, proteins have no net charge and therefore, the maximum protein adsorption from aqueous solution is usually observed at this point. In addition, these interactions between the dye and

protein molecules may result both from the ionization states of several groups on both the ligands (i.e., Cibacron Blue F3GA) and amino acid side chains in human serum albumin structure, and from the conformational state of protein molecules at this pH. It should be also noted that non-specific adsorption is independent of pH and it is observed at the same at all the pH values studied.

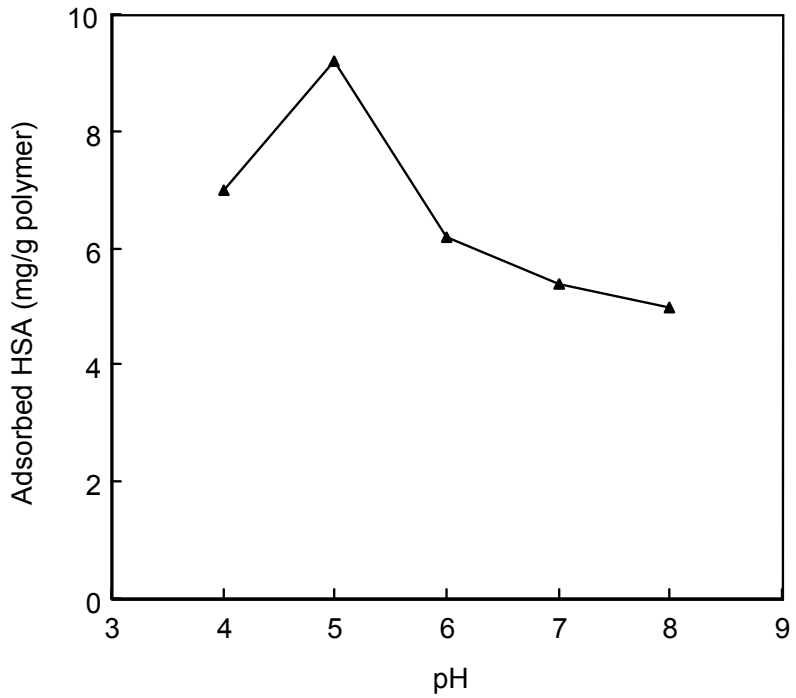

Figure 3.4 Effect of medium pH on the HSA adsorption:
Cibacron Blue F3GA loading: 68.9 μmol/g; HSA concentration: 0.5 mg/ml;
Flow rate: 0.2 ml/min; T: 20^{o}C. Each data is average of five parallel studies.

3.2.2 Effect of the concentration

Figure 3.5 shows the effect of the concentration of HSA on the adsorption. Note that one of the main requirements in dye-affinity chromatography is the specificity of the affinity adsorbent for the target molecule. The non-specific interaction between the support, which is the poly(AAm-AGE) cryogel in the present case, and the molecules to be adsorbed, which are the HSA molecules here should be minimum in order to consider the interaction as specific. As seen in this figure, negligible amount of HSA was adsorbed non-specifically on the poly(AAm-AGE) cryogel, which was 0.2 mg/g.

While dye-immobilization significantly increased the HSA coupling capacity of the cryogel (up to 27 mg/g). The amount of HSA adsorbed per unit mass of the poly(AAm-AGE) cryogel increased first with the initial concentration of HSA then reached a plateau value which represents saturation of the active adsorption sites (which are available and accessible for HSA) on the cryogel. This increase in the HSA coupling capacity may have resulted from cooperative effect of different interaction mechanisms such as hydrophobic, electrostatic and hydrogen bonding caused by the acidic groups and aromatic structures on the Cibacron Blue F3GA and by groups on the side chains of amino acids on the HSA molecules. It should be mentioned that Cibacron Blue F3GA is not very hydrophobic overall, but it has planar aromatic surfaces that prefer to interact with hydrophobic groups in HSA structure.

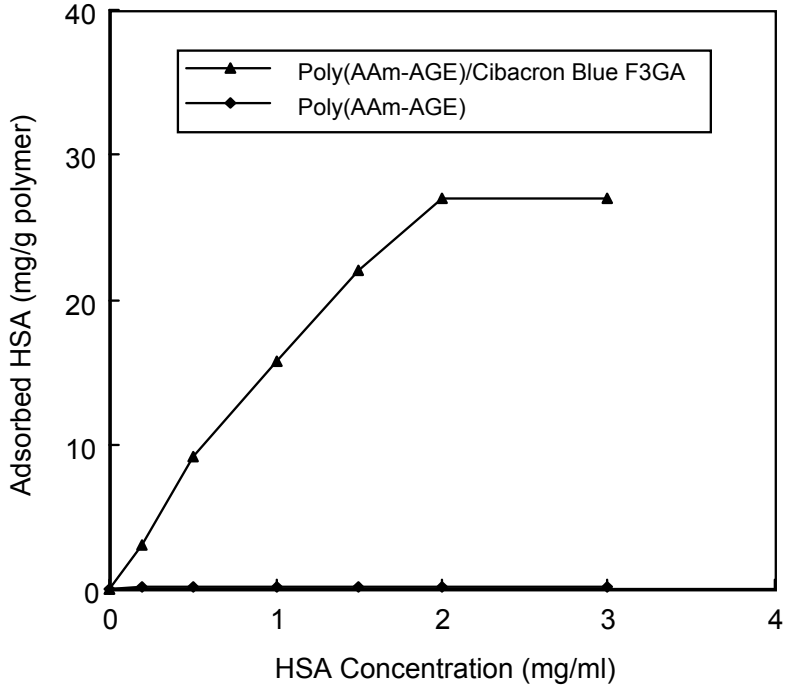

Figure 3.5 Effect of the concentration of HSA on the HSA adsorption: Cibacron Blue F3GA loading: 68.9 μmol/g; pH: 5.0; Flow rate: 0.2 ml/min; T: 20^{O}C. Each data is average of five parallel studies.

3.2.3 Effect of Flow-Rate

The breakthrough curves at different flow-rates are given in Figure 3.6. Results show that the HSA adsorption capacity onto the poly(AAm-AGE)/ Cibacron Blue F3GA cryogel decreases when the flow-rate through the column increases. The adsorption capacity decreased significantly from 9.2 mg/g to 0.6 mg/g polymer with the increase of the flow-rate from 0.2 ml/min to 2.0 ml/min. An increase in the flow rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column. This is due to decrease in contact time between the HSA molecules and the poly(AAm-AGE)/Cibacron Blue F3GA cryogel at higher flow rates. These results are also in agreement with those referred to the literature (Valdman, Erijman, Pessoa, & Leite, 2001). When the flow-rate decreases the contact time in the column is longer. Thus, HSA molecules have more time to diffuse to the pore walls of cryogel and to bind to the ligand, hence a better adsorption capacity is obtained. In addition, for column operation the cryogel is continuously in contact with a fresh protein solution. Consequently the concentration in the solution in contact with a given layer of cryogel in a column is relatively constant. For batch treatment, the concentration of solute in contact with a specific quantity of adsorbent steadily decreases as adsorption proceeds, thereby decreasing the effectiveness of the adsorbent for removing the solute.

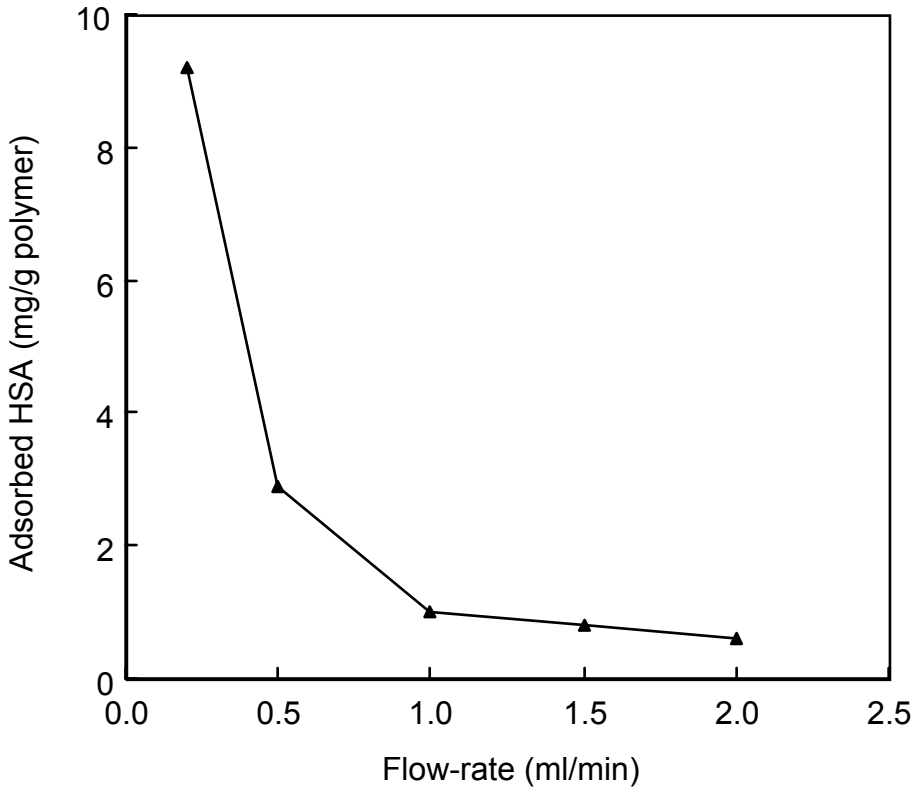

Figure 3.6 Effect of flow-rate on the HSA adsorption: Cibacron Blue F3GA loading: 68.9 μmol/g; pH: 5.0; HSA concentration: 0.2 mg/ml; T: 20^{o}C. Each data is average of five parallel studies.

3.2.4 Adsorption Isotherms

An adsorption isotherm is used to characterize the interactions of each protein molecule with the adsorbent. This provides a relationship between the concentration of the protein in the solution and the amount of protein adsorbed on the solid phase when the two phases are at equilibrium.

The Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which is capable of holding only one molecule. These sites are also assumed to be energetically equivalent, and distant

from each other so that there are no interactions between molecules adsorbed on adjacent sites.

Adsorption isotherms were used to evaluate adsorption properties. The Langmuir adsorption isotherm is expressed by Equation 2. The corresponding transformations of the equilibrium data for HSA gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

$$Q = Q_{max}. b . C_{eq} / (1 + bC_{eq}) \quad (2)$$

where Q is the adsorbed amount of HSA (mg/g), C_{eq} is the equilibrium HSA concentration (mg/mL), b is the Langmuir constant (mL/mg) and, Q_{max} is the maximum adsorption capacity (mg/g). This equation can be linearized so that

$$C_{eq}/Q = 1/(Q_{max}. b) + C_{eq} /Q_{max}. \quad (3)$$

The plot of C_{eq} versus C_{eq}/Q was employed to generate the intercept of $1/Q_{max}.b$ and the slope of $1/Q_{max}$ (Figure 3.7).

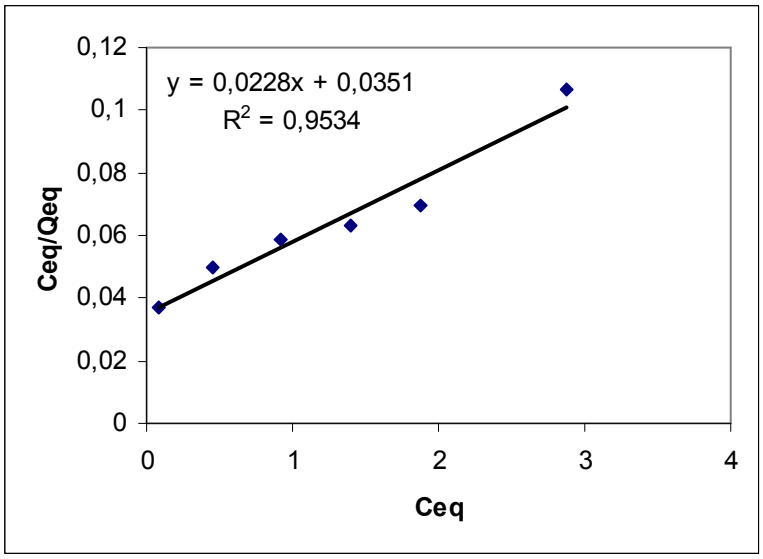

Figure 3.7 Langmuir adsorption isotherms of the poly(AAm-AGE) and Cibacron Blue F3GA attached poly(AAm-AGE) monoliths.

The maximum adsorption capacities (Q_{max}) data for the adsorption of HSA were obtained from the experimental data. The correlation coefficients (R^2) were high for Cibacron Blue F3GA attached poly(AAm-AGE) monolith. Langmuir adsorption isotherm constants, b and Qm and the correlation coefficients were found as 0.650, 43.86 and 0,9534, respectively. The Langmuir adsorption model can be applied in this affinity adsorbent system.

The other well-known isotherm, which is frequently used to describe adsorption behaviour, is the Freundlich isotherm. This isotherm is another form of the Langmuir approach for adsorption on a heterogeneous surface. The amount of adsorbed protein is the summation of adsorption on all binding sites. The Freundlich isotherm describes reversible adsorption and is not restricted to the formation of the monolayer. This emprical equation takes the form:

$$Q_{eq} = K_F (C_{eq})^n \qquad (4)$$

where, K_F and n are the Freundlich constants.

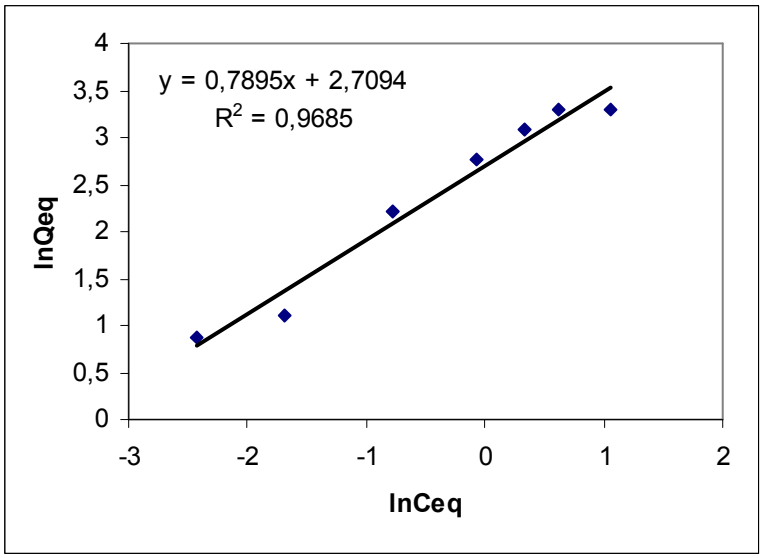

Figure 3.8 Freundlich adsorption isotherms of the poly(AAm-AGE) and Cibacron Blue F3GA attached poly(AAm-AGE) monoliths.

The adsorption isotherms of HSA were found to be linear over the whole concentration range studies and the correlation coefficients were high (Figure 3.8). According to the correlation coefficients of isotherms, Langmuir adsorption model is favorable. Freundlich adsorption isotherm constants, n and K_F and the correlation coefficients were found as 0.7895, 15.02 and 0.9685, respectively. The magnitude of K_F and n values of Freundlich model showed easy uptake of HSA from aqueus medium with a high adsorption capacity of the dye immobilized monolith.

3.2.5 Kinetic Analysis

In order to examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction, kinetic models were used to test experimental data. The kinetic models (Pseudo-first- and second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$dq_t/dt=k_1(q_{eq}-q_t) \quad (5)$$

where k_1 is the rate constant of pseudo-first order adsorption (1/min) and q_{eq} and q_t denote the amounts of adsorbed protein at equilibrium and at time t (mg/g), respectively. After integration by applying boundary conditions, $q_t=0$ at t=0 and $q_t=q_t$ at t=t, gives

$$\log[q_{eq}/(q_{eq}-q_t)]= (k_1t)/2.303 \quad (6)$$

Equation 6 can be rearranged to obtain a linear form

$$\log(q_{eq}-q_t)= \log(q_{eq}) - (k_1t)/2.303 \quad (7)$$

a plot of $\log(q_{eq})$ versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process $\log q_{eq}$ should be equal to the interception point of a plot of $\log(q_{eq}-q_t)$ via t.

In addition, a pseudo-second order equation based on equilibrium adsorption capacity may be expressed in the form

$$q_t/dt = k_2 (q_{eq}-q_t)^2 \quad (8)$$

Where k_2 (g/mg·min) is the rate constant of pseudo-first order adsorption process. Integrating Equation 8 and applying the boundary conditions, $q_t=0$ at $t=0$ and $q_t=q_t$ at $t=t$, leads to

$$[1/(q_{eq}-q_t)] = (1/q_{eq}) + k_2t \quad (9)$$

or equivalently for linear form

$$(t/q_t)= (1/k_2q_{eq}^2) + (1/q_{eq})\, t \quad (10)$$

a plot of t/q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant (k_2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively.

A comparison of the experimental adsorption capacity and the theoretical values which obtained from Figures 3.9 and 3.10 are presented in Table 3.1 The theoretical q_e values estimated from the pseudo-first order were very close to the experimental values and the correlation coefficients were very high. These results showed that this cryogelic dye-adsorbent system was described by the first-order model.

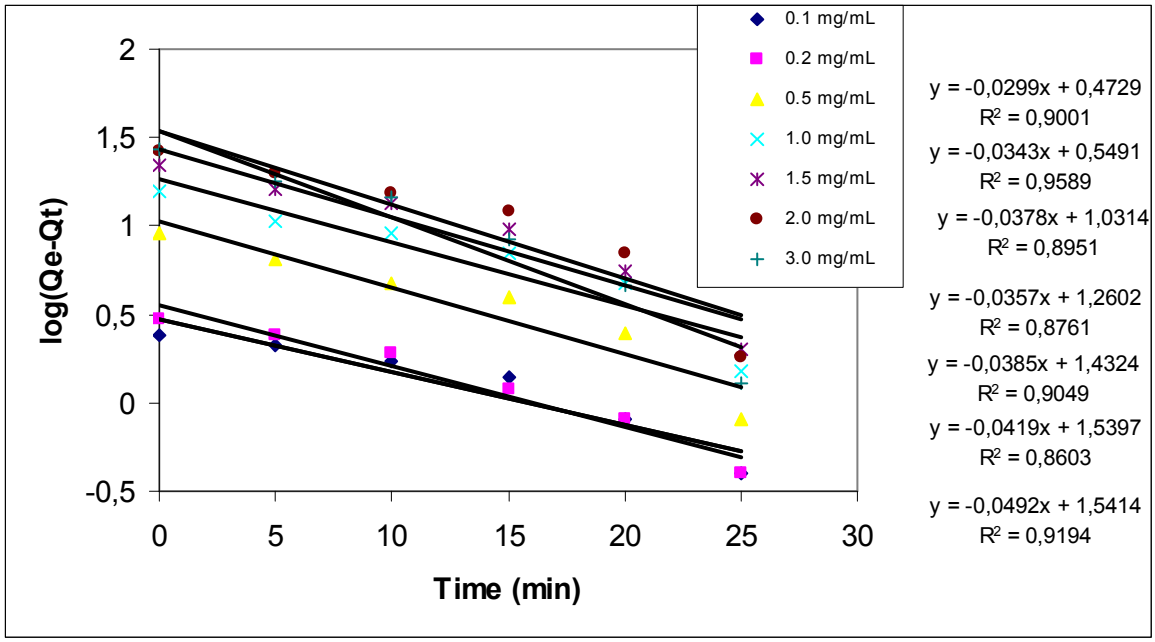

Figure 3.9 Pseudo-first order kinetic of the experimental data for the poly(AAm-AGE)/Cibacron Blue F3GA Cryogel

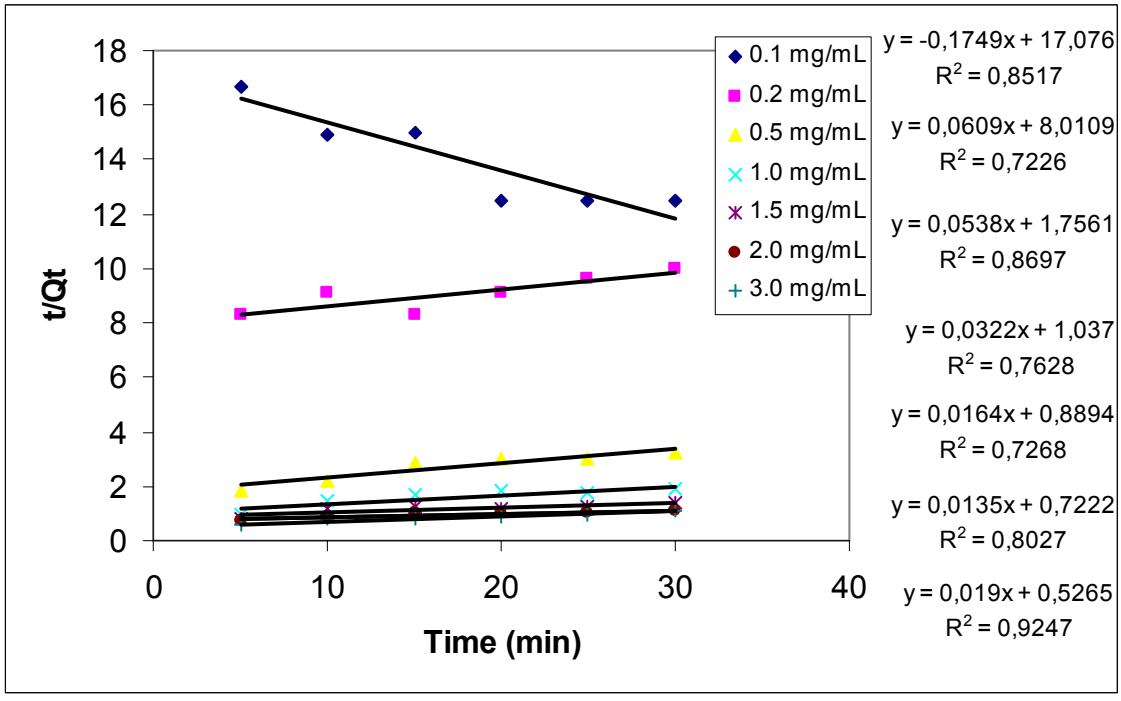

Figure 3.10 Pseudo-second order kinetic of the experimental data for the poly(AAm-AGE)/Cibacron Blue F3GA Cryogel

Table 3.1 The first and second order kinetic constants for poly(AAm-AGE) cryogel.

Initial Conc. (mg/ml)	Experimental q_{eq} (mg/g)	First-order kinetic k_1 (1/min)	q_{eq} (mg/g)	R^2	Second-order kinetic k_2 (1/min)	q_{eq} (mg/g)	R^2
0.1	**2.4**	0.069	2.97	0.900	0.0018	-5.71	0.852
0.2	**3.0**	0.078	3.54	0.959	0.0005	16.42	0.723
0.5	**9.2**	0.087	10.75	0.895	0.0016	18.59	0.870
1.0	**15.8**	0.082	18.21	0.876	0.0001	31.06	0.763
1.5	**22.0**	0.087	27.06	0.905	0.0003	60.98	0.727
2.0	**26.8**	0.096	34.65	0.860	0.0002	74.07	0.803
3.0	**27.0**	0.113	34.79	0.920	0.0007	52.63	0.925

3.2.6 Effect of NaCl concentration

The effect of NaCl concentration on HSA adsorption is presented in Figure 3.11, which shows that the adsorption capacity decreases with increasing ionic strength of the binding acetate buffer. The adsorption amount of HSA decreased by about 93.4% as the NaCl concentration changes from 0.2 to 0.6 M. Increasing the NaCl concentration could promote the adsorption of the dye molecules to the polymer surface by hydrophobic interaction. Moreover, the hydrophobic interactions between the immobilized dye molecules themselves would also become strong, because it has been observed that the salt addition to a dye solution caused the stacking of the free dye molecules. Thus, the numbers of the immobilized dye molecules accessible to HSA would decrease as the ionic strength increased, and the adsorption of the HSA to immobilized dye became difficult. It is also suggested that an increase in NaCl concentration result in the reduction of electrostatic interactions (Yavuz, & Denizli, 2004)

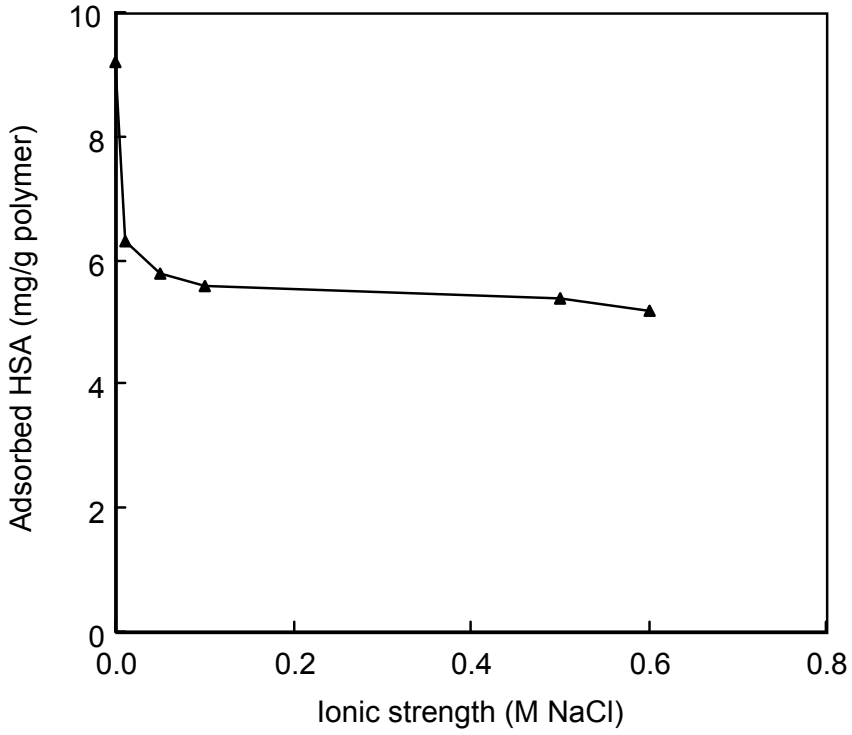

Figure 3.11 Effect of the NaCl concentration on HSA adsorption:
Cibacron Blue F3GA loading: 68.9 μmol/g; HSA concentration: 0.5 mg/ml;
pH: 5.0; Flow rate: 0.2 ml/min; T: 20^{o}C. Each data is average of five parallel studies.

3.2.7 Desorption Studies

Desorption of HSA from the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel was also carried out in continuous system. The desorptions of HSA are expressed in % of totally adsorbed HSA. Up to 98.0% of the adsorbed HSA was desorbed by using 0.1 M Tris/HCl buffer containing 0.5 M NaCl as elution agent. The addition of elution agent changed the charge of the peptide side groups due to their isoelectric points, resulting in the detachment of the HSA molecules from the immobilized dye-molecules. Note that there was no Cibacron Blue F3GA release in this case which shows that dye-molecules are bonded strongly to poly(AAm-AGE) cryogel. With the desorption data given above we concluded that 0.1 M Tris/HCl buffer containing 0.5 M NaCl is a suitable desorption agent, and allows repeated use of the affinity cryogel used in this study.

In order to show the reusability of the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel, the adsorption-desorption cycle was repeated ten times using the same dye-affinity poly(AAm-AGE) cryogel. There was no remarkable reduce in the adsorption capacity of the cryogel (Figure 3.12). The HSA adsorption capacity decreased only 6.4% after ten cycle. By taking into account the different experimental parameters studied above, it should be possible to scale up the process of HSA separation by increasing the cryogel size by dye-affinity chromatography on Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel.

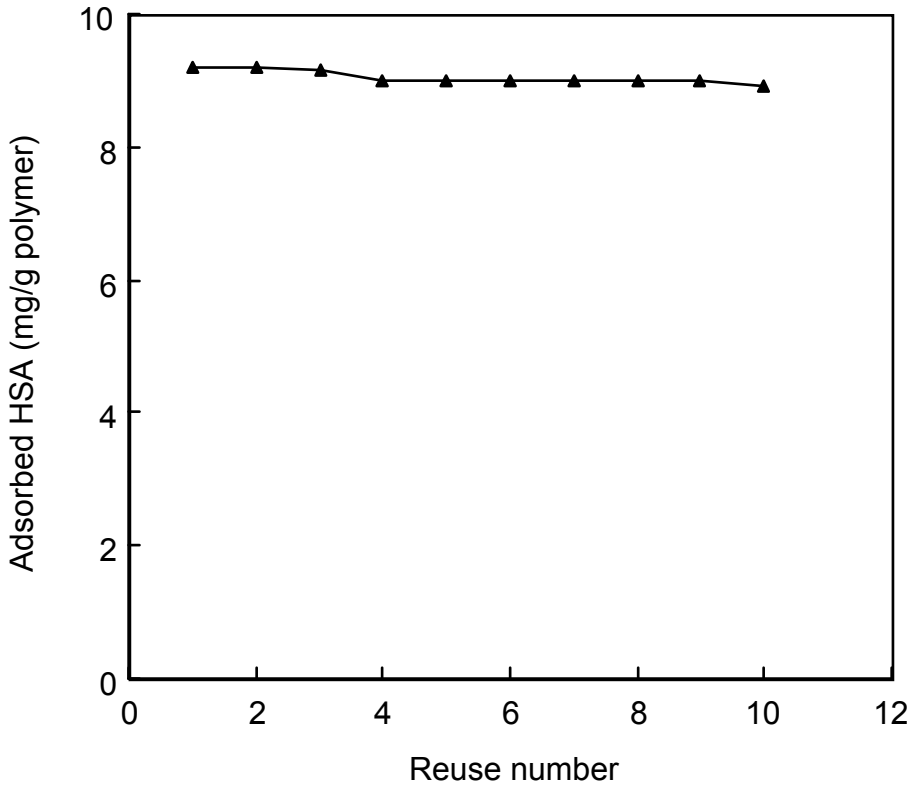

Figure 3.12 Repeated use of dye-attached poly(AAm-AGE) cryogel: Cibacron Blue F3GA loading: 68.9 μmol/g; HSA concentration: 0.5 mg/ml; pH: 5.0; Flow rate: 0.2 ml/min; T: 20^{O}C. Each data is average of five parallel studies.

3.2.8 HSA Adsorption From Human Plasma

Table 3.2 shows the adsorption for human serum obtained from a healthy donor. There was a low adsorption of HSA (1.43 mg/g) on the poly(AAm-AGE) cryogel, while much higher adsorption values (74.2 mg/g) were obtained when the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel were used. The purity of HSA was assayed by SDS-PAGE. The purity of HSA obtained was found to be 92% after purification. It is worth to note that adsorption of HSA onto the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel was approximately 3.0 fold higher than those obtained in the studies in which aqueous solutions were used. This may be explained as follows; the conformational structure of HSA molecule within their native environment (i.e. human plasma) much more suitable for specific interaction with the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel.

Competitive protein adsorption was also carried out and interesting results were obtained in these studies. Adsorption capacities were achieved as 1.7 mg/g for fibrinogen and 4.5 mg/g for γ-globulin. The total protein adsorption was determined as 80.8 mg/g. It is worth noting that adsorption of other plasma proteins (i.e., fibrinogen and γ-globulin) on the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel are negligible. It should be noted that HSA is the most abundant protein in plasma. It generally makes up more than half of the total plasma proteins. It may be resulted that this low adsorption of fibrinogen and g-globulin is due to the high concentration of HSA.

Table 3.2 HSA adsorption from the plasma of a healthy donor;

Cibacron Blue F3GA loading: 68.9 μmol/g; Flow rate: 0.2 ml/min; T: 20^{o}C.

Dilution Agent	Adsorption Capacity (mg/g)
Plasma (undiluted)	74.2 ± 3.25
1/2 diluted plasma	56.8 ± 2.28
1/10 diluted plasma	48.6 ± 2.91

CHAPTER FOUR

CONCLUSIONS

Cryogels are gel matrices that are formed in moderately frozen solutions of monomeric or polymeric precursors. Cryogels play an important role as matrices in advanced separation, e.g., high performance affinity chromatography or capillary electrochromatography of proteins (Bandilla, 2003; Edair, 2003). Cryogels typically have interconnected macropores (or supermacropores), allowing unhindered diffusion of solutes of practically any size, as well as mass transport of nano- and even microparticles. The supermacroporous monolithic cryogel columns present an attractive chromatographic medium with unique properties such as extremely low flow resistance, high mechanical and chemical stability, allowing drying/reswelling or sterilization of swollen monolithic columns. (Plieva, Andersson, Galaev, & Mattiasson, 2004) Cryogelic affinity adsorption is a developing bioseparation technique that has been studied for various proteins such as cytochrome C and bovine serum albumin immunoglobulin G, polynucleotides (Babaç, Yavuz, Galaev, Pişkin, & Denizli, 2006) and lysozyme (Sun, & Chai, 2002). In this study, a supermacroporous monolithic cryogel was produced by copolymerization in the frozen state of monomers, acrylamide (AAm) and allyl glycidyl ether (AGE) with N,N'-methylene-bis(acrylamide) (MBAAm) as a cross-linker in the presence of amonium persulfate (APS)/ N,N,N',N'-tetramethylene diamine (TEMED) as initiator/activator pair. Poly(AAm-AGE) cryogel produced in such a way has non-porous and thin polymer walls, large continuous inter-connected pores (10-100 μm in diameter, supermacro-porous) that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the protein molecules, allowing them to pass easily. The functional epoxy groups on the surface of the pores in monolithic cryogels allowed their modification with the ligand, Cibacron F3GA. Dye-ligands have been considered as one of the important alternatives to natural counterparts for specific affinity chromatography. Dye-ligands are able to bind most types of proteins, especially enzymes, in some cases in a remarkably specific manner. Although dyes are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many

proteins by mimicking the structure of the substrates, cofactors, or binding agents for those proteins (Denizli & Pişkin, 2001). The binding site of a protein is a unique stereochemical arrangement of ionic, polar, and hydrophobic groups in its three-dimensional structure, and where the polypeptide chains probably exhibit greatest flexibility. The triazine dye Cibacron Blue F3GA immobilized to an poly(AAm-AGE) cryogel provided an efficient method to purify HSA, showing high binding capacity and high selectivity, assuring a recovery of about 92%. HSA is a single, non-glycosylated, polypeptide chain containing 585 amino acid residues and has many physiological functions which contribute significantly to colloid osmotic blood pressure and aid in the transport, distribution and metabolism of many endogeneous and exogeneous substances including bile acids, bilirubin, long-chain fatty acids, amino acids (notably tryptophan, thyrosine and cysteine), steroids (progesterone, testosterone, aldosterone, cortisol), metal ions such as copper, zinc, calcium and magnesium and numerous pharmaceuticals (Carter, & Ho, 1994). HSA commonly used for therapeutic purposes such as shock, heavy loss of blood etc., requires relatively high purity for medical use. The aim of this study was to prepare Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel for highly pure HSA adsorption. In conclusion, it was possible to use the Cibacron Blue F3GA-attached poly(AAm-AGE) cryogel in a HSA adsorption-desorption cycle repeatedly.

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