DOKUZ EYLÜL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CIBACRON BLUE F3GA ATTACHED mP(HEMA) BEADS FOR CASEIN PURIFICATION

by

Funda AKDOĞAN

December, 2008

İZMİR

CIBACRON BLUE F3GA ATTACHED mP(HEMA) BEADS FOR CASEIN PURIFICATION

A Thesis Submitted to the
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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **CIBACRON BLUE F3GA ATTACHED mP(HEMA) BEADS FOR CASEIN PURIFICATION** completed by **FUNDA AKDOĞAN** under supervision of **ASSIST. PROF. 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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CIBACRON BLUE F3GA ATTACHED mP(HEMA) BEADS FOR CASEIN PURIFICATION

ABSTRACT

Magnetic poly(2-hydroxyethylmethacrylate) [m P(HEMA)] beads carrying Cibacron Blue F3GA were prepared by suspension polymerization of HEMA. Cibacron Blue F3GA was covalently coupled with magnetic poly(HEMA) microbeads via the nucleophilic substitution reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA molecules under alkaline conditions. Dye-attached mP(HEMA) beads were characterized by Fourier transform infrared spectrometer (FTIR), scanning electron microscopy, elemental analysis, swelling studies. The affinity sorbent carrying 68.3micromol Cibacron Blue F3GA/g was in the 100-140 micrometer particle size range. The swelling ratio of the mP(HEMA) was 62.12 percent. Adsorption studies were performed under different conditions in batch system (i.e., pH, protein initial concentration, temperature, and ionic strength) and than in magnetically stabilized fluidized bed (MSFB) system. The maximum adsorption was observed at pH 5.0, which is the isoelectric pH of beta casein. The maximum amount of beta-casein adsorption from aqueous solution was 57.52 mg/g in batch system and 72.09 mg/g in MSFB system at pH 5.0 (Studied casein concentration: 0.5 mg/ml). Up to 90 percent of the adsorbed beta-casein was removed by 1.5 M NaCl as elution agent. We concluded that Cibacron Blue F3GA attached magnetic poly(HEMA) could be repeatedly applied for beta-casein adsorption without significant losses in the adsorption capacity.

Keywords: Dye –affinity chromatography, Magnetic poly(HEMA), Protein purification, Casein.

CIBACRON BLUE F3GA TAKILI mP(HEMA) KÜRELERİ İLE KAZEİN SAFLAŞTIRILMASI

ÖZ

Cibacron Blue F3GA takılı magnetik poli(2-hydroxyethylmethacrylate) mP(HEMA) mikro küreleri, HEMA manomeri kullanılarak süspansiyon polimerizasyonu ile hazırlandı. Cibacron Blue F3GA, magnetik poli(HEMA) kürelerine alkali koşullarda triazin halkasındaki klorit ve HEMA molekülündeki hidroksil grupları arasındaki nükleofilik substitusyon reaksiyonu ile kovalent olarak immobilize edildi. Boya takılı magnetik poli(HEMA) küreleri, FTIR, taramalı elektron mikroskobu, elementel analiz ve şişme testi ile karakterize edildi. Magnetik mikro küreler, 68.3 mikromol Cibacron Blue F3GA/g içermekte olup 100-140 mikrometre gözenek boyutuna sahiptir. Magnetik P(HEMA) kürelerinin şişme derecesi % 62.12 dir. Adsorpsiyon, kesikli sistemde farklı koşular altında (pH, başlangıç protein konsantrasyonu, sıcaklık, iyonik şiddet) gerçekleştirildi, daha sonra manyetik akışkan yatak sisteminde çalışıldı. Maksimum adsorpsiyon beta-kazeinin izoelektrik noktası olan pH 5 te gözlendi. Sulu çözeltiden adsorplanan maksimum β-kazein miktarı pH 5 te 0.5 mg/ml beta-casein konsantrasyonu için, kesikli sistemde 57.52 mg/g iken aynı koşullarda manyetik akışkan yatak sisteminde 72.09 mg/g dır. Yüzde 90 nın üzerinde adsorplanan beta-kazein, 1.5 M NaCl ajanı ile elue edildi. Adsorpsiyon kapasitesinde önemli bir azalma olmaksızın Cibacron Blue F3GA takılı magnetik poli(HEMA) küreleri tekrar kullanılabilmektedir.

Anahtar Sözcükler: Boya-affinite kromotografisi, Magnetik poli(HEMA), Protein saflaştırma, Kazein.

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CHAPTER ONE
INTRODUCTION

1.1 General Protein Description

Proteins are large biological molecules with molecular weight up to few million Daltons. Proteins are made of amino acids linked into linear chains, called polypeptide chains. Amino acids links between each other by peptide bonds - this peptide bond is formed between the carboxyl and amino groups of neighboring amino acids. Proteins are responsible for many different functions in the living cell. It is possible to classify proteins on the basis of their functions (Protein-general description, 2007).

1.1.1 Protein classification by function

Enzymes**;** proteins that catalyze chemical and biochemical reactions with in living cell and outside. This group of proteins probably is the biggest and most important group of the proteins. Enzymes are responsible for all metabolic reactions in the living cells. Well known and very interesting examples are: DNA- and RNA-polymerases, dehydrogenises etc.

Hormones; proteins that are responsible for the regulation of many processes in organisms. Hormones are usually quite small and can be classifies as peptides. Most known protein hormones are: insulin grows factor, lipotropin, prolactin etc. Many protein hormones are predecessor of peptide hormones, such as endorphin, encephalin etc. It is possible to increase this group of proteins by adding of all protein venoms.

Transport proteins; these proteins are transporting or store some other chemical compounds and ions. Some of them are well known: cytochrome C - electron transport; hemoglobin and myoglobin - oxygen transport; albumin - fatty acid transport in the

blood stream etc. It is possible to classify trance membrane protein channels as a transport proteins as well.

Immunoglobulin or antibodies; proteins that involved into immune response of the organism to neutralize large foreign molecules, which can be a part of an infection. Sometimes antibodies can act as enzymes. Sometimes this group of proteins is considered as a bigger group of protective proteins with adding such proteins as lymphocyte antigen-recognizing receptors, antiviral agents such as interferon, tumor necrosis factor (TNF). Probably the clotting of blood proteins, such as fibrin and thrombin should be classified as protective proteins as well.

Structural proteins; these proteins are maintain structures of other biological components, like cells and tissues. Collagen, elastin, α-keratin, sklerotin, fibroin - these proteins are involved into formation of the whole organism body. Bacterial proteoglycans and virus coating proteins also belongs to this group of proteins. Currently we do not know about other functions of these proteins.

Motor function; these proteins can convert chemical energy into mechanical energy. Actin and myosin are responsible for muscular motion. Sometimes it is difficult to make a strict separation between structural and motion proteins.

Receptors; these proteins are responsible for signal detection and translation into other type of signal. Sometimes these proteins are active only in complex with low molecular weight compounds. A very well known member of this protein family is id rhodopsin - light detecting protein. Many receptors are trans membrane proteins.

Signaling proteins; this group of proteins is involved into signaling translation process. Usually they significantly change conformation in presence of some signaling molecules. These proteins can act as enzymes. Other proteins, usually small, can interact with receptors. Classical example of this group of proteins is GTPases.

Storage proteins; these proteins contain energy, which can be released during metabolism processes in the organism. Egg ovalbumin and milk casein are such proteins. Almost all proteins can be digested and used as a source of energy and building material by other organisms (Protein-general description, 2007).

1.2 Principles of Separation Techniques

One significant aspect of biotechnology research is to use protein engineering techniques to design or modify proteins with optimized properties for specific industrial applications. In order to do this, scientists must be able to isolate and purify proteins of interest so their conformations, substrate specificities, reactions with other ligands, and specific activities can be studied. Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest (Protein-general description, 2007).

Several protein purification methods, with varying degrees of efficiency and specificity, are outlined below.

The degree of protein purity required depends on the intended end use of the protein. For some applications, a crude extract is sufficient. However, for other uses, such as in foods and pharmaceuticals, a high level of purity is required. In order to achieve this, several protein purification methods are typically used, in a series of purification steps.

The first step in purifying intracellular proteins is preparation of a crude extract. The extract will contain a complex mixture of all the proteins from the cell cytoplasm, and some additional macromolecules, cofactors and nutrients. Crude extract may be used for some applications in biotechnology, however, if purity is an issue, subsequent purification steps must be followed.

Intermediate Purification Steps; usually the next step to purifying a protein from a crude extract is by precipitation in a solution with high osmotic strength (i.e. salt

solutions). Nucleic acids in the crude extract can be removed by precipitating aggregates formed with streptomycin sulfate or protamine sulfate. Protein precipitation is usually done using ammonium sulfate as the salt.

Salts in the solution are then removed by dialysis through porous cellulose tubing, membrane filtration, or gel exclusion chromatography (Methods for protein purification, 2007).

All separation procedures depend primarily upon some physical characteristic of the compounds. It is relatively easy to separate substances that have significantly different physical characteristics by simple techniques, such as solvent extraction. However, if the various compounds are similar each other then it are essential that any slight differences between them are exploited in order to achieve separation.

Table 1.1 Classification of separation techniques

Methods based on polarity	• Liquid-solid chromatography (adsorption) • Liquid-liquid chromatography • Gas-liquid chromatography
Methods based on ionic nature	• Electrophoresis, Isotachophoresis • Iso-electric focusing • Capillary electrophoresis • Ion-exchange chromatography
Methods based on size	• Ultracentrifugation • Dialysis and ultra filtration, • Gel permeating chromatography
Methods based on shape	• Affinity chromatography

The polarity of a molecule is a characteristic that significantly affects many of its properties, in particular its solubility and volatility. Separation techniques, such as gas-liquid chromatography, liquid chromatography and adsorption chromatography, all depend to a large extent on polar interactions and slight differences in polarity can be used to facilitate separation.

The rate of migration in an electric field (electrophoresis) and the affinity for ions of opposite charge (ion-exchange chromatography) are extremely valuable techniques in the separation of ionic species.

Molecules that very significantly in their size can be separated by ultra filtration or dialysis, while molecules that are only slightly different in size can often be separated by gel permeation chromatography.

Many molecules show a definite affinity for another molecule based on a shape relationship, e.g. enzymes and their substrates, antibodies and their antigens, and such relationships form the basis of extremely useful and specific methods of separation known generally as affinity chromatography (Holme & Peck, 1989, p.92-94).

1.3 Chromatographic Protein Purification Methods

Chromatographic methods can be applied using bench-top columns or automated HPLC equipment. Separation by HPLC can be done by reverse-phase, ion-exchange or size-exclusion methods, and samples detected by diode array or laser technology.

At the below, figure 1.1 shows the separation principles in general chromatographic purification.

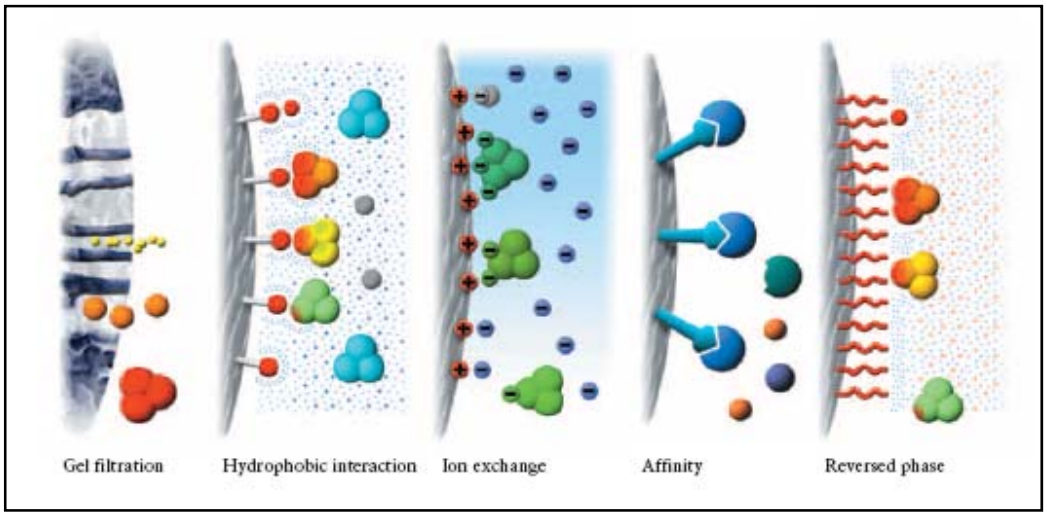

Figure 1.1 Separation principles in chromatographic purification.

Reverse-phase chromatography (RPC) separates proteins based on their relative hydrophobicities. This technique is highly selective but requires the use of organic solvents. Some proteins are permanently denatured by solvents and will lose functionality during RPC, therefore this method is not recommended for all applications, particularly if it is necessary for the target protein to retain activity.

Ion-exchange chromatography refers to separation of proteins based on charge. Columns can either be prepared for anion exchange or cation exchange. Anion exchange columns contain a stationary phase with a positive charge that attracts negatively charged proteins. Cation exchange columns are the reverse, negatively charged beads which attract positively charged proteins. Elution of the target protein(s) is done by changing the pH in the column, which results in a change or neutralization of the charged functional groups of each protein.

Size-exclusion chromatography (gel filtration) separates larger proteins from small ones, since the larger molecules travel faster through the cross-linked polymer in the chromatography column. The large proteins do not fit into the pores of the polymer whereas smaller proteins do, and take longer to travel through the chromatography column, via their less direct route. Eluate is collected in a series of tubes separating proteins based on elution time. Gel filtration is a useful tool for concentrating a protein sample, since the target protein is collect in a smaller elution volume than was initially added to the column. Similar filtration techniques might be used during large scale protein production because of their cost-effectiveness.

Affinity chromatography is a very useful technique for "polishing" or completing the protein purification process. Beads in the chromatography column are cross-linked to ligands that bind specifically to the target protein. The protein is then removed from the column by rinsing with a solution containing free ligands. This method generally gives the purest results and highest specific activity compared to other techniques. (Methods for protein purification, 2007).

1.4 Affinity Chromatography

Affinity chromatography is a method in which bio specific and reversible interactions are used for the selective extraction, separation and purification of biologically active material from crude samples (Wilchek, 2004). These interactions may occur between a

protein and a low molecular weight compound, e.g. an enzyme and inhibitor or between two proteins, such as an antibody and an antigen. In affinity chromatography such biological affinities are exploited for the purification of proteins. One partner of the interaction pair, the ligand, is covalently bound to a solid matrix. The resulting affinity resin, packed into a chromatographic column, is then used to adsorb the protein of interest (the counterligand) from the crude extract that is passing the chromatographic column. With an appropriate elution buffer the purified protein can then be recovered from the affinity (Hochuli, 1992).

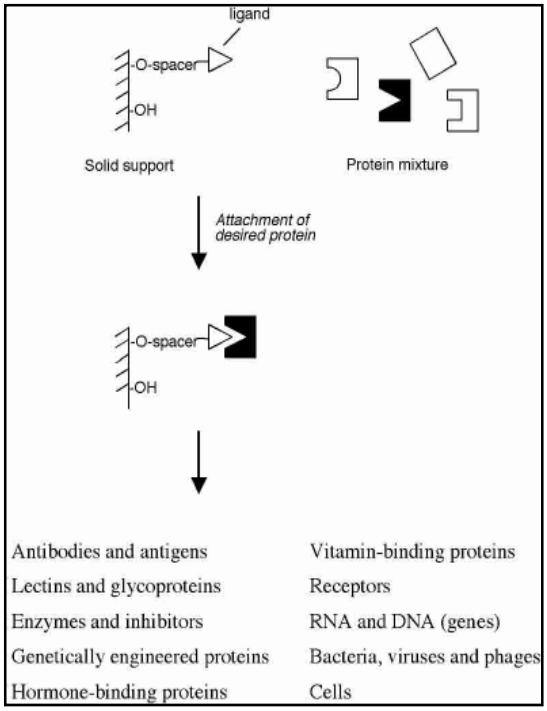

Figure 1.2 Principle of affinity chromatography (Wilchek, 2004)

The above Figure 1.2 shows the principle of affinity chromatography and short list of target materials that have been purified.

Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological

function or individual chemical structure. The technique offers high selectivity, hence high resolutions, and usually high capacity for protein of interest. Purification can be in the order of several thousand-fold and recoveries of active material are generally very high. Target protein is collected in a purified, concentrated form. The technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure substances present at low concentration in large volumes of crude samples and also to remove specific contaminants (Affinity chromatography, principles and methods, 2007).

Specific interactions of molecules are a characteristic of all living cell and of fundamental importance for their functioning. It has been recognized already since almost 30 years that the specific affinity of a certain protein for a biological ligand can be exploited for its purification in a chromatographic manner if certain criteria are fulfilled and the term affinity chromatography has been coined. Since then affinity chromatography represents one of the most powerful tools in purification techniques for studying many biological processes, such as the mechanism actions of enzymes, hormones, protein-protein or cell-cell interaction and others (Telefoncu, 1996, p. 211-212).

Table 1.2 Various techniques derived from Affinity Chromatography

1. Affinity Capillary Electrophoresis	12. Immunoaffinity Chromatography
2. Affinity Electrophoresis	13. Lectin Affinity Chromatography
3. Affinity Partitioning	14. Library - Derived Affinity Ligands
4. Affinity Precipitation	15. Membrane –Based Affinity Chromatography
5. Affinity Repulsion Chromatography	16. Metal - Chelate Affinity Chromatography
6. Affinity TAG Chromatography	17. Moleculer Imprinting Affinity
7. Avidin –Biotin Immobilized System	18.Perfusion Affinity
8. Covalent Affinity Chromatography	19.Reseptor Affinity Chromatography
9. Dye –Ligand Chromatography	20. Tandem Affinity Purification
10. High Performance Chromatography	21. Thiophilic Chromatography
11. Hydrophobic Chromatography	22. Weak Affinity Chromatography

1.4.1 Bioaffinity Chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix. The technique is ideal for a capture or intermediate step in a purification protocol and can be used whenever a suitable ligand is available for the protein(s) of interest. Biological interact ions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, Van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity.

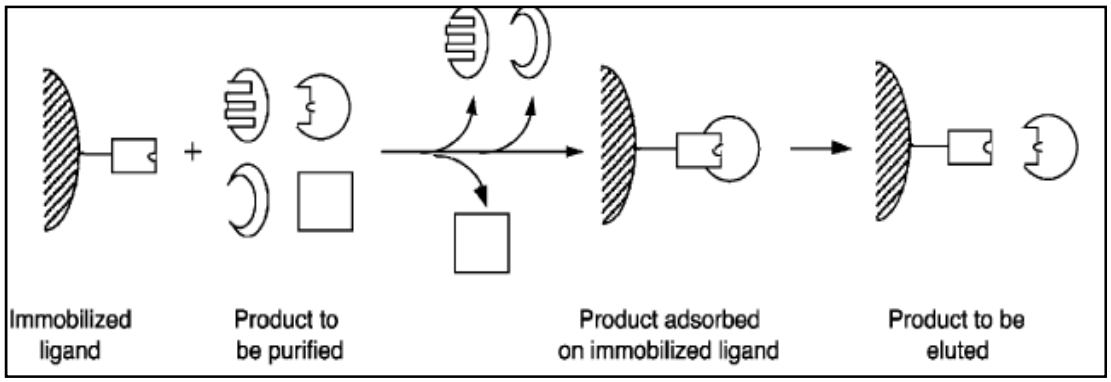

Figure 1.3 Principle of affinity chromatography.

In a single step, affinity purification can offer immense time-saving over less selective multistep procedures. The concentrating effect enables large volumes to be processed. Target molecules can be purified from complex biological mixtures, native forms can be separated from denatured forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances. Successful affinity purification requires a biospecific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules

to be removed in an active form. Any component can be used as a ligand to purify its respective binding partner (Affinity chromatography, principles and methods, 2007).

Some typical biological interactions, frequently used in affinity chromatography are listed below:

- Enzyme - substrate analogue, inhibitor, cofactor.
- Antibody - antigen, virus, cell.
- Lectin - polysaccharide, glycoprotein, cell surface receptor, cell.
- Nucleic acid - complementary base sequence, histones, nucleic acid polymerase,
- Nucleic acid binding protein.
- Hormone, vitamin - receptor, carrier protein.
- Glutathione - glutathione-S-transferase or GST fusion proteins.
- Metal ions- Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces.

Ligands which contain amino acids can be coupled through the ε-amino group of lysine, carboxyl groups of aspartate and glutamate, and the phenolic group of tyrosine. Chemistries which randomly couple these residues also result in random orientation and spacing of the immobilized ligand.

An effective immobilization method must consider the following factors. The functional groups on the ligand that is available for coupling may be one or more of amine, thiol, alcohol, and carboxylic acid. Spacer arms are often needed one small solutes are immobilized so that the immobilized ligand may access the binding site of a macromolecule; these spacers are often substituted hydrocarbon chains, and may alter the binding properties through hydrophobic or ionic interactions (neutral hydrophilic spacers such as polyethylene glycol have been very effective). The pH during immobilizations must be controlled, since this determines the reactivity of the functional

groups and may cause irreversible damage of the ligand or support at extreme values. Finally the density of active groups on the support must be considered: multipoint attachment of large ligands to a support yields high stability, but may distort the binding site and decrease affinity; and improper orientation or spacing of the ligands may also lead to reduced affinity, so that immobilization conditions must be optimized (Mikkelsen & Corton, 2004, p. 280-281)

There are many methods for immobilizations of ligand molecules onto the support matrix, in which usually several intermediate steps are followed. The main points for a successful ligand immobilization are given below. Note that the correct choice of coupling method and conditions depend on both the matrix and the ligand (Denizli & Pişkin, 2001).

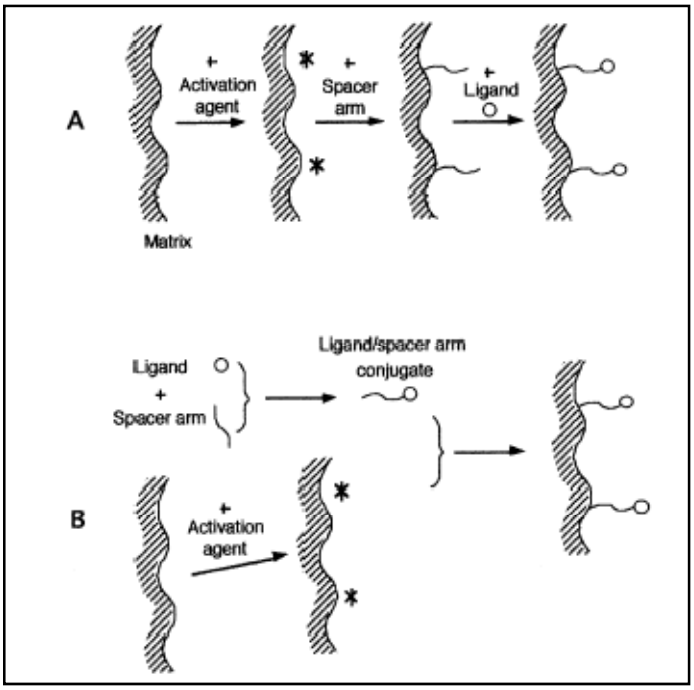

Figure 1.4 Strategies for coupling of ligands to the support matrix; (A. coupling through spacer arm;) B. Coupling through spacer arm-ligand conjugates (Denizli & Pişkin, 2001).

1.4.2 Dye Ligand System

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 (Lillehoj & Malik, 1982; Chase, 1988; Denizli & Pişkin, 1995a; Denizli, Say, & Pişkin, 1995; Denizli & Pişkin, 1995b). 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. 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. 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.

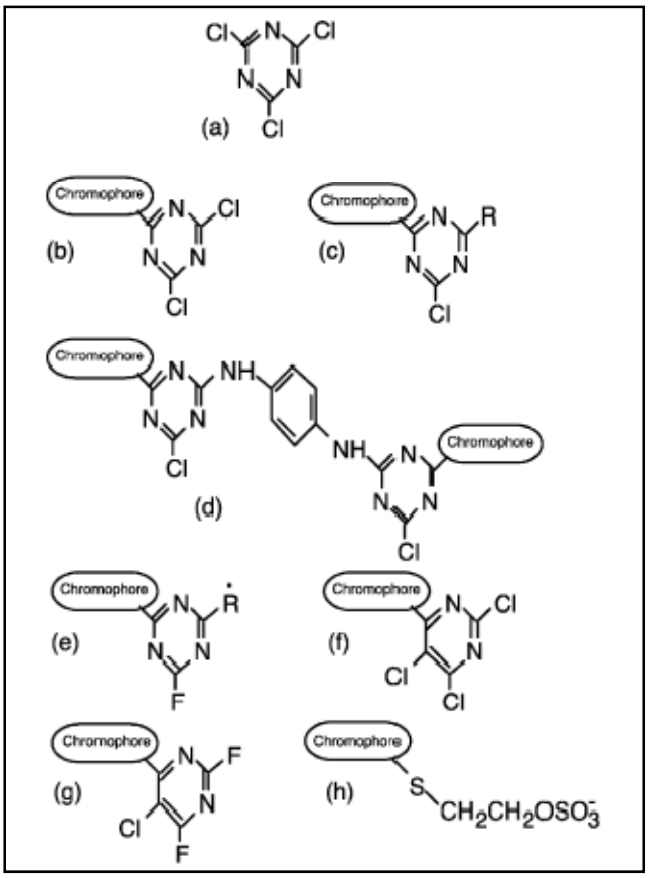

Figure 1.5 Structure of some of the reactive dye molecules; (a) cyanuric chloride; (b) Procion MX series (ICI); (c) Cibacron (Ciba-Geigy) and Procion H (ICI); (d) Procion H-E (ICI); (e) Monofluorotriazinyl, Cibacron, Ciba-Geigy; (f) Trichloropyrimidnyl, Drimarene, Sandoz; (g) Difluorochloro-pyrimidnyl, Levafix, Bayer and Drimarene, Sandoz; (h) Sulfatoethyl sulfone, Remazol, Hoechst (Denizli & Pişkin, 2001).

Today, triazinyl-based reactive dyes are most widely used in protein purification. Cyanuric chloride (1,3,5-trichloro-sym-triazine) is the basic substance used in the synthesis of these dyes (Figure 1.5a.). The presence of electronegative atoms makes the three carbon atoms highly positive, and therefore very susceptible to nucleophilic attacks.

Chromophore molecules are easily attached to this molecule to form the dichlorotriazinyl dyes. The Procion MX series (from Imperial Chemical Industries) is a typical example of this type of dyes (Figure 1.5b.). By further reactions of these molecules with other nucleophilic substituents (such as aniline or sulfanilates), monochlorotriazinyl dye are synthesized. Cibacron (from Ciba-Geig) and Procion H (from ICI), shown in Figure 1.5c, are two examples of monochlorotriazinyl dyes. The only difference between 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.

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 1.5d. 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 1.5e-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 on the matrix at the ligand-immobilization step. One interesting group of dyes not based on triazinyl groups is the Remazol series from Hoechst, which to attach the matrix with vinyl sulfone active groups, have found use as dye-ligands in protein purification (Figure 1.5h).

An important strategy is to tailor-make, or re design the dye structure to improve the specificity of textile dyes for target proteins. This new type of ligand is called biomimetic dye. It carries all the advantages of the parent (unmodified) dye including high specificity (Denizli & Pişkin, 2001).

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. 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 (Denizli & Pişkin, 2001).

Cibacron blue F3GA (CB) is a triazinyl dye widely used as the affinity ligand for dye-ligand chromatography. CB is structurally similar to naturally occurring heterocycles, such as nucleosidephosphate, $NAD^{+,}$ coenzyme A, and folic acid. It has been demonstrated that CB specifically binds to nucleotide binding sites of kinases and dehydrogenases and that some of the enzyme activities are inhibited by CB (Shirai et al.,1998).

Cibracron Blue F3G-A, the most thoroughly studied dye-ligand, binds with a variety of proteins such as adenine coenzyme dependent oxidreductases, phosphokinases, hydrolases, transferases, nucleases, polymerases, synthetases, lyases, decarboxylases, in addition to glycolytic enzymes, and plasma proteins.

1.4.2.1 Procedures for Using Immobilized Dye Ligand

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 (through 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 et al, 1976; Burton et al, 1988; Clonis 1986; Clonis et al 1987; Hey & Dean, 1981). Coupling is achieved at alkaline conditions by nucleophilic substitution of hydroxyl groups with the reactive chlorine on the dye molecules (Figure. 1.6)

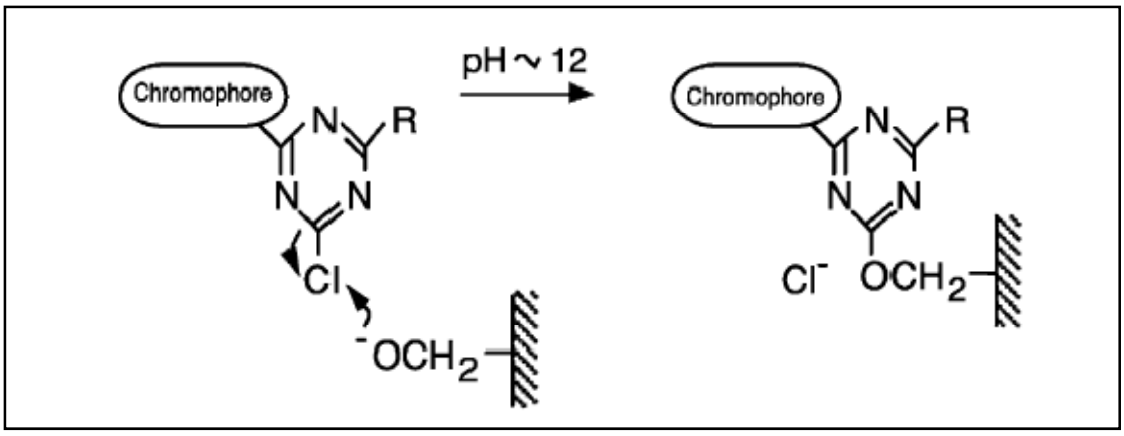

Figure 1.6 Coupling of triazinyl dyes to the matrix bearing hydroxyl groups.

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. Coupling can be achieved at room temperature (20–30 ^{0}C) at pH: 10–12 in about 2–3 days with monochlorotriazinyl dyes (e.g., Cibacron and Procion H series.) However, because of their higher reactivity, 1–2 h may be sufficient for dichlorotriazinyl dyes (e.g., Procion MX-series) at the same conditions. It has been found also that a

similar substitution can be achieved with monochlorotriazinyl dyes at higher temperatures (e.g., 80–90 ^{0}C.)

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).

1.4.2.2 Interactions between dye-ligands and proteins

The binding site of a protein is a unique stereo chemical 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 (Kominska, Dezieciol, & Koscielak, 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 et al, 1985; Lascu et al, 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.

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).

1.4.2.3 Beta Casein

Milk is the most important food for young mammals and a common source of proteins and micro elements for adult people (Kaminski, S., Cieslinska, A., & Kostyra, E., 2007). In milk there are 2 major protein groups: caseins and whey proteins. Caseins account for ca. 80% of bovine milk protein (Niki et al.1994; Martien et al. 1994), whereas both major whey proteins constitute about 14% (McLachlan 2001; Roginski 2003). Bovine milk contains 4 caseins: alpha S1(CSN1S1, 39–46% of total caseins), alpha S2 (CSN1S2, 8–11%), beta (CSN2, 25–35%), and kappa (CSN3, 8–15%) (Eigel et al. 1984; Roginski 2003). There is also gamma-casein, which is a product of degradation of beta-casein (Ostersen et al. 1997; Miller et al. 1990).

β-casein, like other members of the casein family, is well characterized (Swaisgood, 1982). The molecular weight of casein is 23 000–24 000 daltons and it has 209 amino acid residues with very little secondary structure in aqueous solution. Its isoelectric point is at pH 4.9–5.2. Many of the first 50 amino acid groups in the molecular sequence carry a net negative charge at pH 7.0, making this portion of the molecule hydrophilic, whilst the remainder of the molecule is mainly made up of non-polar residues which make the majority of the chain hydrophobic. The distinctly amphiphilic nature of the sequence confers high surface activity, contributing to good foaming and emulsifying actions (Akgöl, Bereli, & Denizli, 2005).

β-Casein is the major component of the casein, which casein is known as a good protein emulsifier. Casein represents about the 80% of the protein in cow's milk and about 30% to 35% of that is β-casein (Akgöl et al., 2005).

Beta-caseins play a key role in the transport of essential ions and nutrients such as calcium and phosphorous. It is known that the beta caseins in milk, when digested, can yield a plethora of bioactive protein fragments, a number of which have been well characterized with regard to potential biological activity.

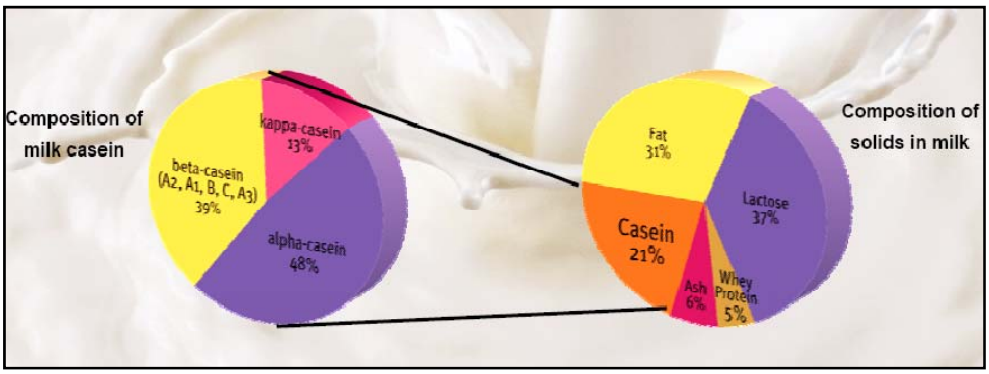

Figure 1.7 Composition of milk

Caseins from milk and β-casein in particular are surface active. Caseins are excellent glues and paint binders and have been used for that purpose since early times (De Kruif,

Grinberg, 2002). Even today, caseins are hard to replace in a number of applications. For instance, beer bottle labels are still glued with casein and shadow masks for TV-tubes are still produced using casein (De Kruif, 2003). The separation of caseins has long been one of the fundamental subjects of casein studies (Swaisgood, 1992). Chromatographic procedures (e.g., DEAE-cellulose) using urea buffers are widely used at present. However, traditional precipitation procedures are still attractive in some respects. For example, there are no requirements for special equipment and there is the capability to scale-up to obtain a large quantity of final products (Igarashi, 1999). β-casein, like other members of the casein family, is well characterized (Swaisgood, 1982).

1.5 Properties and Use of Magnetic Adsorbents

New separation techniques, capable of treating dilute solutions or solutions containing only minute amounts of target molecules in the presence of vast amounts of accompanying compounds in both small and large-scale processes, even in the presence of particulate matter, are necessary (Safarik & Safarikova, 2004). Magnetic micro beads (MMB) used in biomedical applications present usually a core/shell structure. The Magnetic Micro beads aim at interacting with the target molecule through biological recognition (Jaffrezic, Martelet, Chevolet, & Cloarec, 2007).

Table 1.3 Application of magnetic adsorbents on affinity seperation (Safarik & Safarikova, 2004)

Purification of albumin and hemoglobin by magnetic techniques

	Source	**Magnetic carrier**	**Affinity ligand**	**Further details**
Albumin, bovine serum	Commercial preparation	Magnetic agar beads	Cibacron blue3GA	Adsorption experiments
	Commercial preparation	Magnetic cross-linked polyvinylalcohol	Cibacron blue3GA	Adsorption experiments
		Magnetic chitosan microspheres	Cibacron blue3GA	
	Commercial preparation	Magnetic poly(glycidyl methacrylate-triallyl isocyanurate-divinylbenzene) particles		Anion exchange separation
	Commercial preparation	Magnetic poly(ethylene glycol dimethacrylate-co-N-methacryloyl-(L)-histidine methyl ester) microbeads	Cu^{2+}	Elution with 1.0 M NaSCN
Albumin, human serum	Commercial preparation	Magnetic poly(2-hydroxyethylmethacrylate) beads	Iminodiacetic acid charged with Cu^{2+}	Elution with 1.0 M NaSCN
	Human plasma	Magnetic poly(2-hydroxyethyl methacrylate) beads	Cibacron blue F3GA	Elution with 0.5 M NaSCN
	Commercial preparation	Magnetic particles covered with thermosensitive polymer	-	Desorption by decreasing temperature
Albumin, human serum (recombinant, FLAG tagged)	Yeast cells	Magnetic glass beads	Anti-FLAG antibody	Elution with EDTA containing buffer
Glycated hemoglobin	Human blood	Magnetic poly(vinyl alcohol) beads	m-Aminophenyl-boronic acid	Elution with sorbitol
Haemoglobin	Bovine, commercial preparation	Amine terminated iron oxide particles	Iminodiacetic acid charged with Cu^{2+}	Elution with imidazole containing buffer
Haemoglobin A1c	Human blood	Magnetic particles isolated from *Magnetospirillum magneticum* AMB-1	*m*-Aminophenyl-boronic acid	used for affinity immunoassay

Magnetic carriers and adsorbents can be either prepared in the laboratory, or commercially available ones can be used. Magnetic polymer micro beads and a method for preparing the same are provided. The method for preparing the magnetic polymer micro beads includes the following steps: preparing polymer particles; immersing the polymer particles into a solution in order to swell the polymer particles; adding magnetic nano particles to the solution and allowing the magnetic nano particles to enter an interior of the polymer particles; and separating the polymer particles from the solution, wherein the polymer particle is made of polystyrene, or a copolymer containing styrene, and the solution includes a medium polar solvent. The average particle size of the magnetic polymer micro beads of the present invention ranges from sub microns to microns. The magnetic polymer micro beads of the present invention have high magnetization, and the various functional groups can be introduced onto the surfaces thereof.

Such carriers are usually available in the form of magnetic particles prepared from various synthetic polymers, biopolymers or porous glass, or magnetic particles based on the inorganic magnetic materials such as surface modified magnetite can be used (Safarik & Safarikova, 2004).

Such micro beads have an inorganic core *e.g.* iron oxide, surrounded by an outer layer of shell wall that consists of long-chain organic ligands or inorganic/organic polymers. The attachment of bioactive ligands to the surface of the outer shell is the key to bio application of magnetic micro beads (Jaffrezic et al, 2007).

1.5.1 Properties Of Magnetic Fluidized Bed System

There has been several separation approaches performed under magnetic field. The most well known technique is the magnetically stabilized fluidized bed. Magnetically stabilized fluidized bed exhibits combination of the best characteristics of both packed and fluidized bed. These include the efficient fluid-solid mass transfer properties,

elimination of particle mixing, low pressure drop, high feed-stream solid tolerances, good fluid-solid contact, and elimination of clogging and continuous countercurrent operation. Especially, when dealing with highly viscous mediums contact with the magnetic adsorbent in a magnetically stabilized fluidized bed is desirable because of high convective transport rates. Recently, there has been increased interest in the use of magnetic adsorbents in biomolecule coupling and nucleic acid purification. Magnetic adsorbents can be produced using inorganic materials or polymers. High mechanical resistance, insolubility and excellent shelf life make inorganic materials ideal as adsorbent. The main disadvantage of inorganic supports is their limited functional groups for ligand coupling. Magnetic adsorbents can be porous or non-porous. They are more commonly manufactured from polymers since they have a variety of surface functional groups which can be tailored to use in different applications (Yavuz, Say, Andaç, Bayraktar, & Denizli, 2004).

In this study Cibacron Blue F3GA attached magnetic p(HEMA) microbeads were prepared for the adsorption of β- Casein, mP(HEMA) beads were prepared by suspension polymerization. Cibacron Blue F3GA was covalently attached to the mP(HEMA) microbeads via nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA. After ligand immobilization, mP(HEMA) microbeads were used β- Casein adsorption. Effects of some variables on adsorption such as pH, initial β- Casein concentration are reported here.

CHAPTER TWO
EXPERIMENTAL METHODS AND MATERIALS

2.1 Materials

β-casein were supplied from Sigma (St Louis, USA). Cibacron Blue F3GA were supplied by Sigma (St. Louis, MO, USA). 2-Hydroxyethyl methacrylate (HEMA) was purchased from Sigma (St. Louis, MO, USA) and purified by vacuum distillation under a nitrogen atmosphere. Ethylene glycol dimethacrylate (EGDMA, Merck, Darmstadt, Germany) was used as the crosslinking agent. Azobisisobutyronitrile (AIBN, Sigma) was selected as the initiator. Polyvinyl alcohol (PVA) (*Mn*: 100,000, 98% hydrolyzed, Aldrich, Rockford, IL). Magnetite particles (Fe_2O_3, were obtained from Aldrich (USA) All other chemicals were obtained from Merck as analytical grade. All water used in the experiments was purified using a Millipore Corporation (France) Elix 5® reverse osmosis unit with an electrodeionisation (E.D.I.) module (Elix® 5 Water Purification System) followed by a Milli-Q Synthesis A10 Ultrapure® (Milli-Q® Ultrapure Water Purification Systems) organic/colloid removal and ion exchange packed bed system. 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 Preparation of Magnetic PHEMA Beads

The mPHEMA beads were prepared by suspension polymerization. A typical suspension copolymerization procedure of mP(HEMA) beads was performed as below: The dispersion medium was prepared by dissolving 200 mg of poly (vinyl alcohol) (PVA; molecular weight: 50.000) within 50 ml of distilled water. The desired amount of 2,2'azobisisobutyronitrile (AIBN) (0.04 g) was dissolved within the monomer phase 8.0/ 4.0/12.0 ml (EGDMA/HEMA/toluene) with 0.75 g magnetite particles (Fe_2O_3). This solution was then transferred into the dispersion medium placed in a magnetically stirred

(at a constant stirring rate of 600 rpm) glass polymerization reactor (100 ml) which was in a thermostatic water bath. The reactor was flushed by bubbling nitrogen and then was sealed. The reactor temperature was kept at 65°C for 4 h. The temperature was then raised to 90^0C and kept constant by a thermostated water bath during the polymerization time (2h). After polymerization, the mPHEMA beads were separated from the polymerization medium. The residuals (e.g., unconverted monomer, initiator and other ingredients) were removed by a cleaning procedure. Briefly, beads were transferred into a reservoir, and washing solutions (i.e., a dilute HCI solution and a water-ethanol mixture) were recirculated through the system which includes also an activated carbon column, to be assured that the magnetic beads were clean. Purity of the magnetic beads was followed by observing the change of optical densities of the samples (λ: 280 nm) taken from the liquid phase in the recirculation system, and also from the DSC thermograms of the magnetic beads obtained by using a differential scanning microcalorimeter (Mettler, Switzerland). Optical density of uncleaned magnetic beads was 2.63, but after the cleaning operation this value was reduced to zero. In addition, when the thermogram of uncleaned beads was recorded, it had a peak around 60^0C. This peak might originate from AIBN, but after application of the cleaning procedure, no peak between 30–100°C was observed on the thermogram. The dry density of the magnetic beads was measured with pycnometer by dispersing the dry beads in ethanol (Yavuz et al., 2004)

2.3 Cibacron Blue F3GA Immobilization on Magnetic poly(HEMA)

Cibacron Blue F3GA was covalently attached to the mP(HEMA) microbeads via a nucleophilic reaction between the chloride of its triazine ring and the hydroxyl groups of the HEMA molecules under alkaline conditions with the elimination of HCl, resulting in the coupling of Cibacron Blue F3GA to the PHEMA microbeads.

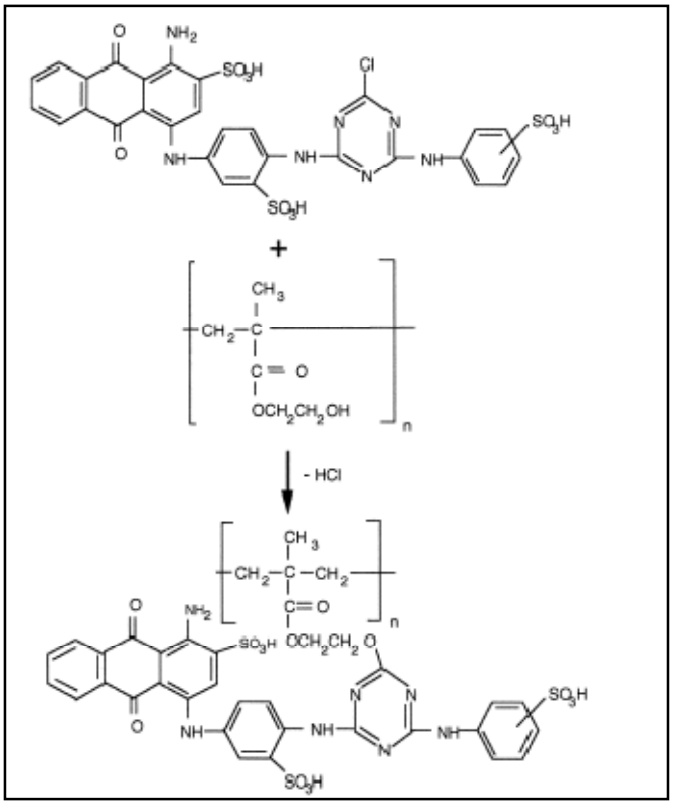

Figure 2.1 Attachment of Cibacron Blue F3GA to PHEMA.

Ten grams of mPHEMA beads was magnetically stirred (at 400 rpm) in a sealed reactor at a constant temperature of 80 ^{0}C for 4 h with 100 ml of the Cibacron Blue F3GA aqueous solution containing 4.0 g NaOH. The initial concentration of the Cibacron Blue F3GA in the medium was 1.0 mg/ml. After incubation, the Cibacron Blue F3GA-attached beads were filtered, and washed with distilled water and methanol several times until all the physically adsorbed Cibacron Blue F3GA molecules were removed. The modified beads were stored at 4 ◦C with 0.02% sodium azide to prevent microbial contamination (Başar, Uzun, Güner, & Denizli, 2007).

The amount of Cibacron Blue F3GA attached onto microbeads was evaluated by using an elemental analysis instrument (Leco, CHNS-932, USA), and measuring the nitrogen and sulfur stoichiometry.

2.4 Characterization of Cibacron Blue F3GA Attachment Magnetic poly(HEMA)

FTIR spectra of the Cibacron Blue F3GA, the magnetic poly(HEMA) beads and Cibacron Blue F3GA-attached magnetic poly(HEMA) beads were obtained by using a FTIR spectro-photometer (FTIR 8000 Series, Shimadzu, Japan). The dry microbeads (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.

To determine the equilibrium water uptake of PHEMA microbeads, approximately 3.0 g of dry polymer sample were put into a cylindirical tube. The height of the bed formed by the dry microbeads (H_d) was measured. Then, 50 ml of buffer solution at a certain pH and ionic strength was added into the tube. The sealed tube was shaken on a rotater with 30 rev. /min for 24 h. At the end of this period; the height of the bed formed by swollen PHEMA microbeads (*H*s) was recorded. The equilibrium water uptake ratio was calculated based on the following expression

$$Equilibrium\ water\ uptake\ ratio = \frac{Hs}{Hd} \times 100$$

Elemental Analysis of Cibacron Blue F3GA attached mP(HEMA) to evaluate the degree of Cibacron Blue F3GA incorporation m(PHEMA) beads were subjected to elemental analysis using a Leco Elemental Analyzer (Model CHNS-932).

The degree of magnetism of the m-PHEMA beads was measured in a magnetic field by using a vibrating sample magnetometer (Princeton Applied Research, Model 150A, USA). The presence of magnetite particles in the polymeric structure was investigated with an electron spin resonance (ESR) spectrophotometer (EL 9, Varian). The presence of magnetite particles in the polymeric structure was confirmed by ESR.

The surface morphology of the polymeric beads was examined using scanning electron microscopy (SEM). The samples were initially dried in air at 25^0 C for seven

days before being analyzed. A fragment of the dried bead was mounted on a SEM sample mount and was sputler coated for 2 minutes The sample was then mounted in a scanning electron microscope (Model: Raster Electronen Microscopy, Leitz- AMR - 1000, Germany). The surface of the sample was then scanned at the desired magnification to study the morphology of Cibacron Blue F3GA attached mP(HEMA) beads.

2.5 Chromatographic Purification Procedures

2.5.1 Beta-Casein Adsorption from Aqueous Solutions

Beta Casein was selected as a model protein for adsorption studies. At first, chromatographic adsorption was studied in batch systems. Following this, studies were carried in optimal conditions and on a magnetically stabilized fluidized bed system by using BioRad economic column (diameter: 1 cm, length: 10 cm). Effects of initial beta Casein concentration, pH of the medium, temperature and ionic strength on the adsorption capacity were studied. The Cibacron Blue F3GA attached magnetic poly (HEMA) microbeads were studied at various pH. The pH of the adsorption medium was varying between 4.0 and 7.0 by using different buffer systems. 0.1 M acetate buffer was used for pH 4.0 – 5.5 and 0.1 M phosphate buffer was used for pH 6.0 – 7.0. The initial concentration of beta casein in the aqueous phase varied between 0.1 and 2.5 mg/ ml. Beta Casein adsorption was studied at different ionic strength, between 0.01 – 1.5 and it was adjusted by NaCl. In the adsorption experiment that were carried out in the batch system for one hour at stirring rate of 200 rpm, 10 ml of the aqueous beta casein solution was added to 50 mg Cibacron Blue F3GA attached mP(HEMA) in a beaker. The beta casein adsorption capacity was determined spectrophotometrically by measuring the initial and final concentrations of beta casein. The adsorption was followed by monitoring the decrease in UV absorbance at 280 nm. The amount of adsorbed beta casein per unit mass of the mP(HEMA) microbeads were calculated by using the following expression.

$$Q = \frac{(C_0 - C) \times V}{m}$$

Here, Q is the amount of β-casein adsorbed onto unit mass of the beads (mg/g); Co and C are the concentration of β-casein in the initial solution and in the aqueous phase after treatment for the certain period of the time, respectively (mg/g); V is the volume of the solution (ml); and m is the mass of the beads used (g).

A flow rate was determined on a magnetically stabilized fluidized bed system (MSFBS) for the optimal conditions. The flow rate of the solution was changed in the range of 0.25-1.0 ml/min And then studied was magnetically stabilized fluidized bed (MSFB) system under different pH values (between pH 4-7) and initial protein concentration (between 0.1-2.5 mg/ml). During the experiment, the magnetic beads in the column were exposed to magnetic field which surrounded the column (Brms ≈24 G,Bp–p ≈33 G, φ = 50 Hz).

2.5.2 Beta Casein Desorption Studies

Desorption experiments of β casein was studied with 1.5 M NaCl solution. In the desorption experiment at batch mode, 10 ml desorption solution was added containing 50 mg beta casein adsorbed magnetic poly (HEMA) particles and stirred for 3 hours at 200 rpm and the final concentration was determined spectrophometrically. In the desorption experiment on magnetic fluidized bed column, the desorption solution was through the column containing magnetic microbeads using peristaltic pump for 1 hour. The desorption medium were measured spectrophometrically in each 15 minute. Desorption ratio was calculated from the amount of beta casein adsorbed on the magnetic beads and the final beta casein concentration in elution medium.

$$Desorption\ ratio = \frac{amount\ of\ beta\ casein\ desorbed\ to\ the\ elution\ medium}{amount\ of\ beta\ casein\ adsorbed\ on\ to\ the\ sorbent}\ x\ 100$$

At the end of the desorption, magnetic poly(HEMA) microbeads were cleaned 50 mM NaOH solution.

In order to test the repeated use of mP(HEMA) microbeads, β casein adsorption-desorption cycle was repeated for five times using the same adsorbent.

CHAPTER THREE
RESULTS AND DISCUSSION

3.1 Characterization of Cibacron Blue F3GA Attachment Magnetic poly(HEMA)

Cibacron Blue F3GA was used as the dye–ligand for specific binding of protein molecules.The mPHEMA beads were prepared by suspension polymerization. The surface morphology and internal structure of nonmagnetic PHEMA and mPHEMA beads are exemplified by the scanning electron pictures in figure 3.1. As seen in Figure 3.1A, mPHEMA beads have a spherical form and a rough surface containing macropores due to the abrasion of magnetite crystals (diameter <20–50 nm) during the polymerization procedure. However, the surface of the non-magnetic spherical PHEMA beads contained no macropores (Figure 3.1B). The pictures in Figure 3.1C and D were taken with broken beads to observe the internal partsof both non-magnetic and magnetic PHEMA beads. The presence of macropores within the bead interior was clearly seen in these photographs. It can be concluded that the mPHEMA beads have a macroporous interior surrounded by a reasonably rough surface, in the dry state. On the other hand, non-magnetic PHEMA beads were in the uniform and spherical shape with smooth surface characteristics.

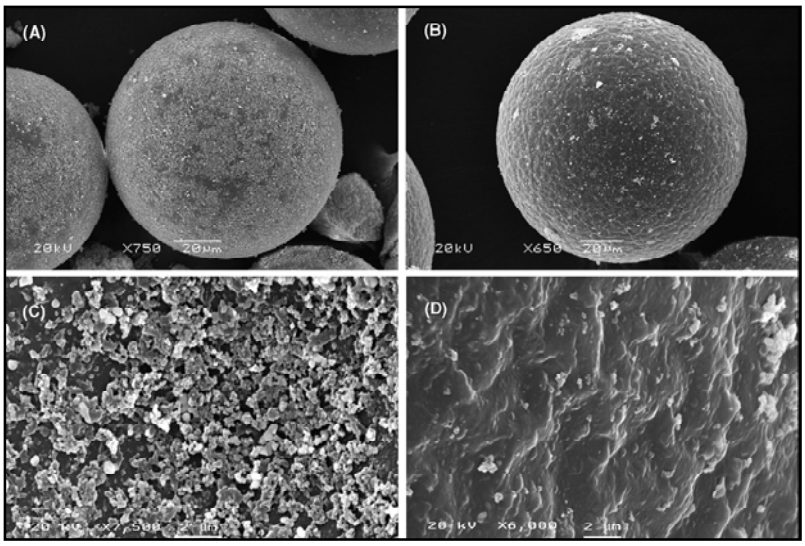

Figure 3.1 SEM pictures of polymeric beads: (A) mPHEMA beads; (B) non-magnetic PHEMA beads; (C) cross-section of mPHEMA beads; (D) cross-section of non-magnetic PHEMA beads.

The equilibrium swelling ratio of the magnetic beads was 62.12 %. These swollen beads had an average diameter within the range 100–140 μm.

Evaluation of the IR spectrum (Figure 3.2b) of cross-linked mPHEMA beads shows that the broad peak of O-H stretching vibration of HEMA monomer at 3452 cm^{-1} has disappeared. Multiple sharp peaks at 3500 cm^{-1} frequency range characterized especially the OH stretching vibration that is added and not added to the hydrogen bonding. However, the point which should be stressed is that; electrostatic interaction also occurs between the Fe^{3+} ion and OH group besides the hydrogen bonds between the OH groups itself. That is why the broad peak of OH in the spectrum of HEMA is not be manifested in the spectrum taken in the presence of Fe^{3+}.

FTIR spectrum of Cibacron Blue F3GA is given in Figure 3.2a. The N–H stretching vibration bands of this dye appear at 3455 cm^{-1} frequency range as multiple bands. The different amine groups of the dye could have a tendency to be free and H-bonded. That is why this spectral situation is regular. The band having moderate intensity at 1739

cm^{-1}, a shoulder at 1650 cm^{-1} and then sharp bands at 1565 cm^{-1} and 1507 cm^{-1} are the spectral evidences that characterize the C=O, C=N and C=C stretching vibrations of the structure. The band at 1226 cm^{-1} and the broad band at nearly 25 cm^{-1} below and above this frequency characterize the S=O stretching vibrations. The band that can be seen obviously at 1085 cm^{-1} belongs to the C-Cl stretching. At the lower wave number such as 1022 cm^{-1} S=O stretching vibration band is also seen in the spectrum. In addition, it is possible to see the C-Cl stretching vibrations of the dye at different wave numbers in the range of 500–700 cm^{-1}.

Figure 3.2c shows the IR spectrum of Cibacron Blue F3GA attached mPHEMA beads. The C=O band is observed at 1727 cm^{-1} as a sharp peak. It implies that the strength of the band at 1650 cm^{-1} is very low. The peaks at 1262 cm−1 and 1158 cm−1 characterize the C-O stretching vibrations. The conversion of the C-Cl stretching vibration of the original Cibacron Blue F3GA at 1085 cm^{-1} to a weak shoulder at 1081 cm^{-1} of the IR bands. In addition the decrease in the intensity of the same stretching vibrations of Cibacron Blue F3GA at 500–700 cm^{-1} frequency interval (Başar et. al, 2007).

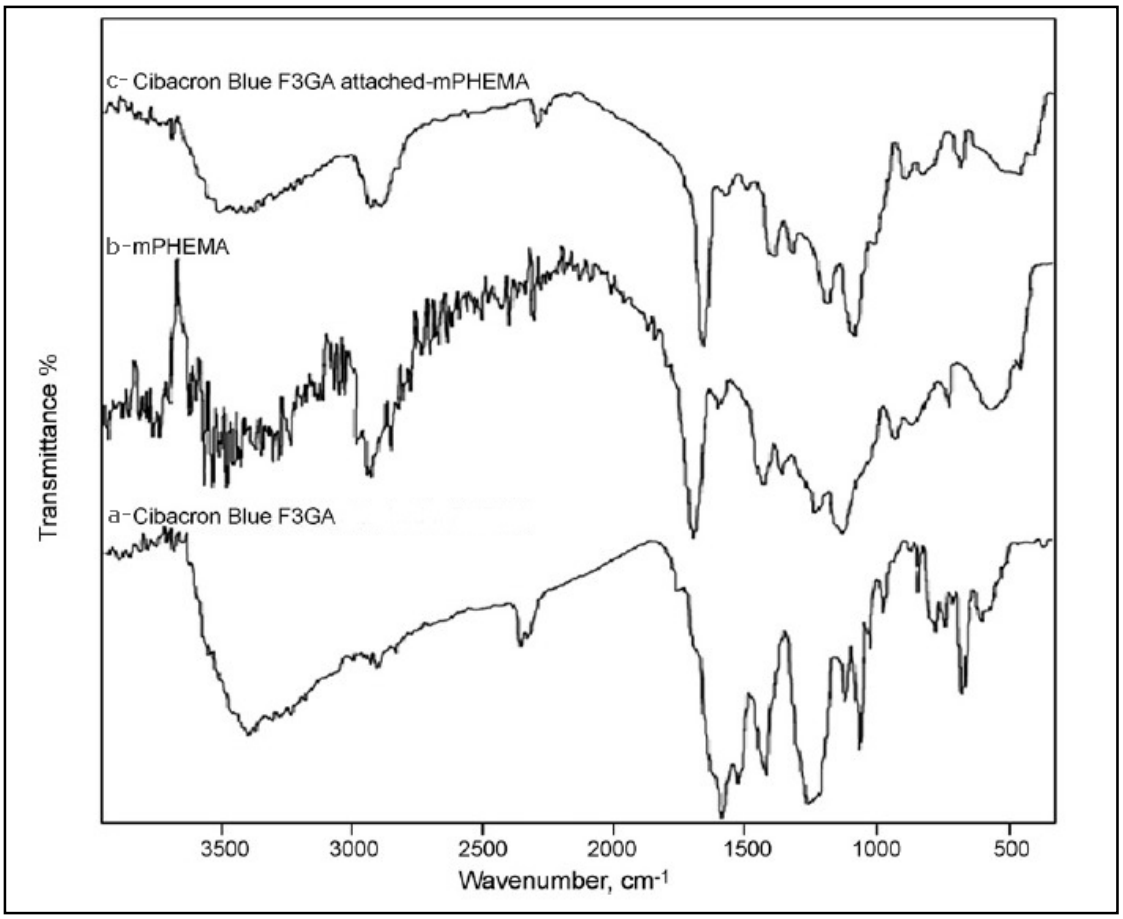

Figure 3.2 FTIR spectra of: (a) Cibacron Blue F3GA; (b) mPHEMA beads; (c) Cibacron Blue F3GA-attached mPHEMA beads.

The amount of Cibacron Blue F3GA attached m-PHEMA beads was calculated from these data on the basis of the sulfur stoichiometry for elemental analysis instrument (% 52.06 C and % 6.96 H).

The degree of magnetism of the m-PHEMA beads was measured in a magnetic field by using a vibrating sample magnetometer (Princeton Applied Research, Model 150A, USA). The presence of magnetite particles in the polymeric structure was investigated with an electron spin resonance (ESR) spectrophotometer (EL 9, Varian). The presence of magnetite particles in the polymeric structure was confirmed by ESR.

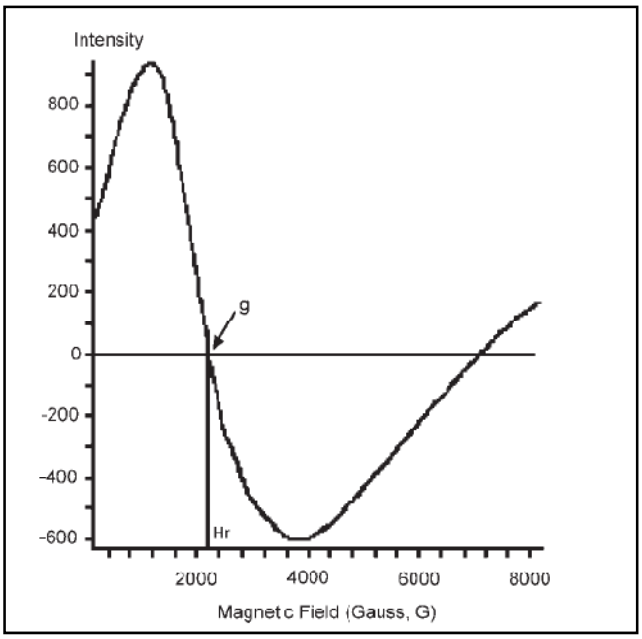

Figure 3.3 The magnetic behavior of m-PHEMA beads.

The presence of magnetite particles in the polymeric structure was confirmed by ESR. The intensity of the magnetite peak against the magnetic field (Gauss) is shown in Figure 3.3. The application of an external magnetic field may generate an internal magnetic field in the sample which will add to or subtract from the external field. The local magnetic field generated by the electronic magnetic moment will add vectorially to the external magnetic field (H_{ext}) to give an effective field (H_{eff}).

$$Heff = H_{ext} + H_{local}$$

As seen in Figure 3.3, mPHEMA beads have a relative intensity of 400. This value shows that the polymeric structure has a local magnetic field because of magnetite in its structure. The g factor given in Figure 3.3 can be considered as a quantity characteristic of the molecules in which the unpaired electrons are located. The measurement of the g factor for an unknown signal can be a valuable aid in the identification of a signal is origin. In the literature the g factor for Fe^{+3} (low spin and high spin complexes) was

determined to be between 1.4-3.1 and 2.0-9.7 respectively (Swartz, Bolton, & Borg, 1972). The g factor was found to be 2.66 for mPHEMA microbeads following equation.

$$g=h\upsilon / \beta H_r$$

Here, h is the Planck constant (6.626 x 10^{-27} erg x s^{-1}), β is the Universal constant (9.274 x 10^{-21} erg x G^{-1}), υ is frequency (9.707 x 10^{9} Hz) and Hr is the resonance of magnetic field (G).

A 2250 Gauss magnetic field was found sufficient to excite all of the dipole moments present in a 1.0 g mPHEMA sample. These values will be an important design parameter for a magnetically stabilized fluidized bed or for magnetic filtration using these magnetic beads. The value of the magnetic field required to stabilize the fluidized bed is a function of the flow velocity, particle shape, size, size distribution and magnetic susceptibility of the beads. In the literature, this value was found to change from 8 to 20 kG for various applications, (Wang, Wang, X., & Feng, 1997) and thus our magnetic beads will need less magnetic intensity in a magnetically stabilized fluidized bed or for a magnetic filter system as mentioned before Akgöl et. al (Akgöl et.al, 2005).

3.2 Beta- Casein Adsorption from Aqueous Solutions in Batch System

3.2.1 Effects of pH

The amount of β- casein adsorbed onto the Cibacron Blue F3GA-attached mPHEMA beads as a function of pH was shown in Figure 3.4. The amount of β- casein adsorbed onto Cibacron Blue F3GA attached mP(HEMA) beads shows a maximum at pH 5.0 (57.52 mg/g adsorbent), which is the isoelectric point of β casein, with a significant decrease at lower and higher pH values. Specific interactions (hydrophobic, electrostatic and hydrogen bonding) between β- casein and dye molecules at pH 5.0 may be resulted both from the ionization states of several groups on both the Cibacron Blue F3GA (i.e., sulfonic acid and amino) and amino acid side chains in β- casein, and from the

conformational state of β- casein molecules (more folded structure) at this pH. Depending on its amino acid composition, a protein can have several charged groups at the pI, the spatial arrangement of which is a function of primary, secondary, tertiary and quaternary structure. Therefore, the interaction of a protein with an affinity system may not occur at its pI value. This could be created from the ionization state of β- casein and could be caused repulsive electrostatic forces between β- casein and the dye molecules. Increase in conformational size and the electrostatic repulsion effects between the opposite charged groups may also cause a decrease in adsorption efficiency.

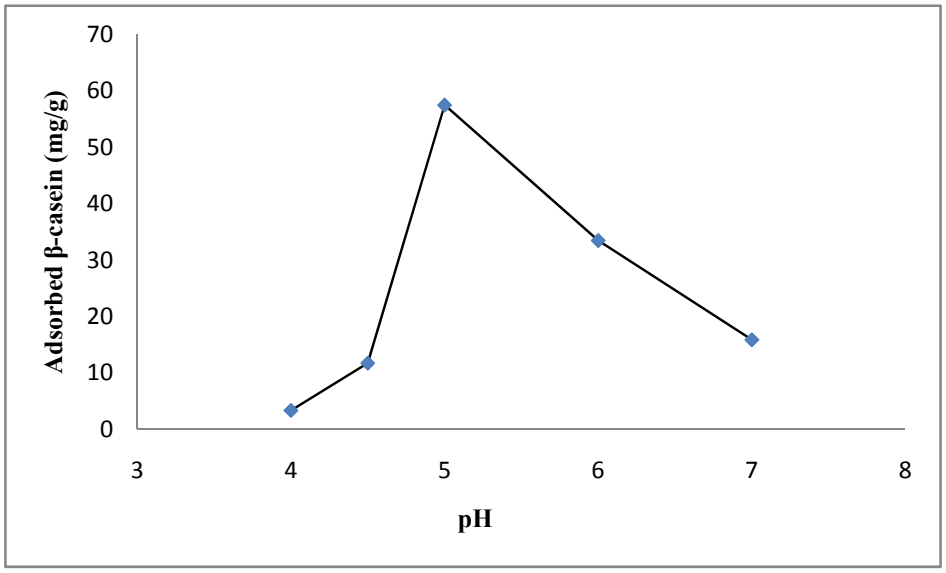

Figure 3.4 Effect of medium pH on the β-casein adsorption on Cibacron Blue F3GA attached magnetic p(HEMA) beads. β-casein concentration: 0.5mg/ml.

3.2.2 Effect of the Initial Concentration

Figure 3.5 shows the effect of the initial concentration of β Casein on the adsorption. The adsorption isotherm of the plain and dye-affinity beads. Note that one of the main requirements in dye-affinity chromatography is the specificity of the affinity adsorbent for the target molecule. Dye immobilization was increased the β Casein binding capacity of the mPHEMA beads. The initial concentrations were varied between 0.1 and 2.5 for

beta casein. The amount of β Casein adsorbed per unit mass of the mPHEMA beads increased first with the initial concentration of β Casein then reached a plateau value which represents saturation of the active adsorption sites on the magnetic beads. The Beta casein adsorption capacity of the microbeads increased up to 138.22 mg β Casein/g. This increase in the β Casein adsorption capacity is due to specific interactions (both electrostatic and hydrophobic) between the Cibacron Blue F3GA and the β Casein molecules. This increase in the β Casein 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 β Casein molecules.

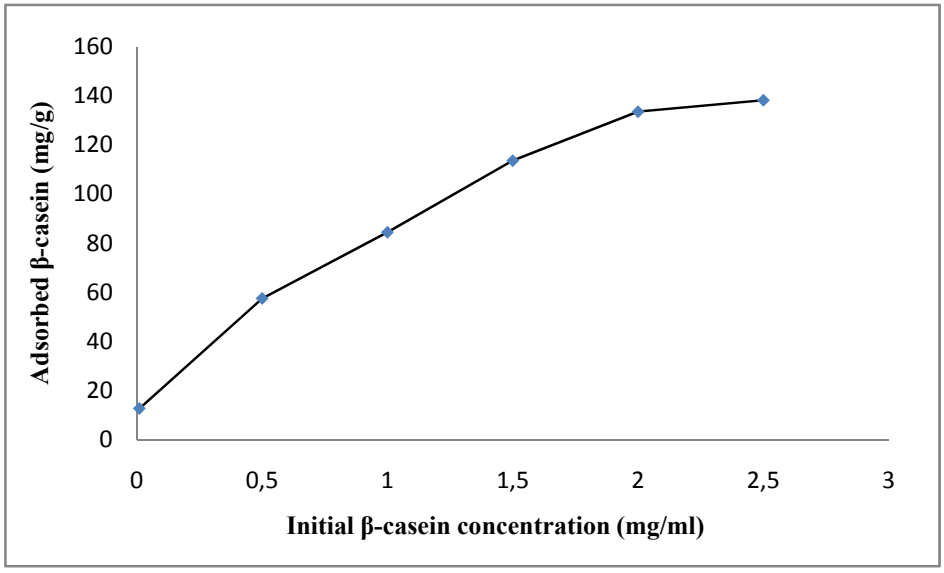

Figure 3.5 Effect of the initial concentration of β-casein adsorption on Cibacron Blue F3GA attached magnetic p(HEMA) beads. pH:5.0

3.2.3 Effect of Ionic Strength

The effect of NaCl concentration on β Casein adsorption is presented in Figure 3.6, which shows that the adsorption capacity decreases with increasing ionic strength of the

binding acetate buffer. The NaCl concentration changes from 0.01M to 1.5 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 β-casein would decrease as the ionic strength increased, and the adsorption of the β-casein to immobilized dye became difficult. It is also suggested that an increase in NaCl concentration result in the reduction of electrostatic interactions.

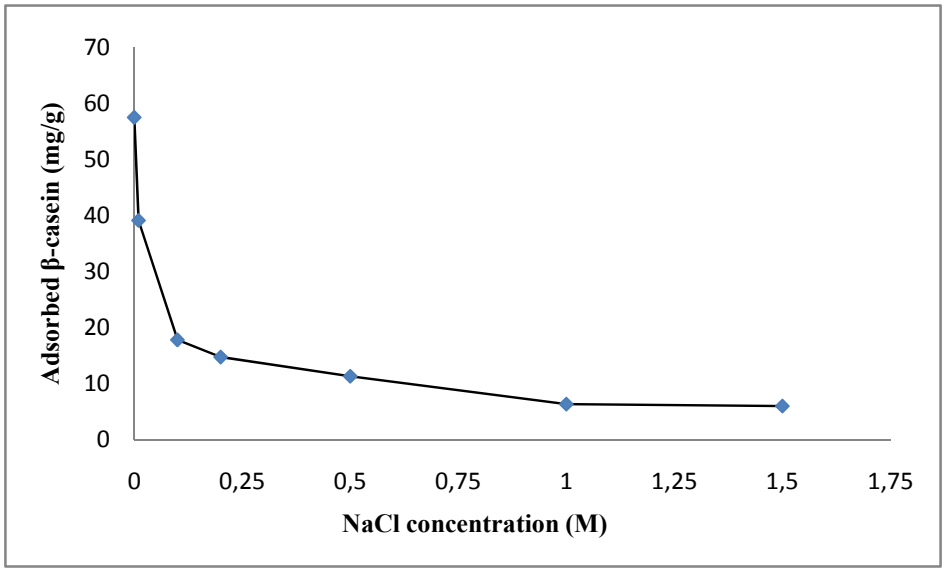

Figure 3.6 Effect of the ionic strength on β-casein adsorption on Cibacron Blue F3GA attached magnetic p(HEMA) beads. β-casein concentration: 0.5mg/ml; pH:5.0

3.2.4 Effect of Temperature

Figure 3.7 represents the effect of temperature on β-casein adsorption. The adsorption experiments were performed at three different temperatures; 25^0C, 37^0C and 45^0C respectively. Adsorption of β-casein decreases inversely with temperature, which bases on the exothermic nature of the process.

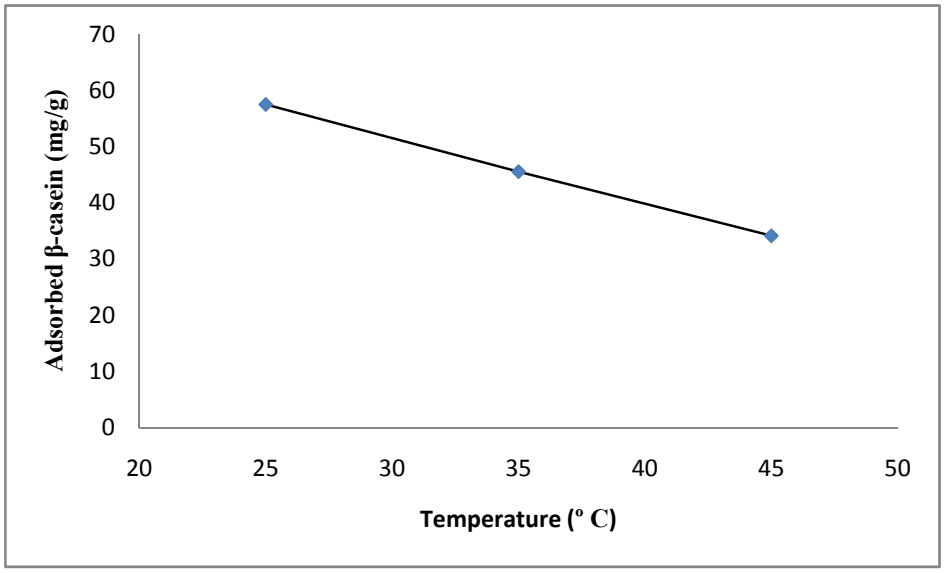

Figure 3.7 Effect of temperature on β-casein adsorption on Cibacron Blue F3GA attached magnetic p(HEMA) beads. β-casein concentration: 0.5mg/ml; pH:5.0

3.3 Beta- Casein Adsorption From Aqueous Solutions On Magnetically Fluidized Bed (MSFB) System

3.3.1 Effect of Flow Rate

The adsorption amounts of β-casein at different flow-rates are given in Figure 3.8. Results show that the β Casein adsorption capacity onto the Cibacron Blue F3GA-attached mPHEMA beads decreases when the flow rate through the column increases (Brms ≈24 G,Bp–p ≈33 G, φ = 50 Hz). The adsorption capacity decreased significantly from 72.09 mg/g to 43.08 mg/g polymer with the increase of the flow-rate from 0.2 ml/min to 1.0 ml/min. An increase in the flow-rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of beads column. This is due to decrease in contact time between the β-casein molecules and the Cibacron Blue F3GA-attached mPHEMA beads 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, β Casein molecules have more time to diffuse to the pore walls of beads and to

bind to the binding sites of adsorbent; hence a better adsorption capacity is obtained. In addition, for column operation the magnetic bed is continuously in contact with a fresh protein solution. Consequently the concentration in the solution in contact with a given layer of beads 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 (Başar et.al, 2007).

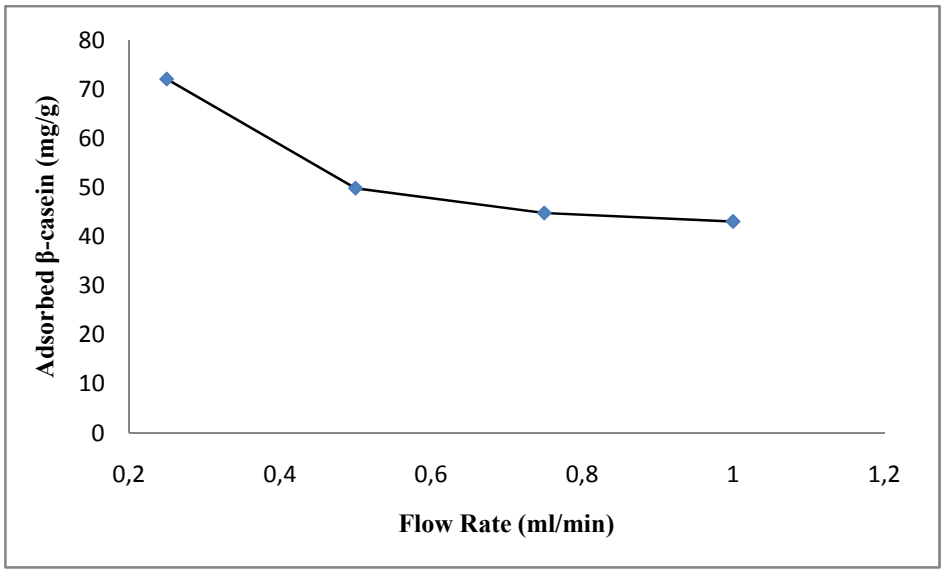

Figure 3.8 Effect of flow rate on β-casein adsorption on Cibacron Blue F3GA attached magnetic p(HEMA) beads. β-casein concentration: 0.5mg/ml; pH:5.0

3.3.2 Effects of pH

Figure 3.9 shows the amount of β- casein adsorbed onto Cibacron Blue F3GA attached mPHEMA beads shows a maximum at pH 5.0, which is the isoelectric point of β casein with a significant decrease at lower and higher pH values as before experiments identically. Adsorption capacity was observed to increase from 57.52 to 72.09 mg/g polymer for beta casein in MSFB system. Specific interactions between β- casein and dye molecules at pH 5.0 may be resulted both from the ionization states of several

groups on both the Cibacron Blue F3GA and amino acid side chains in β-casein, and from the conformational state of β- casein molecules at this pH. The decrease in the beta casein adsorption capacity in more acidic and in more alkaline pH regions can be attributed to electrostatic repulsion effects between the opposite charged groups. It has been shown that proteins have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric points.

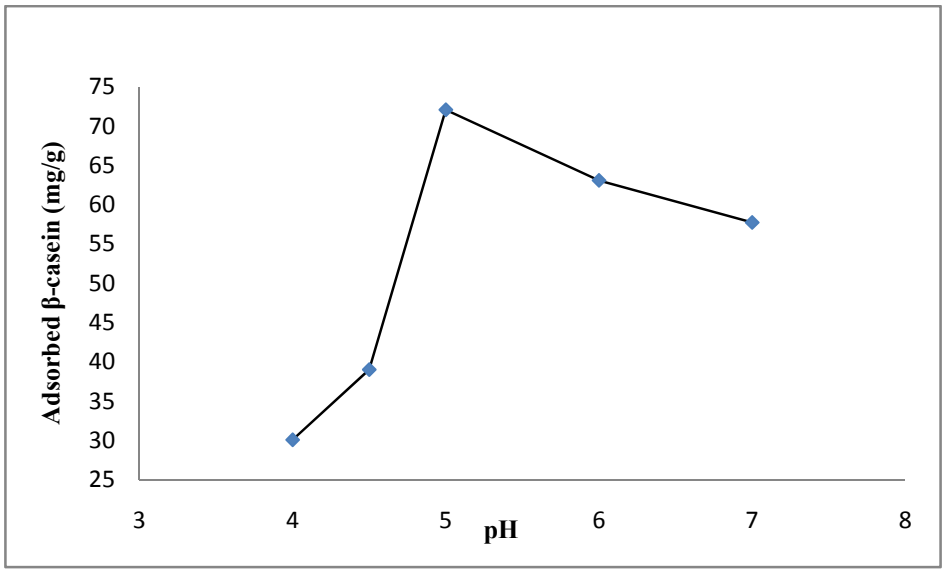

Figure 3.9 Effects of pH on β-casein adsorption. Flow rate: 0.25 ml/min; β-casein concentration: 0.5 mg/ml

3.3.3 Effect of the Initial Concentration

Figure 3.10 shows the effect of the initial concentration of β Casein on the adsorption in MSFB system. The initial concentrations were varied between 0.1 and 2.5 mg/ml for beta casein. The beta casein adsorption capacity of the microbeads increased up to 239.16 mg β Casein/g. This increase in the β Casein adsorption capacity is due to specific interactions between the Cibacron Blue F3GA and the β Casein molecules. This increase in the β Casein 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 β casein molecules.

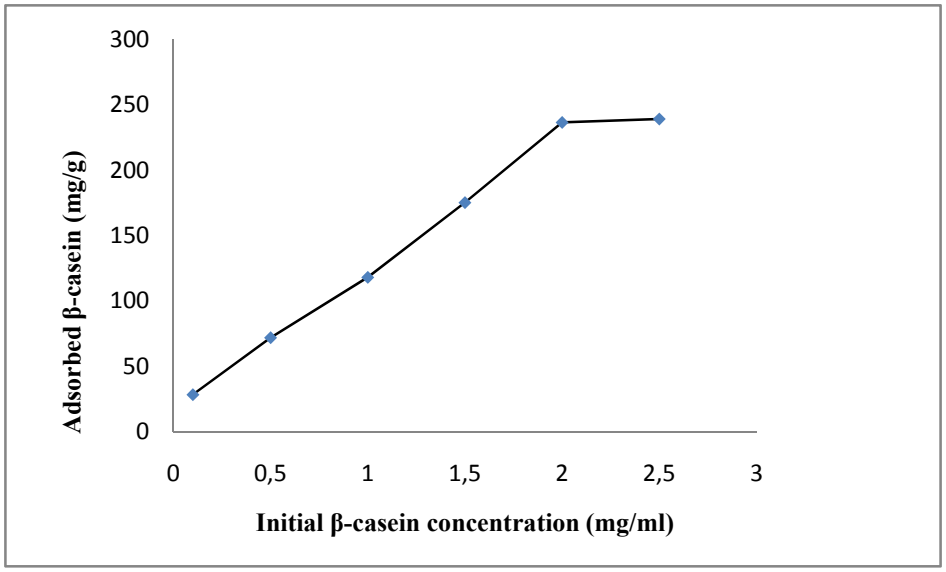

Figure 3.10 Effects of the initial β-casein concentration on adsorption. Flow rate: 0.25 ml/min; pH: 5.0

3.4 Adsorption Isotherms

During the batch and MSFB experiments, adsorption isotherms were used to evaluate adsorption properties. The Langmuir adsorption isotherm is expressed by the equation shown below. The corresponding transformations of the equilibrium data for β-casein gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

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

Where in this equation, Q represents the concentration of bound β-casein in the adsorbent (mg/g), Ceq is the equilibrium β-casein concentration in solution (mg/mL), b

is the Langmuir constant (g/mg) and, Q_{max} is the adsorption capacity (mg/g). This equation can be linearized so that.

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

The plot of $1/C_{eq}$ versus 1/Q was employed to generate the intercept of 1/Qmax and the slope of $1/Q_{max}.b$ (Figure 3.11 and 3.12).

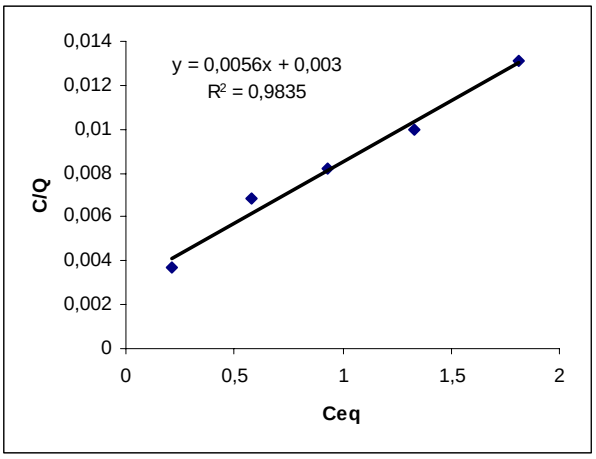

Figure 3.11 Langmuir adsorption isotherm for batch model; pH: 5.0; T: 25^0C.

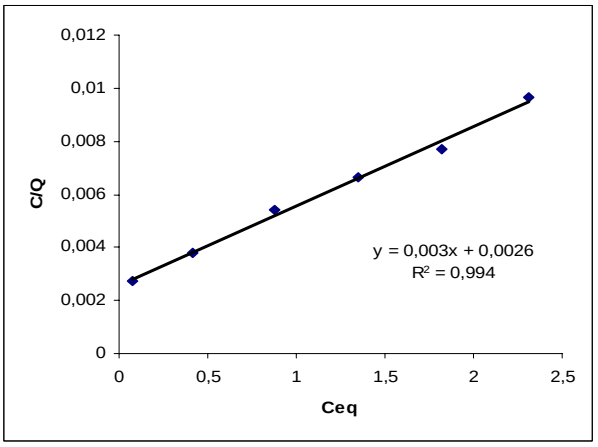

Figure 3.12 Langmuir adsorption isotherm for MSFB model; pH: 5.0; T: 25^0C.

The maximum adsorption capacities (Q_{max}) data for the adsorption of β-casein were obtained from the experimental data. The correlation coefficients (R^2) for batch and column model were 0.9835 and 0.994 at pH 5.0, respectively. The Langmuir adsorption model can be applied in this affinity adsorbent system. It should be also noted that the maximum adsorption capacities (Q_{max}) and the Langmuir constants were found to be 178.57 mg/g (pH 5.0) and 1.861 g/mg for batch system and 333.3 mg/g and 1.155 g/mg for MSFB system, respectively.

Table 3.1 Langmuir isotherm constants for batch and MSFB experiments

	Experimental		*Langmuir model*	
	Q_{exp} (mg/g)	Qmax (mg/g)	b	R^2
Batch	138.22	178.57	1.861	0.9835
MSFB	239.16	333.3	1.155	0.994

3.5 Desorption and Repeated Use

Figure 3.13 shows adsorption capacities for Cibacron Blue F3GA attached magnetic PHEMA microbeads did not change noticeable during the 5 times repeated adsorption-desorption operations. Up to 90% of the adsorbed β-casein was removed by 1.5 M NaCl as elution agent. In the protein separation process, possibility of recycling dye-affinity beads for reuse is an important notice. In this study, adsorption-desorption cycles were repeated 5 times using the same Cibacron Blue F3GA attached magnetic PHEMA microbeads.

The adsorption capacity of the recycled mP(HEMA) can still be maintained at 90.05 % level at the 5 th cycle (Figure 3.13). It can be concluded that the Cibacron Blue F3GA attached magnetic PHEMA microbeads can be used many times without decreasing their adsorption capacities significantly.

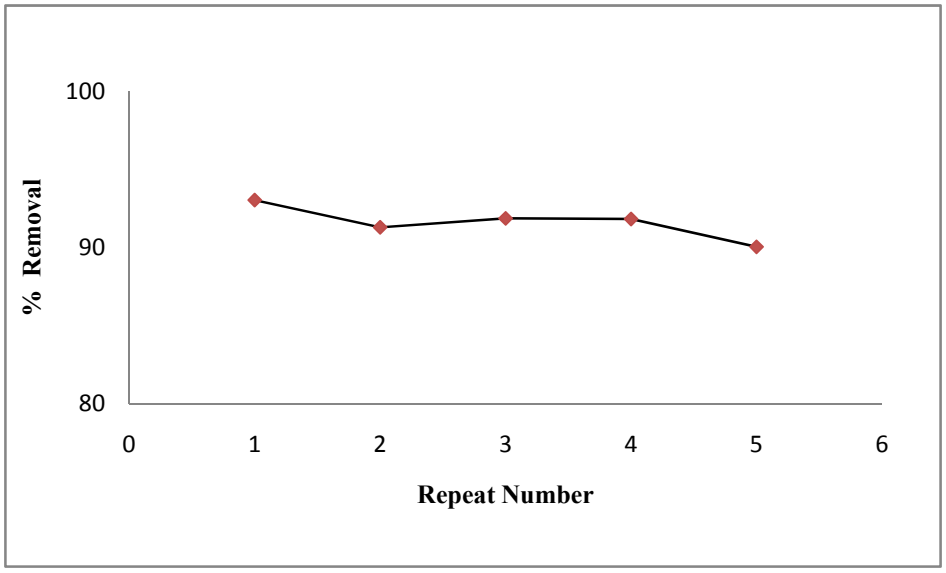

Figure 3.13 Repeated use of Cibacron Blue F3GA attached magnetic PHEMA microbeads. β-casein concentration: 0.5 mg/ml; pH: 5.0 for batch system

CHAPTER FOUR
CONCLUSIONS

In recent years magnetic microbeads have been widely used in the chromatography for fast and effective seperation and purification of biomolecules. The most significant advantage of using magnetic adsorbents in chromatographic applications is the easy removal of them from the reaction media in the presence of magnetic field. Additionally, magnetic supports can easily be stabilized in the fluidized reactors that are externally exposed to magnetic area, which enables continuous separation of biomolecules like enzymes and proteins. Further more the usage of these kinds of materials can seriously reduce the investment and production costs. Biomimetic dye ligands have several advantages compared to biologic ligands in the aspects of high adsorption capacity, rigid, easiness of immobilization and economy. Based on this reason, in this study Cibacron Blue F3GA attached mP(HEMA) microbeads were synthesized with suspension polymerization. Cibacron Blue F3GA attached mP(HEMA) microbeads were analyzed by FTIR, SEM and elemental analysis. In this study, β-casein was selected as model protein in experiments. Adsorption studies were performed under different conditions in batch and MSFB system.

Maximum beta casein adsorption from aqueous solution is 57.52 mg/g for batch system and 72.09 mg/g for MSFB system. Following figure 4.1 gives the comparison of the two systems, batch and MSFB. Results indicate that the adsorption capacity obtained in MSFB system is considerably higher than obtained in batch system. The dye ligand-beta casein dissociation rate in the batch system is higher than association rate in the MSFB system. Cibacron Blue F3GA ligand is found both on the surface and in the pores of the magnetic beads. In the presence of flow, the beta casein solution is forced from the surface into the pores thus eliminating the surface diffusion (Yavuz et. al, 2004).

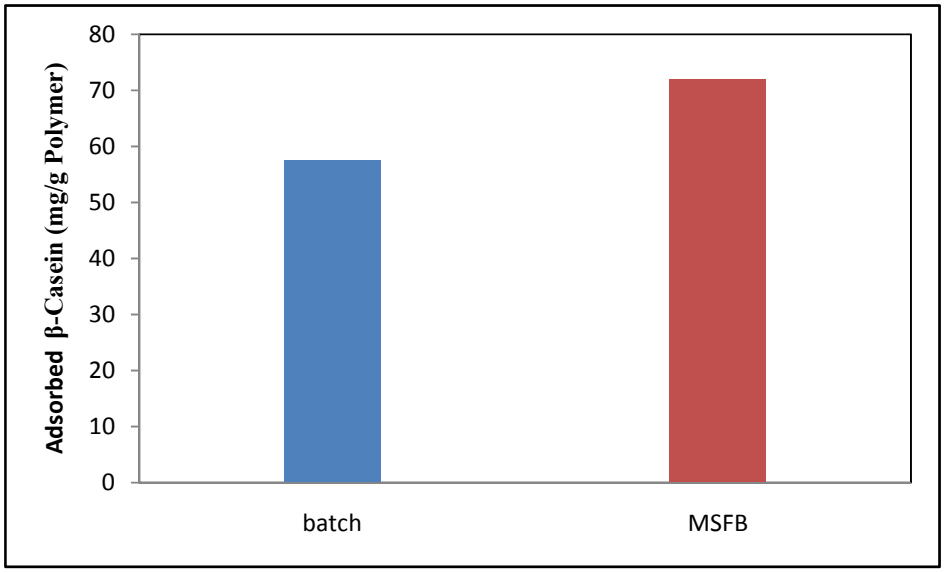

Figure 4.1 Comparison of MSFB and batch system. In both systems β-casein concentration: 0.5 mg/ml; pH: 5.0.

Column-type continuous flow operations appear to have a distinct advantage over batch type operations because the rate of adsorption depends on the concentration of solute in solution being treated. For column operation the adsorbents are continuously in contact with a fresh solution. Consequently the concentration in the solution in contact with a given layer of adsorbent 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 (Başar et.al, 2007). The MSFB column capacity (72.09 mg beta casein/g polymer) was found to be higher than the batch capacity (57.52 mg beta casein/g polymer). This means, in equilibrium binding experiments, maximum adsorption capacity for batch system was % 20.21 lower as compared to the value obtained in MSFB. The higher column capacity may be due to the fact that the continuously large concentration gradient at the interface zones occurred as to passes through the column, while the concentration gradient decreases with time in batch experiment (Başar et.al, 2007).

Finally, we concluded that Cibacron Blue F3GA attached magnetic poly(HEMA) could be repeatedly applied for β-casein adsorption without significant losses in the adsorption capacity.

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