BOYA AFİNİTE KOMPOZİT KRİYOJELLERLE IgG ALTBİRİMLERİNİN SAFLAŞTIRILMASI

SEPARATION OF IgG SUBCLASSES WITH DYE AFFINITY COMPOSITE CRYOGELS

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Submitted to Institute of Graduate Studies

in Science of

Hacettepe University as a partial fulfillment

to the requirements for the award of degree of

MASTER OF SCIENCE

in

CHEMISTRY

ANKARA

2013

Graduate School of Natural and Applied Sciences,

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ÖZET

İmmunoglobulinler (IgG) antibadi izotipleridir. IgG, 150 kDa, toplam vücut miktarının yaklaşık % 80'ini ihtiva eden antikorların en yaygın tipidir. IgG'nin dört altbirimi vardır. Çoğu zaman, IgG'ler IgG_2 altsınıfına ait bakteriyel patojenlere karşı yönlendirilmiştir. Dolayısıyla, bakteriyel enfeksiyon durumunda, zenginleştirilmiş IgG_2 preparatlarını kullanmak avantajlı olmaktadır. Günümüzde, insan tedavisi için uygun zenginleştirilmiş IgG_2 çözeltilerinin hazırlanması için kullanılan bir yöntem yoktur.

Boya-ligand afinite kromatografisi son yıllarda sıklıkla kullanılan kromatografik bir tekniktir. Reaktif boyalar protein saflaştırmada kullanılan afinite ligandlarıdır. Protein saflaştırılması için afinite ligandları olarak reaktif boyaların avantajları; ekonomiktirler ve kolaylıkla bulunabilirler, kolaylıkla immobilize edilirler, aktivasyonları sırasında tehlikeli ve toksik reaktifler oluşturmaz, biyolojik ve kimyasal etkilere karşı kararlıdır, aktivite kaybı olmaksızın depolanabilirler, tekrar kullanılabilirler, yüksek kapasite ile temizlik ve sterilizasyon işlemleri uygulanabilir ve büyük ölçekli uygulamalarda kullanılabilirler. Bu çalışmada IgG moleküllerinin alt sınıflarının saflaştırılması amaçlanmıştır. Bu amaçla poli(2-hidroksietilmetakrilat) (PHEMA) mikroküreler 71-100 µm boyutunda hazırlanarak, bu kürelere Reactive Green HE 4BD ve Reactive Red 120 boyaları takılmıştır. Daha sonra boya takılı küreler PHEMA bazlı kriyojel membranlara gömülmüştür. Kriyojeller, kısmen donmuş monomer veya polimer çözeltileri kullanılarak hazırlanan jel matriksleridir. Genel olarak kriyojeller birbirine bağlı süpermakro gözeneklere sahiptir. Bu özellikleri kriyojellerin nano-mikro ölçek aralığında herhangi bir difüzyon sorunu olmaksızın kullanımına olanak sağlamaktır. Kriyojeller, geniş gözenek boyutu, kısa difüzyon yolu, düşük basınç azalması ve kısa alıkonma süresi de dahil olmak üzere pek çok avantajı sayesinde biyomoleküllerin ayrılmasında kullanılmaktadır. Kriyojellerin düşük yüzey alanları adsorpsiyon kapasitesinin düşük olmasına neden olduğundan, mikroküre gömme yöntemi bu problemi

ortadan kaldırmaktadır. Hazırlanan kompozit kriyojel diskler ile yüzey alanı ölçümleri (BET), taramalı elektron mikroskopu (SEM), Fourier Transform Infrared Spektroskopisi (FTIR), elementel analiz, şişme testleri karakterize edilmiştir. pH, derişim, sıcaklık, akış hızı ve iyonik şiddet gibi parametrelerin kompozit diskler üzerine HIgG adsorpsiyonu miktarı incelenmiştir. RR-CCD ve RG-CCD kolonlarının maksimum adsorpsiyon kapasitesi miktarları sırasıyla 239.8 ve 170 mg/g olarak bulunmuştur. IgG alt birimlerinin RR-CCD ve RG-CCD kolonlarına adsorpsiyonu da hem ticari HIgG çözeltisi ile hem de insan serum örneği ile çalışılmıştır. Kolonların tekrar kullanılabilirliği de test edilmiş olup her iki kolon için de desorpsiyon oranları % 90 civarında bulunmuştur.

Anahtar kelimeler: Kompozit kriyojel, Boya afinite kromotografisi, İmmunoglobulin G, Reaktif boyalar.

Danışman: Prof.Dr. Adil Denizli, Hacettepe Üniversitesi, Fen Fakültesi, Kimya bölümü, Biyokimya Anabilim dalı, Ankara.

SEPERATION OF IgG SUBCLASSES WITH DYE AFFINITY COMPOSITE CRYOGELS

SABINA HÜSEYNLI

ABSTRACT

Immunoglobulin G (IgG) is an antibody isotype. IgG, 150 kDa, is the most common type of antibody comprising about 80% of the body's total amount of antibodies. IgG has four subclasses. Human IgG is composed of four isotypic subclasses termed IgG1 , IgG2 , IgG3 and IgG4. One of the indications of IgG administration is to enhance resistance of the patient to infections. Most often, IgGs directed against bacterial pathogens belong to the IgG2 subclass (Hammarstrom and Smith, 1986) . Hence, in situations of bacterial infections, it would be advantageous to use enriched IgG2 preparations. At present, there is no established method for the preparation of IgG2 -enriched solutions suitable for human therapy.

Dye-ligand affinity chromatography is one of the chromatographic techniques which is widely used in recent years. The advantages of reactive dyes as affinity ligands for protein purification; economical and widely available, ease of immobilization, avoids hazardous and toxic reagents in matrix activation, stable against biological and chemical attack, storage of adsorbent without loss of activity, reusable: with stands cleaning and sterlization, ease of scale up, high capacity, medium specificity.

The aim of this study, purification subunits of IgG (immunoglobulin G) using dye affinity composite cryogels. Poly(hydroxyethyl methacrylate) PHEMA beads were synthesized 71-100 μm sized and Reactive Red 120 (RR) and Reactive Green HE 4BD were attached to these beads. Then the RR-HEMA and RG-PHEMA beads were embedded into the cryogel membranes to obtained RR-PHEMA and RG-PHEMA embedded composite cryogel discs RR-CCD and RG-CCD columns. The high porosity of cryogels makes them appropriate candidates as the basis for such supermacroporous

chromatographic materials. Owing to supermacroporosity and interconnected pore-structure, such a chromatographic matrix has a very low flow resistance. Microsphere embedding method is a useful improvement for the combining the supermacroporosity and low flow resistance properties of the cryogel with large surface area and the high adsorption capacity properties of the microspheres. That was aimed the purification of IgG subunits with composite cryogels. The preapared composite cryogels were characterized by surface area measurments (BET), Scanning Electron Microscopy (SEM), Fourier Transform Infrared (FTIR), elemental analysis, swelling tests. Methods of seperation of IgG were determined. The parameters such as pH, concentration, temperature, flow rate and ionic strenght were investigated on adsorption of HIgG onto composite discs. The maximum adsorption capacity were obtained as 239.8 and 170 mg/g for RR-CCD and RG-CCD columns respectively. The adsorption of IgG subclasses onto adsorbents were also studied from both commercial HIgG solution and human serum. The reusability of the columns were tested and the desorption ratios were obtained about % 90 for both of the columns.

Key words: Composite cryogels, Dye affinity chromotography, Immunoglobulin G, Reactive Dyes.

Advisor: Prof.Dr. Adil Denizli, Hacettepe University, Department of Chemistry, Biochemistry Division, Ankara.

ACKNOWLEDGEMENT

I am very greatly obliged and indebted to Prof. Dr. Adil Denizli, my supervisor, for his valuable guidance, professional advice, constrictive criticism and suggestion during my research.

Especially, I would like to thank to Gözde Baydemir Peşint for all her contributions, supportings and helps in every steps of M. Sc. studies.

Also, I would like to thank Assoc. Prof. Dr. Handan Yavuz Alagöz, Dr. Müge Andaç and Bahar Ergün, they supported me all the time with charity and also want to thank Esma Sarı for her contributions to my thesis.

Especially thanks to Semra Akgönüllü, Nihal Ermiş and Emil Murtuzayev for their support, encouragement and countenance throughout this study.

I wish to thank all of the members of BIOREG group for their help in laboratory, for their collaborating, assisting and providing me a pleasant atmosphere to work in.

Finally, thanks for financial and moral support throughout my life to my dear mother İrade HÜSEYNLİ, dear father Zahid HÜSEYNLİ, who made any sacrifice which is the largest shareholder.

İnfinite thanks...

Sabina Hüseynli

January 2013

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1. INTRODUCTION

Plasma proteins play an important role in the fulfillment of many functions of blood. There are approximately hundred proteins in blood plasma. Blood plasma contains albumins, antibodies (α1, α2, β, γ-globulins), fibrinogen, clotting factors, salts and water. Blood plasma is about 90 % water and 7-8 % proteins, whichare called plasma proteins. These plasma proteins are building blocks of all the body's cell and tissue, including antibodies, hormones and clotting agents. Glycoproteins which are known as a cell molecules made out of carbohydrates and proteins. Glycoproteins are ubiquitous in occurrence, being found in all living organisms. Glycoproteins that contain covalently attached oligosaccharide chains have more than one sugar chain in one molecule (Kobata, 1992). Glycoproteins take places in many cellular event and they play an important role in the recognition of cell surface. Glycoproteins are found in not only the cytoplasm and organelles within the cell the organism, but also the extracellular fluid (Murray, et al., 1996). Antibodies are also glycoproteins. The functions of carbohydrate chains of the eventuating glycoproteins are diverse: they can make increase stabilization of the protein against denaturation by protecting it from proteolytic degradation, enhance its solubility, or serve as recognition signals to facilitate cell-cell interactions. Glycoproteins play an important role in hormone function. Glycoproteins have an substantial tasks in the development and production of biopharmaceutical proteins. They are also important in local antibiotic resistance. Glycoproteins on cell surfaces are essential for communication between cells, for maintaining cell structure and for self-recognition by the immune system. Glycoproteins are important for immune cell recognition, especially in mammals. Immunoglobulins are heterogeneous protein. Immunoglobulin is a mixture of blood proteins called antibodies that are made by the immune system. The immune system is a system of biological structures and processes within an organism that protects against disease. IgG makes up approximately 80% of the serum antibodies. Human IgG is constituted of four isotypic subclasses which are termed as IgG1, IgG2, IgG3 and IgG4 (Roith, 1997). These subclasses are distinguished by having

different heavy chains called D1, D2, D3 and D4, respectively. The relative flexibility of the hinge regions of the IgG subclasses is as follows: IgG3 > IgG1 > IgG4 > IgG2 (Pumphrey**,** 1986).

Reactive dyes are extensively used in affinity columns and in batch systems for the purification of large biomolecular compounds because they are able to bind proteins in such a remarkably specific manner that the protein of interest can be purified from a complex mixture in a single step. Reactive dyes are not as selective as raised antibodies, but their low costs make them attractive for reseachers. Reactive Red 120 dye (RR-120) and Reactive Green HE 4BD selected as dye ligand in this study. Both of the dyes contain two phenolic OH groups and six sulphonic acid groups. The cryogels were selected as afiifnity adsorbent because they are a very good alternative to chromatographic supports. They have many advantages such as large pores, short diffusion path, low-pressure drop and very short residence time for both adsorption and elution. However, large pores result in much lower surface area compared to other chromatographic supports (Baydemir, 2009). This turned in resulted small amounts of ligands available for binding and low adsorption capacity when using cryogels in affinity separations (Arvidsson, 2003). The bead embedding method was applied to overcome this drawback of the cryogels. By this method the surface areas of the cryogel matrix and the ligand amount of the matrix were increase an the adsorption capacity of the matrix were enhanced.

In the present thesis, we aimed to prepare 2 different affinity columns for the recognition of human immunoglobulin and its subclasses (HIgG). The first one was RR attached PHEMA bead embedded cryogel. CB was covalently attached to the PHEMA beads via the nucleophilic substitution reaction between the chloride of their triazine ring and the hydroxyl groups of the PHEMA under alkaline conditions to produce RR-PHEMA beads. The RR-PHEMA beads were embedded into PHEMA cryogels to obtain RR-CCD columns (RR-PHEMA bead embedded composite crogel disc columns). The second one was RG attached PHEMA bead embedded cryogel. RG was also covalently attached to the PHEMA beads via the nucleophilic substitution

reaction between the chloride of their triazine ring and the hydroxyl groups of the PHEMA under alkaline conditions to produce RG-PHEMA beads. The RG-PHEMA beads were embedded into PHEMA cryogels to obtain RG-CCD columns (RG-PHEMA bead embedded composite crogel disc columns).

The obtained RR-CCD and RG-CCD columns were characterized. The adsorption capacities, the binding kinetics of the columns were calculated and the recoveries of the adsorbed HIgG were also investigated. The applications of the RR-CCD and RG-CCD were performed using both commercial IgG solution and a real biological human serum sample.

2. GENERAL INFORMATION

2.1. Plasma Proteins

Plasma proteins play an important role in the fulfillment of many functions of blood. There are approximately hundred proteins in blood plasma. Blood plasma contains albumins, antibodies (α1, α2, β, γ-globulins), fibrinogen, clotting factors, salts and water. Blood plasma is about 90 % water and 7-8 % proteins, whichare called plasma proteins. These plasma proteins are building blocks of all the body's cell and tissue, including antibodies, hormones and clotting agents. They control intravascular osmotic pressure between blood and tissue, and also control a change in the acid-base balance of body fluids. In addition plasma proteins help to control protein reserved for the body. The majority of plasma proteins except for immunoglobulins and proteohormons is synthesized in the liver. γ-globulins are manufactured in plasma cells. The plasma proteins are synthesized on ribosomes bound to the rough endoplasmic reticulum. Albumin which binds ligands and works as a transport protein has a role in the regulation of blood osmotic pressure (75-80%). Albumin accounts for approximately 60% of total plasma proteins. Except for albumin, all of plasma proteins are glycoproteins.

2.2. Glycoproteins

Glycoproteins which are known as a cell molecules made out of carbohydrates and proteins. Glycoproteins are ubiquitous in occurrence, being found in all living organisms. Glycoproteins that contain covalently attached oligosaccharide chains have more than one sugar chain in one molecule (Kobata, 1992). Glycoproteins, glycolipids and proteoglycans which can be described as a ‘glycoconjugate’ are complex carbohydrates (Hughes, 1983). Membranes contain a several types of protein. Enzymes, antibodies, receptors, hormones, except for albumin plasma proteins are the structure of glycoprotein. Glycoproteins take places in many cellular event and they play an important role in the recognition of cell surface. Glycoproteins are found in not only the cytoplasm and organelles within the cell the organism, but also the extracellular fluid (Murray, et al., 1996). Besides their functions in the

immune system, structural glycoproteins have also transport function. The carbohydrate parts that usually constitute 2-30% of the total weight of glycoproteins perform this function by carriying of information which is important for the functioning of many biological recognition systems (Sharon, 1980). Some glycoproteins carry 50-60 % or more carbohydrate. Most glycoproteins display a range of heterogeneity. This heterogeneity is provided both with for to sites of glycosyl chain attachment onto the polypeptide chains as well as the sugar chains themselves (Johannes, et al., 1995). Many proteins which are present in animals, plants, microorganisms and viruses in cells and biological fluids are glycosylated and these proteins known as glycoproteins (Kishino, et al., 1997).

2.2.1. Structure of The Glycoprotein

There are approximately 200 monosaccharides in nature but only 7 major types of them are found in the oligosaccharide chains of glycoproteins. These 7 major types of monosaccharides which have the structure of hexose are Galactose (Gal), Mannose (Man), Glycose (Glc), Fucose (Fuc), N-acetyl glycosamine (GlcNAc), N-acetyl galactoseamine (GalNAc), N-acetyl neuraminic acid (NeuAc). Oligosaccharide chain of glycoproteins also contain five amino acids which are; Asparagine (Asn), Serine (Ser), Threonine (Thr), Hydroxylysine (Hyl), Hydroxyproline (Hyp).

2.2.2. The Classification of Glycoproteins

Based on the nature of the linkage between their polypeptide chains and their oligosaccharide chains, glycoproteins can be divided into four classes: those containing a Ser (or Thr)-GalNAc linkage (1), Ser-Xyl linkage (2), hydroxylysine (Hyl)-Gal linkage (3) and Asn-GlcNAc linkage (4). Containing N-glycosidic bond and containing O-glicosidic bond are the two main groups of glycoproteins which are divided dependent on their type of bonds.Glycoproteins are classified into four prominent groups based on their source, i.e., glycoproteins of mammalian, fish glycoproteins, plant glycoproteins, parasitic and viral glycoproteins (Faillard, 1998). N- and O-linkage glycoproteins are synthesized in the lumen of the endoplasmic reticulum and golgi.

2.2.2.1.Containing N-Glycosidic Bond

The main classes of glycoproteins are N-linkage glycoproteins. All eukaryotic cells express N-linked glycoproteins. Immunoglobulins, ovalbumin, orasomukoid are N-linked glycoproteins. N-linked sugars in glycoproteins are connected to asparagine side-chain amide groups. The N-linked oligosaccharides have a minumum of 5 sugar residues.

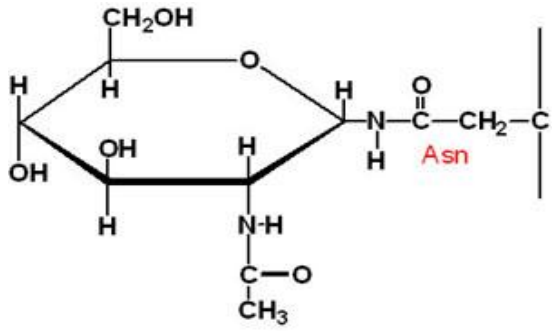

Figure 2.1. Representation of N-glycosidic bond of glycoprotein.

N-linked glycoproteins which include a common pentasaccharide divided into three oligosaccharide class: complex, mannose and hybrid oligosaccharide class (Man3 Glc Nac2). Animal glycoproteins are mixed glycoproteins. All of N-linked glycoproteins made from mannose, and then converted to various types of oligosaccharides. They usually are found in lower eukaryotes, viral glycoproteins. N-linked oligosaccharides are found plasma and membran proteins. All N-linked oligosaccharides are containing 2 GlcNAc residues and 3 mannose units.

2.2.2.2. Containing O-Glycosidic Linkage

0-linked sugar chains are formed by linking hydroxyl group of either a serine or a threonine residue of a polypeptide to one which contains an N-acetylgalactosamine residue at its reducing terminal. Many proteins that are found in membranes, mucins, proteoglycans, collagens are O-linkage

glycoproteins. A lot of membran proteins and secretion proteins (mucins) contain O-linkage glycoproteins (Lennarz, 1980).

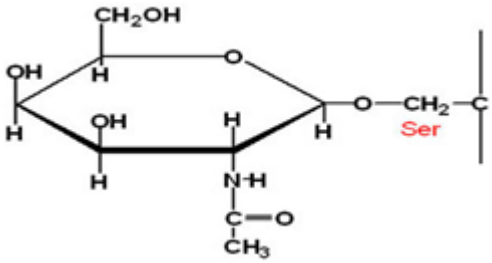

Figure 2.2. Representation of O-glycosidic bond of glycoprotein.

O-linkage oligosaccharides are accessible sialic acid (N-acetyl neuraminic acid), galactose and GalNAc. Because of the presence of sialic acid in O-linked oligosaccharides, they are negatively charged. O-linked oligosaccharides are usually short (1-4 sugar residues).

2.2.3. The Functions of Glycoproteins

Antibodies are also glycoproteins. The functions of carbohydrate chains of the eventuating glycoproteins are diverse: they can make increase stabilization of the protein against denaturation by protecting it from proteolytic degradation, enhance its solubility, or serve as recognition signals to facilitate cell-cell interactions. Glycoproteins have many diffrent function as it shown at Table 2.1. Listed below are several functions of oligosaccharide chains of glycoproteins

i. Taking part in recognition between the cells and molecules

ii. To stabilize the protein against denaturation

iii. Participating in biological activity (hCG)

iv. Embryological development and differentiation

v. Changing the physicochemical properties of proteins

vi. Oligosaccharide chains of glycoproteins increases the solubility and stability of proteins

Table 2.1. Example of glycoproteins and their functions.

Glycoproteins	**Functions**
İmmunoglobulins, interferon	Role in immunological
K-casein	Food store
Transferrin, fibronectin, heparin	Transport
Mucin	Lubricant, protective
Collagen, elastin, fibrin	Structural
Human Chorionic Gonadotropin (HCG)	Hormone
Ribonuclease B, Protrombin	Enzyme

2.2.4. The Importance of Glycoproteins

Glycoproteins play an important role in hormone function. Glycoproteins have an substantial tasks in the development and production of biopharmaceutical proteins. They are also important in local antibiotic resistance. The oligosaccharides which found in glycoproteins are often responsible for varied of biological functions. Glycoprotein components can also use as markers in early detection of cancer and in monitoring progress during treatment has been evaluated. Glycoproteins on cell surfaces are essential for communication between cells, for maintaining cell structure and for self-recognition by the immune system. Glycoproteins are important for immune cell recognition, especially in mammals.

2.3. Immunoglobulins

Immunoglobulins are heterogeneous protein. Immunoglobulin is a mixture of blood proteins called antibodies that are made by the immune system. The immune system is a system of biological structures and processes within an organism that protects against disease. Immune system divided into two classes; adaptive and innate system. Microorganisms or toxins that successfully enter an organism encounter the cells and mechanisms of the innate immune system. The innate response is usually triggered when

microbes are identified by pattern recognition receptors, which recognize components that are conserved among broad groups of microorganisms (Medzhitov, 2007).

The adaptive immune system evolved in early vertebrates and allows for a stronger immune response as well as immunological memory, where each pathogen is "remembered" by a signature antigen (Pancer, et al., 2006).

Table 2.2. Classification of adaptive immunsystem.

Adaptive system	
Immunoglobulins	**Lymphocytes**
IgG	T cells
IgM	B cells
IgA	
IgD	
IgE	

The immunoglobulins are produced by B lymphocytes (B cells) or plasma cells in response to exposure to as antigen. The human immunoglobulins are a group of structurally and functionally similar glycoproteins that confer humoral immunity in humans (Spiegelberg, 1974). They are composed of 82 - 96% protein and 4 - 18% carbohydrate. It is defined that classes of immunoglobulins: IgG, IgD, IgA, IgM and IgE respectively.

IgA makes up approximately 6% of the serum antibodies. The two subclasses of IgA are IgA1 and IgA2. IgA in its basic structure is a monomer of 150 KDa, even though it is a dimer. IgA (mol. wei. 320.000) is an early antibody for bacteria and viruses. IgA appears as a dimer of 2 ‘Y’- shaped molecules and has four epitope-binding sites. IgA is the second most common serum of Ig.

IgD (mol. wei- 180.000) is immunoglobulin class which is present in the blood serum in tiny amounts (approximately 0.2 %). IgD is a monomer and has two epitope-binding sites. IgD exists only as a monomer. IgD does not bind complement.

IgE is an antibody for parasitic diseases. IgE (Mol. wei-200.000) is a monomer and has two epitope-binding sites. Involved in allergic reactions. IgE does not fix complement.

IgM is a pentamer and has ten epitope-binding sites. These immunoglobulins fight blood infections. IgM (Mol. wei-900.000) has a half-life of about five days. IgM normally exists as a pentamer. IgM is the third most common serum Ig. IgG makes up approximately 80% of the serum antibodies. IgG has a half-life of 7-23 days. IgG has two epitope-binding sites.

Bovine serum and lacteal secretions contain three major classes of Igs: IgG, IgM and IgA. The bovine IgG molecule occurs predominantly in two subclasses: IgG1 and IgG2.

2.3.1. Structure of Immunoglobulin G

Immunoglobulin G (IgG) is an antibody isotype. In human serum there are five different classes of antibodies, with different biological properties. Immunoglobulin G (IgG) is the antibody present in highest concentration. IgG arranged in a Y-shape or T-shape typical of antibody monomers. Plasma B cells synthesize and secrete IgG molecules (Kaneko, et al., 2006). Immunoglobulin G (IgG) widely used in clinic for the treatment of diseases related to immunodeficiency (Gürcan, et al., 2007). The basic structure unit of an immunoglobulin molecule, called a monomer, consists of four polypeptide chains linked covalently by disulfide bonds. Immunoglobulin G (Gamma heavy chains) are heavy (~150 kDa) globular plasma proteins. The immunoglobulin protein “backbone” consists of two identical “heavy” (2 x 50 kDa) and two identical “light” (2 x 25 kDa) chains (Schur, 1972; Burton, et al., 1986; Milstein, 1969). Each chain is composed of structural domains called immunoglobulin domains. These domains contain about 70-110 amino acids and are classified into different categories according to their size and function (Barclay, 2003). Hinge region at which the arms of the antibody molecule forms a Y. IgG molecule include 1320 amino acids and each IgG has twoantigenbinding sites.

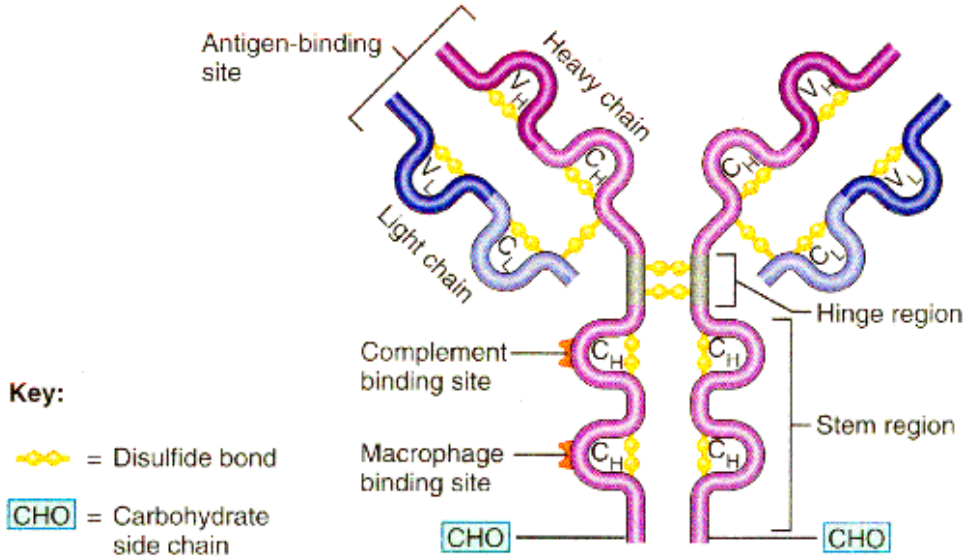

Figure 2.3. Structure of IgG.

Heavy chain; The two heavy chains are linked to each other and to a light chain each by disulfide bonds. Each end of the fork contains an identical antigenbinding site. Each heavy chain has a molecular weight of ~50,000 daltons. There are five antibody isotypes known as IgA, IgD, IgE, IgG and IgM (α, δ, ε, γ, μ). The five type of heavy chain present defines the class of antibody; α and γ contain approximately 450 amino acids, while μ and ε have approximately 550 amino acids. Heavy chain of immunoglobulin consists of a constant and variable region. The variable parts of an antibody are its V regions, and the constant part is its C region. 2 Heavy Chains: IgG VH domain has CH1, CH2, CH3. The heavy chain contains one variable domain (VH) and either three or four constant domains (CH1, CH2, CH3 and CH4, depending on the antibody class or isotype). Variable regions are contained within the amino (NH2) terminal end of the polypeptide chain (amino acids 1-110). Constant regions, comprising amino acids 111-220 (or 440-550).

Light chain; Immunoglobulin light chains are synthesized in heterologous cell. Light chains are of two types, κ and λ. These differences are detected by serological means. Identical light chains (kappa or lambda) that are covalently

linked to a heavy chain. The majority of light chains in serum exist bound to heavy chain. Each light chain has a molecular weight of ~25,000 daltons. 2 Light Chains: VL domain (110 residues), Constant Domain, CL (110 residues). The light chains can also be divided into subtypes : (a) Lambda 1, (b) Lambda 2, (c) Lambda 3, (d) Lambda 4.

2.3.2. Fracture of IgG with Papain and Pepsin Enzymes

Antibody functions can be separated into two proteolytic fragments by cleavage with the enzyme papain in the hinge region. Treatment of antibodies with the proteolytic enzyme papain cleaves the molecule into three fragments each with a MW of 50 kDa. Two of these fragments, the Fab fragments, are identical and contain the antigen-binding activity of the antibody.

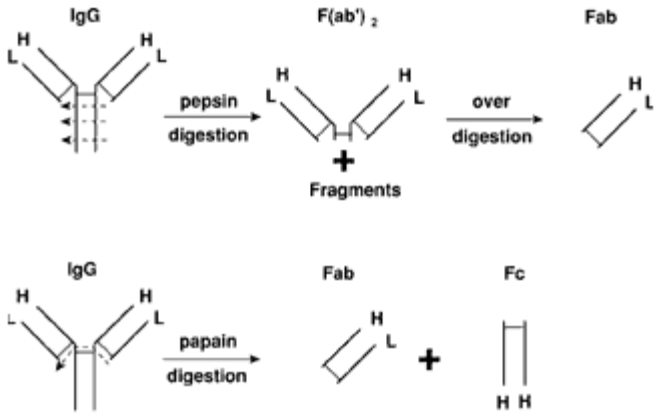

Figure 2.4. Schematic representation of IgG fragments generated by enzimatic digestions.

2.3.3. IgG subclasses

Human IgG is constituted of four isotypic subclasses which are termed as IgG1, IgG2, IgG3 and IgG4 (Roith, 1997). These subclasses are distinguished by having different heavy chains called D1, D2, D3 and D4, respectively (Alberts, et al., 2002).

Table 2.3. Properties of IgG subclasses.

Name	**Percent**	**Complement activator**	**Binds to F_c receptor on phagocytic cells**
IgG1	66%	Second-highest	high affinity
IgG2	23%	Third-highest	extremely low affinity
IgG3	7%	Highest	high affinity
IgG4	4%	No	intermediate affinity

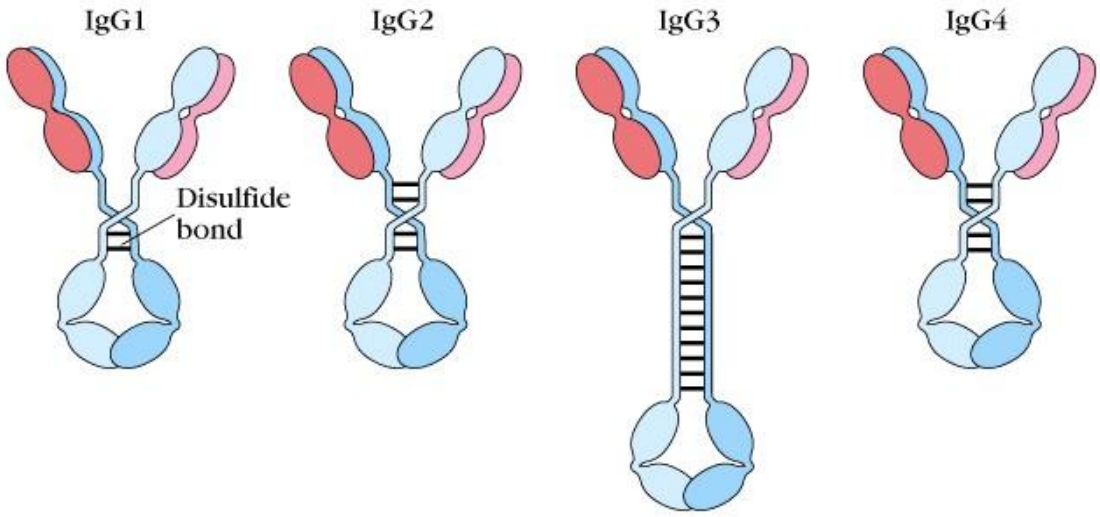

Figure 2.5. IgG subclasses.

While total serum IgG levels can differ considerably between healthy adults, the amount of each subclass is preserved within a relatively narrow range like IgG1, 60-65%; IgG2, 20-25%; IgG3, 5-10%; IgG4, 3-6% (French, et al., 1984). All four subclasses of human serum IgG have a single N-glycosylation site in the constant region of their heavy chain. Plasma and external secretions also have IgG and is expressed on the B-cell membrane. In the early 1960s, following the discovery of the IgG subclasses, there is a wide variety of

respiratory problems associated with IgG subclass deficiency that include recurrent otitis media, sinusitis, pneumonia, chronic obstructive airway disease, bronchiectasis and asthma. The way to test them is posibble by getting a IgG subclass screen in your blood. The Fc region of each IgG subclasses contains a discrete affinity for phagocyte membrane Fc receptors. IgG1 and IgG3 can bind Fc receptors more effectively than IgG2 and IgG4 (Jefferis, et al., 1994). The relative flexibility of the hinge regions of the IgG subclasses is as follows: IgG3 > IgG1 > IgG4 > IgG2 (Pumphrey**,** 1986). The half-life of IgG3 is around nine days while the half-lives of IgG1, IgG2, and IgG4 are almost 21 days (Morell, et al., 1970). These IgG subclasses have 90 to 95 percent homologous amino acid sequences (Jefferis, et al., 1985). While antibody responses to bacterial polysaccharide antigens, such as the capsular antigens of pneumococcus, are consisted mainly of IgG2 molecules, these responses against protein antigens and viral antigens are constituted predominantly IgG1 and IgG3 (Siber, et al., 1980; Spinsanti, et al., 2011). IgG 1 is the most abundant in the four subclasses and reaches 'adult' levels in early childhood. As it's mentioned before, the relative serum concentrations of the human IgG subclasses is as follows: IgG1 > IgG2 > IgG3 = IgG4 (Shakib, et al**.,** 1980; French, 1986). Four subclasses of human IgG (from IgG1 to IgG4) have been identified and characterised on the basis of differences in their structural and biological properties (Giammanco, et al., 2003). Biologically, IgG1 and IgG3 then IgG2 and IgG4, are distinguished by a higher efficiency in fixation of complement and in the capacity of interacting with the Fc receptors on macrophages and therefore as effective opsonins; IgG3, with respect to all the other subclasses, is distinguished by a higher fractional catabolic rate and by a lower half-life (Cushley, 1998). Human IgG has four subclasses, each encoded by a separate C region gene and endowed with unique biological functions which are important on humoral immunity (Jeferis, et al., 1992). IgG2 is generally generated against to carbohydrate antigens as a response (Scott, et al., 1990). One of the indications of IgG administration is to enhance resistance of the patient to infections (Hasnaoui, et al., 1997). Most often, IgGs directed against bacterial pathogens belong to the IgG2 subclass (Hammarstrom, et al., 1986). Hence, in situations of bacterial infections, it would be advantageous to use enriched IgG2 preparations.

Human immunoglobulin G has also commercial value as an important serum protein that produced worldwide on a large scale. Purification of human IgG to homogeneity is a prerequisite for studying their diagnostic and therapeutic applications. A variety of methods for protein precipitation are described in the literature but the most popular method is ethanol precipitation to purify IgG from human plasma on industrial scale (27 tonnes/year) (Muronetz, et al., 2003).

2.3.4 Medical Application of Immunoglobulins

IgG antibodies are essential for fighting bacterial and viral infections. These antibodies are produced for viruses, bacteria, and antitoxins. It is occured in most tissues and plasma. Immunoglobulin G is the most abundant Ig in the serum. Raised IgA prognosticate alcoholic cirrhosis, raised IgM prognosticate viral hepatitis and primary biliary cirrhosis, whereas IgG is elevated in viral hepatitis, autoimmune hepatitis and cirrhosis.

2.4. Purification of Antibodies

Antibody purification includes separation of antibody from serum (polyclonal antibody), various solutions of a hybridoma cell line (monoclonal antibody) (Çanak, 2004; Uzun 2003). Antibody purification methods can be summerized as follows:

2.4.1. Physicochemical Fractionation Antibody Purification

According to these purification methods antibodies separated according to their size, charge or chemical characteristics, these kinds of purifications are called non chromatographic.

2.4.1.1. Size-exclusion Chromatography (SEC)

These techniques generally used for laboratory scale purification and the separation is based on the size of the antibodies. Size exlusion chromatography can not purifiy antibodies alone and generally this method used with other purification techniqus such as precipitation.

2.4.1.2. Precipitation

Precipitation is mostly used for enhance the antibodies from serum or other solutions like cell culture supernatants. The selectivity, yield, purity and reproducibility of precipitation depends on the experimental conditions (time, temperature, pH, etc.). Ammonium sulfate precipitation is a common method for purification of antibodies and it can be used for effective purification for some antibody applications, but generally it is used for preliminary step before column chromatography or other purification applications. Octonoic acid, polyethylene glycol and ethacridine are the other precipitation agents used for antibody purifications.

2.4.1.3. Ion Exchange Chromatography (IEC)

The method is based on the electrostatic attraction between opposite charges on the support and the antibody molecules in a given buffer systems. Two charge modes (anion and cation exchangers) with several ionic forms (strong and weak) are available for resins.

2.4.2. Affinity Purification of Antibodies

2.4.2.1. IgG Purificaiton with Protein A, G and L,

Protein A, protein G and Protein A/G are covalently immobilized to the adsorbent, and they have several antibody-binding domains. The high specificities of these proteins provides excellent selectivities for antibodies especially for IgGs. These are bacterial cell wall proteins produced by Staphylococcus aureus and group G streptococci, respectively. These ligands have the ability to bind to the constant region of many types of immunoglobulins. Protein A and protein G bind most strongly to immunoglobulins at or near neutral pH, but readily dissociate from these solutes when placed in a buffer with a lower pH. These two ligands differ in their ability to bind to antibodies from different species and classes; for example, human IgG_3 binds much more strongly to protein G than protein A, and human IgM shows no binding to protein G but does interact weakly with

protein A. A recombinant protein known as protein A/G, which blends the activities of these ligands, also is available for use in affinity columns.

2.4.2.2. Antigen-Spesific Affinity Purificaiton of Antibodies

Antigen-spesific affinity purification is a process in which the binding affinity of an antigen (Ag) to a parent antibody (Ab) is utilized as a basis of separation. The antibody specific to the protein of interest is immobilized onto a rigid solid support to yield an active immunosorbent. A complex mixture of proteins is then passed over the immunosorbent whereby the antibody captures the protein of interest and the other nonproduct proteins are washed away in the column fall through. The reversible interaction between the antigen and antibody can be disrupted to yield a highly purified product in the column eluate (Verdoliva, 1989). This could be achieved by changes in pH or use of chaotropes such sodium or potassium thiocyanates/ureas as eluents. Due to the customized avidity and specificity, monoclonal antibodies (mAbs) have become indispensable for both protein characterization and purification (Pfeiffer, et al., 1987).

2.4.2.3. Immobilized Metal Chalate Affinity Chromatography

Immobilized Metal Chelate Chromatography (IMAC) can be used fort he purification of antibodies via the interactions of chalated metal ions (Cu2+, Ni2+, etc.) with proteins or peptides. The mammalian IgGs has histidine clusters which is capable to interacts metal ions effectively.

2.4.2.4. Dye Ligand Afinity Chromatography

Azo dyes that are containing one or more azo groups represent by far the largest family of organic dyes. In a reactive dye a chromophore contains a substituent that is activated and allowed to directly react to the surface of the substrate. The dyes contain a reactive group (often trichlorotriazine), either a haloheterocycle or an activated double bond, that, when applied to a fibre in an alkaline dye bath, forms a chemical bond with an hydroxyl group on the cellulosic. Reactive dyes are most commonly used in textile industry for dyeing of cellulose like cotton or flax, but also wool is dyeable with reactive dyes.After Rattee and Stephens invented in 1954 at the Imperial Chemical

Industries Dyestuffs Division sitein Blackley, Manchester, United Kingdom, reactive dyes first appeared commercially in 1956. Dyes are soluble and coloured organic compounds.Reactive dyes are a category of dyes which attach to the substrate by chemical, covalent bonds formed between the anchor group contained in the dye and the functional groups present in the substrate.The first reactive dye was invented in 1954 and marketed by ICI under the trade name Procion MX.

Reactive azo dyes are brightly colored and highly water soluble. Textile industries generally used these dyes for their ability to form covalent bonds with cellulosic moiety of the textiles (Lee, et al., 2004). Application of these dyes not only important in textile industries but also in food, paper and cosmetic industries (Yang, et al., 2005). Reactive dyes are also extensively used in affinity columns and in batch systems for the purification of large biomolecular compounds because they are able to bind proteins in such a remarkably specific manner that the protein of interest can be purified from a complex mixture in a single step. Reactive dyes are not as selective as raised antibodies, but their low costs make them attractive for reseachers. Reactive dyes are usually used supported on agarose, to which they are covalently bonded (Haff, et al**.,** 1978; Guaratini, et al., 2002). Most of the dyes disposed are of non-biodegradable nature and this is other advantage of these dyes.

Reactive Red 120 dye (RR-120) contains two phenolic OH groups and six sulphonic acid groups. It is understood that the sulphonic acid group dissociate at lower pH (below pH=1), the amino groups ionize at pH 3.5 and the phenolic OH groups dissociate at higher pH. RR-120 contains two azo groups as the chromophoric moiety, two chlorotriazine groups as the reactive groups, six sulphonic acid groups and two phenolic OH groups. The UV–vis absorption spectra of RR-120 mainly shows two absorption peaks,one in the UV region at λmax=290 nm and another doublet peak in the visible regionat λmax=512–535 nm.

Certain reactive textile dyes showed properties that have ability to bind to proteins especially those with affinities to various nucleotides (Kopperschlager, et al., 1968; Haeckel, et al., 1968). Also, it's found that Immobilized dyes can bind the proteins from 5 to 60 % in various crude cell extracts. Usage of reactive azo dyes is the largest and the most important class of synthetic organic dyes used in textile industry (Scopes, 1986; Riu, et al., 1997).

Properties of Reactive Dye:

1) Reactive dyes are cationic dyes, which are used for dyeing cellulose, protein and polyamide fibres.

2) Reactive dyes are found in power, liquid and print paste form.

3) During dyeing the reactive group of this dye forms covalent bond with fibre polymer and becomes an integral parts of the fibre.

4) Reactive dyes are soluble in water.

5) They have very good light fastness with rating about 6. The dyes have very stable electron arrangement and can protect the degrading effect of ultra-violet ray.

6) Textile materials dyed with reactive dyes have very good wash fastness with rating about 4-5 due to strong covalent bonds formed between fibre polymer and reactive group of dye.

7) Reactive dye gives brighter shades and has moderate rubbing fastness.

8) Dyeing method of reactive dyes is easy. It requires less time and low temperature for dyeing.

9) Reactive dyes are comparatively cheap

10) Reactive dyes have good perspiration fastness with rating 4-5. Reactive dyes have good perspiration fastness.

Dye affinity chromatography is useful and cheap protein purification method which is based on the high affinity of immobilized dyes for the binding sites on many proteins. Dye-ligand affinity works based on the affinity of the reactive dyes to bind proteins in a selective and reversible manner. The dyes are generally monochlorotriazine compounds. They were originally developed in the textile industry. Dye ligand chromatography is firstly introduced in 1968 by Haeckel et al. experiment about purifying pyruvate kinase using gel filtration chromatography and it's found that BlueDextran (a small dye molecule) co-eluted with the protein (Haeckel, et al., 1968). Affinity chromotography which is one of several types of adsorption chromotography, is suitable for the efficient isolation of biomolecules. The reactive textile dyes which can be described are a group of synthetic ligands that have been widely utilized to purify an astounding array of individual proteins (Lowe, et al., 1984; Lowe, et al., 1981).

C.I. Reactive Red 120 (RR120) and C.I. Reactive GreeneHE 4BD) are two reactive dyes which bearing two azo groups as the chromophoric moiety and two monochloro-s-triazine groups as reactive groups (Guaratini, et al., 2001).

Even though they have inherent problems, they are effective that's why they are widely used (Ingamells, 1993). Reactive dyes are extensively used in dyeing processes in textile industry but the main problem is that about 20–40% of these dyes remain in the effluents (William, et al., 1997; Wu, et al., 1998). Reactive dyes which are highly soluble in water exhibit a wide range of different chemical structures which is primarily based on substituted aromatic and heterocyclic groups (Epolitoa, et al., 2005). Reactive dyes are typically azo-based chromophores combined with various types of reactive groups, which show different reactivity. Reactive dyes have about 20–30% amount of the total dye market share. It was first discovered 35 years ago that these reactive dyes have commercial importance (Vandevivere, et al., 1998; Matyjas, et al., 2003). Classification dyes stuffs are as follows:

- Azo dyes
- Nitro and nitroso dyes

- Dyes polimet
- Arilmetin dyes
- Azo annulene dyes
- Carbonyl dyes
- Sulfur dyes

The most common dyes are azo dyes.The number of azo dyes are equal the sum of all the other classes of dyes. Reactive azo dyes are extensively used in dyeing processes in textile industry. Reactive dyes which contain one or more azo bond (-N=N-), exhibit a wide range of different chemical structures which is primarily based on substituted aromatic and heterocyclic groups (Naveen, et al., 2011; Cho, et al., 2007).

It has been estimated that about 5–50% of more than 700,000 tons of dyes produced yearly ends up in the wastewater that's why dyes released from the textile industry have become a major concern (Hardin, 2007). Because of their advantages in protein purification for both laboratory and large-scale separation, dye-ligand chromatography has gained considerable importance in recent years.

2.5. Cryogels

Cryogels are a very good alternative to chromatographic supports because they have many advantages such as large pores, short diffusion path, low-pressure drop and very short residence time for both adsorption and elution. On the other hand, they are also cheap materials and cryogels can be used as disposables thereby avoiding cross-contamination between batches (Denizli, et al., 2011).

Moderately frozen solutions of monomeric or polymeric precursors are utilized for making cryogels which are gel matrices. Cryogels typically have inter connected macropores (or supermacropores), allowing unhindered diffusion of solutes of practically any size, as well as mass transport of nano an deven microparticles. Osmotic, chemical and mechanical stability of these cryogels

makes them an attractive chromatography matrix wherein the solid support for biological nanoparticles (plasmids, viruses, cellorganelles) and even whole cells. Cryogels that are formed from polymeric precuusors can be considered as one of the effective choices for the immobilization of biomolecules and cell. Cryogels are designed as a result of cryogenic treatment (freezing, storage in the frozen state for a definite time and defrositing) of low or high molecular weight precursors, as well as colloid systems all capable of gelling (Yılmaz, 2008). Production is carried out in partially frozen reaction system where ice crystals perform as pore-forming material (porogen) and the geletion proceeds in non-frozen microphase of the apparently frozen reaction system. shows cyrogel formation (Yılmaz, 2008).

When gelation in microphase is completed, melting of the reaction system results in a system of large pores (the space previously occupied byice crystals) surrounded by walls of dense hydrogel formed in the unfrozen microphase. Cryogels have large pores, as a consequence of that they have low surface area and also low adsorption capacity. Bead embedding method is a useful improvement for the combining the supermacroporosity and low flow resistance properties of the cryogel with large surface area and the high adsorption capacity properties of the microspheres That was aimed the purification of IgG subunits with composite cryogels (Baydemir, et al., 2009).

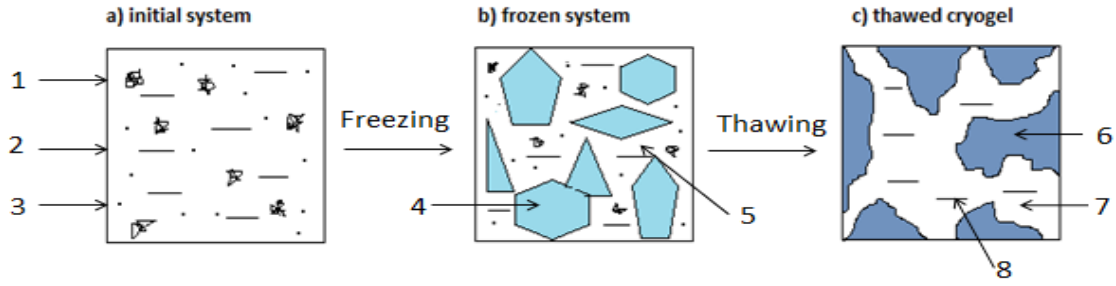

Figure 2.6. Cryogel formation 1) Macromolecules 2) Solution 3) Solvent 4) Polycrystals of freezed solvent 5) Unfreezed solvent 6) Cryogel 7) Macropores 8)Solvent

Figure 2.7. Production of cryogels in different forms.

3. EXPERIMENTAL

3.1. Materials

Human IgG (Sigma Cat. No: I4506), the polymerization reagents; ethylene glycol dimethacrylate (EGMA), hydroxyethyl methacrylate (HEMA), poly (vinyl alcohol) (MW: 100,000, 98 % hydrolyzed), toluene, α-α'-azoisobutyro nitrile(AIBN), ammonium persulfate (APS), methylenebis(acrylamide) (MBAAm), and N,N,N',N'-tetramethylene diamine (TEMED) were supplied from Sigma (St. Louis, USA). Dye ligands, Reactive Green HE 4BD and Reactive Red 120 and all the other chemicals were also supplied from Sigma. Human plasma (Sigma Cat no: H4522) Deionized water was obtained using a Barnstead (Dubuque, IA) ROpure LP®reverse osmosis unit possessing a high flow cellulose acetatemembrane (Barnstead D2731), a Barnstead D3804NANOpure® organic/colloid removal subunit and an ion exchange packed bedsystem.

3.2. Preparation of PHEMA Beads

Poly(hydroxyethyl methacrylate) (PHEMA) were synthesized as follows (Çanak et al., 2004) the stabilizer 400 mg PVAL were dissolved in 400 mL of deionized water and sonicated for half an hour. Then 4 mL of HEMA, 8 mL of EGMA, 12 mL of toluene mixed in another flask for generating the dispersed phase and 0.2 g of AIBN was added and solved in it. Both the dispersed and the stabilizer phases were mixed in a two-necked flask and N_2 gas were pass through the polymerization mixture for 15 min. The two-necked polymerization reactor was submerged in a water bath at 70^0C and the mixture was mechanically stirred at 750 rpm during the polymerization process that takes 8 hour. After the polymerization were completed the reactor was removed from the water bath and cooled down to room temperature. Then, the beads were removed from the suspension medium by filtration and then washed several times using ethanol: deionized water (40:60, V:V) mixture. After the beads were dried and then they were sieved.

beads (20-71 μm- in diameter) were used during this study. The beads were stored in 0.03 % sodium azide solution at 4 ^{0}C in a refrigerator until use.

3.2.1. The Dye Attachment to PHEMA Beads

Reactive Green HE 4BD (RG) and Reactive Red 120 (RR) dyes were used as ligands for the separation of the IgG subclasses. Both of the dyes were covalently attached to the PHEMA beads; the hydroxyl groups of the PHEMA beads reacted with the dye molecules through chloride group, which located in triazine ring of the dyes (Denizli, et al., 2006). Dye attachment procedure was the same for both of the dyes, the procedure can be described as follows; 1 g of PHEMA beads were swelled in 100 mL of the deionized water at 60 ^{0}C while 1 g of the dye were dissolved in 300 mL of deionized water at 60 ^{0}C. Later, they were mixed and 2 g of NaCl were added in it, and this mixture was magnetically stirred for one hour at constant temperature of 60 ^{0}C. Finally 0.2 g of Na_2CO_3 was added in it and the temperature were increased at 80 ^{0}C and mixed for 3 hours. After this procedure the dye attached beads (RR-PHEMA and RG-PHEMA) were filtered and washed several times with methanol and deionized water respectively for removing of unreacted and non-specifically attached dyes from the beads. The washing procedure was repeated until no dye leakage from the PHEMA beads. The leakage of dyes was determined using UV spectrophotometer at 640 nm. The supernatant of the washing mixture were measured after the each washing step and repeated until to obtain zero absorbance value at 640 nm. The dye attachment procedure was performed for each of the Reactive green HE 4BD and Reactive Red dyes in separately. Both Reactive Green HE 4BD and Reactive Red 120's dichlorotriazinyl groups have reactive chloride atoms, these reactive chloride atoms were converted to amino groups by using 2M NH_4Cl at pH 8.5 for 6 h at room temperature. The modified dye attached polymer beads filtered, washed and finally stored at 4 ^{0}C in aqueous solution (Yavuz et al ., 2006).

3.3.Preparation of RR/RG attached PHEMA bead embedded composite cryogel disks

While the 100 mg of dye-attached beads were swelled in 5 mL deionized water in an ice bath, the 6 mmoL of HEMA; 1 mmol of mBAA, were dissolved

in 10 mL deionized water in another flask. Then, the polymerization mixtures were mixed and magnetically stirred for half an hour in ice bath. APS/TEMED initiator pairs initiated the polymerization, and the amount of the initiator pairs was 1% w/v of the total monomers. The final mixture were poured in to the two glass sheets, which are separated each other with 1.5 mm thick spacers. The polymerization mixture in glass sheets was frozen at -12 ^{0}C, 24 hour later the polymerization was completed and it was thawed at room temperature. Same procedure was repeated for the preparation of 6 different beads embedded composite cryogel discs (CCDs).

Table 3.1. The type and the amount of the embedded beads in composite cryogel.

Name of the CCD	**Bead Type**	**Bead Amount (mg)**
RG1-CCD	RG-PHEMA	100
RG2-CCD	RG-PHEMA	200
RG3-CCD	RG-PHEMA	300
RR1-CCD	RR-PHEMA	100
RR2-CCD	RR-PHEMA	200
RR3-CCD	RR-PHEMA	300

Control-CCD	C-PHEMA	100
Non embedded-CD	-	-

The types and the amounts of the embedded beads in composite cryogels were given in Table 3.1 and the names of the prepared composite cryogels were also summarized in it. The composite cryogel sheets were cut by a perforator to obtained circular composite cryogel discs (CCDs) in 2.5 cm diameter. The obtained disks were washed with methanol and deionized water and stored at 4^0C until use.

3.4. Characterization of RG-PHEMA/RR-PHEMA CCD

The swelling ratios (%) of the CCDs were calculated via the following experiment; firstly the dried discs were weighed and recorded as W_d and then put in to a vessel containing deionized water at fixed room temperature for 24 h. After the disks were swelled in deionized water they were removed from the vessel, then weighed and recorded as W_s. Finally the swelling ratio (%SR) was calculated using the following equation,

SR %: [(Ws-Wd)/Wd] x100 (3.1)

The second group of experiment was conducted for the determination of macroporosity of the CCDs roughly. The weight of the swollen CCDs was recorded (W_s) firstly and then the CCDs were squeezed to removed the water from the macropores of the CCDs and recorded as W_{sq}, the the macroporosity was defined as follows,

Porosity %= $[(W_s-W_{sq})/W_s]x100$ (3.2)

SEM examined the surface morphology of the CCDs. The samples were prepared as follows, the swollen CCDs were freezed-dried at 50^0C using a lyophilizer (Chris Alpha 1-2 LD plus, M Christ GmbH, Germany). After the samples were coated with gold- palladium (40:60), the SEM photographs were taken using JEOL JSM 5600 SEM, (Tokyo/Japan). The specific surface areas of the RR-PHEMA, RG-PHEMA beads and the RR-CCD and RG-CCD were obtained using multipoint BET method. (Quantachrome, Nova 2200E, USA). Elemental analysis was used for the identifying the dye amounts into the covalently dye attached PHEMA beads. The measurements were performed using, Leco Elemental Analyzer (Model CHNS-932, USA).

Fourier transform infrared spectra of Reactive Green HE 4BD, Reactive Red 120, PHEMA beads and RG-PHEMA bead and RR-PHEMA beads were obtained using FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The 0.1 gram of dried samples were mixed with 0.1 gram of KBr, and this mixture was transformed into a pellet under the pressure and the FTIR spectrum was recorded using these pellets.

3.5. Adsorption of HIgG from Aqueous Solutions

The adsorption studies were performed according to the interactions of CCDs and HIgG through a continuous recirculating system. 2 pieces of CCDs were placed in a plastic disk holder column, and the solution passed through the column using a peristaltic pump (Watson-Marlow, Wilmington, MA, USA). At the beginning of the each experiment the CCDs were washed with 20 mL deionized water, and then equilibrated with 50 mM phosphate buffer at pH 7.4 for 15 min. The effects of various parameters were investigated on HIgG adsorption on different CCDs. HIgG adsorption was evaluated by analyzing their absorbance at 280 nm using a UV spectrophotometer. Then, the adsorption amount was calculated according to following mass equation,

$$Q=[(Ci-Cf)xV]/m \quad (3.3)$$

In this equation Q is the adsorption amount of the HIgG on to unit mass of CCDs in the unit of mg/g; Ci is the initial HIgG concentration of the aqueous solution in the unit of mg/mL; Cf is the final HIgG concentration of the aqueous solution which obtained after the treatment of the HIgG solution with the CCDs in mg/mL; V is the volume of the aqueous solution in mL; m is the mass of the dried CCDs used during the experiment in (g). Each experiment was performed triplicate for conforming the data.

Firstly the effect of pH on HIgG adsorption amount was determined by changing the solution pH between 4.0 and 8.0. The experiments performed using RG1, RR1 CCDs.

Second group of experiments were performed for the investigation of the effect of the initial concentration of HIgG in aqueous solution on HIgG adsorption amount. It was determined by changing the initial concentration of HIgG in the range of 0.2 mg/mL and 3.5 mg/mL. The 5 mL of the HIgG solutions were passed through the RG1, RR1 and C-PHEMA columns for 2 hour at room temperature. The effect of the amount of the embedded beads on HIgG adsorption amount was also determined. The three different amounts RG-PHEMA embedded RG-CCDs and also three different amounts RR-PHEMA embedded RR-CCDs were synthesized and the adsorption studies were performed using them. The 5.0 mL of HIgG solution (3 mg/mL, pH 7.4 phosphate buffer) was passed through the columns for 2 hour at room temperature. Then, the effect of flow rate on the HIgG adsorption amount was investigated by changing flow rate between 0.5 to 3.0 mL/min. For this purpose the 5.0 mL of HIgG solution (3 mg/mL, pH 7.4 phosphate buffer) was passed through the CCDs for 2 hour at room temperature. Finally, the effect of the salt concentration on HIgG adsorption amount was investigated. 5.0 mL of HIgG solutions (3 mg/mL, pH 7.4 phosphate buffer) containing 0.001 M, 0.01 M and 0.1 M NaCl were prepared and passed through the CCDs for 2

hour at room temperature.

3.6. Desorption of HIgG

Desorption of HIgG was performed using 2M NaCl solution. HIgG adsorbed CCDs were treated with the 15 mL of desorption buffer in a continuous recirculating system. The reusability of the CCDs were studied by performing repeated ten adsorption-desorption cycle using the same CCDs and the desorption ratio (DR %) was calcuted according to the following calculation,

$$DR\ (\%) = \frac{\text{Amount of the desorbed HIgG}}{\text{Amount of the adsorbed HIgG}} \times 100 \qquad (3.4)$$

3.7. Separation of IgG subclasses from commercial IgG solution and human plasma

A typical experiment was conducted for the separation of IgG subclasses from commercial IgG solution, as follows:

The commercial solution of IgG was used for which integrates 66.0% IgG1, 22.0% IgG2, 7.3% IgG3, 4.7% IgG4. 6 mL of this solution was passed through the CCD columns for 2 hours at room temperature. IgG subclasses were detected nepheometrically using a Dade Behring BN2 Nephelometer Analyzer. The analysis performed using a commercial kit provided by Dade Behring, Germany.

The separation of IgG subclasses from human plasma was performed using 10 mL of human plasma, which was obtained from Sigma (Cat no: H4522). The plasma samples were diluted using 50 mM pH 7.4 phosphates buffer with dilution ratio of 1:2 and 1:5. The adsorption studies were performed using the

original human plasma sample and the diluted samples.

4. RESULTS AND DISCUSSIONS

4.1. Characterization

The optical photographs of the synthesized RR-CCDs, RG-CCDs and the control columns were given in Figure 4.1.

The characterization study results of the synthesized polymers summarized in Table 4.1.The swelling properties of the RG-CCD, RR-CCD, control-CCD and the non- embedded CD were given in Table 4.1. The swelling ratios of the RG1-CCD, RG2-CCD, and RG3-CCD were calculated as 79.0, 74.0 and 68.0% respectively. And the swelling ratios of the RR1-CCD, RR2-CCD and the RR3-CCD were calculated as 78.0,73.0, 65.0 % respectively. The swelling ratio of the CCDs is decreased with the increasing amount of the embedded beads. Maximum swelling ratio was observed for the non-embedded CD column (86.0%), which hasn't got any beads in its structure, and it absorbed water more than the other beads embedded columns. The beads allocated in the macropores of the cryogel columns so the macroporosity of the columns were decreased with increasing the amount of embedded beads. As shown in Table 4.1, all the RR attached and the RG attached column's macroporosity ratio were decreased with increasing the amount of the embedded beads, and the non-embedded CD has the maximum macroporosity ratio (76.0 %).

The surface areas of the synthesized columns were analyzed using multipoint BET method. The surface area of the columns increased with increasing the amount of the embedded bead. The surface area of the non-embedded CD was found as 26.0 m^2/g; by the embedding of the beads in to the cryogels, the surface areas of the columns were enhanced up to 192 m^2/g. So it is obvious that the embedding method improves the surface area of the cryogels.

Figure 4.1. The optic photographs of the prepared CCD columns and RG and RR attached beads.

Table 4.1. Physicochemical properties of the columns.

Name of the CCD	Bead type	Bead amount (mg)	Swelling ratio (%)	Macroporosity (%)	Surface Area (m^2/g)
RG1-CCD	RG-PHEMA	100	79.0	70.0	95.0
RG2-CCD	RG-PHEMA	200	74.0	66.0	158.0
RG3-CCD	RG-PHEMA	300	68.0	62.0	192.0
RR1-CCD	RR-PHEMA	100	78.0	72.0	89.0
RR2-CCD	RR-PHEMA	200	73.0	67.0	165.0
RR3-CCD	RR-PHEMA	300	65.0	60.0	186.0
Control-CCD	C-PHEMA	100	77.0	68.0	86.0
Non embedded CD	-	-	86.0	76.0	26.0

The elemental analysis was used for the investigation of the attachment of the Reactive Green HE 4BD and Reactive Red 120 to the PHEMA beads. The attachment of the dyes were found as 71.0 μmol/g and 96.0 μmol/g for the RG-PHEMA and RR-PHEMA beads respectively, according to the sulphur stoichiometry. Both Reactive Red 120 (Figure 4.2) and Reactive Green HE-4BD (Figure 4.3.) has six sulfonic acid groups and the amount of the sulphur originated from only the dye molecules, the other components of the polymers haven't got the sulphure atom.

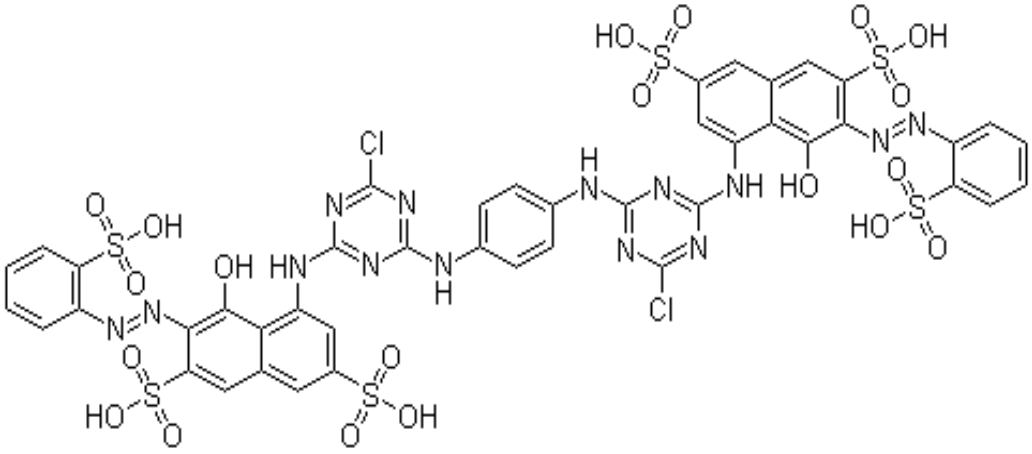

Figure 4.2. Structure of Reactive Red 120 dye (RR-120)

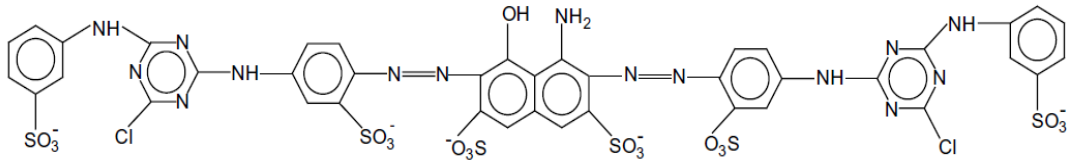

Figure 4.3. Structure of Reactive green HE 4BD

Figure 4.4 and 4.5 show the FTIR spectra of the RG, PHEMA beads, RG-PHEMA beads and RR, PHEMA beads, RR-PHEMA beads respectively in the range of 4000 to 6000 cm^{-1}. The O-H stretching vibration peaks were observed around 3500 cm^{-1} for all of the structures. The broad O-H stretching peak (3514 cm^{-1}) of PHEMA beads was significantly decreased after the dye

(RR/RG) attachments. Dye molecules attached to PHEMA covalently via the specific O-H groups of PHEMA, so the O-H stretching decreased, which confirms the covalent bonding of dye molecules to PHEMA bead. The characteristic C=O stretching vibrations can be seen at 1730 cm^{-1} and 1732 cm^{-1} and 1730 cm^{-1} for PHEMA and RR-PHEMA, RG-PHEMA beads respectively. The bands at 1203 cm^{-1} for RR and 1192 cm^{-1} for RG belong to S=O stretching vibration of the dye molecules. The bands 1045 cm^{-1} for RR and 1036 cm^{-1} for RG represent the C-Cl stretching vibrations.

The surface morphologies of the RR-PHEMA, RG-PHEMA beads and RR-CCD and RG-CCD were determined using SEM. Figure 4.6 A/B, C and D represent the SEM photographs of the PHEMA beads, RR-CCD and RG-CCD respectively. The size of the PHEMA beads is in the range of 20-71μm. In Figure C the RR attached PHEMA beads were embedded into the PHEMA cryogels, and in Figure D, RG attached PHEMA beads were embedded into the PHEMA cryogels; the beads and the cryogel morphologies could be seen from these figures obviously.

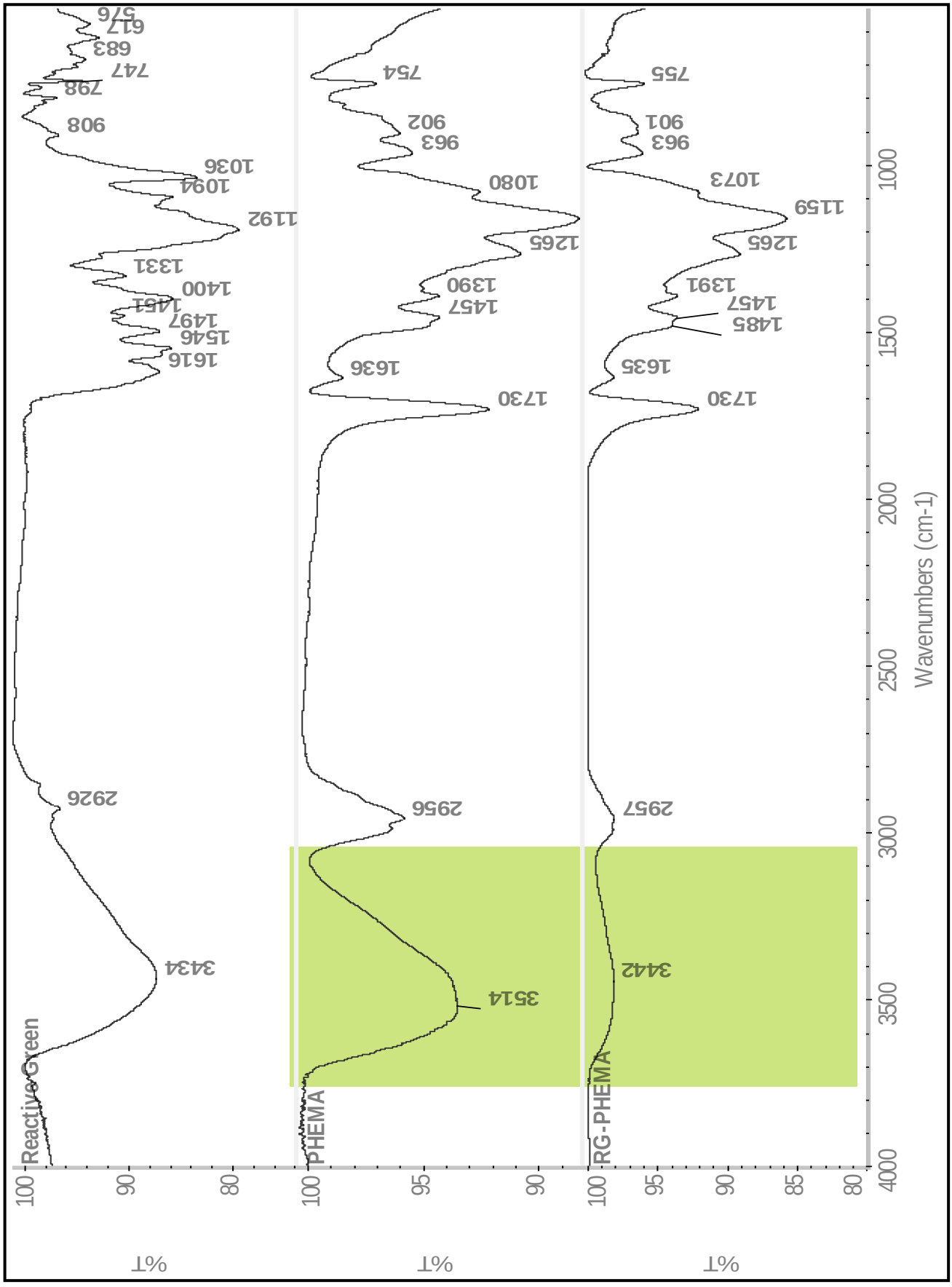

Figure 4.4. The FTIR spectrum of RG dye, PHEMA beads and RG-PHEMA beads.

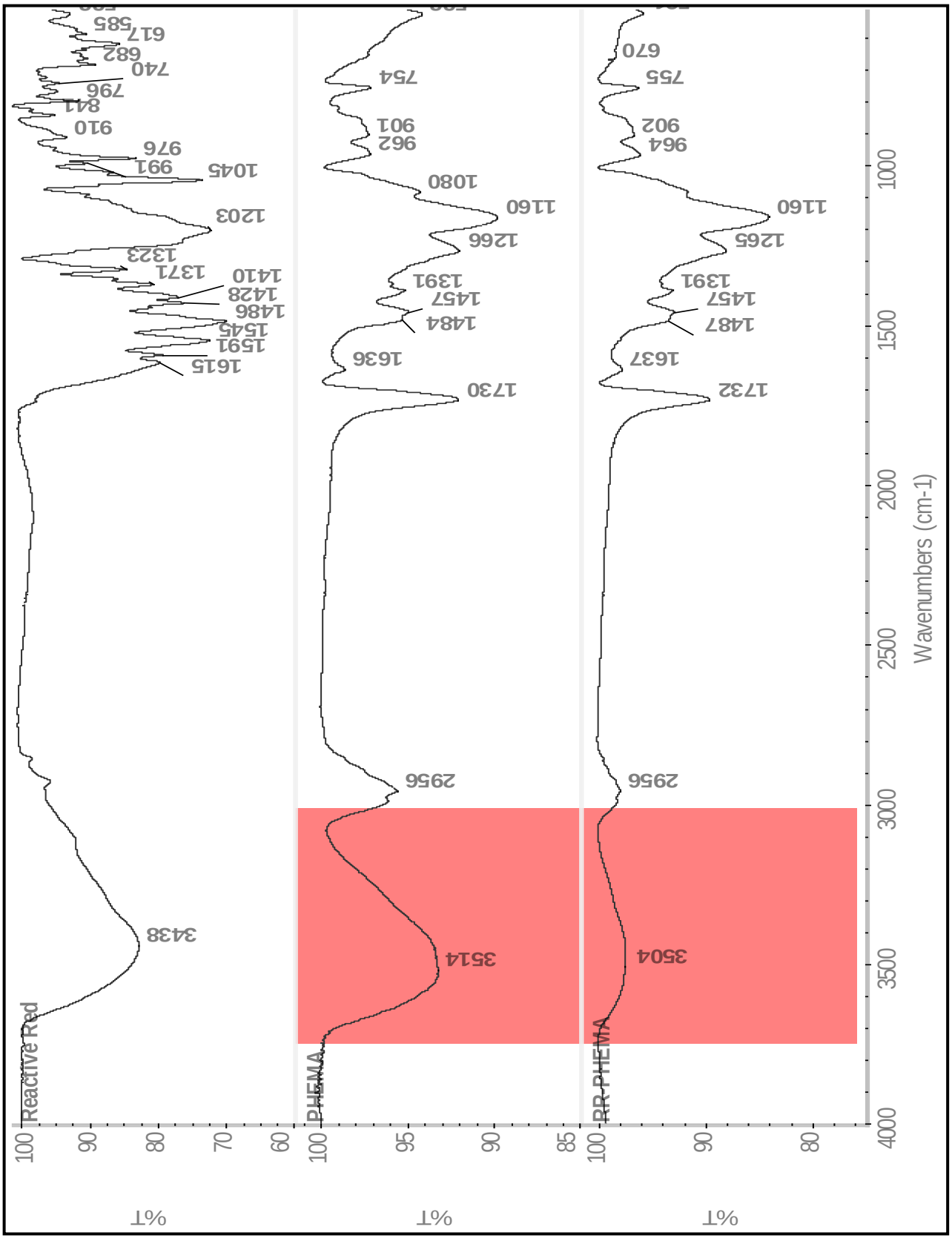

Figure 4.5. The FTIR spectrum of RR dye, PHEMA beads and RR-PHEMA beads.
.beads.

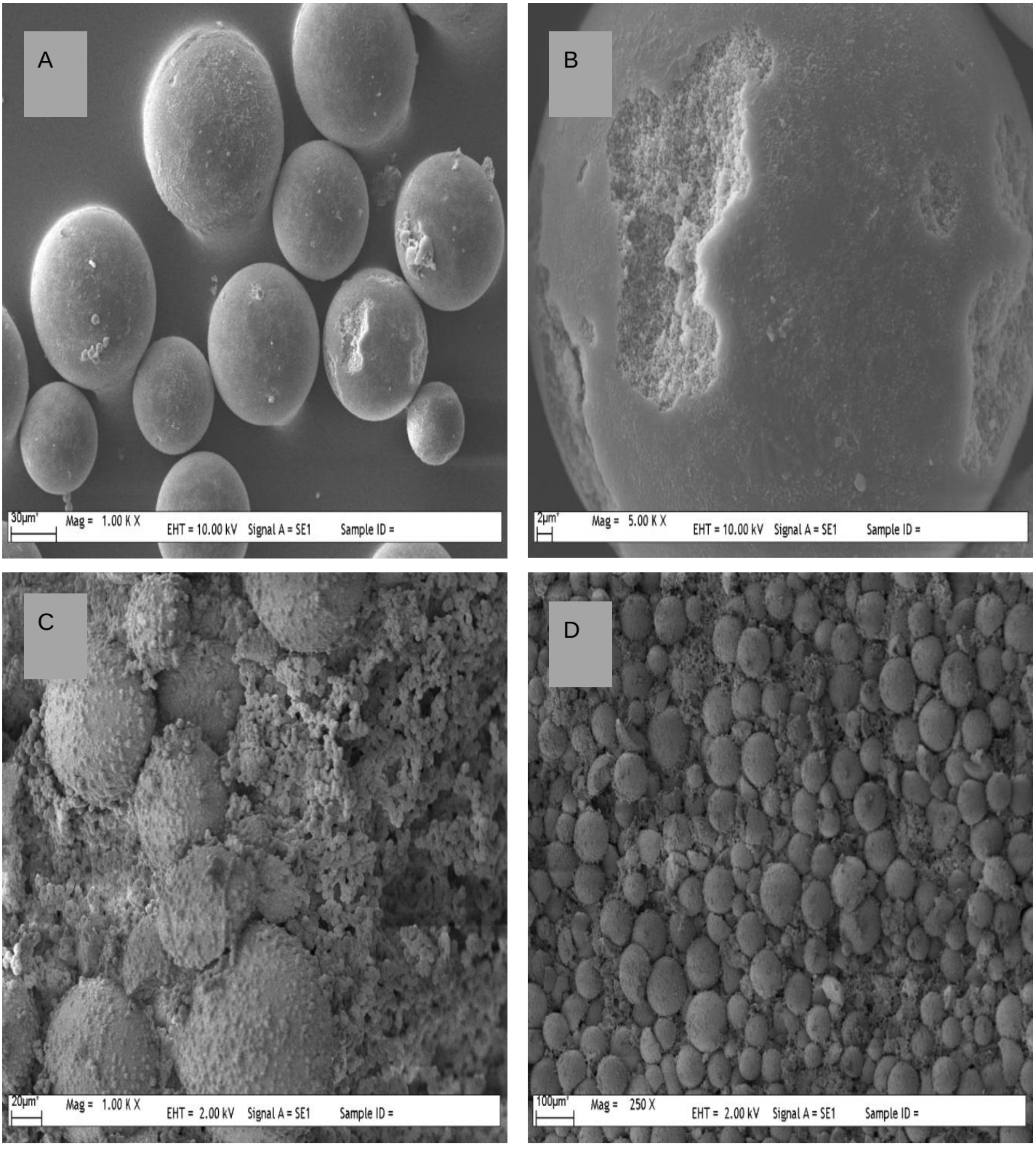

Figure 4.6. The SEM photographs of PHEMA beads (Figure A and B), RR-CCD (Figure C), and RG-CCD (Figure D).

4.2. Adsorption of HIgG from Aqueous Solutions

4.2.1. Effect of pH

Figure 4.7 shows the effect of pH on adsorption amount of HIgG onto RG-CCD, RG-CCD. The maximum adsorption was obtained at pH 6.2 for both of the dye attached discs; adsorption capacity was decreased for the other pH values. HIgG has no net charge at its isoelectric point and most of the proteins bind to the adsorbent around their isoelectronic point. Reactive Green HE 4BD and Reactive Red 120 are dichlorizine dyes and also both of them have six sulfonic acid groups in their structure. The dyes attached to the PHEMA beads by covalent bonding between the chloride groups of dye molecules and the hydroxyl group of the PHEMA beads at a basic pH value. The conformations of the attached RG and RR molecules on pHEMA bead surface may be change by changing pH. So it can be concluded that the adsorption amount of HIgG can change with changing pH depend on this conformational changes. The HIgG molecules adsorbed on to RG-CCD and RR-CCD via both the contributions of electrostatic and the hydrophobic interactions. In the light of these information, the pH value of the aqueous medium is an important parameter for the adsorption of HIgG on the adsorbent, and the optimum pH value was determined as 6.2 for the performing the other adsorption studies.

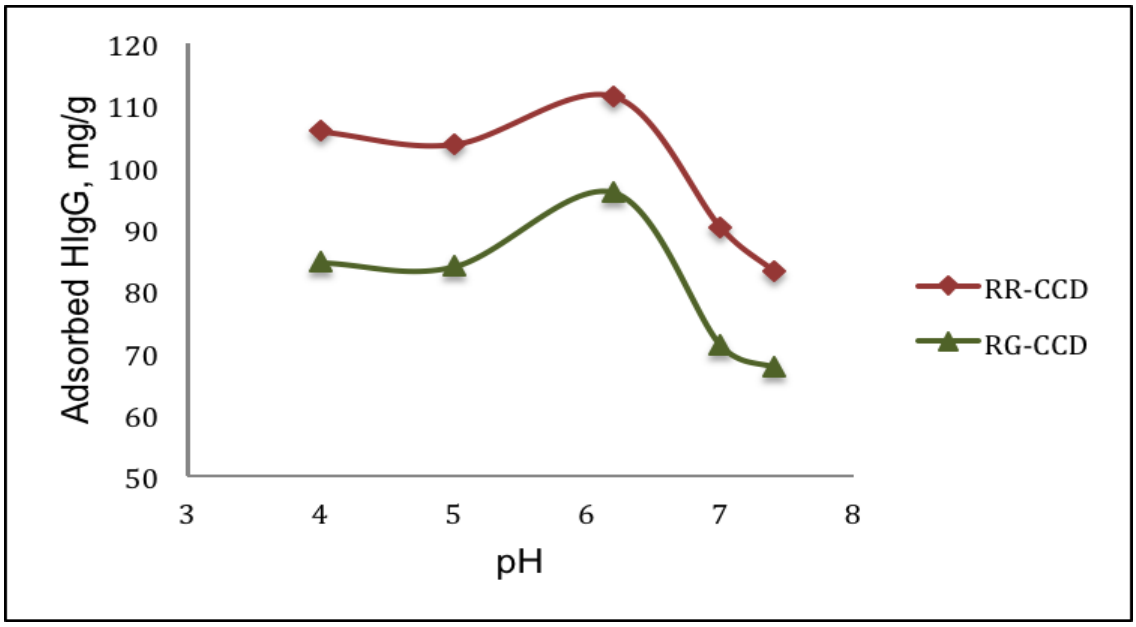

Figure 4.7. The effect of pH on HIgG adsorption amount. Ci: 1 mg/mL, V: 6 mL, mdry: 0.05 g, Flow rate: 0.5 mL/min., Time: 2 h, T: 25^0C.

4.2.2. Effect of Initial Concentration of HIgG

The effect of initial concentration of HIgG on adsorption amount of HIgG was investigated by changing the initial concentration of HIgG in the range of 0.2 mg/mL to 3.0 mg/mL (Figure 4.8). At the beginning, the adsorption amount was increased by increasing the initial concentration of HIgG and then reached a saturation value at 2.0 mg/mL. At this saturation point maximum adsorption capacity was calculated as 170.7 mg/g and 239.8 mg/g for the RG-CCDs and RR-CCDs respectively. The adsorption capacity of the RR-CCD is higher than that of RG-CCD, this can be explained as the following approaches; firstly the dye ligand amount is a very important parameter for the adsorption amount of the HIgG [R].

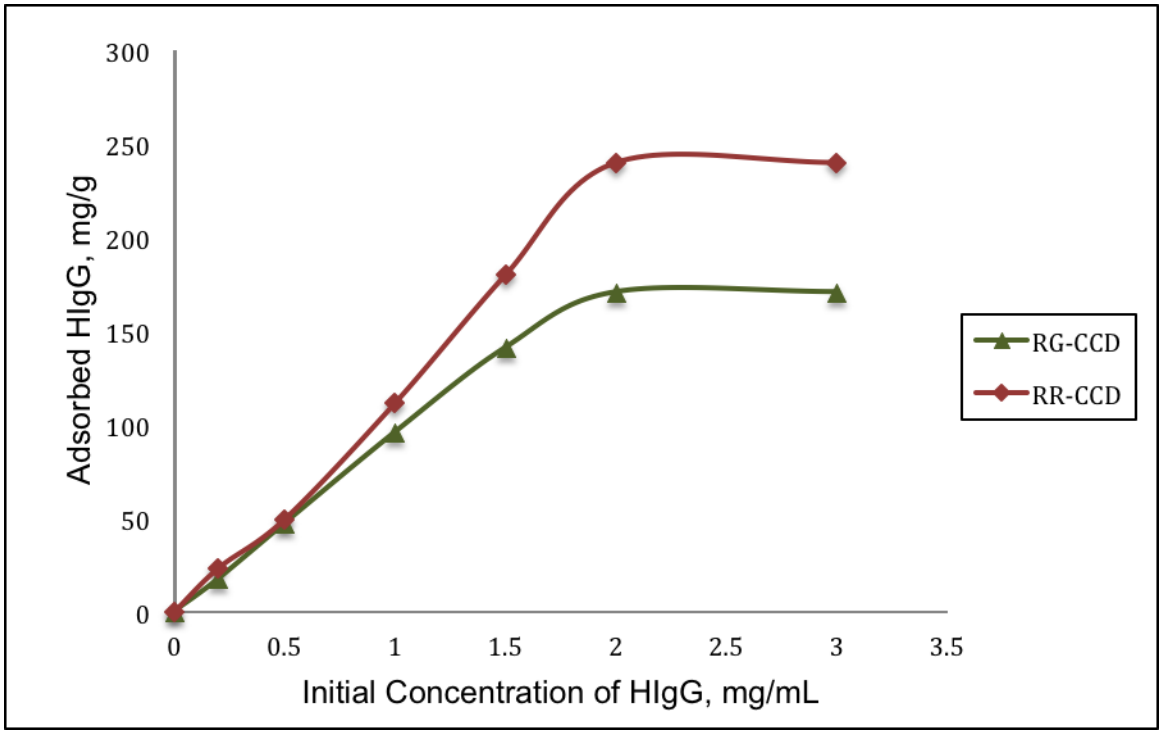

Figure 4.8. The effect of initial concentration of HIgG on HIgG adsorption amount. pH: 6.2, V: 6 mL, m dry: 0.05 g, flow rate: 0.5 mL/min., Time: 2 h, T: 25^0C

As mentioned in the characterization section, the RR attachment amount is higher than RG attachment on to the PHEMA beads according to the elemental analysis results. So it can be concluded that the HIgG adsorption capacity of the RR-CCD

is high because it has high dye ligand amount than RG-CCD. Secondly, both of the dye molecules were covalently bonded to the PHEMA beads in different configurations. The differences in the configurations of RR and RG, which are bonded on the PHEMA beads, bring with the differences in adsorption amount of HIgG. Adsorption studies were performed also using control-CCD and non-embedded-CD columns and the adsorbed HIgG amount was found as 2.65 and 2.15 mg/g respectively. Both of the control columns were not included neither RR nor RG molecules, so there are no specific interactions could take place between the columns and the HIgG molecules. The maximum HIgG adsorption capacities of the control columns were nearly 85-fold and 120-fold less than the RG attached CCD column and RR attached CCD column respectively.

4.2.3. Adsorption isotherms

Langmuir and Freundlich isotherms were calculated for the modeling of adsorption process using equilibrium adsorption data (Labrou and Colonis, 1995). Langmuir adsorption isotherm can be defined as follows:

$$Qe = Q_{max}.b.C_e/(1+b.C_e) \quad (4.7)$$

Which can be linearized as :

$$C_e/Qe = 1/(Q_{max}.\ b) + C_e/Q_{max} \quad (4.8)$$

where, Qe is the theoretical maximum adsorption capacity of Langmuir adsorption

isotherm (mg/g), C_e is the HIgG concentration of solution (mg/mL), b is the Langmuir adsorption constant (mg/mL) which indicates the monolayer adsorption, Qmax is the maximum adsorption capacity (Finette, et al., 1997). A linearized plot of Ce/Qe versus Ce (Figure 4.9) gives Qmax and b.

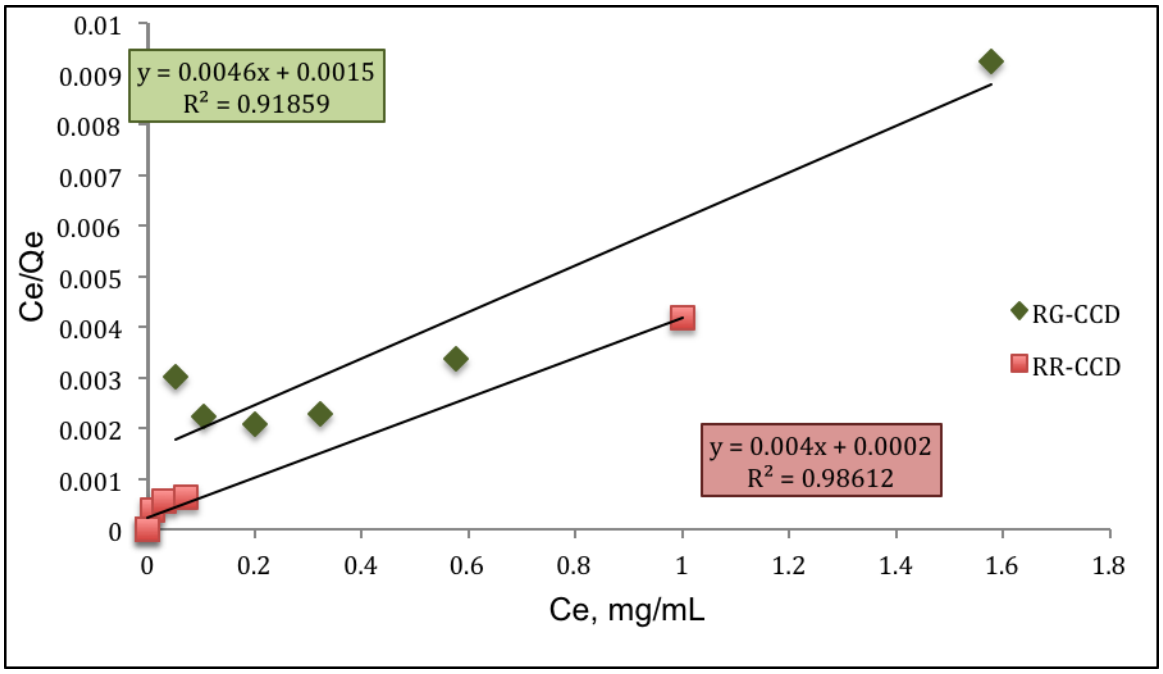

Figure 4.9. The plot of the Langmuir adsorption isotherm for RR-CCD and RG-CCD columns.

Freundlich adsorption isotherm model is used to describe for adsorption onto heterogeneous surfaces in which the adsorbed molecules have different energies (Umpleby, et al., 2001).

$$Qe = Q_f . Ce^{1/n} \quad (4.9)$$

This equation can be linearized as follows;

$$\ln Q_{eq} = \ln Q_f + (n \times \ln C_{eq}) \quad (4.10)$$

where QF is the adsorption capacity calculated using Freundlich model and 1/n is the Freundlich exponent represents the heterogeneity of the system. qF and 1/n can be determined from the linear plot of ln Qe versus ln Ce. The Freundlich isotherm describes reversible adsorption and is not restricted to the formation of the monolayer. A more homogeneous system will have n value approaching unity while a more heterogeneous system will have an n value approaching zero (Umpleby, 2001).

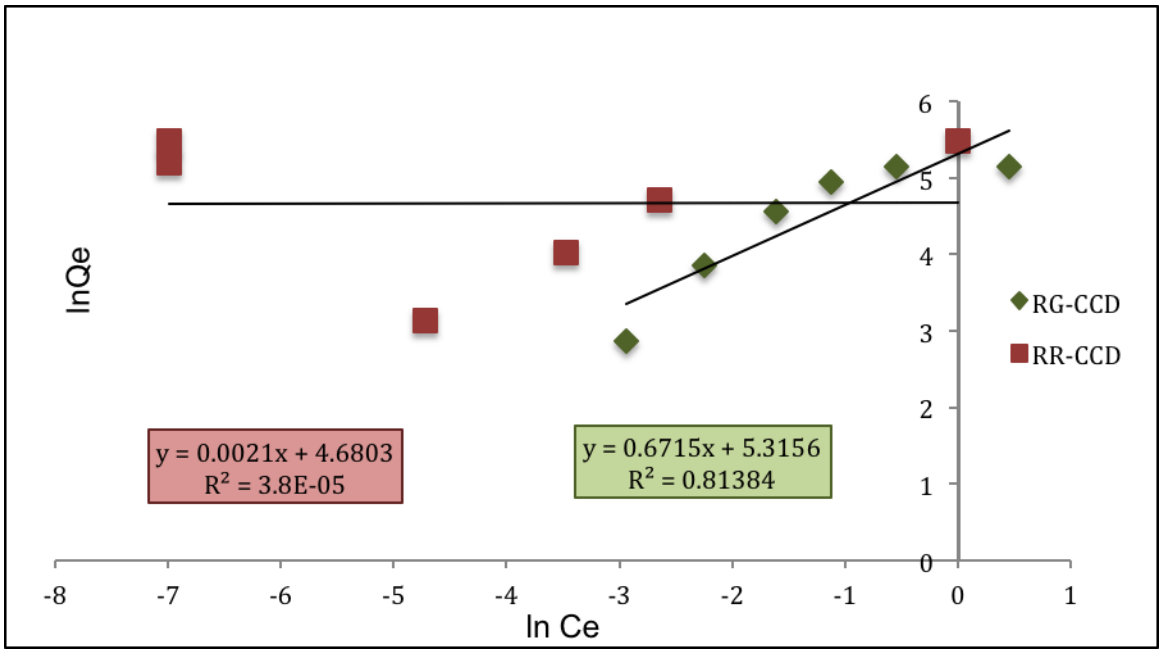

Figure 4.10. The plot of the Freundlich adsorption isotherm for RR-CCD and RG-CCD columns.

Table 4.2. Langmuir and Freundlich constants for RR-CCD and RG-CCD columns.

Name of CCD	Experimental	Langmuir constants			Freundlich constants		
	Q_{exp} (mg/g)	Q_{max} (mg/g)	b (g/mL)	R^2	Q_F (mg/g)	n	R^2
RR-CCD	239.85	250.0	20.0	0.98	107.7	0.0021	$3.8\ 10^{-5}$
RG-CCD	170.72	217.39	3.07	0.92	202.35	0.67	0.81

The experimental data closer to Langmuir rather than Freundlich isotherm, the correlation coefficient (R2) was calculated as 0.98 and 0.92 for RR-CCD and RG-CCD respectively. (Table 4.2). The maximum adsorption capacities were found as 239.85 and 170.72 mg/g for RR-CCD and RG-CCD and Langmuir adsorption capacity values were calculated as 250 and 217 mg/g for RR-CCD and RG-CCD. These results showed that the adsorption behavior of both RR-CCD and RG-CCD were accordance with Langmuir adsorption isotherm rather than Freundlich and it can be concluded that the adsorption of HIgG onto CCDs is a monolayer adsorption.

4.2.4. Effect of Flow Rate

HIgG adsorption of the RG-CCD and RR-CCD decreased with the increase of flow rate. In Figure 5.3. the adsorption amount of the RR-CCD and RG-CCD decreased from 239.8 to 129.8 mg/g and 170.7 to 109.3 mg/g respectively. In lower flow rates the HIgG molecules interacts with both of the RR-CCD and RG-CCD columns sufficiently and the columns adsorbed HIgG easily. Controversially, in higher flow rates the HIgG molecules left the column faster and have not enough time for an efficient interaction with the column so the HIgG adsorption amount of the columns decreased drastically (Figure 4.11). Derazshamshir, et al., 2010)

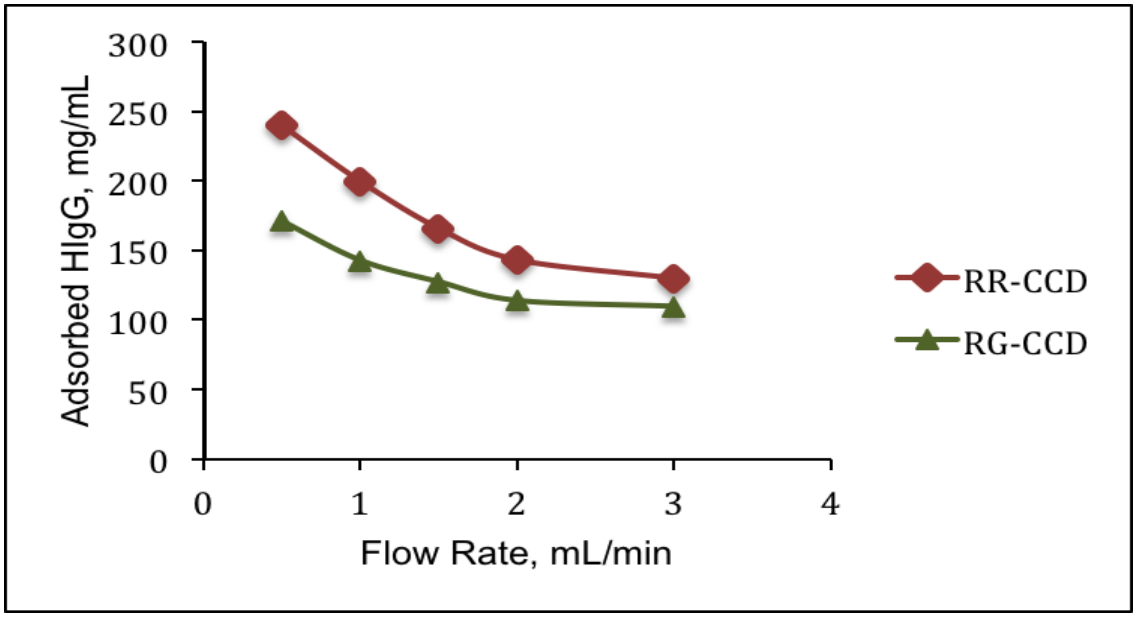

Figure 4.11. The effect of pH on HIgG adsorption amount. Ci: 2.0 mg/mL, pH: 6.2, V: 6 mL, m dry: 0.05 g, time: 2 h, T: 25^{0}C.

4.2.5.Effect of the salt concentration

The effect of salt concentration on adsorption of HIgG was investigated using aqueous solutions of HIgG, included NaCl salt in different concentrations. Figure 4.12 represents that the adsorption capacities of HIgG decreased with increasing the salt concentration for both RG-CCD ang RR-CCD columns. For RG-CCD column the adsorption amount of HIgG decreased from 239.8 to 210.2 and for RG-CCD column it was found to be 171.8 to 163.2 mg/g. When the NaCl concentration increased, the dye molecules interacted with each other rather than HIgG via the

hydrophobic interactions and they also get closer to the polymer surface. So the dye molecules conformations changed and the accessible sides of the dye molecules decreased for the HIgG adsorption. It should be noted that Denizli et al. suggested that when the salt concentration of the medium increased, the sodium ions block the sulfonic acid groups of the dyes and it leads to a decrease in the electrostatic interactions between the dye molecules and the proteins (Denizli and Piskin, 2001). The interactions between the dyes and the HIgG controlled by both the hydrophobic and the electrostatic interactions and the increase in the salt amount causes the decrease in electrostatic interaction but it should be noted that this decrease is not too much for both of the RG-CCD and RR-CCD columns because the hydrophobic interactions support the HIgG adsorption on to the adsorbents.

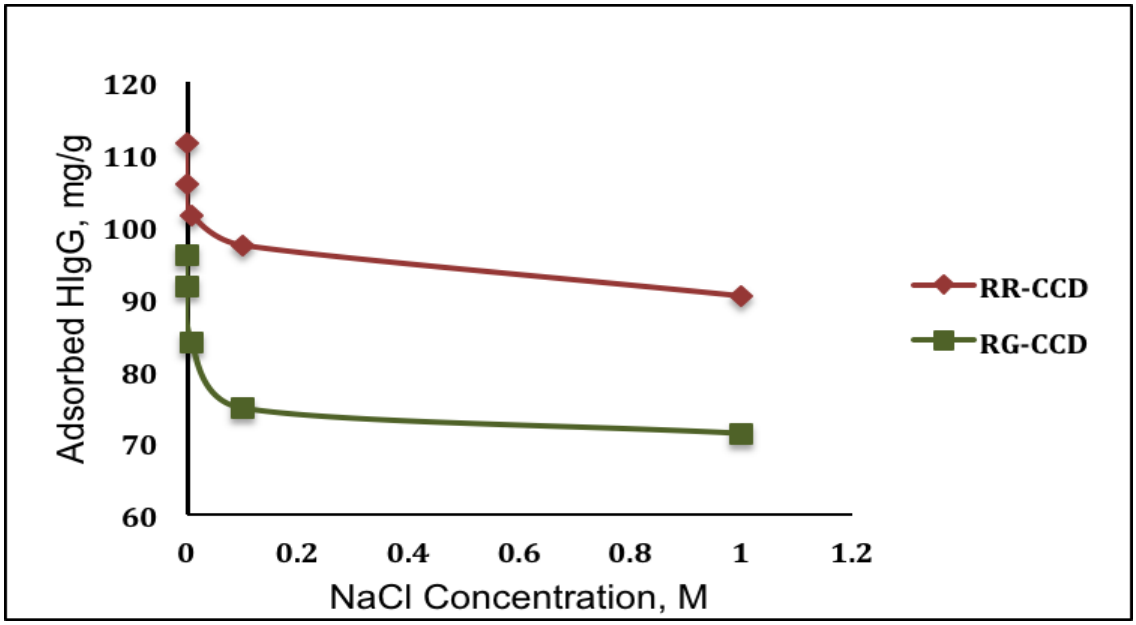

Figure 4.12. The effect of pH on HIgG adsorption amount. Ci: V: 6 mL, m dry: 0.05 g, time: 2 h, T: 25^{0}C.

4.2.6. Adsorption Kinetic Modeling

The adsorption process controlled by kinetic mechanisms, it could be evaluated using pseudo-first-order and pseudo-second-order kinetic models. The linear form of the pseudo-first-order kinetic model described by Lagergren (Cheung, et al., 2001) as follows,

$$\Delta qt/dt=k1(qe-qt) \quad (4.1)$$

where k1 is the rate constant of pseudo-first order adsorption (1/min) and qe and qt represent the amounts of adsorbed protein at equilibrium and at time t (mg/g), respectively. At the beginning of time; t=0, qt=0 and at time t t=t, qt=qt so the equilibrium 4.1 can be rearranged as follows,

$$\log[qe/(qe-qt)]= (k1t)/2.303 \quad (4.2)$$

Equation 4.2. can be linearized as Equation 4.3.,

$$\log(qe-qt)=\log(qe)-(k1t)/2.303 \quad (4.3)$$

a plot of log(qe) versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process log(qe) should be equal to the interception point of a plot of log(qe-qt) via t.

Pseudo-second order equation related with adsorption equilibrium capacity may be defined as follows,

$$\mathbf{\Delta}qt/dt = k2\ (qe-qt)^2 \quad (4.4)$$

where k_2 (g/mg.min) is the rate constant of pseudo-first order adsorption process.

Integrating equation 4.4, q and applying the boundary conditions, qt=0 at t=0 and qt=qt at t=t, leads to

$$1/(qe-qt) = (1/qe) + k_2t \quad (4.5)$$

or equivalently for linear form

$$(t/qt)= (1/k_2qe^2) + (1/qe)\ t \quad (4.6)$$

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

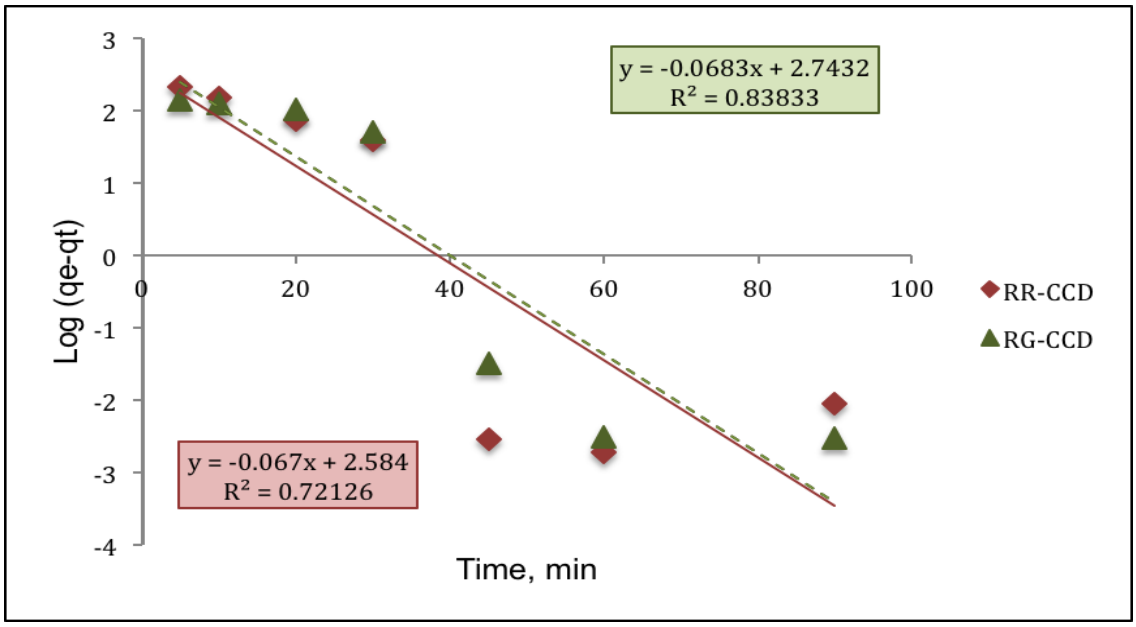

Figure 4.13. Pseudo-first-order kinetic modeling of the experimental data for both RR-CCD and RG-CCD columns.

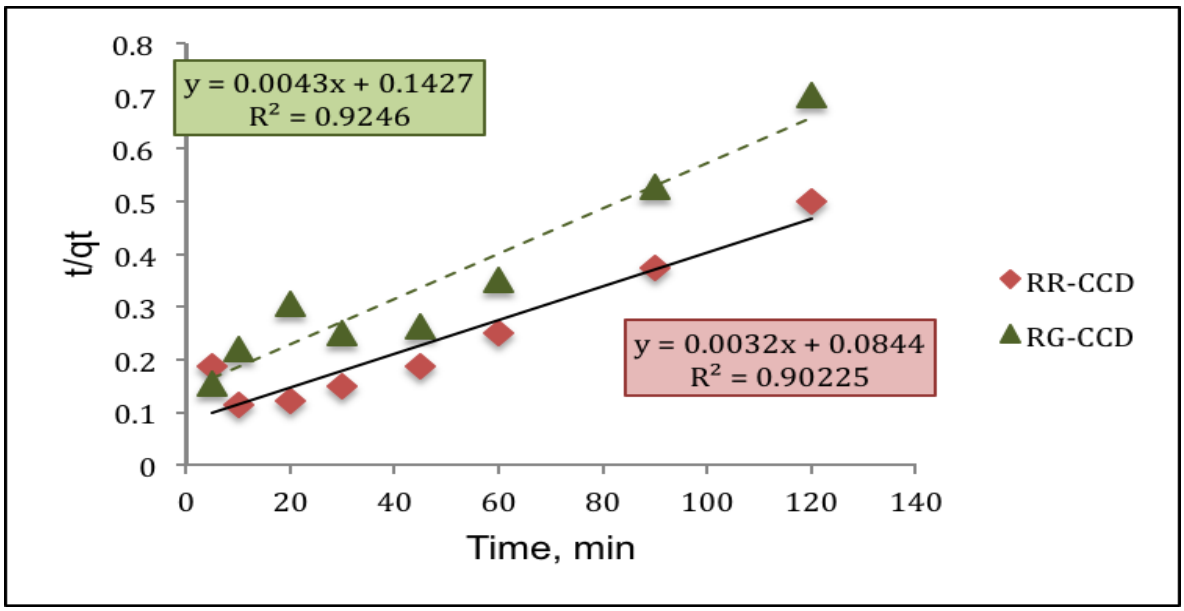

Figure 4.14. Pseudo-second-order kinetic modeling of experimental data for both RR-CCD and RG-CCD columns.

Table 4.3. The first and the second order kinetic constants for RR-CCD and RG-CCD columns.

Name of the CCD	Initial Conc. (mg/mL)	Exp.	First-order kinetic			Second-order kinetic		
		Qeq (mg/g)	k_1 (1/min)	q_{eq} (mg/g)	R^2	k_2 (g/mg.min)	q_{eq} (mg/g)	R^2
RR-CCD	2.0	239.8	0.154	383.7	0.72	$1.2\ 10^{-4}$	312.5	0.90
RG-CCD	2.0	170.7	0.157	553.3	0.84	$1.3\ 10^{-4}$	232.5	0.92

Table 4.3 represents the comparison of pseudo-first-order, pseudo-second-order kinetics values and the experimental adsorption capacity value. According to the correlation coefficients, the correlation coefficient of the pseudo-second-order equation is bigger than that of pseudo-first-order equation. This result shows that the pseudo second-order kinetic modeling is well fitted by adsorption process. As the rate constant for second-order kinetic (k2) is lower than first-order rate constant (k1), which denotes the adsorption rate was controlled by second-order kinetic.

According to these results, it can be concluded that the pseudo-second order adsorption mechanism is dominant and that the HIgG adsorption process controlled by chemical process.

4.2.7. Effect of embedded bead amount on adsorption amount

As seen Table 4.4 the HIgG adsorption amount increased with increasing the amount of embedded-bead in CCDs. The surface area of the composite cryogel and the HIgG adsorption capacity of the RR-CCD and the RG-CCD columns were increased with increasing the amount of the embedded beads. The HIgG molecules interact with RR-CCD and RG-CCD columns through the active binding sites of the dyes. It can be concluded that the increase of the embedded-beads amount, increased the surface

area of the RR-CCD and RG-CCD columns so the accessible active binding sites of the composite discs were also increased. As results, HIgG adsorption amounts increased due to the increase of the amount of the dye attached embedded-beads.

Table 4.4. The effect of the embedded RR-PHEMA and RG-PHEMA beads amount on HIgG adsorption amounts.

Name of the CCD	**RG1-CCD**	**RG2-CCD**	**RG3-CCD**	**RR1-CCD**	**RR2-CCD**	**RR3-CCD**
Bead type	RG-PHEMA	RG-PHEMA	RG-PHEMA	RR-PHEMA	RR-PHEMA	RR-PHEMA
Bead amount (mg)	100	200	300	100	200	300
Surface Area m^2/g	95.0	158.0	192.0	89.0	165.0	186.0
Adsorbed HIgG (mg/g)	97.4	132.6	170.72	101.2	200.9	239.85

4.3. Separation of IgG subclasses from commercial HIgG solution and Human Plasma

The Figure 4.15 and Table 4.5 represents the adsorption amount of both RG-CCD and RR-CCD columns for the IgG subclasses (IgG1, IgG2, IgG3, IgG4), from the commercial IgG solution. The total IgG adsorption amounts were found as 286.1 mg/g and 181.9 mg/g for RR-CCD and RG-CCD columns respectively. Although the IgG_1 component is higher than the other subclasses the adsorption capacity of IgG_2 component is higher than IgG1 for both RR-CCD and RG-CCD columns. In the light of these results it can be concluded that the RR and RG dyes exhibit

higher binding behavior for IgG2 than that of IgG1 and the other subclasses.

The second group of experiments were performed using human serum obtained from Sigma. The similar adsorption behaviour was observed and the adsorption capacities for the human plasma were given in Table 4.5. The greatest adsorption value was observed for IgG2 species as 238.6 and 141.5 mg/g for RR-CCD and RG-CCD respectively.

Table 4.5. The adsorption capacities for IgG subclasses in the commercial IgG solution and plasma sample.

	Name of CCD	IgG1	IgG2	IgG3	IgG4	Total
Commercial IgG solns	RR-CCD	13.96	213.1	23.44	35.66	286.1
	RG-CCD	8.83	135.5	14.9	22.69	181.9
Human Plasma	RR-CCD	17.89	238.6	26.45	42.65	325.6
	RG-CCD	11.26	141.5	18.19	26.41	297.4

4.4. Desorption of HIgG

The regeneration of the RR-CCD and RG-CCD columns was performed using 2 M NaCl solution. This step is very important for reusability of these columns in both laboratory and commercial applications (Yalçın, et al., 2004). Desorption ratios were calculated as 93% for RR-CCD and 92% for RG-CCD columns. After 10 adsorption-desorption cycles, both of the columns maintained of about 90% of their initial HIgG adsorption capacity (Figure 4.15).

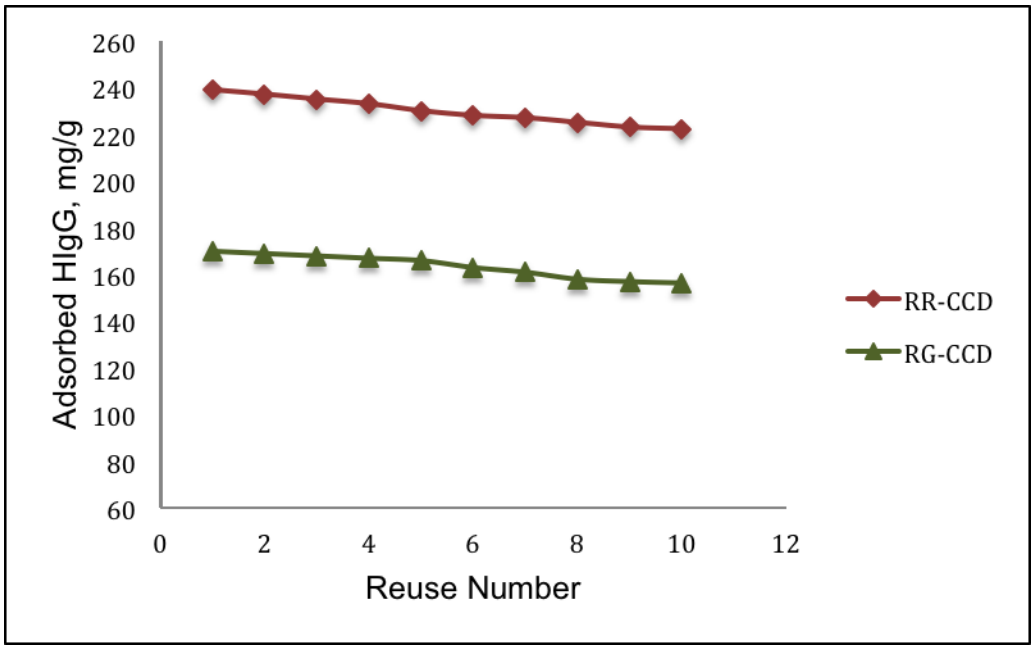

Figure 4.15. Reusability of RR-CCD and RG-CCD columns. Ci: 2mg/mL, V: 6 mL, pH:6.2, Flow rate: 0.5 mL/min, Time: 2h, T: 25 ^{0}C.

5. LITERATURE COMPARISON

A comparison of the maximum adsorption capacity, q_{max}, of RR-CCD and RG-CCD with related literatures is given in Table 4.6. The adsorption capacity of RR-CCD and RG-CCD columns were considerably high when compared with other adsorbents. Differences of IgG adsorption capacities are due to the properties of each adsorbent such as structure, reactive functional groups, ligand loading amount, porosity, pore size-pore size distribution, and accessible surface area (Babaç, et al., 2006).

Table 4.6. The literature comparison of IgG adsorption capacities of RR-CCD and RG-CCD with various adsorbents. (These table adapted from Babaç, et al., 2006)

Comparison of the adsorption capacities for IgG of various adsorbents

Adsorbent	*Ligand*	*Qmax (mg/g)*	*Reference*
Polymethylmethacrylate	**Cu^{2+}**	**54.3**	**Denizli, et al., 2003**
PHEMA	**l-Histidine**	**44.8**	**Özkara, et al., 2002**
PHEMA	**Methacryloylamidohistidine**	**73.8**	**Garipcan and Denizli, 2002**
Eupergit<comma> Affigel	**Protein A**	**20.1**	**Füglistaller, 1989**
PHEMA	**Cu^{2+}<comma> Ni^{2+}<comma> Zn^{2+}<comma> Co^{2+}**	**79.6**	**Özkara, et al., 2003**
PHEMA	**Protein A**	**24**	**Denizli and Piskin, 1995**
Poly(caprolactam)	**Protein A**	**28.3**	**Klein, et al., 1997**
Poly(ethylene)	**Phenylalanine**	**50**	**Kim, et al., 1991**
Sepharose 4B	**l-Histidine**	**0.23**	**Müller-Schulte, et al., 1991**
Poly(ethylene vinyl alcohol)	**l-Histidine**	**77.7**	**Bueno, et al., 1995**
Polysulfone	**Protein A**	**8.8**	**Charcosset, et al., 1995**
Sartobind	**Protein A**	**0.51**	**Langlotz and Kroner, et al., 1992**
Polymethylmethacrylate	**Protein A/G**	**6.6**	**Dancette, et al., 1999**
Poly(vinyl alcohol)	**Protein A**	**13.2**	**Castilho, et al., 2000**
Sepharose 6B	**3-Aminophenol**	**52**	**Teng, et al., 2000**
4-Amino-1-naphthol			
PHEMA beads	**Concanavalin A**	**69.4**	**Bereli, et al., 2005**
Sepharose CL 6B	**Biomimetic ligand**	**7**	**Teng, et al., 1999**
Sepharose 4B	**Biomimetic ligand**	**25**	**Fassina, et al., 1998**
Cellulose	**4-Mercapto ethyl pyridine**	**30**	**Boschetti, 2002**
Poly(ethylene vinyl alcohol)	**Mercapto methyl imidazole**	**16**	**Coffinier and Vijayalaakshmi, 2004**
Cellulose	**2-Mercapto-5-benzimidazole sulfonic acid**	**30**	**Girot, et al., 2004**
poly(AAm-AGE)	**Concanavalin A**	**25.6**	**Babaç, et al., 2006**
Poly(hydroxypropyl methacrylate)	**Reactive Green HE 4BD**	**71.0**	**Yavuz, et al., 2006**
PHEMA Composite Cryogel	**Reactive Red 120**	**239.85**	**This Study**
PHEMA Composite Cryogel	**Reactive Green HE 4BD**	**170.72**	**This Study**

6. CONCLUSION

The average diameter of the, Poly(HEMA-EGDMA) beads obtained mainly falls between 20-71 μm.

The specific surface area of the non-embedded CD was found as 26.0 m^2/g; by the embedding of the beads in to the cryogels, the surface areas of the columns were enhanced up to 192 m^2/g.

Scanning electron micrographs showed that, the polymeric beads have a spherical form and rough surface due to the pores which formed during the polymerization procedure.

The effect of salt concentration on adsorption of HIgG was investigated using aqueous solutions of HIgG, included NaCl salt in different concentrations RG-CCD column the adsorption amount of HIgG decreased from 239.8 to 210.2 mg/g and for RG-CCD column it was 171.8 to 163.2 mg/g.

For the RG-CCDs and RR-CCDs maximum adsorption capacity was calculated as 170.7 mg/g and 239.8 mg/g for optimal conditions; pH 6.0, 0.5 mL/min flow rates.

HIgG adsorption of the RG-CCD and RR-CCD were decreased with the increase of flow rate. Adsorption amount of the RR-CCD and RG-CCD were decreased from 239.8 to 129.8 mg/g and 170.7 to 109.3 mg/g respectively.

Langmuir adsorption capacity values were calculated as 250 and 217 mg/g for RR-CCD and RG-CCD and the correlation coefficients are found higher than those of Freundlich.

The adsorption capacities for individual IgG species in the commercial IgG solution were found to be 4.2 mg/g for IgG1 , 64.5 mg/g for IgG2 , 7.1 mg/g for IgG3 and 10.8 mg/g for IgG4 . Total IgG adsorption was determined as 86.6 mg/g.

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