PREPARATION OF IONIC ACTIVATION OF CRYOGEL MONOLITH COLUMNS FOR PURIFICATION OF Ɛ-POLY-L-LYSINE

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Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2022

PREPARATION OF IONIC ACTIVATION OF CRYOGEL MONOLITH COLUMNS FOR PURIFICATION OF Ɛ-POLY-L-LYSINE

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ACKNOWLEDGEMENTS

This thesis was supported by The Scientific and Technological Research Council of Turkey through TÜBİTAK 1001 The Scientific and Technological Research Projects Funding Program. This research was part of the project “Production of epsilon-poly-L-lysine with continuous bioprocessing” with project number 118M602.

I would like to express my gratitude to my esteemed thesis advisor, Assoc. Prof. Dr. Ali Özhan Aytekin, for his support and guidance. I will always be grateful to him for the opportunities he has given me and given me confidence in what I can do. I am sure I will make him proud of what I learned from him. It was a real honour for me to work with him.

I would like to express my special thanks to Assoc. Prof. Dr. Emrah Nikerel and Assist. Prof. Dr.Bahar Soğutmaz Özdemir for their time, help and the things that learn from them.

I would like to thank dear lab member Neslihan Kayra for her support and interest. I am also grateful to Handegül Altınordu and Eda Kapar for their friendship and support. I would also like to thank each of my friends working in Ferhatpaşa laboratories for their motivation, support and help. It was a pleasure working with all of you.

Although they cannot be with me physically, I am grateful to Emre Erdoğan, Ezgi Yıldırım, Ürün Ukan and Pamir Aral for their friendship and motivation. Special thanks to my faithful friend Özgün İmge Akyıldız for her existence. I would also like to thank my dear friend, Nehir Kızılilsoley, who was always by my side and did not spare her help and made me feel her support constantly. Congratulations today for all your future achievements.

Finally, I am grateful to my beautiful cat Müjgan for being so sweet and unknowingly giving emotional support.

ABSTRACT

PREPARATION OF IONIC ACTIVATION OF CRYOGEL MONOLITH COLUMNS FOR PURIFICATION OF Ɛ-POLY-L-LYSINE

Cryogels are large-pored, high-water-holding polymeric structures produced at sub-zero temperatures. These polymers are used in many fields including bioprocess. The aim of this thesis is primarily to produce and use cryogel monolith columns for the separation and purification of Ɛ-poly-L-lysine molecules. For this reason, cryogel columns were prepared using acrylamide, hydroxyethyl acrylate and hydroxyethyl methacrylate at different temperatures and monomer amounts and catalysts. Afterwards, they were activated with cationic and anionic loadings and their characterizations were made. In these characterizations, scanning electron microscope imaging was performed, swelling capacity and porosity, ion exchange charge, degree of cross-linking were calculated, and chemical structure characterization was performed. In order to understand the binding capacities, static binding and dynamic binding experiments were performed. Sodium chloroacetate was used for anionic loading and iodomethane was used for cationic loading. Among the columns selected in the studies, the Ɛ-PL binding capacity of anionic loaded acrylamide columns was found to be between 0.009 g/mg and 1.691 g/mg. The binding capacities of the cationic loaded hydroxyethyl methacrylate columns for bovine serum albumin were found to be 0.214 g/mg and 1.132 g/mg. Although the binding capacity of the columns is low, they can be used at high flow rates. However, it has been understood as a result of the experiments that the produced columns are not suitable for continuous chromatography and that they are disposable. As a result, more studies are needed on the use of cryogel columns in bioprocesses.

ÖZET

Ɛ -POLİ-L-LİSİN SAFLAŞTIRMASI İÇİN KRİYOJEL MONOLİT KOLONLARININ İYONİK AKTİVASYONUNUN HAZIRLANMASI

Kriyojeller sıfırın altındaki sıcaklıklarda üretilen büyük gözenekli, yüksek su tutma kapasiteli polimerik yapılardır. Bu polimerler biyoproses dahil bir çok alanda kullanılmaktadır. Bu tezin amacı, Ɛ-poli-L-lisin moleküllerinin ayırma ve saflaştırılması için öncelikle kriyojel monolit kolonları üretmek ve kullanmaktır. Bu sebeple akrilamid, hidroksietil akrilat ve hidroksietil metakrilat ile farklı sıcaklık ve monomer miktarları ve katalizörler kullanılarak kriyojel kolonları hazırlanmıştır. Sonrasında katyonik ve anyonik yüklemelerle aktifleştirilip, karakterizasyonları yapılmıştır. Bu karakterizasyonlarda, taramalı elektron mikroskopu görüntülenmesi yapılmıştır, şişme kapasitesi ve porozite, iyon değişim yükü, çapraz bağ derecesi hesaplanmıştır ve kimyasal yapı karakterizasyonu yapılmıştır. Bağlanma kapasitelerinin anlaşılması için, durağan bağlanma ve dinamik bağlanma deneyleri yapılmıştır. Anyonik yükleme için sodyum kloroasetat, katyonik yükleme için iyodometan kullanılmıştır. Yapılan çalışmalarda seçilen kolonlardan, anyonik yüklü akrilamit kolonların, Ɛ-PL bağlanma kapasitesi 0,009 g/mg ile 1,691 g/mg arasında bulunmuştur. Katyonik yüklü hidroksietil metakrilat kolonlarının, bovin serum albumin için bağlanma kapasiteleri 0,214 g/mg ile 1,132 g/mg bulunmuştur. Kolonların bağlanma kapasiteleri düşük olsa da yüksek akış hızlarında kullanılabilmektedirler. Fakat üretilen kolonların sürekli kromatografi için uygun olmadığı ve tek kullanımlık oldukları yapılan deneyler sonucunda anlaşılmıştır. Sonuç olarak kriyojel kolonlarının biyoproseslerde kullanımıyla ilgili daha fazla çalışma yapılması gerekmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

AA	Acrylamide
AC	Affinity chromatography
AEX	Anionic exchange chromatography
AGE	Ally glycidyl ether
AMPS	2-acrylamido-2methyl-1-propane sulfonic acid
APS	Ammonium persulfate
APTMACl	3-Acrylamidopropyl-trimethyl-ammonium chloride
BSA	Bovine serum albumin
CEX	Cationic exchange chromatography
FPLC	Fast protein liquid chromatography
HA	Hydroxyethyl acrylate
HAC	Hydroxyapatite chromatography
HIC	Hydrophobic interaction chromatography
HM	Hydroxyethyl methacrylate
HPLC	High pressure liquid chromatography
IEX	Ion exchange chromatography
IMAC	Immobilized affinity chromatography
MbA	Methylene bis acrylamide
RPC	Reversed phase chromatography
SEC	Size exclusion chromatography
TEMED	N,N,N',N'-Tetramethyl ethylenediamine
Ɛ-PL	Epsilon-poly-L-lysine

1. INTRODUCTION

1.1. HYDROGELS

Hydrogels are of interest in various biomedical applications due to their high water capacity. Gels that swell with the help of water; covalent cross-links, ionic bonds, hydrogen bonds, hydrophobic interactions, polymer chains or combinations of these interactions. Studies on hydrogels started in the 1930's with cross-linking studies on polymers. In the future, many studies have been carried out on the structure, preparation and characterization of hydrogels [1,2].

According to preparation methods, ionic charge and physical structures, hydrogels are evaluated in several categories. According to the preparation methods; it can be classified as homo-polymer, copolymer, multi-polymer and interpenetrating network. Homo-polymers are hydrophilic monomers that form a cross-linked network. Copolymers consist of two co-monomers that are cross-linked and at least one monomer must be hydrophilic to cause swelling in water. Multi-polymers consist of at least three interacting co-monomers. One method for producing interpenetrating network hydrogels is to prepare them with a preformed mesh and a solution [3]. Most preferred is to polymerize a monomer in a different cross-linked hydrogel network. The monomer polymerizes to form a second crosslinking network with the polymer or network [4].

Classification of hydrogels as ionic is done as follows; neutral, anionic, cationic and ampholytic. In ampholytic hydrogels, the net charge can be positive, negative, or neutral [5].

Based on the physical properties of the hydrogels, the classification is made as follows. Amorphous, that is, covalently cross-linked, the molecular chain in these hydrogels is in a random order and semi-crystalline hydrogels that may or may not have covalent bonds. They have ordered molecular chains that are semi crystalline [6].

Also for complexation hydrogels held together by secondary forces; classifications are made according to hydrogen bonds, affinity complexes and hydrophobic bonds [7]. The physical properties of such hydrogels depend on the network density of interactions and

environmental factors. Hydrogels are stable or degradable, and in this degradability they are sub classed enzymatically or hydrolytically [8].

As a result of the structural evaluations made in hydrogels, it is observed that ideal networks are rarely formed. Besides, networks are likely to have functional connections or physical molecular entanglement. It is quite possible to be confused with molecularly defective hydrogels [9].

1.2. CRYOGELS

The term cryogel was coined in an article published in the 1980s, describing a polymeric material prepared by cross-chemical bonding of macromolecules precursors in a moderately frozen environment. This term has emerged from the combination of the Greek words kryos, meaning ice, and the English words gel [10]. Cryogels are obtained from suitable monomers or polymeric precursors in semi-frozen medium, where ice crystals act as porogens and after thawing, interconnected pores serve as templates for size and shape. When examined in microscopic observation, moderately frozen molecules or gelatinous solutions are the unfrozen liquid micro phase test, in which both the semi-frozen solvent and the solute are condensed, and the hetero phase system containing the unfrozen part. While frozen solvent crystals act as a porogen, gelation takes place in unfrozen regions. The conditions required for gel formation are important to determine the different heterogeneous porous structure, physical and chemical properties of the formed polymer and, accordingly, its functional ability [10,11].

Generally, temperatures between -5 and -20 degrees are required for the preparation of cryogels. The solution is prepared and frozen using the monomer, binders and initiators as seen in Figure 1.1.. While the prepared solution freezes, the mixture exists in two states. These are between the ice crystals and the unfrozen micro phase. The reactions take place in this unfrozen liquid medium. The ice crystals increase during freezing and combine with other ice crystals until they form an interconnected order. After the gelation period is completed, the ice crystals are dissolved at room temperature to form a pore. Thus, a highly porous polymer matrix is obtained [10]. The shape and size of the pores formed depend on those of the ice crystals. Because of the low diffusion, advection movement [transport of a

substance by the bulk movement of a liquid] in the matrix pores of the nanoparticles or cells within the appropriate time is required. About 5 percent of this flux does not occur in the liquid between the matrix beads in conventional chromatographic absorbents due to the pore size. The advection flow takes place in monolith columns [12]. To increase this flow, the pores of the column must have a large size. Studies have shown that columns with a pore structure larger than 1 micrometer failed to differentiate due to low binding capacity. Compared to gels, cryogels have large pores that allow advection to occur. These large and interconnected pores and elasticity are the hallmarks of cryogels [4].

The most important feature of cryogels when compared to other gels is that they are highly durable even under possible shape distortions. Generally, cryogels can be compressed by half of their own volume without changing the porous structure compared to standard gels [13]. It is possible to produce cryogels in the form of small pores and thin pore walls by creating a surface area large enough to provide sufficient efficiency in order to bind the desired molecules. This structure can be controlled by the precursors that make up the gel and the chemical reactions that occur. The resistance that liquids encounter during flow is low due to the large and abundant interconnected pores [12,14].

The stretchable pore system of cryogels allows the gel to dry quickly without any deterioration and then swell again. This feature ensures that the gel system can be stored for a long time without making any difference in the operating system. In addition, they swell again quickly after liquid contact [15].

In the preparation of covalent gels, the polymerization of cross linked monomers or crosslinking is achieved with high molecular weight compounds. This is how the covalent cryogels are prepared. In physical cryogels, they are polymers that can gel on their own due to the cooling of the solvent used [10].

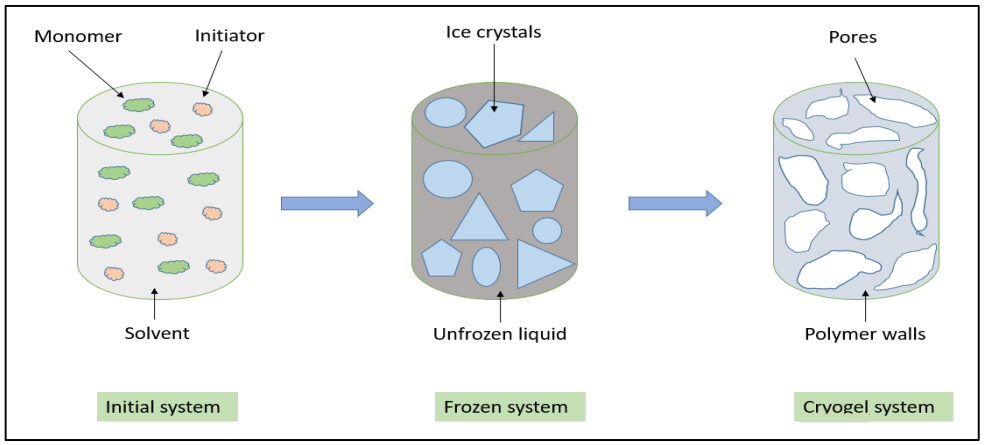


Figure 1.1. Cryogelation process of cryogels

In researches on cryogelization, the difference from traditional gelation is that it occurs at negative temperatures. Based on this information, conventional gelation categories also apply to cryogelization. In colloidal dispersions, freezing of the colloid solution enhances the contact between particles. The chemical bond formation between particles is protected by these contacts, cohesion force and auxiliary substances [16]. In monomeric solutions, frozen water or organic media creates crosslinking polymerization by chemical means. Thus, the production of a slimy gel with a large pore structure occurs. In high molecular weight solutions, the processing of macromolecules on non-frozen systems supports the formation of a large porous and cross-linked matrix. The structure of such super porous spongy gel materials is controlled by the poly crystals used as solvents. In polymers with spontaneous gelation, physical gels are obtained by adding solute material to increase the effect of the solvent or to change the three-dimensional structure of the molecules. In solutions that have low molecular weight or prevent polymeric cross-linking with ions, the gelation cycle is more difficult due to the ion bridges they contain. Because ion exchange reactions are a very fast process and freezing the prepared solution becomes more difficult for this reason. To deal with this, an ionic cross linker whose solubility will increase with the decrease in temperature is used [17].

Cryogel production is a cross-linked polymer matrix containing uncross linked polymer and water. Microporous cryogel membranes have a large surface area and short diffusion path. They can be easily processed due to their mechanical properties. Due to the solution flow in

the pores, the transfer resistance is low. The molecular structure formed is determined by the particle size and each freeze-thaw cycle increases the particle diameter [18].

The method of separation with monolith structures has been mentioned in the literature for more than 50 years. When the first theories were put forward, the gel materials produced could not have much effect as they could not achieve the desired result under pressure. Although more positive results were obtained from polyurethanes with harder and larger pores produced later, they still did not have an effective place in use for chromatographic. Approximately 30 years ago, a pore-like gel containing cross-link was prepared by polymerizing the ammonium sulfate salt, N, N-methylene bis-acrylamide and acrylic acid added to the aqueous environment. The flow obtained after compression of the prepared polymer gave satisfactory results [19]. The cross linked gel was not fully dispersed in the liquid, and when the polymer obtained was compressed, it only provided a monolith-like structure. The first monoliths produced differed in pore size and distribution when the same method was followed for the production of macro pore beads. The modified monoliths were highly permeable and could separate proteins very quickly [20].

Since these properties are remarkable, gel preparation started to be produced in stainless steels afterwards and monolith chromatographic columns emerged. In the following years, this process was further developed and silica-based monoliths with controlled pore were designed. This approach was later accepted by the industry and started to be used commercially [19,21].

Although the first monoliths were hydrogels, a cryotropic gelation technique that forms a hydrophilic monolith with a spongy form that allows the formation of large pores was produced for further development as seen in Figure 1.2.. Cryogels contain two methods that cause monolith formation; Water dissolved monomer polymerization and crosslinking of water dissolved polymers. Unlike hydrogels, prepared solutions must be frozen first [22].

Figure 1.2. Cryogel and hydrogel swollen and dry formations [30]

1.3. FORMS AND TYPES OF CRYOGELS

The porous structure is formed due to the decomposition of the water particles that crystallize during freezing and the non-frozen liquid phase. The frozen liquid mixes with the monomer rich phase. The monomers in this polymerized phase ensure that the structure is continuous and causes the formation of porous walls. When the polymerization reaction is over, if the system is brought to room temperature, the frozen water crystals can dissolve and replace with the mobile phase liquid again [10,16]. The functioning of the mechanism shows the factors affecting the pore structure. First of all, the pore structure of the monoliths obtained due to the polymerization that occurs as the temperature decreases and thus the flow permeability decreases. In cases where the temperature is not low enough, polymerization does not occur and the desired form cannot be obtained. In addition, as the amount of monomer in the aqueous solution increases, the resulting wall thickness increases and the pore diameter decreases. The same is true for polymerization initiator agents. The use of a salt that affects the freezing property causes to obtain harder forms [23].

Cryogels are a very good alternative for protein purification due to the pore size, low flow rate, short diffusion path, short adsorption and elution times. Protein purification is a method of separating the desired protein from mixtures containing non-protein material or other proteins other than the desired protein. This separation is done depending on the size, chemical properties or binding affinity of the desired protein. There are one or more chromatographic analyzes in protein purification [24]. Chromatography is generally focused on the adsorption process. Even solutions at low concentrations are effective. Its basic

principle is to pass the protein-containing solution through columns enriched with various materials. The interaction of each protein with the colon material is different. Therefore, they can be distinguished by the time to pass the column or by the difference in the conditions applied to separate the protein from the column [25].

Organic polymer cryogels are obtained by polymerization from high water content and hydrophilic or hydrophobic monomers or cross-linking polymeric precursors. Although the shapes of the cryogels obtained depend on the vessel in which the reaction is applied, the nature of the environment and the synthesis conditions, they can be formed as monoliths, rods, disks, spherical plates as seen in Figure 1.3., in direct proportion to the processes to be applied later.The porosity structure of cryogels is more important when compared to its shape [26].

The interest in functional cryogels is high due to the increase and expansion of adsorption, that is, the expansion of the applicable areas. A monolith cryogel formed using isopropylacrylamide, a functional monomer commonly used for cryogelation at -12°C, and polyethylene glycol as binder to form pores, swelling rates were higher compared to conventional hydrogels [27].

Some cryogels utilize polysaccharides and compounds with nitrogen and oxygen atoms. Monolith, disc and bead form agarose-alginate cryogels are soft tissue-like biodegradable materials with interconnected large porous structures. Tissue cells showed good proliferation on this material. Although the detailed textural properties of these materials were not analyzed, it was concluded that they are a material that can be used for tissue engineering [26].

Preferred cryogel precursor because of the biocompatibility and functionalizability of protein-based materials. The most preferred are especially collagen and gelatin. It has been noticed that three-dimensional collagen cryogels prepared with dialdehyde starch, which is used as a cross-linker, can be used in biomedical applications due to the preservation of protein structure, large heterogeneous porous structure, fast and high water holding capacity and thermal stability [27].

It is an example of solid-based hard cryogels such as titanium hydrogels synthesized by solid gel polymerization, and the characterization processes are easier than soft cryogels because their structures are more stable compared to them [28].

Hybrid cryogels, which contain both soft and solid forms, contain solids such as activated carbon micro particles. Activated carbon is a good absorbent for low molecular weight organics. However, their biocompatibility is not sufficient for medical applications such as cleaning toxic substances from the blood. Activated carbons can be coated with polymers to increase their biocompatibility. The narrow pores of the activated carbon molecules can be limited to a certain extent, thus reducing the permeability of toxic substances. In fact, activated carbon beads can be added to thin-film polymers or the walls of large-pore cryogels, allowing blood cells to pass through in various medical applications [29].

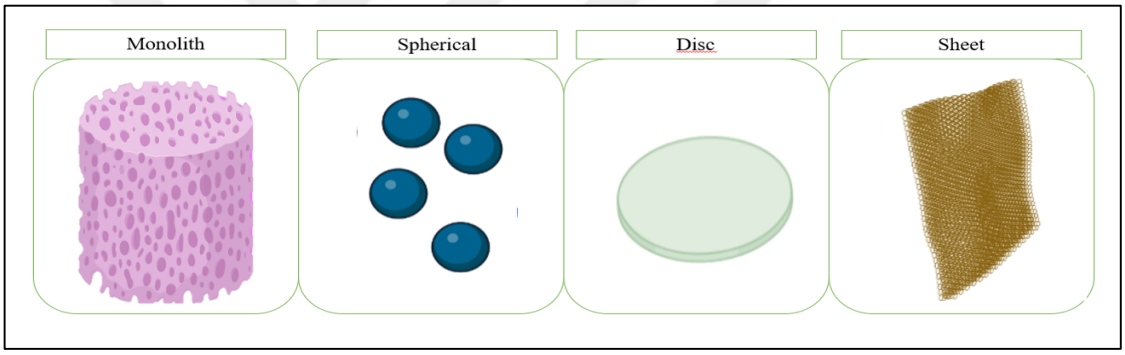


Figure 1.3. Various formation of cryogels

1.4. CHROMATOGRAPHY

Gel filtration or size exclusion chromatography (SEC) separates macromolecules according to their hydrodynamic volume. Columns consist of polymer beads with different sizes of pores. When the mobile phase passes through the column, large-volume particles migrate in a shorter time than small-volume ones, since they do not enter the pores. It is often used as a final purification man because it cannot fully distinguish molecules that have a similar volume [31].

Hydrophobic interaction chromatography (HIC) separates proteins by taking advantage of their hydrophobicity based on hydrophobic interactions between the immobile hydrophobic ligands and non-polar regions on the surface of the proteins. Salt concentration in the mobile

phase increases adsorption and elution is achieved by decreasing the salt concentration of the solvent. Since it has different separation conditions, it is preferred for the purification of protein mixtures that are difficult to separate by other chromatographic methods [32].

Reversed phase chromatography (RPC) is a method that takes advantage of the hydrophobia of proteins. The interaction of the solute with the stationary phase is controlled by changing the mobile phase polarity. It is used for protein and salt separation, as the sample is concentrated in low volume volatile solvent. An organic component is made with the aid of a substance capable of breaking the hydrogen bonding structures of the compound in water, such as acetonitrile or methanol. RPC implementations are more limited. HIC and RPC take advantage of the hydrophobic properties of proteins, but RPC is more nonpolar and more denaturing. Because the binding to the adsorbent is strong, nonpolar solvent is required for protein separation [33].

While proteins perform their biological activities, they often bind or form complexes to certain molecules, ligands. Affinity chromatography (AC) is also based on this principle. When a mixture is passed through the column, the molecule with specific activity for the ligand attaches to the column and other unbound molecules pass through. The solution containing high density ligand is passed through the column and the desired protein is collected from the column. The bond between the absorbent molecule and the ligand can also be reversed. With the AC method, protein separation takes place without any denaturation [34].

Ion Exchange chromatography (IEX) separates proteins through ion interaction, because of their net charge. The column contains resin containing charged groups for adsorption of target proteins. Although it is different in terms of column capacity and separation technique, it is generally similar to affinity chromatography. The column is balanced with a low ionic buffer containing ions opposite to the resin. These ions are displaced by charged molecules absorbed into the resin. The desired bond strength between protein and resin is adjusted by ionic strength, pH and flow rate. Unfortunately, they will come together as some unwanted molecules with the target protein will have the same charge and pi value. In flow mode, the desired protein is washed while the attachment of unwanted molecules is adjusted. Separation process is done with buffers that provide gradual increase of ionic strength or pH value [35].

In order to adsorb proteins with different loads; Anionic Exchange chromatography (AEX) can be used for negatively loaded, and cationic exchange chromatography (CEX) for positively loaded. Hydroxyapatite (HAC) chromatography is an IEX variant that includes both anionic and cationic properties. It contains both positive calcium and negative phosphate ions on its resin. These ions interact with both negative carboxyl groups and positive amino acid groups on the protein. The binding makes the result difficult to predict, but it is a method used to remove nucleic acid [36].

In the separation or purification of biomolecules, a molecule capable of recognizing the desired molecule, namely the ligand, is immobilized on the cryogel. The solution containing the target molecule is passed through the ligand-fed cryogel column, and the ligands capture the target molecule. The target molecule is separated by a specific competitive ligand by adjusting the pH, ionic strength or temperature, and the interaction with the ligand is terminated. There are many different techniques for protein purification. Such as immobilized affinity chromatography (IMAC), hydrophobic interaction chromatography. Protein purification depends on the molecule's properties such as size, net charge, and hydrophobicity [32,36].

1.5. CRYOGELS IN SEPARATION

IMAC is the most common method for cell protein isolation from nucleic acid-containing extracts, based on the selective interaction of metal ions with biomolecules. This separation is based on the interaction of chelated metal ions Cu(II), Ni(II) and electron donor atoms such as sodium and oxygen [37]. Although some metal ions create toxicity problems for the purification of pharmaceutical proteins, low cost, performance, high stability of the ligand are the reasons that make this method common. Also, when working with crude extracts, it is not necessary to remove cells that pass through the column without attachment. For ligand binding, the support matrix must consist of a network permeable to proteins [24,38].

Cryogels in this purification method; It can be successfully applied in the production of enzymes from thin parts of crude cells, in antibody isolation and cell culture. In studies with polyacrylamide-based cryogels and sepharose-based matrices of culture fluids obtained in

continuous cell culture, it was understood that the protein capture of the cryogel column had better selectivity and higher efficiency compared to the other [39,40].

Dye ligands are considered alternatives for specific affinity. These ligands can specifically bind most types of proteins, especially enzymes. Besides being commercially available and inexpensive, they can be easily immobilized on matrices containing hydroxyl groups. Although these dyes are synthetic, they can mimic the structure of the substrate or binding agents and interact with the active sites of proteins [41]. Phema cryogels can be used for the immobilization of Cibacron Blue dye due to its inertness, mechanical strength, stability and compatibility. Poly acrylamide allyl glycidyl ether, another cryogel bound to the same dye, was also used for albumin binding from human blood plasma [42].

Hydrophobic interaction chromatography is the separation of hydrophobic protein on the surface depending on the interaction between polar regions and ligands [43]. When hydrophobic ligands and large porous monolith cryogels were used as the stationary phase, and the epoxy groups between the two media were covalently coupled. The cryogel prepared with hydrophobic hydroxyethyl methacrylate polymer and tryptophan was applied for the separation of lysozyme. It showed high selectivity and high absorption with over 90 percent recovery. These results were obtained by interaction between hydrophobic ligands and non-polar regions on the desired enzyme [44,45].

When macroporous cryogels are used for affinity chromatography, they can be modified by binding various ligands after polymerization to bind to desired proteins. Concanavalin A is generally used for degradation of enzymes by immobilization on matrices [46].

Improving the binding capacity of large-pore cryogels is important for separation and purification. Although this porous structure allows the passage of particles, the surface area can be limited. The limited surface area of the cryogels can be embedded in the monolith cryogel to form a hybrid cryogel with micro or nanoparticles with different properties in order to increase the binding capacity for proteins or other macromolecules [47]. With the cryogels embedded on the matrix and with increased functionality, the bonding capacity increased with the increase of the flow rate. however, these physically located particles can reduce the accessibility of the desired molecule required for interaction and limit its absorption [48].

When the insulin absorption of sodium hydroxide concentration on poly (hydroxyethyl methacrylate) cryogel was investigated, it was noticed that increasing salt concentration decreased the amount of insulin absorbed. The reason for this has been tried to be explained in two ways. salt ions interact with insulin molecules and do not allow them to bind on the colon. or with the increase in ionic strength, it may have caused the formation of a repulsive electrostatic force between the insulin and the colon and decreased absorption [49,50].

The net charge on the molecules is related to pH. The shift of the pH of the substance towards the isoelectric point causes a loss in its net charge. Charged groups with low ionic strength have very low competition, but substances bind strongly. The increase in ionic strength increases this competition and reduces the interaction between the ion exchanger and the sample, causing an increase in competition [14].

1.6. ISOTHERMS

Various models are used to evaluate adsorption events in studies. Generally, experimental data are adapted to isotherm data and appropriate models are used for characterization. With the appropriate model selected, the adsorption parameters are obtained and used to define the adsorption properties [64,65].

The Langmuir isotherm is considered a monolayer continuum. The maximum adsorbent capacity per unit mass, the affinity of the solute to the adsorbent, is determined by the Langmuir constant [64]. Where C is called solute or adsorbate, concentration of Q at equilibrium in solution (mg/L), q mass of solute adsorbed per unit mass of adsorbent at equilibrium (mg/g), Km constant of Langmuir isotherm (L/mg) and qmax, maximum adsorption capacity (mg/g).

In any adsorption experiment, C can be measured and Q can be calculated for a number of different conditions. Then 1/Q can be plotted as a function of 1/C. Most of the adsorption experiments use the above linear form of the equation to evaluate whether the adsorption process satisfies the Langmuir isotherm. If the obtained graph shows significant linearity, it can be assumed that the adsorption process follows the Langmuir adsorption isotherm. As a result, two parameters (Km and qmax) can be obtained using slope and intersection to characterize the adsorption process [64].

The Freundlich isotherm is a multi-layered process in which the amount of adsorbed solute per unit mass is determined. Freundlich parameters are used for the characterization of the process [65]. Similar to the Langmuir adsorption isotherm, the linear form of the equation can be used to evaluate whether the adsorption process satisfies the Freundlich isotherm and to determine the constants. If the experimental results show significant linearity according to the equation, it can be assumed that the adsorption process follows the Freundlich adsorption isotherm. Here Kf is the constant of the Freundlich isotherm and 1/n is the Freundlich exponent. where C is the only variable [65].

In isotherms, the dependent variable, that is, the amount absorbed per unit mass, is expressed as a function of the solute concentration at equilibrium, which is the independent variable, not a function of the initial concentration. this means that the model equation can be used when the solute concentration is known. Besides, it can be used to calculate the initial solute concentration, solution volume and unit mass removal efficiency under given experimental conditions when considering industrial and some practical applications. Unfortunately, there are few studies on estimating yields applicable to real adsorption processes [66,67,68].

Table 1.1. Adsorption isotherms and equations

Model	Equation	Model equation
Langmuir [64]	$Q = \frac{Qmax \times C}{Km + C}$	$\frac{1}{Q} = \frac{Km}{Qmax \times C} + \frac{1}{Qmax}$
Freundlich [65]	$Q = Kf \times C^{1/n}$	$logQ = logKf + \frac{1}{n} logC$

1.7. AIM OF THE STUDY

Cryogels are super-porous gel structures formed by cryogelation process of suitable monomers or polymers at subzero temperatures. These formations are materials that are resistant to deterioration, have high mechanical and chemical stability and water holding capacity, are easy to apply and cost-effective [10]. They are often used in biomedical immobilization, controlled drug delivery, and tissue engineering. When used in the field of chromatography, their binding capacity is impressive [13]. Based on these results in the literature, the aim of this experiment is to achieve the production of an alternative monolith

column that can be used for the purification of poly-L-lysin polymer. Ɛ-poly-L-lysine (Ɛ-PL) is a homopolymer in the range of about 3 to 4 kDa, consisting of 20 to 30 amino acids L-lysine. The production of α-PL is usually made from *Streptromyces* strains. It shows different biochemical properties due to its high cationic property, and therefore, it has different usage areas such as food industry, tissue culture and drug distribution [51].

In this context, several attempts have been made to manufacture monolith columns for Ɛ-PL separation. The monomers to be used in these studies were determined as Acrylamide, Hydroxyethyl acrylate, Hydroxyethyl methacrylate, and progress was continued on these monomers. To prepare the gel formation, selected monomers were mixed with Methylene Bis-acrylamide as well as Ammonium per sulfate (APS) and Tetramethylethylenediamine (TEMED) catalysts and incubated at various subzero temperatures. The other monomer Allyl glycidyl ether will also be used to enhance anionization. Scanning Electron Microscopy (SEM) for pore size, for chemical structure analysis; Fourier Transform Infrared Spectroscopy (FTIR) for strength and porosity; degree of crosslinking for ionic strength; ionic charge analysis for affinity; for static binding affinity and binding mechanism; adsorption isotherms were made.

2. MATERIALS

2.1. INSTRUMENTS

Chemicals which were used in this study were as follows: N,NºMethylene-BisAcrylamide (Sigma Aldrich 146072), Acrylamide (AA) (Sigma Aldrich O1700), 2-Hydroxyethyl methacrylate (HEMA) (Sigma Aldrich 128635), 2-Hydroxymethyl Acrylate (HEAA) (Sigma Aldrich 292818), Ammonium persulfate (APS) (Sigma Aldrich A3678), Tetramethylenediamine (TEMED) (Sigma Aldrich T22500), Potassium phosphate monobasic (Sigma Aldrich 04243), Potassium phosphate dibasic trihydrate (Carlo Erba 471767), Sodium chloride (NaCl) (Sigma Aldrich 31434-R), Hydrochloric acid (HCl) (Sigma Aldrich 3200331), Sodium hydroxide (NaOH) (Sigma Aldrich 221465), Iodomethane (Sigma Aldrich I8507), Sodium chloroacetate 98 percent (C2H2ClNaO2) (Sigma Aldrich A291773), Chondroitin sulfate A sodium salt from bovine trachea (Sigma Aldrich C9819), Sodium phosphate monobasic(NaH2PO42H2O) (Sigma Aldrich S3139), di-Sodium hydrogen phosphate heptahydrate (Na2HPO47H2O) (Sigma Aldrich 1370921000), Ally glycidyl ether (Sigma Aldrich A32608), E-poly L lysine (Sigma Aldrich P7890), Bovine serum albumin (Sigma Aldrich A3733), Lysozyme (Sigma Aldrich).

2.2. EQUIPMENT

Instruments which were used in this study were as follows: Recirculating chiller (Lauda Alpha RA8), Nitrogen gas tank (Habaş), Carousel (Radleys), Laboratory shaker (Radleys), 50 ml Reaction flasks (Radleys RR99070), Freeze dryer (Labconco), Laboratory scale (Shimadzu AUW220D), 10 ml Serological pipettes (Isolab), Micropipettes 1000 µl,100 µl (Thermo Scientific), Micropipette tips, weighing plate, spoon, Burette and stand, Injector 5 ml (Set), 24 Well-plates, Scanning electron microscope (SEM) (Zeiss Evo 40), Sputter coater (Bal-Tec SDC005), Fast protein liquid chromatography (FPLC) (AKTAprime plus), High-performance liquid chromatography (HPLC) (Shimadzu DGU-20A5R), Peristaltic pump (Lead Fluid BT100S), Sonicator (Fisherbrand FB14053), Chromatography empty cartridge (BGB BDL00F-S0004), PH meter (Mettler Toledo S220).

3. METHODS

3.1. PREPARATION OF THE COLUMNS

In the preparation of the columns, the determined amount of monomers is taken into a round-bottomed flask, methylene bis-acrylamide is added, and mixing is started in 15 mL of water at room temperature. Ammonium persulfate (APS) and Tetramethylenediamine (TEMED) are added successively and allowed to mix for a maximum of 30 s. The mixture is brought to the cryogel temperature with a cold water circulator and after waiting for one day, it is dried and a cryogel column is obtained. The names and amounts of the monomers used in the preparation of the column and the preparation conditions are shown in Tables 3.1, 3.2 and 3.3 [15,39].

For cryogels prepared by adding allyl glycidyl ether (AGE), AGE as much as methylene-bis-acrylamide to the monomer mixture before addition of ammonium persulfate and tetramethylenediamine and the mixture was prepared by adding and polymerization was carried out as stated above.

Table 3.1. Reaction conditions for monomer and methylene bis acrylamide prepared cryogel monolith columns

Column name	Monomer type	Monomer amount (mole)	Methylene bis acrylamide amount (mole)	Monomer / methylene bis acrylamide (mole/mole)	Catalyst amount (mg APS; µL TEMED)	Reaction temperature (°C)
C1	AA	0.014	0.002	8.7	20 ; 25	- 12
C2	AA	0.014	0.002	8.7	40 ; 50	- 12
C3	AA	0.014	0.002	8.7	40 ; 50	- 20
C4	AA	0.014	0.002	8.7	20 ; 25	- 16
C5	AA	0.014	0.002	8.7	20 ; 25	- 20
C6	AA	0.035	0.004	8.7	20 ; 25	- 12
C7	AA	0.035	0.004	8.7	40 ; 50	- 12
C8	AA	0.035	0.004	8.7	40 ; 50	- 20
C9	AA	0.035	0.004	8.7	20 ; 25	- 16
C10	AA	0.035	0.004	8.7	20 ; 25	- 20
C11	AA	0.070	0.008	8.7	20 ; 25	- 20
C12	AA	0.070	0.008	8.7	40 ; 50	- 16
C13	HA	0.022	0.002	13.8	20 ; 25	- 20
C14	HA	0.022	0.002	13.8	20 ; 25	- 12
C15	HA	0.011	0.002	6.9	40 ; 50	- 12
C16	HA	0.011	0.002	6.9	40 ; 50	- 20
C17	HA	0.011	0.003	3.5	40 ; 50	- 12
C18	HA	0.011	0.003	3.5	40 ; 50	- 16
C19	HA	0.011	0.003	3.5	40 ; 50	- 20
C20	HA	0.022	0.002	13.8	40 ; 50	- 16
C21	HM	0.011	0.002	6.6	20 ; 25	- 12
C22	HM	0.011	0.002	6.6	20 ; 25	- 16
C23	HM	0.011	0.002	6.6	20 ; 25	- 20
C24	HM	0.011	0.003	3.3	20 ; 25	- 12
C25	HM	0.011	0.003	3.3	20 ; 25	- 16
C26	HM	0.011	0.003	3.3	20 ; 25	- 20
C27	HM	0.021	0.002	13.2	20 ; 25	- 12
C28	HM	0.021	0.002	13.2	20 ; 25	- 16
C29	HM	0.021	0.002	13.2	20 ; 25	- 20
C30	HM	0.021	0.002	13.2	40 ; 50	- 16

C31	HM	0.011	0.002	6.6	40 ; 50	- 16

Table 3.2. Monomer amounts of prepared cryogel monolith columns for systems with two monomers and methylene bis acrylamide

Column name	1st Monomer	1st Monomer amount (mole)	2nd Monomer	2nd Monomer amount (mole)	Total Monomer (mole)	Mole ratio of monomers
C32	AA	0.018	HA	0.011	0.028	1.6
C33	AA	0.028	HA	0.004	0.032	6.5
C34	AA	0.007	HA	0.017	0.024	0.4
C35	AA	0.014	HA	0.013	0.027	1.1
C36	AA	0.021	HA	0.009	0.030	2.5
C37	AA	0.021	HM	0.008	0.029	2.7
C38	AA	0.028	HM	0.004	0.032	7.3
C39	HA	0.017	HM	0.004	0.021	4.5
C40	HA	0.013	HM	0.008	0.021	1.7

Table 3.3. Reaction conditions for prepared cryogel monolith columns with single monomer and systems prepared with methylene bis acrylamide together with allyl glycidyl ether

Column name	Monomer type	Monomer amount (mole)	Methylene-bis acrylamide amount (mole)	Allyl Glycidyl Ether (mole)	Catalyst amount (mg APS ; µL TEMED)	Reaction temperature (°C)
C41	AA	0.014	0.002	0.002	20 ; 25	- 16
C42	AA	0.014	0.002	0.002	20 ; 25	- 20
C43	HA	0.035	0.004	0.004	40 ; 50	- 12
C44	HM	0.035	0.004	0.004	40 ; 50	- 20

3.2. SCANING ELECTRON MICROSCOPE (SEM) OBSERVATIONS

For scanning electron microscopy, samples were cut 3 mm in height and 7 mm in diameter, then washed in 20percent, 40percent, 60percent, 80percent and 100percent (v/v) ethanol solutions, after that washed 3 times with phosphate buffer (pH 7.4) and dried. The samples to be measured were placed on carbon disc slides, then coated with 13 nm gold particles and kept in vacuum and analyzed with Carl Zeiss EVO-40 SEM.

3.3. MODIFICATIONS OF COLUMNS

3.3.1. Anionic loading

Anionic loading of cryogel columns was done with 3 different chemicals. These are 2-acrylamido-2methyl-1-propane sulfonic acid (AMPS), chondroitin sulfate and sodium chloroacetate.

As a first trial, 0.78 mL of AMPS solution (20percent; w/w) and 1 g of Acrylamide are mixed with 5.22 mL of Mba (8.8percent; w/v) and 50 μL of TEMED is added. 0.0054 g (1 mole per 100 mole of monomer) of potassium persulfate is then dissolved in 1 mL of water. The mixture of both solutions is cooled in the refrigerator for 5 minutes. The solutions are then mixed and, after vortexing, they are quickly transferred to syringes and left for 1 day at -18°C for polymerization to occur [14].

3.3.1.1. Sodium chloroacetate

Approximately 2 cm pieces were cut from each column and placed in vials. These column pieces were provided with a continuous system in which 10 ml of distilled water was delivered at a rate of 1 ml/min for 15 minutes. Then, 3 ml of NaOH was added to this water at 1 ml/min for 15 minutes. After that, desired amount of sodium chloroacetate was added at 1 ml/min for 3 hours at 60°C. Finally, these column pieces were washed with 70 percent ethanol and then 90 percent ethanol for 30 minutes at 5 ml/min.

3.3.1.2. Chondroitin sulfate

Approximately 2 cm pieces were cut from each column and placed in vials. These column pieces were provided with a continuous system in which 10 ml of distilled water was delivered at a rate of 1 ml/min for 15 minutes. Then, 3 ml of NaOH was added to this water at 1 ml/min for 15 minutes. After that, desired amount of chondroitin sulfate was added at 1 ml/min for 3 hours at 60°C. Finally, these column pieces were washed with 70 percent ethanol and then 90 percent ethanol for 30 minutes at 5 ml/min.

In the naming of the columns prepared with chondoritin sulfate or sodium chloroacetate (carboxy methylation reaction), the letter "C" is added next to the name of the basic column. In cases where there is a number next to the letter "C", it shows that it has been modified with a different amount of anionic substance. Sodium chloroacetate was reacted with 0.001 mole in C1, 0.009 mole in C9, 0.017 mole in C17 and 0.026 mole in C26.

3.3.2. Cationic loading

Experiments were made with three different chemicals for the cationic loading of the columns. These were methacryloloxy-ethyl-trimethyl-ammonium chloride (META), 3-acrylamidopropyltrimethyl ammonium chloride (APTMACl) and iodomethane. Columns were prepared at different ratios and conditions for META and APTMACl (Table 3.4). An amount of methylene-bis-acrylamide 0.25 mol of the total amount of monomer was added to the reactions. The amount of AGE is the same as the reacted methylene-bis-acrylamide. The monomers were mixed with META or APTMACl and methylene-bis-acrylamide was added under a nitrogen atmosphere, then 20 mg of APS and 25 µL of TEMED are added and the solution is mixed rapidly [14].

Finally, the prepared samples were placed in a chiller that will provide the sub-zero temperature required for polymerization.

Table 3.4. Cationic activation experiment sets with META and APTMACl

Column name	Monomer type	Monomer amount (mole)	META (mole)	APTMACl (mole)	AGE (mole)	Temperature (°C)
C45	AA	0.014	0.014	-	-	-16
C46	AA	0.014	0.014	-	0.007	-16
C47	AA	0.014	0.014	-	-	-20
C48	AA	0.014	0.014	-	0.007	-20
C49	HM	0.021	0.021	-	-	-16
C50	HM	0.035	0.035	-	0.018	-16
C51	AA	0.014	-	0.014	-	-16
C52	AA	0.014	-	0.014	0.007	-16
C53	AA	0.014	-	0.014	-	-20
C54	AA	0.014	-	0.014	0.007	-20
C55	HM	0.021	-	0.021	-	-16
C56	HM	0.035	-	0.035	0.018	-16

In the cationic loading of the columns with iodomethane, the column was prepared by cutting from a height of 3 cm and distilled water is passed through the column at a rate of 1 mL/min for 15 minutes. Then 6 mL of NaOH (10M) was circulated through the column at the same rate for 15 minutes. Iodomethane is heated to 60°C and 0.02, 0.04 and 0.08 moles of iodomethane were passed through the column at the same rate for 3 hours. After processing, the columns are washed with 70 percent and 90 percent ethanol, respectively, at a rate of 5 mL/min for a total of 1 hour, and unbound iodomethane and NaOH are removed from the column. Ethanol was removed by keeping the columns at 65°C for 24 hours.

In the naming of the columns prepared with iodomethane, the letter "I" was added next to the name of the basic column. Where there is a number next to the letter "I", it indicates that it has been modified with a different amount of cationic substance. 0.02 mole of iodomethane was used in I2, 0.04 mole in I4 and 0.08 mole in 18.

3.4. SWELLING CAPACITY

The swelling ratio was calculated according to the volume increase after water is passed through the columns. The columns were cut at different weights (W_i). After their diameters (D_i) and lengths (H_i) were recorded, they were kept in water for 24 hours. At the end of the period, the repeat weights (W_f), diameters (D_f) and lengths (H_f) of the samples were measured [15].

Swelling capacities (percent) were calculated by calculating according to the equation.

$$Swelling\ capacity(percent) = \frac{\frac{Wf}{Wi} \times 100}{\frac{\frac{Df^2}{4} \times Hf \times \pi}{\frac{Di^2}{4} \times Hi \times \pi} \times 100} \tag{3.1}$$

3.5. ION EXCHANGE CHARGE

For ionic charge measurement, samples with and without Ally glycidyl ether treated with anionic and cationic agents were incubated with 2M 50ml NaCl solution for 24 hours. Afterwards, 0.025M HCl and 0.025M NaOH solutions were used for pH balance. The volume of acid or base added to the column (V2) solution, the added volume of the acid or base solution containing only 2M NaCl solution (V1), and the membrane dry weight (Wm) were recorded. These data were used to calculate the ionic exchange charge (IEC) using the equation.

$$Ionic\ charge = (\frac{(V\_Sample - V\_blank)}{(Mass\ of\ column)}) \times 0.025 \tag{3.2}$$

3.6. CROSSLINKING DEGREE

The degree of crosslinking affects the porous size and strength of the gels. For this purpose; Dry column (5 mg) was immersed in ninhydrin solution (1 mL; 1.5percent v/v in ethanol). The first incubation was at 80°C for 25 min then the second incubation was at room temperature for 1 h. Measurement was done at 570 nm. After that the equation was applied.

$$Crosslinking\ degree\ (percent) = \left(1 - \frac{Abs_{blank}}{Abs_{column}}\right) \times 100 \quad (3.3)$$

3.7. POROSITY

The basis of this method is to calculate the increase in weight of the material based on the amount of water remaining in the pores. Columns were placed in a pre-measured dry glass petri dish and kept at 70 °C until constant weight. The dried materials were taken to water and kept for 12 hours and the weight is measured again. The following equation was used to calculate the membrane porosity. Dry material weight (Wi), water-impregnated material (Wf), water density (Dw) and material density (Dm) were determined in the equation. [69].

$$Porosity\ (\varepsilon) = \frac{\frac{Wf - Wi}{Dw}}{\frac{Wf - Wi}{Dw} + \frac{Wi}{Dm}} \quad (3.4)$$

3.8. STATIC BINDING CAPACITY

For a-PL analysis, 10 ml of 1 mg/ml Poly-L-Lysine standard solution was prepared. Each sample was cut into 5, 10, 25 and 50 mg pieces and placed in tubes. The prepared Ɛ-PL solution was added on them and this was enough to cover them with approximately 1 ml. Then the samples were kept at +4°C for 24 hours. After this time, the samples were filtered and analyzed in HPLC [19].

Depending on the unit amount of the column, the amount of Ɛ-PL (Adsorption capacity; Q, mg Ɛ-PL/g column), the amount of unbound Ɛ-PL (c) were summed and determined by the stable binding capacity. Stable binding capacity calculations were made according to the following model equations (Table 1.1). Linearized model equations were defined as y = mx + n; 1/Q versus 1/C was plotted for Langmuir, while LogQ versus LogC was plotted for Freundlich.

For Langmuir, Qmax was calculated by taking the inverse of the point where the equation intersects the y-axis; Then the Km value was calculated by using the Qmax value from the

slope of the graph. 1/Q can be plotted as a function of 1/C. Here C is the solute concentration in solution and the solution volume can be expressed as Vmax. [64].

$$\frac{1}{Q} = \frac{Km}{Qmax} \times \frac{1}{C} + \frac{1}{Qmax} \tag{3.5}$$

For Freundlich, Kf was calculated by taking the inverse logarithm of the point where the equation intersects the y-axis, and the "n" value is calculated by taking the inverse of the slope of the graph [65].

$$logQ = logKf + \frac{1}{n} logC \tag{3.6}$$

3.9. DYNAMIC BINDING CAPACITY

Prepared columns about 2 cm high were placed in the FPLC device. Prepared 20mM sodium phosphate buffer (pH:7.4) was used as binding buffer. Then 50 ml of Ɛ-PL solution was passed at a flow rate of 2 ml/min and 1 ml fractions were collected [20].

3.9.1. Cationic columns

Cationic loaded columns with a height of about 2 cm were placed in the FPLC device. Prepared 20 mM sodium phosphate buffer (pH:6.0) was used as the binding buffer. Then 50 ml of Ɛ-PL solution was passed at a flow rate of 2 ml/min. It was then washed with the prepared elution buffer (sodium phosphate buffer + 0.1 M NaCl) and 1 ml fractions were collected.

3.9.2. Anionic columns

Anionic loaded columns about 2 cm high were placed in the FPLC device. Prepared 20mM sodium phosphate buffer (pH:7.4) was used as binding buffer. Then 50 ml of Ɛ-PL solution was passed at a flow rate of 2 ml/min and 1 ml fractions were collected.

3.10. SEPARATION PERFORMANCE

Columns selected for dynamic coupling were this time processed not only with the Ɛ-PL solution, but also with the prepared protein mixture. This mixture contains 0.1 mg/ml lysozyme, 0.1 mg/ml BSA and 0.1 mg/ml Ɛ-PL. Then 50 ml of mixed solution was passed at a flow rate of 2 ml/min and 1 ml fractions were collected.

3.11. CHARACTERIZATION OF CHEMICAL STRUCTURE WITH FOURIER-TRANSFORM INFRARED SPECTROSCOPY (FTIR)

Infrared spectra were performed using Thermo Scientific Nicolet iS10. After the samples were dried, they were mixed with KBr and shaped by pressing. Then the measurement was taken in the range of 400 – 4000 cm^{-1}. For polymers prepared with AA, HA and HM monomers was summarized according to their molecular states in Table 3.5.

Table 3.5. FTIR wavelength and bond types for synthetic polymers

	Wavelength (cm^{-1})		
Bond type	**AA**	**HA**	**HM**
C-H rock	1301, 1405	747, 848, 898, 1362, 1438	747, 848, 898, 1438
C-N stretch	1045, 1107	1073, 1158, 1221	1076, 1151, 1221
N-O asymmetric stretch	1523	1528	1541
N-H bend	1715	1712	1712
O=C=O	2352	2349	2363
C-H	2930	2925	2931
O-H	3435	3448	3428
Amide N-H stretch	-	-	-

4. RESULTS

4.1. PREPARATION OF COLUMNS

It was thought that monolith columns with different properties would be obtained due to the various structures of Acrylamide (AA), Hydroxyethyl acrylate (HA) and Hydroxyethyl methacrylate (HEMA) monomers. Monolith columns were prepared under different conditions with these three different monomers themselves and their mixtures. Methylene-bis-acrylamide and Allyl glycidyl ether(AGE) were used as binding agents. Monomer and catalyst amounts and polymerization temperature were investigated. In order for the prepared cryogel monoliths to be used as a purification tool, they should not be easily disintegrated, melted and should be homogeneous. Many of the previously prepared columns are not suitable for the desired specifications.

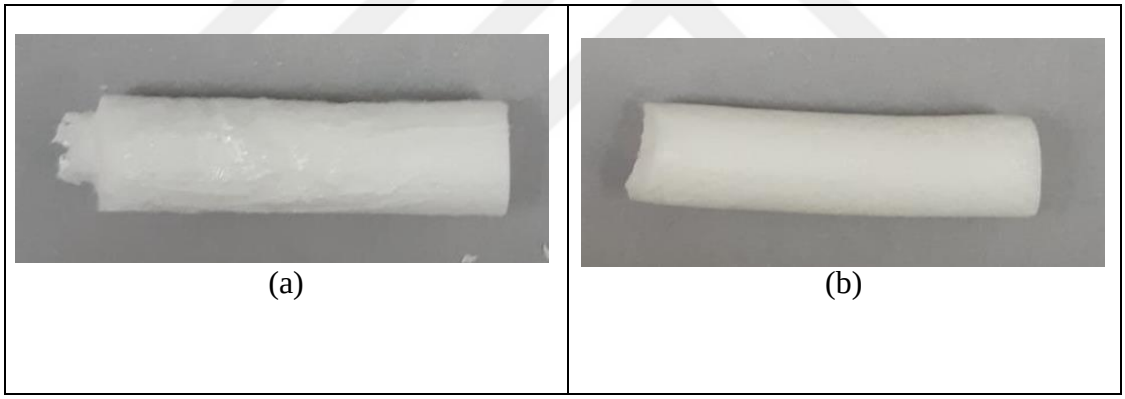


Figure 4.1. Acrylamide column (a) C3, (b) C8

Since Acrylamide contains amino group, it is thought that it will interact less with Methylene bis acrylamide compared to other monomers. The prepared Acrylamide columns are rigid (Figure 4.1.), and it must be cut in order to be suitable for use in the experiments. As the amount of catalyst used increases, its structure becomes harder.

(a)

(b)

Figure 4.2. Hydroxyethyl acrylate columns. (a) C18, (b) C20

The arrangements for the preparation of Hydroxyethyl acrylate are more complex compared to the preparation of acrylamide cryogels. Unlike Acrylamide columns, HA columns are soft and spongy (Figure 4.2.). Adjusting the conditions of HA cryogel preparation seems more difficult than AA cryogel preparation. Unlike the stiffness obtained in AA cryogel columns, HA columns have a spongy texture. It is thought that this feature of HA will increase its holding capacity and allow it to work at different flow rates.

(a)

(b)

Figure 4.3. Hydroxyethyl methacrylate columns. (a) C21, (b) C22

The biggest problem encountered in the preparation process of Hydroxyethyl methacrylate columns is that the formed columns often do not have a stable structure and turn into powder. When the temperature of the cryogel polymerization is kept at -12°C and -16°C, it has been observed that the desired column properties such as hardness and homogeneity are better than other columns (Figure 4.3.).

4.2. SCANNING ELECTRON MICROSCOPY OBSERVATIONS

SEM was used to understand the surface properties and pore structure.

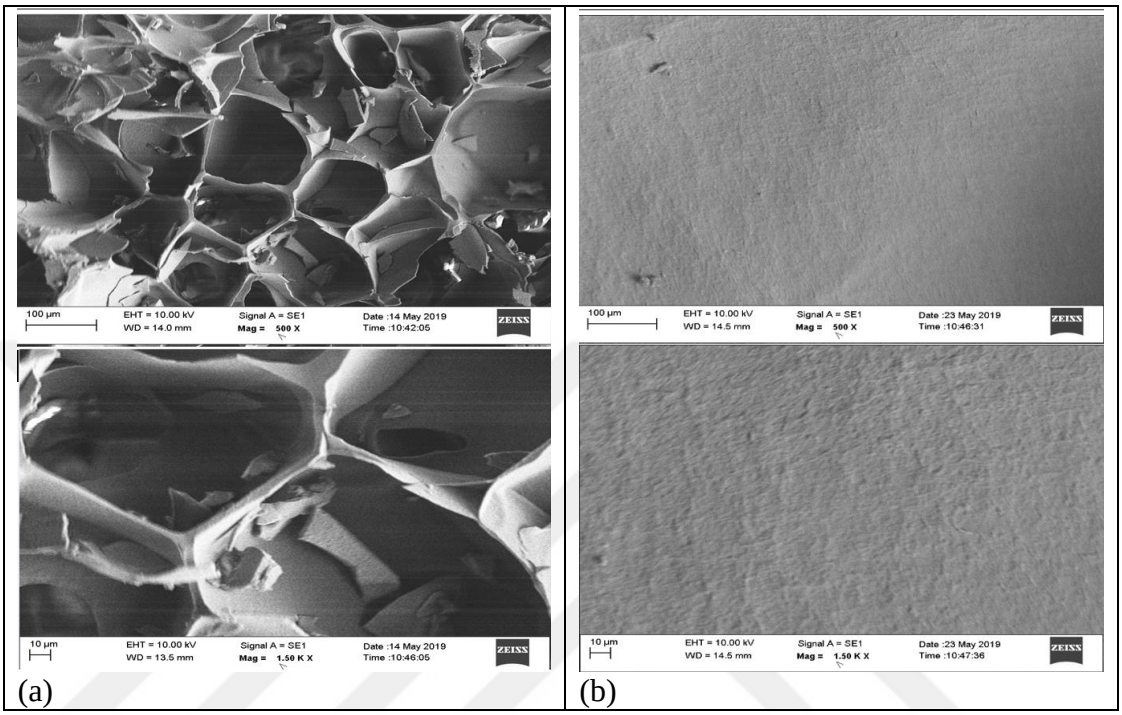


Figure 4.4. SEM visual of sample of Acrylamide columns. (a) C3, (b) C8

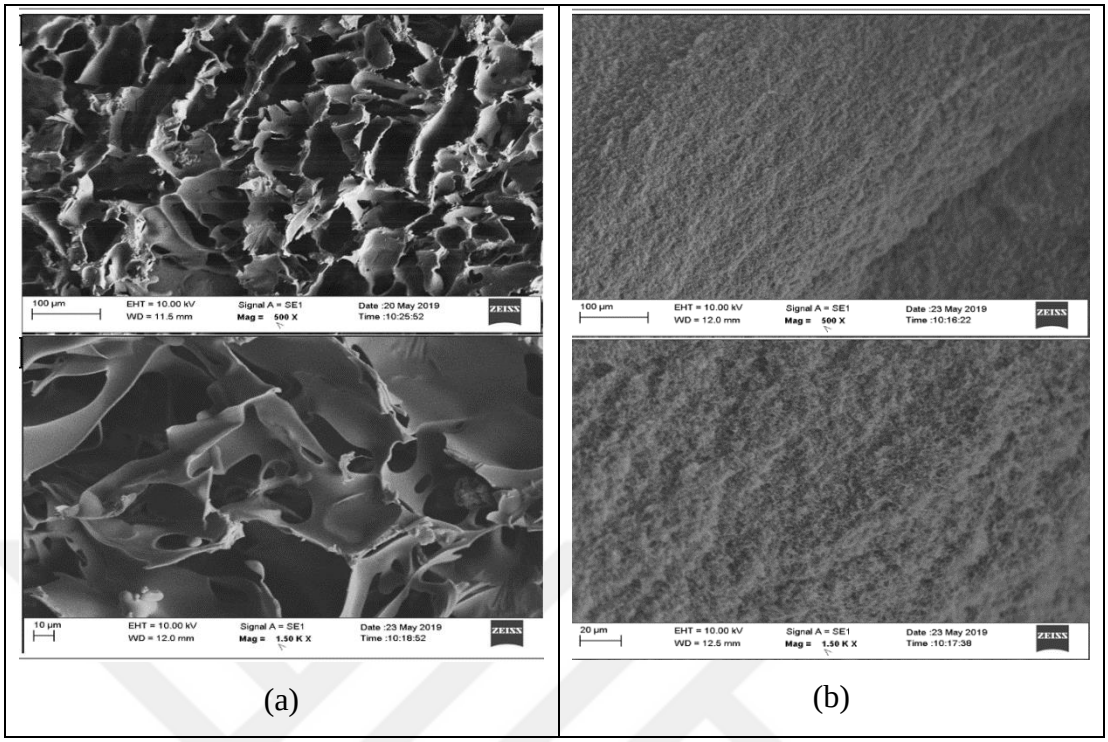


Figure 4.5. SEM visual of sample Acrylamide columns. (a) C4, (b) C9

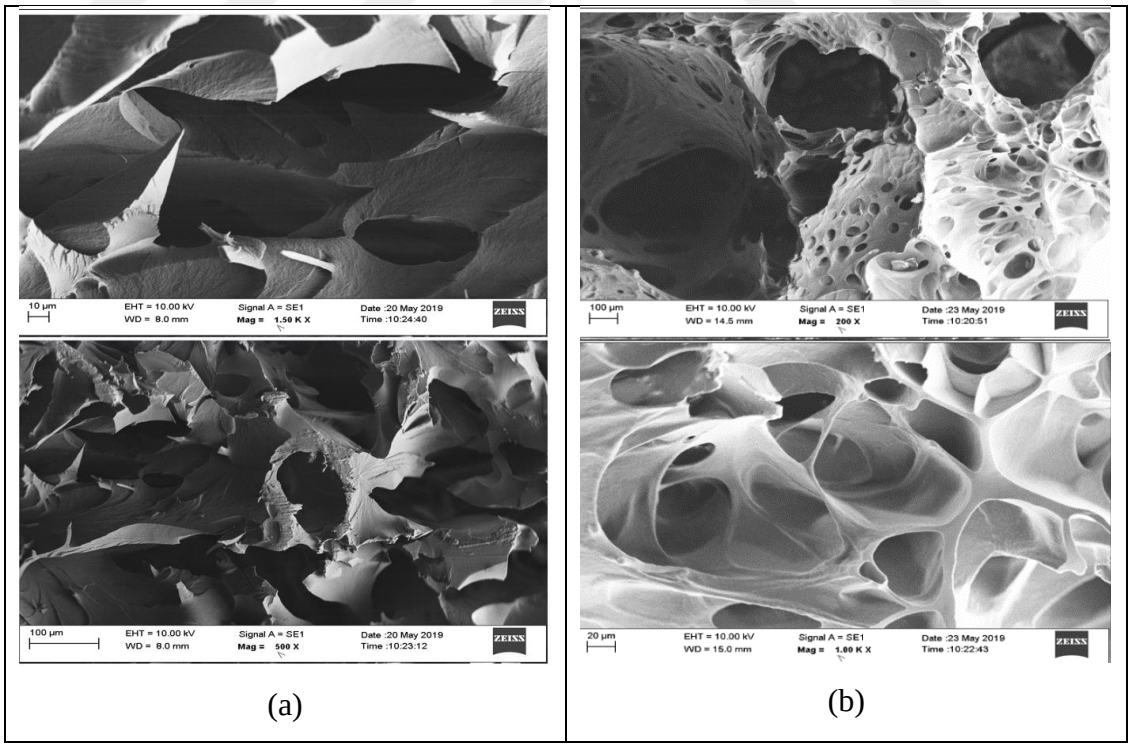


Figure 4.6. SEM visual of sample Acrylamide. (a) C12, (b) C1

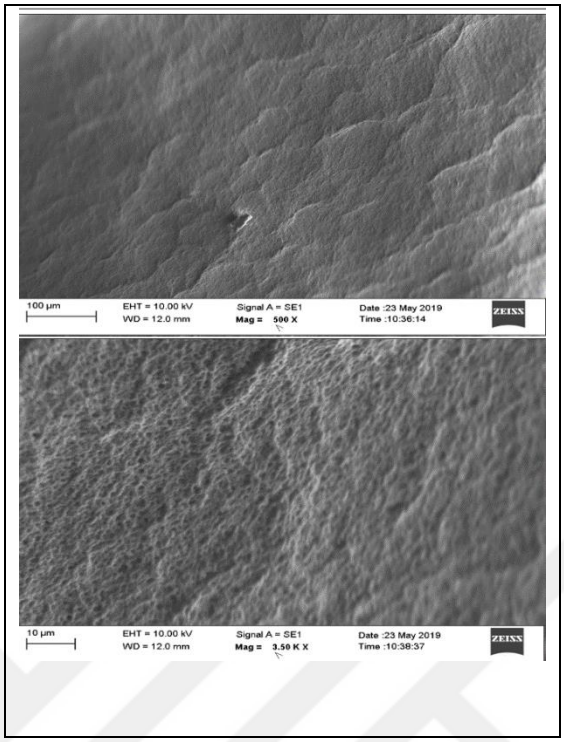


Figure 4.7. SEM visual of sample C7 that Acrylamide column

When acrylamide columns are examined, the effect of the temperatures used on the pore structure can be seen. When the preparation temperature is -12ºC, the presence of various large and small pores is observed (Figure 4.6., Figure 4.7.). It has been understood that cryogels made by reducing the temperature at -16ºC (Figure 4.5.) have a more homogeneous pore structure. When the temperature is lowered further and made at -20ºC (Figure 4.4.), it is seen that the pores are homogeneously distributed and the column surface becomes smooth.

When the amount of monomer is increased, no pore formation is observed and almost smooth surface formation is achieved.

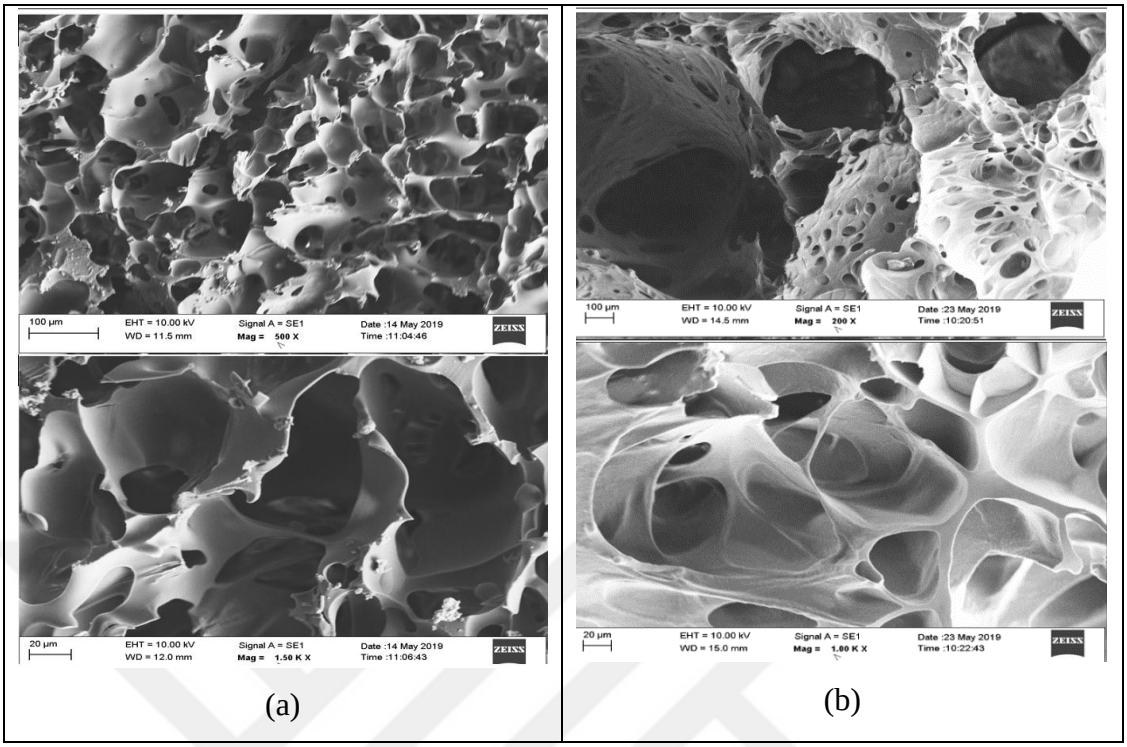


Figure 4.8. SEM results of Hydroxyethyl acrylate columns. (a) C17, (b) C15

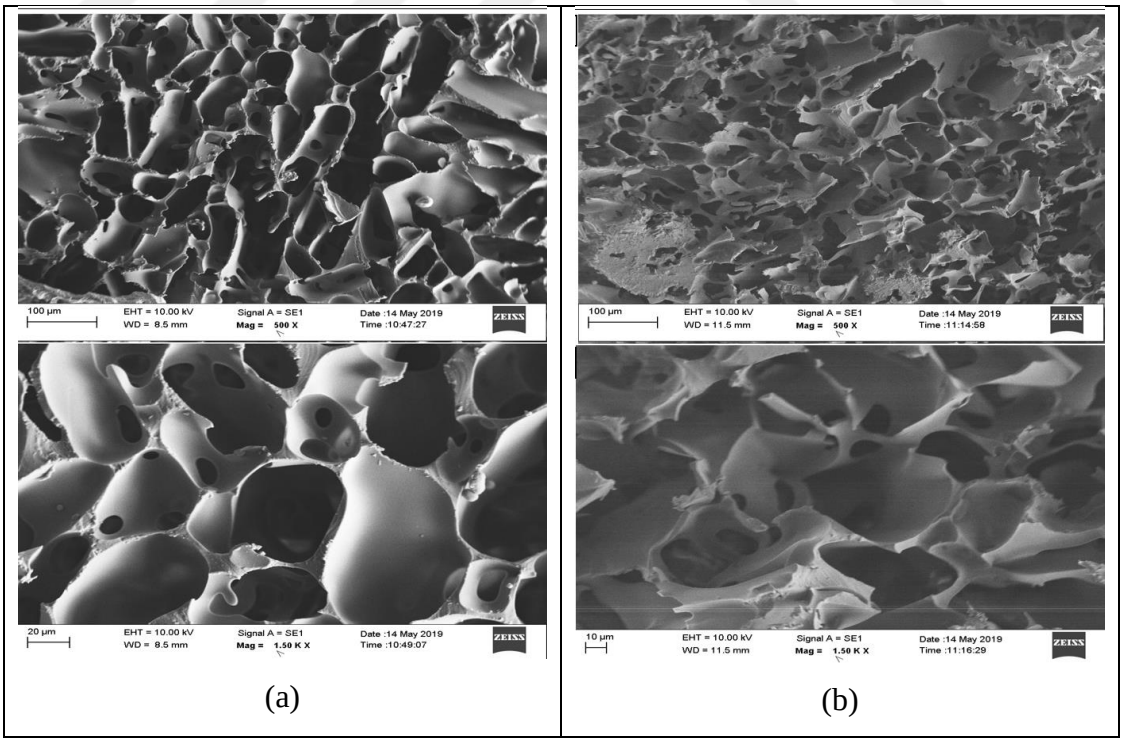


Figure 4.9. SEM results of Hydroxyethyl acrylate columns. (a)C20, (b) C18

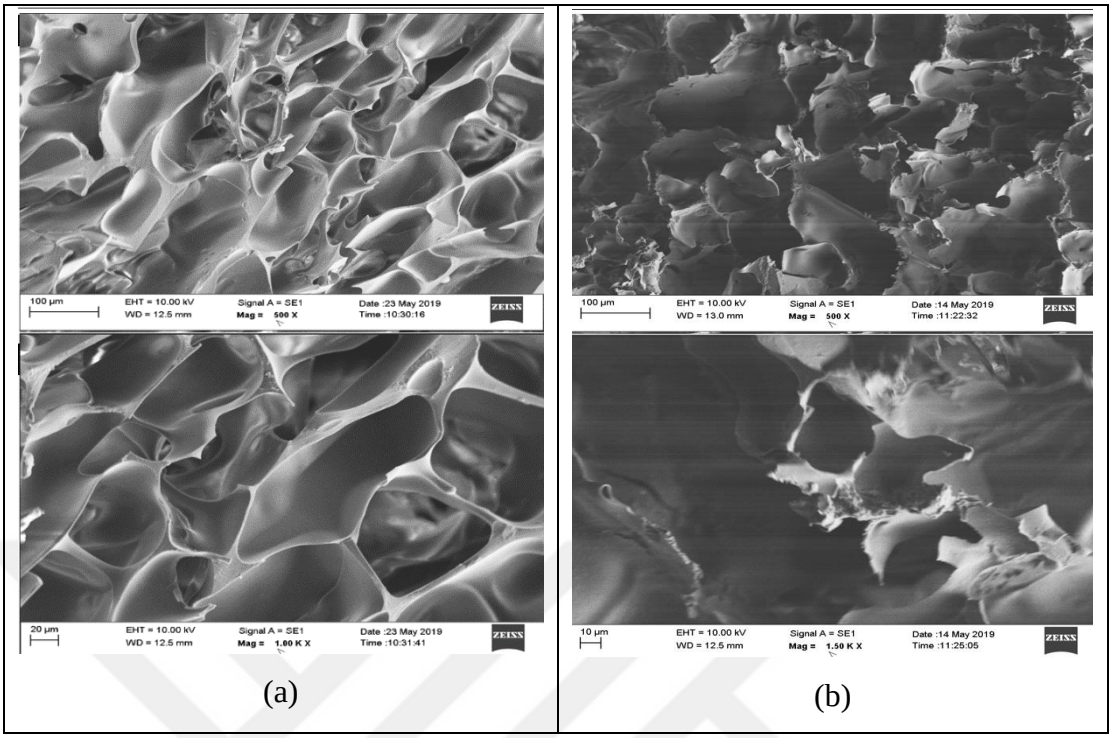
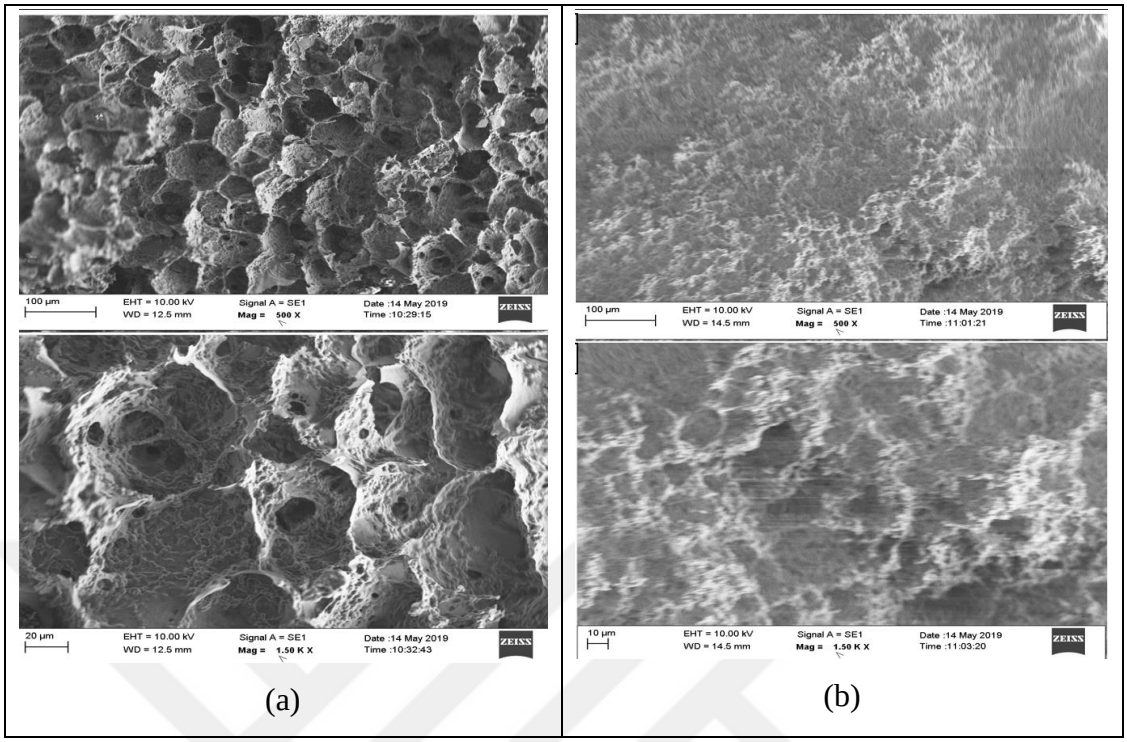


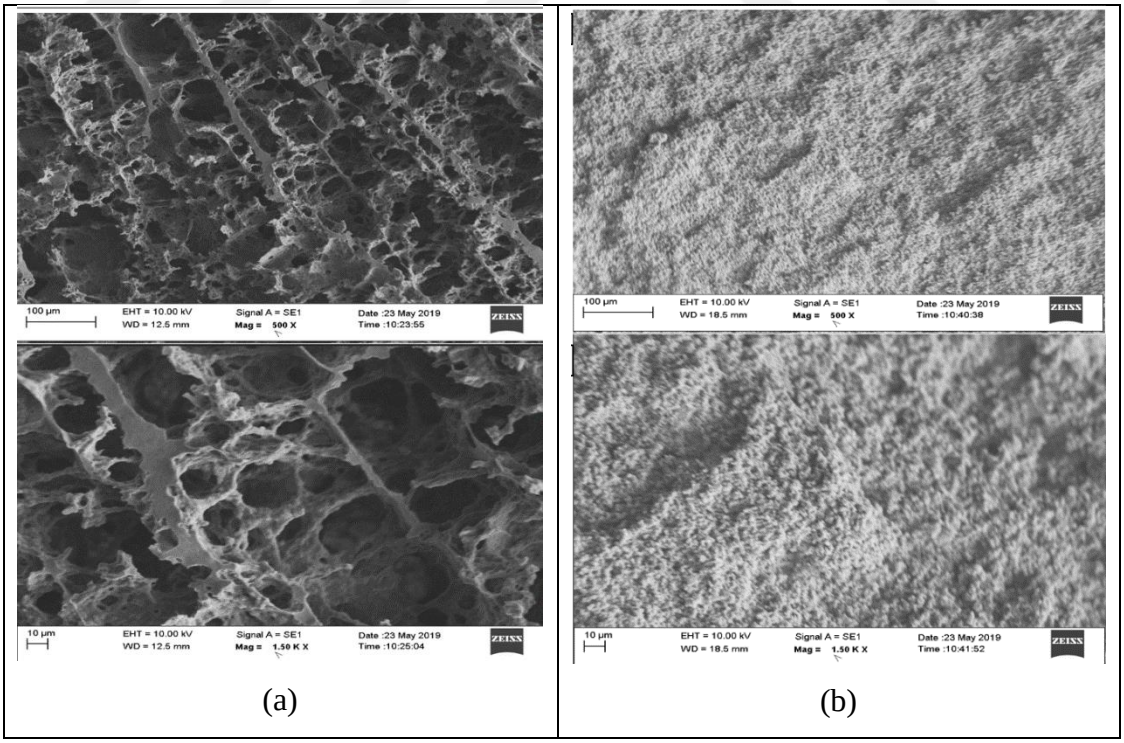
Figure 4.10. SEM results of 1.3ml Hydroxyethyl acrylate columns. (a) C13, (b) C16

In HA cryogels, the roughness of the surfaces decreases as the amount of methylene bis acrylamide used is increased. However, as a result of increasing the amount of HA monomers, both roughness and homogeneous pore distribution decrease in Figure 4.8. and Figure 4.9.. Also, as seen in Figure 4.10., there is no observation of roughness and homogeneously dispersed porosity in polymerizations at -20°C.



(a) (b)

Figure 4.11. SEM results of the Hydroxyethyl methacrylate columns. (a) C21, (b) C24



(a) (b)

Figure 4.12. SEM results of the Hydroxyethyl methacrylate columns. (a) C27, (b) C25

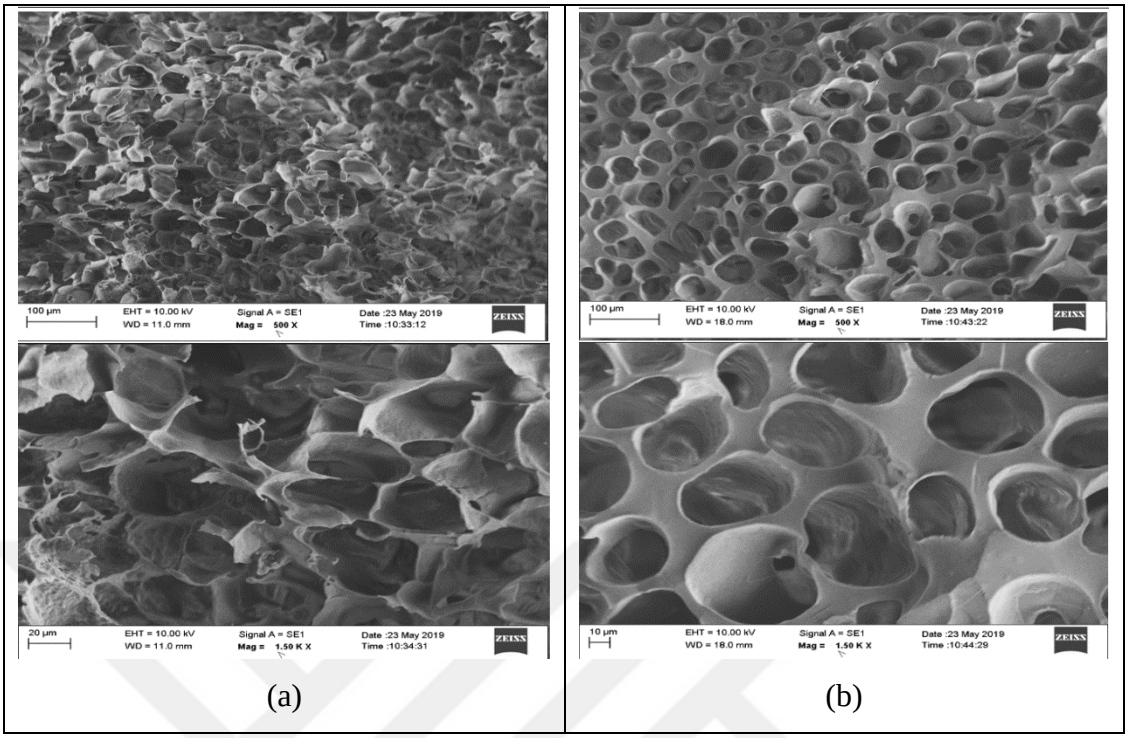


Figure 4.13. SEM results of the Hydroxyethyl methacrylate columns. (a) C22, (b) C28

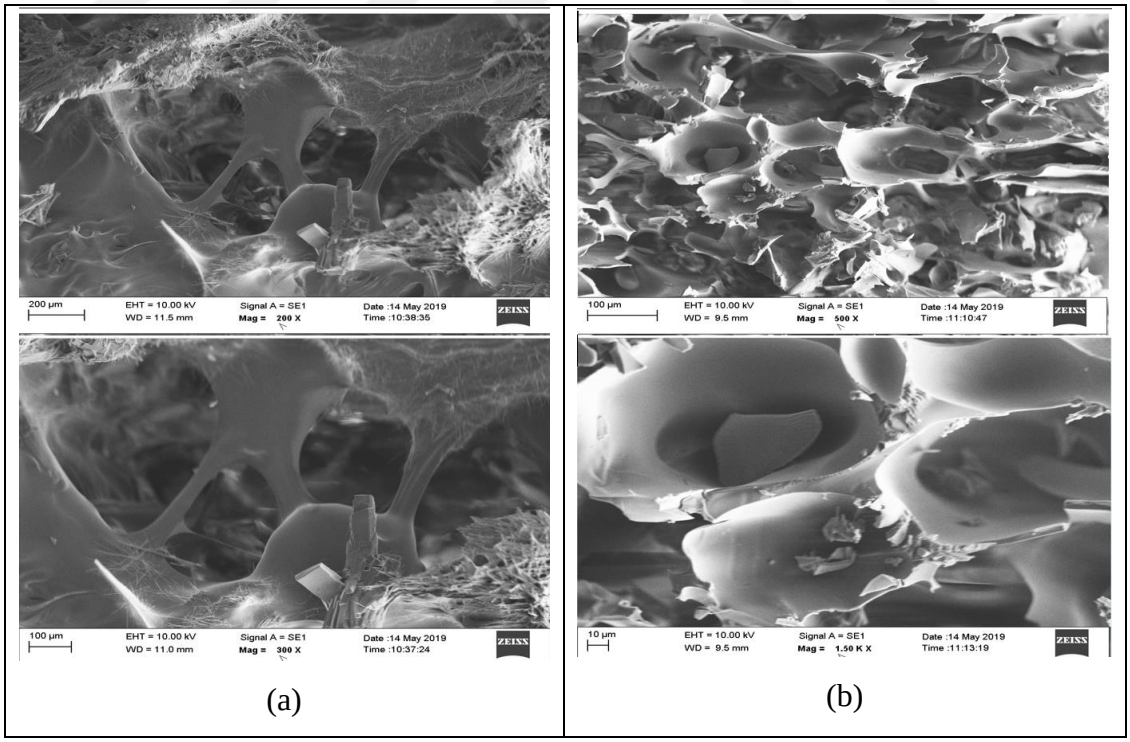
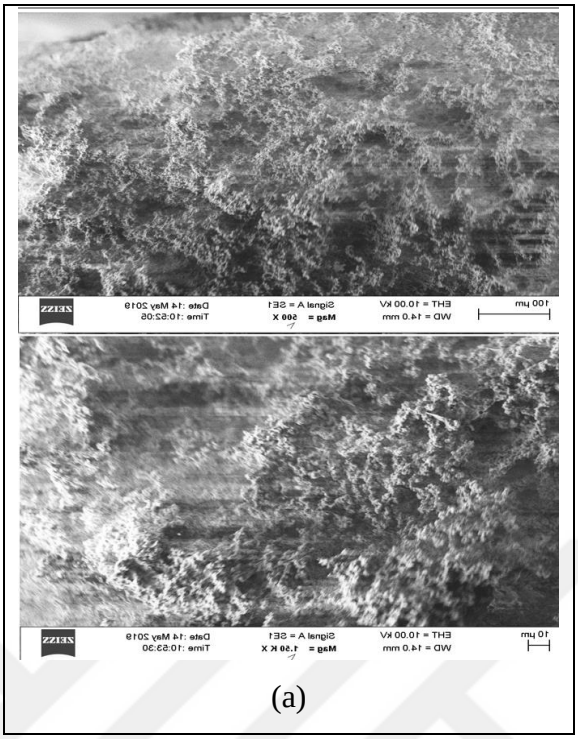


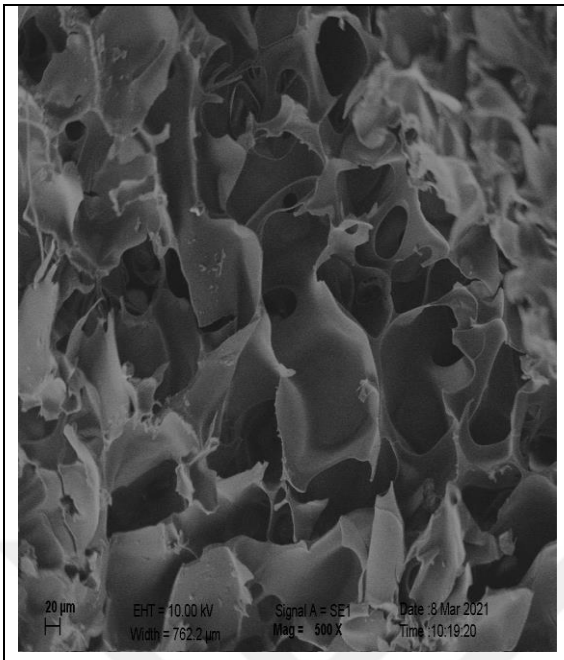
Figure 4.14. SEM results of the 1.3ml Hydroxyethyl methacrylate. (a) C23, (b) C29



(a)

Figure 4.15. SEM results of sample C26 that Hydroxyethyl methacrylate column

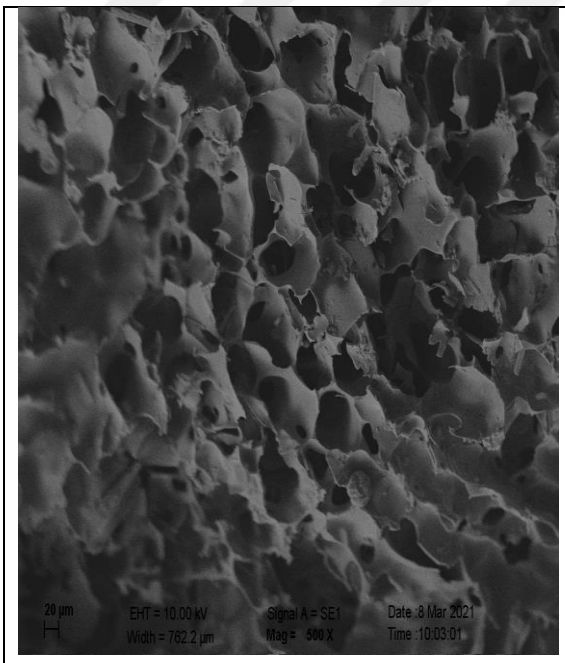
With the increase in the amount of monomer in HM cryogels, the porosity increased considerably. When the polymerization temperature is reduced to -20°C (Figure 4.14.), porosity is obtained, but it is not homogeneous. The best results were obtained at -16°C (Figure 4.13.).



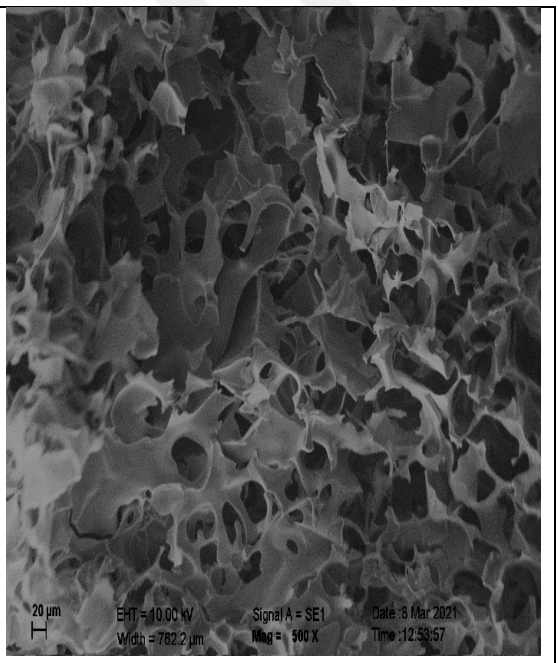
(a)

(b)

Figure 4.16. Columns that prepare with Allyl glycidyl ether. (a) C41, (b) C42C17



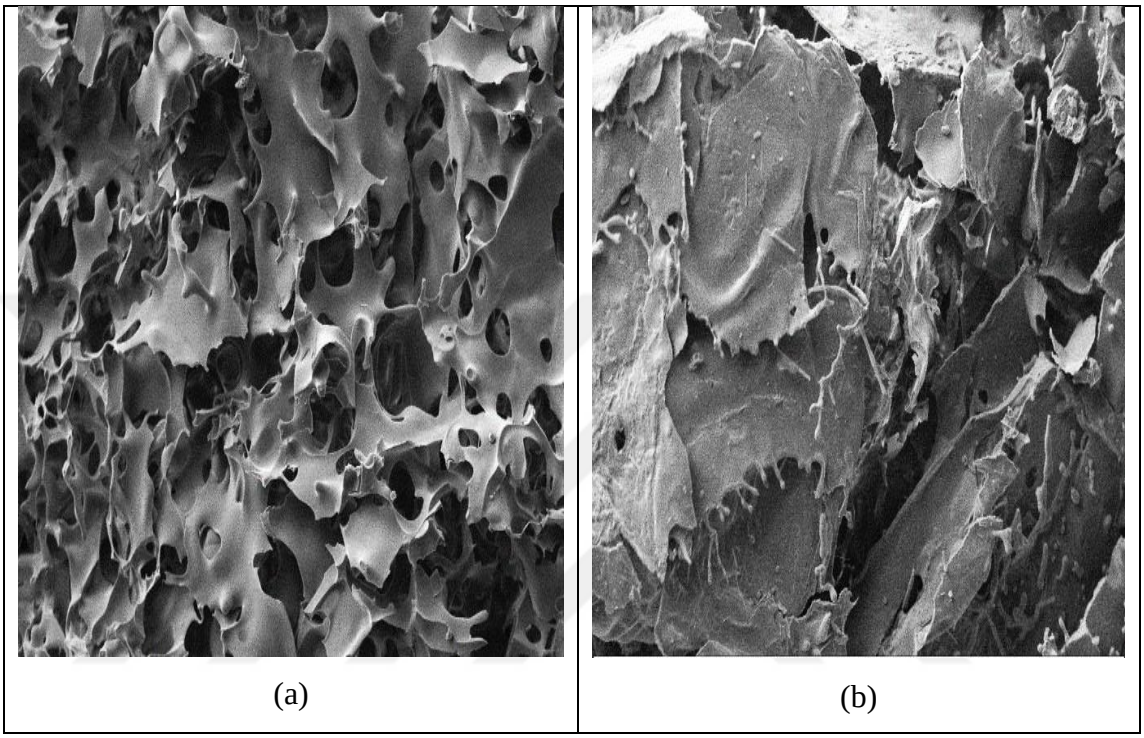
(a)



(b)

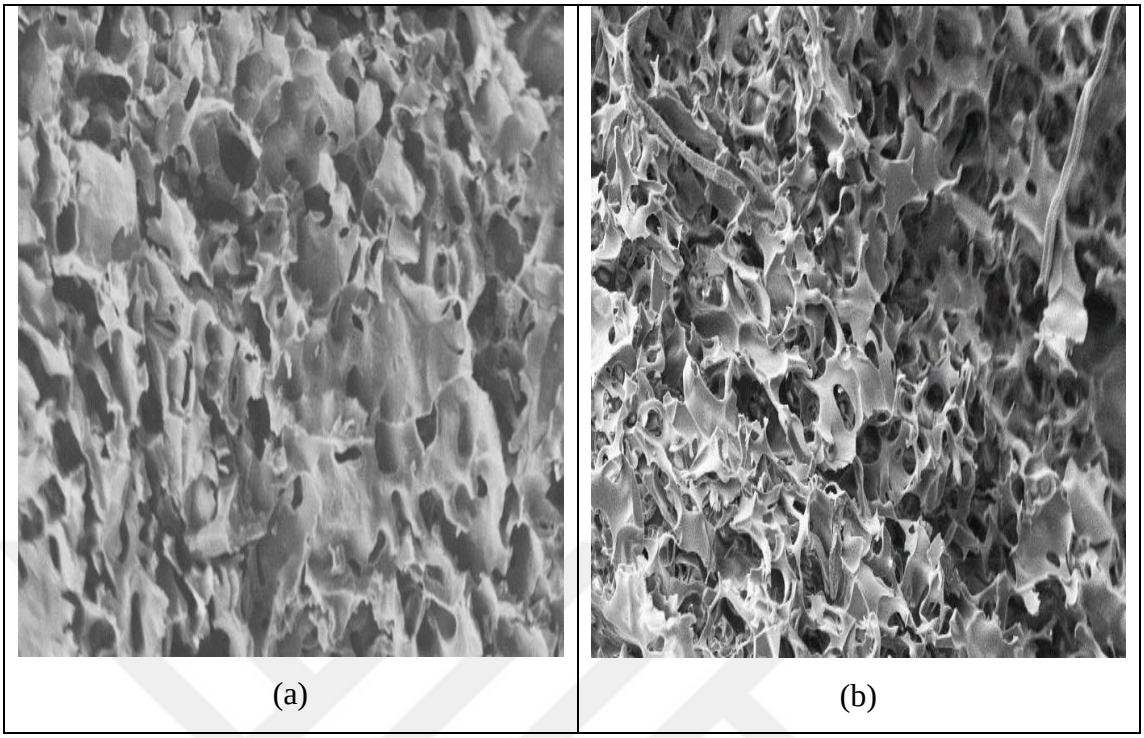
Figure 4.17. Columns that prepare with Allyl glycidyl ether. (a) C43, (b) C22C17

Allyl glycidyl ether monomer was added to increase the pore distribution of the cryogels, and the column structure was tried to be changed. As a result, while the porous structure was observed, it was also observed that they contained smaller pores and channel-like structures were formed.



(a) (b)

Figure 4.18. Columns that prepare with Allyl glycidyl ether. (a) C41I4, (b) C42C17



(a) (b)

Figure 4.19. Columns that prepare with Allyl glycidyl ether. (a) C44I4, (b) C41C17

When the SEM pictures of some of the modified columns are examined, it can be said that the modification made a lot of changes on the pore structure of the column. For anionic modifications, the base varies depending on the column material, and although there is a homogeneous pore distribution in cationic modifications, stratifications are observed on the surface.

4.3. SWELLING CAPACITY AND POROSITY

Table 4.1. Swelling capacity and porosity values of selected columns

Column	Swelling capacity	Porosity (Ɛ)
C4	4.2	0.89
C5	4.1	0.89
C17	5.0	0.88
C28	5.2	0.82
C41	11.7	0.94
C42	5.0	0.92
C43	2.5	0.87

C44	5.0	0.80
C4C17	1.7	0.90
C4C4	1.0	0.93
C5C17	1.2	0.89
C5I4	1.3	0.90
C17C17	3.8	0.85
C17I4	2.4	0.78
C22C9	4.6	0.92
C22C17	0.5	0.88
C22I4	1.8	0.90
C28C5	3.0	0.91
C28C17	1.8	0.70
C28I4	1.7	0.79
C41C5	4.6	0.97
C41C9	4.4	0.91
C41C17	1.6	0.82
C41C26	4.7	0.90
C41I4	3.2	0.93
C42C17	13.8	0.88
C42I4	0.7	0.93
C43C17	3.1	0.89
C43I4	7.1	0.86
C44C17	1.4	0.89
C44I26	1.5	0.86
C44I2	2.4	0.67
C44I4	4.5	0.89
C44I8	3.3	0.81

While the ratio of weight change to volume change gives the swelling capacity, porosity was also measured with weight changes and densities. The porosity values of the columns vary between 0.8 and 0.94. The increase in porosity change seen with the addition of AGE for acrylamide-based columns is that there is no change for HA and decreases for HM. However, it is observed that the swelling capacity for acrylamide can increase approximately 4 times, and the swelling capacity increases in columns (C41 and C42) with AA and AGE. For HA and HM based columns, the swelling capacity was increased by approximately 5 times in both single monomer and AGE based columns.

When the porosity and swelling ratios of the modified columns are examined, it is observed that the porosity decreases by approximately 1-2 percent compared to the basic columns, and the swelling ratio varies according to the modification and basic column material. It is seen that the swelling capacity of C4 and C5 is decreased from 4 and 5 times to 1 to 1.7 times in ionic modifications as AA-based columns. A similar situation exists for columns based on HA and HM. However, no significant changes were observed in the columns using AGE as in the basic columns.

4.4. CROSSLINKING DEGREE

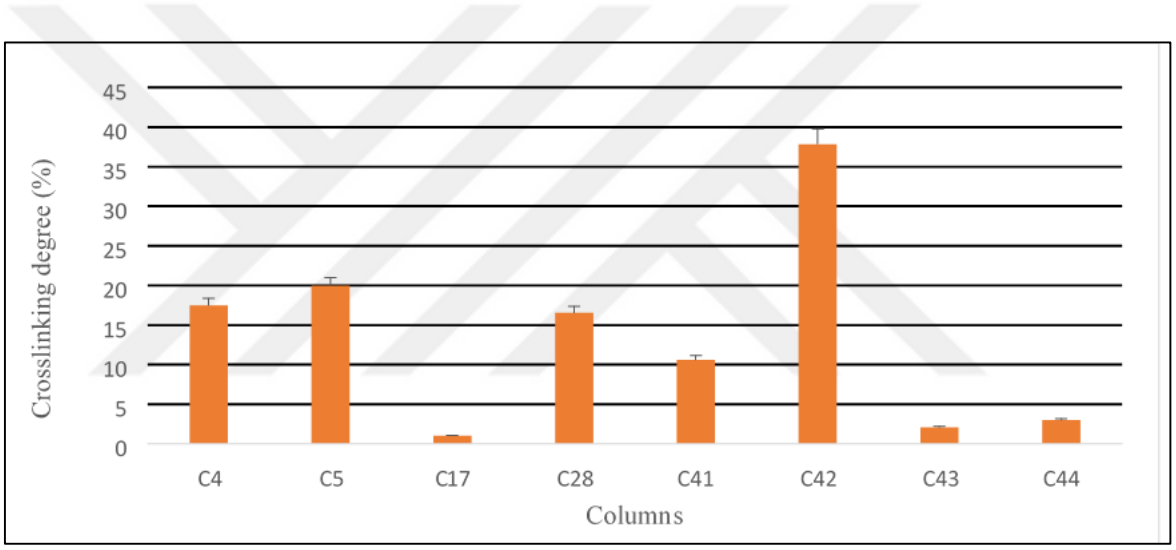


Figure 4.20. Crosslinking degree of selected columns

The degree of crosslinking of the columns shows how effectively the methylene-bis-acrylamide bonded during the polymerization. 18 percent and 20 percent crosslinking was observed for C4 and C5, which are AA-based, respectively, while it was 1 percent for C17, which was HA-based. C28, which is based on HM, also showed approximately 17 percent crosslinking. For C41-C44 containing AGE, it is thought that the polymerization temperature has a significant effect. Although the main difference between C41 and C42 is the polymerization temperature of 4°C, 3 times more crosslinking was observed. While the crosslinking in HA-based columns did not change much, the crosslinking in HM-based C43 decreased to 2 percent when prepared with AGE.

4.5. CHARACTERIZATION OF CHEMICAL STRUCTURE

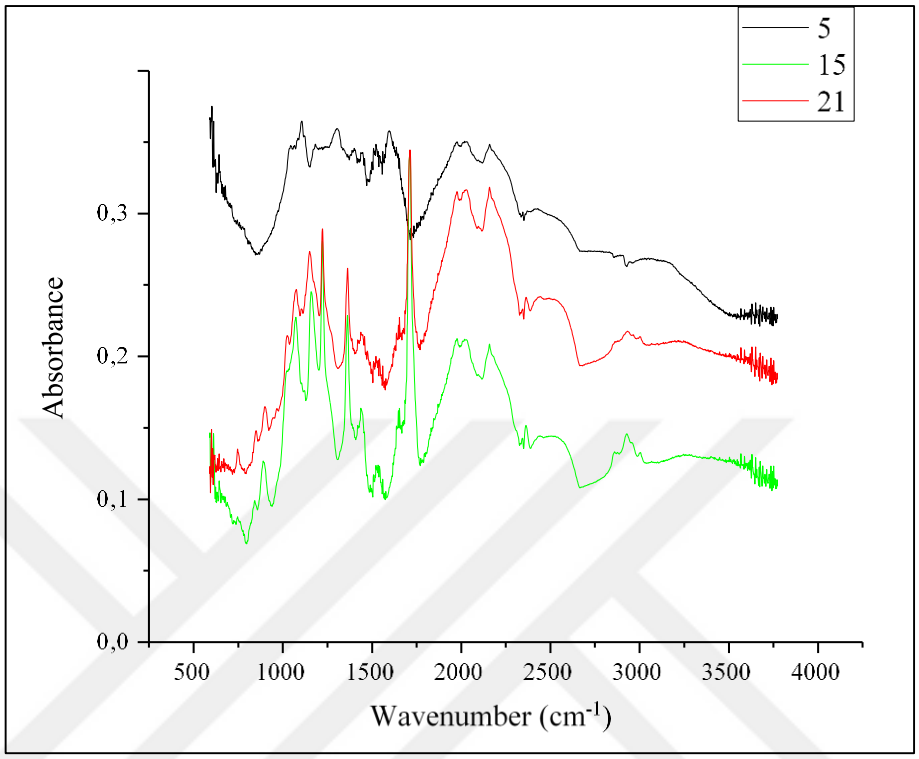


Figure 4.21. FTIR spectrums for C5, C15 and C21

For the prepared columns, measurements were taken with FTIR in order to examine their chemical structures and interactions. FTIR spectra for C5, C15 and C21 are given in Figure 4.20 and for C5, C33, C38 and C39 in Figure 4.21. The N-H bending in HA and HM is due to methylene bis-acrylamide and appears to have a very high energy due to the hydroxyethyl groups. C-H rocking is a high-energy process, it is rarely seen in the FTIR spectrum of acrylamide cryogel, but is seen in many places in HA and HM. This may be why the AA cryogel is rigid. The strength of C-H bonds is also not high in AA compared to others. The O=C=O structure is also seen as a stretch with little energy in all cryogels. While amide N-H stretching is normally seen at 3500 cm-1 and above, it appears as noise in the entire FTIR spectra.

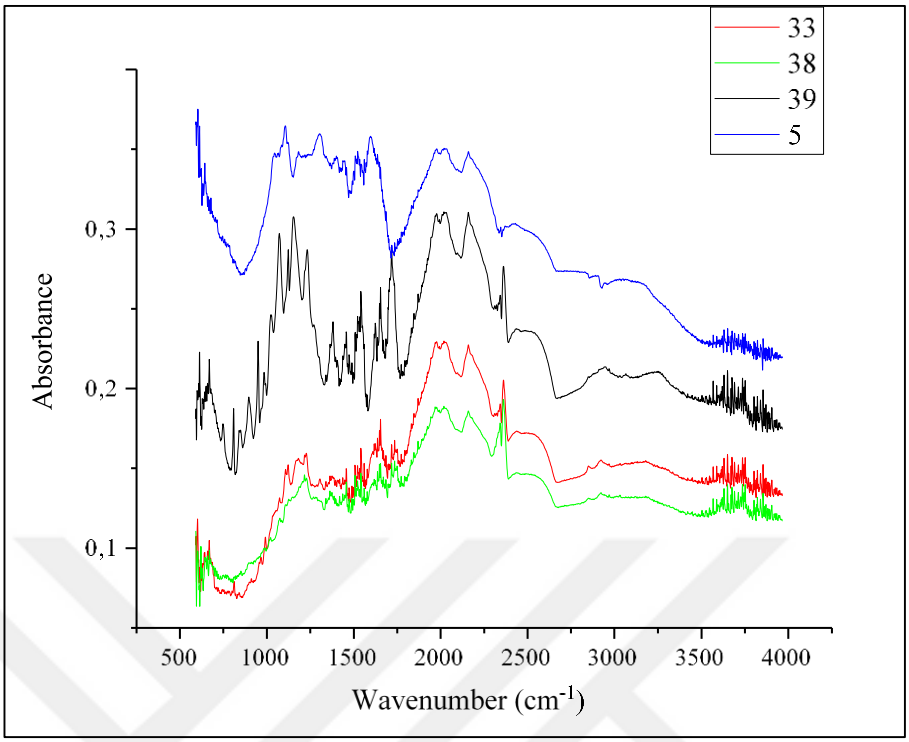


Figure 4.22. FTIR spectrums for C5, C33, C38 and C39

When the FTIR spectra of the mixing of AA and their HA and HM monomers (C33 and C38) and the mixture of HA and HM (C39) are examined comparatively, the O=C=O stretching is observed with high energy in all mixed cryogels. This is proof that the mixture is chemically correct. The shaking of the polymer chain appears to be more severe in the HA/HM mixture than in the mixture with AA. N-H bending is lower in the mixtures made with acrylamide compared to the HA/HM mixture.

4.6. IONIC CHARGE

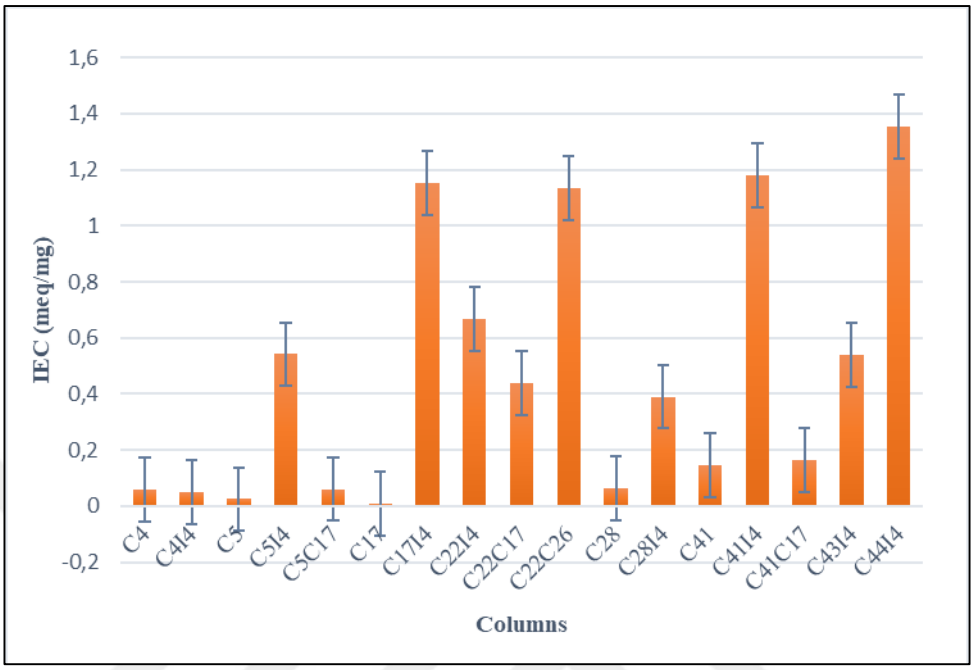


Figure 4.23. Net ion exchange capacities of columns with cationic and anionic modifications

Ionic substances are attached to the amine and/or hydroxyl structures, which are the active groups of the base cryogel columns prepared for the ionic loading of the columns. In the measurement of ionic exchange capacities, negative values were observed in some columns. It is seen that the ion exchange capacities of the cationic loaded columns are generally higher than the columns on which they are based. It is seen that the ion exchange capacity measured at the anionic load loadings is close to the columns on which it is based. It is seen in modified C22 columns that the ion exchange capacity is directly related to the amount of sodium chloroacetate reacted.

4.7. MEASUREMENTS OF STATIC BINDING

BSA in cationic columns and ƐPL in anionic columns were used to measure the stable binding capacities. Calculations were made depending on how much protein was adsorbed from the liquid depending on the column weights.

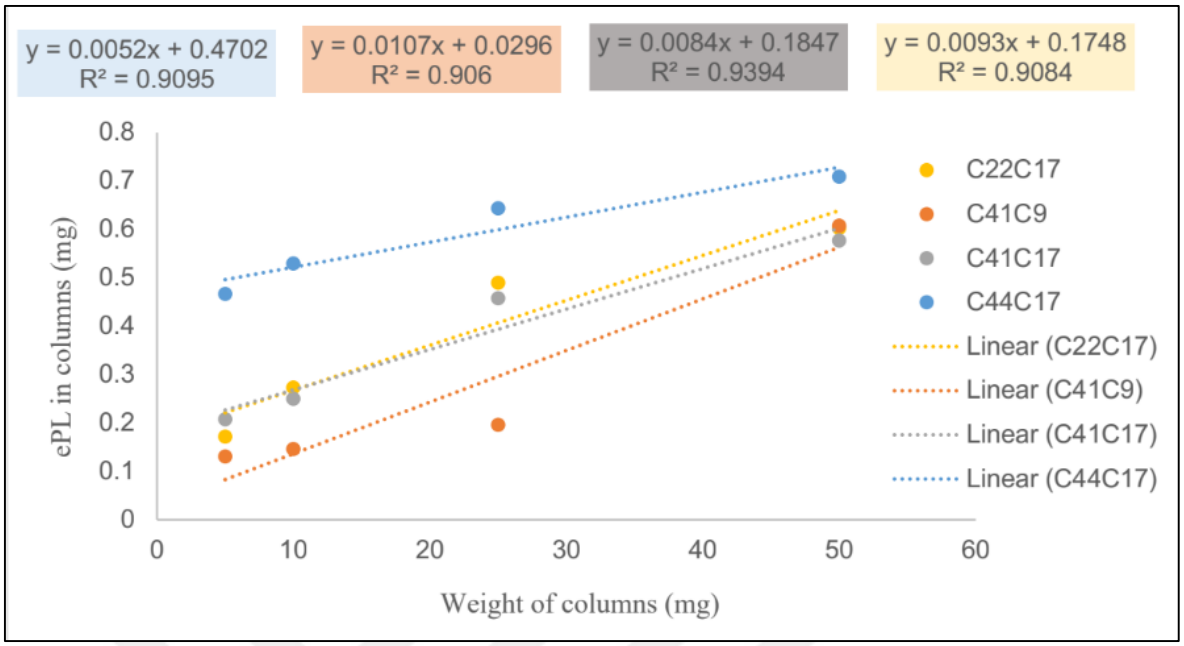


Figure 4.24. Variation of the amount of Ɛ-PL attached to the columns according to the column weight

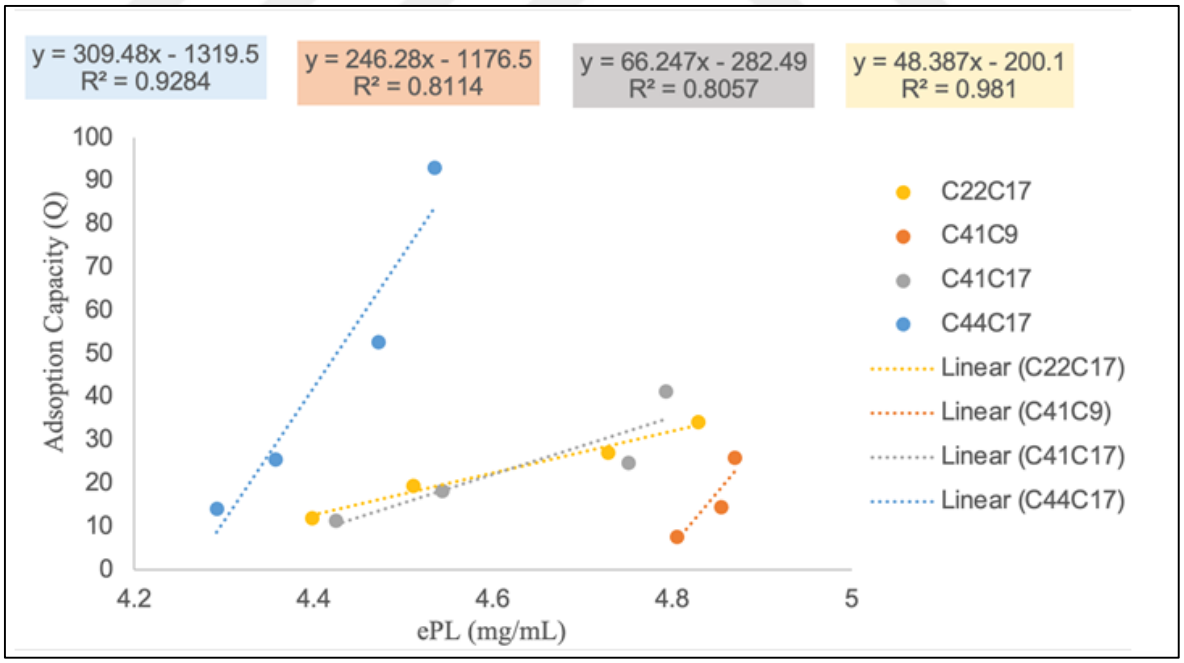


Figure 4.25. Adsorption capacities of the columns for Ɛ-PL

The stable binding capacity of selected anionic columns against Ɛ-PL is shown above. Although there are different ratios for Ɛ-PL bound to the column depending on the column

weight, a similar distribution is seen in all columns. Considering the adsorption capacity, it is seen that it varies significantly depending on the amount of material used for modification, but the same observation cannot be made for the difference in the basic column material.

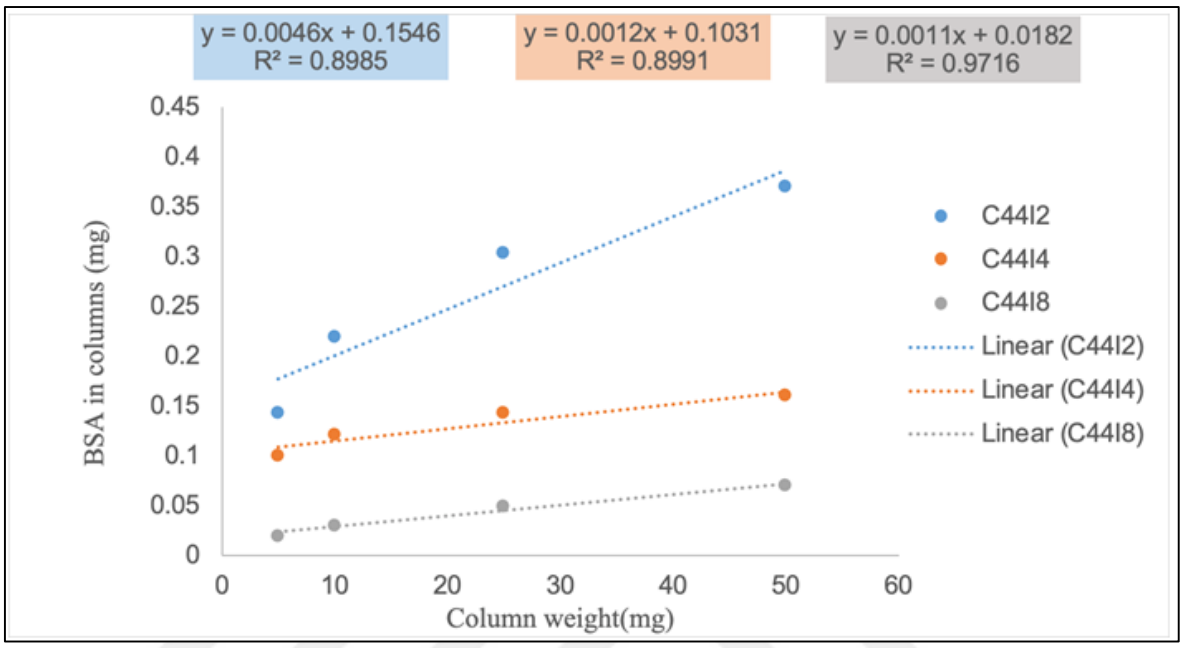


Figure 4.26. Variation of the amount of BSA attached to the columns according to the column weight

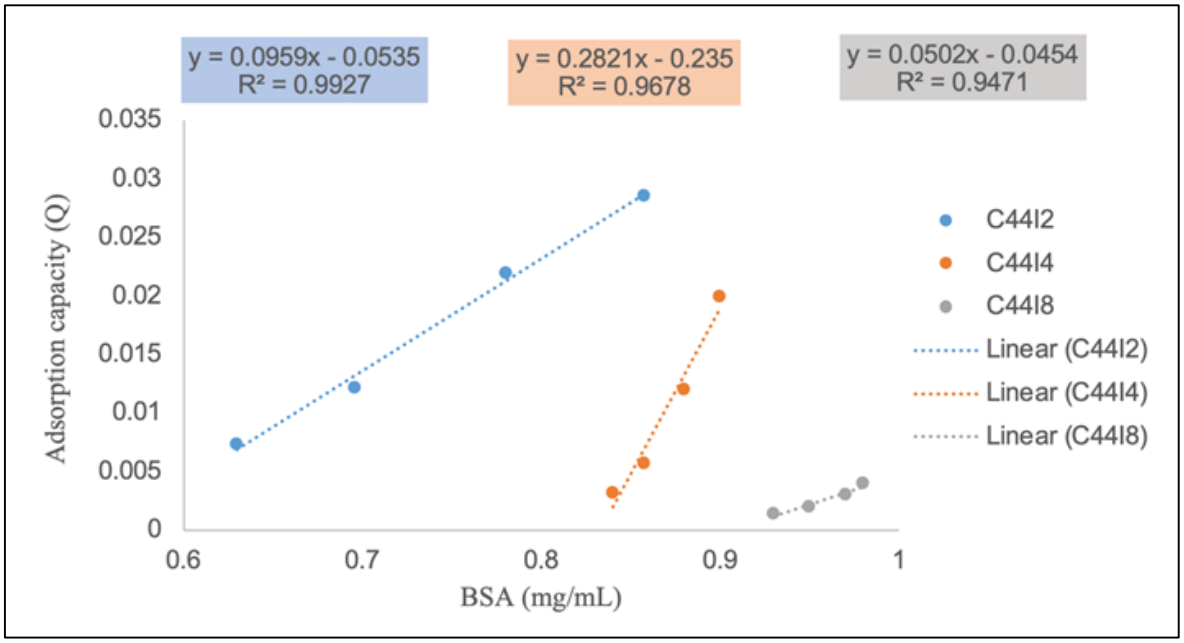


Figure 4.27. Adsorption capacities of columns for BSA

Statistically correct results could be obtained with cationic loaded C44 columns among cationic loaded columns. When the BSA change depending on the amount of column is examined, it is thought that C44I2 has a higher holding capacity than the others, it is seen that the adsorption capacity is higher in C44I4. It was decided to perform dynamic binding studies with C44I2 and C44I4.

Table 4.2. The Langmuir and Freundlich isotherm values

	Langmuir			Freundlich		
Column name	K_m	Qmax	R^2	n	K_f	R^2
C22C17	5.093	2.091	0.895	0.096	$2.65x10^{-06}$	0.957
C41C9	4.902	0.157	0.986	0.012	$2.85x10^{-56}$	0.923
C41C17	5.002	1.597	0.925	0.074	$2.10x10^{-08}$	0.903
C44C17	4.565	0.997	0.932	0.030	$1.58x10^{-20}$	0.994
C44I2	1.010	0.0056	0.948	0.242	0.056	0.972
C44I4	0.906	0.00028	0.928	0.0373	0.352	0.997
C44I8	1.009	0.00012	0.997	0.0507	0.006	0.991

Langmuir and Freundlich isotherms were used to determine the stable binding capacity of the columns and to understand the mechanism. The calculations of the columns, which gave statistically significant results in the stationary bonding test of the columns, according to the isotherms are given in Table 1.1. Accordingly, while C44C17 and C22C17 follow the Freundlich isotherm; C41C columns conform to the Langmuir isotherm. However, cationically charged modified C44 columns follow the Freundlich isotherm.

4.8. MEASUREMENT OF DYNAMIC BINDING CAPACITIES OF COLUMNS

FPLC device was used to measure the dynamic binding capacities for the columns. The amount of protein bound per gram weight of the column is calculated from the flow rate and sample concentration and the amount of column used.

C22C9 and C22C17 were not used in further studies because insignificant impurities were observed in the modifications of C22 in anionic columns despite long-term processes. C41C9 and C41C26 were operated at flow rates of 2 mL/min, while dynamic binding experiment was performed by operating C41C17 at flow rates of 2 and 5 mL/min. As seen in Figure 4.27, different Ɛ-PL arrival times are seen. The highest binding capacity is seen in C41C17, while there is no decrease in binding capacity with increasing flow rates. C41C17 appears to increase in dynamic bonding capacity at higher flow rate.

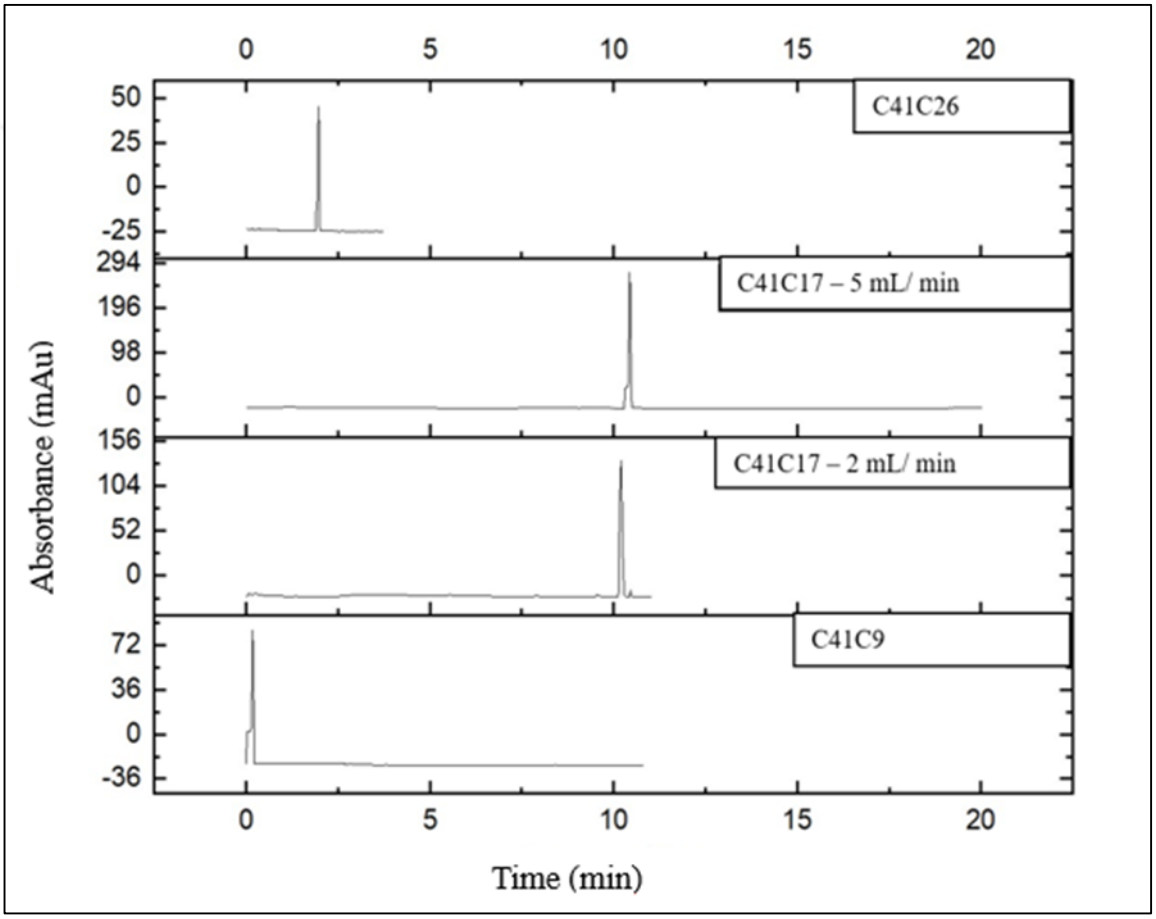


Figure 4.28. Dynamic binding capacities against Ɛ-PL for anionic modified C41 columns

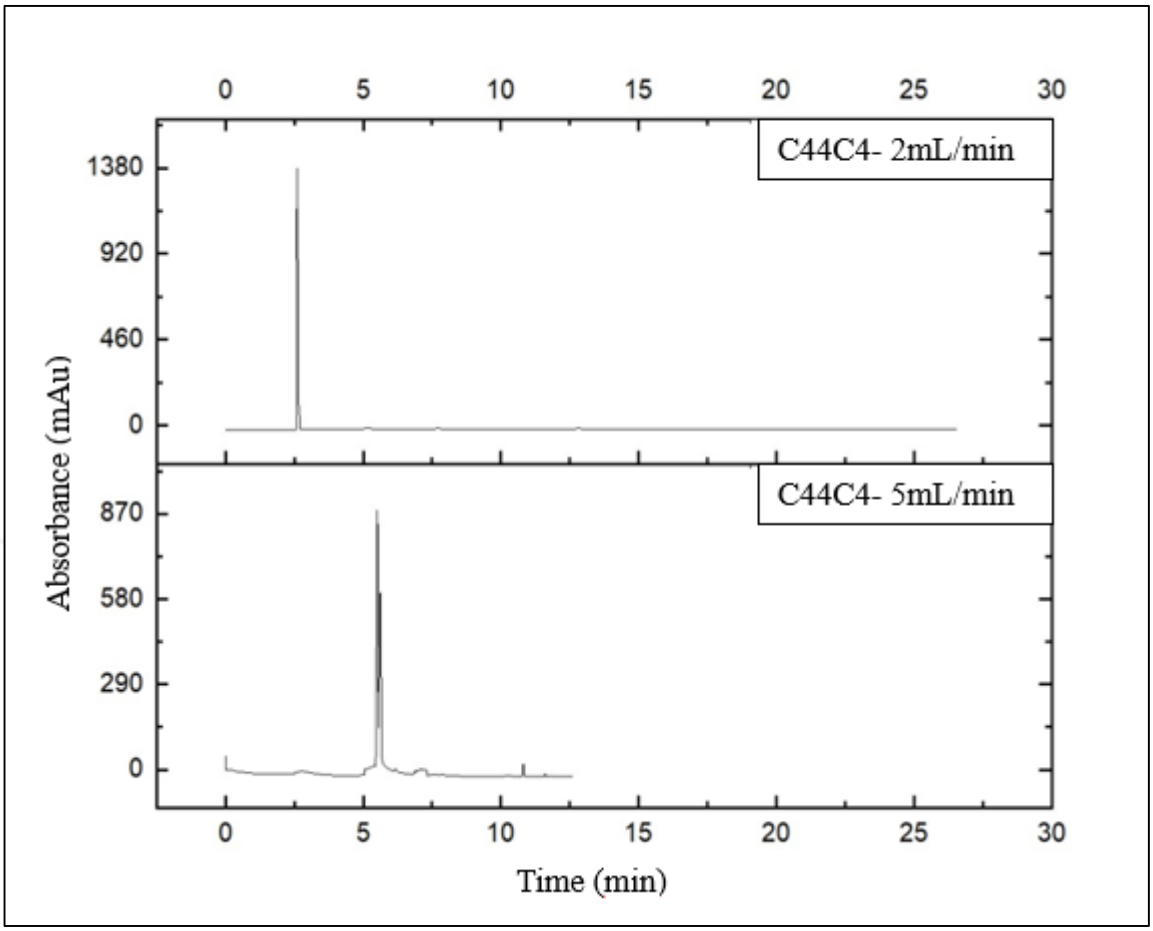


Figure 4.29. Dynamic binding capacities against BSA at flow rates of 2 and 5 mL/min for the C44C4 column

On the other hand, dynamic binding measurement against BSA was performed in cationic columns. For this, the C44I4 column was tested at flow rates of 2 and 5 mL/min. Increased dynamic binding capacity values at increasing flow rates in C41C17 were observed in C44I4. Such a binding capacity is important for high molecular weight BSA.

Table 4.3. Dynamic binding capacities of cryogel columns

Column[*1]	Protein	Dynamic binding capacity (mg protein / g column)
C41C9	Ɛ-PL	0,009
C41C17	Ɛ-PL	0,661
C41C17[*2]	Ɛ-PL	1,691
C41C26	Ɛ-PL	0,103
C44I4	BSA	0,214
C44I4[*2]	BSA	1,132

*1 Flow rate was set as 2 mL/min in the experiment performed in FPLC.

*2 Flow rate was set as 5 mL/min in the experiment performed in FPLC.

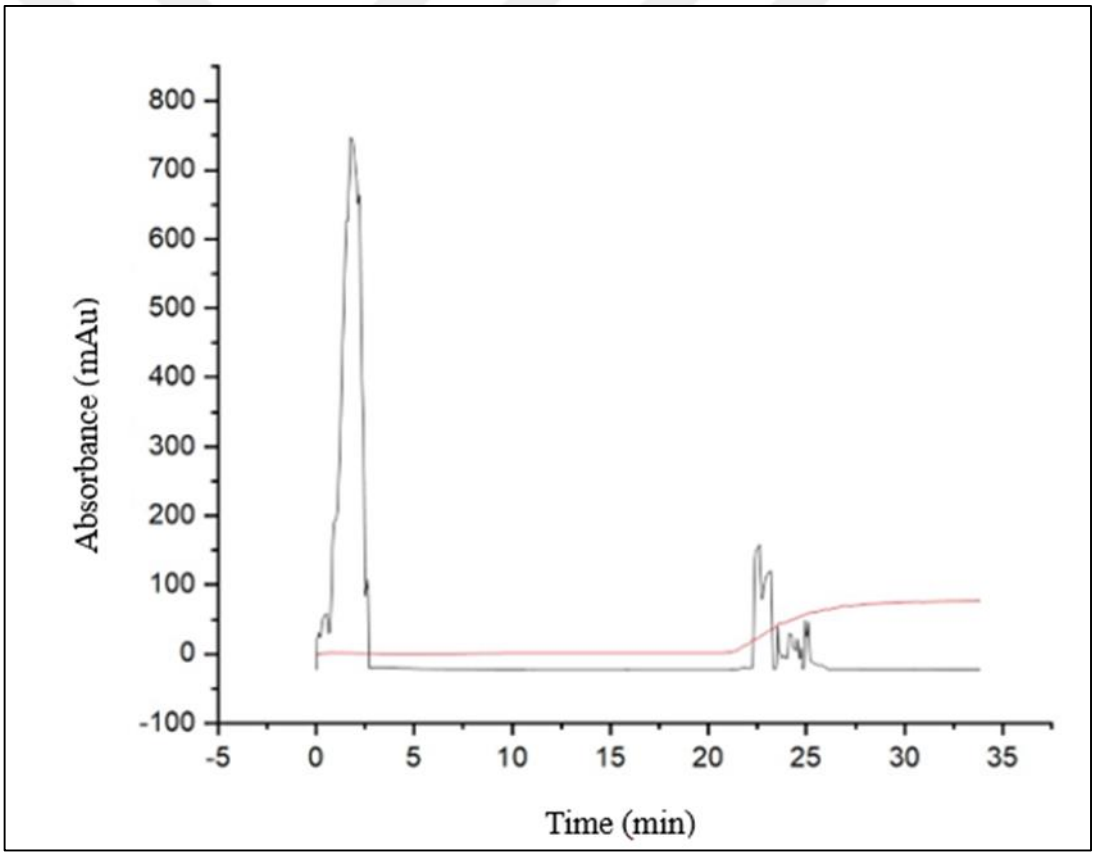


Figure 4.30. Selective separation performance for lysozyme, BSA and Ɛ-PL on the C44I4 column

While FPLC was used for the selective separation performances of the columns, the collected fractions were characterized by HPLC and SDS-PAGE.

Separation studies were attempted for lysozyme, BSA and Ɛ-PL with the C41C17 column used in the dynamic binding experiment. After the dynamic binding experiment, the column was first washed with buffer containing 1M NaCl, then washed with 98 percentage ethanol and 70 percentage ethanol, and then with buffer. Each wash step was performed for at least 5 column volumes. However, impurities are constantly coming from the column and they could not be identified. Therefore, C41C columns were considered to be disposable and selective separation capacity tests were not performed.

In the study performed for lysozyme, BSA and Ɛ-PL in the C44I4 column, according to HPLC and SDS-PAGE studies, lysozyme and Ɛ-PL were in the peak region up to 2.5 minutes, while in the range of 22-25 minutes after gradient washing with NaCl. BSA is coming (Figure 4.30). The contamination condition seen in the C41C17 column was seen in the C44I4 after the first use.

It is not possible to operate the columns, which allow the continuous phase to pass in one go, with continuous chromatography. Therefore, further experiments were not carried out.

5. DISCUSSION

The main purpose of the experiments are the production of the monolith acrylamide based cryogels and purify of the Ɛ-poly-L-lysine according to columns ionic activities (51). For this purpose, 3 different monomers were selected in the preparation of cryogel monolith columns. These are acrylamide (AA), hydroxyethyl acrylate (HA) and hydroxyethyl methacrylate (HM) monomers. The amide structure in AA, the importance of monomer amount and polymerization temperature due to the strong interaction of amine groups can be understood from the experiments. Due to the ethyl group for HA and the hydroxyl groups at its end, it becomes an active column with ionic binding strength. The difference for HM from HA is the methyl group on the vinyl side. This group will keep the polymerization chains away from each other. It is thought that this will affect the pore structure and number [52,53].

Scanning electron microscopy (SEM) results are important in determining the surface properties of the obtained cryogel columns. The changes between parameters in the prepared cryogels show that the surface properties of the cryogel have changed. In the literature, for cryogel polymerization, it must be kept in the reaction for at least 12 hours at a temperature of -12°C to -20°C. Temperatures of -12°C, -16°C and -20°C were applied in the experimental sets. For AA, homogeneous pore distribution and pore sizes were observed depending on the temperature drop. In the polymerizations where HA and HM are used, homogeneous pore distributions are observed up to -16°C, while cryogels prepared at -20°C are not in the desired shape. Changes in the amounts of APS and TEMED alone cannot be clearly detected in SEM images. However, during the experiments, it was observed that polymerization started without taking the cryogel conditions when the time required for adding and mixing to the monomer solution was more than 1 min [54].

Two monomer cryogels in the literature have been presented specifically for the attachment of an active structure [55,56]. In addition to the AA, HA and HM we used for this, two monomer cryogels were tried to be prepared with cationic and anionic monomers. It was thought that the pore diameters and distribution of cryogels prepared with the mixture of basically determined monomers would not allow them to be used in purification systems. Although experiments were carried out at different monomer ratios and under different

conditions, cryogel polymerization was not observed in systems with ionic loaded monomers, while the desired pore diameter and distribution were not encountered in mixtures with basic monomers, and column integrity could not be achieved.

It is aimed to increase the number of regions that can be activated by adding AGE to the cryogel polymerization. The epichlorohydrin structure in AGE has the ability to open in acidic or basic medium and bond with other active groups in the medium. Statistically higher Ɛ-PL binding and ionic charge values were measured in columns where AGE was used in ionic modifications.

Swelling capacity and porosity are important variables for process optimization in column purification. In cases with high swelling capacities, the flow rate and sampling times depending on the column volume should be well adjusted. The porosity value provides the surface area, thus the effective transport or adsorption area for purification. However, this value should be correlated with the pore diameter. In the studies in the literature, it has benefited from the swelling capacity in the adhesion and separation of cell lines and blood proteins [57].

Significant differences were observed for swelling capacity depending on the monomer of the column. For example, it is thought that shrinkage rather than swelling is observed due to methyl groups in HM. However, although the only difference between C41 and C42 is the polymerization temperature, a high swelling capacity is observed. However, the swelling ratio in AA columns (C4 and C5) without AGE was very close to each other. The presence of active groups in AGE has a direct effect on the swelling capacity. The swelling capacity of modified columns loaded with ionic load is much higher than the columns on which they are based. Similar to the presence of active groups in AGE, swelling is observed to be high due to the structures of ionic-loaded columns.

It is seen that the porosity values vary according to the column material. However, it is seen that there are significant differences in porosity after modification. While changes are observed in the porosities around 1-2 percent after modification for AA, there is a decrease in the pores in general in the columns with HA and HM. It is seen that the presence of AGE causes an increase in the pores on AA-based columns, no change in HA, and decreases in HM-based columns after the applied modification. However, although the change in swelling capacity of AGE is the same for AA with respect to porosity, it does not appear to

change in HA and HM. The observation that swelling increases with decreases in porosity means that the polymer chains form surfaces. This can be seen from the SEM images. The degree of crosslinking shows parallelism with swelling and porosity changes.

Cryogel formation was not observed with ionic charged monomers added during polymerization in two different ways applied for ionic loading of cryogel monolith columns. Although the methods in the literature were followed, cryogel polymerization could not be performed in our laboratory conditions. The reason why many of the polymerizations carried out according to the literature could not be realized is thought to be the presence of inhibitors in most of the monomers used and the provision of the polymerization temperature [58,59]. In order to deactivate the inhibitor, the monomer usually needs to be soaked in water or heated. However, these processes cannot be done because heating may cause polymerization. Although monomers are taken into water for cryogel polymerization, insufficient deactivation of the inhibitor may prevent successful polymerization every time. After the formation of the column, ionic loading was performed with its activation and successful results were obtained. Cryogel columns with column integrity were obtained as a result of the reactions applied with iodomethane for cationic loading and sodium chloroacetate for anionic loading. Although there is no direct relationship between the ionic exchange capacity and the efficiency of binding to a particular chemical, it is important for determining the capacity of the prepared columns. It was observed that the obtained values were in parallel with the membrane and resin chromatography columns in the literature (60,61). It has been stated that the Freundlich isotherm is commonly seen as the adsorption isotherm for monolith columns. Langmuir isotherm is seen in C41 columns loaded with anionic charge, but Freundlich isotherm is seen in all other columns. While heterogeneous structure is expected especially after modification, Langmuir isotherm of homogeneous structure was observed in C41C columns. The increase in the binding capacity for C41C17 with increasing flow rate in the FPLC system can be explained by the Langmuir isotherm. However, it can be thought that the increase in the bonding capacity while expected to decrease at increasing flow rates may be due to the effect of the pore distribution of the cryogel column and the pressure applied on it. The increased flow rate will also increase the pressure on the column and cause the pores to be compressed, allowing the protein to make more contact with the column surface and attach to the surface more. The high swelling capacity of the columns confirms this hypothesis. Experiments were made with C41C17 and

C44I4, which were used to determine the selective separation capacities of the columns. C41C17 was not used to determine selective separation capacity as it leaves impurities after first use. It was suspected that the impurities might be from the monomers forming the column, and the impurities were compared spectrophotometrically against the monomer standards, but it was determined that there was no monomer. Adhesion and separation of C44I4 and negatively charged BSA were successfully achieved with increasing salt concentration. It has been understood by experiments that C44I4 can be used as a one-time use like C41C17. In continuous chromatography, two or more columns must be connected and washes performed by repeated passage. If impurities come during the protein binding stage during the second process, it is seen that the purification process by continuous chromatography cannot be performed.

6. CONCLUSION AND FUTURE PERSPECTIVE

The preparation and modification of the cryogel column varies depending on the monomer type and the preparation conditions [10,27,62,63]. While the pore diameters are about 20 – 100 μm for the basic columns, the pore diameters of the modified columns are about 5 μm and surface roughness is observed. It significantly changes its physical structure in structures used as activation media such as AGE. However, it allowed ionic charge modification in columns with AGE to be made without spoiling the column structure. It is important to know the type and content of monomer used in cryogel column preparation and to ensure that the cryogel polymerization conditions are the same each time. It was not possible to obtain directly ionic charged cryogel columns. Monomers and temperature need to be optimized for polymerization. Modification of columns containing epoxy ring-containing structures such as AGE was found to be more appropriate. Methodologically, it is more convenient to develop modifications on the foundation column in terms of operation.

Cryogel columns can be operated at high flow rates. However, this also reduces their binding capacity in general. With continuous chromatography, it is possible to increase the low binding capacity and work at high flow rate. However, it is seen that the columns prepared by cryogel polymerization are not suitable for continuous chromatography because they are disposable. However, single-use purification processes can be performed successfully.

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