ION EXCHANGE MEMBRANE PRODUCTION FROM NATURAL POLYMERS FOR THE PURIFICATION OF IMMUNOGLOBULINS

by

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ION EXCHANGE MEMBRANE PRODUCTION FROM NATURAL POLYMERS FOR THE PURIFICATION OF IMMUNOGLOBULINS

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ABSTRACT

ION EXCHANGE MEMBRANE PRODUCTION FROM NATURAL POLYMERS FOR THE PURIFICATION OF IMMUNOGLOBULINS

Immunoglobulins have become one of the most important products in pharmaceutical biotechnology. Chromatographic separation techniques are widely used in purification steps of immunoglobulins and hence, production cost gets increased. In this project, natural biopolymer bacterial cellulose (BC) is prepared and modified to increase ion exchange capacity to use in purification of immunoglobulin G (IgG) and immunoglobulin A (IgA). Totally, 5 modifications were performed. Hydroxyethyl acrylamide (HEA), epichlorohydrin, ammonium hydroxide (NH3), caffeic acid and propane sultone (SO3) were used for obtaining modified polymers. The membranes chosen to proceed with were, BC, BC-HEA, BC-HEA-NH3, BC-HEA-SO3; based on membrane integrity. Young's modulus values were determined. Fourier's transformation infrared spectroscopy (FT-IR) was used for observing modifications and scanning electron microscopy was used to observe surface and cross-section changes of the membranes. These observations were supported by SEM images. Hydraulic permeability, porosity and pore size of membranes were calculated. Hydraulic permeability values were very high compared to commercial membrane units. There is no protein binding at high hydraulic permeability. The porosity of BC membranes were in the range of 45–95% depending on the modification type. Effective area for protein adsorption was also calculated. Effective area of bacterial cellulose membranes were around $2cm^2$ and between $2–15cm^2$ at constant surface area ($17.35cm^2$) respectively. Effective area directly affect adsorption yield. BC membranes were more preferable for adsorption based on physical conditions. Modifications generally increase the ion exchange capacities of membranes. Before starting to purification experiments of IgG and IgA, fetal bovine serum albumin (BSA) was used for protein affinity experiments. Using the data, feasible adsorption theory was selected for membranes. All BC membranes are fitted to Langmuir isotherm. Protein affinity was calculated using BSA, IgG and IgA proteins. BC showed low protein affinity; however, protein binding yield was higher than other membranes. BC membrane was the best for protein affinity, even it has low ion exchange capacity. BC membranes will be used to purify proteins in following studies.

ÖZET

IMMUNOGLOBULINLERIN SAFLAŞTIRILMASI İÇİN İYON DEĞİŞİM MEMBRANLARININ DOĞAL POLİMERLERDEN GELİŞTİRİLMESİ

Immunoglobulinler, farmasötikal biyoteknolojinin en önemli ürünlerinden biri haline gelmektedir. Dünya çapında yapılan yüksek miktarlardaki üretimlerde en fazla kromatografik saflaştırma adımları kullanılmakta ve bu da üretim maliyetini arttırmaktadır. Yapılan bu projede doğal biyopolimerler olan bakteriyel selülozdan (BC)iyon değişim membranları hazırlanmış ve immunoglobulin G (IgG) ve immunoglobulin A (IgA) için ayırma işlemlerinde kullanılabilmesi incelenmiştir. Çalışmada BC'dan toplamda 5 tane modifikasyon yapılmıştır. Temelde kullanılan aktif maddeler; hidroksietil akrilamid (HEA), epiklorohidrin, amonyum hidroksit (NH3), kafeik asit, propan sülton (SO3) ve bunların kombinasyonları şeklinde olmuştur. Bunlardan membran dayanıklılığını koruyan 8 tanesi; BC, BC-HEA, BC-HEA-NH3, BC-HEA-SO3 seçilerek iyon değişim yükü ve protein ilgisi ölçülmüştür. Young's modulus ile gerilme ve kırılma testleri yapılmış, Fourierin dönüşüm infrared spektroskopisi (FT-IR) ile modifikasyonlardan sonraki değişimler gözlenmiş ve taramalı elektron mikroskobu (SEM) ile de membranların yüzey ve kesit görüntüleri alınarak membran olarak fiziksel ve kimyasal incelemeleri yapılmıştır. Modifikasyonlar sonucunda yüzeyde pürüzlenme gözlenmiş ancak, katman yapısında belirgin bir değişiklik olmamıştır. Hazırlanan membranların hidrolik geçirgenlik deneyleri ile gözeneklilik ve gözenek çapı hesapları yapılmıştır.Ticari olarak kullanılan iyon değişim membranları genellikle 3 – 5 membrandan oluşan üniteler şeklindedir. BC membranlarda gözeneklilik değeri % 45 – 95 arasında modifikasyon tipine göre değişmektedir. Membranların adsorpsiyon için etkili alanları da hesaplanmış ve 17,35 cm^2 yüzey alana sahip membranlarda, gözenekliliğe ve gözenek çapına göre BC membranlarda 2–15 cm^2 arasında değiştiği hesaplanmıştır. Membran için etkili yüzey alanının fazla olması adsorpsiyonun daha verimli olması anlamına gelmektedir. BC membranlar fiziksel olarak adsorpsiyon için daha uygundur. Yapılan modifikasyonlar genel olarak iyon değişim kapasitesini arttırsa da immunoglobulin denemelerinden önce sığır serum albümini kullanılarak deneysel yöntemler oturtulmaya çalışılmış ve membranların protein bağlama ilgilerine uygun adsorpsiyon izotermleri belirlenmiştir. Projenin devamında, BC'nin birden fazla membrandan oluşan üniteleri ile protein ve enzim saflaştırılmasında kullanılması için incelemeler yapılacaktır.

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BC	Bacterial Cellulose
BSA	Bovine Serum Albumin
C	Concentration of the protein which is in balance within the liquid
DMSO	Dimethyl Sulfoxide
L_P	Hydrolic permeability
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K_L	Langmuir adsorption constant
PC	Plant Cellulose
Ɛ	Porosity
q	Protein amount per membrane able to bind
μ	Viscosity

1. INTRODUCTION

1.1. BACTERIAL CELLULOSE

Cellulose which is an organic compound its name is given by Anselme Payen has the molecular formula $(C_6H_{10}O_5)_n$, is one of the most extensive biopolymer found on Earth and consist of β(1→4) linked D-glucose linkages. Cellulose especially composes the cell wall of plants which are eukaryotic or in other words higher plants and concurrently it also plays structural role in the formation for the cell walls of algae and fungi. On the other hand cellulose can also be formed by bacteria (Bacterial Cellulose; BC) as a secondary metabolite which is defined by Louis Pasteur ; a sort of moist skin, swollen, gelatinous and slippery [1].

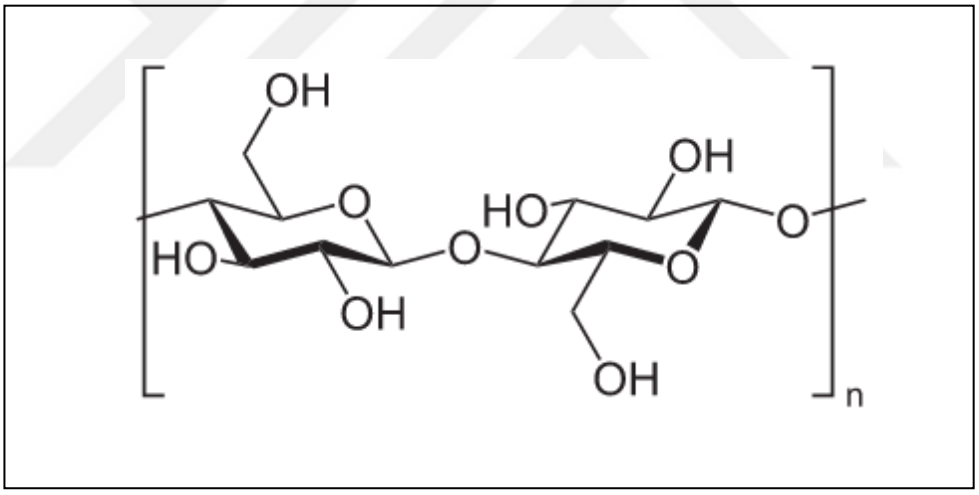

Figure 1.1. Cellulose molecular structure [1]

Cellulose which is a nonaqueous polysaccharide and as like explained previously is mostly generated by veined plants but due to their different properties bacterial cellulose have different utilizations then natural cellulose. Bacteria which can synthesize cellulose as a secondary metabolite are mostly Gram-negative and acetic acid bacteria for example like; Gluconacetobacter, Rhizobium, Agrobacterium, Rhodobacter and Sarcina [2].

1.2. STRUCTURE AND PROPERTIES

When comparing the structural properties of plant cellulose and BC with one another it was seen that their chemical properties are identical which are both formed by unbranched ß-1,4-linked glucopyranose remnants but unlike PC, BC does not embrace pectin, lignin and hemicellulose alongside some other biopolymers [3]. On the other hand BC also differs from natural cellulose due to its high mechanical strength, network structure, water adsorption capacity, crystallinity and mouldability [4]. BC is also biocompatible, biodegradable and well-oriented and has a great macrostructural control [5].

1.2.1. Extracellular matrix imitation

BC transcribes the structural form of collagen in a way that components of their extracellular matrixes are very much alike and they are both primarily mechanical support materials individually for own roots of their tissues. Also they have congener sizes (100 nm in diameter); and as like BC collagen is congregated by pioneer molecules into the chains of the polymer structures [6]. But unlike collagen BC has a hallmark which is immunologic nonreactivity. Because that it is non-recognizable by proteins; as a polysaccharide BC cannot be accepted as a immune stimulant. Besides due to the less amount of endotoxins which is 0.1 EU/ml when it is incubated in water it is a convenient material for microbial processes [7].

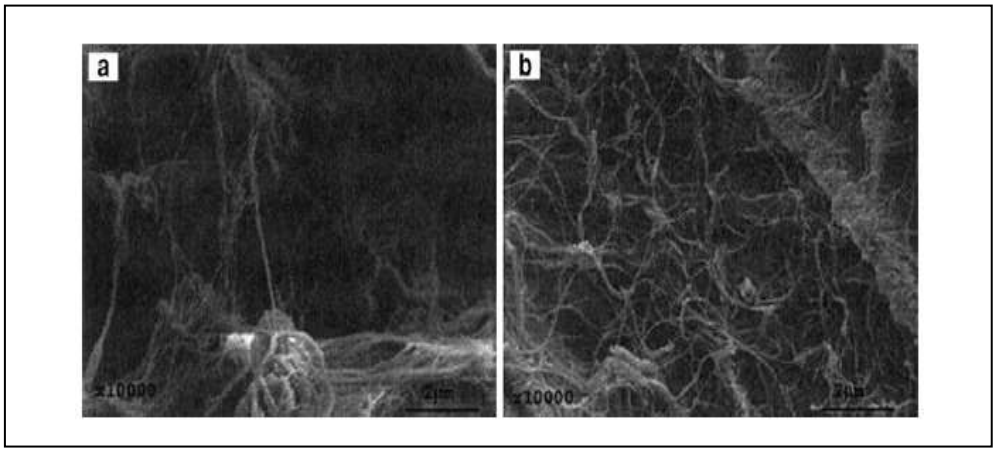

Figure 1.2. SEM Images of cord collagen and bacterial cellulose showing similarity. [8]

1.2.2. Biocompatibility Of Bacterial Cellulose

Another feature of BC which is biocompatibility has been studied in recent years. Findings showed that the implantation sites of the rats even after 12 weeks have shown no tension reaction or in other words foreign body reaction. As a result of the examinations of the implantation site it was noticed that there was no rash, tumescence or any inflammation indications [9].

1.2.3. Biodegradability of Bacterial Cellulose

Cellulose degradation eventuate through the hydrolisation of ß-1,4 D-glucose linkages by cellulase enzyme and besides it is resistivity to degradation due to its dense structure. Humans Gastrointestinal tract's do not secrete enzymes that can degrade cellulose, however fungi and bacteria are able to do this degradation, besides termites and ruminants have cellulose degrading enzyme-secreting bacteria in their Gastrointestinal tracts.

1.2.4. Macrostructural Control Of Bacterial Cellulose

One of the important features of bacterial cellulose is that it can control its structure in a physical way as well as macro-micro and at nano scales too and this features have been significantly helpful for the biomedical applications of Bacterial Cellulose. It is known that BC producing bacteria can form BC in any shape and form and in the meantime it protects its chemistry of its surface, contexture of fibers and also its porosity.

1.2.5. Porosity

Due to its compact structure or in other words density Bacterial Cellulose based materials prevent the migration of the cells which have been implanted to the material so that's why there is a great potential of Bacterial Cellulose for biomedical applications such as ; bone and skin grafting and also for artificial cartilage implementations. Cellular permeability is an important factor for cell integration with a Bacterial Cellulose based matrix, due to its

dense structural form BC acts as an impervious material and able to prevent the invasion of any other cell. Cellular permeability is an important factor for cell integration with Bacterial Cellulose matrix and due to its dense structural form BC acts as an imperious material to be able to prevent the invasion of any other cell to the located area. Pores have specific size and shape and have been used to create pores in molded structures and they get dissolved after the tissue structure is formed. BC is one of the most significant material for artificially porous material production methods, besides gelatin, salt, ice and sugar and paraffin are also used as porogenic. However by using Bacterial Cellulose; it have been investigated that; size of pores may change and interconnectivity may also be changed by using heat treatments.[10]

1.2.6. Orientation

In the case of Bacterial Cellulose culturing is done by using plates; due to its anisotropic contexture; cellulose nano fibers will be superficial and as a result of this network will be formed like gossamer and the substance will still remain quite strong.[11]

1.2.7. Modification

BC modification has great impact in biomedical field. For example for bone scaffolding; to be able to stimulate osteoblast seeding phosphorylation and body fluid treatments have been used for the growth of crystals on BC. Besides this phosphorylated BC may also be used as sorbent matter for materials.[12] Instead of phosphorylation, acetylation causes cellulose acetate formation and due to this formation crystallinity and thermal pertinacity also increases.[13] On the other hand in the case of alkaline treatment BC becomes more suitable for the degradation of cellulose.[14]

1.3. BC PRODUCTION

In the production of BC the goal is to acquire the maximum production yield by using optimum conditions and convenient peculiarities for the method which is going to be applied.

When looking to production methods of BC; static and agitated culture and airlift bioreactors are the most commonly used processes; but for industrial production, semi continuous and continuous methods are being used. [15]

However submerge and aerated production methods also have been used but it was investigated that these two types of production methods cause wild BC strains not to produce cellulose to the accumulation of $Cell^-$ mutants. On the other hand Cel^+ cells are able to form polysaccharides which can dissolve in water but thanks to genetic modification methods and with the selection of appropriate strains; agitated and aerated bioreactors can also be used. However unlike other production methods the produced cellulose will have a retiform structure and limited using areas.[16]

1.3.1. Static Culture

Static culture production method is one of the most commonly used one for BC production. BC is mixed by medium and cultivation was done for 5 to 20 days till the cellulose pellicle is formed. The formed BC layer has a more dense surface at the side which is exposed to air. The effectiveness of the production is straight in the case of the depth less than 4,5 cm. The wall effect is an important limitation in the case of this production method due to the formation of CO_2 bubbles produced by bacteria metabolism.[17] Because of the length of the cultivation time and low production yield, static culture methods are not the most appropriate ones for BC production.[18] But there is a new static culture method used in which the beer fermentation broth's waste and fed-batch production methods for increasing the production yield. It has been noted that instead of chemically prepared mediums, beer fermentation broth's waste is much more suitable for fed-batch culture method and the cellulose nano-fibers are much more dense and thinner when comparing to chemical medium.[19] On the other hand Horizontal Lift

Reactor which combines the virtues of static and continuous methods can also be used for production of nano scale bacterial cellulose and add to this the cost of the production process is relatively low when comparing to other methods which the Erlenmeyer flasks have been used.[20]

1.3.2. Submerged Fermentation

In order to scale up the production submerged fermentation is one of the choices but there are some concerns due to the oxygen insufficiency and disorganized shape of the pellicle, mutant formation and also unproper nutrition transfer. As a solution air-lift bioreactors may be used for the reduction of shear stress and prevent the yield decrease of Bacterial Cellulose.[21]

1.4. BIOREACTOR DESIGN

There are several modifications can be done for handling the most suitable bioreactor. For example like placing a membrane with a surface which can transfer oxygen at the bottom for the reactor. As a result of this change cellulose production can be doubled due to the formation of pellicles at the two sides since the oxygen can reach at the both sides which is facing and not facing the air and the rate of the production will be higher too. Also aerosol bioreactor is a good choice for increasing the BC formation.[22]

Table 1.1. Comparison of different types of bioreactor for BC Production

Reactor Type	Advantage	Disadvantage	Productivity	Production
Airlift Reactor	High cell concentration and productivity; relatively low shear stress	High energy requirement; long biofilm establishment time	0.056-0.016 g/l/h	3.8-8.7 g/l
Rotating Disk Reactor	High cell concentration; Good for aerobic strains	Semi-continuous production; higher risk of contaminations	0.015 g/l/h	5.35 g/l

Biofilm Reactor	High cell concentration and productivity; easy for product separation	Cell fouling constraints in scaled up application	0.82 g (cellulose dry weight/m2/h)	
Fluidized bed Reactor	Uniform particle mixing and temperature gradient; long term production	High energy requirement; long biofilm establishment time; shear stress.	0.08 g/l/h	5.8 g/l
Stirred-tank reactor	High cell concentration and productivity; long-term production	Shear stress of cells; more mixing power needed	0.058-0.23 g/l/h	4.57-13g/l

1.4.1. Airlift Bioreactor

When comparing airlift bioreactor with agitated culture it can be sad that it requires relatively low power supply as a result low cost. With this type of bioreactor, the air which is enriched by oxygen is given from the bottom part of the reactor and circulated in the medium.[23]

1.4.2. Rotating Disk

Rotating disks bioreactors have been created in a way that the half of the disks are exposed to air while the remaining half in the medium. It is quite an advantage so that the BC will get attached on to the disks and this will not cause a reduction of mechanical strength and add to the aeration process will also be done properly.[24] On the other hand when comparing Rotating Disk Bioreactors with Airlift Bioreactors; Airlift bioreactors create homogeneity problems and because of that medium and product are in a mixed state so that separation problems may occur ; besides due to the loss of mechanical strength the applications will also be limited.[25]

1.4.3. Long Term Fermentation

In batch fermentation the main restriction is the time which is required for the new batch production, because that microorganisms acquire time for the adaption to the new environment. Due to this reasons; researches have focused on to create a continuous fermentation process for the minimization of the time needed for sterilization, cleaning and also for the lag phase needed for the cell activation and debris to be able to handle sufficient biomass.[26]

1.5. APPLICATIONS OF BACTERIAL CELLULOSE

Due to inimitable features of cellulose such a water content, high tensile strength, crystallinity, no requirement for delignification and also capacity of production independently for needed size and shape and manageable synthesis properties researchers have focused on its applications.

1.5.1. Filtration

In order to use BC as a filtration material; BC is mixed with different kinds of polymers such as PEG, CMC and cellulose based polymers to the medium at the beginning. On the other hand BC is accepted as a dialysis material when comparing to natural cellulose based dialysis membrane; BC dialysis membranes have higher permeability and although the membrane is thinner the mechanical strength gets higher. [27]

1.5.2. Biomedical Applications

Wound healing mechanism is a agile process and can be accelerated as long as the area kept moisture. BC is an influential wound healing material due to its unrivaled specifications. Investigations have been showed that BC can be used as an accelerator of the healing process of burns, skin injuries for example like for post-surgery disturbance, pain reduction and also for decreasing the role of infection and as a result to decrease the

expenditure of treatments and time.[28] Reasons that BC is a proper wound dressing material is that it has a water absorbance capacity which is 30 percent higher than natural cellulose. Also carboxymethyl cellulose foam reduces the pain and bleeding amount when comparing to regular nasal packaging which is done for sinus surgeries.[29] In cosmetic industry BC is also a good material due to its water absorbance capacity, non-toxicity and reduced allergenic effects.

In lots of biomedical applications, bio adsorption is an important feature because it is wanted for the material to get degraded in time so that substance can be metabolized in human body. In the case of this degradation Bacterial Cellulose's 97 percent turned into glucose in seven days when it is loaded with cellulase enzymes , but also neutral buffers were added for achieving the optimal range for cellulose enzymatic reactions.[30] As a result due to its high water adsorption capacity; elasticity, strength.. etc. Bacterial Cellulose is an excellent material for many biomedical applications. [31]

1.5.3. Acoustic Transducer Diaphragm

The first non-food application acoustic transducers area has been reported to have a potential for the applications of BC. Because that BC have the competency for preserving its shape which can be measured by Young's modulus, it is a convenient material for speaker diaphragms. [32]

1.5.4. Paper Manufacturing

Bacterial cellulose pellicles are suitable for high quality paper production with higher tensile strength due to its retiform structure. BC was also found as an effective material for the pulp paper production. In the case of the addition of Bacterial Cellulose for 15 percent; the fold of the paper gets four times higher. Add to this Young's Modulus value also increases with BC addition. CMC-BC papers also have higher tensile strength comparing to natural paper.[33]

1.5.5. Food Applications

BC used in food industry for paper producing desserts, salads and fabrication foods which contains low calories. For example chocolate drinks are produced with the addition of xanthan gum for increasing the viscosity, but when instead of xanthan gum 3% cellulose is mixed with the drink and analysis were done, it was seen that the viscosity of the cellulose mixed drink remained higher after heat treatment when comparing to xanthan gum. Additionally when cellulose is mixed into ice cream, it increases the shear stress and tends to prevent the flow of ice cream after melting. BC generally recognized as safe and approved by FDA and various formulations were created by using BC as a source in the case of stability, high pH and temperature range conditions are acquired.[34]

1.6. IMMUNOGLOBULINS

Immunoglobulins are one of the most important products of biotechnology. Globulins are secreted by liver and act as immune system agents. Blood consists of albumin and globulin. Globulins are divided into four groups; Alfa 1, Alfa 2, Beta and Gama. In these four groups Gama group of immunoglobulins act as antibody and divided in to five in it self. And have different activities in immune response.[35]

Immunoglobulin G is the one which's amount is highest in the blood an also the most enduring one against corrosion and the most basic one when looking to its structure. IgD on the other hand is the one that have the lowest amount in blood and information's about its biological activity have not been identified yet. IgE acts against allergic reactions and parasites and at the same time acts increases the amount of histamine in blood. Immunoglobulin A on the other hand helps to the secretion of mucous membrane and also can be found in physiologically different parts for example like tears and helps to the protection against foreign substances. Immunoglobulin M is structurally most big immunoglobulin which produced against infections .[36]

Immunoglobulin's infection prevention effects and also the antigen binding sites which is the open site of the Y figure (Fab) and also the tail part (Fc) metabolically triggers the

reactions. For example based on the Fc site; complement system may be activated or with the recognition of the phagocytic cells they can be activated.

Fundamental structures of all immunoglobulins consist of 4 polypeptide rings. Two of these are heavy (H) and two of them are light (L). This structure can also be named as H2L2.

This differentiation can me made because that oligosaccharide groups exist in heavy rings and do not exist in weak ones. This heavy and light rings are bonded to each other with disulphide bonds. Add to this heavy rings bonded each other by dislulphide bridges. These four polypeptide ring's carboxyl site (C) is fixed and amine site (V) forms variable region.

Heavy and light chains include single variable region on the other hand heavy chains consist of 3 and light chains consist of single fixed region. Variable regions constitute specific binding sites for antigens. IgG, IgD and IgE has monomeric form in serum and şn tears and in mucous it tends to have a dimeric structure and as a result this creates four antigen binding sites. IgM consist of 5 H2L2 monomers and this creates 10 antigen binding sites.

Immunoglobulins can be degraded in to different pieces with the help of enzymes and biologically active sites may be formed. Thanks to recombinant technology this biologically active sites can also be formed. Due to the low molecular weight of this biologically active sites they can act much more fastly in cardiovascular and digestive systems and find the unhealthy tissue easily and rapidly. Especially when treating parenchymal tissues to get a rapid respond immunoglobulin sites are used. For this purpose the enzymes which are being used are papain and pepsin. Papain forms 2 Fab and a single Fc site whereas pepsin degrades IgG to 3 different Fab and more than 1 Fc site.

Immunoglobulins which are secreted in blood are active to more than 1 antigen. But for showing high activity for diagnosis and treatment; immunoglobulins should be produced and designed for only one type of antigen. These type of immunoglobulins are named as monoclonal antibody. In health industry due to its stability, activity and production convenience IgG is the most used antibody. Based on the usage area and the feature on demand; normal, dimeric and humanoid and human IgG are also used for different purposes. [37]

Antigens which are produced mostly used in cancer treatment, immune system regulator and also for cardiovascular diseases. Monoclonal antibodies commercial purification composes 40% of production cost and, production composes 25-30% and the rest is consumable and operation expenses. Production is done by mammalian cell lines (CHO: Chinese Hamster Ovary and BHK: Baby Hamster Kidney and etc.), besides recombinant production of IgG by using Saccharomyces cerevisiae and Pichia pastoris are also done commercially.[38] With the improvement of production Technologies production costs have been lowered, besides "Tag" system is also lowers the purification costs. However Histidine amino acid bonded recombinant IgG activity is lower than regular IgG it is mostly used in diagnosis rather than treatment. Most fundamental purification method is chromatography and Protein A, Protein G or Protein A/G affinity columns have been used and after that ion exchangers and ultrafiltration systems IgG is purified. Thanks to the special binders of affinity columns they can make highly selective purification bur unfortunately cost increases and expected life time is much lower comparing to molecular chromatography and ion exchangers.[39] Although Sartorius and Pall produces membrane filters in industry chromatographic methods have been preferred in industry. This is due to the long term uses of these firms products and also the market availability of these two firms columns. However latest research have showed that Millipore and Pall's membranes have the quality to perform as like affinity column chromatography and can be a competitor.[40]

1.7. FILTRATION METHODS AND VARIOUS MEMBRANE TYPES

Membranes are divided into 4 groups based on the physical characteristics of their pores. These 4 groups are:

- Isotropic
- Anisotropic
- Composite
- Multi-layer

membranes. Isotropic membranes pore radius and distribution are homogeneous. Anisotropic membranes pore distribution and radius are heterogeneous and this specificity

increases the permeability and physical durability. Composite membranes on the other hand prepared by using two physically and chemically different membranes.

When filtration processes are examine by their performance there are two different sides. These are flux and capacity based systems. Flux systems are based on the obtaining of pure water and based on the handling of the substances which are needed to prepare buffer solutions and medium. Based on the medium which are needed are protein rich mediums based on the processes aims it can also be examined from flux side. Capacity based systems; generally the substance which is needed to be separated is the substance which is produced; but the important point is this target substance binding and suspension redundancy. To be able to detect the maximum capacity optimum flux should be found by using constant pressure and flux experiments should be performed. [41]

Filtration systems designed on the flat and reverse flow. Reverse flow systems are generally more efficient when comparing to flat flow. Due to the extensiveness of the surface area accumulations and as a result depression can be seen with the higher filtration volume. After reverse-flow based systems the filtrate which is handled is not needed to be cleaned it can be used directly for the further purification processes.

Flat-flow systems have underperformance when comparing to reverse-flow systems but its more economic and have more easy performance methods. It is used for the separation of cell and cell particles after production whereas purification of monoclonal antibodies at the step before chromatography and also for the separation of DNA from mammalian cell cultures. During the filtration process to be able to prevent the membrane congestion and depression filtration assister substances for example like pearlite, diatom earth and active carbon can be used for flat-flow filtration systems**.** [42]

Membrane chromatography based on the modification of microfiltration membranes and formation of a retiform structure and creating multilayered membrane by putting specific number of membranes on top of each other and this form of membranes have highly selective for separation. The membranes which are used for this purpose can have the specificities like ion exchange, affinity, reverse-phase or can be hydrophobic. Although their binding capacities are low; membranes pore structure it creates less mass transfer resistance when comparing to resin based colon chromatography's. This specificity is an

advantage for the separation of high molecular weight proteins and viruses and add to this membrane chromatography is much more appropriate method due to high flow rate, low pressure and relatively short duration time when comparing to colon chromatography. [43]

1.8. MEMBRANE ADSORPTION THEOREMS

Adsorption is concentration change at the layers of the membrane while a solid substance was used for the separation liquid or gas. Adsorption can be divided into two groups based on the physical and chemical properties of the layers of the membranes.
In Physical adsorption the interaction between the substance which is wanted to be bind and the membrane is low. These interactions are commonly based on the Van der Waals forces. Because that physical adsorption is based on molecular interactions based on the specificities of the solvents the process can be carried out reversible. With the increasing heat; molecular energy also increases and the effect of Van der Waals forces decreases and as a result adsorption decreases. Chemical adsorption on the other hand; includes the formation of the chemical bonds. When comparing to physical adsorption it shows quite stronger binding and to be able to perform the reaction reversible solvents solvent specificities may not be enough. Add to this with the increasing heat adsorption velocity also increases. Theorems which explains mathematically the relationship between the constant temperature the substance amount which is hold by the membrane and its concentration are called as Adsorption Isotherms. Basically there are 6 different theorems. Langmuir and Freundlich Isotherms are the most used ones for defining the adsorption and its behavior**.** [44]

When looking to the graph which explains the Langmuir and Freundlich Isotherms; C_U and C_B explains respectively the remaining substance concentration and the concentration of the substance which the membrane have been absorbed.

Langmuir isotherm's (1.1.) equation is

$$x = \frac{qmK_LC}{1 + K_LC} \tag{1.1.}$$

Here:

C: Concentration of the protein which is in balance within the liquid (mg/ml)

q: Protein amount per membrane able to bind (mg);mg/g

K_L:Langmuir adsorption constant

q_m:Langmuir maximum adsorption capacity

Langmuir equality (1.2.); can be prepared based on linearization like below; in response to C C/q graph is drawn and from the slope of the linear graph q_m and K_L can be calculated.

$$\frac{c}{q} = \frac{1}{qm}C + \frac{1}{qmK_L} \tag{1.2.}$$

Freundlich Isotherm's general equation (1.3.) is written below:

$$q = K_F C^{1/n} \tag{1.3.}$$

Here:

C: Concentration of the protein which is in balance within the liquid (mg/ml)

q: Protein amount per membrane able to bind (mg);mg/g

K_F:Frendlich adsorption constant

n:Constant

2. MATERIALS AND METHODS

2.1. MATERIALS

Glucose (Sigma 16301), Peptone (Merck 1072), Yeast Extract (Conda 702), Sodium Diphosphate (Acros Organics 204855000), Citric Acid (Riedel de Haen 27102), NaOH (Sigma 6203), Epichlorohydrine (Fluka 45340), Ammonium Hydroxide (Sigma 221228), DMSO (Sigma 472301), Propane Sultone (abcr AB120923), Chitosan (Sigma 48165), Acetic Acid (Fluka 45731), Sodium nitrite (Sigma 13447), EDAC (Sigma 39391), Cafeic Acid (Sigma 60020)

2.2. METHODS

2.2.1. Preparation of Membranes

In this thesis membranes which are used are BC and its modifications for the modifications of BC membranes Radley Carousel 6 device is used.

Figure 2.1. Radley Carousel 6 Device which is used for modifications of membranes

2.2.2. Preperation of Bacterial Cellulose Membrane

Gluconoacetobacter xylinus stocks were used for Bacterial Cellulose production; as a result of incubation which is explained below in details Bacterial Cellulose was handled as membranes. [44]

Table 2.1. Preparation of Bacterial Cellulose Membrane

Work Flow	Procedure
Preparation Of HS Medium	Hestrin-Schramm medium ((HS; %, v/v), glucose, 2,0; peptone, 0,5; yeast extract, 0,5; sodium diphosphate, 0,27; citric acid, 0,115) prepared and 1M of acetic acid was used for adjusting pH to 5,0. Afterwards medium was sterilized at 121°C by an autoclave.
Static Culture Production	From previously prepared and sterilized HS medium 95 ml were taken and poured into 250 ml previously sterilized Erlenmeyer flasks and then 5 ml of G.*xylinus* was mixed with medium. Then at 30°C for 7 days statically production was done.
Bacterial Cellulose Purification	BC membrane was purified by using 0,3 M of NaOH at 80°C for 1 h.
Bacterial Cellulose Storage	BC membranes which are purified were kept in dH_2O at 4°C for further processes.

2.2.3. Bacterial Cellulose- Epichlorohydrine- Ammonium Hydroxide Modification (BC-EPI-NH3)

Ammonium hydroxide and epichlorohydrine were used for turning the charge of Bacterial Cellulose membranes to cationic.

Table 2.2. Bacterial Cellulose- Epichlorohydrine- Ammonium Hydroxide Modification (BC-EPI-NH_3)

Work Flow	Procedure
BC Membrane Weighing	Bacterial Cellulose Membranes were weighed as 2,5 g.

Epichlorohydrine Activation	12 ml of Epichlorohydrine was dissolved in 1 M NaOH and 200 ml of this solution was poured on to the BC membrane and reaction was performed at 60°C for 2 h.
Washing and pH Adjustment	By washing step it all of epichlorohydrine and relative amount of NaOH were suspended. Afterwards BC was taken in to dH_2O and NaOH was poured by pipette until pH was set as 12.
Binding of Amine Groups	From 29% (a/h) NH_4OH 20 ml were taken and added to the membrane solution and reaction was performed at 60°C for 2 h.
Washing and Storage	Bacterial Cellulose was washed repetitively by dH_2O and its pH is 7,0 and stored in dH_2O at 4°C.

2.2.4. Bacterial Cellulose Hydroxyethyl Acrylamide Modification (BC-HEA)

Hydroxyethyl acrylamide was used for making the Bacterial Cellulose Mebranes cationic and also to form spacer arm.

Table 2.3. Bacterial Cellulose Hydroxyethyl Acrylamide Modification (BC-HEA)

Work Flow	**Procedure**
BC Membrane Preparation	BC membranes were weighed as 2,5 g and put into the dH_2O .
Solution Preparation	Nearly after 10 min 0,228g of ammonium persulphate was added and solution was mixed for another 15 min.
Reaction	With the addition of 3,535 g of Hydroxyethyl acrylamide to the mixture polymerization and BC binding started and reaction was carried out for 1 h at 60°C.
Washing and Storage	After several washing steps with ethanol hydroxyethyl acrylamide which is not bonded as polymer and rested as monomer were was washed away and membrane was stored in dH_2O at 4°C .

2.2.5. Bacterial Cellulose- Hydroxyethyl Acrylamide- Ammonium Hydroxide Modification (BC-HEA-NH3)

To be able to prepare BC-HEA-NH_3 membrane ; preperation was started as like Bacterial Cellulose Hydroxyethyl Acrylamide method and contiued with the addition of ammonium hydroxide.

Table 2.4. Bacterial Cellulose- Hydroxyethyl Acrylamide- Ammonium Hydroxide Modification (BC-HEA-NH3)

Work Flow	Procedure
BC Membrane Preparation	BC membranes dry weight was weighed as 1 g and poured in to dH_2O.
Solution Preparation	After 10 min 0,228 g of ammonium persulphate was added and mixed for 15 min.
Reaction- HEA Binding	3,535 g of hydroxyethyl acrylamide was added and polymerization and BC binding was initiated and reaction was carried out for 1 h at 60°C.
Reaction-NH_3 Binding	Membrane was taken out from the solution and washed by dH_2O for the removal of HEA. Afterwards poured into %29 (a/h) NH_4OH (50 ml) solution. The heat of the solution was adjusted to 60°C and reaction was carried out for 2 h.
Washing and storage	By washing with dH_2O NH_4OH ; which did not bind to membrane was washed away and membrane was stored in dH_2O at 4°C .

2.2.6. Bacterial Cellulose- Propane Sultone Modification (BC-SO3)

To be able to handle anionic BC membranes; prepared BC membranes were kept in DMSO for alcali activation which caused propane sultone to bind –OH groups.

Table 2.5. Bacterial Cellulose- Propane Sultone Modification (BC-SO3)

Work Flow	Procedure
Weighing of BC Membrane	BC membrane was weighed as 2,5 g

Solution Preparation	250 ml DMSO was poured into BC membrane and then 5 g of propane sultone was added and the solution was mixed till propane sultone completely dissolved .
Reaction	0,7 g NaOH was dissolved 2,6 g of dH_2O and poured into the solution and reaction was carried out for 7 h at RT.
Washing and Storage	By several times of washing with dH_2O DMSO and NaOH and unbounded propane sultone were disposed and membrane was stored in dH_2O at 4°C .

2.2.7. Bacterial Cellulose-Hydroxyethyl acrylamide- Propane Sultone Modification (BC-HEA-SO3)

To be able to prepare BC-HEA-SO_3 membrane ; preparation was started as like Bacterial Cellulose Hydroxyethyl Acrylamide method and continued with the addition of propane sultone.

Table 2.6. Bacterial Cellulose-Hydroxyethyl acrylamide- Propane Sultone Modification (BC-HEA-SO3)

Work Flow	**Procedure**
BC Membrane Preparation	BC membranes dry weight was weighed as 1 g and poured in to dH_2O.
Solution Preparation	After 10 min 0,228 g of ammonium persulphate was added and mixed for 15 min.
Reaction- HEA Binding	3,535 g of hydroxyethyl acrylamide was added and polymerization and BC binding was initiated and reaction was carried out for 1 h at 60°C.
Drying	At the end of the reaction BC-HEA membrane was dryed at 50°C for 2 h.
Solution Preparation	Dried BC-HEA was mixed with 30 ml of DMSO
Reaction -SO_3 Binding	After the addition fo 3 g of propane sultone to be able to open a propane sultone ring the solution which is prepared by mixing 2,6 g of dH_2O with 0,7 g NaOH was added and reaction was carried out for 7 h at RT.
Washing and Storage	By several times of washing with dH_2O unbounded NH_4OH was disposed and membrane was stored in dH_2O at 4°C .

3. ANALYSIS

3.1. DETERMINATION OF THE DENSITY OF THE BACTERIAL CELLULOSE MEMBRANES

To be able to determinate the density of the Bacterial Cellulose Membranes Shimadzu density measuring kit (SMK401/SMK301) was used.

3.2. DETERMINATION OF THE MEMBRANE POROSITY

To be able to determinate the membrane porosity (Ɛ) gravimetrical method was applied. This method is based on the water amount which is remained inside pores of the membranes which also causes weight increase. Membranes were placed in to glass petri dishes which were dried before and kept at 70°C till it's weighing was fixed. For bacterial cellulose duration for fixed weighing is 2 days. After the membranes were dried they were placed inside dH_2O for 12 h and then filter paper was used for getting rid of the wet which is located on the surface of the membranes. This process was done very fast. Then weighing was done once more and the equation which located below (3.1.) was used for the calculation of the porosity of the membranes.

$$\varepsilon = (w1 - w2\,Dw)/((w1 - w2)/Dw + (w2/Dp)) \qquad (3.1.)$$

At the equation (3.1) W_1 refers to the weight of the membrane which have absorbed water; W_2 refers to weight of the dry membrane, D_w is the density of water and D_p refers to the density of the bacterial cellulose. The density of the water was accepted as 1g/ml and the density of the bacterial cellulose was determined by the Shimadzu density measuring kit .(SMK401/SMK301)

3.3. DETERMINATION OF THE AVARAGE RADIUS OF THE PORES OF THE MEMBRANES

The average radius of the pores of the membranes were determined based on the filtration velocity. For each membrane 300 ml of water was poured under 1 bar of constant pressure and the duration of the transition and for calculation Guerout-Elford-Ferry equation which is written below was used.

$$Rf = \sqrt{\left(\frac{(2{,}9-1{,}75\varepsilon)8\mu hQ}{\varepsilon PA}\right)} \qquad (3.2.)$$

At the equation μ refers to the viscosity of water; h refers to thickness of the membrane; Q is the volumetric flow rate; P refers to the system pressure and A refers to the membrane surface area. The viscosity of the water (μ; Pas) was accepted as 0,001. Thickness of the membrane was measured by caliper gage. Volumetric flow rate (Q; ml/sec) was determined by measuring the exact duration of the total water to pass. Surface area of the membrane (A; cm^2) was determined by cutting the membranes as circles and the diameters were measured by using sensitive caliper and $A = \frac{\pi D^2}{4}$. (Zeng, Ruckenstein; 1998)

3.4. DETERMINATION OF THE MEMBRANES SURFACE SPECIFICATIONS BY SCANNING ELECTRON MICROSCOPY (SEM)

To be able to have clear image the membranes were kept at 80°C overnight for removal of water . Afterwards membranes were cut as 3x3 cm dimensions and coated with gold for imaging. For SEM imaging Carl Zeiss EVO-40 device was operated under high vacuum and 10 Kv high power. Magnification was done between 500-2500 and it has been tried to have clear image under these circumstances.

3.5. FT-IR IMAGING OF THE CHARACTERIZATION OF THE MEMBRANES AFTER MODIFICATIONS

Membranes were firstly dried and afterwards placed in to Perkin-Elmer Spectrum 100. For each sample absorbance measurements were taken in between 500-4000 cm^{-1} and then Perkin-Elmer OMNIC software program was used for normalization and comparison of the absorbance values.

3.6. DETERMINATION OF THE HYDROLIC PERMEABILITY OF THE MEMBRANES

Steriltech HP4750 was used for determination of the hydraulic permeability (*see Figure 4.0)*. At the beginning firstly 5 ml of water was poured through the membrane at the different pressure levels ranging from 0,1-1 bar and the transition periods were recorded; based on the recorded measurements volumetric flow rates were calculated (J_V; $cm^3/cm^2/min$). Volumetric flowrate unit was taken as cm^3, surface area of membrane was taken as cm^2 and lastly duration was taken as min. . Applied pressure versus volumetric flowrate graph slope gives hydraulic permeability value (L_p; $cm^3/cm^2/min/psi$) . (Zeng, Ruckenstein; 1998).

Membranes were treated with BSA which concentrations changed in between 0,5-2,5 g/L and the experiment followed as it was explained above. Afterwards L_P values were calculated as like the dH_2O experiments.

Figure 3.1. Sterlitech HP4750 which is used for membrane flow experiments

3.7. DETERMINATION OF THE ION EXCHANGE CAPACITY OF THE CATIONIC MEMBRANES

Membranes were treated with 2M , 50 ml of NaCl solution at RT overnight and then they were titrated with 0,025 M NaOH till their pH's reach to 7,0 and the value of NaOH which is needed to achieve 7,0 were recorded as V_2. Membranes which are not treated with 2M , 50 ml of NaCl solution were also titrated with 0,025 M NaOH till their pH's reach to 7,0 and the value of NaOH which is needed to achieve 7,0 were recorded as V_1. Membranes ion Exchange capacity levels were then calculated with the Formula below (3.3.):

$$IEC = \frac{(V_2 - V_1)}{W_m} \times 0{,}025 \qquad (3.3.)$$

3.8. DETERMINATION OF THE ION EXCHANGE CAPACITY OF THE ANIONIC MEMBRANES

Membranes were treated with 2M , 50 ml of NaCl solution at RT overnight and then they were titrated with 0,025 M NaOH till their pH's reach to 7,0 and the value of NaOH which is needed to achieve 7,0 were recorded as V_2. Membranes which are not treated with 2M, 50 ml of NaCL solution were also titrated with 0,025 M NaOH till their pH's reach to 7,0 and the value of NaOH which is needed to achieve 7,0 were recorded as V_1. Membranes ion Exchange capacity levels were then calculated with the Formula below (3.4.):

$$IEC = \frac{(V_2 - V_1)}{W_m} \times 0{,}025 \qquad (3.4.)$$

3.9. DETERMINATION OF THE PROTEIN AMOUNT

Protein amount determination method was done by using quartz cuvettes and measuring the absorbance's at 280 nm. The proteins which are for this experiment IgG and BSA was prepared at different concentrations and measured at 280 nm and the absorbance values were used for creating the Standard graphic; at the end of the experiment with samples absorbance value which is taken at 280 nm were used for protein concentration determination as mg/ml.

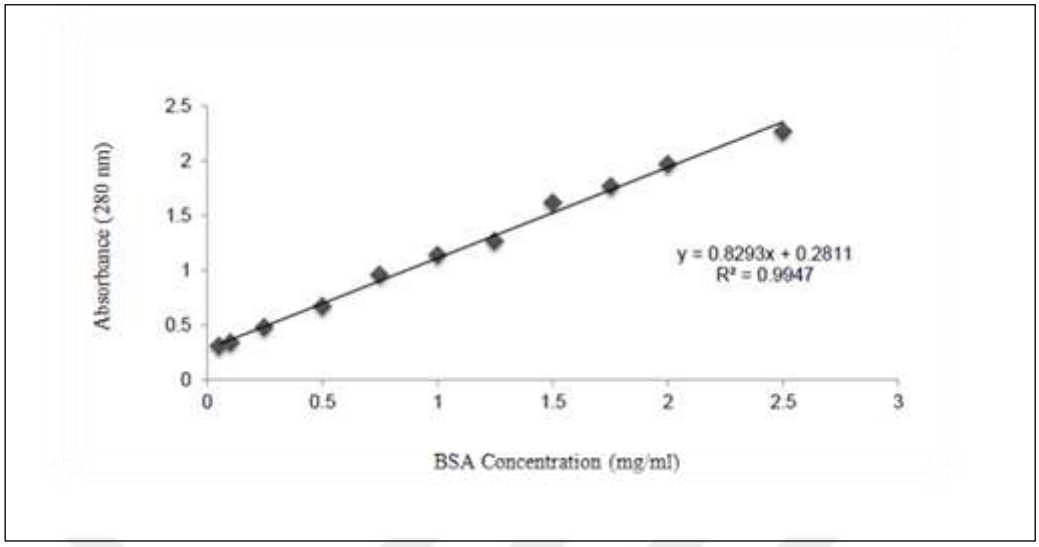

Figure 3.2. Graph which is used for protein amount determination

3.10. STATIC DETERMINATION OF THE MEMBRANES PROTEIN BINDING CAPACITY

Membranes were cut and weighed as 0,1 g and poured in to the distilled water to be able to achieve ionic balance. After 3 hours of incubation membranes were taken out from the water and dried with the help of filter paper. Afterwards the dried membranes were placed into 2 ml's of BSA solution which's concentration is 2,g/l, then for the binding membranes were placed at 4°C for 2 hours. After 2 hours samples were taken from the liquid part and their absorbance's were measured at 260 nm and with the help of the Standard graph the protein amount of the samples were determined. By extraction of starting concentration value from the sample concentration ; the bonded protein amounts of the membranes were calculated. From the graph which shows the membrane concentration versus bonded protein amount; the protein interest of the membranes were determined.

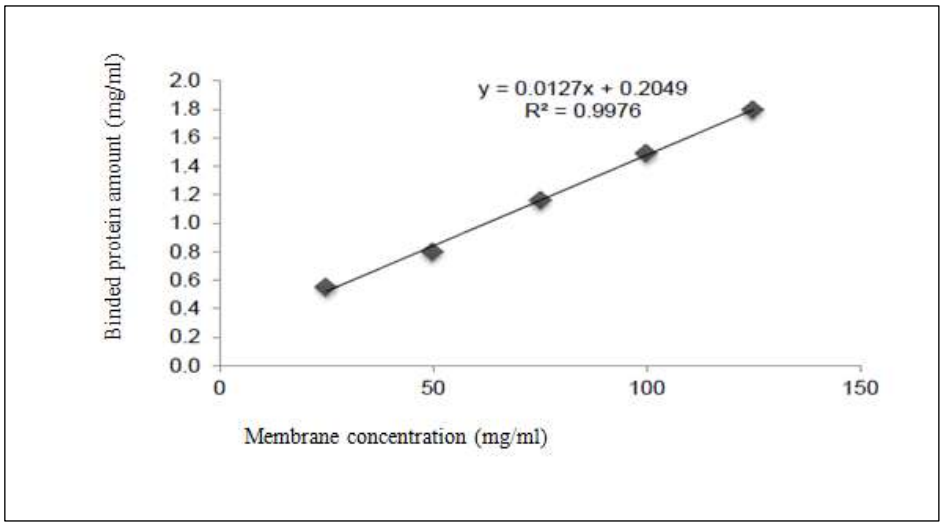

Figure 3.3. Protein interest of the membranes

3.11. DYNAMIC DETERMINATION OF THE MEMBRANES PROTEIN BINDING CAPACITY

Prepared membranes were placed in to Sterlitech HP4750 membrane cell and 300 ml of 1 mg/ml BSA solution was poured on to the membranes. Then under constant pressure of 0,1 bar BSA solution was poured through the membranes and at every 10 ml sample for collected (totally 30 samples). The collected samples absorbance's were measured under 280 nm and their protein concentrations were determined.When the ratio of BSA samples concentration value to the starting concentration 90%; the volume of the BSA solution at that point gives the membrane dynamic capacity .

3.12. TENSION TEST OF THE MEMBRANES: YOUNG' MODULUS

Tension test were done twice for every membrane as wet and dry. For the wet membranes the membranes were taken out of water and cut as 20 x 5 mm dimensions and for the dry membranes they were weighed as 0,2 g and flattened with press machine and these flattened pieces were cut in dimensions of 20 x 5 mm. Afterwards with Instron 5900 Device the tension tests of the membranes were performed. The pieces were placed in between the holder of the device and in a speed of 0,1 N/min the tension and fraction

strengths were measured. With the Formula below (3.5.) their Young's Modulus values were calculated:

$$Young's\ Modulus\ (MPa) = \frac{F/A}{\Delta L/L_0} \quad (3.5.)$$

At the equation F refers to the applied force; A to the surface area of the membranes; L_0 to the starting length of the membranes and ΔL to length difference at the tension moment.

3.13. DETERMINATION OF THE WATER HOLDING CAPACITY OF THE MEMBRANES

Membranes were prepared for the experiment and kept in glass petri dishes which include distilled water for 1 day. Then their weights were measured. To get rid of water membranes were placed on to filter paper for drying fas and then their weights were measured for a second time. For each membrane this process was repeated for 3 times. Afterwards the membranes were incubated at 70°C for nearly 2 days and the dry weight of the membranes were measured.

To be able to calculate the water holding capacity of the membranes the Formula below (3.6.) was used:

$$Water\ Holding\ Capacity\ (\%) = \frac{W_f - W_d}{W_d} \times 100 \quad (3.6.)$$

W_f refers to the wet weight of the membrane (mg) and W_d refers to the dry weight of the membrane.

4. RESULTS

4.1. MEMBRANE PRODUCTION AND MODIFICATION

When looking to the membrane production and its modifications the most important point is the membrane structure formation and its decomposition. Due to this reason at the performed modifications the most significant point was the form and the Young's modulus value.

4.2. BACTERIAL CELLULOSE

Hestrin-Schramm (HS) medium was chosen because that it's the most common and popular medium type for Bacterial Cellulose (BC) production. Although the NRRL B-759 was also existed Gluconoacetobacter *xylinus* was chosen as a bacterial source from the culture stock due to the comparative culture it G.*xylinus* produced high amounts of BC when comparing to NRRL B-759 and also BC was in the pellet form which in this case needed form. At static culture production BC is produced in a film layer of form and this film production makes the sample collection much more easier and the alkali methods which are used for the removal of the other organisms.

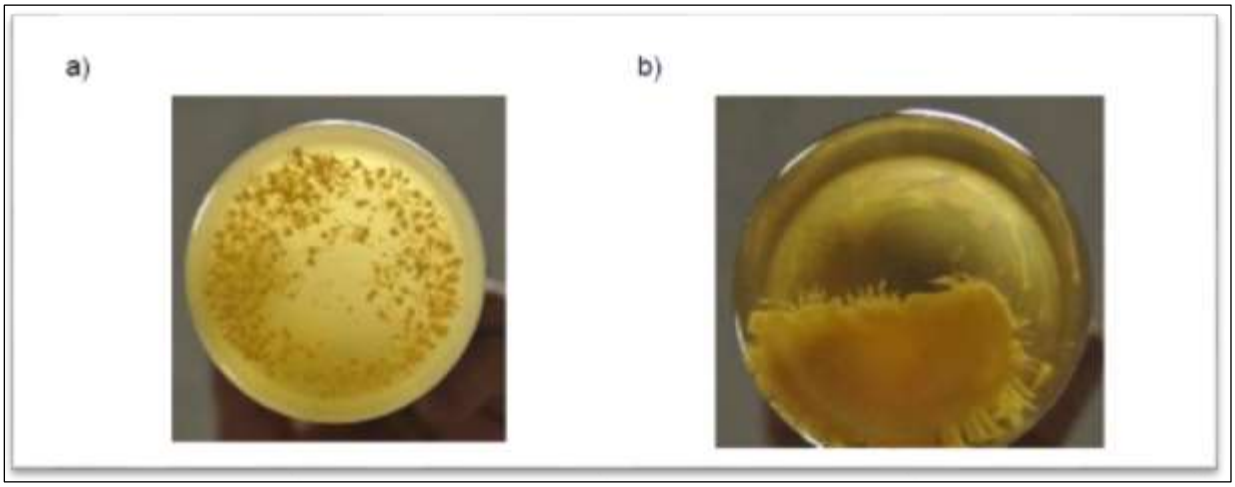

Figure 4.1. Comparison of BC production G.xylinus and NRRL B-759

Although the amount of Bacterial Cellulose is higher in the case of comparative cultures; methods (example: 1-butyl-3-methyl imidazolium) are performed for handling Bacterial Cellulose in a membrane form causes BC decrease in high molecular weight, high crystallinity and homogeneity. To be able to prevent this decrease production is done in static culture.

4.3. BACTERIAL CELLULOSE- EPICHLOROHYDRINE- AMMONIUM HYDROXIDE MODIFICATION (BC-EPI-NH3)

To be able to handle anionic and cationic membranes the produced bacterial cellulose membranes treated with different linking agents with different charges which is in this case ammonium hydroxide and propane sultone. To be able to bind ammonium hydroxide to Bacterial Cellulose as linking agents epichlorohydrine and hydroxyethylacrylamide were used.

Modified Bacterial Cellulose by epichlorohydrine and ammonium hydroxide cannot be used as a membrane. Cultures which have a 10 days of duration were also used for modification. This membrane physically protected its structure but when looking to the structure it can be easily seen that it does not have homogenous distribution. Although it have been treated with lower concentrations of NH_4OH (%15; v/v) membrane structure was protected but the ion Exchange capacity will be lower in future processes when comparing to natural Bacterial Cellulose.

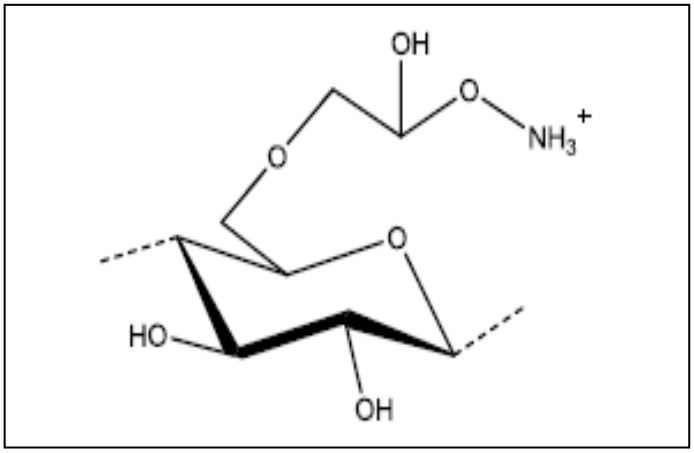

Figure 4.2. BC-EPI-NH3 Molecular Structure

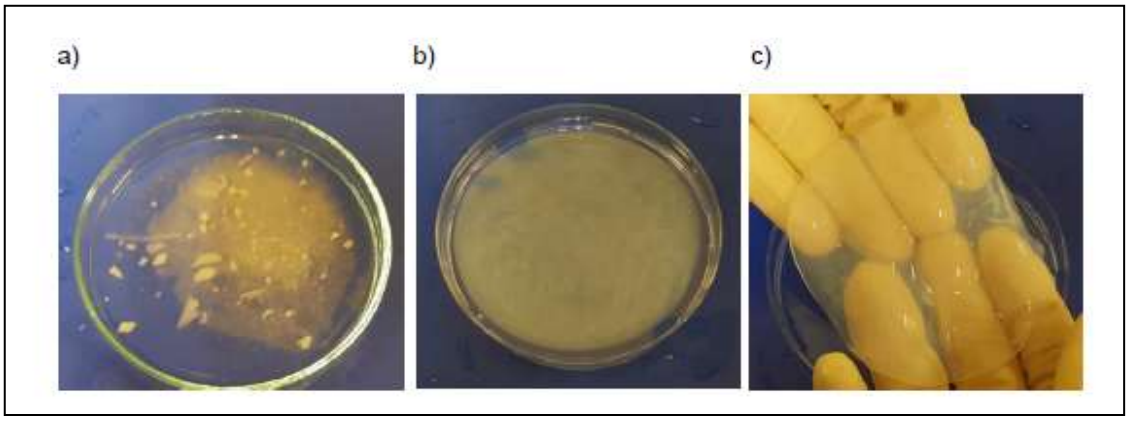

Figure 4.3. Differently produced BC-EPI-NH3 membranes 7 day BC and 29% (v/v) NH4OH; 10 day BC and 29% (v/v) NH4OH; 10 day BC and 15% (v/v) NH4OH

4.4. BACTERIAL CELLULOSE HYDROXYETHYL ACRYLAMIDE MODIFICATION (BC-HEA)

To be able to make Bacterial Cellulose cationic and also to use as a binding source for ammonium hydroxide or propane sultone BC was treated with Hydroxyethyl Acrylamide. Hydroxyethyl Acrylamide when comparing to acrylates, becomes more stable at higher pH ranges and salt concentrations due to its amide group. For performing the reaction ammonium persulphate was used because that ,t causes BC's 6th carbon's hydroxyl group charged and at the same time it polymerizes the hydroxyethyl acrylamide's vinile groups causes to bind BC as polymer.

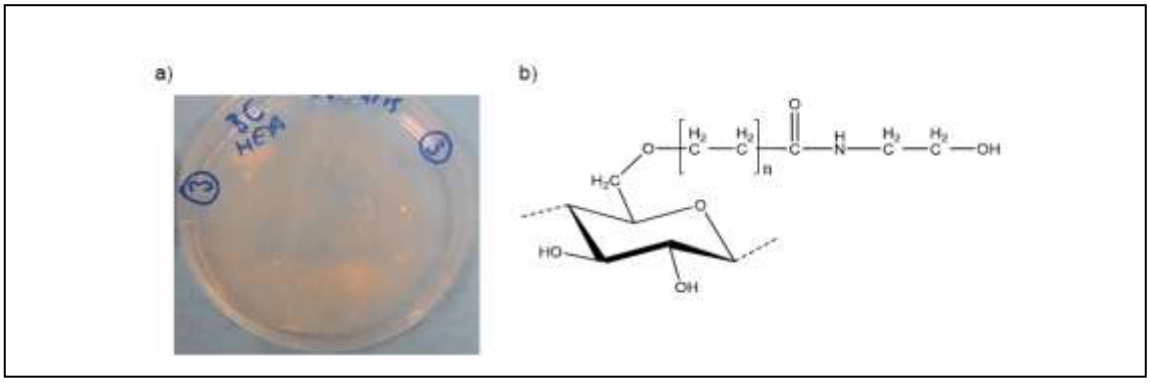

Figure 4.4. BC-HEA picture and molecular structure

4.5. AMMONIUM HYDROXIDE MODIFICATION (BC-HEA-NH3)

In order to increase the cationic charge of the BC ammonium Hydroxide was used. When comparing BC-HEA-NH_3 with BC-EPI-NH_3; BC-HEA-NH_3 has a more homogen membrane form . With the binding of HEA, BC's robustness have been increased and this ,increase prevented the decomposition of membranes with the addition of ammonium hydroxide. As a result of this because that HEA gets polymerized with the accruement of reactions NH_3 binding occurred far away from BC fibers and this causes a decrement at level of BC decomposition.

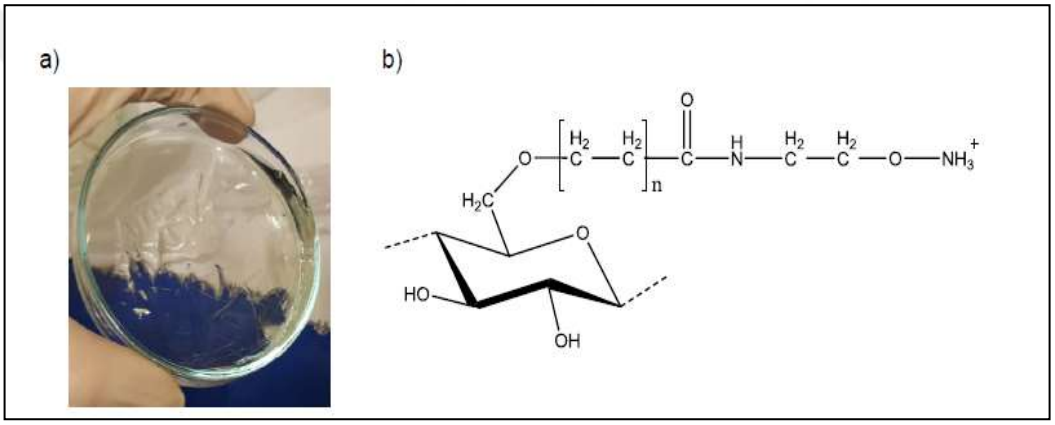

Figure 4.5. BC-HEA-NH_3 picture and molecular structure

4.6. BACTERIAL CELLULOSE- PROPANE SULTONE MODIFICATION (BC-SO3)

In order to make BC anionic charged propane sultone was used. This reaction was performed in the presence of DMSO and Argon gas was applied to be able to inert hydroxyl groups. For the removal of DMSO; membranes were kept inside water for overnight but this caused morphological change and membranes became rigid. This morphological change and rigid structure; membranes became become dysfunctional and cannot be used for the further processes. When the membranes were not kept under DMSO environment ; filter paper was used for the removal of water and membranes were incubated at 50°C for 2 hours , by this method water was removed from the membrane. But at this point it the time of the drying process is really important. When the membranes

kept under 50°C more than 2 hours ; they stick to the glass petri dish and when the membranes tried to be removed from the dish it causes damage. To be able to inert hydroxyl groups; Argon gas is directly given to the BC. Because that BC stands at the top of the solution before Argon gas dissolves it penetrates to BC and this causes heterogenic structures on BC. At the end of the reaction BC becomes in gel form. For the binding of propane sultone alkali environment was created. With the addition of sodium hydroxide propane sultone's ring structure opens and binds burdened to the first structure it faces which is mostly the 6th carbon's hydroxyl group. Although the binding of propane sultone eventuated because that membrane structure is destroyed it was decided to not be used as a purification agent.

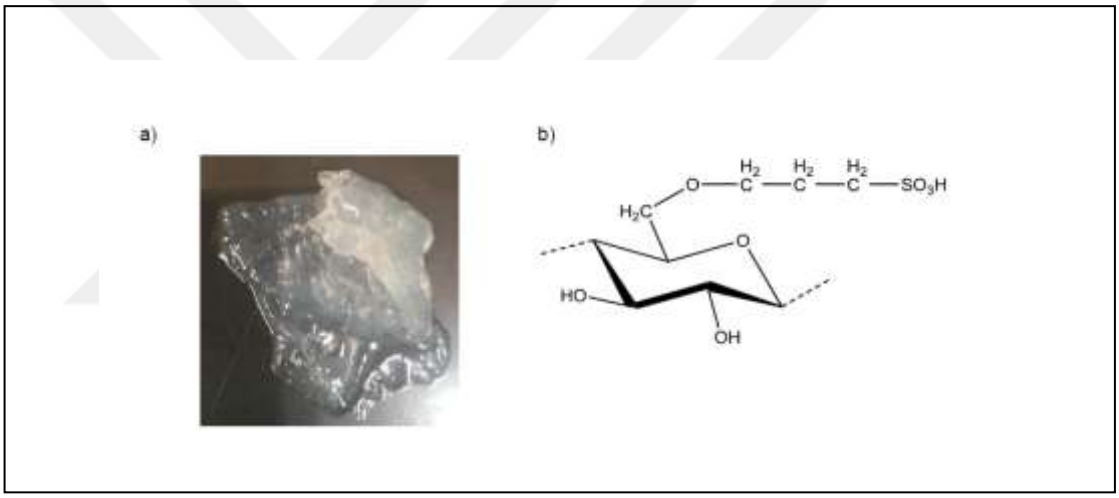

Figure 4.6. BC-SO_3 picture and molecular structure

4.7. BACTERIAL CELLULOSE-HYDROXYETHYL ACRYLAMIDE- PROPANE SULTONE MODIFICATION (BC-HEA-SO_3)

In order to make BC anionic charged and to protect membrane integrity; acrylamide bonded BC was reacted with propane sultone. After drying process BC-HEA look alike like normal BC membrane. After BC-HEA was poured in to DMSO there was no physical change at its structure. On the other hand for the binding of propane sultone to the acrylamide side an inert environment was not needed and because of this there is no need of the feeding of Argon gas to the environment and as a result of this there is no structural change on the membrane.

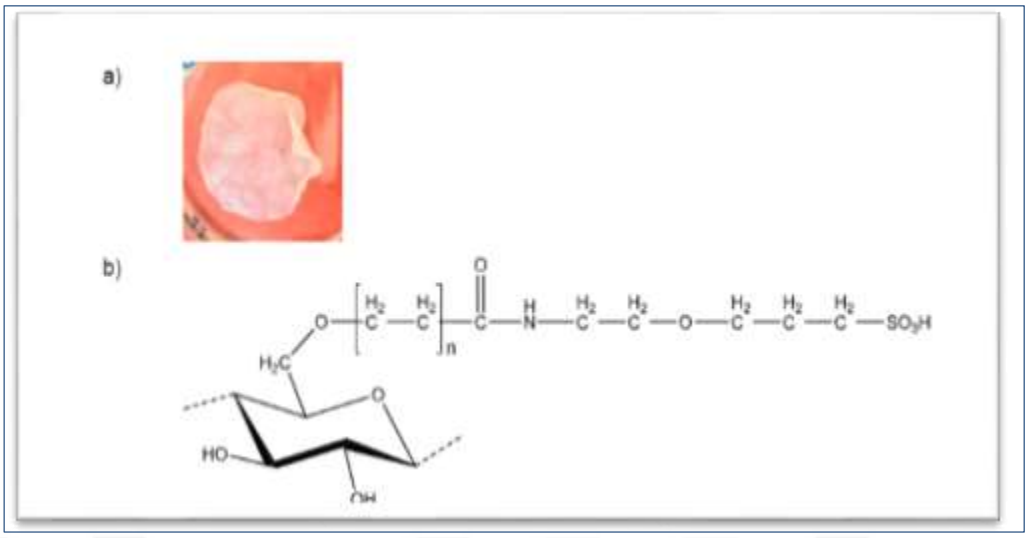

Figure 4.7. BC-HEA-SO3 membrane picture and molecular structure

Based on the experiments which are previously explained ; for identification and comparison of the membranes their durability and Young's modulus values where compared. According to Table 4.1 BC membranes are supportable and can be worked with under high pressure. Their water holding capacity is nearly 50%. When comparing Young's modulus values of wet and dry membranes within each other it can be sad that the wet ones values are higher than dry ones due to the fiber structure of the BC, because that during drying process long fiber structures fractured and this causes breakdown of the membrane during tensile stress tests. The forces which are needed for the membrane to break are also indicated at Table 4.1. Prolongation portion is nearly 10% and breakdown forces are nearly 1/10 of Young's Modulus values.

When looking to the results the membranes which are appropriate for further experiments were identified as: BC, BC-HEA, BC-HEA-NH_3 and BC-HEA-SO_3.

Table 4.1. Identification of the Modified Membranes Polymer's Sufficiency

Polymer Type	**Water Holding Capacit (%)**	**Young's Modulus Value of Wet Membranes (MPa)**	**Young's Modulus Value of Dry Membranes (MPa)**	**Breakdown Force (MPa)**
BC	50.5	382	248	40

BC-EPI-NH_3	49.8	13	-	1
BC-HEA	50.8	311	210	33
BC-HEA-NH_3	51.8	230	172	24
BC-SO_3	50.9	28	-	3
BC-HEA-SO_3	51.1	302	194	31

4.8. PHYSICAL CHARACHTERISTICS OF MEMBRANES

Based on the experiments natural and modified membranes which are suitable for use where classified based on their hydraulic integrity, pore radius and their porosity. These specifications will be furtherly distinctive for the membranes which will be used as polymers in industrial applications and performance.

4.9. CHARACTERIZATIONS OF MEMBRANES BY FT-IR AND SEM ANALYSIS

For the characterization of the membranes FT-IR Analysis and SEM Imaging systems were used. With the FT-IR Analysis the change at the membranes after the modifications can be visualized.

When looking to the BC membrane modifications instead of BC-HEA-NH_3; 2800-3000 cm^{-1} and 900-1400 cm^{-1} ranges absorbance fluctuation patterns look alike. Add to this HEA modifications absorbance fluctuations were recorded in rages between 1600-2700 cm^{-1} when comparing to natural BC. Based on this results it can be concluded that HEA modifications were done successfully.

Generally; C-O-C ether binds at 1058 cm^{-1}, hydroxyl groups at 3415 cm^{-1} and hydrogen bonds in between polymer rings in between 3230-3455 cm^{-1} can be visualized for BC membranes.

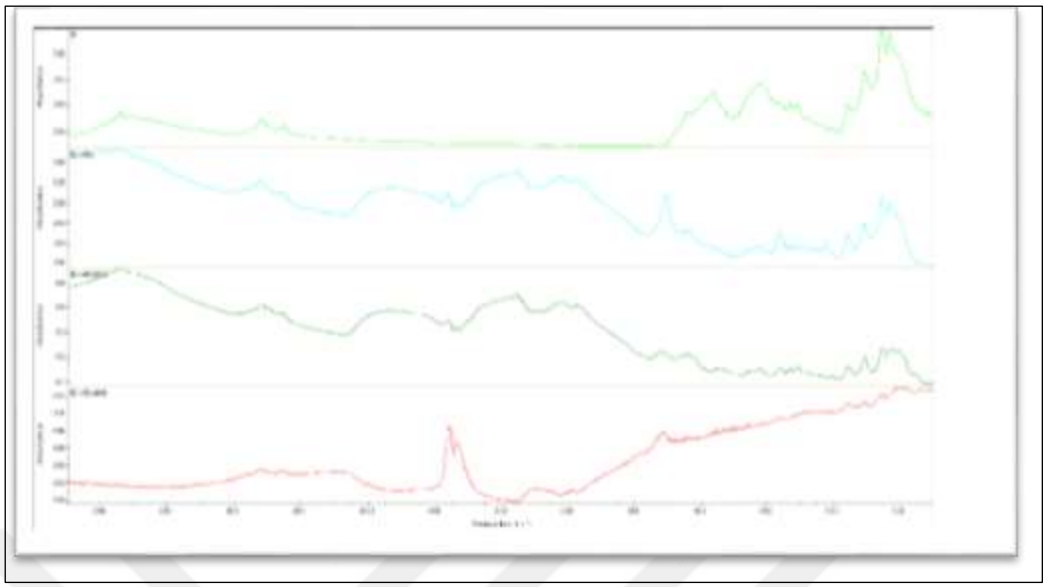

Figure 4.8. FT-IR Spectra of BC Membranes

Membranes surface specifications are directly affected during usage and protein binding because of the physical interaction.

To be able to have the SEM images of the membranes polymers should be totally dry but unfortunately BC membranes stick on the glass and aluminum surfaces and sections cannot be cut ; as a result of this SEM images of the BC membranes cannot be taken. Although the surfaces of BC and BC-HEA membranes were smooth after the modifications with ammonium hydroxide and propane sultone membrane surfaces become flowing and/ dispersed.

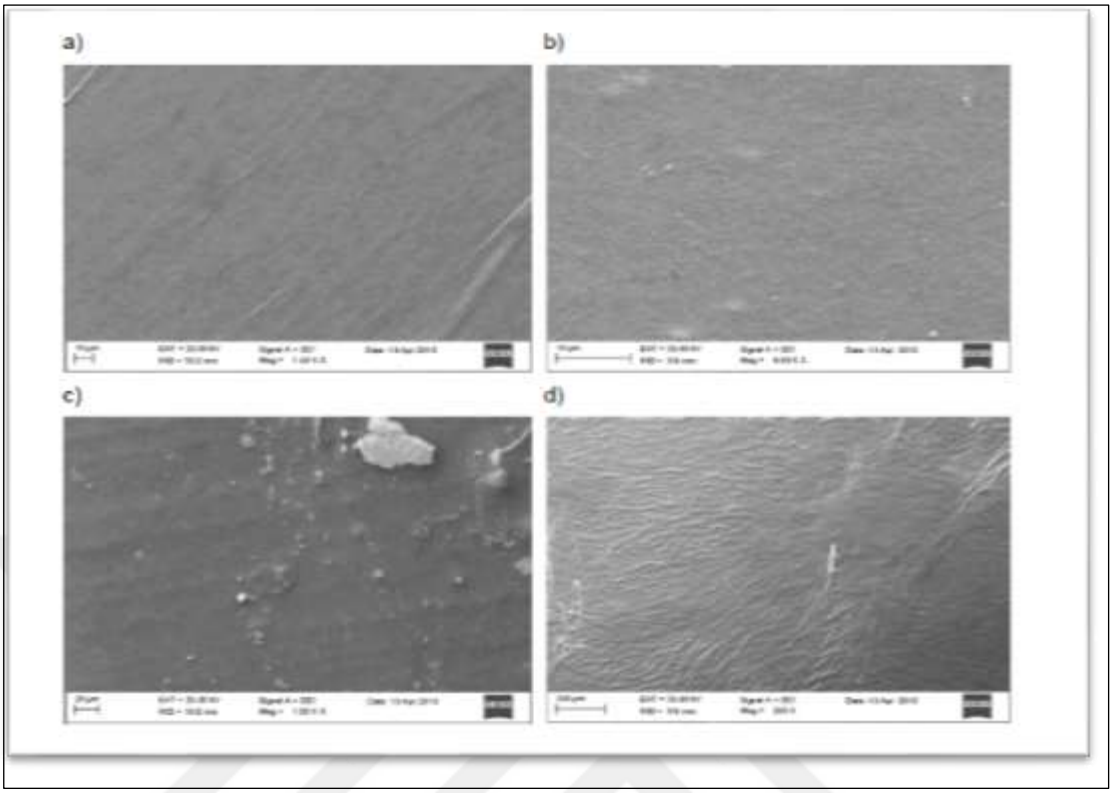

Figure 4.9. SEM Images of BC, BC-HEA, BC-HEA-NH3 and BC-HEA-SO_3

4.10. HYDROLIC PERMEABILITY

Membranes hydraulic permeability values are important data in industrial applications for the calculation of the filtration duration because that it gives the amount of the liquid which passes through the membrane in unit of time under applied pressure.

Permeability of the prepared membranes with respect to water under varied pressures is defined as volumetric flux. The time which fluid passed under different pressure's from the surface area (cm^2) of the membranes under constant volume which is 5 cm^3 is measured and by this way membranes volumetric flux's were measured.

Although there is no pressure at the environment while determining the volumetric flux values of membranes there were still some fluid permeability. Add to this it was also noticed that with the increasing pressure volumetric flux value's changed significantly and as a result of this experiments were continued at low pressure values.

Hydraulic permeability of the liquid which passed through the membranes (L_P) were calculated by using the Formula which is $L_P = J/\Delta P$ from the slope of volumetric flux and pressure values. This linear equation did not started from the zero base line because that under unpressurised environment there is still some flow and this gives us up a volumetric flux value.

Hydraulic permeability of BC-HEA is less than natural BC and all other modifications and this is because of the homogenous structure of the BC is damaged by ammonium hydroxide and propane sultone and effects the membrane's pores. BC-HEA-NH_3 and BC-HEA-SO_3 hydraulic permeability values were higher than natural BC and BC-HEA.

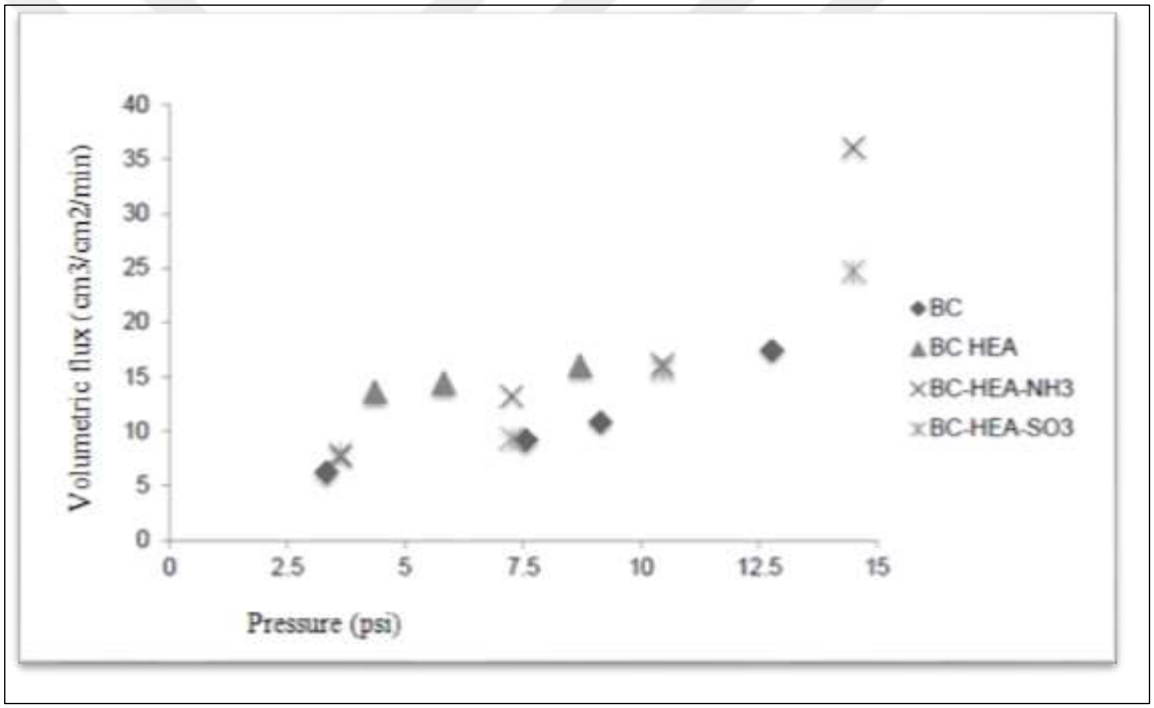

Figure 4.10. Volumetric flux differentiation of modified and natural BC under different pressures

Membranes were placed in Sterlitech HP4750 and for the protein binding experiment 300 ml of distilled water was poured in to the chamber and the duration of the flow was measured.

When looking to the Table 4.2 the time of the flows under 1 bar pressure were recorded and when the durations were examined it was seen that the water passes by really fast. Commercial ion exchange membranes are made by putting 3-5 membranes one after and by this design flow rate is decreased and there is enough time created for protein binding.

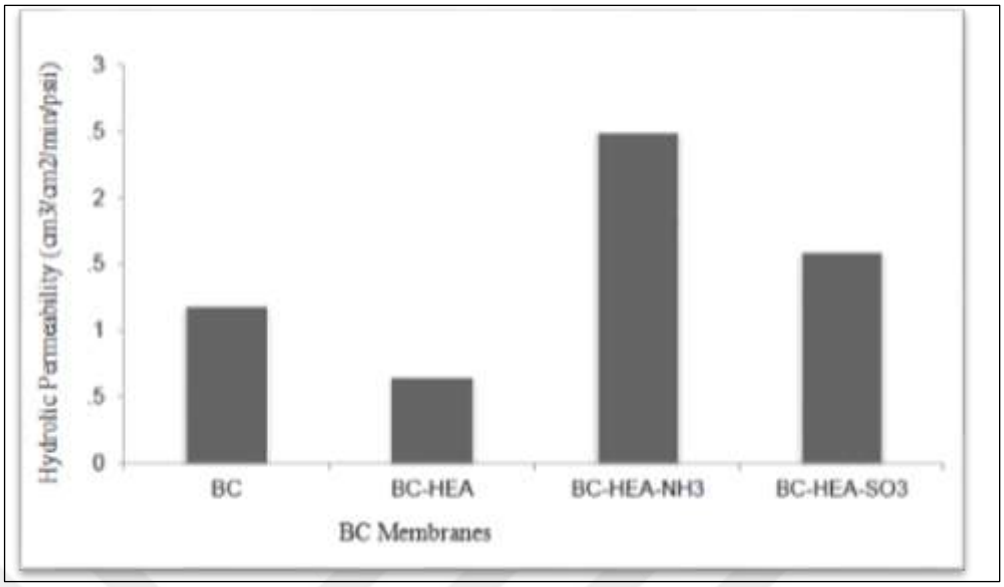

Figure 4.11. Hydraulic Permeability Change of Natural and Modified BC Membranes

Table 4.2. Duration of the passing of the water through the membrane (s)

Membrane Type	Duration of the passing (s)
BC	50.4
BC-HEA	49.8
BC-HEA-SO_3	50.6
BC- HEA-NH_3	50.3

4.11. PORE RADIUS AND POROSITY

Pore radius of the membranes determines , which substances can be separated by using prepared membranes. The separation process which is done based on the electrical charges of the substances and membranes pore radiuses which is changed because of the modifications effects the performance of the filtration. To be able to find the porosity value of the membranes density value's should be 0.742 g/cm^3 .The values of the modified membranes varies in between ±%3 when compared to natural polymers so that for the calculations density value of natural BC was used.

For the pore radius experiments Sterlitech HP4750 membrane stirred cell was used and the durations for the 300 ml passing were recorded and pore radiuses were calculated.

When looking to the pore radiuses of the prepared membranes it was seen that the values were really close to those that have been used at commercial market for ion exchange. Sartorius have been declared that Q and S membranes pore radiuses are higher than 3 µm and BC membranes pores were measured in between 1-2 µm. When looking to the results it can be sad that the based on the modifications which were applied to BC membranes porosity values is changing. When comparing the modifications with one and other it BC-HEA and BC-HEA-NH3 the increase of porosity is %20 and %40 and on other hand when looking to BC-HEA-SO3 its %100.

For membrane performance porosity and pore radius are important parameters whereas surface area for adsorption. Membranes pore number and effective surface values were calculated by the equations below (4.1.), (4.2.) and (4.3.).

$$V_{membrane}\,(cm^3) = Membrane\ Surface\ Area\ (cm^2) \times Membrane\ Thickness\ (cm) \quad (4.1.)$$

$$V_{emptyness}\,(cm^3) = Membrane\ Volume\ (V_{membrane}) \times Porosity \quad (4.2.)$$

$$V_{effective}\ (cm^3) = V_{membrane} - V_{emptyness} \quad (4.3.)$$

Membranes constant surface area was taken as 17.35 cm^2 for its volume of 3.47 cm^3 ($V_{membrane}$) thickness is 0,2 cm .

$$Membrane\ Volume = \frac{\pi \times Pore\ Radius^2}{4} \times Membrane\ Thickness \quad (4.4.)$$

$$Pore\ Number = \frac{V\ empytness}{Pore\ Volume} \quad (4.5.)$$

The surface area which is needed for the binding of the proteins were calculated with the equation below (4.6.).

$$A_{effective} = \pi \times Pore\ radius \times Membrane\ Thickness \times Pore\ number \quad (4.6.)$$

Results of the calculations can be seen in Table 4.3. When comparing these membranes BC-HEA-SO_3's pore number is the high and natural BC's is the lowest.

Table 4.3. Calculations of Pore Number and A effective

Membrane Type	$V_{emptyness}$ (cm3)	$V_{effective}$(cm^3)	Pore Number ($x10^8$)	A effective (cm2)
BC	1.621	1.849	4.26	4.17
BC-HEA	2.170	1.300	7.12	6.23
BC-HEA-NH_3	1.250	2.220	5.82	2.70
BC-HEA-SO_3	3.827	0.183	29.50	15.60

4.12. MEMBRANE ACTIVITIES

4.12.1. Ion Exchange Capacity of Membranes

Protein's surface charges changes based on the solvents pH which they have been kept in and they can bind another charged substance in the medium. Protein interactions of cationic and anionic substances will occur based on the pH of the medium and they are going to bind to the substances. BSA and IgG's pı values are 4,8 and 7,2 and add to this with the increasing ion exchange capacity, binding capacity increases too. To be able to determine the ion exchange capacity, ion balance was created by preparing NaCl mixed solution and for transferring of charges from membrane to water and from water to membrane was done by changing the pH of the solution.

Table 4.4. Ion exchange capacity of BC Membranes

Membrane	Ion exchange capacity (meq/g)
BC	0.023
BC-HEA	0.041
BC-HEA-NH_3	0.012
BC-HEA-SO_3	0.098

Instead of BC-HEA-SO_3 membranes all other membranes which were prepared were cationic charged. BC's ion exchange capacity is relatively low but surprisingly with the

modification BC-HEA-SO_3's ion exchange capacity has been increased 4 times when it is compared to natural BC.

4.12.2. BSA (Bovine Serum Albumin) Interest of Membranes

BSA was used to be able to set the Immunoglobulin binding experiments and also for membrane characterization. Membranes were weighed at different concentrations (25, 50, 75, 100, 125 mg/ml) and poured in to a BSA solutions at a constant concentration (2,5 mg/ml) and the BSA amount after the balance is measured as mg/ml by Standard graphic.

When the results of the experiments were examined it was noticed that BC has no interest to BSA, but modified BC's BSA binding levels increases with respect to membrane concentrations. Although that the concentration of BC is independent, its BSA binding capacity is relatively high. For BC-HEA and BC-HEA-SO3 the membrane concentration for the binding the half of the BSA (1,25 mg/ml) were calculated as 96,6 and 124,2 mg/ml. When the protein affinities of BC's were examined it was noticed that the highest one was hydroxyethyl acrylamide modification.

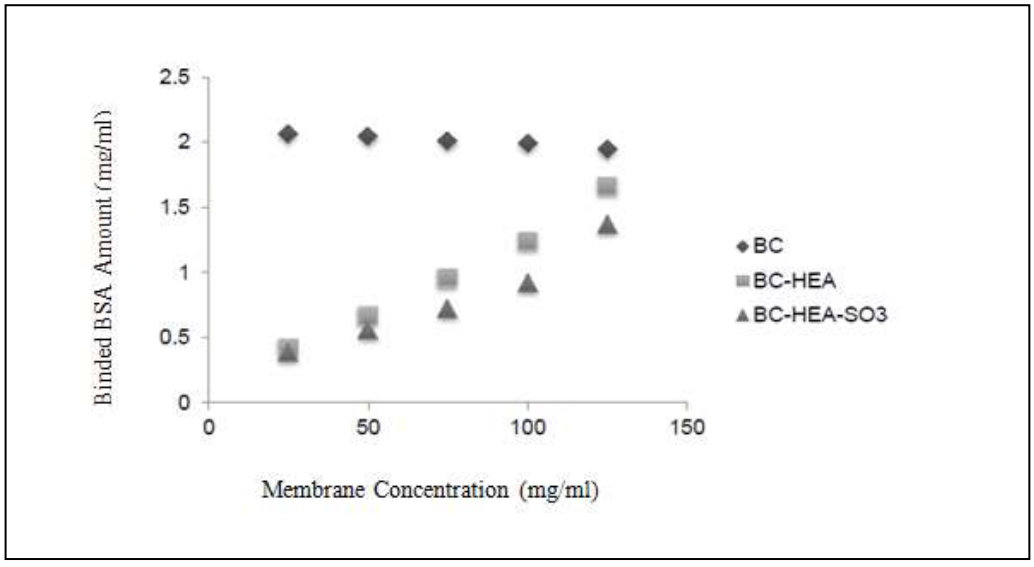

Figure 4.12. BSA Binding Capacities of BC Membranes

Table 4.5. Protein affinity Results of BC Membranes

Membrane	Protein Interest (mg BSA/ g Membrane)
BC	1.1
BC-HEA	12.2
BC-HEA-SO_3	9.3

4.12.3. Membrane Adsorption Isotherms

Langmuir and Freundlich isotherms which are two isotherms that have been used for membrane adsorption mostly were used for the prepared membranes and it was detected that to the which isotherm they fit and based on this their structure were determined.

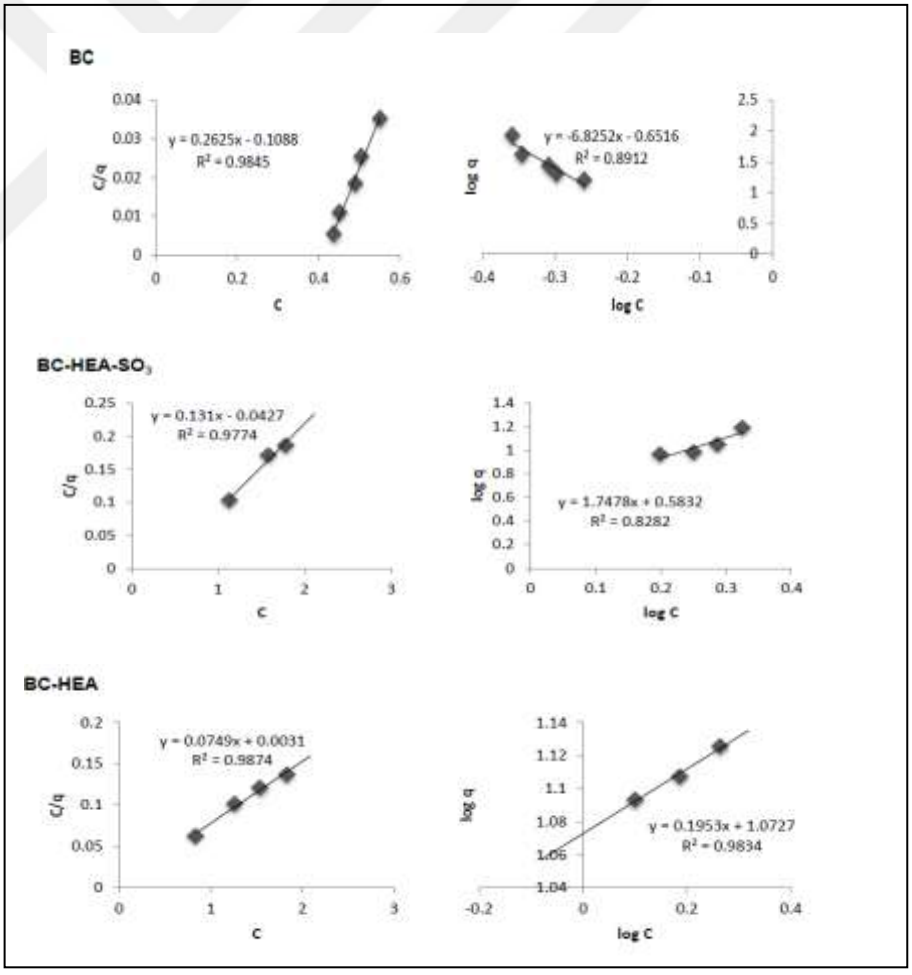

Figure 4.13. Membranes Langmuir and Freundlich Isotherms Model Graphs

Based on the R^2 isotherm values membranes models can be identified. Due to the layer formation of BC Membranes they fit to Langmuir isotherm. When looking to the Freundlich constant which is n value ; it was expected to be higher than 1 for all membranes but although that all membranes n values are higher than 1 it was seen that hydroxyethyl acrylamide modification has much higher value. Same mistake can be seen for Langmuir isotherm too. Langmuir isotherms constant K_L should be higher than 1.

Table 4.6. Langmuir and Freundlich Constants of Membranes

Membrane Type	**Langmuir Isotherm**			**Freundlich Isotherm**		
	K_L	q_m	R^2	n	K_F	R^2
BC	2.413	3.810	0.985	0.146	4.483	0.891
BC-HEA	24.161	13.351	0.987	5.120	11.823	0.983
BC-HEA-SO_3	3.068	7.634	0.977	0.572	3.828	0.828

4.12.4. Membranes Immunoglobulin G (IgG) Interest

To be able to detect the membranes interest to IgG ; IgG was prepared at a concentration of 0,3 mg/ml and its binding activity was examined at different membrane concentrations. For this examination BC's which have the highest interest to BSA were chosen and these membranes were BC, BC-HEA and BC-HEA-SO_3. BC membranes IgG interests were relatively different compared to BSA although that natural BC's BSA interest was low , their IgG binding interest was the highest.

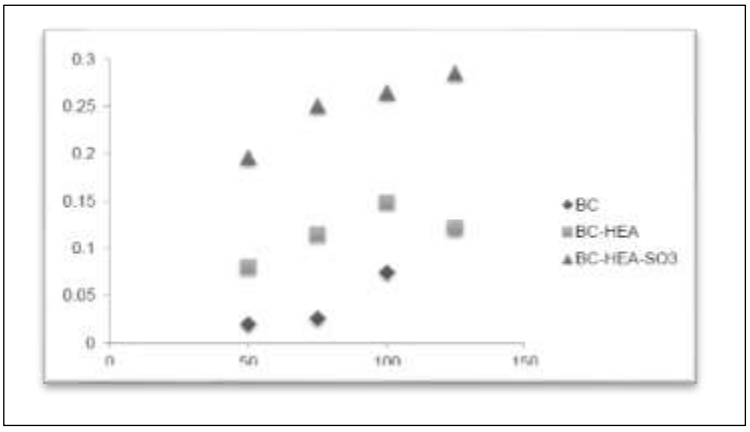

Figure 4.14. BC Membranes IgG Binding Capacities

Table 4.7. IgG Interest of BC Membranes

Membrane Type	Protein Interest
BC	1.9
BC-HEA	1.4
BC-HEA-SO_3	1.4

4.12.5. Membranes Immunoglobulin A (IgA) Interest

To be able to detect the membranes interest to IgA ; IgA was prepared at a concentration of 0,3 mg/ml and its binding activity was examined at different membrane concentrations. For this examination BC and BC-HEA was chosen for comparison and it was seen that hydroxyethyl acrylamide modification of BC caused a decrease at IgA interest.

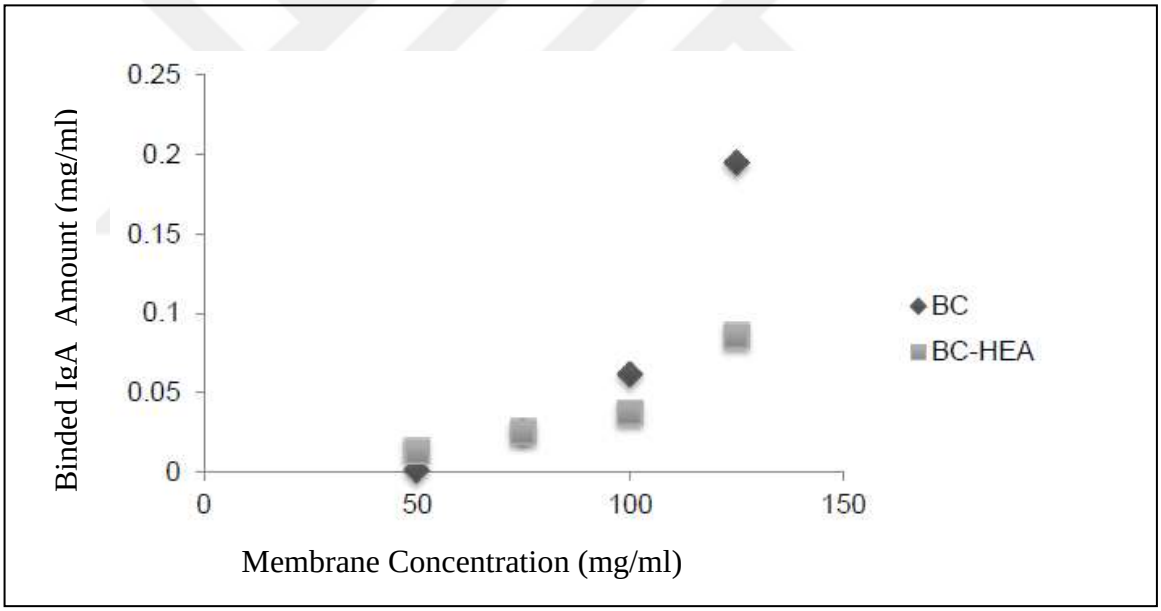

Figure 4.15. BC Membranes IgA Binding Capacities

Table 4.8. BC Membranes IgA Binding Capacities

Membrane Type	Protein Interest mg IgA/g membrane
BC	2,5
BC-HEA	1,2

BC membranes IgA interest were also examined and it was concluded that BC has two times higher interest than BC-HEA.

5. DISCUSSION

As a result of this thesis it should be stated that the membranes which are chosen for the purification of immunoglobulins were determined based on the following requirements:

- Protection of the endurance as a result of modifications
- Identify the hydraulic permeability, pore value and radius of the modified membrane which have protected its endurance and designing the column experiments and determining its characterization
- Identifying the ion exchange capacity and BSA interest of the membranes
- Choose the membranes which have high BSA intensity and examining the immunoglobulin purification

In order to make the purification of immunoglobulins bacterial cellulose and its modified forms were designed as membranes. To be able to increase the membranes ion exchange capacities and their protein interest chemical modifications were applied. These modifications were chosen based on the increase at anionic and cationic charges of the polymers. When looking to the 16 previous experiments, 8 of them were chosen based on the Young's modulus values and surface specifications. When looking to Bacterial Cellulose- Epichlorohydrine- Ammonium Hydroxide Modification (BC-EPI-NH_3); to be able to handle anionic and cationic membranes the produced bacterial cellulose membranes treated with different linking agents with different charges which is in this case ammonium hydroxide and propane sultone. To be able to bind ammonium hydroxide to Bacterial Cellulose as linking agent; epichlorohydrine and hydroxyethyl acrylamide were used. But the modified Bacterial Cellulose by epichlorohydrine and ammonium hydroxide cannot be used as a membrane because that this type of membrane physically protected its structure and have heterogeneous form. Although it have been treated with lower concentrations of NH_4OH (%15; v/v) membrane structure was protected but the ion exchange capacity will be lower in future processes when comparing to natural Bacterial Cellulose. On the other hand when looking to the results of Bacterial Cellulose Hydroxyethyl Acrylamide Modification (BC-HEA); Hydroxyethyl Acrylamide was used to be able to make Bacterial Cellulose cationic and also to use as a binding source for ammonium hydroxide or propane sultone. Due to its amide group Hydroxyethyl Acrylamide when comparing to acrylates,

becomes more stable at higher pH ranges and salt concentrations. For performing the reaction, ammonium persulphate was used because that it causes BC's 6^{th} carbon's hydroxyl group charged and at the same time it polymerizes the hydroxyethyl acrylamide's vinile groups an causes to bind BC as polymer. When looking to the structure of BC and thinking its 3D structure; second and the third carbon's hydroxyl groups are located inside of the polymer and due to this structure binding ratio is relatively low. Bacterial Cellulose-Hydroxyethyl Acrylamide- Ammonium Hydroxide Modification (BC-HEA-NH_3) Hydroxide was used for the increase of the cationic charge of BC. When comparing BC-HEA-NH_3 with other modifications like BC-EPI-NH_3; BC-HEA-NH_3 has a more homogeneous form of membrane and with the binding of HEA, modified BC's robustness have been increased and this increase prevented decomposition of membranes with the addition of ammonium hydroxide. As a result of this because that HEA gets polymerized with the reactions; NH_3 binding occurred faraway from BC fibers and this caused a decrease at level of BC decomposition. Bacterial Cellulose- Propane Sultone Modification (BC-SO_3); propane sultone was used to make BC anionic charged. This reaction was performed in the presence of DMSO and Argon gas was applied to be able to inert hydroxyl groups. But although the DMSO was removed and membranes were kept inside water overnight this presence caused morphological change and membranes became rigid. Membranes become dysfunctional and cannot be used for the further processes. On the other hand Argon gas was used for the insertion of hydroxyl groups. But Argon gas caused the formation of heterogeneous structures on the membrane, because that BC stands at the top of the solution before Argon gas dissolved. For the binding of propane sultone alkali environment was created and with the addition of sodium hydroxide, propane sultone's ring structure opens and binds burdened to the first structure it faces which is mostly the 6^{th} carbon's hydroxyl group. Although the binding of propane sultone eventuated because that membrane structure destroyed, it was decided to not be used as a purification agent. To be able to create Bacterial Cellulose-Hydroxyethyl acrylamide-Propane Sultone (BC-HEA-SO_3) and make BC anionic charged and also to protect membrane integrity; acrylamide bonded BC was reacted with propane sultone. After drying process BC-HEA look alike like normal BC membrane but unlike BC-SO_3 after BC-HEA was poured in to DMSO there was no physical change at its structure. On the other hand for the binding of propane sultone to the acrylamide side an inert environment

was not needed and because of this there is no need of the feeding of Argon gas to the environment and as a result of this there is no structural change on the membrane.

Based on the experiments which are previously explained; for identification and comparison of the membranes their durability and Young's modulus values were compared. When looking to the results BC membranes were supportable and can be worked under high pressure. Their water holding capacity is nearly 50%. When comparing Young's modulus values of wet and dry membranes within each other it can be sad that the wet ones values are higher than dry ones due to the fiber structure of the BC, because that during drying process long fiber structures fractured and this causes breakdown of the membrane during tensile stress tests. Prolongation portion is nearly 10% and breakdown forces are nearly 1/10 of Young's Modulus values. When looking to the results the membranes which are appropriate for further experiments were identified as: BC, BC-HEA, BC-HEA-NH_3 and BC-HEA-SO_3.

FT-IR analysis showed that the modifications were applied successfully. Add to this with the SEM images surface specificities of the membranes were examined. When looking to the surface specificities of the membranes it was examined that although the BC membranes natural forms were smooth, BC modified membranes are in a heterogeneous form. This means that modified forms of BC membranes cannot be used for commercial productions due to high fouling.

BC membranes protein intensity is variable and measured at high values. Although mathematically protein intensity of the BC membranes were low (1,1 mg/ml) experimentally highest bonding level was also examined for BC membranes.

When looking to the BC membrane modifications instead of BC-HEA-NH_3; 2800-3000 cm^{-1} and 900-1400 cm^{-1} ranges absorbance fluctuation patterns look alike. Add to this HEA modifications absorbance fluctuations were recorded in rages between 1600-2700 cm^{-1} when comparing to natural BC. Based on this results it can be concluded that HEA modifications were done successfully.

Generally; C-O-C ether rings at 1058 cm^{-1}, hydroxyl groups at 3415 cm^{-1} and hydrogen bonds in between polymer rings in between 3230-3455 cm^{-1} can be visualized for BC membranes.

Membranes surface specifications are directly affected during usage and protein binding because of the physical interaction. SEM images of the BC membranes cannot be taken because that BC membranes stick on the glass and aluminum surfaces and sections cannot be cut. Although the surfaces of BC and BC-HEA membranes were smooth after the modifications with ammonium hydroxide and propane sultone membrane surfaces become flowing and dispersed.

BC membranes IgG and IgA interest was relatively high due to the high bonding level. However BC-HEA modifications IgA interest decreased as much as half of neutral BC; this means that the interactions of the long fibrils of BC have increased. When proteins lined up in terms of molecular weight BSA <IgG <IgA . When membranes were experimentally examined it was seen that BC binding yield increases with the increased protein's molecular weight.

To be able to use the membranes for future commercial productions; membrane cells should be created with more than one layer. Although that the ion exchange capacity of the membranes were low, protein binding capacity is high due to the long polymer chains of the Bacterial Cellulose. As a result of this for the further experiments should be designed with respect to alterations in the production methods of BC for handling much longer polymer chains or other microorganisms can be used for developing Bacterial Cellulose production.

6. FUTURE STUDY

Future studies will be based on the commercial uses of the prepared membranes and preparations of membrane cells which are formed by several membranes. By this way statically investigated protein binding will also be investigated in a dynamic way and also the selectivity change will be investigated with the usage of other proteins. The low ion exchange charge of the BC membranes and high protein binding capacity is due to the long polymer chains. Due to this specificities for the further studies with the changing at the production methods it will be the aim to handle BC membranes with much longer polymer chains and or with the usage of other types of microorganisms the production processes will be developed.

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