DEVELOPMENT OF ANION-EXCHANGE MEMBRANE CHROMATOGRAPHY FROM REGENERATED CELLULOSE MEMBRANE BY ATTACHING DIFFERENT SPACER ARM LENGTH OF DIAMINE MONOMER

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A thesis submitted in fulfilment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

FEBRUARY 2013

SUPERVISOR'S DECLARATION

I hereby declare that I have checked this project and in my opinion, this project is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering.

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STUDENT'S DECLARATION

I hereby declare that the work in this project is my own except for quotations and summaries which have been duly acknowledged. The project has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Special dedication to God Father, my parents,
siblings and all my beloved friends
for your love and supports.

ACKNOWLEDGEMENTS

I am grateful and would like to express my sincere gratitude to my supervisor, Dr. Syed Mohd Saufi bin Tuan Chik for the knowledge shared, guidance and encouragements which enabled the completion of this thesis. I appreciate his encouragements and support which has been endless throughout the study. I'm grateful for his progressive vision on the subject researched upon, his tolerance for my naïve and careless mistakes. Last but not least, I thank him for the times he spent reviewing my work and giving advices.

I would also like to sincerely thank all course mates and staffs at the labs of Faculty of Chemical Engineering and Natural Resources at University Malaysia Pahang (UMP) who have assisted in many ways to during the progress of this research. I also take this opportunity to thanks all staffs at labs of Faculty of Industrial Sciences and Technology which had kindly lend their lab instruments to enable me to complete my analysis. This has made my days in UMP pleasant and unforgettable. A lot of valuable experience was gain including the lab handling skills which can't be learn from usual curriculum.

Last but not least, I am grateful for my parents and family members for their support throughout these years. They gave strength to me and always listen to my problems which always help me to overcome my exhaust period. My sincere appreciation is also dedicated to those who are involved whether directly or indirectly in the completion of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Acrylic acid
ADP	Adenosine diphosphate
ATRP	Atom transfer radical polymerization
BSA	Bovine Serum Albumin
CA	Cellulose Acetate
DEAE	Diethyl amino ethyl
DNA	Deoxyribonucleic acid
CPES	Carboxylic polyethersulfone
DEEDA	N,N-Diethylethylenediamine
DMAEMA	poly(2-dimethylaminoethyl methacrylate)
EDGE	Ethylene glycol diglycidyl ether
EPI	Epichlorohydrin
IgG	Immunoglobulin G
IPA	Isopropanol
kDa	kilo Dalton
$KMnO_4$	Potassium permanganate
MAETMAC	(2-(Methacryloyloxy)ethyl)-trimethylammonium chloride
MF	Microfiltration
NAD	*Nicotinamide adenine dinucleotide*
NADP	*Nicotinamide adenine dinucleotide phosphate*
NF	Nanofiltration

NMP	N-methyl-2-pyrrolidone
(NTA)-CU^{2+}	Nitrilotriactetate copper
PA	Polyamide
PAN	Polyacrylonitrile
PC	Polycarbonate
PE	Polyethylene
PEGMA	Glycol methacrylate
PES	Polyethersulfone
pH	Potential hydrogen
p*I*	Isoelectric point
PI	Polyimide
Poly(MES)	poly(2-(methacryloyloxy) ethyl succinate
PP	Polyphenol
PPE	Polypropylene
PPO	Polyphenol oxidase
PVDF	Poly(vinyldene fluoride)
RC	Regenerated Cellulose
RNA	Ribonucleic acid
RO	Reverse osmosis
SPM	Sulfopropyl methacrylate
TFC	Thin film composite
UF	Ultrafiltration
UV	Ultraviolet

LIST OF SYMBOLS

J	Flux
v	Volume
A	Area
t	Time
M	Molarity (mg/ml)

PEMBANGUNAN MEMBRAN KROMATORAFI JENIS PERTUKARAN ANION DARIPADA MEMBRAN JENIS REGENERASI SELULOSA DENGAN MENLAMPIRKAN MONOMER DIAMINE YANG MEMPUNYAI PANJANG *SPACER ARM* YANG BERBEZA

ABSTRAK

Teknik kromatografi digunakan secara meluas untuk pemisahan protein. Kromatografi turus terpadat yang biasa digunakan mempunyai beberapa kekurangan. Membran kromatografi adalah alternative teknik yang sesuai untuk pemisahan protein. Monomer tertentu boleh dilekatkan ke atas membran yang tidak bercas untuk mengubahsuainya kepada membran kromatografi. Pengoptimuman parameter-parameter yang terlibat dalam modifikasi secara kimia ini adalah penting untuk menghasilkan membran kromatografi yang berprestasi tinggi dalam aplikasi pemisahan protein. Tujuan kajian ini adalah untuk menghasilkan membran chromatography jenis pertukaran anion daripada membran jenis regenerasi selulosa dengan melekatkan monomer diamine monomer yang mempunyai panjang *spacer arm* yang berbeza. Membran regenerasi selulosa diaktifkan dalam larutan yang mengandungi natrium hidroksida (NaOH) dan epichlorohydrin (EPI). Kemudian, membran itu direndam dalam larutan diamine 1,2-diaminoethane atau 1,4-diaminobutane bagi menghasilkan membrane kromatografi bercas positif. Kepekatan NaOH semasa pengaktifan dari 0.05M ke 0.50M dan kepekatan monomer diamine dari 0.25M ke 2.0M semasa *grafting* telah dikaji. Kepekatan NaOH yang optima ialah 0.20M yang menghasilkan membran kromatografi jenis pertukaran anion berkapasiti 0.310 ± 0.033 mgBSA/cm^2 membran. Kepekatan monomer diamine yang tinggi pada 2.0M 1,4-diaminobutane memberikan membran berkapasiti 0.385 ± 0.027mgBSA/cm^2 membran. Dengan merujuk kepada puncak transmisi graf FTIR, kedua-dua kumpulan berfungsi N-H dan C-N wujud dalam membran yang diubahsuai mengambarkan kejayaan process *grafting*.

DEVELOPMENT OF ANION-EXCHANGE MEMBRANE CHROMATOGRAPHY FROM REGENERATED CELLULOSE MEMBRANE BY ATTACHING DIFFERENT SPACER ARM LENGTH OF DIAMINE MONOMER

ABSTRACT

Chromatography technique is widely used for protein separation. Conventional packed bed column chromatography has several limitations. Membrane chromatography was a suitable alternative technique for protein separation. Specific monomer can be grafted to uncharged membrane to transform into membrane chromatography material. Optimization of parameters involve during this chemical modification is crucial for the development of high performance membrane chromatography for protein separation. The purpose of this research is to develop anion-exchange membrane chromatography from regenerated cellulose membrane by attaching different spacer arm lengths of diamine monomer. Regenerated cellulose membrane was activated in a solution containing sodium hydroxide (NaOH) and epichlorohydrin (EPI). Then, the membrane was immersed in diamine solution of 1,2-diaminoethane or 1,4-diaminobutanhe to produce positively charged membrane chromatography. The concentration of NaOH activation from 0.05M to 0.50M and diamine monomer concentration from 0.25M to 2.0M during grafting were studied. The optimum concentration of NaOH was 0.20M which produced anion exchange membrane capacity of 0.310 ± 0.033 mgBSA/cm^2 membrane. High concentration of diamine monomer at 2.0M 1,4-diaminobutane showed a binding capacity of 0.385 ± 0.027mgBSA/cm^2 membrane. Based on FTIR transmission peak, both N-H and C-N functional groups were detected in modified membrane that indicated the successful of grafting process.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Conventional techniques for separation involve several steps such as impurities removal, isolation, purification and polishing. More than 60% of the total cost of bioprocess in worldwide was due to downstream recovery and purification. High efficiency and high productivity separation techniques were essential to pharmaceutical industry. Besides that, increasing industrial demand of food products for large-scale extraction and purification had cause the separation process to further develop and exploit. In food sector, more advance separation processes have been developed for concentration and fractionation of molecules from raw material such as separation of protein from milk. Many biological active components have potential in nutraceutical applications and the global functional food market was expected to growth approximately 8% per year. At this rate, the market will be valued more than US$100 billion in year 2012 (Smither, 2008).

Chromatography technique was widely used for protein separation in the packed bed configuration. However, conventional packed bed column

chromatography have several limitations such as high pressure drop, long processing times due to slow pore diffusion and complicated scale up procedures (Ghosh, 2003). Sometimes, channelling can occurred due to cracking of packed bed which caused a major problem. Membrane chromatography is becoming more popular as alternative to the packed bed chromatography. It is a combination of chromatographic principle and membrane filtration methods. Membrane chromatography shows several advantages such as low pressure drop, fast protein accessible to the specific functionality in the membrane by bulk convection with only little pore diffusion, easy to scale up and set up.

Highly adsorptive membranes can be prepared in three general steps involving preparation of base membrane, chemical activation of the base membrane and coupling of ligands or specific functional group to the activated membrane (Zheng & Ruckenstein, 1998). Membrane with good performance should include excellent mechanical strength, great oxidative, thermal and hydrolytic stability as well as good-forming properties (Zou et al., 2001).

Various methods used to prepare charged membrane chromatography. Optimization of both preparation method and the chromatography process are necessary in order to obtain higher performance membrane chromatography process. Protein transport by membrane was affected by electrostatic interaction between charged protein and charged membrane. The membrane surface varies according to the type of ligand coupled on it. Molecular structure of charged ligand has impact on the membrane adsorption characteristics. In this study, commercial regenerated

cellulose microfiltration membrane will be modified into anion-exchange by attachment of amine based functional group to create affinity toward anionic protein.

1.2 Problems Statement

Chromatography in a packed bed configuration is commonly used technique for protein separation and isolation. However, several limitation of packed bed column chromatography have been identified such as high pressure drop, long processing times with slow diffusion and complicated scale up procedures (Ghosh, 2002; Kawai et al, 2003). In contrast with column chromatography, membrane chromatography has advantage such as lower pressure drop, higher productivity and easy scale up. Unwanted fouling or clogging is minimized in membrane chromatography and give a promising large-scale production for separation and recovery of protein.

Anion-exchange membrane chromatography with high binding capacity has potential for commercial application in industries. Anion-exchange membrane can be prepared by chemical grafting, UV-grafting, photo-grafting, polymer grafting and etc (Bhattarcharya & Misra, 2004). Negatively charged protein can be separate selectively and effective by using positively charged membrane. Suitable ligand need to be selected to modify existing membrane with positive functional group for adsorption on selective anion charged protein. The parameters involve in synthesis route to produce anion-exchange need to be further study, analyze and characterize to produce high performance membrane chromatography.

Spacer arm lengths of diamine which use as positive charged functional ligand on membrane surface have strong effect on the protein binding capacity. Different spacer arm length, determine by the number of alkyl groups between membrane and functional ligand, having different protein binding and behaviour. The relationship of spacer arm length as one of the ligand properties is essential to study in order to develop membrane with higher performance applications.

1.3 Research Objective

The main objective of this research was to study the effects of parameters involve in preparing anion-exchange membrane chromatography from regenerated cellulose membrane using different spacer arm length of diamine monomer.

1.4 Research Scopes

With the intention of fulfil the objective of the research; the following scopes have been outlined:

i. Study the performance of anion-exchange membrane chromatography with different spacer arm length of diamine monomer which are 1,2-diaminoethane and 1,4-diaminobutane.
ii. Study the effect of NaOH concentration from 0.05M to 0.50M during the activation of regenerated cellulose membrane.
iii. Study the effect of diamine monomer concentration from 0.25M to 2.0M on the protein binding capacity.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Proteins are essential to all living organisms for regulation, function and structure of the body. Development of protein begins with DNA transcribing into RNA and finally RNA translating into proteins. Protein comprised of polymer chains where amino acids linked together by a strong peptide bonds (Hagel et al., 2008). There are more than twenty different types of amino acid use to form protein and each distinguished by functional "R" group. When two amino acids linked together is called a dipeptide and many linked together called as polypeptide. Polypeptide consists of a backbone and side chains. The backbone comprises of amide nitrogen, α- carbon and carbonyl carbon. Some examples of proteins are hormones, enzyme and antibodies. The side chains comprise the functional "R" group. The basic structure of amino acid was shown in Figure 2.1.

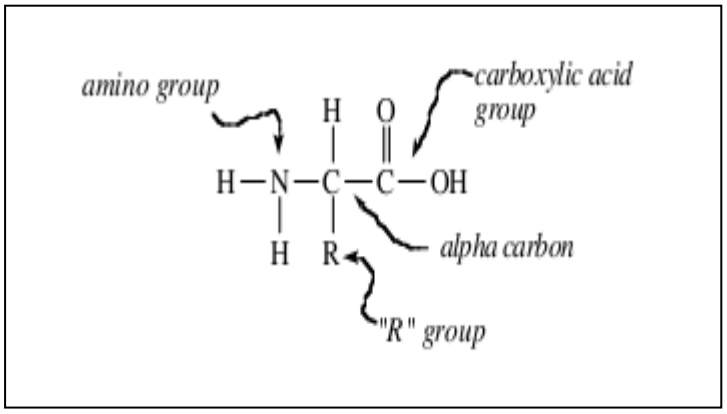


Figure 2.1 Basic structure of amino acid.

Protein is major nutrition needed by human and other living organism. It involves in building body cells and enhances growth, forming blood and maintenance of human body. When protein break down into amino acids, these amino acids functions as major abundant molecule for body parts and act as a precursors to hormones, immune response, cells and molecules repair and co-enzymes (Gregory et al., 2004).

Protein is becoming important in biopharmaceutical and food processing industries. Production of proteins especially for human use is essential to replace the extraction of proteins directly from natural sources to minimize the risk of poisonous impurities enters into human body. Protein-based drugs were growing in ultimate speed for treatment of various diseases in humans and animals. Various single proteins were use in wide range of applications. Bovine serum albumin (BSA) as an example has numerous applications such as act as nutrient in cell and microbial culture. BSA stabilizes some enzymes during DNA suggestion and involve in quantitative determination of other proteins. Immunoglobulin act as an antibody and antigen in medical application especially known as Rho(D) immune globulin

antibodies which against Rhesus factor. Wide applications of protein proven that single protein have huge potential in economic and social development (Petsko & Ringe, 2004).

2.1.1 Properties of Protein

A protein will have a positive net charge at low pH and negative net charge at high pH. This indicate that pH have large influence on the net charge of a protein. This is due to the charged of amino acids and also dissociated carboxylic acid group of a protein. At particular pH where the surface carries no net electric charge is called as isoelectric point (p*I*). Proteins isoelectric point mostly dependes on seven charged amino acids which are glumate (*δ-carboxyl group*), aspartate (β-carboxyl group), cysteine (thiol group), tyrosine (phenol group), histidine (imidazole group), lysine (ε-ammonium group) and arginine (guanidinium group). Positive charges are usually provided by arginie, lysine and histidine, depending on surrounding buffer pH. The protein terminal group (NH_2-COOH) should take into account charge as each of them has its unique acid dissociation constant referred as pK. The net charge of protein is related to the pH of buffer solution. Handerson-Hasselback equation can be used to calculate protein charge in certain pH. The equation is useful for estimating a buffer solution (Henry et al., 2001). Protein can be retarded by cation exchanger above the pI and by anion exchanger below the pI. Proteins normally show absorption at 280nm due to the peptide bond (Hagel et al., 2008).

2.1.2 Bovine Serum Albumin

BSA had a number of amino acid about 583 with molecular weight of 66.5kDa and isoelectric point (p*I*) of 4.7 at 25℃. Isolated BSA was found to be a very functional protein. BSA involves in numerous biochemical applications such as immunoblots, immonuhistochemistry and enzyme linked immunosorbent assay. BSA also functions as a nutrient in cell and microbial culture. BSA acts as a base for preparation of defined fatty acid supplements and help enzyme stabilization during purification stage. BSA prevents adhesion of enzymes to reaction tips and tube surfaces and also acts as a blocking agent to minimize background in protein and deoxyribonucleic acid (*DNA*) labelling (Acton, 2011).

2.2 Methods for Protein Separation

Protein separation can be divided into two different methods that are analytical and preparative scale. Preparative method is more suitable for large scale production in industry. Extraction is one of the techniques that commonly use to break the cells or tissue to obtain the proteins of interest. A few method need to undergo throughout extraction process such as freezing, sonication and filtration. The method depends on fragility of the protein. Soluble protein will be in the solvent after extraction and can be separate from cell membranes by centrifugation.

Precipitation is a common method use to isolate bulk protein with use of ammonium sulfate $(NH_4)_2SO_4$. Different fractions of precipitate protein can be collect by increasing amounts of ammonium sulfate. The process based on some

properties of the solvent such as addition of salts, organic solvents and polymer or by manipulating pH or temperature. Dialysis will be carrying out later to remove ammonium sulfate. Protein will get aggregated when hydrophobic protein groups attracts toward other hydrophobic groups. This method suitable to be use for large volume isolation due to lower operating cost (Deutscher, 1990). Precipitation is the only practical way to separate different types of protein in early days.

Ultracentrifugation is another alternative method for protein separation by using centrifugal force to separate protein mixture. The mixture usually contains varying types and densities of suspended particles suspended inside it. When a vessel containing proteins rotate at high speed for a constant time, a momentum yields an outward force to each particle that is proportional to its mass.

Chromatographic methods widely used in industries where the separation of protein can be done more efficiency and in larger scale. Different protein will interact differently with column material and elution process was required to carry out to recovered bound protein (Charcosset, 1998). There are different chromatographic modes available such as size exclusion chromatography, ion exchange chromatography and affinity chromatography. Chromatography based method has high resolving power and become dominant for protein separation (Hedhammar et al., 2011).

2.3 Chromatographic Methods

Chromatographic methods for proteins separation have been developed for commercial scale production. Chromatography refers to separation techniques that involve retardation of molecules with respect to the solvent front that movement through the material. It refers to resolution of solutes by differential migration during passage through a porous medium. In chromatographic techniques, the separation principle operates in different migration of component between stationary phase and mobile phase. Another component of chromatographic system is inert support or matrix.

The separation of one protein from one to another is mainly base on properties of proteins which different from non-protein contaminants. These properties are size, charge, hydrophobicity and specific biological interaction. Several type of liquid chromatography differ mainly on the types of stationary phase involve in separation of protein. There are various types of interactions available such as ion exchange, hydrophobic, reverse phase and size exclusion chromatography. The different among these interactions is on the mechanism of separation and stationary and mobile phase used to perform the separation. For example, size exclusion chromatography is based on protein size for fractionation and ion exchange chromatography depends on charge of protein (Hedhammar et al., 2011) as shown in Figure 2.2.

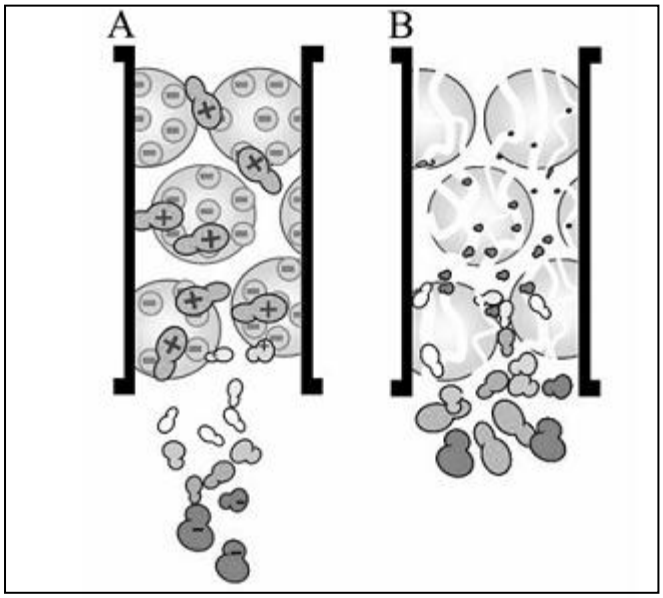


Figure 2.2 Illustrations of (A) ion exchange chromatography and (B) size exclusion chromatography (Source: Hedhammar et al., 2011)

The stationary phase known as adsorbent can either be solid or liquid. A liquid stationary phase will be held stationary by a solid support or matrix. Mobile phase which also known as solvent are normally in gaseous or liquid phase. The advantages and disadvantages for different types of stationary matrix in chromatography process as summarized by Ghosh (2003) are showed in Table 2.1.

Table 2.1 Advantages and disadvantages of four type stationary matrixes in chromatography process (Ghosh, 2003).

Process	Advantages	Disadvantages
Packed bed	• Establish process • High resolution • High binding capacity • Suitable for gradient chromatography	• Slow process • Low reproducibility • Susceptible to column blinding • Variability of column packing
Monolith	• Fast process • High reproducibility • Suitable for gradient chromatography • Predominance of convective transport • Low pressure drop	• Expensive
Membrane	• Fast process • High reproducibility • Predominance of convective transport • Low pressure drop • Inexpensive • Disposal device	• Unsuitable for gradient chromatography • Low binding capacity
Fluidized bed	• Fast • Suitable for feed containing particles	• Poor resolution • Poor reproducibility • High energy consumption • Breakage of chromatographic media

2.3.1 Ion-exchange Chromatography

Ion-exchange chromatography separated protein mixture by depending on the relative number of charged side chains and overall protein charge. Anion proteins have a p*I*< 7.0 while cation proteins will have p*I*> 7.0. An anion protein will bind to cation resin in the process known as anion-exchange chromatography. Cation protein will attract toward anion resin within a supporting matrix in cation-exchange chromatography.

Functional groups for cation-exchange chromatography can be divided into weak acidic group such as carboxylmethyl or strong acidic group such as metyl sulfonate and sulfopropyl group. Functional group or resin for anion-exchange chromatography usually is amino group such as polyethylene amine.

Bound protein to the ion exchanger material can be recovered by alter the elution buffer used. Two general methods are available by changing the pH of eluting buffer or increase the ionic strength of elution buffer.

2.3.2 Affinity Chromatography

Affinity chromatography involves attachment of protein covalently to the specific ligand on the chromatography matrix. This ligand had very high specificity toward the protein of interest. Binding must be tight enough for sufficient process but this may cause problems for recovery process. An example of affinity chromatography is separations of immunoglobulin G subclasses from serum or cell culture supernantants by using Protein A, Protein G or a mix of these two proteins as

ligand. Another example is separation of glycoproteins and some antigens by bind them with α-D-mannose, α-D-glucose and related sugar by using the interaction of OH functional groups. Enzymes suitable with this separation method since co-factors such as NAD, NADP, and ADP are ligand use in affinity chromatography (Whilford, 2005).

2.3.3 Size Exclusion Chromatography

Size exclusion chromatography separated protein by based on their molecular size. Proteins which are large in molecule size are suitable to separate using size exclusion method. A series of pores in constant size was formed by covalent cross-linking polymers. In order to obtain smaller pore size, high level cross linking polymer needed. Only small protein molecule is able to enter into the region while large protein molecule will be elute in the void volume and fail to interact with the pores. This process is an effective way to separate proteins according to different sizes.

Size exclusion chromatography normally used as an effective method to eliminate impurities from protein mixture. Size exclusion chromatography is differs from other chromatography because the stationary phase surface does not react with mixed sample (Barth et al., 1998).

2.3.4 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography separates proteins with difference in hydrophobicity. Protein mixture with high ionic strength solution will bind to the hydrophobic chromatographic column. Proteins can be separate with high resolution in high yield but high concentration of salt for eluent is inconvenient for large scale purification.

Hydrophobic interaction chromatography has been successfully use to separate hydrophobic proteins such as r-protein A and antibody by using low salt concentration. Hydrophobic interaction chromatography at low salt concentration was found to compatible with other modes of liquid chromatography (Kato et al., 2002).

2.3.5 Membrane Chromatography

Membrane chromatography also known as membrane adsorption uses adsorptive membrane that carries specific functions similar to chromatography media. Membrane chromatography have advantages over column chromatography due to low pressure drop, high flow rate operation, convective mass transfer, low clogging tendency with easily scale up and packing procedure (Kawai et al., 2003). Transportation of solutes to the binding sites mainly occurred by convection with only small pore/film diffusion as comparing to packed bed chromatography column. Thus, the mass transfer was reduced greatly (Ghosh, 2002). Figure 2.3 shown transportation of solute by convection and pore diffusion in membrane chromatography.

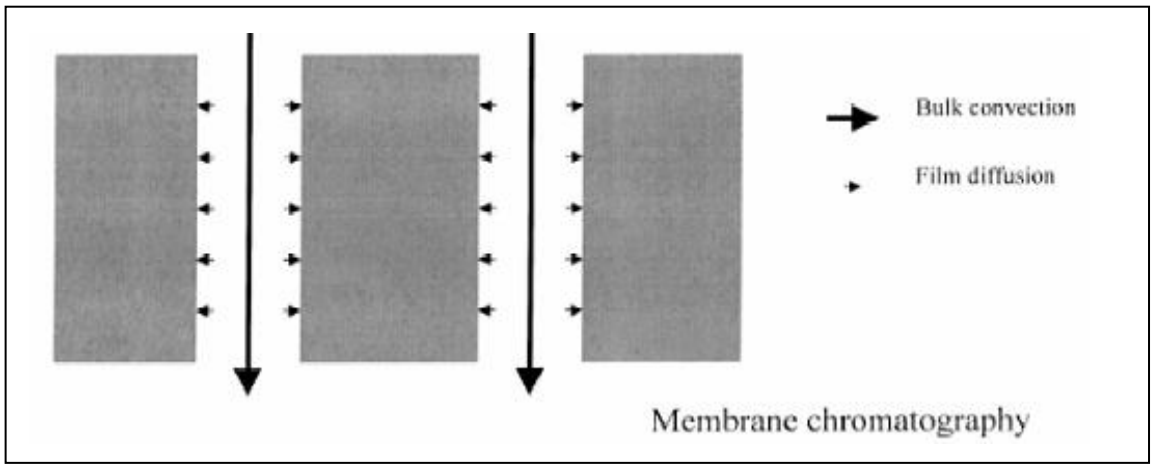


Figure 2.3 Transportation of solute by convection and pore diffusion in membrane chromatography.

Membrane technology and column chromatography combined together to form advantageous of high performance membrane chromatography.

The binding capacity of membrane chromatography can be improve by making a three dimensional hydrogel on internal membrane surface to immobilized ligands (Gebauer et al., 1997). The pore size of membrane and its distributions influence the binding capacity. If the pore size happens to be too large, the radial concentration gradients within the pores could lead to early breakthrough which will cause dynamic capacity to be less than static capacity (Wickramasinghe et. al., 2006).

In membrane chromatography, flow distribution was concern. Three general types of membrane absorbers used for protein separation that is flat sheet, hollow fibre and radial flow.

2.4 Membrane Technology

Membrane is defined as selective barrier between two phases. It can explain as a phase that acts as a barrier to prevent mass movement but regulated passage of one or more species. The selectivity of membrane depends on size, shape, electrostatic charge, diffusivity, physicochemical interactions, volatility and solubility. Driving force in membrane processes are transmembrane pressure, concentration gradient, chemical potential, osmotic pressure, electric field, magnetic field, partial pressure and pH gradient.

Membrane process has several advantages such as eliminating the use of additives, operated as a compact module separating operation, flexibility and relatively low capital cost (George & Thomas, 2001). Membrane can be classified into several types according to driving force in its application. Table 2.2 shows the type of driving force used in membrane process.

Table 2.2 Classification of membrane process according driving force (Nath, 2008)

Driving Force	Membrane Process
Pressure driven	• Reverse osmosis (RO) • Microfiltration (MF) • Ultrafiltration (UF) • Nanofiltration (NF) • Pervaporation • Membrane gas separation
Concentration gradient driven	• Dialysis • Membrane extraction
Electrical potential driven	• Electrodialysis
Temperature different	• Thermo osmosis • Membrane distillation

One of the limitations of membrane based process is concentration polarization which can foul and reduce the efficiency of the membrane. Concentration polarization occurs due to the accumulation of solute at the membrane fluid interface. The membrane performance in term of selectivity and flux will be decrease. Fouling can caused loss of flux when all variable are held constant. Fouled membrane normally will be recovered by cleaning it under turbulent flow.

Membrane structure can be porous or non-porous. For porous membrane, viscous flow and size exclusion or mass influence the transport rate and selectivity of separation process. As for non-porous membrane, membrane materials and interactions will affect the transport rate and selectivity of protein separation process (George & Thomas, 2001).

Membrane technology is able to remove small amount of substances by yielding large amount of purified product. Removal of small amount of substances can be done by retention of small fraction by membrane or selective permeation of small fraction through the membrane. More than two components can be separate by membrane even in low amounts in a solution. Fractionation of biomolecules can be done by using UF, MF or NF membrane (Ulbritch, 2006) .Membrane technologies were established in large scale. MF use for particle removal is various industries while UF use in water treatment and purification process. Membrane process classification based on size of solute to be separated is shown in Figure 2.4.

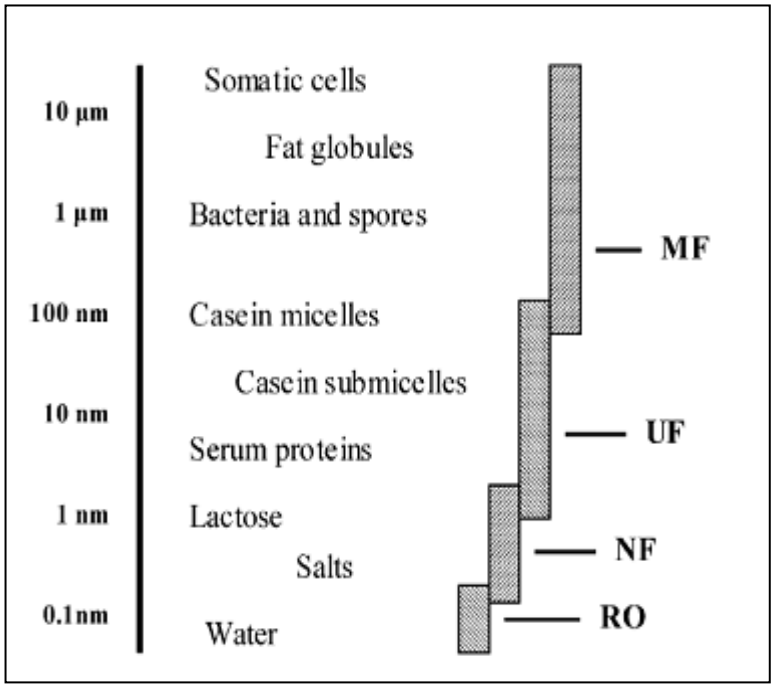


Figure 2.4 Membrane process classification based on size indication (Source: Brans et al., 2004) MF: microfiltration, UF: ultrafiltration, NF: nanofiltration, RO: reverse osmosis

2.4.1 Membrane Types and Materials

Membrane is classified to inorganic and polymeric type. The interface of membrane may be molecularly homogeneous and may be chemically or physically heterogeneous. By convention, filter is usually limited to structures that separate particulate suspensions larger than 1 to 10 µm.

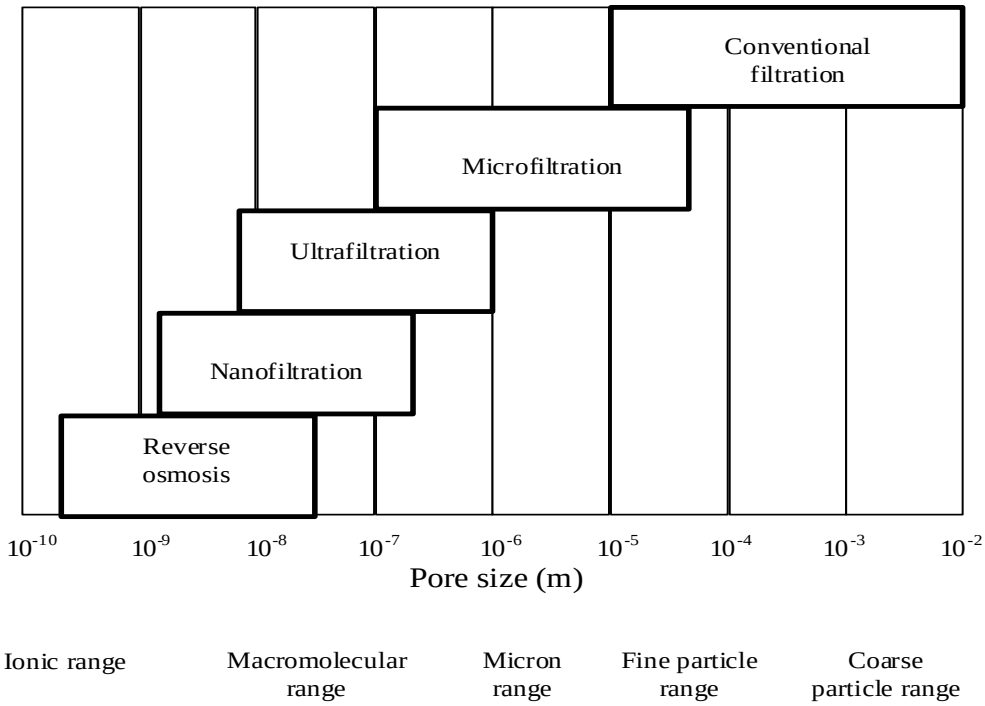


Figure 2.5 Membrane characterizations with pore size.

There are various types of materials used as a membrane chromatography. Polymeric and inorganic polymers as well as mixed matrix and composite are materials widely use in separation of proteins. Examples of polymeric membranes are polycarbonate (PC), polyimide (PI), polyamide (PA), polypropylene (PPE) and polyethylene (PE). Inorganic membrane can be classified into carbon, zeolites, ceramic, metal and glass.

Cellulose is a basic material which used in preparation of membrane. Cellulose membrane has limitations due to its small pore size and not suitable for high pressure drop chromatographic process. Cross linking may increase the stability of cellulose membrane. Regenerated cellulose and cellulose acetate membrane may obtain from phase inversion. These membranes have hydrophilic surfaces and low

nonspecific protein binding. Cellulose derivative membranes contain reactive hydroxyl group and various ion-exchange groups suitable to couple to cross-linked regenerated membrane such as sulfonic acid and diethylamine groups. Epichorohydrin (EPI) mixed with NaOH at 50 ℃ was use to cross-link with cellulose membrane for approximate 3 hours to obtain chemically and mechanically stable membrane. Larger pore sizes were also acquired (Zeng & Ruckenstein, 1999).

Polysulphone membrane has good film-forming and thermal resistance characteristics. Stability of polysulphone is high and it can be easily modify without change polymer properties. Besides that, microporous polyamide is also a good choice of material selection. Narrow pore size and mechanical rigidity is advantages of microporous membrane. Adsorptive membranes can be prepared by grafting a monomer or copolymer into a base membrane (Charcosset, 1998).

2.4.2 Spacer Arm Length

The spacer arm length is the length of hydrocarbon chain exists between functional ligand and specific solid support. Low molecular ligand might show poor function due to low steric availability. From previous studies, spacer arm length will affect the performance of the separation process. Spacer longer than 12 atoms will offer favorable results at high ligand concentration. However, the possibility of arm folding increase with long spacer which will lead to reduction in capacity and this is unfavorable condition. According to DePhillips et al (2004), greatest retention occur for the adsorbent with no spacer arm length by examine the effect of spacer arm on protein retention on a cation-exchange adsorbent. Besides that, optimum spacer arm

length for a binding system should have no active center and ensure the functional group easy to access to molecules (Zou et al., 2001).

Mehta and Zydney (2009), examined the effects of spacer arm length on performance characteristics of electrically charged ultrafiltration membrane using cytochrome c. Membrane generated by 1-amine 6-hexanol contain single secondary amine leading to significantly less protein retention than membrane having two amine group. Several researches have been done to prove that tentacle type ligands can provide higher binding capacities than conventional ion exchange resins. The flexibility of tentacles (polyelectrolyte chains) allow the binding occur at multipoint interactions between proteins and charge groups on the functional ligands.

2.4.3 Membrane Modification

Surface modification technique is gaining interest in separation field because modified membrane is able to minimize limitations of existing membrane. Fouling will reduce membrane flux and may chemically degrade the material of membrane. Increase of hydrophilicity will create better fouling resistance. Increasing of surface roughness will enhance fouling resistant. Porosity, pore size, hydrophilicity, contact angle and roughness will affect the performance of membrane.

Blending, curing and grafting are techniques use for polymer modification. Blending is mixing of two or more polymers in requisite properties by physical means. Grafting is a method where monomer bond to polymer chain covalently. Curing is a method which produce smooth surface by fill in the valleys in the surface.

Carboxylic polyethersulfone (CPES) was prepared by using blending CPES to PES at ratio of 1:10, 1:4 and 1:2 based on weight basis. The increased of blended concentration caused the fluxes of blended hollow fiber membrane increased (Wang et al., 2011). Selective base membrane and suitable modification process (operating conditions) will change the properties and functional group of the membrane surface (Rana & Matsuura, 2010). Figure 2.6 shows the schematic view of polymer modification methods.

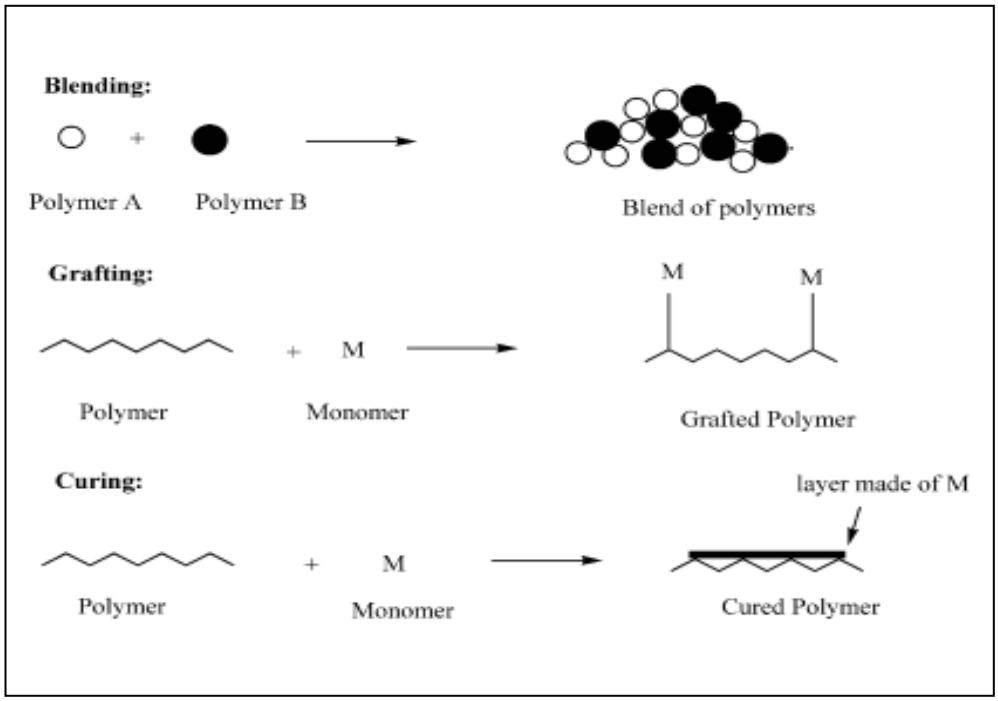


Figure 2.6 Schematic view of polymer modification methods (Source: Bhattacharya & Misra, 2004).

2.4.4 Grafting

Grafting is a method where monomer is bonded to a functional of polymer chain. There are several techniques of grafting such as chemical treatment, radiation, enzymatic grafting and plasma-induced techniques. Grafting maybe initiated by chemical means either via free radical path or ionic path. Grafting may proceed by radiation method with the ions form through high-energy irradiation to form polymeric ion. Carlmark and Malmstroem (2002) have done surface grafting by immobilized 2-bromoisobutyryl bromide with hydroxyl group on a filter paper. The modified paper then immersed into solution containing methyl acrylate, Cu(I)Br, tris-2-(dimethyl amino)ethyl amine, sacrificial initiator and ethyl acetate. The filter paper represented the cellulose and surface grafting done by controlled living radical polymerization which combined the conventional free radical and ionic polymerization. Schematic route for this reaction is shown in Figure 2.7.

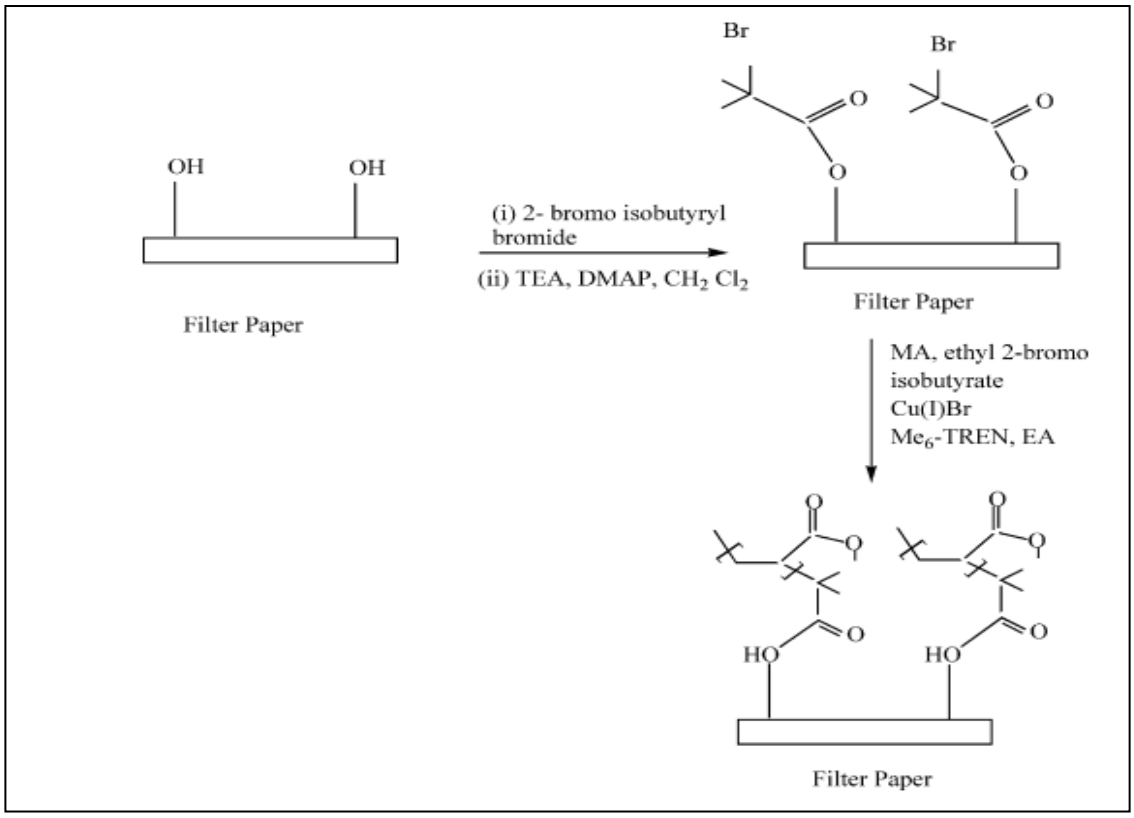


Figure 2.7 Schematic presentation of surface grafting on cellulose (Source: Carlmark & Malmstroem, 2002).

Radiation method was effective to prevent impurities. Radiation process is free from contaminants and the purity of the product can be maintained. Direct preparation of anion-exchange membrane has done by attachment of poly((2-(methacryloyloxy)ethyl)-trimethylammonium chloride) onto the surface of hydrolyzed PP membranes by using photo-grafting method. Ion-exchange group will either expand or shrink in grafted chains depend on the ionic strength and pH. The reduction of swelling and improving of stability shown by cross-linked grafted chains (He &Ulbricht, 2008).

Enzymatic grafting is a new technique where still not apply widely. The process involve enzyme to initiates the chemical grafting reaction. According to Cosnier et al (2002), thionine and toluidine blue bounded irreversibly to the

backbone of poly(dicarbazole) and grafting of polyphenol oxidase (PPO) on polydicarbazole has been done.

2.4.5 Role of Cross Linker in Membrane Modification

Cross link is a covalent or ionic bond that links one polymer chain to another. The cross linking promote a difference in the polymer's physical properties. The modification of mechanical properties of a membrane depends strongly on the cross linked density. Cross link can be formed by chemical reaction. The chemical reaction can be initiated by heat, pressure or by radiation. The mixing of un-polymerized (unmodified membrane) to specific chemicals called as cross linking reagents forms cross links. Cross linking can also be induced by exposure of membrane to a radiation source. Normally, ultraviolet light will be use as the radiation source (Ulbricht, 2006).

Some common cross linkers are N-hydroxysuccinimide ester, formaldehyde and epichlorohydrin. The cross linkers will promote nucleophilic attack of amino group and subsequent covalent bond by cross linker. Currently, membrane separation technology was facing instant challenge to achieve high selectivity while retaining the productivity. Cross-linking modification is one approach to improve the separation properties.

Polyimide membrane was immersed in 10%(w/v) of p-xylenediamine in methanol for a period of time. The modified membrane was found to be useful for gas separation of hydrogen and nitrogen. The selectivity of carbon dioxide toward

methane for cross linked film was found to be higher than the ideal selectivity (Tin et al., 2003). Through the reaction of ammonia as functional cross linking agent, the pore structure of membrane could be stabilized in a three dimensional network. The chemical stability was increased that the membrane can be use for separation of strongly acidic or alkaline aqueous solution (Ulbricht, 2006).

Cross linking agents contain at least two reactive groups including amines and sulfhydryls. Functional groups that can act as cross-linking agents are primary amines, carboxyls and carboxylics acids. Protein molecules have many of these groups hence the proteins can conjugated using cross linking agents. The selectivity of cross linking basically based on chemical specificity, spacer arm length, water solubility and cell membrane permeability.

2.4.6 Modification of Existing Membrane

Many attempts have been made to enhance the performance of membrane by membrane modification. Different types of chemical modification have been carried out by using different monomer attached toward different base membranes. The modification adapted new interaction to membrane to attract its desired molecules. Most modification done was on the ion-exchange interaction where the installation of charged monomer to the surface of membrane further enhances the binding of selective molecule which is in opposite charge. Table 2.3 shows selected membrane modification processes that have been done.

Table 2.3 Selected membrane modification process.

Author	Base Membrane	Ligand/ Interaction	Chemical Modifications
He & Ulbricht (2008)	Hydrophilized PP microfiltration membrane (cut-off pore diameter 0.2 μm)	Anion-exchange	• Attachment of poly((2-methacryloyloxy)ethyl)-trimethylammonium chloride via photo-grafting techniques. • Two different grafting routes are use: Synergist immobilization and adsorption. • For adsorption method, membrane soaked into benzophenone solution in methanol. For synergist method, membrane immersed in DEEDA. • Both membrane immersed in MAETMAC solution followed by 15min UV radiation
Fregar et al. (2002)	TFC polyamide membrane	Anion-exchange	• A redox system composed by potassium persulfate and potassium metabisulfite use to generate radicals. • Polymer was initiated by attachment of monomer than undergo propagation. • Hydrophilic monomers included Acrylic (AA), methacrylic acid, polyethylene gycol methacrylate (PEGMA), sulfopropyl methacrylate (SPM). • A cross linker was added to the reaction solution.

Table 2.3 "Continued"

Author	Base Membrane	Ligand/ Interaction	Chemical Modifications
Mehta & Zydney (2008)	Ultracel composite regenerated cellulose membrane (30kDa and 100kDa cut off)	Anion-exchange	• Hydroxyl group of membrane coupling with epichlorohydrin in specific condition. • The membrane disk was immersed in NaOH solution. • The membrane was removed, rinsed and immersed with diamine solution with pH approximately 11.
Zeng & Ruckenstein (1998)	Chitosan membrane	Anion-exchange	• The macroporous Chitosan membranes were cross-linked with ethylene glycol diglycidyl ether (EDGE) aqueous solutions. • The membrane was washed and kept buffer overnight. • Ion-exchange capacity was determines using potentiometric titration method. • Membrane rinsed with CO_2-free distilled water, treated ith anhydrous ethanol and air dried.
Wickramasi-nghe et al. (2008)	Regenerated cellulose (RC) membrane	Anion-exchange	• Initiator molecules anchored to the membrane pore surfaces in a first step. • Atom transfer radical polymerization (ATRP) was used in second step to graft poly(2-dimethylaminoethyl methacrylate) (DMAEMA)

Table 2.3 "Continued"

Author	Base Membrane	Ligand/ Interaction	Chemical Modifications
Cheng et al. (2010)	P84 co-polyimide	Anion-exchange	• P84 polymer used to prepare anion-exchange membranes by: (1) Phase inversion where P84 powder dissolved in NMP and cast onto a piece of non-woven cloth. The non-woven cloth immersed in IPA and then immersed into methanol. (2) Amination with diamine where the membrane soaked into ethylene diamine/diamine butane/methanol solution. (3) Methylation with methyl iodide. (4) All membranes were post treated by soaking in HCl and wash with deionized water.
Rohani & Zydney (2009)	Ultracel™ composite regenerated cellulose membrane (30kDa molecular weight cut-off)	Cation-exchange	• All membrane was soaked in isopropanol to remove any wetting agents and then rinsed with deionized water. • Positively charged membrane was generated by chemically attachment of quaternary amine to hydroxyl group by using proprietary solution chemistry by Millipore. • Chromatography experiment performed by using a binary mixture of lysozyme and cytochrome c.

Table 2.3 "Continued"

Author	Base Membrane	Ligand/ Interaction	Chemical Modifications
Rohani et al. (2010)	Ultracel™ composite regenerated cellulose membrane (100kDa molecular weight cut-off)	Cation-exchange (amine group)	• All membrane soaked in isopropanol to remove any storage agents. • Amine ligands attached to glucose monomers in membranes by: a)1,10-diaminodecane b)1,4-diaminobutane c)pentaethylenehexamine d)1,6-diaminohexane followed by 2-aminoethyltrimethyl ammonium chloride e)2-aminoethyl trimethyl ammonium chloride
Zhai et al. (2002)	Microfiltration membrane	Hydrophilic	• Thermally-induced molecular grafted copolymerization of 4-vinylpyridine (4VP) with ozone preactivated poly(vinyldene fluoride) (PVDF) in N-methyl-2-pyrrolidone (NMP) solution to produce 4VP-g-PVDF copolymer. • 4VP-g-PVDF copolymers with different graft concentrations were cast into microfiltration membranes by phase inversion.
Wang et al. (2011)	Polyethersulfone (PES) membrane	Ion-exchange	• Acetylating reaction steps, PES was dissolved in N-methylpyrrolidone (NMP) with Acetyl chloride was as acetylating agent and $AlCl_3$ use as catalyzed. • Oxidating reaction, PES-$COCH_3$ was dissolved in NMP solutions which consist of $KMnO_4$, NaOH and double distilled water. • Functional polyethersulfone (PES) was prepared by blending method with ratios of CPES to PES 1:10, 1:4 and 1:2(weight ratio).

CHAPTER 3

METHODOLOGY

3.1 Materials

Regenerated cellulose (RC) membrane with pore size of 0.45μm was purchased from Whatman Ltd. Epichlorohydrin (EPI), 1,2-diaminoethane and 1,4-diaminobutane were purchased from Merck. All reagents were under analytical grade and use without any further purification. Different concentrations of NaOH in a range of 0.05M to 0.5M were used as activation or hydrolysis agent for the modification process. 0.1M Hydrochloric acid (HCl) was used as pH adjuster when necessary.

Bovine serum albumins (BSA) with purity range of 95 to 99% was supplied from Sigma-Aldrich and used as model protein for binding. BSA had an isoelectric point of 4.7 at 25℃ and molecular weignt ъ6,120. 20mM sodium phosphate buffer pH 7.0 was used as binding buffer, which prepared from monosodium phosphate monohydrate ($NaH_2PO_4.1H_2O$) and sodium dihydrogen phosphate heptahydrate ($NaH_2PO_4.7H_2O$). Elution buffer was a 1.0M of sodium chloride (NaCl) dissolved in binding buffer.

3.2 Modification of Regenerated Cellulose Membrane

Modification process was performed on 47mm diameter of RC membrane disk. The membrane disk was handled carefully with limited contact to the edges of the membrane to avoid any damage on the membrane. Each membrane was soaked in ultrapure water for at least 45 minutes to clean the membrane surface. The water was replaced changed for two times with fresh water. The membrane was sonicated in an ultrasonic cleaner for 15 minutes to remove impurities bond to the membrane surface. The membranes were then stored in 0.1M NaOH solution overnight for membrane swelling.

Figure 3.1 shows the schematic reaction involves in the modification process. Membranes disk was removed from NaOH and clean with soft tissue to remove excess solution on the surface. Clean membrane was immersed in solution containing 10mL of 0.1M NaOH and 5mL of EPI for cross-linking reaction. The reaction was carried out in an incubator shaker with temperature adjusted to 45℃ and agitated at 150rpm for 2 hours (Step 1 in Figure 3.1). The membrane was removed and rinsed with ultrapure water. Then, the membrane was wiped with soft tissue to remove excess water on the membrane surface.

Cross-linked membranes disk was immersed in 20mL of a 1.0M diamine solution with pH adjusted to 11.0± 0.2 by addition of small amount of 1.0M HCl as needed. The reaction was allowed to progress at 45℃ with agitation at 150rpm for 12 hours (Step 2 in Figure 3.1). Membrane was removed from the solution and rinsed with ultrapure water for at least 1 hour (Mehta and Zydney, 2008).

The above procedure was repeated using different concentration of NaOH from 0.05M to 0.50M (step 1 Figure 3.1) to study the effect of hydrolysis to the membrane efficiency binding capacity. In another experiment, the concentration of 1,4-diaminobutane monomer was varied from 0.25M to 2.0M (step 2 Figure 3.1) to study the effect of monomer concentration to the membrane binding capacity.

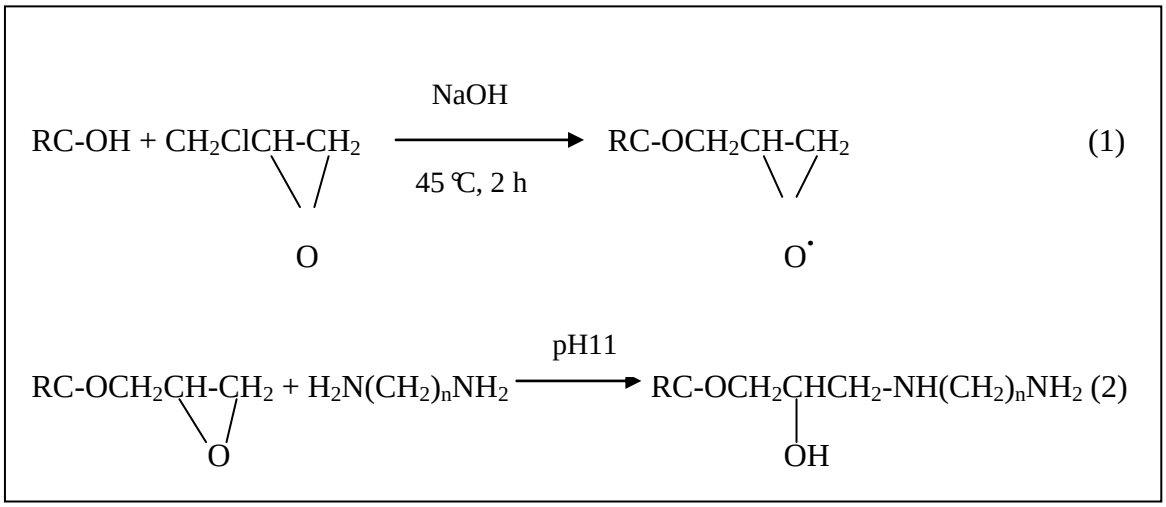


Figure 3.1 Schematic reaction chemistry for coupling of EPI and diamines to cellulose membrane to prepare anion exchange membrane chromatography (Source: Mehta and Zydney, 2008).

3.3 Weight of Membrane

The weight of membrane before and after modification was measured to determine the weight changed. Biotron model cleanvac 12 Freeze dryer was using to dry the wet membrane. Original weight of membrane was taken for the membrane after washing step. The weight change was calculated according to Equation 3.1:

$$\text{Weight change (\%)} = \frac{\text{Weight of modified membrane} - \text{Weight of original membrane}}{\text{Weight of modified membrane}}(100)$$

Equation 3.1

3.4 Protein Binding and Elution

Membrane disk was cut into a rectangular shape of 1cm x 2cm dimension for binding experiment. The weight of membrane was measured. Modified membrane was equilibrated for 3 hours in 1.5ml of sodium phosphate buffer pH 7.0. Equilibrated membrane was then incubated with 1.5ml of 2mg/ml BSA solution dissolved in binding buffer for 12 hours at room temperature. The membranes were removed from BSA solution and wiped gently with soft tissue to remove excess protein solution on surface. All steps were carried out using 2.0ml centrifuge tube and rotated on rotator as shown in Figure 3.2 at 15rpm speed.

The bound membrane was incubated with 1.5ml of elution buffer for 3 hours to elute the bound BSA. After elution process, the membrane was removed and wiped with soft tissue to remove excess solution and stored in phosphate buffer pH 7.0 for further used if necessary. All solution after protein binding and elution step was collected for further analysis.

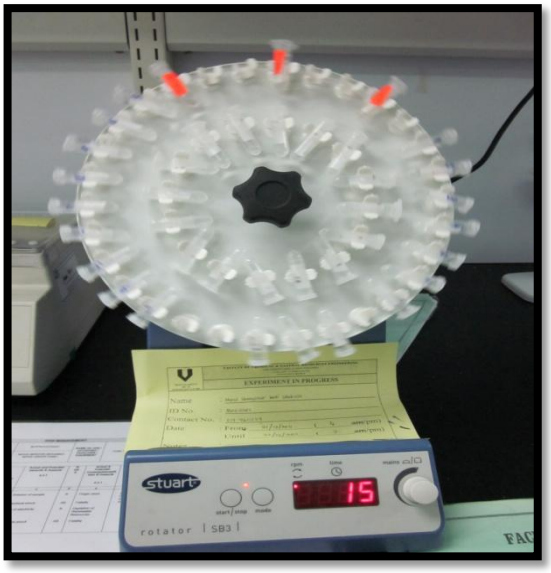


Figure 3.2 Centrifuge tube rotated on rotator at 15rpm

3.5 Protein Concentration Analysis

UV-vis spectrophotometer was used for quantitative determination of BSA proteins concentration. The protein absorbance at 280nm was measured using Hitachi U-1800 model Uv-vis spectrophotometer. The BSA concentration was calculated based on the absorbance-concentration curve developed from known BSA concentrations.

Three sets of BSA standard curve were prepared in sodium phosphate buffer solution. The BSA concentrations in standard are 1.0mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0.0625mg/ml and 0.03125mg/ml.

3.6 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was used to analyze the functional group in the membrane samples. FTIR spectrum of membrane sample at different stages of modification process was collected to track the progress of modification process. Dried membrane samples were cut into small pieces and subjected to FTIR measurement on Nicolet Avatar 370 DTGS FTIR model. Unmodified membrane was also analyzed by FTIR as a control experiment. The peak transmission at different wavelength was analyzed from FTIR spectrum which is representing by different functional groups.

3.7 Water Flux Test

Original membrane and modified membrane were cut into 2.6cm diameter circle shape for water flux measurement. Membranes were immersed in ultra pure water and undergo ultrasonic cleaned around 2 minutes before water flux test. The membranes were fixed tightly into Amicon stirred cell Model 8010 as shown in Figure 3.3.

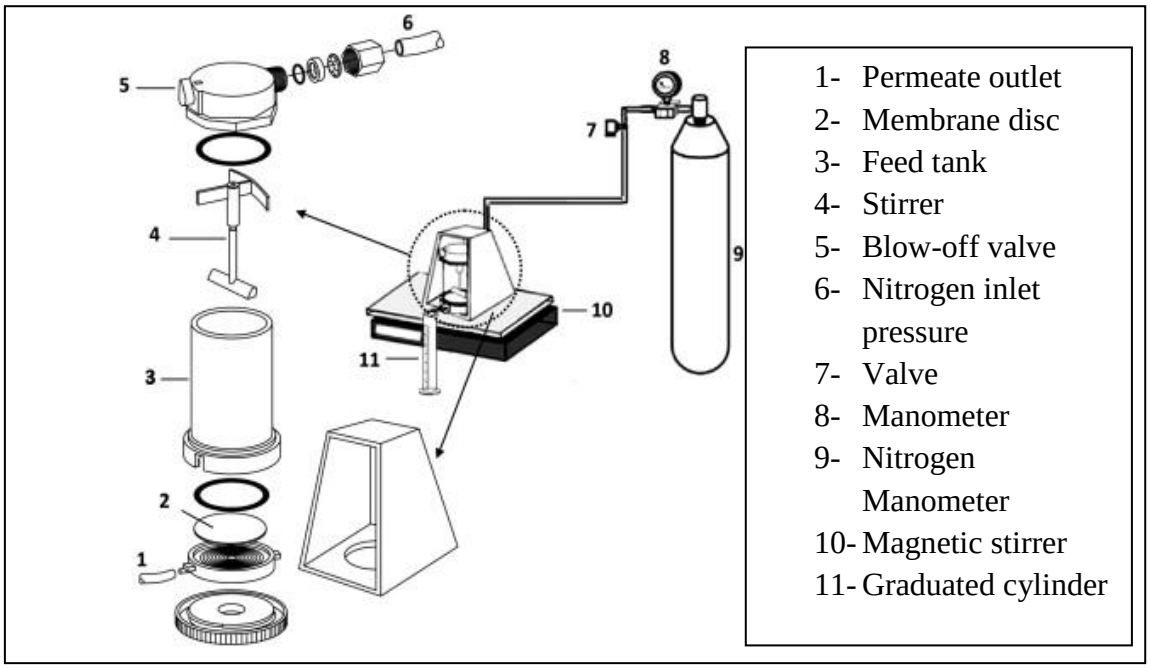


Figure 3.3 Schematic diagram of the Amicon stirred cell and experimental set-up (Source: El-Abbasia et al, 2011)

The stirred cell was filled in with clean de-ionized water from the upper inlet hole (gas inlet hole) and stirred around 200 to 300 rpm speed. No pressure is needed during this test due to the membrane used was in microfiltration (MF) range that had big pore size. Water could penetrate through MF membrane without pressurized

condition. The time taken to permeate 5 ml water through the membrane was taken. The flux was calculated using equation 3.2.

$$\text{Flux, } J = \frac{V}{A.t} \qquad \text{Equation 3.2}$$

where v is volume of permeated in litre, A is the area of membrane in m^2while t is the time taken in hour. The flux calculated was in the unit of $\left(\frac{L}{m^2 \cdot h}\right)$.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Membrane Performance Modified with Different Monomer

The concentration of protein after binding and elution fraction was calculated using the standard curve of bovine serum albumin (BSA) shown in Appendix A (Figure A.1). The equation of y=0.6444x was obtained with the y represent the value of absorbance at 280nm and x represent the BSA concentration in mg/ml. The standard curve follows the Beer-Lambert law showed a linear relationship between absorbance and concentration of the protein (Skoog et al., 2007).

Membrane with rectangular dimension of 1cm x 2cm was incubated with 1.5ml of 2mg/ml BSA solution. The binding capacity of membrane was calculated as mass of BSA bound per area of membrane (mgBSA/cm^2membrane). As a control experiment, unmodified regenerated cellulose (RC) membrane was also tested for protein binding. The binding capacity and elution recovery of unmodified RC was shown in Table 4.1.

Unmodified RC membrane showed relatively low binding capacity of 0.022 ± 0.008 mg BSA/cm^2 membrane. RC membrane does not have enough active functional group to interact with negatively charged BSA. The only active functional group exist in original RC membrane is hydroxyl (OH) group but it is less chemically active state.

RC membrane was chemically grafted with two different spacer arm length of diamine monomers of 1,2-diaminoethane and 1,4-diaminobutane. Spacer arm length refer to the length of hydrocarbon chain exist between the functional ligand and solid support. Both 1,2-diaminoethane and 1,4-diaminobutane are primary amines where one organic group is attached to the nitrogen. 1,4-diaminobutane monomer has longer spacer arm length than 1,2-diaminoethane.

Table 4.1 Protein binding capacity and elution recovery of various type of RC membrane

Monomer Type	Binding capacity (mg BSA/cm^2 membrane)	Protein recovery (%)
Unmodified RC membrane	0.022 ± 0.008	81.6 ± 14.5
1,2-diaminoethane	0.192 ± 0.014	62.0 ± 8.7
1,4-diaminobutane	0.298 ± 0.041	53.3 ± 10.9

The binding capacity of RC membrane modified with 1,4-diaminobutane monomer is higher than 1,2-diaminoethane monomer as shown in Table 4.1. The binding capacity was increase about 55.21% when the number of carbon increased by 2 carbons chain from 1,2-diaminoethane to 1,4-diaminobutane.

Compared to unmodified RC membrane both modified membrane showed an increased about 88.51% and 92.62% BSA binding capacity for 1,2-diaminoethane and 1,4-diaminobutane monomer respectively. The capacity increased due to the addition of positively functional group of primary amines modified RC membrane which can be able to bind negatively charged BSA. Figure 4.1 shows the molecular structure of RC membrane at different stages of modification process.

As comparison, BSA binding by using 1.2 μm and 5.0 μm nylon-poly(2-(methacryloyloxy) ethyl succinate (poly(MES)) membrane modified with nitrilotriactetate (NTA)-CU^{2+} achieved 80 ± 2 mgBSA/cm^3 and 24 ± 4mg/cm^3 BSA respectively with BSA used was 5mg/ml (Jain et al., 2012). This binding capacity was higher as compared to this study BSA binding capacity by using RC membrane grafted with 1,2-diaminoethane and 1,4-diaminobutane which are 0.257mg BSA/cm^3 and 0.398mg BSA/cm^3 with BSA used was only 2mg/ml.

Wang et al. (2011) prepared anion exchange membrane by blending carboxylic polyethersulfone (CPES) with PES at different ratio CPES to PES 1:4 and 1:2. BSA binding capacity obtained was 9.50 ± 0.4 μg/cm^2 and 7.61 ± 0.2 μg/cm^2 for CPES to PES ratio 1:4 and 1:2 respectively (Wang et al., 2011). As compared to RC membrane modified in this study, BSA binding capacity of 191.75 ± 14.49 μg/cm^2 and 298.09 ± 41.26 μg/cm^2 was achieved which is higher than functional CPES membrane.

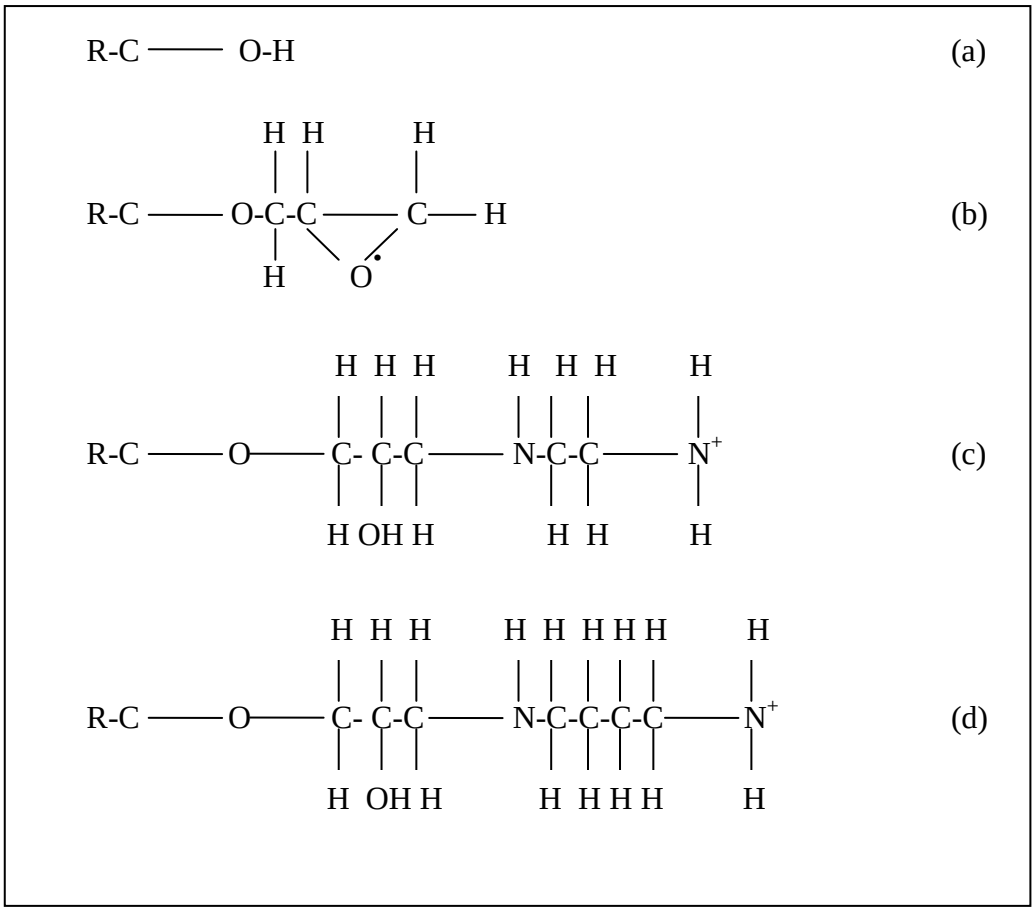


Figure 4.1 Chemical structure for RC membrane at various stages of modification process: (a) original, unmodified RC membrane, (b) RC membrane after epichlorohydrin (EPI) cross-linked, (c) modified RC membrane with 1,2-diaminoethane and (d) modified RC membrane with 1,4-diaminobutane
R: alkyl group

4.2 Characterization of Modified RC Membrane Chromatography

Infrared (IR) spectroscopy obtained from FTIR analysis was studied to determine the successful of membrane modification steps. Table 4.2 summarized the important functional group exist in original membrane; membrane cross-linked with EPI and membrane modified with diamine monomer based on the FTIR spectrum showed in Appendix C1 to C4.

Peak for N-H and C-N for primary and secondary amines was in range of 3300 to 3555 while peak for C-N group was between 1020 to 1220 and 1250 to 1360 for aromatic amine (Solomans & Fryhle, 2008). No transmission peak for N-H and C-N found in original membrane sample indicated that no amine group exists in the original unmodified RC membrane structure. Both N-H and C-N functional groups were identified in modified membrane as a proved that diamine group was successfully grafted to the membrane structure.

Table 4.2 IR transmission peak from FTIR spectrum of original membrane, membrane cross-linked with EPI and membrane modified with diamine monomers

Type of Membrane	Molecular Motion	Wavenumber (cm^{-1})
Original	O-H	3250.5/1101.8
	CH_2	2892.4
	C-H	1740.5/1426.5/1378.7/1319.6/1264.2
	C=C	1644.5
	C-O	1205.2
Membrane with EPI cross linker	O-H	3133.5
	C-C	1646.5
	C=O	2150.3
	C-O	1275.3/1238.4/1074.0/1205.2
	C-H	1360.5/896.85/714.14/645.5
Membrane grafted with 1,2-diaminoethane	N-H	3542.07
	C-N	3117.33/1336.57/1315.80/ 1232.41/1199.36/1119.92
	C=O	1644.65
	C-O	1274.92
	C-H	2895.32/1419.78
	CH_3	1370.57
Membrane grafted with 1,4-diaminobutane	N-H	3542.32/3466.21
	C-N	1232.42/1199.33/1315.79/1079.5
	O-H	3247.50
	C=O	1644.76
	C-H	2896.5/1420.0
	C-O	1274.92/1315.79
	CH_3	1370.41

The change in membrane weight represents the progress of modification steps involve in preparing membrane chromatography. Figure 4.2 showed the weight of unmodified membrane and membrane modified with 1,4-diaminobutane monomer.

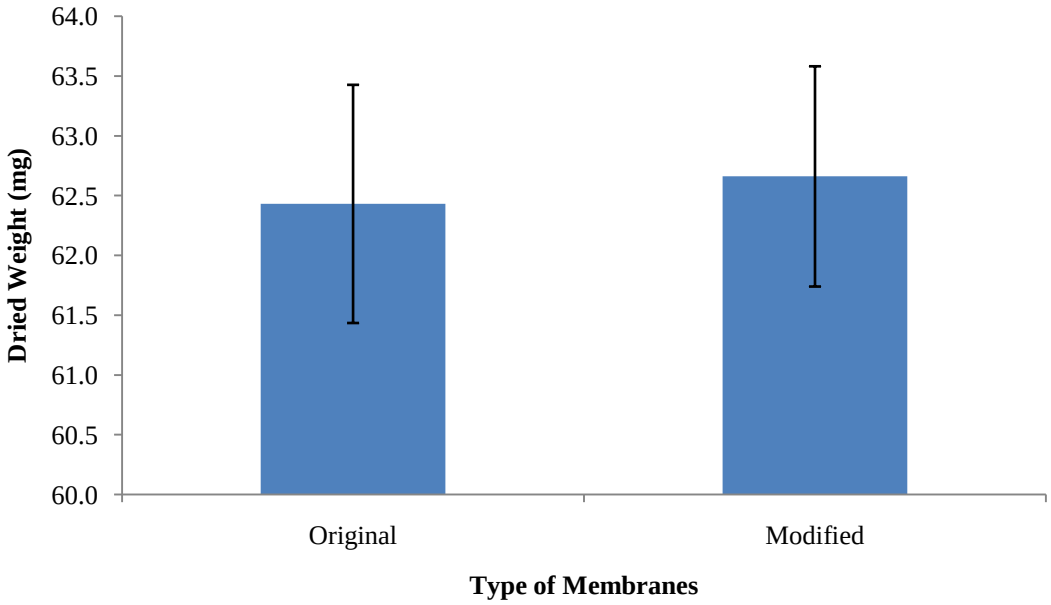


Figure 4.2 Weight of unmodified membrane and membrane modified with 1.0M 1,4-diaminobutane

Modified membranes were gained weight increment about 0.1mg to 0.4mg which was about 0.36±0.17%. Although this increment was low, but it showed that new molecule or atom of cross-linker or grafting monomer was successfully attached on the membrane structure after modification.

Water flux is the volume of water passing through the membrane per unit time per unit membrane surface area. For a modified membrane, a layer of cross-linker probably was formed on surface that gave resistance for water to pass through the membrane. Hence, the water flux for modified membrane was expected to be

smaller than unmodified membrane. Figure 4.3 shows the water flux for original membrane and membrane grafted with 1,4-diaminobutane.

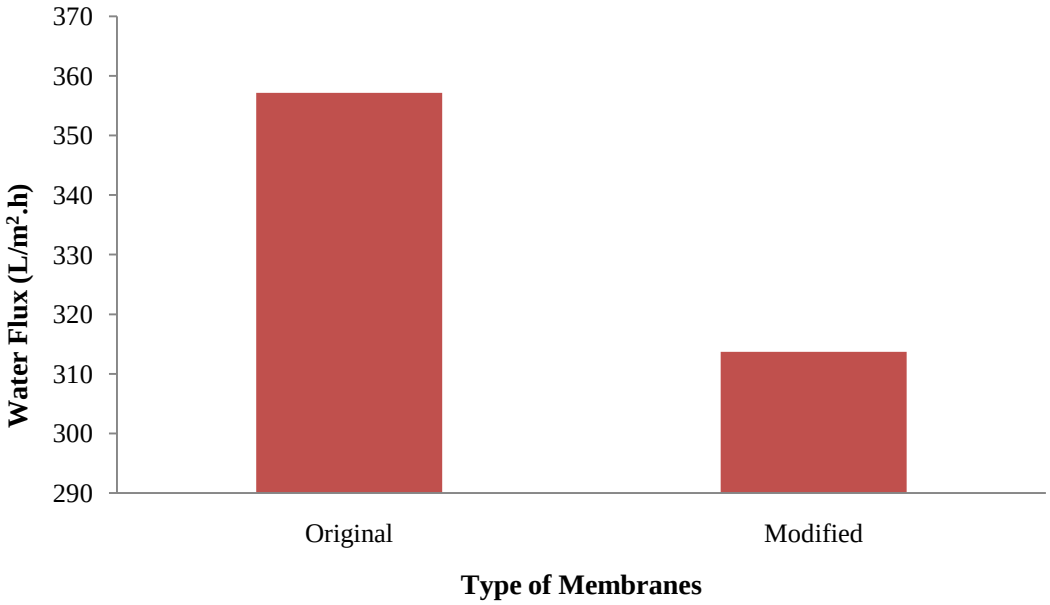


Figure 4.3 Water flux for original membrane and membrane modified with 1,4-diaminobutane

The original pore size of RC membrane was 0.45 µm. This range of pore size indicated that RC membrane used was in microfiltration (MF) range. After modification, the pore size of membrane was reduced due to the attachment of new cross-linker and monomer on the surface on membrane. Cross-linker that formed on the membrane surface formed act like a layer of barrier. The reduction of pore size caused the water molecule to have more difficulties when passed through the MF membrane. Hence, time taken for collection of 5ml water was longer for membrane reacted with cross-linker and grafted with monomer.

4.3 Effect of NaOH Concentration during Activation on the Performance of Membrane Chromatography

During activation step, NaOH is functioned as a hydrolysis agent and swelling agent. In NaOH hydrolysis, H^+ ion was removed from the RC membrane to create an active RCO^- site for coupling with EPI. EPI cross-linked RC membrane later was grafted with diamine ligand to produce anion exchanger membrane chromatography. In this study, the concentration of NaOH varied from 0.05M to 0.50M, while other parameters were keep constant. The cross-linked membrane was grafted with 1.0M of 1,4-diaminobutane.

Table 4.3 and Figure 4.4 showed the data and plot of the performance of anion exchanger membrane chromatography activated using different concentration of NaOH.

The binding capacity was increased as the concentration of NaOH increased from 0.05M to 0.20M, and then decreased from the concentration of 0.25M to 0.50M. Optimum NaOH concentration achieved was at 0.20M which gave the highest binding capacity of 0.310 ± 0.033 mg BSA/cm^2membrane. After optimum concentration, the binding capacity does not show much impact as the concentration increased from 0.25M to 0.50M. Higher concentration of NaOH created more active site on membrane surface for reaction with cross-linker EPI, however it was limited to the available OH group in the specific area of RC membrane used.

Table 4.3 Binding capacity and protein recovery 1,4-diaminobutane membrane chromatography activated using different concentration NaOH

Concentration of NaOH (M)	Average binding capacity (mg BSA/cm^2 membrane)	Average protein recovery (%)
0.05	0.148 ±0.009	54.5 ±3.0
0.10	0.278 ±0.013	57.6 ±4.7
0.15	0.300 ±0.023	56.9 ±4.4
0.20	0.310 ±0.033	61.3 ±5.6
0.25	0.182 ±0.027	57.7 ±6.5
0.30	0.201 ±0.060	59.8 ±16.1
0.50	0.203 ±0.039	53.5 ±9.0

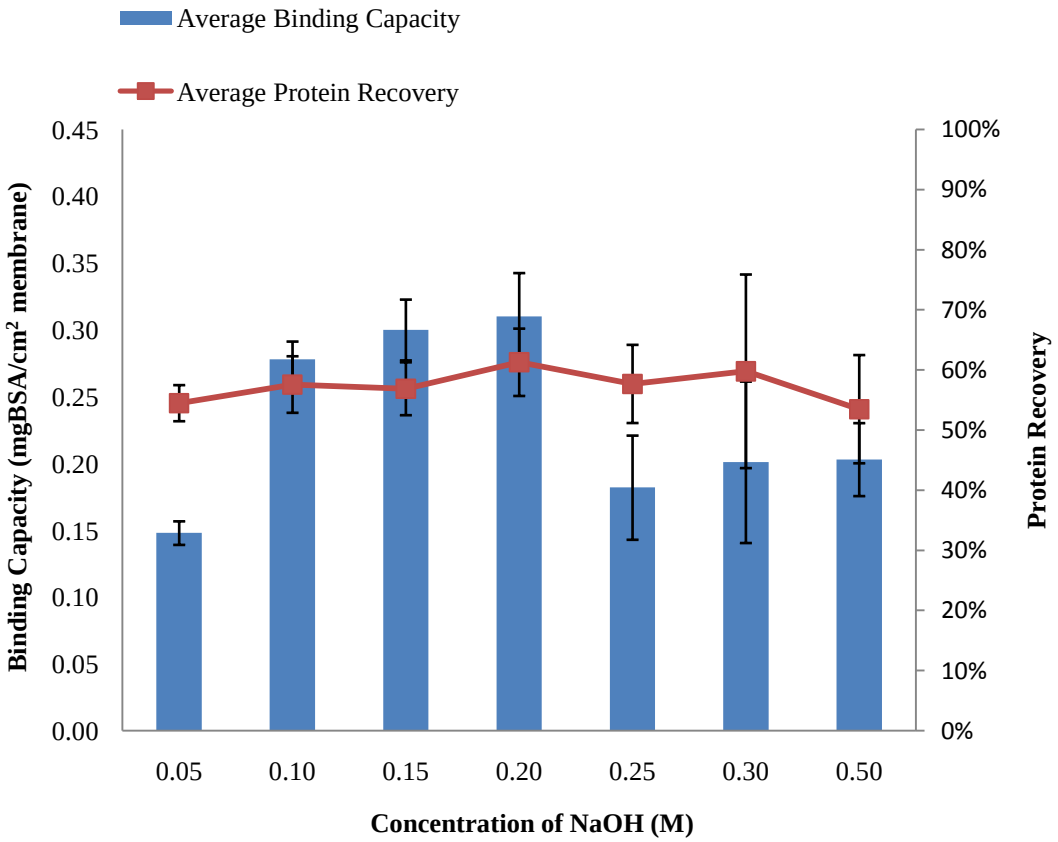


Figure 4.4 Binding capacity and protein recovery 1,4-diaminobutane membrane chromatography activated using different concentration NaOH

The percentage of protein elution recovery with the membrane activated using different concentration of NaOH showed a comparable recovery percentage within 53.5% to 61.3%. This signified that the modification of membrane does not affect the protein elution recovery too much.

FTIR was also used to analyze all membrane samples activated using different NaOH concentration as shown in FTIR spectrums in Appendix C, Figure C.5 to C.11. The peak for following particular functional groups of N-H and C-N were presented in all spectrums at the wavelength range of 3300 to 3555cm^{-1}, 1020 to 1220cm^{-1} and 1250 to 1360cm^{-1}. This proved that all NaOH concentrations ranges used in this experiment were able to swell and hydrolyzed the RC membrane for subsequent attachment of diamine group in grafting step.

4.4 Effect of 1,4-Diaminobutane Monomer Concentrations on the Performance of Membrane Chromatography

The effect of diamine monomer concentration from 0.50M to 2.00M on the protein binding capacity was studied at the optimum NaOH activation concentration of 0.20M achieved in the previous part. The protein binding at different diamine monomer concentration was shown in Figure 4.5 and Table 4.4.

Table 4.4 Binding capacity and protein recovery for different concentration 1,4-diaminobutane

Concentration of 1,4-diaminobutane(M)	Average binding capacity (mg BSA/cm^2 membrane)	Average protein recovery (%)
0.25	0.057 ±0.017	83.6 ±12.8
0.50	0.130 ±0.036	68.2 ±14.1
1.00	0.377 ±0.028	48.1 ±6.6
1.50	0.378 ±0.029	50.1 ±3.6
2.00	0.385 ±0.027	48.9 ±4.2

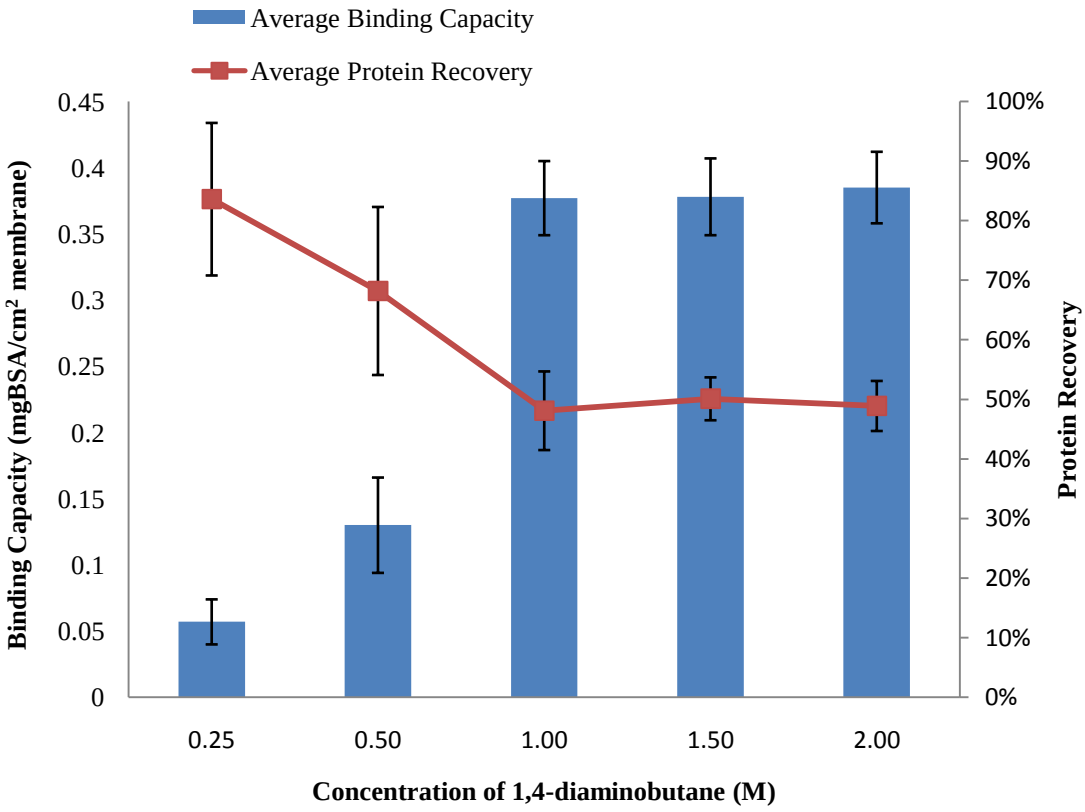


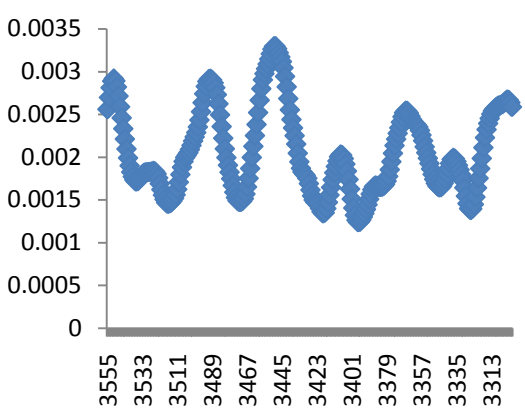
Figure 4.5 Binding capacity and protein recovery for different concentration 1,4-diaminobutane

By increasing diamine monomer concentration, the number of diamine molecules in the solution also increased. More diamine molecule can react with the cross-linker to form ion exchanger site on the membrane. The optimum concentration of 1,4-diaminobutane was found at 2.0M which gave the highest binding capacity of 0.385 mg BSA/cm^2 membrane. Protein binding was increased significantly when the monomer concentration increased from 1.0M to 2.0M. This drastic change occurred due to the available active site (RCO^-) on the membrane were progressively attached with the monomer molecules. When all the available active site was reacted, it will reach the maximum capability of monomer grafting onto the membrane. An increase on monomer concentration after this point will not increase significantly the binding capacity.

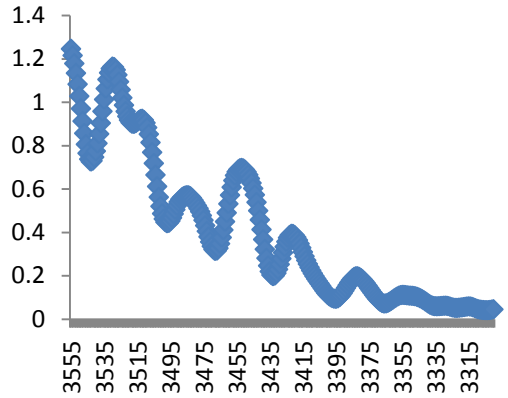
FTIR analysis was conducted to validate the existence of N-H and C-N functional groups in modified membrane. The wavelength for N-H group is 3300 to 3555cm^{-1} while for C-N group is between 1020 to 1220cm^{-1} and 1250 to 1360cm^{-1}. Based on the FTIR spectrum shown in Appendix C, Figure C.12 to C.16, the important peak for membrane grafted with different monomer concentration were tabulated in Table 4.5 below.

Table 4.5 IR transmission peak for representative groups of membrane modified with different concentration of 1,4-diaminobutane

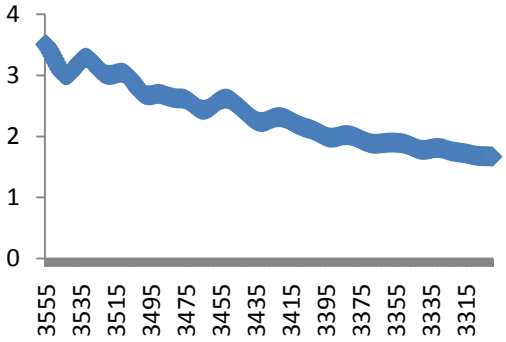
Concentration (M)	Molecular Motion	Wavenumber (cm^{-1})
0.25	N-H	3395.5/ 3496.8/ 3542.77
	C-N	1155.5/1199.34/ 1275.09/ 1315.81/ 1336.35
0.50	N-H	3464.88/ 3543.27
	C-N	1156.97/ 1199.26/ 1275.14/ 1315.79/ 1336.45
1.00	N-H	3542.76
	C-N	1155.0/ 1199.32/ 1315.83/ 1275.24/ 1336.22
1.50	N-H	3467.25/ 3495.49/ 3542.79
	C-N	1155.0/ 1199.33/ 1275.32/ 1315.82/ 1336.18
2.00	N-H	3431.91/ 3467.37/3542.15
	C-N	1155.00/ 1274.67/ 1199.11/ 1233.01/ 1274.67/ 1316.22



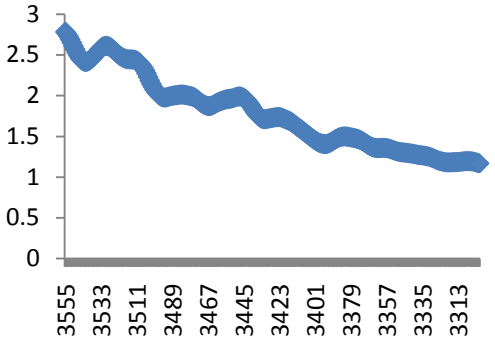
(a) Original Membrane



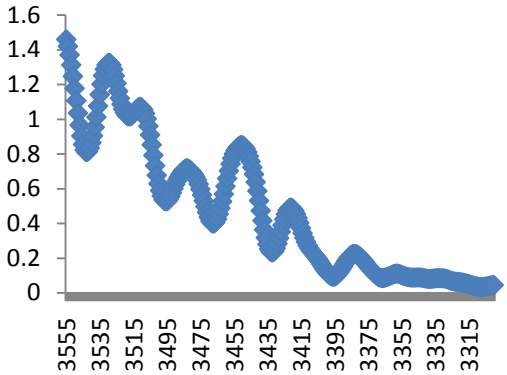
(b) 0.25M 1,4-diaminobutane



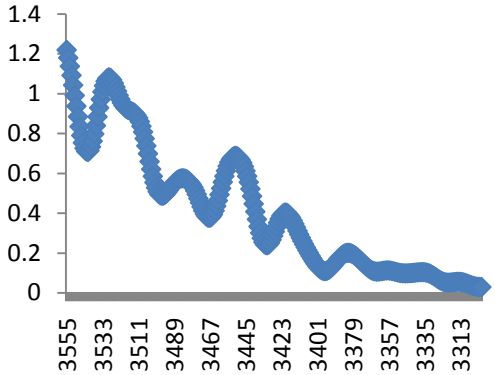
(c) 0.50M 1,4-diaminobutane



(d) 1.0M 1,4-diaminobutane



(e) 1.5M 1,4-diaminobutane



(f) 2.0M 1,4-diaminobutane

Figure 4.6 IR transmission peak for N-H functional group range 3300 to 3555cm^{-1} for (a) original membrane and membrane modified with (b) 0.25M 1,4-diaminobutane (c) 0.50M 1,4-diaminobutane (d) 1.0M 1,4-diaminobutane (e) 1.5M 1,4-diaminobutane (f) 2.0M 1,4-diaminobutane

For better visualization, the FTIR spectrum for N-H peak was zoomed as shown in Figure 4.6. For original membrane, the transmission peak within the N-H functional region was very low where all peaks were below 0.0035%T. After modified with 1,4-diaminobutane as monomer, there was a drastic change in the N-H region due to diamine group bind to the structure of membrane. It was clearly shown that membrane modified with 0.50M and 1.0M of 1,4-diaminobutane have higher percentage of transmission peak.

However, the binding capacity revealed different result where highest concentration 2.0M was supposed be the most effective. Hence, it was believe that there was other functional group such as C-N also contributed to the interaction with opposite charged protein. The existing of functional group C-N also proven that diamine binds successfully to the RC membrane structure.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In the current study, regenerated cellulose (RC) membrane was modified into anion-exchange membrane chromatography using different spacer arm length of diamine monomers. Binding effectiveness of two primary diamines which are 1,2-diaminoethane and 1,4-diaminobutane was compared. Membrane modified with longer spacer arm length 1,4-diaminobutane was found to have higher average binding capacity of 0.298 ± 0.041 mgBSA/cm^2 membrane. The study of different spacer arm length of diamine monomer and its performance as anion-exchange membrane chromatography was important to maximize the membrane toward the protein of interest.

The effect of NaOH activation agent in hydrolysis or membrane swelling was studied. Optimum concentration of NaOH was found to be 0.20M which gave the average binding capacity of 0.310 ± 0.033 mgBSA/cm^2 membrane. Further increased of NaOH concentration does not increase the protein binding.

A few membrane characterization techniques were applied to prove the success of the modification process. One of them is by measuring the membrane weight changed. Modified membrane showed a weight increased about $0.36 \pm 0.17\%$ which indicated that the membrane stru res undergo addition or alteration on it surface molecular structure. Membrane modified with 1,4-diaminobutane also showed lower water flux compared to original unmodified membrane. The pore size of modified membrane was altered into small size and produced a resistance for water to pass through the membrane.

FTIR analysis gave a more convincing result for the modification of RC membrane into anion-exchange membrane. The infrared transmission peak obtained for all modified samples shown the increasing and existing of N-H and C-N functional group. These functional groups do not exist or exist in a very minor transmission peak for original membrane. The increasing of N-H and C-N transmission percentage indicated that diaminie monomers do bind to the active site on membrane surface.

Different concentration of 1,4-diaminobutane monomer affect the effectiveness of BSA protein binding on anion exchanger membrane chromatography. Higher monomer concentration produced more active positive site on membrane surface to enable high protein binding. 2.0M of 1,4-diaminobutane was found to have highest binding capacity of $0.385 \pm 0.027 mgBSA/cm^2$ membrane.

5.2 Recommendations

In order to improve the result and further develop the scopes of this research, the following recommendations have been outlined:

1. The time of reaction for membrane to react with cross linker epichlorohydrin (EPI) can be study to maximize the reaction between the cross linker and membrane structure.
2. The time of reaction for membrane to react with diamines monomers can be manipulate to optimize the number of diamines molecules bind to cross linker on the membrane surface.
3. Other diamines such as 1,6-diaminohexane and even secondary and tertiary diamines may be used for a more precise comparison of effect of spacer arm length.
4. Membrane with different configuration or pore size maybe used for comparison on pore size reduction after grafting.

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APPENDIX A Standard Curve for Bovine Serum Albumin using UV-Vis Spectrophotometer

Table A.1 Absorbance for three sets of BSA serial dilution

Concentration (mg/ml)	Absorbance			Average	Standard Deviation
	Set 1	Set 2	Set 3		
1.00	0.708	0.533	0.681	0.6407	0.0942
0.50	0.357	0.296	0.324	0.3257	0.0305
0.25	0.183	0.136	0.175	0.1647	0.0251
0.125	0.086	0.065	0.091	0.0807	0.0138
0.0625	0.051	0.043	0.064	0.0527	0.0106
0.03125	0.011	0.029	0.053	0.0310	0.0211

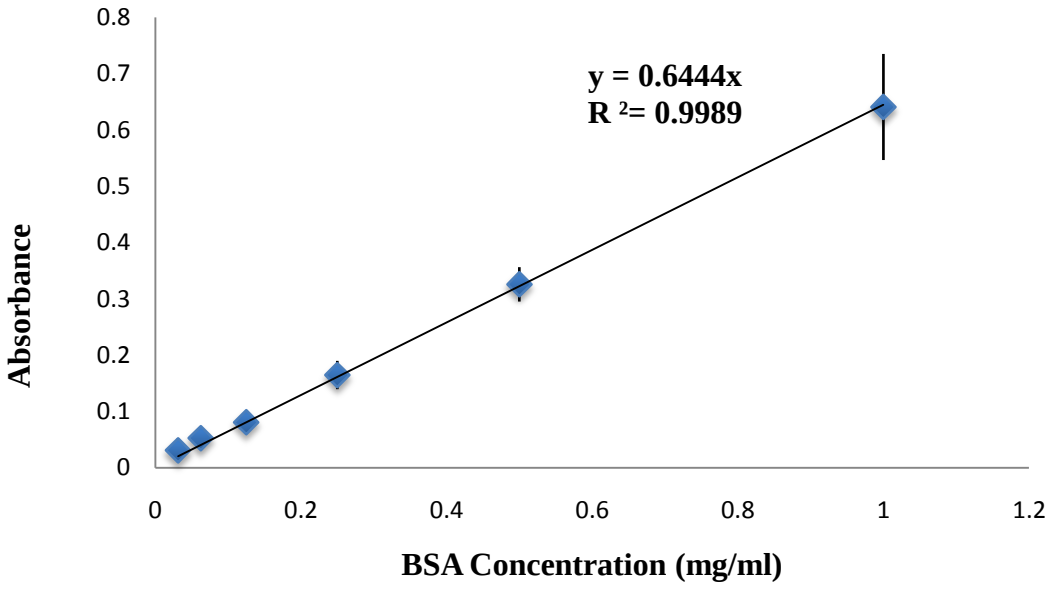

Figure A.1 Standard curve of average absorbance for three sets of BSA serial dilution.

APPENDIX B Calculation of Protein Binding Capacity, Protein Recovery and Weight of Original Membrane and Modified Membrane

Table B.1 Protein binding capacity and elution recovery for original membrane

Membrane Sample	Amount of Feed Protein (mg)	Amount of Bound Protein (mg)	Binding capacity (mg BSA/cm^2 membrane)	Protein recovery (%)
a	2.6443	0.0582	29.0968	88.0
b	2.6443	0.0279	13.9665	92.0
c	2.6443	0.0466	23.2775	65.0
Average	2.6443	0.0442	22.1136	81.7
Standard Deviation	0	0.0153	7.6320	0.1445

Table B.2 Protein binding capacity and elution recovery for membrane modified with 1,2-diaminoethane and 1,4-diaminobutane

Membrane Sample	Binding capacity (mg BSA/cm^2 membrane)		Protein recovery (%)	
	1,2-diaminoethane	1,4-diaminobutane	1,2-diaminoethane	1,4-diaminobutane
a	187.3836	311.9181	65.0	56
b	195.5307	378.2589	65.0	46
c	174.5810	256.0521	59.0	41
d	199.0224	289.8045	71.0	57
e	178.0726	297.9516	62.0	71
f	128.7281	325.8845	50.0	40
g	196.6946	271.1825	74.0	65
h	219.9721	253.7244	51.0	50
Average	191.7482	298.0971	62.1	53.3
Standard Deviation	26.7157	41.2639	8.5930	11.1066

Table B.3 Dried weight of original membrane and membrane after modified with 1.0M of 1,4-diaminobutane

Membrane Sample	Original weight (mg) (a)	After modification (mg) (b)	Weight different $\frac{(\mathbf{b})-(\mathbf{a})}{(\mathbf{b})}$ (%)
1	62.4	62.5	0.16
2	63.7	63.9	0.31
3	62.4	62.7	0.48
4	63.8	63.9	0.16
5	61.7	62.0	0.48
6	62.0	62.2	0.32
7	60.8	61.2	0.65
8	62.7	62.9	0.32
Average	62.44	62.66	0.36
Standard Deviation	0.996	0.921	0.17

APPENDIX C Graph of FTIR Spectra Transmission versus Wavelength

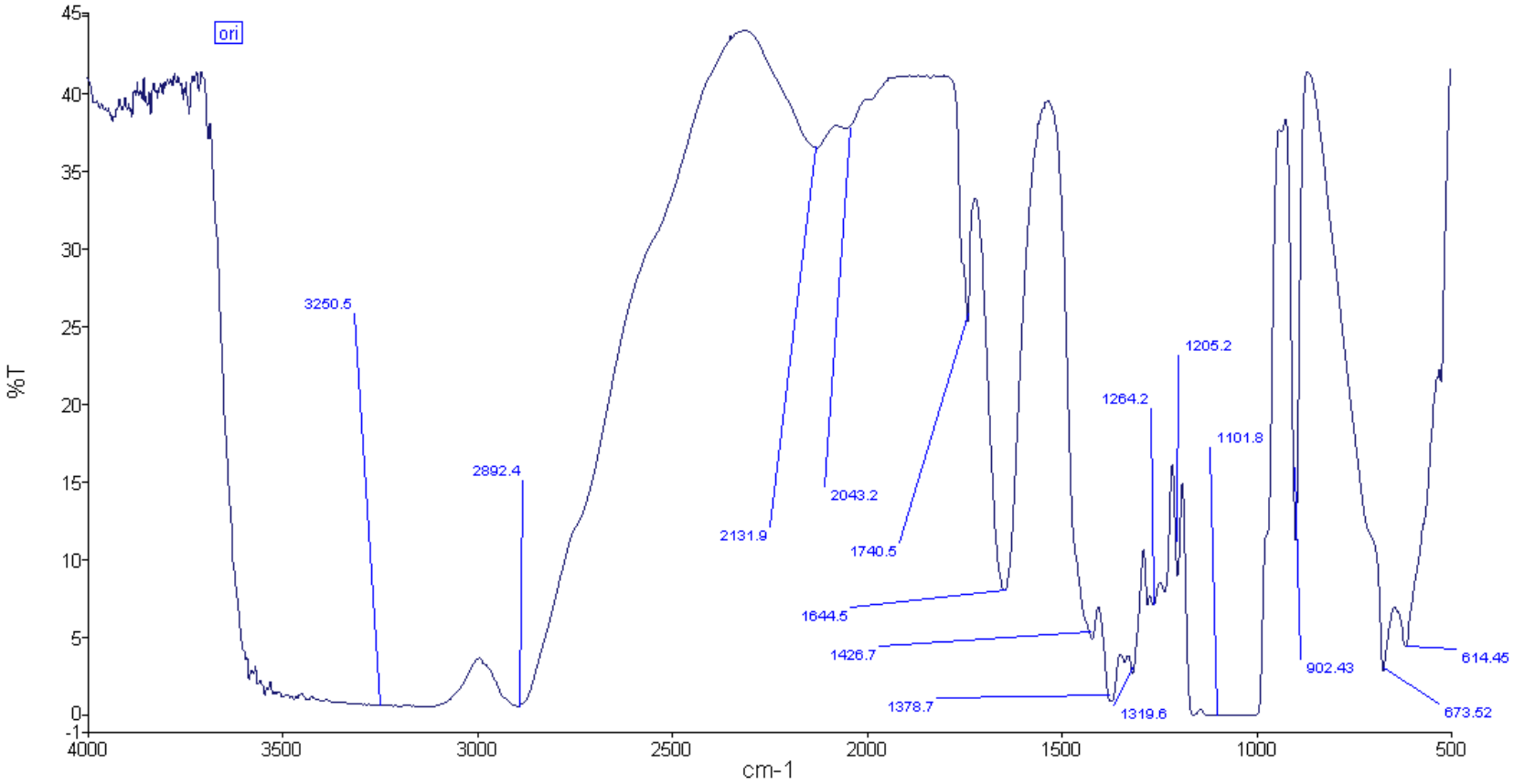

Figure C.1 FTIR result for original membrane

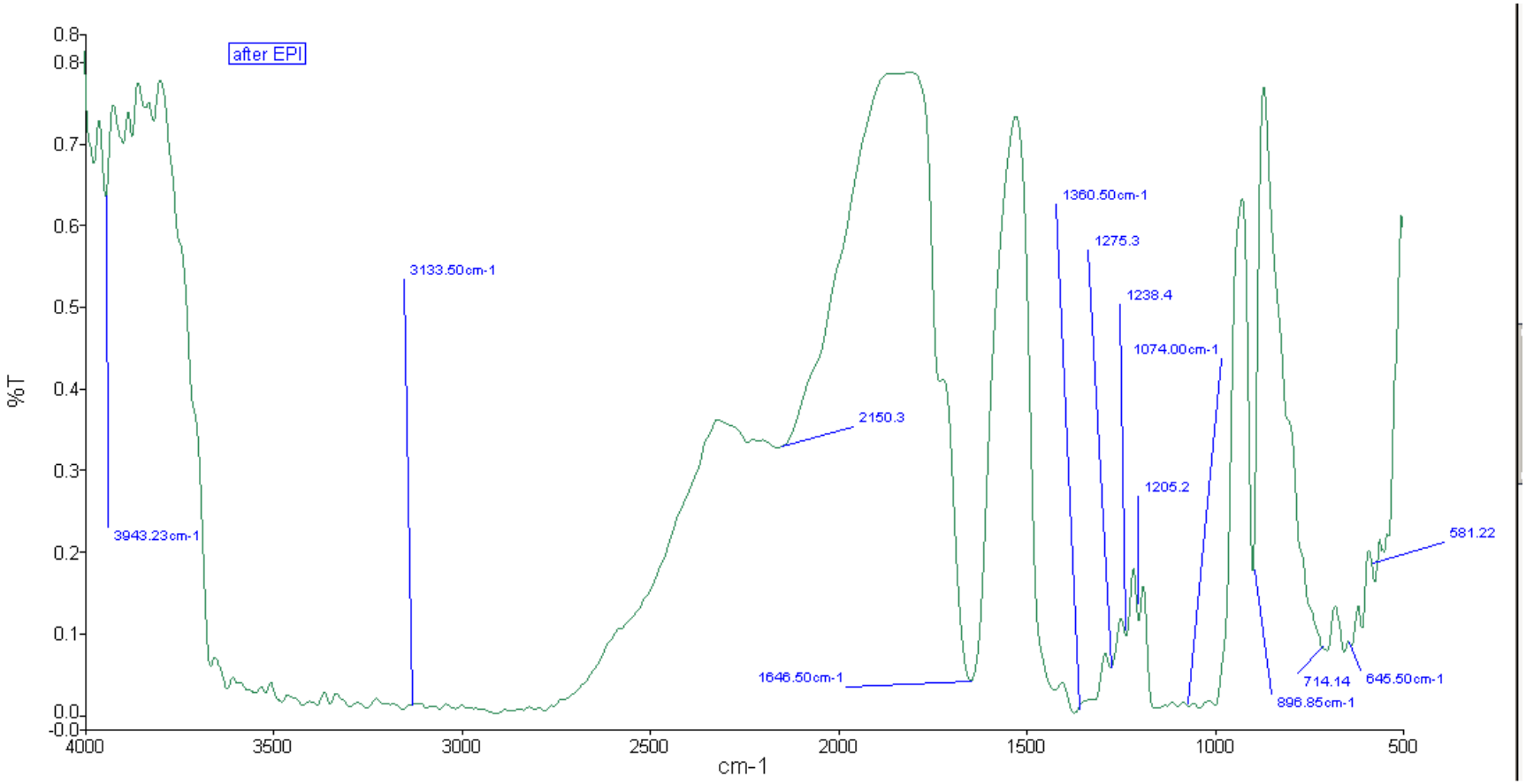

Figure C.2 FTIR result for membrane immersed in Epichlorohydrin (EPI)

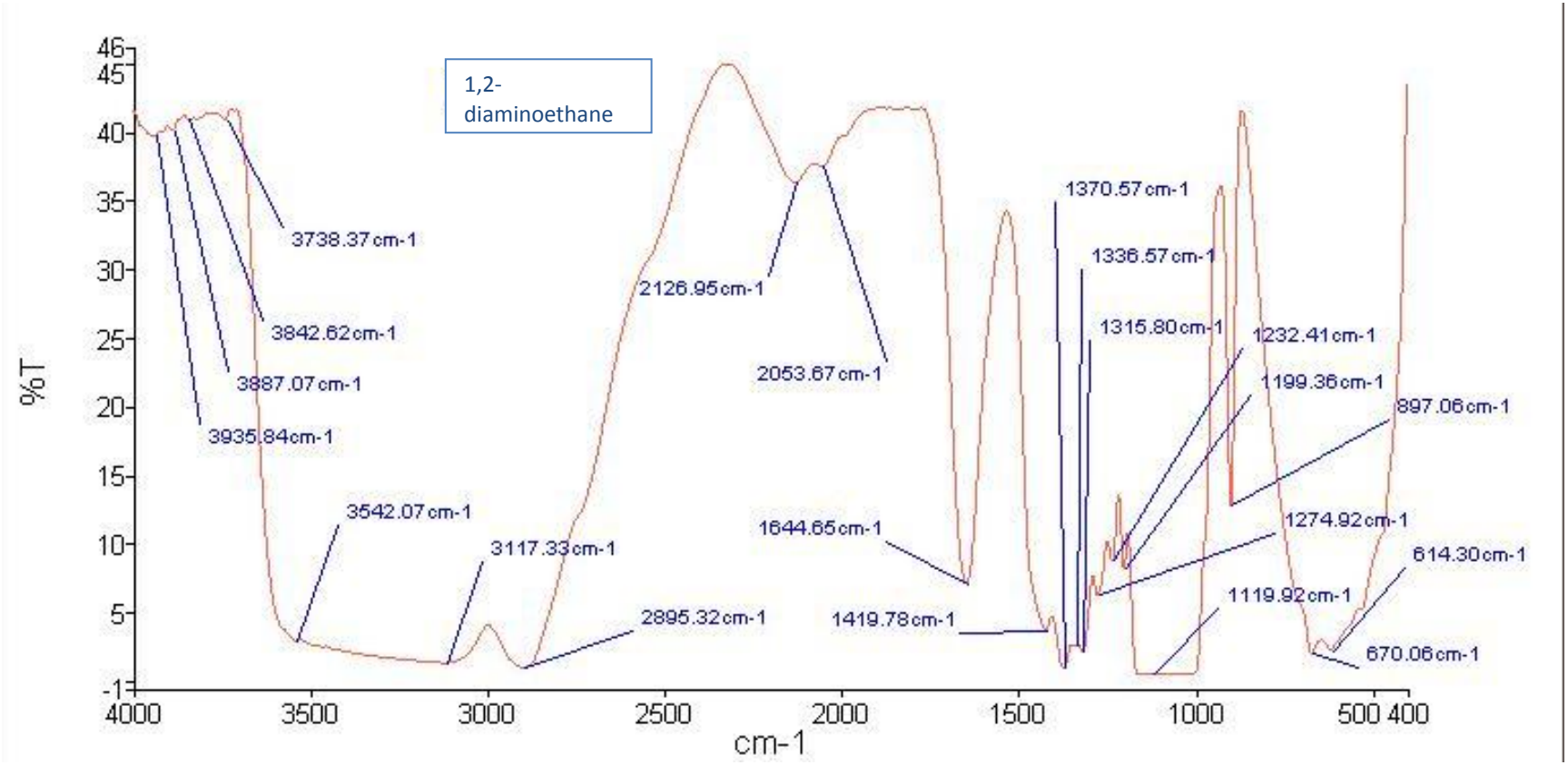

Figure C.3 FTIR result for membrane modified with 1.0M of 1,2-diaminoethane

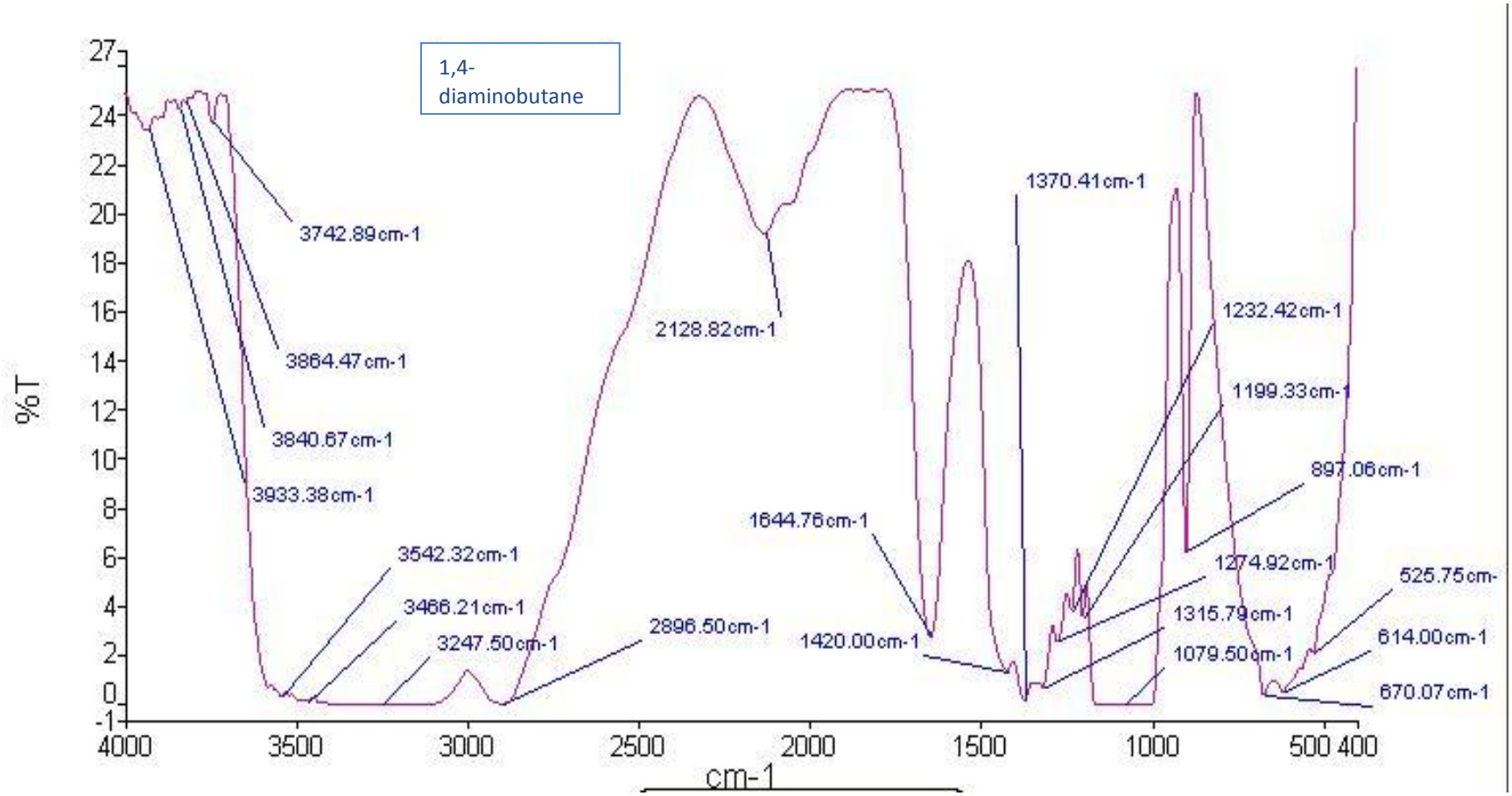

Figure C.4 FTIR result for membrane modified with 1.0M of 1,4-diaminobutane

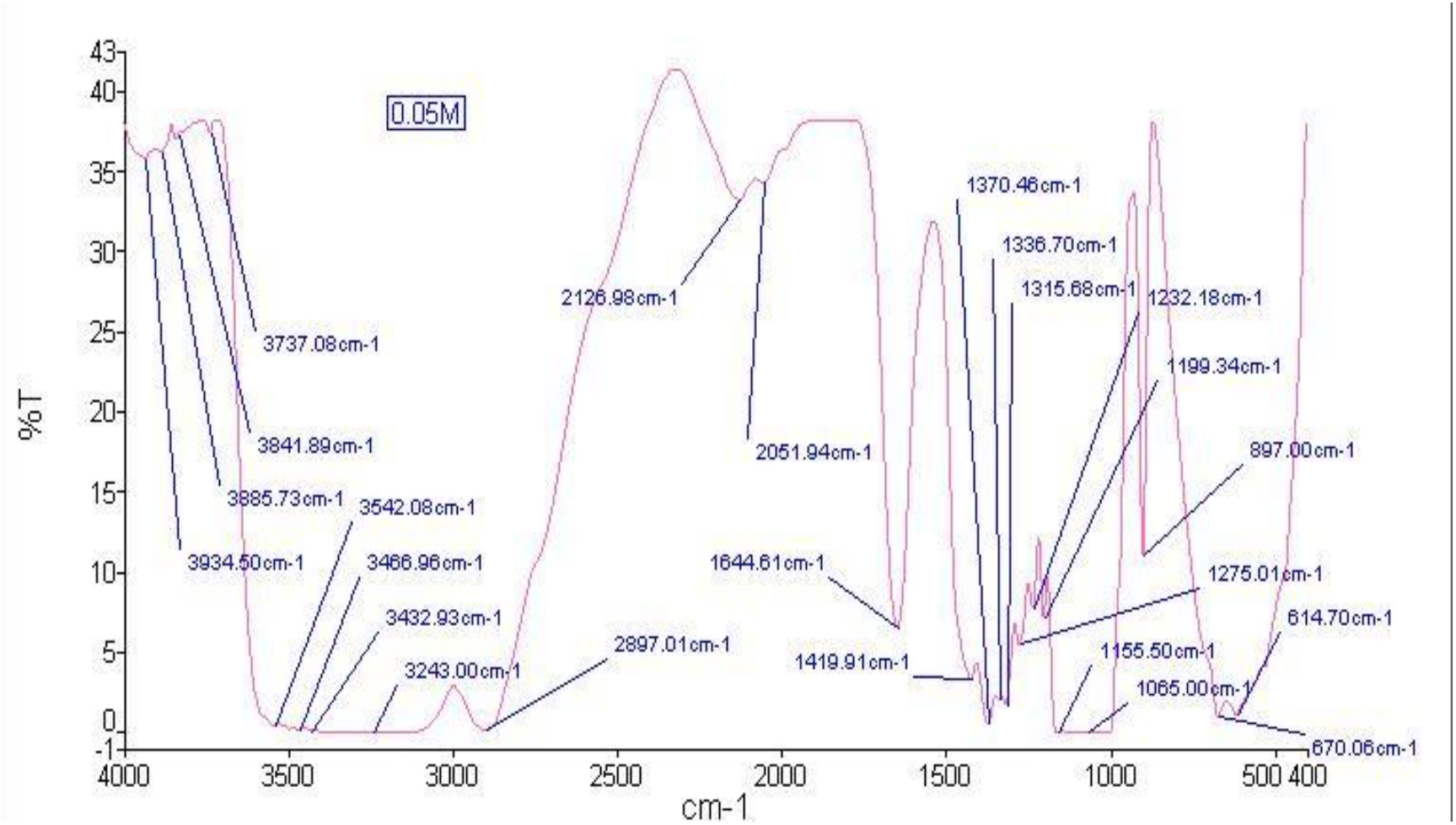

Figure C.5 FTIR result for membrane activated with 0.05M NaOH and grafted with 1.0M of 1,4-diaminobutane

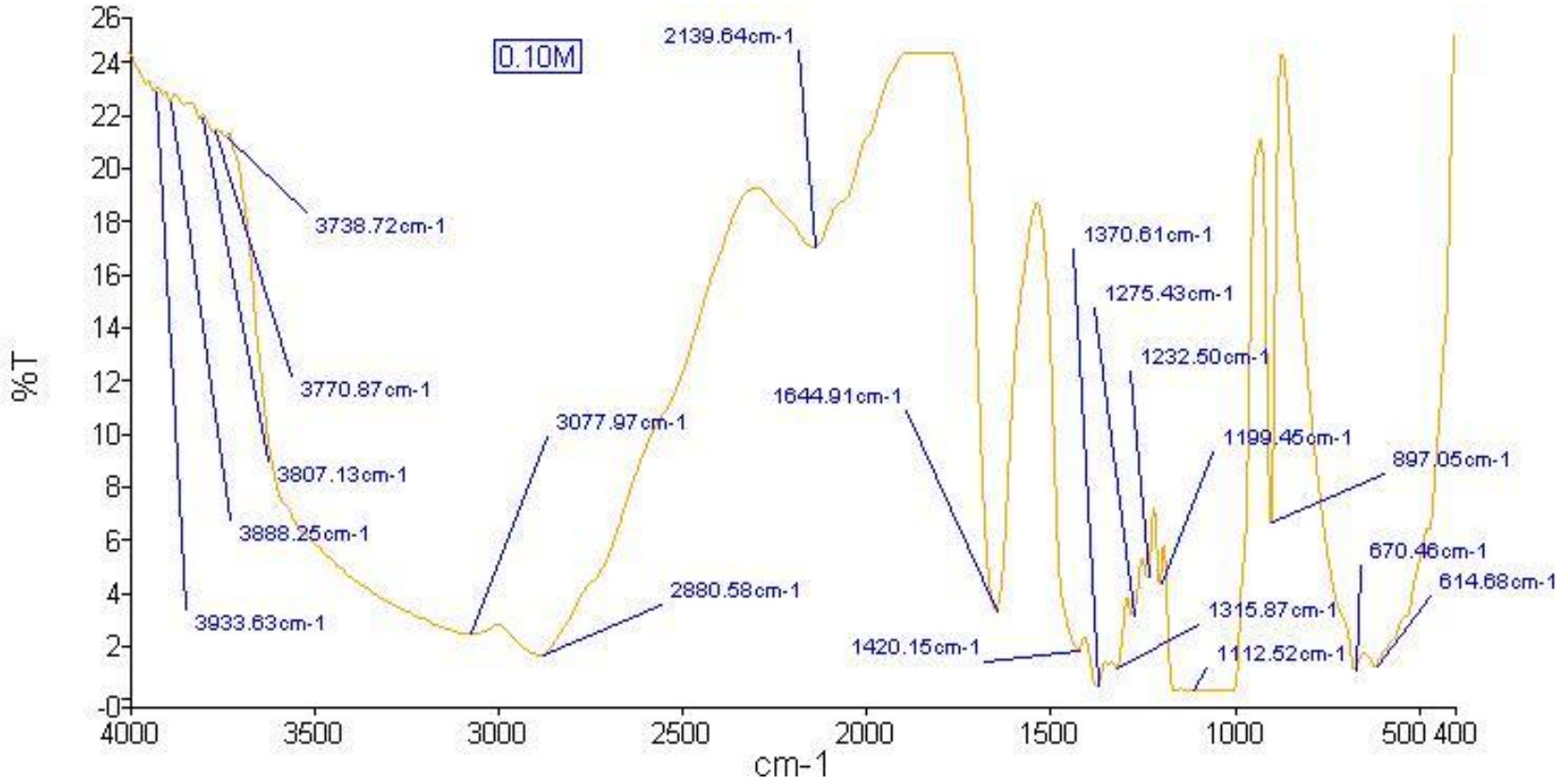

Figure C.6 FTIR result for membrane activated with 0.10M NaOH and grafted with 1.0M of 1,4-diaminobutane

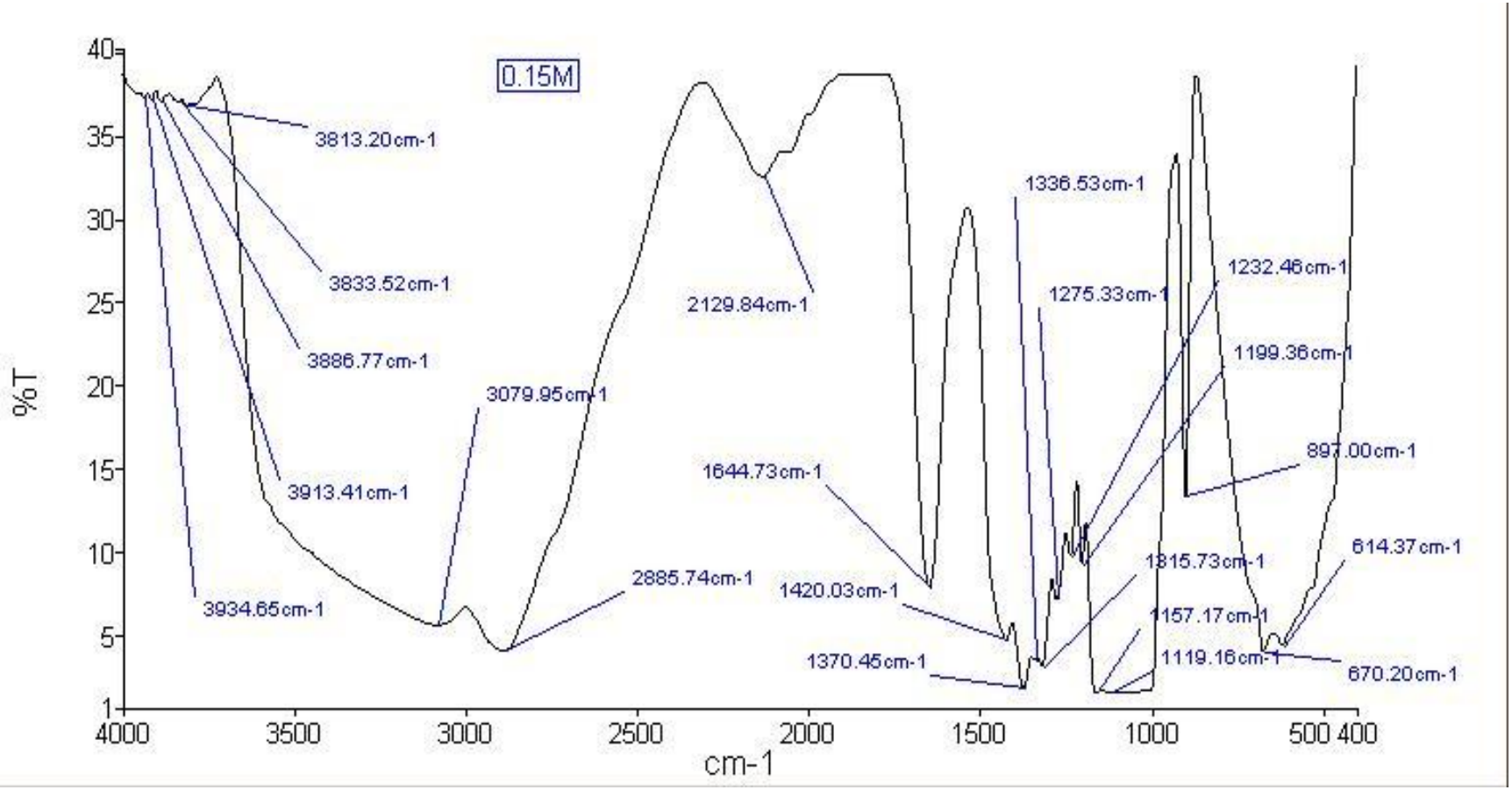

Figure C.7 FTIR result for membrane activated with 0.15M NaOH and grafted with 1.0M of 1,4-diaminobutane

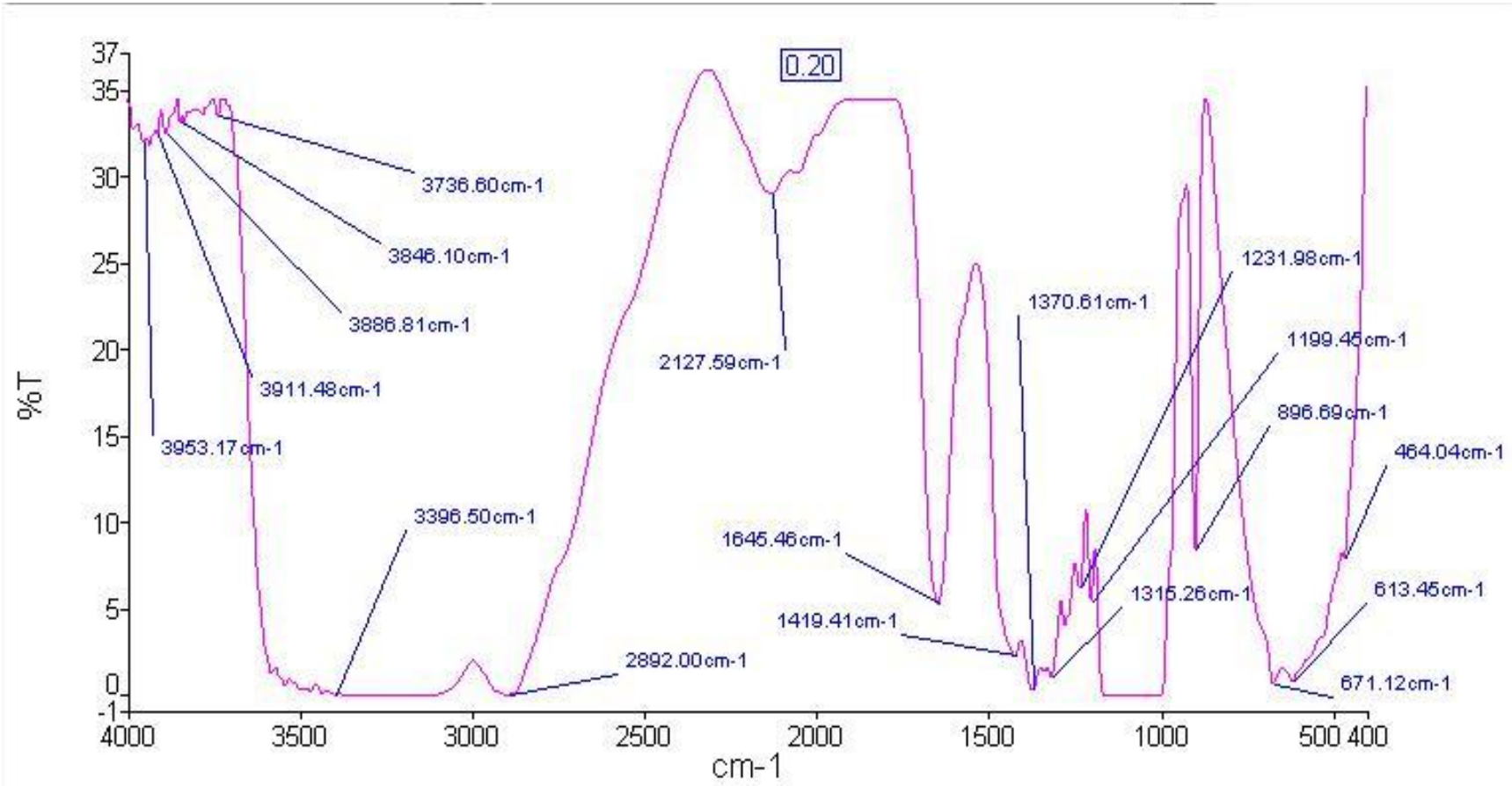

Figure C.8 FTIR result for membrane activated with 0.20M NaOH and grafted with 1.0M of 1,4-diaminobutane

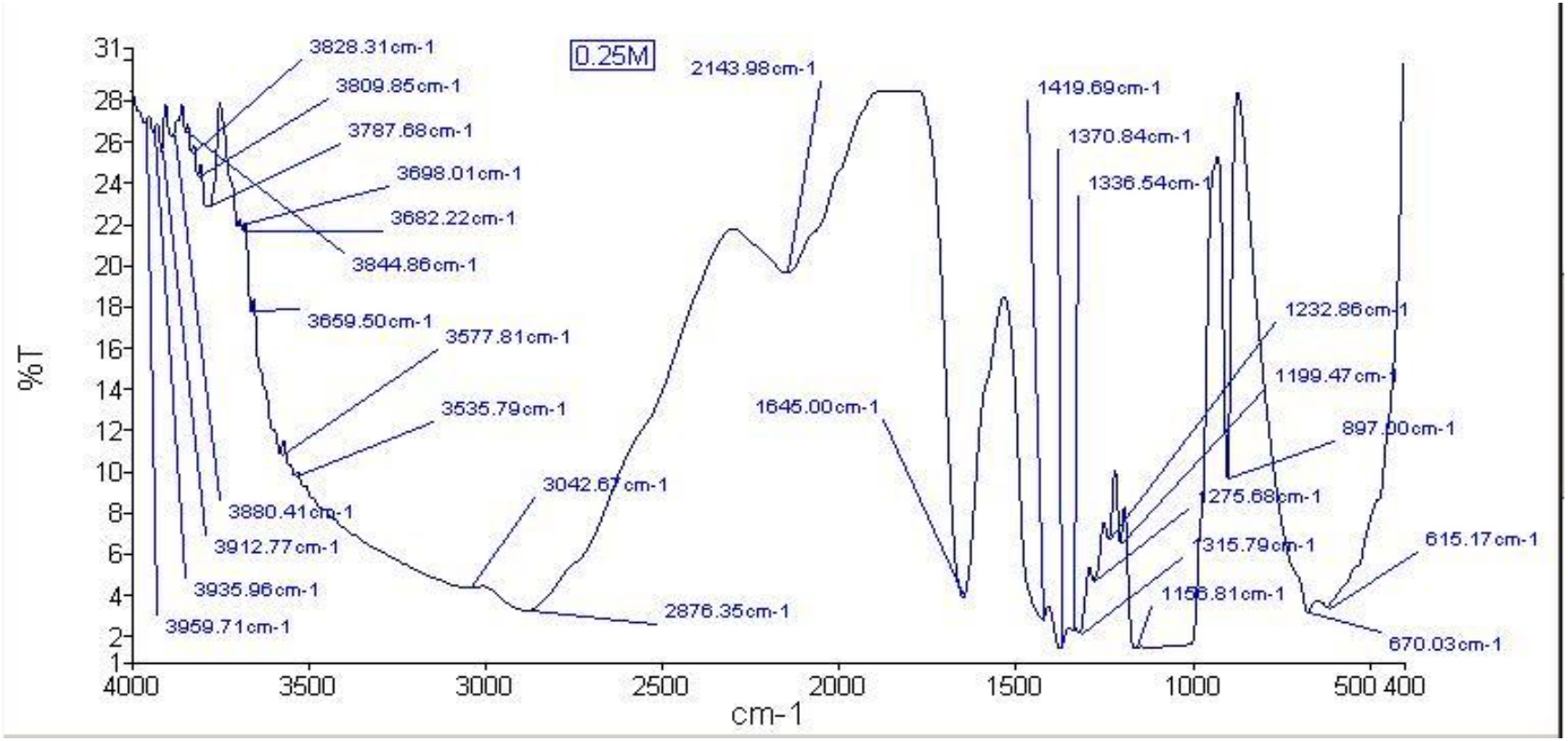

Figure C.9 FTIR result for membrane activated with 0.25M NaOH and grafted with 1.0M of 1,4-diaminobutane

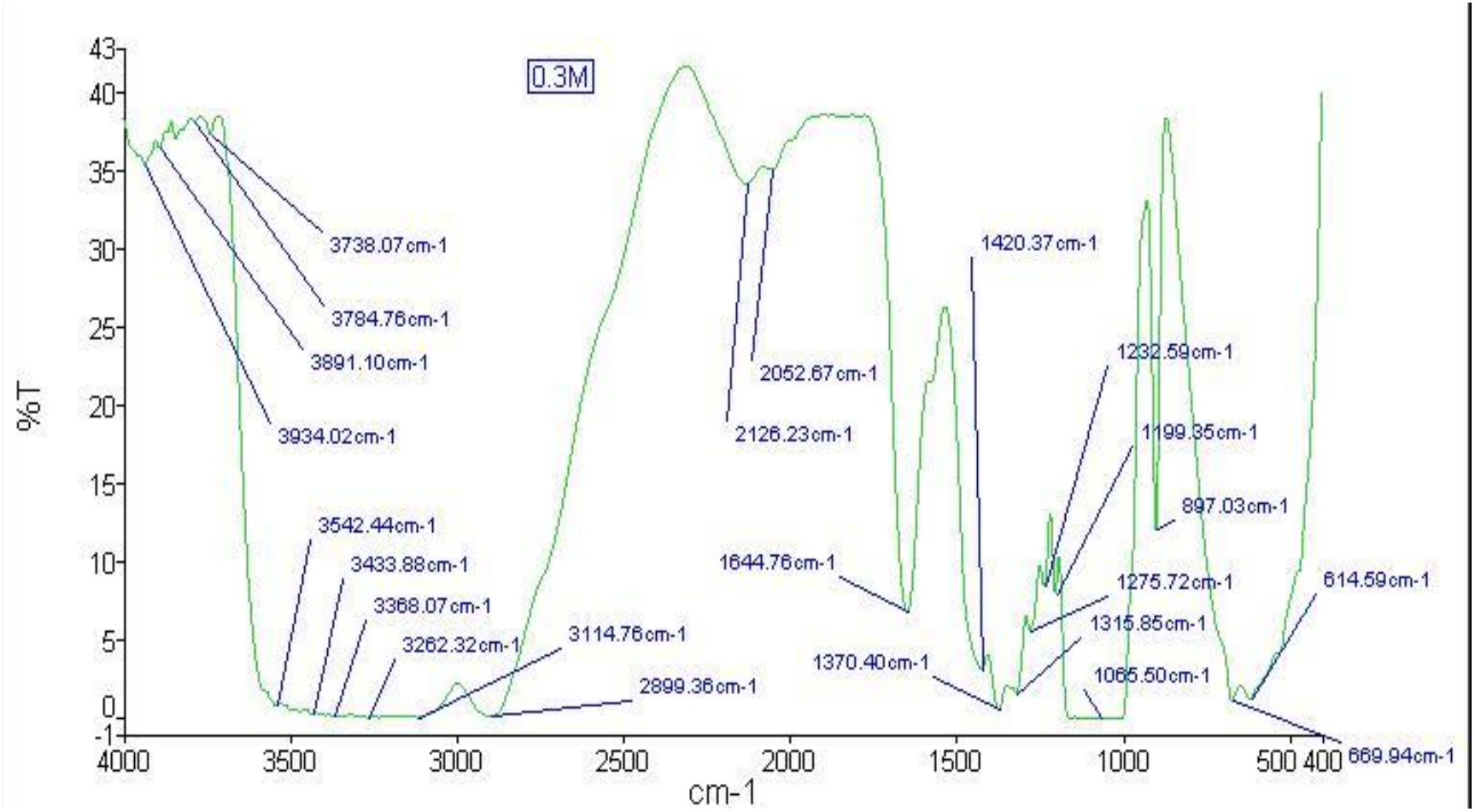

Figure C.10 FTIR result for membrane activated with 0.30M NaOH and grafted with 1.0M of 1,4-diaminobutane

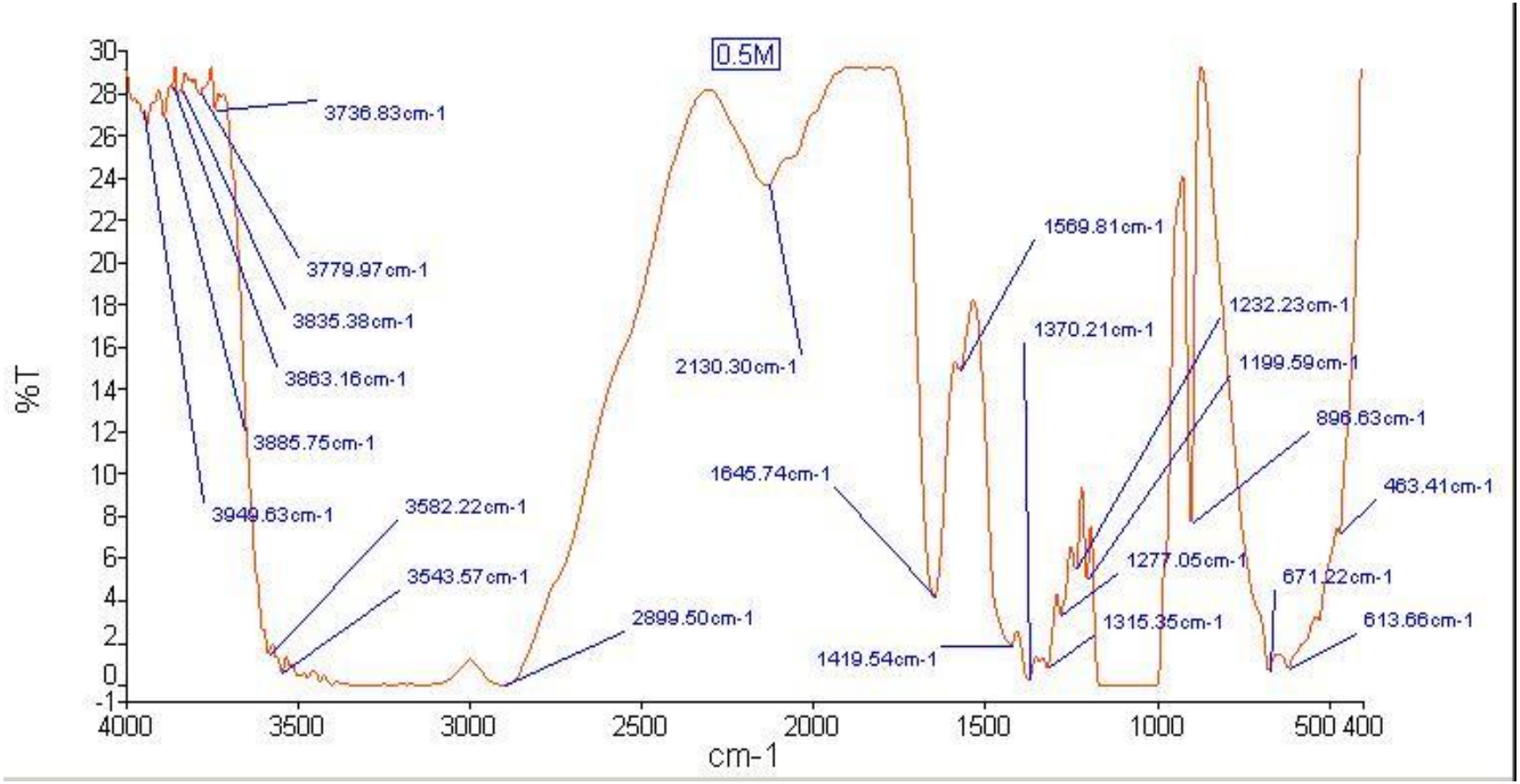

Figure C.11 FTIR result for membrane activated with 0.50M NaOH and grafted with 1.0M of 1,4-diaminobutane

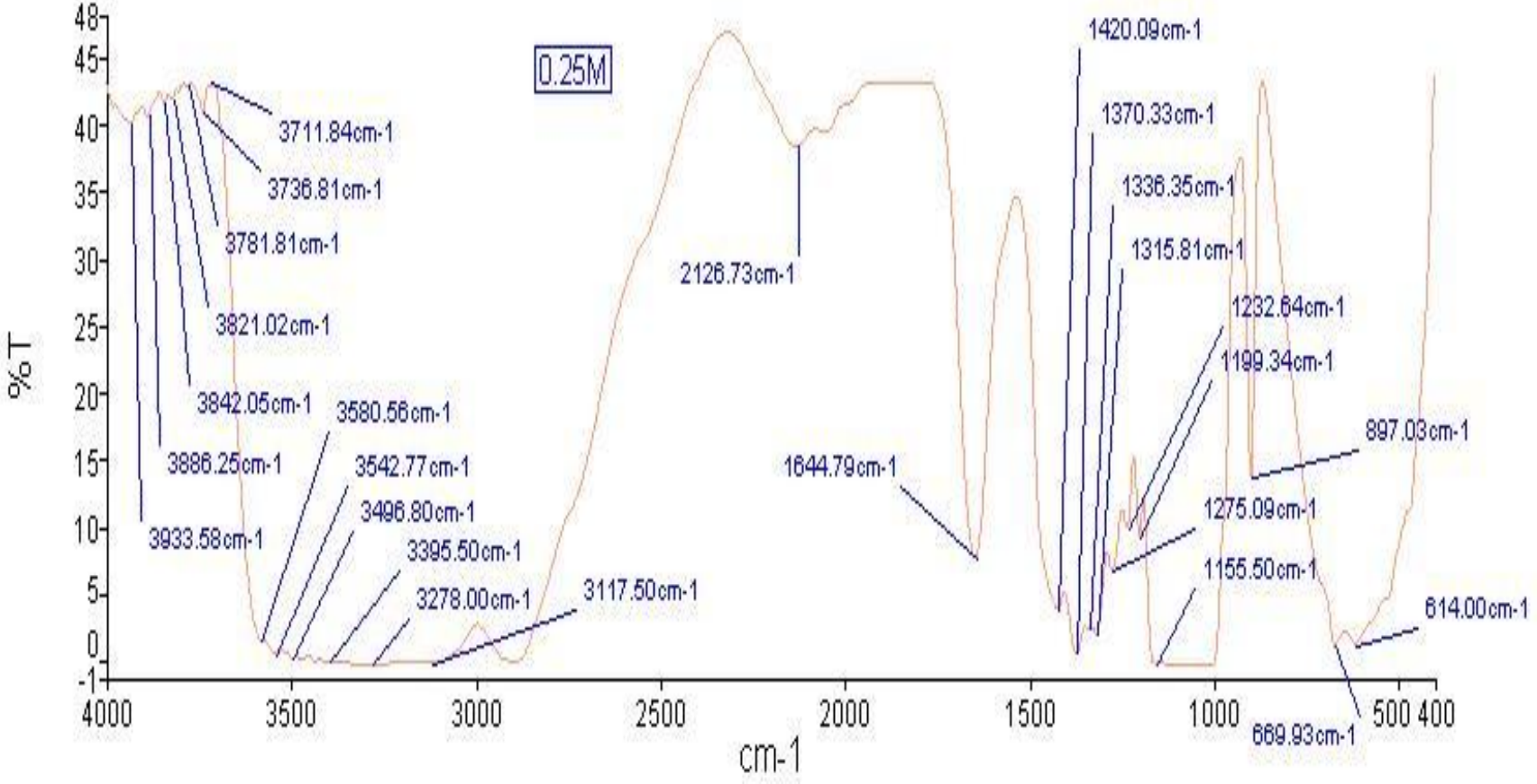

Figure C.12 FTIR result for membrane activated with 0.20M NaOH and grafted with 0.25M of 1,4-diaminobutane

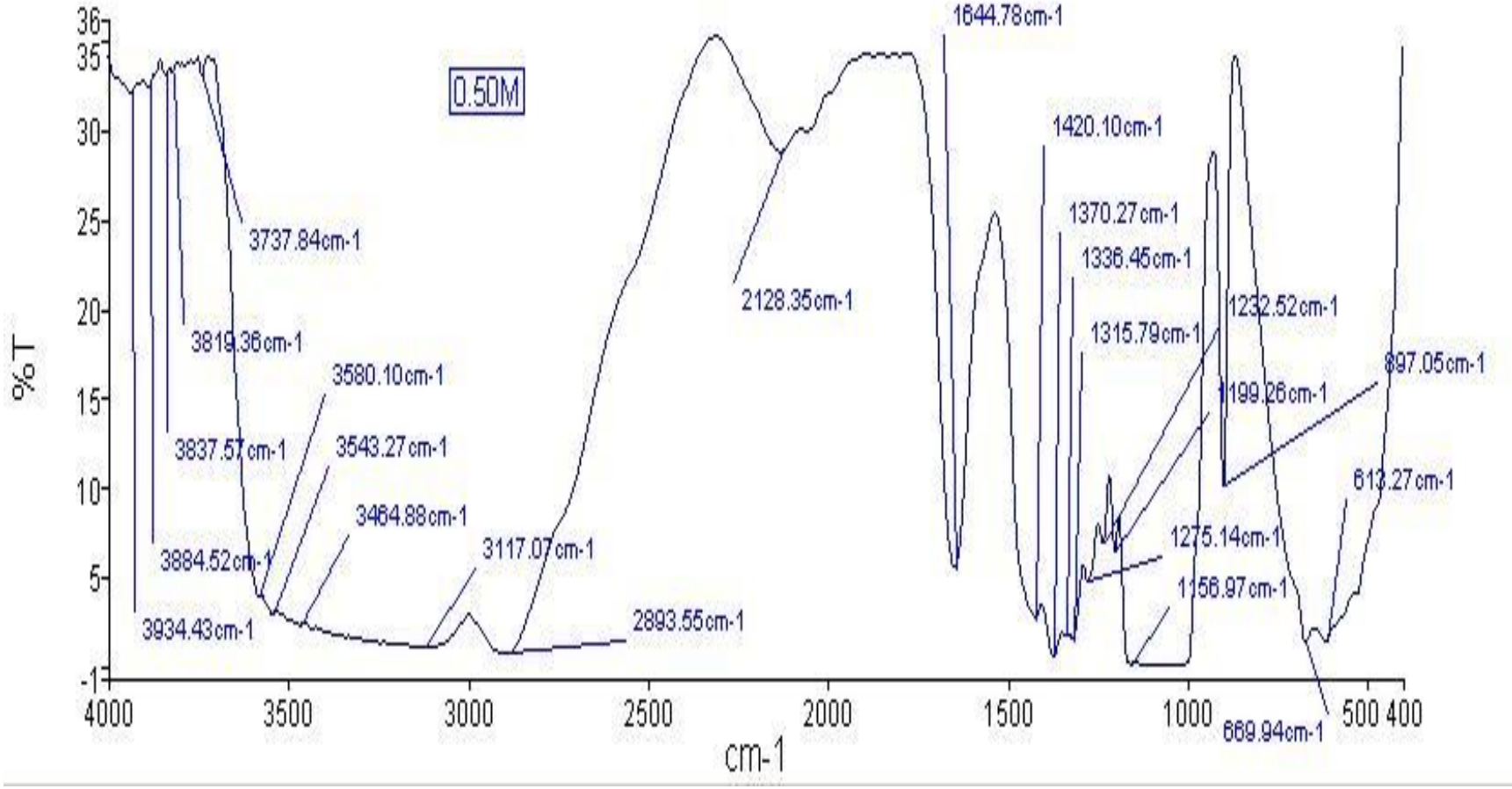

Figure C.13 FTIR result for membrane activated with 0.20M NaOH and grafted with 0.50M of 1,4-diaminobutane

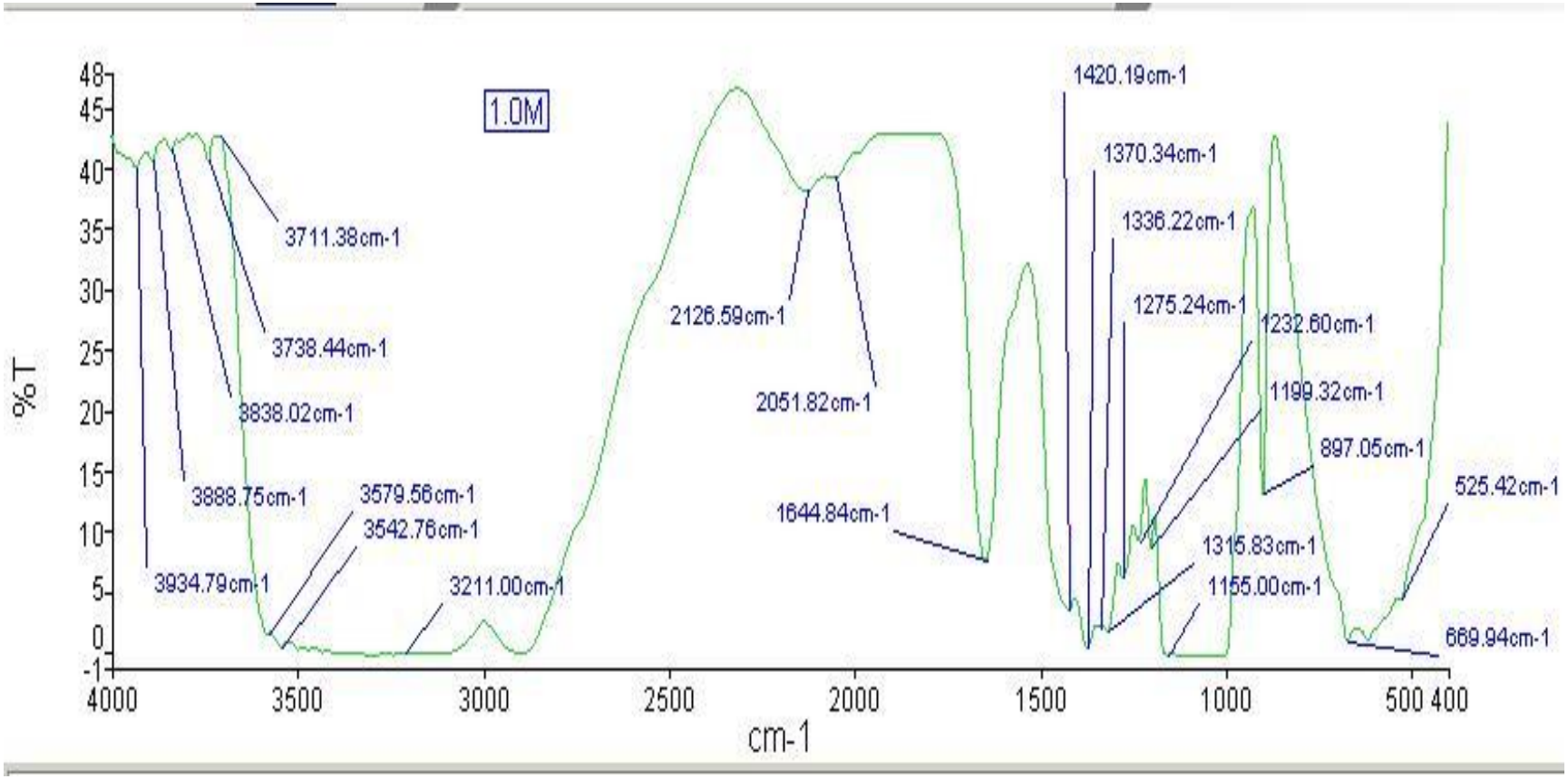

Figure C.14 FTIR result for membrane activated with 0.20M NaOH and grafted with 1.00M of 1,4-diaminobutane

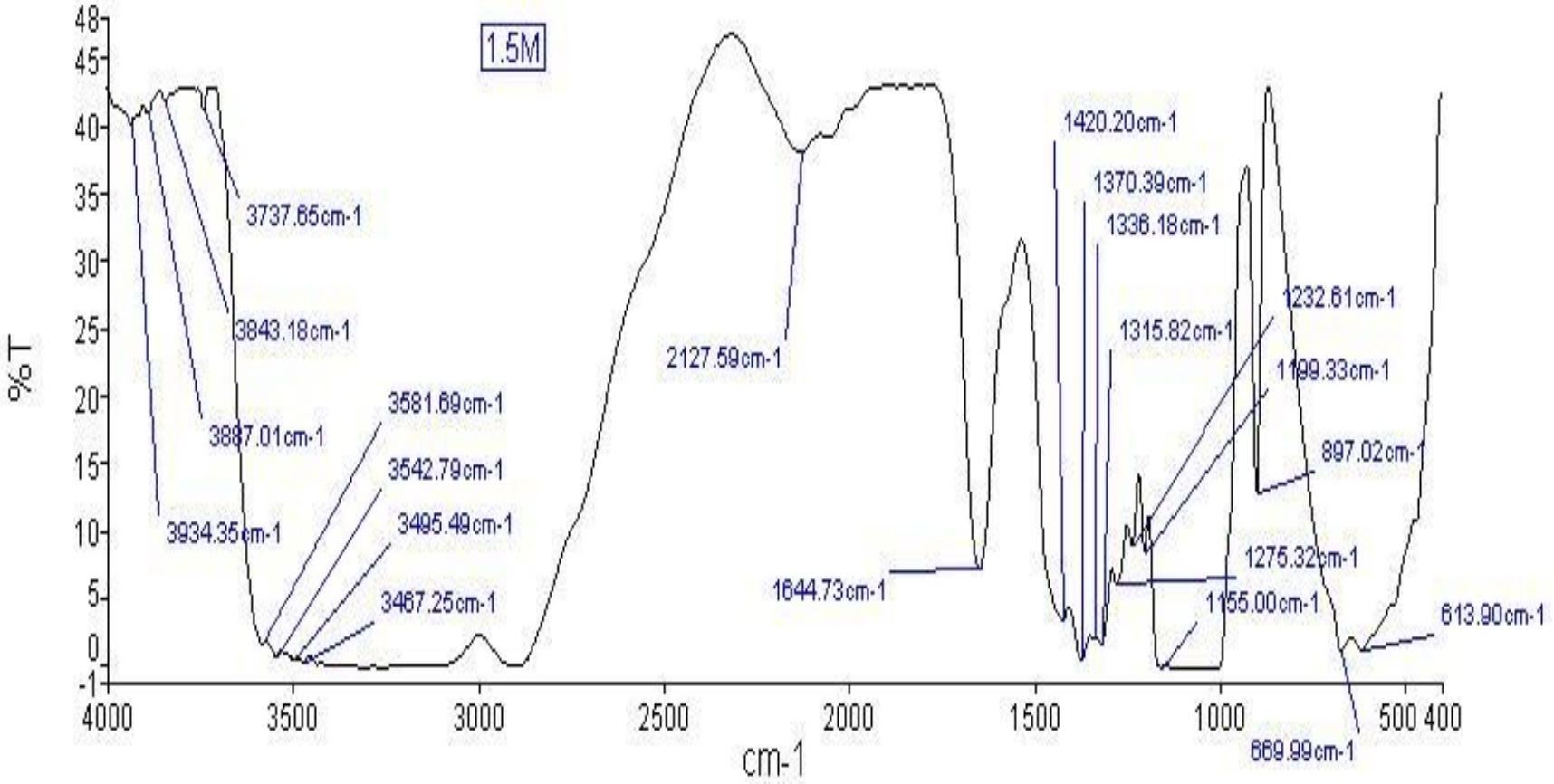

Figure C.15 FTIR result for membrane activated with 0.20M NaOH and grafted with 1.50M of 1,4-diaminobutane

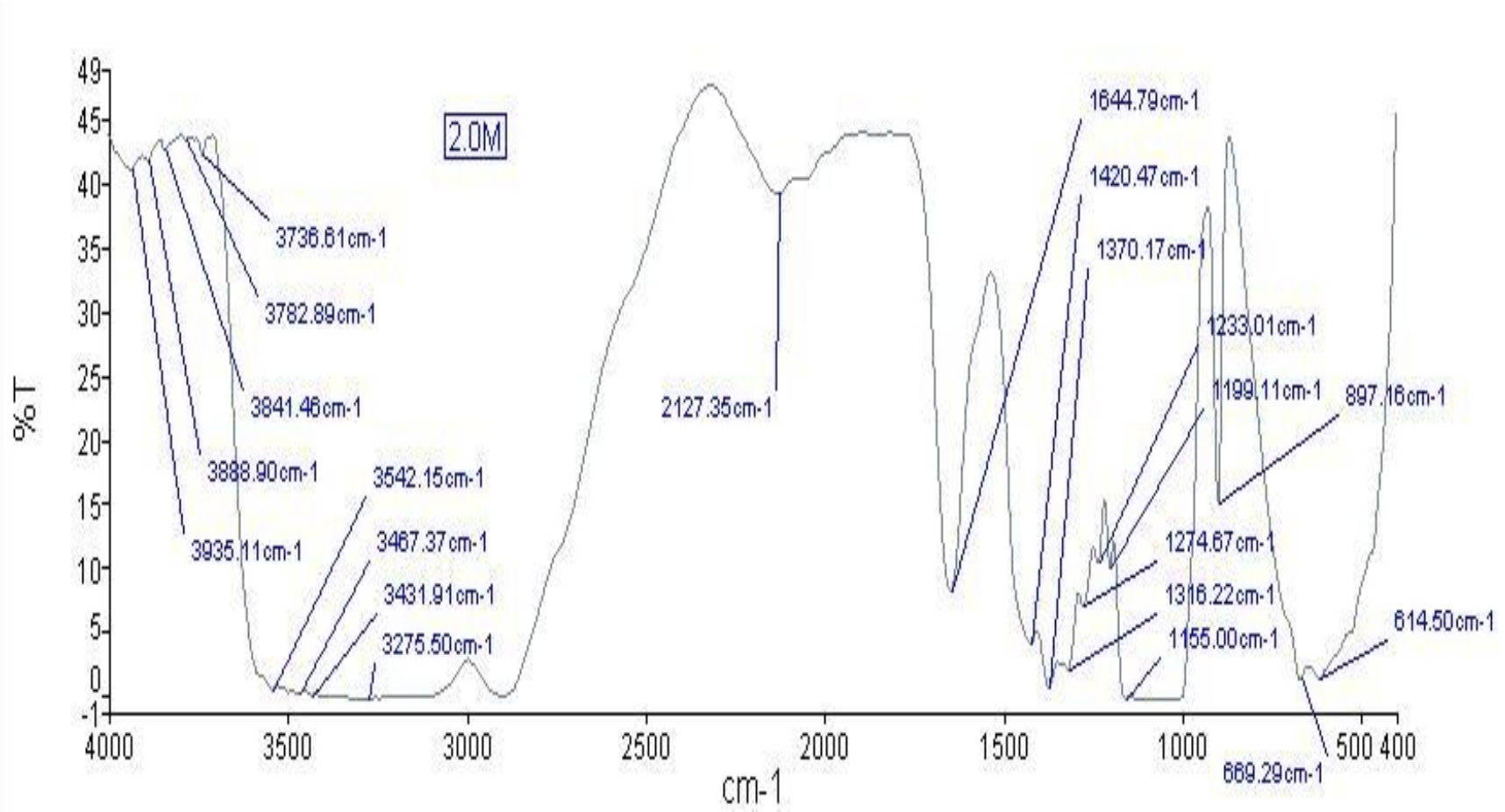

Figure C.16 FTIR result for membrane activated with 0.20M NaOH and grafted with 2.00M of 1,4-diaminobutane