# PROTEIN IMMOBILIZATION ON GLUTARALDEHYDE ACTIVATED NYLON MEMBRANES: EFFECTS OF THE ACTIVATION CONDITION

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**by**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| BuChE | Butyrylcholinesterase |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| BOD | Biochemical oxygen demand |
| DNA | Deoxyribonucleic acid |
| DoE | Design of Experiment |
| FTIR | Fourier Transform Infrared Spectroscopy |
| GA | Glutaraldehyde |
| HCl | Hydrochloric acid |
| HRP | Horseradish peroxidase |
| IUPAC | International Union of Pure and Applied Chemistry |
| MA | Malathion |
| Mab | Monoclonal antibody |
| $N_2$ | Nitrogen gas |
| PANi | Polyaniline |
| PCR | Polymerase chain reaction |
| PM | Pirimiphos-methyl |
| PVA | Poly (vinyl alcohol) |
| PVDF | Polyvinylidence fluoride |
| RNA | Ribonucleic acid |
| SEM | Scanning Electron Microscopy |
| TGA | Thermogravimetric analysis or thermalgravimetric analysis |

**PROTIN YANG BERGERAK KE ATAS MEBRAN NILON YANG TELAH DIAKTIFKAN DENGAN GLUTARALDEHYDE: KESAN KE ATAS SYARAT PENGAKTIFAN**


## ABSTRAK

Protin yang bergerak ke atas membran nilon yang telah diaktifkan dengan glutaraldehyde telah dikaji dalam kajian ini. Pertama, membran nilon diaktifkan dengan glutaraldehyde dan kemudian protin akan melekat ke atasnya. Morfologi membran dicirikan dengan mengimbas mikroskopi electron (SEM), analisis FTIR dan analisis Termogravimetri (TGA) untuk memeriksa kehadiran glutaraldehyde. Kajian awal telah dijalankan untuk mengkaji kesan tiga faktor (masa untuk proses membran diaktifkan, pH dan kepekatan GA) ke atas protin yang melekat manakala sampel membran ditetapkan pada 16% berat polimer nilon dan 84% berat campuran pelarut dan bukan pelarut. Hasil daripada kajian itu, ia boleh dapati bahawa syarat GA-pengaktifan optimum adalah pada 40 minit masa untuk proses membran diaktifkan, pH 7.5 dan 1% berat bagi kepekatan GA. Sebagaimana faktor campuran (kepekatan polimer dan campuran pelarut dan bukan pelarut) dan faktor-faktor proses (masa untuk proses membrane diaktifkan dan pH) adalah saling berkaitan, oleh itu reka bentuk eksperimen (DoE) telah digunakan. Syarat-syarat GA-pengaktifan optimum diperoleh daripada metodologi reka bentuk campuran proses yang melintasi reka bentuk (DoE) pada 25% berat polimer, 75% berat campuran pelarut dan bukan perlarut, larutan GA pada pH 9.0 dan pada 70 min masa untuk proses membrane diaktifkan, dengan protin yang melekat diramalkan pada 2410.19 $\mu g/cm^3$. Pengesahan eksperimen ini telah diajalankan pada keadaan optimum dan keputusan protin yang melekat ditemui pada 2411.57 $\mu g/cm^3$ dengan sisihan piawai

0.057%. Hasil eksperimen menunjukkan sisihan piawai yang kecil yang bermaksud model boleh digunakan untuk meramalkan protin yang bergerak pada glutaraldehyde diaktifkan ke atas membran nilon.

**PROTEIN IMMOBILIZATION ON GLUTARALDEHYDE ACTIVATED NYLON MEMBRANES: EFFECTS OF THE ACTIVATION CONDITIONS**


## ABSTRACT

Protein immobilization on glutaraldehyde (GA) activated nylon membranes has been studied in this work. Nylon membranes was first activated with GA and then immobilized with the protein. The morphology of the membranes was characterized by scanning electron microscopy (SEM), FTIR analysis and thermogravimetric analysis (TGA) to check the presence of glutaraldehyde. The preliminary study was carried out to investigate the effect of three factors (incubation time, pH and concentration of GA) on protein binding while the membrane sample is fixed to 16 wt% of nylon polymer and 84 wt% of mixture solvent and non-solvent. From the study, it can be found that the optimum condition of GA-activation which are incubation time is at 40 min, at pH 7.5 and 1 wt% of GA concentration. Since the mixture factors (concentration of polymer and mixture solvent and non-solvent) and process factors (incubation time and pH) were inter-related, therefore Design of Experiment (DoE) was applied. The optimum GA-activation conditions was obtained from a crossed mixture-process design methodology (Design of Experiment) at 25 wt% of nylon polymer, 75 wt% of mixture solvent and non-solvent, GA solution at pH 9.0 and GA activation time of 70 min, with protein binding predicted at 2410.19 $\mu g/cm^3$. The experimental validation was conducted at this optimum condition and the protein binding result was found at 2411.57 $\mu g/cm^3$ with the standard deviation of 0.057%. The experimental result shown a small standard deviation which means the model can be used further to predict the protein that immobilized on glutaraldehyde activated nylon membranes.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Protein immobilization

Immobilization can be defined as the attachment of molecules to a substrate surface resulting in reduction or loss of mobility (Rusmini et al., 2007). Proteins should be attached onto a surfaces without affecting conformation and function for fully retain biological activity. There are many immobilization techniques which are based on the following mechanisms; chemical, covalent and bioaffinity immobilization.

For chemical immobilization, proteins can adsorb on surfaces via intermolecular forces which could be the ionic bonds, hydrophobic interactions or polar bindings. Thus, the layer is likely to be heterogeneous and randomly oriented since each molecule can form many contacts in different orientations to minimize the repulsive interactions with the substrate. However, its disadvantage are random orientation and weak attachment of protein (Rusmini et al., 2007, Kim and Herr, 2013). This is because protein may be removed by some buffer or detergents when performing the immobilization.

Usually proteins are covalently bound to the immobilization support through accessible functional groups of the exposed amino acids (Rusmini et al., 2007). The protein-substrate covalent bonds are mostly formed between side-chain-exposed functional groups of the proteins, which resulting a high surface coverage. However, the covalent reaction is usually slow, so that the protein and surface required long incubation times (Kim and Herr, 2013). The disadvantages of covalent linkage are

reduced activity of proteins, toxic reagents and complicated chemistry (Kim and Herr, 2013).

For bioaffinity immobilization, biochemical affinity reactions offer a gentle oriented immobilization of proteins, providing an important advantage over other immobilization techniques (Rusmini et al., 2007). This is because bioaffinity offers better accessibility to binding partners than random orientation while a covalent bond can be formed on active sites of proteins that causes in reduced activity (Kim and Herr, 2013). The difference between bioaffinity and covalent bond is the used of reagent. For covalent bond, the immobilization surface is activated via reactive reagents while for bioaffinity is needed specific binding phenomena existing in nature and the bioaffinity reagent used as an intermediate binding molecule between the surface and proteins (Kim and Herr, 2013). The bioaffinity bonding has not only provided oriented and homogeneous attachment; but also possible to detach proteins and make repeated use of the same surface.

## 1.2 Gluataraldehyde activation

Activation of supports using glutaraldehyde is one of the most popular techniques to improve the immobilization of biomolecules on the membrane or porous support (Betancor et al., 2006b). This is because this method is quite simple, efficient and allow to improve protein stability. Glutaraldehyde is used to introduce chemically crosslinking in between proteins and the membrane support to improve the protein adsorption capacity on the membrane surface. Indeed, chemical crosslinking is one of the most effective and simplest methods to bind the

biomolecules onto the membrane surface (Shaimi and Low, 2016) due to the no support is required to prevent protein loss in the substrate solution.

As for the porous platform for biomolecules immobilization, membranes such as mixed cellulose, poly (vinylidene fluoride) (PVDF) are of the suitable candidate due to their hydrophobic characteristic that can interacts with proteins (Shaimi and Low, 2016, Akashi and Kuroda, (2014)). Among all, nylon membranes have high mechanical rigidity compared to nitrocellulose membranes (Narang et al., 2011) and it is an inherently hydrophilic membrane. Because of its hydrophilic characteristics, it can increase the number of reactive sites for protein immobilization (Narang et al., 2011). The mechanism of protein binding for nylon is by electrostatic interaction that involved positively charged and negatively charged functional groups (Kim and Herr, 2013). However, nylon membrane is positively charged. Despite the superior material characteristics, nylon still lacks desirable surface-reactive functionalities of negatively charged, that would facilitate immobilization of proteins (Farahmand et al., 2015).

## 1.3 Problem Statement

Biosensors are increasingly becoming practical and useful analytical tools in medicine, food quality control, environmental monitoring and research. It uses a biological recognition element (bio-receptor) and a transducer in direct special contact with the target analyte to provide selective quantitative or semi-quantitative analytical information. (Shaimi and Low, 2016).

The most concern in bio-sensing application is about the sensitivity and capacity of protein immobilization. Since then the development of biosensors has

continued and diversified and the International Union of Pure and Applied Chemistry (IUPAC) now defines a biosensor as a self-contained integrated device that provides quantitative analytical information using a biological recognition element (biochemical receptor) in contact with a transduction element (Thévenot et al., 2001). A bioanalytical system requires additional processing steps (such as addition of reagents) and a single-use biosensor (e.g. pregnancy test, glucose meter test strip) is disposable and cannot monitor analyte concentration continuously.

Protein immobilization studies with high protein binding sensitivity are of primary concern for any biosensing applications. In order to develop well-functioning biosensors, the binding capability of the immobilized protein needs to be retained and nonspecific (unwanted) bindings of proteins must be reduced (Shaimi and Low, 2016). Nonetheless, the protein has some problem such as may unfold, whereby the binding site may be disrupted and the protein may lose its binding ability. These often lead to the weak of protein binding. Biosensors are promising tools for detecting directly many chemical and biological parameters. If the biosensor can't achieve require protein immobilization, the biological recognition element cannot capture the analyte that is immobilized on the membrane. In order to enhance the protein immobilization in biosensor, many ways have been proposed including a crosslinking agent. Since glutaraldehyde has found widely used for protein immobilization, it also found that it is the most effective crosslinking agent (Migneault et al., 2004, Silva et al., 2007) due to of high molecule stability.

A nylon is mostly used in many biomedical application because of its advantages, such as high internal surface area and the possibility of product separation via transmembrane transport in the case of reactions with product inhibition (Zeng et al., 2014). Thus, it advantages make a membrane an excellent

support for protein immobilization. Two different techniques which are physical adsorption and covalent immobilization was performed in analyte-substrate intraction in order to create a unique structure that offers considerably high surface area for biomolecular immobilization (Farahmand et al., 2015). Nevertheless, nylon has a low concentration of strongly reactive groups leading to a low ligand density and weak interaction of the enzyme with the polar surface. Therefore, to overcome these problems, partial acid hydrolysis of the nylon membrane surface is used to generate reactive amino groups without loss of mechanical strength, which can be coupled with protein using glutaraldehyde to increases reactive sites and reduces nonspecific adsorption.

Different parameters such as time, concentration and pH also affect the glutaraldehyde immobilization. As the concentration of glutaraldehyde can affect the formation of intra and intermolecular interactions between the proteins, so various amount of glutaraldehyde were used. How longer time is needed for glutaraldehyde immobilization also is a concern for biosensing application. It is possible that glutaraldehyde could denature most of the enzyme if their contact time is too long. As protein and enzyme activity is sensitive at pH, suitable range pH must be determined in order to prevent protein to denature. This is because changes in pH will affect the chemistry of amino acid residues and can lead to denaturation. Adjusting the pH for buffer solution to maintain the pH and the activity in the protein.

## 1.4 Objectives

I. To explore cross-linking of glutaraldehyde on the membrane surface in concentration, reaction time and pH condition.

II. To characterize the glutaraldehyde activated nylon membrane.

III. To evaluate the interactive and independent factors of the activation conditions through statistical tool.

## 1.5 Scope of Study

Nylon membrane with good mechanical rigidity and a narrow pores size distribution was studied to investigate the amount protein binding of the membrane. Nylon membrane is activated by using glutaraldehyde to improve the protein adsorption capacity on the membrane surface. By determine the protein binding, this study also can explore cross-linking of glutaraldehyde on the membrane surface. The protein used in this studied was bovine serum albumin (BSA) for immobilization. Thus, the presence of protein in membrane was detected by using bicinchoninic acid assay (BCA). The use of glutaraldehyde was studied by investigate the effect of time incubation, pH and concentration of glutaraldehyde to cross-linking with the protein. Then, the optimization is evaluated using the Design Expert (DOE) which is D Optimal design that used statistical tool in crossed. Further investigation was arranged to characterize the glutaraldehdye activated nylon membrane by using FTIR, SEM and TGA to check the morphology of the membrane. Therefore, from the analysis of the data, we can know the best activation condition and the effect of the condition for nylon membranes in protein immobilization.

## 1.6 Thesis organization

This thesis consists of five chapters. Chapter one consist of introduction about protein immobilization and glutaraldehyde activation on nylon membrane. Chapter one will state the problem statement and research objective in this thesis. Scope of study will be included in chapter one to state what will be study in this thesis. In chapter two literature reviews about protein detection using membrane, nylon membrane in protein immobilization and interaction of protein with the nylon membrane. Use of glutaraldehyde also study in chapter two. In chapter three, the method to carry out experiments to study different effect of parameter will be mention. The type of characterization will be used in this study will be included in chapter three too.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Protein detection using membrane

Pathogenic bacteria or viruses causes extensive illnesses and mortality around the globe. Then it can causes infections such as contaminated water and food supplies. In that perspective, the development of highly sensitive biosensors is a crucial to detect the cause of illness in humans in order to allow appropriate treatment.

A biosensor is design to detect or quantify the presence of a specific biological analyte which integrates with a bio-recognition element and a transduction system. Shaimi and Low (2016) states that principle of biosensor analysis is based on the specific interactions between a substance of interest (target analyte) and the biological recognition element (capture analyte) that is immobilized on the lateral flow membrane.

Membranes are commonly used in biomedical applications that are used as filters for the concentration and isolation of cells, viruses and bacteria, detection of proteins, DNA and RNA in western, Southern and northern blots respectively (Hurk and Evoy, 2015). The membranes are being incorporated into various sensors and biosensors that are being used to detect different compounds including protein, DNA, RNA, bacterial cell, virus particles and pathogens.

For example, composite membrane can be a devices to monitor the presence of bacterial pathogens such as E.coli 0157 and Yersinia pestis (Hurk and Evoy, 2015) as shown in Figure 2.1. The liquid sample containing the E.coli is placed on

the glass fiber membrane sample application pad. Then the solution flows towards the cellulose membrane absorption pad. Along its path HRP conjugated polyclonal antibody (HRP-pAb) enters the solution as it is released from the glass fiber conjugate release pad. While some of the HRP-pAb binds to the E.coli. The pathogens with attached HRP then binds to the monoclonal antibody (mAb) bound to the nitrocellulose membrane signal generation pad. Some unbound HRP-pAb binds to the pAb to HRP-pAb as a control. A reaction then take place with a substrate solution which is catalysed by the HRP to produce a visible output.

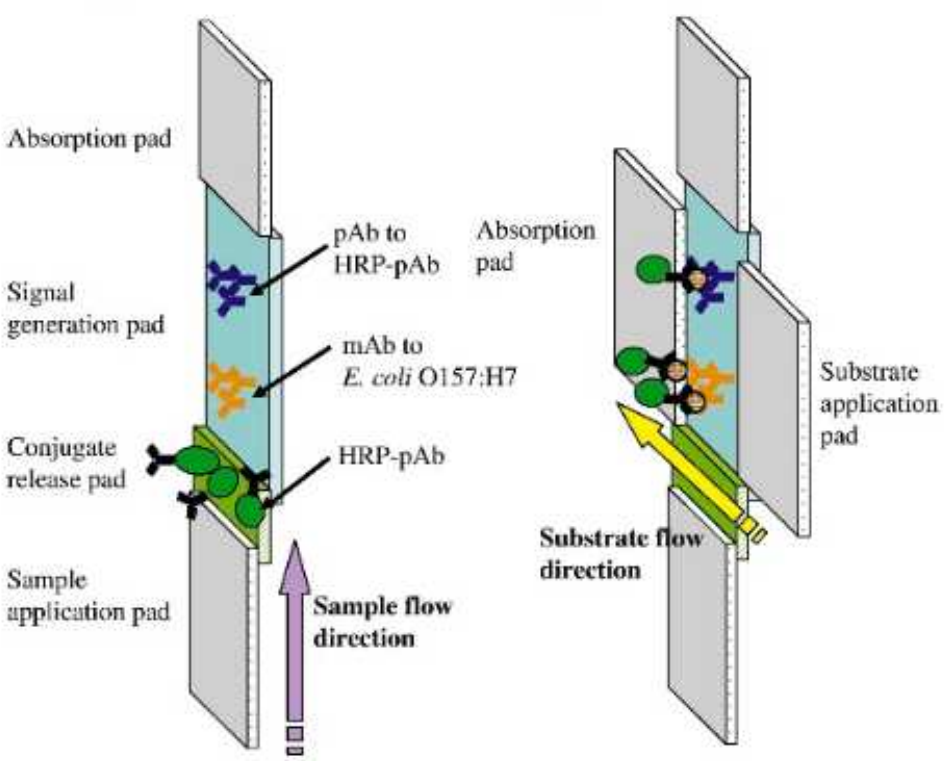


Figure 2.1: A composite membrane sensor (Hurk and Evoy, 2015).

A similar design of composite membrane biosensor but the difference is instead of the visual output however, electrodes were also included beside the capture pad (Hurk and Evoy, 2015). Then polyaniline or iron oxide nanoparticle conjugated antibodies were used to detect the antigen, and form and electrical circuit

as shown in Figure 2.2 (Hurk and Evoy, 2015). The pathogen was introduced in solution to the cellulose. Along the way the conductive material-conjugated antibodies were released from the fiberglass conjugate pad and bound to the pathogen. These pathogens then bound to the antibodies linked to the nitrocellulose capture pad and increased the conductivity of the circuit.

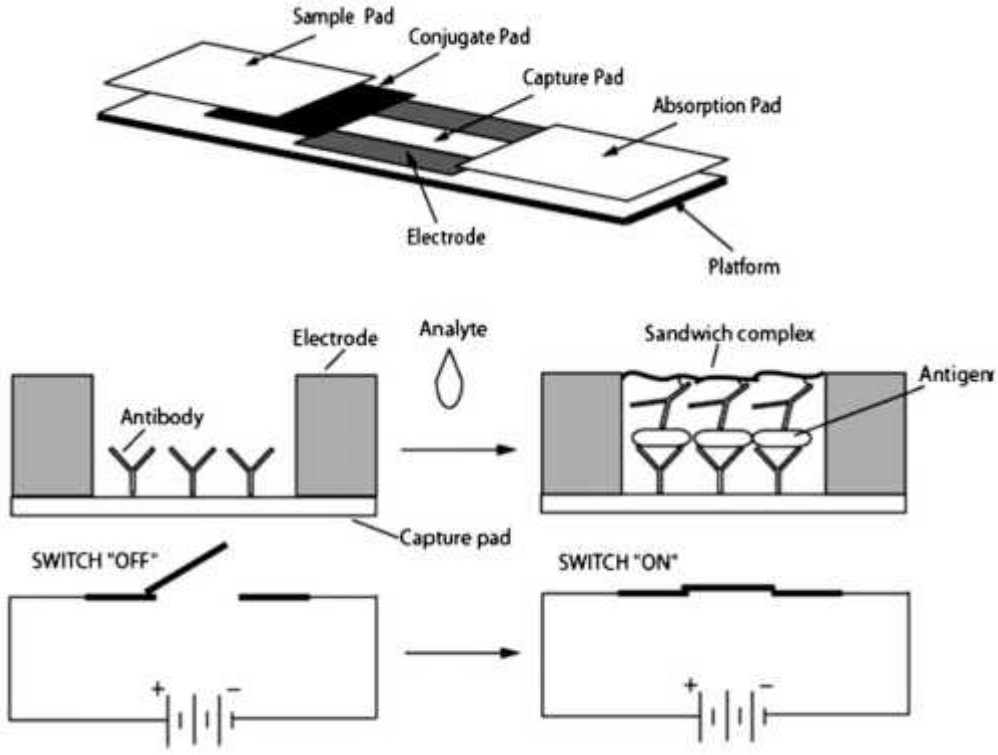


Figure 2.2 : Composite membrane biosensor with electrode(Hurk and Evoy, 2015).

In order to specifically detect the pathogens of interest, it is necessary to use a sensing molecule or molecules which bind only the pathogen or component of the pathogens interest such as antibodies are the most common reagent for specific detection of biomolecules (Hurk and Evoy, 2015). Hurk and Evoy (2015) Reported that the use of specific DNA or RNA probes for oligonucleotide hybridization with extracted DNA or RNA from the pathogen of interest. The sensitivity of biosensor

was increased by using PCR to extend the probe DNA to the length of the pathogen DNA.

## 2.2 Membrane definition and classification

Membrane may be defined as an imperfect barrier or an interphase between two phases which restricts the transport of various substances from one phase to another in a rather specific manner (Piskin, 1986). It also is an interphase between two adjacent phases acting as a selective barrier, regulating the transport of substances between the two compartments. For instance, membranes were used in water treatment, enzymatic catalysis, controlled drug release, oil refinement and gas separation, for the development of biosensors and also protein binding (Algieri et al., 2014).

A membrane can be classified as a natural or synthetic such as ceramic and polymeric membrane. As of synthetic membrane, its structure can be homogeneous or heterogeneous according to their micro-level structure as shown in Figure 2.3. For homogeneous, it considered as a continuous media without any pores in it (Piskin, 1986). However, there are also some kinds of openings which permit the transfer of permeating molecules. It also can be prepared as symmetric or asymmetric in structure. Asymmetric may be integral or composite which refers to two different polymer layer composed in one membrane. While for heterogeneous consist of a solid matrix with defined pores which have a diameter ranging from 5 nm to 50 nm (Piskin, 1986). It also can be prepared as a symmetric or asymmetric in structure.

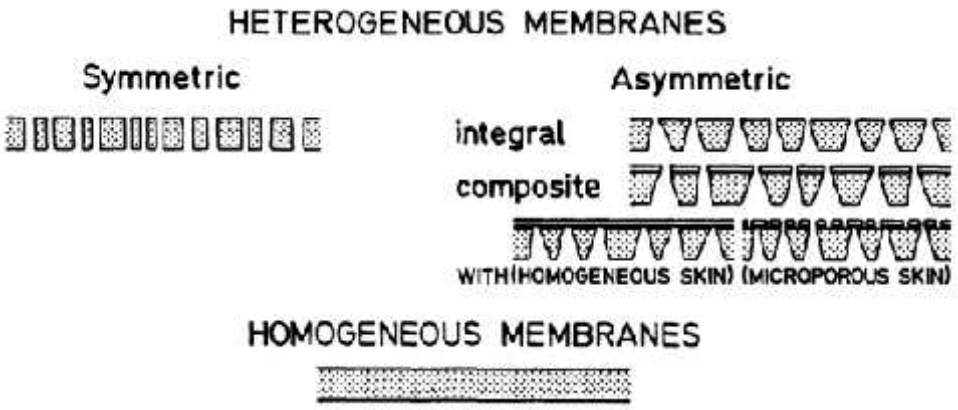


Figure 2.3: Membrane structure (Piskin, 1986).

Membranes are commonly used in a variety of biomedical applications such as used as filters for the concentration and isolation of cells, viruses and bacteria (Hurk and Evoy, 2015). Common materials for membranes for biomedical applications include nitrocellulose, cellulose acetate, nylon and polycarbonate membranes. As of biomedical applications, the membranes are served as the detection platform that can provide a large specific area for bio molecular immobilization due to their highly porous structures (Farahmand et al., 2015). For example, various polymeric materials have been used for immobilization of biomolecules and subsequent virus detection including dengue in biosensor devices. Immobilized protein based biosensors present a fast and reliable alternative due to their simplicity, specificity, fast response and reusability, which make them cost effective (Narang et al., 2011).

Synthetic polymers such as gained high attraction for technical as well as for medical application for various reasons. A wide range of physical and chemical properties can be achieved based on the monomer units, polymerization reaction and formation of co-polymers consisting of different components at adjustable concentrations (Maitz, 2015). Nylon has been used in clinical application because of its high tensile strength and can be used for suture materials in general surgical

implants. Nylon is classified on non-resorbable suture materials is used to treat slow-healing tissue and tissue with high mechanical exposure such as skin or tendons. It also can be used as scaffolds for ligament and tendon repair. This is because of nylon provide better mechanical stability than the biological scaffolds, however its non-degradation and persistence in the body causes problems.

It also can classified according to the number of carbon atom in their monomeric units such as nylon 6,6 and 1,6-diamine hexane. Nylon is a generic name for certain types of thermoplastic polymers and known as polyamides. These polyamides are produced by the condensation reactions between a diamine $NH_2$-$(CH_2)_6$-$NH_2$ and a dibasic acid, $CO_2H$-$(CH_2)_4$-COOH (Vojdani and Giti, 2015).

## 2.3 Nylon membrane for bio-sensing

Biosensors have become very important tools for the detection of chemical and biological compounds for clinical, environmental and food monitoring (Algieri et al., 2014). The wide range of applications in different fields of these devices is due to their excellent high specificity, sensitivity and rapid response. Another important aspect of biosensors is the method that is used to link the capture molecule to the surface, in this case a membrane. This is important because it can substantially affect the sensitivity and specificity of the biosensor.

Based on Coller and Bundy (2003), two parallel studies were conducted and the biosensors were constructed by immobilizing the enzymes on nylon membranes. First, nerve agent-surrogates malathion (MA) and pirimiphos-methyl (PM) were detected based on their inhibition of the enzyme butyrylcholinesterase (BuChE). For second study, numerous metal ions of biomedical interest were detected based on

inhibition of the enzyme urease (Ur). In order to measure the analytes, BuChE and Ur were immobilized onto nylon membranes. Since the detection levels for metals using this sensor are equal to or lower than values sometimes found in body fluids adjacent to metal implants, this type of biosensor also can be also applied in biomedical uses. Coller and Bundy (2003) suggest to outperform nylon-based sensors, it need to construct sensors that use hydrogels to encapsulate the sensing agents in an active form.

Nylon nanofibrous membrane-based biosensors has been introduced by Scampicchio et al. (2010) as shown in Figure 2.4. It also can be applied in various sectors, such as medical, pharmaceutical and food for the detection of glucose. This is where the glucose oxidase is covalently tethered at the nanofibers. The resulting of this biosensing is inherent permeability to the cosubstrate diffusion (Scampicchio et al., 2010) and does not require any activation step of the polyamide (Jia et al., 2006). The advantages of using nylon nanofibrous which is minimally affects the kinetic of the biocatalysis while keeping unaltered the selectivity of the biorecognition event (Scampicchio et al., 2010).


Figure 2.4: Schematic picture of the nylon nanofibrous biosensing unit coupled with a glassy carbon electrode. Also shown a scanning electron microscopy detail of the nanofibrous structure (Scampicchio et al., 2010).

Moreover, nylon membrane has been used for biosensor is to shorten detection time and achieve a lower detection limit. So, the biosensor has utilized a nylon microporous membrane as a matrix for an immunofilter that provides a large surface are for antibody immobilization as shown in Figure 2.5. The smaller diameter of the membrane`s pores was expected to dramatically increase the interactions between pathogens and antibodies and the opportunities of capturing individual pathogens by the immobilized antibodies (Liu et al., 2007).

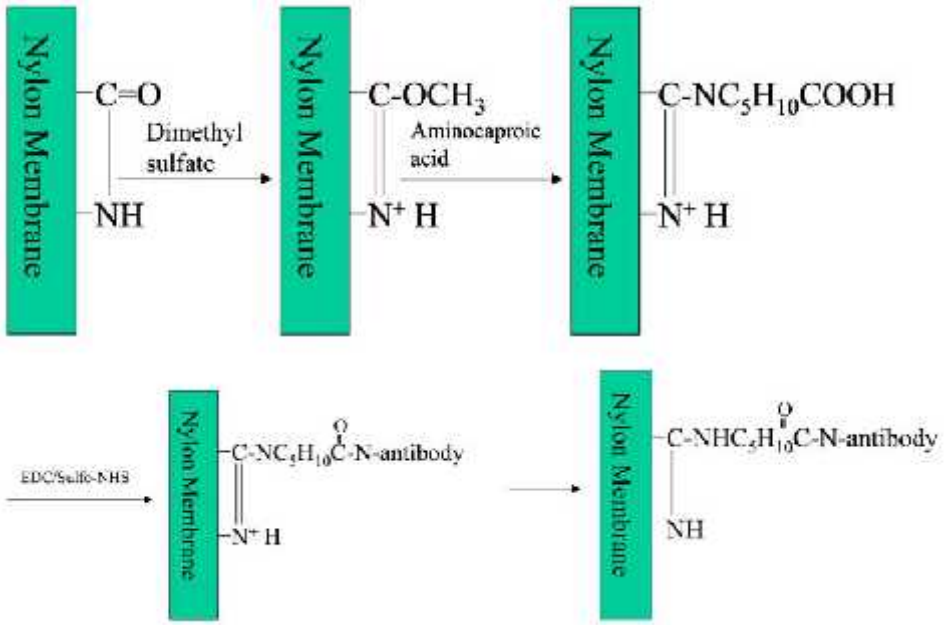


Figure 2.5: The schematic representation of the immobilization of an antibody onto the micro-porous nylon membrane (Liu et al., 2007)

For some applications the membranes may even be used in reverse. One group, for example, used a nylon membrane on the bioelectrode surface in a catalase activity sensor to prevent biofouling by bacterial buildup on the sensor (Serra et al., 2008). This is because the micro-organisms environment seems to affect the stability of the biosensor (Serra et al., 2008). Measurement has carried out and the results demonstrate the effective prevention of surface fouling using the nylon membrane as stated by Serra et al. (2008).

### 2.3.1 Chemical and Physical Properties

Nylon is an inherently hydrophilic membrane, thus, it will helps prevent nonspecific adhesion. On the other hand, synthetic polyamides nylon have been widely used as affinity supports for nylon membranes and protein immobilization (Screenivasulu Reddy et al., 2002). This is because of their characteristics which are thermoplastic polymer with high mechanical strength, superficial hardness and resistance to abrasive conditions caused by the intermolecular hydrogen bond interactions established between the amide groups (Lozano and Iborra, 1997). It is made of repeating units linked by peptide bonds (or amide bonds) bound by secondary amide linkage (Lozano and Iborra, 1997) and frequently referred to as polyamide, as schematically shown in Figure 2.6. It is also has inter- and intra-molecular hydrogen bonding through its amide group as shown in Figure 2.7. Generally, nylon membrane consisted of a microporous structure and positively charged. It is cationic and maintains its positive charge over a wide pH range (Narang et al., 2011).

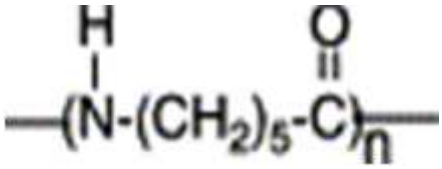

Figure 2.6: Chemical structure of Nylon-6 (Screenivasulu Reddy et al., 2002).

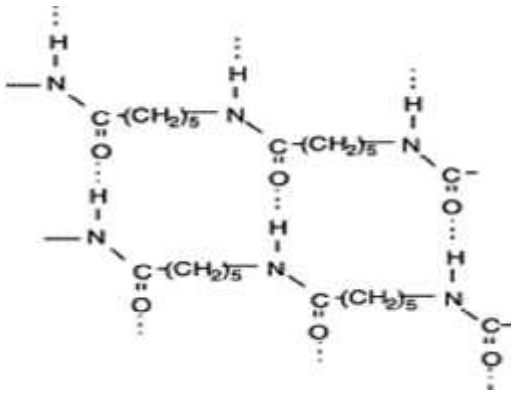

Figure 2.7: Hydrogen-bonding networks of Nylon-6 (Screenivasulu Reddy et al., 2002).

As a support for protein immobilization, nylon is nonporous and provides advantages and disadvantages with respect to the porous supports. Because of nylon have hydrogen bonding, proteins will attach to microporous structure of membrane by means of physical interaction (Farahmand et al., 2015). The physical interaction mainly via three fundamental forces which are hydrophobic interactions, hydrogen bonding and ionic attraction.

Microporous polyamide membrane (nylon) is an engineering plastic that offers narrow pore size distribution and good mechanical rigidity (Farahmand et al., 2015). It also provide a large specific area for biomolecular immobilization due to its characteristic which is highly porous structure. Nylon is a material that popular choice that have been used in biomedical applications. However, nylon lacks desirable surface-reactive functionalities such as amine ($-NH_2$), hydroxyl (-OH) and carboxyl (-COOH) groups that would facilitate immobilization of protein. Analyte-substrate interaction was performed via two different techniques which are physical adsorption and covalent immobilization. Moreover, the surface functional groups of membranes and different “functionalization” techniques is important factor to consider. However, most of the surfaces are prone to “aging effect”. Therefore, resulting in unstable surface chemistries and the performance in bio-activation is inefficient (Hosseini et al., 2015, Abdel-Hamid et al., 1999).

### 2.3.2 Application of Nylon

Nylon 6 is a widely used synthetic polymer because it has combination of strength, flexibility, toughness and abrasion resistance. To improve nylon`s performance, it is necessary to introduce specific functional groups on its surface in

pre-determined locations, densities and patterns (Jia et al., 2006). For example, selective introduction of amine groups to nylon surfaces is likely to open up new possibilities for nylon. This is because of amine-enriched surfaces play an important role in processes such as the removal of heavy metal ions from aqueous solutions, prevent biofouling to occur and for the covalent immobilization of biomolecules such as DNA and polysaccharides.

Because of nylon characteristics such as excellent stability in human body fluid, good mechanical strength and thermal stability that make it to be used in biomedical applications (Esfahani et al., 2015). Since the chemical structure of nylon 6 contains carboxyl and amine groups ($CO(CH_3)_5NH$), it can be easily combined with ceramic particles that making them suitable for many biochemical applications.

Gene expression analyses by hybridization of probes derived from mRNA to cDNA target arrayed on a nylon membranes have been performed with increasing frequency and success over the last decade (Cox, 2001). Southern (1975) was realised that whole DNA libraries could be arrayed on membranes as targets for hybridization with labelled probes to identify genomic or cDNA clones. It means that individual clones are applied as spots either in the form of bacterial colonies, PCR products or plasmids (Cox, 2001). By using nylon membrane based cDNA arrays, information on gene expression is high. A variety of arrays are available such as those including key gene from different cell states such as apoptosis and cancer or general collections from different genomes such as human or mouse. The advantages of by using nylon is appreciated as a relatively economical alternative to other gene expression technologies and can be assembled rapidly, or added to, on an ad hoc basis (Cox, 2001). For generating arrays, the cost are moderately high but now it give a valuable profit due to the main characteristic of these system; the large

amount of data generated, since the expression of many thousands of genes are measured in parallel (Cox, 2001).

### 2.3.3 Advantages of Nylon

Membranes are commonly used in variety of biomedical applications. Common materials for membrane include nitrocellulose and polycarbonate and such membranes are being incorporated into various sensors and biosensors in particular. It can be used to detect different compounds including proteins, DNA and RNA, bacterial cell and virus particles (Hurk and Evoy, 2015).

Generally, nylon membrane offers narrow pore size distribution and good mechanical rigidity. It widely used in biomedical applications because of its properties such as lightweight, low production costs, strength and durability (Farahmand et al., 2015). Charge of the membrane is positively charged. Since nylon is a crystalline polymer, its characteristic effect the lack of solubility of nylon in solvents as well as high heat resistance and high strength coupled with ductility. Other than that, it also has higher elasticity, toxicological safety for patients and metal allergy (Vojdani and Giti, 2015).

Membranes such as nitrocellulose (Gershoni and Palade, 1983, Zaluzec et al., 1994), PVDF (polyvinylidene difluoride) (Gershoni and Palade, 1983, Zaluzec et al., 1994), activated paper (Gershoni and Palade, 1983), activated nylon (Gershoni and Palade, 1983, Zaluzec et al., 1994), or glass fiber (Zaluzec et al., 1994) have been used successfully to bind transferred proteins. These membranes can be classified as being uncharged (PVDF), anionic nitrocellulose, glass fiber or cationic (charge

modified PVDF), charge modified nylon or modified glass fiber (Zaluzec et al., 1994)

. Table 2.1 compares the relative binding affinity of nylon, nitrocellulose and PVDF for radiolabeled sheep antirabbit igG by protein binding capacity (Zaluzec et al., 1994). For nitrocellulose, it is the most commonly used membrane support. However, the nitrocellulose membrane give a disadvantages on protein binding where the proteins are not covalently bound and the membrane is brittle when dry. It has been tested that only a small proteins tend to move through nitrocellulose membrane and only a small fraction of the total amount that actually binds (Kurien and Scofield, 2003). Amongst the membranes, nylon has excellent mechanical strength. However, it can only bind a small amount of proteins. Therefore, to solve this problem, in this work, the activation of nylon that contain positively charge is proposed, which is expected to produce higher non-specific binding (Kurien and Scofield, 2003).

Table 2.1: Characteristics of protein adsorption to immobilization membrane supports (Zaluzec et al., 1994).

| Membrane Type | Protein binding capacity ($\mu g/cm^3$) |
|---|---|
| Nylon | 80 |
| Nitrocellulose | 80 – 100 |
| PVDF | 170 - 200 |

For examples, Dhall et al. (2013) has study about the screening of different supports for the immobilization of a bacterial consortium for the development of a BOD biosensor. The different supports has been used such as PVA (polyvinyl alcohol) + nylon cloth, agarose, nitrocellulose and a nylon membrane to compare their stability, viable and most effective for the BOD biosensor. After testing of

different type of supports for the BOD biosensor, it can be found that microbes immobilized on the nylon membrane exhibited a maximum stability compared to other membranes such as nitrocellulose and PVA (polyvinyl alcohol). This is because the nylon membrane acts as a better support for immobilizing microorganism to be used been proved that for the BOD biosensor. Moreover, it is a positively charged membrane which can entrap and adsorb the negatively charged bacteria effectively.

## 2.4 Protein-Nylon interaction for protein immobilization

In most cases, membrane needs to be activated. Membrane activation is to increase the potential of protein binding ability. Usually membrane activation could be carry out by using methanol. The role of methanol is to help good transfer of proteins to membrane, prevent membrane swelling from heating and to enhance the protein adsorption on the membrane. Thus, many methods have been established in order to improve the immobilization of protein.

Protein can be immobilized to a nylon membrane via physical adsorption (physisorption), bioaffinity interaction, covalent bond or the combinations of these three mechanisms (Kim and Herr, 2013, Zhang et al., 2013) as shown in Figure 2.8.

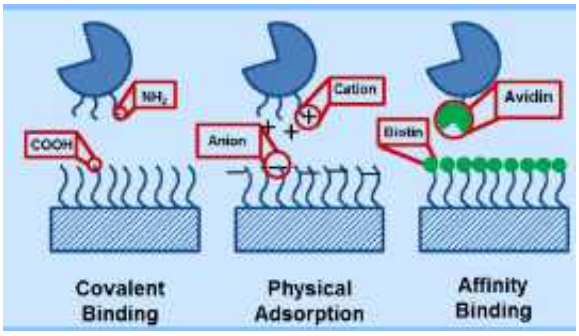


Figure 2.8: Illustration of representative examples of covalent binding (amide bond formed by carboxyl and amine groups), physical adsorption (ionic interaction), affinity binding (biotin-streptavidin interaction) (Jia et al., 2006).

The simplest method among method mentioned above is physical adsorption which is physisorption. By this method, protein can be adsorbed to various surfaces via intermolecular forces such as electrostatic, hydrophobic, van der Waals, hydrogen bonding interactions (Zhang et al., 2013, Jesionowski et al., 2014). For example, Piletsky et al. (2003) has been studied that Polypropylene (PP) membranes modified with polyaniline (PANI) show an adhesion mechanism of combined electrostatic and hydrophobic interactions and demonstrate high affinity and compatibility toward different proteins. Sometimes, physical adsorption happen instantaneously compared to other bonds that required a substantial incubation time. Therefore, it can be used as an intermediate immobilization step of a multistep assay sequence such as Western blot (Kim and Herr, 2013).

However, this techniques is weak, the adsorbed layer of protein is not stable as compared to covalent bond (Rusmini et al., 2007, Daly et al., 2005). The protein may remove by some buffers or detergents when performing the assays (Rusmini et al., 2007). Thus, the intermolecular forces are also highly dependent on environmental conditions such as pH, ionic strength, temperature and surface condition. For examples, Betancor et al. (2006b) has found that if the solution with high ionic strength is used, immobilization will proceed slower, but the protein will be immobilized directly via a covalent attachment. At high ionic strength, immobilization would occur through the region with the most reactive amino groups by altering the ionic strength.

Bioaffinity interaction has more advantages over physical adsorption. This is because it yield relatively stronger, highly specific oriented protein immobilization (Kim and Herr, 2013). The advantages of this method are the minimal of protein leakage and the immobilized protein is having a better accessibility to the binding

partners than the random orientation strategies. Moreover, the bioaffinity interactions can be reversed using chemical treatment, pH change and heat treatment. Usually, it is used in conjunction with other immobilization mechanisms such as antibodies and antigens, nucleic acids and nucleic acid-binding proteins and avidin and biotin (Mohamad et al., 2015) where, the bioaffinity reagent is used as an intermediate binding molecule between the surface and proteins.

As for the covalent bond between the substrate and protein, this method is frequently used in microfluidic assays (Kim and Herr, 2013). Covalent binding is used to bind the protein to the functional group of cross-linking agent via chemical bond. However, the selection of suitable immobilization conditions also important because it may maximize a covalent attachment such as reaction time, pH value and temperature (Mateo et al., 2007). For example, the use of more drastic condition such as pH over 8, higher glutaraldehyde concentrations yielded an uncontrolled reaction that generated the polymerization of glutaraldehyde in solution (Betancor et al., 2006b). This mechanism required the activation of the immobilization surface via reactive reagents. It can be done by the activated surface reacts with amino acid residues and resulting in formation an irreversible linkage. For example, protein is immobilized to nylon membrane through covalent linkage formed between the glutaraldehyde and free amino groups on the nylon and also free amino groups on the surface of the protein (Inman and Hornby, 1972). The unreacted active functional groups are blocked or deactivated. However, the disadvantages of covalent linkage is its slow covalent reactions and usually require long incubation times.

Although nylon membrane could be interact with protein through these three methods, however, the binding may not be strong or easily washed out. Therefore, among three methods, covalent bonding is often preferable (Hurk and Evoy, 2015)

and one of the simplest methods to bind the biomolecules onto the membrane surfaces (Shaimi and Low, 2016) which is by using glutaraldehyde as cross-linking reagents (Shaimi and Low, 2016, Hurk and Evoy, 2015) and preventing them from being washed off.

## 2.5 Glutaraldehyde to activate membrane

Glutaraldehyde activation supports for protein immobilization is one of the oldest activation methods but still popular due to it can be used in wide range application. It also popular due to its advantages. There are due to their simplicity of the procedure, high efficiency and possibility of application a variety of natural and synthetics supports, such as alginates, chitosan, gelatin, nylon, silica or graphite (Bezbradica et al., 2014). Glutaraldehyde is one of the most effective protein crosslinking reagents.

Glutaraldehyde activation can be formed at usual conditions of support activation such as structures of glutaraldehyde exits in its water solution, its mono- and dehydrates can be found as well as cyclic hemiacetal and oligomers (Bezbradica et al., 2014). Most of the protein were traditionally immobilized on glutaraldehyde activated supports. Thus, it suggested that the linkage on glutaraldehdye activated supports are performed through a different mechanism involving the reaction with cycled forms of the glutaraldehyde (Bezbradica et al., 2014).

For example, Shaimi and Low (2016) has studied the use of glutaraldehdye to chemically cross-link with the cellulose compound in mixed cellulose membrane and the protein molecules. Thus it creates the high protein immobilization to the