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Development of a piezoelectric biosensor based on PVDF films

Dissertação para obtenção do Grau de Mestre em Engenharia Química e Bioquímica

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Resumo

A presente dissertação tem como base um biosensor piezoeléctrico baseado em ondas acústicas que se propagam em filmes finos do polímero fluoreto de polivinilideno (PVDF), utilizado para produzir um dispositivo ressonante oscilatório. A essência deste dispositivo consiste num filme composto por um polímero piezoeléctrico, PVDF, responsável pela oscilação do filme, e por uma membrana porosa, Immobilon, um tipo especial de PVDF com capacidade de ligação a proteínas que pode actuar como receptor biológico.

Foi estudada a possibilidade de se utilizar um filme composto apenas por PVDF piezoeléctrico. Devido ao carácter extremamente hidrofóbico do PVDF, pretendia-se efectuar a modificação da superfície do polímero através da imobilização de uma camada funcional à sua superfície, de modo a melhorar a hidrofilicidade e biocompatibilidade do filme. Para tal, foi efectuado um método de imersão, aplicado tanto a PVDF piezoeléctrico não poroso como a membranas porosas de PVDF. Foram estudas três possíveis soluções para o revestimento, nomeadamente, soluções de polietilenoglicol (PEG), 3,4-Dihidroxi-DL-fenillalanina (DL-DOPA) e goma arábica. A hidrofilicidade das membranas foi caracterizada através da medição de ângulos de contacto e a sua composição elementar foi estudada por análise elementar. Marcou-se a proteína BSA com o fluoróforo isotiocianato de fluoresceína – FITC, para que se realizassem ensaios qualitativos e quantitativos para o estudo da sua ligação a membranas de PVDF. Os resultados obtidos indicaram que seria com uma solução de PEG aplicada a uma membrana porosa de tamanho de poro 0.2 µm que se obteria o melhor sistema a aplicar no sensor. A resposta do sensor foi testada utilizando um sistema composto por PVDF/Immobilon (0.2 µm) /PVDF para a detecção de PEG e BSA, sendo possível detectar a presença de ambos os compostos no meio líquido utilizado no sensor.

Termos chave: Biosensor, PVDF, piezoelectricidade, membranas de PVDF, modificação de superfície

Abstract

The core of this work is a piezoelectric biosensor in which acoustic waves are launched in very thin PVDF polymer films to produce an oscillatory resonant device. The essence of the device consists in a polymer film system made of a piezoelectric polymer, PVDF, responsible for the film oscillation and a porous membrane, Immobilon, a special type of porous PVDF with protein binding capacity which can act as a biosensitive area.

The possibility of using a film system composed only by PVDF was studied. Due to its strong hydrophobic nature, surface modification was aimed to be performed by coating a functional layer on the membrane surface, in order to improve the hydrophilicity and biocompatibility of PVDF. An immersion method was preformed and applied to porous and non-porous PVDF membranes. Three distinct coating solutions were studied, namely, polyethylene glycol (PEG), 3,4-Dihydroxy-DL-phenylalanine (DL-DOPA) and Gum Arabic solutions. Hydrophilicity improvements of the membranes were characterized by water contact angle measurements and its elemental composition was studied by elemental microanalysis. BSA protein marked with FITC fluorescein was used to perform quantitative and qualitative assays in order to study its adsorption to coated and uncoated PVDF membranes. Globally, the best results were obtained when a solution of PEG was utilized with 0.2 μm microporous membrane. The sensor response was tested with polyethylene glycol (PEG) and bovine serum albumin (BSA), using a film system composed by PVDF/0.2 μm Immobilon/PVDF, which was able to respond to the presence of both compounds in liquid medium.

Keywords: Biosensor, PVDF, piezoelectricity, PVDF membranes, surface modification

Table of contents

Index of Figures

Index of Tables

Acronyms

BSA	Bovine serum albumin protein
CBB	Coomassie Brilliant Blue
DI	Deionized
DL-DOPA	3,4-Dihydroxy-DL-phenylalanine
FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
GA	Gum Arabic
PEG	Polyethylene glycol
PVDF	Polyvinylidene Fluoride
QCM	Quartz Crystal Microbalance
Tris	Tris(hydroxymethyl)aminomethane

Chapter 1 Introduction

1.1 Biosensors

In today's world, there is a great need of detecting a wide diversity of compounds. Several common chemical and biological materials can be extremely dangerous to human health and to the environment. For example, industrial and agricultural use of chemicals can lead to food and water supplies contamination and food deterioration can lead to bacteriological contamination. Also, the potential threat of bioterrorism must be considered, due to the possible deliberate release of biological toxins. As a result, there is the need for a tool that can rapidly, accurately and reliably detect contaminating bio-agents. Biosensors are low-cost high efficient devices for this purpose which currently are in the focus of research activities in sensorics and analytical chemistry [1-2].

The biosensor field has grown enormously since the first demonstration of the biosensor concept by Leland C. Clark Jr., in 1962, with the development of an amperometric enzyme electrode for glucose. Since then, research communities from chemical, biological, material, physical sciences and engineering and computer sciences have come together to develop more reliable, robust and accurate biosensing devices. Therefore, the definition of biosensor varies with the field of application and was not uniform in the literature a few years ago.

A biosensor can be defined as "an analytical device, which converts the concentration of the target substance, the analyte, into an electrical signal through a combination of a biological or biologically derived recognition system either integrated within or intimately associated with a suitable physic-chemical transducer" [3].

The general structure of biosensors consists in a combination of two parts: the *bioreceptor* and the *transducer*. The bioreceptor, a biologically sensitive element, interacts with the analyte selectivity, whereas the transducer generates a signal as a result of the former interaction, which carries information about the concentration of the analyte. The bioreceptor is highly specific to the analyte to which it is sensitive and consequently it does not recognize other analytes [4]. A general block diagram of a biosensor is described above in Figure 1.1.

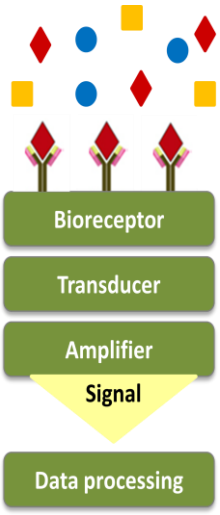


Figure 1.1 General block diagram of biosensors

Biosensors can be distinguished either by their type of biorecognition system or their transducer mechanism. The classification is shown in Figure 1.2.

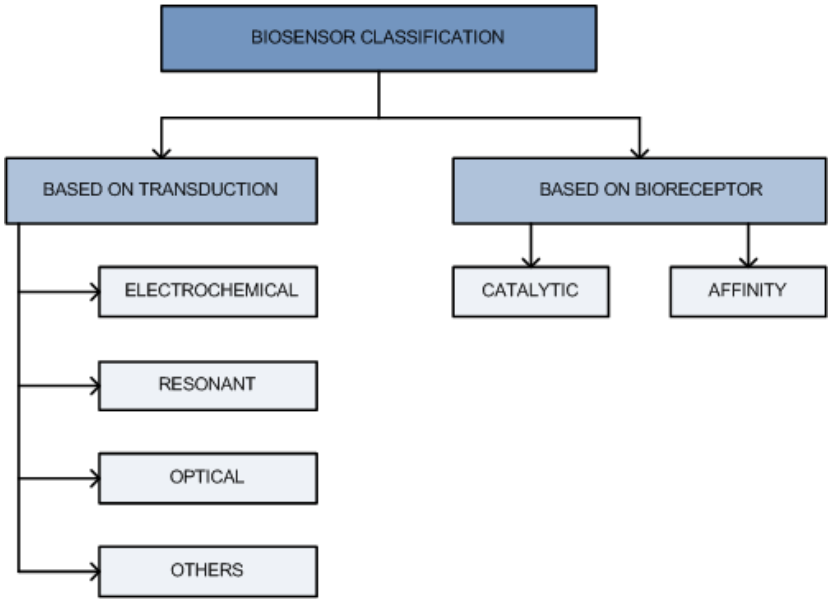


Figure 1.2 Biosensor classification.

1.1.1 Biorecognition Systems

The main distinguishing feature of a biosensor is the recognition system for the target analyte. To chose these biological elements, the nature of the target analyte must be taken into consideration, as well as its concentration in the sample, the presence or absence of interfering substances, and whether the measurement is discrete, real-time or continuous [3].

According to the type of the bioreceptor, biosensors can generally be distinguished into the following groups:

- *Catalytic biosensors* that use immobilized enzymes, microorganisms or living tissues as as bioreceptors. The use of an enzyme as a biorecognition element utilizes its selectivity to bind with a specific substrate and catalyze its conversion to a product. This enzyme–substrate-catalyzed reaction makes it possible for a signal to be produced by the transducer, which can be based on the reaction products, the charge exchange or heat generation.
- *Affinity biosensors* are based on specific chemical binding. The analyte may simply bind to the biological material present on the biosensor, for example, antibodies, neuro-receptors and nucleic acids. These biosensors are based on affinity interactions by separating an individual or selected range of components from complex mixtures of biomolecules [5]. In immunosensors this would mean the antigen-antibody reaction. Each antibody recongnizes its antigen with great specificity. Neuro-receptors are neurologically active compounds such as insulin, other hormones and neuro transmitters that act as messengers via ligand interaction. In DNA sensors, the chemical selective chemical binding is the hybridization of molecule clusters with DNA molecules to form a double structure [4].

1.1.2 Transduction mechanisms

Biosensors can be classified based upon the transduction methods they employ. The transduction process transforms the physical, chemical or biological response of biorecognition into an electrical, optical or any other form of signal with high sensitivity. Transduction can be accomplished via a great variety of methods. Most forms of transduction can be categorized in one of three main classes: 1) optical detection methods, 2) electrochemical detection methods and 3) mass detection methods. Each of these three main classes contains many different subclasses, creating a nearly infinite number of possible transduction methods or combination of methods.[6]

1.1.2.1 Electrochemical biosensors

The underlying principal of electrochemical biosensors is that many chemical reactions produce or consume ions or electrons which cause some change in the electrical properties of the solution which can be sensed out and used as a measuring parameter. Electrochemical

biosensors can be classified based on the measuring electrical parameters as: *conductimetric*, where the measured parameter is the electrical conductance/resistance of the solution; *amperometric*, where current is the measured parameter; and *potenciometric*, where the measured parameter is oxidation or reduction potential of an electrochemical reaction. This class of biosensors is mainly used for detection of hybridized DNA, DNA-binding drugs, glucose concentration, etc. [1].

1.1.2.2 Gravimetric resonator biosensors

In resonant biosensors, an acoustic wave or piezoelectric transducer is coupled with a bio-element. When the analyte molecules get attached to the membrane, the mass of the membrane changes. The resulting change in the mass subsequently changes the resonant frequency of the transducer. This frequency change is then measured [1, 5].

1.1.2.3 Optical biosensors

In the most commonly used form of an optical biosensor, the transduction process induces a change in the phase, amplitude, polarization, or frequency of the input light in response to the physical or chemical change produced by the biorecognition process. For this type of biosensor, light is the output transducer signal measured. Optical transduction offers the largest number of possible subcategories of all three of the transducer classes. This is due to the fact that optical biosensors can be used for many different types of spectroscopy (e.g., absorption, fluorescence, phosphorescence, Raman, SERS, refraction, dispersion spectrometry, etc.) with different spectrochemical properties recorded. These properties include: amplitude, energy, polarization, decay time and/or phase [6].

Electrochemical, optical and acoustic transducers account for well over 90 % of the published literature in biosensors. However, new types of transducers are constantly being developed for use in biosensors as, for example, approaches based on thermal and magnetic principles.

1.1.3 Applications of Biosensors

Biosensors can be applied in any circumstances where analysis requires a rapid, direct, accurate, and a fool-proof read-out from a sample, proximal to where the analytical sample is taken.

Its major applications sectors are biomedical [4], industrial, food and beverage, agricultural [7], environmental [8-9], forensic, security and defence [10], and toxicity monitoring. For example,

sensors have been developed for measuring the concentration of biologically active, physiologically important compounds, such as glucose, urea, cholesterol levels in blood, testing of food flavour and components, allergens, meat quality, and fish freshness, monitoring of air, water and soil, herbicides, insecticides, detergents, fermentation and bioreactor processes, immunoreaction tests with immunosensors and fast genetic analysis using DNA chips. [11-12]

1.1.4 Immobilization techniques

One key issue of biosensor fabrication is the appropriate attachment of the biological elements onto the transducer surface (immobilization). The biorecognition elements are normally immobilized on a solid support, usually a membrane, polymer, copolymer, or semiconductor material. [5].

The choice of the optimum immobilization technique to a particular application depends on various factors such as the transduction principle, the nature of the biological receptor and the nature of the analyte to be detected. Also, it depends on the way the biosensor is to be used and on its surface chemistry [13]. Numerous immobilization methods based on physical or chemical processes have been developed as, for example, physical adsorption at a solid surface, cross-linking between molecules, covalent binding to a surface, and entrapment within a membrane, surfactant matrix, polymer or microcapsule. In addition to these conventional methods, sol-gel entrapment, electro-deposition, photo-polymerization and bulk modification have been also used.

1.1.5 Piezoelectric gravimetric biosensors

Piezoelectric sensors have been developed based on very established theories in electricity, mass, and viscoelasticity and with commercially available instruments, such as quartz crystal microbalance (QCM). Piezoelectric sensors have shown their advantages over other sensors in terms of sensitivity, versatility, label free, low cost, and simplicity. They have been applied to the biomedical area mainly in the forms of immunosensors and genosensors for rapid detection of bacteria, viruses and proteins, and DNA/RNA hybridization, respectively [14-15].

Since the discovery of piezoelectricity in some simple crystals (e.g. quartz and topaz) by Jacques and Pierre Curie in the late 19th century, this phenomenon has kept minds excited. The Curies observed that a mechanical stress applied to the surfaces of various crystals, caused a corresponding electrical potential across the crystal, whose magnitude was proportional to the applied stress. Also, they verified the converse piezoelectric effect in which application of a voltage across these crystals caused a corresponding mechanical strain. These are the direct and reverse piezoelectric effects. Etymologically, the word “piezoelectricity'” derives from the Greek word “piezo”, which means stress [16].

Many types of materials (quartz, tourmaline, lithium niobate or tantalate, oriented zinc oxide or aluminium nitride) exhibit the piezoelectric effect, but the properties of quartz make it the most common crystal type used in analytical applications. Most piezoelectric gravimetric biosensors are based on quartz crystal microbalances (QCM) or surface acoustic wave devices. Due to its piezoelectricity the crystal can be made to oscillate, by simple electronic circuitry, in a shear mode at a natural frequency which is inversely proportional to the crystal thickness. The addition of mass bounded to its surface reduces the frequency [17].

The polymer polyvinylidine fluoride (PVDF) can exhibit, when specially treated, piezoelectric properties and thus, has been reported to be used to replace the quartz crystal in gravimetric biosensors [17-18].

1.1.5.1 Piezoelectric biosensors based on acoustic waves in thin PVDF film

1.1.5.1.1 PVDF

Polyvinylidene Fluoride (PVDF) is a highly non-reactive thermoplastic fluoropolymer, which generally possesses distinction chemical stability against most of the chemicals, including a wide range of harsh chemicals such as halogens and oxidants, inorganic acids, as well as

alcohols and aliphatic, aromatic and chlorinated solvents [19-20]. However, its excellent chemical stability is of a particular concern when exposed to strong base solutions or esters and ketones.

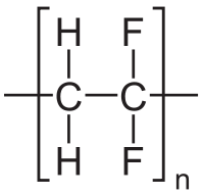


Figure 1.3 Chemical formula of PVDF

PVDF is a semicrystalline polymer, usually containing 59 wt % fluorine and 3 wt % hydrogen [21]. As shown in Figure 1.3, the macromolecular linear chain structure of PVDF is $-CH_2CF_2-$. The spacial arrangements of CH_2 and CF_2 groups along the polymer chains and the strong interaction between both groups contribute to unique properties of PVDF. The bond between fluorine, which is highly electronegative, and the carbon atom is extremely strong (460 kJ/mol) and the C-C bond of the main polymer is surrounded by fluorine and hydrogen atoms, resulting in excellent chemical, thermal and mechanical stabilities [19-20].

Crystallinity can vary from about 35% to 70%, depending on the method of preparation and thermo-mechanical history and its degree affects PVDF's toughness and mechanical strength. The characteristics of PVDF vary according to molecular weight, molecular weight distribution, extent of irregularities along the polymer chain and crystalline form. PVDF exhibits a complex crystalline polymorphism not observed in other synthetic polymers. Its chains can crystallize into several crystalline phases known as α, β, γ and δ [22-23]. The most common polyforms of PVDF are the alfa and beta forms. The chain configuration of the α form is a trans-gauche conformation, in which hydrogen and fluorine atoms are located alternately on each side of the chain (Figure 1.4) [24]. The β crystalline form consists of all the fluorine atoms on one side of the chain, and the hydrogen atoms on the other side (Figure 1.4) — the 'zigzag' chain structure. This structure is the key to high piezoelectric and pyroelectric activity because the net dipole moment is very high and perpendicular to the chain direction [25-27].

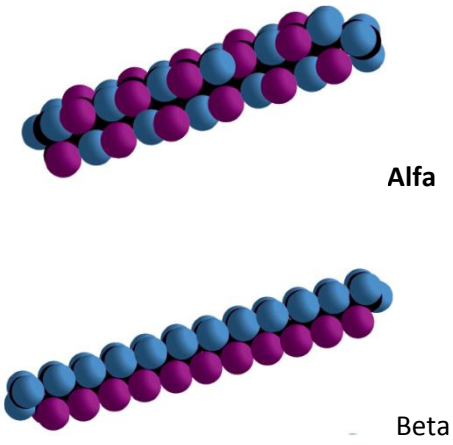


Figure 1.4 PVDF-α form and PVDF-β form (Carbon, Hydrogen, Fluorine)

In recent years, PVDF has received great attention, especially as a membrane material regarding its outstanding properties, which make it suitable for a wide range of applications. PVDF membranes have been applied in water purification, medical purification, gas separation, food and beverages filtration, battery separators and fuel cell membranes. PVDF films can be applied in automotive and building industry, piezoelectric films and greenhouse. Piezoelectric PVDF films can particularly be used in medical and non-destructive evaluation transducers, loudspeaker, sonar detection, vehicle detection, and literally hundreds of other types of sensors.

PVDF membranes can be prepared by various methods such as phase inversion, use of inorganic particles [28], sintering [29-30] and track etching [31]. The main method for its preparation is phase inversion due to its simplicity, flexible production scale and low cost of production.

1.1.5.1.2 Hydrophilic modification of PVDF membrane

Despite PVDF's excellent chemical, thermal and mechanical stabilities, there are some critical problems which limit further developments and applications of PVDF membranes. Its low surface energy and critical surface tension result in the poor wettability of PVDF membrane.

Due to the strong hydrophobic nature of PVDF, it is very important to improve the hydrophilicity of PVDF membranes. Different modification ways have been reported in literature in recent years. Hydrophilic modification techniques can be catalogued into surface modification and blending modification. Surface modification is achieved by coating or grafting a functional layer on the membrane surface, on the top and/or bottom of the surface,

excluding the pores inside the membrane, due to the limited diffusion ability of the modifying agents into the membrane pores. Blending modification is used to achieve the desired functional properties along with the membrane preparation, accomplishing both preparation and modification process in a single step.

Surface modification of PVDF membranes can be classified into two categories: surface coating and surface grafting. Surface coating is the simplest way of improving the surface of PVDF membranes temporarily through coating or depositing a thin film functional hydrophilic layer onto its surface. However, the instability of the coated layer can be a problem, because it can be washed way along the operation and cleaning process due to the weak physical interaction between PVDF and coated layer. Chemical treatments (e.g. sulfonation or crosslinking) can be performed on the membrane surface to anchor the coated laye, while surface grafting can conquer the instable problem completely by the grafting polymerization of monomers, immobilizing the functional chains, brushes or layers onto the membrane surface through covalent bonding interaction[20]. Covalent attachment offers a long-term chemical stability of grafted chains in contrast with physically surface coating method and can be achieved by means of UV photo irradiation, plasma, high energy irradiation and controlled polymerization [32-33]. Examples of different surface coating approaches are listed in Table 1.1.

Table 1.1 PVDF membrane modified by surface coating

Surface coating	PVDF membrane	Coated layer
Original **Modified**	PVDF 0.2 μm PVDF/ poly(DOPA)	3,4-Dihydroxyphenylalanine (DOPA) [34]
Original **Modified**	PVDF 0.22 μm, Millipore PVDF/ Chitosan	Chitosan [35]
Original **Modified**	HFP-707 (Koch) PVDF/PVA	Poly(vinyl alcohol) (PVA) [36]
Original **Modified**	PVDF 0.22 μm, Millipore SPVDF	Sulfonation [37]

Figure 1.5 illustrates an example of the route of surface coating modification for DOPA coated layer and heparin immobilization:

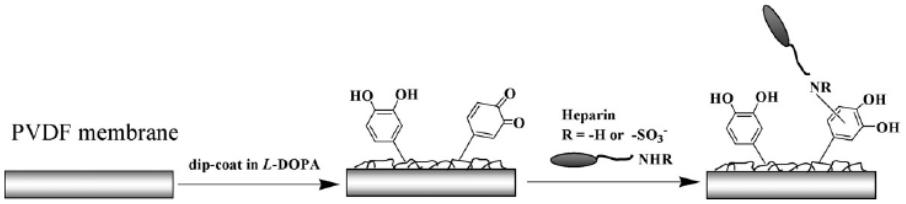


Figure 1.5 Surface modification of PVDF porous membrane via poly(DOPA) coating and heparin immobilization [38]

Examples of versatile ways to modify PVDF membranes surface by surface grafting are listed in Table 1.2:

Table 1.2 PVDF membranes modified by surface grafting

Surface coating	PVDF membrane	Monomer	Grafting way
Original **Modified**	PVDF 0.65 µm, Millipore PVDF-g-PEG	Poly(ethylene glycol) (PEG)	plasma [39]
Original **Modified**	PVDF PVDF-g-PEGMA	PEGMA	electron beam [40]
Original **Modified**	PVDF 0.45 µm, Millipore PVDF-g-poly(2-vinylpyridine)	2-Vinylpyridine	ATRP [41]
Original **Modified**	PVDF PVDF-g-PVP	N-vinyl-2-pyrrolidinone (NVP)	UV [42]

Figure 1.5 illustrates an example of a mechanism of plasma surface grafting of PEG onto PVDF membrane:

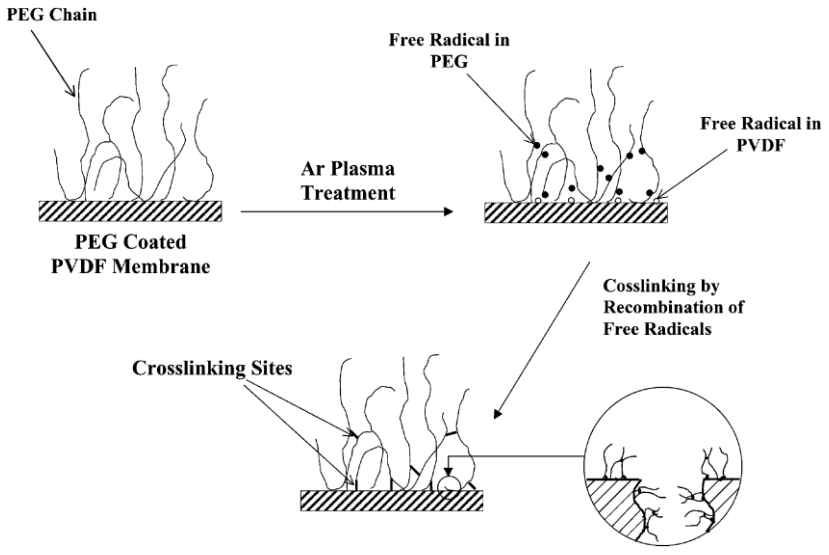


Figure 1.6 Schematic representation of the plausible mechanism of plasma grafting of PEG onto the PVDF microporous membrane [39]

1.2 The Piezoelectric Biosensor

The piezoelectric biosensor, developed at CENIMAT, has a principle of operation identical to a QCM. This device uses a piezoelectric polymer film system made of PVDF, used to produce a mass sensitive oscillatory resonant device.

1.2.1 Operating Principal and Oscillating Circuit

A generic piezoelectric film is clamped between two pairs of electrodes, as shown in Figure 1.7. One set of electrodes acts as the transmitter and the other set as the receiver.

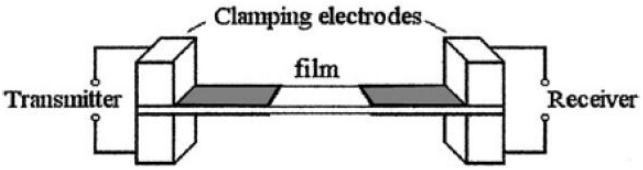


Figure 1.7 - A piezoelectric film clamped between two pairs of electrodes

In operation, a voltage signal is applied to the transmitter which launches a longitudinal acoustic wave through the film, due to the piezoelectric effect. When the wave reaches the other side, a charge is produced in the set of electrodes forming the receiver, due to the inverse piezoelectric effect. The acoustic wave is reflected and returns to the transmitter end where it is again reflected. An oscillatory resonant system can be obtained by driving again the transmitter at this precise time, resulting in the enhancement of the acoustic wave. The film will have a resonance frequency dependent on both its length and properties, given by Equation 1-1:

$$f_o = \left(\frac{1}{2L}\right)(\rho\, s_{11}^E)^{-1/2}$$

Equation 1-1

where L is the length between the clamping electrodes, ρ is the density of the film and $s_{11}^E$is the elastic compliance.

The deposition of a small mass in the surface of the film changes the film density ρ , and therefore there will be a change in the resonance frequency, given by Equation 1-2:

$$\frac{\Delta f}{f} = -\frac{1}{2}\frac{\Delta \rho}{\rho}$$

Equation 1-2

where Δf is the change in the original frequency f produced by a change in density ρ of $\Delta\rho$. To express the bounded mass in terms of surface density, ngcm^{-2}, the above equation can be easily changed to:

$$\frac{\Delta f}{f} = -\frac{1}{2}\frac{\Delta m}{m}$$

Equation 1-3

where Δm is the added mass to the surface of the film with a surface mass of m of the PVDF film expressed in the same units. The negative sign indicates that as Δm increases the frequency decreases [17].

In order to obtain an oscillatory resonant device, this set-up must be connected to an appropriate electronic circuit, as shown in Figure 1.8:

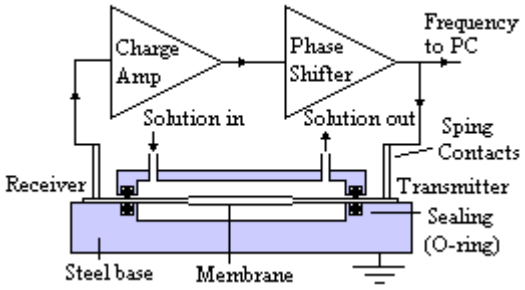


Figure 1.8 Oscillator electronics schematic

The membrane can be totally immersed in a liquid solution and its extremities are left outside for electrical contacts. The oscillatory circuit is composed by the membrane (film), a charge amplifier and a phase shifter.

The charge produced in the receiver, goes to the charge amplifier where it is converted to a voltage and fed to the phase shifter. The phase shifter guarantees that the signal applied to the transmitter is at the correct phase in order to reinforce the acoustic wave travelling back and forth trough the film. A self-sustained oscillation in the film is achieved when the correct phase and sufficient gain in the feedback loop are ensured.

The output of the circuit is its frequency of oscillation, which is monitored by a frequency meter and register by a PC with appropriate software (eg. MATLAB, WaveStar^{TM}).The voltage signal applied to the transmitter is continuously monitored by an oscilloscope[43].

1.2.2 Polymer film system

The essence of this device is a polymer film system made of different forms of PVDF, namely a piezoelectric form and a porous form of PVDF: Immobilon, from Millipore Co. The polarized PVDF has good piezoelectric properties and it is responsible for the film mechanic oscillation while the porous forms of PVDF acts as a biosensitive area.

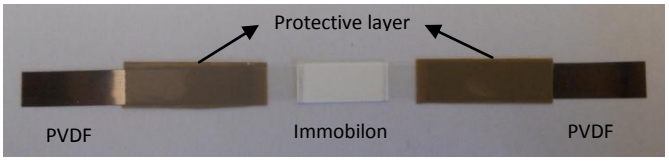


Figure 1.9 Polymer film system

A schematic of the fabrication process of both film systems is shown at Chapter 2 – Materials and Methods (2.4.4.3).

Although this film system has been applied successfully in protein detection experiments [43], it presents some irregularities in the junctions between PVDF and Immobilon and, consequently, improvements must be studied in order to minimize the noise in the output signal of the biosensor.

1.3 Aims of the Work

The main goal of this project was to improve the film system applied to a Piezoelectric Biosensor, developed at CENIMAT [43-45]. A film system made of different forms of PVDF, namely a piezoelectric form and a porous form of PVDF has been utilized, where the polarized PVDF has good piezoelectric properties and is responsible for the film mechanic oscillation and the porous form of PVDF acts as a biosensitive area. As the constructed films presented some irregularities in the junctions between PVDF and Immobilon, a film system constructed only with PVDF was preferred.

Thus, it was intended to functionalize non porous piezoelectric PVDF film, with a biocompatible coating by means of a simple immersion method, which could be used for film mechanic oscillation and, simultaneously, to act as biosensitive area, when applied to the sensor. Due to PVDF's low surface energy and high hydrofobic character, surface modification was aimed to be performed in order improve the hydrophilicity and biocompatibility of non-porous PVDF films and biomolecular recognition systems were intended to be applied at the biosensor.

Chapter 2 Materials and Methods

2.1 Instrumentation

Sessile drop experiments for the determination of the contact angles were carried out at room temperature with a CAM 200 optical contact angle and surface tension meter (KSV Instruments, Ltd.). The images were analyzed using the KSV CAM Optical Contact Angle and Pendent Drop Surface Tension Software Version 4.00. To reach the required pH for the buffer solutions a pH 211 microprocessor pH meter (HANNA Instruments) was used. To perform qualitative fluorescence assays, a Fluorescence Microscope Olympus BX 51 with an objective U-RFL-T (40x amplification), U-MWB (λ_{exc} = 460-490 nm; λ_{em} = 515 -570 nm), an Olympus U-RFL-T lamp, an objective Uplam FLN, and Cell F software for monitoring, were used. Image J was utilised for analysis of data obtained from fluorescence microscopy. Incubation procedures were carried out in a IKA KS 4000 ic control Incubator shaker (shaking incubator) or using a Boekel Big SHOT III™ High Capacity Hybridization Oven Model 230402. Membranes were washed by centrifugation using a ScanSpeed Mini Personal Microcentrifuge. Solid reagents were weighed in an Acculab ATILON digital balance. FTIR spectra were performed in a Satellite FTIR Mattson Spectrometer. Absorbance and fluorescence intensity were measured on Tecan's Infinite®F200 multimode microplate reader. Black 96 well plate (flat bottom), 96 well transparent microplates (flat bottom) from Sarstedt and 96 well-UV star plate, half area, F-form from Greiner bio-one were used. When performing assays at the biosensor, the frequency of oscillation was registered by MATLAB® and WaveStar™ Software for Oscilloscopes.

2.2 Chemicals

PVDF Solef was obtained from Solvay and Immobillon^{TM} PVDF membranes were purchased from the Millipore Co, Ltd.: Immobilon^{P} (0.45 μm) and Immobilon^{PSQ} (0.2μm). 3,4-Dihydroxy-DL-phenylalanine (DL-DOPA) (D9503) was purchased from Sigma-Aldrich and used as received. Tris(hydroxymehyl)aminomethane (Tris) (154563) and Epichlorohydrine (99%) (541780-187) were obtained from Aldrich. Polyethylene glycols (PEG) with molecular weights 200Da was supplied by Fluka. Bovine serum albumin (BSA) (A7906), Fluorescein isothiocyanate, Isomer I (FITC) (F7250), Gum Arabic from acacia tree (69752) and Glutaric dialdehyde (50 wt % in water) (536104-217) were purchased from Sigma. Sodium Chloride (1.316.591.211), Disodium-hydrogen Phosphate 2-hydrate (12.507), Sodium-di-hydrogen Phosphate 1-hydrate (1.319.651.211) and Sodium Hydroxide (1.316.871.211) and Toluene (CAS 108-88-3) were supplied by Pancreac. Methanol (> 99 %) (8388,5) was obtained from Roth. All chemicals were

analytical grade and used without further purification. Deionized (DI) water was used for preparing solutions.

2.3 Chromatographic materials

PD-10 desalting columns were used for isolation of the labeled protein, BSA-FITC.

2.4 General Methods

2.4.1 Immersion method for surface coating/modification of hydrophobic PVDF membranes

Non-porous PVDF and Immobilon membranes were cut to 1 cm x 0.5 cm and each membrane was weighted. Each membrane was immersed in 1 ml of a solution, in a microtube, with gentle stirring for 24 hours, at room temperature. Then, the samples were washed thoroughly for 30 min with the respective solvent and dried in a vacuum excicator, at room temperature. The samples were characterized by water contact angle measurements, elemental analysis and qualitative and quantitative fluorescence assays were performed.

The solutions utilized for the immersion method are listed below:

- *DL-DOPA solution*: The amount of DOPA was varied to obtain three DL-DOPA solutions with different concentrations, a 2.0 g/L, a 3.0 g/L and a 15.0 g/L DL-DOPA solution. DL-DOPA was dissolved in a mixture of Tris solution (10 mM, pH 8.5) and ethanol as solvent (V_{tris}:$V_{ethanol}$=7:3).
- *PEG solution*: 5 wt %, 9 wt % and 25 wt % PEG solutions were prepared by dissolving PEG in chloroform or in DI water.
- *Gum Arabic solution*: 40 mg/ml and 80 mg/ml Gum Arabic solutions were prepared, by dissolving Gum Arabic in water, at 4°C.

In the specific case of Gum Arabic solutions, the PVDF membranes were immersed in solution, for 24 hours at 4°C, and not at room temperature

Negative control assays were also performed, by immersion of membranes in its respective solvents.

Table 2.1 summarizes the immersion solutions used for both positive and negative control.

Table 2.1 Immersion solutions for positive and negative control

Positive Control	Negative Control
DL-DOPA in Tris (10 mM, pH 8.5)	Tris (10 mM, pH 8.5)
Gum Arabic in DI water	DI water
PEG in chloroform / DI Water	Chloroform / DI Water

2.4.2 Conjugation of BSA to FITC

2.4.2.1 Conjugation Procedure

The conjugation started by dissolving 5 mg of BSA protein in 1 ml of PBS buffer (15 mM sodium phosphate, 150 mM NaCl, pH 7.4) in a reaction vial labelled “5:1”. 1 mg of FITC was added to 2 ml of 0,1 M carbonate-bicarbonate buffer pH 9 and vortexed until all FITC was dissolved (20:1 dilution). A 5:1 dilution of FITC in 0.1 M carbonate-bicarbonate buffer (0,25 ml of 20:1 solution + 0,75 ml of 0,1 M sodium carbonate-bicarbonate solution) was prepared. 250 µl of the 5:1 FITC dilution were added dropwise to the BSA solution, while stirring the reaction vial completely covered with aluminum foil to protect from light. The reaction mixture was incubated for 2 hours at room temperature with gentle stirring.

2.4.2.2 Isolation of labelled protein

A PD-10 column was supported over a suitable beaker. The cap was removed from the top of the column, the lower tip of the column was cut open and the excess of liquid was allowed to flow through. The column was equilibrated with 30 ml of PBS solution (15 mM sodium phosphate, 150 mM NaCl, pH 7.4) until the absorbance at 280 and 495 nm equalized zero. The reaction mixture (2.4.2.1) was then applied to top of the column gel bed and the flow through was collected (Fraction 1) in microtubes covered with aluminum foil to protect from light. The column was eluted with 10 ml of PBS, collecting 1.0 ml fractions (10 x 1 ml). The absorbance of each fraction was monitored at 280 nm and 495 nm. The fractions with A_{280}>0,4 were pooled and stored at 4°C. The column was washed with 50 ml of PBS solution to remove unbound fluorophore, and the absorbance was measured reading zero. The column was washed thoroughly with a 20 %(v/v) ethanol solution and stored capped at 2-8 % with 1 ml buffer above the gel.

2.4.2.3 Determination of Fluorescein/Protein Molar Ratio (F/P)

For the determination of the F/P ratio, it was necessary to first determine the absorbance of the conjugate sample (pool) (2.4.2.2) at 280 nm and then at 495 nm (dilution of 1:10). From the absorbance readings (A_{280} and A_{495}) of the conjugate sample, the F/P of the FITC-BSA conjugate was calculated according to the equation:

$$Molar\ \frac{F}{P} = \frac{A_{495} \times 0{,}61}{A_{280} - (0{,}35 \times A_{495})}$$

2.4.3 Characterization techniques

2.4.3.1 Water contact angle measurements

Water contact angle measurements were conducted to investigate the effect of the coating on the membrane hydrophilicity, using the sessile drop method measured by a contact angle goniometer.

A water droplet was deposited by a syringe pointed vertically down onto the PVDF membrane sample surface and an image was captured by a high resolution camera, which was analysed using an image analysis software.

2.4.3.2 Folin-Ciocalteu colorimetric assay

A PVDF membrane sample was placed on a filter paper and it was pulverized with Folin-Ciocalteu reagent.

2.4.3.3 Coomassie Brilliant Blue staining of BSA proteins on PVDF

A CBB solution (0.5 % Coomassie brilliant blue, 40 % methanol, 10 % acetic acid in milli-Q water) and a destaining solution (30 % methanol and 10 % acetic acid in milli-Q water) were prepared in advance.

Each PVDF membrane was separately stained in 1.5 ml of the CBB solution for 30 seconds and then distained until the background was “transparent”. The membranes were washed 3-5 times with H_2O.

2.4.3.4 BSA-FITC labeling of functional groups

2.4.3.4.1 Labeling of free hydroxide groups present in PVDF coating

After performing the immersion method, epoxy groups were introduced by means of the reaction between the hydroxide groups present in PVDF coating, with epichlorohydrin. A volume of 0.6 ml of DI water and 0.4 ml of 10 M NaOH were added to each microtube, containing a PVDF membrane (0.5 cm X 0.5 cm). Then, 72 µl of epichlorohydrin were added and the microtubes were incubated for 2 hours at 34 °C with orbital shaking. After incubation, the membranes were washed 10 times with DI water.

A volume of 100 µl of BSA-FITC conjugated (0.5 mg/ml), prepared as described at 2.4.2, and 400 µl of PBS buffer (15 mM sodium phosphate, 150 mM NaCl, pH=7.4) were added to each microtube containing a membrane. The samples were incubated overnight at 35 °C. At the end of the incubation period, the samples were washed 5 times with PBS buffer and the respective washes were kept for posterior absorbance reading.

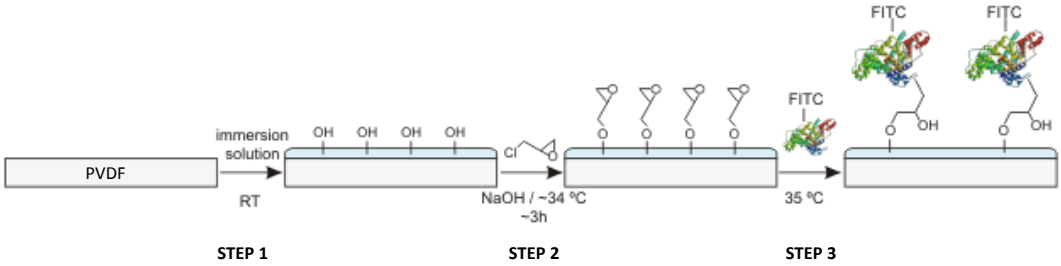


Figure 2.1 BSA-FITC labelling of free hydroxide groups

A calibration curve of BSA-FITC solution was performed and the absorbance of the washes was measured, at 280 nm and 495 nm. The control experiments preformed are summarized at Table 2.2.

Table 2.2 Control Experiments for –OH labelling with BSA-FITC

Control experiments	
Positive control - P	Step 1, Step 2, Step 3
Negative control - N1	Step 1, Step 3
Negative control – N2	Step 2, Step 3
Negative control – N3	Step 3
Negative control – N4	Pristine PVDF

2.4.3.4.2 Labeling of free -NH_2 groups present in PVDF coating

After performing the immersion method, a volume of 1 ml of 5 % (v/v) glutaraldehyde in PBS solution was added to each microtube, containing a PVDF membrane (0.5 cm X 0.5 cm) with NH_2 groups present in its coating and then, it was incubated for 1 hour at room temperature, with gentle stirring. After incubation, the membrane was washed 3 times with 1 ml of PBS buffer.

A volume of 100 µl of BSA-FITC conjugated (0.5 mg/ml), prepared as described at 2.4.2, and 400 µl of PBS buffer (15 mM sodium phosphate, 150 mM NaCl, pH=7.4) were added to each microtube tube containing a membrane. The samples were incubated for 1 hour at room temperature, with gentle stirring. At the end of the incubation period, the samples were washed 3 times with PBS buffer and kept in PBS to posterior fluorescence characterization.

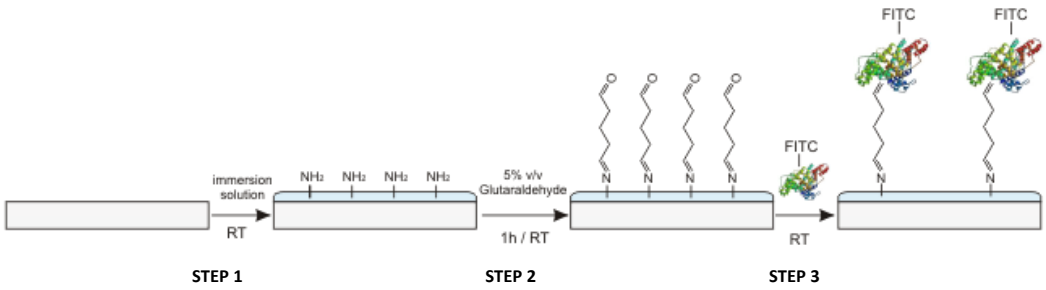


Figure 2.2 BSA-FITC labelling of free amine groups

The control experiments preformed are summarized at Table 2.3.

Table 2.3 Control Experiments for –NH2 labelling with BSA-FITC

Control experiments	
Positive control – P’	Step 1, Step 2, Step 3
Negative control – N’1	Step 1, Step 3
Negative control – N’2	Step 2, Step 3
Negative control – N’3	Step 3
Negative control – N’4	Pristine PVDF

2.4.4 Piezoelectric Biosensor experiments

2.4.4.1 Equipment's instructions

The film system is placed in the reaction chamber and the chamber is placed inside the biosensor's box. The temperature sensor is connected to the oscilloscope and the thermocouple is connected to the base of the biosensor. The heating unit and the temperature controller are turned on. The tubing is connected to the peristaltic pump and the pump is turned on pressing the "START" button to fill the reaction chamber with the chosen solution. During this process, the reaction chamber must be slightly sloped. The electrodes are connected to the film system and the power supply is turned on. The frequency meter, the oscilloscope, the amplifier (x 50) and the charge amplifier are turned on. The "OPERATE" button of the charge amplifier is turned on. The WaveStar^{TM} Software for Oscilloscopes is used to capture frequency and temperature measurements and save the experimental data. When an experiment is concluded, the "OPERATE" button of the charge amplifier is turned off and the connections to the film system are disconnected. The thermocouple is disconnected to the base of the biosensor and both heating unit and temperature controller are turned off. The reaction chamber is emptied with the peristaltic pump and the reaction chamber is sloped during this process. DI water is allowed to flow through the tubing in order to wash the tubing and the chamber. Further cleaning of the chamber occurs when the film system is removed.

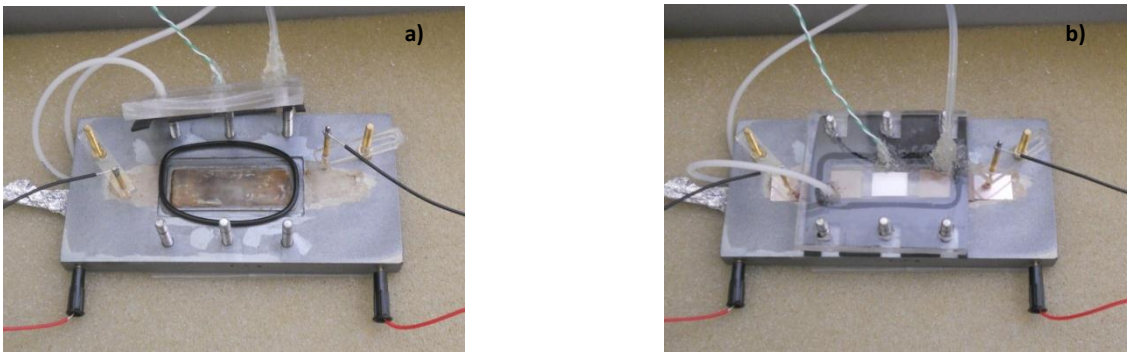


Figure 2.3 a) Opened reaction chamber b) Film system placed inside the reaction chamber

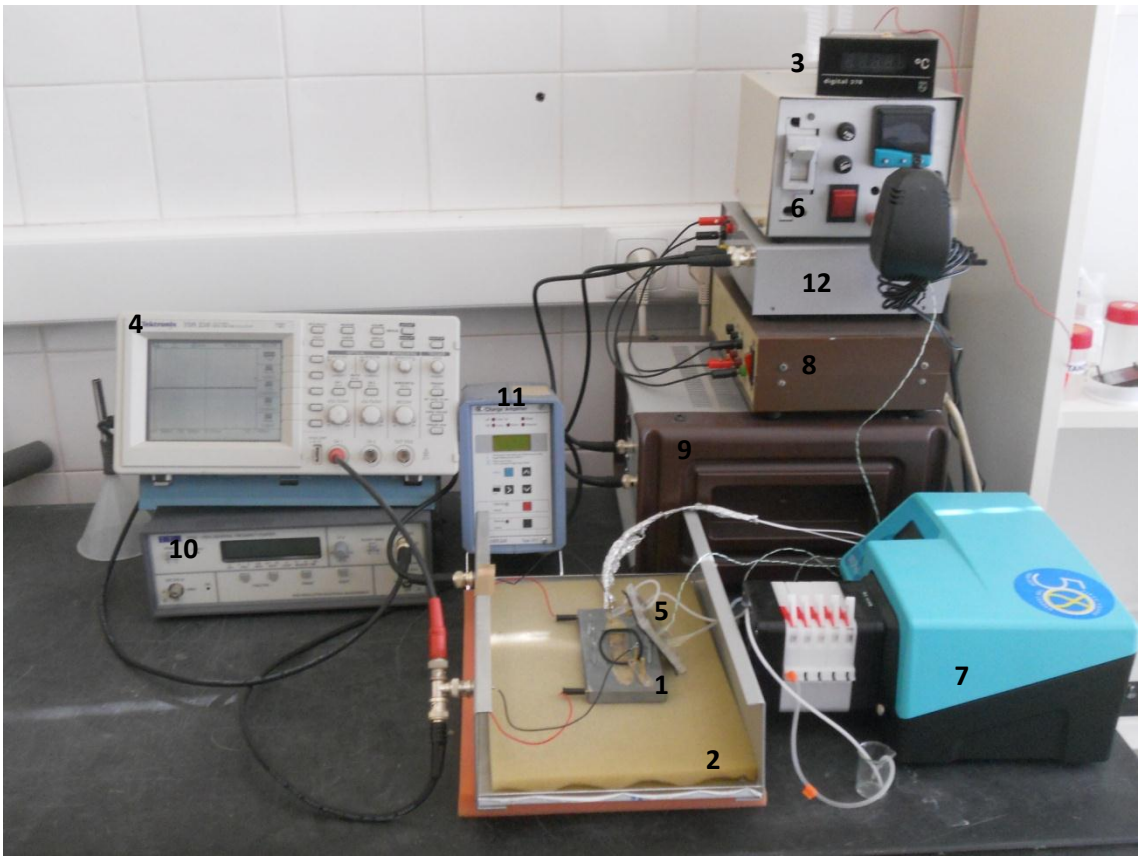


Figure 2.4 Piezoelectric Biosensor apparatus

Legend:

1 – Reaction chamber
2 – Biosensor's box
3 – Temperature sensor
4 – Oscilloscope
5 – Thermocouple
6 – Temperature controller
7 – Peristaltic pump
8 – Power source
9 – Amplifier
10 – Frequency meter
11 – Charge amplifier
12 – Phase shifter

2.4.4.2 General procedure for experiments at the piezoelectric biosensor

Firstly, the reaction chamber was filled with water (or the respective solution solvent, e.g. PBS buffer for BSA assays). After stabilizing the temperature at 30 °C, the sensor oscillated for a few minutes, only with H_2O in the chamber. At this stage, there was no flux in the chamber. Then, 1ml of a solution was allowed to flow at a flow rate of 0.17 ml/min, for a period of 30 min (Figure 2.5), in closed circuit (Figure 2.5).

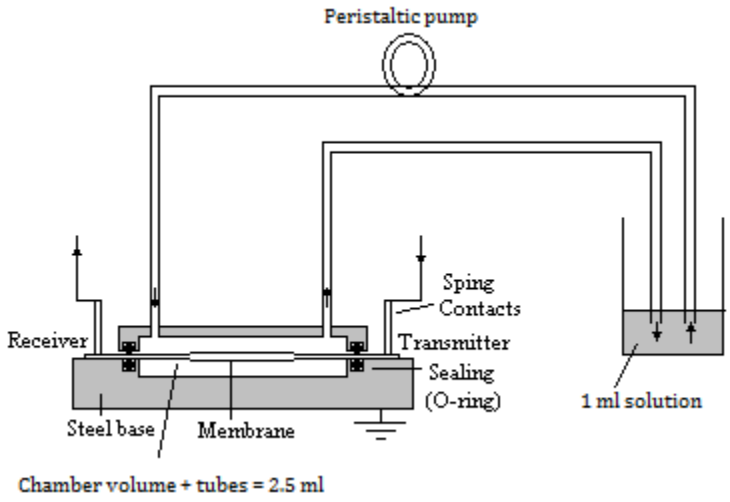


Figure 2.5 Closed circuit scheme for input of solution

After that period, the flux was stopped and the frequency was allowed to stabilize. Then, a washing procedure with water (or the respective solution solvent, e.g. PBS buffer for BSA) was carried out, for 20 min in open circuit, as shown in Figure 2.6.

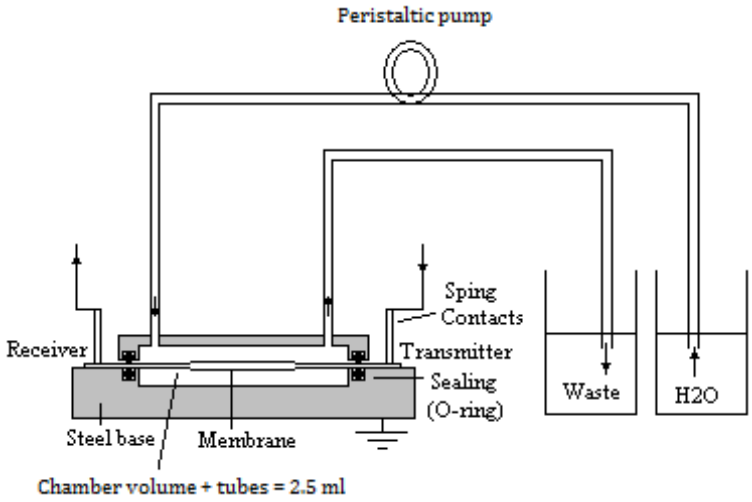


Figure 2.6 Open circuit scheme for washing procedure

For every stage of the described procedure, the frequency of oscillation was acquired via MATLAB® and WAVESTAR™ software.

2.4.4.3 Film system preparation

The polymer film system was made with different forms of PVDF, namely a piezoelectric form and a porous form of PVDF, Immobilon, which can have different porosity: Immobilon^{P} (0.45 µm) and Immobilon^{PSQ} (0.2µm). Figure 2.7 shows a schematic of the fabrication process of both film systems:

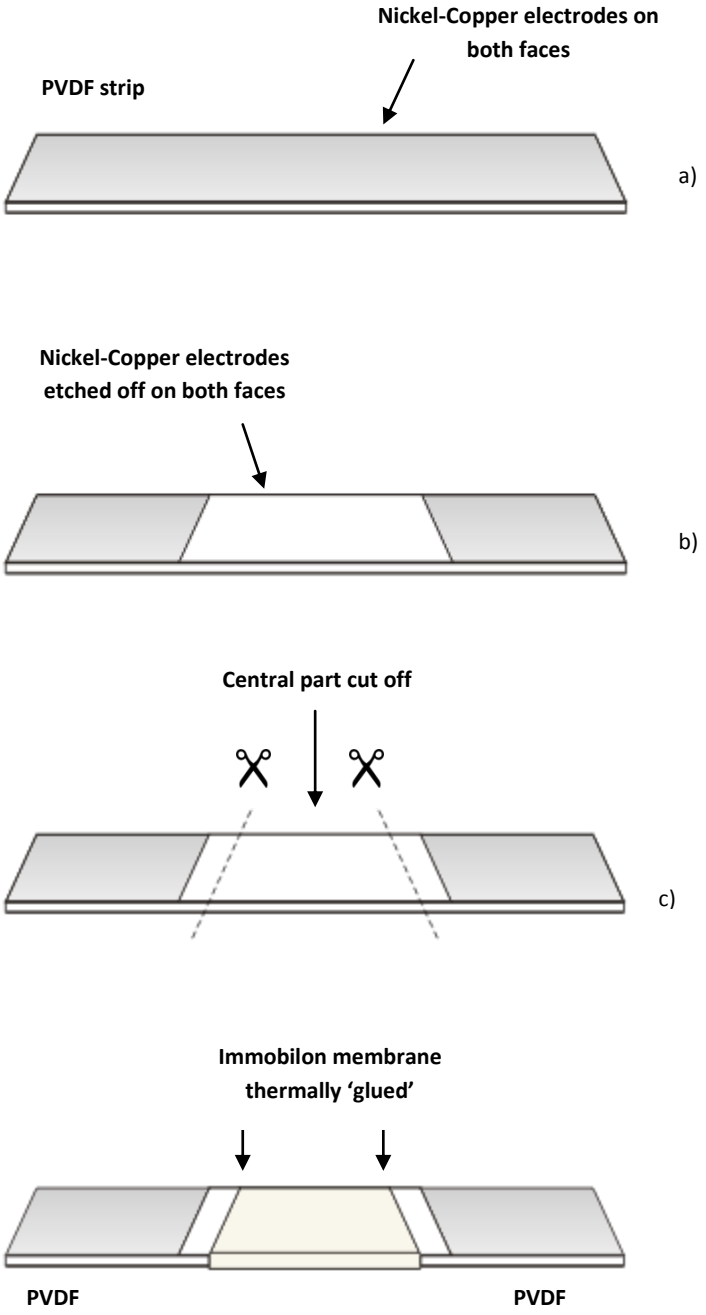


Figure 2.7 Fabrication process of the polymer film systems

At first, a polarized PVDF strip was cut into the intended dimensions (Fig. 2-7a). The central part of the electrodes was then etched off on both faces of the strip (Fig.2-7b). After cutting off part of the central area (Fig.2-7c), a piece of Immobilon was thermally glued to this central part at a suitable temperature, while a small pressure was being applied at the seams between PVDF and Immobilon (Fig.2-7d). Experiments were carried out with two different types of polymer film systems: a PVDF film system (Fig.2-7b) and a PVDF/Immobilon/PVDF film system (Fig.2-7d).

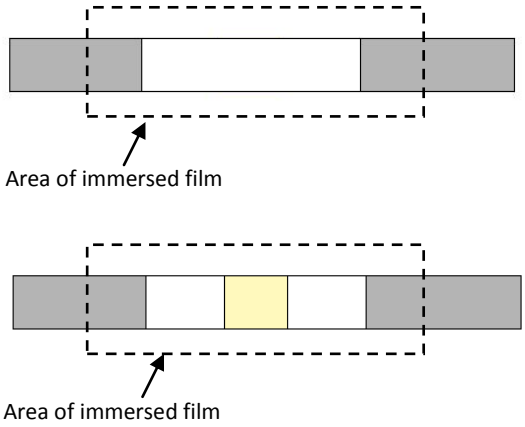


Figure 2.8 Area of immersed film

As it can be seen in Figure 2.8, a small area of electrodes (in grey) will be in contact with solution. In order to avoid that the electrodes are corrupted quickly, a protection is needed. Therefore, a plastic layer is placed on top of the electrodes. The final appearance of both film systems is shown in Figure 2.9.

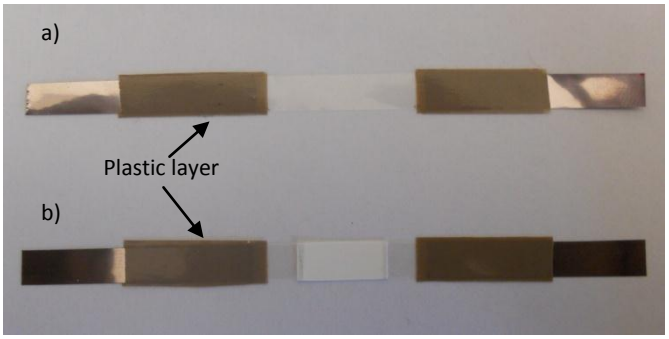


Figure 2.9 a) PVDF film system b) PVDF/Immobilon/PVDF film system, with plastic layer protection

Chapter 3 Surface modification of PVDF

3.1 Introduction

In order to study the possible formation of coatings onto PVDF films, containing functional groups, experiments were carried out with three distinct solutions, using the following compounds: DOPA, Gum Arabic and PEG.

DOPA is an important component of mussel adhesive proteins (MAPs). DOPA and its derivatives were reported to exhibit powerful interfacial adhesion strength and recently it has been used to perform surface modification for both organic and inorganic materials. In particular, the strong adhesive behavior of poly(DOPA) on microporous PVDF has been reported [34, 38].

DL-DOPA was used to study the possible formation of an adhesive DOPA coating onto PVDF films. As shown in Figure 3.1, DL-DOPA contains reactive groups that can provide an important platform for further surface functionalization.

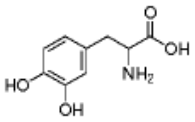


Figure 3.1 Chemical structure of DL-DOPA

Polyethylene glycol (PEG) is a polyether compound with many applications, commercially available over a wide range of molecular weights from 200 g/mol to 10,000,000 g/mol. While PEGs with different molecular weights find use in different applications and have different physical properties due to chain length effects, their chemical properties are nearly identical. PEG has unique properties such as hydrophilicity, flexibility, nontoxicity and non-immunogenicity, which make it appropriate to be used for coatings, creating a hydrophilic biocompatible layer on a wide variety of surfaces (ex. polymers, magnetic nanoparticles, etc.).

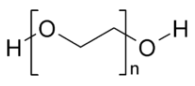


Figure 3.2 Chemical structure of PEG

Gum Arabic (GA), the gum exudate from stems and brunches of Acacia trees, is extensively used in food, pharmaceuticals and cosmetics industry because of its unique properties like emulsification, film forming, and encapsulation [46-47]. GA is a branched-chain, complex polysaccharide, either neutral or slightly acidic, and it has been described as a complex mixture of calcium, magnesium and potassium salt of a polysaccharidic acid (arabic acid), with rhamnose and glucuronic acid end units and containing a small proportion (ca. 2%) of protein [48-49].

An immersion method for PVDF coating surface modification was applied to three different types of PVDF: a non-porous type of PVDF and two different microporous PVDF (known as Immobilon) with 0.2 µm and a 0.45 µm porous size. For each type of PVDF, the concentration of DOPA, PEG and Gum Arabic solutions was varied. As DOPA, PEG and GA are hydrophilic compounds, its immobilization at the membranes would raise the number of functional groups which have affinity to water, originating more hydrophilic membranes, that could provide an important platform for further functionalization.

3.2 Results and Discussion

3.2.1 Surface modification PVDF with different porosity (non-porous, 0.2 µm and 0.45 µm) by immersion method in DL-DOPA, PEG and Gum Arabic solutions

3.2.1.1 Water contact angle measurements

After performing the immersion method (2.4.1), the changes of PVDF surface hydrophilicity were evaluated by water contact angle measurements. The results are showed in Table 3.1.

Table 3.1 PVDF hydrophilicity evaluation for different coating solutions and porous type

Water contact angle (°)			
Coating solution	**Non-porous PVDF**	**0.2 µm PVDF**	**0.45 µm PVDF**
Pristine PVDF	**94 ± 6**	**105± 4**	**103 ± 6**
3.0 g/L DOPA	75 ± 7	112 ± 9	110 ± 9
15.0 g/L DOPA	77 ± 5	99± 12	101 ± 12
5 wt % PEG	61 ± 3	63 ± 2	88 ± 3
9 wt % PEG	56 ± 11	80 ± 2	71 ± 10
25 wt % PEG	64 ± 3	78 ± 3	89 ± 2
40 mg/ml GA	68 ± 4	94 ± 1	94 ± 12
80 mg/ml GA	64 ± 8	85 ± 3	83 ± 11

To better visualize the relation between the coating solution and type of used membrane in hydrophilicity improvements, the results are also presented in Figure 3.3.

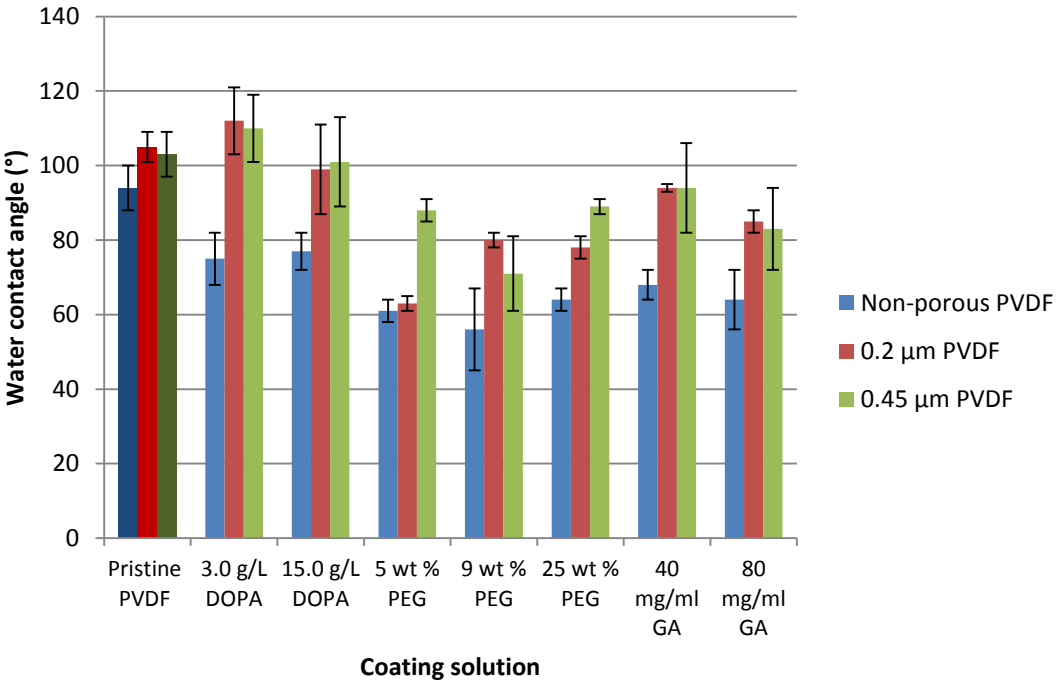


Figure 3.3 Water contact angle results

The results for water contact angle measurements confirmed that pristine non porous PVDF, 0.2 µm and 0.45 µm PVDF membranes have a hydrophobic surface, with contact angles higher than 90° (94 ± 6°, 105 ± 4° and 103 ± 6°, respectively).

When 3.0 g/L and 15.0 g/L DOPA solutions were used, it can be observed that hydrophilicity improvements were only verified for the non porous PVDF, where the water contact angle decreased from about 94° to approximately 75° and 77°, respectively. To both porous types of PVDF, water contact angle measurements remained higher than 90°, i.e., the membranes maintained a hydrophobic surface. Considering that DOPA molecules are very small, it is possible that a certain amount of DOPA could be immobilized only inside the porous of the membranes and not at its surface. Therefore, the surface hydrophobic character would not be modified for porous membranes.

The results depicted in Table 3-1 show that the highest hydrophilicity improvements were obtained with PEG solutions, for each of the three types of PVDF. For non porous PVDF, it is observed that the water contact angle decrease is similar, independently to the increase of concentration of PEG. This can be due to the type of interaction between the molecules and the surface, which is believed to be only electrostatic. When comparing the porous membranes, it can be seen that except for 9 wt % PEG, it was for the 0.2 µm PVDF that the best hidrophilicity improvements were obtained. This can be related to the porous size of the

membranes. For higher porous size (0.45 µm) it is possible that a higher amount of PEG molecules can be immobilized at the bulk of the membrane and less at the surface, when compared to 0.2 µm porous size membrane. Results for 9 wt % should be repeated. Also, it with be interesting to perform more assays with different concentrations of PEG, using different types of porous membranes, in order to better understand the relation between the increase of concentration of PEG according to the porous size of the membrane and its impact in hydrophilicity improvements.

For the Gum Arabic solutions, improvements were only considerable when the non porous PVDF membranes were used, with a decrease of 94° to about 68° and 64° to the 40 mg/ml and 80 mg/ml GA solutions, respectively.

It is believed that the surface hydrophilicity improvements obtained when non porous PVDF was used, for all the solution, can be due to electrostatic interactions between the coating compounds and its surface.

In summary, the highest surface hydrophilicity improvements were obtained when PEG solutions were utilized, for each of the three types of PVDF: non-porous, 0.2 µm and 0.45 µm PVDF. Particularly, the best results were obtained when non porous PVDF samples were immersed in PEG solutions and when a 5 wt % PEG solution was utilized with 0.2 µm porous size membrane.

It can be observed that, in some cases, the experimental error obtained was high, which could be caused by a non uniform coating onto the surface of the samples, for example, or irregularities at the samples. Also, water contact angle measurements can be difficult to interpret the porous membranes because of capillarity forces within pores and roughness. Studies about time dependence of water contact angle on the porous membranes should be performed better evaluate hydrophilicity improvements.

3.2.1.2 Elemental microanalysis results

The elemental composition of the samples was determined by elemental microanalysis, as shown in Table 3.2, to verify if there was a significant change in the percentage of carbon, nitrogen or hydrogen after the immersion method in the respective solution.

Non-porous PVDF results show that there was no significant change in the composition of the immersed samples when compared to pristine PVDF membranes. For PVDF membranes with a porosity of 0.2 µm it can be seen that there was a variation on the percentage of carbon and hydrogen, when PEG solutions were utilized, when compared to pristine 0.2 µm PVDF (% C = 39.38 and % H = 3.08). It is observed that the % H increases with the increase of concentration of PEG solution, which indicates that the amount of incorporated PEG increases with the increase of PEG concentration. In the specific case of 5 wt % PEG solution, both percentages of hydrogen and carbon increased, after the immersion procedure. For both 9 wt % and 25 wt % PEG solutions, a similar behavior would be expected, however, their correspondent percentages of carbon decreased to 29.57 % and 31.28 %, respectively, while the amount of hydrogen increased. In fact, it can be seen that for the 25 wt % PEG solution the percentage of hydrogen was raised to more than the double when compared to the 0.2 µm PVDF control. The results for these two membranes were unexpected. It is believed that there could have been a problem with these samples, related with their handling during the experimental work or to the elemental analysis procedure and, consequently, these analysis should be repeated.

When using the 0.45 µm PVFD membranes, there was an increase of both carbon and hydrogen percentages, correspondent to the utilization of 9 and 25 wt % PEG solutions, which might indicate that a certain amount of PEG was incorporated at this type of PVDF.

When Gum Arabic and DOPA solutions were used, there were no remarkable variations on the elemental composition of the samples, for the three types of PVDF. In the specific case of DOPA, the atomic percentage of nitrogen remained null, indicating that there was no DOPA coated at the surface of the samples or immobilized in the bulk of the porous membranes.

A Folin-Ciocalteu assay was performed to the samples that were immersed in DOPA solution, for the three types of PVDF. This reagent can be used for colorimetric assays of phenolic and polyphenolic compounds. There was no color appearance at the immersed membranes, confirming, once again, that there were no polyphenol groups present at the surface of PVDF membranes.

Table 3.2 Elemental microanalysis comparisons between DOPA, Gum Arabic and PEG modified PVDF with different porosity

PVDF porosity	Samples	Nitrogen %	Carbon %	Hydrogen %
Non-porous	**Unmodified PVDF**	**0.00**	**41.91**	**3.36**
	3.0 g/l DOPA solution	0.00	39.63	3.75
	15 g/l DOPA solution	0.00	39.43	3.01
	5 wt % PEG solution	0.00	40.44	3.66
	9 wt % PEG solution	0.00	41.31	3.63
	25 wt % PEG solution	0.00	40.50	3.50
	40 mg/ml Gum Arabic solution	0.00	43.00	3.63
	80 mg/ml Gum Arabic solution	0.00	39.51	3.60
0.2 µm	**Unmodified 0.2 µm PVDF**	**0.00**	**39.38**	**3.08**
	3.0 g/l DOPA solution	0.00	39.35	3.76
	15 g/l DOPA solution	0.00	39.70	3.79
	5 wt % PEG solution	0.00	43.25	4.81
	9 wt % PEG solution	0.00	29.57	5.96
	25 wt % PEG solution	0.00	31.28	6.77
	40 mg/ml Gum Arabic solution	0.00	39.22	3.63
	80 mg/ml Gum Arabic solution	0.00	39.41	3.34
0.45 µm	**Unmodified 0.45 µm PVDF**	**0.00**	**39.72**	**3.13**
	3.0 g/l DOPA solution	0.00	39.53	3.76
	15 g/l DOPA solution	0.00	39.33	3.63
	5 wt % PEG solution	0.00	40.22	3.53
	9 wt % PEG solution	0.00	43.03	4.88
	25 wt % PEG solution	0.00	44.91	5.54
	40 mg/ml Gum Arabic solution	0.00	39.49	3.31
	80 mg/ml Gum Arabic solution	0.00	39.49	3.38

3.2.1.3 Conclusions

In an overall observation, it can be verified that the most considerable water contact angle decreases were obtained when PEG solutions were utilized for each of the three types of PVDF membranes, suggesting that surface hydrophilicity improvements were achieved.

However, the surface hydrophilicy results obtained by water contact angle measurements were not entirely supported by elementary microanalysis. For example, when non porous PVDF was used, there were no significant changes on the composition of the membranes, on the contrary to what would be expected considering the water contact angle results, where decreases (hydrophilicity improvements) were registered for all solutions applied.

Results were in accordance for the following systems: 5 wt % PEG – 0.2 µm PVDF and 25 wt % PEG – 0.45 µm PVDF, where both carbon and hydrogen percentages increased after performing the immersion procedure. In the case of 9 wt % PEG – 0.2 µm PVDF and 25 wt % PEG – 0.2 µm PVDF, the amount of hydrogen was considerably raised, although, the carbon amount inexplicably decreased.

Although results obtained with PEG solutions suggest that there was a possible adsorption/incorporation of PEG to the porous types of PVDF, water contact angle measurements and elementary microanalysis are limited techniques to infer about the possible coating of compounds onto PVDF surface or its immobilization inside the bulk of the porous membranes, and they are not enough to choose or exclude a solution by themselves. Therefore, a complementary strategy for selection of the better system, according to the porosity of PVDF and the coating solution, was adopted. BSA was market with FITC and fluorescence assays were preformed in order to select the system that would show the best response to the adsorption/immobilization of the conjugated BSA-FITC.

3.2.2 Quantitative and qualitative study of BSA-FITC adsorption at modified/coated PVDF membranes

Fluorescein isothiocyanate (FITC) is widely used to attach a fluorescent label to proteins, reacting with amino terminal and primary amines in proteins. As described at 2.4.3.4, FITC was used for the labeling of BSA, to be further coated on PVDF surfaces. In order to remove unbound BSA-FITC from the surface of PVDF membranes, the membranes were washed with PBS buffer and the washes were kept. The absorbance of the washes was read directly in a microplate reader to quantify the amount of BSA-FITC that was unbounded to the surface of the samples and the samples were observed with a fluorescence microscope. Positive and negative control experiments are summarized in Table 3.3 or in more detail in Table 2.2 Control Experiments for –OH labelling with BSA-FITC and Table 2.3 Control Experiments for –NH2 labelling with BSA-FITC.

Table 3.3 Control experiments for labelling assays with BSA-FITC (X = step preformed)

Control experiments	Pristine PVDF membrane	Coating solution	Epoxyactivation or Glutaraldhyde	BSA-FITC
P	X	X	X	X
N1	X	X	-	X
N2	X	-	X	X
N3	X	-	-	X
N4	X	-	-	-

P-Positive control, N-Negative control

3.2.2.1 Quantitative determination of unbounded BSA-FITC onto the surface of PVDF

The results for the amount of BSA-FITC unbounded onto PVDF samples are shown in Figure 3.4, according to the type of PVDF used and coating solution.

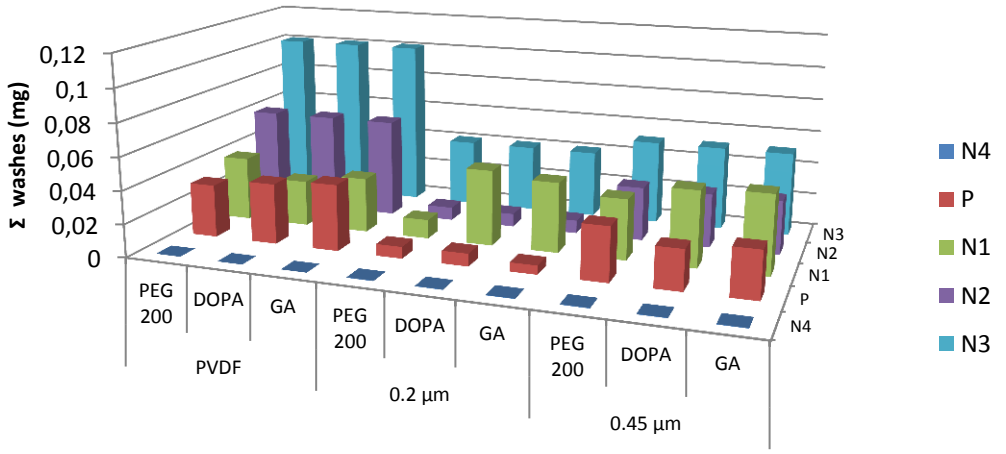


Figure 3.4 Comparison between the amount of BSA-FITC unbounded onto PVDF surface according to the porosity type and coating solution.

As observed, the results for all N4 samples (pristine PVDF) were null. BSA-FITC was not used and, therefore, the absorbance was zero, as it was expected. When comparing N3 results, it can be seen that the amount of BSA unbounded is higher for PVDF than for both Immobilon types of PVDF. Without surface modification, PVDF does not have the ability to immobilize protein, although, porous PVDF membranes show that capacity. The results indicate that 0.2 µm membranes have a higher protein adsorption capacity and a higher retention than 0.45 µm membranes. In fact, in literature, it can be found that 0.45 µm has a BSA binding capacity of 215 µg/cm^2 and 0.2 µm has a BSA binding capacity of 340 µg/cm^2. The same behavior was observed when N2 control was performed, although, the amount of BSA unbounded was lower than for N3 control. This can indicate that the use of epicholorohydrin or glutaraldhyde can contribute to the improvement of BSA adsorption to PVDF surface, through the establishment of covalent bonds.

When observing N1 control, it can be seen that, in the case of PVDF samples, the amount of unbounded BSA-FITC was lower that for N2 and N3. This might indicate that the existence of a coating compound at the surface of the membranes improved the ability of BSA to be physically adsorbed to the membranes. In the case of 0.2 µm, the possible existence of a coating compound seems to have no significant influence when compared to N3 control, when DOPA and Gum Arabic solutions were used. As for PEG, improvements can be visualized comparing to N3 but when compared to N2, the change was not significative indicating that both PEG and epichlorohydrine have a similar influence on the immobilization of protein. For 0.45 µm, the results obtained for N1 for all three coating compounds were identical to N3, meaning that whether the coating compound were not successfully attached to the membranes or that its presence does not improve the immobilization of BSA to the membranes. Positive control experiments related with PVDF do not show a considerable variation to N1. This can be justified by a possible unsuccessful attachment of epicholohydrine or glutaraldehyde to the coating compound, and consequently, results are similar to N1. Positive controls for 0.2 µm show the lowest values obtained for unbounded BSA, however, similar to N2 which is indicative of the unspecific binding of BSA to the membrane.

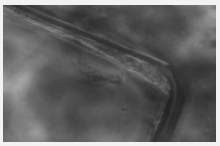
Results from positive control show that the lower amount of unbounded BSA-FITC was obtained when 0.2 µm porous membrane was utilized for the following coating solutions: PEG 200, Gum Arabic and DOPA , although, the best result was obtained for PEG.

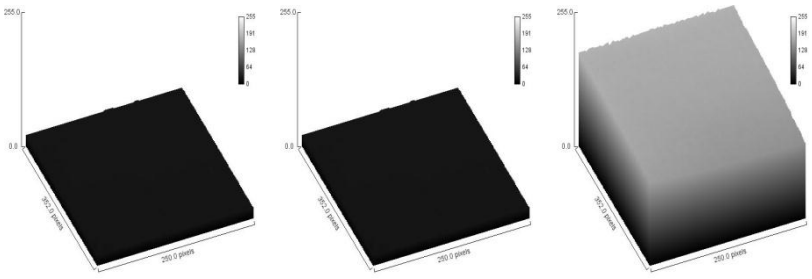
3.2.2.2 Fluorescence Microscopy study of BSA-FITC adsorption onto PVDF

The fluorescence microscopy study of BSA-FITC adsorption at PVDF membranes was performed in order to qualitatively assess if there was BSA-FITC coated at the surface of the membranes, in accordance to the results obtained by the quantitative method presented above. The fluorescence photographs were taken with exposure periods of 500 ms, the time necessary for the observation of the BSA-FITC solution alone.

Firstly, it was important to verify if pristine PVDF membranes, *per se*, presented background fluorescence. While observing N4 control samples (pristine), it was found that non porous PVDF and the 0.2 µm pore size PVDF membrane did not present background fluorescence, whereas 0.45 µm pore size PVDF (Immobilon-P) showed high background fluorescence and, therefore, it was not adequate for use with fluorescence probes (Table 3.4 , Figure 3.5). As a consequence, the results obtained for 0.45 µm pore size PVDF were compromised and will not be considered at this stage for discussion. The possibility of a contamination was raised, although several confirmation experiments were preformed with pristine membranes from different sheets which were not kept near a possible source of FITC, always presenting a similar result for background fluorescence. Also, in literature, a reference was found referring to similar experimental observations when using these types of membranes for fluorescence probes.

Table 3.4 Fluorescence microscopy results for pristine membranes (N4)

Control	Type of PVDF	Black and White	Fluorescence
N4	PVDF		
	0.2 µm		
	0.45 µm		

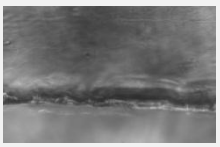
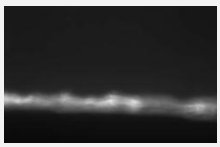
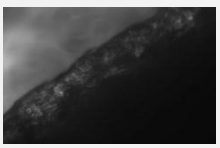


PVDF 0.2 µm 0.45 µm

Figure 3.5 Fluorescence background of PVDF membranes

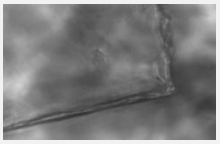
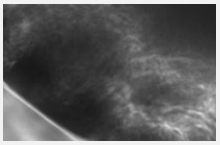
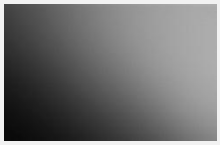
When visualizing N3 samples, it can be seen that non porous PVDF exhibits some fluorescence at the margins of the sample. Probably, a small amount of BSA-FITC was retained at its margins as a consequence of irregularities of the cut of the sample. Absorbance results indicated that there was a higher amount of unbounded BSA-FITC to PVDF than to 0.2 µm membrane. The results are confirmed by fluorescence microscopy, as it can be seen that PVDF show lower fluorescence than 0.2 µm sample (Table 3.5).

Table 3.5 Fluorescence microscopy results for N3 control

Control	Type of PVDF	Black and White	Fluorescence
N3	PVDF		
	0.2 µm		

According to the quantitative results obtained to N2 control, it would be expected that N2 control for PVDF exhibit higher fluorescence that for N3 control, due to a lower unbounded BSA-FICT amount, however, as before, PVDF samples only show little fluorescence at its margins and not onto its surface. In respect to 0.2 µm sample, it would also be expected to visualize higher fluorescence at the N2 control, when compared to N3. In fact, expectations were met (Table 3.6).

Table 3.6 Fluorescence microscopy results for N2 control

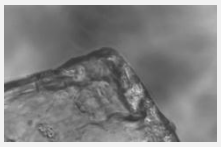
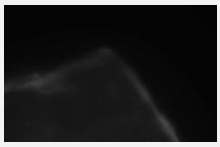
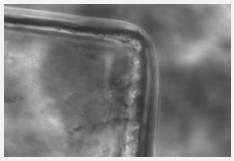
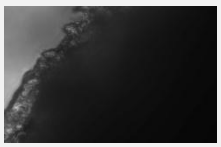
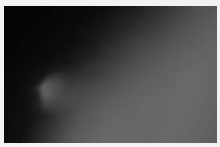
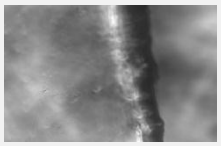
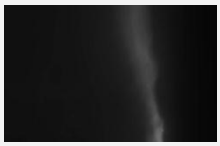
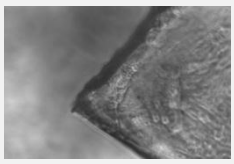
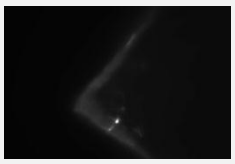
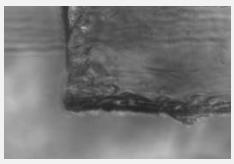
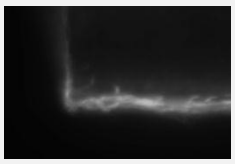
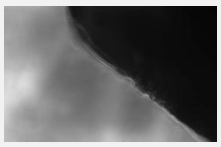
Control	Type of PVDF	Black and White	Fluorescence
N2	PVDF		
	0.2 µm		

Fluorescence microscopy results for N1 and P control are presented in Table 3.7. Considering the quantitative results for unbounded BSA-FITC, where there was no significative variation of results for N1 and P controls to PVDF assays, similar microscopy results were also expected. As before, for all fluorescence microscopy experiments with PVDF, fluorescence is only present at the margins of PVDF samples and not at its surface.

Actually, the highest microscopy fluorescence result was obtained for the positive control, when PEG was utilized at a 0.2 µm microporous membrane. When DOPA and GA were used, positive control showed an improvement of fluorescence at the surface, although, when N1 was performed, there was no fluorescence at all.

With an overall observation, it can be seen that PVDF samples, for all controls and coating compounds, only presented some fluorescence at the edges and never at the surface. The presence of BSA coated at PVDF was, therefore, not verified. The fluorescence at the margins can be explained by the irregularities caused by the cut of the membranes, which could retain BSA-FITC. The highest fluorescence result was obtained for the positive control of PEG when applied to 0.2 µm membranes, confirming absorbance results, that showed that this system corresponded to the lower amount of unbounded BSA, i.e., to a higher retention of BSA-FITC to the membrane.

Table 3.7 Fluorescence microscopy results for N1 and Positive control P

		N1 control		P control	
Coating solution	**Type of PVDF**	**Black and White**	**Fluorescence**	**Black and White**	**Fluorescence**
PEG	PVDF				
	0.2 µm				
DOPA	PVDF				
	0.2 µm				
GA	PVDF				
	0.2 µm				

Previous water contact angle measurements revealed that hydrophilicity improvements were achieved when utilizing PEG as coating solution for 0.2 µm membranes. Also, elementary microanalysis revealed that, after the immersion method, the amount of carbon and hydrogen had significant variations. Both characterization techniques indicated that PEG was successfully immobilized to 0.2 µm membranes, although, information about how it was immobilized could not be given by these two techniques. It was more likely that PEG was physically adsorbed to its surface or entrapped inside its porous. Both quantitative and qualitative results obtained for fluorescence assays with PEG and 0.2 µm membranes, reveal that the lower amount of unbounded BSA-FITC was obtained when the positive control procedure was executed (Figure 3.6). Fluorescence microscopy image confirmed that there was a high amount of BSA-FITC at the sample. Thus, it is believed that the positive control procedure was effectively achieved, promoting the specific binding of BSA to the epichlorohydrin bound to PEG, although some unspecific immobilization of BSA must not be set aside.

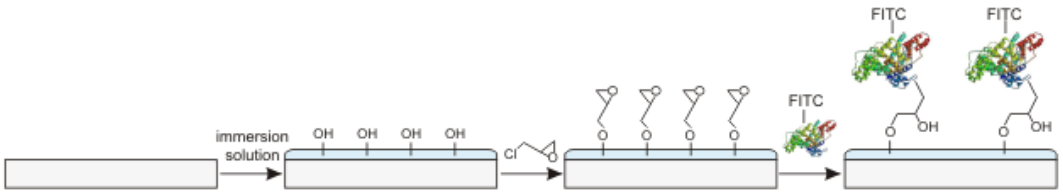


Figure 3.6 Positive control (P) for fluorescence assays

Chapter 4 The Piezoelectric Biosensor

4.1 Results and Discussion

According to the results obtained for surface modification of PVDF the PVDF/Immobilon^{PSQ} (0.2 µm)/PVDF film system was selected to be studied at the biosensor. Also, the sensor itself was used as a "characterization technique" to support the results presented above. Unfortunately, the use of epichlorohydrine and solvents such as chloroform were not compatible with the biosensor construction material, therefore, control experiments such as P and N2 could not be applied to the sensor.

Taking these limitations in consideration and the results obtained above, it was chosen to test the response of the sensor to a PEG solution and BSA solution. The response of the biosensor was registered by measuring the frequency output during the experiments.

4.1.1 Detection of PEG and BSA using a PVDF/Immobilon^{PSQ} (0.2 µm)/PVDF film system

The following experiments were performed with a PVDF/Immobilon^{SQ} (0.2 µm)/PVDF film system. Although it is known that the time axis would preferably be in minutes or hours, the frequency results are given as a function of time in seconds, to allow a better visualization of the frequency output acquired during the experiments.

Experiment A.1

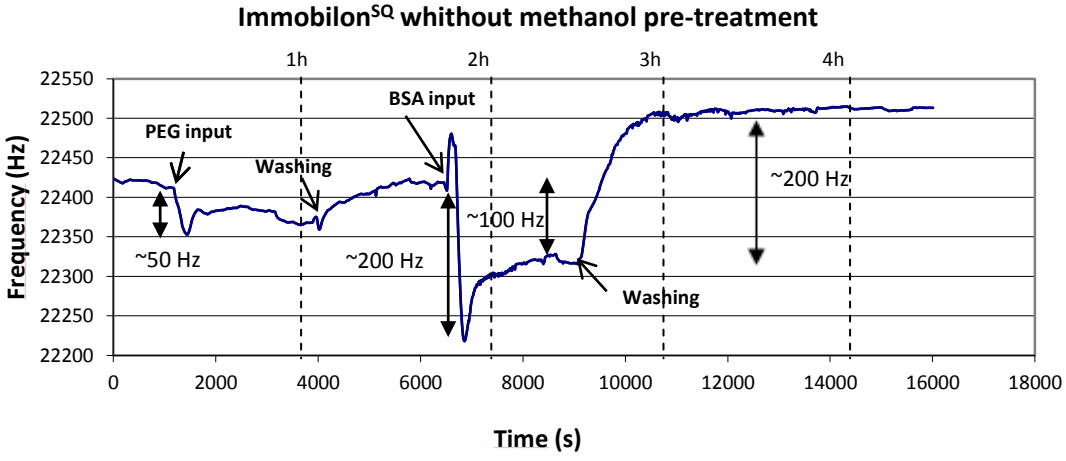


Figure 4.1 Detection of PEG and BSA using a PVDF/0.2um/PVDF film system

Initially, the sensor oscillated only with water inside the reaction chamber, at 30 °C. At 1172 s, 1 ml of 10 wt % PEG 200 was introduced and flowed, in closed circuit, for about 30 min. A frequency drop of ~ 50 Hz was registered almost at time of input of the solution. The flux was stopped at 3145 s and the frequency stabilized at less ~25 Hz than the initial frequency. At 3885 s a washing procedure with DI water was executed for about for 30 min, and the frequecy increased; returning to the initial frequency value. The washing procedure ended at 5780 s. At 6454 s, 1 ml of BSA-FITC was introduced and flowed, in closed circuit, for 30 min, and an abrupt frequency drop was registered (~200 Hz). The flux was stopped at 8631s and the frequency stabilized at less ~ 100Hz than the starting value. At 9066 s, a washing procedure with PBS buffer was preformed and the frequency increased (~ 200 Hz). The procedure ended at 10829 s.

The results show that the device is sensitive to PEG, reacting with a drop of frequency which could be related to the deposition of mass in the surface of the film. Although, it can be seen that after the washing procedure, the frequency returned to the initial value, indicating that the PEG was washed away and that there was no PEG adsorbed to the membrane. This can be easily explained because PEG is soluble in water or it can be related to the short period of incubation of PEG.

A similar behaviour was registered when the solution of BSA-FITC was introduced in the sensor's reaction chamber. However, the frequency drop registered was higher than the observed when PEG was introduced, which can be related to the difference of the molecular weights of these compounds. BSA molecules (66 776 Da) have a superior molecular weight when compared to PEG 200 (200 Da). Consequently, the deposition of a higher amount of mass on the piezoelectric film causes a higher frequency decrease. The oscillation frequency can also be affected by physical properties of the adjacent media such as density or viscosity of the used solution. After performing the washing procedure, that proceeded the input of BSA, a frequency increase was observed. Again, this behaviour indicates that, as PEG, BSA was washed way due to the weak physical interaction between BSA and hydrophobic PVDF.

After performing this experiment, it was planned to remove the porous membrane from the film system in order to observe it at the fluorescence microscope. As BSA-FITC was used, it would be a manner of confirming if there was or was not a certain amount of immobilized BSA at the membrane, as a complementary strategy to the sensor results. However, by that time,

the fluorescence microscope lens was damaged and it could not be used. The following experiments at the sensor were performed with BSA which was not marked with FITC, although, a different strategy for analysing the membrane after the sensor experiments was thought: a Coomassie Blue staining protocol was intended to be applied at the porous membrane in order to confirm the presence of BSA at the membrane, by appearance of blue colour.

Experiment A.2

An identical experiment to Experiment A.1 was preformed introducing a variation: higher incubation time for PEG. Figure 4.2 shows the obtained frequency variation when overnight incubation of PEG was carried on.

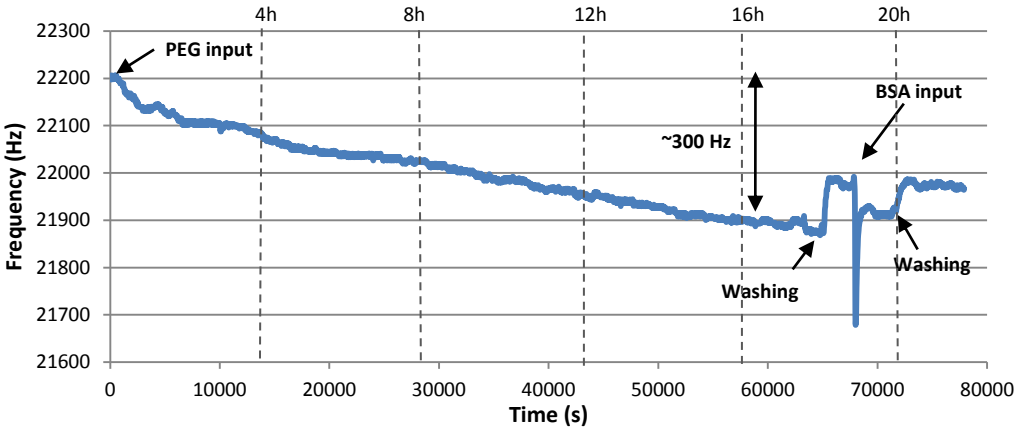


Figure 4.2 Detection of PEG and BSA using a PVDF/0.2um/PVDF film system - Overnight experiment

PEG solution was introduced to the sensor and flowed, in closed circuit, overnight. The frequency decreased along time and stabilized at about 21900 Hz, corresponding to a difference of ~300 Hz, when compared to the initial frequency. When performing the washing procedure, a frequency increase (~100 Hz) was registered, although, on the contrary to the previous experiments, it did not return to the initial value. When BSA was added, an immediate frequency drop was observed, however, after a short period, the frequency increased to a value of frequency ~100 Hz lower than when BSA was introduced. After stabilization, a second washing procedure with PBS was executed and a frequency increase was observed.

The fact that, after the first washing procedure, the frequency did not return to the initial value can indicate that only a low amount of PEG was washed away. The behavior of frequency

when BSA was introduced was similar to the obtained at Experiment A1. The sensor detected its presence in solution, reacting with an abrupt frequency drop and then the frequency increased. After the second washing procedure, the frequency increased to a value similar to the registered before the introduction of BSA, so it is thought that BSA was washed way. However, the final frequency stayed lower than the initial, which can indicate that a certain amount of PEG was immobilized at the membrane.

The porous membrane was then removed from the film system and a Coomassie Blue Staining protocol was applied to the membrane. Results were observed at a microscope, and blue spots were not visualized, indicating that either there was no BSA at the membrane or that this staining method may not be sensitive enough to detect low amounts of immobilized protein.

Conclusions

The main difference between Experiment A.1 and Experiment A.2 was the time of PEG incubation. Comparing the results presented in Figure 4.1 and Figure 4.2, it can be seen that in both experiments, a frequency drop was registered after the introduction of PEG in the chamber. In the first case, for a short period of incubation (about 30 min), the frequency dropped approximately 50 Hz. In the second case, for a period of incubation of about 15 hours, a 300 Hz drop was registered. For Experiment A.1, after the first washing procedure, the frequency returned to a value similar to the one before de addition of PEG, indicating that PEG could have been washed way, although, in Experiment A.2, the frequency increased but stabilized at a value of about 200 Hz inferior to the initial, which could mean that only a certain amount of PEG was washed way. Taking these results in consideration, it can be concluded that a higher incubation time promotes the immobilization of PEG at the membrane indicating that PEG was successfully immobilized at Experiment A.2. In relation to BSA, the frequency behavior was identical in both cases decreasing abruptly when the solution was introduced and increasing to a value 100 Hz inferior to the initial (BSA input) in both experiments. However, it could not be confirmed if there was BSA immobilized at the membrane, after the washing procedures.

4.1.2 Detection of BSA using a PVDF/Immobilon^{PSQ} (0.2 µm)/PVDF film system, with methanol pre-wetting

After performing the above experiments, it was found in literature that protein binding to PVDF membranes could be achieved by simply bringing the protein into contact with the membrane. However, this could only be achieved if the membrane was previously wetted in methanol [50]. The following experiment was accomplished after previously wetting the porous part of the film system in methanol. It was intended to prove this information, by acquiring the frequency response simultaneously to the entrance of an aqueous solution of BSA in the reaction chamber, which would be in contact with the membrane pre-wetted in methanol.

As PVDF is a hydrophobic polymer which won't wet-out in aqueous solution, the porous membrane was wetted in methanol in order to become compatible with aqueous systems, which was, in this case, a BSA solution. When the membrane was wetted in methanol, a change in its appearance from white opaque to semi-transparent occurred, indicating that the wetting was complete. The alcohol was removed from the membrane by extensive rinsing in water, and the membrane was placed at the biosensor reaction chamber and a PBS buffer was allowed to flow in order to equilibrate the membrane.

Figure 4.3 shows the sensor response to the input of BSA, after pre-wetting the membrane with methanol. Based on Equation 1-3, it would be expected that the frequency decreased with the input of BSA solution, although it can only be applied when there is only deposition/binding of mass over the film surface. On the contrary, the sensor responds with an increase of the frequency of oscillation. This can be due to the changing of the elastic properties of Immobilon.

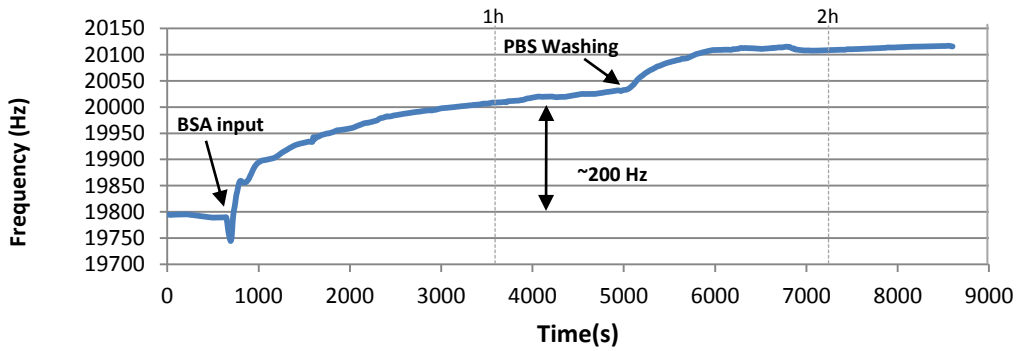


Figure 4.3 Response to the input of BSA after methanol pre-treatment

Firstly, a frequency drop was observed which indicated that, for low BSA concentration, the binding of BSA was occurring only at the surface of the membrane. A few seconds after, an abrupt increase was verified probably due to the changing of the elastic properties of Immobilon, caused by the penetration of BSA molecules into the bulk of the membrane, as a consequence of a higher protein concentration inside the reaction chamber.

Conclusions

In conclusion, it was experimental proved that after wetting the porous membrane with methanol, the membrane has the ability to bind to BSA. The sensor responded with an increase on the frequency of oscillation which indicates that the BSA molecules may be immobilized inside the porous of the membrane and not only at its surface. It can be said that methanol promotes the entrapment of BSA molecules to the membrane, which was detected by a frequency increase, possibly caused by its elastic deformation as a consequence of porous deformation by the entrance of BSA molecules to the bulk of the membrane. Therefore, it would be very important to study the electromechanical properties of the membrane. For example, the elastic properties of PVDF can be investigated by performing Young Modulu's measurements in order to study the stiffness variations of the PVDF membranes [51]. It is known that if the stiffness of the membrane increases, Young Modulu's increases and consequently the elastic compliance decreases. Thus, it is believed that the frequency increase observed when BSA was introduced is a consequence of the decrease of elastic compliance, caused by the entrance of BSA molecules to the bulk of the membrane.

4.1.3 Sensor modifications

The frequency of oscillation is sensible to temperature variations. Therefore, there was the need to control the temperature inside the reaction chamber and to monitor temperature during experiments, to verify if the difference of temperature between the reaction chamber and the solution to be added could have a significant influence in signal acquisition and if the changes in room temperature during overnight experiments would have a considerable contribution to frequency variations.

In order to minimize the referred difference of temperature, a modification to the input of solution was introduced, at the sensor. A coil tube was placed above the heating unit allowing the solution to entry the reaction chamber through the coil tube at a determined temperature, equal to the experimental temperature. Also, to control and measure the exact temperature, a thermocouple was placed inside the reaction chamber. Thus, it was possible to enable the simultaneous acquisition of temperature and frequency.

Figure 4.4 exemplifies the simultaneous acquisition of temperature and frequency during an overnight assay with PEG.

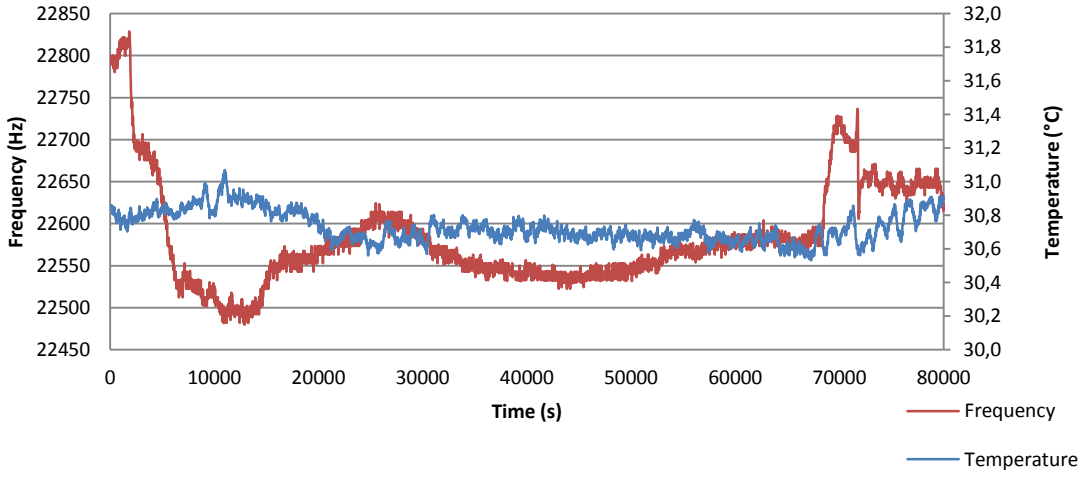


Figure 4.4 Simultaneous acquisition of frequency of oscillation and temperature

Chapter 5 Conclusion remarks and Future Work

The aim of this work consisted in the surface modification of piezoelectric PVDF in order improve its hydrophilicity and biocompatibility, to be applied at a piezoelectric biosensor, however it was not achieved. It was intended to utilize a simple strategy for surface modification of piezoelectric PVDF, which would not interfere with its piezoelectric properties, by coating of functional groups onto the surface. An immersion method was applied to piezoelectric PVDF and also to Immobilon, a porous type of PVDF. Water contact angle measurements were used to study hydrophilicity improvements at the surface of the membranes and elemental analysis was utilized to study their composition. Quantitative and qualitative assays were performed with BSA-FITC to infer about the adsorption of BSA to coated and uncoated PVDF membranes. In accordance to the immersion method results, a system composed by a coating solution and a type of PVDF was chosen to be applied and testes at the sensor. Table 5.1 resumes the experimental work allowing to quickly view where positive and negative results were obtained, leading to the choice of the system to be applied at the biosensor:

Table 5.1Summary of positive and negative results

		Characterization technique		
		Water contact angle measurements	Elementary microanalysis	BSA-FITC binding
Coating solution	Porosity	Hydrophilicity improvements	Elemental composition	Qualitative and quantitative study of the amount of bind BSA-FITC to the membranes
DOPA	PVDF	+	-	-
	0.2 um	-	-	+
	0.45 um	-	-	-
PEG	PVDF	**+**	-	-
	0.2 um	**+**	**+**	**+**
	0.45 um	+	+	-
Gum Arabic	PVDF	+	-	-
	0.2 um	-	-	+
	0.45 um	+	-	-

5.1 Surface modification by coating of functional layers

Water contact angle measurements are a convenient way to assess the hydrophilicity and wetting characteristic of polymer surface. This characterization technique was applied to PVDF polymer membranes, although, such measurements were complex to interpret. Capillarity

forces, heterogeneity, roughness and irregularities at the membranes can influence results and difficult its interpretation. Nevertheless, it was considered that the highest surface hydrophilicity improvements were obtained when PEG solutions were utilized, for each of the three types of PVDF: non-porous, 0.2 µm and 0.45 µm PVDF. Herafter, studies about time dependence of water contact angle on the porous membranes should be performed and could be very helpful in the interpretation of results since it is known that higher hydrophilic porous membranes not only have a smaller initial water contact angle but also a quicker decrease rate of the static contact angle. The decay along time of water contact angles can be resultant from the increasing of hydrophilicity of the membrane surface or from the hydrophilicity improvement in the membrane bulk, which facilitates the diffusion of water to the membrane matrix [52]. Thus, theses studies could provide important information about hydrophilicity improvements, complementary to static angle measurements.

The elemental composition of the samples was studied by elementary microanalysis. These results were not always consistent with the water contact angle measurements results. Significant changes in the composition of the immersed samples were only registered for the following systems: 5 wt % PEG – 0.2 µm PVDF and 25 wt % PEG – 0.45 µm PVDF, where both carbon and hydrogen percentages increased after performing the immersion procedure. In the case of 9 wt % PEG – 0.2 µm PVDF and 25 wt % PEG – 0.2 µm PVDF, the amount of hydrogen was considerably raised, although, the carbon amount inexplicably decreased.

Although results obtained with PEG solutions suggest that there was a possible adsorption/incorporation of PEG to the porous types of PVDF. water contact angle measurements and elementary microanalysis revealed to be limited techniques to infer about the possible coating of the used compounds onto PVDF surface. BSA was marked with FITC and fluorescence assays were preformed in order to select the system that would show the best response to the absorption of the conjugated BSA-FITC. In an overall observation of the results obtained, PEG revealed to be an interesting system to be applied at the piezoelectric biosensor. While performing fluorescence assays at the fluorescence microscope, it was concluded that Immobilon^{PSQ} (0.2 µm pore size) was suitable for fluorescence probes on the contrary to Immobilon-P (0.45 µm pore size), which presented fluorescence background. Herafter, fluorescence probes can be performed using a type of 0.45 µm pore size PVDF membrane optimized for fluoresce applications, namely, Immobilon-FL membrane (from Millipore), which is described as presenting extremely low background that improves

sensitivity of all fluorescence detection protocols and that is compatible with all commonly used fluorescent probes at all excitation and emission wavelengths [50].

It is believed that PVDF surface modification results could be more clarifying if alternative and complementary characterization techniques were used. For example, the microstructure and composition of the membranes could be characterized by attenuated total reflectance (ATR) FT-IR, X-ray photoelectron spectroscopy (XPS), and thermogravimetric (TG) analysis. Also, surface morphology studies to characterize the surface and cross-sectional morphology of the membranes by scanning electron microscopy (SEM) would be useful to obtain information about the membrane morphology and to understand if the coating was only on the membrane surface or also within its pores. In addition, alternative compounds and methods for PVDF surface modification should be studied.

The selected method to perform PVDF surface modification was based in surface coating by immersion of PVDF membranes in a coating solution. It was intended to apply a simple modification method that would improve the surface hydrophilicity and biocompatibility of PVDF by coating or depositing thin film functional layers onto its surface.

During experimental work, it was concluded that this method was very limited and did not ensure the achievement of the aims of the work. Specially in the case of non-porous PVDF, results indicated possible surface hydrophilicity improvements through coating or thin film deposit, although, this improvement seemed to be more likely temporary due to the weak physical interaction between PVDF and the coated layer, which can be washed way along the handling and washing procedures. To overcome the instability problem, future work could include experimental testing of different ways to modify the surface of non porous piezoelectric PVDF. Covalent attachment strategies by means of, for example, UV irradiation, plasma, high energy irradiation and controlled polymerization could be studied, as they are referred in literature as possible methods to provide a long-term stability of grafted chains onto these polymer membranes. Blending modification can also be suggested as an alternative to surface modification, which could be used to achieve the desired functional properties. Although, as both preparation of the membrane and modification process are performed in a single step, in the case of the polymer PVDF, studies should be realized in order to ensure that its piezoelectric properties are maintained, so it could successfully be applied at the biosensor.

Future work could also include experiments with different compounds where experimental conditions like temperature, incubation time and concentration could be varied and the

suggested characterization techniques could be used. For example, chitosan would be an interesting compound to be studied. Chitosan is a hydrophilic biopolymer derived from shells of shrimp and other sea crustaceans, with a wide range of commercial uses and biomedical applications [53-54]. Its chemical structure is represented in Figure 5.1:

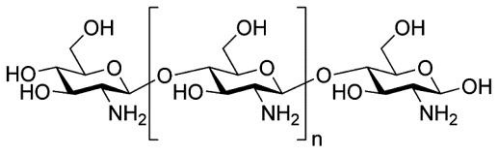
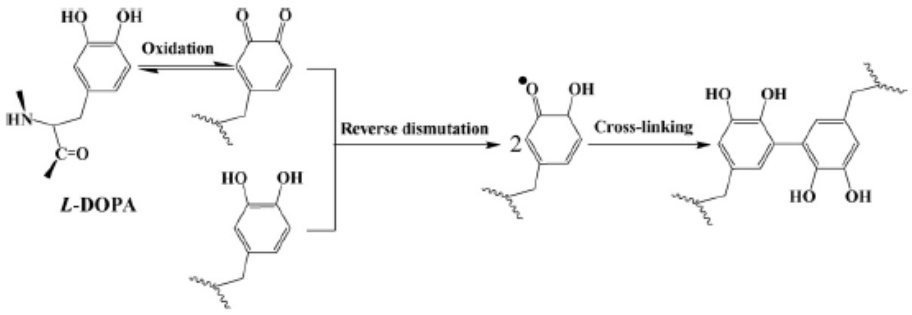


Figure 5.1 Chemical structure of Chitosan

Chitosan is a biocompatible and non toxic polyelectrolyte with reactive functional groups, high adsorption capability and gel-forming capability. Its application as a modifying agent of PVDF microporous membranes has been reported in literature [35].

Experiments with DL-DOPA could also be repeated and studied in more detail. A DL-DOPA layer on membrane surface, containing carboxyl, hydroxyl and amino groups strongly adhered to the membranes could construct a platform for the further functionalization of PVDF membranes. Water contact angle results indicated that the hydrophilicity of the PVDF porous membranes was not improved and elemental microanalysis revealed that its elemental composition was not changed, although, in literature, it was found that the employment of ethanol as solvent of reaction solution would allow DOPA to self-polymerize not only on membrane surface, but also on membrane pore walls. A possible reaction mechanism for the case of L-DOPA is shown in Figure 5.2.



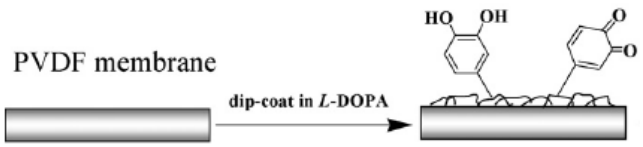


Figure 5.2 L-DOPA self-polymerization on PVDF membranes [38]

Possibly, in this work, the cross-link reaction between DL-DOPA molecules did not occur and the route described above was not followed. On the other hand, when the immersion procedure was carried out, it was observed that the initial transparent solution became black, which might indicate that the oxidation step occurred. Contrary to what would be expected, hydrophobic PVDF membranes were not modified by coating DOPA layer.

5.2 Piezoelectric Biosensor

According to the results obtained for surface modification of PVDF the PVDF/Immobilon^{PSQ} (0.2 µm)/PVDF film system was selected to be studied at the biosensor. The sensor response was tested with PEG and BSA. Results show that the device is sensitive to both PEG and BSA, responding with a variation on the frequency signal. When PEG was introduced, the frequency dropped. In theory, the deposition of a higher amount of mass on the piezoelectric film causes a higher frequency decrease, so it is possible that this frequency drop occurred as a consequence of the deposition of PEG on the membrane surface. Unfortunately, biosensor experiments confirmed that PEG can be easily washed way from the membrane surface. More work shall be done in order to achieve a strong hydrophilic biocompatible layer on PVDF surface. It would be very interesting to perform a durable immobilization of PEG at piezoelectric PVDF, which would allow applying a pertinent biomolecular recognition system to the piezoelectric biosensor. Future work should also include the study of biomolecular recognition system based in PEG, to permit specific detection of determined analytes.

Several factors can influence the oscillation frequency including the physical properties of the adjacent media, like density or viscosity of air or liquid. Studies about the variation of the oscillation frequency with the density or viscosity of the solution used in experiments applied to the developed piezoelectric sensor would be very interesting and helpful to the optimization of its operation conditions.

When studying the protein binding capacity to porous PVDF, it was found that methanol improved the entrapment of BSA to the membrane bulk, causing the opposite response of

what would be expected. Actually, when BSA is added after treating the membrane with methanol, the biosensor responds with an increase of the frequency output. This abrupt increase was most likely caused by the changing of the elastic properties of Immobilon, as a consequence of the penetration of BSA molecules into the bulk of the membrane. In order to better understand these results, studies about the elastic properties of PVDF can be performed.

Experiments were also preformed with a film system composed only by PVDF but, as they were not reproducible, they were not considered valid to present. Also, when studying the BSA capacity of binding to PEG, it was chosen to use Immobilon^{PSQ} membranes instead of Immobilon-P considering that the binding capacity is determined by the internal surface area of the pores and that Immobilon-P^{SQ} transfer membrane is higher than the internal surface area of Immobilon-P transfer membrane, resulting in higher adsorptive capacity.

Although the initial aim of the work was not accomplished, due to the experimental limitations already discussed, the course of experimental work lead to very interesting results. For example, it was found that the immersion of porous PVDF in methanol is a very simple way of improving its hydrophilicity towards proteins without the need of more complicated surface modification procedures and that a film system composed by porous PVDF was successfully applied to the detection of BSA, confirming that it has binding capacity to BSA molecules. However, the binding is not specific and so it would be very interesting to test the sensor towards different proteins and to try to obtain calibration curves for each type of tested proteins.

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