POLYACRILONITRILE CARBON NANOTUBES (PAN-CNT) NANOCOMPOSITES FOR BIO FUEL CELLS CATHODE

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
MECHANICAL ENGINEERING

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2011

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ABSTRACT

This thesis presents the morphological and electrochemical characterization of composite membranes prepared from Polyacrylonitrile (PAN), Multi-Walled Carbon Nanotubes (MWCNT), and Catalase composites for applications as bioelectrodes in fuel cells. The composite membranes were prepared by solvent casting and electrospinning. We have studied the effect of processing parameters and the concentration of carbon nanotubes in the morphological characteristics and electrochemical response of the bioelectrode. The electrochemical characterization was conducted using cyclic voltammetry, while morphological characterization of the membranes was performed using optical microscopy and scanning electron microscopy (SEM). The enzymatic loading in the electrode was measured using Bradford method. Catalase was immobilized by covalent bonding and the electrochemical response of the bioelectrodes was tested in presence of hydrogen peroxide (H_2O_2). The electrochemical characterization allows concluding that electrospun fibers with concentrations of 5% and 2% of MWCNT are the best material of the evaluated to be used as bioelectrodes supports.

RESUMEN

En este trabajo se presenta la caracterización morfológica y electroquímica de fibras y membranas preparadas a partir del compuesto Poliacrilonitrilo (PAN) y nanotubos de carbono de múltiples paredes (MWCNT), para una posible utilización como biocatodo en celdas de combustible usando la enzima catalasa catalizador. Las fibras fueron preparadas por "electrospinning", mientras que las membranas fueron preparadas por "solvent casting". Se evaluó la incidencia del contenido de MWCNT en la morfología y en la respuesta electroquímica de las fibras y membranas. La caracterización electroquímica fue realizada mediante voltametría cíclica y la caracterización morfológica fue realizada a través de microscopia óptica y microscopia electrónica de barrido (SEM). La carga enzimática en los electrodos fue determinada usando el método de Bradford. La caracterización electroquímica permitió establecer que de los evaluados, el mejor material para ser usado como soporte en los biocátodos son las fibras con las concentraciones de 2% y 5% de MWCNT.

To my family, dear friends and all the people that allowed me to have a pleasant time far away of home.

ACKNOWLEDGEMENTS

During the development of my graduate studies at the University of Puerto Rico, several persons and institutions collaborated directly and indirectly with my research. Without their support it was have been difficult for me, to finish my thesis work. I wish to dedicate this section to recognize their friendship, company and support.

I want to start expressing my gratitude to my advisor, Dr. Ricky Valentín. He gave me the opportunity to research under his guidance and supervision from him. I received motivation; encouragement and support during my studies. A special acknowledgement to Dr. Surinder Singh and Dr. Rubén Díaz, for their support, instruction and collaboration and also their confidence to allow me use their equipment in the development of my work.

A very special acknowledgment to my dear friend M.Sc. Andrés Velasco for his unconditional friendship, company, guidance and support, Also to M.Sc. Mariana Mendoza for her encouragement and wise advices.

Here in Mayaguez, I had the opportunity to meet wonderful people who became my friends; they are Eng. Jairo Boada, Eng. Amanda Blagg, Gustavo Otero, my partners during my master studies, Lic. Jessamine Hernández and Lic. Yolanda Perez, I am very thankful with them.

I want to give an acknowledgment to the NANOmaterials Processing Lab – Engineering Science and Materials Department, of the University of Puerto Rico, and also to José Almodovar for their contribution to the development of my work and also to ARGONE national lab for providing the funds for this research.

At last, but the most important, I would like to thank to my family, for their unconditional support, encouragement and love.

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1. INTRODUCTION

The improvement of power generation devices is playing an important role in recent years due to the quick depleting and the cost of the non-renewable fossil fuels. Currently, one of most promising devices for clean and efficient power generation is the fuel cell. Fuel cells are electrochemical devices that directly convert the chemical energy of the fuels into electrical energy [1], avoiding the energy losses from producing heat and mechanical work common in traditional engines and power plants.

There are several types of fuel cells, which include Solid Oxide Fuel Cell (SOFC), Molten Carbonate Fuel Cells **(**MCFC), the Phosphoric Acid Fuel Cell (PAFC), the Alkaline Fuel Cells (AFC) and the Proton Exchange Membrane Fuel Cells (PEMFC).

The proton exchange membrane (PEM) fuel cells uses a proton-conducting polymer membrane as electrolyte and offer several advantages over the other previously mentioned types of fuel cells that include operation at low temperature and the possibility to use different types of catalyst and fuels [1 - 3].

Catalyst made of transition metals makes possible the reversibility of some redox reactions, which are irreversible at common unmodified electrodes and also decreases the overpotentials of many important electrochemical reactions. Platinum is the most used catalytic material but is very scarce and expensive, which increases the cost of the fuel cells [2].

An alternative that can substitute platinum for catalysis application is the utilization of biocatalyst, such as enzymes. The use of enzymes as catalytic materials generates a new challenge in nanomaterials development, because they require a suitable substrate for their immobilization.

A wide variety of nanomaterials such as mesoporous media, nanoparticles, nanofibers, and nanocomposites have been used as supports for enzyme immobilization and have manifested a great potential for the stabilizing, activating, and increasing the lifetime of enzymes. Some of the characteristics expected from these supports included: high electrical conductivity, high surface area, high porosity, and high compatibility with the enzyme. Nanofibers have been successfully used for this purpose because they provide a large surface area for the attachment of enzymes, which enhances the enzyme loading in the electrode. Also the high porosity of the fibers contributes to increasing the catalytic activity and lifetime of enzymes, improving the power density generation of the biofuel cells [32].

Nanofibers with carbon nanotubes embedded inside their structures also have demonstrated to be a good choice of material for this application [52, 64]. However, the short lifetime and catalytic activity of the enzymes and the low power generation of these devices have been a serious limitation for their massive utilization [2, 4, 52]. Our hypothesis is that fuel cell lifetime and catalytic activity can be increased by enzymatic electrodes made of composite materials constituted by electrospun Poliacrylonitrile (PAN) nanofibers and multiwall carbon nanotubes (MWCNT) will increase the catalytic activity of the fuel cells.

Chapter 2, presents a literature review related to biofuel cells, techniques for enzyme immobilization and development of new materials for biofuel cells electrodes. The chapter focuses in the fuel cells constructed with nanofibrous membranes containing carbon nanotubes and fuel cells that use redox enzymes as catalyst materials. Also, it includes a brief revision of the theoretical background and basic concepts required to understand the performance of the biofuel cells and the role of the enzymes as catalyst for chemical reactions, and the expected properties of the materials to be used as electrodes supports in this devices.

Chapter 3, discusses the methods for the preparation of the electrodes supports, and techniques for the characterization of these. Also it describes the procedure followed for the preparation of the membranes by electrospinning and casting, the immobilization of the catalytic enzymes and the characterization of the constructed bioelectrodes.

Chapter 4 and Chapter 5 are dedicated to the presentation of the results of this research and to the conclusions, respectively.

2. LITERATURE REVIEW

This chapter is divided into two sections. The first section introduces the technological background and current progress on enzymatic fuel cells, focusing on the development of electrode materials. The second section reviews the strategies for synthesizing composite nanomaterials, particularly nanofibers with carbon nanotubes.

2.1 FUEL CELLS

Fuels cells are electrochemical devices for power generation. In a fuel cell, electrons and protons are generated in the anode due to the oxidation of the fuel, which is promoted by a catalyst material; the electrons are forced to travel through an external circuit toward the cathode of the cell. In the cathode, another catalytic process allows the combining of the electrons generated in the anode with the protons and oxidant (typically O_2) to form waste products like water and carbon dioxide [1].

Fuel cells are typically classified according to the types of the electrolytes that they use. There are alkaline fuel cells (operating temperature 60 - 200 °C), phosphoric acid fuel cells (120 - 210°C), molten carbonate fuel cells (650°C), solid oxide fuel cells (600 - 1000°C), and proton-exchange membrane (PEM) fuel cells (40 - 90 °C). PEM fuel cell has the widest application range of all types of fuel cells. The base of a PEM fuel cell is the membrane electrode assembly (MEA) that is composed of an anode, a cathode, and the PEM. The PEM allows only protons been transport from the anode to the cathode, this membrane typically made of Nafion is a physical barrier to separate the anode from the cathode, and to prevent the hydrogen and oxygen from mixing.

A fuel such as hydrogen is oxidized at the anode to form protons and electrons (see Equation 1). An oxidant such as oxygen (typically from air) is reduced at the cathode by combining with protons and electrons to form water (Equation 2).

$$2H_2 \rightarrow 4H+ + 4e- \quad \text{(Equation 1)}$$

$$O_2 + 4H+ + 4e- \rightarrow 2H_2O \quad \text{(Equation 2)}$$

The redox reactions normally do not occur at the operation temperatures of the PEM fuel cells. Catalyst materials such as platinum are used to initiate the redox reaction. The catalyst particles are in direct contact with protons, electrons, and reactants and directly participate in the electrochemical reactions. Thus, the efficiency of a fuel cell depends of the total active area of the catalyst particles that participate in the redox reactions. The regions in that redox reactions are often called catalyst-electrolyte-reactant three phase boundaries; these regions are shown in the Figure 1.

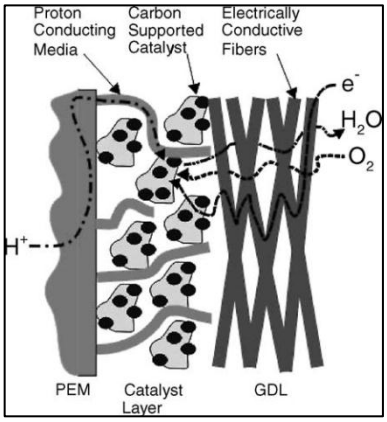


Figure 1. Representation of three-phase boundary

Precious metals such as platinum and palladium and their alloys are often used as catalyst materials in fuel cells. These catalyst materials can operate at high temperatures and in the

extreme conditions of acid or basic media. The catalyst weaknesses include low selectivity; they are capable to catalyze in the same surface anodic and cathodic reactions causing a short circuit. They also are limited resources and the massive use of these during the last years has increased their cost.

As a less expensive alternative to these precious catalyst materials, the enzymes are highly selective catalyst and they can only catalyze a limited number of reactions per type. For example, the oxido-reductase enzymes (or redox enzymes) only catalyze electron transfer reactions making them proper catalyst materials to provide an efficient electrical contact between the catalyst and the electrode surface. These types of enzymes are particularly useful for the construction of the denominated mediator electron transfer electrodes. The fuel cells that use enzymes as catalyst materials are called bioenzymatic fuel cells.

2.2 ENZYMATIC BIOFUEL CELLS

Enzymatic biofuel cells use enzymes as catalyst; their temperature operation range is between 20℃ - 40℃ and a PH near to neutral. Also, the variety of reactions able to be catalyzed by enzymes allows the use of a wide range of substance like fuels in these devices. Bioenzymatic electrodes can be located at both sides of the biofuel cells [61], generating the membrane-less fuel cells, or only one of the electrodes can be of the enzymatic type, giving in this case the hybrids biofuel cells. The enzyme based biofuel cell uses the same operation principle that PEM fuel cells, in these, the fuel is enzymatically oxidized in the electrode surface at the bio-anode, producing protons and electrons; the electrons are conducted by an exterior electrical circuit toward the bio-cathode. Oxidant material (usually oxygen or peroxides) reacts with electrons and protons to generate water.

2.3 ENZYMES

Enzymes are proteins that mediate all reactions carried out by living organisms. They increase the rate (catalyze) these reactions without undergoing any net change in the enzyme. Enzymes bind temporarily to one or more of the reactants of the reaction that they catalyze, lowering the activation energy required for the reaction and thus increasing its rate.

Enzymes exhibit many advantages over conventional chemical catalysts like platinum, including their specificity and selectivity for particular reactions and also between similar parts of molecules (regiospecificity) or optical isomers (stereospecificity) [50, 51, 52, 59]. They can catalyze only the reactions of very narrow ranges of reactants (substrates), these substrates can consist of small quantity of closely related classes of compounds, a single class of compounds, or a single compound. The shape, charge and characteristics of hydrophilic and hydrophobic of enzymes and substrates are responsible for this specificity.

2.3.1 Enzyme Catalysis

An enzyme increases the rate of a chemical reaction this because the enzymes binding preferentially to the transition state of its specific substrate. The high affinity of the enzymes by the intermediate state of a substrate, influence the specificity and in the catalytic reaction of the enzymes. The enzymes decrease the activation energy of the chemical reactions, as is shown in Figure 2.

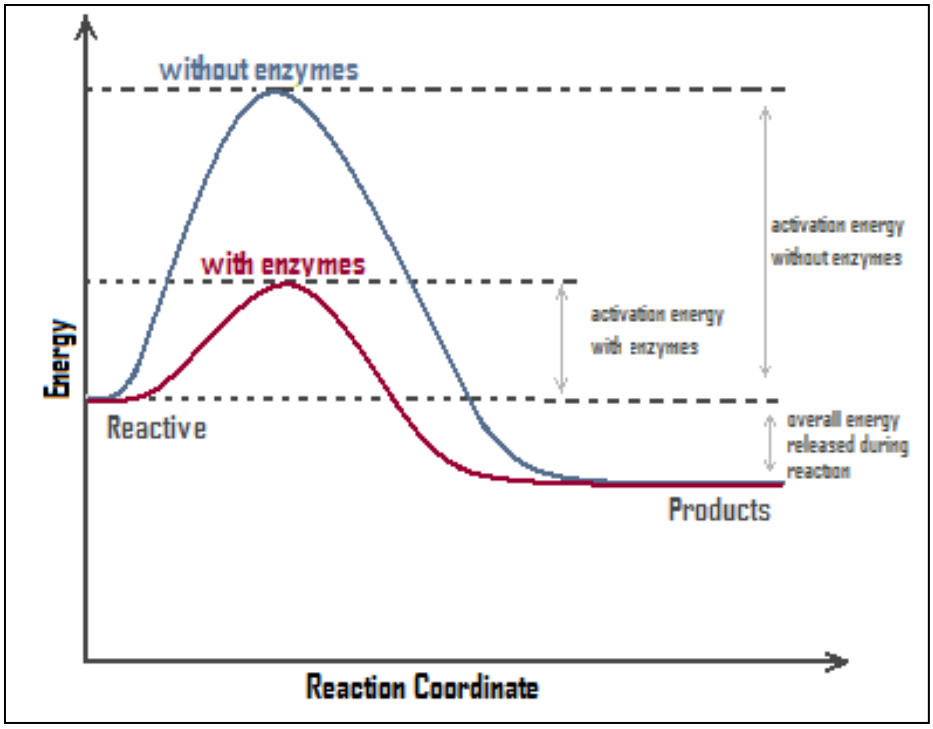


Figure 2. Effect of the enzymes in the activation energy of the chemical reactions

This decrease in the activation energy of the reactions is achieved by the enzymes in the following ways:

- Providing catalytically active groups for the development of a specific reaction mechanism.
- Binding several substrates in a preferred orientation that favored the catalysis of the reaction, which reduces the entropy change of the reaction. Considering entropy change alone overlooks this effect.
- Using the differential binding energy of the substrate in its transition state, compared with its normal state, in this case the transition state is stabilized (e.g. straining the shape of a substrate by binding the transition-state conformation of the substrate/product molecules, the enzyme distorts the bound substrate(s) into their transition state form, thereby reducing the amount of energy required to complete the transition).

- Temporarily reacting with the substrate to form an intermediate complex that provides an alternative pathway to complete faster the reaction.
- Increasing the temperature which speeds up reactions, However, if heated too much, the shape of the enzyme could be denaturized and only when the temperature comes back to normal does the enzyme regain its shape. Interestingly, this entropic effect involves destabilization of the ground state, and its contribution to catalysis is relatively small.

2.3.2 Redox Enzymes in Biofuel Cells

The use of isolated enzymes for energy conversion was originally reported by Kimble at 1960. In 1980, direct electron transfer was observed by Barton between some enzymes and electrodes [64]. It was only in 2006 that the direct electron transfer was applied in fuel cells when researchers developed bio-cathodes for the reduction of oxygen using laccase and bilirubin oxidase and bioanodes employing glucose oxydase [82, 89].

Numerous redox enzymes have been used as catalyst at the anode and cathode of biofuel cells. In the anode side glucose oxydase [70], glucose dehydrogenase, alcohol dehydrogenase [61], microperoxydase 11, lactate dehydrogenase; and lacasse, billirubin oxydase, cytochrome c, chloroperoxosases, horseradish peroxidase, microperoxidase 8 [61], at the cathode side.

Most of these redox enzymes have the active centers embedded inside them, surrounded by thick insulating protein shells. As a consequence of this, electrons cannot be efficiently transferred between the enzyme (catalytic material) and the electrode surface, in these cases chemical mediators are required to develop this function, giving place at the called mediated electron transfer. The widely used glucose oxydase is an example of this type of enzyme [74]. Some enzymes like peroxidase, catalase, laccase, and azurin, etc. are exceptions of mediated

electron transfer. In these enzymes the active redox centers are located close to the surface of protein.

The catalytic behavior of immobilized enzymes is influenced mainly by the properties of the substrate and by the conformation and orientation of the immobilized enzymes [64]. The first affects the accessibility from the enzyme reaction centers to the substrate and the second determines the efficiency of the enzyme reaction centers [32]; thus the properties and morphology of the supports are very important for the enzyme immobilization and stability. In general, biocompatibility (between enzyme and support) is required and highly porous supports are of preference. The electrospun nanofibers have a large surface area to volume ratio that can provide a large amount of bound enzymes, and a high porosity can enhance the enzyme activity through decreasing the diffusion resistance of substrate [32], in the same way an increase in the surface area of the electrodes supports and also in the quantity of catalyst material allows to increase the active points in the fuel cells, improving the efficiency of this devices.

The method for the immobilization of enzymes plays an important role in the lifetime and catalytic activity of enzymes. Physical adsorption gives weak bonding between enzymes lead to the formation of randomly oriented layers. The preferential orientation of the active site toward and closer the electrode is required in order to improve the efficiency of the bioelectrodes [64 - 75].

2.3.3 Catalase

Catalase is a type of oxidoreductase enzyme that speeds up the breakdown of hydrogen peroxide into oxygen and water.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad \text{Equation 3}$$

Catalase is found in the peroxisomes of cells. And it has 4 polypeptide chains with each chain containing 500 amino acids each (giving a total of approximately 2000 amino acids per catalase). Catalase breaks down and detoxifies hydrogen peroxide at a very high rate. it has one of the highest rates of all known enzymes: about 40,000,000 molecules peroxide per second. If there is a single change in the protein sequence of catalase, such as in its active site, then it cannot break down hydrogen peroxide. Cells, in this case, would not exist because the toxic nature of hydrogen peroxide, so it is crucial that catalase be intact to be active. In addition to the required protein sequence there are other factors that affect the activity of catalase, are temperature and pH.

Temperature affects the activity of most enzymes by speeding up a reaction, but high temperatures can change or damage the molecular structure of proteins, leading to its deactivation. A pH reading is a measure of how many hydrogen ions are in a solution. Lower PH numbers mean more hydrogen ions present in the solution. A change in the pH environment of an enzyme causes it to exist in either an acidic solution (more hydrogen ions; pH 1.0-5.0) or basic solution (less hydrogen ions; pH 9.0-14.0).

In both cases, the changes produced in the chemical bonds of the enzyme molecule result in a change in shape that decreases enzyme activity. The majority of enzymes, like catalase, exist in solutions that are neutral (solutions that are pH 7.0). As long as catalase remains at 37 degrees Celsius and at physiological pH (7.0), it will remain active.

Proteins, such as catalase, control important biochemical reactions in the cells. DNA and protein sequences are organized very systematically for individual enzymes and will affect its activity if altered. Such an alteration can change the rate of reaction or inactivate the enzyme. In the case of catalase, very toxic conditions occur, and cells die as a result of peroxide build up.

2.3.4 Direct Electron Transfer

One of the critical challenges for the development of enzymatic biofuel cells is the lower electron conduction between biocatalysts and the electrodes. The redox active centers of most redox-active biological molecules are embedded within the glycoprotein. As a consequence of this, electrons cannot be efficiently transferred between the enzyme and electrode. Mediators and redox enzyme are been required to increase the electron conduction in the electrodes; this is called mediated electron transfer. Usually mediators are low molecular weight redox active compounds and redox polymers, which participate in an active way in catalytic reactions, being oxidized or reduced in the process and transferring the resulting electrons from or to the electrode‘s surface.

Direct Electron Transfer (DET) between an enzyme and electrodes has been observed with several enzymes, such as cytochrome c, laccase, hydrogenase, and several peroxidases, including microperoxidases, azurin and blue cooper protein [12, 26, 61]. For DET, close contact between the active sites of the enzymes and the surface of the electrodes is required. For bioelectrodes that uses laccase as catalyst for direct reduction of oxygen, a critical distance between the enzyme active site and the electrode surface is near to 20 Angstroms [26]. This requires a proper orientation of the enzymes in the electrode surface [51]. The orientation of the enzymes can be controlled using suitable molecules as linkers during the enzyme immobilization process.

2.3.5 Selectivity

Selectivity is one of the most remarkable characteristics of the enzymes for their uses as catalytic material in fuel cells. An example of the importance of the selectivity of the catalyst

enzymes in a biofuel cell can be found in PEM fuel cells that uses methanol as fuel, and Nafion (which is permeable to methanol) as polymer electrolyte membrane. Methanol is a suitable fuel for portable fuel cells [61], which can be enzymatically oxidized to CO_2 (in the anode side). Using a series of three different enzymes (enzyme cascade) among which are included the dehydrogenases, the alcohol can be completely oxidized. If platinum is used in place of the enzymes, the methanol can be permeate trough the Nafion layer toward to the cathode side, where this could be oxidized by the platinum, resulting in an important loss of cathode potential. However, if reducing enzymes are used as catalytic materials in place of platinum, in the cathode side, the anodic oxidation of the alcohol cannot proceed because the reducing enzymes do not catalyze this reaction.

Other important example of the enzyme selectivity is the DMFC (direct methanol fuel cells); which possess catalytic enzymes in both electrodes of the cell, so the separation between electrodes (Nafion membrane) was eliminated. The elimination of the Nafion in this device is due to the low probability that the product reactions of each electrode interact with each other. The membrane-less fuel cell could contribute to the miniaturization of bio fuel cells.

Despite of the numerous advantages of the bio fuel cells, they have serious limitations for the practical applications of these devices; two critical issues are short lifetime and poor power density, both of which are related to enzyme stability, electron transfer rate, and enzyme loading. Many nano-structured materials, such as nanoparticles, nanofibers, and nanotubes, have been demonstrated to be efficient hosts for enzyme immobilization [26 -50].

2.3.6 Stability

In most cases, the stability of biocatalysts determines the lifetime of biofuel cells. Most enzymatic fuel cells usually last only a few days [53]; however the immobilization can help to extend the lifetime of enzymes. The half lifetime of the native parent enzyme is only 7 to 8 h in solution whereas an active lifetime of more than 45 days was achieved after immobilization [61].

The immobilization of the enzymes on the electrode surface is considered as one of the critical steps to guarantee the efficiency of the enzyme electrodes. Different chemical, biochemical or physical principles such as chemical cross-linking, magnetic interactions, entrapment or encapsulation within polymers, and the formation of paste materials are generally followed in order to immobilize the enzyme on the electrode surface.

2.3.7 Immobilization

The enzymes are formed only in living cells, but many enzymes can be separated from the cells, immobilized in other substrates and can continue to function in vitro [52]. The enzyme immobilization onto electrodes surfaces can be achieved by chemical or physical methods. Most of the enzyme-based biofuel cells reported so far have been constructed by physically immobilization of enzymes. Ethanol/ O_2 biofuel cells constructed with this bioelectrode, generated a power density as high as 2.04 mW/cm^2 [52]. However, some studies suggest that this immobilization method could clog the pores of the electrodes, limiting the mass transport between the enzyme and the electrode (fuels and ionic species) [64].

Supports for enzyme immobilization must be inexpensive, inert, physically strong and stable. The nature of support will also have a considerable effect on an enzyme's expressed activity and apparent kinetics. It will increase the enzyme specificity whilst reducing product

inhibition, shift the pH optimum to the desired value for the process, and discourage microbial growth and non-specific adsorption.

The main methods used for the immobilization of biocatalysts are:

- Entrapment in polymers
- Adsorption of the charged biocatalyst onto oppositely charged support materials
- Covalent attachment to chemically activated supports.

Also combinations of these techniques can also be used, such as adsorption of the biocatalyst to a charged support followed by cross-linking place.

2.3.7.1 Immobilization by entrapment

This method is based in the occlusion of an enzyme inside a restrictive structure tight enough to prevent the protein of diffusing into the surrounding medium, while still allowing penetration of substrate. This immobilization method is recommended to use in polymeric supports with low molecular weight which allows the diffusion of enzymes trough the matrix, however resistance to diffusion can affect in an adverse way the kinetics of the enzyme.

Enzymes can be entrapped inside fibers; for this an emulsion prepared mixing a solution containing the enzyme and a polymeric solution is extruded through a spinneret; droplets of enzyme solution being trapped inside the fibers, however the fiber entrapped enzymes exhibit diffusion limited kinetics and are best suited for enzyme systems that work on low molecular weight substrates. Also the applicability of this method is limited by the use of some non-water miscible solvents that can cause the inactivation of enzymes.

Entrapment is carried out by mixing the biocatalyst into a monomer solution, followed by polymerization initiated by a change in temperature or by a chemical reaction. The polymer is

formed either in particulate form, or as a block which can be disrupted to form discrete particles. The most common methods of entrapment use polyacrylamide, collagen, cellulose acetate, calcium alginate or carrageenan as the matrices.

2.3.7.2 Immobilization by adsorption

This is considered the easiest way to reach the immobilization of proteins and enzymes and is capable of high enzyme loading (about one gram per gram of matrix). The adsorption can be reached by only putting in contact the surface of the support with a solution of that contain the enzyme of interest under appropriated conditions of pH and ionic strength, followed after by a sufficient incubation period, by washing off loosely bound and unbound enzyme, will produce the immobilized enzyme in a directly usable form. This also requires also that the adsorbent support possess a high affinity with the enzyme in order to avoid its denaturalization. A disadvantage of this method is the relatively weak binding forces between the enzyme and the support [64]. The driving force causing this binding is usually due to the combination of hydrophobic effects and the formation of several salt-links per enzyme molecule.

The particular choice of adsorbent depends principally upon minimizing leakage of the enzyme during the use. Although the physical links between the enzyme molecules and the support are often very strong, they may be reduced by many factors including the introduction of the substrate. Examples of suitable adsorbents are ion-exchange matrices, porous carbon, clay, hydrous metal oxides, glasses and polymeric aromatic resins.

2.3.7.3 Immobilization by crosslinking

Most used agents of coupling is glutaralgehyde , which is a bifunctional reagent which may be used to cross-link enzymes or link them to supports. It is particularly useful for producing immobilized enzyme membranes for use in biosensors, by cross-linking the enzyme plus a non-catalytic diluent protein within a porous sheet (e.g. lens tissue paper or nylon net fabric).

2.3.7.4 Immobilization by covalent binding

This is the most widely used method for immobilizing enzymes. The enzymes are very firmly bound, but are chemically modified and so many are denatured during immobilization. Immobilization of enzymes by their covalent coupling to insoluble matrices is an extensively researched technique. Only small amounts of enzymes may be immobilized by this method (about 0.02 gram per gram of matrix) although in exceptional cases, as much as 0.3 gram per gram of matrix has been reported.

The strength of binding is very strong, however, and very little leakage of enzyme from the support occurs. The most commonly used method for immobilizing enzymes on the research scale (i.e., using less than a gram of enzyme) involves Sepharose, activated by cyanogen bromide. This is a simple, mild and often successful method of wide applicability. Sepharose is a commercially available beaded polymer which is highly hydrophilic and generally inert to microbial attack.

EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride), is another coupling agent of common use to enzyme immobilization by covalent bonding, the structure of this chemical compose is shown in Figure 3.

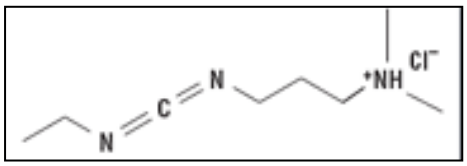


Figure 3. Representation of EDC molecule

The EDC is a zero-length crosslinking agent used to couple carboxyl groups to primary amines. This crosslinker has been used in diverse applications such as forming amide bonds in peptide synthesis, attaching haptens to carrier proteins to form immunogens, and creating amine-reactive NHS-esters of biomolecules by mean of the reaction with NHS (see Figure 4). The basic mechanism of EDC is reacting with a carboxyl to form an amine-reactive intermediate which can be hydrolyzed to regenerate the carboxyl, if it does not encounter an amine to react.

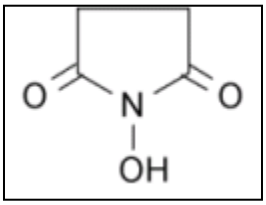


Figure 4. Representation of NHS molecule

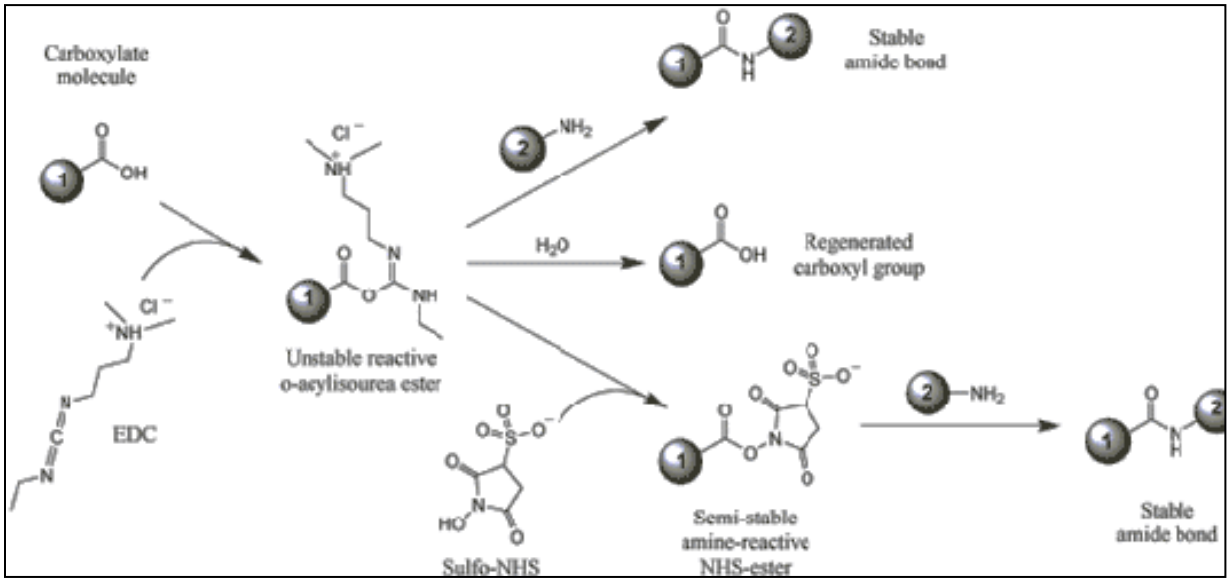


Figure 5. Mechanisms of reaction of EDC

EDC possess several mechanisms of reaction, which are shown in Figure 5. EDC reacts with a carboxyl group on molecule 1 to form an amine-reactive O-acylisourea intermediate. This intermediate compose is unstable and can react with an amine group on molecule 2, to link the two molecules through a stable amide bond. Other reaction way of the intermediate compose is the hydrolysis for this reason this ester has a short-live in aqueous solution. The addition of NHS (see Figure 4) stabilizes the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions [42, 43]. The amine-reactive Sulfo-NHS ester intermediate has sufficient stability to permit two-step crosslinking procedures, which allows the carboxyl groups on one protein to remain unaltered.

Covalent coupling via an amide bond formation at an electrode surface can be achieved without carbodiimide coupling reagent, if the self-assembled monolayer includes active ester groups. These groups can react spontaneously with amino groups of organic compounds (e.g. amino groups of lysine residuals of protein/enzyme molecules).

The incorporation of a carbon catalyst-support into the PEM fuel cell electrodes brings in both advantages and disadvantages. The advantages include the increased catalyst dispersion and the reduced catalyst loading. The disadvantages comprise of the promotion of peroxide formation that accelerates the ionomer decay rate, and the problems associated with the carbon corrosion.

Functionalizing the surface of carbon by suitable groups can add new advantages such as reducing the catalyst particle size and enhancing the thermal stability and also allows the immobilization of catalytic redox enzymes in the surface of the electrodes. The enzyme catalase

is an example of redox enzymes that has been immobilized by covalent binding, over several substrates that includes PAN nanofibers in the cathode side of biofuel cells.

2.4 ELECTRODES FOR FUEL CELLS

The ideal support material for fuel-cells catalysts should have the following characteristics: high electrical conductivity, adequate water-handling capability at the electrode, and also good corrosion resistance under oxidizing conditions [41]. Carbons are the de facto standard negative electrodes of commercial fuel cells because of a number of good features:

- High specific capacity coupled to a sufficiently electrode potential for ion insertion and removal.
- Excellent cyclability due to their dimensional stability during cycling.
- Most carbons are inexpensive and abundant materials.

Carbonaceous materials possess all the mentioned properties and has been used for the construction of some of the different layers in fuel cells, a good example of this are the carbon cloths widely used as gas diffusion layers, this due its high reactant and product permeability, high thermal and electrical conductivity also good mechanical resistance and high porosity (>70%).

In the case of supports for electrodes in fuel cells, carbon materials attract a growing interest due to their specific characteristics such as high surface area ratio where the catalyst is easy to disperse as well as high electrical conductivity, chemical stability under the fuel cells operation and also the possibility to control, up to certain limits, the porosity and surface chemistry, for this application carbon nanofibers has been recently used. Also the presence of carbon nanotubes in the fibers and in the carbon nanofibers, enhance their electrochemical

properties and act as a support site for enzyme immobilization, in the case of bioelectrodes for fuel cells [27, 32, 37, 38]. Table 1 summarizes some examples of enzymes immobilization onto substrates of carbonaceous materials.

Table 1. Use of carbonaceous materials as substrates for enzymes immobilization

Substrate	Achievement
Carbon black or graphite powder	• Hydrogenase and laccase were adsorbed on carbon black particles to construct composite electrodes (Tarasevich et al., 2002).
The enzymes GOx or HRP was adsorbed on synthetic graphite particles.	• A glucose/H_2O_2 biofuel were developed cell using Ferrocene-modified composite electrodes. The electrodes were prepared by spray-printing of the dispersion of graphite particles. (Pizzariello et al., 2002)
Use of CNT as filler in polymer nanofibers to immobilize catalase and horseradishperoxidase.	• Multiwalled carbon nanotubes (MWCNTs) were filled into poly(acrylonitrile-co-acrylic acid) nanofibers for covalent immobilization of catalase. • When the mass ratio of MWCNTs to the polymer was 30%, the activity of the immobilized catalase was increased by 47% without reduction in the enzyme loading. • This composite nanofibrous support was also used to immobilize another redox enzyme, horseradish peroxidasa.
Immobilization of catalase onto the nanofibers from MWCNT co-electrospun with poly(acrylonitrile-co-acrylic acid) bearing metalloporphyrin pendents	• The incorporation of Porphyrin that is used like an electron donor, seem to have a synergic effect with the MWCNT which act as conductor of electrons. • The results showed that the activity of the immobilized catalase was enhanced most when both MWCNTs and metalloporphyrin were incorporated into the nanofibers. • Despite the fact of activity enhancement for redox enzymes, there is no evidence to directly relate this effect with the electrical conductivity.
Hybrid biofuel cell that use a biocathode of horseradish peroxidase (HRP) immobilized on graphite and use H_2O_2 as an oxidizer.	• Design of hybrid biofuel cell formed by the battery type Zn anode and the biocathode of horseradish peroxidase immobilized on graphite. The cell yields the open circuit voltage V_{oc} of 1.68 and 1.57 V and the short-circuit current density of $800\mu A\ cm^{-2}$ at pH 6. The biofuel cell operated at 1.5 V for 6 days. (Gomez et al, 2010)
CNTS inside the carbon matrix (nano-particles were inserted to a carbon cloth) results in better enzymes immobilized and more reproducible output currents	• Mediator-free bilirubin oxidase (BOD) modified CNT-carbon electrodes, the glucose-air battery showed the open-circuit potential of ca.0. 5 V, and a peak power of 0.075 to 1 mW·cm−2 between 0.25–0.3 V.

Carbon black is the most used support materials for electrocatalysts, however other carbonaceous materials such as graphite nanofibers (GNFs) and carbon nanotubes (CNTs) have been investigated as catalyst supports.

2.4.1 Carbon Nanotubes

Oxidized carbon nanotubes embedded on electrospun nanofibers were used in bioelectrodes to: improve the electrical conductivity of the fibers and act as bridges for the electron transfer between enzymes and electrodes [12]. CNTs have specific chemical groups that allow the immobilization of the enzyme above electrospun membrane. Also their intrinsic electrical conductivity allows obtaining thinner fibers that subsequently increases the surface area and porosity, which favors the enzyme immobilization.

For the development of electrodes for biofuel cells and for biosensors, Ju et al. [22] evaluated the electrochemical properties of electrospun PAN / MWCNT carbon nanofibers electrodes coated with the conductive polymer polypyrrole to help in the immobilization of the enzymes on the surface of electrodes [64, 65]. They found that the use of CNT helps to decrease the diameter of the fibers of 220 nm to 230 nm, also the polypirrol coating create a good charge-transfer complex with the aligned nano-sized Activated Carbon Nanofiber/CNT achieving improvement in the capacitance of the electrode.

For the immobilization of catalase, electrodes based on electrospun PAN and poly(acrilonytrile-co-N-vinyl-2-pyrrolidone) (PANCNVP) nanofibers and MWCNT were prepared by L. S. Wang et al [31, 32]. The results of this research indicate that the electrospun membranes specially those filled with MWCNT, are suitable matrices for catalase immobilization because the increase in the quantity of immobilized enzyme of 24.45 mg/g to 29.81 mg/g (mg of enzyme per g of fiber), and also in the activity retention of the electrode of

32.4% to 42.5% which is evidence of a good behavior in the decomposition of H_2O_2 (catalase decompose the H_2O_2 in H_2O and O_2). The improvements in enzyme retention of activity are attributed by the authors to several causes, such as morphological features induced in the fibers by the CNTs, which can enhance the access to/from the enzyme active centers. Also the authors attributed the improvements in the catalytic activity to the facilitation of electron transfer process due to the conductivity of the CNTs.

Wan et al [12] and H. Shou et al [35], report improvements in the electrochemical behavior of electrodes containing carbon nanotubes developed using catalase immobilized onto their surfaces. Wan and co-workers covalently immobilized CAT onto electrospun nanofibers (diameter = 180 nm) of acrylonitrile-based copolymers containing porphyrin pendants, either in the presence or absence of MWCNTs, founding that the specific activity and the retention activity have an increasing in the presence of MWCNTs [34].

Yu Xin and collaborators also studied the use of CNT in the construction of bioelectrodes, successfully immobilizing the enzyme horseradish peroxidase at the ends of single-wall carbon nanotube forest electrodes. The lifetime for the electrodes of several weeks, stored in buffer at 4˚C with only 20% loss of peak current [69].

Vaze et al, use CNTs in the construction of a biocatalytic anode for glucose oxidation [70], the obtained electrode has essential attributes of an anode in a mediator less biocatalytic fuel cell and showed electrochemical activity for 6 days. The enzyme appear to have direct electron transfer assisted by CNT.

In the same way, SWCNT were used to modify the surface of gold electrodes [73] by Gooding et al. They attached well aligned oxidized SWNCT in a normal orientation respect to the surface of the electrode and the enzyme microperoxidase11 was covalently immobilized at

the edges of the CNTs. The authors reported direct electrons transfer between enzyme and electrode, mainly at the ends of the CNTs.

The use of carbon nanotubes dispersed in polymer nanofibers for this particular application addressed other advantages related to the possibility to modify the chemical properties of the surface of carbon nanotubes, which allows adhering of several types of materials, enzymes and microbes to improve the catalytic properties of this materials supports.

In general exist two ways to functionalize CNT, covalent and non- covalent modification. Covalent functionalization implies a permanent modification of CNT structure generating reactive groups in its surface, which can react later to form covalent bonds with others molecules. Most protocols for covalent functionalization are based in the oxidation of CNTs under different conditions. In all cases the ends and side-walls become rich in oxygenated functions, mainly carboxylic groups.

Depending on how drastic is the treatment; it is possible not only to increase the density of oxygenated functional groups, also to break the tubes or even to shorten them, which can be cause detriment of their properties. Common methods for CNT oxidation involve treatment in acidic solutions of sulfuric, nitric and hydrochloric acids, either concentrated or diluted, and have been used at room temperature or under refluxing, with or without sonication for different times [18, 29, 36] also other oxidizing agents such as $KMNO_4$, H_2O_2, K_2CrO_7 are used for this purpose [44].

The amount and type of oxygen-containing functional groups created in the CNT depends on the treatment methods. In the case of refluxes using concentrated nitric acid, formation of acid groups likes carboxyl; phenol and lactol are reported [47].

Refluxes using a mixture of nitric and sulfuric acid creates in the CNT surface carboxylic, carbonyl and phenolic groups [44, 46].

Davis [12] provide the first example of direct electron transfer to proteins using CNT modified electrodes, in this research the electrodes were made using MWCNTs, which were functionalized using nitric acid, and then mixed with nujol, bromoform, mineral oil or water. Cytochrome –c and azurin were adsorbed onto and/or within the tubes, the adsorbed enzyme retained its catalytic activity.

Also was found that the proteins adsorption occurs mainly at the walls and the ends of tubes, while that electron transfer is achieved predominantly at the ends of CNT.

Previous investigations has been developed employing randomly entangled nanotubes, which give a poorly defined electrode surface and a poorly defined protein immobilization. Aligned nanotubes electrodes will provide a more controlled surface on which to immobilize and communicate with redox proteins, and electrospinning is a suitable technique to reach the alignment of CNT inside polymeric fibers [18], which also have high porosity and high active surface area.

Improves in the enzyme activity was observed in enzyme-polymer-single wall carbon nanotube (SWNT) composites [57], in comparison to similar enzyme-containing composites without using SWNTs. This can be attributed to the SWNTs, possesses a high specific surface area which allows adsorbing enzyme molecules and retaining the enzyme within the polymer matrix.

2.4.2 Nanofibers

Electrospinning is a technique that utilizes an external electrostatic field to generate fibers of several materials with micrometric and sub-micrometric diameters (nanofibers). The fibers obtained by this method exhibit a large surface area to volume ratio, good mechanical strength, high flexibility and high porosity [13 - 20]. One important advantage of this technique is the possibility to generate fibers composites, mostly based on polymer precursors. One of the most used polymers for the preparation of polymer composites is Poliacrylonitrile (PAN), it is soluble in dimethilformamide (DMF), a polar solvent that is also a good media for dispersion and suspension of Carbon nanotubes (CNT). Fibers based in PAN are also a good precursor to obtaining carbon nanofibers [14, 17, 21, 24].

Electrospun nanofibers provide a large surface area for the attachment or entrapment of enzymes. In the case of porous nanofibers, they can reduce the diffusional path of the substrate from the reaction medium to the enzyme active sites because of the reduced dimension in size, leading to better enzyme activity.

Electrospinning can generate non-woven mats or well aligned arrays of nanofibers with controllable compositions and sizes in a matter of minutes [54 - 56]. Electrospun nanofiber mats are durable and easily separable and can also be processed in a highly porous form to relieve the mass-transfer limitation of the substrate through the mats.

2.5 PREPARATION OF NANOFIBER-CNT COMPOSITE MEMBRANES

Properties of polymer-CNT composites are strongly influenced by factors, such as polymer-CNT interactions; dispersion and orientation of CNT in the polymeric matrix [77, 78], loadings of CNT and also the processing method of the composite [78]. In general, uniform

dispersion of nanotubes within the polymer matrix is required for improved electrical and thermal properties at a lower CNT content and it also contribute to improve mechanical properties [79].

Polyacrylonitrile (PAN) is an attractive polymer thanks to its ability to form long conjugated imine chains by polymerization of its pending nitrile groups during the stabilization stage that take place at 180℃ in the carbonization process to create carbon fibers. The ability to form a conjugated system also makes it an excellent candidate for various applications that rely on the nature of the conjugated chain, such as formation of complexes and electric conductivity. Also the nitrile groups can be easily converted to carboxyl groups in presence of NaOH, which allows the immobilization of proteins in the surface of this polymer.

The introduction of CNT into PAN results in a composite material with unique properties, thanks to a feasible p electron interaction of the graphitic structure of CNT with the nitrile groups.

The interaction between the CNT and PAN was modeled as charge-transfer mechanism of CNT π-electrons from the highest occupied molecular orbital (HOMO) to the empty nitrile π^* orbital of PAN [48, 49].

Probably several different intermolecular forces contribute to the mechanism of CNT π-electron bonding with polyacrylonitrile through its –C≡N groups, such as dispersion and dipole-induced-dipole forces (particularly at long range).

The charge-transfer complex formed from the combination of the highest level of polyacrylonitrile molecule (π_zCN) and π-electrons of CNT will vary in accordance with the PAN unit count, its configuration, and the electronic structure of the tubes. The latter depends highly on tube chirality, length, diameter, structural distortion etc.

2.5.1. Solvent Casting

Solvent casting is a common and easy fabrication method for CNT- polymer nanocomposites. The processing by solvent casting involves the preparation of a suspension of nanotubes in a polymer solution, followed by evaporation of the solvent to produce a composite nanotube-polymer film. The films prepared by solvent casting commonly contain nanotube agglomerates generated by the aggregation of CNT during solvent evaporation.

Drop casting is a variation of the solvent casting method, which involves dripping a polymer-CNT suspension above a hot surface [79], to increase the rate of the solvent evaporation, then minimizing the agglomeration of CNT and bundle presence in the composite film.

Some studies on PAN/ multiwall carbon nanotube (MWNT) films shows that uniform dispersion of MWNT can be achieved at CNT loadings ranging from 1 to 25 wt %. Huina and collaborators prepared PAN –CNT composite films of about 25 μm thickness by mean of casting process, and compare the effect of eight different types of carbon nanotubes at nanotube loadings of 5, 10, and 20 wt %. This research reveals that the CNT structure and morphology (metallic impurity, surface area, and diameter) can affect the overall properties of polymer-CNT composites. In general DWCNT and MWCNT, exhibit relatively poor interaction with PAN compared to SWCNT analyzed in this research.

Another method for the preparation of composites polymer - CNT membranes is electrospinning.

2.5.2 Electrospinning

Electrospinning is an electrostatic technique for the preparation of fibers at nano and micro scale and also of mats and membranes constituted by fibers. This technique allows the preparation of fibers with diameters ranging from several micrometers to ten of nanometers using for this a wide variety of materials that include polymer, ceramics and composites [8].

A large surface area to volume ratio, good mechanical strength, high flexibility, high porosity, are some of the main characteristics shows by the fibers obtained via electrospinning.

Electrospinning has been also recognized as an efficient technique for the fabrication of polymer/ CNT composite materials [81]. Good dispersion of nanotubes in the spinning solution is required to achieve a good degree of axial orientation of individual CNTs in the fibers, which allows to obtain fibers of uniform diameter and also to improve its mechanical properties.

Electrospun nanofibers with diameter ranging from 100 nm to 200nm were prepared using PAN and oxidized MWCNTs as precursors; the fibers were collected using a rotating drum. Concentrations of 10 % wt and 20 %wt of MWNTs with respect to the PAN polymer were used. Transmission Electron Microscopy (TEM) and 2D WAXD studies reveal that MWCNT are aligned along the fiber axis of the nanofibers, moreover the degree of alignment for the oxidized MWNTs in the nanofibers results to be significantly greater than the PAN matrix crystals. TEM images also reveal the formation of a net of MWCNT connected inside the fibers, which can be confirmed by the presence of electrical conductivity, because the electrical conductivity requires the formation of a percolating network of MWNTs [81]. The measured electrical conductivity values were up to 0.1 and 0.5-1.0 S/cm for PAN/MWNT (90/10) and (80/20) respectively [77].

The surfaces of the fibers exhibited roughness because some nanotubes were located across the transverse direction of the nanofibers and break its surface, increasing the diameter of the fibers in these points. These defects could be associated with the concentration of the MWNTs, the DMF evaporation rate, and the resulting packing of the MWNTs in the PAN matrix during electrospinning [77].

2.5.2.1 Electrospinning Parameters

During the electrospinning process an electric field is applied to a polymer solution loaded in a syringe fitted with a needle; the needle usually act as one of the electrodes which is connected the high voltage power source. The other electrode, in which the fibers are collected, can be a simple metal plate or a more elaborated device. The electric field is applied between the electrodes and when the applied electrical field overcomes the surface tension of the polymer droplet in the tip of the needle, a charged jet of polymer is ejected, which is collected in the target like fibers mesh; most of the solvent is evaporated in high electrical field during electrospinning [15, 16, 18]. The Figure 6, Shown the basic scheme of electrospinning setup.

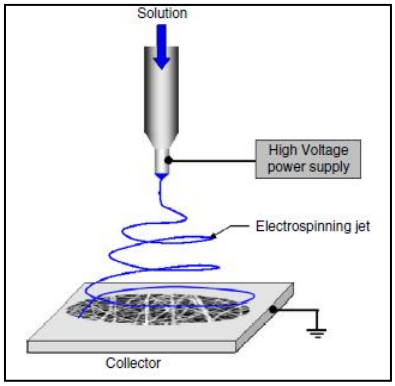


Figure 6. Basic electrospinning setup (extracted from reference [19])

Usually a direct current power supply is used in electrospinning and the negative electrode is connected to the needle tip, however it has been found that the polarity has not influence in the quality of the generated fibers [16]. The electrostatic charges that remain in the collected fibers are responsible of the repulsion between them, which hinder their alignment.

The morphology (diameter, homogeneity, beads presence) are influenced by process parameters such as polymer solution rate (low solution rate such as 1 μL/h is required to reach nanometric fibers diameter), voltage, distance of needle to target (collector), solution properties (viscosity, solvent concentration, surface tension, polymer molecular weight, conductivity) and environmental conditions (temperature, humidity and air flow) [13, 14, 86]. Table 2 summarizes the effect of electrospinning parameters in the characteristic of the fibers and their effect over other variables in the process.

The fibers obtained by the electrospinning process are normally randomly oriented in the form of non-woven mats. This randomly alignment has useful applications in filtration, tissue scaffolds, wound dressing, etc. However, for many other applications (nanoelectronic devices and sensors), a continuous single nanofiber or aligned fiber bundles are preferred.

Several techniques have been developed to align electrospun nanofibers and some breakthroughs have been achieved [9 - 12]. One of most used devices to collect aligned electrospun fibers is the drum collector; in this device the fibers are collected in a mandrel that is rotated at high velocity. Some researchers [18] have reported that higher molecular orientation in the polymer is achieved when the electrospun fibers are collected in a high speed rotation drum. A variation of this technique is the rotating mandrel with sharp pin inside and wire drum rotating [9, 10]. Also is common the use of auxiliary electrodes and parallel conducting collectors for improve the alignment of the fibers [8, 10].

Table 2. Parameters of electrospinning process (extracted from reference [86]).

Parameter	Effect
Needle to Collector Distance	• Exponentially inverse to the volume charge density • Inversely proportional to bead formation density • Inverse to the electric field strength • Inversely proportional to fiber diameter
Flow Rate	• Directly proportional to the electric current • Directly proportional to the fiber diameter • Inversely related to surface charge density • Inversely related to volume charge density
Voltage	• Inversely proportional to surface charge density • Direct effect on bead formation • AC potential improved fiber uniformity • Inversely related to fiber diameter
Concentration of Polymer	• Directly proportional to the fiber diameter • Power law relation to the fiber diameter • Cube of polymer concentration proportional to diameter • Parabolic— upper and lower limit relation to diameter
Ionic Strength	• Directly proportional to charge density • Inversely proportional to bead density
Solvent	• Effects volume charge density • Directly related to the evaporation and solidification rate
Temperature	• Inversely proportional to viscosity • Uniform fibers with less beading
Viscosity	• Parabolic relation to diameter, and spinning ability

A central collector is a variation of parallel conducting collectors, in this device conducting electrodes are imbedded in a non-conducting media, such as silicon, the electrodes are connected to the power source in an alternating way, achieving fibers with complex forms such as mesh and with good alignment [13, 14, 16]. Li et al, using a central collector obtained PVP electrospun nanofibers in the form of mesh and several layers of fibers rotated at 60° relative to each other.

A new method for minimizing the repulsion between charged electrospun fibers obtained is “biased AC electrospinning” reported by Sarkar [19, 20], this researcher employ a

combination of AC and DC potentials to obtain a highly aligned fibers mats of several polymers. This work suggest that the use of the magnitude of the DC bias must be less than half the total amplitude of the AC potential and that the frequency should be between 500 and 1000 Hz during the process.

The Figure 7a, corresponds to a scheme of the setup used by Sarkar [19, 20]. Figure b, and figure 7c, shows the effect of electrostatic charges in the fibers, repulsion in the first case and attraction in the other.

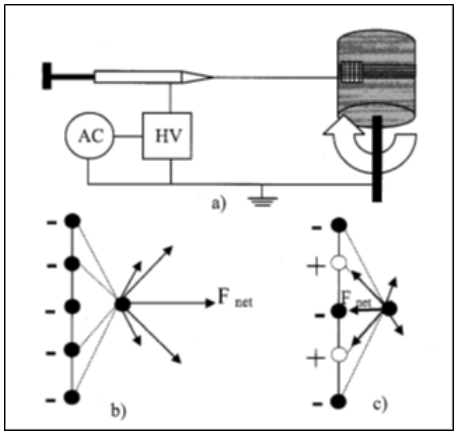


Figure 7. Scheme setup Sarkar's experiment. (Taken from reference [19]

Voltage and distance to needle to target are another parameters that affect the structure of the electrospun nanofibers, higher voltage and longer distances has shown to be key points to obtain more ordered polymer chain structures.

2.6 CHARACTERIZATION OF MEMBRANES AND MATERIALS

The main membrane properties required for any practical application are morphology, molecular structure and mechanical properties. These properties can be measured trough several techniques, some of them are discussed next.

2.6.1 Ultraviolet- Visible Espectroscopy (UV - VIS)

The absorption of an incident beam of ultra violet (UV: 190-380 nm) or visible light (VIS: 380-750 nm) for a sample, give place to a molecular excitation of short lifetime. When the excited species return to their initial state, the absorbed energy can be transformed into heat or light. If the absorbed energy is high enough new substances could be formed in this process.

UV-Vis spectrum is the representation of the variation of the absorbance (A) with frequency of the incident light. If the initial power (P_0) is known (before passing to the sample), and the final power (P), after passing through the sample, is possible to calculate the transmittance (T), by mean of:

$$T = \frac{P}{P_0} \qquad \text{Equation 4}$$

The absorbance can be calculated as the negative logarithm of transmittance.

$$A = -\log(T) = -log\left(\frac{P}{P_0}\right) \qquad \text{Equation 5}$$

Figure 8. Beckman Coulter DU 800 UV/Visible Spectrophotometer

Depend on the nature of the sample; several measurement techniques of UV-spectroscopy can be used. For clear solids or clear liquids, the technique to use is transmittance; for translucent or opaque solids, diffuse reflectance must be used; for turbid samples diffuse

transmittance is used and for reflecting optical surfaces is recommended the use of specular reflectance.

2.6.1.1 Absorptions of UV Chromophores

The group of atoms producing a characteristic absorption is called a chromophore. A specific grouping of atoms produces a characteristic absorption band at specific wave lengths. However the intensity and location of these absorption bands will change with structural changes in the group of atoms and with solvent changes.

Table 3. Characteristic absorption bands for some chemical functional groups

Chromophore	Absorption band (nm)
Nitriles (R-C = N)	160 [a]
Alcohols (R-OH)	180 (175-200)"
Ketones (R-C--O-R')	180, 280 [a]
Amines, primary (R-NH2)	190" (200-220)
Aldehydes (R-C----O-H)	190, 290 [a]
Carboxylic acids (R-C--O-OH)	205
Esters (R-C--O-OR')	205
Amides, primary (R-C--O-NH z)	210

a. Absorptions below the cutoff for common solvents would not be observed in solvent solution measurements.

2.6.1.2. APPLICATION OF UV-VIS SPECTROSCOPY FOR ENZYMES CHARACTERIZATION

Presence of enzymes can be detected using Uv vis spectroscopy, each type of enzyme have associated a characteristic absorbance peak. Table 4, gives a summary of characteristic wavelengths for the analysis of enzymes using UV- vis spectroscopy.

Table 4. Characteristic wavelengths of some enzymes (extracted of reference [84])

Enzyme name	Reaction type and assay wavelength(nm)
Lactate dehydrogenase	Direct absorbance at 350
Alcohol dehydrogenase	Increased abs. NADH at 340
D-Amino acid oxidase	Decreased abs. NADH at 340
L-Amino acid oxidase	Change in abs. at 436
CataIase	Liberation of H20 2 abs. at 240
Glucose oxidase	Color reaction at 436
Glucose phosphate isomerase	Increased abs. NADPH at 340
Lipase	Increased abs. at 300
Peroxidase	Decrease in abs. NADH at 340

2.6.1.3 USES OF UV-VIS SPECTROSCOPY FOR THE CHARACTERIZATION OF CNT AND PAN-CNT COMPOSITES

Chemical changes in compounds can be detected using UV- vis spectroscopy. For oxidized MWNTs dispersed in DMF, the larger absorption bands are expected at 283nm and 1020 nm range, Furthermore, the absorption band observed in the UV-vis absorption spectra at 1020 nm has been reported as evidence of the presence of bundles of MWNTs in the samples [77].

UV vis spectroscopy has been also used to detect the formation of complexes in composites materials, such is the case of electrospun fibers of PAN- MWCNT, where the presence of extensive fine absorption bands in UV/vis spectra suggest the formation of charge-transfer complexes between the surface-oxidized MWNTs and the PAN molecules, the absorption structure detected via UV/vis spectroscopy indicated that charge transfer complexes formed between the surface-oxidized nanotubes and negatively charged functional groups in

PAN during electrospinning, leading to a strong interfacial bonding between nitrile groups the nanotubes and surrounding polymer chains.

As a result of the highly anisotropic orientation and the formation of complexes, the composite nanofiber sheets possessed enhanced electrical conductivity, mechanical properties, thermal deformation temperature, thermal stability, and dimensional stability. The electrical conductivity of the PAN/MWNT composite nanofibers containing 20 wt % nanotubes was enhanced to 1S/cm.

2.6.2 Electron Scanning Microscopy (SEM)

An incident electron beam that focused in a sample produce a variety of signal after it collides with the surface. The response of the surface include secondary electrons, back scattered electrons, characteristic x-rays, light, transmitted electrons and specimen current. The secondary electron signal can be used to produce an image of the surface.

Contrast within the image is due to the spatial variations in intensity of the transmitted electron beam through the specimen, as the beam is raster scanned over the specimen. The image may be produced in a number of ways – from variations in the intensity of secondary electrons back-scattered from the specimen through to X-ray emission produced by inelastic collisions of the primary beam with bound electrons in the specimen [85].

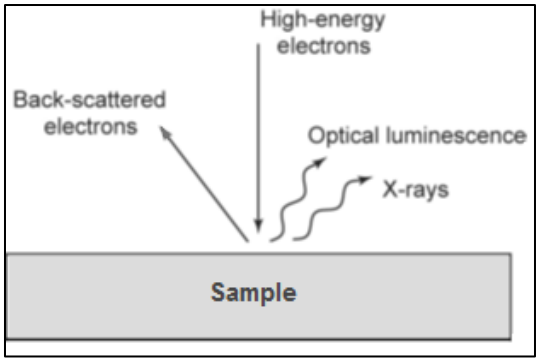


Figure 9. Effects of the incidence of electron beam in a sample.

Electrospun fibers require a special preparation before to conduct SEM characterization. First the nanofibers sample must be dried to eliminate the excess of solvent; then the sample must be cut and attached to a conducting stub by mean of a carbon tape.

Polymer nonconductive fibers require of a conductive coating usually of gold of about 25 nm, which is made using techniques such as sputtering. The diameter determination is conducted using an estimative of the average diameter of the fibers over a determinate region of the sample, high magnifications, near to 15.000X are recommended to improve the accurate of the measurement [13].

2.6.3 Cyclic Voltammetry (CV)

Voltammetry is an amperometric technique, in which a scanning of potential is applied to a specimen to obtain current as response. This current is measured and plotted against the applied voltage. These plots are kneed as voltammograms (see Figure 10).

There are many variations of voltammetry exists, such as: polarography (DC Voltage) linear sweep, differential staircase, normal pulse, reverse pulse, differential pulse and more.

Cyclic voltammetry is one of the most widely used forms and it is particularly useful to obtain information about the redox potential and electrochemical reaction rates of analyte solutions.

During the test, the applied voltage is swept between two values (V1 and V2) at a fixed rate, however, when the voltage reaches V2 the scan is reversed and the voltage is swept back to the voltage V1. The voltage is measured between the reference electrode and the working electrode, while the current is measured between the working electrode and the counter electrode.

The scan rate is a critical factor, since the duration of a scan must provide sufficient time to allow for a meaningful chemical reaction to occur.

As the voltage is increased toward the electrochemical reduction potential of the analyte, the current will also increase. With increasing voltage past the reduction potential, the current decreases, having formed a peak as the analyte concentration near the electrode surface diminishes, since the oxidation potential has been surpassed. As the voltage is reversed to complete the scan toward V1, the reaction will begin to reoxidize the product from the initial reaction. This produces an increase in current of opposite polarity as compared to the forward scan, but again decreases, having formed a second peak as the voltage scan continue toward V1. The reverse scan also provides information about the reversibility of a reaction at a given scan rate.

The shape of the voltammogram for a given compound depends not only on the scan rate and the electrode surface, which is different after each adsorption step, but can also depend on the catalyst concentration. For example, increasing the concentration of reaction specific

enzymes at a given scan rate will result in a higher current compared to the non-catalyzed reaction.

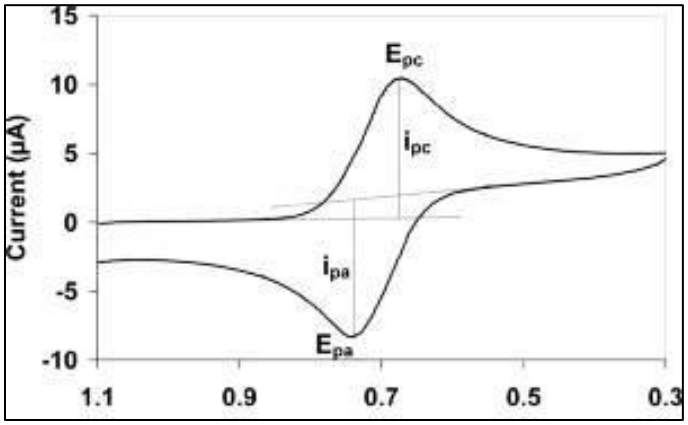


Figure 10. Anodic and cathodic peaks in a Voltammogram

The important parameters in a cyclic voltammogram are the peak potentials (Epc , Epa) and peak currents (ipc , ipa) of the cathodic and anodic peaks, respectively see Figure 10. If the electron transfer process is fast compared with other processes (such as diffusion), the reaction is said to be electrochemically reversible, and the peak separation is:

$$\Delta E\text{p} = E\text{pa} - E\text{pc} = 2.303\, RT/nF \qquad \text{Equation 5}$$

Thus, for a reversible redox reaction at 25 °C with n electrons ΔEp should be 0.0592/n V or about 60 mV for one electron.

The formal reduction potential (Eo) for a reversible couple is given by

$$E_0 = \frac{E_{pc} - E_{pa}}{2} \qquad \text{Equation 6}$$

For a reversible reaction, the concentration is related to peak current by the Randles–Sevcik expression (at 25 °C):

$$I_p = 2.686 * 10^5 n^{\frac{3}{2}} A_c{}^0 D^{\frac{1}{2}} V^{\frac{1}{2}} \qquad \text{Equation 7}$$

Where Ip is the peak current in amps, A is the electrode area (cm^2), D is the diffusion coefficient ($cm^2\ s^{-1}$), Co is the concentration in mol cm^{-3}, and n is the scan rate in $V\ S^{-1}$.

2.6.4 Determination of Enzymatic Loading (Bradford Method)

Bradford it is a method for protein determination which allows the detection of quantities in the scale of microgram of protein also is accurate, rapid and very reproducible.

The Bradford method consists in the measurements of shifts in the absorbance spectra of the dye coumassie blue caused by their binding to the protein under study [82].

The free Coumassie blue exist in four different ionic forms, but the form which binds to the protein usually has an absorbance maximum at 590 nm. Thus, the quantity of protein can be estimated by determining the amount of dye measuring the absorbance of the solution at 595 nm [83].

Bradford method consider two types of assay, the standard assay, which is suitable for measuring between 10 and 100 μg of protein, and the microassay, which is useful to detects between 1 and 10 μg of protein. The latter, is also more susceptible to interference from other compounds.

New approaches have been used in the development and improvement of biofuel cells, these included Membrane-less biofuel cells [61], where the enzymes are used in both anode and cathode electrodes.

Another approach is the genetic modification of proteins for electrochemical applications that consist in to improve characteristics of the enzymes such as the electron transfer capacity through the exposition of the active site closer the surface. Also modify some groups of proteins

in order to improve characteristics such as thermal resistance, has been reached by genetic modification.

The most recent approach is the use enzymes cascades or co-immobilization of several enzymes, to increase the degree of oxidation and also the efficiency of fuels [52, 61] in biofuel cells.

3. EXPERIMENTAL METHODS

Composite cast PAN-MWCNT membranes and composite electrospun PAN-MWCNT membranes were prepared following the procedure depicted in Figure 11.

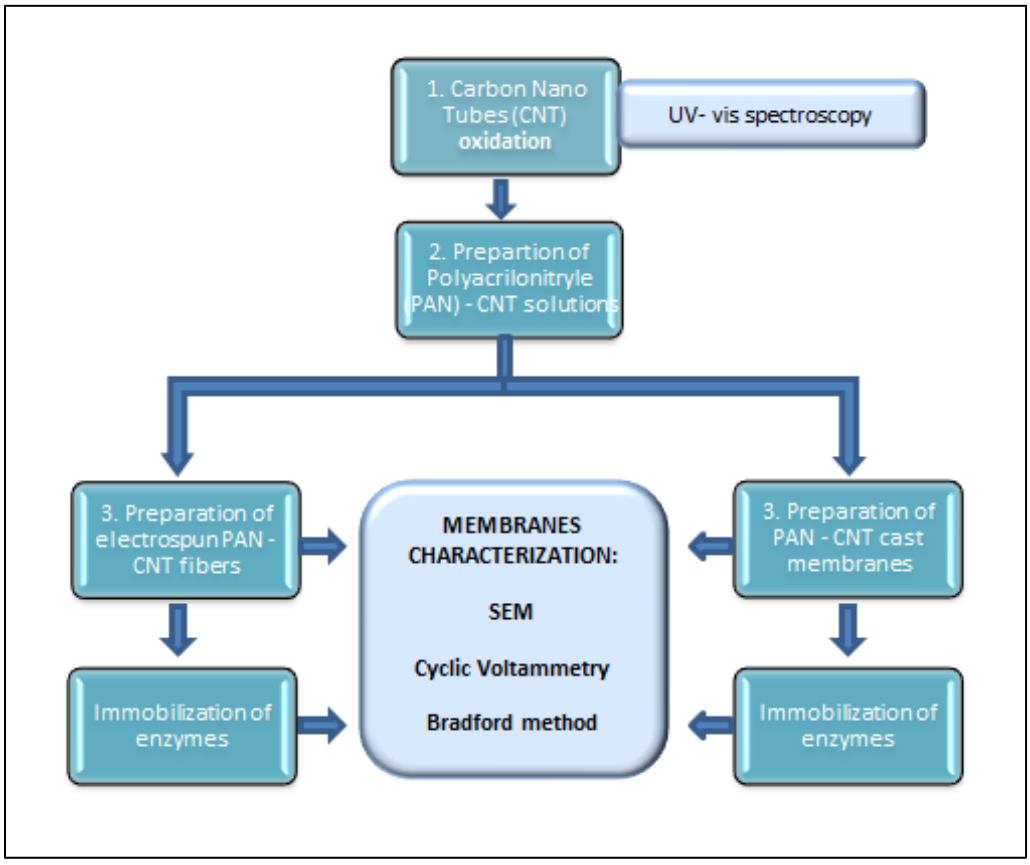


Figure 11. Experimental procedure for the preparation and characterization of membranes

Polyacrylonitrile (PAN) and N,N-dimethylformamide (DMF) and MWCNT (diameters of 110–170 nm and lengths between 5 - 9 microns) were purchased from Sigma-Aldrich Chemical Co.

3.1 OXIDATION OF CNT

During the first step, MWCNTs were added to a concentrated mixture 3:1 v/v ratio of sulfuric and nitric acid, respectively. One gram of MWCNT was mix with the 3:1 v/v of sulfuric and nitric acid. The solution was sonicated for 4.5 hours using an ultrasonic cleanser (Fisher scientific FS20H, USA) of 60W. The oxidized MWCNTs were rinsed with distilled water to reach a PH of 7, followed by filtration using a vacuum pump to remove the remaining acid.

The MWCNTs were dried at 60 °C during 24 hours using a vacuum oven. UV-Vs spectroscopy was employed to characterize the functional groups generated in MWCNT during the oxidation process. The process of oxidation of MWCNT is described in Figure 12. The final product of the process is shown in Figure 13.

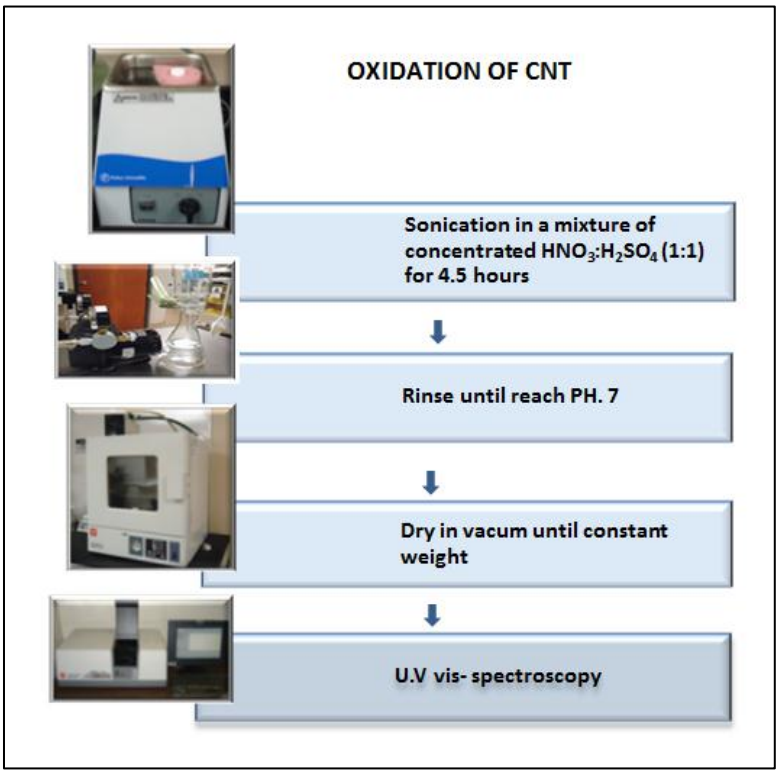


Figure 12. Process for oxidation of CNT

Figure 13. Oxidized MWCNT

3.2 PREPARATION OF PAN/MWCNT SOLUTIONS

Three concentrations of MWCNT (0.5, 1, 2 % and 5% wt) were used in the preparation of the solutions. The initial step in the preparation of the solutions is the dispersion of the MWCNT in the DMF solvent. For this the oxidized MWCNT were sonicated in DMF for 60 minutes using a ultrasonic cleaner (Fisher scientific FS20H, USA), and then homogenized using a homogenizer (VWR VDI 12, USA) for 3 minutes and finally sonicated again for 60 minutes. Three concentrations of the PAN solutions (5%, 7% and 10%) were prepared by simple dissolution of the polymer in DMF at 60 ˚C under constant agitation.

The dispersed MWCNT were placed in a heater plated with agitation and heated to 60 ˚C. PAN was slowly added to the MWCNT- DMF dispersion and heated under constant agitation until the excess of solvent was eliminated by evaporation and the solution reached the expected concentration. In all cases 5% by weight of PAN was used. An excess of solvent was required in the preparation of the solutions in order to obtain a good dispersion of MWCNT. The process for preparation of the PAN - MWCNT solutions is depicted in Figure 14.

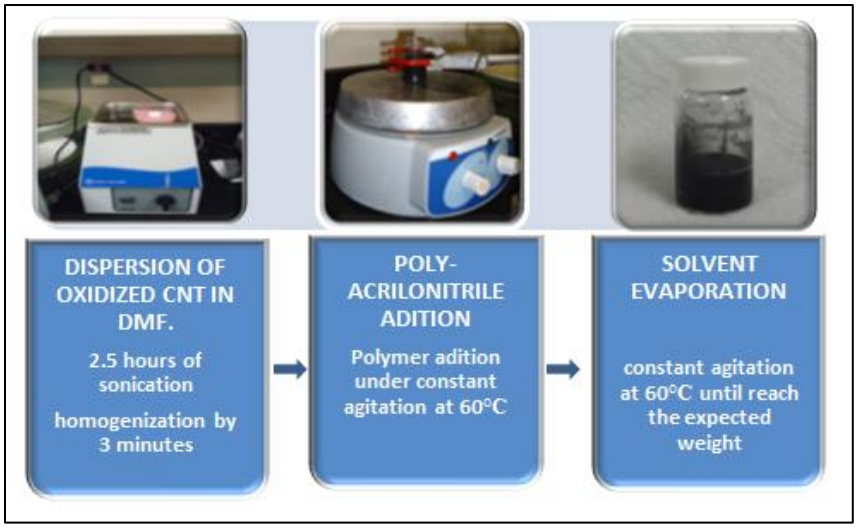


Figure 14. Preparation of PAN-CNT solutions

3.3 PREPARATION OF ELECTROSPUN FIBERS

Fibers of PAN and fibers of the PAN-MWCNT composite were prepared using a variable high voltage power supply (Matsuada, Japan). The solutions were dispensed using a BD 3 ml syringe and a blunt tip needle of gauge 23 . The anode of the high voltage power supply was clamped to a syringe needle tip, and the cathode was connected to a central collector. The equipment is presented in Figure 15. The electrospun fibers were collected over a carbon foils bonded to the aluminum collector (see Figure 16). The applied voltage was 10 kV, the distance between the needle and collector was 20 cm. Several flow rates were used to evaluate their effect in the properties of the fibers. The Table 5 summarizes the flow rates employed in the preparation of the fibers.

Table 5. Flow rates used in the preparation of electrospun fibers

Type of solution	**Flow rate (µL/h).**		
PAN	10		
PAN-MWCNT (1 - 2 % MWCNT)	50	70	100
PAN-MWCNT (5% MWCNT)	100	200	300

a.  b. 

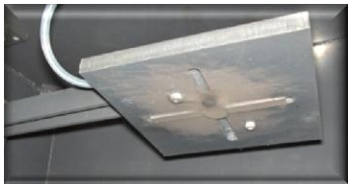
c.  d. 

Figure 15. Electrospinning setup: a. electrospinning chamber, b. power generator, c. central collector, d. pump.

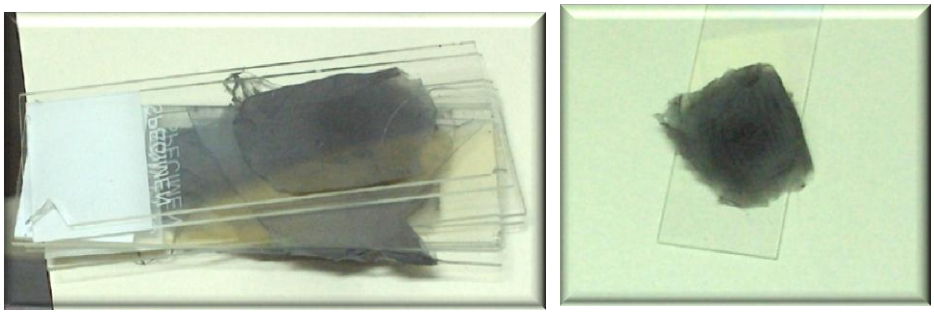


Figure 16. Details of collected fibers.

3.4 PREPARATION OF CAST MEMBRANES

Solutions of 2% and 5% MWCNT were used for the preparation of cast membranes. All the membranes were prepared using 1 mL of solution, which were placed in a polymeric die of 2cm of diameter (see Figure 17 and Figure 18). The membranes were dried during 6 hours in a vacuum oven and finally removed from the die.

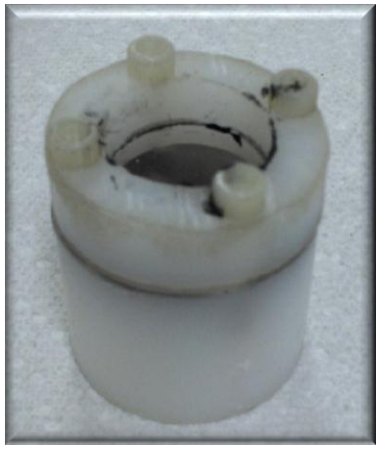


Figure 17. Dye for cast membranes preparation

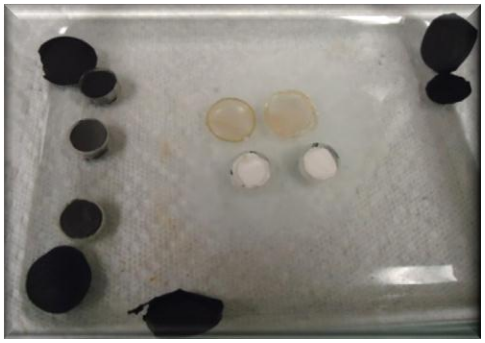


Figure 18. PAN and PAN-MWCNT cast membranes

3.5 ACTIVATION OF FIBERS AND MEMBRANES

The electrospun fibers and membranes were cuts into samples of approximately 1 cm^2 and then immersed in a solution of NaOH 1.0 M at 40 ^oC for 10 minutes. The purpose of the immersion is to create carboxyl groups of the nytrile groups present in the polymeric matrix of the PAN fibers. After the activation process, the fibers were washed with deionized water. The carboxyl groups of the activated fibers were regenerated using a solution of HCl 2.0 M. Finally, the fibers were washed with deionized water and then rinsed with phosphate buffer solution (PBS 50 mM) to eliminate the acid residues.

3.6 PREPARATION OF CATALASE-PAN-MWCNT COMPOSITE ELECTRODE

Catalase, EDC and NHS were purchased from Sigma-Aldrich Chemical Co. The PAN coated with catalase (referred as catalase-PAN-MWCNT) was prepared by covalent linking, as summarized in literature [42]. The PAN/MWCNT were activated by immersion in a EDC:NHS solution (1:1 molar ratio) and stirred for two hours at room temperature. Later, the samples were submerged in a catalase solution (0.1 mg/ml) for 60 min and washed 20 times with PBS 50 mM solution.

3.7 SEM

The images concerning to fibers morphology were recorded in a JEOL - JSM-5410 LV Scanning Electron Microscopy (Figure 19).

Figure 19. JEOL - JSM-5410 LV Scanning Electron Microscope, University of Puerto Rico, Mayaguez Campus.

The fibers for SEM characterization were collected in carbon tape fixed on aluminum stubs to avoid deformation of the fibers as they were collected. The non-conductive PAN fibers were coated with a layer of gold for the SEM characterization. However, the conductive PAN-MWCNT did not required gold coating.

3.8 CYCLIC VOLTAMMETRY

Cyclic voltammetry was performed using a three electrode setup. The electrolyte was a solution of PBS 0.2 M. Successive additions of H_2O_2 0.01M were made to determine the catalytic properties of the immobilized catalase. The potential scanning was conducted between -0.2 V - 1.0 V, at a scan rate of 2 mV/s.

All the fibers used for electrochemical characterization were collected for 60 minutes. The electrodes and separator were soaked in deionized-distilled water (17.7 Mohm) and then with PBS prior to cell assembly. All electrochemical measurements were run at room temperature. A calomel saturated electrode was used as reference electrode and a rod of carbon glassy was used as counter electrode.

The samples were placed in a hermetic holder to maintain constant the exposed area of the sample under evaluation. Details of the setup used for cyclic voltammetry are shown in Figure 20.

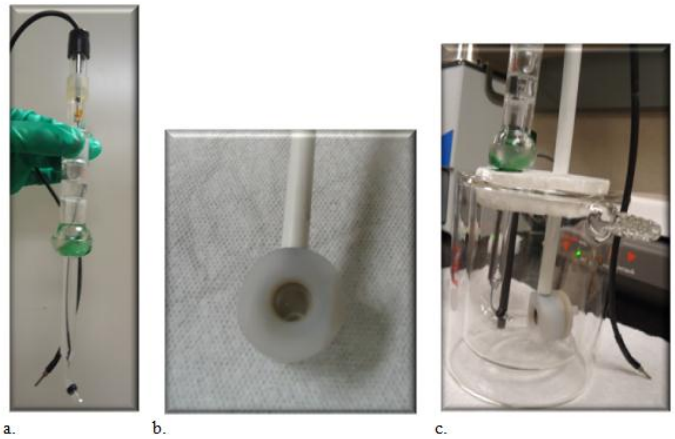


Figure 20. Details of the components of voltammetry cell. a. Calomel saturated electrode, b. holder for working electrode, c. complete assembly of the cell.

4. EXPERIMENTAL RESULTS

4.1 PAN NANOFIBERS

4.1.1 Electrospinning flow rate and PAN-DMF load effect

Flow rates of 5µL/h, 10µL/h and 15 µL/h of a PAN-DMF solution with concentrations of PAN of 5% wt., 7% wt, and 10% wt. were used to synthesis PAN nanofibers. The best result was obtained with the 10% wt. Figure 21, shows the effect of flow rate in the PAN nanofibers at 5% wt. The nanofibers obtained at 5µL/h (see Figure 21) are free of beads and have a smaller diameter compare to those obtained at 15 µL/h (Figure 21a) and 10µL/h (Figure 21b).

Using the software IMAGE J, the average diameter of the 5µL/h fibers was estimated to be 625 nm and 1µm for those obtained at 15µL/h. The diameter is a measured of the average 50 fibers diameters at random points for each image.

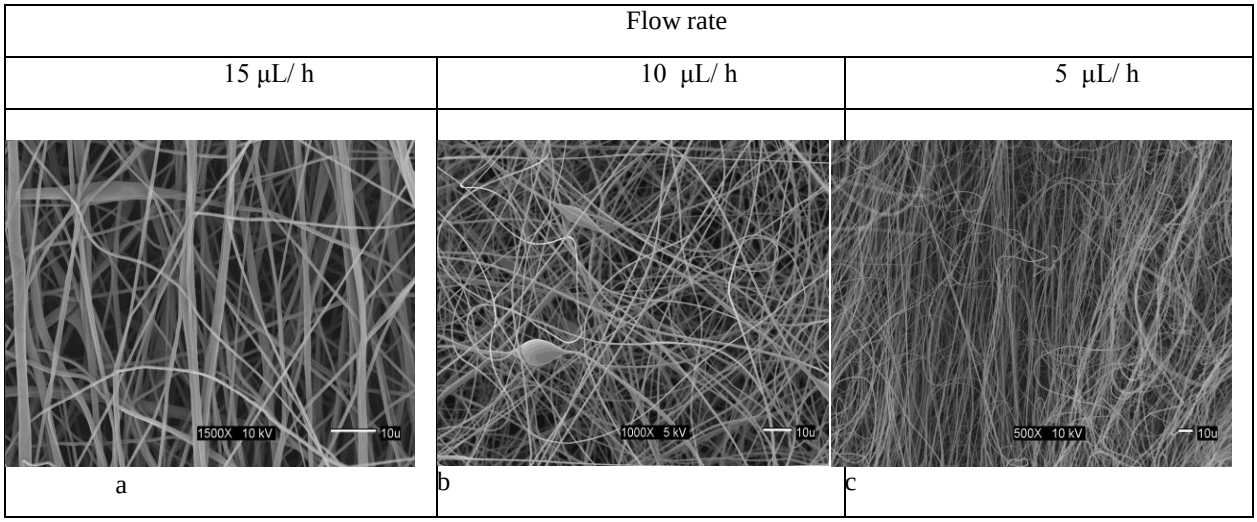


Figure 21. PAN nanofibers 5% wt. a) obtained at 15 µL/h b) obtained at 10 µL/ c) obtained at 5 µL/h central collector.

Figure 22, presents some regions of the 10% wt PAN fibers obtained at 5 µL/ h. formation of fibers with average diameters of 450 nm can be appreciated. These fibers proved to be more homogeneous and free beads than those obtained from the PAN solutions at 5% and 7%.

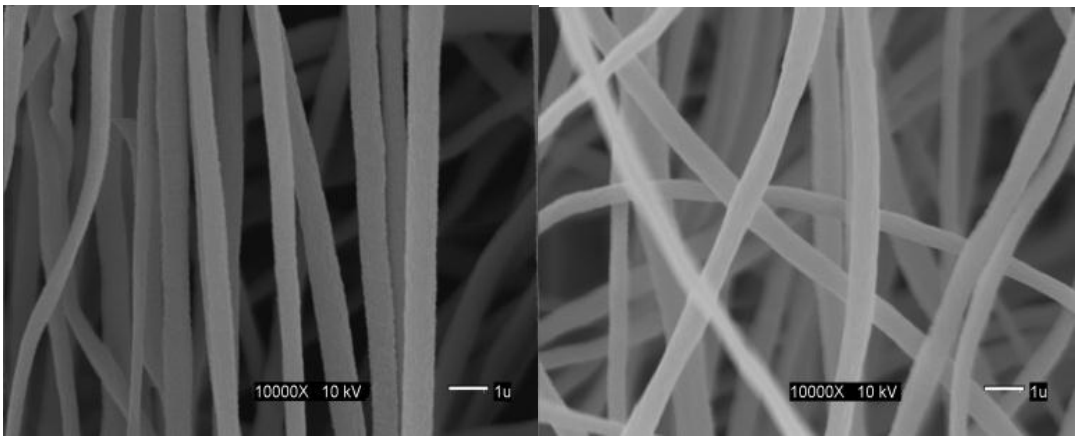


Figure 22. PAN nanofibers 10% wt. obtained at 5 µL/h under magnifications of 10000x

4.2 PAN-MWCNT NANOFIBERS COMPOSITES

4.2.1 Nanotubes oxidation

The time of oxidation was selected to be 4.5 hours because is reported to be the minimum time required to reach the oxidation of MWCNT without significant damage in the structure and also detriment of their electrical properties [74]. The oxidation of MWCNT was verified with UV spectroscopy. The presence of the peak near to at 260 nm in the UV-vis absorption spectrum indicate the existence of carboxyl group (–COOH) on the MWCNT surface as a result of the oxidation process. The UV-vis spectrum is shown in Figure 23.

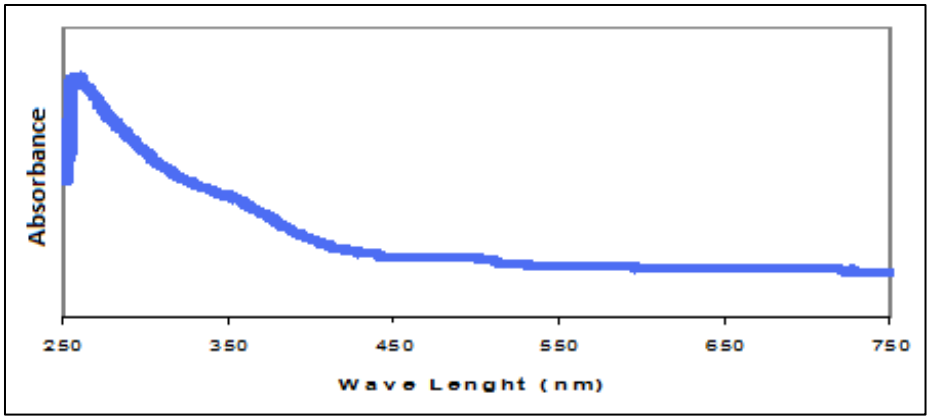


Figure 23. UV. Spectrum of acid treated MWCNT at 4.5 hours of treatment

4.2.3 Effect of DMF on the alignment of the nanotubes

All the PAN-MWCNT fibers precursors solutions were prepared using solvent (DMF) in excess. The weight ratios (DMF:MWCNT) evaluated were 75:1, 150:1, 200:1, 250:1, 360:1. The results reveal that the DMF: MWCNT ratio during the preparation of the solutions played an important role in the final quality of the fibers. At ratios DMF: MWCNT below 150:1, the fibers were so weak that they cannot be collected without breaking them. Also these fibers exhibited a poor dispersion of MWCNT, which can be seen as clusters in the fibers in Figure 24.

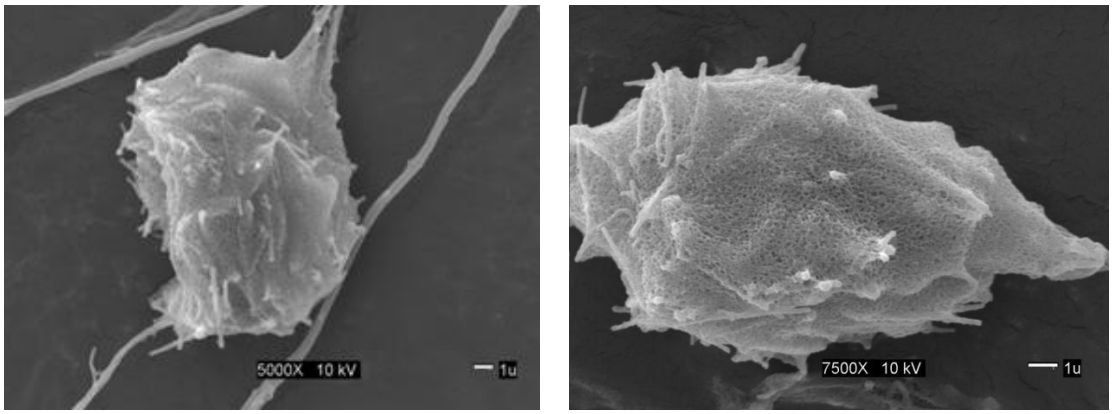


Figure 24. SEM image of clusters presence in PAN-MWCNT fibers with 3% MWCNT. Magnification 5000x and 7500x.

Between 200:1 and 250:1 DMF: MWCNT ratios, non-uniform dispersion of carbon nanotubes was observed in the fibers as shown in Figure 25.

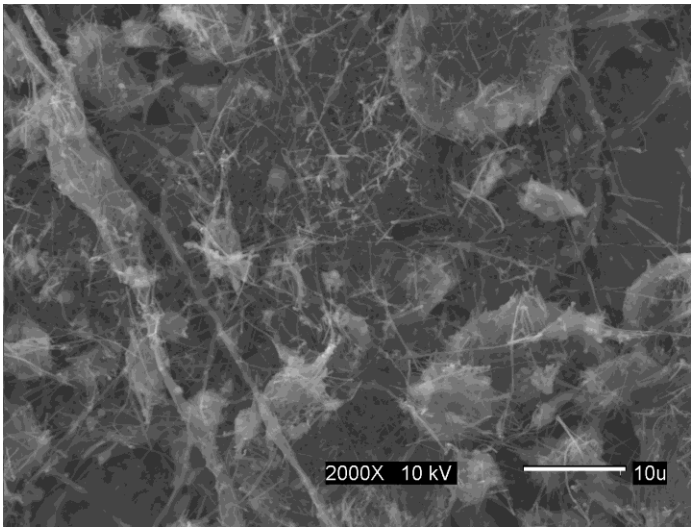


Figure 25. SEM image of clusters presence in PAN-MWCNT fibers with 5% MWCNT. Magnification 2000x.

The poor dispersion of nanotubes are the result of the formation of fragile, non-uniform and randomly aligned fibers, despite of the use of a central collector (used to improve the alignment of the fibers).

However, using DMF: MWCNT ratio 360:1, well aligned nanofibers with good dispersion of MWCNT were observed at all evaluated MWCNT concentrations 0.5%, 1% , and 2% (see Figure 26 to Figure 30).

4.2.3 Effect of the MWCNT concentration

Figure 26 shows that there are no significant differences between the diameters of the fibers obtained at 0.5 %, 1% and 2% concentrations of MWCNTs. The average diameter of the fibers obtained at these concentrations was aproximately to 4 μm (at 100 μL/h). Protrusion and entangled of MWCNT can be appreciated in the fibers obtained at 0.5% MWCNT and 70 μL/ h

(Figure 27a) and a considerable dispersion in the diameter of the fibers can be observed too (see Figure 27a and Figure 27b). A non-uniform diameter is observed along each fiber. A similar behavior was observed at all the concentrations of MWCNT and the flow rates evaluated.

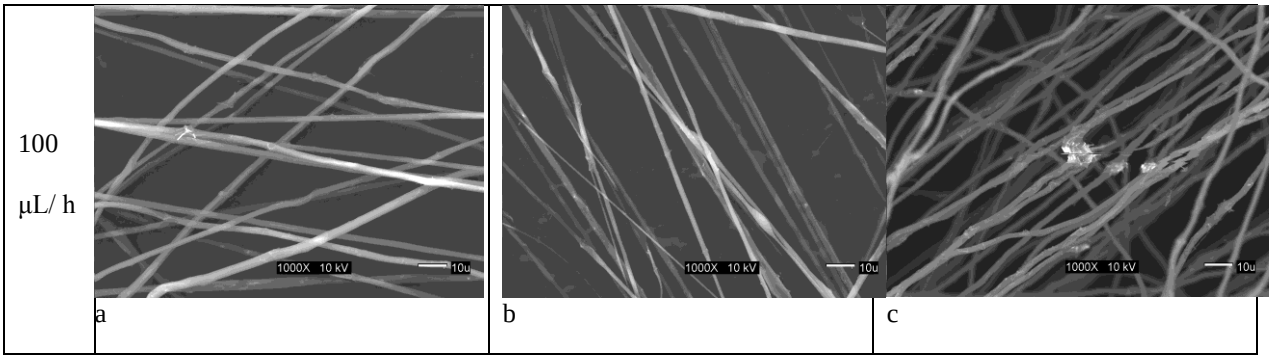


Figure 26. SEM images at 1000x of MWCNT-PAN fibers obtained at 10 KV DC, 5 % wt PAN, 100 µL/ h; (a) 0.5% wt MWCNT; (b) 1 % wt MWCNT; (c) 2 % wt MWCNT

Random orientation of MWCNT inside the fibers can be observed at a larger magnification in

Figure 27. This is opposite to the expected alignment along the axis of fibers due the flux of the solution during the electrospinning process. This random orientation of MWCNT inside the fibers is the main cause of the non-uniform diameter exhibited by the samples, however the protrusion could be considered as a favorable feature due to this increase the superficial area of the fibers, enabling the posterior enzyme immobilization. The average diameter for the fibers obtained at 100µL/h, is presented in Table 6.

Table 6. Diameter of fibers at 100µL/h

Electrospun fibers obtained at 100µL/h		
MWCNT content %Wt	Average diameter (µm)	Standard deviation
0.5%	2.120	0.377
1%	1.624	0.516
2%	2.267	0.520

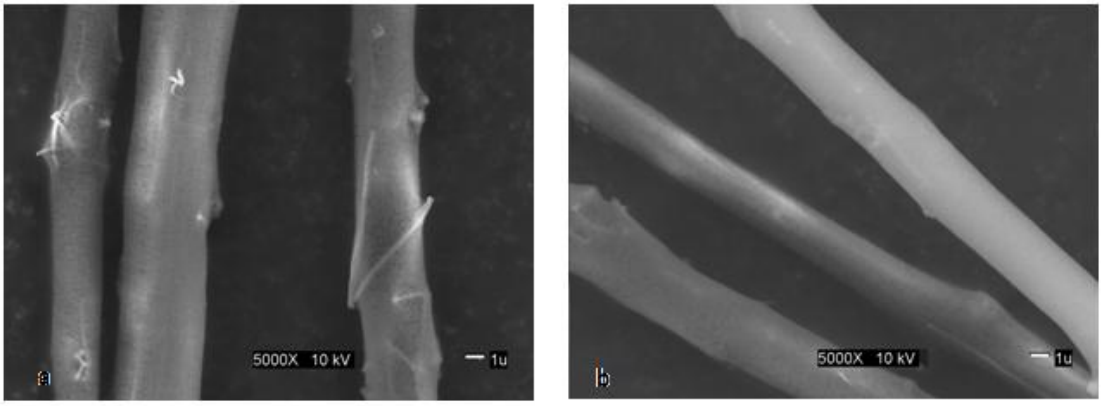


Figure 27. MWCNT-PAN fibers 70 µL/ h; (a) 0.5% wt MWCNT; (b) 1 % wt MWCNT

4.2.4 Flow rate effect in MWCNT-PAN fibers

A small decrease in the diameter of the fibers can be appreciated by increasing the flow rate of the solutions during electrospinning process, as is shown in Figure 28 and Figure 29. The SEM pictures show that the fibers obtained at 100 µL/ h (Figure 28c and Figure 29c) appear to be thinner compared to those obtained at 50 µL/ h (Figure 28a and Figure 29a) and 70 µL/ h (Figure 28b and Figure 29b). This behavior is opposite to the expected increase in the diameter of the fibers due to a greater volume of solution would be carried away of the needle tip during electrospinning process, increasing in this way the feed rate of the solution.

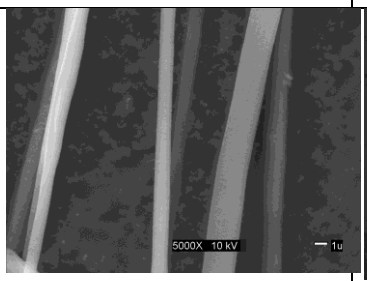
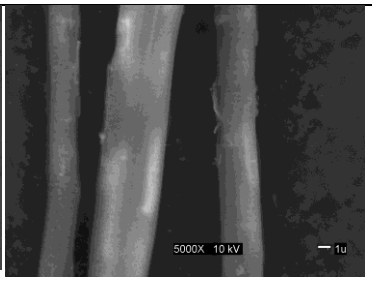
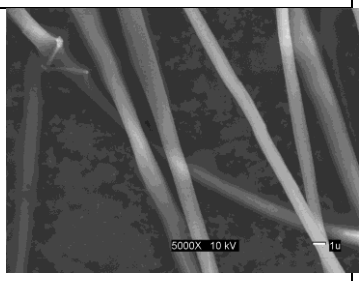
FLOW RATE	50 µL/ h	70 µL/ h	100 µL/ h
0.5%	 a	 b	 c

Figure 28. SEM images of MWCNT-PAN fibers 0.5% wt at 5000x; (a) 50 µL/ h; (b) 70 µL/ h; (c)100 µL/h

The average diameter for the fibers obtained with 1% of MWCNT is shown in Table 7.

Table 7. Average diameter of electrospun fibers with 1% MWCNT

Electrospun fibers 1% MWCNT		
Flow rate	**Average diameter (μm)**	**Standard deviation**
100 μL/h	1.624	0.516
70 μL/h	3.277	0.671
50 μL/h	3.794	0.543

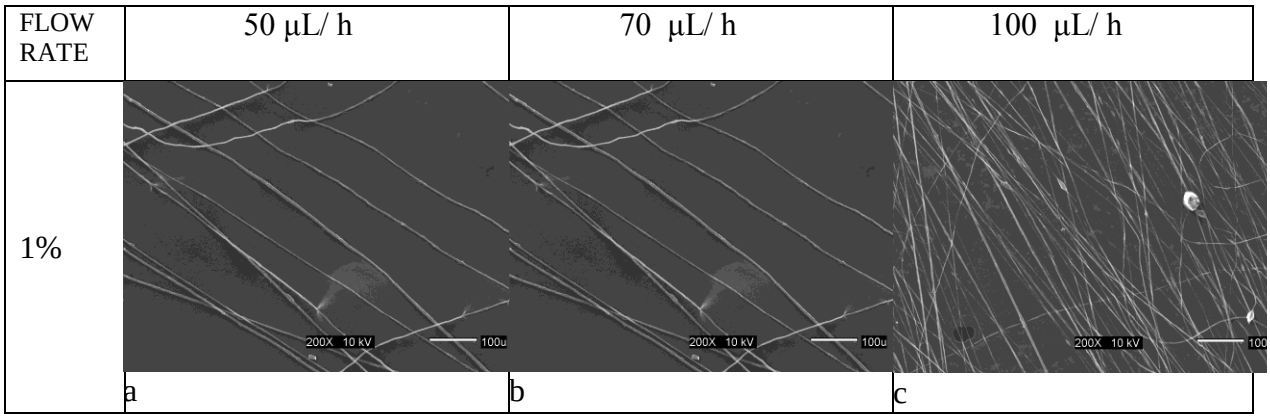


Figure 29. SEM images of MWCNT-PAN fibers 1% wt at 200x; (a) 50 μL/ h; (b) 70 μL/ h; (c)100 μL/ h

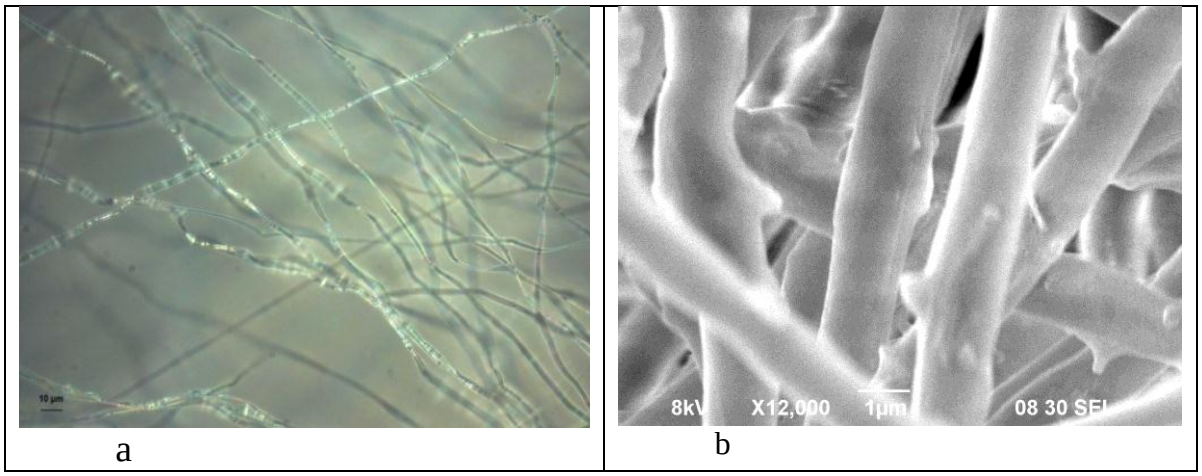


Figure 30. Optical and SEM micrograph of MWCNT-PAN fibers obtained at 200 μL/ h; 5% wt MWCNT; a) magnification 1800X, b) magnification 12000X

The solutions with 5% MWCNT created fibers with an average diameter 1.4 nm with SD of 0.16 nm, which is significantly smaller than those obtained at 0.5% MWCNT, 1% MWCNT

and 2% MWCNT. It can be assumed that this increase in the concentration of MWCNT led an increase in the conductivity of the solutions and therefore decreased the fiber diameter. Similarly to the fibers obtained at 1% and 2% MWCNT, the fiber diameters were not homogeneous and non-uniform along each fiber with the presence of protrusion in the fibers (Figure 30b).

4.3 CAST MEMBRANES

Under the optical microscope, the prepared cast membranes have a smooth and brilliant appearance that suggests that the MWCNT are covered by the polymer with low presence of protrusion of MWCNT at the surface, which is evident in the SEM micrograph (see Figure 31.b). Clusters of MWCNT can be appreciated in the optical micrograph under 1800x magnification of the PAN-MWCNT cast membranes (Figure 31.a). These clusters are also distributed in the whole surface of the membrane and are evidence of a faulty arrangement of the nanotubes inside the membranes.

In comparison, the presence of clusters is not evident in the fibers, despite the fact that the solution used in the cast membrane preparation is the same as the used for the preparation of electrospun nanofibers. The formation of clusters can be attributed to the time (6 hours) required for the complete evaporation of the solvent during the conformation of the cast membrane, which could give place to the regrouping of the MWCNT in the membrane.

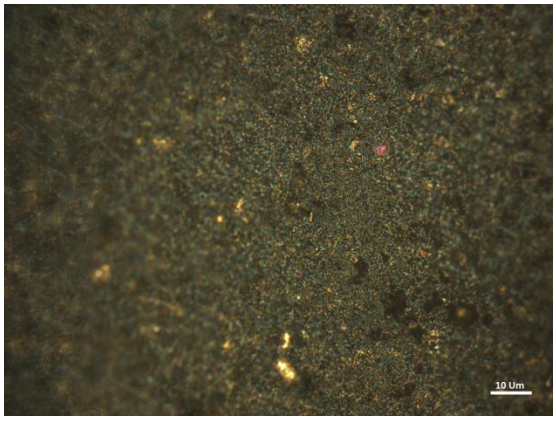
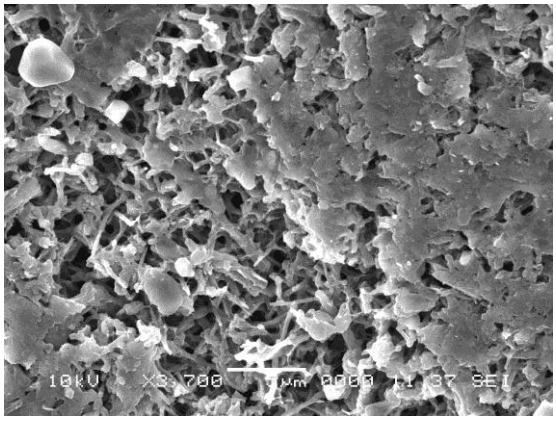
a.  b. 

Figure 31. Cast Membranes: a. Optical micrograph with of 5% MWCNT at 1800x; b. SEM micrograph with of 5% MWCNT at 3700x.

4.4 ELECTROCHEMICAL CHARACTERIZATION OF MEMBRANES

Cyclic voltammetry was performed using a three electrode setup. The electrolyte was a solution of (PBS 0.2 M), the potential scanning was conducted between -0.2 V to 1.0 V, and the scan rate was 2 mV/s.

The final fiber membranes were obtained with flow rates of 200 μL/ h and were collected during 60 minutes in order to obtain a thicker membrane.

The voltammograms shown in Figure 32 corresponding to fibers with content of MWCNT between 0 - 1%, in this plot is evident the absence of cathodic or anodic peak for the samples at all conditions. However, is observed an increase in the current that is dependent of an increase in the content of MWCNT.

This tendency to an increase of the current density with the increase of the content of MWCNT is also observed in the fibers with the maximum load of 5% MWCNT (see Figure 33). The maximum current density for the 5% MWCNT case is 2.16 mA.

In the same way, a tendency to the increase of the hysteresis area of the voltammograms with the increase in the content of MWCNT can be observed too in Figure 32.

The hysteresis area of the voltammograms is related to the active area of the electrode.

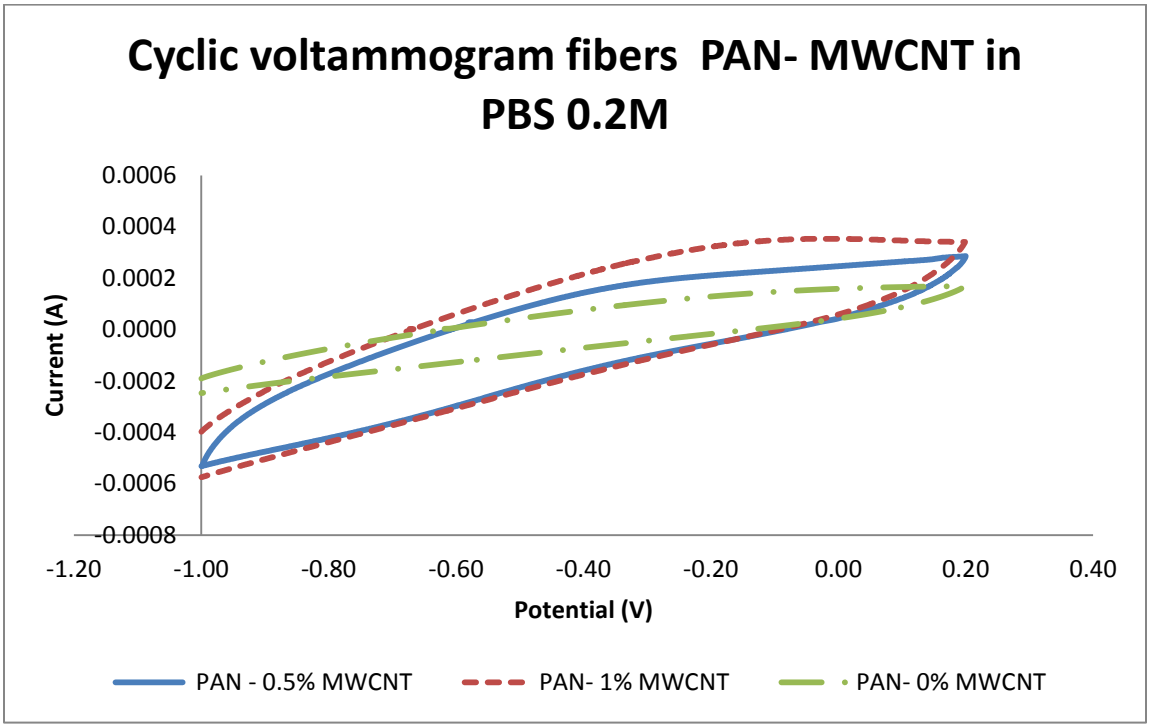


Figure 32. Voltammograms for PAN-MWCNT fibers in PBS 0.2M

The cyclic voltammetry test conducted to PAN – MWCNT nanocomposite membrane at 2% and 5% MWCNT show a similar electrochemical behavior for both (Figure 33 - solid and dashed line respectively). In the same way, no difference in the electrochemical behavior of the fibers at 5% MWCNT was observed in presence of H_2O_2 (-dot line).

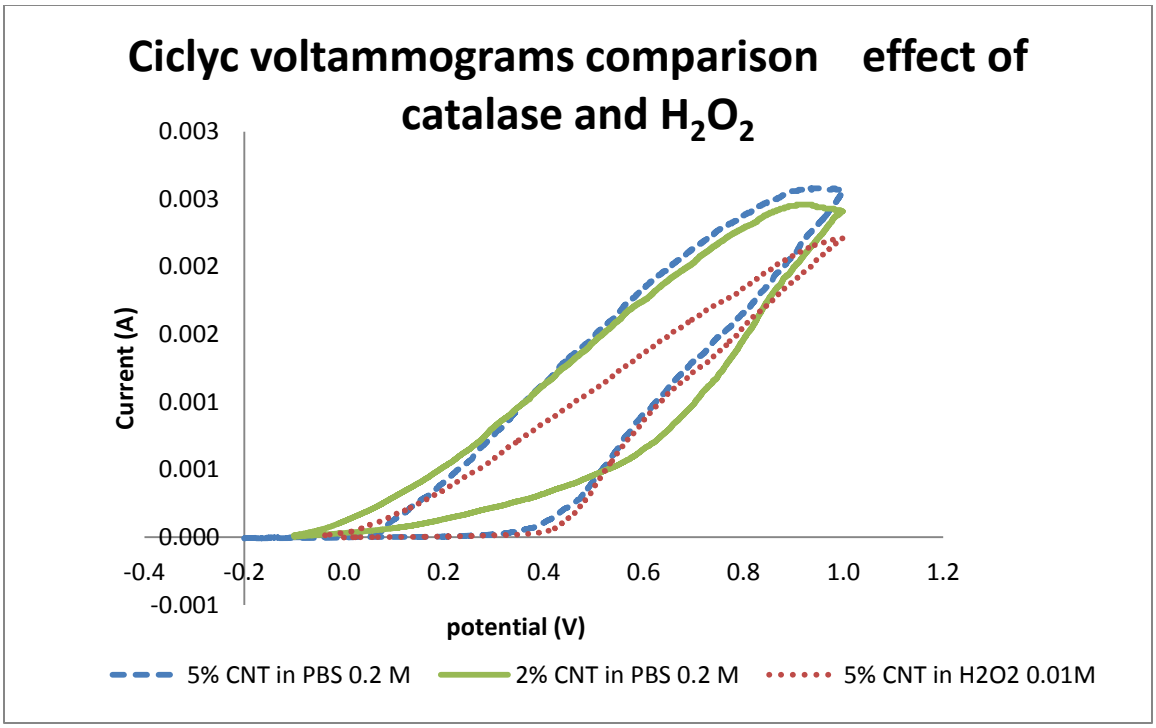


Figure 33. Voltammograms of the fibers at 2% MWCNT and 5% MWCNT.

In contrast to the observed in the case of the nanocomposite electrospun fiber membranes, increasing the load of MWCNT do not appear to have a mayor effect in the electrochemical response of the cast membranes (see Figure 34). In all the cases, the electrochemical behavior of the cast membranes appear to be similar to the response of the electrospun fibers with 0% of MWCNT (see Figure 34). This suggests that the cast membranes have a low electrical conductivity despite the presence of MWCNT.

The low area of hysteresis of the voltammograms of the cast membranes indicates that these possess a low effective area, make them inadequate for applications as electrodes in which a high effective area is required to the attachment of the catalyst. The high presence of MWCNT clusters in the cast membranes (see Figure 31) can contribute to the reduction of its conductivity hen compare to the fibers membranes in which the MWCNT are well dispersed (see Figure 26 to Figure 30). This effect is expected given that the conductivity in CNTs occurs mainly at their

ends, for this reason, they must be in contact through their ends to guarantee improvements in electrical conductivity.

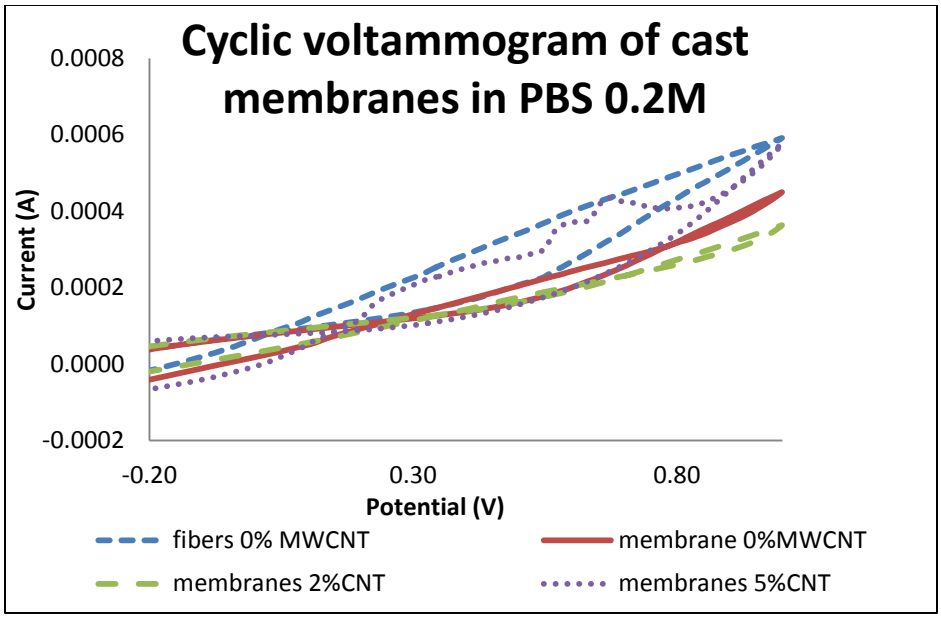


Figure 34. Voltammograms for PAN-MWCNT cast membranes in PBS 0.2M

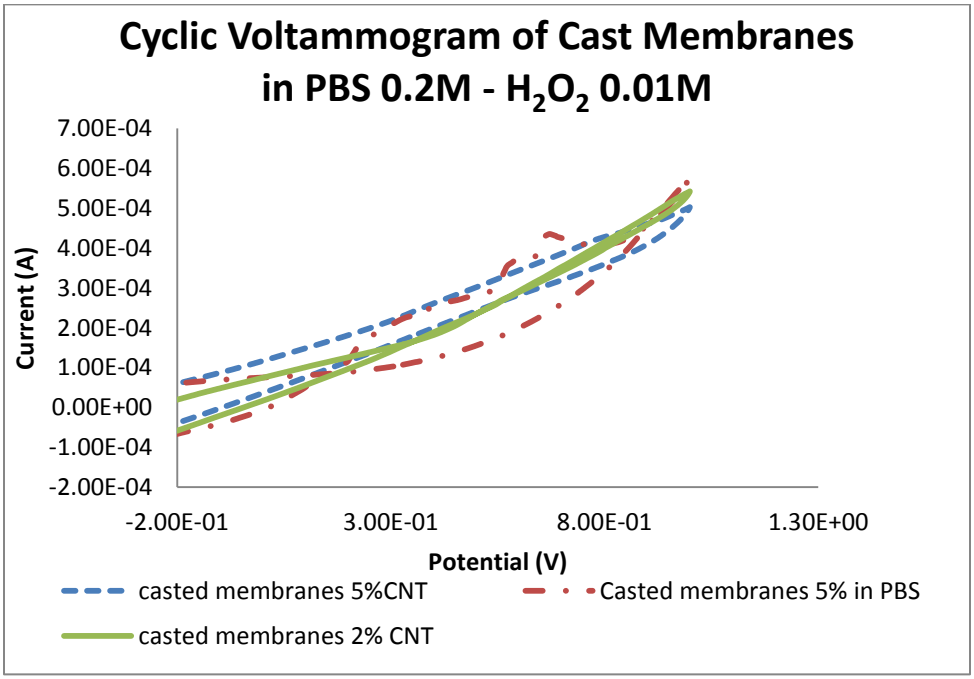


Figure 35 Voltammograms of cast membranes in PBS 0.2M – H_2O_2 0.01M

4.5 TESTING FOR CATALASE IMMOBILIZATION

Voltammetry test in (PBS + H_2O_2) of the nanocomposite membranes with 5% MWCNT and **c**atalase immobilization was performed. The increase in cathodic current (Figure 36) with catalase immobilized fibers in presence of H_2O_2 suggests the potential application of composite PAN-MWCNT nanofibers for bioelectrode, however the observed denaturation observed in the enzymes suggest a short lifetime of this under the test conditions.

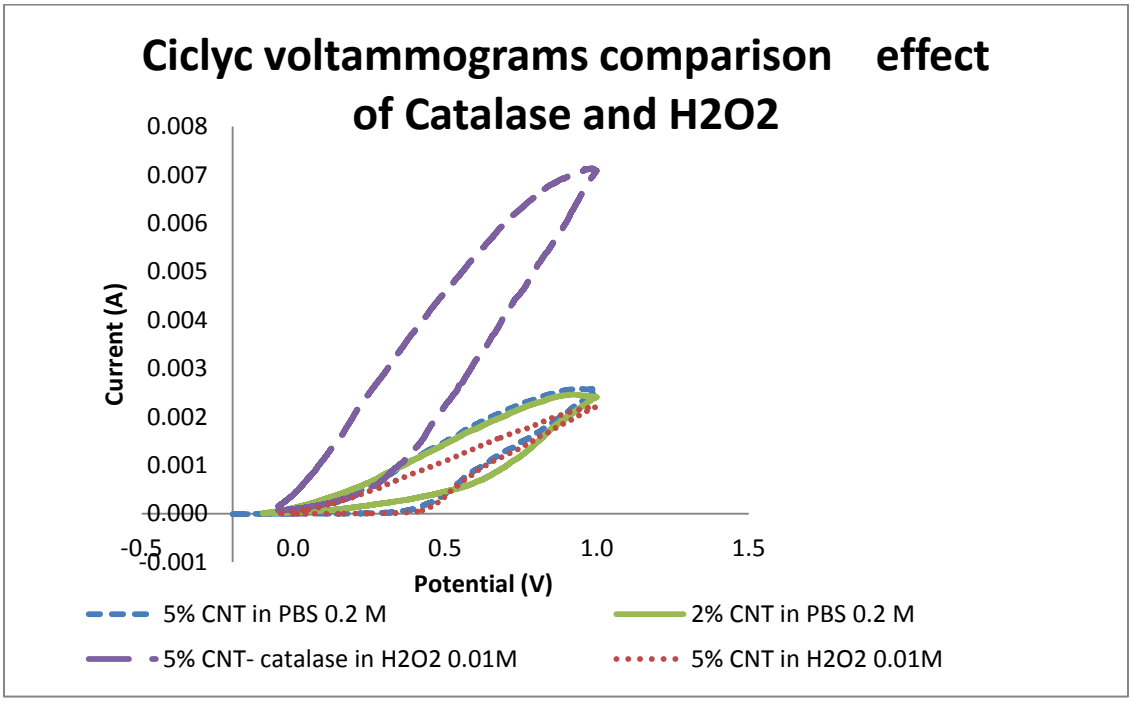


Figure 36. Effect of catalase and H_2O_2on electrochemical behavior of nanocomposite fibers

Cyclic voltammetry results shown that all the cast membranes are inadequate support for catalase immobilization due to a low effective area. Loading MWCNT in the cast membranes, do not improve their electrochemical response even in the presence of catalase. The electrochemical behavior observed for cast membranes were in all cases similar to the observed in bare PAN membranes.

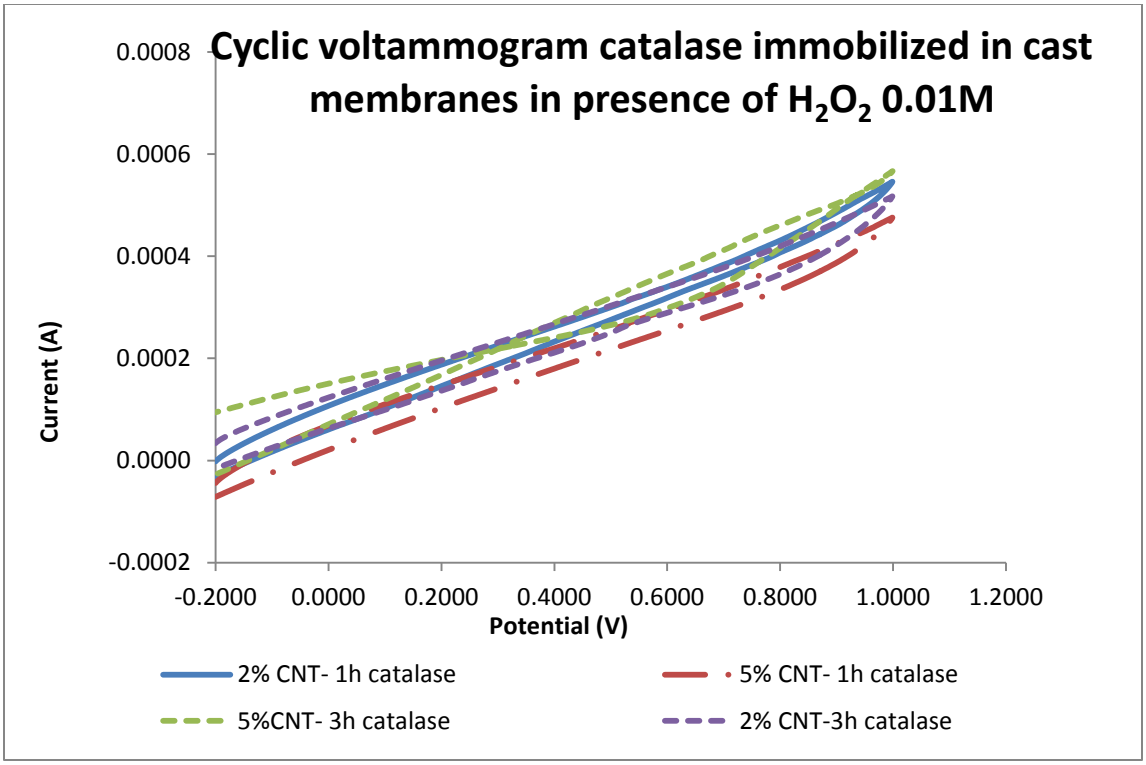


Figure 37. Effect of catalase on electrochemical behavior of cast membranes

The increase in current reached with the immobilization of catalase in the fibers was near to 10 times superior (Figure 38) to the reached with the catalase immobilized in cast membranes, in which the immobilization of the enzyme appear do not have any effect. This neutrality in the behavior in the cast membranes is due to the embedment of the MWCNT in the polymer matrix of the casting that insulates them and avoids the contact with the enzymes. Also the low superficial area of the membranes could be contributed to reduce the load of immobilized enzymes.

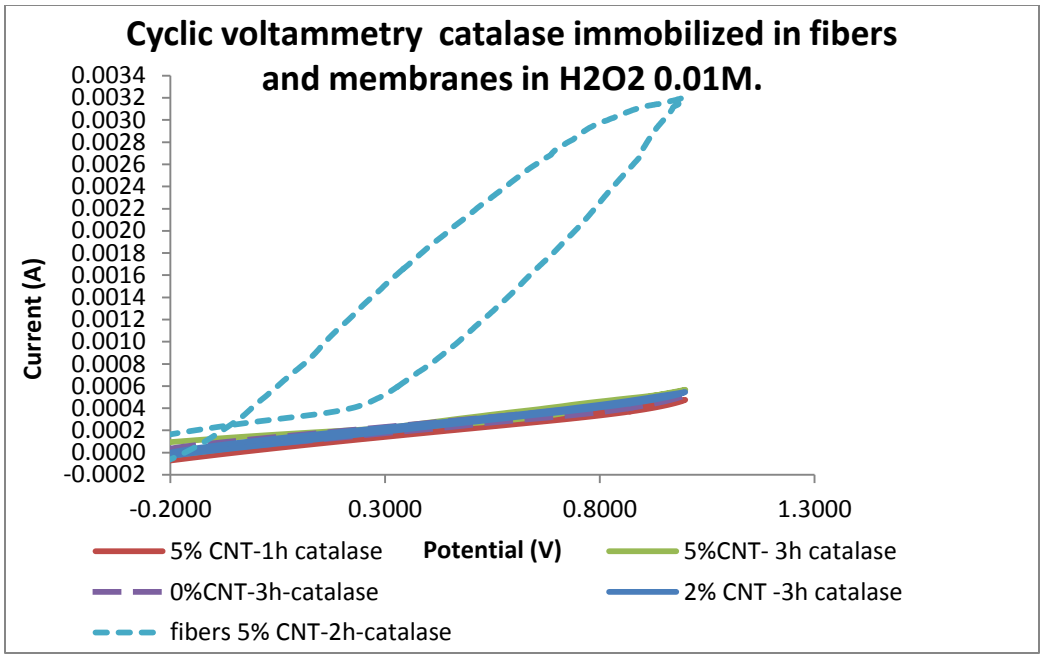


Figure 38. Cyclic voltammograms comparison of the electrochemical responses of fibers and cast membranes with immobilized catalase.

SEM images of the membranes presented in Figure 39. Let to evidence the formation of precipitates can be appreciated on the surface of the fibers. These precipitates can be attributed to the breaking of the bonds that retain the globular configuration of the enzymes generating a new filament structure. This phenomenon is known as enzyme denaturation and could be generated from a change in the PH of the media when performing the voltammetry test. The denaturuzation of enzymes is not desired phenomenon, because the catalytic properties of enzymes are vanished. In some cases when the initial conditions of the media are restored the denaturation can be reversed [22].

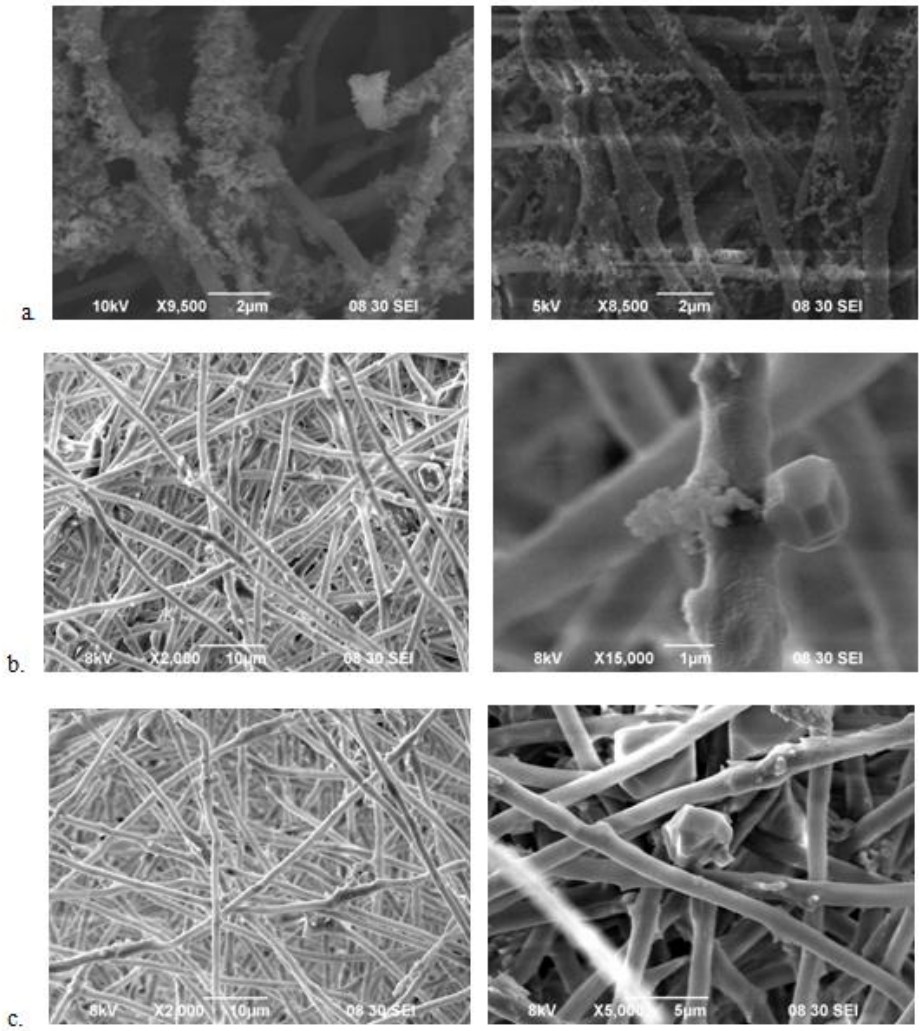


Figure 39. SEM images of enzyme immobilized in fibers

4.6 DETERMINATION OF INMOBILIZED CATALASE USING BRADFORD METHOD

In base of the calibration chart traced using bovine serum albumin (see Figure 40), was possible to obtain a lineal model which allow quantifying the presence of catalase in the solutions under evaluation.

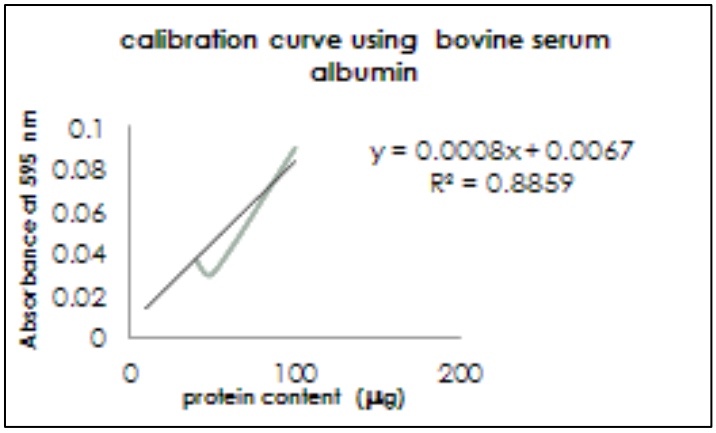


Figure 40. Calibration curve for absorbance of brilliant blue at differences concentrations of protein

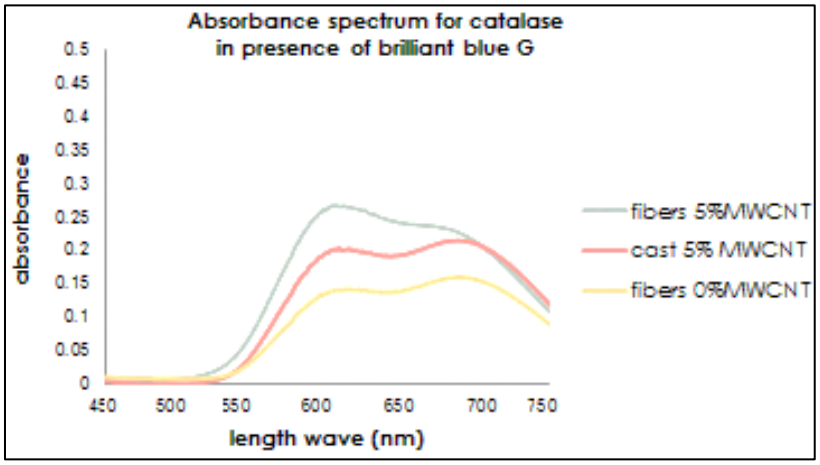


Figure 41. Absorbance of brilliant blue in presence of catalase

Of the reading of the absorbance at 595 nm (see Figure 41) and calculating the difference of the initial catalase concentration, the content of catalase immobilized in the fibers is presented in Table 8.

Table 8.Catalase content according Bradford assay

Membrane type	absorbance at 595 nm	Catalase Content (mg/g)
fiber 5%	0.2607	3.852
cast 5%	0.1982	1.655
fibers 0%	0.1414	3.145

5. CONCLUSIONS AND FUTURE WORK

5.1 CONCLUSIONS

- Nanocomposite membranes of PAN-MWCNT were synthesized by electrospinning reaching loadings as higher as 50% of MWCNT, which is greater than the maximum loading reported till now (35%). Morphological characteristics and electrochemical behavior of the membranes were evaluated and compared against cast membranes of the same material. The test conducted for both materials allows to concluding that the nanocomposite membranes made of fibers appear to have better electrochemical behavior compared with the cast nanocomposite membranes.

- The nanocomposite fibers were synthesized using electrospinning process, and the synthesis parameters were optimized by controlling the flow rate, applied voltage, MWCNT concentrations and solvent to PAN ratio, to obtaining fibers of diameters lesser than 1 μm.

- SEM results revealed that precursor solutions with concentrations below of 2% MWCNT, do not affect in a significant way the diameter of the electrospun fibers and also the electrochemical behavior of the membranes.

- Enzyme Catalase was covalently immobilized above PAN-CNT nanocomposite fibers and cast membranes. The immobilization of catalase improves the electrochemical response of the composite fibers in presence of H_2O_2, however, this do not have the same effect in the cast membranes.

- The comparison of the enzyme electrochemical response indicates the importance of the electrospinning process over simple film casting process. The higher enzyme loading as suggested by Bradford assay could be attributed to porous surface morphology and alignment of CNT in the composite membranes.

- Overall this work shown a successful synthesis of new PAN-CNT nanocomposite and its electrochemical activity; however efforts are still required to achieve the practical goals.

5.2 FUTURE WORK

Increase the lifetime of the enzymes in the bioelectrodes is one of the biggest issues to solve in order to achieve a massive utilization of biofuel cells. The denaturation observed for the enzyme catalase during cyclic voltammetry tests in this work, is precisely one of the aspects to continue investigating. The main objective at this respect is to determinate which are the main variables to cause the denaturation of the enzyme in our bioelectrode. Some of the variables to evaluate are the applied voltage during C.V, PH and also the immobilization method.

The nature of the support is another important topic to continue the investigation in order to obtain better bioelectrodes for biofuel cells. At this respect, is suggested to evaluate the effect of the graphitization of the composite electrospun fibers. With the graphitization increases in the conductivity of the fibers are expected, however this process generate very brittle fibers, which are difficult to handle, making necessary the development of new procedures to prepare the bioelectrode support.

Also to evaluate the viability of the PAN-MWCNT electrospun fiber composite, as supports for another type of enzymes is another matter to continue as a future work. In this research, the electrospun composite support was evaluated as bio-cathode; however, enzymes

such as dehydrogenases type could give the opportunity to evaluate this electrode support as bio-anode too.

Electrospun PAN-MWCNT fibers of diameter smaller than 300 nm (see Appendix 3) were obtained using a most powerful power supplies, as a future work the effect of the diameter reduction in the bioelectrodes behavior must be evaluated.

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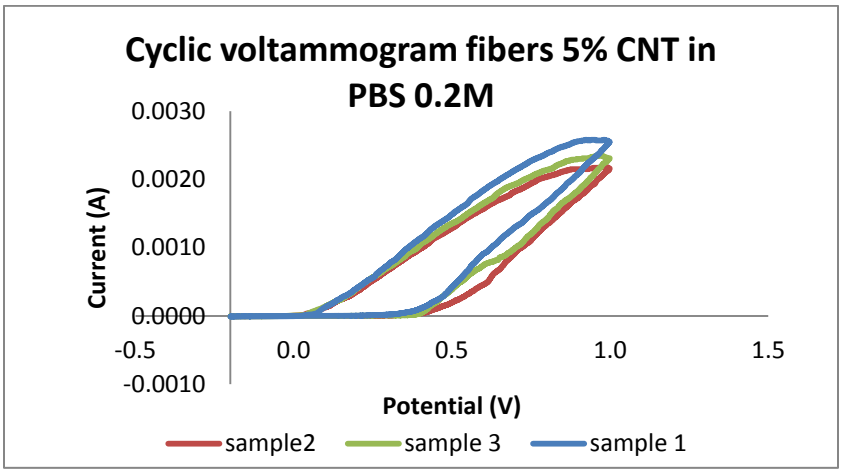
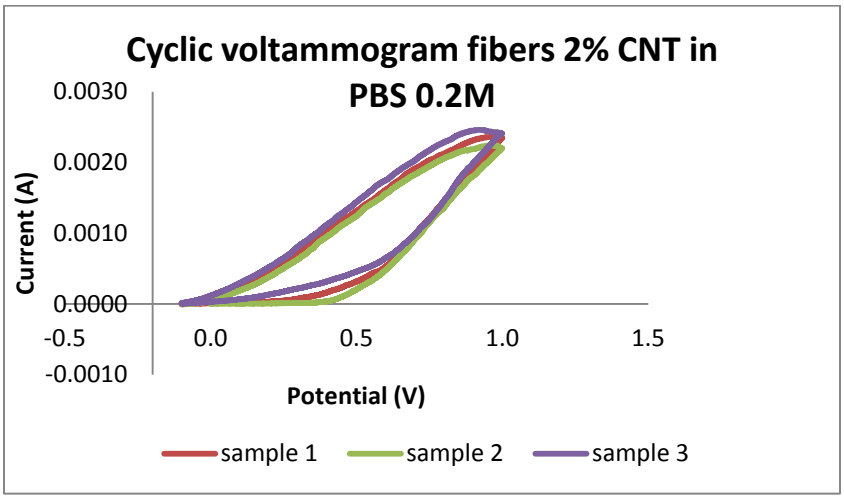
7. APPENDIXES

APPENDIX 1. CYCLIC VOLTAMMOGRAMS FOR FIBERS

a.



b.



c.

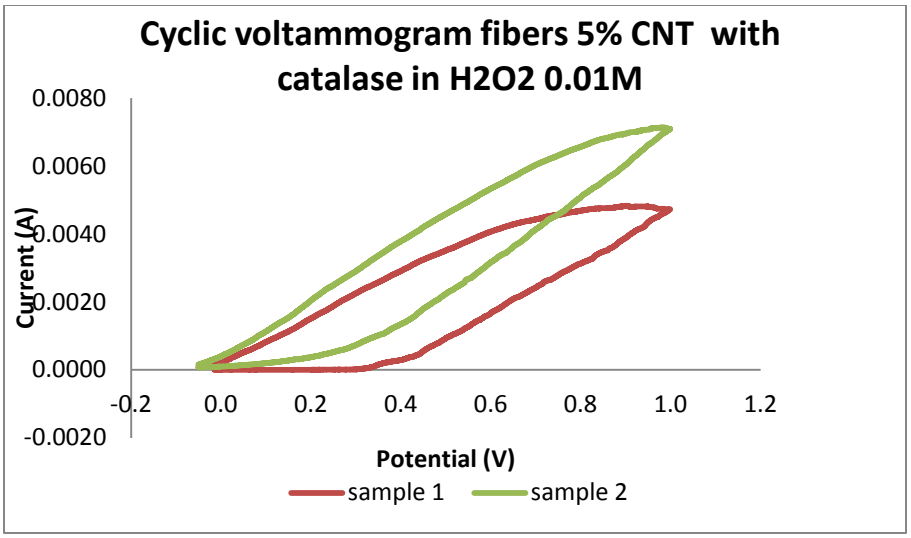


Figure 42. Cyclic voltammograms for electrospun fibers a. 5% MWCNT, b. 2% MWCNT, c. 5% MWCNT – catalase.

APPENDIX 2. HYSTERESIS AREA OF VOLTAMMOGRAMS

The hysteresis area was calculated in base of the difference of the area under the curves of the superior and inferior portion that compound the complete voltammograms curves.

Polynomial tendency were used to obtain the equation of the curves, which integral was evaluated between -0.2 to 1. The equations and areas are shown in appendix 2.

Type of membrane	**Superior curve equation**	**Inferior curve equation**	**Total area**
Cast membranes 2% CNT	$y = 0.0002x^3 - 0.0002x^2 + 0.0004x + 0.0001$	$y = 0.0007x^5 - 0.0012x^4 + 0.0006x^3 - 6E-05x^2 + 0.0004x + 6E-05$	0.000024
Cast membranes 5% CNT	$y = -0.0001x^4 + 0.0004x^3 - 0.0003x^2 + 0.0004x + 7E-05$	$y = 0.0007x^5 - 0.001x^4 + 0.0005x^3 - 0.0001x^2 + 0.0004x + 2E-05$	0.000031
Fibers 0% CNT	$y = 0.0001x^6 + 7E-05x^5 - 0.0003x^4 - 0.0001x^3 + 0.0003x^2 + 0.0005x + 7E-05$	$y = -0.0004x^6 - 9E-05x^5 + 0.0005x^4 + 0.0004x^3 - 7E-05x^2 + 0.0002x + 8E-05$	0.000069
Fibers 1% CNT	$y=-.0006x^2+0.00009x+0.0004$	$y=0.0006x^3+0.0009x^2+0.001x+0.00009$	0.000072
Fibers 2%cnt	$y = -0.0032x^5 + 0.0055x^4 - 0.0058x^3 + 0.0044x^2 + 0.0014x + 0.0001$	$y = -0.0261x^6 + 0.0548x^5 - 0.0353x^4 + 0.0079x^3 + 0.0009x^2 + 0.0002x + 3E-05$	0.000642
Fibers 5% CNT	$y = -0.0046x^3 + 0.0061x^2 + 0.0011x - 4E-06$	$y = 0.0424x^6 - 0.0989x^5 + 0.068x^4 - 0.0062x^3 - 0.003x^2 + 0.0002x + 2E-05$	0.00068
Fibers 5% CNT with catalase	$y = -0.0083x^6 + 0.0212x^5 - 0.0192x^4 + 0.0042x^3 + 0.0032x^2 + 0.0011x + 9E-05$	$y = -0.0369x^6 + 0.0719x^5 - 0.0468x^4 + 0.0144x^3 - 0.001x^2 + 2E-06x + 1E-05$	0.00064

APPENDIX 3. PAN-50% MWCNT FIBERS OBTAINED AT 25 KV AND 20 Cm.

Thinner fibers (average diameter 0.202 nm with SD 0.097 nm) than those obtained at 10 kV (average diameter 1.361 nm with SD of 0.166 nm) are shown in Figure 43. For future work is suggested also to evaluate the effect of diameter of fibers in the enzymatic load and electrochemical response of the bioelectrodes.

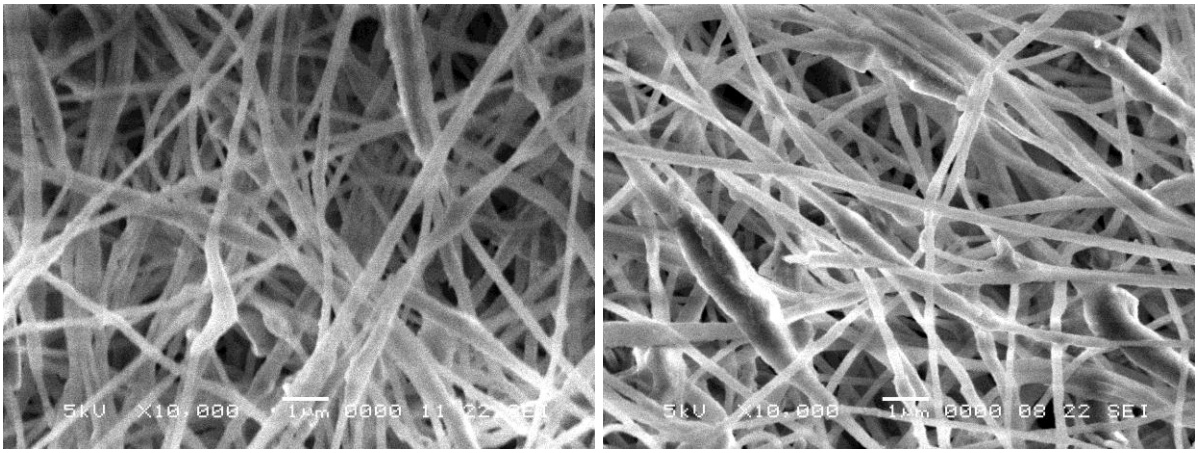


Figure 43. . SEM images of PAN -5% MWCNT fibers at 25 KV and 20 Cm.

APPENDIX 4. INMOBILIZATION OF CATALASE BY CROSSLINKING USING GLUTARALDEHYDE

To compare the effect of immobilization technique in the response of the electrode, the enzyme catalase was also immobilized via crosslinking using glutaraldehyde. The procedure to immobilization is described in Figure 44.

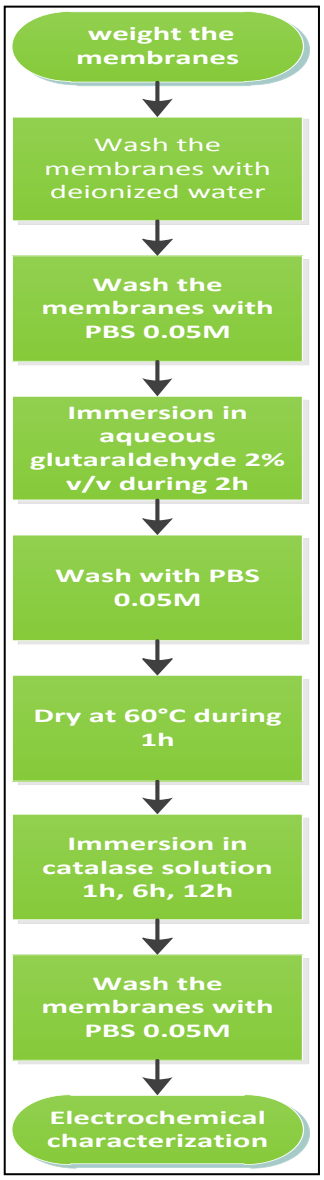
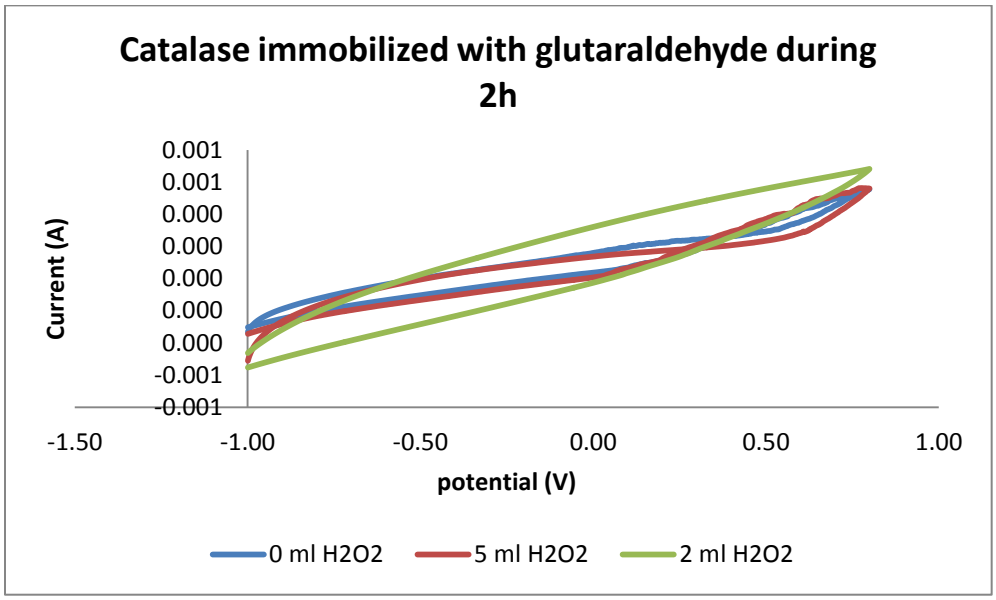


Figure 44. Immobilization of enzyme with glutaraldehyde

ELECTROCHEMICAL CHARACTERIZATION OF ELECTRODES OBTAINED BY IMMOBILIZATION OF CATALADE WITH GLUTARALDEHYDE

Cyclic voltammetry test was conducted in the same way than the performed for testing of nanofibers y cast membranes, using PBS 0.2 M as electrolyte and H_2O_2 0.1M as analyte.

a.



a.

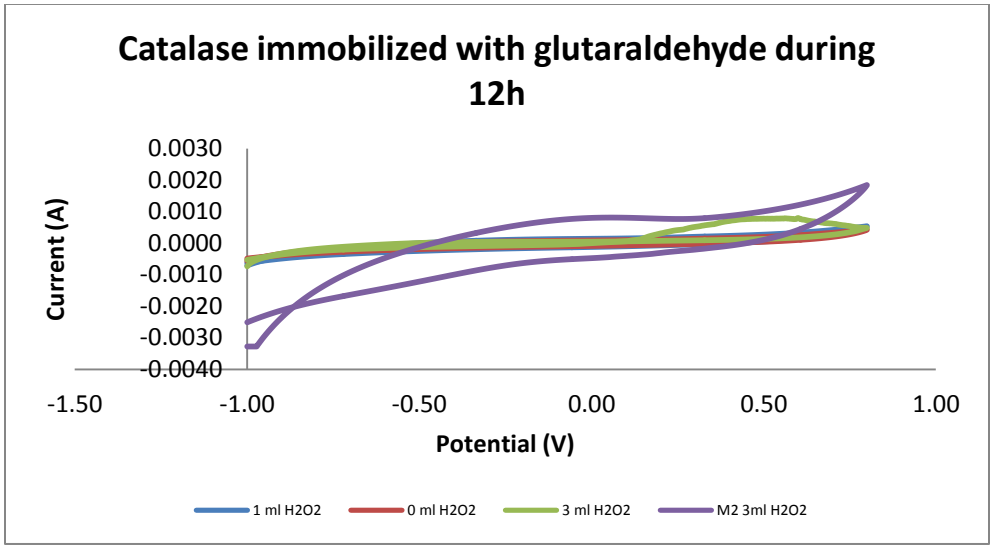
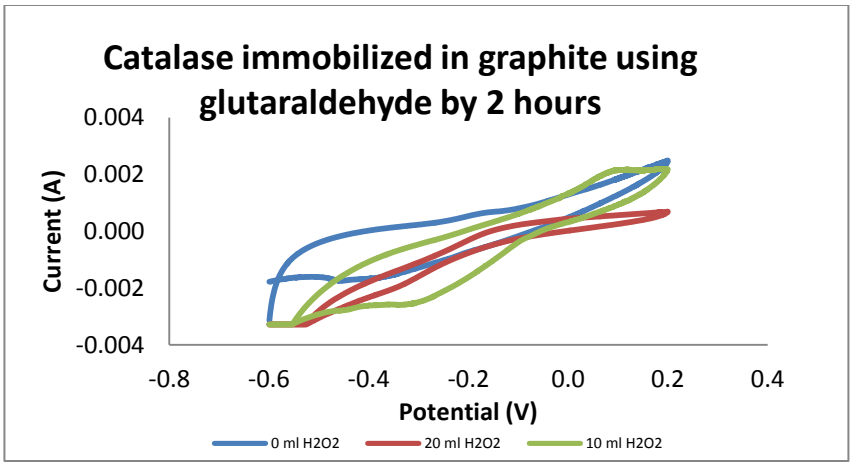


Figure 45. Cyclic voltammograms for electrodes prepared with catalase immobilized with gluraldehyde a.2 Hours b. 12 hours

a.



b.

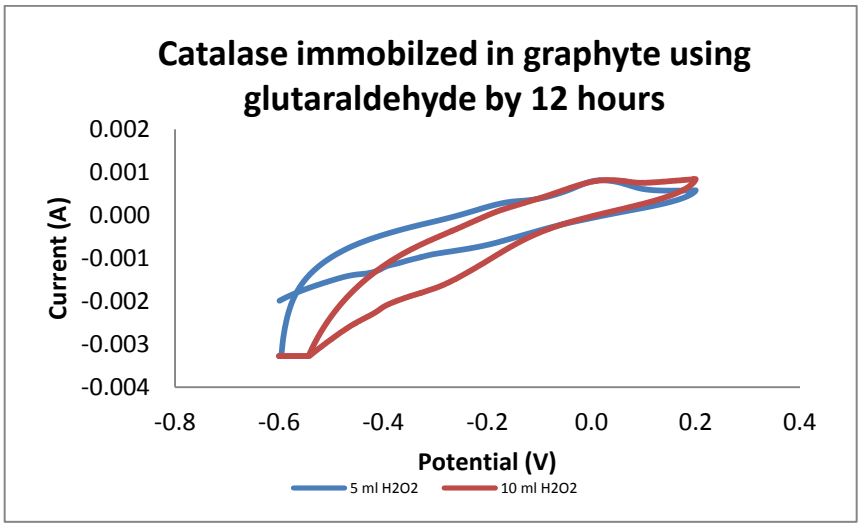


Figure 46. Cyclic voltammograms for graphite electrodes prepared with catalase immobilized with gluraldehyde a.2 Hours b. 12 hours

The results obtained using glutaraldehyde (Figure 45) are comparable with those obtained using EDC-NHS as coupling agents during enzyme immobilization, however the use of a more conductive type of enzyme support as graphite improves the electrochemical response of the electrode.

Compared with PAN-MWCNT electrospun fibers, electrodes prepared using graphite, appear to have a better electrochemical response in presence of H_2O_2. Presence of slightly evident Anodic peak at -0.4 V and a catholic peak at 0 V can be appreciated in Figure 46.