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Biocatalytic Fibers For Bioremediation

Dissertação para obtenção do Grau de Mestre em Bioquímica

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[Biocatalytic Fibers For Bioremediation]

"When you see a good move, look for a better one."

Emanuel Lasker.

Agradecimentos

“ We can know only that we know nothing, and that is the highest degree of human wisdom.”

“By, leo Tolstoy “

Depois deste ano intenso, se há algo que ficou bem claro para mim, é que a autocorreção é uma ferramenta bastante forte que devemos usar ao longo da nossa carreira.

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A vocês e todos os que referi dedico esta dissertação.

Resumo

As enzimas podem exibir excelentes propriedades catalíticas, nomeadamente alta atividade em condições ambientais variadas, alta especificidade e seletividade. Apesar destes aspectos positivos, demonstram algumas limitações que podem inibir a sua aplicação a nível industrial. A imobilização de enzimas em fibras poderá proporcionar aos materiais catalíticos obtidos boas propriedades mecânicas, uma elevada área superficial, resistência à temperatura, alcalóides e humidade.

Este trabalho consiste na produção, através da técnica de electrospinning, de membranas biocatalíticas contendo uma lacase, para biorermediação enzimática por esta enzima.

O plano de trabalho inicial consistia na produção de fibras coaxiais, com uma camada exterior de sílica, conferindo resistência e protecção, e um núcleo constituído por uma gelatina iónica, resultante do cross-linking entre gelatina e um líquido iónico, contendo a lacase. Para a preparação das camadas de sílica, seleccionou-se o processo sol-gel, utilizando como percursores tetrametoxisilano (TMOS) ou tetraetoxisilano (TEOS), mediante catálise ácida com HCl e utilização de metanol como co-solvente. Para conferir porosidade e flexibilidade à estrutura de sílica, adicionou-se solução de álcool polivinílico (PVA) à solução de electrospinning, que foi testada na produção de fibras. Entre as várias formulações testadas, a formulação (em razões molares) 1:1,8:1.1:7,9E-03 TMOS:H_2O:metanol:HCl conduziu a fibras de aspecto regular e com baixa dispersão de tamanhos, tal como revelado por microscopia electrónica de varrimento. Igualmente com vista à preparação de fibras co-axiais, testaram-se formulações para o núcleo das fibras. O controle de temperatura disponível não permitiu impedir que a solução de gelatina de porco e líquido iónico dihidrogenofosfato de colina solidificasse na agulha, inviabilizando o processo de electrospinning. Para ultrapassar esta dificuldade, foi feita uma diminuição da concentração de gelatina, que resultou na produção de electrospray, em vez de fibras. De seguida foi feita uma alteração na natureza da gelatina, que foi substituída por gelatina de peixe, cuja dissolução ocorre à temperatura ambiente. No entanto, o cross-linking desta gelatina com o líquido iónico não foi eficiente, e a membrana de fibras à base de gelatina obtida dissolvia-se imediatamente em água.

Optou-se então por produzir uma membrana em camadas, constituída nomeadamente por uma camada de fibras à base de sílica em cada uma das superfícies, e uma zona interior compreendendo fibras reactivas à base de um po-

límero diferente da gelatina. Tando já sido feita a optimização da produção de fibras de sílica pelo processo sol-gel, o foco foi agora a produção das fibras bior-reactivas do interior da membrana. Testaram-se dois polímeros, nomeadamente policaprolactona (PCL) e PVA. No sentido da preservação da actividade enzimática, testaram-se solventes diferentes para a PCL, mas em nenhum dos casos se obteve evidência de actividade da lacase imobilizada nas fibras obtidas. Já nas fibras à base de PVA, a enzima manteve-se activa. Procedeu-se então à produção de membranas de fibras com camadas superficias de sílica e interior de PVA com enzima. Estas membranas exibiram actividade catalítica, permitindo cumprir o objectivo do trabalho.

Palavras chave: Electrospinning, sílica, fibras biocatalíticas, PVA, lacase, biorremediação.

Abstract

Enzymes can exhibit excellent catalytic properties, namely high activity at different environmental conditions, high specificity and selectivity. Despite these advantages, they exhibit some limitations that can hinder their application in industry. The immobilization of enzymes on fibers can lend good mechanical properties, high surface area, and resistance against high temperature, alkaloids and humidity to the resulting catalytic materials.

This work consists in the production, using electrospinning, of biocatalytic membranes containing laccase with a view to enzymatic bioremediation.

The initial work plan consisted in the production of coaxial fibers with an outer layer of silica, lending strength and protection, and a core containing an ionic gelatin resulting from the cross-linking of gelatin and an ionic liquid, containing the laccase. For the preparation of silica layers, it was selected the sol-gel process using as precursors tetrametoxysilane (TMOS) or tetraethoxysilane (TEOS) through acid catalysis with HCl, using methanol as co-solvent. To confer flexibility and porosity to the silica structure, a polyvinyl alcohol (PVA) solution was added to the electrospinning solution. Among the various formulations tested, the composition (in molar ratios) 1:1,8:1.1:7,9E-03 TMOS:H_2O:methanol:HCl led to regular fibers with low size dispersion, as revealed by scanning electron microscopy. Also for the preparation of co-axial fibers it was tested formulations for the core of the fibers. The temperature control available did not prevent the solidification of the solution of porcine gelatin and ionic liquid choline dihydrogenphosphate in the needle, making it impossible to carry out the electrospinning process. To overcome this difficulty, the concentration of gelatin was decreased, which resulted in the production of electrospray instead of fibers. Then a change was made in the nature of gelatin, which was replaced by fish gelatin, whose dissolution occurs at room temperature. However, the cross-linking of this type of gelatin with the ionic liquid was not efficient and the membrane obtained dissolved immediately in water.

It was then decided to produce a multi-layered membrane, consisting of a layer of silica-based fibers on each side, and an inner section comprising reactive fibers based on a different polymer than gelatin. Given that the production of silica fibers via the sol-gel process had already been optimized, the focus was on the production of the inner layer of bioreactive fibers. Two polymers were tested, namely polycaprolactone (PCL) and PVA. In order to preserve enzyme activity, different solvents were used to dissolve PCL, but in no case was there evidence of activity of the laccase immobilized within the fibers. On the other

hand, the enzyme remained active in PVA-based fibers. Thus membranes were produced with silica surface layers and a core of PVA with enzyme. These membranes exhibited catalytic activity, allowing the main goal of the work plan to be fulfilled.

Keywords: electrospinning, silica, biocatalytic fibers, PVA, laccase, bioremediation.

Nomenclature

TMOS Tetraethyl orthosilicate; tetraethoxysilane

TEOS Tetramethyl orthosilicate; tetramethoxysilane

PVA Poly (vinyl alcohol)

ILs Ionic Liquids

SEM Scanning electron microscopy

PCL Polycaprolactone

ABTS 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACN Acetonitrile

Choline DHP Choline dihydrogen phosphate

DCM Dichloromethane

DMF *N*,*N*-Dimethylformamide

CL Chloroform

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Chapter 1

1 Introduction

1.1 Bioremediation

The human commitment to create the leading-edge civilization that we live in has led to a sizable industrialization and a massive advance in technology. However this growing affects considerably worldwide environment, and one of the major preoccupations is water pollution. Water is a huge resource on earth but only 3% of it is without salt, two-thirds of which are in the form of glaciers and ice caps.[2]

In the previous decades, there have been numerous undertakings to create cost-effective and eco-friendly options to conventional waste treatment methods. Among all the alternatives bioremediation has emerged as the utmost desirable approach to clean up the environment and to restore its original status.[3]

Microbial remediation is the application of microorganisms to eliminate hazardous wastes. This process can exist on its own in the environment, such as natural attenuation or intrinsic pathways of habitats. Bioremediation can be used at the scene of infection (*in situ*) or on contamination removed from the original site (*ex situ*). If the contamination takes place in the soil or sediments, it can involve land tilling in order to expose the nutrients and make oxygen more highly available to the microorganisms.[2]

Some examples of bioremediation strategies (Figure 1) are phytoremediation (plants), biosorption (dead microbial biomass), bioaugmentation (artificial introduction of viable population), bioaccumulation (live cells) and some others that have been used to render harmless various contaminants, with proportionately low cost, low technology techniques and high public acceptance.

Enzymatic remediation is an alternative to microbial remediation, which relies on the action of biocatalysts capable of degrading the target pollutants. Enzymes can be used intracellularly, or as isolated enzymes.[4]

1.2 Phenolic compounds

Among the water pollutants that cause concern are the phenolic compounds, which are released into the natural water resources from a variety of chemical industries, such as coal and petroleum refining, phenol manufacturing, resin paint, plastic and varnish, textile units using organic dyes, pharmaceutical, among others. [5]

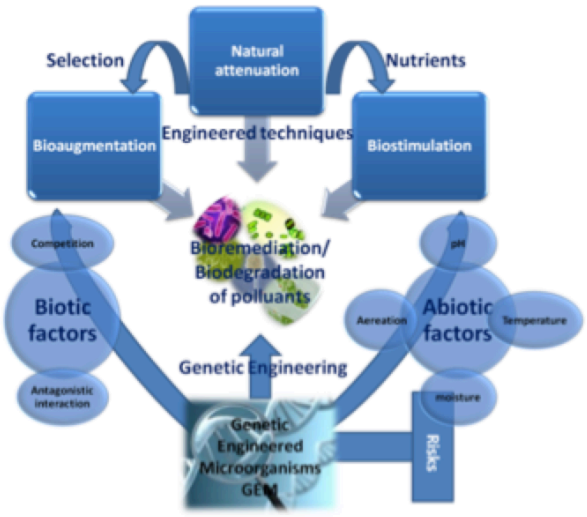


Figure 1.1 Bioremediation of pollutants utilizing biodegradation abilities of microorganisms include the natural attenuation, although it may be enhanced by engineered techniques, either by addition of selected microorganisms (bioaugmentation) or by biostimulation, where nutrients are added. [76]

Phenol (C_6H_5OH), also known as benzenol or monohydroxybenzene, is the simplest member of the group of phenolic compounds. It is an organic compound characterized by a hydroxyl (-OH) group attached to a carbon atom that is part of an aromatic ring. The hydroxyl group makes it soluble in water, and the aromatic ring lends it high hydrophobicity that makes it soluble in organic compounds as well, which is the main factor for phenol penetration in organisms.[6]

Phenols and phenolic compounds have been demonstrated to be the greatly resistant and obstinate to natural degradation, and hence persistent in the environment. This persistence enables long range transportation to different locations, bioaccumulation in human and animal tissue. The infection in humans affects several biochemical functions, creates central nervous system disorders and may lead to a coma collapse. It may also attack the muscular system, provoking pain, convulsions, weakness and tremors. Exposure to phenol may cause also skin burning and erosion. In humans eyes it can cause conjunctional swelling, corneal whitening and finally blindness. Studies have proven that chronic administration of phenols to animals leads to pathological changes in skin, oesophagus, lungs, liver, kidneys and also urogenital tract.[7] The demonstrated changes are mainly induced by lipid peroxidation that is responsible for damage and finally degradation of cell membrane. Other studies point to mutagenic activity of phenol. This compound led to synthesis inhibition and replication of DNA in Hela cells and damage reparation of DNA on diploid

human fibroblasts. E.g. hydroquinone (1.4-dihydroxyphenol) causes damages to chromosomes in human lymphocytes, increasing deletion ratio.[6]

Several phenolic compounds are included in the Environmental Protection Agency (EPA) Priority Pollution List. However there are phenolic compounds, such as oleuropein or hydroxytyrosol, which exist in virgin olive oil, that have a positive effect on certain physiological parameters, such as plasma lipoproteins, inflammatory markers, antimicrobial activity and bone health. Other species with positive effects on human health are phenolic compounds from green tea, such as those from catechin derivatives, flavonols and phenolic acids, whose bioactivity is associated to reduction of severe illnesses such as cancer, cardiovascular and neurodegenerative diseases.[8]

Phenolic dyes are an important group of phenolic compounds. These dyes can be of natural origin or produced synthetically, and may be used to impart colour to various materials, such as textiles, paper, leather, hair, animal hair, photography, and also in food since due to their chemical structure, dyes are resistant to the disappearance of colour by exposure to light, water and other chemical compounds.[9]

1.3 Remediation of phenolic compounds

For phenol remediation there are several approaches, such as adsorption using activated carbon, wet oxidation, also called wet air oxidation, which uses de properties of oxygen to remove organic and inorganic compounds at elevated temperature and pressure, photochemical oxidation, based on supplying energy to organic contaminants by UV irradiation,[10] biological treatments that require the involvement of a specific microorganism to degrade the organic pollutant, enzymatic treatment, and also chemical oxidation that involves the use of chemical agents to eradicate or convert the contaminants to harmless or less toxic compounds.

Phenolic compounds are normally extremely strong reducing agents, and therefore their oxidation takes place in a short time.[11]

1.3.1 Biodegradation of phenolic compounds

Wastewater treatment via a biological pathway has been shown to be an economic alternative to other types of treatments, since the cost of biological material can be 5 to 20 times lower than that of chemical treatments. The microorganism survives with carbon as a source of energy and inorganic salts or nutrients to reproduce and maintain its metabolic activities and cycles.

There are several parameters that affect the biodegradability of an organic pollutant, such as its chemical structure and concentration, pH, temperature, availability of oxygen in case of aerobic processes.[12] The biodegradation procedure leads to conversion of these contaminants to harmless or simple inorganic compounds, such as water and CO_2. One example of a large scale bioremediation process is the clean-up of spilled oil in the Alaskan shoreline of Prince Williams.[13]

The microorganisms most used in remediation are bacteria and fungi. Biodegradations based on free, suspended microbial cells or biofilms have proved to be very efficient because of higher cell concentration per unit volume of bioreactor and higher cell resistance to the toxic effect of phenolics. This method is very promising because it can use natural immobilized cells or biofilms. The latter are superior as a result of large biofilm support surface, hydrodynamic conditions, high mass transfer rates of both oxygen and substrate, and very good contact between the solid and liquid phase.[14]

A relevant fungal bioremediation pathway involves enzymatic systems with suitable mechanisms of action for the target compounds, which under optimal culture conditions are produced fast and in adequate amounts. The demanding step in aromatic compounds metabolism is the destruction of the resonance structure by hydroxylation and division of the benzoid ring, which is carried out by a dioxygenase-catalysed reaction in the aerobic system. A good example of enzymes suitable for this degradation includes oxygenases hydroxylases, peroxidases, laccases, tyrosinases and oxidases, all belonging to the class of oxido-reductases.

Enzyme bioremediation has become an attractive approach for removing chemicals that are harmful to the environment [15]. The performance of enzymes in the degradation of dyes has been widely studied, owning to the fact that enzymes can efficiently cleave the dye structure. [16].

1.3 Laccases

Laccases have been studied since the nineteenth century. In 1883 a laccase was isolated from the Japanese lacquer tree *Rhus vernicifera.* In 1896, Bertrand and Laborde showed the presence of a laccase in a fungus.

Most of the laccases used today are isolated from ascomycetes, deuteromycetes and basidiomycetes fungi, namely from white rot mushroom from wood, such as laccases from *Trametes versicolor*, used in this work, from *Trametes hirsuta*, or *Trametes ochracea* .[17]

1.3.1 Structural and molecular properties

Laccase is a blue copper protein, also known as a polyphenol oxidase, which catalyzes the oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, amino phenols, methoxy phenols, aromatic amines and ascorbate, via the concerted transfer of four electrons from the target species to molecular oxygen, which is thereby reduced to water.

A common laccase reaction is shown in Figure 1.2, where diphenol goes through a 4-electron transfer oxidation reaction that yields an oxygen-centered free radical, which subsequently can be oxidized to a quinone via another 4-electron transfer to molecular oxygen. The radical can also go through a non-enzymatic coupling reaction (polymerization) with the quinone.[17]

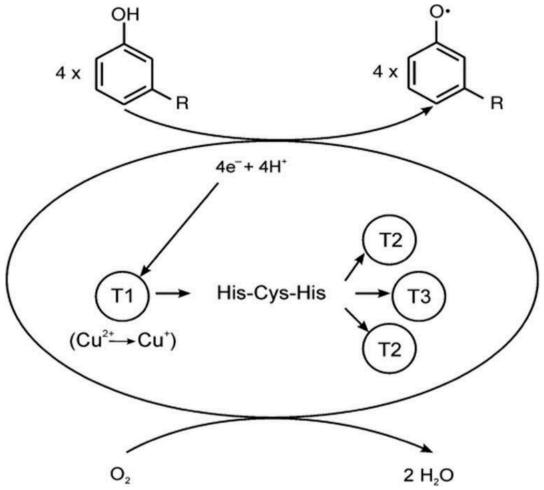


Figure 1.2 Laccase catalytic cycle. [1]

Structurally, a laccase molecule contains four copper atoms per monomer explicar, allocated in three different sites, which play a crucial role in the catalytic mechanism. The four copper atoms can be divided in three different binding sites, indicated as T_1, T_2 and T_3, with different functions (Figure 1.3). T_1 is reduced by the oxidized substrate, and then transfers electrons to the tri-nuclear copper center T_2/T_3, where a third electron transfer step takes place, to molecular oxygen. [18], [19]

The structural orientation of the T_1 copper is organized via two nitrogen atoms from two histidines (His) and a sulphur atom from a cysteine residue (Cys), which is responsible for the blue color of the enzyme. There is a fourth residue involved, often a phneyalanine (Phe). The T_2 copper chain involves two histidines, and T_3 in-

volves six histidines. In the T_2/T_3 coper center there occurs the reduction of oxygen and water release. [20]

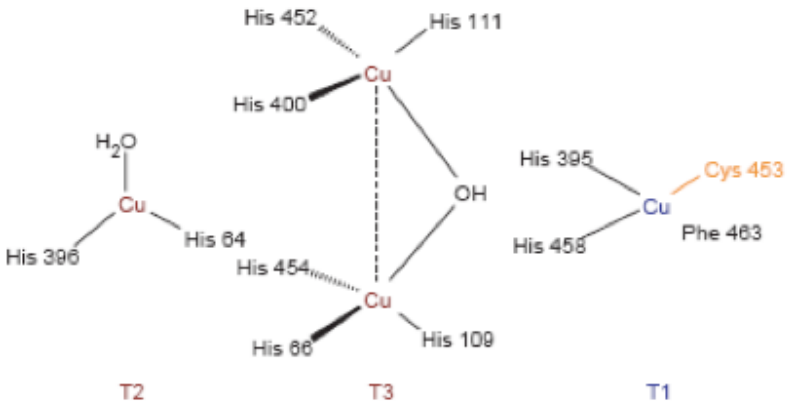


Figure 1.3 - Schematic representation of the copper centers in a laccase.[20]

Fungal laccases have an isoelectric point ranging from 3 to 7, while plant laccases, have an isoelectric point of around 9. This leads to lower pH ranges for optimum catalytic function by fungal laccases than for plant laccases. Fungal laccases function well under acidic conditions, with an optimum pH of around 3.6 to 5.2. Table 1 shows the properties of laccases from different sources.[21]

1.3.2 Mediators

The oxidation rate of a substrate by laccase depends on the difference in redox potential between the substrate and the enzyme, and is favored when the laccase has a higher redox potential and the substrate has a lower redox potential.

When the substrate has a higher redox potential, or when it is of larger size, it may still be oxidized by laccase with the help of a mediator. A mediator is a low molecular weight compound whose purpose is to carry electrons to and from the enzyme, thereby facilitating the oxidation of the substrate (Figure 5). A good mediator must be a good substrate for the enzyme and its reduced and oxidized forms must be stable but must not interfere with the overall enzymatic reaction.[20]

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was the first efficient mediator reported for laccases, and it is most commonly used for these enzymes. This and other laccase mediators are included in Figure 1.4

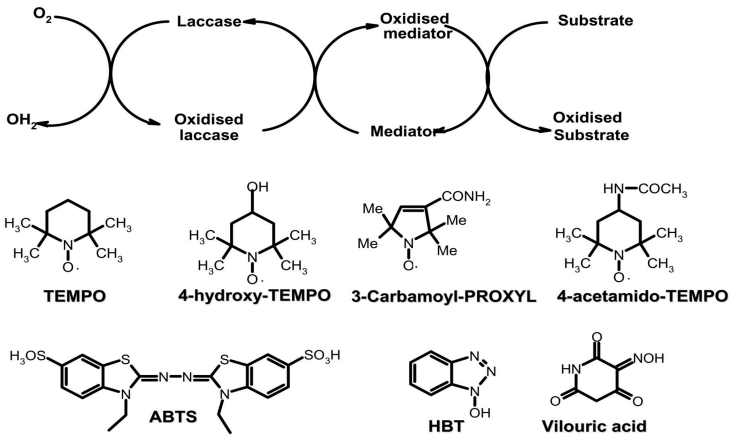


Figure 1.4 Catalytic cycle of a laccase-mediator oxidation system and some example of laccase mediators. [20]

ABTS acts as a co-oxidant that cooperates with the enzyme electron transfer system, and is chemically oxidized via two steps involving the radicals $ABTS^{+}$ and $ABTS^{2+}$ (Figure 1.5). This mechanism is responsible for the green-blue color indicative of enzyme activity. [22]

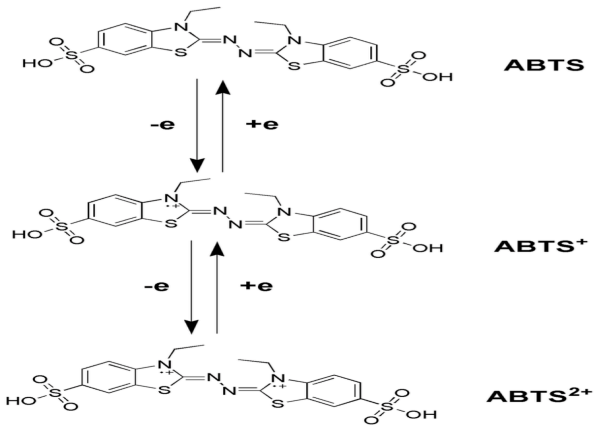


Figure 1.5 Oxidation of ABTS in presence of laccase.[22]

1.4 Enzyme immobilization

The main deficiencies of many important enzymes for their use in industrial applications are their low stability and productivity, and high production costs.

The most frequently used stabilization method is immobilization, which allows for enzyme reutilization, facilitation of separation, and of reaction control. Enzyme immobilization can affect optimum pH and temperature for enzyme function, kinet-

ics parameters, and may strengthen protein structure. Higher thermostability of the enzyme allows conducting processes at higher temperatures and so it reduces reaction time. The advantages and disadvantages of enzyme immobilization are shown in below.[23]

Advantages
Easier separation and recovery of the enzyme and products
Reusability
Increase of thermal stability and resistance against denaturing agents
Reaction can be stopped more easily
Continuous operations can be easier to achieve
Higher flexibility in the design of bioreactors
Prevents the contamination caused by the protein in the final product
Microbial contamination is easier to control
Disadvantages
Lower enzymatic activity caused by the immobilization process
Increase of the Michaelis-Menten constant

Enzyme immobilization was performed for the first time by Nelson and Griffin in 1916. They adsorbed the enzyme invertase on activated carbon and alumina, with the retention of its catalytic activity.[24] However, the development of enzyme immobilization processes started almost fifty years later. In 1969, in Japan, the first industrial process using immobilized enzymes for the production of L-aminoacids was implemented, and soon after, in 1972, in the US, a process was launched using immobilized glucose isomerase for the isomerization of glucose to fructose and production of the so-called high fructose corn syrup. From then on, many new immobilization methods have been reported in the literature.

Different methods based on physical and chemical mechanisms are used for enzyme immobilization on solid materials and gels.[25] The chemical methods include covalent bonds between the enzyme and the support, cross-linking between the enzyme and the support, and enzymatic cross-linking by multifunctional agents.

The physical methods involve adsorption, entrapment of enzymes in insoluble polymeric gels (polymeric entrapment) or in micelles (encapsulation).[26]

Table 1.2 shows a scheme of the classification methods used for enzyme immobilization, with their particular advantages and disadvantages.[27] ,[28],[29].

Table 1.2 Immobilization methods

Immobilization Method	Advantages	Disadvantages
Chemical		
Covalent bond	Dissipated organization of enzymes	Full interaction between the enzyme and any external interface Possible inactivation of the enzyme by any external agent
Single covalent bond with cross-linker	Same mass used for enzyme and support. Facilitated linkage, because of geometrical shape.	Enzyme is attached, but there is no evidence of substantial increase of the enzyme stability Enzyme can present deactivation by conformational changes induced by distorting agents
Multipoint covalent bond with cross-linker	Increase of the enzyme stability by reducing the conformational changes of the enzyme induced by any distorting agent (heat, organic solvents, extreme, pH values)	High need of geometric congruence between the enzyme and the support The mass of the support is considerably higher than the mass of the immobilized enzyme
Enzyme crosslinking	The mass of the enzyme aggregate can be comparable to the mass of the enzyme Simplicity in operation	Interaction between any external interface and the enzyme is conditioned by its position in the aggregate Poor reproducibility and mechanical stability Handling problems
Physical		

Adsorption	No need to functionalize the support (or a more simple method is needed) Immobilization takes place by electrostatic or hydrophobic binding, without the need of adding more compounds	If the substrate is very large or hydrophilic, the near presence of the hydrophobic support surface may generate some steric hindrances, reducing the activity of the enzyme Attachment is weaker than by covalent binding
Entrapment		
Encapsulation	Retains the enzyme in its natural form in capsules of different size Reduces the contact of the enzyme with the external interface (depends on the diffusion of different compounds through the capsule)	Enzymatic retention hardly depends on the porosity of the capsule Enzyme can present deactivation by conformational changes induced by distorting agents
Polymeric entrapment	Retains the enzyme in its natural form, useful for flat surfaces Reduces the contact of the enzyme with the external interface (depends on the diffusion of different compounds through the layers)	Enzyme can present deactivation by conformational changes induced by distorting agents Possible appearance of nanoenvironments that can alter the operation of the enzyme

1.4.1 Immobilization of laccase

Various approaches for laccase immobilization have been studied, such as adsorption, covalent binding, crosslinking and entrapment.

E.g. laccase from Pleurotus sajor-caju was immobilized by adsorption by Rubenwolf et al,[30] for application in a biofuel cell cathode. Rahman et al [31], immobilized a laccase on gold nanoparticles through covalent binding to build a catechin sensor[31]. Laccase from the white rot fungus Coriolopsis polyzona was immobilized for the first time through the formation of cross-linked enzyme aggregates to eliminate endocrine disrupting chemicals, by Cabana et al,[32]. Laccase for decolorisation of a dye was entrapped in lens-shaped, micron-sized semipermeable hydrogel capsules of polyvinyl alcohol [33]

1.4.2 Biocatalytic fibers

Fibers obtained by electrospinning provide a very large area of contact, and this has led to the use of electrospun fibers, or electrospun fiber membranes, to immobilize enzymes. For example Ling Ge et al [34] immobilized glucose oxidase within electrospun nanofibrous membranes for food preservation. A novel food packaging material was obtained by immobilizing glucose oxidase in PVA/CS/tea extract electrospun nanofibrous membrane.[34]

Immobilization via encapsulation often involves diffusion limitations for the chemicals or metabolites that must be exchanged between the enzyme and its surroundings. Immobilization within thin-walled fibers, on the other hand, facilitates the diffusion of chemicals species into and out of the enzyme microenvironment. This and the already referred large surface area are advantages of this immobilization approach, which has been explored in the encapsulation of bacteria [35]. These authors used co-axial fibers, with an outer, protective shell made of silica, and a core made of a polymer suitable for the biological material. For example a laccase immobilization approach in fibers used by Dai Yunrong et al [36], with four types of laccase-carrying electrospun fibrous membranes (LCEFMs), with high laccase-catalytic activity and sorption capacity, were fabricated by emulsion electrospinning. These LCEFMs were composed of beads-in-string structural fibers, with nanoscale pores distributed on the surface and active laccase encapsulated inside. This obtained structure could protect laccase from external disturbance, resulting in that all of the four LCEFMs retained more than 70% of activity relative to free laccase, and after glutaraldehyde treatment, their storage and operational stabilities were definitely improved. [37]

When compared to the use of free enzymes, membrane bioreactors such as those indicated above allow additionally for better product recovery, reuse of enzyme, and continuous operation of the reactor [27]. The operating conditions, such as substrate concentration, immobilization matrix, types of immobilization, and characteristics of the membrane considerably influence the performance of membrane bioreactors [38].

1.5 The sol-gel process

The sol-gel process can be described as a chemical route to synthesize glassy or ceramic materials at relatively low temperatures, based on wet chemistry processing, which involves preparation of a sol, its gelation, and then removal of the liquid within the porous gel.[39]

Use of inorganic ceramic and glass materials in the sol-gel process began as early as the mid-1800s by Ebelmanl and Graham who studied silica gels. Hydrolysis of tetraethyl orthosilicate (TEOS; $Si(OC_2H_5)_4$) under acidic conditions yielded SiO_2 in the form of a "glass-like material".

In the 1950 and 1960s, Roy and co-workers used the sol-gel method to synthesize a large number of novel ceramic oxide matrices, involving AI, Si, Ti, Zr, etc.[40]

During the last few decades, the sol-gel method has been widely used as an alternative technology to prepare a variety of substances in such forms as monoliths, powders, tubes and fibers. [41]

Sol gel processing involves generation of colloidal suspensions (sols), which are subsequently converted to viscous gels, and then to solid materials. A sol is defined as a stable dispersion of solid colloidal particles in a liquid phase. Colloids are classified as solid particles with diameters of 1-100 nm which are not affected by the gravitational force, while a gel is considered as an interconnected network with pore size in sub-micrometer dimensions, and polymeric chains whose average length is greater than 1 μm.[42]

1.5.1. Sol-gel reaction mechanism

Since this thesis involves the synthesis of silica based fibers using the sol-gel method, the sol-gel process will be explained in terms of silica.

It is difficult to divide a sol-gel process into different stages, as different processes mostly occurring simultaneously. However, the occurrence of the following major stages is acknowledged:

- Hydrolysis
- Condensation
- Poly-condensation
- Aging
- Drying

1.5.2.1 Hydrolysis

It consists in a reaction between water and the silica precursor, when hydroxyl ions get attached to the silicon part of the precursor. The major silica precursors used are tetramethyl and tetraethyl orthosilicate (TMOS), (TEOS). The first path of hydrolysis of these precursors is slow and to accelerate it an acid or a basic catalyst is used (Figure 1.6). Mineral acids, ammonia, acetic acid, HF, KF, amines, KOH, titanium alkoxides and vanadium alkoxides are known catalysts of the sol- gel process. [43]

Different extents of hydrolysis reaction in the acid and base catalyzed processes can be justified on the basis of electronic effects. The ethoxy groups have less of an electron donor character than the alkoxy groups, and therefore the reaction rate decreases in acid catalyzed reactions as the positively charged transition state becomes less stabilized when more alkoxy(-OR) groups are replaced by hydroxyl (-OH) groups. [44]

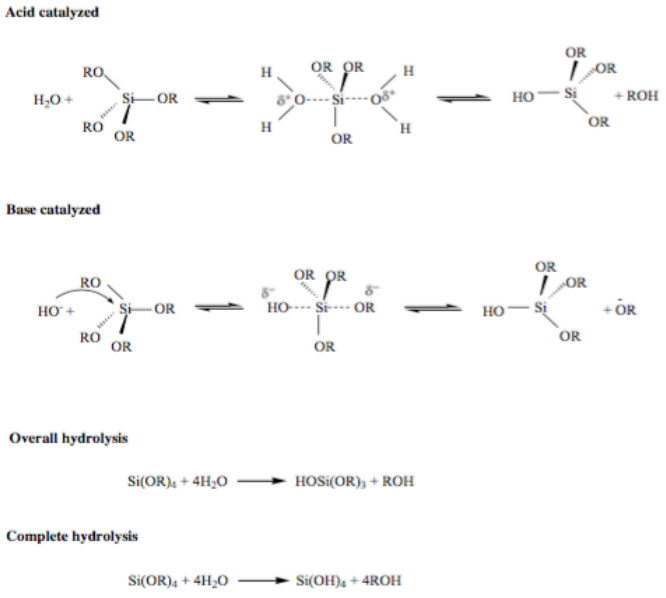


Figure 1.6 Acid and base catalyzed hydrolysis reactions in the sol-gel process. Depending on the type and amount of water and catalyst present, hydrolysis may go to completion so that all the –OR groups are completely replaced by –OH, or stop while the alkoxide is only partially hydrolyzed.[39] [41]

1.5.2.2 Condensation

Generation of siloxane bonds requires a polymerization that can be alcohol condensation (equation 2.1) or water condensation (equation 2.2)

$$Si(OR)_3OH + HOSi(OR)_3 \rightleftharpoons (RO)_3SiOSi(OR)_3 + H_2O \qquad \text{Eq.2.1}$$

$$Si(OR)_3OR + HOSi(OR)_3 \rightleftharpoons (RO)_3SiOSi(OR)_3 + ROH \qquad \text{Eq.2.2.}$$

Condensation takes place to maximize the number of siloxane linkages and minimize the number of terminal hydroxyl (silanol) groups through internal condensation. Although the sequence of condensation requires both depolymerization and presence of monomer (whether already present in the system or generated as a result of depolymerization), the rate of depolymerization is found to be reduced in alcohol-water systems than in aqueous media. The initial condensation reaction is followed by polycondensation that involves formation of more siloxane linkages and a stronger network.[41]

1.5.2.3 Gelation

It is generally observed that the gelation process begins with the formation of fractal (a seemingly irregular structure formed by repeated subdivisions of a basic form and having a pattern of regularity) aggregates that grow until they impinge on each other. These clusters link together through chemical, hydrogen or van der Waals' bonds forming a network which ultimately results in the generation of a giant three dimensional spanning cluster that extends throughout the sol and coexists with the sol phase that contains many small clusters.[39]

At this point, the mixture has a high viscosity but low elasticity and it still holds many sol particles entrapped and entangled in the spanning cluster. The gelation point of any system is easy to observe qualitatively but quite difficult to measure analytically. Generally, gel point or gelation time is defined as the point at which a sol can support stress elastically. Although the reactions that bring about gelation in silica continue long after the gel point because of the large concentration of labile hydroxyl groups on the surface of the silica network [45], it is established that the sharp increase in viscosity that accompanies gelation essentially freezes in a particular polymer structure at the gel point. This "frozen in" structure may change a lot with the passage of time depending on the pH, temperature, solvent and the drying conditions. [46]

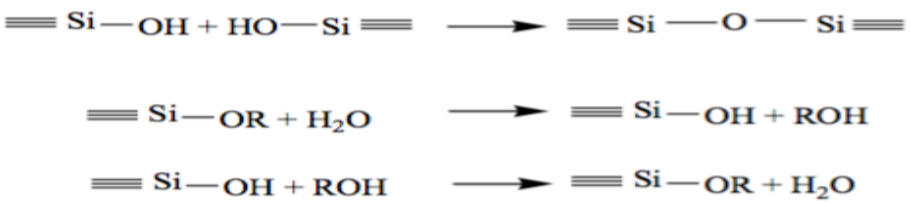
1.5.2.4 Aging

While a gel maintains solvent in its pores, its structure and properties continue to change long after the gel point. This process is called aging. Aging of a gel, also called syneresis, involves maintaining the cast object for a period of time (hour, days or months) completely immersed in either the mother liquor or some other specific solvent used to control the properties of the final product. During aging, polycon-

densation continues, which increases the thickness of inter particle necks and decreases the porosity. The strength of the gel thereby increases with aging. An aged gel is considered quite capable to resist cracking during drying [44].

1.5.2.5 Polymerization

Increase in the connectivity of the network produced by condensation is identified as polycondensation or polymerization. In alkoxide based systems, usually the hydrolysis reaction goes to completion in a short span of time, especially when catalyzed by acidic species. Through Nuclear Magnetic Resonance (NMR) and Raman studies of the silica gels synthesized in alcoholic solutions, it is discovered that the number of bridging bonds keeps on increasing long after gelation [47]. Since the chemical reaction is faster at high temperatures, aging can be accelerated through hydrothermal treatment that increases the rate of condensation reaction. In addition to condensation (eq. 2.3), aging can also result in further hydrolysis (eq. 2.4) or re-esterification, which is the reverse reaction (eq. 2.5) and can be suppressed by using excess water. [41]



Eq.2.3

Eq.2.4

Eq.3.5

1.5.2.6 Drying

Drying of a gel is a crucial step that is mainly governed by the capillary pressure of the solvent, interfacial tension of the solvent, the pore radius and the thickness of a surface adsorbed layer. All these parameters are quite critical as it is the gradient in the capillary pressure during evaporation that causes collapse in the surface structure. The smaller pores are capable to induce greater damage to the gel because of their enormous capillary pressure [48]. The capillary pressure can only be reduced by using solvents with low surface tension or by preparing a gel with larger pore equation 3.5. However, there are chances for a reversible shrinkage, which might occur during drying. The gel springs back during the last part of drying. This reversible shrinkage occurs if gels have stiffness high enough so they are not forced beyond yield by capillary pressure during drying or if the inner surface of the wet gel has been modified i.e., by silylation, to hinder siloxane bond formation during drying. [41]

1.6 Electrospinning

1.6.1 Nanofibers

In the last years there has been a high increase in the use of nanostructured materials in several fields, motivated by the good performance of these materials as well as ease of manufacturing with good control of material properties.

The large surface to mass ratio is one of the important properties of nanofibers. Until now nanofibers have been produced commercially with silica, or alumina, and silica has demonstrated good performance as regards chemical stability and thermal capacity.[49]

1.6.2 Electrospinning process

Electrospinning was first studied by Formhals in the 1930s, and gained a considerable attention in the 1990s.

Electrospinning is a synthesis method that, like drawing, phase separation, template synthesizes, and self assembly, can be used to fabricate, easily and efficiently, fibers with size ranges around 10 to 100 nm.

A typical setup for the electrospinning process consists of three major components:

1. A high-voltage supplier: A direct current voltage in the range of 5–35 kV is necessary to generate electrospun fibers. Alternative current potentials are also used, but in a less widespread way.
2. A capillary tube with a spinneret (needle or pipette of small diameter): The spinneret is connected to a syringe in which the polymer solution or melt is hosted. The syringe is usually associated to a pump to control the rate of fluid flow. As shown in Figure 1.7 the capillary tube and the needle may be arranged vertically, but more often they are arranged horizontally to minimize the effect of gravity on drop formation.
3. A metallic collector: Commonly the collector is a conductor metallic screen. With this type of collecting device, the fibers are generally deposited as a random network. However, for many applications it is desirable to have aligned fibers or a specific arrangement of accumulated fibers. By using patterned electrodes, conductive substrates separated by a nonconductive gap, disc collectors, or other methods, varying degrees of fiber alignment can be achieved.

Many nanofibers assemblies and their associated methods are presented in reference.[49]

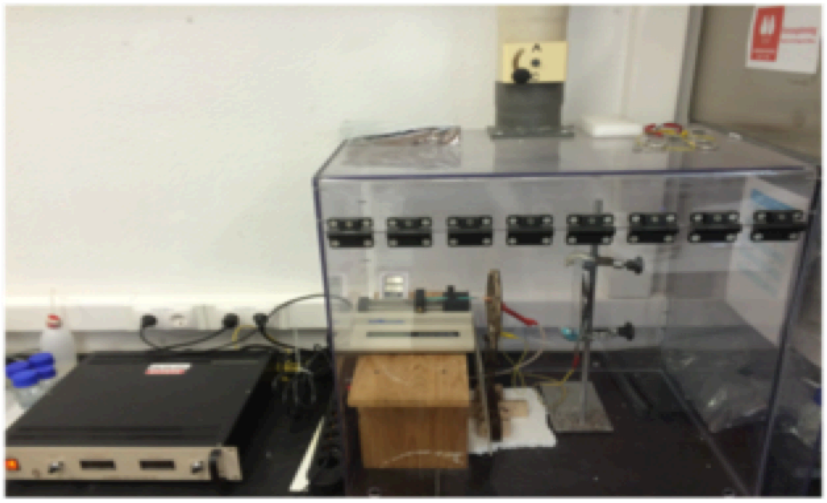


Figure 1.7 Electrospinning setup

1.6.3 Electrospinning parameters

To synthesize a fiber with certain specifications it is necessary to control some parameters, such as solution composition and concentration, viscosity, applied field, flow rate, collector distance, and ambient condition.

The solution composition has an important effect on electrospinning, related to its concentration. If the solution has a lower concentration, an electrospray process can occur, because there are no conditions for fiber elongation.[50] Likewise, if the concentration is higher than required, it is not possible to form Nano scale fibers. The viscosity of the solution is related to its concentration. If the solution has a high viscosity, no fibers can be produced because the solution cannot be pumped through the needle, whereas a low viscosity leads to electrospray.[51]

The distance between the end of the needle and the collector affects the solvent evaporation rate, enabling fiber production. If this distance it too short, there is no time for solidification, whereas a higher distance may provide disruption of the polymer elongation and lead to formation of beads.

The flow rate is another important parameter, which affects the morphology of the fibers. With a very slow flow rate, the fibers obtained are thin, because of complete evaporation of the solvent before reaching the collector. A high flow rate withdrawal from the nozzle produces a thick fiber with beaded structure, due to the inability of the jet to dry before reaching the collector. [52]

A crucial aspect to consider in the electrospinning process is the applied voltage. The applied voltage turns the droplet that comes from the nozzle into a jet, which lands as a fiber on the collector. If the applied voltage increases, so does the electrostatic repulsive force on the fluid jet that mostly results in contraction of the fiber diameter. One might assume that the voltage of the electrospinning setup controls the fiber diameter, but the latter varies also with the nature and concentration of the polymer, and the tip to collector distance. [53]

The environmental conditions also affect the solution properties, as well as the solvent evaporation rate, which in turns results in changes in fiber morphology. A higher temperature, on the other hand, results in faster evaporation of the solvent, making the fibers thinner. The humidity values also influence fiber morphology, since a more humid atmosphere results in slow solvent evaporation that leads to the formation of thicker fibers, while a less humid atmosphere accelerate the evaporation rate, which may become so fast that the solute clogs the needle of the spinneret.[49]

1.6.4 Co-axial Electrospinning

A coaxial fiber is essentially built from two different materials: one on the outside of the fibers, denominated the "shell", and the other on the inside of the fibers, called the "core". Coaxial fibers can have different mechanical, physical and degradation properties in the core and in the shell, depending on their respective compositions.[54]

The only difference between the normal electrospinning setup and the coaxial electrospinning setup is the needle apparatus. In terms of the material selection, the solvents used in each solution must be immiscible, and the shell solution must be highly electrospinnable. The voltage and the distance between the collector must also be optimized for the coaxial electrospinning process. The increase of voltage in coaxial electrospinning could provide a separation of the core and shell, despite a low voltage also could produce a good fiber production. [55]

1.7 Ionic liquids and ion jelly

1.7.1 Ionic Liquids

One of the approaches in this work is to use an ionic liquid in the manufacture of co-axial fibers.

Studies on ionic liquids started in the nineteen-century with the formation of an ionic liquid in the course of a Friedel-Crafts reaction that produced a "red oil",

later discovered to be composed of a carbocation and a tetrachloroaluminate anion. In 1914 an ionic liquid was synthesized for the first time (ethylammonium nitrate), by Paul Walden. Interest in ionic liquids grew, as it became clear that they could be useful for many applications, such as electrolytes for batteries and metal electroplating systems. [56]

Ionic Liquids are substances fully made of ions, with a melting point below 100 ºC. Ionic liquids are a complex network of ions mediated by non-specific interactions (electrostatic charges) and specific interactions (hydrogen bonding). The asymmetric form of the ions and their bulky/non-linear association creates a reduction in bond strength between the ions and a corresponding reduction in the columbic interactions, which results in a lowering of the salt melting point [57]. A subclass of these materials known as '*room temperature ionic liquids*' (RTILs) have moved to the forefront of ionic liquid research due to their melting temperatures being below 30°C. These liquids exhibit the most favorable properties for solvent use due to their formation of stable, non-volatile liquids in ambient room conditions while also maintaining their stability over large temperature ranges. Ionic liquids differ from other

classes of solvents due to the binary, charged nature of the liquids when compared to atomic or molecular solvents.[58]

Ionic liquids can be recycled, reducing chemical waste and increasing the lifetime of the liquids, further adding to the 'green' aspects of ionic liquids [59]. For example, the hydrophobic nature of certain ionic liquids allows for the addition of hydrophobic compounds that, once reacted, form hydrophilic products. The addition of water then allows for the removal of pure products and the removal of the ionic liquid for use once again a reaction medium. [60]

The vast numbers of available ions results in a theorized number of possible ionic liquids to be over 1014 combinations[61].For this reason, ionic liquids have become known as "designer" [56], whose customizability allows for the ability to endow specific properties upon the final liquid product.

In this work, it was used choline di-hydrogen phosphate (choline DHP) as the ion jelly ionic liquid, which is shown to give stability to biological substances, namely proteins such as ribonuclease and cytochrome c. The structure of choline DHP is shown in Figure 1.10.

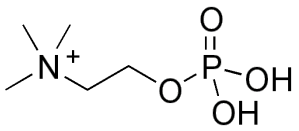


Figure 1.8 cholineDHP structure
(http://www.churchoffreethought.org/freethought_101.php)

Ionic liquids can lend conductivity to polymeric materials, such as those used for enzyme-entrapment. This can be particularly useful when the enzyme catalytic mechanism involves charge transfer, as in the case of reactions catalysed by laccases. There are some reports on ionic liquids decreasing enzyme activity, but this may be circumvented by the increase in enzyme stability that they impart. [62].

1.8 Ion Jelly

The ion jelly interpretation starts with a combination of ionic liquid (IL) with a gel polymer, and can be divided into three major types: gelation of ILs within polymers/ biopolymers, in situ polymerization of vinyl monomers in ILs, and polymerization of ILs containing polymerizable groups. These approaches are very interesting ways to achieve higher ionic conductivity without liquid components. [63], [64] [65], [66].

The procedure used in this work is based on gelation, which is a simple method that permits a good compromise between the retention of the IL and its mobility inside the polymeric network. This approach combines the mechanical properties of polymers with physico-chemical properties that are relevant for current solid-state polyelectrolytes in energy devices, such as dye-sensitized solar cells, supercapacitors, lithium ion batteries, and fuel cells [67].

1.9 Multi layered fiber membrane reactor

The initial objective of the work plan behind this thesis was to follow an approach similar to that of Tong et al. (2014) and fabricate, using coaxial electrospinning, reactive fibers containing a laccase for phenol bioremediation, comprising an ion jelly core entrapping the enzyme, and a silica-based, mechanically resistant, porous shell.

The choice of silica was motivated by its thermal stability, relative inertness, low cost and biocompatible sol-gel transition at room temperature. Silica was combined with polyvinyl alcohol (PVA), which optimizes the sol-gel solution in different aspects. PVA acts as a thickening agent, providing viscosity adjustment to the elec-

trospinning solution, protects the biological material from the gelling silica precursors, adds flexibility to the membrane, and increases its porosity.

The choice of the ion jelly for the core was motivated by the good performance of oxido-reductases immobilized within this material.

Several difficulties that arose during the implementation of the workplan described above led to a different concept of fiber membrane, based on the production of a membrane layer by layer. The outermost top and bottom layers were made of silica-PVA fibers, and the inner layer, containing the enzyme, was no longer made of ion jelly, but was fabricated with wither polycaprolactone (PCL), or PVA.

Chapter 2

2 Materials and methods

2.1 Enzymatic studies

The enzymatic studies were performed with methyl red indicator with pH limit (4,4-6,2) (Merck), choline dihydrogen phosphate, 98% (ABCR), acetonitrile (Carlo Erba), 1-butyl-3-methylimidazolium dicyanamide, 98% (io-li-tec), 1-butyl-3-methylimidazolium tetrafluoroborate, 99% (io-li-tec), 1-ethyl-3-methylimidazolium ethyl sulphate, 99% (io-li-tec), sodium phosphate buffer prepared in lab with concentration of 100 mM, pH 5 and 6.

Enzyme: laccase from *Trametes versicolor* that did not require any step of purification and was obtained from sigma Aldrich (DE), (0.5 U/mg).

Enzyme mediator: 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt (≥98% HPLC) was supplied by Sigma-Aldrich (DE).

2.1.2 enzyme activity in aqueous solution

UV/Visible spectroscopy was used (Beckman coulter, DU 800).

To one cuvette 930 μL of 0.5 mM ABTS in 100 mM phosphate buffer at pH 6 was pipetted, and 50 μL of mM phosphate buffer at pH 6 was added to set the absorbance = 0 line ((λ = 418 nm). Previously homogenized 20 μL of a 2.58 mg/mL laccase solution was added and the absorbance was measured every 5 s for 210 s, keeping the solution homogenized (ε = 36000 mM^{-1} cm^{-1}).

2.1.3 enzyme activity in non-aqueous solution

0.5 mM ABTS in 100 mM phosphate buffer at pH 6 was used to set the absorbance = 0 line ((λ = 418 nm). Then 3 mg of laccase was added and thoroughly mixed. Stopping agitation made the enzyme precipitate very quickly. Then 1 mL of the solution was withdrawn into a cuvette and absorbance was measured. This step was repeated every 1 min for 8 minutes, and in between samples mixing continued.

2.1.4 effect of ACN in $ABTS^{+}$ radical stability

After completing an aqueous solution laccase activity assay as indicated earlier, the cuvette was set aside and periodically absorbance was measured. To obtain an

accurate measurement, the solution in the cuvette was diluted. After color stabilization occurred, 200 μL of the solution was mixed with 1.5 mL of ACN. Periodically, during 1 h, absorbance was measured to see if the blue colour imparted by $ABTS^{\cdot}$ disappeared through addition of a nonaqueous solvent.

2.1.5 enzymatic degradation of a dye

A 24 mg/L of methyl red solution was prepared in 10 mM phosphate buffer at pH 6, and was diluted so that its absorbance was close to 1,2.

To one cuvette 920 μL of diluted methyl red solution was pipetted, to set the absorbance = 0 line ((λ = 430 nm). Previously homogenized 80 μL of a 24.6 mg/mL laccase solution was added and the absorbance was measured every min for 15 min, keeping the solution homogenized (ε = 23360 mM^{-1} cm^{-1}).

2.2 PVA Silica solutions for electrospinning

2.2.1 Chemicals

TMOS (tetramethyl orthosilicate; Si(OCH3)4; wt = 152.25 g/mol; density = 1.032 g/cm3; ≥ 98%), TEOS (tetraethyl orthosilicate; Si(OC2H5)4; wt = 208.33 g/mol; density = 0.933 g/cm3; ≥ 99%), methanol (wt = 32.04 g/mol; density = 0.792g/cm3; 99.8%), ethanol (wt = 46.07 g/mol; density = 0.789 g/cm3), polyvinyl alcohol (PVA; 87-90% hydrolyzed, wt = 30000-70000 g/mol, taken to be 50000 g/mol in all calculations) , Enzyme: laccase from trametes versicolor from sigma Aldrich (DE), (0.5 U/mg), hydrochloric acid (HCl; wt = 36.46 g/mol; density = 1.19 g/cm3; ≥ 38%)

2.2.2 2.2.2 TMOS-based sol-gel

The procedure by Tong et al [35], was adapted for this work. The relative amounts of the various components of the sol-gel solution were varied and these changes were assessed by the ability to produce fibers by electrospinning.

PVA concentration was set as 18 wt% PVA was dissolved in deionized water at 60 ºC while stirring in a water bath for 50 min. A typical sol-gel solution with 1000 μL TMOS and different concentrations of methanol and water was prepared and thoroughly mixed. Then a HCl solution was added dropwise to a concentration of 4M. The solution was stirred at 60 ºC in a water bath for one hour. Afterwards 0,65 g of PVA per each ml of TMOS solution was slowly added to the TMOS solution and was thoroughly mixed for one hour by stirring in a water bath at 60 ºC.

Table 2.1 Concentrations used to prepare sol-gel solution with PVA.

Solu-tions	TMOS (µL)	Methanol (µL)	H2O (µL)	HCL (µL)	PVA (18%)
1.	1000	300	215	13,3	0,65 g per 1 ml of TMOS
2.	1000	215	300	13,3	0,65 g per 1 ml of TMOS
3.	1000	257	257	13,3	0,65 g per 1 ml of TMOS

2.2.3 2.2.3 TEOS-based sol-gel

For the preparation of TEOS-based sol-gel solutions, the procedure adopted was also based on the work of Pizarda et al [68]. The procedure was the same as in section 2.2.2 with the required alteration of solutions regarding that for TEOS it is hydro-lysed by ethanol.

Table 2.2 Concentration used to prepare sol-gel solution with PVA.

Solu-tions	TEOS (µL)	Ethanol (µL)	H2O (µL)	HCL (µL)	PVA (18%)
1.	1000	203	203	13,3	18 (0,65 g per 1 ml of TEOS)
2.	1000	175	240	13,3	18 (0,65 g per 1 ml of TEOS)
3.	1000	240	175	13,3	18 (0,65 g per 1 ml of TEOS)

2.2.4 Parameters variation

In this section the concentrations and percentage of HCl and PVA used were varied, while following exactly the same procedures as described in sections 2.2.2 and 2.2.3. Table 5 shows the changes in parameters.

Table 2.3 Variation of HCL and PVA concentration for sol-gel solution.

HCL (M)	0,001	0,01	0,1	1	2	4
PVA wt (%)	8	10	14	16	18	25

2.2.5 TEOS and TMOS established sol-gel with PVA solutions with enzyme

To prepare silica fibers with entrapped enzyme, a procedure as described in the previous sections was carried out. Then a 4 mg/mL of enzyme with 100 mM phosphate buffer at pH 6.0 was prepared. 100 μL of the enzyme solution was added to the silica solution, mixed well with the tip, and introduced in the electrospinning syringe right before the start of the electrospinning process.

2.3 Coaxial electrospinning solutions for core-shell fibers

2.3.1 Chemical used

In addition to the chemicals referred earlier, the following chemicals were also used: sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$; wt = 137.99 g/mol; ≥ 98%), from Sigma, gelatin from porcine skin (90-110 Bloom), from Oxoid, 250 BLOOM, the water used was Millipore water.

2.3.2 Core solution of the fiber based on Ion jelly

Based on previous work by Santos et al [65], and with some adaptations to create good conditions for electrospinning, the solutions for the core of the fibers had different concentrations of gelatine, choline DHP mixed with deionized water or 100 mM phosphate buffer at pH 7. The various components were mixed in a water bath at 60 ºC under stirring.

Table 2.4 Concentration used for ion jelly solution

Solutions	Gelatin (μL)	Choline DHP (μL)	H_2O (μL)	Phosphate buffer, pH7 (μL)
1.	166	112	1000	1000
2.	50	39	1000	1000
3.	85	57	1000	1000
4.	100	64	1000	1000
5.	125	77	1000	1000
6.	200	90	1000	1000
7.	250	125	1000	1000

2.3.3 Core solution of the fiber based on Ion jelly with fish gelatine

The procedure and formulations were similar to those described in the previous section. Fish gelatin powder was used instead and there was no need to heat the solution, because fish gelatin dissolves at room temperature under stirring for 30 min.

2.3.4 solution based on Ion Jelly and enzyme

After preparing one of the core solutions described earlier, there was a waiting time of around 30 min until the temperature of the solution dropped to around 35 ºC. To the gelatin solution was then added 100 μL of a 10 mg/mL of enzyme in 100 Mm phosphate buffer at pH 7, well mixed with the tip. The resulting solution was introduced in the syringe and immediately the electrospinning process started.

2.3.5 silica-shell/ Ion jelly-core fibers

The next step was using the solutions tested earlier in co-axial electrospinning. The sol-gel solution was prepared with 5.0 mL of TMOS, 1.50 mL of methanol, 1,225 mL of water and 67 μL of HCl (4M), in a water bath at 60 ºC, under magnetic

stirring, for 60 min. Then the PVA solution at 18% was prepared, and 0,6 g for each ml of TMOS were added to the sol-gel solution. The resulting mixture was stirred and kept in a water bath at 60 ºC for 1 h 30 min.

Then the gelatin solution was prepared. After several experiments, the following formulations were selected, due to better electrospinning characteristics:

1 - 255 mg of porcine gelatin, 171 mg of choline DHP and 3 ml of deionized water, dissolution at 60 ºC with stirring for 60 min.

2 - 300 mg of fish gelatin, 100 mg of choline DHP and 2 mL of deionized water, dissolution at 60 ºC with stirring for 60 min.

2.4 Multi Layer membrane production

2.4.1 Chemical used

Poly(vinyl alcohol), 95% hydrolysed, Natural Gelatin from Germany, Polycaprolactone average M_n 80,000, laccase from *trametes versicolor* obtained from sigma Aldrich (DE), (0.5 U/mg), TMOS (tetramethyl orthosilicate; Si(OCH3)4; wt = 152.25 g/mol; density = 1.032 g/cm3; ≥ 98%), TEOS (tetraethyl orthosilicate; Si(OC2H5)4; wt = 208.33 g/mol; density = 0.933 g/cm3; ≥ 99%), methanol (wt = 32.04 g/mol; density = 0.792g/cm3; 99.8%), ethanol (wt = 46.07 g/mol; density = 0.789 g/cm3), polyvinyl alcohol (PVA; 87-90% hydrolyzed, wt = 30000-70000 g/mol, taken to be 50000 g/mol in all calculations)

2.4.2 multilayer membrane preparation

The top and bottom layers were prepared as in 2.3.5, but the total volume of solution for each layer was 5 times lower (proportion for 1 mL TMOS).

For the inner layer, two different polymers were tested, as seen in the next sections.

2.4.3 PCL fibers for multilayer membranes, with and without enzyme

The PCL solutions were prepared according to Xiaohong Qin et al,[69] and some alterations made, using dichloromethane (DCM), dimethylformamide (DMF) or chloroform, and the amounts of compounds used are shown in the table.

To ensure polymer dissolution, the mixtures were left under stirring for 24 h at room temperature.

To prepare fibers with enzyme, to the PCL solution was added 100 μL of 30 mg/mL of laccase solution in 100 mM phosphate buffer pH 6.

Table 2.5 Preparation of PCL solution with different solvents and concentrations.

PCL Solutions	PCL (mg)	DMC (μL)	DMF (μL)	Chloroform (μL)
1.	130	500	500	-
2.	130	750	250	-
3.	100	-	-	1000

2.4.4 PVA fibers for multilayer membranes, with and without enzyme

The PVA solutions were prepared according to Zs. K. Nagy et al.[70] PVA was dissolved in 5 ml of deionized water at a concentration of 18% w/w (1,125 g added to 5 ml of water). To ensure complete dissolution, the mixtures were left under stirring for 4 h at 60 ºC.

To prepare fibers with enzyme, to the PVA solution was added 100 μL of 30 mg/mL of laccase solution in 100 mM phosphate buffer pH 6.

2.5 Electrospinning experimental SET-UP and condition

2.5.1 conventional electrospinning setup

A Glassman EL 30kV high-voltage source was used directly linked to the metallic needle. To inject the solution to a fixed collector, a KDS100 pump from KD Scientific was used. The needle diameters can be varied depending on the requirements and solution composition. In this work, all mixtures were electrospun using 25G needles from ITEC (0.26 mm inner diameter). The collector was fixed and covered with aluminum foil. The set-up was placed in an acrylic box, in order to control the environmental parameters. The temperature and humidity inside the acrylic box were controlled by a commercial electric heater and air-conditioning in the laboratory.

2.5.2 TMOS sol-gel fibers with PVA

Table 2.6 Electrospinning setup for TMOS fibers with PVA.

Solution from section 2.2.2	Temperature (°C)	Flow rate (mL/h)	Distance (cm)	Humidity (%)	Voltage (kV)	Deposition time (h)
1.	22-28	0,4	18	25-30	18-20	1
2.	22-28	0,4	15	25-30	18-20	1
3.	22-28	0,3	18	25-30	18-20	0.5

2.5.3 TEOS sol-gel fibers with PVA

Table 2.7 Electrospinning setup for TEOS fibers with PVA.

Solution from section 2.2.3	Temperature (°C)	Flow rate (mL/h)	Distance (cm)	Humidity (%)	Voltage (kV)	Deposition time (h)
1.	25-30	0,2	18	25-30	18-20	1
2.	25-28	0,3	15	25-30	18-20	1
3.	25-30	0,3	18	25-30	18-20	0.5

2.5.5 Parameters variation (HCL concentration and PVA %); TEOS and TMOS established sol-gel with PVA solutions with enzyme

In this section the electrospinning setups were exactly as used in previous set-up for TMOS/TEOS fibers production.

2.5.6 Ion jelly fibers with porcine gelatin

Table 2.8 Electrospinning setup for Ion jelly solutions.

Solution from section 2.3.2	Temperature (◦C)	Flow rate (mL/h)	Distance (cm)	Humidity (%)	Voltage (kV)	Deposition time (h)
1.	40-45	0,2	18	35-45	18-20	1
2.	40-45	0,3	16	35-45	18-20	0,3
3.	40-45	0,3	16	35-45	18-20	0,3
4.	40-45	0,3	16	35-45	20-22	0,3
5.	40-45	0,3	17	35-45	18-20	0,5
6.	40-45	0,3	18	35-45	18-20	1
7.	40-45	0,3	18	35-45	18-20	1

2.5.7 Ion jelly fibers with fish gelatin

Table 2.9 Electrospinning setup for Ion jelly solutions with fish gelatin.

Solution from section 2.3.3	Temperature (◦C)	Flow rate (mL/h)	Distance (cm)	Humidity (%)	Voltage (kV)	Deposition time (h)
1.	30-35	0,2	18	35-45	18-20	1
2.	30-35	0,3	16	35-45	18-20	0,22
3.	30-35	0,3	16	35-45	18-20	0,2
4.	30-35	0,3	16	35-45	20-22	0,2
5.	30-35	0,3	17	35-45	18-20	0.5
6.	30-35	0,3	18	35-45	18-20	1
7.	30-35	0,3	18	35-45	18-20	1

2.5.4 Co-axial electrospinning setup

This procedure is implemented using mostly the same set-up as for conventional electrospinning, but with a different need apparatus, a special co-axial block from Linari Engineering. Two different needles are used, requiring one pump for each needle and a specific tubing material that is used in biomedical applications.

For the sol gel solution it was used the outer needle, and for the core solution, the inner needle.

Table 2.10 Co-axial electrospinning setup.

	Temperature (°C)	Distance (cm)	Humidity (%)	Voltage (kV)	Deposition time (h)	Flow rate (mL/h) Shell	Flow rate (mL/h) Core
1.	35-40	18	35-45	22	1	0.2-0.4	0.5-0.7
2.	30-35	18	32-38	22	1	0.2-0.3	0.5-0.8

2.5.3 Polymer fibers for multilayer membranes

Table 2.11 PCL fibers for multilayer membranes electrospinning setup.

PCL Solutions	Temperature (°C)	Flow rate (mL/h)	Distance (cm)	Humidity (%)	Voltage (kV)	Deposition time (h)
1.	Rom temperature	0,2	15	25-30	16	1
2.	Rom temperature	0,2	15	25-30	16	1
3.	Rom temperature	0,3	15	25-30	17	1

Table 2.12 PVA fibers for multilayer membranes electrospinning setup.

PVA Solutions	Temperature (°C)	Flow rate (mL/h)	Distance (cm)	Humidity (%)	Voltage (kV)	Deposition time (h)
(18 %)	Rom temperature	0,6	15	30-35	18-20	1

2.6 Enzymatic studies with immobilized laccase

2.6.1 Ion jelly films with choline DHP

Prior to producing fibers with ion jelly cores, the core solutions were used to prepare films. The ionic liquid and gelatin with 100 mM phosphate buffer at pH 6 were mixed for 1 h in a water bath at 60 ºC. The solution was then allowed to cool down to around 35 ºC, at which point 100 μL of a 1 mg/mL enzyme solution were added and well mixed with the tip.

To perform this study, five solutions from table 2.4 were used, namely solutions 1, 3, 5, 6 and 7.

Enzyme activity was tested by adding 100 μL of 0,5 mM of ABTS dissolved in 100 mM phosphate buffer in pH 6.

2.6.2 PCL or PVA fibers

As a quick colorimetric assay, a small portion of the membrane containing enzyme was detached and a small amount of ABTS solution was deposited on it, and observed for blue colour development.

2.6.3 Multilayer membrane tests

2.6.3.1 Modified Lowry test

This method gives the amount of protein in the membrane.

For this assay it was weighed 6,9 mg of membrane, which was ground to powder form. To this powder was added 1 ml of NaOH 1M solution, and the mixture was immersed in a 100 °C bath for 10 min, which brought about total dissolution of the powder. The resulting solution was cooled down in an ice bucket.

The Lowry solution consists of a the mixture of three prepared solutions: 250 μL of solutions 2 and 3, solution A to a final volume of 25 mL. In this mixture the solution A is used as a solvent solution and solutions 2 and 3 as solute solutions.

1. **Solution A** mixture of sodium carbonate (30 g/L) and sodium hydroxide (4 g/L)
2. **Solution 2** sodium and potassium tartrate 2% (p/v)

3. **Solution 3** pentahydrate copper sulfate 2% (p/v)

To 2 mL Eppendorfs were pipetted 0, 20, 50, 100, 150 and 200 μL of standardized BSA solution at 200 μg/ml, and in the first five cases deionized water was added to make up 200 μL volume. To an additional 2 mL Eppendorf was pipetted 200 μL of the membrane solution. To all 7 eppendorfs were added 1 mL of Lowry solution, and after vortexing followed by a 10 min wait, 200 μL of Folin-Ciocalteau solution was added. Again all solutions were vortexed, followed by a 40 min wait before absorbance was measured at 750 nm.

2.6.3.2 membrane activity test with ABTS

Two pieces of membrane were cut off for testing enzyme activity, and were immersed in 100 mM phosphate buffer at pH 6, or ACN, containing 0.5 mM ABTS, and checked for the appearance of the characteristic blue colour.

2.6.3.3 Enzyme leaching test

20 mg of membrane was detached and immersed in 5 ml of 100 mM phosphate buffer pH 6, for 3 days. Afterwards the membrane was withdrawn, and 200 μL of the solution were collected for the Lowry assay. Then, 100 μL of 5 mM ABTS solution were added, and absorbance was measured.

2.6.3.4 activity of entrapped enzyme in aqueous and nonaqueous media

For this assay 20 mg of the membrane were immersed in 5 ml of 100 mM phosphate buffer at pH 6. Then, 5 mM ABTS solution was added, so that the concentration of ABTS was the same as in the previous assay, and absorbance was measured

2.7 Fiber characterization using Scanning Electron Microscopy

Is a type of electron microscope that creates images by the electrons emitted when the primary electrons coming from the source strike the surface and are inelastically scattered by atoms in the sample. SEM images have a characteristic 3-D appearance and are therefore useful for judging the surface structure of the hybrids.[71]

The microscope used in this study was a Zeiss Auriga. The samples were set in a carbon conductive tape purchased from Agar Scientific.

Figure 2.1 SEM disc with coated samples.

2.7.1 Energy dispersive spectroscopy (EDS)

This method is combined with SEM, which detects chemical elements and produces a profile of the regions of each material detected. The instrument used was, Oxford instrument EDS.

2.7.2 Membrane solubility test

The solubility of the membranes in aqueous medium was measured by immersing 20 mg of membrane in 10 mL of deionized water for 3 days, recovering the membrane and drying it in an oven at 40 °C for 2 days. The weight of the membrane was measured, and compared to the weight before immersion in water. The latter was tested for laccase activity with ABTS.

Chapter 3

Result and Discussion

3.1 Free enzyme studies

3.1.1 Laccase activity in aqueous medium

Figure 3.1 shows one of the assays to determine laccase activity in aqueous medium, using ABTS as substrate.

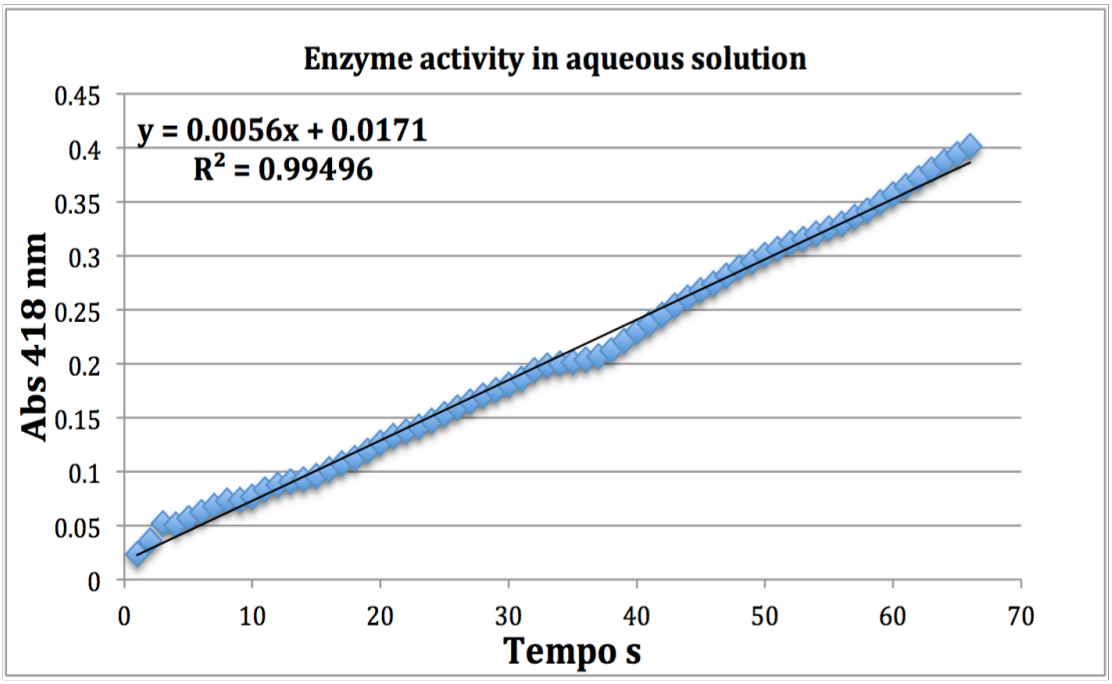


Figure 3.1 Absorbance as a function of time for the oxidation of ABTS in 100 mM phosphate buffer at pH 6.

The data in the figure together with data obtained for replicate measurements led to an enzyme activity of 3,0 nmol $s^{-1}mg^{-1}$ (= (slope /(ε x b)) x reaction volume/mass of enzyme, in appropriate units).

3.1.2 Enzyme activity in non-aqueous medium

Acetonitrile (ACN) was used instead of aqueous buffer. From replicate measurements, enzyme activity was calculated to be 0,06 nmol s-1 mg-1. This shows that ACN had a negative effect on enzyme activity, although the enzyme reacted promptly to give the characteristic blue colour. Figure 3.2 shows the data obtained for one of the assays conducted in this solvent.

This result can be explained by the polarity, which affects the solvation. Since ABTS is a polar compound is known that this is easily stabilized also in a more polar medium, in this case in aqueous medium.

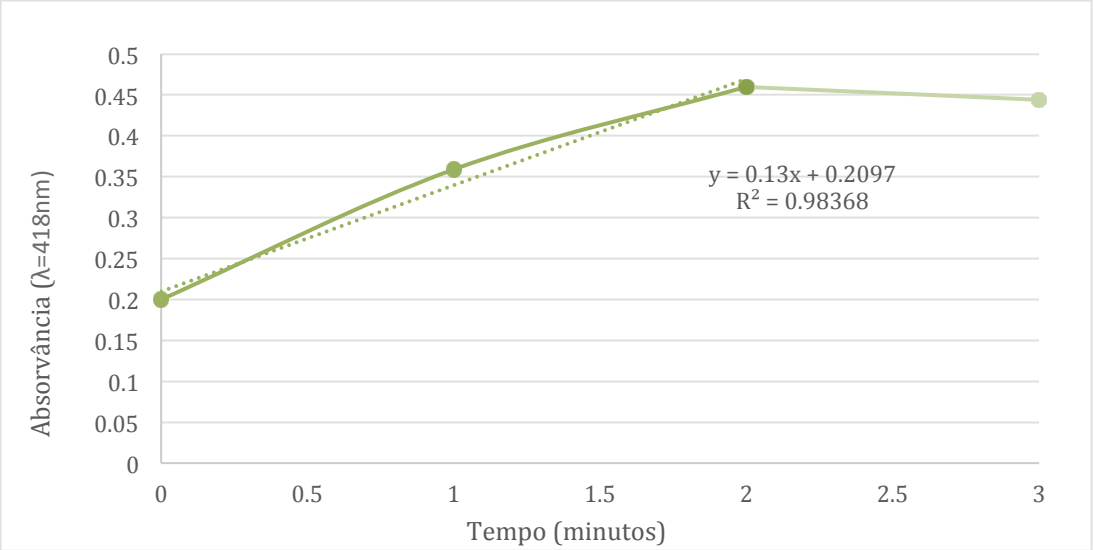


Figure 3.2 Absorbance as a function of time for the oxidation of ABTS in acetonitrile.

3.1.3 Effect of ACN in ABTS^{+} radical stability

Since experiments were performed to determine the enzyme activity in ACN, it was necessary to evaluate whether the radical ABTS·+ becomes unstable and, therefore, causing a decrease in absorbance in a non-aqueous medium. To this end, ACN was added to the radical formed in an aqueous medium. From the results obtained it was found that the solvent does not affect the stability of the radical during the time scale of the measurements of enzyme activity in ACN. This shows that the lower enzymatic activity measured in ACN is due to intrinsically lower activity of the enzyme in this solvent.

3.1.4 Enzymatic degradation of a dye

The quantification of dye degradation by laccase over time is shown in Figure 3.3. The results obtained lead to an enzyme activity of methyl red degradation. Using the same method as used previously the enzyme activity is 0,18 nmol $s^{-1}mg^{-1}$.

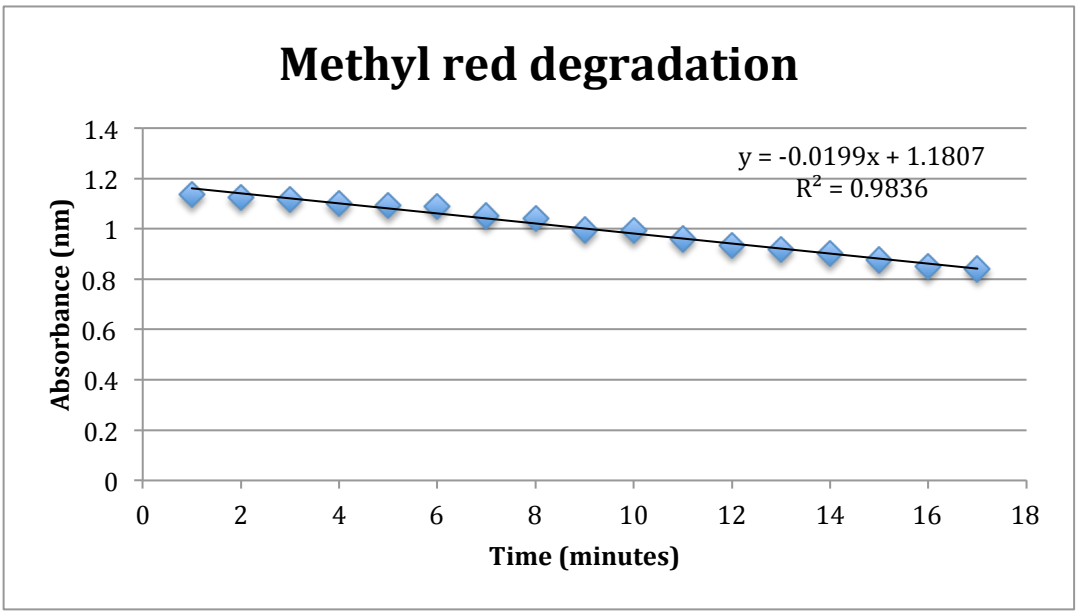


Figure 3.3 graphic representation of the absorbance over time of the enzymatic degradation of methyl red dye by the Trametes versicolor laccase at room temperature.

3.2 PVA Silica solutions for electrospinning

Synthesis of PVA silica hybrid nanofibers and effect of intermolecular interactions on the reduced water solubility of the resultant fibers are elucidated at this stage of research. The main objective of this study was to produce a variety of less hydrophilic nanofibers by using different mass ratios of PVA and silica.

3.2.1 TMOS-based sol-gel

For this study the concentrations of the silica precursors, HCl and PVA were maintained, while the water and methanol concentrations were changed, based on further work of Thong [35]. Solution 1 of table 2.1 was set as reference because with this solution it was possible to obtain the best fibers. Solvents may alter the electrostatic and hydrogen bond interactions; therefore a proper selection of solvents is required. The choice of solvents used in this work was limited because they must dissolve TMOS. Methanol was the chosen solvent for TMOS because it is an organic, polar solvent.

According to the procedure from table 2.1, solutions 1 and 3 lead to good sol-gel formation without solidification but for solution 2 containing higher concentration of water than methanol, solidification occurred upon PVA addition because the increase in water amount makes the solution more viscous. The high concentration of HCl acting as acidic catalyst accelerates the process, turning the normal step of

hydrolysis process, which is quite slow to a better and faster formation of sol-gel. The isoelectric point of silica is around pH ≈ 2.2, and all the solutions used present a pH around ≈ 1.

Due to the low viscosity of solution 2, there was no formation of fibers in this case, but rather electrospray.

3.2.2 2.2.3 TEOS-based sol-gel

Another silica precursor tested was TEOS, mixed with water, HCl and ethanol. All the sol-gel solutions present pH≈1 like the TMOS solutions. The molar ratio of ethanol and water was modified so as to obtain conditions allowing fiber formation by electrospinning[46]. Solution 1, with the same amount of water and ethanol, presents a very low viscosity and leads to spray formation; the second solution, with increased water molar ratio, leads to solidification and aggregation after PVA addition, making impossible the electrospinning of the solution.

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3.2.3 PVA and HCl variation

This section provides tests of two crucial parameter for fibers production, which is the percentage of PVA and the concentration of HCl.

Different concentrations of HCl were used, from 0,001 M to 4 M for the TMOS and TEOS assays, while keeping the concentration of the PVA solution at 18 %, shown to be optimal for electrospinning, as seen below. A great concern is the time it takes before the sol-gel solution solidifies. Solutions with concentrations between 0,001 and 0,01 M led to solidification before PVA was added. Solution with 0,1 and 1 M HCl concentration solidified right after the addition of PVA. The 2 M solutions afforded a longer period before solidification, but not long enough for coaxial-electrospinning because this procedure requires a long process of preparation and the sol-gel solution must not be allowed to solidify in the needle. The 4 M HCl solution remained liquid for about 24 hour, and was selected for this thesis.

The sol and gel phases are controlled by hydrolysis and condensation reactions. Condensation takes place to maximize the number of siloxane linkages and minimize the number of terminal hydroxyl (silanol) groups through internal condensation. Although the sequence of condensation requires both depolymerization and presence of monomer (whether already present in the system or generated as a result of depolymerization), the rate of depolymerization is found to be much reduced in alcohol-water system than in aqueous media [72]. Initial condensation reac-

tion is followed by polycondensation that involves formation of more siloxane linkages and a stronger network. Bu using a 4 M HCl, the sol-gel solutions present a pH around 1 which is lower than the isoelectric point, and adequate for the purpose of this thesis.

In the current study, an electrospinning system was developed where the volume ratio of the PVA solution to the TMOS solution and their reaction time can be precisely controlled such that the optimum viscosity of the solution can be maintained throughout the whole electrospinning process, thus allowing continuous formation of silica/PVA nanofibers into reactive mats. The optimized reaction time should be the time it takes for the sol to reach an optimum viscosity for electrospinning of uniform nanofibers. The concentration of PVA was varied from 8 % to 22% to find which was the perfect amount of PVA necessary for fiber formation. For all solutions was used the same amount of HCL and the procedure was exactly as in sections 2.2.2 and 2.2.3, where the best ratio of methanol/ethanol, water, and HCl were selected.

Fibers made by using 8 to 14 % concentrations of PVA were very thin, and became thicker for 16 % PVA, which provided good electrospinning conditions, but did not lead to strong membranes.

The 18 % PVA concentration was the ideal amount and led to the production of excellent fibers, easy to electrospin, with no need of additional changes in electrospinning parameters. The membranes obtained were easy to remove from the collector, and exhibited good elasticity.

When using the 22 % PVA solution, solidification occurred in about 1 hour after the addition of PVA, it was difficult to do electrospinning because of high solution viscosity, and the time frame for the process was 1 hour, to avoid solidification of the solution in the syringe.

3.2.4 TEOS and TMOS established sol-gel with PVA solutions with enzyme

This study was just a confirmation assay, with the conditions set previously. The solution chosen for electrospinning has too low a pH for laccase to remain active. Indeed no enzyme activity was detected using the ABTS assay.

3.3 Coaxial electrospinning solutions for core-shell fibers

After solutions for preparing fiber shells were tested, studies proceeded with solutions for the fiber cores.

3.3.1 Core solution fibers based on Ion jelly

Depending on the evaluation of IL with laccase in section 2.2.4, the IL chosen for ion jelly fabrication was choline dihydrogen phosphate. Gelatin is not considered a suitable polymer for electrospinning because it has a high degree of hydrogen bonding, which makes electrospinning difficult. To electrospin gelatin highly polar solvents, low pH or high temperature are required, which are not good conditions for an enzyme. Based on Santos and co-workers [65], the concentration of gelatin and ionic liquid were varied. It was found that even a small decrease in gelatin concentration leads to a strong decrease in viscosity and consequently to electrospraying, instead of electrospinning. Low gelatin concentrations will force the polymer solution to break up into droplets due to its higher surface tension and lower viscosity. On the other hand, high gelatin concentration results in an increased viscosity leading to the solidification of solution or too short a timeframe for coaxial electrospinning.

Table 3.1 Core solution composition, using porcine gelatin.

Solutions	Gelatin (μL)	Choline DHP (μL)	H_2O (μL)	Phosphate buffer, pH7 (μL)	Notes
1.	166	112	1000	1000	Electrospray formation
2.	50	39	1000	1000	Very low viscosity, impossible to electrospin
3.	85	57	1000	1000	Low viscosity, impossible to electrospin
4.	100	64	1000	1000	Low viscosity, impossible to electrospin
5.	125	77	1000	1000	Electrospray formation
6.	200	90	1000	1000	Solidifies after 25 min
7.	250	150	1000	1000	Solidifies after 5 min

3.3.2 Core solution fibers based on Ion jelly with fish gelatin

After a great number of assays and modifications to the previous solutions, an alternative was found avoiding the need to use high temperatures in the electrospinning process to prevent solidification of the sol-gel solution or more aggressive pH conditions: to use fish gelatin.

Commonly collagen from fish gelatin has a lower number of proline and hydroxyproline aminoacids than mammalian gelatin, which could be the reason why it does not need a high temperature to denature. Fish gelatin has a much higher water solubility and exhibits thermally reversible gel formation ability. Normally gelling and melting points for porcine and bovine gelatins range from 20 to 25 °C and 28 to 31 °C, respectively, while the gelling and melting points for fish gelatins range from 8 to 25 °C and 11 to 28 °C, respectively.[73]

The same range of solutions was prepared for fish gelatin as for porcine gelatin, but now there was no need of a heating process and the gel formation was much faster, the whole process taking around 45 min under stirring.

Table 1.3 Core solution composition, using fish gelatin.

Solutions	Gelatin (μL)	Choline DHP (μL)	H_2O (μL)	Phosphate buffer, pH7 (μL)	Notes
1.	166	112	1000	1000	Fiber formation
2.	50	39	1000	1000	Electrospray
3.	85	57	1000	1000	Electrospray
4.	100	64	1000	1000	Fiber formation
5.	125	77	1000	1000	Fiber formation
6.	200	90	1000	1000	Fiber formation
7.	250	150	1000	1000	No fiber formation

3.3.3 solution based on Ion Jelly and enzyme

When using porcine gelatin, no fibres were obtained, but rather electrospray. When using solutions with enzyme, the material obtained tested negative for enzyme activity. The high temperatures required to keep the gelatin solution liquid may be behind this result.

On the other hand, the use of fish gelatin allowed the production of fibers without any heating or acidic auxiliary help. As shown in the table, solutions 6 and 7 produced fibers. But when the fibers were tested for solubility in water, the fibers dissolved immediately. The presence of the ionic liquid did not lend resistance to solubilisation in water, possible due to lack of efficient cross-linking between the ionic liquid and the gelatin.

3.3.4 silica-shell/ Ion jelly-core fibers

Initially this section was to be the main goal of this dissertation.

The porous and mechanically resistant shell, made of silica-based fibers, was produced, but for the biocompatible cores it was not possible to obtain any positive results. Even after carrying out a great number of assays with porcine gelatin, there was always a parameter that could not be controlled before or after the solution was inserted in the syringe, making fiber formation impossible when using the coaxial block. Either solidification of the electrospinning solution occurred before fibers started to be electrospun, or it occurred prematurely, providing too little time for producing a membrane using this methodology.

3.4 Multi Layer membrane production

I suggested this approach of making a membrane composed by two protecting layers, prepared by using the silica-PVA "shell solution", and an inner layer, composed by PVA fibers containing laccase. The two protecting layer were electrspun for one hour each and the inner solution for 4 hours, which resulted in a 140 mg membrane. One can conclude that a major part of the electrospinning solution was lost in the process.

Figure 3.5 shows the membrane produced and also a sample for SEM, which reveals the different layers of the membrane.

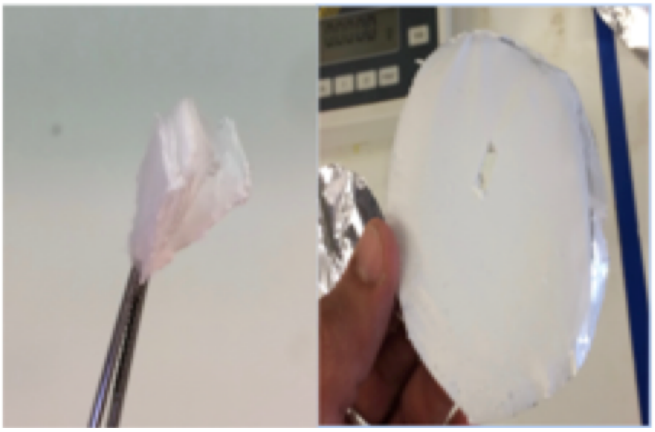


Figure 3.5 Multi layer membrane and a piece of membrane cut off for SEM analysis.

3.5 Film preparation

3.5.1 Choline DHP Ion jelly

To see if there was any enzyme activity in the ion jelly materials, 5 ion jelly-based solutions were prepared and deposited on a Petri dish, and allowed to form films at room temperature, for 24 hours. To each film 100 μL of 0,5 mM ABTS solution were added. As Figure 3.6 shows, the characteristic blue color appeared, which is indicative of enzyme.

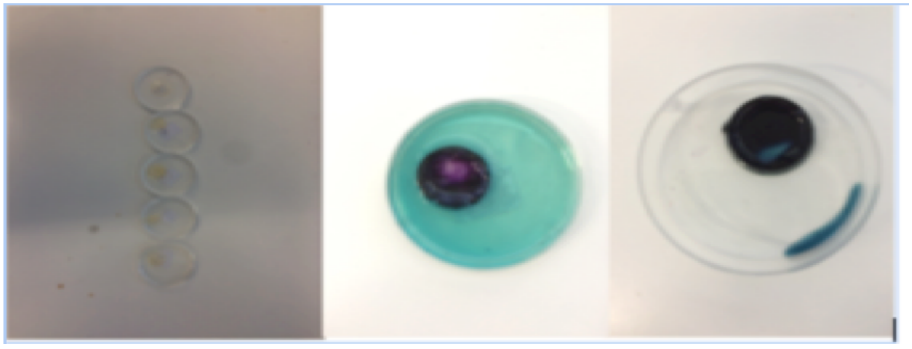


Figure 3.6 Ion jelly-based films with entrapped laccase testing positive for enzyme activity using the ABTS assay.

3.5.2 PCL and PVA films

Unfortunately for PCL films there was no sign of enzyme activity after the ABTS solution addition. On the other hand, when the enzyme substrate was added to the PVA films containing enzyme, there was an instant bluish colour formation, representing enzyme activity.

PVA was dissolved in water, whereas PCL was dissolved in organic solvents. To understand the above results better, enzyme activity assays in DMF and in DCM could have been conducted. Milstein et al. used laccase from *Trametes versicolor* to oxidize a different substrate and observed no enzyme activity in DMF, and very low enzyme activity in dichloroethane, more than ten times lower than in acetonitrile.

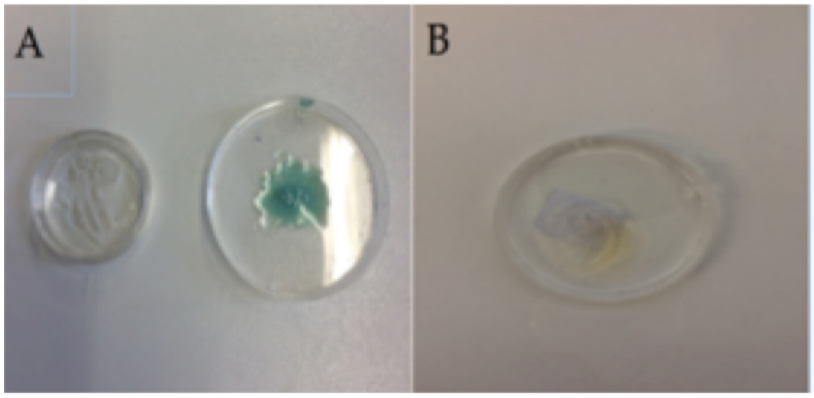


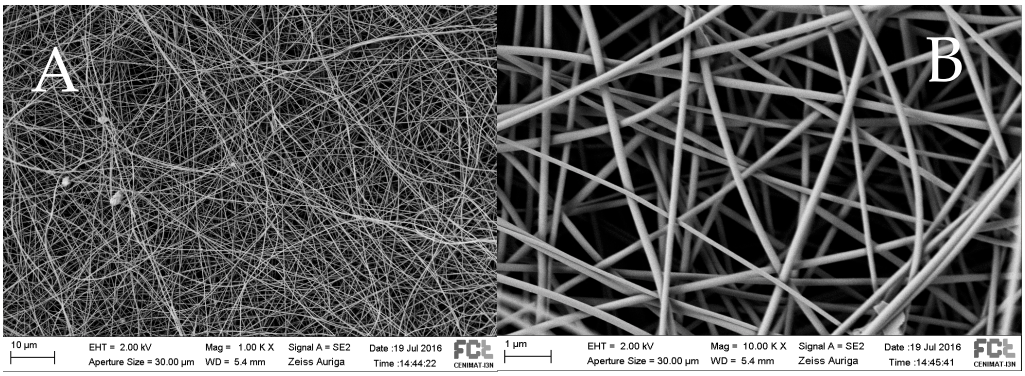
Figure 3.7 a) PVA films with entrapped laccase testing positive for enzyme activity using the ABTS assay. b) PCL films with entrapped laccase that yielded negative ABTS assays.

3.6 Fiber characterization

3.6.1 SEM

3.6.1.1 TMOS Fibers

The SEM micrograph of the silica-based fibers with PVA, produced from solution 1, table 2.1 is presented in figure 3.8. Uniform fibers with no beads were obtained, as it can be seen from figures 3.8 A and 3.8 B. Fibers with a diameter of $169 \pm 23\,nm$ and a narrow diameter distribution were obtained, as it can be seen from figure 3.8 C.



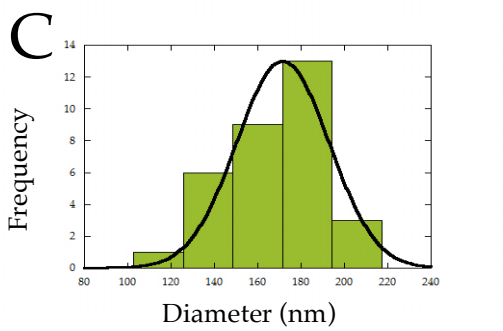


Figure 3.8 SEM images of TMOS fibers produced solution 1 (table 3.1): A) 1000 x amplification; B) 10000 x amplification. C) Fibers diameters distribution.

3.6.1.2 TEOS Fibers

In this assay was optimized the [68]protocol, hybrid silica-PVA nanofibers via sol-gel using TEOS as the silica precursor, which afterward was adapted for this study using TMOS instead of TEOS. The figure 3.8 presents the SEM images of TEOS fibers obtained from solution 3 (table 3.2.). Uniform fibers with no beads were obtained, as it can be seen from figures 3.9 A and 3.9 B. Fibers with a diameter of $204 \pm 83{,}3\ nm$ and a narrow diameter distribution were obtained, as it can be seen from figure 3.9 C.

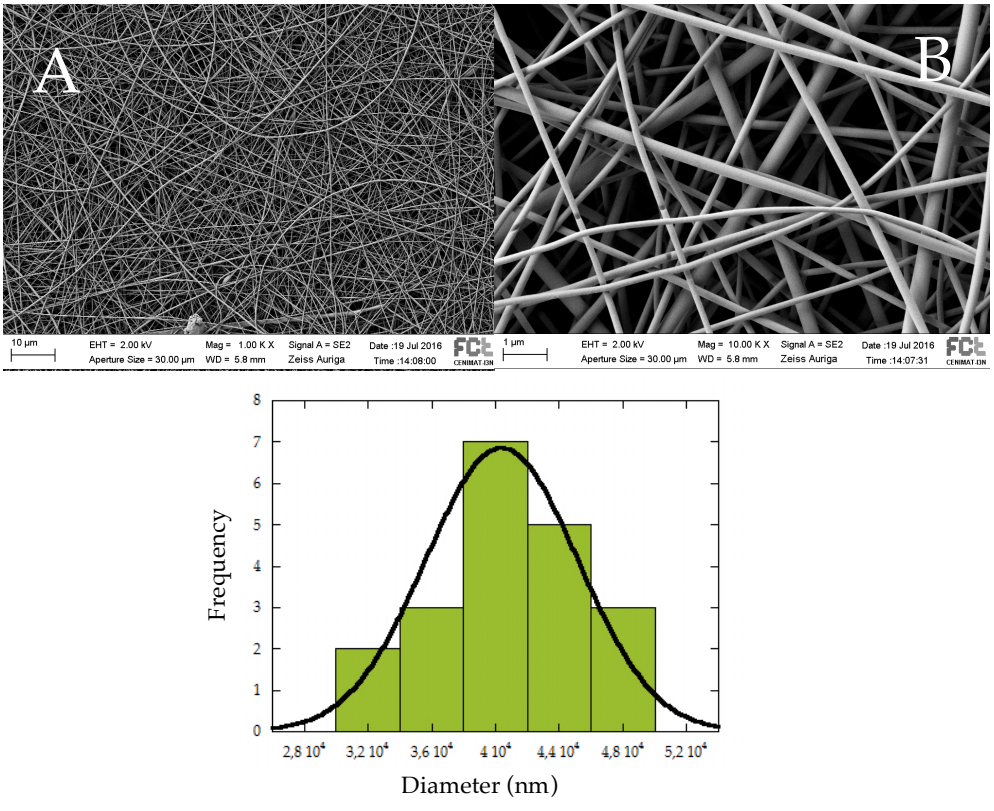


Figure 3.9 SEM images of TEOS fibers produced solution 3 (table 3.2): A) 1000 x amplification; B) 10000 amplification. C) Fibers diameters distribution.

3.6.1.4 PVA and HCl variation

These two parameters are essential for the sol-gel. After this study it was concluded that only HCL at 4 M concentration made the sol-gel in stable phase and easily electrospinnable. So the HCL concentration was kept at this value for every solution made in this thesis. The PVA range used in this assay goes from 8% to 25%, the figures below representing the comparison between the lower and the highest amount of PVA for both silica precursors.

As is shown in figure 3.10 fibers produced from solutions with low concentration of PVA present a bead-on-a-string morphology. The formation of beads is related with the lower viscosity and higher surface tension of the electrospinning solution. Lower viscosity is responsible for the inability of the jet to undergo extensional flow and contributes to the formation of beads. However the most important factor determining the formation of the bead-on-a-string morphology in electrospun membranes is the surface tension of the electrospinning solution, which is mainly dictated by the type and amount of solvent. In the present study the solvent used was always the same and we have changed its amount by changing the concentration of the polymer in solution. The increase of polymer concentration leads to the decrease of the surface tension of the solution allowing the electric force to overcome it and to produce fibers without defects. Uniform fibers are produced by electrospinning from a solution with 25% of PVA, which have a diameter of 462.54 $\pm$ 27,93 nm and a narrow diameter distribution as it can be seen from figure 3.11.It is also noticeable an increase of the fibers porosity with the increase of PVA in the electrospinning solution.[68]

TMOS

PVA 8%

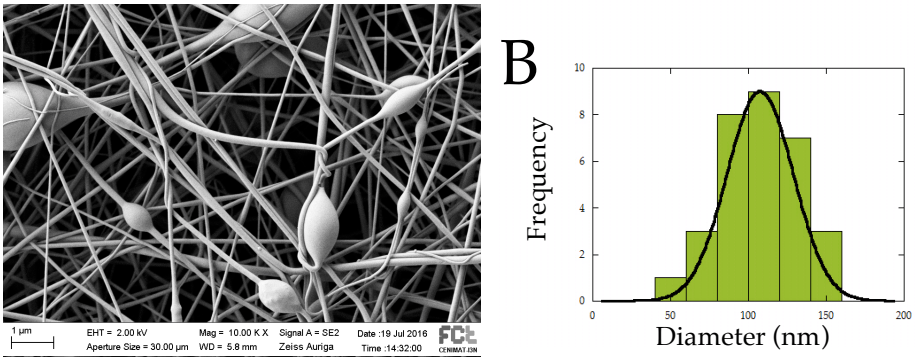


Figure 3.10 A) SEM image of TMOS fibers produced from a PVA 8% solution (table 3.3)

PVA 25%

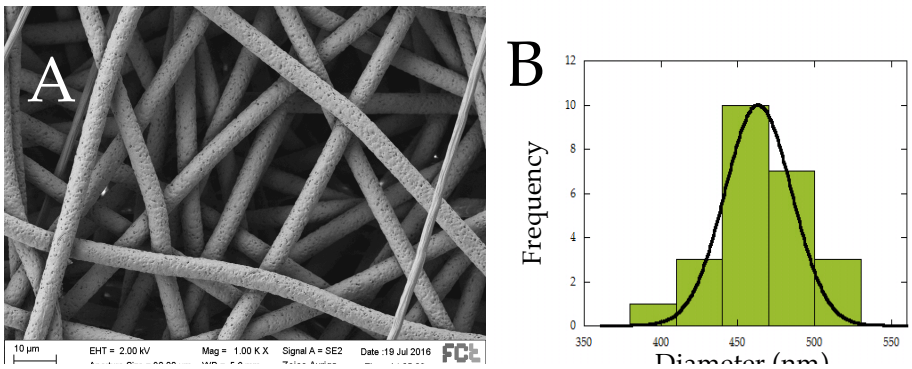


Figure 3.11 SEM image of TMOS fibers produced from a PVA 25% solution (table 3.3), B) Fibers diameter distribution.

The variation of PVA for TEOS assay result quite exactly as for TMOS, for the lower viscosity solution there is a formation of beads. Uniform fibers are produced by electrospinning from a solution with 25% of PVA, which have a diameter of $462{,}54 \pm 27{,}93nm$ and a narrow diameter distribution as it can be seen from figure 3.11. The change in silica precursor, from TMOS to TEOS, has also an effect in fiber smoothness: as it can be seen from figure 3.13, fibers produced using TEOS have no pores on the surface.

TEOS

PVA 10 %

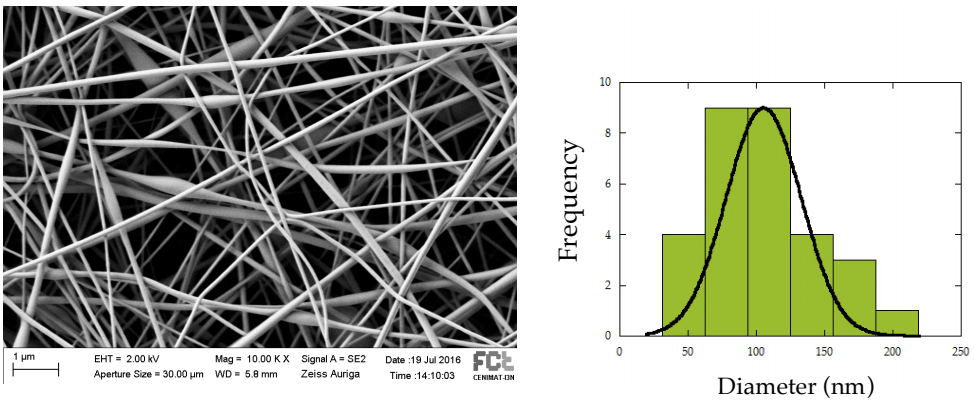


Figure 3.12 SEM image of TEOS fibers produced from a PVA 8% solution (table 3.3), B) Fibers diameter distribution.

PVA 25%

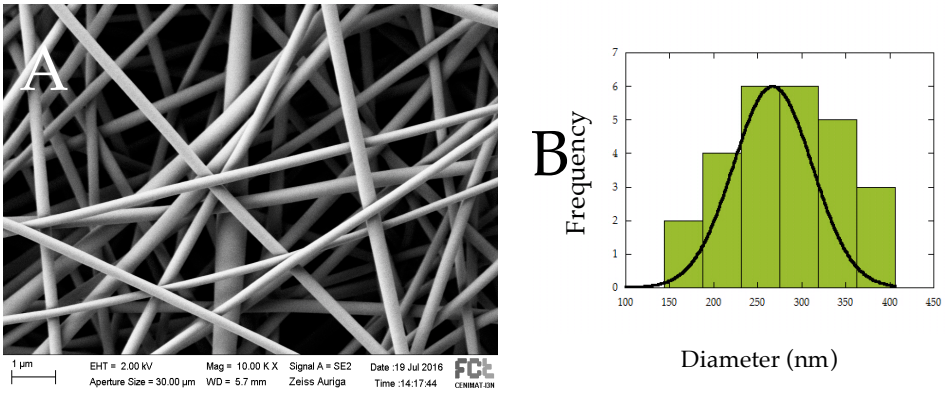


Figure 3.13 SEM image of TEOS fibers produced from a PVA 25% solution (table 3.3), B) Fibers diameter distribution.

3.6. PCL Fibers

To produce nanofibers, one of the most important variables to control is polymer solution concentration. Nevertheless, the value of concentration is dependent on the molecular chain length, the chemical nature of the polymer and the solvents selected for the polymer solution. When a specific polymer of certain average molecular weight and molecular chain length is used in electrospinning, the range of solvents for the polymer solutions plays a crucial role in defining the value of concentration. Therefore, the selection of a suitable solvent is fundamental to this process when we are working with enzymes. And for all these reasons were used three different solvents for PCL.

Figure 3.14 presents the SEM of PCL with 50% of dichloromethane (DCM) and N,N-dimethylformamide (DMF). It can be observed there is a regular fiber formation, without any beads, round shape and the average diameters is 246,45 nm and a standard variation ≈ 46,21, which demonstrates that there is a regular diameter of fibers.

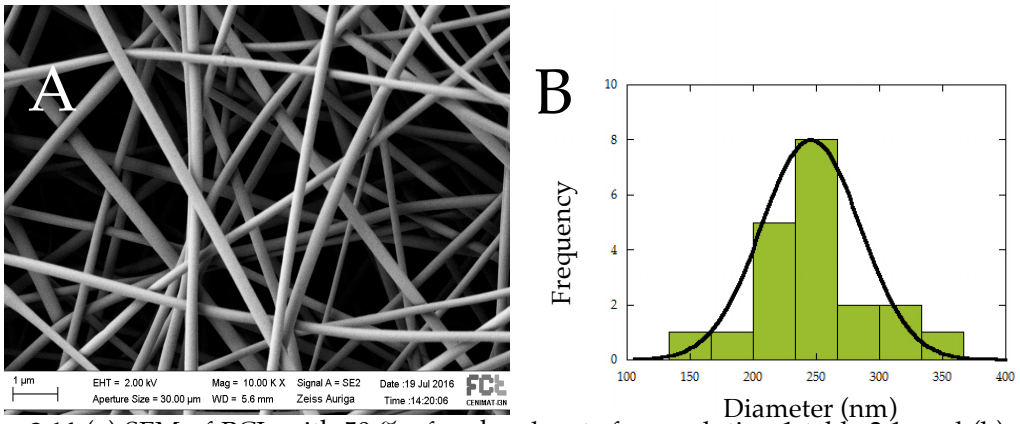


Figure 3.14 (a) SEM of PCL with 50 % of each solvents from solution 1 table 2.1, and (b) represent the standard variation of the fibers diameters.

Furthermore DCM and CF have higher solubility for PCL, but they can not produce electrospun nonwoven mats with higher quality, due to their higher volatility. Furthermore, fibers diameter and fiber size distribution were much larger when DCM and CF were used. This fact is shown in figure 3.15 with average diameters 302,62 nm and 3.16 with average diameters of 2273,3 nm representing the SEM of PCL fibers with chloroform and with higher amount of DCM that represented in figure 3.14.

According to Tang et al at [74] higher molecular weights, due to reduced solvent evaporation and increased solution viscosity, yield relatively wet fibers that flatten upon impact with the collector. The flattened, fused fibers can be attributed to the high molecular weight of the electrospinning solution that can be a result of either the increased solution viscosity due to higher polymer content. This factor is recognized in the SEM images, showing fibers that do not have the circular and round shape seen in the PCL fibers with 50% of DMC/DMF.

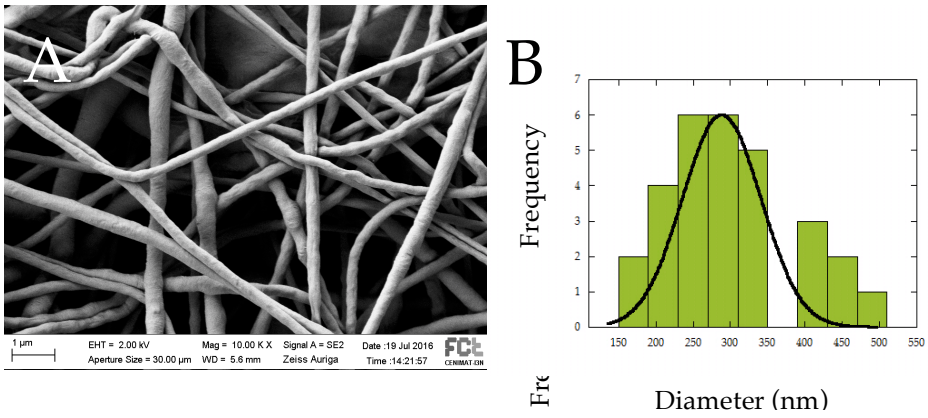


Figure 3.15 (a) SEM of PCL with 75 % of DMC and 25% DMF of solvents, solution 1 table 2.1, and (b) represent the standard variation of the fibers diameters.

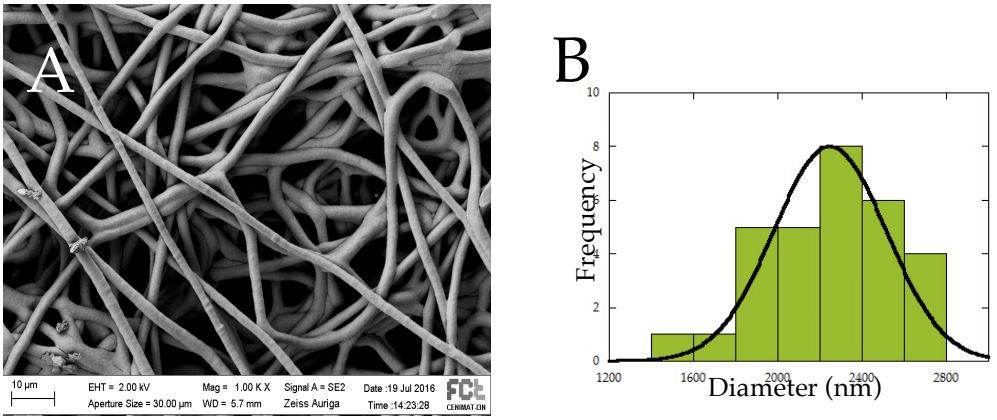


Figure 3.16 (a) SEM of PCL with 10% chloroform, solution 1 table 2.1, and (b) represent the standard variation of the fibers diameters.

2.6.1PVA fibers

The PVA fibers do not present uniform fiber formation as PCL. From the SEM image it is possible to detect some fiber fusion.

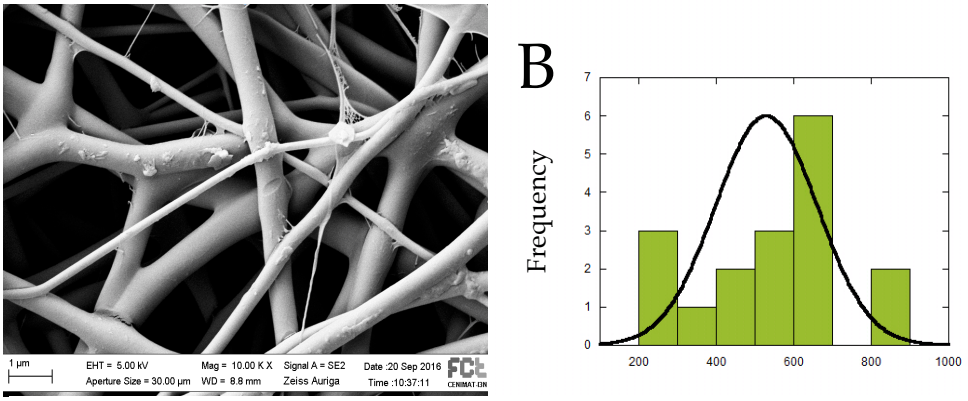


Figure 3.17 a) SEM of PVA fibers, and (b) represent the standard variation of the fibers diameters.

2.6.1 Gelatin form porcine and fish electrospray

Figure 3.18 could not be characterized, because there is high fiber fusion formation. A high amount of solvent that is not able to evaporate during the process of fiber deposition leads to the fusion of fibers, and also the solution is ejected so rapidly from the needle that there is no time form the fibers to dry and form during the trajectory to the collector.

2.6.1. EDS of multi Layer membranes

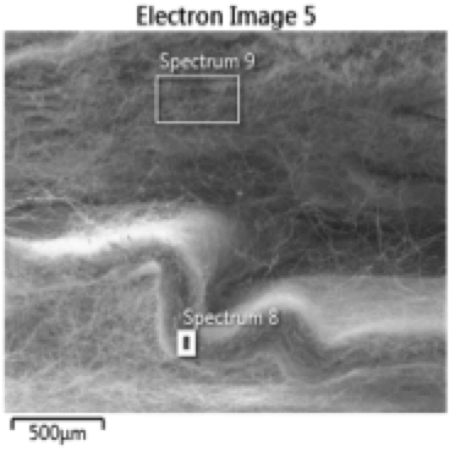


Figure 3.19 Multi layer membrane

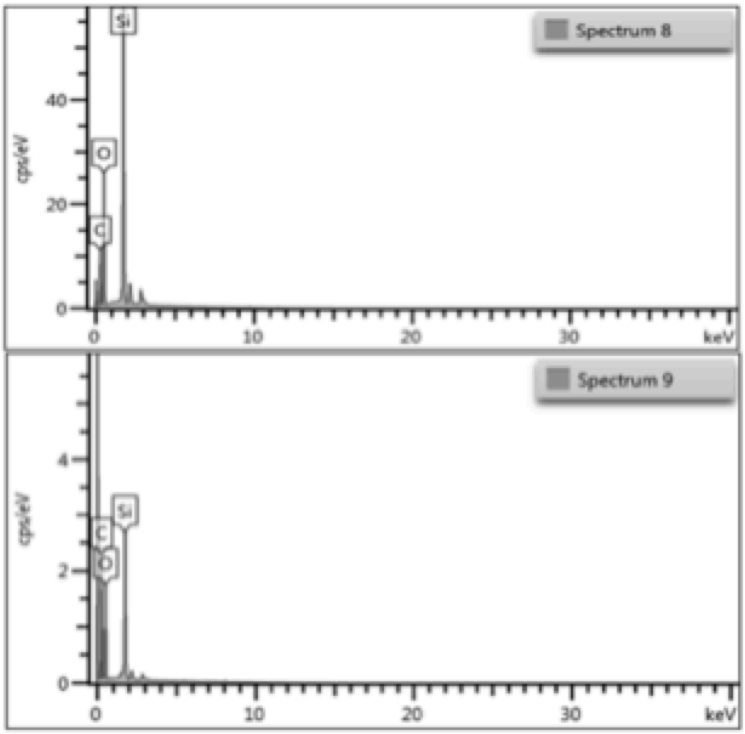


Figure 3.20 EDS analysis of the multi layer membrane

The EDS method detects the chemical elements present in the sample analysed. Figure 3.19 shows the membrane obtained, detailing the protecting layer and the inner layer, respectively marked with spectrum 8 and spectrum 9. The following figure 3.20 shows the result of EDS analysis, with both spectra revealing a high amount of silica, and spectrum 9 showing a comparatively higher amount of carbon, which is consistent with the presence of PVA. The high levels of silica found in the inner part of the membrane may have an artificial input from the cutting of the membrane for the SEM analysis.

3.7 Enzymatic assays

3.7.1 Modified Lowry test

The method is based on both the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu^+, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions results in a strong blue color, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/mL.

The results obtained are shown in figure 3.21. Using the slope from the line and the absorbance of the membrane solution which was 0,4310 ,it was possible to calculate the amount of enzyme in the solution tested, which is 340 μg. This value most be divided by the total amount of the membrane used for this assay, which was 6.9 mg, and the result of enzyme loading in 1 mg of membrane used was 0,049 mg. Assuming the membrane was homogeneous, there were 6,89 mg of enzyme in 140 mg of membrane.

It was expected to obtain around 3,0g of membrane, considering that the water and methanol evaporate during the electrospinning process. And if each mL of PVA solution contained 100 μL of a 30 mg/ml solution of enzyme, the estimated amount of enzyme is 12 mg. Therefore, the loading of enzyme in the membrane appears to be about one order of magnitude higher than aimed for.

This may be due to the fact that the membrane is not homogenous, and the portion detached for the assays had a proportionately higher amount of enzyme.

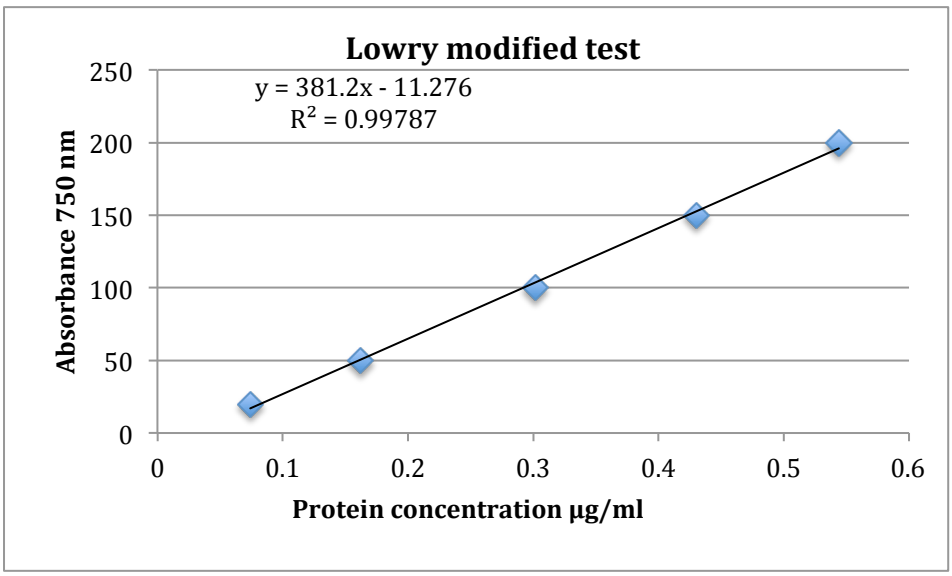


Figure 3.21 Absorbance as a function of protein concentration.

3.7.2 Membrane activity test with ABTS

The following image 3.22 shows the results provided by this assay. The membrane immediately turned blue after being immersed in an aqueous solution containing ABTS, but failed to do so when the medium was ACN. As seen earlier, the enzyme is active in ACN, but its activity is much lower than in aqueous medium, which may be behind the negative result obtained for the membrane in ACN.

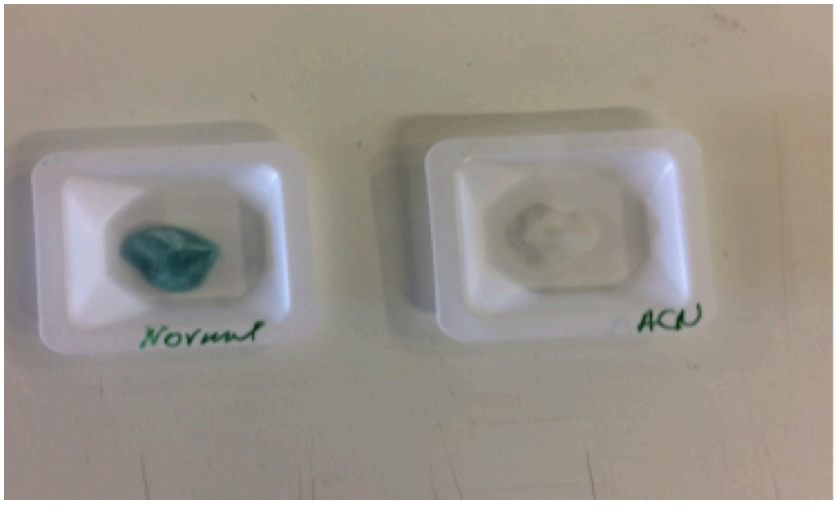


Figure 3.22 Membranes tested for enzyme activity when immersed in aqueous and non-aqueous media containing ABTS.

3.7.3 Enzyme leaching assay

in this section is was estimated the amount of enzyme leached from the membrane, after immersing 20 mg of membrane for 3 days in 5 ml of 100 mM of phos

phate buffer pH 6. After removing the membrane, 200 uL of solution were used to perform the Lowry assay. Then 100 $L of 5 mM of ABTS were added, and absorbance was measured. The results obtained indicate that the leached enzyme remained active. The Lowry assay allowed to conclude that 28% of enzyme leached in 72 hours.

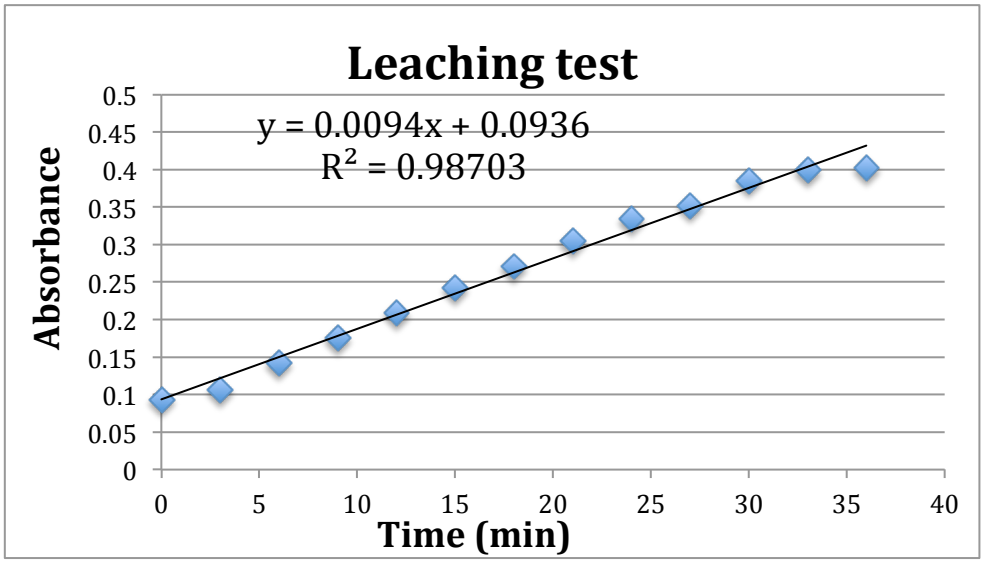


Figure 3.23 Enzyme leaching assay.

3.7.4 Entrapped enzyme activity in aqueous medium

In this assay, 20 mg of membrane were immersed in aqueous medium with ABTS and tested for enzyme activity. Using the concentration of ABTS used in the previous assay, it was possible to calculate the enzyme activity in the membrane to be 3,6 nmol s^{-1} mg^{-1} which was quite similar to that of the free enzyme, showing that the entrapped enzyme inside the PVA fibers is active, and able to be used for phenol remediation.

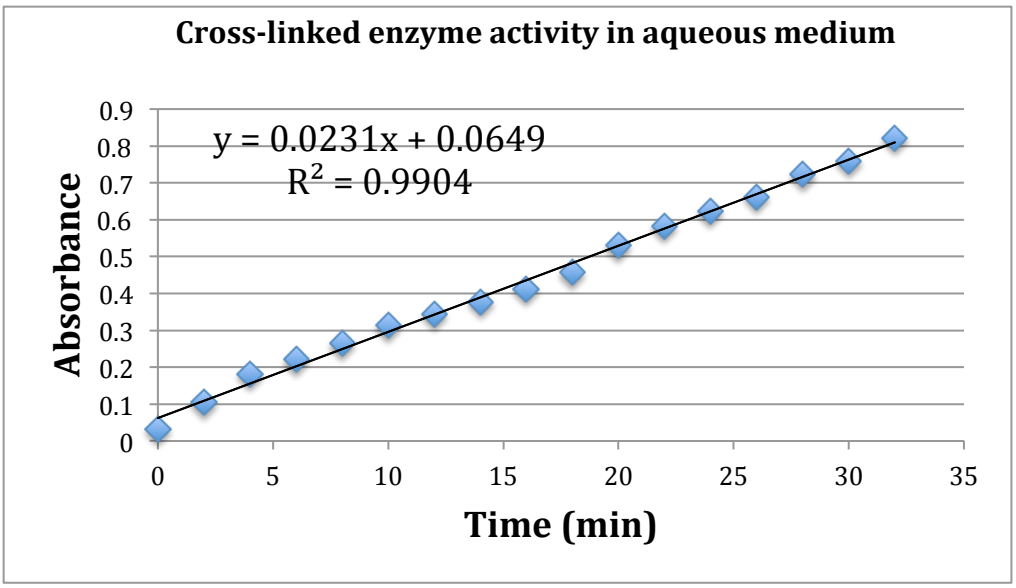


Figure 3.24 Entrapped enzyme activity in aqueous medium

3.7.5 Solubility of multilayer membrane

The result of this assay demonstrated that the membrane did not dissolve after being kept for 3 days in water, as evidenced by the similar weight of the dry membrane before the assay (20 mg), and the weight of the membrane after recovery from the medium and drying. Nor did the protecting layer or "the shell", which is composed by silica-based fibers with PVA, appear to deform after drying for another 24 h in an incubator.

The acid catalyzed hydrolysis of TMOS and TEOS leads to condensation processes that result in the formation of a highly divided grid gel of siloxane linkages. The surface of silica terminates in silanol groups, which contribute to hydrogen bonding. Subsequently PVA also has its terminal –OH groups simply accessible for bonding, hydrogen bonding between PVA and silica is promising, which might result in the formation stronger bonds for interfacial interactions, rather than just physical blends of silica and PVA. If hydrogen bonding is the lead interaction, doubtless the silica : PVA hybrids would be water soluble or would hydrate or swell when exposed to water, whereas if covalent bonds formed which crosslink PVA, the PVA-silica complex would be rendered insoluble in water. [75].

Chapter 4

Conclusion

This dissertation focused on the immobilization of a laccase on membranes obtained by electrospinning. The initial work plan suffered many changes as work evolved. It was intended to use coaxial electrospinning to fabricate reactive fibers comprising a biocompatible core entrapping the enzyme and a silica-based porous shell, offering mechanical, thermal and pH stability, as well as chemical inertness, at low cost.

However, the coaxial method was very difficult to implement, especially in what concerned the core of the fibers. It was attempted to make this core with ion jelly, obtained from the cross-linking of gelatin with an ionic liquid. However, it was not possible to implement a solution to meet all the requirements of the process, that is, a solution that did not require a temperature too high for the preservation of the catalytic activity of the enzyme, but at the same time high enough to maintain the solution fluid enough for electrospinning, providing an adequate window for making fibers before solidification occurred.

Several modifications to the protocol for making ion jelly were carried out, and when the source of the gelatin was changed to fish gelatin that does not require heating to dissolve, fibers were obtained. However, these fibers were readily soluble in water, which was not an envisaged characteristic for this work.

The fabrication of silica fibers was comparatively easy. Many different combinations of experimental variables were tested, and it was possible to make fibers and improve their properties through those manipulations, both when using as silica precursor tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS). To overcome the brittleness of silica, the silica precursor was always mixed with polyvinyl alcohol (PVA), a biocompatible polymer that lends porosity to the fibers. Scanning electron microscopy revealed that these fibers were without beads, had a rounded shape, and low dispersion in diameter. The electrospinning solution in this case displays pH values around 1, making impossible the use of enzyme in it.

To try and find a solution that was somehow analogous to that of fibers with a shell/core structure, I suggested an approach consisting on the production of a multilayer membrane, with three layers, of which 2 protecting, outer layers made from silica fibers, and an inner layer made from polymer-based fibers. For these, two different polymers were used, namely PCL and PVA. Experiments performed with PCL showed that the enzyme was not active, possibly because of contact with the solvents used to dissolve PCL. PVA was a good choice in this respect, due to its known biocompatibility, and it was possible to obtain good PVA fibers. The concern

was the fact that PVA is water soluble, although the ease of solubility in water depends on the molecular weight of the polymer. Indeed after the fabrication of the membrane, a piece of it was immersed in aqueous medium for an extended period of time and observed afterward, and apparently the integrity of the membrane had been preserved. Also it tested positive for enzyme activity.

After this positive outcome, the membrane was produced at larger scale, using 2 mL of silica solution for the two protecting layers and 4 mL of the PVA solution for the inner layer. However, only a 140 mg membrane was obtained, showing that there were major losses of solution during the electrospinning process.

The membrane was tested in aqueous and nonaqueous media. Unlike in aqueous medium, in ACN it did not reveal activity, possibly because the enzyme is considerably less active in ACN than in aqueous buffer, and the amount of enzyme in the membrane is also small.

Nevertheless the main goal of the dissertation was achieved, with the enzyme entrapped, in active form, on a fiber membrane that does not readily dissolve in water and from which enzyme leaching is moderate.

Bibliography

[1] P. Baldrian, "Fungal laccases-occurrence and properties," *FEMS Microbiol. Rev.*, vol. 30, no. 2, pp. 215–242, 2006.

[2] L. Science and S. Sharma, "Bioremediation: Features, Strategies and applications," *Asian J. Pharm. Life Sci.*, vol. 2, no. 2, pp. 202–213, 2012.

[3] C. O. Elekwachi, "Global Use of Bioremediation Technologies for Decontamination of Ecosystems," *J. Bioremediation Biodegrad.*, vol. 5, no. 4, p. 1000225, 2014.

[4] A. Singh and O. Ward, "Biotechnology and bioremediation - An overview," *Biodegrad. Bioremediation*, vol. 2, pp. 1–5, 2004.

[5] C. I. Nair, K. Jayachandran, and S. Shashidhar, "Biodegradation of phenol," *African J. Biotechnol.*, vol. 7, no. 25, pp. 4951–4958, 2008.

[6] J. Michałowicz and W. Duda, "Phenols - Sources and toxicity," *Polish J. Environ. Stud.*, vol. 16, no. 3, pp. 347–362, 2007.

[7] Z. Kyselova, "Toxicological aspects of the use of phenolic compounds in disease prevention," *Interdiscip. Toxicol.*, vol. 4, no. 4, pp. 173–183, 2012.

[8] N. S. Gad and A. S. Saad, "Effect of Environmental Pollution by Phenol on Some Physiological Parameters of Oreochromis niloticus," *Glob. Vet.*, vol. 2, no. 6, pp. 312–319, 2008.

[9] R. C. Senan and T. E. Abraham, "Bioremediation of Textile Azo Dyes by Aerobic Bacterial Consortium Aerobic Degradation of Selected Azo Dyes by Bacterial Consortium," *Biodegradation*, vol. 15, no. 4, pp. 275–280, 2004.

[10] O. Gimeno, M. Carbajo, F. J. Beltrán, and F. J. Rivas, "Phenol and substituted phenols AOPs remediation," *J. Hazard. Mater.*, vol. 119, no. 1–3, pp. 99–108, 2005.

[11] Y. C. Zhao, X. Y. Yi, M. Zhang, L. Liu, and W. J. Ma, "Fundamental study of degradation of dichlorodiphenyltrichloroethane in soil by laccase from white rot fungi," *Int. J. Environ. Sci. Technol.*, vol. 7, no. 2, pp. 359–366, 2010.

[12] A. Kunamneni, I. Ghazi, S. Camarero, A. Ballesteros, F. J. Plou, and M. Alcalde, "Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers," *Process Biochem.*, vol. 43, no. 2, pp. 169–178, 2008.

[13] Z. Nixon and J. Michel, "Predictive modeling of subsurface shoreline oil encounter probability from the Exxon Valdez oil spill in prince William sound, Alaska," *Environ. Sci. Technol.*, vol. 49, no. 7, pp. 4354–4361, 2015.

[14] O. Milstein, B. Nicklas, and a Hüttermann, "Oxidation of aromatic compounds in organic solvents with laccase from Trametes versicolor," *Appl. Microbiol. ...*, vol. 250, pp. 70–74, 1989.

[15] Y. Gao, Y. B. Truong, P. Cacioli, P. Butler, and I. L. Kyratzis, "Bioremediation of pesticide contaminated water using an organophosphate degrading enzyme immobilized on nonwoven polyester textiles," *Enzyme Microb. Technol.*, vol. 54, no. 1, pp. 38–44, 2014.

[16] M. Fernández-Fernández, M. Á. Sanromán, and D. Moldes, "Recent developments and applications of immobilized laccase," *Biotechnol. Adv.*, vol. 31, no. 8, pp. 1808–1825, 2013.

[17] Shraddha, R. Shekher, S. Sehgal, M. Kamthania, and A. Kumar, "Laccase: microbial sources, production, purification, and potential biotechnological applications.," *Enzyme Res.*, vol. 2011, p. 217861, 2011.

[18] U. N. Dwivedi, P. Singh, V. P. Pandey, and A. Kumar, "Structure-function relationship among bacterial, fungal and plant laccases," *J. Mol. Catal. B Enzym.*, vol. 68, no. 2, pp. 117–128, 2011.

[19] Y. Dai, J. Niu, J. Liu, L. Yin, and J. Xu, "In situ encapsulation of laccase in microfibers by emulsion electrospinning: Preparation, characterization, and application," *Bioresour. Technol.*, vol. 101, no. 23, pp. 8942–8947, 2010.

[20] S. Riva, "Laccases: blue enzymes for green chemistry," *Trends Biotechnol.*, vol. 24, no. 5, pp. 219–226, 2006.

[21] R. Chandra and P. Chowdhary, “Properties of bacterial laccases and their application in bioremediation of industrial wastes.,” *Environ. Sci. Process. Impacts*, vol. 17, no. 2, pp. 326–42, 2015.

[22] L. P. Christopher, B. Yao, and Y. Ji, “Lignin Biodegradation with Laccase-Mediator Systems,” *Front. Energy Res.*, vol. 2, no. March, pp. 1–13, 2014.

[23] P. Wasserscheid and W. Keim, “Ionic Liquids—New ‘Solutions’ for Transition Metal Catalysis,” *Angew. Chemie Int. Ed.*, vol. 39, no. 21, pp. 3772–3789, 2000.

[24] U. T. Bornscheuer, “Immobilizing enzymes: How to create more suitable biocatalysts,” *Angew. Chemie - Int. Ed.*, vol. 42, no. 29, pp. 3336–3337, 2003.

[25] V. K. Gochev and a I. Krastanov, “Fungal Laccases,” *Biotechnology*, vol. 13, pp. 75–83, 2007.

[26] U. Guzik, K. Hupert-Kocurek, and D. Wojcieszyńska, “Immobilization as a Strategy for Improving Enzyme Properties-Application to Oxidoreductases.,” *Molecules*, vol. 19, no. 7, pp. 8995–9018, 2014.

[27] M. . Rao, R. Scelza, R. Scotti, and L. Gianfreda, “Role of Enzymes in the Remediation of Polluted Environments,” *J. soil Sci. plant Nutr.*, vol. 10, no. 3, pp. 333–353, 2010.

[28] C. Altinkaynak, S. Tavlasoglu, N. ??zdemir, and I. Ocsoy, “A new generation approach in enzyme immobilization: Organic-inorganic hybrid nanoflowers with enhanced catalytic activity and stability,” *Enzyme Microb. Technol.*, vol. 93–94, pp. 105–112, 2016.

[29] S. Datta, L. R. Christena, and Y. R. S. Rajaram, “Enzyme immobilization: an overview on techniques and support materials,” *3 Biotech*, pp. 1–9, 2012.

[30] O. Fokina, J. Eipper, S. Kerzenmacher, and R. Fischer, “Selective natural induction of laccases in Pleurotus sajor-caju, suitable for application at a biofuel cell cathode at neutral pH,” *Bioresour. Technol.*, vol. 218, pp. 455–462, 2016.

[31] M. A. Rahman, H. B. Noh, and Y. B. Shim, “Direct electrochemistry of laccase immobilized on Au nanoparticles encapsulated-dendrimer bonded conducting polymer: Application for a catechin sensor,” *Anal. Chem.*, vol. 80, no. 21, pp. 8020–8027, 2008.

[32] H. Cabana, J. P. Jones, and S. N. Agathos, “Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals,” *J. Biotechnol.*, vol. 132, no. 1, pp. 23–31, 2007.

[33] R. Stloukal, J. Watzková, and B. Gregušová, “Dye decolorisation by laccase immobilised in lens-shaped poly(vinyl alcohol) hydrogel capsules,” *Chem. Pap.*, vol. 68, no. 11, pp. 1514–1520, 2014.

[34] L. Ge, Y. S. Zhao, T. Mo, J. R. Li, and P. Li, “Immobilization of glucose oxidase in electrospun nanofibrous membranes for food preservation,” *Food Control*, vol. 26, no. 1, pp. 188–193, 2012.

[35] H. W. Tong, B. R. Mutlu, L. P. Wackett, and A. Aksan, “Silica/PVA biocatalytic nanofibers,” *Mater. Lett.*, vol. 111, pp. 234–237, 2013.

[36] Y. Dai, J. Yao, Y. Song, X. Liu, S. Wang, and Y. Yuan, “Enhanced performance of immobilized laccase in electrospun fibrous membranes by carbon nanotubes modification and its application for bisphenol A removal from water,” *J. Hazard. Mater.*, vol. 317, pp. 485–493, 2016.

[37] Y. Dai, J. Niu, L. Yin, J. Xu, and J. Xu, “Laccase-carrying electrospun fibrous membrane for the removal of polycyclic aromatic hydrocarbons from contaminated water,” *Sep. Purif. Technol.*, vol. 104, pp. 1–8, 2013.

[38] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. C. Rodrigues, and R. Fernandez-Lafuente, “Strategies for the one-step immobilization-purification of enzymes as industrial biocatalysts,” *Biotechnol. Adv.*, vol. 33, no. 5, pp. 435–456, 2015.

[39] H. Jiang and C. Blouin, “Insertions and the emergence of novel protein structure: a structure-based phylogenetic study of insertions.,” *BMC bioinformatics*, vol. 8. p. 444, 2007.

[40] J. J. Roy and T. E. Abraham, “Preparation and characterization of cross-linked enzyme crystals of laccase,” *J. Mol. Catal. B Enzym.*, vol. 38, no. 1, pp. 31–36, 2006.

[41] L. L. Hench and J. K. West, “The sol-gel process,” *Chem. Rev.*, vol. 90, no. 1, pp. 33–72, 1990.

[42] J. Livage, C. Roux, J. M. Costa, J. F. Quinson, and I. Desportes, “Immunoassays in sol-gel matrices,” *J. Sol-Gel Sci. Technol.*, vol. 7, no. 1–2, pp. 45–51, 1996.

[43] L. Spanhel and M. A. Anderson, “Semiconductor Clusters in the Sol-Gel Process: Quantized Aggregation, Gelation, and Crystal Growth in Concentrated ZnO Colloids,” *J . Am. Chem. SOC*, vol. 113, pp. 2826–2833, 1991.

[44] A. Podhorodecki, M. Nyk, J. Misiewicz, and W. Strek, “Optical investigation of the emission lines for Eu3+ and Tb3+ ions in the GaN powder host,” *J. Lumin.*, vol. 126, no. 1, pp. 219–224, 2007.

[45] T. W. Zerda and G. Hoang, “Effect of Solvents on the Hydrolysis Reaction of Tetramet hyl Ort hosilicate,” no. 19, pp. 372–376, 1990.

[46] S. S. Choi, S. G. Lee, S. S. Im, S. H. Kim, and Y. L. Joo, “Silica nanofibers from electrospinning/sol-gel process,” *J. Mater. Sci. Lett.*, vol. 22, no. 12, pp. 891–893, 2003.

[47] I. Introduction, “T H E Chemistry of Tetracyanoethylene,” vol. VI, no. Iv, pp. 63–90, 1967.

[48] W. N. I. Wan Mohd Zawawi, A. F. Mansor, N. S. Othman, N. A. Mohidem, N. A. N. Nik Malek, and H. Mat, “Synthesis and characterization of immobilized white-rot fungus Trametes versicolor in sol???gel ceramics,” *J. Sol-Gel Sci. Technol.*, vol. 77, no. 1, pp. 28–38, 2016.

[49] B. Sun, Y. Z. Long, H. D. Zhang, M. M. Li, J. L. Duvail, X. Y. Jiang, and H. L. Yin, “Advances in three-dimensional nanofibrous macrostructures via electrospinning,” *Prog. Polym. Sci.*, vol. 39, no. 5, pp. 862–890, 2014.

[50] V. Beachley and X. Wen, “Effect of electrospinning parameters on the nanofiber diameter and length,” *Mater. Sci. Eng. C*, vol. 29, no. 3, pp. 663–668, 2009.

[51] J. Anu Bhushani and C. Anandharamakrishnan, “Electrospinning and electrospraying techniques: Potential food based applications,” *Trends Food Sci. Technol.*, vol. 38, no. 1, pp. 21–33, 2014.

[52] N. Bhardwaj and S. C. Kundu, “Electrospinning: A fascinating fiber fabrication technique,” *Biotechnol. Adv.*, vol. 28, no. 3, pp. 325–347, 2010.

[53] Z. Li and C. Wang, “One-Dimensional nanostructures,” pp. 15–29, 2013.

[54] H. Qu, S. Wei, and Z. Guo, “Coaxial electrospun nanostructures and their applications,” *J. Mater. Chem. A*, vol. 1, no. 38, pp. 11513–11528, 2013.

[55] T. T. T. Nguyen, O. H. Chung, and J. S. Park, “Coaxial electrospun poly(lactic acid)/chitosan (core/shell) composite nanofibers and their antibacterial activity,” *Carbohydr. Polym.*, vol. 86, no. 4, pp. 1799–1806, 2011.

[56] H. D. B. Jenkins, “Ionic liquids-an overview,” *Sci. Prog.*, vol. 94, no. 3, pp. 265–297, 2011.

[57] M. Galiński, A. Lewandowski, and I. Stepniak, “Ionic liquids as electrolytes,” *Electrochim. Acta*, vol. 51, no. 26, pp. 5567–5580, 2006.

[58] R. Ratti, “Ionic Liquids: Synthesis and Applications in Catalysis,” *Adv. Chem.*, vol. 2014, no. 3, pp. 1–16, 2014.

[59] I. Hemeon, N. W. Barnett, N. Gathergood, P. J. Scammells, and R. D. Singer, “Manganese dioxide allylic and benzylic oxidation reactions in ionic liquids,” *Aust. J. Chem.*, vol. 57, no. 2, pp. 125–128, 2004.

[60] D. R. MacFarlane, J. M. Pringle, K. M. Johansson, S. a Forsyth, and M. Forsyth, “Lewis base ionic liquids.,” *Chem. Commun. (Camb).*, no. 18, pp. 1905–1917, 2006.

[61] R. Bini, C. Chiappe, V. L. Mestre, C. S. Pomelli, and T. Welton, “A rationalization of the solvent effect on the Diels–Alder reaction in ionic liquids using multiparameter linear solvation energy relationships,” *Org. Biomol. Chem.*, vol. 6, no. 14, p. 2522, 2008.

[62] H. Tateishi-Karimata and N. Sugimoto, “Structure, stability and behaviour of nucleic acids in ionic liquids,” *Nucleic Acids Res.*, vol. 42, no. 14, pp. 8831–8844, 2014.

[63] R. M. Couto, T. Carvalho, L. A. Neves, R. M. Ruivo, P. Vidinha, A. Paiva, I. M. Coelhoso, S. Barreiros, and P. C. Simões, “Development of Ion-Jelly?? membranes,” *Sep. Purif. Technol.*, vol. 106, pp. 22–31, 2013.

[64] P. Vidinha, N. M. T. Lourenço, C. Pinheiro, A. R. Brás, T. Carvalho, T. Santos-Silva, A. Mukhopadhyay, M. J. Romão, J. Parola, M. Dionisio, J. M. S. Cabral, C. a M. Afonso, and S. Barreiros, “Ion jelly: a tailor-made conducting material for smart electrochemical devices.,” *Chem. Commun.*

(Camb)., no. 44, pp. 5842–5844, 2008.

[65] R. Dos Santos, Â. Rocha, A. Matias, C. Duarte, I. Sá-Nogueira, N. Lourenço, J. P. Borges, and P. Vidinha, "Development of antimicrobial Ion Jelly fibers," *RSC Adv.*, vol. 3, no. 46, p. 24400, 2013.

[66] A. F. R. Pimenta, A. C. Baptista, T. Carvalho, P. Brogueira, N. M. T. Lourenço, C. A. M. Afonso, S. Barreiros, P. Vidinha, and J. P. Borges, "Electrospinning of Ion Jelly fibers," *Mater. Lett.*, vol. 83, pp. 161–164, 2012.

[67] C. M. M. Duarte and P. Vidinha, "Ionic Liquids Gelation with Polymeric Materials : The Ion Jelly Approach," 2010.

[68] T. Pirzada, S. A. Arvidson, C. D. Saquing, S. S. Shah, and S. A. Khan, "Hybrid Silica − PVA Nanofibers via Sol − Gel Electrospinning," 2012.

[69] X. Qin and D. Wu, "Effect of different solvents on poly(caprolactone) (PCL) electrospun nonwoven membranes," *J. Therm. Anal. Calorim.*, vol. 107, no. 3, pp. 1007–1013, 2012.

[70] Z. K. Nagy, K. Nyúl, I. Wagner, K. Molnár, and G. Marosi, "Electrospun water soluble polymer mat for ultrafast release of donepezil HCL," *Express Polym. Lett.*, vol. 4, no. 12, pp. 763–772, 2010.

[71] A. Fidalgo, M. Emı, and L. M. Ilharco, "Chemical Control of Highly Porous Silica Xerogels : Physical Properties and Morphology," *Chem. Mater.*, vol. 15, no. 8, pp. 2186–2192, 2003.

[72] T. Pirzada, "Synthesis of Polymer Silica Hybrid Xerogels and Nanofibers through Sol Gel Processing and Electrospinning," 2012.

[73] A. A. Karim and R. Bhat, "Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins," *Food Hydrocoll.*, vol. 23, no. 3, pp. 563–576, 2009.

[74] H. W. Tong, B. R. Mutlu, L. P. Wackett, and A. Aksan, "Manufacturing of bioreactive nanofibers for bioremediation," *Biotechnol. Bioeng.*, vol. 111, no. 8, pp. 1483–1493, 2014.

[75] H. Zou, S. Wu, and J. Shen, "Polymer / Silica Nanocomposites : Preparation , Characterization , Properties , and," pp. 3893–3957, 2008.

[76] N. T. Joutey, W. Bahafid, and H. Sayel, "Biodegradation: Involved Microorganisms and Genetically Engineered Microorganisms," *Biodegrad. - Life Sci.*, pp. 289–320, 2013.