



**Diogo da Silveira
Vieira da Silva**

**Imobilização de β -galactosidase em membrana
nanofibrosa**



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica e Química dos Alimentos, realizada sob a orientação científica do Doutor José António Teixeira Lopes da Silva, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

Dedico este trabalho aos meus pais.

o júri

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palavras-chave

Electrospinning, nanofibras, álcool polivinílico, imobilização, enzimas, β -galactosidase.

resumo

O objectivo deste trabalho consistiu no desenvolvimento de matrizes nanofibras por electrospinning para a imobilização da enzima β -galactosidase. Para tal, foi utilizado álcool polivinílico (PVA), um polímero sintético solúvel em água, para a produção das matrizes. Para permitir a utilização destas matrizes em meio aquoso, as membranas sofreram crosslinking por imersão em glutaraldeído, sendo as fibras caracterizadas por microscopia electrónica de varrimento (SEM). A concentração de 100 mmol/L de glutaraldeído revelou ser eficiente para manter a morfologia das nanofibras após imersão em água durante várias horas. A percentagem de imobilização enzimática foi determinada espectrofotometricamente, obtendo-se um valor médio de 83%. Estes resultados permitiram fazer um estudo comparativo da actividade e estabilidade da β -galactosidase livre e imobilizada. A actividade máxima da enzima imobilizada nas fibras foi de 3,86% em relação à enzima livre registando ainda uma retenção da actividade de aproximadamente 70% após 4 ciclos de reutilização.

Tanto as matrizes nanofibras produzidas como os resultados de actividade e estabilidade enzimática obtidos mostram potenciais vantagens relativamente aos métodos enzimáticos tradicionais. No entanto, há ainda a necessidade de melhorar a actividade catalítica das formas imobilizadas para permitir uma aplicação rentável a nível industrial.

keywords

Electrospinning, nanofibers, polyvinyl alcohol, entrapment, enzymes, β -galactosidase.

abstract

The aim of this work was the development of nanofibrous matrices by electrospinning for the entrapment of β -galactosidase enzyme. Therefore, it was used polyvinyl alcohol (PVA), which is a water-soluble synthetic polymer, to electrospin the matrices. The nanofibrous membranes were crosslinked by immersion in glutaraldehyde in order to be used in an aqueous medium. Fibers were characterized by Scanning Electron Microscopy (SEM). A glutaraldehyde concentration of 100 mmol/L was efficient to maintain fibers morphology after water immersion during several hours. The enzymatic immobilization yield was spectrophotometrically determined, with an average value of 83%. Based on these results, a comparative study between free and immobilized β -galactosidase activity and stability was made. The maximum activity of the entrapped enzyme was 3.86% of the free form while retaining approximately 70% of the initial activity after 4 reutilization cycles.

The nanofibrous matrices produced and the enzymatic stability and activity results show the potential advantages of this new method regarding the traditional enzymatic procedures. However, it is necessary to enhance the obtained catalytic activity for the immobilized enzyme forms in order to achieve a profitable industrial application.

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Introduction

The main purpose of this thesis is the development of nanofibrous matrices which can be used as a support to immobilize enzymes. It is also our objective to achieve competitive advantages relatively to the traditional enzymatic methods used nowadays in the food industry, using either free or immobilized enzymes.

The use of immobilized enzymes has some theoretical advantages that must be studied and verified. Contrary to free enzymes, the use of immobilized forms allows reutilization, easier purifications and continuous enzymatic processes. However, there are some drawbacks associated to the immobilization process which are difficult to predict for a particular enzyme-support system and thus must be extensively studied. Among these disadvantages, the limitation to diffusional transfer processes, which reduces severely the catalytic efficiency, is the most concerning issue.

The performance of immobilized enzymes largely depends on the structure of supports. Among many types of carriers already investigated, nanostructured supports are believed to be able to retain the catalytic activity as well as ensure the immobilization efficiency of enzyme to a high extent. In many reports, nanoparticles and nanotubes are used as immobilization supports in order to reduce this disadvantage, but they have also a great degree of dispersion and the possibility of reutilization is considerably low. Therefore, this is an area of great interest and much research is needed in order to find new and innovative supports and immobilization techniques.

Electrospinning provides a simple and versatile method to make nanofibrous supports. Compared with other nanostructured supports (e.g. mesoporous silica, nanoparticles, nanotubes), nanofibrous supports show many advantages for their high porosity and interconnectivity, what in combination with the high surface-to-volume ratio can provide valuable matrices for catalytic processes, with many and accessible binding sites and high enzyme loads. It is also supposed that due to its own properties, any diffusional limitations are minimized and reutilization is possible.

Following these ideas, this thesis is based on the production by electrospinning, functionalization and characterization of nanofibrous membranes, and on the study of the activity and stability of the immobilized enzymes.

β -Galactosidase was the enzyme chosen to be immobilized. This enzyme is used in some food industries, more precisely in milk and derivatives industry. β -Galactosidase is responsible for the

enzymatic lactose degradation which is of great importance in order to produce lactose-free products. However, its free-form use prevents enzyme reuse which turns this process highly expensive and its frequent substitution by chemical and thermal processes. The production of a stable and active nanofibrous membrane with immobilized β -galactosidase would be a great achievement for many industrial processes. It was focused on this objective that this thesis was made, showing for the first time an active and immobilized β -galactosidase in an electrospun nanofibrous membrane.

In chapter 1 of this thesis, we describe the preparation of the electrospun PVA mats, their water insolubilization by crosslinking with Glutaraldehyde and their characterization.

In chapter 2, we describe the entrapment of β -galactosidase in PVA nanofibers and the activity studies made with free and immobilized enzyme.

Finally, we will present the final conclusions and some perspectives for future work.

1 Electrospinning of PVA Nanofiber Matrices

1.1. Literature review

1.1.1. Nanotechnology

The origin of the word nanotechnology is derived from the Greek word *nanos* or *nannos* that means “little old man” or “dwarf”. As one can see, since the early days nano is related to things in a small scale and is now used as a metric prefix. Nano means one billionth of a unit or 10^{-9} m. Since it is difficult to measure or even to imagine we can say that a single human hair has around 80000 nanometers in width.

The importance of nanotechnology as an emerging technology has been recognized worldwide, with large and expensive investments in nanotechnology research over the past few years. Nanotechnology has attracted much attention recently, and it can be applied to all aspects of science and engineering, as well as to life. But what is nanotechnology? There are many definitions of the term, but one of the most accepted definitions is from El-Naschie [1]:

“The naive and direct answer to the frequently posed question what exactly is Nanotechnology is to say that is a technology concerning processes which are relevant to physics, chemistry and biology taking place at a length of one divided by 100 million of a meter”

Nanotechnology main purpose is to create new materials and develop new products and processes based on the growing capability of modern technology to see and manipulate atoms and molecules. As referred previously, nanotechnology is not a specific technology but instead a group of techniques, based on physics, chemistry, biology, materials engineering and computer science. All these aim to extend Man capability to manipulate all kinds of materials till reach atom limit [2].

The nanostructuring of surfaces is one of the most important advantages of this technology once it leads to exceptional effects, for example, the Lotus (self-cleaning) effect. The nanoscale is also particularly relevant for biological systems, because dimensions of proteins, viruses, and bacteria fall in this size range. Comparison with the diameters of these objects shows that the diameters of electrospun fibers can span a relatively wide range (Figure 1).

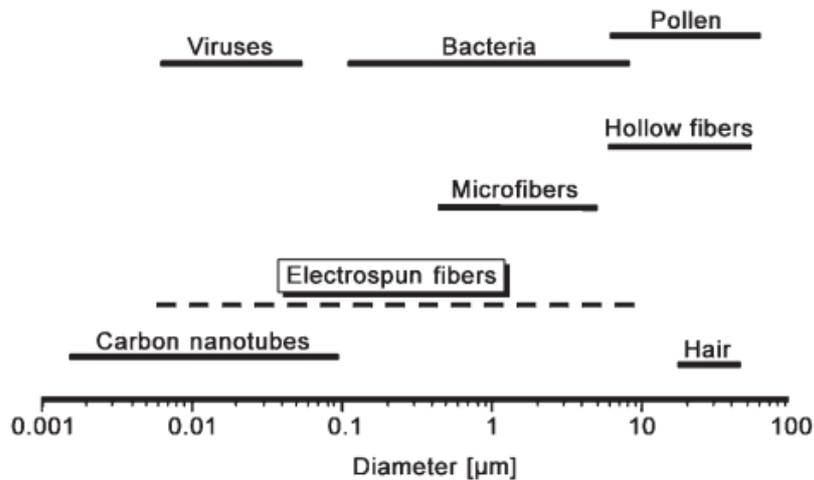


Figure 1 - Comparison of the diameters of electrospun fibers to those of biological and technological objects [3].

Nanostructured systems are promising for diverse applications, such as the transport and targeted release of drugs and active agents in organisms, tissue engineering, the surface modification of implants, and in microelectronics. In many of these applications, nanoparticles and carbon nanotubes are currently in the spotlight. Nanorods of metals, metal oxides, or semiconductors and nanofibers of polymers have only recently gained importance [3].

1.1.2. History of electrospinning

Electrospinning is an old technique. This technique is used to produce fibers which can vary between micrometers to less than 100 nanometers in diameter. To obtain these fibers it is applied an electric induction on a solution or a fluid. The fibers are collected as porous matrices which can be used in a great number of fields, as chemistry, biology, medicine or engineering [2].

We can say that electrospinning is related to the William Gilbert findings in the late 1500s, when he set out to describe the behavior of magnetic and electrostatic phenomena. His work is an early example of what would become the modern scientific method. One of his more obscure observations was that when a suitable charged piece of amber was brought near a droplet of water it would form a cone shape and small droplets would be ejected from the tip of the cone – the first recorded observation of electrospaying [4].

In 1745, Bose described aerosols generated by the application of high electric potentials to drop fluids. In the late 1800s, electrodynamics was used to explain the excitation of dielectric liquid under the influence of an electric charge. Lord Rayleigh investigated the question of how many

charges were needed to overcome the surface tension of a drop. This probably led to the invention of electrospinning to produce fibers in the early 1900s by Cooley and Morton [3, 5].

J.F. Cooley is associated with the first description of a process recognizable as electrospinning. In 1902 he filed a United States patent entitled "*Apparatus for electrically dispersing fibers*". This patent describes a method for using a high voltage power supplies to generate a yarn. At this stage it was recognized that to form fibers instead of droplets some specific conditions must be achieved: (i) the fluid must have sufficiently high viscosity, (ii) the solvent should be volatile enough to evaporate to allow regeneration of the solid polymer, and (iii) the electric field should have a strength within a certain range.

The next relevant academic development was achieved by John Zeleny, who published work on the behavior of fluid droplets at the end of metal capillaries [4].

From 1934 to 1944, Formhals came out with several innovative set-ups to produce yarns made out of electrospun fibers including designs that do not require a spinneret. With these works, Formhals published a series of patents, describing his setups for the production of polymer filaments using an electrostatic force. In fact, many recent electrospinning setups can be traced back to the patents more than half a century ago such as using multiple spinnerets and using parallel electrodes to produce aligned fibers. About 50 patents for electrospinning polymer melts and solutions have been filed in the past 60 years [6]. Based on Zeleny's work, Sir Geoffrey Ingram Taylor produced, between 1964 and 1969, the theoretical underpinning of electrospinning. Taylor's work on electrostatics was performed during his retirement after a broad career with significant contributions to the fields of fluid mechanics and solid mechanics and also development of supersonic aircraft. Taylor's work contributed to electrospinning by mathematically modeling the shape of the cone formed by the fluid droplet under the effect of an electric field. This characteristic droplet shape is now known as the Taylor cone [4].

Before 1990, there was very little academic research and publications on electrospinning. Nevertheless, there was some research on the behavior of thin liquid jets in an electric field. One can say that electrospinning was re-discovered in 1995 by Doshi and Reneker who, whilst investigating electrospraying, observed that fibers could be easily formed with diameters on the nanometer scale. From that moment, there have been further theoretical developments and more intensive studies on the driving mechanisms of the electrospinning process. One example is the extensive work made by Reznik and co-workers on the characterization of the Taylor cone and the subsequent ejection of a fluid jet. The investigation done by Hohman and co-workers [7] is another one, which was based in

the relative growth rates of the numerous proposed instabilities in an electrically forced jet once in flight. Also important has been the work by Yarin and co-workers [8] that endeavours to describe the most important instability to the electrospinning process, the bending instability.

The interest in electrospinning is been growing in the past years and in global scale. More than 200 universities and research institutes worldwide are studying various aspects of the electrospinning process (Figure 2). There are several reasons that can explain this emergent attention but the possibility to combine fundamental and application-oriented research from different science and engineering disciplines is one of the most fascinating reasons. These research efforts usually target complex and highly functional systems, which could certainly be applied on a commercial level. Fiber systems in which the macroscopic properties can be targeted through modifications on the molecular level are of particular interest.

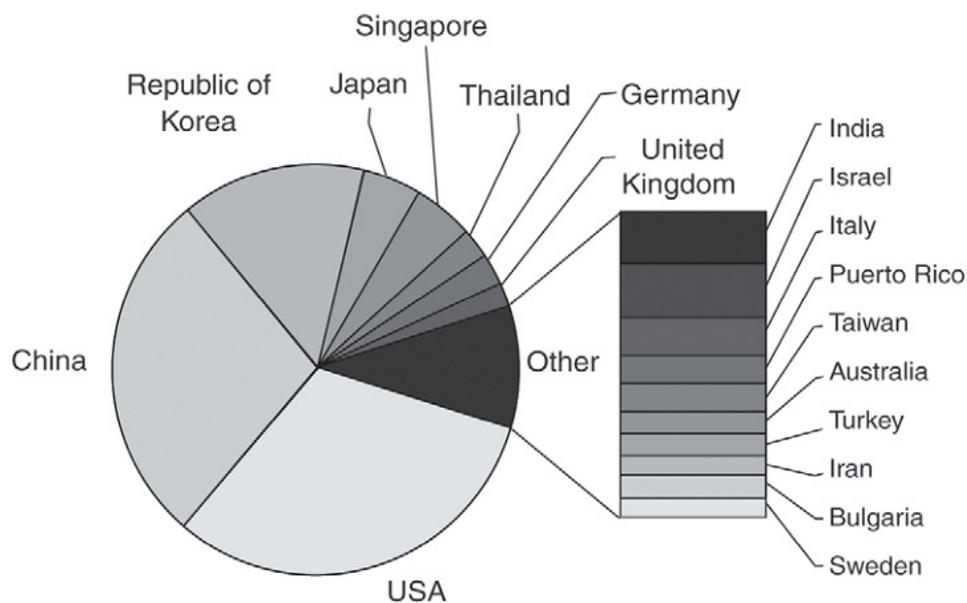


Figure 2 - Breakdown of journal papers with the keyword "Electrospinning" by country of origin [4].

Since so many workgroups became alert to the extensive range of applications presented by electrospinning in the most diverse scientific areas, a multitude of new and interesting concepts, developed at great speed. This rapid development is reflected by the skyrocketing numbers of scientific publications and patents (Figure 3).

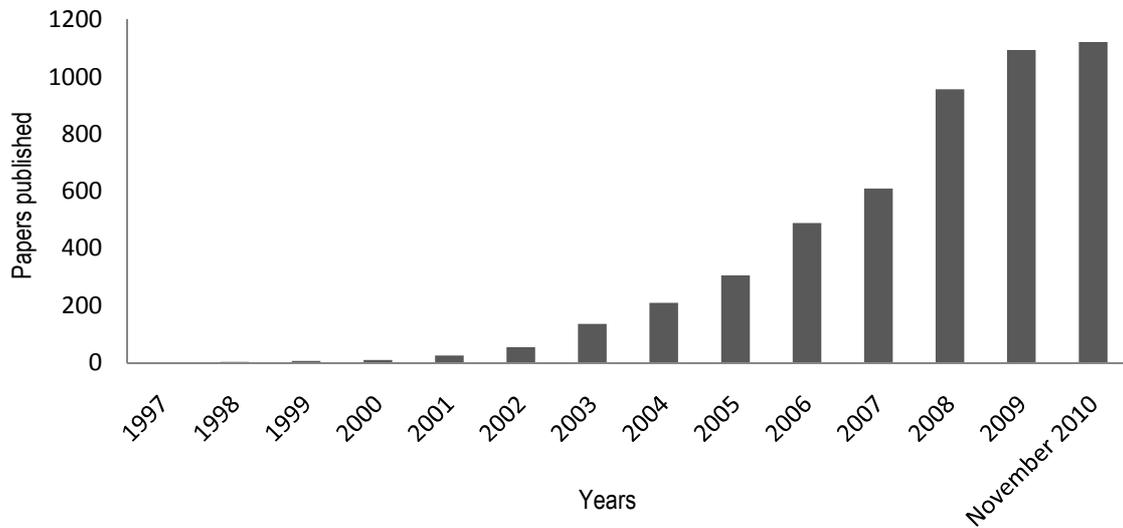


Figure 3 - Number of papers published with the keyword "Electrospinning" in a given year.

1.1.3. Electrospinning: Setup, Mechanisms

The formation of nanofibers through electrospinning is based on the uniaxial stretching of a viscoelastic solution. To understand and appreciate the process that enables the formation of various types of nanofibers, the setup and the principles of electrospinning have to be analyzed.

1.1.3.1. The basic setup for electrospinning

There are various approaches to produce nanofibers. The following can be used: drawing technology for producing micro/nanofibers using a micropipette with a diameter of a few micrometers; template synthesis of carbon nanotubes, nanofiber arrays and electronically conductive polymer nanostructures; and thermally induced phase separation method for producing nanoporous nanofibers.

Electrospinning is the cheapest and the most straightforward way to produce nanomaterials [9]. Electrospinning setups can be quite simple as we can see in Figure 4 that shows schematic illustrations of basic setups. Currently, there are two standard electrospinning setups, one vertical and another horizontal, with both consisting of three major components: a high-voltage power supply, a spinneret (a metallic needle) and a collector (a grounded conductor) [6, 10].

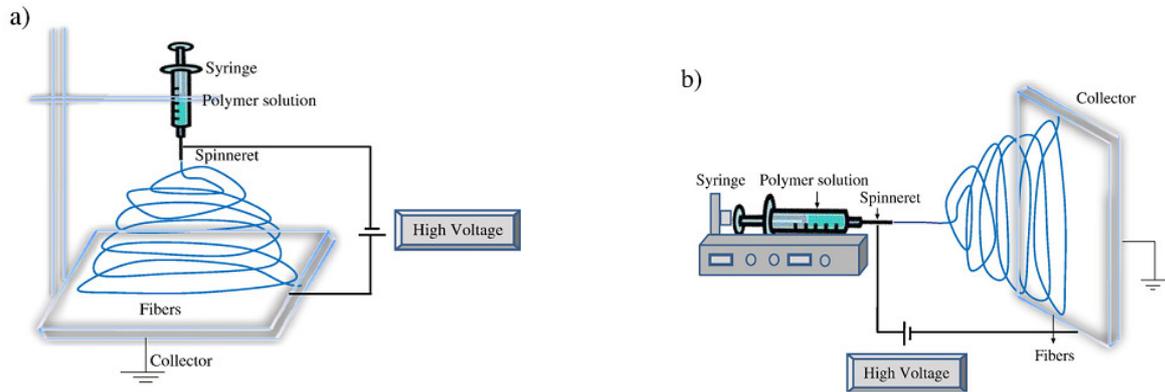


Figure 4 - Schematic diagrams of electrospinning apparatus setups: (a) Typical vertical setup and (b) horizontal set up [6].

Electrospinning is usually conducted at room temperature under atmosphere conditions. Most of the polymers are dissolved in appropriate solvents (water or organic solvents) before the process. The polymer fluid is then introduced into the capillary tube for electrospinning. Due to unpleasant or even harmful smells emitted by some polymers, the process should be conducted within a ventilated chamber [6].

After filling the syringe with the polymer solution, it is mounted on a syringe pump that will control the flow rate of the spinning solution and in an angle sufficient to prevent discharge of the fluid from the syringe under its own weight. A wire electrode is connected to the solution (or to the spinneret, often a metallic needle) and when a high voltage (usually in the range of 1 to 30 kV) is applied, the pendent drop of polymer solution at the nozzle of the spinneret will become highly electrified. Under such conditions, the drop will experience two major types of electrostatic forces: the electrostatic repulsion between the surface charges; and the Coulombic force exerted by the external electric field. Repulsion between charges at the free surface then works against surface tension and fluid elasticity to deform the droplet into a conical shape, called the Taylor cone. Beyond a critical charge density, this cone is unstable and a jet of fluid is emitted from the tip of the cone. This charged jet then seeks a path to ground. As it does so, the fluid filament is accelerated and undergoes a stretching and whipping process, leading to the formation of a long and thin thread. As the liquid jet is continuously elongated and the solvent is evaporated, its diameter can be greatly reduced from hundreds of micrometers to as small as tens of nanometers. A suitable collector electrode is used to direct this path to ground and the charged fibers are often deposited as a randomly oriented, non-woven mat [11].

1.1.3.2. Electrospinning Mechanism

As seen previously, the electrospinning setup is extremely simple, although the spinning mechanism is rather complicated. Electrospinning involves complex electro-fluid-mechanical issues.

Recently, many experimental observations demonstrated that the thinning of the jet that occurs during the electrospinning process is caused by the bending instability associated with the electrified jet. As we can see in Figure 5 (left), it is obvious that the jet was initially a straight line and then became unstable. It appears that the cone-shaped, instability region is composed of multiple jets. However, a closer examination establishes that the conical envelope contains only a single, rapidly bending or whipping thread (Figure 5, right) [10].

Bending instability promotes that a straight section of the jet turns sideways and forms loops in the horizontal plane. The loop diameters increase with time during the motion towards the counter electrode (with velocities on the order of meters per second). During this process, the jet is highly stretched and reduced. Along these reduced fibers, bending occurs again and is followed by the formation of a new state of coils. This procedure is repeated until the fibers solidify or become resistant towards these instabilities, owing to their extreme thinness. As a consequence of these instabilities, fibers with diameters down to a few nanometers can be generated stably and without decomposition of the jet into droplets. The bending, stretching and coiling which may occur in the jet are reflected in the morphologies of the evolving fibers [3].

Many papers report bending instability during electrospinning, which provide a better understanding of the mechanism responsible for the electrostatic process. Yarin *et al.* [8] made an extensive study on the electric force and the polymer jet in the electrospinning process. Their theoretical work demonstrates that during the electrospinning process most charge carriers in organic solvents and polymers have lower mobilities and for that reason the charge moves through the liquid for larger distances only if given enough time. After the initiation from the cone, the jet undergoes a chaotic motion or bending instability and is directed through the electric field towards the oppositely charged collector, which collects the charged fibers.

Shin *et al.* [12] studied the electrically forced jet and associated instabilities. From their work, it is possible to conclude that in order to electrospun solid nanofibers, the instability associated with the fluid is a key step, especially the whipping jet. The experiments that were done indicate that the whipping jet is caused by interactions between the external electric field and the surface charges of the jet. Those interactions are also responsible for stretching and acceleration of the fluid

jet in the instability region prior to impact on the collector and may be also responsible for any diameter reduction in electrospinning.

Deitzel *et al.* [13] have studied the effect of processing variables on the morphology of Poly(ethylene) Oxide (PEO) nanofibers and have confirmed the importance of the jet stability. During the electrospinning of PEO solution, voltage increase originated a change in the shape of the jet. This shape change, which corresponded to a decrease in the stability of the initial jet, was associated with a relevant increase in the number of bead defects along the PEO fibers.

These and many other reports are of big importance because they may assist experimentalists in the design of new setups that may provide a better control over the diameter and structure of electrospun nanofibers.

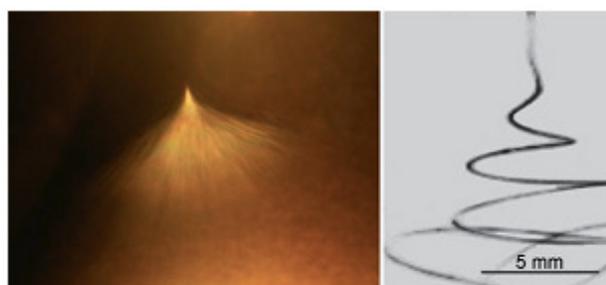


Figure 5 - Left: Instability region of a liquid jet electrospun from an aqueous solution of poly (ethylene oxide) (PEO) solution during electrospinning. Right: high-speed photograph of jet instabilities [3].

1.1.4. Effects of various parameters on electrospinning

Parameters affecting electrospinning of polymer solutions are of great interest and will be discussed in this section. The parameters affecting electrospinning and the obtained fibers may be broadly classified into three main groups: polymer solution parameters, processing conditions and ambient conditions. With the understanding of these parameters, it is possible to come out with setups to yield fibrous structures of various forms and arrangements. It is also possible to create nanofibers with different morphology by varying these parameters.

1.1.4.1. Polymer solution parameters

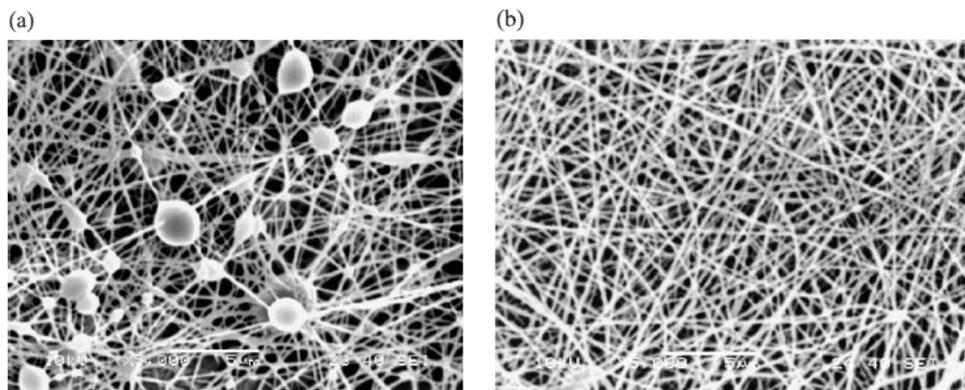
The properties of the polymer solution have the most significant influence in the electrospinning process and on the resultant fiber morphology. Solution parameters include those resulting from the intrinsic properties of the polymer, such as its chemical structure, molecular weight

and concentration, the characteristics of the solvent (e.g. volatility, dielectric constant), and properties of the resulting solution, such as viscosity, conductivity, and surface tension.

Concentration and molecular weight effect

The amount of solubilized polymer and its molecular weight (Mw) have a significant effect on the rheological properties, electrical conductivity, dielectric strength and on the surface tension of the solution. The preceding properties are some of the major parameters governing the electrospinning process which can produce a variety of structures including beads, beaded fibers, complete fibers and flat fibers. Several works have studied the effect of molecular weights and solution concentrations for different polymers and solvents, to highlight the range of structures that may be produced by electrospinning. In this work, our main interest was on the polyvinylalcohol (PVA) properties during the electrospinning process, thus we review below the available information regarding this polymer to illustrate concentration and molecular weight effects on the electrospun fibers.

Koski *et al.* [14] studied the effect of polymer average molecular weight on the fiber structure of electrospun PVA with Mw ranging from 9000 to 186,000 g/mol. In the Mw range of 9000 - 13,000 g/mol, the fibrous structure was not completely stabilized and a bead-on-string structure was obtained, indicating the resistance of the jet to extensional flow. As the molecular weight increases to 13,000 - 23,000 g/mol, a fibrous structure is stabilized. Flat fibers were observed at a molecular weight of 31,000 – 50,000 g/mol as can be seen in Figure 6.



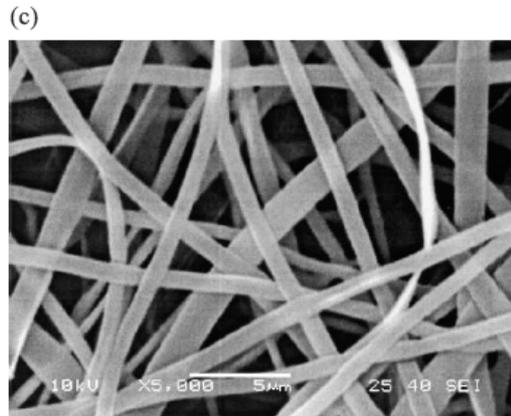


Figure 6 - SEM Photographs showing the typical structure of electrospun PVA fibers obtained for PVA samples with various molecular weights: a) 9000-10,000 g/mol; b) 13,000-23,000 and c) 31,000-50,000 g/mol (solution concentration: 25 wt.%) [14].

As the solution concentration increases, the fiber diameter and interfiber spacing increase and there is a gradual shift from circular to flat fibers. In low molecular weight samples, this shift from circular to flat fibers occurs at a higher concentration than in high Mw polymers (Figure 7). For example, Koski *et al.* [14] concluded that flat fibers begin to appear at a concentration of 31 wt.% PVA for samples with molecular weight range 13,000 – 23,000 g/mol, whereas for PVA samples with Mw 50,000 – 89,000g/mol completely flat fiber structures were observed at 13 wt.% PVA.

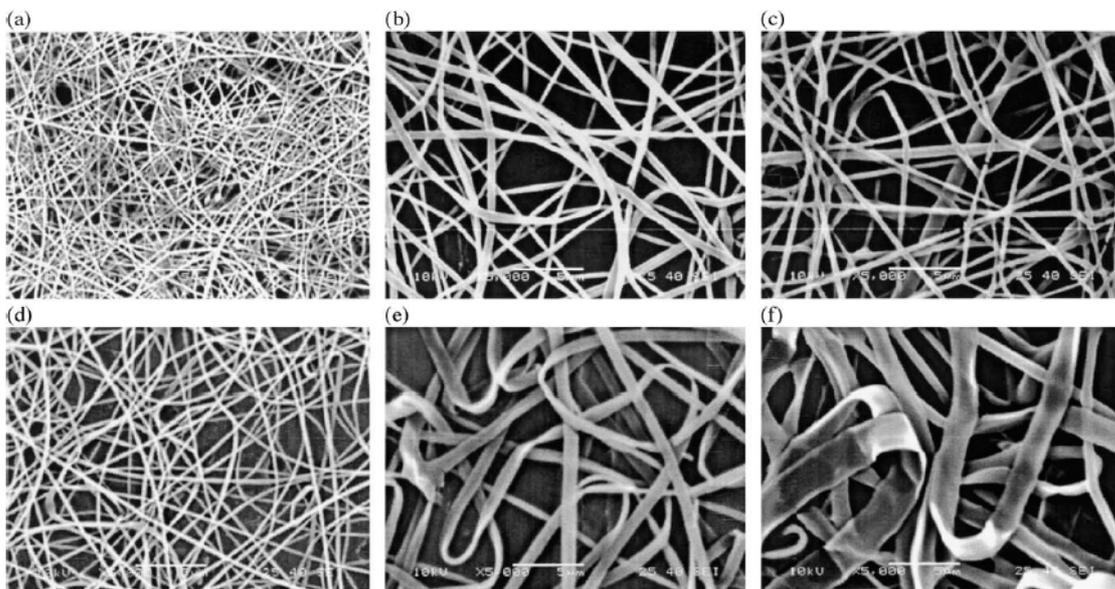


Figure 7 - SEM Photographs showing the effect of solution concentration on the morphology of electrospun PVA fibers. Molecular weight = 13,000 - 23,000 g/mol; a) 21 wt.%; b) 27 wt.%; c) 31 wt.%. Molecular weight = 50,000 - 89,000 g/mol; d) 9 wt.%; e) 13 wt.%; f) 17 wt.% [14].

Tao *et al.* [15] studied the molecular weight dependent structural regimes during the electrospinning of PVA. The polymer molecular properties have shown to play a vital role in determining fiber initiation and stabilization. This way, at any molecular weight, the effect of concentration on the breakdown of the solution jet can be described by two critical concentrations, C_i and C_f which define the morphological transition from bead-only structure to bead-free fibers. Below C_i , only beads are produced due to insufficient chain entanglements in the solution. Above C_i , a combination of beads and fibers is observed and when the concentration is increased above C_f , complete bead-free fibers are produced. C_i is typically near the entanglement concentration C_e , at which chain entanglements within the solution become significant. Hence, C_i is a transition concentration at which fibers begin to emerge from the beads and C_f is the concentration at which a fibrous structure is stabilized. Attending to these assumptions and based on experimental procedures, they achieved the transitions points (C_i and C_f) for various PVA molecular weights, which are plotted in Figure 8 [15].

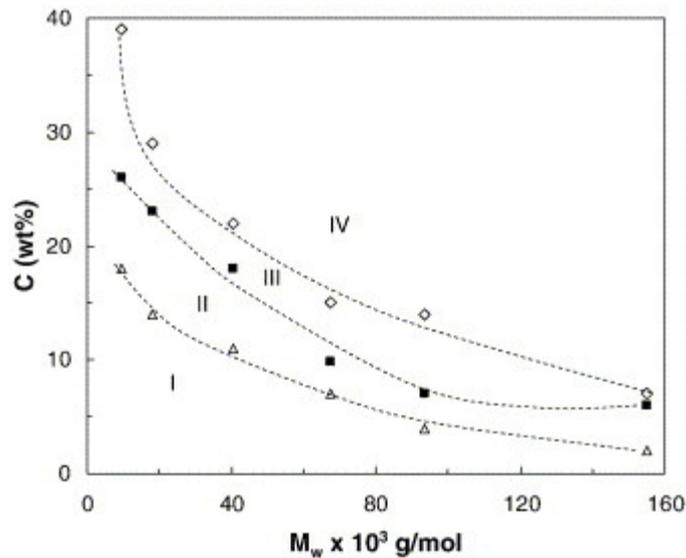


Figure 8 - Regimes for various morphologies observed in the electrospun PVA. I: beads, II: beaded fibers, III: complete fibers and IV: flat fibers. The symbols and the accompanying lines correspond to the transition point from one structure to the other [15].

In another study on morphology of electrospun PVA mats, Zhang and co-workers [16] have evaluated some solution parameters, such as polymer concentration, for a PVA sample with a degree of polymerization of 1700 ± 50 , reaching similar conclusions to those previously mentioned. At 6%, spindle-like beads were seen and with increasing concentration, the morphology was changed from beaded fiber to uniform fiber structure. Above the concentration of 8.3%, the polymer solution did not form fibers but formed big droplets falling on the collection target. This way, and as

seen previously, one needs to exceed a critical concentration of polymer to achieve extensive chain entanglements necessary to produce electrospun fibers. Below this concentration, chain entanglements are insufficient to stabilize the jet and the contraction of the diameters of the jet driven by the surface tension cause the solution to form beads or beaded fibers. At higher concentration, the viscoelastic force which resisted rapid changes in fiber shape originates the formation of uniform fibers. However, there is also a maximum polymer concentration above which the solution viscosity is too high precluding the liquid jet development and originating the formation of drops and not uniform fibers [16].

Viscosity

Generally, when a polymer of higher molecular weight is dissolved in a solvent, the solution viscosity will be higher than that of a solution of the same polymer but of a lower molecular weight. One of the conditions necessary for fiber formation during the electrospinning process is that the solution must consist of polymer chains of sufficient molecular weight and that the solution must be of sufficient viscosity [17].

It has been found that with very low viscosity solutions there is no continuous fiber formation and when the viscosity is very high, the spinning solution tends to accumulate at the tip of the syringe, occurring some solidification which will difficult the ejection of jets from the polymer solution. Therefore there is a requirement of optimal viscosity for electrospinning.

Viscosity, polymer concentration and molecular weight of polymer are correlated to each other. The solution viscosity has been strongly related to the concentration of the solution and the relationship between the polymer viscosity and/or concentration and fibers obtained by electrospinning have been extensively reported. Lee *et al.* [18] in their study about the influence of the PVA molecular weight found that increasing viscosity reduced the split ability. The increase on the solution viscosity originated long spindle-like beads and larger fibers with a bigger diameter. Cengiz *et al.* [19] also reported the influence of viscosity on the spinning capability. Spinnable PVA solutions were related to high viscosity and a pronounced shear-thinning behaviour (PVA with molecular weight in the range 80.000-150.000). Nien *et al.* [20] also showed the importance of the solution viscosity for the electrospinning process and final PVA nanofiber morphology. They have also reported that with an increase in solution concentration (from 4 to 12%), nanofibers became uniform and beads disappeared. By increasing the polymer concentration, viscosity also increased

which showed that higher viscosities may produce better nanofibers. This may occur because high viscosity polymer solutions typically exhibit longer stress relaxation times, which could prevent the fracturing of the ejected jets during electrospinning. For solutions of low viscosities, the surface tension is the dominant factor and just beads or beaded fibers are formed. In this work, they have also shown a relevant correlation between viscosity and applied voltage. In a solution with higher viscosity, there is a higher polymer chain entanglement. For this reason, there must be applied a higher force to allow it to be ejected from the syringe tip and produce nanofibers. This way, a general conclusion can be made: to electrospun a uniform nanofiber with a low-viscosity solution, a low voltage should be applied, while a high voltage should be used with high-viscosity solutions. The effects of viscosity and voltage on the electrospinning are competitive.

Surface Tension

Surface tension, more likely to be a function of solvent composition of the solution, plays a critical role in the electrospinning process and in the resulting fibers. The initiation of electrospinning requires the charged solution to overcome its surface tension. However, as the jet travels towards the collection plate, the surface tension may cause the formation of beads along the jet. Surface tension has the effect of decreasing the surface area per unit mass of a fluid. In this case, when there is a high concentration of free solvent molecules, there is a greater tendency for the solvent molecules to congregate and adopt a spherical shape due to surface tension. A higher viscosity will mean that there is a greater interaction between the solvent and polymer molecules thus when the solution is stretched under the influence of the charges, the solvent molecules will tend to spread over the entangled polymer molecules thus reducing the tendency for the solvent molecules to come together under the influence of surface tension (Figure 9).

Different solvents may contribute to different surface tensions. Generally, the high surface tension of a solution inhibits the electrospinning process because of instability of the jets and the generation of sprayed droplets. The formation of droplets, bead and fibers depends on the surface tension of the spinning solution and a lower surface tension of the solution helps electrospinning to occur at a lower electric field. However, not necessarily a lower surface tension of a solvent will always be more suitable for electrospinning. Basically, surface tension determines the upper and lower boundaries of the electrospinning window if all other variables are held constant [6, 17].

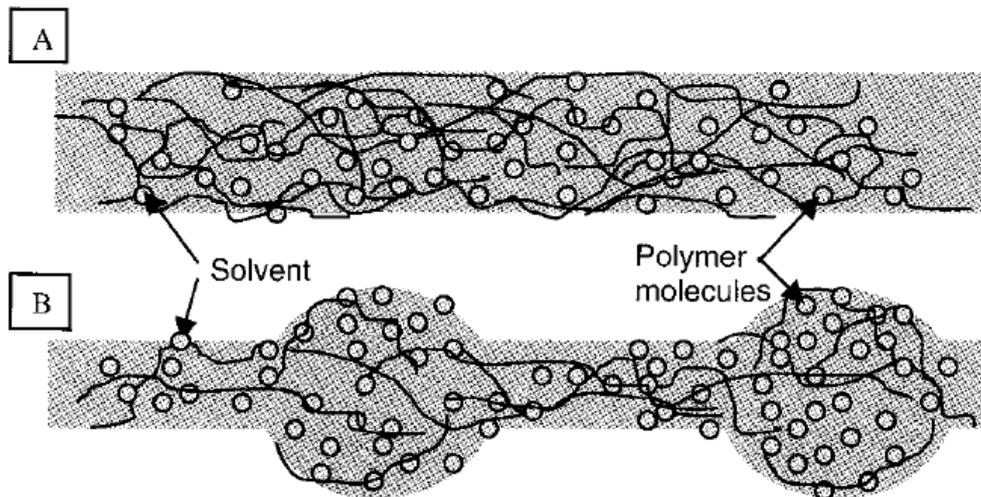


Figure 9 - [A] At high viscosity, the solvent molecules are distributed over the entangled polymer molecules. [B] With a lower viscosity, the solvent molecules tend to congregate under the action of surface tension [17].

Solution conductivity and surface charge density

Polymers are mostly conductive, with a few exceptions of dielectric materials, and the charged ions in the polymer solution are highly influential in jet formation. Solution conductivity is mainly determined by the polymer type, solvent used, and the availability of ionisable salts. It has been found that with the increase of electrical conductivity of the solution, there is a significant decrease in the diameter of the electrospun nanofibers whereas a low conductivity of the solution, results in an insufficient elongation of a jet by electrical force to produce uniform fiber, and beads may also be observed. It has been shown by Hayati *et al.* [21] that highly conductive solutions are extremely unstable in the presence of strong electric fields which results in a dramatic bending instability as well as a broad diameter distribution. Generally, electrospun nanofibers with the smallest fiber diameter can be obtained with the highest electrical conductivity [6].

1.1.4.2. Processing parameters

Other important factors affecting the electrospinning process and the characteristics of the electrospun fibers, as referred previously, are the various external factors related to the electrospinning process. This includes mainly the supplied voltage, the feed rate, the distance between the needle tip and collector and the rotational speed of collector. These parameters have a certain influence in the fiber morphology although, in general, they are less significant than the solution parameters [17].

Applied Voltage

To apply a voltage to a polymeric solution is the fundamental principle behind the electrospinning technique. It is the voltage that will cause the jet stretching and fiber formation. For a given polymer/solvent system there is a critical voltage responsible for fiber formation[22].

Some studies have been done to understand how the effect of the electric field strength interferes with fiber morphology. Supaphol *et al.* [23] working with PVA reported that for a given concentration, increasing the applied electrical potential caused the number of the as-spun fibers (or beaded fibers) per unit area to increase, which is in line with the observed decrease in the diameters of the as-spun fiber mats with increasing the applied electrical potential. Interestingly, they stated that increasing the applied electrical potential also caused the size of the beads to decrease and their shape to be more elongated (i.e., spindle-like), what they assumed as an indication of the increased stretching force exerted on the jet segment. Probably, the increase in the applied electrical potential would cause the number of charges carried within a jet segment to increase, hence an increase in both the electrostatic and the Coulombic repulsion forces. The increased Coulombic repulsion force should cause the diameters of the as-spun fibers to decrease (due to an increasing stretching force exerting on the jet segment), while the increased electrostatic force should cause the diameters of the as-spun fibers to increase (due to both the increase in the speed of the jet segment and the increase in the mass flow rate, the phenomena that cause the onset for the bending instability to occur closer to the screen collector).

Based on these assumptions, one can assume that the observed decrease in the fiber diameters with the initial increase in the applied electrical potential could be due to the contribution from the increase in the Coulombic repulsion force, while the observed increase in the fiber diameters with further increase in the applied electrical potential could be due to the contribution from the increase in the electrostatic force [23].

Ding *et al.* [24] also reported on the voltage influence on the electrospinning process and fiber morphology. They have verified experimentally that the shape of the initiating drop changes with spinning conditions like voltage, viscosity and feed rate. The electrostatic force was gradually increased with increasing the voltage causing the reinforcement of the droplet split ability. They have shown that the average diameter of PVA fibers slightly decreased with increasing applied voltage. As illustrated in Figure 10, they also showed that the electrospinning throughput increased smoothly with increasing voltage from 7 to 19 kV. Increasing the voltage caused the rate at which the solution

was removed from the capillary tip to exceed the rate of delivery of the solution to the tip needed to maintain the conical shape of the surface.

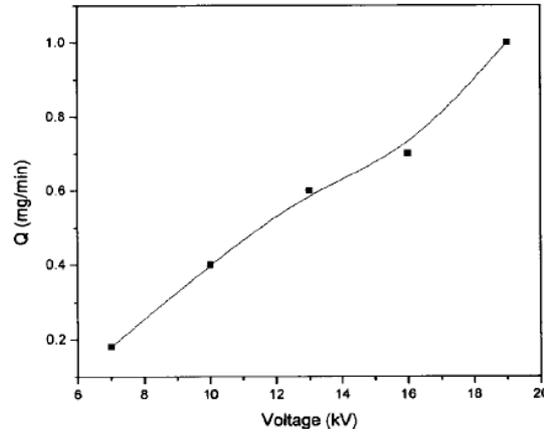


Figure 10 - Throughput as a function of voltage [24].

This way, both studies exemplify what is general accepted, i.e. that in the electrospinning process the voltage applied to the solution is a crucial element. Only after the attainment of a certain voltage threshold, the necessary charges on the solution are induced along the electric field and the electrospinning process can be initiated leading to fiber formation.

Feed Rate

Solution feed rate is decisive for the amount of polymer solution that is ejected from the tip of the syringe at every moment during the electrospinning process. This way, changing this parameter will affect the resulting electrospun fibers. An increase in the flow rate will correspond to an increase in the fiber diameter and the appearance of beads in the non-woven mat. This is due to a higher quantity of spinning solution that reaches the needle tip resulting in a more difficult solvent evaporation. On the other hand, with slow feed rate, there is a good solvent evaporation avoiding these problems and therefore is more desirable.

Feed rate and voltage are also associated. For a stable Taylor cone to be maintained there must be a correct relation between voltage and feed rate. When the last one is increased to the same rate that the solution is carried away by the spinning solution jet, there must be a corresponding increase in the charges in order to maintain jet stability [6, 17, 25].

Tip to collector distance

The distance between the tip and the collector (TCD) has been examined as another approach to control the fiber diameters and morphology. It has been found that a minimum distance is required to give fibers sufficient time to dry before reaching the collector; otherwise, with distances that are either too close or too far, beads have been observed. The effect of TCD on fiber morphology is not as significant as other parameters and this has been observed with electrospinning of different polymers.

Barhate *et al.* [26] used polyacrylonitrile to prepare electrospun fibrous mats and studied, among other parameters, the influence of the TCD on fibers morphology. They showed that the extent of drying, deposition and orientation of fibers can be affected by increasing the tip-to-collector distance. A densely packed membrane was obtained, instead of a porous nanofibrous membrane, when the distance between the spinneret and the collector was less than 8 cm indicating inadequate drying of the jet. A tip-to-collector distance of 10 cm has improved the drying of the jet and collection of the nanofibers. An increase in the tip-to-collector distance from 10 to 16 cm did not influence the average diameter of the nanofibers; this revealed that the fibers were sufficiently dried in transit between the spinneret and the collector.

The deposition of the fibers was mostly confined to the middle portion of the mandrel (collector) when the deposition distance was 16 cm, which indicates that the area of the envelope cone created by the electrically driven bending jet was not adequate to cover the entire length of the drum. This is a problem, since the resulting mat was not homogenous.

Barhate *et al.* [26] also observed that by further increase the TCD (13, 14 and 16 cm), there was a tendency for the fibers to deposit unevenly on the collector (the fibers were being deposited more at the right portion of the collector). The extent of uneven deposition of the fibers increased as the TCD was increased from 13 to 16 cm, what was attributed to the different rates of drying and charge decay experienced by the nanofibers before depositing.

Effect of collector

As referred previously, for the electrospinning process to initiate there must be an electric field between the source and the collector. Usually, the collector plates used are aluminum foil, conductive paper, conductive cloth, wire mesh, pin, rotating rods, rotating wheels and others are

common. These are electrically grounded so that there is a stable potential difference between the source and the collector [6].

When a non-conducting material is used, charges on the electrospinning jet will accumulate very quickly on the collector, resulting in a fewer fibers deposited. These fibers have a lower packing density compared to those collected on a conductive surface, due to the repulsive forces of the accumulated charges on the collector as more fibers are deposited,. For a conductive collector, charges on the fibers are dissipated thus allowing more fibers to be attracted to the collector. As a result, the fibers are packed closely together. A static or moving collector has also an obviously effect on the electrospinning process. Rotating collectors have been used to collect aligned fibers and it was found that they assist in yielding fibers that are dried. This is very useful because certain solvents have high boiling points that may result in the fibers being wet when they are collected. The rotating collector will give the solvent more time to evaporate and also increase the rate of evaporation of the solvents. This will improve the morphology of the fiber where distinct fibers are required [17].

1.1.4.3. Ambient Conditions

Besides solution and processing parameters, ambient conditions may also affect the electrospinning process and electrospun nanofiber morphology. Factors as humidity or temperature can have an impact on the resulting fibers.

An increase in temperature is associated with a global diameter decrease. This reduction may be due to a diminished viscosity of the polymer solutions which is always interconnected with temperature rising in an inverse relationship.

Ambient humidity which is always affecting spinning process also induces a morphology change in final nanofibers. High humidity is related with the formation of pores on fiber surface and a continuous increase in ambient humidity may lead to the coalescence of these pores. High humidity can also help discharge of the electrospun fibers. On the other hand, when a low humidity is surrounding an electrospinning process, volatile solvents may evaporate rapidly. When this evaporation is faster, there is a quick loss of solvent from the tip of the needle, limiting electrospinning process to a few minutes, before the needle tip is clogged [6, 17].

1.1.5. Polymers used in electrospinning

There are a great number of polymers used nowadays in electrospinning, which are capable of producing nanofibers with quality and used for many applications. These nanofibers have been produced from different synthetic or natural polymers, or in many cases from a blend of both. From several years ago, nanofibers are being developed using proteins [27], nucleic acids [28] and polysaccharides [29].

Polymers that occur naturally have usually higher biocompatibility when compared to synthetic ones, which are of great value for biomedical applications. The most used and reported natural polymers employed in electrospinning are collagen [30], elastin [31], gelatin [32], and chitosan [33] among others. The use of these polymers is of great value for medical applications. However, partial denaturations have been reported recently which is of great concern, once the properties of the polymers are lost and it is unknown how this can affect their applications, for example, in tissue engineering.

On the other side are synthetic polymers, which offer many other advantages over natural ones. They can be customized to give a wider range of properties such mechanical ones and a desired degradation rate, which is not possible with natural polymers. Till date, many synthetic polymers have been reported on the fabrication of nanofibers such polystyrene and poly(styrene-co-maleic anhydride) [34], poly(ethylene terephthalate) [35], polyacrylonitrile [36], poly (lactic acid) [37] among many others, used for bone tissue engineering, wound dressings or drugs controlled release. For a more complete understanding, Table 1 lists different polymers used in electrospinning and their applications.

Table 1 - Different polymers used in electrospinning and their applications.

| Polymer | Application | References |
|-----------------|-------------------------|--------------------------------|
| PVA | Biomaterials | Ding <i>et al.</i> [24] |
| PEO | Nanofibrous Scaffolds | Szentivanyi <i>et al.</i> [38] |
| Cellulose | Affinity membrane | Ma <i>et al.</i> [39] |
| Polystyrene | Skin tissue engineering | Sell <i>et al.</i> [40] |
| Chitosan | Biomedical Applications | Jayakumar <i>et al.</i> [41] |
| Gelatin | Wound healing | Powell <i>et al.</i> [42] |
| Hyaluronic acid | Medical implant | Um <i>et al.</i> [43] |
| Silk | Biomedical applications | Zarkoob <i>et al.</i> [44] |

Another form to enhance and develop nanofibrous electrospun mats is related with electrospinning of copolymers. The use of two or more polymers instead of one often contributes to the improvement of the final polymeric materials. These improvements include tailoring of thermal stability, mechanical strength and barrier properties and have therefore being highly pursued for many applications in different fields of research. When properly implemented, the performance of electrospun scaffolds based on copolymers can be significantly improved as compared to homopolymers. Some works report mixtures of polymers that allowed producing nanofibers with desired mechanical properties, morphology, structure, pore size, distribution and biodegradability. For example, the elastic poly (ethylene-co-vinyl alcohol) (PEVA) nanofibrous mat becomes stiffer after poly (glycolide) (PGA) is added for blend electrospinning. The thermal stability of poly methyl methacrylate (PMMA) can be improved through copolymerization of methyl methacrylate (MMA) with methacrylic acid (MAA). The glass transition temperature of poly (methacrylic acid) (PMAA) is higher than PMMA and it also exhibits a higher degradation temperature due to formation of anhydride upon heating [6].

Although so many polymers are available nowadays and numerous combinations are possible between polymers in order to achieve the desired characteristics of the final materials, this work is based on the use of PVA to produce nanofibers for enzyme encapsulation. For this reason is now important to understand the characteristics of this polymer and how they can be relevant for different applications.

1.1.5.1. Poly (vinyl alcohol)

PVA is a water-soluble polyhydroxy synthetic polymer (Figure 11) and the largest volume synthetic resin produced in the world. The excellent chemical resistance, physical properties and complete biodegradability of PVA resins (with water and carbon dioxide as degradation products) have led to their broad practical applications [45].

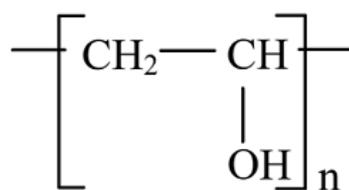


Figure 11 - Repetition unit of PVA [2].

PVA has been mainly used as paper coating, adhesives and colloid stabilizer. In recent years, much attention has been focused on the biomedical applications of PVA hydrogels including contact lenses, artificial organs and drug delivery systems [46].

Molecular structure and physical properties of PVA

Contrasting to the majority of vinylic polymers, PVA is not synthesized by polymerization of its own monomer. The vinyl alcohol monomer only exists under acetaldehyde form and every try to produce PVA by polyaldolic condensation hadn't success till date. This way, PVA is obtained industrially by the partial or total saponification of poly (vinyl esters) such as poly (vinyl acetate) (PVAc) and poly (vinyl pivalate) (PVPi). Generally, PVAc has been used as the precursor of PVA, where acetate groups are removed [47].

Usually, the industrial process used for PVA production is the acetaldehyde monomer polymerization in methanol. After this process, occurs a deacetylation in order to obtain poly (vinyl alcohol). This procedure can be done in solution, suspension or emulsion in an alkaline medium, usually by esterification in methanol in the presence of high amounts of sodium methoxide as catalyst, leading to the production of PVA and methyl acetate (Figure 12).

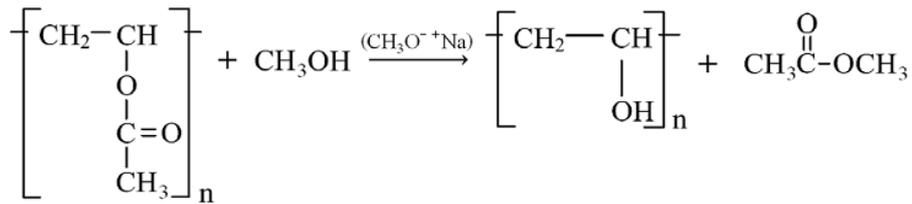


Figure 12 - Hydrolysis of PVAc to produce PVA [2].

Each PVA monomer is isomeric. A hydroxyl side group is connected to the backbone of PVA. These groups can be a source of hydrogen bonding, which readily forms between PVA chains in aqueous solutions. When PVA is industrially synthesized, the percentage of acetate groups converted to hydroxyl groups determines the degree of hydrolysis (DH) of PVA. PVA can also have different degrees of polymerization (P_n). For high hydrolysis PVAs, the hydroxyl groups on one polymer chain can form hydrogen bonding with hydroxyl groups of another chain as can be seen in Figure 13 a). Consequently, the polymers will line up with each other leading to chain orientation. The acetyl groups in PVA that has undergone partial hydrolysis act as spacers, which limit the crystallinity by preventing molecular chains from close approach (Figure 13 b).

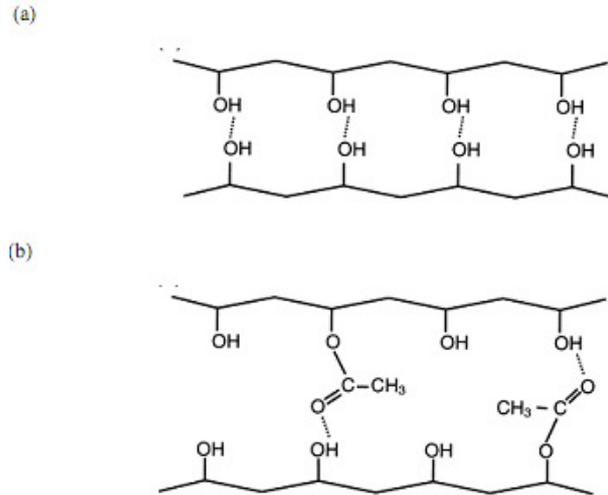


Figure 13 – a) At high hydrolysis many secondary hydrogen bonds are established. B) At low hydrolysis, acetate groups act as spacers and restrict the level of hydrogen bonding [48].

Due to the difficulty of carrying the reaction to completion without more drastic treatment, there is always an appreciable proportion (commonly, 2 mol% or less) of residual groups from the precursor poly (vinyl acetate) [48].

When this polymer has a DH between 87 and 89 mol% it has a lower mechanical and water resistance than a PVA with a DH between 98 and 99.9%. Moreover, the higher de DH, the higher is the amount of crystallinity. The crystalline unit cells predominant in the PVA are orthorhombic, monoclinic and hexagonal [46].

Water dissolution degree diminishes when DH and P_n increases. Usually, to achieve the total dissolution of PVA it is needed an approximate temperature of 90 °C, although when PVA have a DH less than 88%, it can be dissolved at lower temperatures [2]. Once that the extent of both inter and intra chain hydrogen bonding and solute-solvent hydrogen bonding is mainly determined by DH in the PVA chains, other polymer properties can be affected. Thus viscosity and solubility can be related to PVA degree of hydrolysis. This effect can be seen in Figure 14.

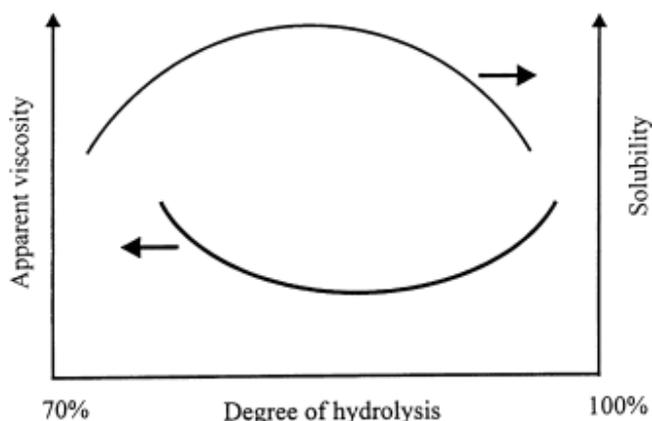


Figure 14 - Diagram of the interrelationship between apparent viscosity and DH, and between solubility and DH for aqueous PVA solution [49].

Due to the presence of acetate groups in the PVA chains, intra and inter chain hydrogen bonding are reduced. Consequently, the solubility of the PVA is enhanced because the increase of degree of hydrogen bonding between PVA chains and water molecules. The apparent viscosity shows a decrease with initial decrease of degree of hydrolysis and then an increase with further decrease of degree of hydrolysis. On the contrary, the solubility shows a maximum with variation of extent of hydrolysis. The viscosity and DH curves are functions of polymer molecular weight, solute concentration, storage time of the solution and ambient temperature. It is also worth mentioning that the interactions between solute and solvent will also decrease with further increase of the acetate group fraction in the PVA chains due to the increasingly hydrophobic influence of the additional

acetate groups. As result of this effect, the PVA usually becomes insoluble in water when the DH falls bellow ca 70% [49].

Poly (Vinyl Alcohol) Nanofibers

Many experiments to produce PVA nanofibers have been reported in the past years with different applications purposes. Some of these works have already been mentioned in section 1.1.4.1 PVA electrospun fibers obtained from PVA solutions (PVA with M_w 65000 g/mol) with different concentrations are illustrated in Figure 15 [24].

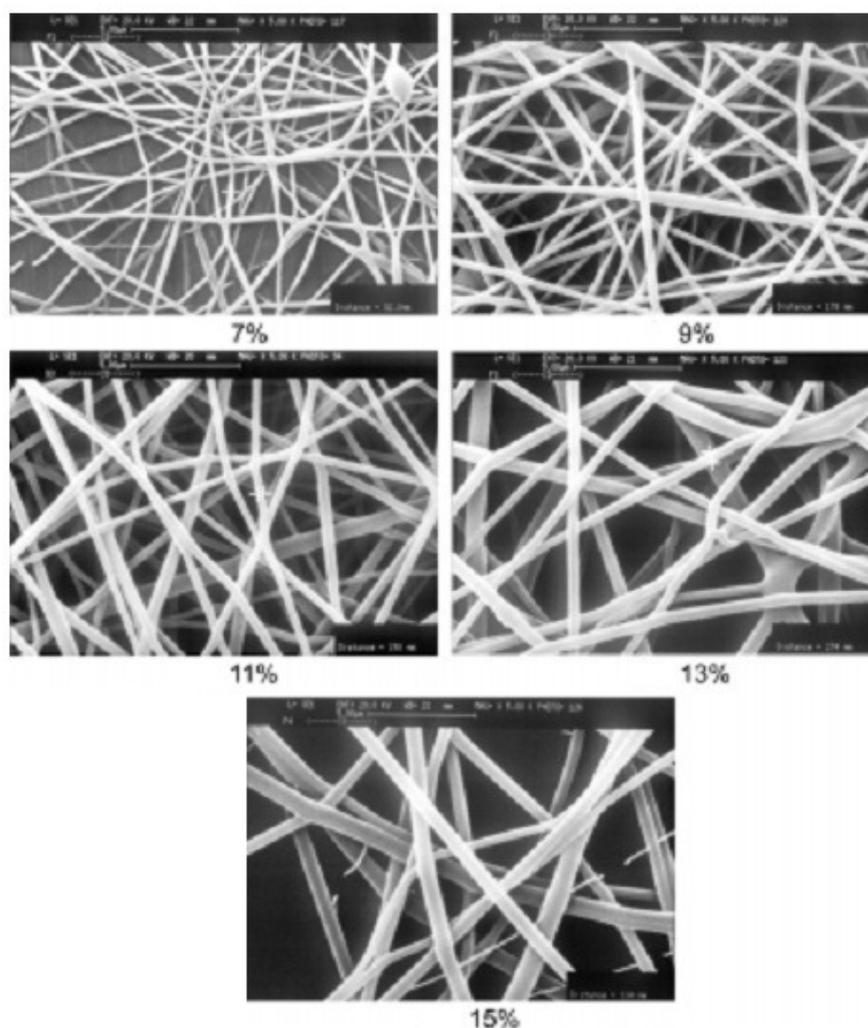


Figure 15 - SEM images of PVA fibers as a function of concentration.

As observed, the diameter of PVA fibers increased as the concentration increased. Supaphol *et al.* [23] also reported the production of PVA nanofibers matrices (PVA with Mw 72000 g/mol) from polymer solutions with different concentrations. As seen in Figure 16 and according to other references, they concluded that if the concentration was too low, it would occur the formation of droplets while a continuous raise of concentration would lead to complete fibers. Based on these two studies and others referred previously, one can predict an approximate concentration for the production of any fiber matrices attending to the Mw and DH of the PVA in use.

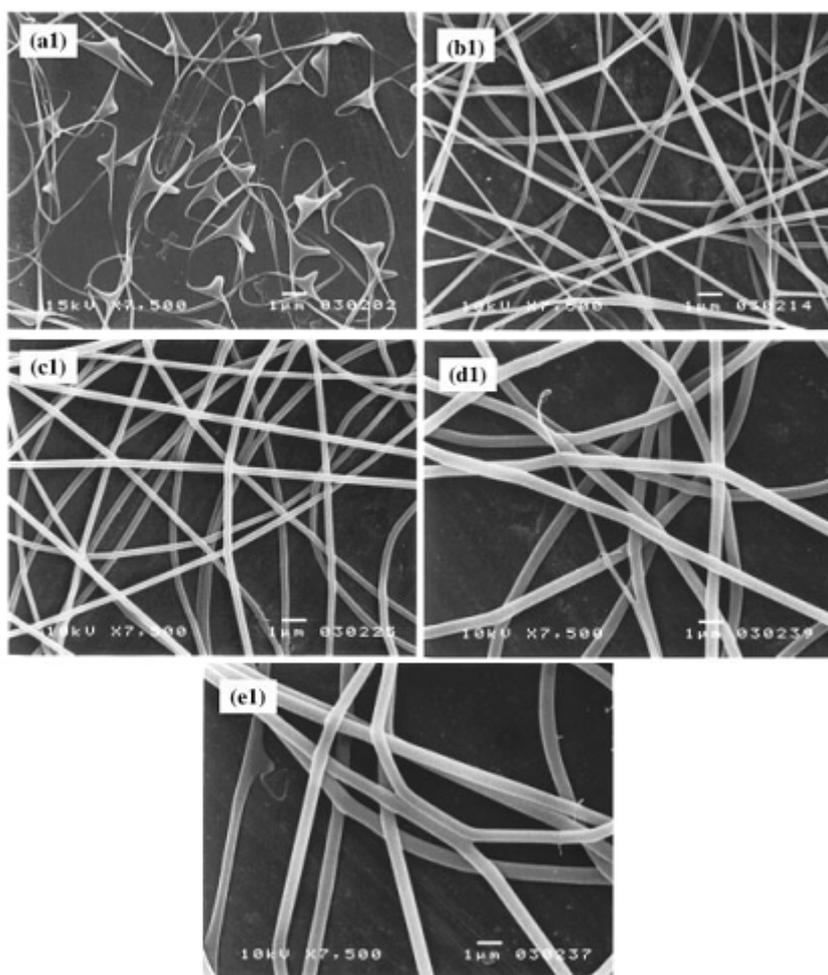


Figure 16 - SEM images of [23] the spun fibers from (a) 6, (b) 8, (c) 10, (d) 12, and (e) 14% (w/v) PVA solutions (adapted) [23].

Due to PVA non-toxicity, non-carcinogenicity and good biocompatibility and to all these studies on PVA nanofibers an extensive field of applications erupted easily. For example, Kenawy *et al.* [50] developed a new system for delivery of ketoprofen (a non-steroidal anti-inflammatory drug)

based on electrospun fibers of partial and full hydrolyzed PVA. Wang *et al.* [51] used PVA fibers to successfully immobilize lipase enzyme, which can be used as a bioreactor. They conclude that the use of PVA nanofibers is a simple and versatile approach applicable to other proteins or bioactive compounds. Jin *et al.* [52], used electrospun PVA nanofibers containing silver nanoparticles for use in antimicrobial applications.

As can be seen from these examples, electrospinning and PVA are at this moment important factors of development in many fields of technology that contribute effectively for our daily life. Although, PVA polymer will form excellent nanofibers by electrospinning under suitable conditions, a major drawback for certain applications is that they will disintegrate immediately upon contact to water. However, PVA is a highly functional polymer, which gives multifold opportunities for tuning of physical properties by chemical treatment.

Water-stable PVA nanofibers: crosslinking methods

Due to PVA structure where many hydroxyl groups are available to make hydrogen bonding with water, nanofibers mat produced have great hydrophilicity. This means that when the electrospun nanofiber membrane is immersed in water, it dissolves, blocking a variety of promising applications. Therefore, it is necessary to crosslink the PVA polymer and stabilize the electrospun nanofiber membranes in wet conditions.

Essentially, there are two main strategies to achieve the insolubilization of PVA. One makes use of the native functionalities of the PVA. All multifunctional groups capable of reacting with the –OH group may be used as a crosslinker of PVA. Thus, dialdehydes, dicarboxylic acids, dianhydrides among others are good options to bring PVA membranes water-insoluble [53].

One of the most used and successful crosslinkers is glutaraldehyde (GA) [50, 53-58]. In recent years, GA has gained increasing attention as a PVA crosslinking agent because of the absence of thermal treatment needed to drive the reaction. Additionally, it is also well known that GA can bind nonspecifically to biomolecules, such as proteins. Because the crosslinker has two active sites, it can be successfully used to bind proteins and PVA together. This feature enables the development of tailored structures to be used in biosensors once the constraints imposed by the biomolecules are considered.

The procedure commonly used to crosslink PVA membranes with GA is based on the immersion of the film in an alcoholic or acetone solution containing the crosslinking agent and a

mineral acid, such as HCl or H₂SO₄. Alcohol or acetone are used as a medium to swell the membrane, which allows the diffusion of the dialdehyde molecules and the protonic ions, which are used to catalyze the reaction. The mechanisms proposed for the reaction indicate that there is an optimum rate between the reactants that favors the crosslinking of the polymer, whereas higher amounts of the aldehyde can lead to the branching of PVA, as illustrated in Figure 17 [59]. As can be seen, the reactions between GA and PVA can be intramolecular and/or intermolecular crosslinks, either should render PVA insoluble.

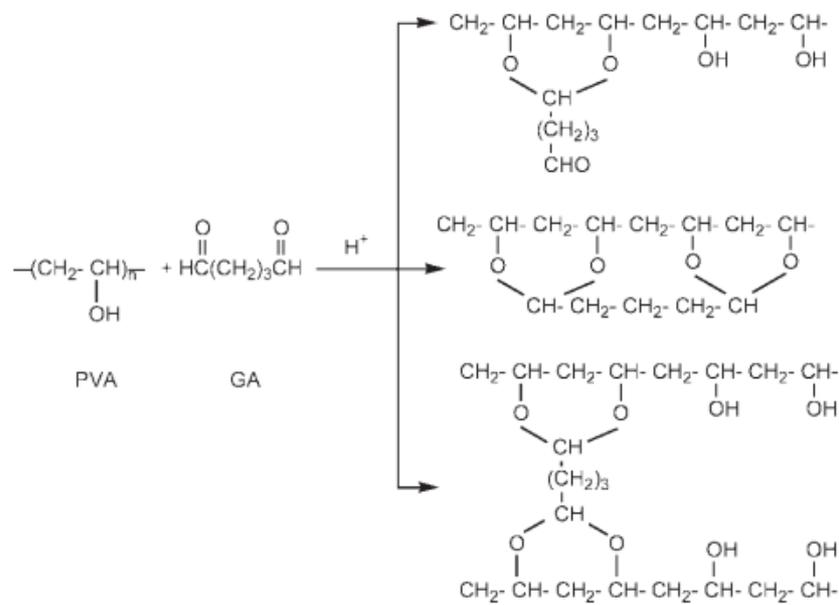


Figure 17 - Reactions of PVA with GA[58].

Yao *et al.* [60] used methanol as a crosslinking agent for PVA. They have found that soaking a PVA mat in methanol for at least 12 h preserves the integrity of the mat when it is immersed in water. Figure 18 shows a comparison between a wet electrospun PVA mat prior to and after methanol treatment. It is speculated that methanol treatment induces the increase of the degree of crystallization and therefore the number of physical crosslinks among the electrospun PVA fibers. The methanol addition removes any residual water within the fibers, which allows PVA-water hydrogen bonding to be broken and replaced by intermolecular polymer hydrogen bonding. This fact induces an additional crystallization of the mat. From their reported data, methanol treatment was not only helpful in the maintenance of nanofiber structures in water but also significantly increased the mechanical strength of the mat. When methanol-treated PVA mat was immersed in water, it

swelled significantly. The material became softer yet affording the characteristics of a mechanical stable hydrogel.

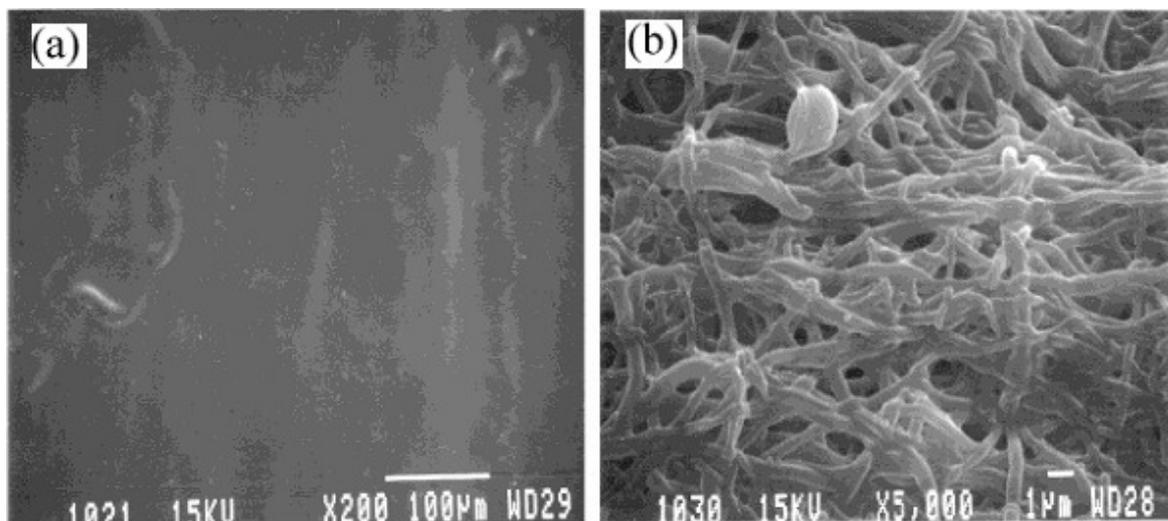


Figure 18 - SEM images of a) PVA mat without methanol treatment after immersion in water for 1 h and b) PVA mat with methanol treatment after 3 weeks of immersion in water [60].

Zeng et al. [61] have used the crosslinking of PVA by esterification with poly(acrylic acid) (PAA) in order to improve water resistance of PVA matrices. The water stability of electrospun PVA fibres was improved significantly by annealing with PAA. Better results were obtained for high-molecular weight PAA, originating PVA-PAA composites with excellent water-resistance even at low PAA content and low annealing temperatures. Some of the results obtained are shown in Figure 19. No change in fiber morphology was observed after 1 day in water at 20 °C, whereas a slight swelling of the fibers was observed after exposure to steam for 1h, indicating excellent water-stability of the annealed PVA/PAA fibers.

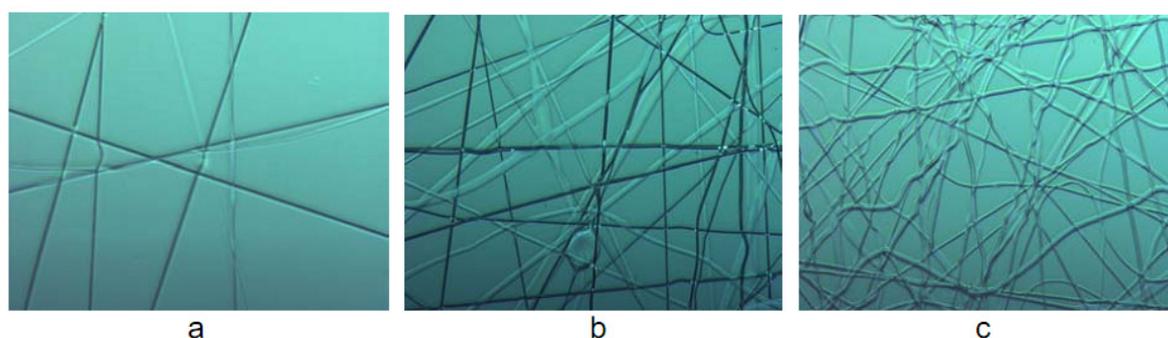


Figure 19 - Optical micrographs of annealed PVA/PAA (75:25) fibers; a) before immersion in water, b) after immersion in water at 20 °C for 1 day, c) after immersion in water steam (95 °C) for 1h [61].

These results clearly show the potential for the improvement of water-stability of PVA fibers by annealing in the presence of PAA. However, the addition of PAA and the esterification will certainly also lead to additional property changes such as hydrophilicity, OH functionality and acidity, which in turn will affect other chemical, physical and biological properties such as biocompatibility, thermal stability, electrostatic properties, among others [61]. So, it is elementary that more research is done to reduce collateral changes in PVA properties.

A second strategy is based mainly on photocrosslinking methods which demand a previous step of PVA derivatization. Zeng *et al.* [62] tried to synthesize a photocurable PVA derivate with later photocrosslinking of electrospun nanofibers. This way, they produced a PVA derivate containing thienyl acrylate substituents (PVA-Thio) by means of an analogous reaction between PVA and thienyl chloride with DMF as solvent. When these PVA-Thio fibers were exposed to UV radiation for different times, it was assumed that it was induced an intermolecular crosslinking by [2+2] cycloaddition reaction of the vinyl double bonds (Figure 20). To test the water stability of the crosslinked fibers, PVA-Thio fibers were exposed to steam (95 °C) for 1h. The PVA-Thio fibers without UV radiation disappeared immediately once contacting with steam, whereas PVA-Thio fibers after exposure to UV radiation for 3 min could endure the steam and displayed shrunk and slightly welled morphology. Longer irradiation time resulted in fibers with better water stability. No shrinking and swelling were observed for PVA-Thio fibers irradiated for 10 min (Figure 21).

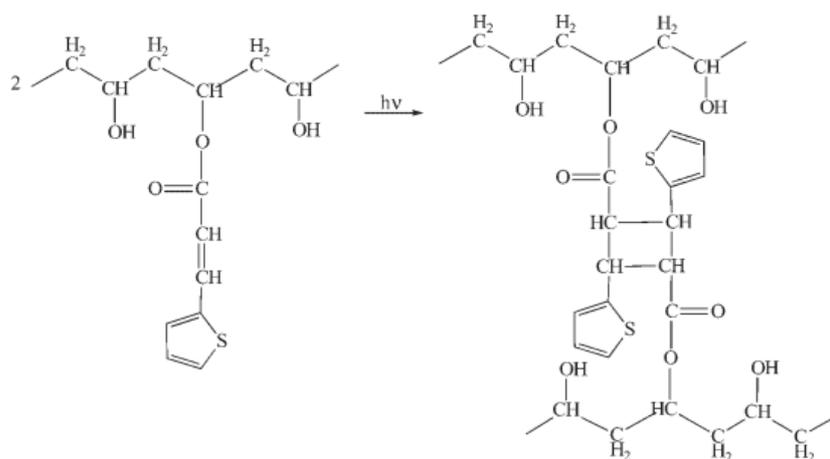


Figure 20 - UV induced crosslinking of PVA-Thio [62].

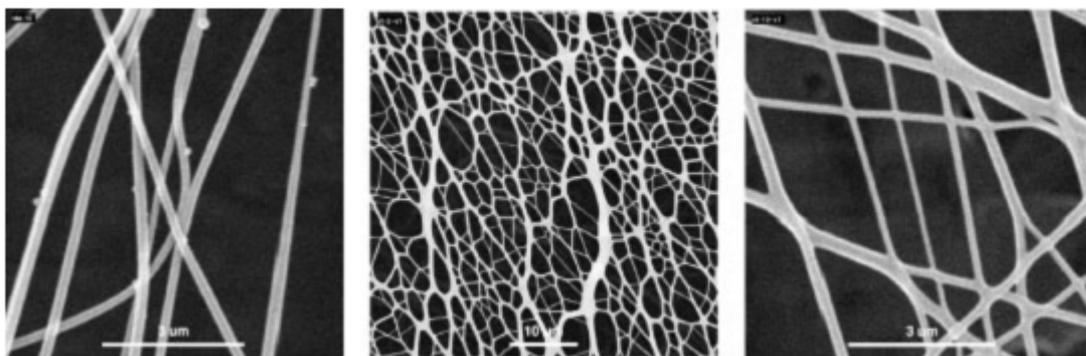


Figure 21 - SEM images of PVA-Thio fibers: a) crosslinked before steam treatment, b) UV-radiated 3 min, steam 1h and c) UV-radiation 10 min, steam 1h [62].

Liu *et al.* [63] also used photocrosslinking to prepare water-insoluble fibers of PVA. To do this, they also synthesized a PVA derivate with a pendent styrylpyridinium groups (SbQ) which undergoes cycloaddition reactions, crosslinking the PVA backbones. They concluded that UV radiation significantly decreases the water solubility of PVA-SbQ- $h\nu$ fibers and lowers their mass loss to about 17.5 % without changes in fiber morphology during water immersion.

Based on these results, one can consider that the UV treatment is a viable and a potential approach to produce PVA nanofibers that can be water-stable, allowing them to be used in many fields as drug delivery systems or implant applications [63]. However, when using photocrosslinking, the need to work with a PVA derivate instead of PVA by itself can be a limitation or undesirable in some cases. Also, it is time consuming to prepare a PVA derivate before using it. Therefore, for most applications the more simple and direct crosslinking methods are usually the chosen ones, mainly using GA and *ex situ* crosslinking methods.

1.2. Materials and Methods

1.2.1. Materials

Poly(vinyl alcohol) (PVA) ($M_w = 85\text{-}124$ kDa, 99+% hydrolyzed), Triton X-100, and the crosslinking agent glutaraldehyde (50 wt.% in water) were obtained from Sigma-Aldrich Química SA (Portugal) and used as received. Acetone (Pro Analysis) was obtained from Panreac and hydrochloric acid (min. 37%) was obtained from Riedel-de Haën. These chemicals were used without further treatment. Deionised water was used as solvent.

1.2.2. Electrospinning system

In every experience the electrospinning system was composed by the following components:

- Harvard Apparatus PHD 2000 Syringe Pump, with two syringe capability and an adjustable flow rate between $0,0001 \mu\text{L}/\text{min}$ and $220,82 \text{ mL}/\text{min}$;
- Spellman CZE 1000R high voltage power supply with a variable power between 0 and 30 kV and a continuous electric current between 0 and $300 \mu\text{A}$;
- A rotational aluminum mandrel (10 cm in diameter), connected to a Siemens motor equipped with a velocity changer (controlled velocity between 0 and 3200 rpm);
- A Plastic syringe (20 mL) equipped with a metal needle.

1.2.3. Solution preparation for electrospinning

Aqueous PVA solutions (10 wt.%) were prepared by dissolving PVA powder (99+ hydrolyzed, number average MW 85000-124000) in deionized water at 90°C with constant stirring for 6 h. After the solution cooled down to room temperature, Triton X-100, as a surfactant, was added to a final concentration in solution of 1.5 wt. %. The mixture was stirred for 20 min before electrospinning.

1.2.4. Operation parameters of the electrospinning system

For the electrospinning of PVA solution it was chosen a combination of operating parameters as described next:

- Applied Voltage: 21 kV;
- Speed Rotation of the collector: 109 RPM;

- Tip to collector distance: 12 cm
- Feed Rate: 1,2 mL/h

These parameters were chosen as the more appropriated to obtain uniform fibers, free of beads or other defects, based on previous works, references and by a macroscopic and microscopic analysis of the PVA matrices obtained by changing these parameters in preliminary tests.

1.2.5. Crosslinking of electrospun PVA fibers

A crosslinking treatment was necessary to achieve PVA fibrous mats with satisfactory water-resistance. We have tested two different crosslinking methods, taking into consideration that the final objective was to incorporate an enzyme in the electrospun fibers, therefore with the need to avoid severe treatments, such as high temperatures, that could affect the enzyme functionality. The objective was to evaluate crosslinking methods that would allow to preserve the enzyme activity while preserving the structure and morphology of the nanofibrous mat while in contact with water.

One of the tested methods was based on methanol treatment [60]. Electrospun PVA mats were immersed in methanol for 24 h. Then the methanol-treated PVA was dried in a ventilated hood at room temperature for 24. In order to test water-stability, the PVA mat was immersed during several hours in water and dried in a petri dish for 24 h in a ventilated hood.

The other crosslinking method was based on the reaction of glutaraldehyde (GA) with the PVA hydroxyl groups [56, 57]. The procedure was performed by immersion of electrospun PVA mats in acetone solution with 30, 45, 60 or 100 mmol/L GA and 0,01 mol/L HCl, for 24 h, in a plastic container with orbital shaking. Then, the crosslinked PVA mat was dried in a petri dish in a ventilated hood at room temperature for 24 h before use. To test the water-resistance and morphological changes resulting from the water contact, after drying, the PVA mat was immersed in water for 5 h in a beaker and dried in a petri dish for 24 h in a ventilated hood.

1.2.6. Morphologic characterization of PVA matrices

To examine the fiber morphology and the fiber organization within the PVA mats, samples of the fibrous PVA mat were placed in a microscopy metal support with double-layer carbon adhesive tape, sputter-coated with a thin layer of gold. They were analyzed by scanning electron microscopy (SEM) at 4 and 15kV (Hitachi SU-70) and at 25 kV (Hitachi S4100).

The average fiber diameter and distribution were determined by measuring the diameter of 100 fibers using an image analysis program (Image J 1.43u).

1.3. Results and discussion

1.3.1. Fiber formation

Fiber production is a process based on try and error when operating conditions are unknown. It is typically a multivariate problem if one desires to evaluate the effect of the several variables that influence electrospinnability of a certain polymer-solvent system and the final characteristics of the electrospun fibers. This optimization was not the main objective of this work although some variables were analyzed in order to obtain electrospun fibrous mats with satisfactory characteristics for enzyme immobilization and handling.

Polymer concentration was fixed at 10 wt.%. This value was selected based on previous group works and in available literature. Figure 22 shows the morphology of electrospun fibers obtained from a 10 wt.% PVA solution. One can observe that PVA nanofibers were electrospun without any beads or flat fibers, indicating that complete and uniform tubular-like fibers were produced. These results indicate that a polymer concentration of 10 wt.% and the selected electrospinning conditions are appropriated for an efficient electrospinning of ultra-thin fibers, in accordance with other previously reported work [15, 23, 24]

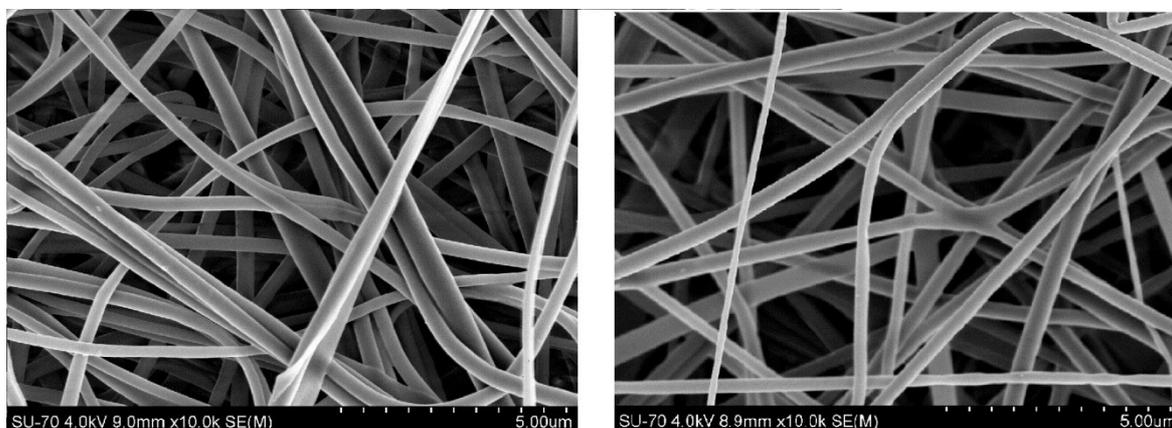


Figure 22 – SEM images obtained at different locations of an electrospun PVA mat obtained from a 10 wt.% PVA solution.

The presence of beads or granules resulting from discontinuities in polymer solution during electrospinning, due to low polymer concentrations, and the effect of polymer concentration on the final fiber size and morphology are well-documented in literature, as previously mentioned (1.1.4.1).

Using SEM images and Image J, it was possible to analyze the fiber diameter distribution (Figure 23). It is clear that the majority of the fibers, approximately 71%, have a diameter between 100

and 200 nm. Also with a relevant frequency (27%) there are fibers with diameters ranging from 200 to 300 nm. With a frequency of only 2% but relevant to mat characterization there are fibers from 300 to 400 nm. The average fiber diameter and distribution will be important to understand how the crosslinking methods can affect fibers and how it can influence the nanofiber mat applications.

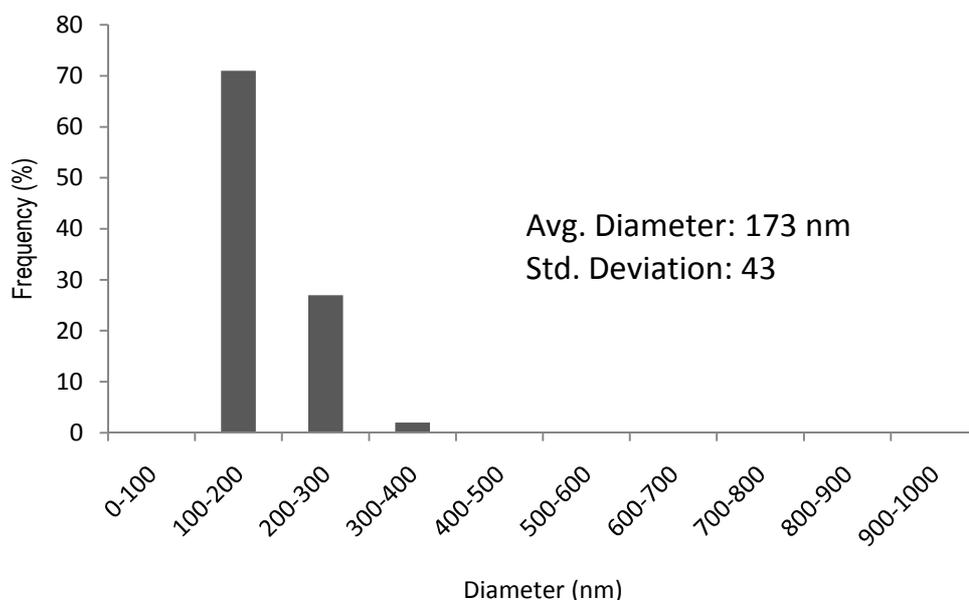


Figure 23 – Fiber diameter distribution of PVA nanofibers.

A semi-empirical evaluation of the effect of the solution feed rate, based on the direct observation of the electrospinning jet and characteristics of the deposited fibers, revealed that a feed rate of 1.2 mL/h allowed to maintain a constant and relatively fast electrospinning process, sustaining a perfect Taylor cone without lowering fiber quality. When the solution feed rate is above the maximum value possible for the production of a nanofiber mat, the electrospinning system is unable to produce polymer stretching. The result is the formation of junction zones and spherical agglomerates that appear in the nanofibers web [64]. Based on the SEM analysis, there is a complete absence of spheric agglomerates on the final fibrous mats indicating that a correct feed rate was used.

Tip to collector distance is another parameter that can be discussed. In the space between the needle and the collector, solvent evaporation occurs. Like this, a minimal distance is necessary to prevent that liquid solvent reaches the collector. When this distance is not used, solvent drops appear or nanofibers can emerge together, instead of being deposited as individual fibers [65]. This distance should be a compromise between the satisfactory solvent evaporation and jet dispersion,

i.e. for low tip-to-collector distances fibers can collapse due to solvent still reaching the collector, but for high distances some problems can also occur due to high jet dispersion. Figure 24 shows that occasionally and in some parts of the electrospun membrane some fibers are still together when reaching the collector. However, even considering that this distance is not the ideal one to achieve a complete solvent evaporation, higher distances caused a large dispersion of the polymer jet due to the surrounding environment. Therefore, a tip to collector distance of 12 cm was selected to have a balance between jet dispersion and solvent evaporation.

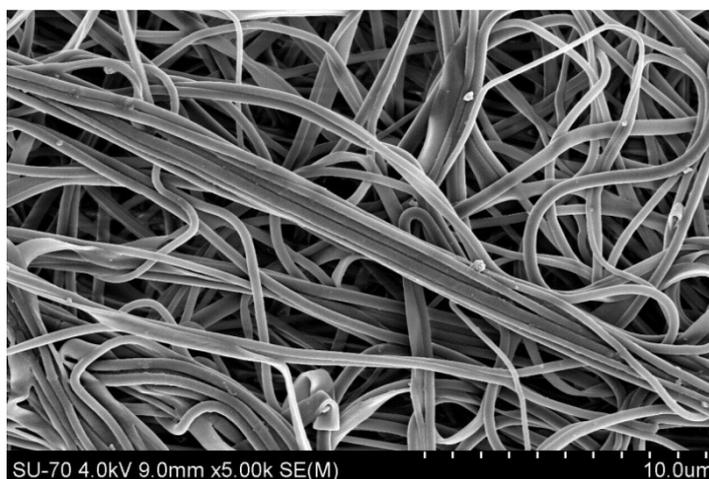


Figure 24 – SEM images of PVA nanofibers reaching the collector together.

The applied voltage was also changed, maintaining the feed rate at 1.2 mL/h and the tip to collector distance at 12 cm. It was noticed that a Taylor cone started to form for a minimum value of 12 kV. However, it wasn't stable and the jet wasn't directed to the collector. By increasing the voltage, the jet stability and orientation increased until reaching 21 kV, which led to a less polymer loss. For voltages higher than 21 kV, the polymer jet became unstable again and voltage failures were observed. This way, a voltage of 21 kV was used for every electrospinning process that has been done.

1.3.2. Effects of Crosslinking of the electrospun PVA nanofibers

When an electrospun PVA mat is immersed in water, the mat shrinks and becomes transparent, gelatinous and gradually dissolves. Methanol treatment was the first option to overcome this problem. PVA mat was immersed in methanol for 24 h and it was tested for water-resistance. In Figure 25 it is possible to see the result of the methanol treatment.

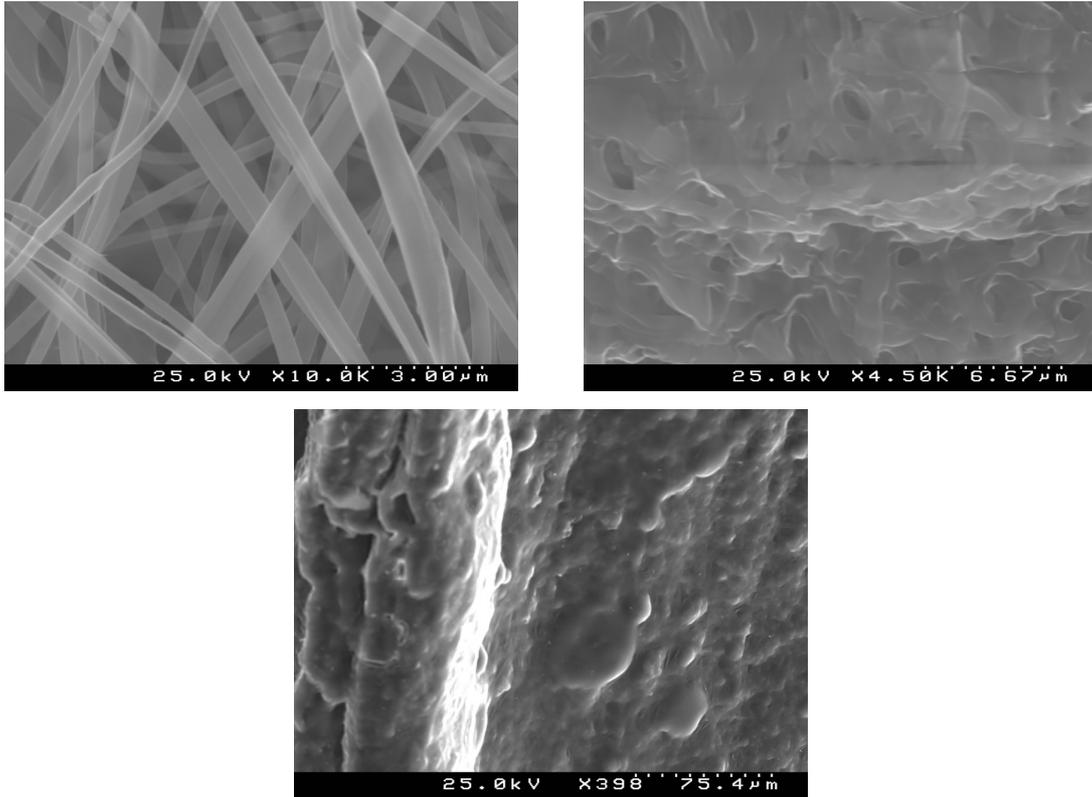


Figure 25 - SEM images of (a) PVA mat without methanol treatment, (b) after immersion in methanol and (c) after immersion in water.

Based on these images it is clear that the results are not in accordance with those previously reported for similar PVA matrices and crosslinking treatments [60]. After methanol immersion, some nanofibers could still be distinguished but a large merging between fibers occurred originating an almost dense and low porosity mat. Moreover, the immersion in water completely destroyed the nanofibrous structure. These treatments were run several times resulting always in fiber loss.

Since the previous approach did not allow PVA stabilization in water, one has tried to stabilize the electrospun mat towards disintegration in water by soaking them in different concentrations of glutaraldehyde solutions. The applied treatment was based on the use of acetone, as the solvent medium to the crosslinking agent, which penetrates the fibers, swelling them and expanding all the membrane. This phenomenon allows the diffusion of the GA molecules between the fibers and into the fibers where it will react with PVA hydroxyl groups by intra and intermolecular reactions. Based on these facts, it would be expected that PVA nanofibers undergo some swelling, increasing moderately their diameter, reducing mat porous structure but allowing the desired water

resistance. Figure 26 shows the result of crosslinking reactions in comparison with the original electrospun PVA mats.

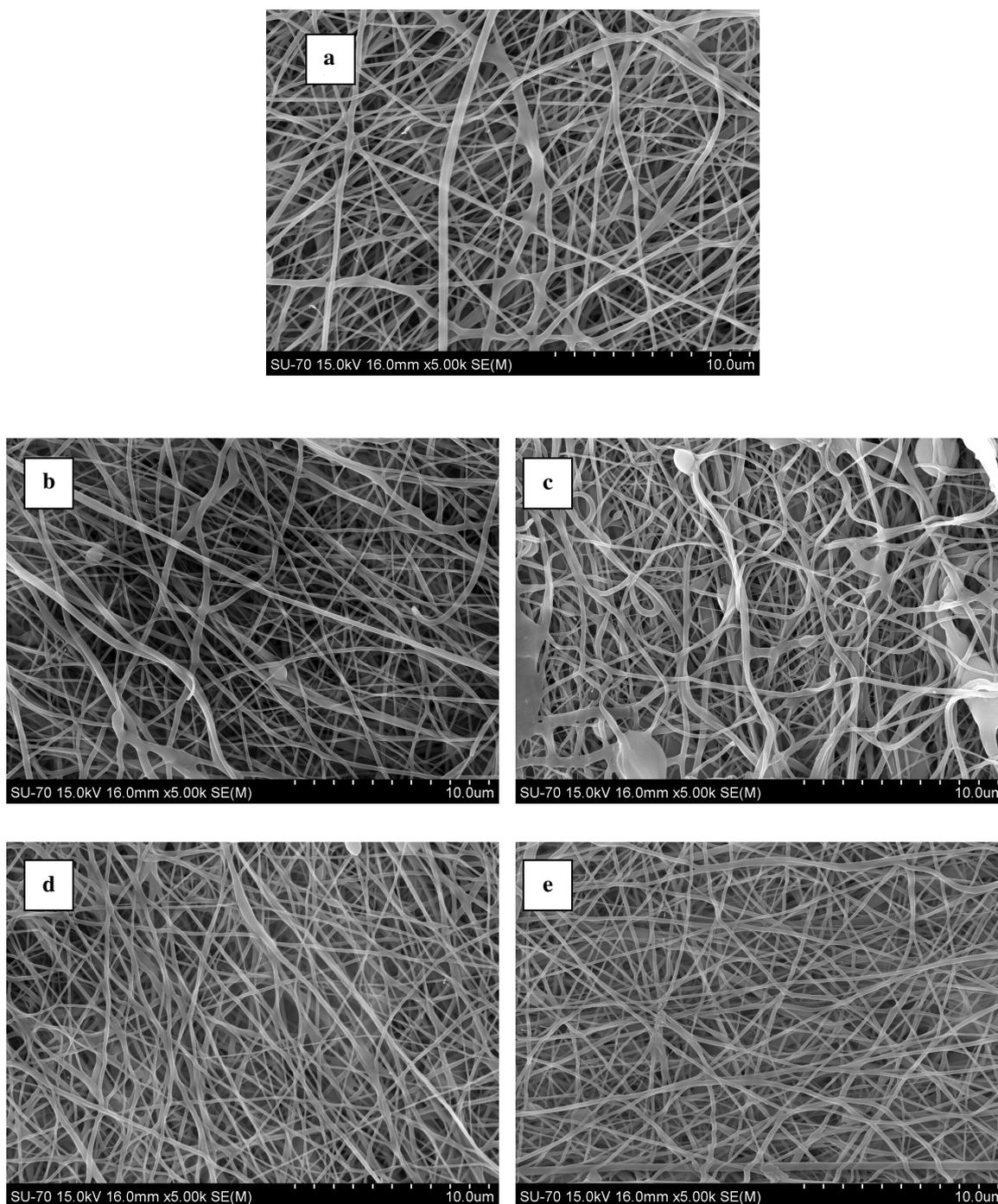


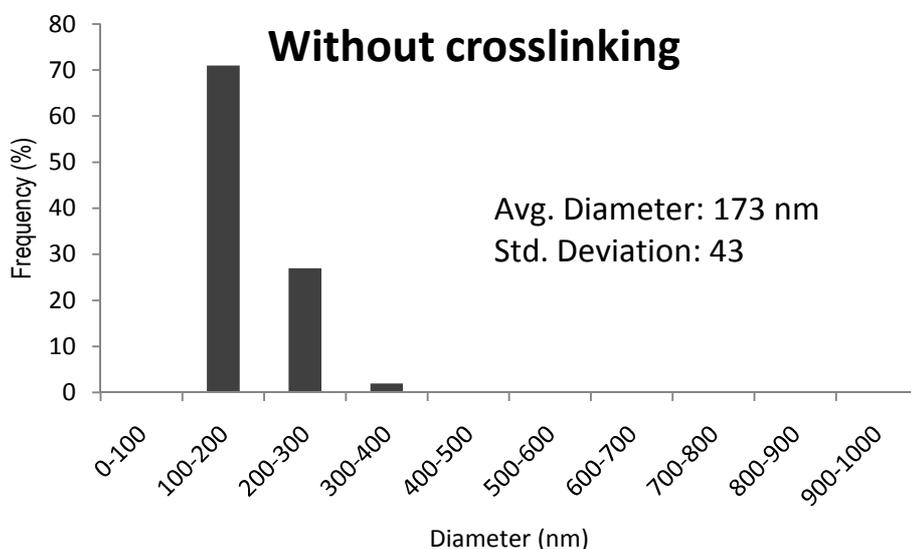
Figure 26 – SEM images of crosslinked PVA nanofibers with different GA concentration for 24 h: a) 0 mM, b) 30 mM, c) 45 mM, d) 60 mM and e) 100 mM.

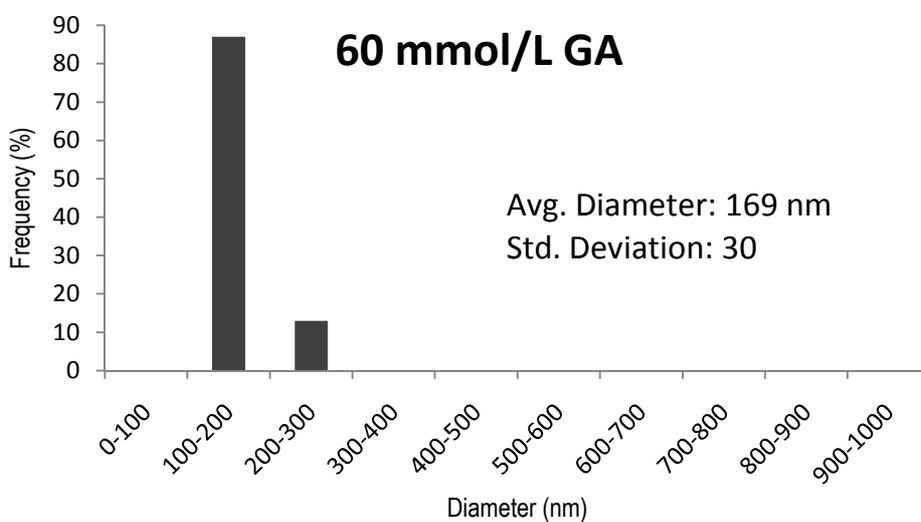
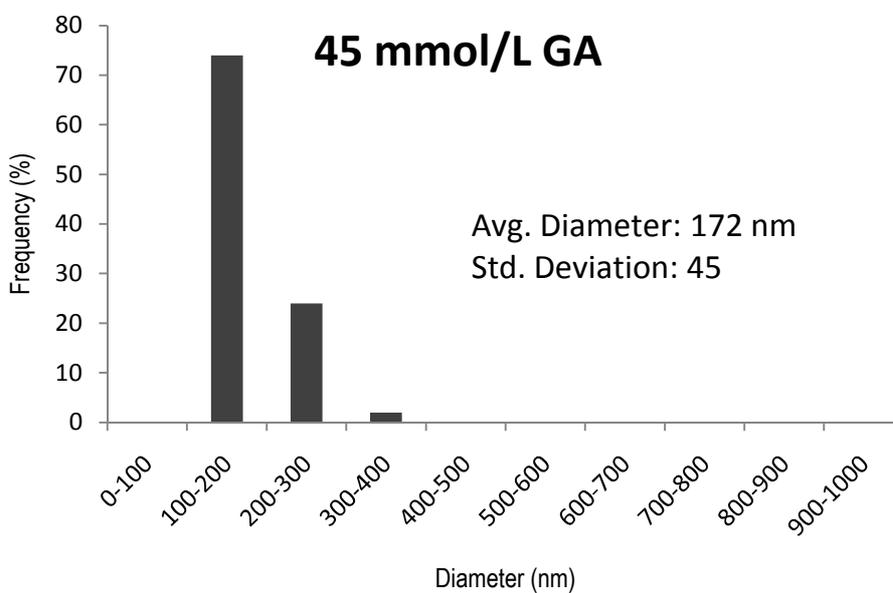
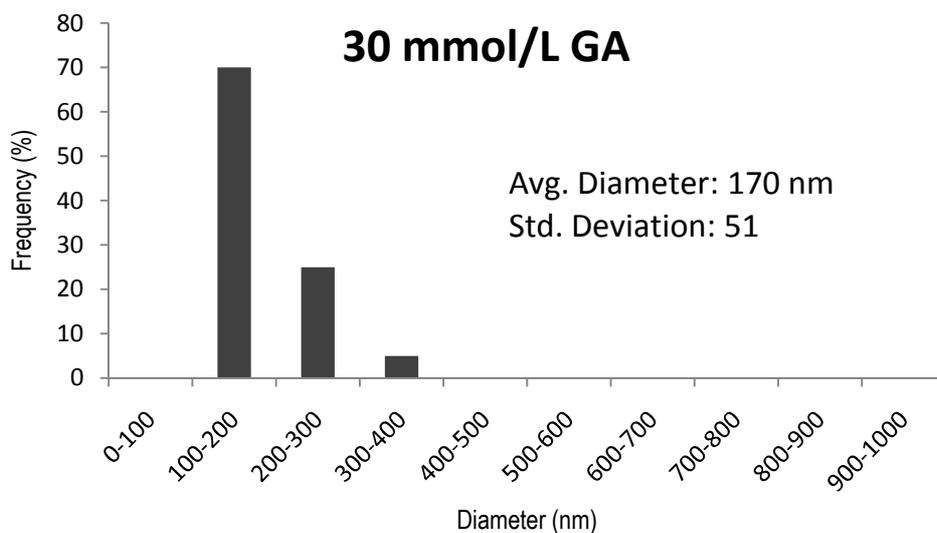
As can be seen by the figures above, comparing to the original PVA which did not suffer crosslinking, membrane porosity remained very similar and nanofibers morphology did not change.

This fact was not expected once theoretically the crosslinking reaction may cause the fiber structure to be more compacted and fibers to fuse which is related with fiber swelling and linkage between PVA fibers –OH groups. A reduced porous structure affects diffusion of molecules in and out the membrane. This fact, could be highly limiting to enzyme activity.

Although exists a great preservation of the mat structure, it can be seen that for the lowest concentrations of GA used (30 and 45 mmol/L) there is some fiber fusion and a few beads. With an increase of GA concentration, these “defects” disappeared showing that there is a visible direct relation between GA concentration and morphology maintenance.

Although crosslinking doesn't seem to have a great effect in morphology change, it is necessary to analyze any diameter change. Diameter is very important since the superficial area is one of the most relevant advantages in nanofibers. In Figure 27 is possible to see fiber diameter distribution after crosslinking. Comparing with PVA mat without crosslinking, the treatment used for water stabilization does not seem to affect to a great extent the average diameter and distribution. For a GA concentration of 30, 45 and 60 mmol/L, the average fiber diameter remains very similar to that of PVA fibers without crosslinking. Distribution of diameter size was also very similar. More than 70% of the fibers have a diameter between 100 and 200 nm, while the rest of the fibers (almost 30%) have a diameter between 200-300 nm. However, for a GA concentration of 100 mmol/L, the average diameter is 197 nm which represents a slight increase. There is a decrease in the frequency of fibers between 100 and 200 nm (57%) and a bigger number of fibers with a size between 200 and 300 nm, (42 %) which explains this difference. The higher GA concentration used may establish more inter and intramolecular bonding resulting in the expansion of the fibers.





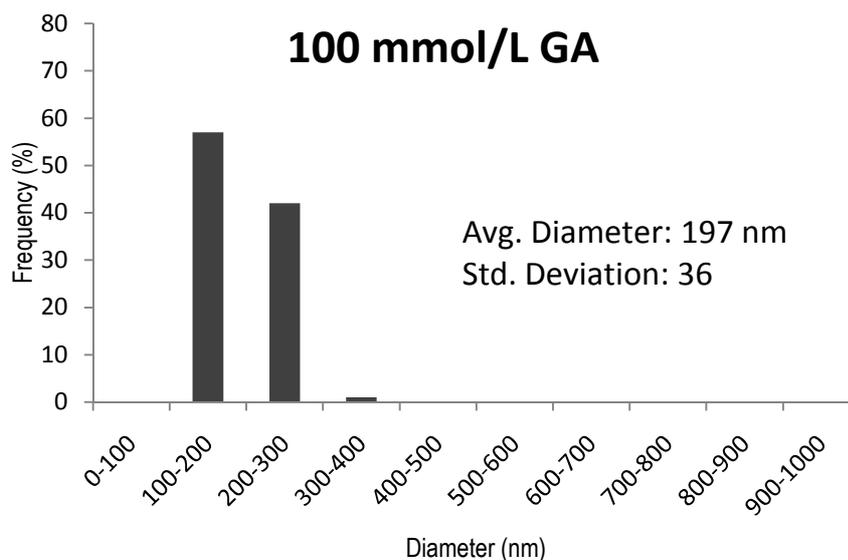


Figure 27 - Fiber diameter distribution of crosslinked PVA nanofibers according to GA concentration.

Based on the results one can conclude that the crosslinking treatment used was not prejudicial to the maintenance of the fiber morphology and porous structure and if it allows water-resistance, it can be considered a useful method to have an active enzyme entrapped in PVA nanofibers in aqueous medium. This way, the next step was to evaluate the nanofibers water-resistance after GA treatment.

1.3.3. Effect of soaking in crosslinked PVA nanofibers

The capability to crosslink PVA fibers reported in the previous section must be complemented with an effective water resistance of the crosslinked fibers, *i.e.*, after soaking the PVA mat in water, fiber morphology and porous structure must be essentially preserved. In Figure 28, there is a visual comparison of a wet electrospun mat with and without GA treatment after water exposure for 5 h at 37 °C. As expected, SEM images revealed that the fibrous morphology in the PVA mat without any crosslinking treatment was completely destroyed when the mat was immersed in water. A GA-treated mat appears to retain a significant degree of fibrous character after water exposure.

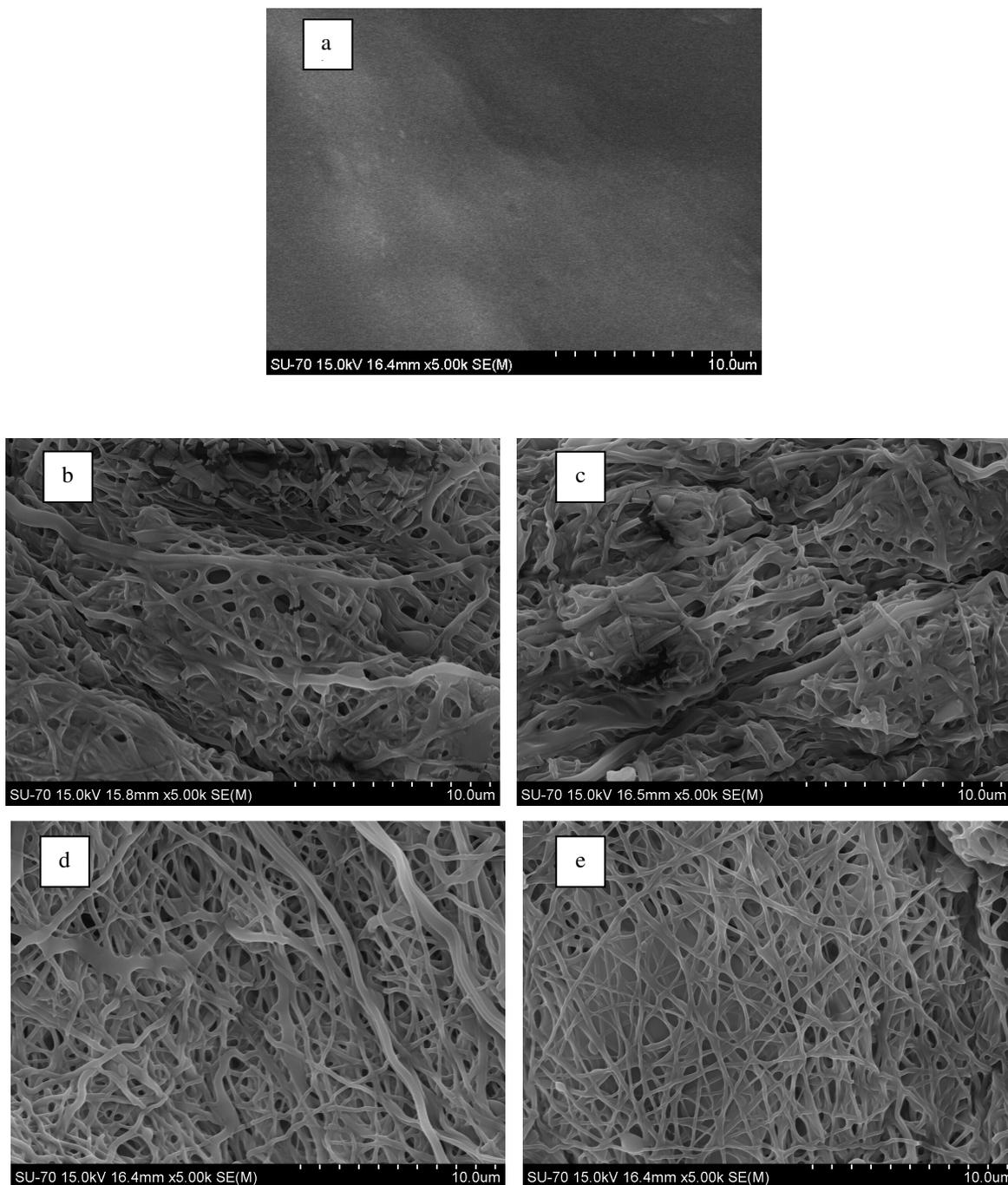


Figure 28 – SEM images of (a) original electrospun PVA mat without crosslinking and electrospun PVA mat crosslinked with (b) 30, (c) 45, (d) 60 and (e) 100 mmol/L of GA after immersion in water.

It is clear that the retained fiber structure of the membrane is highly dependent of the GA concentration used during crosslinking. For the lowest concentrations as 30 and 45 mmol/L, it is visible that extended water uptake have occurred, which lead to fiber fusion, morphology changes and a reduced porosity through all the membrane. Lower concentrations of the crosslinker (30 and 45 mmol/L) prevents that enough links between the GA and PVA hydroxyl groups occur. Since that

many hydroxyl groups are still available to bond with water molecules, membrane has a higher water uptake resulting in fiber merging which will also affect membrane porosity (Figure 28 b) and c)). However, for higher GA concentrations it is clearly visible a decreased water uptake. As GA concentration is augmented, the degree of hydroxyl-GA bonding increases, preventing bonding with water molecules. For 60 mmol/L, fiber merging is less extent and the porosity is larger (Figure 28 d)), and with 100 mmol/L GA the best result was achieved. PVA fibers demonstrate a remarkable resistance to water uptake that can be seen in the fewer number of merged fibers, and a remarkable preservation of the fiber morphology and porous structure. According to what has been said and although some natural swelling, this is an excellent result attending to the goal of this work, where PVA-enzyme mat will be used in an aqueous medium.

1.4. Conclusion

Based on the morphologic results previously discussed, it can be concluded that it was possible to produce a PVA nanofiber membrane through electrospinning. Based on a set of processing and polymer solution parameters we have obtained uniform fibers without the presence of beads or flat fibers and with an average diameter of 170 nm.

The two water stabilization methods chosen had produced different results. It was not possible to achieve a PVA mat stable in water with the methanol treatment although it has been reported previously. However, the second approach which used glutaraldehyde as crosslinking agent in an acetone medium revealed to be efficient and enhanced nanofibrous membrane resistance to water. A direct relationship between GA concentration and membrane water-resistance effect was clear. After exposure to GA and immersion in water, nanofibers maintained their structure, although with some expected swelling. A gradual increase in GA concentration allowed to gradually decrease PVA water-uptake. This way, a GA concentration of 100 mmol/L was chosen as the best to crosslinking PVA nanofiber mat.

Having the knowledge to stabilize PVA fibers in water without any severe loss of the polymer fibrous mats, it was possible to move forward to next part of this work where β -Galactosidase will be added to PVA nanofibers. This new stage will be analyzed and discussed in the next chapter.

2 Immobilization of β -Galactosidase in PVA nanofiber matrices

In the previous chapter, the preparation of electrospun PVA mats was analyzed and the obtained fibrous mats were characterized. In this second chapter, the immobilization of the enzyme β -galactosidase within the PVA nanofibers will be studied. First, it will be reported the simultaneous production of PVA+enzyme nanofibers, focused on fiber morphology changes and entrapment efficiency. Then, a parallel study about the properties of both the free and immobilized enzyme activity and stability will be done, including measurements of enzyme activity, stability and reusability.

First of all, a brief literature review will be presented, related to the characteristics of the enzyme, β -galactosidase, and available methods for enzyme immobilization, focusing mainly on the utilization of electrospun nanofibers as polymeric supports.

2.1. Literature Review

2.1.1. β -Galactosidase

β -Galactosidase (β -D-galactohydrolase, EC 3.2.1.23) is an enzyme that hydrolyzes D-galactosyl residues from polymers, oligosaccharides or secondary metabolites [66]. Even though the term "lactase" is obsolete, many authors continue to use this nomenclature in preference to the trivial name β -galactosidase. β -Galactosidase is widely used in food industry, being the hydrolysis of lactose one of the more important areas of application, to remove this disaccharide from milk products for lactose intolerant people.

The enzyme is also capable of catalyzing synthesis of certain oligosaccharides via the galactosyl transfer reaction. The β -D-galactosyl transfer occurs preferentially at the primary alcohol of D-glucose with the formation of various di- and oligosaccharides.

Traditionally, much of the work has been done on the β -galactosidase obtained from *Escherichia coli*. However, this enzyme is widely distributed in nature and can be found in plants, animal organs, yeast, bacteria and fungi. β -galactosidase from different sources varies considerably

in many of its properties although the specificity of the enzyme remains essentially the same. Several of these β -galactosidases have been purified, sequenced and extensively characterized.

The enzymes commercially available are derived from safe sources, principally, the yeast *Kluyveromyces fragilis*, *Kluyveromyces lactis*, and *Candida pseudotropicalis*, the fungi *Aspergillus niger* and *Aspergillus oryzae* and a *Bacillus* species closely related to *Bacillus stearothermophilus*.

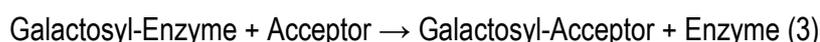
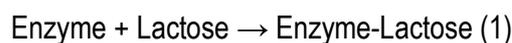
At a molecular level, β -galactosidase (from *E.coli*) has a molecular weight of 464 kDa and is made up of four identical subunits, each containing 1023 aminoacids residues and a binding site for magnesium, which is an activator [67]. Aminoacid sequences have been established for β -galactosidases from several other bacteria and comparasion with *E.coli* enzyme shows extensive homologies and highly conserved regions.

The enzyme obtained from *Aspergillus oryzae*, the source used in this work, showed a pH-optimum of 4.8 or 4.5, with lactose or *o*-nitrophenyl β -D galactopyranoside (ONPG) as the substrate, respectively, an optimum temperature of 46 °C, and an apparent molecular weight of about 105 kDa [68].

Various methods are available for determining β -galactosidase activity. The activity can be determined readily by lactose as a substrate and determining the resulting glucose or galactose. Because of transfer reactions, it may be advisable to determine both appearance of glucose and disappearance of lactose. Alternatively, *o*-nitrophenyl- β -D-galactopyranoside, abbreviated as ONPG, can be used as the substrate, and progress of the reaction can be followed by estimating the chromogen *o*-nitrophenol. In fact, the use of this substrate is one of the best procedures available to evaluate the β -galactosidase activity [69].

2.1.1.1. Mechanism of catalysis by β -Galactosidase

Early studies with the enzyme suggested that a minimum of three steps were involved in β -gal action mechanisms, the last of which allows for hydrolysis or transferase activity:



When the sugar acceptor is water, free galactose is formed by hydrolysis. When the acceptor is another sugar, the result is galactosyl-oligosaccharide formation.

The effect of pH on the activity and the inhibition of enzyme thiol groups suggested that the active site of neutral pH enzymes contains a thiol group acting as a general base and an imidazole group acting as a nucleophile to facilitate splitting of the glycosidic bond.

The mechanism indicates that the enzyme will transfer galactose to nucleophilic acceptors containing a hydroxyl group. Transfer to water produces galactose; transfer to another sugar produces di-, tri- and higher galactosyl-saccharides, collectively termed oligosaccharides. These in turn become substrates for the enzyme and are slowly hydrolyzed. In this scheme galactosyl transfer is the general reaction and hydrolysis can be regarded as a special instance of galactosyl transfer to water. Under most conditions, hydrolysis predominates due to the high concentrations of water, and oligosaccharides can be increased by using higher substrate (acceptor) concentrations and/or by decreasing the water content [70]. Figure 29 shows a proposed reaction mechanism for the action of β -galactosidase on lactose.

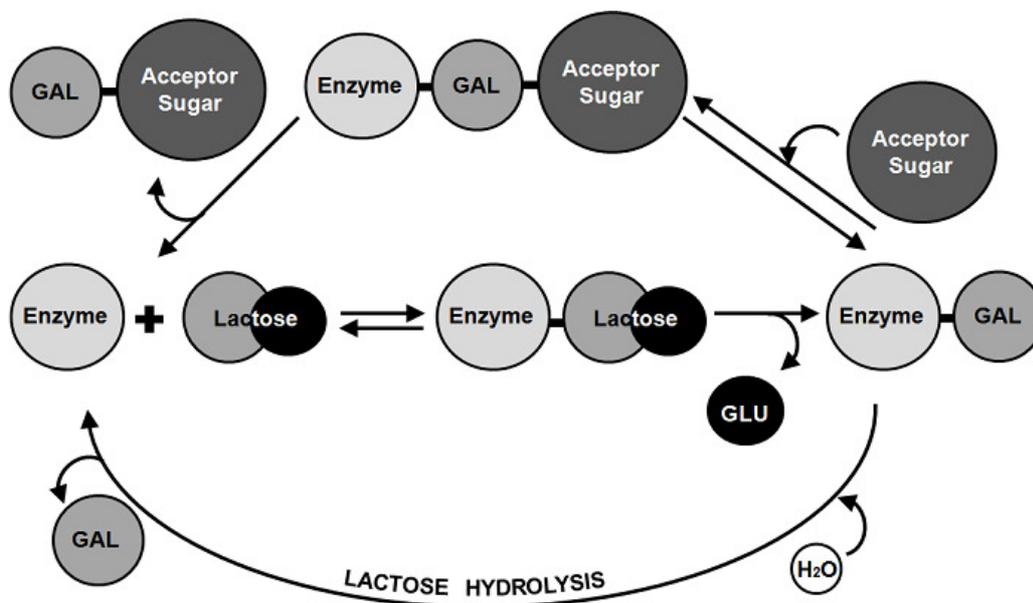


Figure 29- A proposed reaction mechanism for the action of β -galactosidase on lactose.

GAL:galactose [69].

2.1.2. Lactose: an industrial issue

The use of β -D-galactosidase has been suggested for the production of lactose free products, such as milk or milk derivatives. Mammal milk contains about 3-8% (w/v) lactose and 70-80% of the solid components in cheese whey is lactose [71].

A large number of people do not digest lactose properly due to a lack or inactivity of the intestinal β -galactosidase and they suffer from intestinal dysfunctions if their diet contains lactose.

Also, if compared to other sugars, lactose has low solubility and sweetness, being a hygroscopic sugar with a strong tendency to adsorb flavours and odours. Without a proper control, these characteristics may cause defects in refrigerated foods, as ice-creams, while the hydrolysis of this sugar would be interesting for reducing costs in food edulcorants, energy and avoidance of the sandy texture due to the crystals of lactose. Finally, this sugar is scarcely biodegradable compared to its hydrolysis products, glucose and galactose, so solutions containing lactose can not be disposed of without expensive treatments.

When lactose is undesirable, the hydrolysis of lactose is a promising process in the food industry for the development of new products with no lactose in their composition and for the recycling of whey to human and cattle food resources.

To perform the hydrolysis of lactose in milks and whey acids or enzymes can be employed as catalysts. The acid procedure is carried out with the acid in solution or with acid ion-exchange resins at 150 °C and the environment pH value fluctuates between 1 and 2. The procedure is scarcely adequate for the hydrolysis of lactose contained in foods due to secondary reactions among the acid and proteins and fats, the development of nasty odours and flavours and the reduction of the nutritional properties of the milk so treated. Enzymatic hydrolysis of lactose is, on the contrary, technically convenient, as final products not only retain, but increase the alimentary properties of milk. Moreover, as the reaction conditions of temperature and pH are much softer, energy and material savings in the process are achieved [72].

As can be seen, lactose hydrolysis in glucose and galactose by β -galactosidase is one important process in the food industry, due to the potential beneficial effects on assimilating the foods containing lactose, as well as the technological and environmental advantages of industrial applications [73]. There are basically two different ways to use β -galactosidases: the soluble enzyme is normally used for batch processes while the immobilized form lends itself to continuous operation [74].

Enzyme immobilization provides many important advantages over use of enzymes in soluble form, namely, enzyme reusability, continuous operation, controlled product formation, and simplified and efficient processing [75]. The main challenges in enzyme immobilization include not only containment of a large amount of enzyme to be immobilized while retaining most of its initial activity but also the performance of immobilized enzyme in actual production process in industrial-type reactors. Continuous reactors provide high productivities and minimize downstream time, enzyme

cost and capital investment, and offer an attractive design for enzyme reactors, especially when dealing with unclarified substrate fluids [76].

Immobilization of enzymes has been extensively studied on a wide range of matrices; apparently, the suitability of support and method of immobilization vary from enzyme to enzyme and their intended use [77]. An appropriate immobilized system is yet to be developed once that is difficult to obtain simultaneously high activity, high stability and optimal mechanical properties [78]. In the next section, immobilization methods will be discussed.

2.1.3. Enzyme Immobilization

As seen previously, there are several reasons for using an enzyme in an immobilized form. In addition to more convenient handling of the enzyme, it provides for its easy separation from the product, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitates the efficient recovery and reuses of costly enzymes, in many applications a strict condition for economic viability.

2.1.3.1. Types of immobilization

Basically, three traditional methods of enzyme immobilization can be distinguished: binding to a support (carrier), entrapment (encapsulation) and crosslinking.

(i) *Support binding* can be physical, ionic or covalent in nature. However, physical bonding is generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentration and high ionic strength. Ionic binding is generally stronger and covalent binding of the enzyme to the support even more so, which has the advantage that the enzyme cannot be leached from the surface. However, this is also a disadvantage: if the enzyme is irreversibly deactivated both the enzyme and the support are rendered unusable. The support can be a synthetic resin, a biopolymer or an inorganic polymer [79].

(ii) *Entrapment via* inclusion of an enzyme can occur in a polymer network such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule. The physical restraints generally are too weak, however, to prevent enzyme leakage. Hence, additional covalent attachment is often required. Enzyme leakage during repeated use is the major limitation of this technique due to the small molecular size. In addition, a disadvantage of the method is diffusion limitations. Entrapment methods are classified into five major types: lattice, microcapsule, liposome, membrane and reverse micelle.

(iii) *Crosslinking* method utilizes a bi- or multifunctional compound, which serve as the reagent for intermolecular crosslinking of the biocatalyst. In case of β -galactosidase immobilization, crosslinking is often used in combination with other immobilization method, mainly with adsorption and entrapment. For example, β -galactosidase from *K. fragilis* was immobilized on silanized porous glass modified by glutaraldehyde binding. The coupling efficiency was very high so that more than 90% of the enzyme was active and 87.5% of the protein was bound to the support [74].

2.1.3.2. Types of support

Various nanostructures, generally providing a large surface area for the immobilization of enzyme molecules, have been actively developed for enzyme stabilization. In this section it will be discussed the role of those nanostructures in stabilizing the enzymes. An overview on nanoparticles, nanofibers, nanostructures via sol-gel enzyme encapsulation and single-enzyme nanoparticles will be presented.

Nanoparticles

Improvement of biocatalyst efficiency can be achieved by manipulating the structure of carrier materials for enzyme immobilization. Nonporous materials, to which enzymes are attached to the surfaces, are subject to minimum diffusion limitation while enzyme loading per unit mass of support is usually low. On the other hand, porous materials can afford high enzyme loading, but suffer a much greater diffusional limitation of substrate.

Reduction in the size of enzyme-carrier materials can generally improve the efficiency of immobilized enzymes. In the case of surface attachment, smaller particles can provide a larger surface area for the attachment of enzymes, leading to higher enzyme loading per unit mass of particles. In the case of enzyme immobilization into porous materials, much reduced mass-transfer resistance is expected for smaller porous particles owing to the shortened diffusional path of substrates when compared to large-sized porous materials.

Recently, a growing interest has been shown in using nanoparticles as carriers for enzyme immobilization. The effective enzyme loading on nanoparticles could be achieved up to 10 %wt due to a large surface area per unit mass of nanoparticles. Overall, nanoparticles provide an ideal remedy to the usually contradictory issues encountered in the optimization of immobilized enzymes: minimum diffusional limitation, maximum surface area per unit mass, and high enzyme loading.

In addition to the promising performance features, the unique solution behaviours of the nanoparticles also point to an interesting transitional region between heterogeneous and homogeneous catalysis. Theoretical and experimental studies demonstrated that particle mobility, which is governed by particle size and solution viscosity, could impact the intrinsic activity of the particle-attached enzymes [80].

Nanofibers

Nanoparticles are desirable from several perspectives. However, their dispersion in reaction solutions and the subsequent recovery for reuse are often found a daunting task. It appeared that the use of nanofibers would overcome this limitation while providing the advantageous features of nanosize materials. Electrospun nanofibers provide a large surface area for the attachment or entrapment of enzymes and the enzyme reaction. In the case of porous nanofibers, they can still reduce the diffusional path of substrate from the reaction medium to the enzyme active sites due to the reduced dimension in size. Electrospun nanofiber mats are durable and easily separable, and can also be processed in a highly porous form for relieved mass-transfer of substrate through mats [80].

PVA nanofibers produced via electrospinning were examined for the immobilization of acetylcholinesterase (AChE). With the huge specific surface area provided by the nanofiber, immobilized AChE retained 40% of its initial activity. After immobilization, there was a 1.7-fold increase in K_m value for immobilized enzyme and in addition, immobilized AChE could retain most of the activity at a lower pH values when compared with the free enzyme. This system, showed also a good storage stability and a better reusable stability [81].

Another possible approach is an enzyme aggregate coating on the polymer nanofibers surface. This approach employs GA treatment that crosslinks additional enzyme molecules and aggregates onto the covalently attach seed enzyme molecules, Figure 30 .

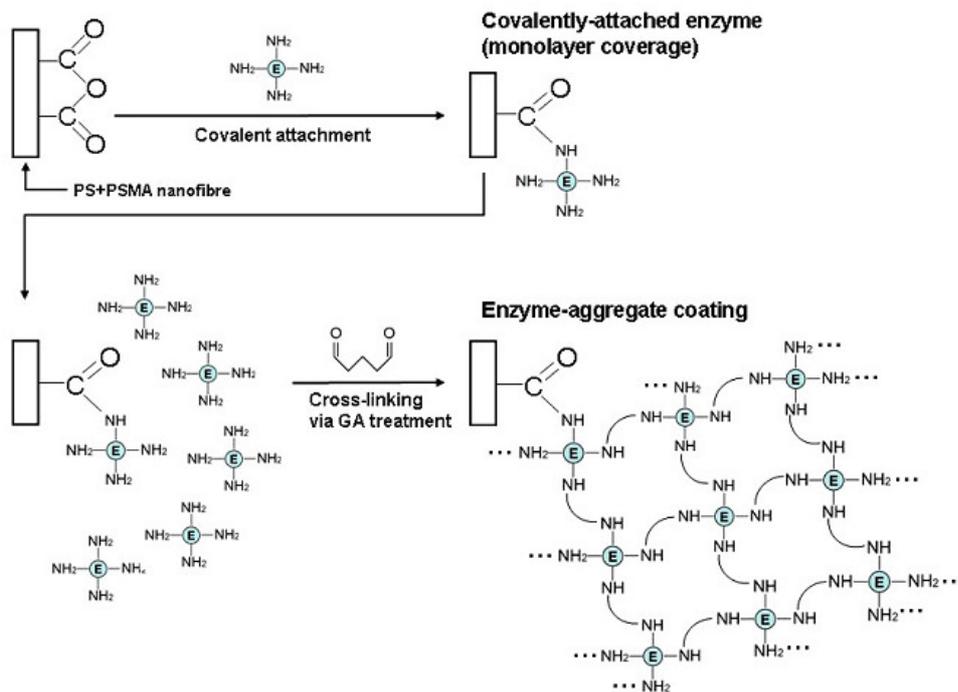


Figure 30 - Preparation of covalently attached enzymes and enzyme-aggregate coatings on polystyrene and poly(styrene-co-maleic anhydride) fibers using GA as crosslinker [34].

It was demonstrated that the enzyme aggregate coating on nanofibers improves not only the enzyme activity but also the enzyme stability. These active and stable biocatalytic nanofiber mats were highly durable and could be easily recovered from a solution even after more than a one month incubation in a rigorous shaking condition [34].

Finally, another option is available for enzyme immobilization in nanofibers. Nakane *et al.*[82] produced PVA-Lipase nanofibers directly from electrospinning. The lipase is physically entrapped among the PVA chains and distributed throughout the nanofibers without an immobilization procedure, as coating or crosslinking. This non-woven mat on nanofibers was used as the catalyst for the esterification of (Z)-3-hexen-1-ol. These nanofibers showed higher activity for esterification than lipase powder and PVA-film-immobilized lipase. This is due to the high specific surface area of the nanofibers and the high dispersion state of lipase molecules in the PVA matrix. The activity of the nanofibers became higher with increased specific surface area, indicating that esterification occurs in the neighbourhood of the nanofiber surface.

Nanostructures via sol-gel enzyme encapsulation

Enzyme encapsulation via sol-gel approach has been one of the most popular methods for enzyme immobilization since the first report in 1994 when encapsulated enzymes into sol-gel matrices maintained their activities. In a typical synthetic protocol, tetramethylorthosilicate (TMOS) or tetraethylorthosilicate (TEOS) is hydrolyzed into “sol” and the addition of enzyme solution into “sol” initiates condensation reaction leading to “gel”, where enzymes are encapsulated in silicate matrices. In this approach, various pores and channels are formed in the final silicate matrices, ranging from 0.1 to 500 nm in size. It requires a careful optimization process to prevent the leaching of encapsulated enzymes. However, once the enzyme leaching is prevented, the sol-gel approach results in a fairly stable form of enzyme immobilization since the close fit of the enzyme molecule within the sol-gel pore likely prevents unfolding and denaturation of encapsulated enzymes. Since enzymes are added from the early stage of silicate formation, more tight confinement of encapsulated enzyme is anticipated in this sol-gel approach than with the approach of enzyme immobilization into pre-formed mesoporous silica [80].

Single enzyme nanoparticles (SENs)

In this method, each enzyme molecule is surrounded with a porous composite organic/inorganic network of less than a few nanometers thick. Converting free enzymes to SENs can result in significantly more stable catalytic activity while the nanoscale structure of the SEN does not impose a serious mass-transfer limitation on substrates. The synthesis of SENs is also different from enzyme modification such as surface aminoacid modification or polymer attachment, which generally does not provide long-term enzyme stabilization. Figure 31 shows a schematic comparison of SENs with enzyme modification and encapsulation approaches.

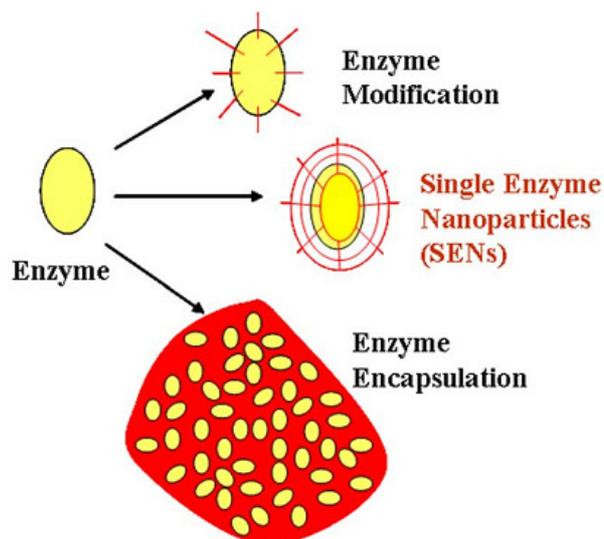


Figure 31 - Comparison between SEN approach with enzyme modification and encapsulation [80].

The preparation of SENs begins from the enzyme surface, with covalent reactions to anchor, grow, and crosslinking a composite organic/inorganic network around each enzyme molecule. The procedure for SEN synthesis is shown in Figure 32. A vinyl-group functionality is grafted onto the enzyme surface by covalently modifying the amino groups of lysines on the enzyme surface with acryloyl chloride. Using a tiny amount of surfactant, these modified enzymes are solubilized into hexane as ion-pair form. By this way, this approach enables the next polymerization step to start from the surfaces of the enzyme molecules. Silane monomers containing both vinyl groups and trimethoxysil groups are added to the reaction mixture. Free radical vinyl polymerization is initiated in this homogeneous solution to produce linear polymers that are covalently bound to the enzymes surface. By extracting these intermediates back into the water phase, the pendant groups are hydrolyzed and condensed to each other, yielding a crosslinked composite network around each separate enzyme molecule. This silicate polymerization consisting of hydrolysis and silanol condensation represents the second polymerization step.

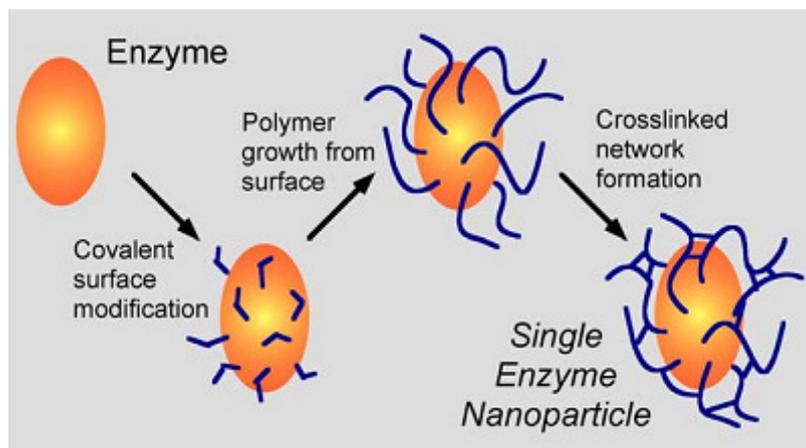


Figure 32 - SEN synthesis[80].

In this unique structure of SENs, the enzyme is attached to the hybrid polymer network by multiple covalent attachment points. The thickness of the network around each enzyme molecule is less than a few nanometers. The network is sufficiently porous to allow substrates to have an easy access to the enzyme active site [80].

2.1.3.3. Studies on enzyme immobilization in nanofibers

As seen so far, different nanostructures materials have been used as supports, such as, nanoparticles and nanofibers. Nanofibers as an excellent support is attributed to: (i) a variety of polymers can be electrospun and meet different requirements as supports, (ii) the high porosity and the interconnectivity of electrospun supports endows them with a low hindrance for mass transfer, and (iii) the nanofiber surfaces can be modified to benefit enzyme activity [83]. Several papers have reported immobilization of several enzymes on different polymer nanofibers

One of the most studied enzymes is lipase. Nakane *et al.*[84] physically entrapped lipase among the PVA chains and throughout nanofibers in order to study flavour ester synthesis in a non aqueous medium. One first difference noticed is the diameter increase. The diameter of lipase-immobilized nanofiber is larger than pure PVA nanofiber. An increase in spinning solution viscosity (from 350 mPa.s to 380 mPa.s) is seen as responsible for this fact. This change, results in a smaller surface area for enzyme-immobilized nanofiber (3.75 m²/g) relatively to pure PVA nanofiber (4.06 m²/g). Although this is not good since the high surface area is one of the most important advantages in nanofibers use, this was only a slight decrease. Regarding to ester synthesis, the conversion of lipase-immobilized nanofiber increases with increasing reaction time, and exceeds 80% after 72 h.

The difference in dispersion states between lipase powder and immobilized ones will affect esterification rates. In lipase powder, enzyme molecules are engulfed by impurities that are insoluble in organic solvents. On the other hand, in PVA nanofibers lipase molecules have a relatively good dispersion once lipase powder is dissolved in PVA aqueous solution before electrospinning. This way, the esterification rate for lipase-immobilized nanofiber is faster than for lipase powder.

Another parameter studied was reusability. After nine runs, the immobilized-lipase retains about 65% of the activity of the first run whereas the activity level of lipase powder drops steeply after two runs. This decreased may be due to water (a reaction product) adsorption onto the lipase powder which will prevent esterification. Water may decrease contact area between enzyme and substrate and lead to enzyme aggregation. This does not occur in immobilized lipase once PVA support readily absorbs water. Finally, lipase powder is not suitable for reuse, because it forms a gelatinous mass while the nanofiber can be recovered very easily from the reaction solution

Sakai *et al.*[85] studied transesterification by entrapped-lipase in PVA nanofibers in a non-aqueous medium. A comparison was made between lipase entrapped in the nanofibrous mat and non-treated lipase activity. The initial transesterification rate was 5.2-fold faster than that for non-treated lipase. This faster transesterification achieved may be interpreted as a consequence of the high dispersion of lipase in PVA nanofibers. Contrary to this, non-treated lipase which is a crude-purification product contains impurities which are insoluble in an organic solvent. As seen on the previous study, this fact increases substrates and products diffusion resistance, which results in a lower transesterification process. Since immobilized-lipase is not affected by this phenomenon, it is clearly shown that the entrapping of lipase in PVA electrospun fibers is useful for transesterification processes.

Li *et al.*[36] immobilized lipase for soybean oil hydrolysis. They prepared a polyacrylonitrile (PAN) nanofibrous membrane and activated it by amidination reaction for lipase covalent binding resulting in small protein aggregates. Although there is not an enzyme entrapment (lipase is not present in spinning solution), due to the large specific surface area provided by the nanofibers, with covalent binding it was achieved a protein loading of 21.2 mg/g material of the membrane. The protein activity was determined to be 32.27 ± 0.44 U/mg which is equivalent to 87.5% activity retention. A variation of the activity centre caused by a structural conformation change of the lipase during covalent binding may be responsible for the enzymatic activity decrease. Immobilized lipase also showed a good biocatalyst activity. The conversion of hydrolyzed oil reached 72% after 10 min and 85% after 1.5 h under optimal operating conditions.

Since reusability is one of the major advantages of using immobilized enzymes Li *et al.*[36] also compared the effect of reuse between PAN nanofibers and other supports. The retention activities of zeolite supports is about 10% in palm oil hydrolysis after being reused seven times and porous accurel EP400 powder maintains 55% of its sunflower oil hydrolysis after being reused four times. Using immobilized lipase in a PAN matrice, the conversion of soybean hydrolysis was 72% and still retained 65% of the original conversion after being reused 20 times which can be considered very high attending to the other supports results.

Several enzymes beside lipase have been studied. One example is the entrapment of glucose oxidase (GOX) in electrospun polylactide (PLA) fibers used to activate the lactoperoxidase (LP) system in milk [86]. Due to a larger surface area and more porous structure of the electrospun fibers, GOX activity (13.05 ± 1.26 U/mg GOX) was significantly higher than compared to a cast membrane (0.67 ± 0.09 U/mg GOX). However, due to a possible limited diffusion of reactants and steric hindrance on the active sites of the immobilized GOX, activity was about two times lower than for the free enzyme. Regarding enzyme stability, GOX-in-PLA was more stable under storage condition tested (4°C). After storage for 26 days, immobilized GOX retained 65% of the original activity, while the activity of the free GOX was reduced to less than 10%. A multiple dipping test with the fiber mats, resulted in a 37% activity decrease during the first reuse. Possibly, a significant leaching loss occurred. Thereafter, repeated reuses of the fiber mat resulted in a continuing loss of GOX activity. It is possible to understand that a proportion of GOX was immobilized and remained active in the PLA fibers. Once more, nanofibers show that they have a potential as support for enzyme usage.

Wu *et al.*[87] immobilized cellulase in nanofibrous PVA membranes. PVA and cellulase were dissolved together and electrospun into nanofibers with diameter of around 200 nm. This way, enzymes were entrapped within PVA nanofibers. In order to maintain a water-stable nanofiber mat, a crosslinking with GA vapour was done. With water-resistant nanofibers it was possible to evaluate cellulase activity in an aqueous medium which was not the case in the previous studies shown. A SEM analysis shows that the addition of cellulase to PVA nanofibers increased fiber irregularity and diameter from 200 to 500 nm. It is also possible to see in Figure 33 that shuttle-shape beads are dispersed throughout the fibers. It was expected that protein aggregates could form during electrospinning which would induce beads formation and increase fiber diameter once they are immobilized inside them.

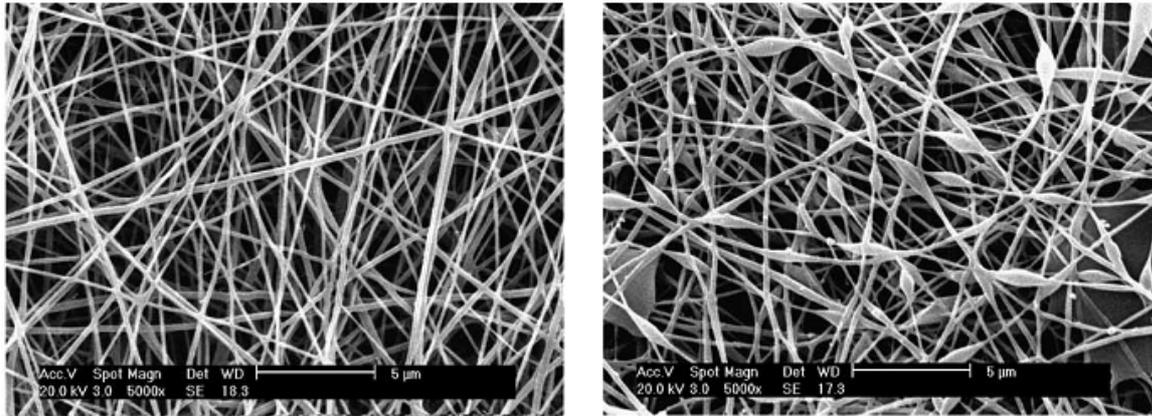


Figure 33 – SEM images of electrospun PVA/cellulose fibers from PVA solutions with (a) 0%, (b) 10% of cellulase[87].

According to enzyme activity analysis, immobilized cellulase activity was over 65% of that free enzyme, and was nearly two times higher than in casting films from the same solution which they also analyzed. Attending to the activities of other immobilization supports, they considered this value as quite high. Due to a high surface area and porous structure there is a great exposure of enzymes at fiber surface and reduced diffusion resistance which will undoubtedly enhance enzyme activity. It was seen in previous sections that these two characteristic are part of the main advantages of nanofibers over other possible supports. However, the enzyme bonding to polymer chain limits substrate access which results in the differences registered to the enzyme free form.

The reusability of entrapped cellulase was also reported. From the data collected it was noticed that enzyme relative activity compared to its initial value decreased along with the reusing times. At the end, cellulase retained over 36% of its initial activity after six cycles of reuse. This loss can be a consequence of some cellulase leakage during measuring cycles which were only physically bonded (weaker than chemically). Changes in fiber morphology resulting from water uptake can also reduce enzyme activity over time. Anyway, when comparing to cellulase free form, where recovery is very difficult or with low percentages, it can be considered that such nanofiber reusability can be a great advantage for industrial application.

These are only a few examples of the work that has been developed over the years in enzyme immobilization in nanofibers. Beside the reports cited above, others using peroxidase [88], alkaline phosphatase [89], luciferase [90] or α -chymotripsin [34] have been published. The results obtained show that nanofibers have a great potential as support for enzyme immobilization and several advantages over other supports.

2.1.3.4. Studies on β -Galactosidase Immobilization

As seen in previous sections, many enzymes were immobilized in different polymer nanofibers but no studies regarding β -galactosidase immobilization in nanofibers has been reported. However, many other kinds of immobilizations were tried previously with this enzyme, with satisfying results.

For example, Ates *et al.* [91] entrapped β -galactosidase in cobalt alginate beads with a relative activity of 83%. Enzyme leakage was avoided by treatment with GA solution following which the relative activity was stable. This method yielded high activity and good physical-chemical characteristics. Baran *et al.* [92] entrapped β -galactosidase on poly(2-hydroxyethyl methacrylate) (pHEMA) membrane bulk. An increase in β -galactosidase loading resulted in a consistent increase in membrane activity. The K_m value for the entrapped enzyme was higher than that for free enzyme. The optimum reaction temperature and pH were the same for free and immobilized β -galactosidase. After 15 successive uses the retained activity of the entrapped enzyme was 95% and the storage stability of the enzyme was found to increase upon immobilization.

In the work of Woudenberg-van Oosterom *et al.* [93], β -Gal from *Aspergillus oryzae* and *Kluyveromyces fragilis* were adsorbed on phenol-formaldehyde resins of the Duolite type followed by crosslinking stabilization. The maximum adsorption percentage for *K.fragilis* was 71% with A-7 resin and 94% for *A.oryzae* with S-761 resin. These values show that the immobilization procedure was really effective, only a minor loss occurred. However, the activity yield obtained was relatively low for both β -Gal. A maximum of 23% for *K.fragilis* and 54% for *A.oryzae* were achieved which corresponds to an efficiency of 32 and 59% respectively. The immobilization process was also reflected in the reactions catalyzed by β -Gal specifically on the condensation of galactose and glycerol and the glycerolysis of some galactosyl donors. For the condensation of galactose by *K.fragilis* β -Gal a slow reaction was registered since almost 31 h were needed to achieve an equilibrium conversion of approximately 30%. Also for glycerolysis of galactose, both β -Gal registered very slow reactions. β -Gal from *A.oryzae* and *K.fragilis* required 24 h for only a product conversion of 19 and 23% respectively. However, for ONPG substrate, β -Gal from *A.oryzae* took 45 minutes to achieve 99% of product while 360 minutes were needed to obtain 96% of product using β -Gal from *K.fragilis*. These results show that there is some drawbacks in the immobilization process that limits enzyme-substrate interaction. Nevertheless, it is important to register that the enzyme was efficiently fixed and remained active for different substrates which is an excellent result and a start point for many optimizations.

Ladero *et al.* [72] made a kinetic study of the hydrolysis of lactose and ONPG with β -galactosidase from *E.coli*. In this study, the enzyme was covalently immobilized on a silica-alumina support.

Tanriseven *et al.* [71] entrapped β -galactosidase in fibers composed of alginate and gelatin hardened with glutaraldehyde. Immobilization resulted in 56% relative activity retained for 35 days without decrease. The optimum conditions were also not affected by entrapment, and the optimum pH and temperature for free and immobilized enzymes were 4.5 and 50 °C respectively. Immobilized β -galactosidase was more stable at high pH and temperatures.

In another study (Albayrak *et al.* [75]), *A.oryzae* β -galactosidase was immobilized on cotton cloth for galacto-oligosaccharides (GOS) production from lactose. Cotton cloth is an inexpensive fibrous matrix, with high porosity, large specific surface area and an excellent mechanical strength and for that reason it is assumed as a good matrix for enzyme immobilization. One of the first obvious results regards the substrate diffusion across the matrix. It was noted that the kinetics of the GOS formation was almost the same for reactions carried out with the enzyme immobilized on cotton cloth and free enzyme in solution. This fact suggests that there isn't a remarkable diffusion limitation or alteration imposed by the cotton cloth which is definitely related to its characteristics. Since high porosity and large specific area are the main properties of cotton cloth which are also for nanofibers, this result gives good perspectives for nanofiber immobilization. Thermal stability was also affected in a positive way. Immobilized enzyme was stable at 40 °C under continuous operational conditions, and was much more stable than the free enzyme. Immobilized β -Gal had also an estimated half-life of more than 1 year at 40 °C and 48 days at 50 °C. The thermal stability was also increased by 25-fold by immobilization on the cotton cloth, as compared with the free enzyme. A possible conclusion is that the cotton matrix has a protective effect over the enzymes which allow more aggressive conditions which wouldn't be tolerated by free-enzymes. These excellent outcomes allowed testing it in a plugflow reactor, resulting in a stable and continuous operation for 2 weeks without any problem. Consequently, the continuous enzyme reactor had a high productivity which was several-fold higher than previous reports. A year later they reported once more the use of cotton cloth as an immobilization matrix but with the formation of polyethyleneimine-enzyme aggregates [76]. This innovation allowed the arrangement of enzymes in multilayers. As a result, a larger amount of enzyme was immobilized, with 90 to 95% of efficiency, naturally increasing the reactor productivity.

Chitosan-immobilized β -galactosidase was also described [77]. In this report, a comparative study between immobilized and free *A.oryzae* β -gal was made concerning stability, catalytic

efficiency and enzymatic characteristics. First, the immobilization process as expected limited the mass-transfer diffusion, which decreased the activity yield from 100% (free enzyme) to 18.4% (chitosan-immobilized enzyme). Although and as seen in previous examples, the immobilization had a protective effect to the enzymes. The chitosan-immobilized enzyme was thermally more stable than the free one. Immobilization led also to an enhancement in the half-lives of the enzyme at different temperatures employed. The inactivation constant of the immobilized β -gal at 60 and 65 °C was higher than that of free enzyme, also indicating that a higher thermostability was achieved due to immobilization. Chitosan-immobilized enzyme was reused for four cycles without a significant activity loss which couldn't be done with a free β -Gal.

Mariotti *et al.*[73] studied the hydrolysis of whey lactose by immobilizing β -Gal on silica with glutaraldehyde crosslinking. It was observed that the immobilization without washing the GA before enzyme addition and followed by the treatment with GA allowed a high activity, initially and after incubation. On a long term operation, using lactose solution the half-life of the biocatalyst was estimated to be 12 months. This result confirms one more time the possibility to obtain good performances with an immobilized β -Gal.

Neri *et al.* [69] immobilized β -galactosidase from *Kluyveromyces lactis* onto a polysiloxane–polyvinyl alcohol magnetic composite using glutaraldehyde as the activating agent.

Hronska *et al.*[94] immobilized β -Gal in high elastic and stable lens-shaped PVA capsules and used for lactose hydrolysis. Carrying out the hydrolysis at temperatures between 15 and 45 °C, the reaction degree obtained was 80 to 100%. The operational stability of the enzyme was also tested during 20-repeated batch hydrolysis and β -Gal retained more than 85% of its initial activity after nine repeated uses. Another relevant fact is the temperature at which immobilized enzyme worked. The lower temperature at which milk hydrolysis occurred (15 °C) with good results could decrease manufacturing costs and also contaminating risks. Therefore, it is once again shown that enzyme immobilization is a great advantage when comparing with free enzymes.

Many other studies were done regarding β -Gal immobilization but none reported the use of electrospun fibers as a matrix. This way, the work reported on this thesis may be considered the first where β -Galactosidase is entrapped in water-stable PVA nanofibers and a comparative evaluation of the activity and stability is described.

2.2. Materials and Methods

2.2.1. Materials

Poly(vinyl alcohol) (PVA) (Mw = 85-124 kDa, 99+% hydrolyzed), Triton X-100, the crosslinking agent glutaraldehyde (50 wt.% in water), β -galactosidase from *Aspergillus oryzae* (EC 232-864-1) and 2-nitrophenyl β -D-galactopyranoside (ONPG) were obtained from Sigma-Aldrich Química SA (Portugal) and used as received. Acetone (Pro Analysis) was obtained from Panreac and hydrochloric acid (min. 37%) was obtained from Riedel-de Haën. These chemicals were used without further treatment. Deionised water was used as solvent.

2.2.2. Solution preparation for electrospinning

Aqueous PVA solutions (10 wt.%) were prepared as referred in section 1.2.3. PVA powder (99+ hydrolyzed, number average MW 85000-124000) was dissolved in deionized water at 90 °C with constant stirring for 6 h. After the solution cooled to room temperature, Triton X-100, as a surfactant, was added to a final concentration of 1.5 wt. %. The mixture was stirred for 20 min. Finally, β -galactosidase was added in order to obtain a 5 wt. % concentration and stirred to complete homogenization.

2.2.3. Viscosity and Surface Tension measurements

For the measurement of viscosity and surface tension, a 10 wt.% PVA and 10 wt.% PVA/ 5 wt.% β -galactosidase solutions were prepared as referred previously in 2.2.2. The viscosity was measured in a constant stress rheometer (AR 1000 Rheometer, TA Instruments) and the surface tension in a Contact Angle System OCA-20 (Dataphysics).

2.2.4. Operation parameters of the electrospinning system

Based on the results obtained previously, the operation parameters selected for the electrospinning of PVA/ β -Gal solutions were:

- Applied Voltage: 21 kV;
- Speed Rotation of the collector: 109 RPM;
- Tip to collector distance: 12 cm
- Feed Rate: 1.2 mL/h

2.2.5. Crosslinking of electrospun PVA fibers

The crosslinking method was based on the reaction of glutaraldehyde (GA) with the PVA hydroxyl groups successfully reported in the previous chapter. Although a GA concentration of 100 mmol/L was defined as the optimum concentration, the procedure was once again performed by immersion of electrospun PVA mats in acetone solution with 30, 45, 60 or 100 mmol/L GA and 0,01 mol/L HCl for 24 h. Then, the crosslinked PVA/ β -Gal mat was dried in a petri dish in a ventilated hood at room temperature for 24 h before use. To test the water-resistance and morphological changes resulting from the water contact, the PVA mat was immersed in water for 5 h in a beaker and dried in a petri dish for 24 h in a ventilated hood.

2.2.6. Morphologic and characterization of PVA matrices

To examine the fiber morphology, samples of PVA mat were placed in a microscopy metal support with double-layer carbon adhesive tape. Then, they were sputter-coated with a thin layer of gold and analyzed with a scanning electron microscope (SEM, Hitachi SU-70) at 15 kV. The average fiber diameter and distribution were determined by measuring the diameter of 100 fibers using an image analysis program (Image J 1.43u).

2.2.7. Determination of enzyme loading efficiency

The PVA/ β -Gal membranes after crosslinking were incubated in citrate-phosphate buffer (pH 5.0) at 37 °C. The amount of released β -Gal from the membrane was determined by measuring the residual enzyme present in the reaction medium by the bicinchoninic acid (BCA) assay to determine total protein. Bovine serum albumin (BSA) was used as standard protein. It was supposed that all enzymes that weren't entrapped could be released from the membrane and got into the buffer. Regular aliquots were taken from the reaction medium until no protein increase was measured. The enzyme loading efficiency was determined by dividing the amount of protein in solution by the amount of the initial immobilized protein.

2.2.8. Measurement of β -galactosidase activity

The enzyme activity assays which measured ONPG hydrolysis were performed based on the procedure of Tanaka *et al* [68]. An ONPG solution (4.5 mmol/L) in a citrate-phosphate buffer (0.1 mol/L pH 5.0) was incubated with immobilized (2.5 mg) or free β -galactosidase (0.015 mg/mL) at 37 °C with a reaction mixture of 200 rpm on a magnetic stirrer. Samples (1.0 mL) were withdrawn at 20

minutes intervals and absorbances were read at 420 nm in an UV-Visible spectrophotometer till become stable. After each assay, the PVA/ β -Gal mat was washed with buffer and stored at 4 °C overnight and reused again in the same conditions described above. β -Gal solutions and PVA/ β -Gal mats were prepared in the same day and assayed simultaneously.

2.3. Results and discussion

2.3.1. PVA- β -Galactosidase nanofibers formation

The electrospinning of the PVA/ β -Galactosidase solution occurred in the same conditions referred for the electrospinning of PVA nanofibers. This way, any differences registered in fiber morphology, membrane porosity or diameters change could be an effect of the enzyme entrapment. Once more through SEM analysis it was possible to examine the electrospun nanofibers. In Figure 34 it is shown the resulting fibers of the electrospinning process.

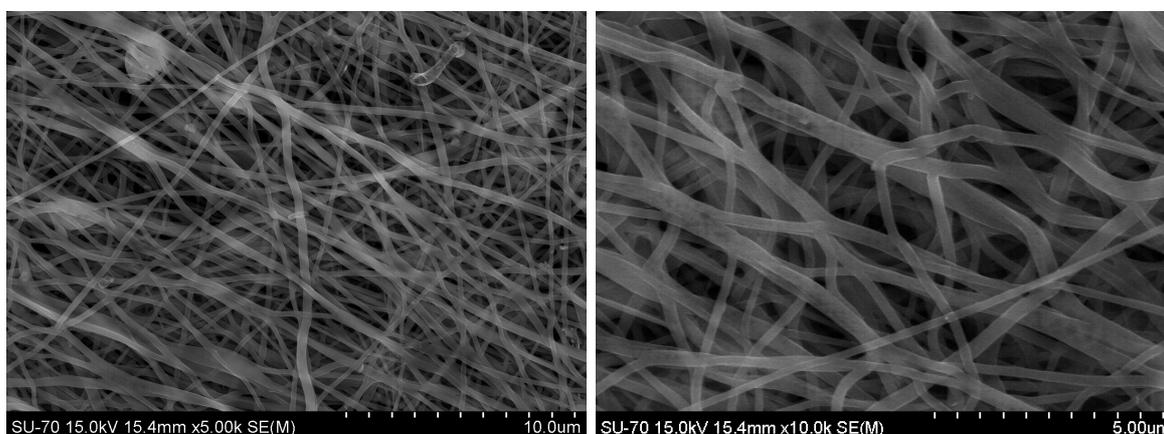


Figure 34 - SEM images of electrospun PVA/ β -Galactosidase nanofibers.

From the collected images it is possible to see that the presence of β -gal in the electrospinning solution did not change the morphology of the obtained fibers. No beads or flat fibers were noticed in the fiber mat and for that reason one can assume that only uniform fibers were electrospun. From the images it is also worth to note that there are not any granules or fiber aggregates in the mat. Thus it is possible to assume that the electrospinning solution wasn't chemically changed by the presence of the enzyme and the electrospinning conditions used were appropriated. To confirm this, the viscosity and surface tension for the PVA and PVA/ β -Gal electrospinning solutions were measured. As can be seen from Figure 35, both viscosities were very similar, approximately 1 Pa.s, although the flow behaviour showed slight differences. For low shear rates (resting state) the solution containing the enzyme is slightly more viscous. As shear rate starts to increase, both solutions achieve the same viscosity but for high shear rates, the PVA/ β -Gal is slightly less viscous. Higher shear rates are experienced by the electrospinning solution during ejection from the needle in order to produce fiber stretching. This way, it is at higher rates that one

most compare solutions viscosities. However, even if any significant difference existed between the viscosities of both solutions, this difference was not enough to produce any significant effect on the resulting fibers morphology.

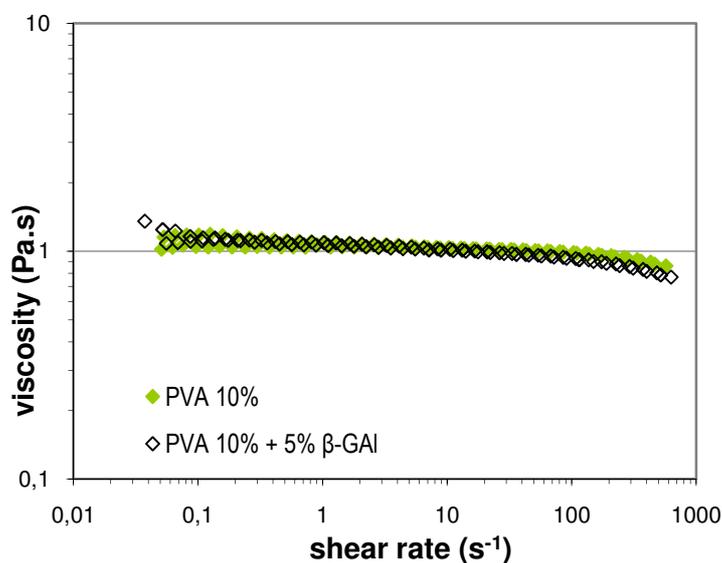


Figure 35 - Graphic representation of the viscosity behavior of PVA 10% (green) and PVA/β-Gal (white) solutions.

Surface tension of both solutions was also measured. For the PVA solution an average value of $75.51 \pm 0,34$ mN/m was obtained while for the PVA/enzyme solution $74.80 \pm 0,19$ mN/m was registered. The slight difference did not cause any significant effect of fiber morphology.

However, fiber diameter is a relevant parameter and must be analyzed. In Figure 36 it is possible to see that the average diameter had a high increase which can be associated to the presence of β-Gal. Fiber size distribution also changed. In PVA fibers, the highest frequency is related to fibers with a diameter between 100 and 200 nm while for PVA/β-Gal fibers between 200 and 300 nm dominate. Once those enzymes are entrapped within the fibers it is normal and predictable that the diameter could increase.

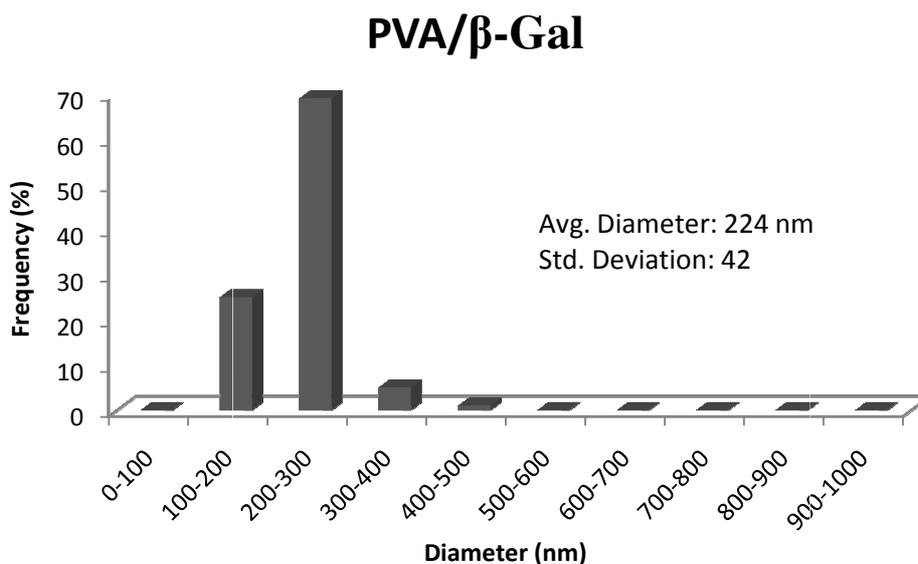
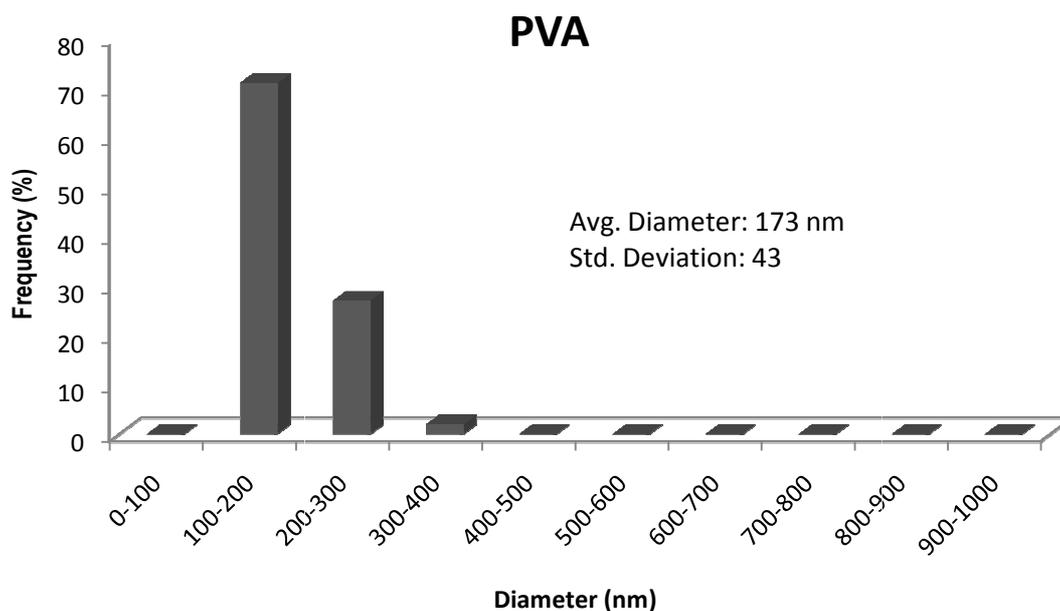


Figure 36 - Fiber diameter distribution of PVA and PVA/ β -Gal nanofibers.

2.3.2. Effect of crosslinking and water immersion

The crosslinking of the PVA/ β -Gal membrane was once more done with the use of glutaraldehyde as previously described. Even though this method was successful for water resistance of the PVA mat, it was necessary to know if the presence of the enzyme would interfere with the crosslinking process and how it could affect the enzyme activity. This way, it was again

tested the water resistance of the membrane in increasing concentrations of glutaraldehyde. In Figure 37 it is possible to see the effect of crosslinking in PVA/ β -Gal nanofibers morphology.

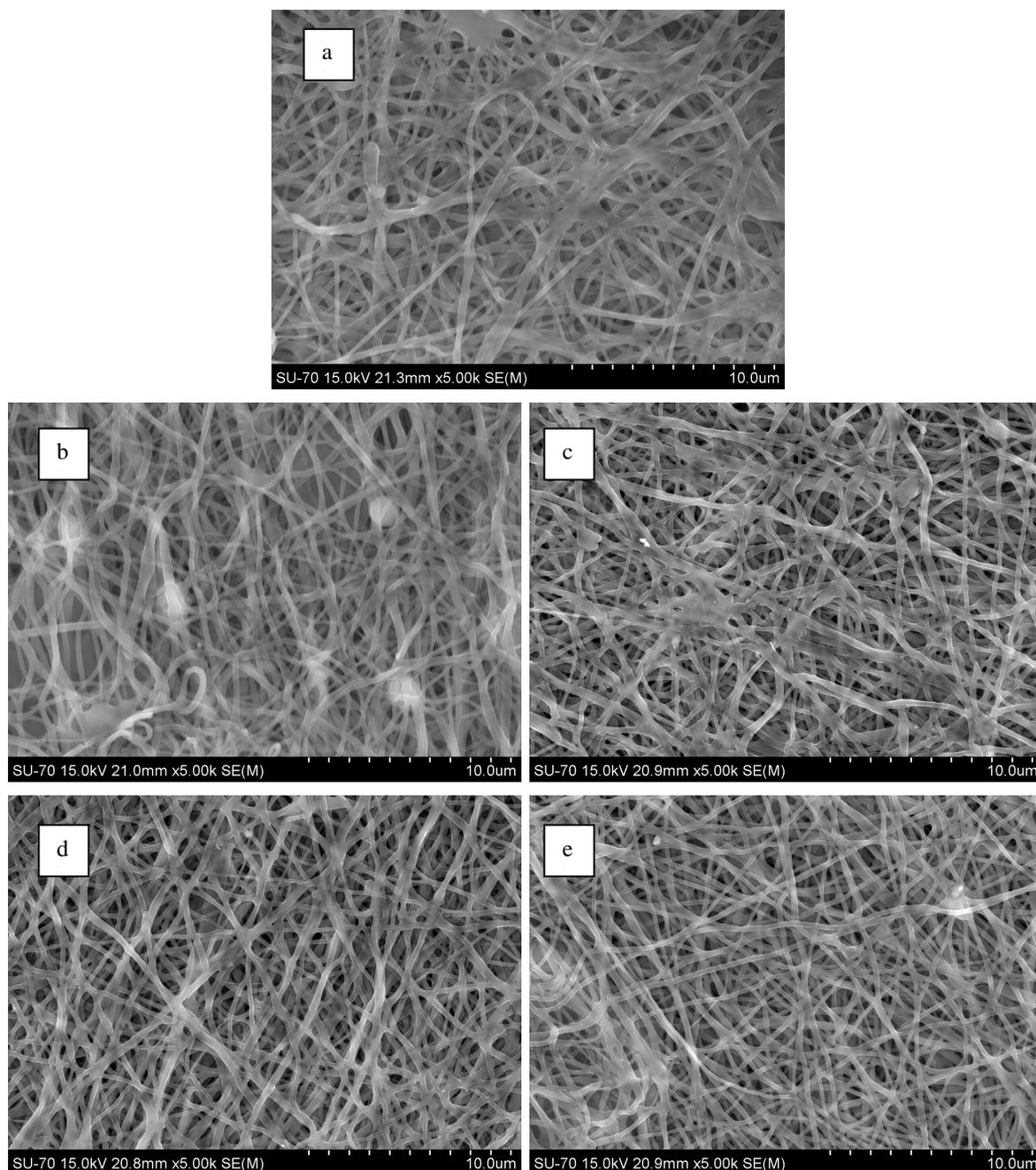
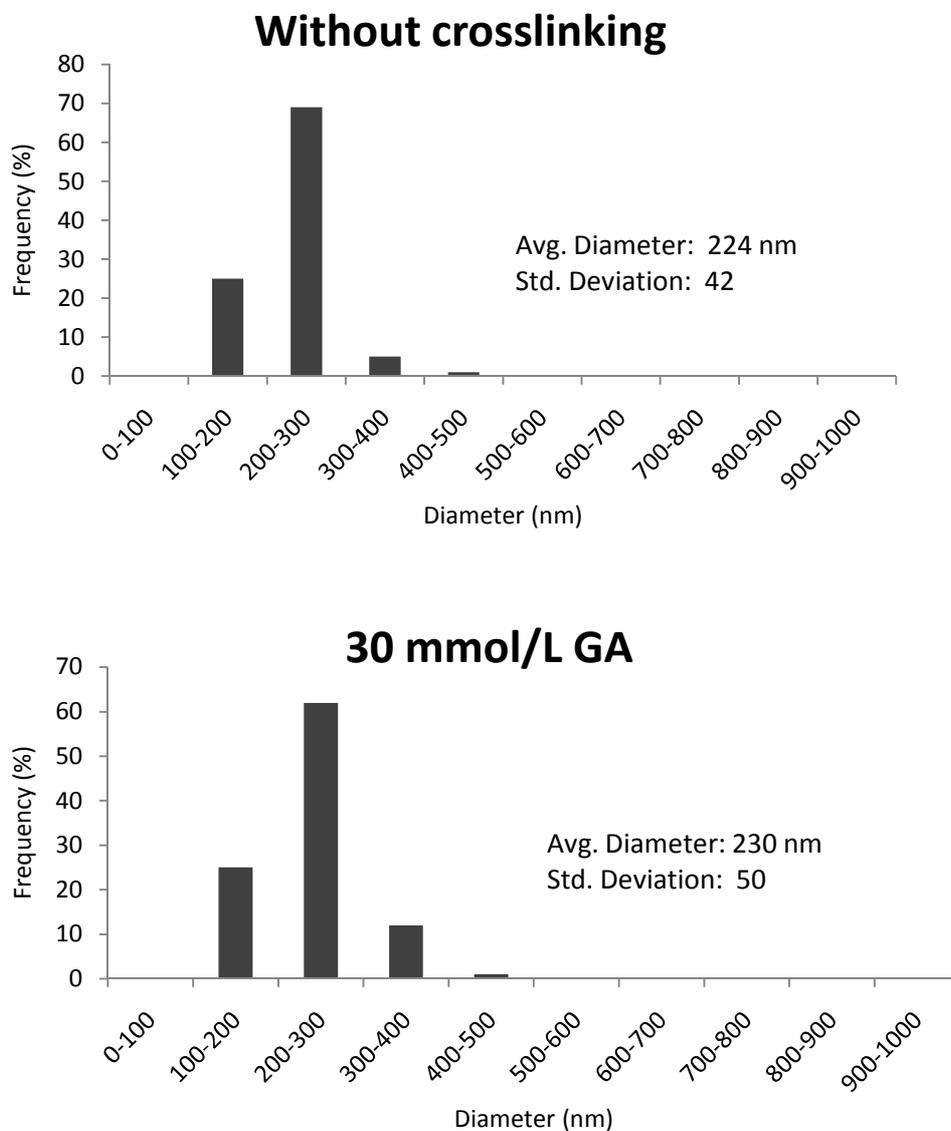


Figure 37 - SEM images of crosslinked PVA/ β -Gal nanofibers with different GA concentration for 24 h: a) 0 mM, b) 30 mM, c) 45 mM and d) 60 and e) 100 mM.

It is perceptible that the nanofibers have a relatively normal morphology and the membrane porosity is conserved. However, it is possible to see that occurred some aggregation and fiber fusion

comparing when the enzyme was not present. The possible presence of enzymes at the fiber surface may affect the bounding of GA to the PVA hydroxyl groups. It may lead to a competition between hydroxyl and amine groups of β -gal and hydroxyl groups of PVA for the crosslinker. This way, glutaraldehyde bounding to PVA could be affected and decrease the crosslinking effect. The effect of increased GA concentration in crosslinking solution could also be analyzed. Some beads and larger fibers that aggregated were noticed in lower concentrations, while with higher concentrations no beads are visible and the fibers were thinner, close to non-crosslinked fibers. In order to confirm it, a diameter study was made. In Figure 38 is shown the average diameter and diameter distribution of the crosslinked fibers compared to non-crosslinked fibers.



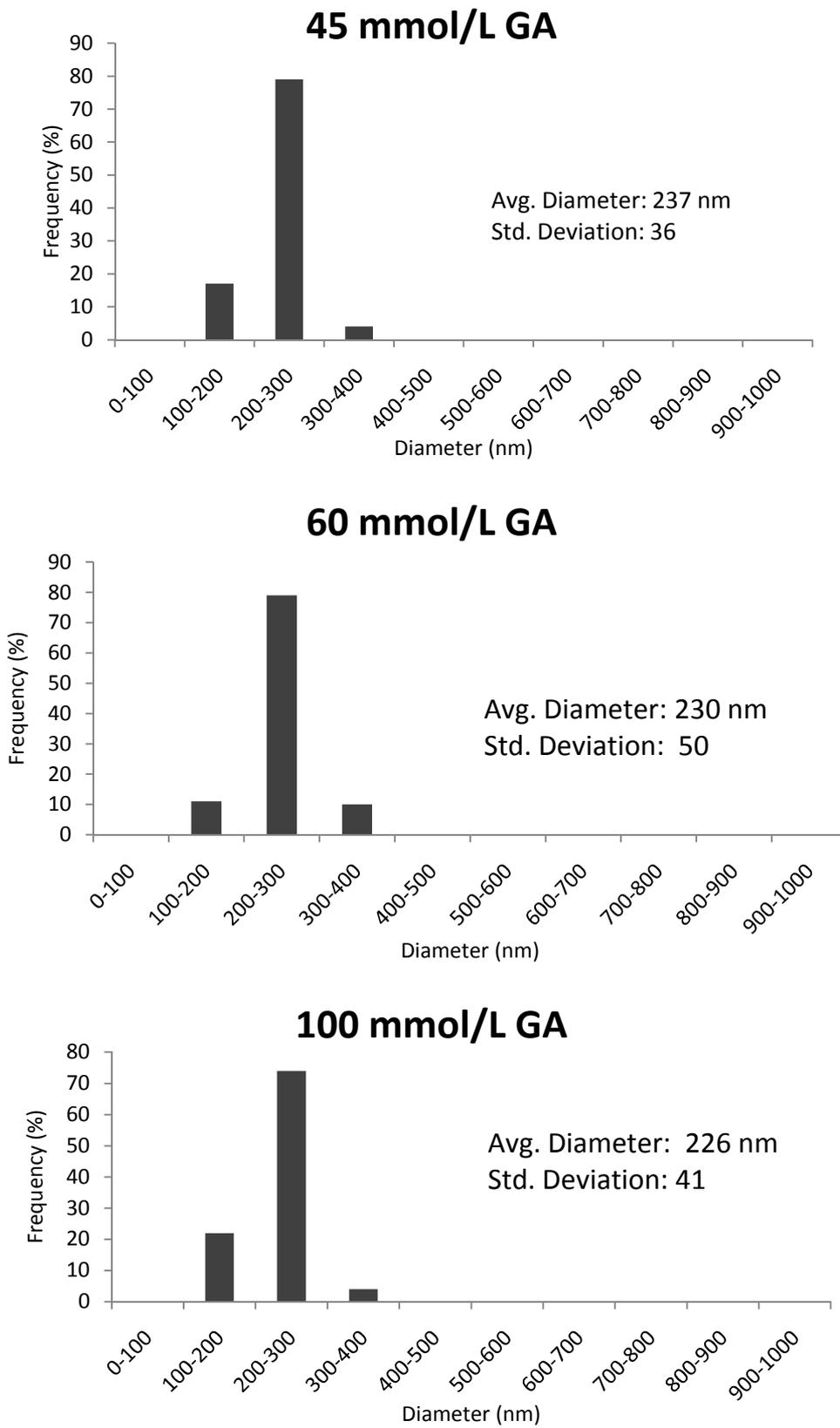
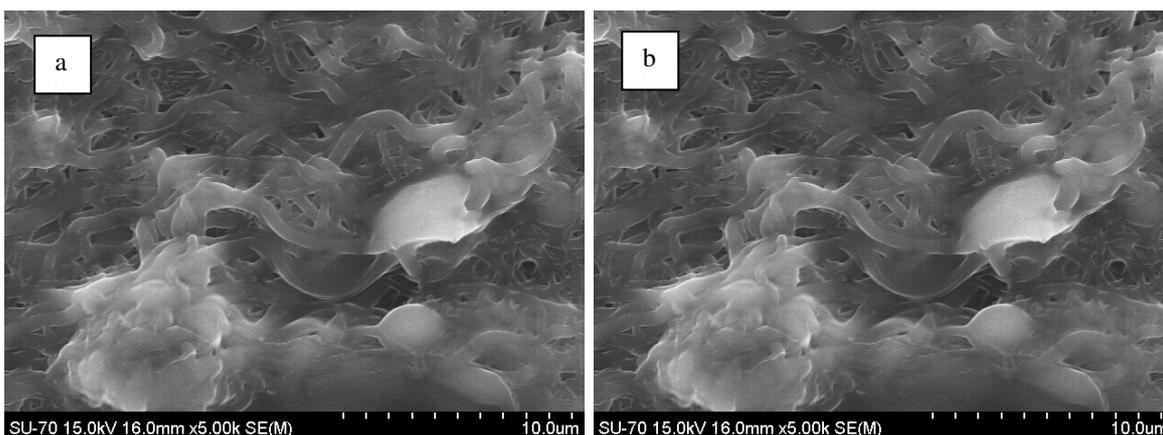


Figure 38 - Fiber diameter distribution of crosslinked PVA/ β -Gal nanofibers according to GA concentration.

From the graphics analysis, one can see that there is a slightly increase in the average diameter with the raise in GA concentration. However, this is not seen with a concentration of 100 mmol/L. The diameter remains almost the same of the non-crosslinked fibers. Since the difference in the average diameter between crosslinked and non-crosslinked fibers is only of a few nanometers and fiber distribution it's almost the same, it can be assumed once more that there is no significant influence of the crosslinking treatment.

After the crosslinking procedure, PVA/ β -Gal mat was immersed in water to test water resistance. From the SEM images (Figure 39) it is clear that these fibers hadn't resisted to water dissolution as seen previously for PVA fibers only, having more difficulty to retain the proper morphology and membrane porosity. GA concentration is once more related to the degree of morphology retention. With concentrations of 30, 45 and 60 mmol/L, the water uptake was very high, resulting in fiber swelling and a great fiber aggregation, decreasing extremely the membrane porosity. These results show once more that the enzyme may interfere with crosslinking reactions due to bounding between GA and β -Gal. However, due to higher concentration of GA this effect is relatively suppressed and there is a reduced fiber aggregation resulting in more spaces through the membrane when immersed in water. With a GA increase to 100 mmol/L, PVA/ β -Gal fibers resisted more to the water uptake due to high degree of linkage between PVA hydroxyl groups and GA. The fibers have a uniform morphology and it seems that fiber aggregation is minimal. It will be vital to minimize diffusional limitations during ONPG hydrolysis.



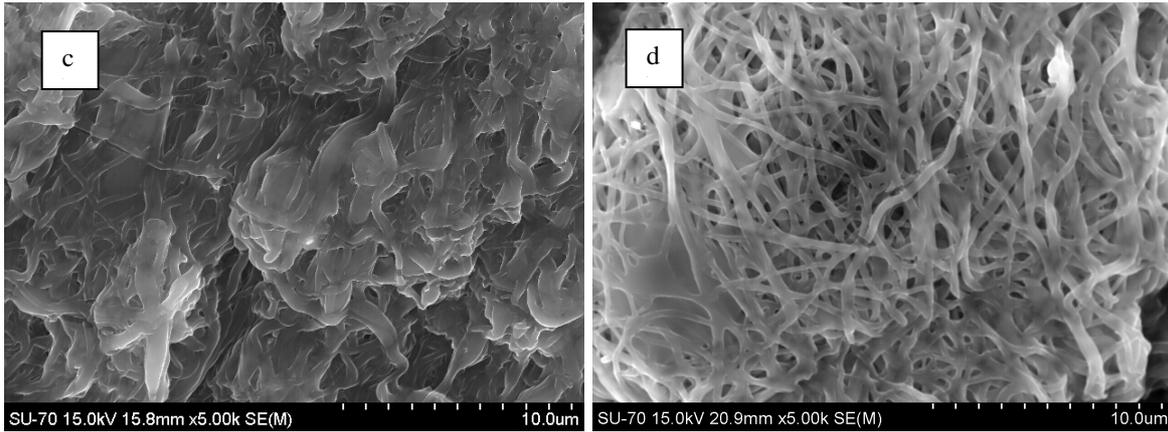


Figure 39 – SEM images of electrospun PVA mat crosslinked with (a) 30, (b) 45, (c) 60 and (d) 100 mmol/L of GA after immersion in water.

2.3.3. Enzyme loading efficiency

Although the obtained results show that water disintegration was relatively overcome with a GA concentration of 100 mmol/L, during PVA/ β -Gal mat immersion always occurred some water uptake and some fiber degradation, even if minimal. This way, some enzyme leaching can occur which will affect enzyme loading and the final mat activity. Thus, the enzyme loading after crosslinking and water immersion was determined and the results are shown in Table 2. In this table, it is possible to evaluate three different parameters with time. First, one can see the β -gal concentration (mg of enzyme per milliliter of immersing solution) where PVA mat was immersed. This value allows calculating the absolute quantity (mg) of enzyme that exists in the solution. Knowing the quantity of enzyme that is present in the PVA/ β -gal mat it is possible to estimate the yield of immobilization, *i.e.*, the enzyme loading efficiency:

$$\frac{\text{Quantity of } \beta\text{-gal in solution}}{\text{Initial quantity of } \beta\text{-gal in PVA mat}} \times 100\% \quad (1)$$

Table 2 - Enzyme loading efficiency

| Time of immersion (min) | [Protein] (mg/mL) | Protein in Solution (mg) | % immobilization |
|----------------------------|----------------------|-----------------------------|------------------|
| 30 | 0,08 | 0,412 | 83,53 |
| 60 | 0,08 | 0,417 | 83,33 |
| 220 | 0,084 | 0,421 | 83,14 |
| 280 | 0,08 | 0,415 | 83,38 |

As can be seen in Table 2, there is some leakage of enzyme from the nanofibrous membrane. Although, the immobilization yield (enzyme loading efficiency) is approximately 83% which represents a high value since PVA nanofibers could undergo some disintegration although their great stability in water after the crosslinking procedure. It is also highly relevant that only after 30 minutes, the yield of immobilization it is the same than for 280 minutes of immersion. Based on this, one can assume that the proteins found in solution were at the surface of the membrane. If some leakage of proteins entrapped within the fibers were occurring during the immersion, a constant increase in quantity of protein in solution should be registered which is not the case. Based on this behaviour it seems that enzymes were rapidly removed when in contact with water, so the immobilization wasn't as strong as the entrapment in the fibers which points to a superficial binding. This way, it was defined that before any enzymatic assay, PVA/ β -Gal mat should be immersed in water for removing any superficial-bond enzyme.

2.3.4. Activity of immobilized β -Galactosidase

One of the work's objectives was the possibility to compare free and immobilized β -Gal activity and stability. Before even know the assay's results some outcomes were already expected. It was estimated that free enzyme would have a higher activity than immobilized one. The diffusional limitations imposed to an enzyme entrapped within a fiber are higher than for a free enzyme which can interact without any limitation with the substrate. Since the assays were always done using the same enzyme source (enzyme solution for free form and PVA mat for immobilized form), stored at the same conditions, it was possible to analyze enzyme activity through time, *i.e.*, enzyme stability since the solution or nanofibrous mat was made.

Figure 40 shows the changes of absorbance at 420 nm with time for four samples till complete ONPG hydrolysis. The enzyme activity was determined from the slope of the initial minutes where a constant absorbance raise was registered (black line).

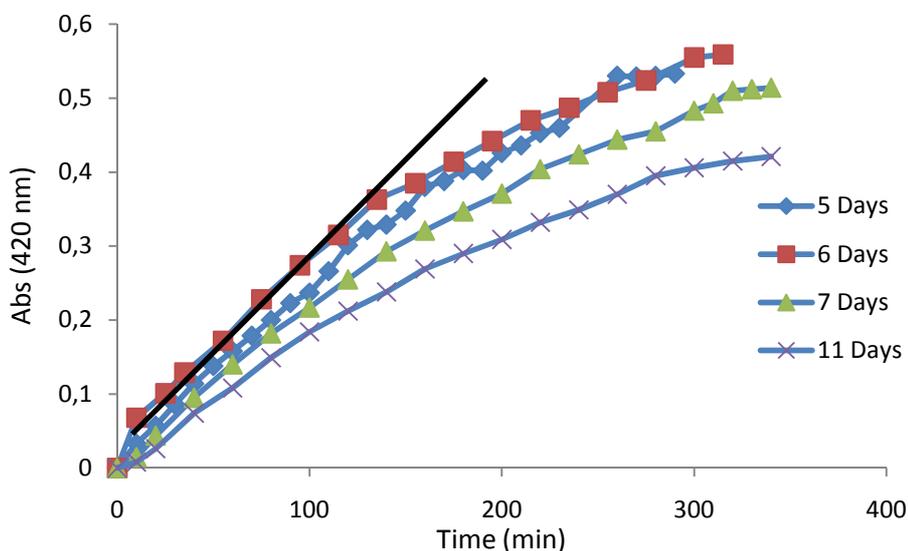


Figure 40 - Change in the absorbance with time for four assayed samples in different days after electrospinning.

In order to obtain the quantity of hydrolyzed substrate per minute (enzyme activity), the following equation was used where ϵ stands for molar extinction coefficient:

$$Enzyme\ activity = \frac{slope}{\epsilon} \times Volume \quad (2)$$

Finally, to determine β -gal activity per mass of used enzyme (enzyme specific activity), the following equation was used:

$$Enzyme\ specific\ activity = \frac{Enzyme\ activity}{mg\ of\ enzyme} \quad (3)$$

In Table 3 it is possible to see the behaviour differences between the two forms of β -Gal some days after electrospinning the PVA mat electrospinning and β -Gal solution preparation.

Table 3 - Activity of free and immobilized β -Galactosidase

| Days | Free β -Gal | | | Immobilized β -Gal | | |
|------|---------------------------------------|---|--------------------------|---------------------------------------|---|--------------------------|
| | Activity (mmol min ⁻¹) | Specific Activity (mmol min ⁻¹ mg ⁻¹) | Retained activity (%) | Activity (mmol min ⁻¹) | Specific Activity (mmol min ⁻¹ mg ⁻¹) | Retained activity (%) |
| 0 | 7.36x10 ⁻⁵ | 9.81x10 ⁻⁴ | N.A. | N.A | N.A | N.A |
| 5* | 6.24x10 ⁻⁵ | 8.33x10 ⁻⁴ | 84.9 | 7.84x10 ⁻⁶ | 3.19x10 ⁻⁵ | 3.25 |
| 6 | 6.96x10 ⁻⁵ | 9.27x10 ⁻⁴ | 94.5 | 8.98x10 ⁻⁶ | 3.79x10 ⁻⁵ | 3.86 |
| 7 | 6.94x10 ⁻⁵ | 9.26x10 ⁻⁴ | 94.3 | 6.31x10 ⁻⁶ | 2.51x10 ⁻⁵ | 2.55 |
| 11 | 6.49x10 ⁻⁵ | 8.65x10 ⁻⁴ | 88.2 | 4.32x10 ⁻⁶ | 1.78x10 ⁻⁵ | 1.81 |

N.A.: Not applicable; * Problems with spectrophotometer

As expected, the free enzyme had a much higher activity than immobilized one. The diffusion limitations had a high influence on the catalytic activity of the enzyme, once the ONPG substrate was unable to enter and exit the PVA nanofibers without restraints. The negative influence of the electrospinning and crosslinking processes on enzyme activity can not be discarded as a cause of the observed decrease in activity for the immobilized enzyme.

This way, β -Gal was unable to quickly hydrolyze the substrate and the retained activity was significantly lower. The maximum retained activity when comparing with the free form was only 3.86 %. From this result, it is obvious that an extensive work is necessary to minimize the diffusional limitations across the fibers. The combination of other polymers with PVA or a different crosslinking method could reduce this apparent barrier for substrates and products. Using a polymer mixture maybe it would be possible to introduce some bigger porous along the fibers or weaker crosslinked areas that could be used as exchange points for ONPG and the resulting products. In order to verify the reproducibility of these results, another membrane was electrospun and submitted to the same activity assays conditions and the results are shown in Table 4.

Table 4 - Activity of immobilized β -Galactosidase (Free enzyme activity: 9.81x10⁻⁴ mmol min⁻¹/mg)

| Days | Immobilized β -Gal | | |
|------|---------------------------------------|---|--------------------------|
| | Activity (mmol min ⁻¹) | Specific Activity (mmol min ⁻¹ /mg) | Retained activity (%) |
| 5 | 7.70x10 ⁻⁶ | 3.03x10 ⁻⁵ | 3.09 |
| 6 | 7.18x10 ⁻⁶ | 2.81x10 ⁻⁵ | 2.86 |
| 8 | 5.70x10 ⁻⁶ | 2.22x10 ⁻⁵ | 2.26 |
| 11 | 4.04x10 ⁻⁶ | 1.56x10 ⁻⁵ | 1.59 |

These results confirmed a satisfactory reproducibility of the assays. Once more, the mat activity was much lower than the free form enzyme and the maximum retained activity was 3.09 % which is approximately the same from the preceding test.

From the tables above it is also possible to analyze the stability of the enzymes in the two assayed forms. It is obvious that the immobilization process clearly decreases the enzyme stability over the time, once the activity is significantly lower only 11 days after the moment of electrospinning. Probably it occurred some fiber degradation over the time that limited even more the interaction between β -Gal and ONPG resulting in great decay in activity retention. Another explanation is the simple protein denaturation during storage. However, free enzyme solution remained with high activity although a slow decrease in the percentage retention which shows that protein denaturation is only minimal at storage conditions. This way, PVA degradation is possibly the best explanation to these results.

Besides these results, another objective was established in this work. As referred in section 2.1.3. one of the most important advantages of immobilization is the enzyme reusability. This way, PVA/ β -Gal mat was tested over several reuse cycles. In Figure 41, it is shown the behavior of the two used mats when are reused. After the first reuse cycle, the activity had only a slightly decrease. With two more reuse cycles, the first used mat stabilized at approximately 75% of retained activity. On the other side, the second mat used had a lower activity retention. After one cycle, the activity only dropped to 95%, but with two more reuse cycles, it stabilized at 65%, which is slightly lower than the previous mat. However, both results testify that the nanofibrous membrane could be reused with a high percentage of retained activity. The decreased activity can be explained by some morphology loss which reduced porosity limiting even more material diffusion and also by some enzyme leakage. The accumulation of ONPG products (ONP and galactose) inside the nanofibrous membrane over the time may act as inhibitors for the enzyme, avoiding hydrolysis of new ONPG molecules.

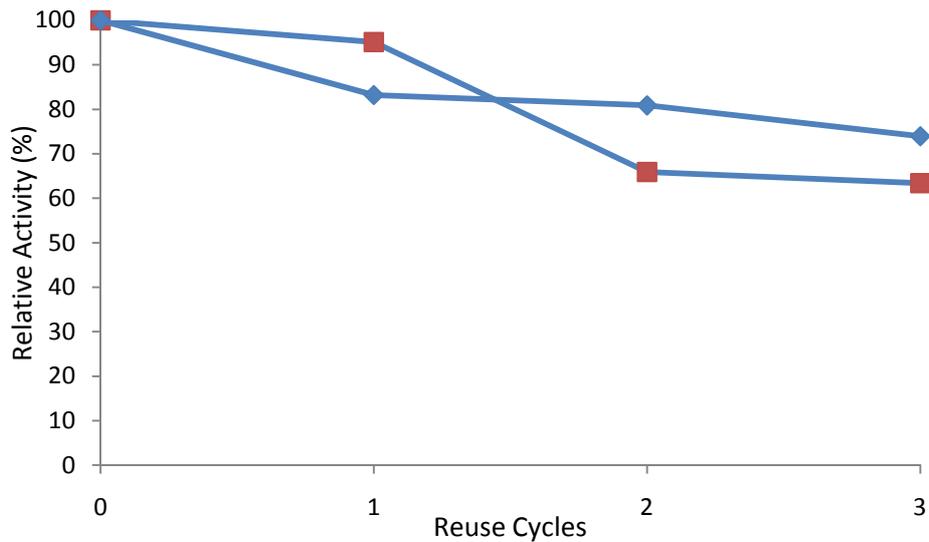


Figure 41 - Reusability of the two PVA/Gal mats tested.

2.4. Conclusion

In this chapter it is possible to conclude about two distinct parts of the work. In the first place, one can evaluate if there is any effect of the enzyme entrapment in PVA fibers morphology. The water resistance of the mat was also verified after enzyme immobilization which is highly relevant to the main purpose of this work. In second place, the performed activity assays with immobilized and free β -Gal allowed us to compare both enzyme forms, pointing advantages and disadvantages of the two methods. These results will be very helpful predicting and directing further studies on this field.

Regarding the enzyme immobilization, it was confirmed that β -Gal did not induce severe changes in the fiber morphology. Electrospinning was performed without any difficulty which is attested by the use of the same optimized processing parameters. The resulting PVA mat was composed of stable, non-flat and bead-free fibers, which is always the desirable result of any electrospinning process. However, it is worth to note that fiber average diameter had a small increase with the addition of β -Gal to PVA fibers. This increase was assumed to be exclusively related to protein aggregates within the fibers and not with any chemical change of the electrospinning solution, specifically viscosity and surface tension. β -Gal presence in the PVA mat did seem to have an effect on crosslinking reactions with PVA. The presence of the enzyme at the surface of the fibers, may limit GA-PVA hydroxyl groups bonding, which induced a high water uptake and fiber fusion and disintegration. Higher concentrations of glutaraldehyde overcome this problem,

drastically reducing water uptake. Thus, it was possible to have a PVA/ β -Gal water-stable mat which could be assayed in an aqueous medium.

The activity assays, were very important to understand the behaviour of an immobilized enzyme and its usability in catalytic processes. As expected, β -Gal revealed a high catalytic rate when in solution, hydrolyzing all available substrate in few minutes. In the immobilized form, several hours were needed to achieve a full hydrolysis. As could be seen, activity was highly affected by diffusional limitations, resulting in low activity retention. Stability of the immobilized form was also lower than for the free form. The immobilized enzyme tended to lose activity as more storage days were passed, while the free form had a more preserved activity. Although, these are concerning disadvantages that extremely limit any real application, it was possible to achieve a great result with PVA/ β -Gal mat reuse. After some cycles of reuse of the immobilized enzyme, it was possible to achieve an elevated retained activity, ranging from 60 to 70%. Since it is difficult and costly to recover any free enzyme after a catalytic procedure, this result points the use of immobilized β -Gal in nanofibers as an attractive model in the future to many industrial processes use nowadays. However, the balance between low activity, lower stability and a good reusability of the entrapped enzyme may still favour the use of the free form.

Final considerations

In a final overview, this thesis work allowed to understand how nanofibers are produced through electrospinning, the basic process fundamentals and how it can be affected by processing and solution parameters.

Through crosslinking reactions with glutaraldehyde, it was possible to render PVA nanofibers mat water resistant with a wide range of applications.

It was possible to understand the addition effect of a complex molecule as a protein, to a polymer in order to produce nanofibers through electrospinning.

It was studied the activity, stability and β -galactosidase behaviour when in immobilized and free form with interesting and important results which can potentiate many different applications in the future

Finally, some activity problems associated with enzyme immobilization were recognized which must be overpassed in order to produce efficiently nanofiber mats entrapping enzymes.

Future perspectives

In the future it will be of extremely importance to minimize the barriers that are limiting β -Galactosidase activity. This way, the use of other polymers such as chitosan or particles as silica with PVA could enhance nanofibrous porosity, augmenting diffusional transport across the fibers wall. Since only crosslinking with glutaraldehyde solution was effectively used, there must be tested other crosslinker agents. This may give us at least three advantageous informations. It will allow us to understand if glutaraldehyde is the best agent to render the PVA mat water-insoluble, if it is blocking enzyme activity and at the same time if it is possible to substitute it for a safer, non-toxic and food compatible agent.

Following the activity assays that were done, it would be relevant to study the kinetic parameters of the immobilized β -Gal, *i.e.*, the maximum velocity of the enzymatic reaction (V_m) and

the dissociation constant of the enzyme-substrate complex (K_m). Since all the assays that were done in this work used ONPG as substrate, it would be very interesting to repeat these and future studies using Galactose and Lactose as substrate.

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