UCLouvain

epl

École polytechnique de Louvain

Impact of some Layer-by-layer assembly conditions on the enzymatic activity of glucose oxidase immobilized in nanopores

Authors : **Steicy de Campos Poles**

Supervisors: **Christine DUPONT-GILLAIN**, **Sophie DEMOUSTIER-CHAMPAGNE**

Readers : **Arnaud DELCORTE**, **Christine DUPONT-GILLAIN**, **Delphine MAGNIN**, **Sophie DEMOUSTIER-CHAMPAGNE**

Academic year 2020–2021

DEDICATION

Eu gostaria de dedicar essa tese à minha família, especialmente para meus pais que sempre fizeram de tudo (e mais) para investir na minha educação e na dos meus irmãos.

Também gostaria de dedicar ela à minha avó Carminha, que eu desejaria que estivesse aqui para me ver concluir meus estudos. Sua doçura de ser continua presente nas nossas vidas.

ACKNOWLEDGMENTS

I would like to acknowledge and express my gratitude to my supervisors Christine Dupont-Gillain and Sophie Demoustier-Champagne, who made this work possible. With their knowledge and patience, they have advised and guided me through all the stages of my master thesis project and especially oriented me while I had doubts on the most optimal route for the experimental path, and its discussions for this master thesis dissertation. They were always comprehensive regarding my unskilled scientific background, for which they have constantly provided me constructive feedbacks and allowed me to improve myself while keeping me motivated. Additionally, both of them have inspired me on pursuing and increasing my knowledge in the biotechnology field through the courses they have lectured.

I would also like to thank the Post-doctorate Ievgen Kurylo who has patiently guided and encouraged me throughout the project. Especially thanking him for orienting me in the experimental work, which demands very delicate and precise work and those would have not been successful without his guidance.

Those people have been essential for the success of this thesis, which has made me learn and mature deeply as an engineer.

RESUME

Enzyme immobilization is a prosperous approach to enzymatic application because it enhances its *in vitro* performance by increasing stabilization, providing higher operational time, better control of the process, and enables its reuse. In specific, the elaboration of enzymatic nanotubes is especially interesting due to the creating of a nano-confinement environment (slightly mimicking the in vivo environment), the creating of a protective shell that will isolate the enzyme from direct contact with the medium, and the possibility of tailoring the nanotube's components and parameters. These nanotubes may be prepared by a template-assisted synthesis combined with a bottom-up Layer-by-layer (LBL) assembly technique to create nanotubes inside the pores of a nanoporous membrane. The LBL technique consists of depositing alternating layers of materials that are oppositely charged, in this project this is performed by immersing the deposition support (polycarbonate nanoporous membrane) in the solutions of the materials (weak polyelectrolytes (PE)).

The enzyme chosen to be immobilized in the assembled nanotubes was the Glucose Oxidase (GOx - negatively charged polyelectrolyte), this choice was mainly moved because it is a well-studied enzyme, with a high number of literature references and its 3D structure is well determined by science, and it was partially moved because of its importance in the industry and its vast fields of application. The second deposition material chosen was Branched-Polyethyleneimine (bPEI - positively charged polyelectrolyte), a choice motivated due to literature resources that prove that this polyelectrolyte combined with GOx, and LBL, will prevent the enzyme desorption.

The aim of this thesis is to analyze how the assembly parameters influence the enzymatic activity of the (bPEI/GOx) nanotubes and possibly finding a most optimal assembly setup to maximize the catalyst performance. Three parameters were chosen as the variables: PE assembly concentration, pore diameter of the membranes, and number of bilayers deposition. To measure the GOx activity of the samples (i.e., membrane containing nanotubes) a cascade reaction was performed between GOx and Peroxidase. Cascade reaction means that the product of the first reaction is reagent of the second reaction, in this case, the product of the second enzyme is a colored product, which color intensity (i.e., spectrometry absorbance) is measured because it is directly correlated with GOx's activity. The samples' activity measurement was performed in static (no flows) and dynamic (income and outcome flows) regimes, to obtain combined & complementary information on the GOx activity behavior.

Results have shown that, for static applications, the decrease of the pore diameter decreases the specific activity (i.e., activity per GOx mass) results, possibly due to diffusional restriction for the substrate to enter the pore. This result is no longer observed in the dynamic, possibly due to the flow. Regarding the PE influence, samples assembled with higher & even PE concentration (both GOx and PEI with same solution concentration) present higher specific activity results (static and dynamic) than samples assembled with lower & even PE concentrations. Regarding the bilayers parameter, for the static and dynamic applications the increase of bilayers has provoked a decrease of the specific activity for samples assembled with high & even PE concentrations and has provoked the contrary effect on samples assembled with low & even PE concentrations. The PE and bilayer influence responses are due to the same reason, samples assembled with high & even PE concentrations present layers with a high amount of GOx, this dense layer block the substrate access to the deeper GOx layers and will decrease the activity. For low & even PE concentrations the layers are less crowded, allowing the substrate to diffuse and increasing the total activity; the increase of the specific activity is possibly a response to the nanoconfinement effect and due to an enhanced GOx immobilization configuration (e.g., facilitating the contact between enzyme-substrate). Samples were also assembled with high & uneven PE concentration, however, the interpretation of its results was limited to hypothetical explanations.

This project has concluded that the assembly parameters indeed affect the specific activity of the immobilized enzymes and that the optimal setup to maximize enzyme activity may be achieved by the combination of parameter factors (just one factor is not sufficient). However, further studies and analysis are needed for the achievement of this maximum enzymatic activity due to assembly scenario.

TABLE OF CONTENT

Dedication II

Acknowledgments III

Resume IV

Table of content V

List of Abbreviations VIII

1. Introduction 1

1.1 Importance of enzymes and drawbacks 1

1.2 Why it is interesting to immobilize enzymes 1

1.3 Current approaches and limitations of enzyme immobilization 2

1.4 Thesis added value 3

2. Literature review 5

2.1 Layer by Layer technique (LBL) 5

2.1.1 Layer by layer: assembly types 5

2.1.2 Layer by layer: Interactions and parameters 6

2.1.3 Layer by layer in pores 9

2.2 Enzymes: Composition and structure 12

2.2.1 Chemical composition 12

2.2.2 Structure 12

2.3 Enzymes: Mechanisms and activity parameters 14

2.3.1 Reaction mechanism and types of reaction 14

2.3.2 Factors affecting the enzymatic reaction 16

2.4 Enzymes: Immobilization and confinement effect 19

2.4.1 Types of immobilization methods 19

2.4.2 *In vivo* and *in vitro* confinement effects 21

2.4.3 Immobilized Enzymes' activity and parameters 22

2.4.4 Immobilization in Layer by layer & Nanotubes 22

2.5 Glucose oxidase (GOx) and cascade reaction 23

2.5.1 Composition and structure 23

2.5.2 Characteristics 23

2.5.3 Enzymatic reaction mechanism 24

2.5.4 Cascade reaction with peroxidase and GOx activity measurement 25
3. Context, objectives, and strategy 26
4. Materials and methods 30
4.1 Materials 30
4.1.1 Buffer 30
4.1.2 Polyelectrolytes (PE) 30
4.1.3 Track-etched polycarbonate membranes (PCm) 30
4.1.4 Bicinchoninic acid (BCA) protein assay 30
4.1.5 POD mix solution 31
4.1.6 D-Glucose 31
4.2 Methods 32
4.2.1 Layer-by-layer templating build-up of nanotubes in porous membrane 32
4.2.2 Bicinchoninic acid (BCA) protein assay 33
4.2.3 Measurement of the enzyme's activity in static conditions 35
4.2.4 Measurement of the enzyme's activity in dynamic conditions 37
5. Results and discussion 40
5.1 Complementary study: Scanning electron microscopy (SEM) 40
5.2 Bicinchoninic acid (BCA) protein assay 41
5.2.1 BCA: Standards absorbance per enzyme concentration calibration curve 41
5.2.2 BCA: Equivalent GOx mass per area of sample and per total internal area results 43
5.2.3 BCA: Partial discussion of results 48
5.3 Measurement of the enzyme's activity in Static conditions 50
5.3.1 Static activity: Set 1 results 51
5.3.2 Static activity: Set 3 results 54
5.3.3 Static activity: Comparision set 1 & day 8 and set 3 results 55
5.3.4 Static activity: Partial discussion of results 56
5.4 Measurement of the enzyme's activity in dynamic conditions 59
5.4.1 Dynamic activity: Set 1 results 60
5.4.2 Dynamic activity: Set 2 results 64
5.4.3 Dynamic activity: Set 3 results 65
5.4.4 Dynamic activity: Comparison set 1, set 2, and set 3 results 66
5.4.5 Dynamic activity: Partial discussion of results 68

5.5 Discussion of results and Comparison of activity measurements results (Static and Dynamic) 71
6. Conclusion 79
7. References I
8. Appendix VIII
8.1 Enzymes: PKA of amino acids VIII
8.2 Layer-by-layer ilustration of techniques IX
8.3 Diffusional restriction that affect the activity X

LIST OF ABBREVIATIONS

BCA: Bicinchoninic acid protein assay.

BL: Bilayers, meaning one layer of glucose oxidase and one layer of branched-Polyethylenimine (e.g., 4BL = 4 bilayers).

bPEI or PEI: branched Polyethylenimine.

GOx: Glucose Oxidase enzyme.

LBL: Layer-by-Layer assembly.

PCM: Polycarbonate membrane.

PE: Polyelectrolyte (in this project case they are the GOx and PEI).

PODmix: Mixture of peroxidase, 4-Aminoantipyrine, and p-Hydroxybenzoic acid.

Sample: Polycarbonate membrane with embedded nanotubes

STDS: Standards

1. INTRODUCTION

1.1 IMPORTANCE OF ENZYMES AND DRAWBACKS

Enzymes are natural catalysts (i.e., biocatalyst) and their importance has been continuously growing in the past decades. This growth occurs not only due to its ability to enhance the reaction rate without disturbing the equilibrium reactant-product but also due to other factors such as: its enantio- and stereo-selectivity, the reactions' product purity, and the fact that they are ecofriendly (biodegradable and used with ecofriendly solvents such as water).

All those factors have contributed to the increase of their participation in the industry in the most diverse fields of application, for example: food, biofuel production, pharmaceuticals, cleaning products, disease diagnosis, chemical synthesis, animal feed, and so on (Cacicedo et al. (2019); Mohamad et al. (2015)). In 2020, the global enzyme market was valued at around 10 billion dollars and it is expected to reach 13.4 billion dollars by 2027 (Expert market research; ReportLinker (2021)).

However, this biocatalyst also presents downsides, such as: the elevated cost of its production and purification; its sensitivity to process conditions; its low stability; and its partial or full inhibition. Long-term operational stability, recovery, and enzyme reuse still are ongoing challenges to be achieved (Cacicedo et al. (2019); Mohamad et al. (2015)).

1.2 WHY IT IS INTERESTING TO IMMOBILIZE ENZYMES

Intensive studies and research are being performed by scientists all over the world on the topic of enzyme immobilization. This confinement technique is promoted with the aim of increasing the stability of the enzymes and their lifetime while trying to maintain their performance (Cacicedo et al. (2019)).

In addition, the immobilization also has the purpose of promoting: (1) higher operational time of the enzymes; (2) the re-use of the catalyst; (3) of simplifying the manipulation allowing better control of the reaction process (such as removing the catalyst to stop one reaction); (4) of decreasing product purification process (since the catalyst removal is facilitated); (5) prevention of microbial contamination (Cacicedo et al. (2019); Mohamad et al. (2015)); Guisan et a. (2020)).

In general, all these new improvements brought by the immobilization of the enzymes contribute to a total decrease of the industrial cost of bio-catalyzed reactions; and reduces the consumption of energy and chemicals while lowering waste generation (Cacicedo et al. (2019); Mohamad et al. (2015); Homaei et al. (2013)).

1.3 CURRENT APPROACHES AND LIMITATIONS OF ENZYME IMMOBILIZATION

Enzymes are immobilized by binding them onto surfaces or by encapsulation, this process may be done through physical methods or chemical methods. Both methods and their techniques are illustrated in Figure 1. In short, physical methods use physical forces to immobilize enzymes and chemical methods will do the immobilization through covalent or ionic bonds (Dwevedi (2016)).

All methods present advantages and drawbacks and the choice of method will depend on the desired application and on the enzyme chosen for this application. In general, the methods from the chemical category present more stability, however, they are usually more expensive and the chemical bond (especially the covalent bond) may lead to conformation changes and a decrease in enzymatic efficiency (Dwevedi (2016)).

The physical method may be performed by adsorption, entrapment, or microencapsulation techniques. Regarding the pros and cons, the entrapment and microencapsulated enzymes may present diffusional problems and it may be limited to certain types of enzymes; the adsorption method is the cheapest one and with no enzyme type specification but it is frequently reversible and may be sensitive to environmental conditions (Dwevedi (2016; Homaei et al. (2013)). Therefore the method and technique should be chosen carefully to match the experimental setup, its limitation and the applications of the enzyme. The techniques will be further discussed in the Literature Review Section 2.4.1 Types of immobilization methods.

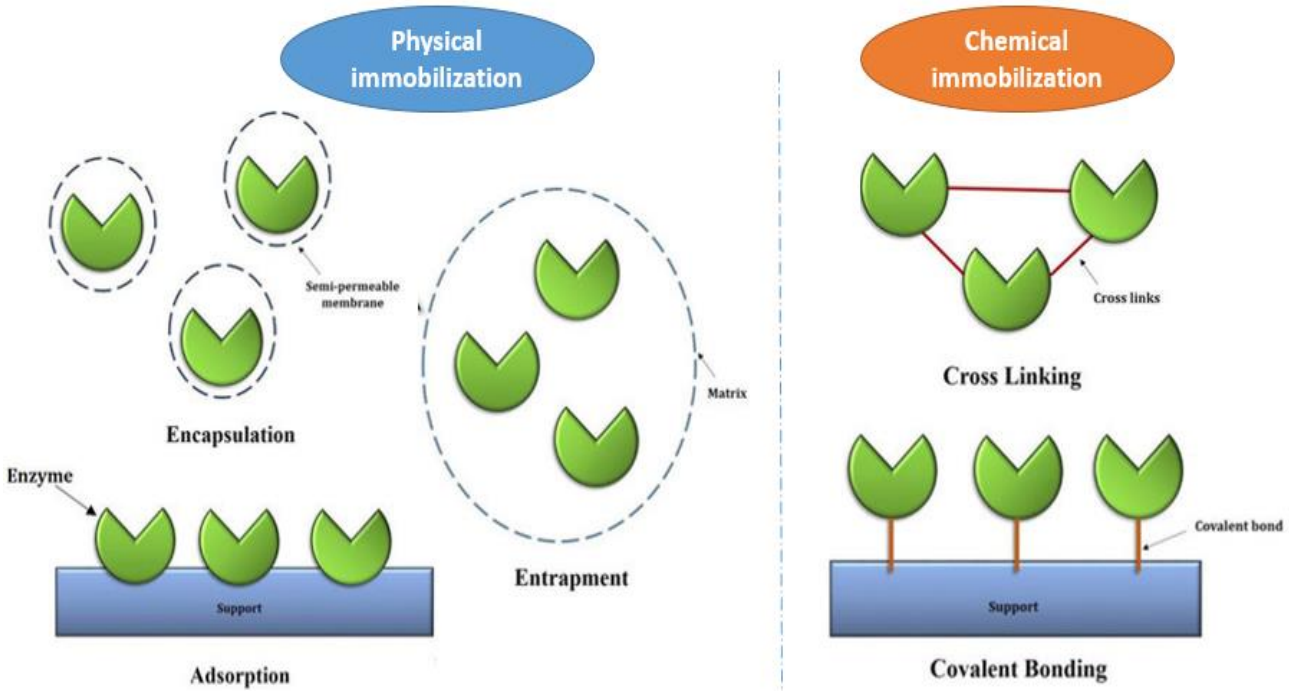


Figure 1: Techniques of enzyme immobilization (Modified from Jun et al. (2019)).

Before, the support (binding surface or inclusion matrix) was only used to insolubilize the enzymes and therefore help its separation and its reuse (Xie et al. (2009)). But, scientists have discovered that this attachment support may also influence the enzyme's performance. The supports' physical and chemical properties (e.g., size, shape, hydrophobicity, surface chemistry, and water attraction) have effects on enzyme's immobilization and on its catalytic properties (Xie et al. (2009)).

This discovery opened a whole new field of research regarding designing specific supports for various bio immobilization and separation procedures and also aiming at the optimization of the immobilized enzyme (quantity, stability & performance) (Xie et al. (2009)).

1.4 THESIS ADDED VALUE

For this thesis, the immobilization method chosen was the adsorption through electrostatic attraction. The technique applied was the Layer-by-Layer, which consists in alternatively depositing layers of enzyme and an oppositely charged material over a support (called polyelectrolytes).

A porous support was immersed in the aqueous solutions of the charged cationic and anionic solutions and in between deposition a rinsing step was performed to remove non-adsorbed excess. The adsorption inside the support's pores creates nanotubes containing the immobilized enzymes (Nguyen et al. (2017)).

This method and technique were chosen due to their high biocompatibility, its simplicity, and the possibility of changing the setup parameters (e.g., support's pore size, the concentration of the polyelectrolyte solutions, enzyme, and material) (Nguyen et al. (2017)).

The materials chosen as aqueous solutions were Glucose Oxidase (GOx) enzyme and Polyethylenimine (PEI), and the porous support is a polycarbonate membrane (PCM).

The purpose of this master thesis is to study how changes in the nanotube parameters will affect the performance (i.e., activity) of the glucose oxidase immobilized enzyme. The parameters chosen were: pore size of the nanotubes, concentration of the polyelectrolyte's solutions, and amount of nanotube's layers (more precisely, amount of bilayers GOX-PEI). An illustration of the nanotubes and the three affected parameters may be found in Figure 2.

This thesis is part of Ievgen Kurylo Post-Doctorate thesis on "Nano-confinement effects on the enzymatic activity of bioactive layer-by-layer assemblies in nano-pores". His work focuses on the enzyme's activity in static condition reaction (i.e., the reaction's reactants and the catalyst are added at the same time and place and the product accumulates over time until total reagent consumption) and this master thesis also analyses the the activity effects in dynamic conditions reaction (i.e., the reactant is a constant flow in contact with the enzyme, and the product is collected throughout the reaction's time).

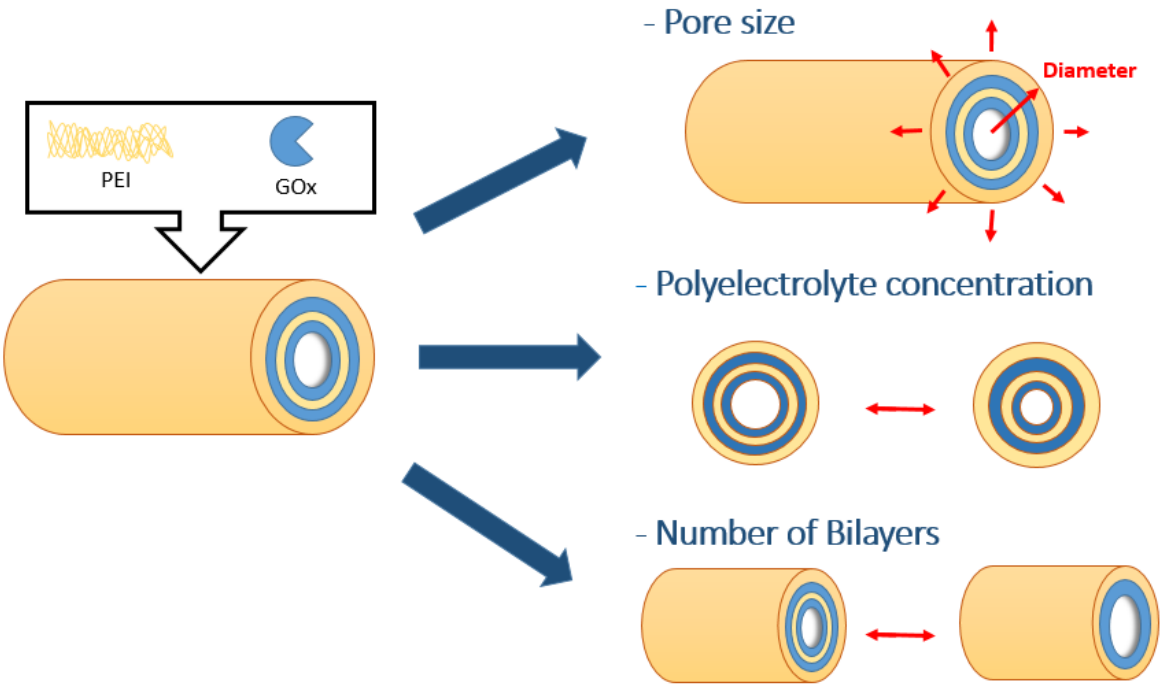


Figure 2: An example of a nanotube GOX-PEI and the parameters that will be varied in order to better understand their effect on the catalytic reaction's efficiency.

2. LITERATURE REVIEW

2.1 LAYER BY LAYER TECHNIQUE (LBL)

The layer-by-layer technique enables the production of multilayer samples by alternatively depositing positively and negatively charged particles in the form of layers. It has many advantages, such as simplicity; possibility to coat independently of size or shape of support; creating multilayer system with tailored design and properties (order, size, composition, stability, surface, porosity, and others) (Zhang and al. (2007); Johnston et al. (2006)).

The assembly demands two types of materials: the *substrate (or support)* where the deposition will be performed on; and the *layering material.* The substrate may be present in several forms such as a particle, surface, membrane, or fibers; and the *layer material* may be a protein, DNA, lipids, polymers, nanoparticles, and others (Richardson et al. (2015)).

2.1.1 LAYER BY LAYER: ASSEMBLY TYPES

Although assembly technique was first discovered in 1966 by Iler, it only began to be implemented in the 1990s when it was fully supported by other layer characterization techniques that determined the amount of substance adsorbed (e.g., quartz crystal microbalance) or provided structural information of the deposition (e.g., energy transfer, neutron reflectivity, X-Ray). (Zhang and al. (2007); Richardson et al. (2016); Decher (1998)).

Afterward, the technique was constantly developed until present applications, which may be divided into three categories: conventional; unconventional, and quasi-LBL (Richardson et al. (2016)). Only the first type is relevant for this work and will be discussed.

The conventional category may be performed in five main assembly techniques: immersive, spin, spray, fluidic, and electromagnetic assembly. A schematic representation of all those techniques may be found in Appendix 8.2 Layer-by-layer ilustration of techniques.

The *spin* technique coats a spinning substrate, which facilitates the coating and drying. The *spraying* type does the layers deposition using a spray. The *fluidic* case deposits the layers by the means of fluidic channels/capillarity. The *electromagnetic* assembly performs the coating by electro-deposition using a magnetic driving force (Richardson et al. (2016)).

This thesis's samples were prepared by the *immersive* assembly, which consists of manually and alternatively immersing the substrate into the oppositely charged layering materials solutions. In between these deposition steps, washing is performed to remove the adhering solution not adsorbed. The assembly is performed with high concentration polyelectrolytes solutions which will be responsible for the reversing of the surface's charge. A scheme of the immersive LBL process may be seen in Figure 3.

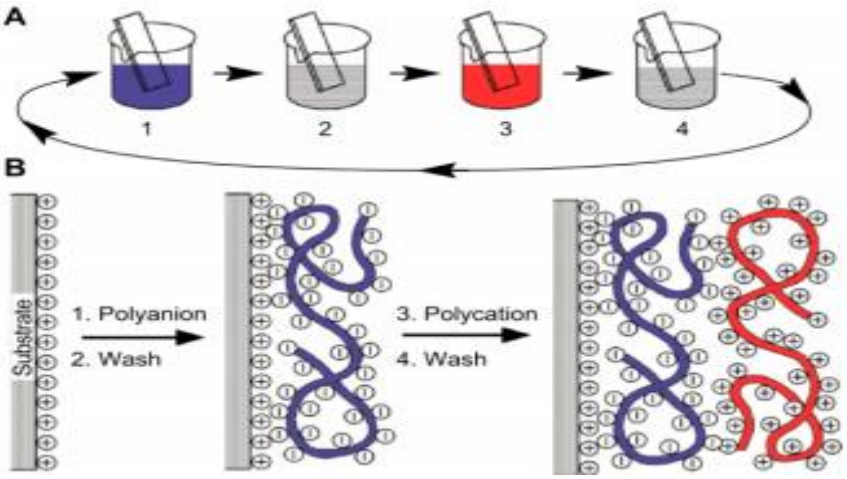


Figure 3: Schematic representation of the steps perormed in an immersive Layer-by-layer assembly. The charged planar substrate is immersed in the opposite charged polymer solutions, alternately. In between steps, washing is performed. (Modified from Richardson et al. (2016)).

In the Figure 3 example, a positively charged substrate is immersed in a high concentration solution of polyelectrolyte negatively charged (polyanion). With the immersion the negative components deposit on the substrate forming the first layer. The washing is then performed to eliminate not adsorbed particles. The surface becomes negatively charged. Following, the process is continued by the adsorption of a positive layer by immersing the sample in the concentrated polycation solution, positively charging the surface, and forming the first bilayer (Zhang and al. (2007); Richardson et al. (2016); Lvov et al. (1995)).

2.1.2 LAYER BY LAYER: INTERACTIONS AND PARAMETERS

For the case of immersive LBL assembly, the interaction between layers occurs by three main forces, the most relevant one is the electrostatic interaction. But layers may also be assembled based on the other two forces: hydrogen bonding (in case of presence of hydrogen bond acceptor and donor) or by covalent linkage (usually done to increase stability). (Zhang and al. (2007)).

The electrostatic interaction is based on the attraction of the two opposite charges of the assembled polyelectrolytes. This force does not require the exact matching position of the groups and allows different building layers to be adsorbed. Due to X-Ray analysis, it was also possible to confirm that the layers are interpenetrated to some extent (Zhang and al. (2007)).

Therefore, the assembly highly depends on the electrostatic attraction of the polyelectrolytes, which depends on their charge density and deposited amount of PE (Elzbieciak et al. (2009)). Those two may be affected by environmental conditions, such as: pH of solutions; nature of solvent; ionic strength; adsorption time, and charge density. Besides, they may be manipulated to tailor the physic LBL structure in thickness, roughness, swelling, and others (Borges et al. (2014); Zhang and al. (2007)).

2.1.2.1 EFFECT OF PH, IONIC STRENGTH, AND TYPE OF POLYELECTROLYTES

The three parameters are presented together because they mutually affect each other. For starters, the choice of polyelectrolyte should be design so that both weak polyelectrolytes[1] have opposite charges in the set experimental conditions.

Secondly, each one of the weak polyelectrolytes presents its own acid dissociation constant (pKa) value and each PE solution has its own ionic strength. Here is how they are defined:

1. The pKa measures how strongly a proton is being held to a functional group (i.e the strength of a Bronsted acid) in an aqueous solution. A small pKa means that the acid donates protons easily in an aqueous solution and therefore will dissociate into its ions. (Chemistry LibreTexts – pKa values(2019); Helmenstine (2020); ChemTalk).
    - When the pH is higher than the pKa the functional group is majorly deprotonated, and pH is lower the group is majorly protonated. So, the operational pH and species should be chosen carefully to correspond to the formation of both polycation and polyanion PEs (Chung et al. (2002); Chemistry LibreTexts (2019)– Distinguish between pH and pKa (2019)).).
    - Specifically for protein, the pH chosen should take into consideration the pKa of all the amino acid functional groups (amino, carboxyl, and side-chain) in order to obtain the desired final charge.

2. The ionic strength is the measurement of the ions' concentration in a solution (Eun (1996)).
    - It is possible to add salts in the solution to change the ionic strength and therefore changing the LBL assembly. The added salt creates a shied screen over the deposited PE and may lead to a more coiled PE conformation increasing the thickness of the bilayers (Zhang et al. (2017)). In this project, this parameter will not be explored.

As mentioned, the pH has control over the protonation degree of a solution. This regulates charge density along the chain and will also influence the conformation and interactions of the deposited material (Elzbieciak et al. 2009). Additionally, the protonation degree also influences the repulsion between specifies and therefore on the arrangement of the layer (straight or coil form) (Tang et al. (2006)).

[1] Weak polyelectrolyte is a specie that contains weak acid and basic groups and which ionization varies with the environment. Strong polyelectrolytes contain strong groups and the ionization is fixed at the time of synthesis. (Nová (2017)).

Mendelsohn and al. performed studies of the pH influence on the weak polyelectrolytes PAA (poly acrylic acid) and PAH (poly allylamine hydrochloride) on a Layer-by-Layer assembly. In the study, the pKa value of the polyanion PAA is 5.0 and for the polycation PAH it is 9.0. It was observed that when both polymers are fully charged the layers are flat due to the strong repulsion. And at a pH where the components are partially charged the layers will have a more loop conformation, forming thick layers with internal charge pairing. These two results regarding the flat and loop conformation may be seen in Figure 4.

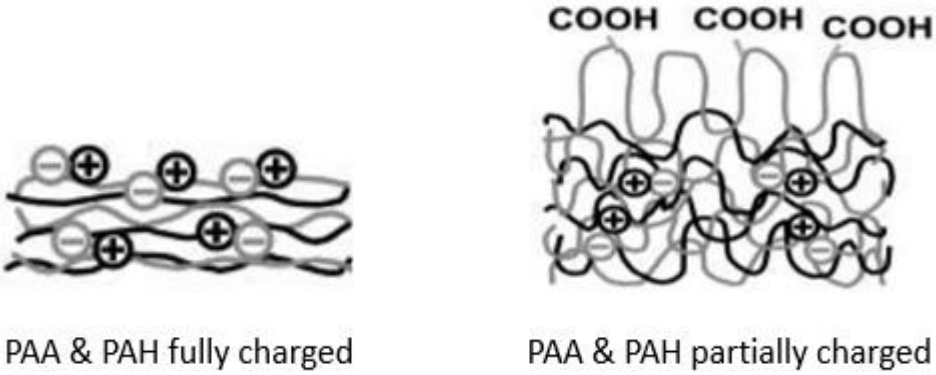


Figure 4: Schematic representation of how the bilayer profile changes with pH and the degree of ionization of the weak polyelectrolytes (Modified from Mendelsohn et al. (2003)).

In conclusion, it is possible to understand why in this thesis project it is important to choose a pH where both components used are correctly charged and why this pH should be constant for all the experiments in order to not change the charge of the functional groups or the profile of the layers (i.e., coil or flat).

2.1.2.2 EFFECT OF NUMBER OF BILAYERS AND POLYELECTROLYTE'S CONCENTRATION

Garg et al. (2008) studied the effect of varying the concentration of both PE in a LBL assembly performed on a quartz crystal coated with silica forming films. The PE chosen were: PAH (poly(allylamine hydrochloride)) and the sodium salt of the polymeric dye PCBS. The assembly was performed at PE concentrations 1, 5, and 10 mM, and afterward, the amount of adsorbed material was measured through three techniques: quartz crystal microbalance with monitoring of dissipation (QCM-D), ellipsometry measurements, and absorbance.

The results of their experiments showed that the increase of PE concentration from 1 to 5 mM has increased the thickness per bilayer and the amount of both polymer and PAH adsorbed per layer. However, an increase of concentration from 5 to 10 mM doesn't provoke big changes in results, suggesting that there exists an adsorption saturation limit (such as the adsorption isotherm plateau).

In their study, they have also mentioned that the increase of the number of bilayers does not necessarily have a linear growth effect on the thickness since it may be influenced by poor solvent quality (e.g., mixing water solvent with ethanol), desorption of PEs out of the formed bilayer and due to insufficient rinsing. And they believe that the degree of charge of the species (and therefore charge reversibility of LBL) directly impacts the stability of the formation of the films and therefore the maximum number of homogeneous bilayers. For example, when both PEs are fully charged they have a flat conformation (see Figure 4) making it possible to form stable homogeneous layers of more than 600 bilayers. (Poptoshev et al. (2004); Garg et al. (2008))

However, when only one PE concentration is changed the assembly will occur differently than if both PE solutions' concentrations are equally altered. Ali et al. (2010) studied the influence of keeping one PE concentration constant (0.01M) and varying the other PE concentration (from 0.0001M to 0.1M), and repeated this test for both PEs. For both PEs a curve of adsorption per PE concentration was found and it was observed the existence of an adsorption peak (i.e., concentration in which this PE will have a higher adsorption rate) and after this peak, the increase of concentration did not alter the amount of PE adsorbed. In other words, if one PE concentration is kept constant and the other PE concentration is located at the same place of this adsorption peak this would result in a huge difference of adsorption between the two PEs and therefore the LBL would have drastically different results than the case where both PEs concentrations are varied equally. Therefore, varying one concentration and not the other may bring unexpected results if this adsorption x variation of concentration curve is unknown.

2.1.2.3 EFFECT OF TEMPERATURE

Shi et al. (2003) investigated how the temperature affects the LBL assembly of multilayers films made of PDDA (poly(dimethyldiallyl ammonium chloride)) and PSS (poly(styrenesulfonate)). They have discovered that, for their project, both layers' thickness increase with the increase of temperature possibly due to factors such as: effects on polymer conformation; changes in electrostatic interaction, and dehydration (that may cause aggregation of the polymers). Therefore, to avoid temperature influence on the LBL assembly, the best option is to always perform the assembly in the same conditions of temperature.

2.1.3 LAYER BY LAYER IN PORES

The LBL may be performed in different types of geometries and architectures. Nanotubes may be assembled by combining the LBL with pored membranes used as substrate (usually composed of alumina or polycarbonate) and afterward, the membrane may be dissolved to release the tubes, this whole process may be seen in Figure 5.

These nanotubes have applications in fields such as biosensors, disease treatments, enzymatic bio-reactors, bio-separators, or more complex stimuli-responsive nanotubes (Tian et al. (2006); He et al. (2009).

The major advantage of this technique is the possibility to create nanotubes while controlling their composition and dimensions, as per example the diameter (since the external diameter is equal to pore size) and its length (equal to the thickness of the template). However, some other dimensions are harder to control, as for the wall thickness and the inside diameter of the tubes (Hou et al. (2004)). The composition and dimensions may be tailored for specifically designed application or possibly to alter the tubes' performance (such as an enzymatic activity that will be investigated in this thesis)

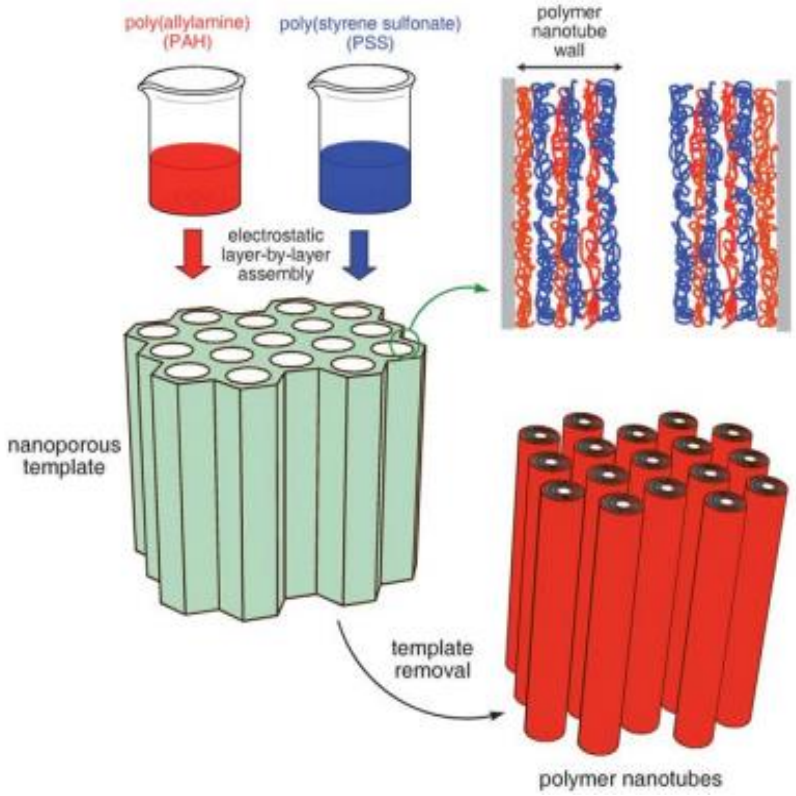


Figure 5: Representation of the bottom-up formation of nanotubes using the Layer-by-Layer technique based on electrostatic interactions. The assembly is followed by template removal and release of the nanotubes (Retrieved from Azzaroni et al. (2011)).

The environment parameters that affect the LBL assembly on planar structures (such as pH, polyelectrolyte, ionic strength, and temperature) also influence the formation of nanotubes, but in a slightly different way. In fact, Roy et al. (2010) created LBL multilayer nanotubes using Sodium poly(styrene sulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) and they investigated how the pore diameter, molar mass of PEs, and solution's ionic strength can affect the mechanism of multilayer growth regarding the layers' thickness, the diffusion rate of PE in the pores and the interaction of the layers with the pore surface.

The results of their experiences have shown that the LBL buildup in nanotubes may be observed in two different regimes:

a) **Regime 1:** if the pore radius is large when compared to the hydrodynamic radius of the polymer (i.e. the size of the PE chain in solution), the PE diffusion inside the pore is not a parameter that limits the buildup and the assembly will take place in the pore walls just like it occurs on flat surfaces. And just as for the LBL on flat surfaces, for nanopores the layer grows linearly and the layer thickness will also increase with the increase of the ionic strength of the PE solution (due to a more coiled conformation of the layers).
b) **Regime 2:** if the pore radius is in the same order of magnitude hydrodynamic radius of the PE, the diffusion of the PE in the pore is a limiting parameter (presenting slower kinetics). In this regime, the pore is almost completely filled by the PEs adsorbed and the confinement leads the PE chains to entangle (spider-web-like structure), leading to the formation of a dense gel structure (Roy et al. (2010)).

Additionally, it was also observed that the formation of the second regime mainly depends on the initial pore diameter. It is possible to have the second regime in larger pores when increasing both ionic strength and molar mass, which will cause an increase of the hydrodynamic radius. Alem et al. (2007) have suggested that this non-direct correlation with the ionic strength or molar mass indicates that the polymer chains entangle and form the gel.

One last important observation to be mentioned is that it is known that PE swells when they are in solution (around 20 to 40%), the assembly is performed in this swell state and the drying state will cause a shrinking, this effect may be seen in Figure 6. Specifically for the second regime, the drying causes the gel to collapse in a tube of wall thickness proportional to the pore diameter (Roy et al. (2010); Alem et al. (2007)).

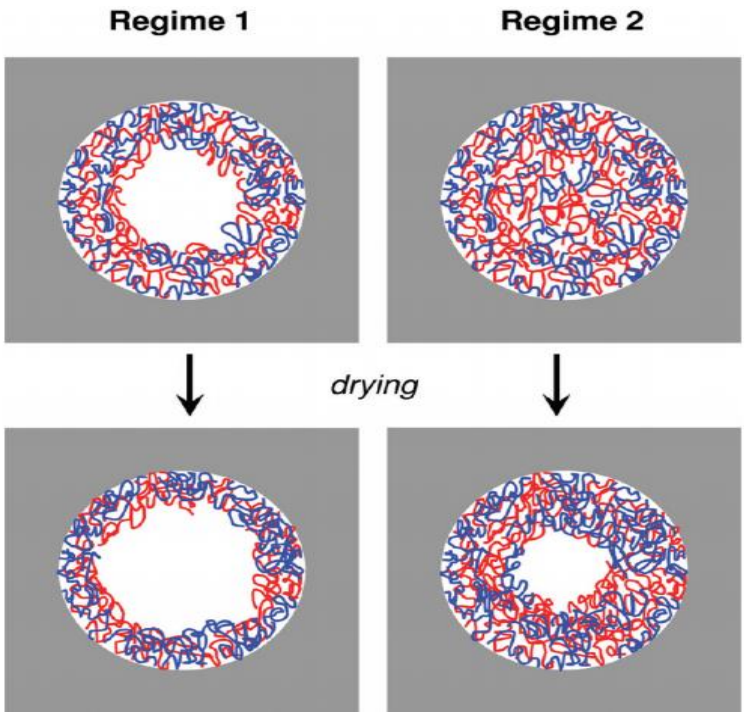


Figure 6: Schematic representation of multilayer LBL buildup for the regime 1 and 2 before and after drying (Retrieved from Roy et al. (2010)).

2.2 ENZYMES: COMPOSITION AND STRUCTURE

Enzymes are biological catalysts that generally consist of globular proteins. Like any other protein, they are organized in structures: primary, secondary, tertiary, and quaternary. The composition of the amino acid units influences the 3-D structure of the enzyme due to the bonding throughout the main chain (protein backbone) and due to its side chain. The folding and obtaining of the 3D form of the enzyme are done spontaneously.

The elaboration of this discussion was based on the 3rd chapter of the book "Enzymes: A practical introduction to structure, mechanism, and Data Analysis" by Copeland, R. (2000).

2.2.1 CHEMICAL COMPOSITION

Protein enzymes may be composed of 20 types of occurring amino acids. These units may be linked and form the polypeptide chain, a connection responsible for creating the protein backbone. The chemical formula of those amino acid units is illustrated in Figure 7.

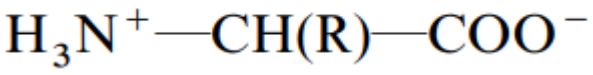


Figure 7: General chemical formula of amino acids. From left to right, four groups are present: amine group; chiral carbon; side-chain; and carboxyl group (Retrieved from Copeland (2000)).

The R represents the side chain of the amino acid unit; the central carbon is known as "alpha carbon" and since it is a chiral center there exist two enantiomer forms (L and D). The peptide bond bonds the units by condensing two amino acids (bonding one amino and carboxyl group) and liberating one molecule of water, the process is illustrated in Figure 8.

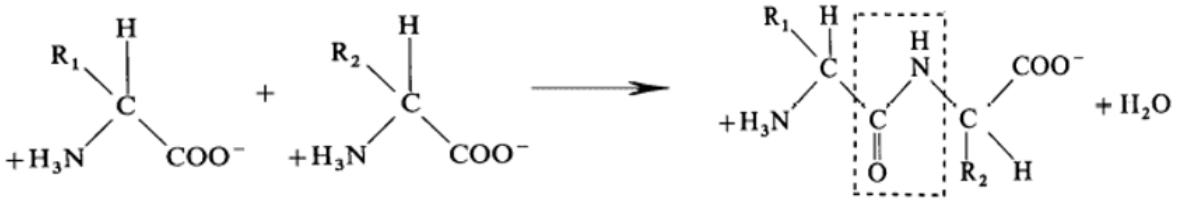


Figure 8: Representation of the peptide bond condensation mechanism. (Modified from Copeland (2000)).

2.2.2 STRUCTURE

The *primary structure* of a protein is the arrangement order of the amino acids in the polypeptide chain. The peptide bond neutralizes the amino acids terminals during the condensation and only the side chains and the outermost terminals remain charged: the amino-terminus, and the carboxy-terminus. The amino acids are distinguished through their side chains and their location may be numbered (starting from N-terminus). The *secondary structure* refers to the organization of protein domains into two different possible structures: the α-helices (coiling) and β-pleated sheets (stretched strands).

The *tertiary structure* refers to the 3-D shape of the protein. The protein backbone and its amino acid units interact in diverse ways. The main interaction is through the side chain, which hydrophobicity may affect folding and packing (due to repulsion, size, and shape of side chain) and may promote protein stabilization through bonding (e.g., hydrogen, covalent, salt bridge). The hydrophobicity acts on the folding by clustering non-polar molecules that fold to shield from the polar solvent, minimize repulsion and to obtain a less energetic structure, and possibly forming an active center. The arrangement of the amino acids in the active site defines its topology concerning hydrophobicity, electrostatic character, and stereochemistry.

The *quaternary structure* refers to the organization of the folded enzyme in subunits. These polypeptide folded subunits are associated mostly by non-covalent bonds and may have two different purposes: being an active site (or binding pocket, interacts with the substrate) or helping on the structure's stabilization. Denaturation of the enzyme is the loss of activity caused by the loss of its native conformation, for poly-chain, it is mainly due to the quaternary active site deformation, for single-chain polymers this occurs in the tertiary structure.

Figure 9 illustrates the four protein structures. Other non-protein chemical groups (cofactors) may also be incorporated in the enzyme's active site and perform roles for the protein (e.g., oxi-reduction at the active site). The protein is a small and detailed 3D molecule, it may be observed through (Philips 1966), new NMR, or magnetization transfer methods.

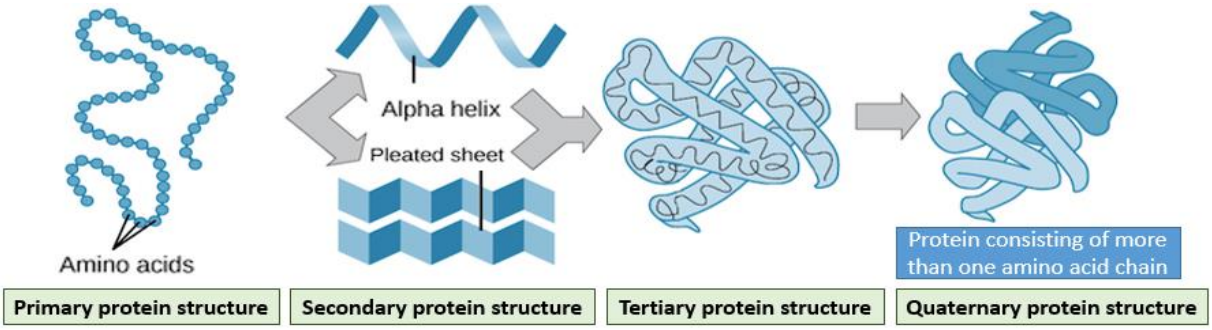


Figure 9: Protein structures (Modified from Clark et al. (2018)).

2.2.2.1 ENZYMATIC STRUCTURE STABILITY TO AVOID DENATURATION

The enzyme structure stability depends on diverse factors, such as pH, temperature, ionic strength, and composition of their solution. The optimal stability conditions of the protein are not necessarily equal to the ones that optimize its activity.

For stability reasons, the enzymes' storage should be done at -70ºC for long-term cases or at a conventional freezer -20ºC if diluted in 50% glycerol solution. Re-freezing cycles are discommended. The enzyme can be stably stored at 4ºC for several days as long as it does not stay a long period and that is conserved in a small volume with high concentration conditions.

Regarding enzyme inactivation during activity essays, it may occur due to the instability of the protein in the present conditions or due to spontaneous inactivation resulted from a catalytic turnover (by covalent adduct formation or by the destruction of binding active site).

2.3 ENZYMES: MECHANISMS AND ACTIVITY PARAMETERS

Enzymes perform catalysis, which has two main key features: accelerating the reactions' rate by lowering the reaction's activation energy and acting on specific molecules, and producing specific products. The catalysis takes place in the "substrate-binding cavity".

The kinetics of the reaction will not be discussed since the mechanism is in focus. The production of this topic was fully based on chapters four to seven of the book "Enzymes: A practical introduction to structure, mechanism, and Data Analysis" by Copeland, R. (2000).

2.3.1 REACTION MECHANISM AND TYPES OF REACTION

The enzymatic reaction occurs in four steps. It is important to recall that the enzyme "facilitates" the reaction but it is not a substrate, meaning that at the end of the reaction the enzyme is recovered with the product. The general mechanism steps of the enzymatic reactions may be found in **Figure 10**, however, it is important to mention that there exist some more specific equations to represent specific types of reactions (e.g., Michaelis–Menten representing single-substrate reactions).

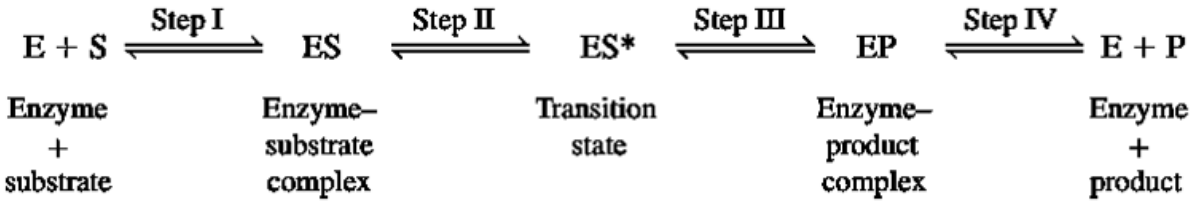


Figure 10: Schematic representation of the enzyme catalysis reaction. The E represents the enzyme, the S the substrate, and the P the product. (Modified from Denniston et al. (2020)).

The **first step** of the catalysis is the binding of the enzyme's active site with the substrate. This results in a reversible formation of the enzyme-substrate binary complex ES (also called Michaelis complex). The binding occurs by non-covalent forces (such as hydrogen bonding, Van der Waals interactions, electrostatic interactions, hydrophobic forces, or others) and it depends on the relative affinity between the two substances.

The stronger the affinity/strength of interaction the stronger is the binding and the lower is the dissociations constants. This last one may be correlated to the Gibbs free energy of binding. This affinity is usually high and therefore an excess of substrate concentration is demanded so the reaction occurs freely.

The 3D binding may be imagined as a key fitting into a lock, and for that to occur the two substances must complement themselves in the matters of: stereo-chemical fitting, being electrostatically complementary (avoid repulsion), and in the arrangements of hydrophobicity and hydrogen bonding interactions (to enhance binding). The attachment occurs by a minimum of three contact points, allowing the enzyme to be stereo-, regio-, and enantio-selective.

It is also worth mentioning that an enzyme may present multiple binding sites (i.e., more than one pocket available to the ligand) which may behave independently or dependently and they may also present different affinities to the ligand.

After the formation of the ES binary complex the **second step** begins, which consists of forming the stabilized enzyme-transition binary complex ES‡, this stable transition state consequently lowers the energy barrier, allowing the enzymatic reaction to be more favorable than an un-catalyzed one. On the other hand, the non-catalyzed reaction requires the formation of the transition state (without a transition binary complex) and this step is the main energetic barrier for obtaining the product. This required activation energy may be calculated using the Arrhenius equation (taking the temperature into consideration).

This lowering of the energy barrier may be done by mechanisms of five major types: (1) *bringing the reactants closer*, for the case where there is a binding of the participant substrates in the active site; (2) *covalent catalysis*, where there is a covalent intermediate between the substrate and enzyme; (3) *acid/base catalysis* acting as Bronsted-Lowry acid and base; (4) by adjustments on the *enzyme's conformation*; (5) by having a *pre-organized active site* that is fit to the substrate in its transition state conformation.

Following this non-spontaneous second step, the next two final steps occur spontaneously (i.e., negative Gibbs free energy variation). The **third step** of the mechanism is the transition of the stabilized enzyme-transition binary complex ES‡ to the enzyme-product complex EP. The final **fourth step** is the release of the product and the enzyme's re-availability.

If the reaction arrives at a state where the rate of formation of ES is the same as the generation of the products this state is the "steady-state phase". For example, in this period is possible to kinetically measure activity and study substrate specificity (Lorsch (2014)).

Figure 11 represents the whole enzymatic reaction in terms of free energy.

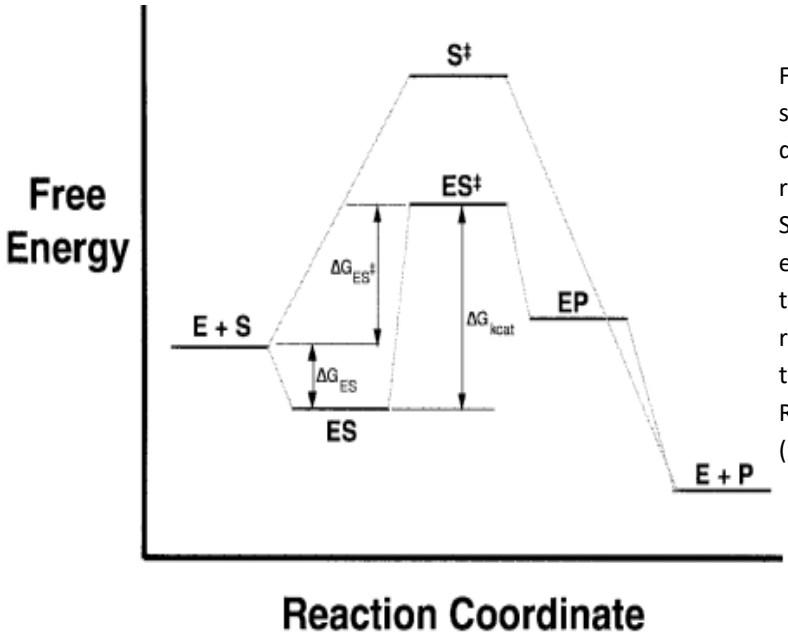


Figure 11: Enzyme catalysis reaction steps represented by an energy level diagram. The un-catalyzed reaction is represented by: E+S > S‡ > E+P. Spontaneous steps have negative free energy variation. It is noticeable that the activation energy to the catalyzed reaction (ES to ES‡) is smaller than for the un-catalyzed reaction (E+S to S‡). Reason why the first one occurs faster. (Retrieved from Copeland (2000)).

2.3.2 FACTORS AFFECTING THE ENZYMATIC REACTION

The activity of enzymes depends on their folding, for which structure stability depends on several other factors such as temperature and pH. Not only should the conditions of the parameters be chosen in accordance with the enzyme's optimum stable state (non-denaturation) but also be chosen to match the enzyme's optimum operation state (activity efficiency). The enzymes' activity is measured in the *standard international unit (IU),* corresponding to the amount of enzyme required to catalyze one micromole of the substrate per minute. The specific activity of an enzyme corresponds to the number of activity per milligram of the protein under specific solution conditions.

2.3.2.1 EFFECT OF SUBSTRATE CONCENTRATION

The product appearance curve is a mirror of the substrate depletion curve. The obtained product curve may be divided into three parts: *at high substrate concentration* the product curve grows without dependence of substrate concentration; *in the middle part* the velocity of reaction depends on this concentration in a curvilinear form; and finally, at low substrate concentration the product finds its stabilization due to its substrate dependency. Both substrate depletion and product production curves may be found in Figure 12.

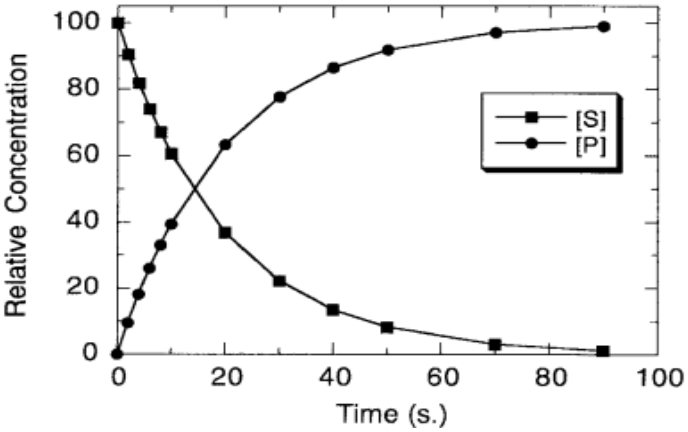


Figure 12: Curves of product appearance and loss of substrate concentration during the time of the enzymatic reaction (Retrieved from Copeland (2000)).

To optimize the enzyme's operation one should work with a very high concentration of substrate so the conversion advances freely without the jeopardizing caused by the reduction of substrate. In this case, all the enzymes are present in the form of ES complex therefore the reaction rate depends on the rate of conversion of ES into EP and the product release.

2.3.2.2 EFFECT OF ENZYME CONCENTRATION

Since the reactions are performed in high substrate concentration conditions, the product appearance is proportional to the concentration of enzyme. Therefore, an increase in the enzyme's quantity results in a linearly proportional increase of the reaction's rate. The increase takes place until the point where substrate depletion becomes significant, after this point the increase of enzyme is no longer in linear proportion to the reaction rate.

2.3.2.3 EFFECT OF PH

The pH to which the enzymes are submitted may provoke a deformation of the protein. Therefore, depending on the deformation degree, the enzyme may lose its ability to bind with substrate and may become inactive (denaturation). Most enzymes have a stable structure at the solution's pH around 7.4 with a range of 4 to 5 pH units (e.g., pH 5.4 to 9.4). But even inside this stable conformation range, another pH factor may still affect activity: the pKa.

The pKa value is related to the acid dissociation constant of a substance. In a pH lower than the pKa the substance becomes protonated. Amino acids have three chargeable groups: N-term (with pKa around 9.5), C-term (with pKa around 2.15[2]), and side chains (Nelson et al. (2001)). Therefore, pH changes may affect the protonation of the three groups.

Often, enzymatic reactions begin with the transfer of a protein proton to the substrate. And generally, throughout the reaction, a nucleophilic attack on the substrate or the stabilization of an intermediate positively charged occurs. For those to occur the proton donor and the stabilizing protein (catalytic nucleophile) should be protonated. Therefore, in that case, the external pH value must be chosen in a way that is in agreement with the pKa of the proton donor and the pKa of the catalytic nucleophile (Nielsen et al. (2003)).

The enzymes are usually diluted in buffer, a solution that resists change in pH. The adequate buffer is chosen mainly in accordance with the pH range corresponding to the stable state (stable structure) of the enzyme. For example, in this thesis the buffer used is MES which pKa value is 6.15 at 25ºC, the buffer will provide good buffering capacity according to the MES' pKA and therefore between pH 5.15 and 7.15, which is perfect to match the glucose oxidase's optimal pH value 5.75 (pH to avoid denaturation) (Singh et al. (2013)).

For any enzyme, it is possible to obtain the profile curve of reaction's velocity varying with pH (represented in Figure 13). The velocity depends on the optimal enzyme's pH range.

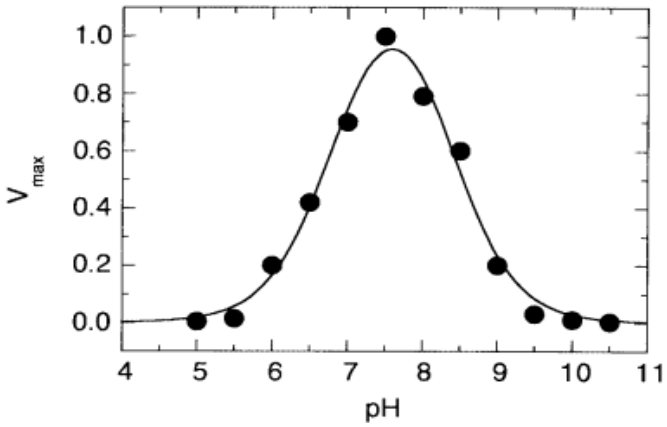


Figure 13: Velocity of reaction in function of the pH for a typical enzymatic reaction (Retrieved from Copeland (2000)).

[2] pKa values of amino and carboxyl group were obtained by calculating the average pKa value of the groups based on the table of the book "Principles of Biochemistry", the full values may be found in the appendix.

2.3.2.4 EFFECT OF TEMPERATURE

There is a widely known chemical rule that an increase of 10ºC in temperature doubles the rate of a reaction (demonstrated by the Arrhenius equation). Part of this enhancement theory may also be applied to the case of enzymes. However, another factor that interferes with this is the possible denaturation of this substance caused by the increase in temperature. Both factors determine the optimum temperature of the enzymatic reaction (for stability and for operation).

Most experiments are conducted at 25 or 37ºC (since it is the physiological temperature). By measuring the variation of activity per temperature change it is possible to obtain the optimal curve just as it may be observed in Figure 14.

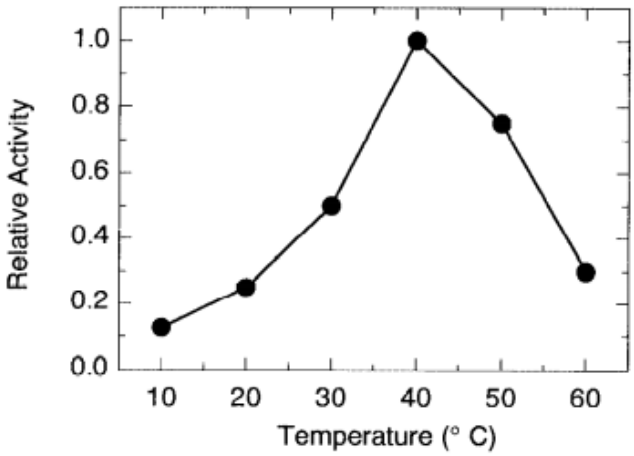


Figure 14: Typical curve profile of enzyme activity as a function of temperature (Retrieved from Copeland (2000)).

2.4 ENZYMES: IMMOBILIZATION AND CONFINEMENT EFFECT

The industrial application of free enzymes in solution is usually hampered by the lack of long-term stability and the difficulties of recovering (of both the enzymes and the product) and re-using this expensive bio-catalyst. The immobilization of enzymes is a good solution to overcome these drawbacks (Sheldon (2007)).

This immobilization also brings some other advantages, such as: facilitates the handling of the enzyme which increases the controlling of the process (e.g., the reaction can be stopped by enzyme removal); facilitates its separation from the product (reducing product treatment's price); increases enzyme stability during storing or during operation; increases resistance to environmental changes; reduces the overall production cost (e.g., recycle of enzyme and no product purification); and it allows the creation of multi-enzyme systems (Sheldon (2007)).

These improved enzymatic results occur due to the creation of a microenvironment for the enzyme to act and due to structural changes inside the enzyme molecule (i.e. these changes do not involve deformation of binding site and denaturation). The structural changes that may increase stability for the immobilization case can be conformation changes such as: higher rigidity, reduced entropy for unfolding, compact packing, and low conformation strain or may also be structural changes due to variations in intramolecular interactions and bonds of the protein. It is important to recall that these structural changes aren't the ones that cause the binding site to deform and the enzyme to denature (like it was discussed in previous sections) or simply that the conformation is small. (Homaei et al. (2013), Secundo (2013))).

However, the activity of the immobilized enzymes is usually lower than for the free enzymes in the solution. This happens mainly due to the difficulty of accessing the substrate (Homaei et al. (2013)). This is the reason why more research should be done in order to improve and optimize the current immobilization methods and increase activity performance and the main motivation of this project.

2.4.1 TYPES OF IMMOBILIZATION METHODS

The immobilization of a biocatalyst can be done using either cells (containing the catalyst) or using enzymes. The enzyme immobilization consists of confining the enzyme by binding it onto a surface or by enclosing it into a matrix (Datta et al. (2013)).

As it was mentioned in the Introduction section, there are two main entrapment types, which are based on the interactions of the forces between enzyme-(surface or matrix): Physical and chemical (Dwevedi (2016)).

The **physical method** consists in attaching or adsorbing the enzymes to the matrices via physical forces (e.g., Van der Waals, hydrogen bonding, and hydrophobic interactions). Some examples of techniques are: adsorption, entrapment, and microencapsulation (Dwevedi (2016))

1. Adsorption: Consists in attaching the enzyme to the support material by weak non-covalent bonds (i.e. Van der Waals, electrostatic and hydrophobic interactions). The solid support enters in contact with an enzymatic solution for a time period (under proper enzymatic conditions) and the enzyme is adsorbed on the support. The advantages are that this method is simple, cheap, and surface regeneration possibility. However, it is time reagent and time consuming, with low homogeneity and they may be sensible to the surface choice. Besides, the weak forces may lead to reversibility and enzyme leakage. And the leakage may also be activated by changes in the environmental conditions that affect the interaction strength (e.g., pH, ionic strength, polarity, and ionic strength) (Nguyen et al. (2017)).
    a. Electrostatic binding LBL: The adsorption may be done by electrostatic force using the Layer-by-layer method. Which consists in alternatively depositing the support in oppositely charged solutions (one of them being the enzyme solution). The method is very easy and controllable, allowing to modify layer thickness and structure. However, it has the disadvantages of overcharging of the surface with the adsorbed reagents (or accumulated product) which may jeopardize the diffusion and change pH stability (Nguyen et al. (2017)).
2. Entrapment: It is the cross-linking of the enzyme to a polymer lattice in every direction. The polymeric matrix allows the entry of substrate and release of product. This method is simple, doesn't change the enzymatic properties, improves its stability, reduces denaturation, and has minimal enzyme requirement. However, it may be applied to a limited number of enzymes, may have enzyme leakage, and for membranous matrix, the substrate and products have to be small and the matrix has to be in balance with the environment (Dwevedi (2016); Datta (2013); Nguyen et al. (2017)).
3. Encapsulation: Refers to the confinements of an enzyme within a semi-permeable polymer membrane with controlled porosity. Protecting the enzyme from the contact with the bulk and avoiding leaching out. The porosity has to be chosen with respect to the size of the product, substrate, and enzyme. If the enzyme diameter is close to the one of the reaction's participants the encapsulation should not be chosen because the enzymes will leak out of the membrane. Besides this problem, clogging of pores due to high enzyme concentration may also occur (Dwevedi (2016); Nguyen et al (2017); Jegannathan et al. (2008)).

And the **chemical methods** attach the enzymes via covalent or ionic bonds (non-reversible). Examples: covalent attachment, cross-linking, and ionic binding (Dwevedi (2016)).

1. Covalent: Covalent bonds are used to attach the enzyme to the matrix. The method is based on non-essential amino acids (i.e., that do not participate in the active site), and tries to promote minimal conformation changes. It has the advantage of increasing the enzymatic resistance to environmental changes. However, sometimes there are big conformation changes that occur, and also the bond is sometimes made with the amino acids from the active site causing denaturation (Dwevedi (2016)).

2. Cross-linking: Several irreversible covalent bonds are formed between the matrix and the enzyme using reagents. This method is simple but the active site may be possibly deformed (Dwevedi (2016)).
3. Ionic binding: A charged matrix and the enzymes have ionic interactions. Similar to the adsorption, this method's interactions will also depend on pH, the concentration of enzymes, and temperature. However, the variation of ionic strength and pH may cause the enzyme to detach from the matrix (Dwevedi (2016))

Regarding the immobilization supports, they can be natural polymers (e.g., alginate, chitosan, collagen, gelatin), synthetic polymers (e.g., ion exchange resins with a porous surface for enzyme trapping), or inorganic materials (e.g., ceramics, silica, glass) (Nguyen et al (2017)).

Another way to classify the immobilization techniques is: surface-confinement (on a support) and volume-confinement (in a network or compartment) (Küchler (2016)).

2.4.2 *IN VIVO* AND *IN VITRO* CONFINEMENT EFFECTS

Enzymatic immobilization and confinements are not innovative ideas, because those already exist in nature. Enzymes occur naturally in a confined biological environment, such as cells. They present a very specific structure, stability, and catalytic properties. Those complexities cannot be exactly reproduced in vitro conditions Not only that, in the biological medium most enzymatic reactions occur in a confined environment (i.e. on a surface, in a small volume, or at the interface) and crowded environment (Küchler et al. (2016)).

In vivo enzymatic catalysis have leverage over in vitro due to cellular ability to form multi-enzyme complexes with a well-defined spatial location that favors enzyme's availability to the substrate and due to its *de novo synthesis* (replacing enzymes not active, released, or degraded) (Küchler et al. (2016)).

So as it may be seen, the in vitro reaction is far less effective than the in vivo one. Therefore, to decrease their activity difference the in vitro should operate in a setup that maximizes the enzyme's activity. This requires optimal localization (by immobilization), enzyme quantity, storage, and operational stabilities (Küchler et al. (2016)).

When comparing in vitro applications of enzymes usually enzymes free in solution present higher activity because they have fewer constraints to substrate access. However, technique improvements have been made through the years, making it that immobilized enzymes have a higher activity (& stability) than the ones of the bulk (Küchler et al. (2016)).

Besides, the support characteristics should also be chosen in a way to maximize this enzymatic performance. Parameters such as size, shape, pore size, hydrophobicity affect the immobilization and performance. This thesis aims to comprehend this topic (Xie et al. (2009)).

2.4.3 IMMOBILIZED ENZYMES' ACTIVITY AND PARAMETERS

The success of the catalysis depends on the enzyme's active site availability to substrate. Surface-immobilized catalysts may face access restrictions due to physical constraints (e.g., entering pores, or bad orientation of the active site). And the volume-immobilized faces difficulties of substrate accessing the compartment or diffusion through the compartment (Küchler (2016)).

The parameters for enzyme activity are also influenced by the same ones as non-immobilized enzymes: substrate and enzyme concentration, pH, time, and temperature.

Some parameters influence the immobilized enzymatic performance in a not-so-direct way. For example, for free enzymes in solution, the increase of enzyme will increase the total amount of activity (if there is enough substrate available), but for immobilized enzymes the increase of enzymes may cause protein-protein interaction, decreasing the stretching flexibility of the enzyme's conformation (making it difficult for the enzyme to modulate itself in a suitable conformation to substrate catching and product releasing), resulting in steric hindrance and inactivation of enzymes (Zhang et al. (2013)).

2.4.4 IMMOBILIZATION IN LAYER BY LAYER & NANOTUBES

As mentioned before, the layer by layer assembly is one type of surface adsorption technique, based on the electrostatic interaction force of oppositely charged polyelectrolytes. It is advantageous to perform immobilization with LBL due to its malleability to construct multi-layers and due to the mild condition (e.g., aqueous) used in the technique, which helps minimize protein denaturation and aids on substrate mobility (Sakr (2013)).

Another bright side of the technique is using less enzyme than the cross-linking methods and the possibility of creating homogeneous film layers with high protein density (as it was mentioned in section 2.1.2.2 Effect of number of bilayers and polyelectrolyte's concentration) (Sakr (2013)) (Ferreira (2004); Garg et al. (2008)). However, overcharging of the LBL (surface, substrate, or product) may cause kinetics distortion and change the enzyme's stability. (Nguyen(2017)).

If the LBL technique is performed on porous membranes one can obtain multi-layer nanotubes. The quantity of layer should not be too big to not limit diffusion of the substrate and consequently decreasing the specific activity. Besides that, another factor that may reduce activity is the interpenetration of the PE layers since it reduces the availability of the active sites. (Sakr (2013))

2.5 GLUCOSE OXIDASE (GOX) AND CASCADE REACTION

2.5.1 COMPOSITION AND STRUCTURE

Glucose oxidase is a bi-substrate enzyme and may be originated from two possible fungal sources: Aspergillus or Penicillium. Although the second one possesses higher kinetics advantages, this thesis experiments used the A. niger type due to its high availability of research sources in literature and its price advantage (Bankar et al. (2009)).

This glycoprotein is composed of two identical 80-kDa subunits linked by two bisulphite bridges and two non-covalent linked FAD (flavin adenine dinucleotides) coenzymes. The coenzymes act on the electron carrying during the catalysis mechanism (Wong et al. (2008); Wilson et al. (1992)). Figure 15 contains two representations of the GOx's structure:

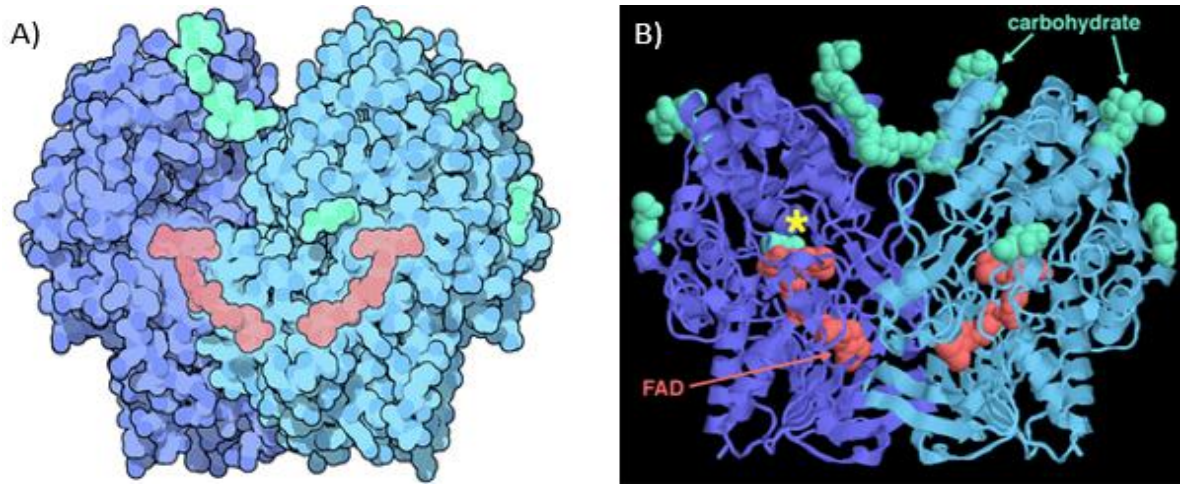


Figure 15: Representation of the Glucose Oxidase enzyme. The two subunits may be differentiated by dark and light blue, the red color represents the FAD coenzyme and the green represents some outsider proteins. A) Representation of the GOx. B) More detailed GOx representation created with RasMol, the (*) represents the active binding site, which is small when compared to the protein size (Modified from Goodsell (2006), the Scripps Research Institute).

2.5.2 CHARACTERISTICS

The A. niger enzyme has a molecular weight ranging from 130 to 175 kDa. The enzyme catalyzes the oxidation reaction of β-d-glucose[3] and it is specific to its beta isoform. Meaning that it is not suitable for the alfa-anomer substrate. The enzyme has an optimum pH in the range of 3.5 to 6.5, which explains its suitability to dilution in MES buffer (Better explained at session 2.3.2.3 Effect of pH).

[3] β-d-glucose is one of the main energy sources for living organisms, its chemical formula is C6H12O6 and the β stands for the beta isoform and the D refers to "dextrorotatory" which is one of the two stereoisomers of the glucose. (National Center for Biotechnology Information (2021)).

GOx has an isoelectric point (pI) of 4-5. Meaning that the protein is electrically neutral at this pH range. In a simple explanation, this point is equal to the pH vale where all the protein groups' charges sum will be zero, in a pH lower than this value the charge of amino acids will be predominantly positive and at higher pH, and if lower it will be negative (Bankar et al. (2009)).

It is a globular protein slightly elongated, its average diameter is 8 nm and specific volume 0.75 mL/g (Wilson et al. (1992)). It has major industrial importance in several commercial applications, such as: improvement of flavor and color in food, removal of oxygen in canned products to prevent rancidity. It is also used to determine glucose in urine and blood to detect diabetes. Those were just a few examples of the importance of this enzyme in the fields such as food, clinical, biotechnology, chemical, and others (Bankar et al. (2009)).

2.5.3 ENZYMATIC REACTION MECHANISM

The oxidation of β-d-glucose occurs in two steps and the oxygen is used as an electron acceptor for the enzyme. The first step is called the reductive step, where the GOx catalyzes the oxidation of the β-d-glucose ($C_6H_{12}O_6$) to forms the product d-glucono-δ-lactone ($C_6H_{10}O_6$). This product is then hydrolyzed (without enzyme interference) to gluconic acid. In this step, the FAD ring is reduced to FADH2. The second step is called the oxidative step, here the enzyme is re-oxidized by oxygen, which leads to the production of H_2O_2 (Bankar et al. (2009)). The whole reaction catalyzed by GOx is represented in Figure 16.

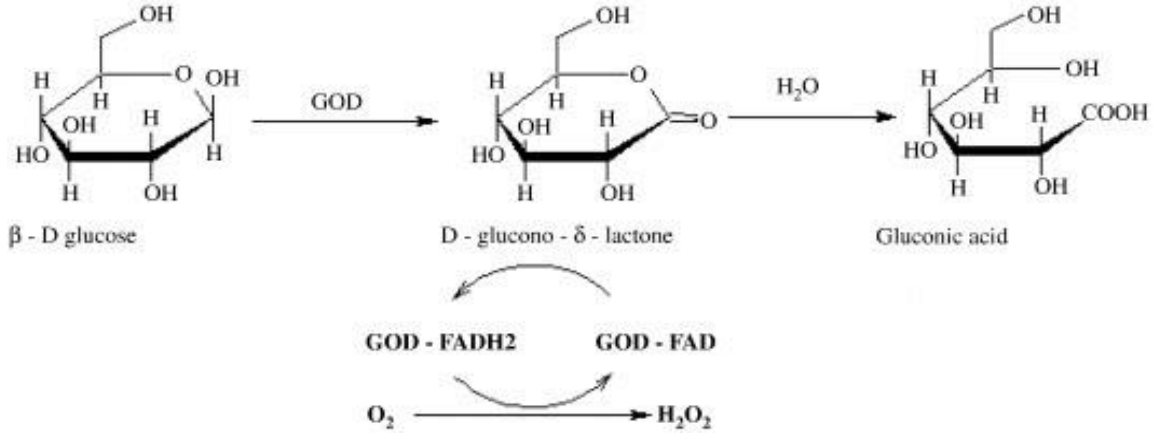


Figure 16: Representation of the catalyzed oxidation performed by the glucose oxidase on β-d-glucose. Done in two steps: on the top, it is the reductive step; and on the bottom the oxidative one. (Image by Bankar (2009)).

It is also important to mention that the spontaneous generation of gluconic acid results in a decrease of the pH and this may jeopardize the reaction. Besides, the accumulation of hydrogen peroxide inactivates the enzyme by modifying groups located near the active site (Wong et al. (2008)).

2.5.4 CASCADE REACTION WITH PEROXIDASE AND GOX ACTIVITY MEASUREMENT

Cascade reaction is the name given to two consecutive enzymatic reactions where the product of the first is the reagent of the second one. In this thesis, the multi-enzyme system used is: glucose oxidase and horseradish peroxidase (also referred to HRP in literature). The real important reaction is the first one since it will be the one where the immobilized GOx enzyme will participate, the second reaction is done because it produces a colored product which color may be correlated with the activity of the immobilized GOx enzyme.

The reagents of the first catalyzed reaction (GOx) are glucose, oxygen, and water; and the reagents of the second catalyzed reaction (horseradish peroxidase) are hydrogen peroxidase (product of the first reaction) and the dye molecules 4-Aminoantipyrine and p-Hydroxybenzoic acid, this last two were added to the reaction to participate in this second catalyst reaction (Yang (2017))(Yamanaka (1992)).

In this cascade reaction, firstly the GOx performs the oxidation of β-d-glucose using gas oxygen and water to produce D-gluconic acid and hydrogen peroxide. Following, the peroxidase does the oxidation of the dye molecules using the hydrogen peroxide (product of the first reaction). This second oxidation produces water and a colored product called quinoneimine with a maximum absorption wavelength at 510 nm (information later used as a parameter for activity analysis). The full cascade reaction may be found in Figure 17.

It is possible to correlate the colorimetric analysis results of the rate of formation of this quinoneimine product with the amount of production of H_2O_2 (from the first reaction) and therefore with the activity of the GOx enzymes. This is the most common technique applied for the activity measurement of glucose oxidase. (Yang (2017))(Yamanaka (1992)).

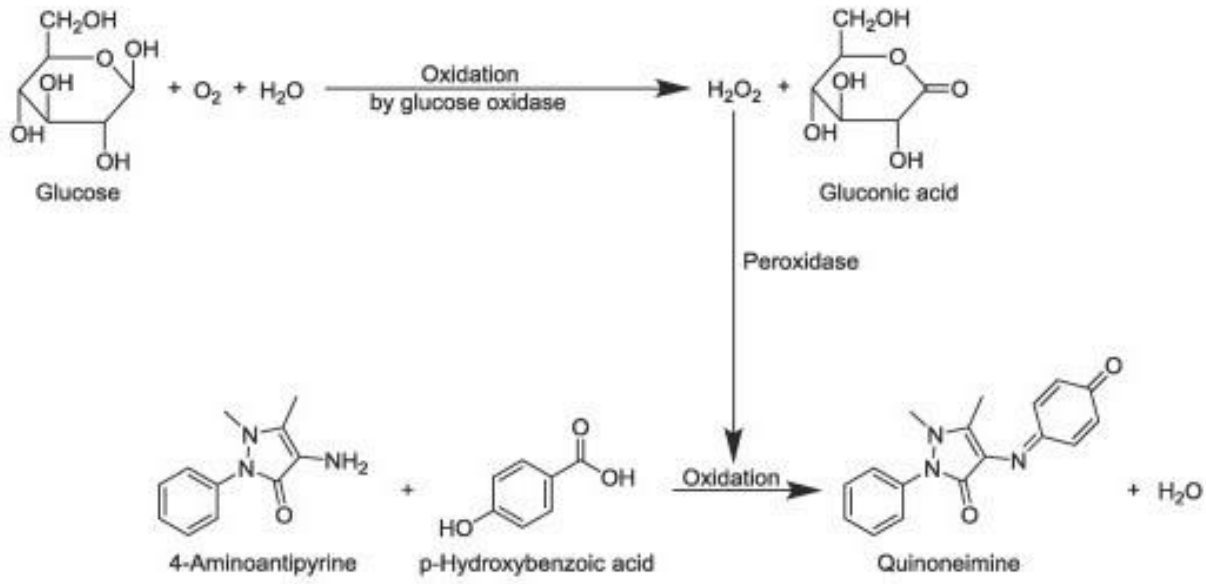


Figure 17: Schematic diagram of the cascade enzymatic reaction of GOx and Peroxidase. The product H2O2 of the first reaction is one of the reagents of the second reaction. The peroxidase reaction produces quinoneimine, a colored substance. (Image retrieved and adapted from Lim et al. (2019)).

3. CONTEXT, OBJECTIVES, AND STRATEGY

In biological systems, enzymes are often surface- and volume-confined, and their activity is timely and spatially defined, which is believed to be at the basis of their great efficiency. Mimicking the *in vivo* organization of enzymes is therefore expected to lead to major advances in the design of *e.g.* bioreactors, biofuel cells, biosensors, etc. Elucidating the role of confinement and spatial distribution on enzyme activity is a key step towards the design of highly bioactive enzyme-based systems. Previous studies highlighted some interesting features of enzyme confinement in nanochannels, but further investigations are still needed to gain a better and deeper understanding of the influence of nanoconfinement effects on enzymatic activity in synthetic nanostructures. Among the different available strategies of enzyme encapsulation, the elaboration of enzymatic nanotubes is a particularly interesting approach. A particularly efficient and highly versatile strategy to prepare tubular nanomaterials with tailored properties consists is the template-assisted synthesis combined with layer-by-layer (LbL) assembly into the nanopores of a membrane (Roy et al. (2010); Alem et al. (2007)).

In this context, the main objective of the master thesis was **to contribute to the evaluation of the impact of the LbL assembly conditions and enzyme confinement on the enzyme loading in nanotubes, and the biocatalytic efficiency**.

The experiments analyzed the effect of different parameters on the catalyst's performance, the parameters chosen were: concentration of polyelectrolytes (0.1 mg/mL and 1.0 mg/mL); diameter of nanotubes (250, 400, and 800 nm); and the nanotube bilayer structures (2 and 4 bilayers).

The strategy followed to achieve this goal was executed in three steps:

1. **Elaboration of enzymatic nanotubes through Layer by layer assembly and determination of the enzyme loading**

Glucose oxidase (GOx) was selected for this work as it is a robust and well-studied enzyme with a known 3D structure, readily available substrate, and has considerable importance for many applications. Branched-Polyethylenimine (bPEI) is used as a counter-charged polyelectrolyte (polycation) due to its well-known ability to be readily LbL-assembled with GOx with no substantial desorption of the enzyme, knowledge obtained from previous scientific articles (Lvov et al. (1996)). Branched polyethyleneimine (bPEI)/glucose oxidase (GOx) nanotubes were prepared by LbL assembly within porous polycarbonate membranes (PCM). The bPEI/GOx assembled nanotube is illustrated in Figure 18.

The LBL buildup of the nanotubes was performed by alternatively immersing the porous PCM into the solutions of both GOx and PEI polyelectrolytes. Since the membrane is negatively charged the first immersion should be done in PEI. Then, in order to explore

the impact of the assembly parameters on the biocatalyst the bPEI/GOx nanotubes were grown in PCM of various pore diameters (250, 400, and 800 nm), with different polyelectrolyte concentrations (0.1 mg/mL and 1.0 mg/mL), and different number of (bPEI/GOX) bilayers (2 and 4 bilayers).

Bicinchoninic acid (BCA) assay was employed to estimate the amount of enzyme loaded per sample area of the different LbL assemblies. Nanotubes were kept embedded within the membranes for further biocatalytic tests.

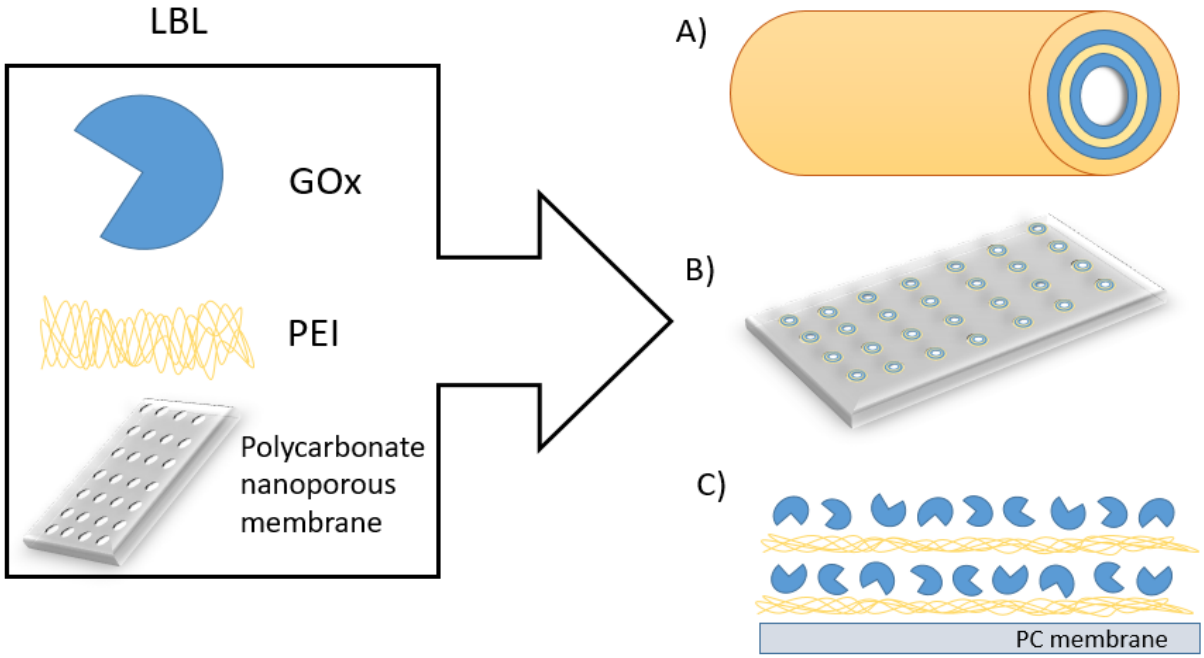


Figure 18: Schematic representation of the multilayer nanotubes localized inside a porous membrane. The materials used in the LBL technique are GOx, PEI, and PC membrane. A) Nanotube made of two bilayers of polyelectrolyte. B) The membrane containing the nanotubes in the place of its pores, the membrane is not dissolved so all experiments are done using the membrane with the tubes. C) The multilayer structure of the nanotubes in the case of a tube made of two bilayers.

2. Measurement of the enzyme's activity under static conditions:

The GOx activity of different (bPEI/GOx) nanotubes embedded in the membranes was measure by a colorimetric assay based on the oxidation of p-hydroxybenzoic acid and 4-aminoantipyrine by the generated H_2O_2, the full reaction is represented in Figure 19.

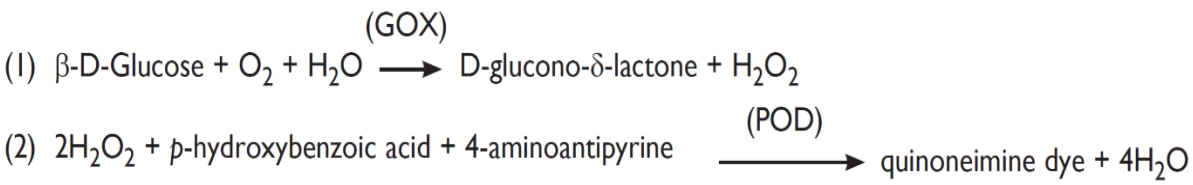


Figure 19: Cascade reaction and its two steps, first with the GOx enzyme and secondly with peroxide.

More specifically, to obtain this colorimetric results a circular piece is removed from each sample (i.e., the membrane containing the nanotubes) and inserted in a solution of glucose and POD mix (containing peroxidase) in order to perform the cascade reaction. The colored product of this bi-enzymatic reaction is analyzed in a spectrometer and its intensity results correlate with the amount of H_2O_2 available, which is directly correlated with the activity of the GOx biocatalyst. Therefore, the analysis results provide us information regarding the GOx activity in the units of absorbance divided by the mass of GOx (from BCA) per reaction time. An illustration of the analysis is found in Figure 20.

Performing the reaction in static conditions means that there is no flow of substrate coming in or product coming out of the reaction, therefore the absorbance (and activity) is the result of a product accumulation over time until all substrate consumption.

Shortly, this step is done to obtain the results of the GOx activity per quantity of enzyme for each sample and allowing us to study further and comparing the optimality of the parameters (concentration, diameter, and BL).

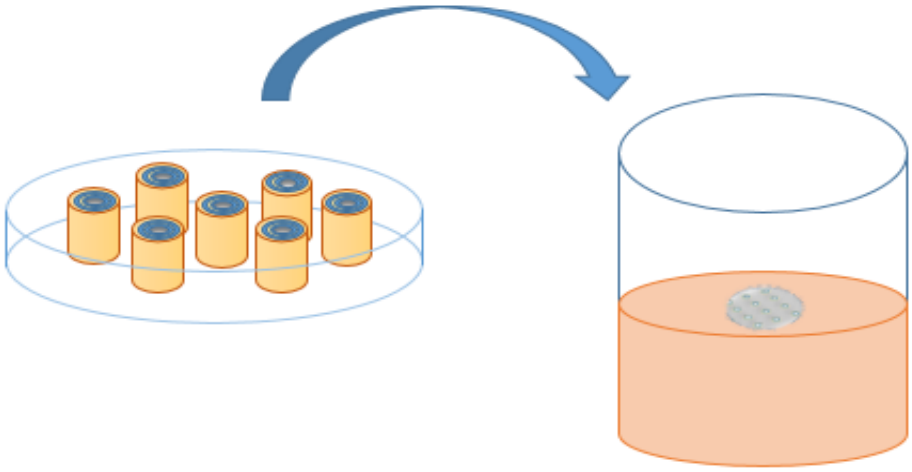


Figure 20: Schematic representation of the activity measurement in static conditions. A circular piece of the sample (left) is inserted into a solution of glucose and peroxidase. The product of the cascade reaction is a colored product called quinoneimine and is analyzed in the spectrometer

Measurement of the enzyme's activity under dynamic conditions

This step is performed by pumping the glucose through a sample piece, promoting the oxidation of the glucose and producing an outcome containing the H2O2 product. Then, this outcome will be mixed with the second reaction's substrates and peroxide (POD mix) to form the cascade reaction. A colorful product (quinoneimine) will be produced and will be analyzed through spectrometry, its absorbance results are correlated with the activity of the GOx enzyme. The analysis results obtained are given in absorbance divided by the mass of GOx (obtained from BCA results) per time of collecting. The dynamic activity measurement of GOx process is represented in Figure 21.

Performing the reaction in dynamic conditions means that there is a flow of substrate coming in and a flow of product coming out of the reaction, therefore the activity is the product is collected throughout the reaction time.

Shortly, this step also has the purpose of obtaining the activity amount per mass of GOx enzyme for each sample, the final results are compared to study the influence of the different assembly parameters.

The static and dynamic tests are different but may be seen as complementary. The static activity analysis provides results of the total amount of product resulted from the enzymatic reaction over time, per example, as it would be seen in a batch industrial application. And the dynamic activity provides us information about the amount of product resulted from the reaction for each time of reaction, providing us further information if this static reaction results are constant over time. In other words, the static case does not provide precise information regarding how the activity behaves through time (constant or not), and results are limited only until spectrometry saturation. And in the dynamic case, it is possible to find more specific information on how the enzyme will behave in long-term applications if the activity behaves constantly or not, and if there is possible enzyme desorption and denaturation occurring.

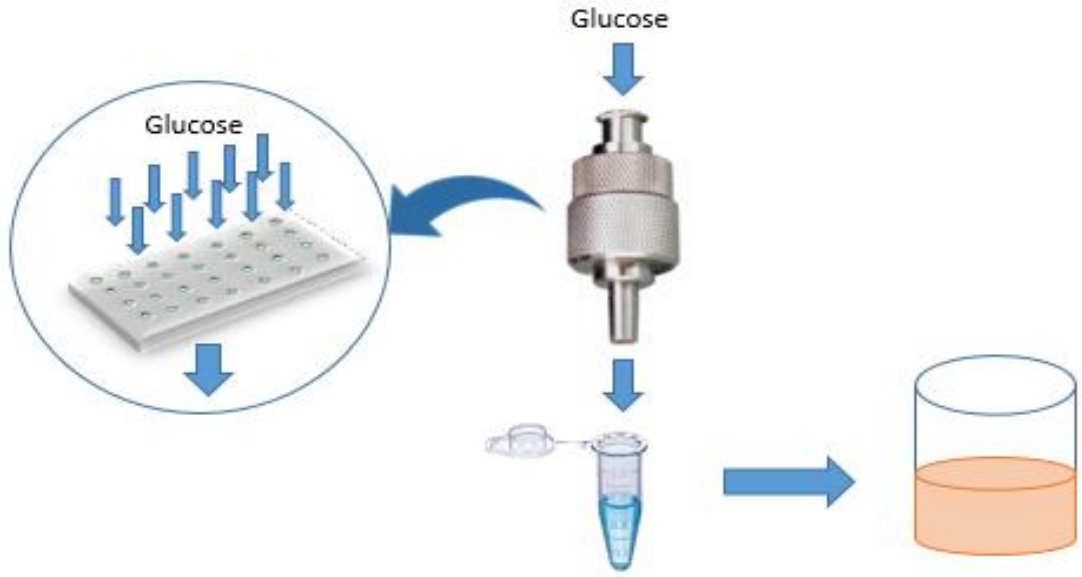


Figure 21: Schematic representation of the activity measurement in dynamic conditions. A rectangular piece of the membrane sample (containing the nanotubes in the pores) is inserted in a filter holder. The glucose solution is pumped inside the filter holder and the reacted outcome is collected. This collected solution is mixed with the reactants of the glucose-peroxidase cascade reaction to be analyzed through spectrometry.

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 BUFFER

The buffer solution (10 mM) was prepared by diluting 1,920 g of MES (2-(4-morpholino)ethanesulfonic acid, monohydrate, purchased from Acros Organics™) in 900 mL of water. In sequence, the pH was adjusted to 6.5 with NaOH 0.5M. The solution was prepared in atmospheric conditions and was stored in the same conditions.

4.1.2 POLYELECTROLYTES (PE)

Branched-Polyethyleneimine (bPEI, average M_w ~ 25,000 by LS) and glucose oxidase (GOx from Aspergillus niger, Type X-S, lyophilized powder, 167870 units/gram) were purchased from Sigma-Aldrich™ and used as positive and negative polyelectrolytes, respectively.

The polyelectrolytes solutions were prepared in three concentrations: both PE concentrations equal to 0.1 mg/mL (referred to as **set 1**); both concentrations equal to 1.0 mg/mL (referred as **set 2**. For the PE preparation of **set 3,** an error occurred during the dilution of GOx, therefore this set presents an unknown concentration of GOx higher than 1.0 mg/mL and PEI concentration equal to 1.0 mg/mL. Their dilution is done in the buffer. The solutions should be stored at a temperature around 5ºC.

4.1.3 TRACK-ETCHED POLYCARBONATE MEMBRANES (PCM)

Track-etched polycarbonate membranes with nominal diameters of 250, 400, and 800 nm, a pore density of 4.10^7 $cm^{-2,}$ and a thickness of 25 µm (24 µm for 800 nm PCM) were provided by the company It4ip (ipPore™, Louvain-la-Neuve, Belgium, http://www.it4ip.be/). They are all negatively charged and were stored in ambient conditions.

4.1.4 BICINCHONINIC ACID (BCA) PROTEIN ASSAY

The reagents A and B needed for the Body composition assay were purchased from Thermo Scientific™ Pierce™.

4.1.5 POD MIX SOLUTION

POD mix is the name of the mixture of peroxidase, 4-Aminoantipyrine, and p-Hydroxybenzoic acid and it is used for executing the cascade reaction glucose oxidase-peroxidase. The solution was prepared following the instruction of Megazyme's glucose oxidase assay kit.

The materials used were:

1. Bottle 1: Buffer (12 mL, pH 7.0) plus p-hydropxybenzoic acid and sodium azide (0.09% w/v) as a preservative.
2. Bottle 2: Peroxidase plus 4-aminoantipyrine and stabilizers; lyophilized powder.

And the given instructions were:

1. Dilute bottle 1 to approximately 150 mL of distilled water.
2. Dissolve the content of bottle 2 in the solution obtained in the first step. Check the pH and adjust it to 7.0 using 1 M HCl or 1 M NaOH. Adjust the volume to 200 mL. This solution is stable for approximately 3 months in a dark bottle at 4ºC.

All the materials and instructions information were retrieved from the Megazyme Glucose Oxidase Assay Procedure manual (2020). After its preparation, the solution bottle should be sealed with Parafilm® and stored at 5ºC.

4.1.6 D-GLUCOSE

The D-Glucose (MM 180.16 g/mol) used for the experiments was from two different sources. In the case of **set 1 (PEs concentration = 0.1 mg/mL)** the source was from Gibco™ and the solution had a concentration of 200 g/L or 1.11 mol/L. The **set 2 (PEs = 1.0 mg/mL)** and **set 3 (PEI concentration=1.0 mg/mL & GOx concentration>1.0 mg/mL)** glucose source was also from the Megazyme Glucose Assay kit, the solid glucose was diluted (following the instructions) in buffer to obtain a concentration of 90 g/L or 0.50 mol/L.

4.2 METHODS

4.2.1 LAYER-BY-LAYER TEMPLATING BUILD-UP OF NANOTUBES IN POROUS MEMBRANE

The (bPEI/GOx) nanotubes embedded in PCM were fabricated according to the procedure shown in Figure 22. PE multilayers were deposited by alternately dipping the PCM in bPEI and GOx solutions. As previously mentioned, the PEI is the positively charged PE and GOx is the negatively charged PE, both in buffer at pH 6.5. The steps were performed as following:

1. The porous membrane is completely immersed in a compartment containing the solution of polyelectrolyte. As the pore walls of the membrane are negatively charged the build-up started with the positively charged bPEI adsorption. Following, ultrasonication was applied for 10 minutes to facilitate the diffusion of the PE inside the pores, followed by an additional 20 minutes of immersion in the bPEI solution.
2. The membrane was removed from the bPEI solution and rinsed twice in MES buffer.
3. The membrane was then immersed in GOx solution, submitted for 10 min to ultrasounds, and rest 20 minutes more in solution before rinsing with buffer.
4. The membrane was removed from the GOx solution and rinsed twice in MES buffer.
5. To avoid the clogging of the membrane pore, the PCM surfaces were decrusted after each 2 bilayer deposition (so, 4 PE deposition steps). For that purpose, a cell scraper was used to remove the LbL film formed on the two external surfaces of the PCM.

All those 6 steps were performed in ambient conditions and repeated until the desired number of bilayers was achieved. After the LBL build-up, the samples were quickly immersed in distilled water (to remove any excess of buffer), were dried in paper and stored in a Petri dish, and stored in a cold room at 5ºC.

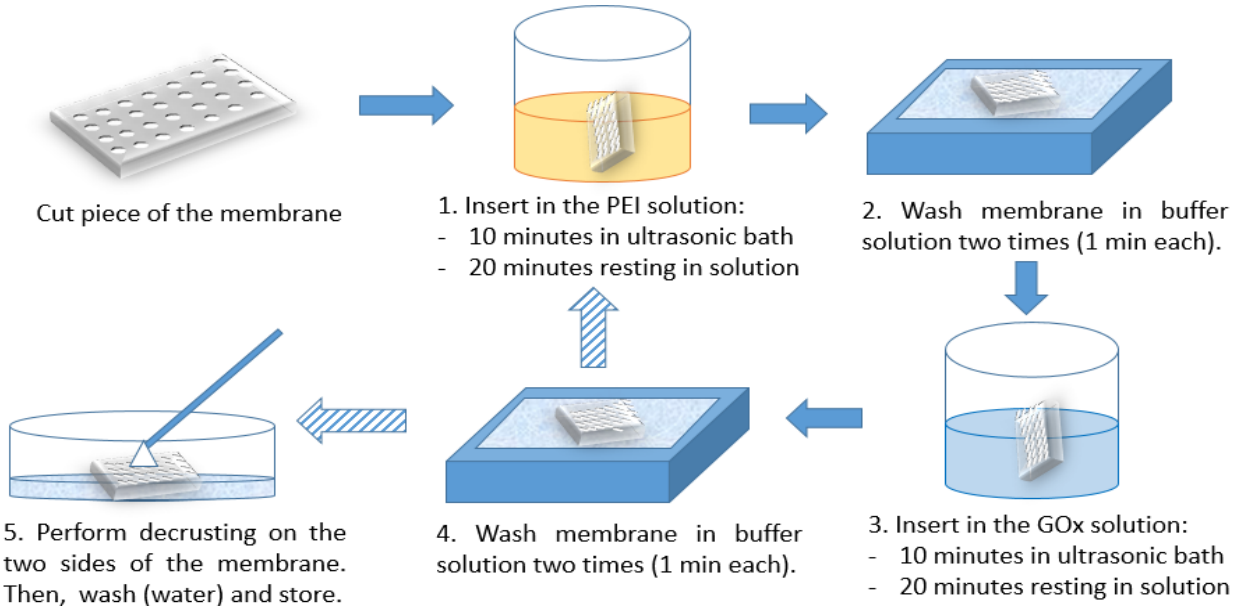


Figure 22: Layer by layer assembly of the PEI and GOx nanotubes in the pores of the polycarbonate membrane.

Three sets of samples were prepared, each one containing 6 samples in order to include all 3 types of diameter (250, 400, 800 nm) and 2 types of bilayers (2BL and 4BL), these six types of samples are shown in Table 1. The sets are distinguished by their PE concentration: the **set 1** was assembled at PEs (PEI and GOx) concentration equals 0.1 mg/mL; the **set 2** was assembled with PEs concentration equal to 1.0 mg/mL; and during the PE solutions preparation of **set 3** an error occurred and this set was assembled at PEI concentration 1.0 mg/mL and GOx concentration higher than 1.0 mg/mL (in the same magnitude order but its precise value it is unknown).

Table 1: The 6 types of samples produced by the LBL assembly

LBL samples assembled for the three sets		
250 nm 2 bilayers	400 nm 2 bilayers	800 nm 2 bilayers
250 nm 4 bilayers	400 nm 4 bilayers	800 nm 4 bilayers

4.2.2 BICINCHONINIC ACID (BCA) PROTEIN ASSAY

This analysis was performed to determine the amount of enzyme adsorbed in the sample per area (of the membrane or total internal area of the pores). This information is of great importance to correlate the activity per amount of enzyme and analyze which nanotube configuration and PE concentration is optimal.

Data were acquired following this protocol:

1. A 5x5 mm^2 square piece of each one of the samples and insert it in a 3 mL glass bottle.
2. Insert 40 μL of water in each one of these bottles.
3. In other bottles insert 40 μL of standard solution. The standard concentrations chosen were: 0 (water), 0.015, 0.045, 0.075, 0.105 and 0.135 mg of GOx/mL. The concentrations are obtained by diluting the enzyme in water.
4. In each one of the bottles insert 400 μL of the A/B reagent with a proportion of 50:1.
5. Close the bottles with their lids and seal them with Parafilm® in order to avoid evaporation interference in the results.
6. Insert the tubes in a water bath at 60 ºC for 1 hour.
7. Later, let the bottles rest 20 min on top of an ice bag, to promote water condensation and to obtain the real concentration of the samples.
8. Remove 200 μL of each bottle (samples and standards) and pour them into a 96-well plate. Measure light absorbance at the wavelength of 562 nm (as recommended by the provider ThermoScientific™).
9. Remove the remaining solution of the bottles and repeat the process using the same samples. Therefore, adding 40 μL of water, 400 μL of A/B reagent, and incubating, cooling, and analyzing 200 μL of the solution. The process is repeated to maximize protein recovery. The final BCA absorbance A_{562} is a sum of the two obtained absorbance results.

Regarding the data treatment, the final results are also obtained in steps:

1. The absorbance results of the standards were used to establish a calibration curve: A_{562} vs. concentration of GOx.
2. Using the calibration curve the concentration of GOx in each analyzed sample was determined.
3. The obtained concentration values in mg of GOx/mL are then multiplied by 40 µL equivalent to the water volume inside of the glass bottles to obtain the mass of GOx in the inserted sample piece.
4. Finally, the mass quantity is divided by the surface area. This may be applied in two different ways:
    a. The equivalent mass is divided by 25 mm^2 to obtain the final result in the unit of mass of GOx per area of the membrane (containing the nanotubes). The absorbance results of samples with different diameters may not be compared, because the immobilization occurs inside of the tube areas, and samples' membranes present different internal areas and pore density.
    b. The equivalent mass is divided by the total internal surface area of all the pores.

A resume of the steps performed for BCA data obtaining and treatment may be found in Figure 23.

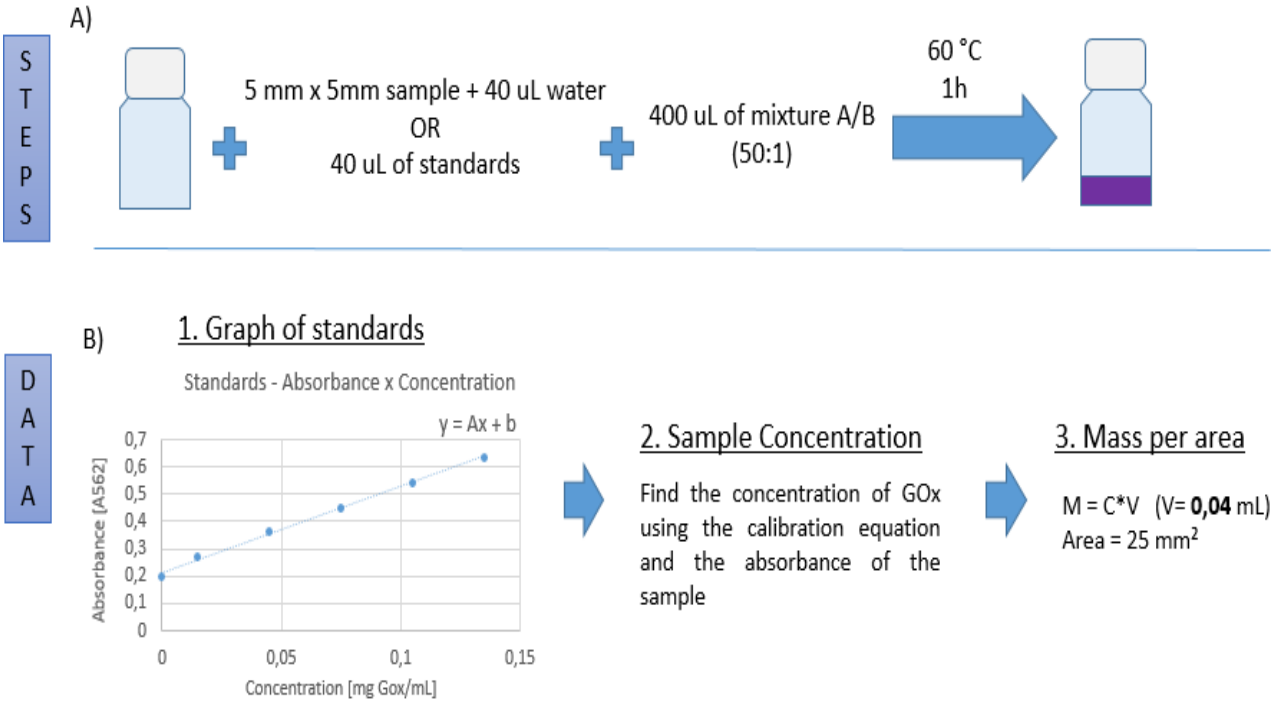


Figure 23: Schematic representation of the BCA test. A) Steps to prepare the colored product analyzed by the spectrometer. B) Steps of the data treatment and how to obtain the final result in mass of GOx per area.

4.2.2.1 BCA TOTAL INTERNAL SURFACE AREA OF A MEMBRANE

To complete the data treatment step 4.b the total internal surface area of the membrane should be determined. As mentioned in Section 4.1.3 Track-etched polycarbonate membranes (PCm), all polycarbonate membranes have pore density 4.10^7 cm^{-2} and thickness of 25 µm (for diameter 250 and 400 nm) or 24 µm (for 800 nm). Based on those values it is possible to find the total internal area by calculating the area of one pore (cylinder) and multiplying by the pore density. The internal area per pore is calculated by Equation 1: **Internal area per pore**.

$$Internal\ area\ per\ pore = \pi * diameter * height$$

Equation 1: Internal area per pore

The total internal areas calculated for each type of pore diameter are gathered in Table 2. It is important to remember that the results are given in total internal surface per 1 cm^2 of the membrane and in the BCA test 0.25 cm^2 of the sample was used, therefore the total internal surface should be multiplied by 0,25 before use.

Table 2: Results for the internal surface area inside the PC membranes.

Pore diameter d [nm]	Pore density D, [cm^{-2}]	Pore height, H [µm]	Internal surface area per pore [$µm^2$]	Total internal surface per 1 cm^2 of membrane [cm^2]
250	4.10^7	25	20	7,9
400	4.10^7	25	31	12,6
800	4.10^7	24	60	24,1

4.2.3 MEASUREMENT OF THE ENZYME'S ACTIVITY IN STATIC CONDITIONS

This analysis has the purpose of determining the sample's activity performance (activity of the GOx enzymes per mass of GOx) per time of the reaction, with information it is possible to compare the performance of the samples and analyze which nanotube configuration (PEs concentration, diameter, and the number of the bilayers) is the most optimal for the efficiency of the enzyme.

The reaction is performed in static conditions (i.e., no flow currents entering in or going out) and therefore the reaction (cascade reaction) will take place over time and accumulate the product produced until total substrate consumption. Performing the measurement in static conditions has the advantages of simulating a batch application of the nanotubes (which is a very common reactor to be used with enzymes) and allows us to do the comparison between the sample's different assembly parameters with their GOx performance (activity per enzyme quantity).

However, it presents the disadvantage that, due to the accumulation of product and the saturation of the product's absorbance (limitation of spectrometry analysis), it is not possible to obtain detailed information of how the enzymes' activity behaves throughout the time of reaction (e.g., if the activity of enzymes at reaction time 15 minutes is equal to its activity at 45 minutes of reaction), therefore not providing important information on how the immobilized enzymes behave in long-term applications and if there is any modification occurring throughout the reaction's time (e.g., enzyme desorption or enzyme denaturation).

The steps performed to acquire the data for the measurement of the enzyme's activity for static reaction conditions are illustrated in Figure 24 and explained following:

0. Place a 96-well plate on top of an ice bag and performed all steps in this condition. Reducing the temperature slows down the reaction that will take place, and with this, it is possible to consider that all the samples reactions were triggered at the same time.
1. Insert 50 µL of water in the wells of the 96-well plate. The number of wells filled should be the same amount as the number of samples plus one for the blank.
2. Extract a circular piece of each sample using a Punches Harris Uni-Core™ of TIP 1.0 mm (Patent No. 709308) and insert it in the well containing water. Repeat this process three times for each sample.
3. Insert 200 µL of POD mix in each well (containing sample or blank).
4. Insert 50 µL of Glucose (0.50 mol/L) in each well and immediately analyze the 96-well plate in the spectrometer at a wavelength of 510 nm. This wavelength is chosen because it corresponds to the maximum absorption of the quinoneimine. This step provides us the absorbance results of the samples, which may be correlated with the quantity of activity of the GOx enzymes.

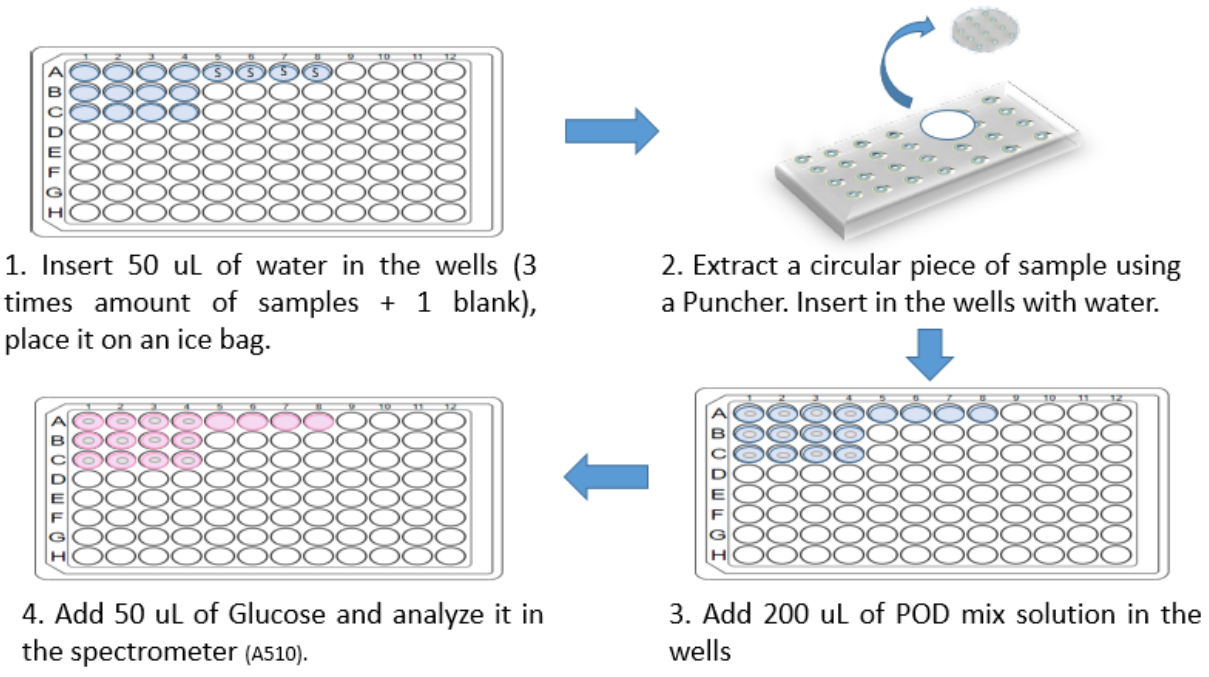


Figure 24: Representation of the steps performed for measuring enzymes' activity in static conditions.

Regarding the data treatment, the final enzyme's activity results of the samples were obtained by the following steps:

1. Subtract the absorbance value of the blank (zero concentration of GOx, composed of 50 μL water + 200 μL POD mix + 50 μL of Glucose) from the absorbance data of the samples in order to obtain the pure absorbance value due to GOx activity.
2. Divide the absorbance results of the samples by the circular area that was extracted from the samples to perform the experiment. The circle had a diameter of 1.0 mm and therefore surface area equals 0.00785 cm^2.
3. Divide the obtained absorbance per area of sample results by the BCA results of GOx mass per sample area.
4. The final results are obtained in the units of absorbance A_{510} per GOx mass. This absorbance corresponds to the amount of quinoneimine produced, which is proportional to the produced H_2O_2 and therefore may be correlated with the activity of the immobilized GOx enzyme. The performance of the enzymes may finally be compared between samples (with different parameters and from different sets).

The data acquirement and treatment were performed for the samples of **set 1** and **set 3.** For the case of set 1, the static activity measurement was performed two times, the first time was performed on the same day as the samples were assembled and the second was performed 8 days later. For the set 3, the analysis was performed the next day after the samples' LBL assembly. Unfortunately, the measurement of the enzyme's activity in static conditions was not performed for the samples of set 2.

4.2.4 MEASUREMENT OF THE ENZYME'S ACTIVITY IN DYNAMIC CONDITIONS

This is the main analysis of this thesis. This step has the goal of measuring the GOx enzyme's activity of the different types of samples assembled (regarding the concentration of PE, diameter, and number of bilayer) for each time of the glucose oxidation reaction. Allowing us to analyze how the immobilized enzymes behave over the reaction time (if they present peaks or possible decrease of activity due to denaturation or desorption) and allow us to compare the activity results between samples with differently assembly parameters in order to achieve an assembly optimization for applications in dynamic conditions. The dynamic conditions activity measurement was performed on the samples of set 1, set 2, and set 3.

The analysis in dynamic conditions simulates a real-life sample's application in a CSTR reactor. As mentioned, the collecting throughout the time provides more specific information on how the immobilized enzymes behave than the analysis in static conditions. Additionally, the activity analysis over a long period may also be correlated with the re-using of the samples for applications performed on the same day.

Regarding data **obtaining**, the steps necessary for this process are the following:

1. A laboratory peristaltic pump (Glison band) and a filter holder are used to perform this experiment. The first step is cleaning the pump tubes by pumping a solution of Hellmanex™ (diluted in water in a volume proportion of 2%) and cleaning the filter holder by inserting it in the solution of Hellmanex™ 2% and immersing it in the ultrasonic bath for 20 min, this is done to assure an effective cleaning and no clogging of pores. Following, abundantly rinse both tubes and filter holder with water. Finally, the stainless steel filter holder should be attached to the end of the tube and air should be pumped to dry both the tubes and filter holder.
2. A rectangular piece (12 mm x 5 mm) of the sample (i.e., the membrane containing nanotubes) is cut and inserted in the filter holder. The sample is on the support screen and the gasket is deposited on top of the sample so it does not move.
3. Buffer is pumped in the complex (i.e., pump, tubes, and filter holder with sample) for 25 min. to assure stable conditions of the sample before the reaction. This buffer is pumped at the speed of 2.05 rpm which, according to the equipment user's guide, corresponds to approximately 1.0 ml/min for medium tubes (Gilson User Guide). Then, air should be pumped into the complex to assure that there is no buffer remaining.
4. A solution of glucose diluted in buffer (0.05 mol/L or 9.0 g/L) is pumped into the complex at the speed of 2.05 rpm. The glucose will be oxidized by the GOx embedded in the nanotubes inside of the pores of the membrane, to produce gluconolactone and hydrogen peroxide products. When the first drop of those products comes out of the filter holder the collecting and chronometer starts and this is our reference time zero. Following, the time collecting was performed in plastic test tubes (1,5 mL) throughout the analysis time (i.e., glucose oxidation reaction time) in two different ways:
    a. For the set 1 and set 3 the collecting was the following:
        i. 1 mL (or approx. 1 minute) of the outcome was collected 5 times
        ii. Then, 1 mL was collected after every 4 minutes, therefore a 5 minutes cycle that is repeated 8 times. For example, at minute 5 the outcome is collected until minute 6, then 4 minutes of outcomes are not collected, and, finally, at minute 10 the outcome is collected until minute 11 (because 1 mL is approximately equal to 1 minute).
        iii. Therefore the time collecting range are:
            1. 0-1; 1-2; 2-3; 3-4; 4-5 for the 5 first collecting.
            2. 9-10; 14-15; 19-20; 24-25; 29-30; 34-35; 39-40; 44-45, for the 8 remaining collecting. Therefore, 13 collecting per sample.
    b. For The set 2 the collecting was the following:
        i. 0.5 mL (or approximately 30 seconds) of the outcome was collected 45 times, without any pause between collectings.
5. 50 µL of the collected outcome is reacted with 150 µL of POD mix in the 96-well plate. A 50 µL of glucose solution is also reacted with POD mix to be used as the blank. Finally, the plate is analyzed in the spectrometer at a wavelength of 510 nm.

A scheme of the measurement steps may be found in Figure **25**.

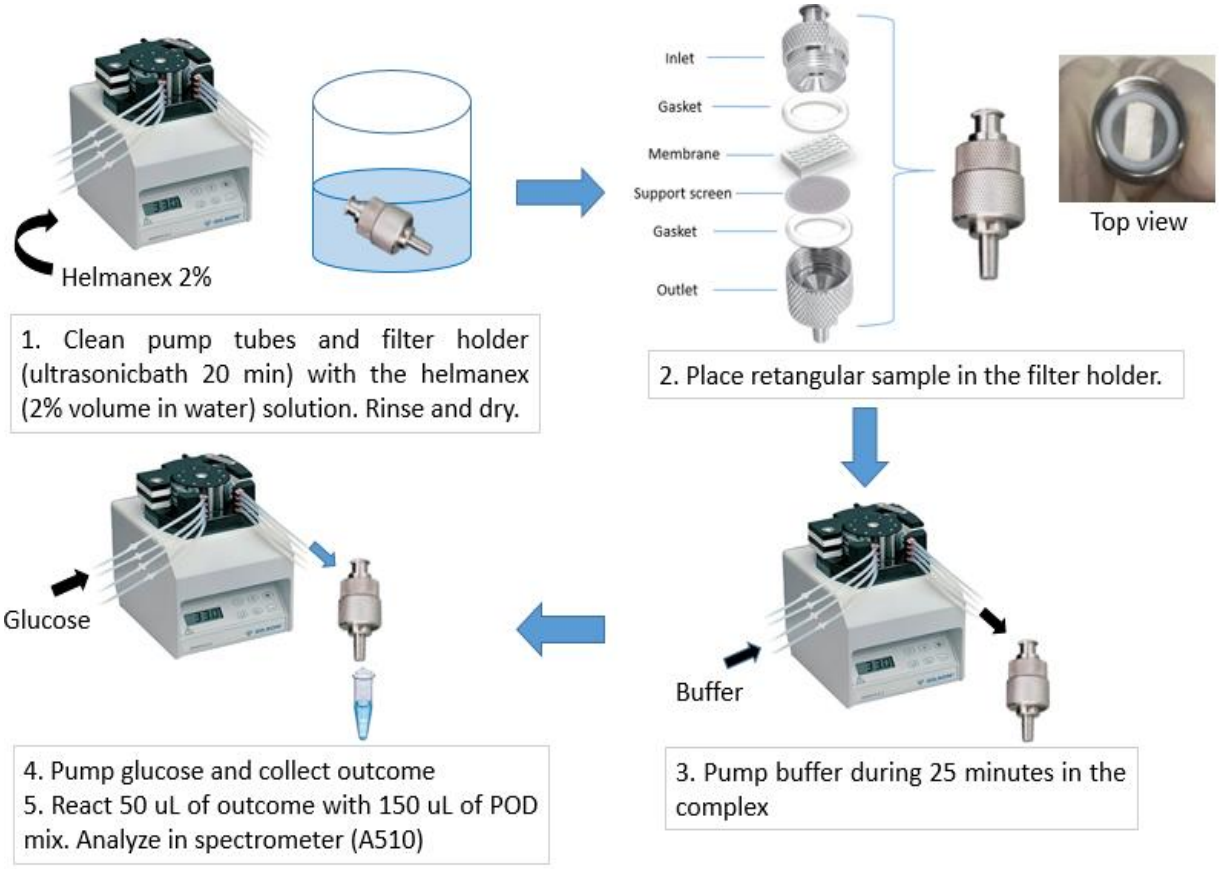


Figure 25: Schematic representation of the steps executed to perform the measurement of the activity of enzymes in dynamic conditions

Regarding data treatment, after obtaining the absorbance results they should be divided by the area of the sample (i.e. 12mm x 5mm). The obtained value should then be divided by the BCA result of GOx mass per area of the sample in order to obtain the final absorbance per mass of GOx result. The absorbance value measured by the spectrometer corresponds to the amount of quinoneinime produced (by the second reaction of the cascade), which is proportional to the amount of H_2O_2 present (product of the first reaction and reagent of the second reaction), which is proportional to the activity of the GOx enzyme. Therefore, the absorbance result may directly be correlated with the activity of GOx.

Another remark to be made is that the final result of both static and dynamic activity measurements are given in absorbance per mass of GOX, allowing us to compare them later.

5. RESULTS AND DISCUSSION

The (bPEI/GOX) nanotubes were created inside of a PC porous membrane using the immersive LBL assembly technique. Three parameters (PE concentration, pore diameters, and the number of bilayers) were chosen as variables to verify their influence on the enzymes' activity. In total, the samples (i.e., the PC membrane containing the nanotubes) were divided into three sets, based on their LBL assembly PE concentration. The sets are: **set 1** (PEs concentration=0.1 mg/mL), **set 2** (PEs concentration=1.0 mg/mL) and **set 3** (PEI concentration=1.0 mg/mL & GOx concentration>1.0 mg/mL). And each set contains 6 samples: 250 nm-2BL; 250nm-4BL; 400nm-2BL; 400nm-4BL; 800nm-2BL and 800nm-4BL.

Before any experiments, the first step performed was the SEM analysis in order to verify if the nanotubes were being successfully assembled. Following, the BCA analysis was performed in order to quantify the amount of enzyme deposited in the sample's area (i.e., membrane containing the nanotubes), this step is performed so it is possible to, later on, correlate the activity per amount of enzyme, and also to verify the success of the assembly.

Finally, the immobilized GOx's activity is measured through a reaction in static conditions and in dynamic conditions (this last one being the focus of this thesis), allowing us to understand how the change of the parameters will affect the enzyme's performance.

5.1 COMPLEMENTARY STUDY: SCANNING ELECTRON MICROSCOPY (SEM)

To prove that the nanotubes assembly was occurring successfully, one set of samples had its membrane dissolved to obtain the free tubes and analyze them through SEM. The results obtained by the SEM (represented in Figure 23) proves the efficiency of the assembly method. This entire step was performed by Ievgen Kurylo.

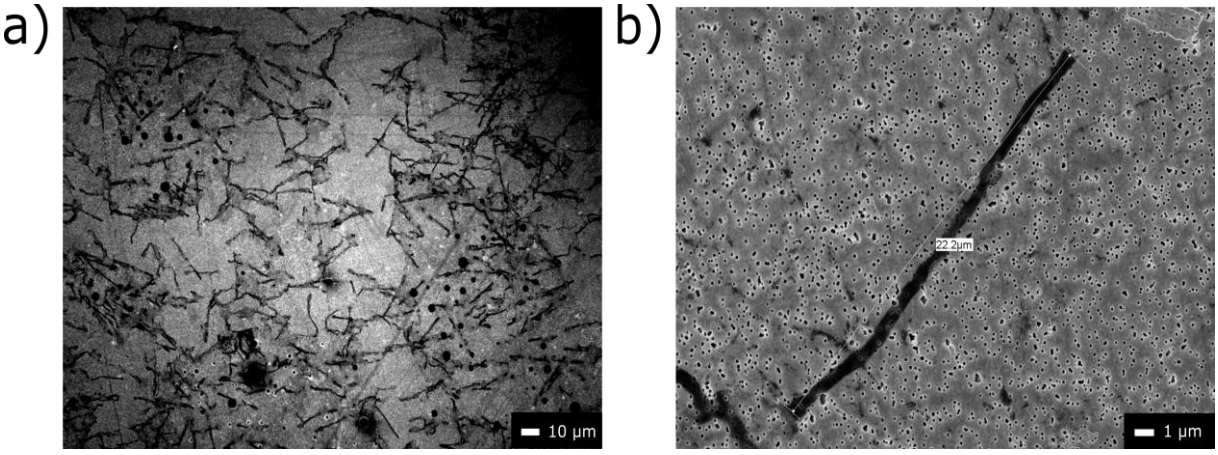


Figure 26: SEM analysis of the 6 bilayers free (bPEI/GOX) nanotubes after membrane dissolution into dichlorometane (Retrieved from Ievgen Kurylo Post Doctorate thesis)

5.2 BICINCHONINIC ACID (BCA) PROTEIN ASSAY

This process quantifies the amount of GOx enzyme adsorbed per area of sample (area of the membrane containing the nanotubes, or total internal area of nanotubes in the sample's area). The required data was collected by immersing a piece of sample in a solution containing water (40 µL), reagent A and reagent B (400 µL proportion 50/1) inside a glass bottle, the same preparation was also performed on known concentration of GOx solutions (standards). Then, all the glass bottles were heated and the final colored solution's absorbance was measured through spectrometry and correlated with the amount of protein present (i.e., GOx enzyme). The process is performed twice to maximize protein recovery and therefore the total absorbance of each sample is a sum of both the absorbance data of both tests. The mass of the sample is determined by correlating the samples' absorbance data with the calibration curve obtained by the absorbance results of the standard solutions of GOx.

The final result of the sample's mass of GOx per area is an important information for determining the activity performance of the samples based on their amount of enzyme.

5.2.1 BCA: STANDARDS ABSORBANCE PER ENZYME CONCENTRATION CALIBRATION CURVE

The first step of the data treatment is to obtain the calibration curve through the standards' GOx concentrations and its absorbance results. The quadruplicate standards' absorbance results may be found in Table 3. In theory, each standard should have been analyzed six times (analysis performed on the three sets and two times per set) but since there was a problem in the enzyme's dilution in set three (and therefore the diluted GOx was not correct), its absorbance results were not taken into consideration for the calibration curve.

Table 3: Absorbance values of the standard and average absorbance

Standard Concentrations [mg GOx/mL]	Absorbance results A562 [AU]				Absorbance average [AU]	Standard deviation [AU]
	Test 1	Test 2	Test 3	Test 4		
0	0,231	0,166	0,190	0,213	0,200	± 0,027
0,015	0,288	-	0,270	0,280	0,279	± 0,009
0,045	0,394	0,361	0,365	0,389	0,376	± 0,017
0,075	0,522	0,452	0,447	0,468	0,473	± 0,035
0,105	0,561	0,520	0,546	0,598	0,556	± 0,033
0,135	0,661	0,667	0,640	0,675	0,661	± 0,015

The absorbances average values of the standards were plotted in an absorbance per GOx concentration curve. The calibration curve was obtained by inserting the linear tendency curve that better fits the standards 'absorbances, the obtained curve and its equation may be found in Figure 27**Erreur ! Source du renvoi introuvable.** In the next steps, this calibration curve will be important to find out the equivalent GOx concentration and GOx mass present in the analyzed piece of the samples based on the sample's absorbance results.

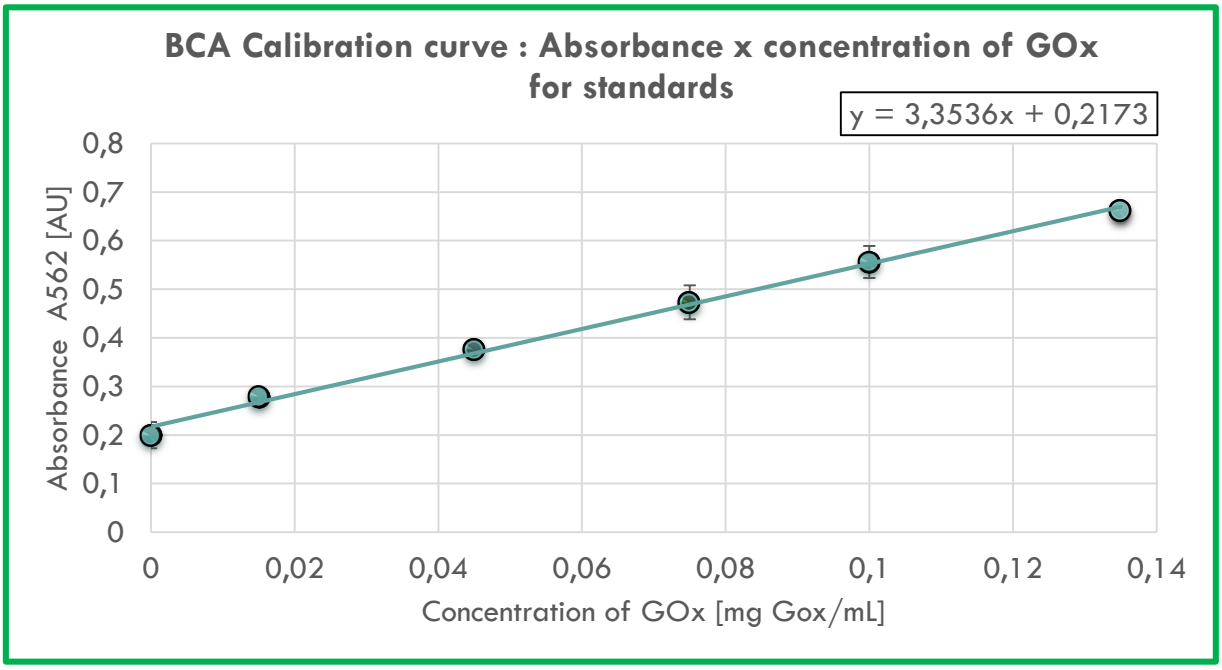


Figure 27: Calibration curve of the Absorbance x concentration of GOx for the standard solutions. On the top right, it is possible to find the equation of the tendency linear curve

From the calibration curve, it is possible to notice that the points indeed follow a linear growth tendency, this linear curve equation was represented in terms of absorbance and GOx concentration in Equation 2: BCA standards calibration curve equation.

$$Absorbance\ A562 = 3{,}3536 * GOx\ Concentration + 0{,}2173$$

Equation 2: BCA standards calibration curve equation

It is also important to mention why the blank point (i.e. zero enzyme concentration) presents an absorbance result, this non-zero absorbance value is actually normal for the BCA test. The Thermo Scientific™ Pierce™ provider normally orients the user to exclude this value of the other standard results before doing the calibration curve (Information found in User Guide BCA Protein Assay Kit) but in this analysis data treatment, this recommended step was not performed, which is not a problem because by applying the obtained calibration curve equation on the sample's absorbance results the blank absorbance will be subtracted.

5.2.2 BCA: EQUIVALENT GOX MASS PER AREA OF SAMPLE AND PER TOTAL INTERNAL AREA RESULTS

The calibration curve provides the amount of absorbance per concentrations of GOx information. With the absorbance results of the samples, it is possible to obtain the equivalent GOx concentration of the samples by simply inserting their total absorbance results in Equation 2: **BCA standards calibration curve equation** of the calibration curve. Finally, this equivalent concentration should be multiplied by water the volume in which the samples were inserted (0.04 mL) and should be divided by the area of the sample, either the area of the membrane containing the nanotubes (25 mm^2) or the total internal area of all the nanotubes in the sample piece (25 mm^2 times the total internal area per cm^2 of membrane from Table 2).

It is important to mention that the interpretation of both result forms has some major differences. By **dividing the GOx mass by the sample area** it is possible to do some primary comparison on how the enzyme's deposition occurred for samples assembled with different PE concentrations and BLs, this result may also provide information on the amount of sample preparation needed for a known industrial application, per example. However, the GOx mass per sample area results of samples with different pore diameters may not be compared because the samples with different pore sizes present different total internal pore areas, which is the only area where the enzymes are only deposited (assured by the decrusting step).

And **Dividing the GOx mass by the total internal area of the sample piece** provide us definitive results regarding the adsorption of the enzyme in the samples and allow us to compare the samples regarding the three different assembly parameters (PE concentration, number of bilayers, and pore diameter) because in this case the enzyme mass is actually divided by the area where the adsorption occurred.

Each one of the three sets of results will be analyzed and the main observations are focused on observing the correlations between the different assembly parameters and the amount of GOx absorbed per area (of sample or total internal area):

(1) Correlation between the number of bilayers and mass of GOx adsorbed:

Evaluated by comparing the amount of GOx adsorbed results of samples (from the same set) with the same pore diameter and different number of bilayers (e.g., samples 250 nm 2BL and 4BL; samples 400 nm 2BL and 4BL; samples 800 nm 2BL and 4BL)

(2) Correlation between diameter and mass of GOx adsorbed:

Evaluated by comparing the enzyme adsorption of samples (from the same set) that were assembled with the same number of bilayers and different diameters (e.g., samples assembled with 250, 400, and 800 nm with 2BL; and the same case for 4BL).

(3) Correlation between polyelectrolytes concentration and mass of GOx adsorbed:

Evaluated by comparing the amount of GOx absorbed of samples from different sets with the same diameter and number of bilayers (e.g., comparing the samples 250 nm 2 BL of the set 1 and set 2).

5.2.2.1 BCA: SET 1 RESULTS

The absorbance results of the **set 1 (PE concentration = 0.1 mg/mL)** and its final results calculations for the equivalent concentration and the equivalent GOx mass divided by the area of the sample and by total internal area may be found in **Table 4**: BCA absorbance results and equivalent mass of enzyme per area calculation for each sample assembled with PEs concentration 0.1 mg/mL in set 1..

Table 4: BCA absorbance results and equivalent mass of enzyme per area calculation for each sample assembled with PEs concentration 0.1 mg/mL in set 1.

BCA Set 1: Mass of GOx per area (of sample or by total int. area)						
Sample (diameter and BL)	**First extraction A562 [AU]**	**Second extraction A562 [AU]**	**Total absorbance A562**	**Equivalent Concentration [mg/mL]**	**Eq. Mass of GOx per area of sample [µg/cm²]**	**Eq. Mass of GOx per total internal area of pores [µg/cm²]**
250 nm 2 BL	0,490	0,217	0,707	0,146	**23,36**	**2,96**
250 nm 4 BL	0,664	0,234	0,898	0,203	**32,48**	**4,11**
400 nm 2 BL	0,754	0,266	1,020	0,239	**38,30**	**3,04**
400 nm 4 BL	0,842	0,261	1,103	0,264	**42,26**	**3,35**
800 nm 2 BL	0,821	0,239	1,060	0,251	**40,21**	**1,67**
800 nm 4 BL	0,869	0,278	1,147	0,277	**44,36**	**1,84**

From the results of Table 4 it is possible to perform two main observations:

(1) **Correlation between the number of bilayers and mass of GOx adsorbed:**

When comparing samples with the same pore diameter and different BLs it is possible to observe that the results of GOx mass per area (of the sample and per total internal area) increase with the increase of the number of bilayers, which is trivial. Additionally, the samples with diameters 400 and 800 nm had a 10% increase of GOx, while the samples with 250 nm had an increase of 39% of GOx's mass.

(2) **Correlation between diameter and mass of GOx adsorbed:**

It is possible to compare the mass of GOx per total internal area results of samples that contain the same number of bilayers and different diameters and observe that there is a general tendency that the membranes with small pore diameter tend to present higher enzyme adsorption per pore area, this trend was observed for both amounts of BLs. In other words, it is possible to observe that for 2BL and 4BL the ascending result order based on the sample's diameter is: 800, 400, and 250 nm.

5.2.2.2 BCA: SET 2 RESULTS

The absorbance, equivalent GOx concentration, and its equivalent mass results divided by the analyzed area of the **set 2 (PE concentration =1.0 mg/mL)** may be found in Table 5**.**

Table 5: BCA absorbance results and equivalent mass of enzyme per area calculation for each sample assembled with PEs concentration 1.0 mg/mL in set 2.

BCA Set 2: Mass of GOx per area (of sample or total int. area)						
Sample (diameter and BL)	**First extraction A562 [AU]**	**Second extraction A562 [AU]**	**Total absorbance A562**	**Equivalent Concentration [mg/mL]**	**Eq. Mass of GOx per area of sample [µg/cm²]**	**Eq. Mass of GOx per total internal area of pores [µg/cm²]**
250 nm 2 BL	0,630	0,242	0,872	0,195	**31,2**	**3,95**
250 nm 4 BL	1,208	0,339	1,547	0,396	**63,4***	**8,03***
400 nm 2 BL	0,700	0,268	0,968	0,224	**35,8**	**2,84**
400 nm 4 BL	0,778	0,269	1,047	0,247	**39,6**	**3,14**
800 nm 2 BL	0,941	0,290	1,231	0,302	**48,4**	**2,01**
800 nm 4 BL	1,595	0,344	1,939	0,513	**82,1**	**3,41**

By observing the results of the set 2 (Table 5) it is possible to perform two observations:

(1) **<u>Correlation between the number of bilayers and mass of GOx adsorbed:</u>**

The increase of bilayers increases the amount of enzyme deposited (for both types of results) and, just as for the set 1, the samples with diameter 250 nm had the biggest deposition increase. The mass increases were the following: for 250 nm there was an increase of 100%; for 400 nm a 10% increase; and for 800 nm a 70% increase. The results of the sample 250 nm 4BL have an (*) because they are much higher than for the other samples, therefore it would be ideal to re-do the assembly in order to verify veracity.

When comparing with the observation of set 1 it is noticeable that the mass increase results of the sample with diameter 400 nm is the same for both sets, and for the samples 250nm and 800nm the mass increase in set 2 is much higher.

(2) **<u>Correlation between diameter and mass of GOx adsorbed</u>**:

Globally, there seems to exist a tendency that samples with smaller pore diameters present higher enzyme adsorption per total internal area, this is observed for both 2BL and 4BL and the same trend was noticed in the set 1 results.

5.2.2.3 BCA: SET 3 RESULTS

The BCA results for **set 3 (PEI concentration = 1.0 mg/mL and GOx concentration >1.0 mg/mL)** may be found in Table 6**.** As mentioned in the Materials and Methods section, for the set 3 a problem occurred during the preparation of the GOx polyelectrolyte solution for the LBL assembly and therefore the exact real concentration of the enzyme PE for the build-up is unknown, but it is still possible to know that it is bigger than 1.0 mg/mL in the same order of magnitude. Fortunately, this conflict situation will not cause any further error propagation or problems in this thesis because by knowing the GOx mass per area results (shown in Table 6) it is possible to calculate the essential activity per mass information necessary for the project (independently of the GOx polyelectrolyte concentration).

Table 6: BCA absorbance results and equivalent mass of enzyme per area calculation for each sample assembled with PEI concentration 1.0 mg/mL and unknown GOx concentration higher than C=1.0 mg/mL in set 3.

BCA Set 3: Mass of GOx per area (of the sample or by total int. area)						
Sample (diameter and BL)	**First extraction A562 [AU]**	**Second extraction A562 [AU]**	**Total absorbance A562**	**Equivalent Concentration [mg/mL]**	**Eq. Mass of GOx per area of sample [µg/cm²]**	**Eq. Mass of GOx per total internal area of pores [µg/cm²]**
250 nm 2 BL	0,453	0,213	0,666	0,134	**21,4**	**2,71**
250 nm 4 BL	0,511	aprox. = 0,240	0,751	0,159	**25,5**	**3,22**
400 nm 2 BL	0,592	0,225	0,817	0,179	**28,6**	**2,27**
400 nm 4 BL	0,681	0,231	0,912	0,207	**33,1**	**2,63**
800 nm 2 BL	0,805	0,266	1,071	0,255	**40,7**	**1,69**
800 nm 4 BL	0,818	0,261	1,079	0,257	**41,1**	**1,71**

The second BCA extraction of the 250 nm 4 BL sample was not a success, therefore the result was found by a proportion approximation: first, the first extraction value of the 250 nm 4BL sample was divided by the absorbance of the 250 nm 2BL (first extraction as well), this ratio rudely estimates how much the absorbance increases with the increase of bilayer; then the absorbance value of the second extraction of 250 nm 2BL was multiplied by this ratio, representing the absorbance value the 4BL sample would have if the proportionality ratio continues and is used as an approximated results for the second extraction of the 250 nm 4BL.

Regarding the Table 6 results for the set 3 samples, it is possible to perform two observations:

(1) **Correlation between the number of bilayers and mass of GOx adsorbed:**

Increasing the number of bilayers increases the mass deposited (observed by both types of areas). The samples assembled with 400nm diameter presented an increase of 16% of the mass (result similar to the ones of set 1 and set 2 for the same diameter) and the ones with 800 nm diameter only had a 1% mass increase with the increase of BL, which is not logical and therefore or a problem occurred during the BCA measurements (more probable) or there was a problem with the build-up (less probable since the LBL as the same for all the samples). The increase of GOx mass in the samples with 250 nm diameter will not be analyzed due to the approximation performed for the second extraction of the 250 nm 4BL sample.

(2) **Correlation between diameter and mass of GOx adsorbed:**

When comparing the results of samples with the same number of bilayers and different diameter it is possible to observe that for both 2BL and 4BL there is a tendency that the decrease of the diameter provokes an increase in the amount of GOx absorbed per total internal area, which is the exact same observation done for the results of set 1 and set 2.

5.2.2.4 BCA: COMPARISON SET 1, SET 2, AND SET 3 RESULTS

Finally, the results of the three sets are compared and compiled in Table 7 to study how varying the PE assembly concentration influences the mass per total internal area results (this comparison could have also been performed with the results divided by sample's area).

Table 7: Compilation of equivalent mass per sample area results for all three sets of samples.

Sample	Set 1 (C= 0.1 mg/mL): Eq. Mass of GOx per total internal area of pores [µg/cm²]	Set 2 (C= 1.0 mg/mL): Eq. Mass of GOx per total internal area of pores [µg/cm²]	Set 3 (GOx>1.0 mg/mL): Eq. Mass of GOx per total internal area of pores [µg/cm²]
250 nm 2 BL	**2,96**	**3,95**	**2,71**
250 nm 4 BL	**4,11**	**8,03***	**3,22**
400 nm 2 BL	**3,04**	**2,84**	**2,27**
400 nm 4 BL	**3,35**	**3,14**	**2,63**
800 nm 2 BL	**1,67**	**2,01**	**1,69**
800 nm 4 BL	**1,84**	**3,41**	**1,71**

From Table 7 it is possible to compare the results of the set 1 and set 2 and observe:

(3) Correlation between polyelectrolytes concentration and mass of GOx adsorbed:

By comparing the GOx deposition results of samples from two different sets that have the same assembly configuration, it is possible to observe that for samples with diameters 250 and 800 nm there is a trend that the increase of the PE concentration (set 2) results in an increase in the GOx mass per area results for both 2BL and 4BL cases. The same trend was not observed for the samples with diameter 400 nm, for this case the deposition presented higher results in the set assembled with lower PE concentration (set 1).

The set 3 will not be compared with the set 1 and 2 because its exact GOx assembly concentration is unknown and the concentration of the PEI and GOx solutions are different (uneven), which may result in a different enzyme adsorption and therefore its results may not be compared with the other sets. However, it is possible to observe that for this set the overall GOx adsorption results were lower than for the other two sets.

5.2.3 BCA: PARTIAL DISCUSSION OF RESULTS

The trends observed regarding the influence of the assembly parameters on the GOx adsorbance results of the different samples (and of different sets) were the following:

(1) **Correlation between the number of bilayers and mass of GOx adsorbed:**

For all three sets of samples, it was observed that the increase of bilayers increases the amount of GOx deposited per area (of sample or total internal area), which was expected and is trivial.

Additionally, for both set 1 and set 2:

a) The GOx mass increase per bilayer increase was higher for the samples assembled with the diameter 250 nm (100% increase for set 2 and 70% increase for set 1);
b) For samples assembled with 800 nm, the GOx mass increase from 2BL to 4BL was 70% for set 2 and 10% for the set 1;
c) For samples with diameter 400 nm the GOx mass increase (from 2BL to 4BL) was 10% for both sets.
d) This higher GOx absorption observed in the set 2 for (a) and (b) could be an indication that samples with diameter 250 and 800nm & assembled in higher PE concentration present a higher growth of layers (i.e., more deposition of electrolytes). The samples with a diameter 400 nm appear to grow faster in low PE.

For set 3 the growth of samples with a diameter 250 nm could not be measured and for the 800 nm the growth percentage had an irregular result of 1%, indicating that probably a problem occurred during the BCA analysis for the 800 nm 2BL and 4BL samples. And the growth of the samples with 400 nm was 16%, similar to the one observed for sets 1 and 2.

(2) **Correlation between diameter and mass of GOx adsorbed:**

When comparing the GOx mass per total internal area results of the samples with the same number of bilayers and different diameters the same trend was observed for both 2BL and 4BL for all three sets of samples: the decrease of pore diameter increases the amount of GOx adsorbed. When comparing this trend with the theory of the two types of regimes in section 2.1.3 Layer by layer in pores) 2.4.4 Immobilization in Layer by layer & Nanotubesit is possible to presume that this trend occurs because of the gel effect and spider-web-like deposition forming in the nanotubes with lower diameter, and therefore a higher PE amount can be adsorbed.

(3) **Correlation between polyelectrolytes concentration and mass of GOx adsorbed:**

For the set 1 and set 2, when comparing the GOx per area (area of sample or internal area) results of the samples with the same assembly conditions (diameter and BLs) it is possible to observe that:

a) For the samples with diameters 250 nm and 800 nm, there is a trend that the samples assembled with higher PE concentration (set 2) present a higher amount of GOx deposited, this was observed for both 2BL and 4BL;
b) For the samples with 400 nm, the trend observed was the opposite, samples assembled with lower PE concentration (set 1) had higher GOx absorption results.

It is also possible to notice that in the observation (1) **Correlation between the number of bilayers and mass of GOx** the point (d) commented that: for the samples with diameter 250 and 800 nm, the increase of GOx amount per increase of bilayer was bigger when the samples were assembled with higher PE. And now at the observation (3) **Correlation between PE concentration and mass of GOx** point (a) notices that: for the diameters 250 and 800 nm, when comparing the samples with the same BL and diameter (e.g., 250 nm 2BL from set 1 and set 2) the ones assembled with higher PE concentration present higher GOx deposition. Combining these two observations it is possible to indeed prove that the samples with diameter 250 nm and 800 nm present a higher growth of layer (GOx deposition amount per layer).

For the set 3, it was observed that the overall GOx deposition results were lower than for the other two sets, possibly indicating that the assembly with uneven PE concentrations occurs differently than for even PE concentrations. but it was not possible to perform any trends observation because the GOx's assembly concentration exact value is unknown.

It is important to remember that samples that present the higher GOx adsorption per area do not necessarily present more activity because this will depend on the efficiency of the activity per amount of GOx mass results.

5.3 MEASUREMENT OF THE ENZYME'S ACTIVITY IN STATIC CONDITIONS

This process measures the GOx's activity per mass amount in a reaction performed in static conditions. Providing information on how the sample's immobilized enzyme behaves in a reaction without flows, carried over time while the substrate is constantly being consumed and the product is accumulating. This measurement simulates a real-life batch reaction application of the samples and allows us to compare how the different assembly parameters will affect the performance of the samples' immobilized enzyme. However, it does not provide any specific information if variations are occurring during the enzymatic reaction (e.g., peaks, denaturation, or desorption) and it is limited by absorbance saturation (in the spectrometer) or substrate consumption.

The measurement was made by extracting a 1mm diameter circular piece of the sample (i.e., PC membrane with embedded nanotubes) and inserting it in the wells of a 96-well tray containing water in each well. Following, the POD mix and glucose were added. The entire preparation was carried on an ice tray to slow down the beginning of the reactions so that all the measurements are triggered at the same time. The plate was then analyzed in a spectrometer at wavelength A_{510}, this absorbance data may be correlated with the activity of the GOx enzyme. Finally, the blank's absorbance was removed from the absorbance results, the absorbance of the values was divided by the sample's area (0,782 mm^2) and then this final value was divided by the BCA results of GOx mass per area of the sample. The final results are obtained in absorbance A_{510} per GOx mass.

The measurement was performed two times on set 1 samples (on two different days), and it one time on the set 3 samples. The test was not performed on set 2. The analysis of the results are majorly focused on observing if the assembly parameters are or not correlated with the changes in GOx activity, for which four main points are examined:

(1) **<u>Correlation between the number of bilayers and activity per GOx mass results</u>**:
Evaluated by comparing the activity (absorbance per GOx mass) results of samples from the same set with same pore diameter and different number of bilayers (e.g., samples 250 nm 2BL and 4BL; samples 400 nm 2BL and 4BL; samples 800 nm 2BL and 4BL)

(2) **<u>Correlation between diameter and activity per GOx mass results:</u>**
Evaluated by comparing the activity results of samples (from the same set) that were assembled with the same number of bilayers and different diameters (e.g., samples assembled with 250, 400, and 800 nm with 2BL; and the same case for 4BL).

(3) **<u>Correlation between the time interval of analysis and the analysis activity per GOx mass:</u>**
The results from the same samples analyzed on day 1 and day 8 are compared to see if storing the samples affects the GOx activity performance of the samples. (e.g., comparing the samples 250 nm 2 BL of the set 1 analyzed on day 1 and day 8).

(4) **<u>Correlation between polyelectrolytes concentration and activity per GOx mass:</u>**
Evaluated by comparing the samples of different sets with the same diameter and number of bilayers (e.g., comparing the samples 250 nm 2 BL of the set 1 and set 2).

5.3.1 STATIC ACTIVITY: SET 1 RESULTS

The static activity measurement was performed two times for the set 1 samples, the first one was performed on the same day as the samples were assembled (this results will be named set 1 – day 1) and the second measurement was performed 8 days later (results referred as set 1 – day 8), both experiments were performed using the same samples and following the same protocol, the only difference between them is the time interval.

5.3.1.1 STATIC ACTIVITY: SET 1 DAY 1 RESULTS

As mentioned, after obtaining the absorbance values the blank should be removed from the results, the blank absorbance result obtained for this set is equal to 0.04 AU. Following, the remaining absorbance value was divided by the area of the circular piece of the sample and correlated with the BCA test (Table 4), the final average result of the triplicate test (with its standard deviation) is presented in a graph over time of reaction in **Figure 28**.

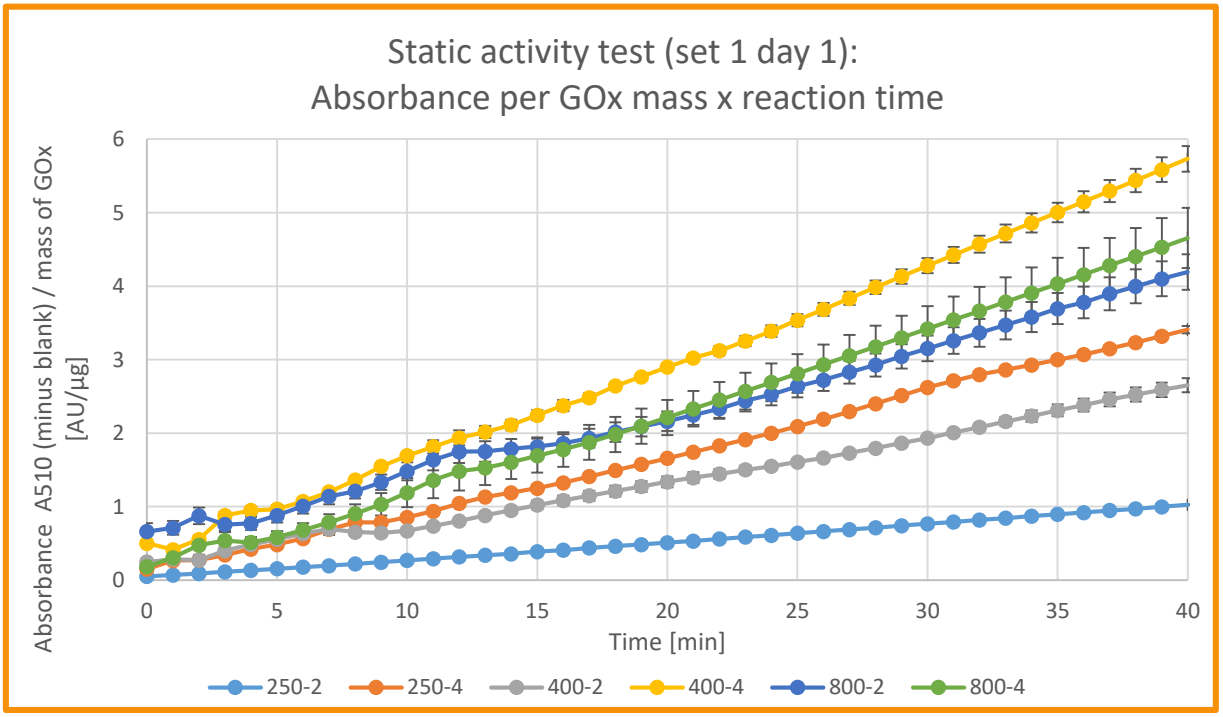


Figure 28: Static test for set 1 day 1, the results are given in absorbance (minus blank) /mass of GOx x Reaction time. The triplicated results are shown with their standard deviation.

From Figure 28 we should observe the influence of the two assembly parameters:

(1) **<u>Correlation between the number of bilayers and activity per GOx mass results</u>**:

By comparing samples with the same diameter and different number of BLs it is noticeable that the increase of the bilayers has a tendency of increasing the activity results for the samples with diameters 250 and 400 nm. The same tendency was not observed for the sample with a diameter of 800 nm since the results for 2BL and 4BL are very similar and are often located inside their standard deviation range.

(2) **Correlation between diameter and activity per GOx mass results:**

It is possible to identify an ascending order of activity results based on the sample's diameters for the 2 types of bilayers. For the 2BL the ascending order are the samples: 250, 400, and 800 nm. And for samples with 4 BL, the order is: 250, 800, and 400nm. The two ascending orders are not the same, therefore at this stage is still not possible to find any possible correlation or tendency. However, based on the curve it is possible to notice that the activity of samples with higher pores is higher than for smaller pores.

5.3.1.2 STATIC ACTIVITY: SET 1 DAY 8 RESULTS

The blank absorbance value obtained for this measurement was also equal to 0.04 AU. Identically as before, the blank was subtracted from the samples' absorbance and then the absorbance was correlated with the amount of GOx enzymes based on the BCA results (Table 4). The final triplicate average absorbance per mass of GOx over time of reaction results (and standard deviation of the results) for all the samples may be found in **Figure 29**.

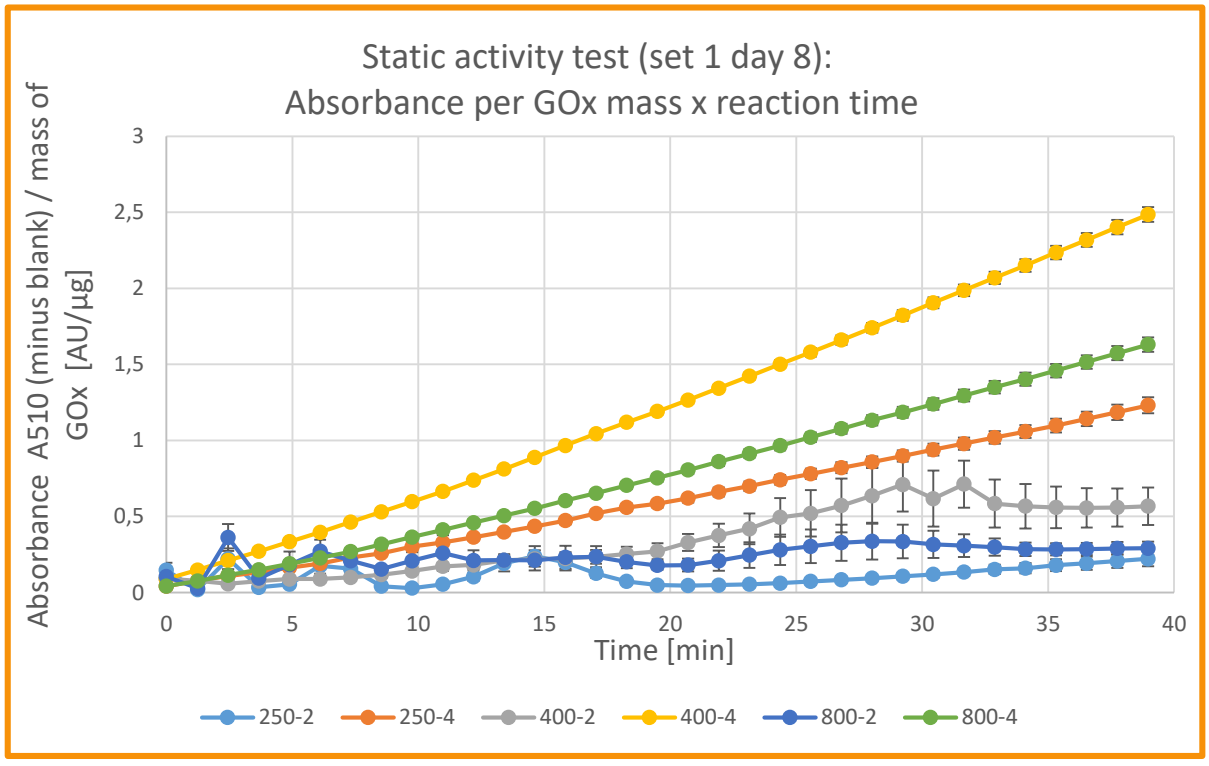


Figure 29: Static test for set 1 day 8, the results are given in absorbance (minus blank) /mass of GOx x Reaction time. The results were performed in triplicate and their standard deviation is shown in the graph.

The first thing to be commented on about Figure 29 is that this graph is represented in a Y-axis two times smaller than the one of set 1 day 1 results, this is done so it is possible to observe the results in more details.

Regarding the results of set 1 day 8, two main observations are made:

(1) **Correlation between the number of bilayers and activity per mass of GOx results**:

When comparing samples with the same diameter and different BL it is possible to notice that for all three types of diameters there is a tendency that the increase of the BL also results in an increase of the absorbance per GOx mass results, suggesting that samples with more bilayers present a tendency of having a higher GOx activity performance.

(2) **Correlation between diameter and activity per mass of GOx results:**

The ascending results order for the samples assembled with 2BL, based on their diameter, is: 250, 800, and 400 nm; and for 4BL the order is: 250, 800, and 400 nm. The order is the same between the 2BL and 4BL (and it is also equal to the order observed for the set 1 day 1 4BL samples), possibly indicating a trend between diameter and activity.

5.3.1.3 STATIC ACTIVITY: COMPARISON SET 1 DAY 1 AND DAY 8

The results of set 1 day 1 and set 1 day 8 samples regarding GOx's activity per enzyme quantity over the time of reaction were merged into one graph in Figure 30.

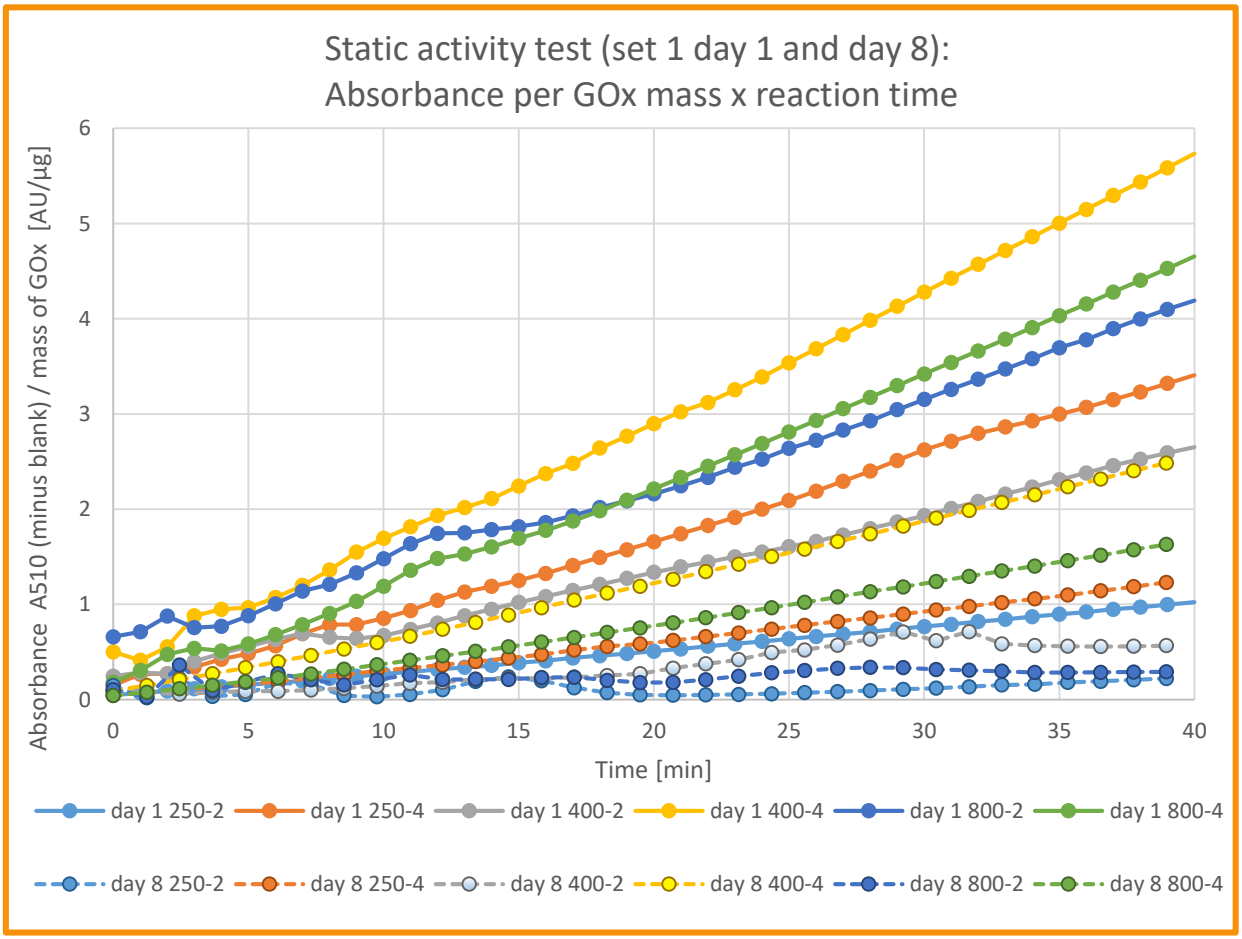


Figure 30: Static test for set 1 day 1 and day 8, the results are given in absorbance (minus blank) /mass of GOx x Reaction time.

The Figure 30 results of the set 1 day 1 and day 8 are compared regarding the interval:

(3) **Correlation between the time interval of analysis and the analysis activity GOx mass:**

When comparing samples with the same assembly parameters analyzed in two different days (day 1 and day 8) and comparing their activity results (e.g., comparing sample 250 nm 2BL from set 1 analyzed in day 1 and day 8) it is possible to observe that: the samples analyzed in the day 1 present higher activity performance results than the ones observed on day 8. This may also be easily noticed by observing that the range of results in the day 1 samples is higher than for day 8. This activity performance decrease between day 1 and day 8 analysis may have occurred due to denaturation of the immobilized enzymes after storing or due to the dehydration of the samples (decreasing the amount of water absorbed between the layers and jeopardizing substrate entering in contact with GOx).

Another detail to be observed is that both day 1 and day 8 almost present the same ascending order of samples' results, the only sample that does not follow the order (and that has suffered the most dramatic reduction of activity) was the sample 800 nm 2BL.

5.3.2 STATIC ACTIVITY: SET 3 RESULTS

The activity measurement was performed 1 day after the assembly of the set 3 samples. Again, the blank absorbance measured was 0.042 AU, this value was subtracted from the samples' absorbances before they were divided by the mass quantity of GOx. The set 3 results of the static activity measurement are represented in Figure 31.

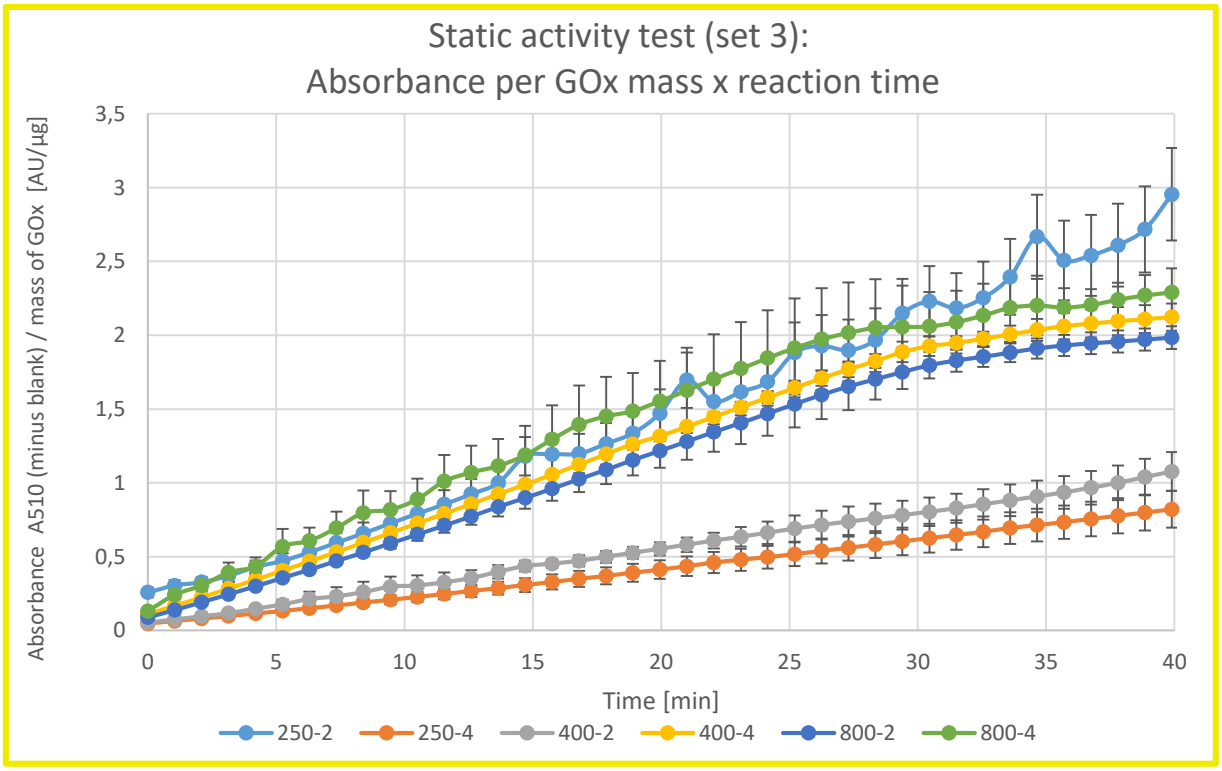


Figure 31: Static test for set 3, the results (with dev.) in absorbance (minus blank) /mass of GOx x Reaction time

Regarding the static activity results of set 3, the same observations are performed:

(1) **Correlation between the number of bilayers and activity per GOx mass results:**

When comparing the samples that have the same diameter and a different number of bilayers it is possible to observe that for the diameter 250 nm and 400nm the increase of BL tends to decrease the absorbance per GOx mass results, this trend is the opposite of the one observed for the set 1 (day 1 & 8) results. The samples 800 nm 2 BL and 4BL presented very close results and therefore are not distinguished.

(2) **Correlation between diameter and activity per mass of GOx results:**

In samples with the same number of bilayers and different diameters it is possible to obtain the ascending order of activity performance results based on the sample's diameter. For the samples assembled with 2BL, the ascending order of results is: 400, (800, and 250 nm), the () indicates that samples present very similar results. For the samples of 4BL, the ascending order is: 250, (400, and 800 nm). Since there is no reproducibility between the order observed for 2BL and 4BL samples, therefore it is presumable that when only observing the results of set 3 that no correlation can be found.

5.3.3 STATIC ACTIVITY: COMPARISION SET 1 & DAY 8 AND SET 3 RESULTS

The activity per GOx mass results of set 1 (day 1 & day 8) and set 3 samples are compared in the same in Figure 32.

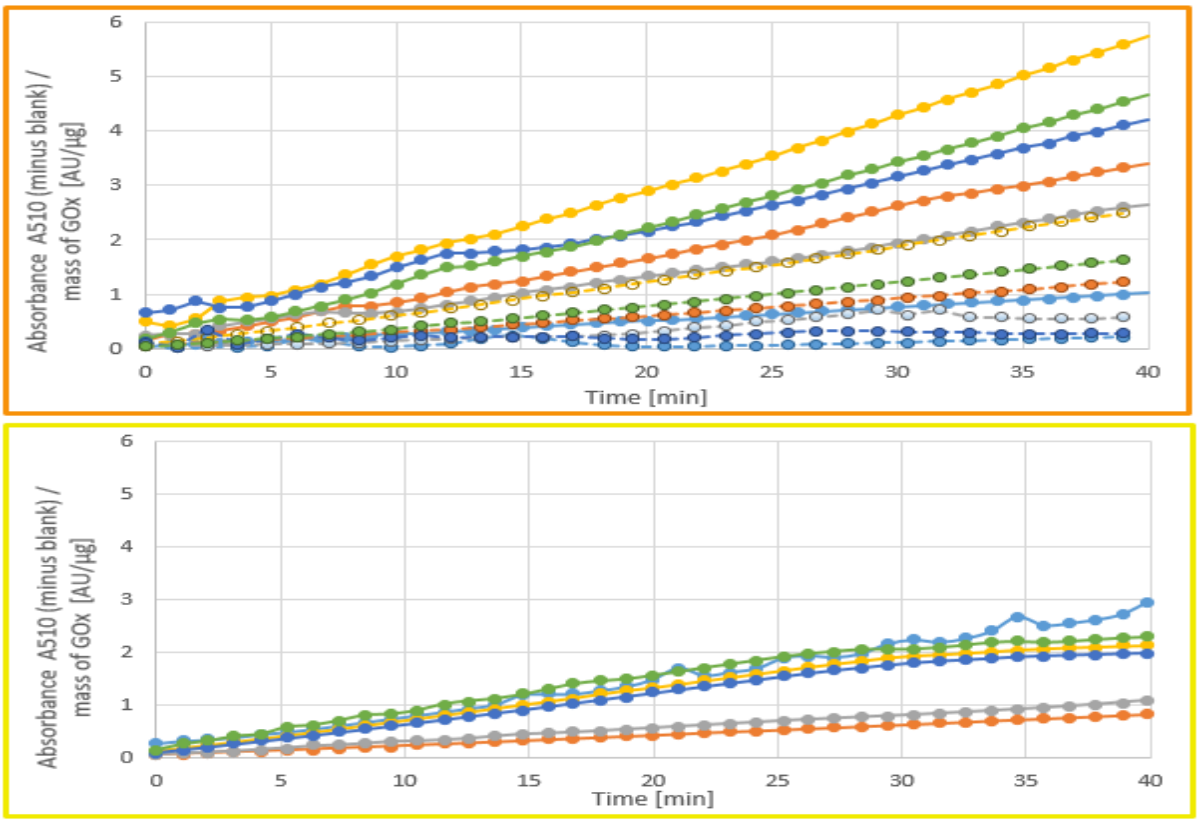


Figure 32: Static test results for set 1 (day 1 and day 8) and set 3, graphs shown for range comparison.

By comparing the results range for the set 1 and set 3 it is possible to do observations regarding the influence of PE concentration on the absorbance per GOx mass results. Here, the PE comparison is discussed for both sets but it is important to recall that the set 3 presents an uneven concentration of PEI (C=1.0 mg/mL) and GOx (unknown concentration higher than 1.0 mg/mL), and the set 1 presents an even PE concentration (C=0.1 mg/mL). Therefore, in reality, the two sets are not 100% comparable since they present different variations (even and uneven), and different concentrations and the uneven concentration of PE may possibly provoke unpredicted changes in the assembly profile.

(4) **Correlation between polyelectrolytes concentration and activity per GOx mass results:**

When comparing the results of set 1 day 1 and day 8 (observation number (3), section 5.3.1.3 Static activity: Comparison set 1 day 1 and day 8) it was observed that the time interval between analyses provokes a general decrease in the activity's results. Therefore, in principle, the results of set 3 (analyzed 1 day after the assembly) should be compared with the results of set 1 day 1 (analyzed on the same day as assembly) because they both had a small interval between assemblies and analysis.

However, when comparing the range of the sets' results (set 1 day 1, day 8 and set 3) it is possible to observe that the results range of set 3 is located between 0 until 3.0 [AU/µg], which is the same range as the results of set 1 day 8 results and is 2 times smaller than the one for set 1 day 1 results. Meaning that the activity results of set 3 (analyzed performed one day after assembly) are a lot smaller than the results of set 1 day 1 (same day analysis), which can be caused by three possible reasons: or this range is smaller than the range of set 1 day 1 because the set 1 and set 3 are not comparable due to PE concentration with an uneven variation; or the increase of GOx concentration decreases the activity performance per GOx amount; or the one day time interval is already sufficient to cause the enzyme's specific activity to decrease (e.g., layers dehydration or denaturation of the enzymes).

When comparing the overall ascending results order of the samples it was not possible to find any reproducibility between set 1 and set 3 results.

5.3.4 STATIC ACTIVITY: PARTIAL DISCUSSION OF RESULTS

The final wrapped up conclusions regarding how the assembly parameters (PE concentration, diameter, and BL number) and how the time interval between analyses affect the activity results of the GOx enzymes are the following:

(1) **Correlation between the number of bilayers and activity per mass of GOx results:**

When comparing samples from the same set with the same diameter and different bilayers it was possible to observe that for the set 1 (day 1 & day 8) there is an overall tendency that the increase of bilayers increases the activity performance (i.e.g, activity per Gox mass) of the enzyme. The set 3 comparison has observed the opposite trend, the samples with a smaller number of bilayers have shown a higher specific activity of GOx.

Therefore, there are two possible trend conclusions regarding how the number of bilayers affects the enzyme's activity: (1) or indeed there is a trend that samples assembled with smaller PE concentration (set 1) present higher activity for higher bilayers number and the opposite is observed for samples assembled with higher PE concentration (set 3); (2) or there is no real and reproducible correlation between the number of bilayers and the activity results because the set 1 and set 3 may not be 100% compared. To confirm which one of these conclusions is true more analysis and repetitions should be performed.

(2) **Correlation between diameter and activity per mass of GOx results:**

In order to analyze if there is a correlation between the diameter of the samples and the activity of GOx (per mass of enzyme), we compare samples from the same set that present the same number of bilayers and different diameters and then we order the activity results in an ascending order based on the sample's diameters:

a) Samples with 2 bilayers ascending order of results described by sample's diameter:
   a. Set 1 day 1: 250, 400, and 800 nm
   b. Set 1 day 8: 250, 800, and 400 nm
   c. Set 3: 400, (800, and 250 nm), () means that the results are highly similar.

b) Samples with 4 bilayers ascending order of results described by sample's diameter:
   a. Set 1 day 1: 250, 800, and 400nm
   b. Set 1 day 8: 250, 800, and 400 nm
   c. Set 3: 250, (400, and 800 nm)

The first observation to be made is that for the set 1 day 1 results the ascending order for 2BL and 4BL is not the same and therefore, there is a possibility that the increase of bilayers has impacts differently on the activity performance of the samples with different diameter. The same point is observed for the set 3 2BL and 4BL. Only the results of set 1 day 8 present the same samples ascending order for both 2BL and 4BL

The second conclusion is that by comparing the ascending activity order of samples from different sets or different analysis days with the same number of bilayers it is not possible to observe any repetition or tendency between the diameters and the order. However, there appears to exist an overall tendency that smaller diameters present lower activity results than higher diameters (possibly due to diffusional restrictions inside of smaller pores). In conclusion, the ascending order of samples results is not fully reproduced between samples with different BLs in the same set and between samples with the same BL of different sets, but still, there may exist an overall trend that samples with smaller diameter present lower activity results than samples with high diameter, which is possibly caused by the diffusional restrictions that the substrate face to access smaller pores.

(3) **Correlation between the time interval of analysis and the analysis activity per mass of GOx results**

It is possible to analyze the influence of the analysis time interval on the activity performance by observing the range of the activity results of set 1 results of day 1 (performed on the same as the assembly) and day 8. When comparing, it observed that the range of results has decreased 50% after 8 days, leading us to the hypothesis that the storing of samples causes the enzyme's effectiveness to decrease, this may occur due to denaturation of dehydration of the layer (which jeopardizes substrate diffusion necessary for the contact between the substrate and the enzyme) (See Appendix 8.3 Diffusional restriction that affect the activity)..

The final point to be observed is that both results present the same ascending order of the samples (see the color disposition in Figure 30: Static test for set 1 day 1 and day 8, the results are given in absorbance (minus blank) /mass of GOx x Reaction time.), except for the sample 800 nm 2 BL. Which indicates that the activity reducing due to time interval occurs in the same way for almost all the samples (except sample 800 nm 2BL).

(4) **Correlation between polyelectrolytes concentration and activity per GOx mass results:**

The correlation between PEs assembly concentration and the activity performance of the enzyme is analyzed by comparing the results of sets 1 and 3. When comparing the **range of results** of the sets, it was observed that the range of set 3 (analysis was performed one day after the assembly) is more close to the range of the set 1 day 8 results than of the set 1 day 1 results. This may be due to three main reasons: (1) increasing the PEs concentration decreases enzyme's activity, or (2) one day of storing is enough to decrease the enzyme's activity (due to denaturation or dehydration), or (3) the set 1 and set 3 present different trends and may not be compared because the set 1 presents even PE concentration of 0.1 mg/mL and the set 3 presents uneven PE concentration (where PEI concentration is 1.0 mg/mL and GOx concentration is higher than one), and therefore since there are two variables in the PE concentration and the two sets are not 100% comparable. More experiments with samples assembled with different PE assembly concentrations should be performed to verify which trend is repetitiously observed in the analysis results.

When comparing the **overall ascending order** of the sample's results of the sets was:

A. Set 1 day 1**:** 250nm-2BL; 400nm-2BL; 250nm-4BL; (800nm-4BL and 800nm-2BL); 400nm-4BL; the parenthesis means that the samples had close results the difference of values is located inside of the standard deviation.
B. Set 1 day 8: 250nm-2BL; 800nm-2BL; 400nm-2BL; 250nm-4BL; 800nm-4BL; 400nm-4BL
C. Set 3: 250nm-4BL; 400nm-2BL;(800nm-2BL; 400nm-4BL; 800nm-4BL; 250nm-2BL)

By analyzing the ascending order of the two sets it is not possible to find any repetition or any optimal parameter setup.

5.4 MEASUREMENT OF THE ENZYME'S ACTIVITY IN DYNAMIC CONDITIONS

This process measures the activity of the enzymes in a reaction carried in dynamic conditions, which means that the reactants and products of the reaction are in a flow regime and the product is collected throughout specific times of the reaction. The dynamic reactions provide us precise information about the reactions' efficiency that the static analysis does not provide, such as: (1) further knowledge of how the enzymes act over time of reaction (e.g. if the GOx's activity is constant, or if the rhythm decreases or increases with time); (2) provide us possible information of how the immobilized enzymes would behave during re-use.

To perform the dynamic reaction activity test, a piece of the sample (i.e., membrane containing the nanotubes) was inserted inside of a clean filter holder. Next, the buffer was pumped through the sample to stabilize the sample's conditions and to promote some enzyme desorption (because we do not want to cause the desorption of the enzyme in the collected outcome). Following, the glucose flow was pumped through the filter holder containing the membrane, a flow of product leaves the filter holder at the same time. This product flow is collect at specific times of the reaction to be later reacted with POD mix to obtain the information on the amount of product being produced (i.e. enzyme's activity) at specific times of the reaction.

The final result is given in the unit absorbance A510 per mass of glucose oxidase. To obtain it in this form the absorbance result (with the glucose blank subtracted from the sample's results) from the spectrometry measurement is divided by the area of the sample and, then, the results of the BCA mass of enzyme per area of the sample are applied in order to obtain the final result in absorbance per GOx mass.

The measurement of the enzymes' activity in dynamic conditions was performed for the three sets of samples, and regarding the results, three main observations were performed on each set to analyze the correlation (or not) between the assembly parameters and the activity results:

(1) <u>Correlation between the number of bilayers and activity per mass of GOx results:</u>

Evaluated by comparing samples from the same set with the same pore diameter and different number of bilayers (e.g., samples 250 nm 2BL and 4BL; samples 400 nm 2BL and 4BL; samples 800 nm 2BL and 4BL)

(2) <u>Correlation between diameter and activity per mass of GOx results:</u>

Observed by comparing samples (from the same set) assembled with the same number of bilayers and different diameters. (e.g., samples assembled with 250, 400, and 800 nm with 2BL; and the same case for 4BL).

(3) <u>Correlation between polyelectrolytes concentration and activity per GOx mass results:</u>

Evaluated by comparing the samples of different sets with the same diameter and number of bilayers. (e.g., comparing the samples 250 nm 2 BL of the set 1 and set 2).

5.4.1 DYNAMIC ACTIVITY: SET 1 RESULTS

The glucose solution had to be prepared multiple times, therefore the absorbance of the glucose blank was measured for each sample of the set 1 and this value was subtracted from the sample's absorbance results, for this set the average set 1 blank absorbance result had a value of 0.04 AU. After subtracting the blank from the sample's absorbance results the final absorbances were divided by the area of the sample (1,2 x 0,5 cm^2). This obtained value was divided by the BCA mass of GOx per sample area results for set 1 (Table 4) and then finally the result of the dynamic test was obtained in absorbance per mass of GOx.

The activity measurement was performed twice (analysis performed in the same day and conditions) for the samples: 250 nm 4 BL and 800 nm 4BL, this section will first analyze the error range (measured in standard deviation) between the two measurements of the same samples before discussing the final activity results obtained for all the samples of set 1.

5.4.1.1 DYNAMIC ACTIVITY: SET 1 ERROR RANGE CALCULATION

The two measurement results of the sample 250 nm 4BL, its average value, and its standard deviation may be found in Figure 33.

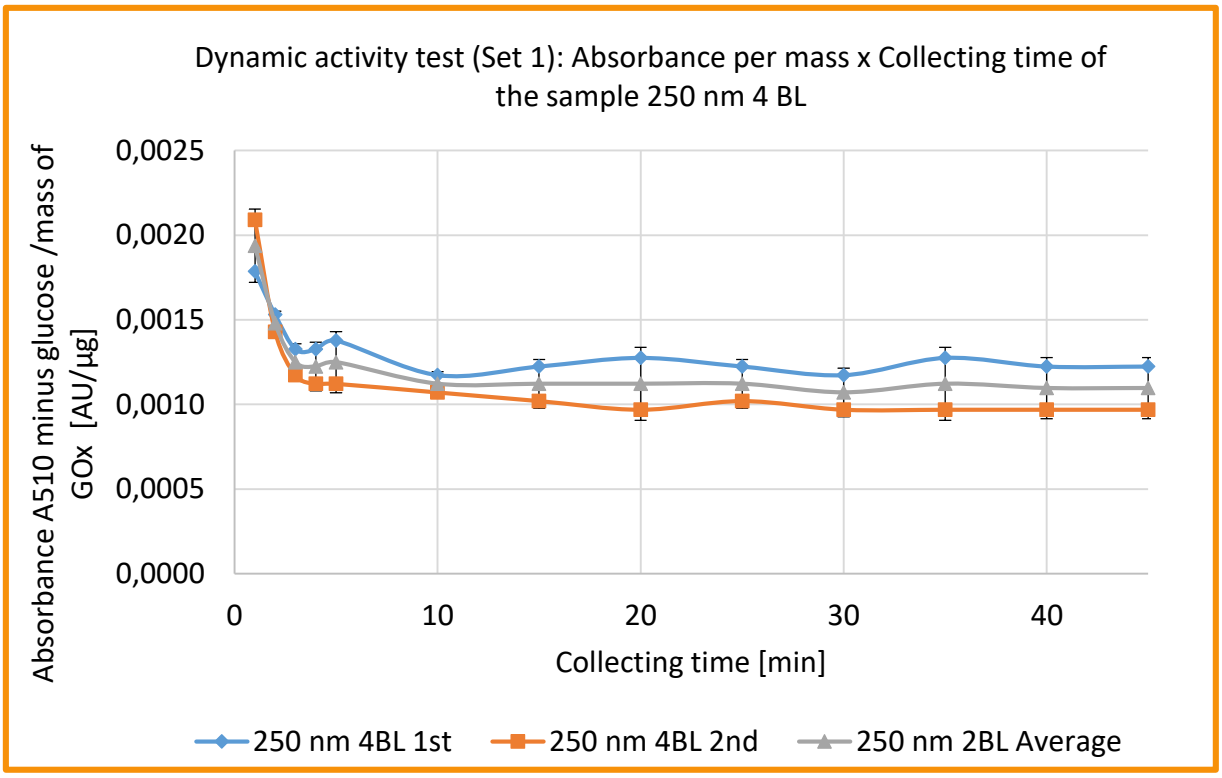


Figure 33: Dynamic test results for both measurements of sample 250 nm 4BL (set 1), the results are given in absorbance (without glucose) /mass of GOx x Collecting reaction time.

The error average was measured and its value is ±0.00015, this value may seem small but the activity per mass results are also small, therefore the error range mighty be an important factor when comparing the results of different samples. For example, at the time 20 minutes, the standard deviation is at its maximum and its value is ±0,000 while the average absorbance per GOx mass is 0.0011 [AU/µg], therefore the error corresponds to 18% of the value which is a significant amount, and may be even more significant when comparing the results difference of different samples.

The same process was repeated for the sample 800 nm 4BL and its activity per GOx mass results, its average, and its standard deviation may be found in Figure 34.

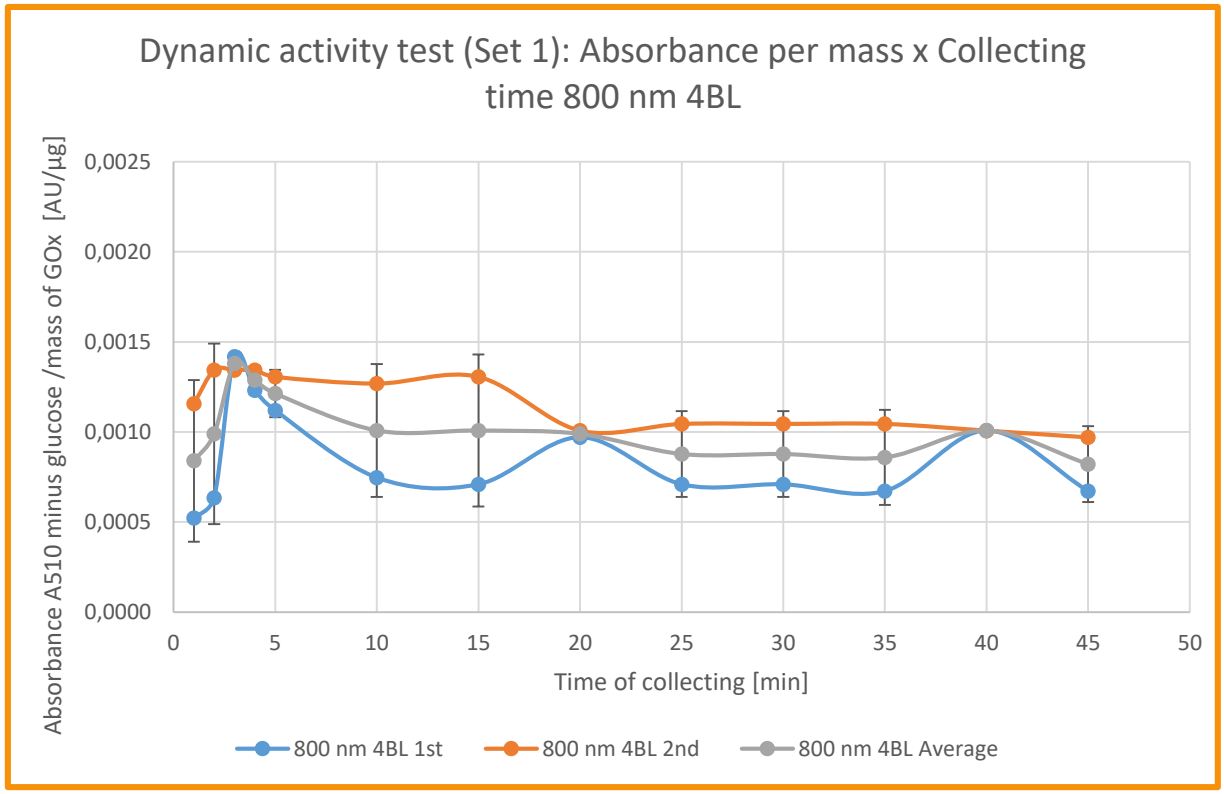


Figure 34: Dynamic test results for both measurements of sample 800 nm 4BL (set 1), the results are given in absorbance (without glucose) /mass of GOx x Collecting reaction time

The average standard deviation for the 800 nm 4BL results is ±0.00023, which is significant considering that the results of the measurements are in the Y-axis range 0.008 and 0.0013 [AU/µg].

Further, by comparing both standard deviations it is possible to obtain an average value of 0.00019, this value will be used as a "hypothetical error deviation value" of the other samples since their standard deviation was not measured. It is important to mention that this is just a hypothetical value and this approach is far from being precise (or correct).

5.4.1.2 DYNAMIC ACTIVITY: SET 1 SAMPLES RESULTS

The dynamic activity per GOx mass curves obtained from the results of the samples of set 1 and the average results of the samples 250 nm 4BL and 800 nm 4BL (with its average deviation) may be seen in Figure 35.

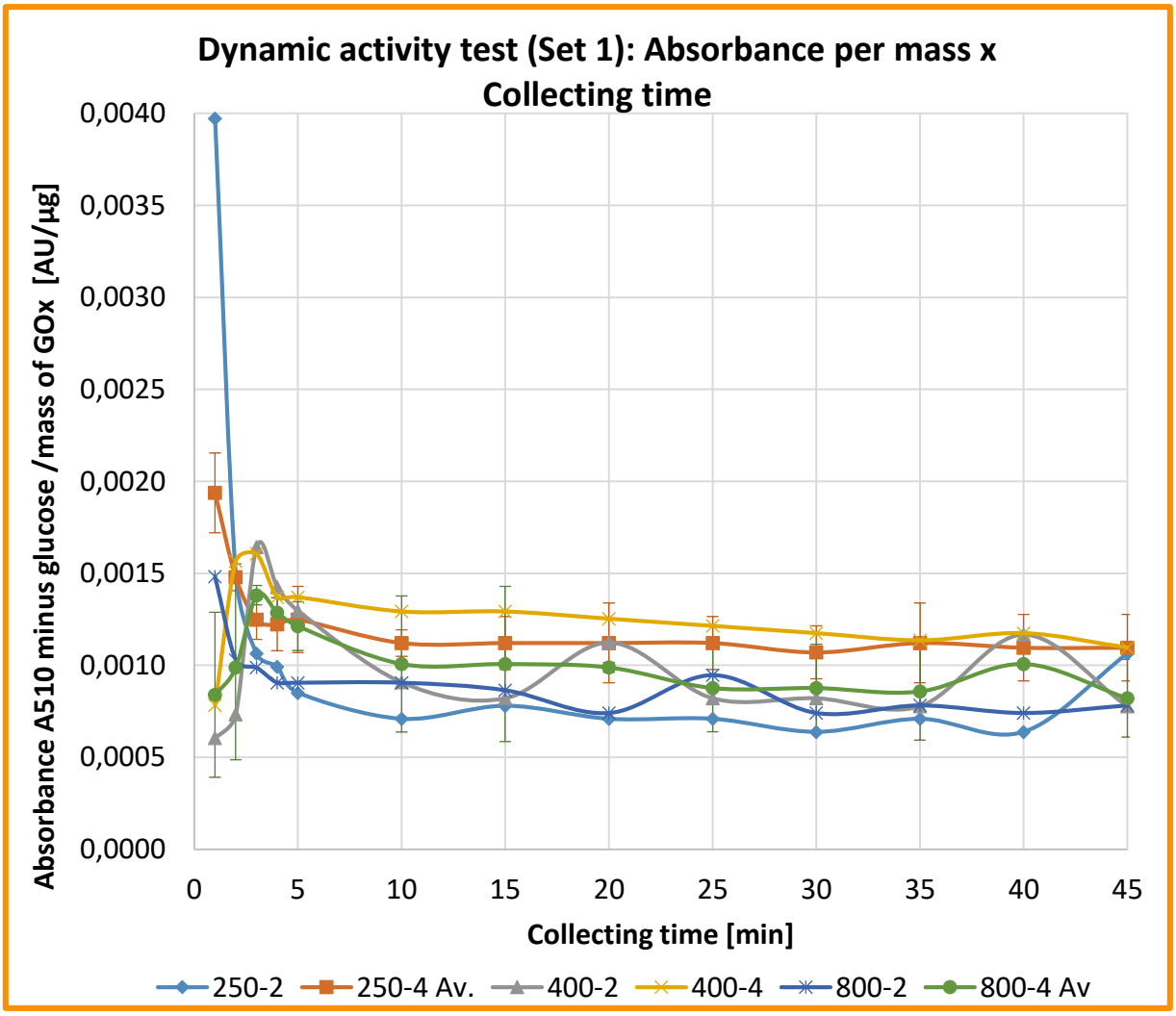


Figure 35: Dynamic test for set 1, the results are given in absorbance (without glucose) /mass of GOx x Collecting reaction time

From Figure 35, the first point to be observed is that during the first minutes of reaction there is a big variation in results for all the samples and after about 5 minutes the reaction reaches a more stable pattern (even though oscillations are still observed). During the first minutes all samples have reached their maximum value of absorbance per mass of enzyme, some samples' results have started at their maximum (250 nm 2BL & 4BL and 800 nm 2BL) and some samples' results have started low then increased until their maximum (400 nm 2BL & 4BL and 800 nm 4BL). This maximum point at the start is possibly due to enzyme desorption in the collected outcome or possibly due to the high availability of the enzyme. And the stability reached after 5 minutes is possible due to the product consumption & enzyme occupation occurring at the same rate as product formation (i.e., "steady-state phase").

The second point to be observed is that, after 5 minutes, the samples' "plateau" result curves are relatively close to each other and they often interpenetrate each other while the results oscillate. This oscillation is a possible indicator of the error range of the results of the samples, and it is possible to notice that the range of oscillation is often as big as the difference of results between samples. Therefore, without obtaining the results' standard deviation for all samples it is questionable if there is a real difference of absorbance per GOx mass results between samples or if the results are due to the error range of the experiment (e.g. error range due to fluctuation of results and due to manual work during collecting).

And finally, by analyzing the curves starting at 5 minutes (due to stabilization) it is possible to make the following remarks regarding the possible correlation between the assembly parameters and the activity results:

(1) **Correlation between the number of bilayers and activity per mass of GOx results:**

When comparing the activity results of samples with the same diameter and different number of bilayers it is possible to notice that for each type of diameter there is a slight increase in the activity results for samples with more BLs. However, this increase is really slight may be located inside the standard deviation of the samples' results.

When comparing the samples 250 nm 2BL and 4BL it is observed that that difference is not inside the standard deviation of the sample 250 nm 4BL but since we do not have the deviation of the sample 250 nm 2BL it is not possible to confirm if the activity result variation between the samples is, in fact, significant. The same increase is seen for 400nm.

Comparing the samples 800 nm 2BL and 4BL, this time, this slight difference of absorbance per GOx mass observed between the samples is located inside the standard deviation of the sample 800 nm 4BL, and therefore it is most probable that this slight deviation is not a real tendency since it may just be due to standard deviation of the results.

(2) **Correlation between diameter and activity per mass of GOx results:**

By comparing the results of samples with the same BL number and different diameters it was possible to observe that for both bilayers (2BL and 4BL) there is no concrete correlation between the sample's diameters and the activity performance.

In the graph, the results of 250nm-2BL, 400nm-2BL, and 800nm-2BL are highly similar and the curves often interpenetrate each other during their own oscillations.

Regarding the samples assembled with 4BL, the results ascending order based on the sample's diameter is: 800, 250, 400 nm, the difference of results is slight. However, it is observed that the difference of results between the samples 250nm-4BL and 400nm-4BL is located inside of the standard deviation of the sample 250nm-4BL; and the standard deviation of the sample 800nm-4BL is big, the results of the samples 250-4BL and 400-4BL are all located inside of this deviation, therefore, it is not possible to judge if they present a real result variation.

Therefore, in practice, we may presume that there is no real result difference between samples and there is no correlation between the diameter and the GOx activity performance.

5.4.2 DYNAMIC ACTIVITY: SET 2 RESULTS

The dynamic results for the **set 2** samples were obtained the same way as for set 1, subtracting the blank from the sample's absorbance results, dividing it by the sample area, and then correlating this area with the mass of GOx per sample area result from the BCA analysis, the final result is shown in a graphic in Figure 36.

For the set 2, the absorbance average value found for the blank (glucose plus POD mix) was 0.08 AU, there is no specific reason why the absorbance value is the double of the one for set 1 and this should not have an effect on the result since the value is removed from the sample results before the comparison between different sets.

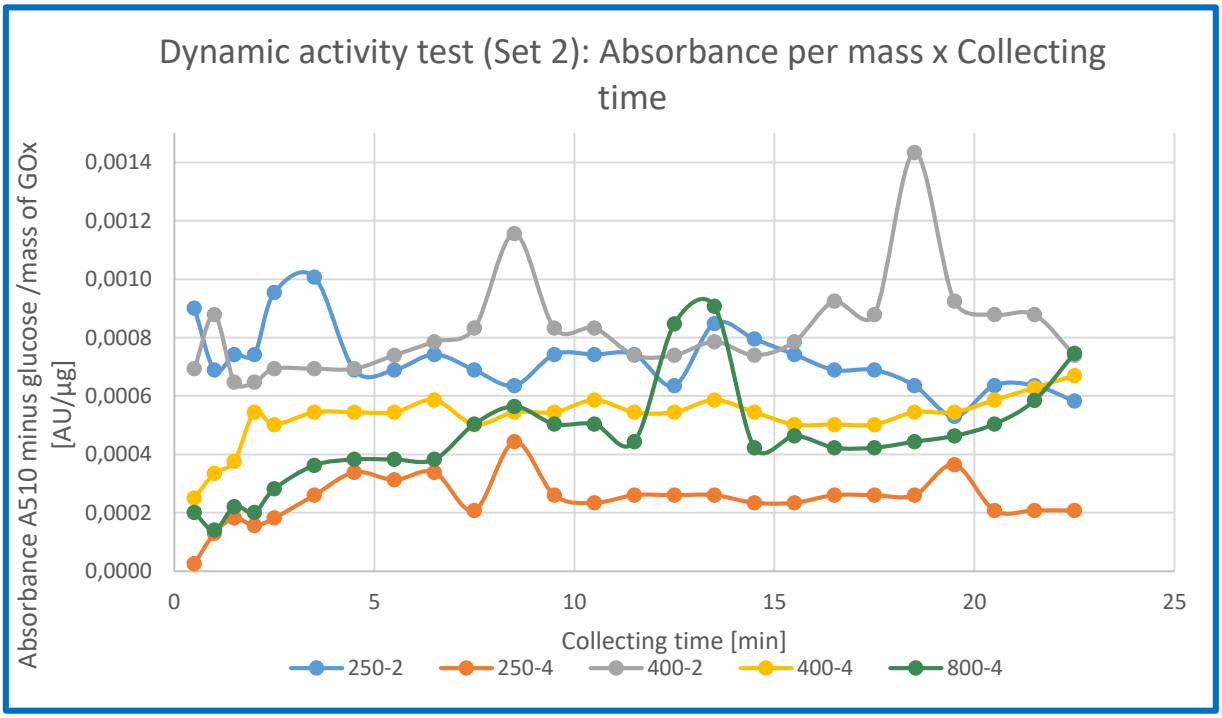


Figure 36: Dynamic test for set 2, the results are given in absorbance (without glucose) /mass of GOx x Collecting reaction time

The observation of the tendencies correlated with the change of parameters is performed starting from the point 5 minutes since at this point this was the observation start point for set 1 (where the curves became more stable). However, it is noticeable that the curves of set 2 present higher oscillations than for set 1 and are therefore less stable, this may be due to the smaller Y-axis range (and therefore the oscillations are amplified) or possibly due to the increase of the number of points analyzed (time collecting points), which increases manual work and could have consequences on the error propagation in the results.

(1) **Correlation between the number of bilayers and activity per mass of GOx results:**

It is possible to observe that for the diameters 250 and 400 nm there tendency that samples assembled with 2BL present higher absorbance per GOx mass results than the samples assembled with 4BL. For the 250 nm the difference of the value is higher than for the 400 nm. However, it is worth recalling that the standard variation was not calculated for this set of samples and this error range might be big enough to invalidate the real difference of activity results between the samples (as it was discussed in Section 5.4.1.1 Dynamic activity: Set 1 error range calculation)

(2) **Correlation between diameter and activity per mass of GOx results:**

Samples assembled with the same number of bilayers and different diameters did not present a concrete tendency between the diameters and activity results. For the samples with 2BL, the results of 250 nm and 400 nm are too often interpenetrated, therefore no real comparison can be made. And for the ones assembled with 4BL the ascending order of activity based on the diameter of the samples was: 800, 400, 250 nm, however, the curve results of the samples are often interpenetrated and the difference of results is slight. There is no correlation between the ascending order of results of 2BL and 4B and no correlation was found between diameter and activity. Besides, the slight difference of results may be located inside of the deviation range of the sample's results.

5.4.3 DYNAMIC ACTIVITY: SET 3 RESULTS

The set 3 data treatment was performed in the same manner as the other two sets (subtract blank, divide by area, compare with BCA). For this set the average blank absorbance was 0.04 AU. The dynamic activity measurement results of set 3 are located in Figure 37.

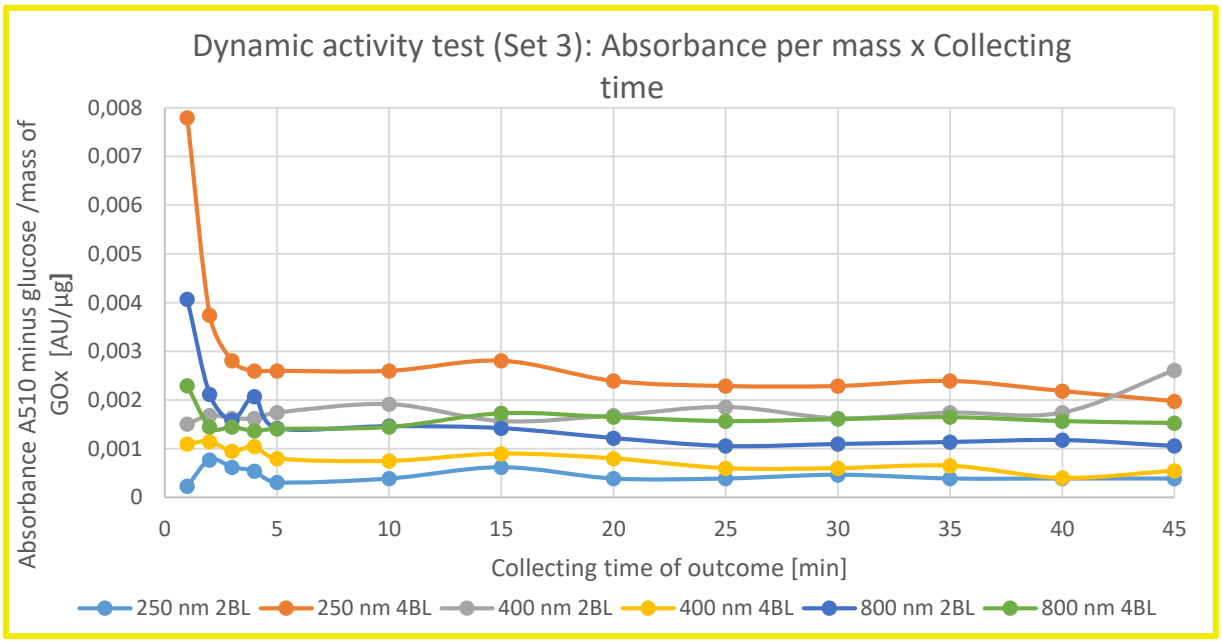


Figure 37: Dynamic test for set 3, the results are given in absorbance (without glucose) /mass of GOx x Collecting reaction time

It is worth mentioning that the set 3 results in Figure 37 are presented on a bigger Y-axis scale than for the set 1 and set 2. The results from the different sets will be compared among themselves in the same Y scale later. Just as for set 1 and set 2, the correlation between the parameters and the activity results will be observed after 5 minutes (where the results start to become more stable).

(1) **Correlation between the number of bilayers and activity per mass of GOx results:**

By comparing the curve results of samples with the same diameter and different number of bilayers it is possible to observe that for the samples with 250nm diameter the increase of bilayers has considerably increase the absorbance results; for the samples with 400 nm diameter the sample with 2BL presents higher absorbance per GOx mass results; and the samples with 800 nm present highly similar results but the results of the sample with 4BL are, generally, slightly higher.

Again, the ideal situation would be to obtain the standard deviation of the results to obtain concrete information on how relevant is the difference of results between samples.

(2) **Correlation between diameter and activity per mass of GOx results:**

By comparing the samples with the same BL number and different diameters it may be observed that the overall ascending results order for the 2BL are the samples with the diameters: 250, (800, and 400 nm), the 800 and 400 are indicated inside of the () because their difference is slight. And for the 4BL the ascending order is: 400, 800, and 250 nm. When comparing both ascending orders no observation can be made, and no concrete tendency is observed between the diameter of the nanotubes and the absorbance per GOx mass results. Besides, since the standard variation of the samples is unknown, the real relevance of the difference of results is unknown.

5.4.4 DYNAMIC ACTIVITY: COMPARISON SET 1, SET 2, AND SET 3 RESULTS

Finally, the results of the set 1, set 2, and set 3 are presented in the same scale to observe if there is a correlation between the PE assembly concentration and the absorbance per GOx mass results. The three graphs are represented in Figure 38.

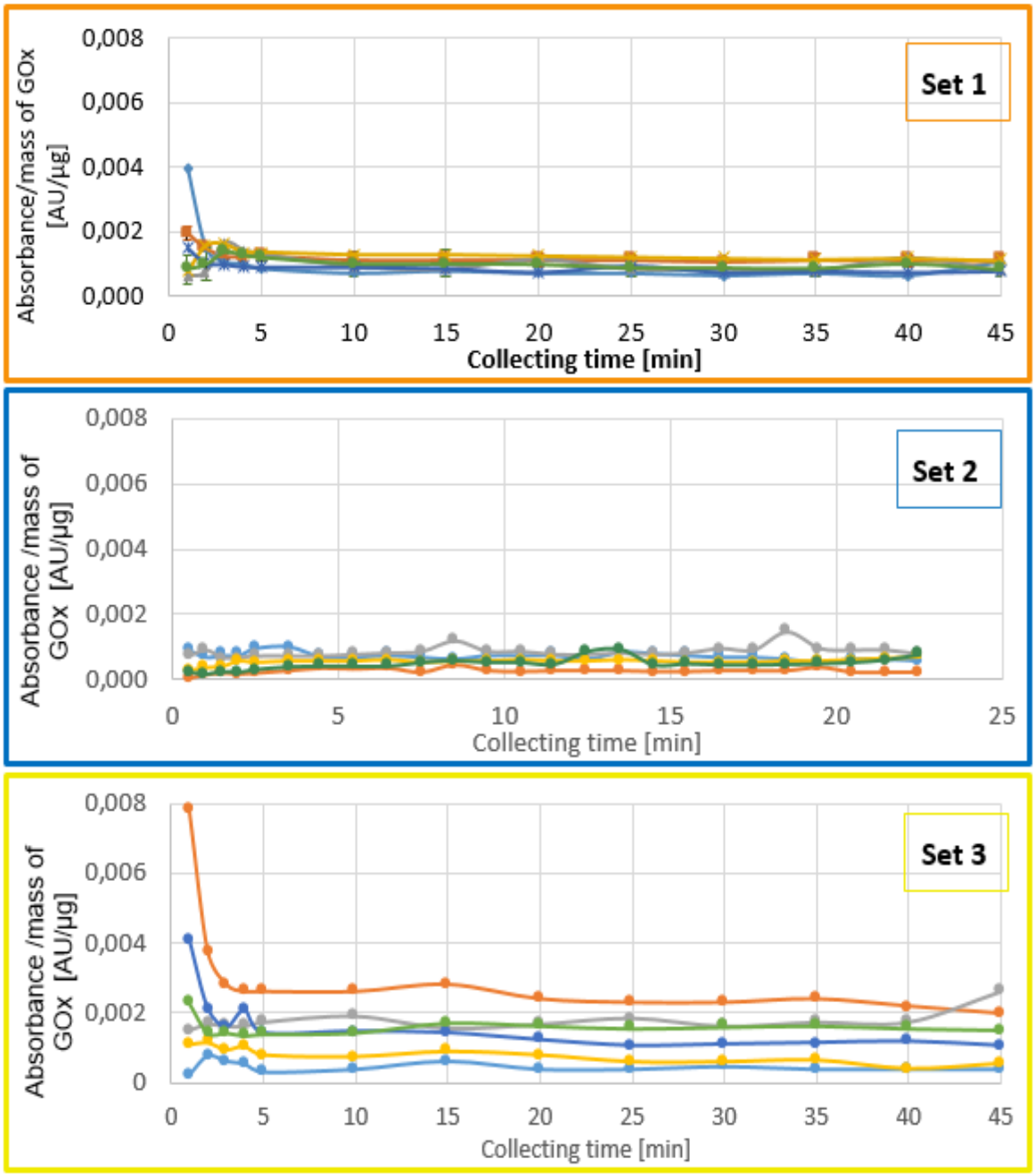


Figure 38: Dynamic test results for set 1, set 2, and set 3 samples. The absorbance per GOx mass results are represented in the same Y-axis range for the three sets. The collecting time X-axis is not in the same range for all the samples. Graph shown for range comparison purposes.

In Figure 38, the graphs of set 1 and 2 Y-axis were adjusted to be presented on the same scale as the one of set 3 in order to compare the overall range of the results (during the "stable period" after 5 minutes) of the different sets. It is possible to observe that the set 3 range is the biggest (i.e range size) and highest (between 0.0005 to 0.0028 AU/µg), the second-highest in the set 1 range (0.0007 to 0.0013 AU/µg) and, finally, the lowest range is the one of set 2 (mainly between 0.0005 and 0.001 AU/µg).

(3) **Correlation between polyelectrolytes concentration and activity per GOx mass results:**

By comparing the range of the results of the different sets and their PE concentration during LBL assembly it is possible to observe that, the increase of PE concentration from 0.1 mg/mL (set 1) to 1.0 mg/mL (set 2) provoked a slight decrease of the results range of about 0.00025 units. However, this value is about the same size as the standard deviation values of the set 1 samples 250-4BL and 800nm-4BL, therefore one should wonder if this range variation is indeed significant.

Differently, the set 3 curves presented a more significant increase in the results of absorbance per GOx mass when compared to the curves of set 1 and set 2. As mentioned, the set 3 was assembled with PEI concentration 1.0 mg/mL and an unknown concentration of GOx higher than 1 (but still in the same order of magnitude). This higher range indicates that possibly the key for optimizing the activity of the immobilized GOx enzyme may be varying the concentration of the polyelectrolytes unevenly. However, this hypothesis needs to be confirmed by performing new experiments with different and uneven PE concentrations.

When comparing the ascending order of results based on the samples' parameters it is not possible to find a reproducing order or tendency, this order will be better elaborated in the next section 5.4.5.

5.4.5 DYNAMIC ACTIVITY: PARTIAL DISCUSSION OF RESULTS

This section contains the final conclusions obtained by correlating the variation of the assembly parameters (PE concentration, diameter, and BL number) with the variation of the activity of GOx (represented by absorbance per GOx mass) of all three sets of results, these findings are resumed in three main correlation points.

It is also important to mention that it was observed that the activity results curves were unstable during the first 5 minutes of the collecting time, in this period a maximum value of activity was observed for all most samples, this may be due to enzyme desorption or due to high availability of enzymes. For that the reason, all three correlations points are analyzed based on the results starting at 5 minutes where the curves became more stable (possibly being the steady-state of the reaction). After this time the curves still present oscillations, and these may be possible indications of the error range of the sample's results.

Additionally, one other important point to remember is that the analysis of two samples of set 1 was reproduced in order to calculate the standard deviation of their results, and what was observed is that this standard deviation may be considered high when the difference of results between the samples is slight. Therefore, during this wrap-up when the word "slight" is used the reader should understand "the results are similar and it is highly possible that their difference is inside of their standard deviation range".

As mentioned before, the ideal situation would be to re-measure the absorbance results of all the samples to measure their standard deviations and correctly evaluate the impact of the variation of results when comparing different samples.

(1) **Correlation between the number of bilayers and activity per mass of GOx results:**

When comparing samples of the same set with the same diameter and different number of bilayers it was possible to observe that for the set 1 (PE assembly concentration 0.1 mg/mL) samples with the diameters 250 and 400 nm presented an increase of activity with the increase of bilayers. The difference of results between 800 nm 2BL and 4BL was located inside of the standard deviation of the sample 800nm 4BL and therefore this difference is not considered since it may just be due to fluctuation of results.

The results of set 2 (PE assembly concentration 1.0 mg/mL) presented the opposite trend, for the samples with 250 and 400 nm diameter the results of the samples with 2BL were higher than for the ones with 4BL.

The set 3 samples' results presented two opposite trends, for the diameter 250 nm there was an increase with the increase of the bilayers and for the 400 nm the trend was the opposite. The results of the sample 800nm were too similar to be compared.

In conclusion, it is still not possible to conclude if: (1) the samples with smaller PE concentration (set 1) increase activity with the increase of BLs and the samples with higher PE concentration (set 2) observe the opposite trend; or if (2) there is no real trend because both trends were observed in the set 3 results.

More repetitions are needed to verify the validity of the trends.

(2) **Correlation between diameter and activity per mass of GOx results:**

Samples (from the same set) with the same number of bilayers and different diameters are organized based on their results of absorbance per GOx mass order, the results are ordered to analyze if there is a correlation between the diameter of the samples and the activity of GOx (per mass of enzyme).

a) Samples with 2 bilayers ascending results order described by sample's diameter:
    a. Set 1: results too similar, no real ascending order
    b. Set 2: results too similar and too interpenetrated
    c. Set 3: 250, 800, and 400 nm.

b) Sample 4 bilayers ascending results order described by sample's diameter:
    a. Set 1: 800, 250, and 400nm (all located inside of the standard deviation of the 800nm-4BL sample, therefore, it is possible that this order is not accurate).
    b. Set 2: 250, (800, and 400nm); the parenthesis represents "slight difference".
    c. Set 3: 400, 800, 250

When comparing the order results of the 2BL and 4BL of set 3, it is possible to observe that they do not present the same ascending order, and one probable explanation for this could be that the increase of the bilayers will present different effects on the activity of samples assembled with different diameters.

When comparing the ascending activity order of samples from different sets and the same number of bilayers, it is possible to observe that the order is not repeated for both 2BL and 4BL cases. In fact, all the three orders of the sets of samples with 4BL are completely different.

Therefore, no correlation was found between the diameter and the activity of the immobilized GOx.

(3) **Correlation between polyelectrolytes concentration and activity per GOx mass results:**

When comparing the **results range** of the sets, it was possible to observe that the set 1 range was about 0.00025 units higher than the ones for the set 2, this increase may possibly be a trend that samples assembled with lower polyelectrolyte concentration present a higher activity result. However, this shift of 0.00025 is small and similar to the two standard deviation measured values, therefore, if the samples' results deviation values were similar to the ones measured this shift value will not be highly significant.

The range of set 3 was higher than for the other two sets, possibly indicating that assembling samples with an uneven concentration of PE may be the optimal condition to obtain higher GOx activity.

When comparing the **overall ascending order** of the sample's results of the sets the obtained order was:

A. Set 1**:** 250nm-2BL; (800nm-2BL; 800nm-4BL; 400nm-2BL); 250nm-4BL; 400nm-4BL; the parenthesis indicates that the results are similar and often interpenetrate.
B. Set 2: 250nm-4BL; 800nm-4BL; 400nm-4BL; (250nm-2BL; 400nm-2BL)
C. Set 3: 250nm-2BL; 400nm-4BL; 800nm-2BL; (800nm-4BL; 400nm-2BL); 250nm-4BL.

By observing all three orders it is not possible to find an optimal parameter setup and neither observe any tendency.

5.5 DISCUSSION OF RESULTS AND COMPARISON OF ACTIVITY MEASUREMENTS

This project has the goal of analyzing how the activity per amount of immobilized GOx (in nanotubes) will be impacted by the variation of different assembly parameters of the nanotubes: PE concentration, diameter of pores, and the number of bilayers.

The layer by layer immersive method was applied on porous membranes to create nanotubes inside of its pores (i.e., embedded nanotubes), the polyelectrolytes chosen were: Glucose oxidase (GOx) and Branched-Polyethyleneimine (bPEI). The assembly was performed with 3 types of PE concentrations, creating three sets of samples: set 1 (even PE concentration = 0.1 mg/mL); set 2 (even PE concentration = 1.0 mg/mL) and set 3 where an error occurred during the preparation of GOx solution (PEI concentration = 1.0 mg/mL and GOx concentration higher than 1.0 mg/mL). Each set contains 6 samples, to include the 3 different pore diameters (250, 400, and 800 nm) and the 2 different numbers of bilayers (2BL and 4BL).

The first step of the results analysis was confirming if the nanotubes were successfully assembled. For that, the doctorate Ievgen Kurylo has performed a Scanning electron microscopy analysis on free nanotubes (i.e., nanotubes freed by dissolving the membrane) and has confirmed the assembly success, the photo of the nanotubes captured by SEM may be found in Section 5.1 Complementary study: Scanning electron microscopy (SEM).

The second step was to perform the Bichinchoninic acid (BCA) protein assay to measure the amount of GOx immobilized per sample area or total internal area of the sample (for all the samples of the three sets). This is a piece of necessary information for the next step (activity measurements), making it possible to correlate the total activity with the amount of GOx present in the analyzed piece of sample. The BCA test has concluded 4 important points:

(1) The increase of the BLs increases the amount of deposited GOx (for all three sets), and this increase is higher for samples with a smaller diameter (250nm) and for samples assembled with higher & even PE concentration (set 2 only, set 3 not included).
(2) For all three sets, smaller pore diameters present more GOX adsorbed per area. This may be explained by the gel-effect theory cited in section 2.1.3 Layer by layer in pores.
(3) Increasing (evenly) the PE concentration (from set 1 to set 2) increases the amount of deposited enzyme per area. However, this trend was not valid for the sample with a pore diameter of 400 nm.
    a. The set 3 GOx mass per area results are smaller than for set 1 and set 2, indicating that the assembly with uneven PE concentrations occurs differently than the assembly for even concentration, this was also explained in the theory of Section 2.1.2.2 Effect of number of bilayers and polyelectrolyte's concentration.
(4) It was observed that the diameters 250 nm and 800 nm had a more rapid layer growth (i.e., amount of GOx deposited by layer) when assembled with higher PE concentration (set 2).

Finally, the activity measurements of GOx were performed in both static and dynamic conditions (both performed using the same assembled samples) and the tests provided the final measurement in terms of absorbance A_{510} per GOx mass over reaction time. The tests measure the product solution's absorbance, which corresponds to the amount of quinoneimine (product from second cascade reaction), which is proportional to the amount of H_2O_2 present (product of the first reaction and reagent of the second), directly proportional to the activity of GOx, therefore the activity is proportional to the absorbance A_{510}.

In the static conditions, there are no flows, and therefore the absorbance results are a constant accumulation of the product, the measurement will occur until the limit of the spectrometer or until total substrate consumption. The static condition has the disadvantage of not providing information on how the GOx activity of the samples changes with time (if there are peaks or if it decreases over time). The static may simulate a batch reactor.

In the dynamic conditions, there is a flow of both substrate and product, the product will be collected at specific times of the reaction and will have the sample's GOx activity measured. This measurement has the advantage of proving specific information on how the activity behaves over long-time operations (e.g., if the sample's GOx activity at the first minute of the reaction is the same as the one measured after 1 hour). This test simulates a CSTR operation and may indicate how the sample will behave when re-used. It is also important to mention that in this test the standard deviation for all results was not measured, and the difference between results is often slight so the validity of the observed trends was questioned.

Regarding the results observed in sections 5.3 Measurement of the enzyme's activity in Static conditions and 5.4 Measurement of the enzyme's activity in dynamic conditions, the correlations of the effect of the parameters on the activity results (for the samples of the 3 sets) were organized in three main discussion points, which will be discussed in the next three sub-subsections (5.5.1; 5.5.2 and 5.5.3). One additional parameter was discussed in section 5.5.4, the period of time between analyses.

5.5.1 **(POINT 1)** CORRELATION BETWEEN NUMBER OF BILAYERS AND ACTIVITY PER GOX MASS

The static measurement has observed that there were two main possible conclusions regarding the correlation between the number of BL and activity:

I. There is a trend that samples assembled with smaller PE concentration (set 1) present an increase of activity with the increase of bilayers, the opposite trend is observed for samples assembled with higher PE concentration (set 3);
II. There is no real and reproducible correlation between the number of bilayers and the activity results because the set 1 and set 3 may not be 100% compared

The conclusion also mentioned that more experiments should be performed with different PE concentrations to confirm the veracity of trend I.

The dynamic measurement has found two possible conclusions regarding the correlation between the number of BL and activity:

I. There is a trend that the samples assembled with smaller PE concentration (set 1) present an increase of GOx activity with the increase of BLs, and the samples assembled with higher PE concentration (set 2) observe the opposite trend. (The results were inconclusive for set 3 results)
II. There is no real trend because this was not reproduced in the results of set 3, where both increase (for diameter 250nm) and decrease (diameter 400nm) were observed with the increase of the BLs.

Here as well, the conclusion mentioned that more experiments should be performed with different PE concentrations.

Combining the two trend conclusions observed for both static and dynamic activity measurements it is possible to notice that there is a trend repetition mentioning that: samples assembled with smaller PE concentrations present an activity increase with the increase of the bilayers, and samples assembled with higher PE concentration observe the opposite trend.

Both trends may be explained by the results of the BCA test. In the results of that section it was observed that, when comparing set 1 and set 2, the samples assembled with higher PE concentration presented more amount of GOx per area and a higher bilayer growth. In other words, the results show that at higher PE assembly concentration the layers contain more GOx deposited than the layers of smaller PE concentration.

The denser layers (set 2) jeopardizes the diffusion of the substrate through the layers, making it harder for the substrate to access the GOx layers located deeper in the nanotubes (i.e., closer to the membrane walls). Therefore, increasing the number of bilayers will increase the amount of GOx per area, but the enzymatic reaction itself will only remain on the outermost layers because they cannot access the deeper layer, which will make the final absorbance per GOx mass result decrease. Besides, by increasing the number of bilayers their circular area will decrease, which may also have a consequence of making the layers denser and increase their diffusional restrictions (See Appendix 8.3 Diffusional restriction that affect the activity).

For the samples assembled with lower PE concentrations (set 1), the explanation follows the same logic. The layers formed with lower PE concentration are less dense (containing less PE molecules absorbed per area and per layer) and, therefore, the substrate will be able to diffuse through the layer to access layers localized deeper, increasing the overall activity results. However, it is not only the overall activity that is increasing, but the activity per GOx mass result is also increasing as well, this may be a result of the confinement effect or possibly due to configuration of the enzyme (e.g., since there is less amount of GOx enzymes per layer, the enzymes have space in the layer to be immobilized in an optimal configuration where its active binding site is more available to the substrate).

However, this trend that high PE concentration presents thicker layers and therefore less diffusion and less activity per GOx amount is not applicable for the set 3 results. By observing the BCA results it is possible to notice that this is the set with the lowest amount of GOx per layer of the nanotube, even if it is the set with the highest total PE concentration. Therefore, it is possible to presume that in this set there is another factor that is causing the activity to decrease with the increase of the bilayers, and this factor is a result of the uneven PE assembly concentration. This case will be further discussed in section 5.5.3.1.

5.5.2 **(POINT 2)** CORRELATION BETWEEN DIAMETER AND ACTIVITY PER GOX MASS

The static activity measurement of the samples has obtained three main results when observing the influence of the sample's diameter on the activity results:

I. When comparing the results' ascending order (based on the sample's diameters) of samples with different bilayers, it was observed that this order is not the same for the samples (from the same set) with 2BL and 4BL (e.g., in set 1 observe the ascending order of results for 2BL and compare with the order of 4BL) and therefore there was no trend observed between the diameter and the activity regarding the increase of bilayers.

II. When comparing the results' ascending order (based on the sample's diameters) of different sets based on samples with the same amount of BLs (e.g., comparing all the samples with 2 BL of the set 1 and set 2), no order repetition was observed in both 2BL and 4BL cases and therefore no trend between diameter and activity. For the 2BL case, the trend of smaller pores having more activity was strongly present.

III. There exists a possible trend that samples with smaller diameters present lower activity results than samples with a higher diameter (specially observed for the set 1 results) due to the higher diffusional accessibility of the substrate inside big pores (i.e., it is easier for substrates to enter big pores and they suffer limitations when entering small pores) (See Appendix).

Based on those results, the only possible trend reached was: there is a tendency that small pores tend to present lower activity results than big pores, and there is no ascending samples' results order reproducibility between samples with different bilayers of the same set and or of different sets.

The dynamic measurement has also one conclusion for the diameter's influence:

I. No correlation was found between diameter and activity results. This is concluded because there is no diameter results trend between 2BL and 4BL samples in the same set; and no diameter trend between samples with the same BL from different sets.

By combining the conclusions of the activity test and dynamic test we may conclude that for a first-time batch application of the samples with a smaller diameter will indeed present a smaller activity than the samples with higher diameter due to diffusional restriction for the substrate to enter the pore. However, for samples applications in dynamic conditions, there is no correlation between the diameter of the samples and the activity results.

The explanation as to why the static trend is not valid for dynamic applications may be explained by recalling how the activity measurements are performed. The first possible explanation is that the mechanical pumping of the reactants inside of the pores decreases the diffusional limitation of the small pores, and therefore there is no longer a trend between pore size and activity results for the dynamic conditions.

A second possible explanation is that, for the dynamic test, the buffer is pumped through 20 minutes in the sample, which causes GOx desorption; additionally, the test analysis are performed starting from 5 minutes because before this time the results are unstable (possibly due to enzyme desorption in the collecting product or due to the high availability of the enzymes). Therefore, it may be the case that this general enzyme desorption of all the samples has seemed to decrease the diffusion advantage that big pores presented and therefore the trend between the diameter and activity is no longer valid.

This desorption explanation is also the main explanation (besides the product accumulation) as to why the results of the static test are so much higher than for the dynamic test, simply because all this enzyme desorption did not occur.

5.5.3 **(POINT 3)** CORRELATION BETWEEN POLYELECTROLYTES CONCENTRATION AND ACTIVITY PER GOX MASS RESULTS

The static measurement has obtained two main results when correlating the PE concentration with the activity results of the samples:

I. When comparing the range of results of different sets, it is possible to observe that the set assembled with lower PE concentration (set 1 day 1) presented higher activity per GOx mass results than the set assembled with a higher PE concentration (set 3).

II. When comparing the ascending order of sample's results of the sets, no reproducibility was found nor any optimal parameter setup

Therefore, the only trend that was possibly seen is regarding the result (I) trend.

The dynamic measurement obtained three main results as well:

I. When comparing the range of results of different sets it is possible to observe that the set assembled with lower PE concentration (set 1) presented a small increase of activity than the set assembled with a higher PE concentration (set 2). This is not valid for the set 3.

II. The set 3 presents the higher results range, indicating that changing the PE concentrations unevenly may be the key to increasing the activity of the enzyme.
III. When comparing the ascending order of samples' results of the sets no reproducibility was found nor any optimal parameter setup.

Therefore, the conclusion of the dynamic test is: the increase of PE concentration decreases the range of activity results of the samples. This is not valid for uneven PE concentrations (set 3).

By combining the conclusions of static and dynamic tests we may observe that there is a repetition of trends that: smaller PE assembly concentrations tend to present higher activity results than samples assembled with higher PE concentration. However, this trend is no longer observed for samples assembled with high uneven PE concentration (set 3).

This results trend may be explained based on the conclusion of (point 1) Correlation between the number of bilayers and activity per GOx mass. As mentioned, the set assembled with higher PE concentration (set 2) presents layers with a higher amount of GOx than for the set with a lower PE concentration (set 1). Therefore, the samples assembled with higher PE concentration will present very dense layers and higher diffusional restrictions and the substrate will not have the ability to diffuse in the layers and reach the other GOx layers localized deeper in the nanotube (i.e., closer to the membrane's wall), which will decrease the activity. While for samples assembled with lower & even PE, the layers are not dense (low amount GOx per layer) and therefore the substrate can pass through one layer to access the layers located deeper, increasing the total activity. Besides that, for low PE samples the activity per GOx mass is also increasing, which may be due to the confinement effect or possibly due to configuration effects.

Therefore, the samples assembled with lower PE concentration will present smaller diffusional restrictions and the substrate will have the ability to diffuse in the layers and reach the other GOx layers localized deeper in the nanotube (i.e., closer to the membrane's wall), which will increase their activity. While for samples assembled with high even PE the layers are very dense (with a lot of GOx per layer) and therefore the substrate cannot pass through one layer to access the layers located deeper, and the activity will be smaller since the reaction is occurring on the outermost layers only.

5.5.3.1 POINT 3.1 CORRELATION BETWEEN UNEVEN PE CONCENTRATION (SET 3) AND ACTIVITY RESULTS

As seen previously, the **set 3** does not present the same trend as the other sets:

I. The BCA test reveals that, even with high PE concentrations, the set 3 has the lowest density of GOx per layer.
II. The point 1 shows that, for the set 3 static test the increase of layers will decrease activity; and the set 3 dynamic test has shown inconclusive results.
III. The point 3 reveals that, for the static test the set 3 has presented lower activity than for the set with a lower PE concentration (set 1). And the dynamic set 3 test has shown that the set 3 has presented a higher range of results than the set 1 (smaller PE concentration) and set 2 (even and high PE concentration).

No similar experiment was found in any literature, therefore I will explain my hypothesis as to why the set 3 results present odd results.

First, the BCA shows (I) show that the GOx layers of the set 3 are less dense (and therefore present less diffusional restriction), but we do not have any information on how the uneven concentration of GOx and PEI has affected the PEI layer.

Second, the point 1 (or II) has shown that the increase of layers has decreased the activity, which means that the substrate is not able to access the deeper GOx layers (i.e., closer to the membrane wall). This is not logical because the BCA has shown that the GOx layers are not dense so they shouldn't present diffusional restriction; therefore my hypothesis here is that the uneven PE concentration has affected the PEI layer, creating layers with diffusional restriction (possibly due to denser layers).

Third, the point 3 has proven that the static activity test the set 3 is lower than the activity of set 1. This is coherent with the hypothesis made, because the dense PEI layers do not allow the substrate to access deeper GOx layers and therefore the reaction will only occur in the outermost layers of GOx, and each one of these layers presents a small amount of GOx deposition per layer (from BCA results), making the final absorbance per GOx mass activity results be small.

However, in the point 3 the dynamic activity test has shown that the set 3 presents higher activity than the other two sets. Following the hypothesis, it is possible that the buffer flow has caused PEI desorption, making the PEI layers no longer present high diffusional restriction. Therefore, the activity will only depend on the restrictions caused by the GOx layers, and since this restriction is low (not dense layers) the substrate can access more easily the deeper GOx layers, and therefore the total activity will increase. Its activity per GOx mass results range are also increasing, this may possibly be due to the confinement effect or due to the configuration effects. Not only the specific activity is increasing but it is the highest of the three sets, which may possibly indicate that layers less crowded (with less GOx amount) are more optimal for the activity results.

Of course, to support this theory the results of the dynamic test for set 3 should have shown that samples with 4BL should have higher activity than the ones with 2BL (because of the diffusion to deeper layers). This is not fully observed in the results, however, it is important to also remember that the standard deviation of results is unknown, therefore the order of samples' results can not be fully confirmed (since the standard deviation is unknown), and that the buffer flow will cause the desorption of both PEI and GOx, and this desorption can be higher on samples with higher BLs and therefore they will not necessarily present higher activity results when compared to the samples with 2BL.

5.5.4 **(POINT 4)** CORRELATION BETWEEN TIME INTERVAL BETWEEN ANALYSIS AND ACTIVITY PER GOX MASS RESULTS

This analysis was performed for the static measurements but not for the dynamic measurements. The static results have shown a 50% reduction of results range between the analysis performed on the same day as the samples were assembled and after 8 days of assembly. This decrease is most likely due to the desorption of the enzyme during storing and due to dehydration (which decreases substrate accessibility to the enzyme's active site) (See Appendix 8.3 Diffusional restriction that affect the activity).

The dynamic activity measurements were not performed on the same day as the sample's assembly and usually, they were performed on different days for different samples (because they took a long time and it was impossible to perform them at the same day), therefore, it is possible that this factor is influencing on the dynamic results in an unknown ways.

6. CONCLUSION

The final conclusion of this master thesis work will be highlighted in this conclusion section. The assembled samples (i.e., membranes with embedded nanotubes) are divided into three sets based on the PE assembly concentration; each set presents 6 samples (with 3 different diameters and 2 different numbers of bilayers). The set 1 presents an even & low PE concentration (i.e., meaning that both GOx and PEI solutions have the same concentration and this concentration is low); the set 2 presents an even and high PE concentration; and the set 3 presents an uneven high PE concentration where the concentration of GOx is higher than the concentration of PEI. The main goal of this thesis project is to analyze how the variation of these three parameters will affect the activity of the immobilized GOx enzyme.

First, the success of the (bPEI/GOx) nanotubes layer-by-layer immersive assembly was confirmed by the Scanning electron microscopy analysis performed on free nanotubes.

Secondly, the Bicinchoninic acid (BCA) protein assay measured the amount of GOx enzymes present in the samples. This analysis has concluded that: (1) The increase of bilayers increases the mass of GOx deposited in the sample - trivial; (2) Smaller pores tend to present more GOx adsorption per area – explained by the gel-effect theory; (3) Evenly increasing the PE concentration (set 2) increases the amount of GOx absorbed per layers (valid for 250 nm and 800 nm)– creating more dense layers, which may cause diffusional restrictions; (4) The set 3 (unevenly high PE) has presented the smallest amount of GOx deposited per layer - indicating that the assembly with uneven PE concentrations occurs differently than for even concentrations.

The activity measurement of the samples was performed in both static and dynamic condition. Both activity results were correlated to all three different assembly parameters to understand their influence on the enzyme immobilization performance, the activity results were measured in units of absorbance per mass of GOx, this absorbance corresponds to the amount of the quinoneimine present (product of the second enzymatic reaction of the cascade), which is proportional to the amount of H_2O_2 present (reagent of second reaction and product of the first reaction), which is proportional to the activity of the immobilized GOx enzyme. The static (no flows) provided the information on how much of the product was being accumulated over reaction time; and the dynamic test has provided more detailed information on how this product is being produced over different reaction times (constant or varies). The analyzed result of the dynamic test is that during the first 5 minutes of the reaction a maximum activity is observed and afterward the reaction becomes stable, this maximum may have occurred due to the high availability of the enzymes or due to the enzyme desorption in the product outcome.

Regarding the influence of the number of bilayers on the activity of GOx, it was observed that for samples assembled with low and even concentrations of PE (set 1) the increase of bilayers will increase the activity of the GOx enzyme; and for samples assembled

with a high and even concentration of PE (set 2) the increase of bilayers will decrease the activity. Both trends occur because the GOx layers of low PE (set 1) present a lower quantity of enzyme than the ones assembled in high PE. Therefore, the layers with high enzyme density will present diffusional restriction, which will now allow the substrate to reach the GOx layers located deeper (i.e., nearer to the membrane walls) and therefore the reaction and activity will remain on the outmost layers, and the final activity per mass of GOx results will be lower. The samples assembled with low (an even) PE concentration are not dense, do not present the diffusional restriction and therefore the substrate may access deeper GOx layers, increasing the total activity results with the increase of the layers. However, the specific activity (i.e., activity per mass of GOx) is increasing as well, this may be a result of the confinement effect or due to a more optimal configuration immobilization of enzyme.

Regarding the influence of samples' pore diameters on the activity results, it was observed that for the static measurement the pores with smaller diameter presented smaller activity results than pores with high diameter. This trend is mainly justified by the diffusional restriction of the substrate to go inside of smaller pores (in other words, it is more difficult for the substrate to enter smaller pores). This trend is no longer present in the dynamic test, which may occur because with the flow conditions the substrate entering in the pore will no longer be restricted or due to the enzyme desorption of the samples (which may have different results impacts for different pore diameters).

Regarding the influence of the PE assembly concentration and its effects on the activity results, both static and dynamic results have shown that samples assembled with lower & even PE concentration (set 1) present higher total activity results (and specific activity results) than the samples assembled with higher & even PE concentration (set 2). Again, samples assembled with the higher (and even) PE concentration will present more GOx crowded layers (with a higher amount of enzyme) and therefore will present diffusional limitations, not allowing the substrate to access other layers and decreasing the overall activity per GOx mass results.

A final hypothesis was created to explain the static and dynamic results of the set where the samples were assembled with a high and uneven concentration of PE (set 3). This set of samples are the ones with the lowest amount of GOx per layer (from BCA) and therefore with the lowest diffusional restrictions to reach the deeper layers. However, the static test shown that the increase of bilayers has decreased the activity and also that this is the test with the smallest value of the absorbance per GOx mass, therefore the hypothesis is that there exists a diffusional restriction caused by the PEI layer and therefore the assembly performed with uneven PE concentrations occurs differently than the one with even assembly. The dynamic test has shown that this set presents an increase of the activity results of GOx activity per mass, this change of results may be due to the desorption of PEI with the pumping. However, not only was the activity per mass results increased, but they were also higher than

for the set 1, indicating that layers with less amount of GOx are more optimal for activity, possibly due to the configuration of the enzyme of due to confinement effect.

Finally, the conclusion is that indeed the parameters affect the activity per GOx mass results, however, this influence does not depend on one parameter only (e.g., the influence of the BL number also depends on the PE concentration) and their impact may also depend on the type of application of sample (static and dynamic regimes).

It is also important to mention that other parameters may be studied, such as type of PEs; concentration of glucose, pH, the flow rate of the dynamic measurement, time of adsorption, temperature, and others.

7. REFERENCES

Ahmad, R., & Khare, S. K. (2018). Immobilization of Aspergillus niger cellulase on multiwall carbon nanotubes for cellulose hydrolysis. *Bioresource technology*, *252*, 72-75. https://doi.org/10.1016/j.biortech.2017.12.082

Alem, H., Blondeau, F., Glinel, K., Demoustier-Champagne, S., & Jonas, A. M. (2007). Layer-by-layer assembly of polyelectrolytes in nanopores. *Macromolecules*, *40*(9), 3366-3372. https://doi.org/10.1021/ma0703251

Ali, S. W., Rajendran, S., & Joshi, M. (2010). Effect of process parameters on layer-by-layer self-assembly of polyelectrolytes on cotton substrate. Polymers and Polymer Composites, 18(5), 237-249. https://doi.org/10.1177/096739111001800501

Azzaroni, O., & Lau, K. A. (2011). Layer-by-layer assemblies in nanoporous templates: nano-organized design and applications of soft nanotechnology. *Soft Matter*, *7*(19), 8709-8724. https://doi.org/10.1039/C1SM05561E

Bankar, S. B., Bule, M. V., Singhal, R. S., & Ananthanarayan, L. (2009). Glucose oxidase—an overview. *Biotechnology advances*, *27*(4), 489-501 https://doi.org/10.1016/j.biotechadv.2009.04.003

Borges, J., & Mano, J. F. (2014). Molecular interactions driving the layer-by-layer assembly of multilayers. *Chemical reviews*, *114*(18), 8883-8942 https://doi.org/10.1021/cr400531v

Cacicedo, M. L., Manzo, R. M., Municoy, S., Bonazza, H. L., Islan, G. A., Desimone, M., ... & Castro, G. R. (2019). Immobilized enzymes and their applications. In *Advances in enzyme technology* (pp. 169-200). Elsevier. https://doi.org/10.1016/B978-0-444-64114-4.00007-8

Cang-Rong, J. T., & Pastorin, G. (2009). The influence of carbon nanotubes on enzyme activity and structure: investigation of different immobilization procedures through enzyme kinetics and circular dichroism studies. *Nanotechnology*, *20*(25), 255102. https://doi.org/10.1088/0957-4484/20/25/255102

Chemistry LibreTexts. (2019). Distinguishing between pH and pKa. Retrieved July 19, 2021, from https://chem.libretexts.org/@go/page/136801

Chemistry LibreTexts. (2019). pKa Value. Retrieved July 19, 2021, from https://chem.libretexts.org/@go/page/17429

ChemTalk. What is pKa. What is pKa? | ChemTalk (chemistrytalk.org)

Chung, A. J., & Rubner, M. F. (2002). Methods of loading and releasing low molecular weight cationic molecules in weak polyelectrolyte multilayer films. *Langmuir*, *18*(4), 1176-1183. https://doi.org/10.1021/la010873m

Clark, M. A., Douglas, M. C., & Choi, J. (2018). Chapter 3-4 Proteins. In Biology 2e. Houston, Texas: OpenStax. (Protein structure image adapted from National Human Genome Research Institute) .Chapter URL: https://openstax.org/books/biology-2e/pages/3-4-proteins.

Copeland, R. A. (2000). *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis* (2nd ed.). Wiley-VCH. https://doi.org/10.1021/jm020467f

Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. 3 Biotech, 3(1), 1-9. https://doi.org/10.1007/s13205-012-0071-7

Decher, G., Eckle, M., Schmitt, J., & Struth, B. (1998). Layer-by-layer assembled multicomposite films. *Current opinion in colloid & interface science*, *3*(1), 32-39. https://doi.org/10.1016/S1359-0294(98)80039-3

Denniston, K. J., Topping, J. J., Dorr, Q. D. D., Caret, R. L. (2020). Chapter 19: Enzymes. In General, organic, and biochemistry (10th ed., pp. 666–666). McGraw-Hill Education.

Dwevedi, A. (2016). Basics of enzyme immobilization. In Enzyme immobilization (pp. 21-44). Springer, Cham. https://doi.org/10.1007/978-3-319-41418-8_2

Elzbieciak, M., Zapotoczny, S., Nowak, P., Krastev, R., Nowakowska, M., & Warszynski, P. (2009). Influence of pH on the structure of multilayer films composed of strong and weak polyelectrolytes. *Langmuir*, *25*(5), 3255-3259. https://doi.org/10.1021/la803988k

Eun, H. M. (1996). Enzymes and Nucleic Acids: General Principles. *Enzymology Primer for Recombinant DNA Technology. Elsevier Inc. All*, 1-108. https://doi.org/10.1016/B978-012243740-3/50004-1

Expert market research. Global Enzymes Market: By Type: Protease, Amylase, Lipase, Cellulase; By Industry Type: Industrial, Special; By Application; Regional Analysis; Historical Market and Forecast (2016-2026); Competitive Landscape; Industry Events and Developments https://www.expertmarketresearch.com/reports/enzymes-market

Feng, W., & Ji, P. (2011). Enzymes immobilized on carbon nanotubes. *Biotechnology advances*, *29*(6), 889-895. https://doi.org/10.1016/j.biotechadv.2011.07.007

Ferreira, M., Fiorito, P. A., Oliveira Jr, O. N., & de Torresi, S. I. C. (2004). Enzyme-mediated amperometric biosensors prepared with the Layer-by-Layer (LbL) adsorption technique. *Biosensors and Bioelectronics*, *19*(12), 1611-1615. https://doi.org/10.1016/j.bios.2003.12.025

Garg, A., Heflin, J. R., Gibson, H. W., & Davis, R. M. (2008). Study of film structure and adsorption kinetics of polyelectrolyte multilayer films: effect of pH and polymer concentration. Langmuir, 24(19), 10887-10894. https://doi.org/10.1021/la8005053

Garlet, T. B., Weber, C. T., Klaic, R., Foletto, E. L., Jahn, S. L., Mazutti, M. A., & Kuhn, R. C. (2014). Carbon nanotubes as supports for inulinase immobilization. *Molecules*, *19*(9), 14615-14624. https://doi.org/10.3390/molecules190914615

Gilson. *Peristatic Pump User's Guide, figure 6.* MINIPULS3_UG_LT801121-17.pdf (gilson.com)

Goodsell DS (2006) Glucose oxidase. Molecule of the month: PCSB Protein Data Bank https://doi.org/10.2210/rcsb_pdb/mom_2006_5

Guisan, J. M., López-Gallego, F., Bolivar, J. M., Rocha-Martín, J., & Fernandez-Lorente, G. (2020). The science of enzyme immobilization. In Immobilization of Enzymes and Cells (pp. 1-26). Humana, New York, NY. https://doi.org/10.1007/978-1-0716-0215-7_1

Han, U., Seo, Y., & Hong, J. (2016). Effect of pH on the structure and drug release profiles of layer-by-layer assembled films containing polyelectrolyte, micelles, and graphene oxide. *Scientific reports*, *6*(1), 1-10. https://doi.org/10.1038/srep24158

He, Q., Cui, Y., Ai, S., Tian, Y., & Li, J. (2009). Self-assembly of composite nanotubes and their applications. *Current opinion in colloid & interface science*, *14*(2), 115-125. https://doi.org/10.1016/j.cocis.2008.09.005

Helmenstine, Anne Marie, Ph.D. (2020, August 25). pH and pKa Relationship: The Henderson-Hasselbalch Equation. Retrieved from https://www.thoughtco.com/the-ph-and-pka-relationship-603643

Homaei, A. A., Sariri, R., Vianello, F., & Stevanato, R. (2013). Enzyme immobilization: an update. *Journal of chemical biology*, *6*(4), 185-205. https://doi.org/10.1007/s12154-013-0102-9

Hou, S., Harrell, C. C., Trofin, L., Kohli, P., & Martin, C. R. (2004). Layer-by-layer nanotube template synthesis. *Journal of the American Chemical Society*, *126*(18), 5674-5675. https://doi.org/10.1021/ja049537t

Jegannathan, K. R., Abang, S., Poncelet, D., Chan, E. S., & Ravindra, P. (2008). Production of biodiesel using immobilized lipase—a critical review. Critical reviews in biotechnology, 28(4), 253-264. https://doi.org/10.1080/07388550802428392

Jesionowski, T., Zdarta, J., & Krajewska, B. (2014). Enzyme immobilization by adsorption: a review. *Adsorption*, *20*(5-6), 801-821. https://doi.org/10.1007/s10450-014-9623-y

Johnston, A. P., Cortez, C., Angelatos, A. S., & Caruso, F. (2006). Layer-by-layer engineered capsules and their applications. *Current opinion in colloid & interface science*, *11*(4), 203-209. https://doi.org/10.1016/j.cocis.2006.05.001

Jun, L. Y., Yon, L. S., Mubarak, N. M., Bing, C. H., Pan, S., Danquah, M. K., ... & Khalid, M. (2019). An overview of immobilized enzyme technologies for dye and phenolic removal from wastewater. *Journal of Environmental Chemical Engineering*, *7*(2), 102961. https://doi.org/10.1016/j.jece.2019.102961

Küchler, A., Yoshimoto, M., Luginbühl, S., Mavelli, F., & Walde, P. (2016). Enzymatic reactions in confined environments. *Nature nanotechnology*, *11*(5), 409. https://doi.org/10.1038/nnano.2016.54

Lim, J., Zhang, X., Ferruzzi, M. G., & Hamaker, B. R. (2019). Starch digested product analysis by HPAEC reveals structural specificity of flavonoids in the inhibition of mammalian α-amylase and α-glucosidases. *Food chemistry*, *288*, 413-421. https://doi.org/10.1016/j.foodchem.2019.02.117

Lorsch, J. R. (2014). Practical Steady-State Enzyme Kinetics. *Methods in Enzymology*, 3–15. https://doi.org/10.1016/b978-0-12-420070-8.00001-5

Lvov, Y., Ariga, K., Ichinose, I., & Kunitake, T. (1995). Assembly of multicomponent protein films by means of electrostatic layer-by-layer adsorption. *Journal of the American Chemical Society*, *117*(22), 6117-6123. https://doi.org/10.1021/ja00127a026

Lvov, Y., Ariga, K., Ichinose, I., & Kunitake, T. (1996). Molecular film assembly via layer-by-layer adsorption of oppositely charged macromolecules (linear polymer, protein and clay) and concanavalin A and glycogen. *Thin solid films*, *284*, 797-801. https://doi.org/10.1016/S0040-6090(95)08449-5

Mendelsohn, J. D., Yang, S. Y., Hiller, J. A., Hochbaum, A. I., & Rubner, M. F. (2003). Rational design of cytophilic and cytophobic polyelectrolyte multilayer thin films. *Biomacromolecules*, *4*(1), 96-106. https://doi.org/10.1021/bm0256101

Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnology & Biotechnological Equipment, 29(2), 205-220. https://doi.org/10.1080/13102818.2015.1008192

National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 64689, beta-D-Glucose. https://pubchem.ncbi.nlm.nih.gov/compound/beta-D-Glucose

National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 9033, Aziridine. https://pubchem.ncbi.nlm.nih.gov/compound/Aziridine.

Nelson, D. L., & Cox, M. M. (2004). *Lehninger Principles of Biochemistry* (4th ed.). W. H. Freeman. https://doi.org/10.1002/bmb.2005.494033010419

Nguyen, H. H., & Kim, M. (2017). An overview of techniques in enzyme immobilization. *Applied Science and Convergence Technology*, *26*(6), 157-163. https://doi.org/10.5757/ASCT.2017.26.6.157

Nielsen, J. E., & McCammon, J. A. (2003). Calculating pKa values in enzyme active sites. *Protein Science*, *12*(9), 1894–1901. https://doi.org/10.1110/ps.03114903

Nová, L., Uhlík, F., & Košovan, P. (2017). Local pH and effective p KA of weak polyelectrolytes–insights from computer simulations. *Physical Chemistry Chemical Physics*, *19*(22), 14376-14387. https://doi.org/10.1039/c7cp00265c

Park, C., & Raines, R. T. (2001). Quantitative Analysis of the Effect of Salt Concentration on Enzymatic Catalysis. *Journal of the American Chemical Society*, *123*(46), 11472–11479. https://doi.org/10.1021/ja0164834

Phillips, D. C. (1966). *The Three-Dimensional Structure of an Enzyme Molecule* (Vols. 215, issue 5, pp. 78–90). Scientific American. https://doi.org/10.1038/scientificamerican1166-78

Poptoshev, E., Schoeler, B., & Caruso, F. (2004). Influence of solvent quality on the growth of polyelectrolyte multilayers. *Langmuir*, *20*(3), 829-834. https://doi.org/10.1021/la035485u

ReportLinker (2021). Global Enzymes Market By Source, By Type, By Reaction Type, By Application, By Regional Outlook, Industry Analysis Report and Forecast, 2021 – 2027. Global Enzymes Market By Source, By Type, By Reaction Type, (globenewswire.com)

Richardson, J. J., Björnmalm, M., & Caruso, F. (2015). Technology-driven layer-by-layer assembly of nanofilms. Science, 348(6233). https://doi.org/10.1126/science.aaa2491

Richardson, J. J., Cui, J., Björnmalm, M., Braunger, J. A., Ejima, H., & Caruso, F. (2016). Innovation in layer-by-layer assembly. *Chemical Reviews*, *116*(23), 14828-14867. https://doi.org/10.1021/acs.chemrev.6b00627

Roy, C. J., Dupont-Gillain, C., Demoustier-Champagne, S., Jonas, A. M., & Landoulsi, J. (2010). Growth mechanism of confined polyelectrolyte multilayers in nanoporous templates. Langmuir, 26(5), 3350-3355. https://doi.org/10.1021/la903121e

Sakr, O. S., & Borchard, G. (2013). Encapsulation of enzymes in Layer-by-Layer (LbL) structures: latest advances and applications. *Biomacromolecules*, *14*(7), 2117-2135. https://doi.org/10.1021/bm400 198p

Secundo, F. (2013). Conformational changes of enzymes upon immobilisation. Chemical Society Reviews, 42(15), 6250-6261. https://doi.org/10.1039/C3CS35495D

Sheldon, R. A. (2007). Enzyme immobilization: the quest for optimum performance. *Advanced Synthesis & Catalysis*, *349*(8-9), 1289-1307. https://doi.org/10.1002/adsc.200700082

Shi, J., Hua, F., Cui, T., & Lvov, Y. M. (2003). Temperature effect on layer-by-layer self-assembly of linear polyions and silica multilayers. Chemistry letters, 32(4), 316-317. https://doi.org/10.1246/cl.2003.316

Singh, J., & Verma, N. (2013). Glucose oxidase from Aspergillus niger: Production, characterization and immobilization for glucose oxidation. *Adv. Appl. Sci. Res*, *4*(3), 250-257. Corpus ID: 8597568. Glucose oxidase from Aspergillus niger:Production, characterization and immobilization for glucose oxidation | Semantic Scholar

Spahn, C., & Minteer, S. D. (2008). Enzyme immobilization in biotechnology. *Recent patents on engineering*, *2*(3), 195-200. https://doi.org/10.2174/187221208786306333

Tang, Z., Wang, Y., Podsiadlo, P., & Kotov, N. A. (2006). Biomedical applications of layer-by-layer assembly: from biomimetics to tissue engineering. *Advanced materials*, *18*(24), 3203-3224. https://doi.org/10.1002/adma.200600113

Thermo Scientific™ (2020). User Guide: Pierce™ BCA Protein Assay Kit. Catalog Numbers 23225 and 23227. Doc. Part No. 2161296. Pub. No. MAN0011430 Document Connect (thermofisher.com)

Tian, Y., He, Q., Cui, Y., & Li, J. (2006). Fabrication of protein nanotubes based on layer-by-layer assembly. *Biomacromolecules*, *7*(9), 2539-2542. https://doi.org/10.1021/bm060412l

Wilson, R., & Turner, A. P. F. (1992). Glucose oxidase: an ideal enzyme. *Biosensors and Bioelectronics*, *7*(3), 165-185. https://doi.org/10.1016/0956-5663(92)87013-F

Wohlfahrt, G., Trivić, S., Zeremski, J., Peričin, D., & Leskovac, V. (2004). The chemical mechanism of action of glucose oxidase from Aspergillus niger. *Molecular and cellular biochemistry*, *260*(1), 69-83. https://doi.org/10.1023/B:MCBI.0000026056.75937.98

Wong, C. M., Wong, K. H., & Chen, X. D. (2008). Glucose oxidase: natural occurrence, function, properties and industrial applications. *Applied microbiology and biotechnology*, *78*(6), 927-938. https://doi.org/10.1007/s00253-008-1407-4

Xie, T., Wang, A., Huang, L., Li, H., Chen, Z., Wang, Q., & Yin, X. (2009). Recent advance in the support and technology used in enzyme immobilization. *African Journal of Biotechnology*, *8*(19). https://doi.org/10.5897/AJB2009.000-9431

Yamanaka, S. A., Nishida, F., Ellerby, L. M., Nishida, C. R., Dunn, B., Valentine, J. S., & Zink, J. I. (1992). Enzymatic activity of glucose oxidase encapsulated in transparent glass by the sol-gel method. *Chemistry of materials*, *4*(3), 495-497. https://doi.org/10.1021/cm00021a001

Yang, K. L. (2017). Combined cross-linked enzyme aggregates of horseradish peroxidase and glucose oxidase for catalyzing cascade chemical reactions. *Enzyme and microbial technology*, *100*, 52-59. https://doi.org/10.1016/j.enzmictec.2017.02.007

Zhang, D. H., Yuwen, L. X., & Peng, L. J. (2013). Parameters affecting the performance of immobilized enzyme. Journal of chemistry, 2013. https://doi.org/10.1155/2013/946248

Zhang, J., & Jiang, S. P. (2017). Self-Assembly in Fuel Cells. In Comprehensive Supramolecular Chemistry II (Vol. 9, pp. 274-295). https://doi.org/10.1016/B978-0-12-409547-2.12642-3

Zhang, X., Chen, H., & Zhang, H. (2007). Layer-by-layer assembly: from conventional to unconventional methods. *Chemical Communications*, (14), 1395-1405. https://doi.org/10.1039/B615590A

8. APPENDIX

8.1 ENZYMES: PKA OF AMINO ACIDS

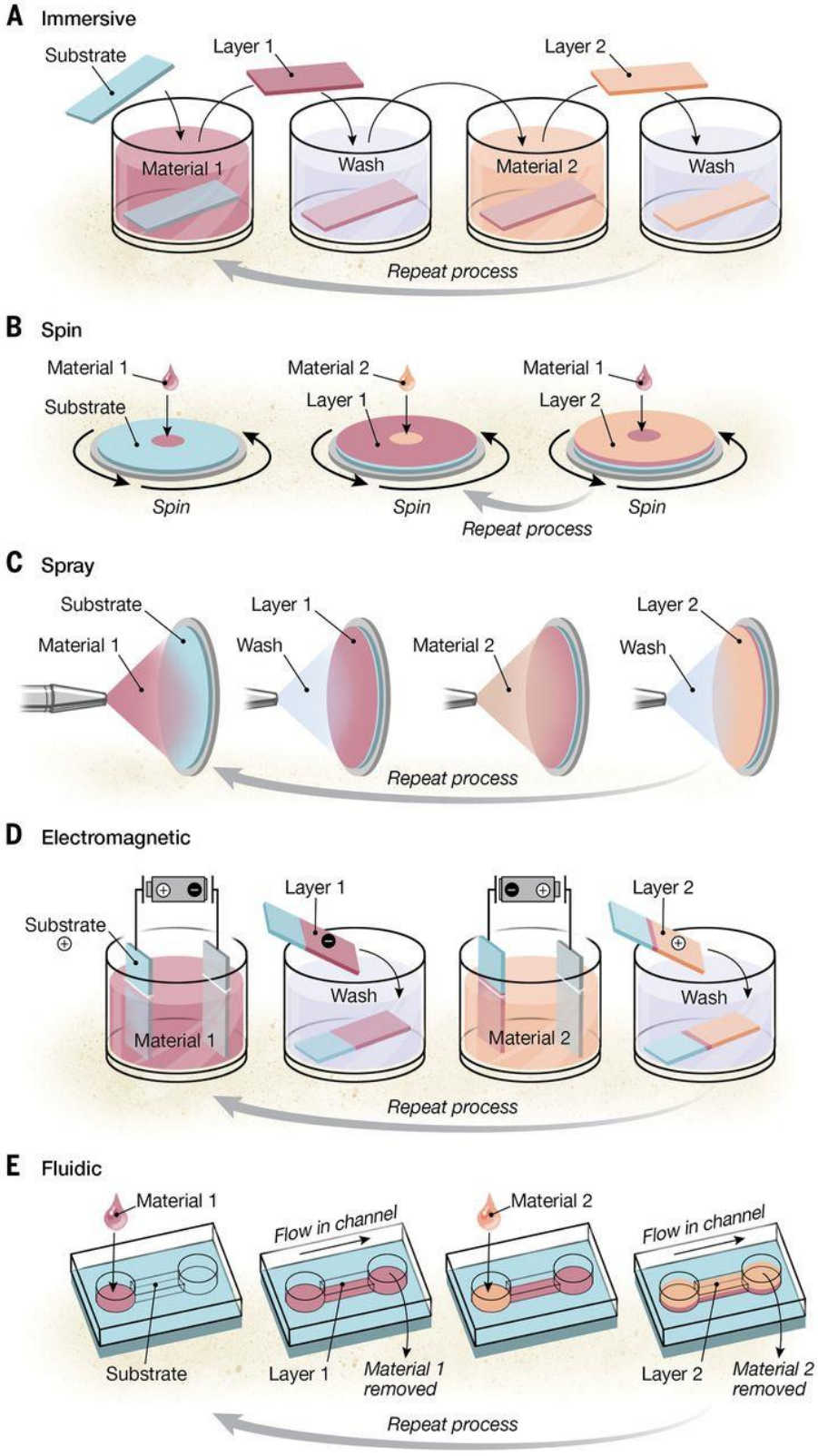
TABLE 3–1 Properties and Conventions Associated with the Common Amino Acids Found in Proteins

Amino acid	Abbreviation/ symbol	M_r	pK_a values: pK_1 (—COOH)	pK_2 ($—NH_3^+$)	pK_R (R group)	pI	Hydropathy index*	Occurrence in proteins (%)†
Nonpolar, aliphatic R groups								
Glycine	Gly G	75	2.34	9.60		5.97	−0.4	7.2
Alanine	Ala A	89	2.34	9.69		6.01	1.8	7.8
Proline	Pro P	115	1.99	10.96		6.48	1.6	5.2
Valine	Val V	117	2.32	9.62		5.97	4.2	6.6
Leucine	Leu L	131	2.36	9.60		5.98	3.8	9.1
Isoleucine	Ile I	131	2.36	9.68		6.02	4.5	5.3
Methionine	Met M	149	2.28	9.21		5.74	1.9	2.3
Aromatic R groups								
Phenylalanine	Phe F	165	1.83	9.13		5.48	2.8	3.9
Tyrosine	Tyr Y	181	2.20	9.11	10.07	5.66	−1.3	3.2
Tryptophan	Trp W	204	2.38	9.39		5.89	−0.9	1.4
Polar, uncharged R groups								
Serine	Ser S	105	2.21	9.15		5.68	−0.8	6.8
Threonine	Thr T	119	2.11	9.62		5.87	−0.7	5.9
Cysteine	Cys C	121	1.96	10.28	8.18	5.07	2.5	1.9
Asparagine	Asn N	132	2.02	8.80		5.41	−3.5	4.3
Glutamine	Gln Q	146	2.17	9.13		5.65	−3.5	4.2
Positively charged R groups								
Lysine	Lys K	146	2.18	8.95	10.53	9.74	−3.9	5.9
Histidine	His H	155	1.82	9.17	6.00	7.59	−3.2	2.3
Arginine	Arg R	174	2.17	9.04	12.48	10.76	−4.5	5.1
Negatively charged R groups								
Aspartate	Asp D	133	1.88	9.60	3.65	2.77	−3.5	5.3
Glutamate	Glu E	147	2.19	9.67	4.25	3.22	−3.5	6.3

*A scale combining hydrophobicity and hydrophilicity of R groups; it can be used to measure the tendency of an amino acid to seek an aqueous environment (− values) or a hydrophobic environment (+ values). See Chapter 11. From Kyte, J. & Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157,** 105–132.

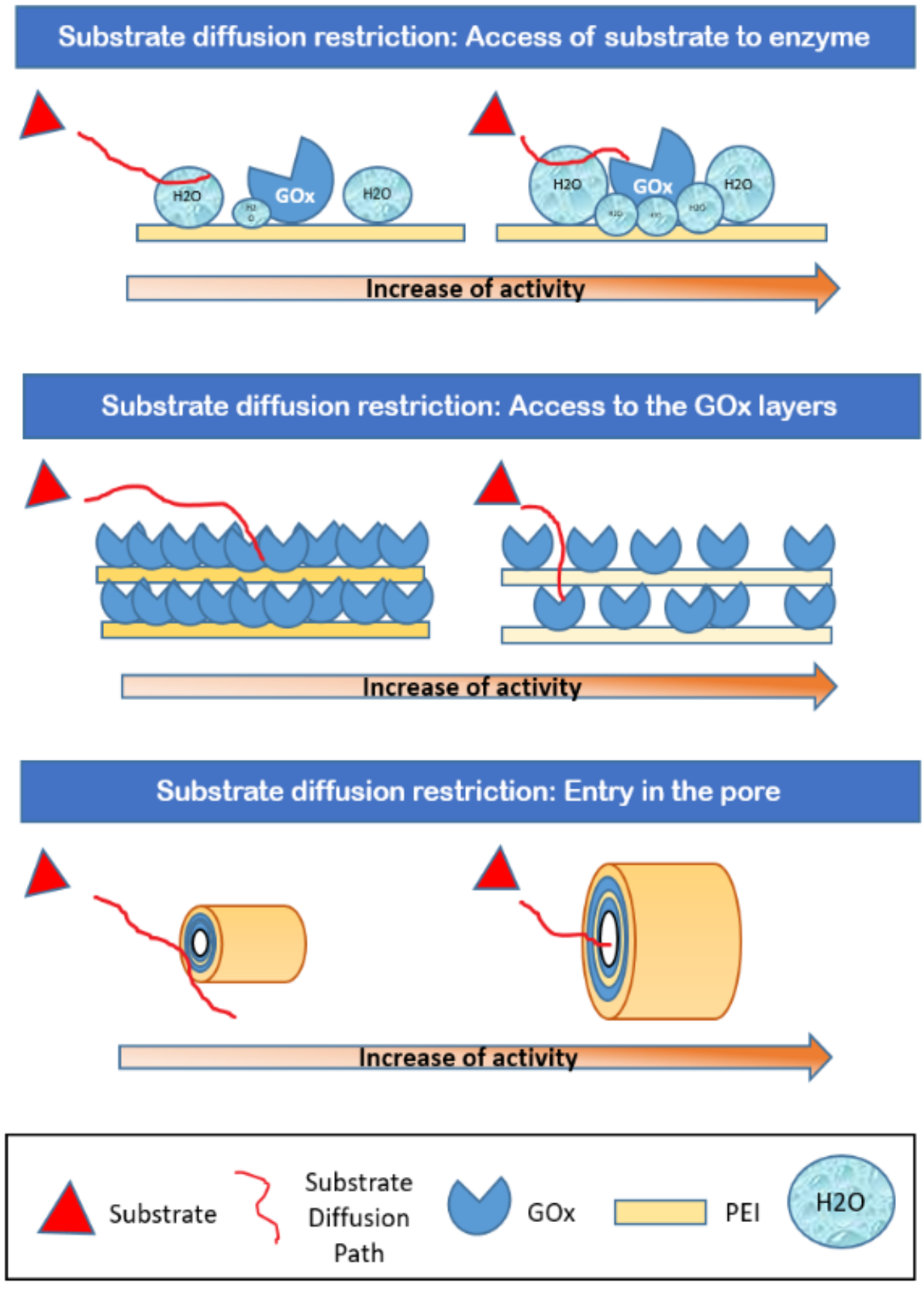
Source: Nelson, D. L., Cox, M. M. (2001). BOOK Lehniger Principles of Biochemistry. Fourth edition.

8.2 LAYER-BY-LAYER ILUSTRATION OF TECHNIQUES



(Richardson et al. (2015))

8.3 DIFFUSIONAL RESTRICTION THAT AFFECT THE ACTIVITY



UNIVERSITÉ CATHOLIQUE DE LOUVAIN
École polytechnique de Louvain
Rue Archimède, 1 bte L6.11.01, 1348 Louvain-la-Neuve, Belgique | **www.uclouvain.be/epl**