

Faculté des bioingénieurs

Development of an enzyme immobilization system combining LbL and Avidin-biotin interaction

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LIST OF ABBREVIATIONS

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AC: Alternating current
BCA: Bicinchoninic Acid
DTT: Dithiothreitol
HABA: 4'-Hydroxyazobenzene-2-carboxylic acid
GOS: Galactosyl-oligosaccharides
GOx: Glucose oxidase
IEP: Isoelectric point
LbL: Layer-by-Layer
LB: Langmuir-Blodgett
L-DOPA: L-3,4-hydroxyphenylalanine
MOPS: (3-(N-morpholino)propanesulfonic acid)
NHS: N-hydroxysuccinimide
PAA: Poly(acrylic acid)
PAH: Poly(allylamine hydrochloride)
PBS: Phosphate-buffered saline
PDADMAC: Poly(diallyldimethylammonium chloride)
PE: Polyelectrolyte
PEG: Polyethylene glycol
PSS: Polystyrene sulfonate
PVS: Polyvinyl sulfonate
QCM: Quartz Crystal Microbalance
REIMs: Reactor with enzymes immobilized on membranes
SAMs: Self-assembled monolayers
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TMPyP: Tetrakis(4-methylpyridinium)porphyrin
wp: multi-well plate

I. INTRODUCTION

1. LAYER BY LAYER ASSEMBLY

Over the last decades, the interest in developing functionalized surface at the nanoscale has kept growing. The ability to precisely tailor nanoscale surface properties such as polarity, morphology, topology and roughness is of major importance for a wide range of research fields such as biomaterials, drug delivery, tissue engineering, biosensing, implantable materials etc...

In the search for simple, inexpensive, and versatile surface modification tools Layer-by-layer (LbL) assembly technology has emerged. It is a simple, cost effective, flexible and versatile bottom-up strategy to modify surfaces and prepare functional multilayer assemblies.¹

1.1. Concept

The fabrication of multi-layered thin film consists of the nanoscopic assembly of different materials in a single device using low-cost techniques.² The nature of the assembled materials can range from small organic molecules or inorganic compounds^{3,4} to macromolecules⁵⁻⁸ and biomacromolecules such as proteins and DNA⁹⁻¹¹ or even colloids¹²⁻¹⁴. This technique can be applied to almost any solvent accessible surface from planar surfaces to microcapsules and even cells¹⁵⁻¹⁷.²

Layer-by-Layer assembly is obtained by successively layering materials on top of each other onto a surface.¹⁸ In most cases, electrostatic interactions are the main forces driving multi-layered thin film assembly but this is by no means a prerequisite.² Many other interactions have been used successfully to build multi-layered thin films including donor/acceptor interactions^{19,20}, hydrogen bonding²¹⁻²⁶, adsorption/drying cycles^{27,28}, covalent bonds²⁹, stereocomplex formation³⁰⁻³² or specific recognition^{11,33-41} (see 1.3).² Since electrostatic interaction is not the only driving force for building a multi-layered thin film, many different materials can be used in the LbL assembly. However, even though multi-layer thin film assembly is a complex phenomenon resulting from the combination of many driving forces, electrostatic interactions remain, in most cases, the most significant one. The charge of the materials is thus an essential factor for successful LbL construction. Polyelectrolytes (PE) are widely used materials for this method.⁴² The first step consists of immersing the substrate into a solution of charged polyelectrolytes (Figure 1). Note that in most cases the charge of the surface is negative, therefore, the assembly process usually starts with a polycation solution. These polycations adsorb onto the negatively charged surface and generate an overcompensation of the surface charge.

Following a washing step to get rid of the loosely attached PE the now positively charged surface is ready to adsorb a layer of negatively charged PE (Figure 1). Once again, the adsorption of polyanions onto the surface results in an overcompensation of charge and the surface now becomes negatively charged. The growth of the multi-layered thin film depends on this overcompensation of charge. Larger overcompensation of charge will result in a greater adsorption of the oppositely charged PE at the next step and thus in a thicker multi-layered thin film. These two steps followed by washes can be repeated until the desired film thickness is obtained.¹⁸ This technique offers the possibility of designing ultra-thin ordered films in the range of 10 nm up to several tens of μm with a precision of 1 nm and a good knowledge of their molecular composition.⁴³

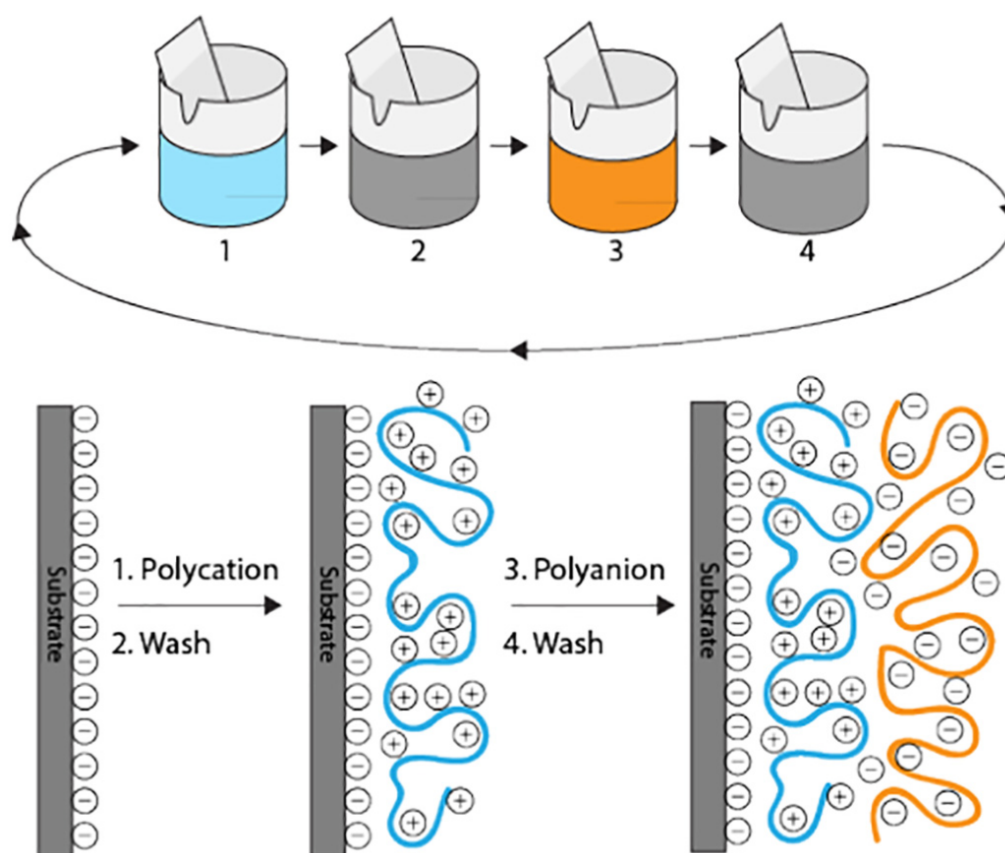


Figure 1 - **Schematic of the Layer-by-Layer deposition process.** Steps 1 and 3 represent the adsorption of a polyanion and polycation, respectively, and steps 2 and 4 are washing steps. Counterions are omitted for clarity. The polyanion conformation and layer interpenetration are an idealization of the surface charge reversal with each adsorption step.¹⁸

1.2. Different assembly techniques

LbL assembly has witnessed considerable growth in the past two decades. Many different assembly technologies have been developed to meet the requirements for diverse applications and assembly conditions. These assembly technologies are of importance because they affect both the process properties (time, scalability and manual

intervention) and the resultant physicochemical properties of the film (thickness, homogeneity and film organization).⁴⁴ Three of the major technology categories for LbL assembly are shown in Figure 2 and described below.

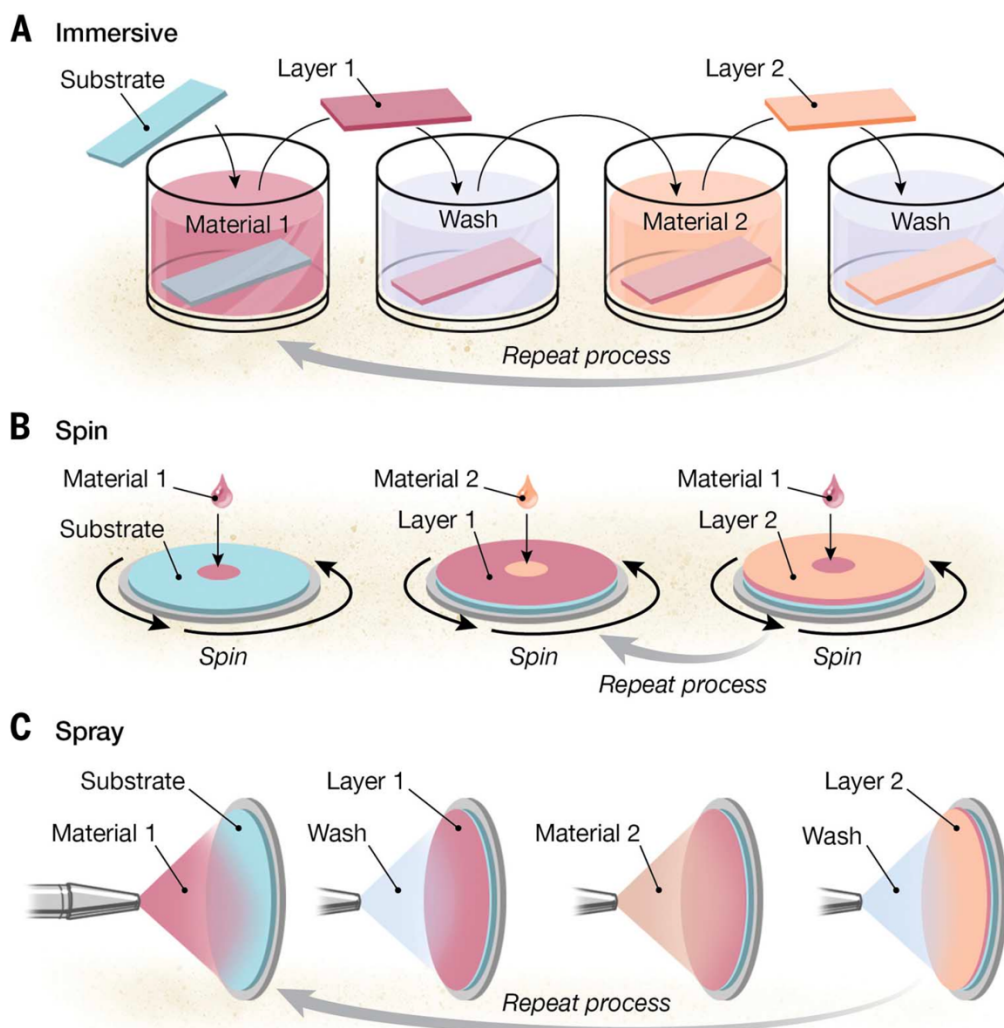


Figure 2 - **Schematics of three of the five major technology categories for LbL assembly.** Adapted from ⁴⁴

Immersive assembly

Immersive assembly, illustrated in Figure 2a, is the most commonly used LbL assembly technique and is often used as standard against which other techniques are compared. This technology is already described in 1.1. The benefits of this technique include the ease of use and versatility of material and template choice. Material can be immobilized on substrate of any shape or size. The films resulting from this assembly method typically present an interpenetrated structure with relatively thick layers. An inconvenient of this technique is that it is time consuming: the substrate generally needs to be immersed for several minutes in each bath.^{42,45} Methods such as roll-to-roll assembly⁴⁶ or dewetting⁴⁷ have emerged to reduce the assembly time. Another inconvenient of this method is

that it requires more material than other technologies, especially for immersing large substrates at industrial scale. ⁴⁴

Spin assembly

Spin assembly, illustrated in Figure 2b, uses a rotating substrate to deposit layers and remove excess coating material. The solution is deposited onto a spinning or stationary substrate then spun. It typically produces thinner, more organized and more stratified multilayers than immersive assembly. ^{48,49} The assembly process is much faster (~30 sec) and depends on the spin speed. Moreover, by controlling the spinning speed of the substrate, it is possible to tune film thickness. Higher spin speeds lead to thinner films. ⁵⁰ Films resulting from spin assembly can be transparent which makes them ideal as optical coating. ^{49,51} This method, however, is not adapted to coat complex shapes and is only adequate for flat surfaces. Moreover, at higher ionic strengths and lower spin speeds, the film presents a thicker layer where the solution was cast compared with the edges of the substrate. ^{50,52,44}

Spray assembly

In this method illustrated in Figure 2c, the coating solutions are aerosolized to be sprayed onto the substrate. This assembly method is very fast (~6 sec per layer)⁵³ and is very easy to implement at the industrial scale for large substrates. ^{54,55} It is also possible to coat complex 3D objects such as membranes with this method. ⁵⁶ Like for spin assembly, spray assembly results in films with more distinct layers than the ones obtained by immersive assembly. ^{55,57} Moreover, the film properties can be controlled by parameters such as the suspension concentration, spray flow rate, spray duration, resting duration, washing and orientation of the spraying. It is thus easy to tailor the film properties as desired. ⁴⁴

1.3. Molecular interactions driving the LbL assembly of multilayers

Just as many types of assembly techniques exist, many different interactions can serve as LbL assembly driving force. In the following section, the different types of driving forces are described. Note that to this day it is still unclear how these different interactions affect the assembly process but there's a high probability that these interactions combine to drive the LbL assembly.

Electrostatic interactions

The complexation of two oppositely charged PE is known to be athermal. ^{58,59} The change of free energy observed upon complexation of two PE is mainly due to the release of counterions which generates an increase in entropy. ⁶⁰ Upon complexation, PE chains

form ion pairs and release their counterions as shown in Figure 3. Electrostatic neutrality is conserved and no chemical bonds are formed.⁶¹ Since the counterions located around the PE chains have an important role in the complexation of the PEs, the ionic strength of the PE solutions will have an influence on the formation of the PE complexes. Moreover, changing the salt concentration in PE solution post complexation influences swelling as shown in Figure 4c. If more salt is added, the PE multilayer can even decompose.⁶¹

In the presence of an additional PE, overcompensation occurs near the surface leading to an excess charge reversal which allows the adsorption of the next PE.⁶¹ Since ions in PE solutions are well hydrated, release of water of hydration should also be considered in the PEs complexation mechanism.⁶² The effect of water release is more detailed in the next section about *Hydrophobic interactions*.

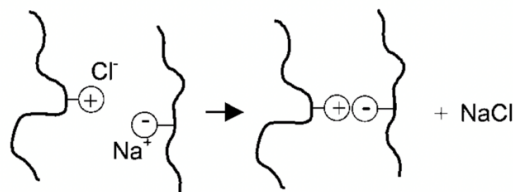


Figure 3 - **Complexation between positive PE and negative PE releasing counterions.**⁶¹

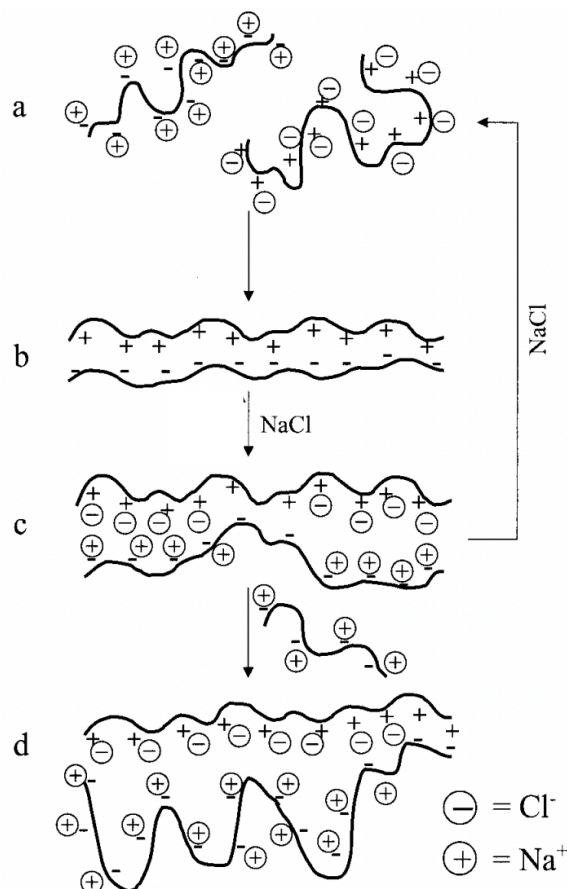


Figure 4 - **Charge balance in PE multilayers from formation (b) to swelling (c) to overcompensation (d).**⁶¹

Hydrophobic interactions

The role of hydrophobic interactions in the LbL assembly can be illustrated through the following example. Large dye molecules, like Tetrakis(4-methylpyridinium)porphyrin (TMPyP), show a greater tendency to self-assemble compared with smaller dye molecules. The smaller molecule, methylviologen, has twice as high positive charge density per atom as TMPyP. Nonetheless, it was observed that methylviologen does not adsorb onto PSS while TMPyP does.⁶³ An optimum ratio between the number of hydrophobic and charged group needs to be respected for the self-assembly to occur. If a molecule is too hydrophilic, even though it is small and highly charged, it won't form a thermodynamically stable complex with the polyelectrolyte.⁶⁴

Kotov proposed a model assessing the contribution of hydrophobic interactions to the LbL assembly of multilayer thin films.⁶⁴ In this model he considers the hydrated ion as a charged core surrounded by a layer of partially immobilized water molecules, the hydration shell. Outside this shell, electrostatically attracted oppositely charged ions form an ionic atmosphere. The LbL assembly is separated in 3 steps (1) the removal of the ionic atmosphere around the (+) PE and (-) PE, (2) the partial removal of the hydration shell around (+) PE and (-) PE and (3) the formation of the (+)PE/(-)PE film. The electrostatic interactions as well as the hydrophobic interactions are considered in this model. It was shown that when hydrophobic parts of the polyelectrolyte chains establish contact, great entropic changes are observed due to (1) the removal of the ionic atmosphere around both PE, (2) the reorientation of water molecules previously oriented by charged PE, (3) the loss of mobility of the polyelectrolyte chains and (4) the release of water molecules located around both PE. In conclusion, along with the electrostatic forces, short-range hydrophobic forces can be identified as one of the important factors determining the ability of a compound to self-assemble via LbL technique.⁶⁴

Biologically specific interactions

Biologically specific interactions include many different types of molecular interactions such as electrostatic, hydrophobic interactions, and hydrogen bonding. These types of interactions ensure high specificity and functionality to the target molecule. The main specific interactions used for LbL assembly over the years are avidin-biotin, antibody-antigen, lectin-carbohydrate and DNA hybridization.¹ Special emphasis will be placed on avidin-biotin interaction in section 2.1 given its relevance in the context of this master thesis.

1.4. Factors controlling film properties

The LbL assembly mechanism relies on a combination of driving forces. Consequently, many factors influence film properties such as pH, ionic strength, nature and concentration of assembly materials. By varying these parameters, the multi-layered film can be assembled so that its properties best match its application.

pH

In the case of PE multilayer assembly, pH influences the build-up only if one of the PE is weak. As the pH changes, the weak PE dissociates partially or totally leading to a weakly or strongly charged PE respectively. This change in charge density affects the PE conformation (see *ionic strength*) and thus has an effect on the multilayers build-up and structure.⁶⁵

When using proteins as assembly material, the choice of pH is crucial since the protein net charge can either be positive or negative whether the pH is below or above the isoelectric point. When the pH of the solution is set above the IEP of the protein, the protein will bear a negative charge. The negatively charged protein then needs to be immobilized by LbL assembly by alternating adsorption with a positive PE. For the same protein, if the solution's pH is set below the IEP of the protein, the protein will bear a net positive charge and it will have to be immobilized using a negatively charged PE. The further the pH is set from the IEP of the protein, the higher the protein absolute charge. At a pH close to the IEP, the protein bears both positive and negative charges resulting in a small net charge. Since the LbL assembly strongly depends on electrostatic interactions, a small net charge will be detrimental for LbL growth. It is preferred to set the pH of the protein solution a few units above or below the IEP to have the greatest net charge and thus good immobilization of the enzyme using an oppositely charged PE. It was shown that the amount of protein immobilized on an oppositely charged multilayer surface correlates with the absolute charge of the protein. Setting the pH away from the IEP of the protein lead to a better LbL assembly all the while immobilizing greater amount of protein onto the multilayer film. However, depending on the application, it is also important to set the pH at a value which won't denature the protein or form protein aggregates. Furthermore, the pH value at which the multilayer film is assembled is in most cases not the same as the pH of the final application. This could undermine the stability of the film leading to the destruction of the film. This is in most cases undesired unless the aim of the film is for drug release.¹⁸ The challenge is therefore to choose a working pH that provides a sufficient net charge for good protein immobilization without

affecting its biological properties all the while resulting in a suitable film stability for the desired application.

Ionic strength

Along with pH, another critical factor influencing film thickness and amount of deposited material is the ionic strength of the medium. Dubas and Schlenoff showed that film thickness, for a 10 bilayer film of PSS/PDADMAC (Polystyrene sulfonate and Poly diallyldimethylammonium chloride), increases linearly with salt concentration between 10^{-2} and 2 M.⁴⁵ These results coincide with the observations by Lösche et al. on a PSS/PAH (polyallylamine hydrochloride) multilayer film and with the results of Decher et Schmitt on the same system.^{66,67} This can be explained by the conformational change of the adsorbed polyelectrolyte in the presence of salt. At low ionic strength, the polyelectrolyte adsorbs in a “flat” conformation whereas at high ionic strength the adsorbed polyelectrolyte chains form a “loopy” conformation, as illustrated in Figure 5.

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This change in conformation is explained by the screening effect of the ionic groups fixed on the PE chains.⁶⁶ Without salts in the PE solution, the charged groups on the PE chain repel each other leading to a linear conformation. When salt is added to the PE solution, salt ions have a screening effect on the charged groups of the PE chain. PE thus becomes more flexible and adopts a more coiled structure.¹⁸

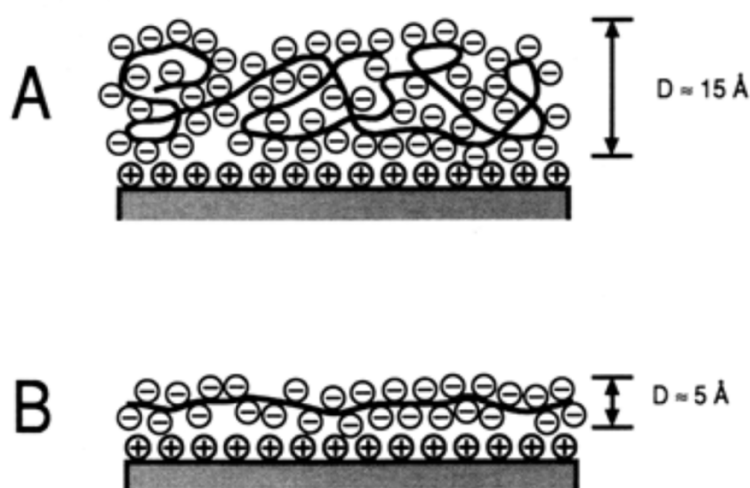


Figure 5 - **Schematic view of the structure of the adsorbed PE depending on the ionic strength.** (A) is an adsorbed PE chain in the “loopy” conformation. This is the case when the assembly solution has a high ionic strength. (B) is an adsorbed PE chain in the “flat” conformation. This is the case when the PE assembly solution has a low ionic strength.⁶⁶

When the adsorbed polyelectrolyte is in the “loopy” conformation, the charge reversal of the substrate will be more easily achieved because some segments of the polyelectrolyte aren’t attached to the substrate and therefore excess ionic side groups are exposed to the solution interface. Moreover, this “loopy” conformation increases the surface roughness of the film which increases the surface available for the adsorption of the next PE or protein.¹⁸

Additionally, increasing ionic strength in a solution of proteins to be adsorbed leads to greater adsorption of proteins on the substrate because lateral repulsion between proteins decreases due to the screening effect of the salt ions.¹⁸ It is important to note however that at high ionic strength, excessive charge screening hinders film growth by alternate adsorption. The impossibility to grow a LbL film at high ionic strength could also be due to a decrease strength of the entropic contribution of ion pairing leading to a decrease of electrostatic attraction.¹⁸ In conclusion, film thickness increases linearly with the ionic strength of the solution from which the polyelectrolytes are adsorbed up to a certain point.⁶⁶

Layering material concentration

During LbL assembly, the PEs or proteins adsorb onto a surface through diffusion driven kinetics. The rate of diffusion will depend on parameters such as temperature and concentration of the layering material solutions. Increasing protein concentration in the assembly solution allows to reach the equilibrium faster. Different protein concentrations also allow to reach different equilibrium states. It was observed that, keeping all other parameters constant, by increasing the protein concentration, more protein was immobilized on the substrate. This could be caused by an incomplete surface saturation at lower protein concentrations.¹⁸

Nature of polyelectrolyte

As seen earlier, to obtain an efficient LbL assembly, PE should be flexible to form loops at the interface and adapt as best as possible to the surface of the protein layer. Flexibility of PE can be evaluated using the persistence length (l_p). l_p is divided into the intrinsic persistence length (l_p^0) and the electrostatic persistence length (l_p^e). l_p^0 represents the stiffness coming from steric hindrance and hybridization state of the atoms in the PE chain while l_p^e is the stiffness due to electrostatic repulsion between charged repeating units of the PE chain. To ensure good flexibility of the PE, both values should be small. l_p^0 depends strictly on the nature of the PE while l_p^e can be tuned by the salt concentration of the PE solution as explained in 1.4 *salt concentration*. l_p^e can also be minimized by

choosing a PE with fewer number of charged repeating units (e.g tuning the ionization degree of chitosan or weak PE).¹⁸

The ability of the PE to form hydrogen bridges with the protein also impacts multilayer growth. It was observed by Lee et al. that the growth of a κ -casein/PSS multilayer film was linear while that of a κ -casein/PAA film was first exponential then linear. This difference in film growth was explained by the formation of hydrogen bonds between the protonated carboxylic acids of PAA (at pH 3) and the amide bonds of κ -casein. Consequently, much thicker layers were obtained in the κ -casein/PAA construction.⁶⁸

Drying

Finally, it was observed that drying the multilayer film between each adsorption step ensures LbL growth. Exponential growth of the film is observed when drying compared to linear growth when not drying. This change in growth is due to the structural change of the film upon drying. Rearrangement of the PE layer following the drying step allows further adsorption of the same PE.¹⁸

1.5. LbL films characterisation

When assembling multi-layered films, different types of characterization are required. First, one must confirm the correct deposition of the layered materials. Then, after confirmation of film deposition, it is possible to determine film properties, such as thickness and porosity, and quantify the growth process.⁶⁹

Depending on the type of substrate used and on the intended application, different characterization methods can be used. Whether the film is assembled on a planar or a particulate substrate, the characterization methods will vary. This work focuses on film assembly on planar substrates and only the characterization methods on the latter are described.⁶⁹

Assessing film growth

Quartz Cristal Microbalance (QCM) is the most widely used and accessible method to assess the LbL assembly process and in situ film growth. By measuring the changes in frequency and the dissipation time of a quartz crystal resonator it is possible to evaluate the LbL assembly in situ and ex situ as illustrated in Figure 6. The small size of its instrumentation, ease of use and applicability to almost any type of layering material contributes to its popularity for LbL characterization.⁶⁹

Using the QCM, it is possible to quantify the mass of deposited material or even the mass of the film itself since QCM is capable of measuring subnanogram levels of materials.⁶⁹ QCM also gives information about the viscoelastic properties of the deposited film.

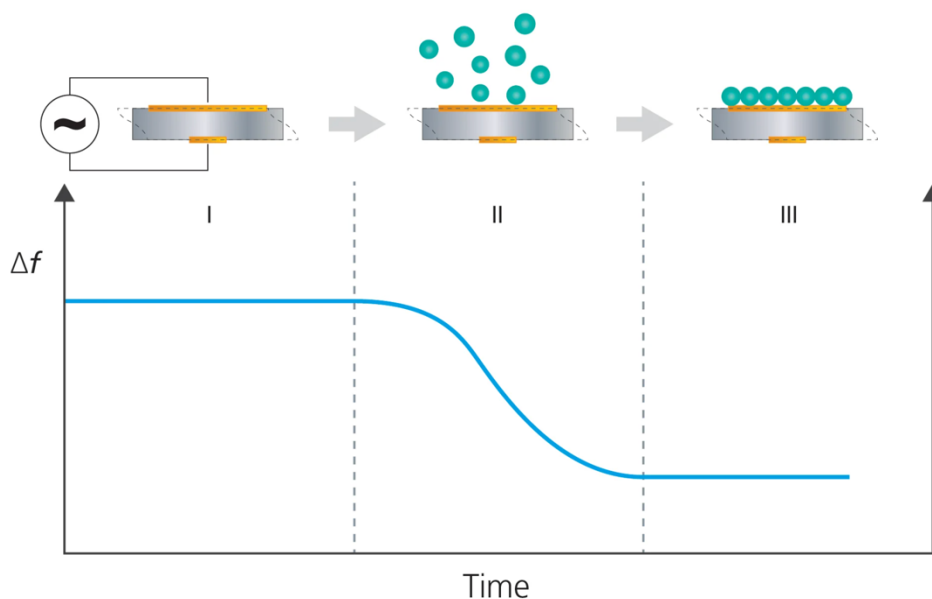


Figure 6 – **Schematic illustration of the QCM working principle.** (I) bare sensor surface and stable baseline of Δf . (II) molecules adsorb to the sensor surface which results in a frequency decrease. (III) the surface uptake has been completed and the frequency response has stabilized. The frequency shift between I and III can be used to quantify the mass uptake at the sensor surface.⁷⁰

Many other methods can be used to characterize film growth. Optical methods include ellipsometry, surface plasmon resonance (SPR) and dual polarization interferometry (DPI). Other part of the light spectrum can be used to assess film growth such as UV-vis spectrometry if the adsorbed materials have specific adsorption bands and if the substrate is transparent, fluorescence when using fluorescent building blocks, X-rays (SAXS, XRD, XPS), in the IR-range FT-IR spectroscopy and finally surface-enhanced Raman spectroscopy (SERS) can be used to characterize LbL films containing metallic nanoparticles and carbon nanotubes.⁶⁹

Examining film morphology

The structure and morphology of LbL thin film can be evaluated using various microscopy techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM). Film roughness can be monitored by water contact angle analysis.⁶⁹

Internal film structure

LbL films are typically porous. Nuclear magnetic resonance cryoporometry (NMR) as well as positron annihilation spectrometry (PAS) and XRR have been used to determine the

porosity and the internal structure of the film. Infrared spectroscopy, sum frequency generation vibrational spectroscopy and XPS can also be used to assess the porosity of the multi-layered thin film.⁶⁹

Film activity

In addition to the structural characterization of the film, different activity assays can be carried out to evaluate the efficiency of the film. Each technique is directly adapted to the assembled film. For example, BCA assays are carried out to quantify protein concentration in the film. Enzymatic assays are performed when enzymes are immobilized in film and antibacterial activity for antimicrobial films.

1.6. Protein multilayer assembly using LbL

For a successful protein multilayer assembly, it is important to alternate the adsorption with a “soft” linear or branched PE layer. These flexible linear PEs penetrate between protein globules and act as an “electrostatic glue”.⁴³ By forming a cover around the protein surface and creating bridges between the protein molecules, linear PEs can stabilize the layer. This is crucial for a good immobilization of the protein in the LbL multi-layered thin film. By respecting the proper alternation of negatively and positively charged species, it is possible to assemble multi-layered thin films with protein, PEs, nanoparticles and more.⁴³ The most common charged species used for the immobilization of protein are PEI, PAH, PDADMAC (polycations) and PSS, DNA and heparin (polyanions).^{15,71–73} As mentioned earlier in section 1.4, the pH of the protein solution must be set apart from the IEP so that the proteins are sufficiently charged for a good adsorption on the oppositely charged PE.

The surface of the substrate on which the protein is adsorbed can affect the stability of the protein. It is preferred to treat the surface beforehand with the alternate adsorption of PEI/PSS.^{34,72,74,75} These surfaces can then be immersed alternatively in the protein and oppositely charged PE solutions with intermediate washing steps. By monitoring the adsorption kinetics with a QCM it was shown that each step eventually reaches adsorption saturation and is reproducible.^{72,74} The time required for the protein to reach adsorption saturation will depend on the charge of the protein (at the experimental pH) and on its size but in general, protein adsorption time ranges between 10 to 20 min.⁴³

Packing density, orientation and hydration of the proteins in a layer are factors influencing protein monolayers thickness.⁴³ Moreover, the amount of protein adsorbed at each step depends on the thickness of the underlying PE layer. The thickness of the PE layer can

be tuned by controlling the ionic strength of the polyelectrolyte solution used during adsorptions steps. The higher the ionic strength, the thicker the polyelectrolyte layer adsorbed onto the film (see 1.4 *salt concentration*). A thicker PE layer provides a greater surface area for protein immobilization, but also more protein is needed to overcompensate the charge of the underlying film. As a result, more protein is immobilized in the multilayer film.⁷⁶

1.7. Enzyme immobilization using LbL

Immobilization of enzymes in LbL films presents many practical advantages for microreactors and biosensors development. A major advantage of the LbL technique is its mild assembly conditions which allow to preserve the protein folding and activity.⁷⁷ Moreover, entrained water, important to maintain enzymatic activity, is present in LbL films.⁷⁸

In most cases of protein immobilization in multi-layered thin film, no denaturation of the protein is observed meaning the activity of enzymes is preserved^{74,79,80}. In some cases, immobilization of enzymes in a multi-layered film even enhances enzymatic stability. This could be due to the fact that inside multilayers, enzymes are protected from microbial attacks. Experiments on glucose oxidase (GOx) showed that in the multilayer with PEI, the enzyme demonstrated increased temperature stability and preserved its activity at a higher pH than in solution.⁴³

Following immobilization of the enzyme in a multi-layered thin film, it is expected to observe a decrease of specific enzymatic activity. This means that for a similar amount of enzyme, the observed activity of the immobilized enzyme is lower than if it were free in solution. For GOx, the immobilized enzyme exhibits 20-30% of its activity in solution. The activity of the enzyme/PE film increases linearly with the number of layers constituting the film until the film bioactivity reaches a saturation. This saturation is due to diffusion limitations of the substrate inside the film. Activity increases up to a point where the film thickness becomes so important, the substrate faces difficulties to reach the enzymes located in the lower layers of film, resulting in saturation of the total activity. Diffusion coefficients for some organic molecules in polycation/polyanion multilayers were determined by Möhwald and Klitzig⁸¹. Substrate transport through the multilayer and compactness of the film will affect the maximum number of layers required for best enzymatic activity of the film.

2. BIOTIN-AVIDIN INTERACTION

Among the various types of biological interactions that can be exploited for producing multilayer films, the one between avidin, a protein found mostly in egg whites, and biotin (vitamin B7) presents many interests. The aim of the following section is to briefly introduce how this interaction works, and how it can be used as a strategy for enzyme immobilization.

2.1. Concept

Avidin is a glycoprotein isolated from egg-whites. As seen on Figure 7, it is formed of 4 identical subunits each possessing a binding site for biotin. Avidin has a combined molecular mass of 66 kDa and has an IEP of 10 which makes it a positively charged protein at pH lower than 10.⁸² Tryptophan and Lysine residues are involved in the formation of the binding pocket.⁸³



Figure 7 - **Molecular model of Avidin.** The four subunits (dark blue, purple, blue and red) are identical and each consist of 128 amino acids. Four biotin in total, represented in yellow, are bound within the binding pockets of the avidin subunits.⁸⁴

Biotin, shown in Figure 8, is a well-defined molecule of simple structure and ubiquitous in nature. It is a key component in many processes involving carboxylation reactions. It functions as a cofactor and a transporter of CO₂.⁸⁵

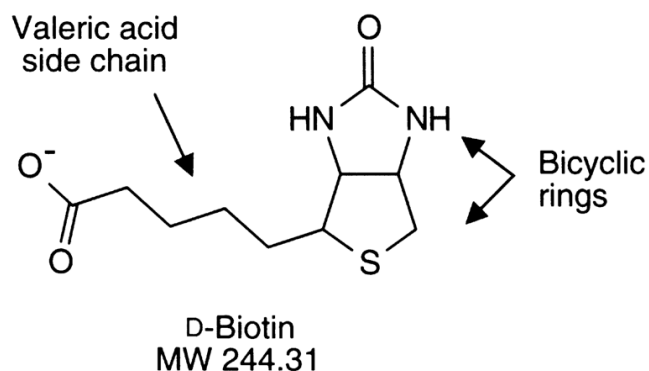


Figure 8 - Molecular representation of D-biotin. ⁸⁶

The binding between biotin and avidin occurs between the bicyclic ring of biotin and the four binding pockets of avidin. The valeric acid of biotin is not involved in the interaction with avidin. ^{82,87} It is possible to modify the biotin's valeric acid group without altering the binding between biotin and avidin. Protein biotinylation can occur directly by attaching D-biotin to the protein via its valeric acid side chain or by derivatizing D-biotin at the carboxylate to create the desired spacer arm and reactive group. ⁸⁶

The Biotin-Avidin interaction is among the strongest noncovalent affinities known. The dissociation constant for the biotin-avidin complex is as low as 1.3×10^{-15} M. Moreover, the complex presents a resistance to denaturation to the most extreme conditions. The structural integrity and activity of the complex is maintained under concentrations as high as 8 M urea or 3 M guanidine hydrochloride. ⁸⁸ Extreme pH, presence of denaturant or detergent and extreme temperature don't prevent the complex formation. ⁸⁹ The biospecific interaction between biotin-avidin resembles that of antibody-antigen or receptor-ligand but has a greater affinity constant. ⁸⁶

The great binding affinity of biotin for avidin and the high stability of the complex has made it a useful tool in many applications such as immunoassay ⁹⁰, imaging ⁹¹, protein purification ^{92,93} and protein immobilization ⁹⁴.

2.2. Use of biotin avidin interaction for enzyme immobilization

The idea behind this strategy is to use previously immobilized avidin to serve as an anchor for biotinylated enzymes. ⁹⁵⁻⁹⁹ The first step consists of immobilizing Avidin using the LbL assembly method. As seen above, LbL relies strongly on electrostatic interactions. Since the IEP of Avidin is high, the protein is very positively charged at the working pH of 5 and can easily be immobilized using a negatively charged PE like PSS. ⁹⁶ The second step requires to biotinylate the enzyme to immobilize. Thanks to the biotin-Avidin interaction, the biotinylated enzyme binds to the avidin previously immobilized in the multilayer film.

2.3. Protein biotinylation techniques

The popularity and diversity of applications in which the biotin-avidin interaction can be used lead to the development of a great variety of techniques to biotinylate proteins and macromolecules. The standard biotinylation technique consists of using biotin derivatives which contain reactive moieties able to couple with functional groups in proteins or molecules. The functional groups targeted for biotinylation are amine residues, carboxylates, sulfhydryls and carbohydrate groups.¹⁰⁰ The basic design of biotin reagents consists of the bicyclic biotin ring on one end and the reactive group on the other, as shown in Figure 9. The two groups are separated by a spacer arm built off the valeric acid side chain of the biotin. This spacer arm is important since the biotin is buried deep inside the binding pocket of avidin. By extending the biotin group away from the modified protein, it ensures better interaction with avidin.¹⁰¹ The spacer arm also affects the rate of binding of biotinylated compounds to avidin. Longer spacer arms lead to faster rate of interaction.¹⁰² Three different types of spacer arms can be used: (1) aliphatic spacers, (2) Polyethylene glycol (PEG) based spacers and (3) cleavable or non-cleavable. The aliphatic arm consists of hydrocarbons which makes it very hydrophobic and sometimes leads to the aggregation and precipitation of biotinylated proteins in solution. To circumvent this issue, it is important to keep the biotinylation level at a minimum or to use hydrophilic PEG-based spacers.⁸⁶

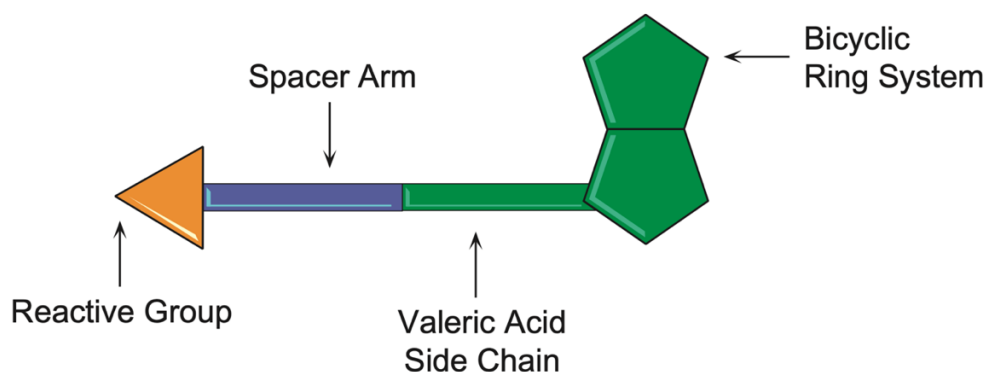


Figure 9 - **Basic design of a biotinylation reagent.** The bicyclic ring is represented in green, the spacer arm in blue and the reactive group in orange.⁸⁶

Since biotin is a small molecule, its covalent attachment to proteins and macromolecules will not significantly affect their physicochemical properties.¹⁰³ Once biotin is covalently attached to a protein, the latter will interact strongly with avidin.⁸⁶

The main biotinylation techniques are introduced in the following sections.

Amine-reactive biotinylation reagents

Amine-reactive biotinylation reagents react with primary amines in proteins and molecules to form a covalent bond. Two types of reagents can be used: N-hydroxysuccinimide (NHS) esters and carboxylates. NHS esters react spontaneously with amines to form amide bonds while carboxylates are coupled to amines via a carbodiimide-mediated reaction.⁸⁶

NHS-Biotin, shown in Figure 10, results from the activation of the valeric acid carboxylate group of biotin to an NHS ester.⁸⁶

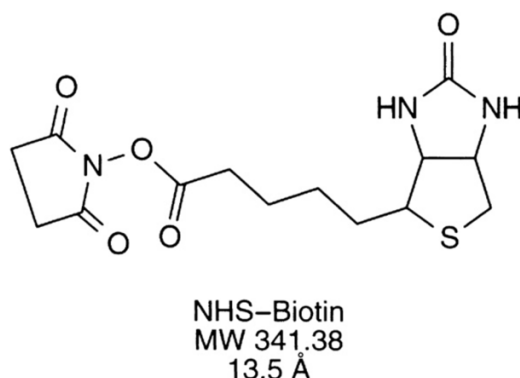


Figure 10 - Molecular representation of an NHS-Biotin.⁸⁶

Through a nucleophilic attack of an amine on the carbonyl group of the NHS ester, the NHS group is released and a stable amide bond is formed between the protein and biotin, as shown in Figure 11. The reaction is carried out under mild alkaline conditions and the biotin incorporation efficiency is usually high.⁸⁶

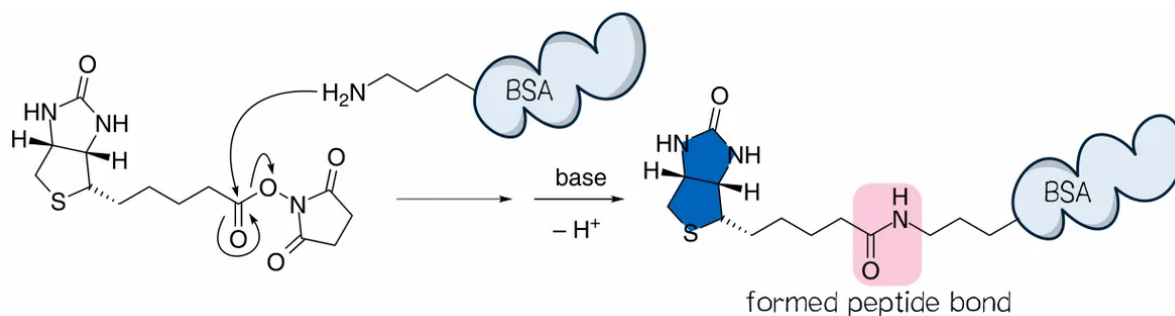


Figure 11 - Nucleophilic attack of the protein's amine on the carbonyl group of the NHS ester to form an amide bond between the protein and biotin.¹⁰⁴

Note that NHS-biotin is insoluble in water. It must first be dissolved in organic solvent to facilitate dissolution. Moreover, NHS-biotin insolubility can lead to protein precipitation or precipitation of the buffer salt. To circumvent this problem, it is possible to use Sulfo-NHS-biotin, a water-soluble analog of NHS-biotin that contains a negatively charged sulfonate group on its NHS ring.⁸⁶

Other amine biotinylation reagents exist but are less common. NHS-*iminobiotin* is an analog of NHS-biotin that has less affinity for binding avidin. It is used in cases where reversible binding of biotin to avidin is sought. Sulfo-NHS-SS-biotin is a long-cleavable biotinylation reagent. Once the reagent has reacted with the protein it is possible to cleave the two at the di-sulfide bridge using dithiothreitol (DTT). This comes in handy to purify molecules using affinity chromatography on a column of immobilized avidin by using milder conditions than the ones required to disrupt the biotin-avidin interaction. ¹⁰⁵

Other biotinylation reagents

Apart from amines, other functional groups can be targeted for biotinylation such as sulfhydryl, carbonyl or carboxyl groups. The biotinylation reagents vary in their reactive group to target different functional groups on the protein. By doing so, the biotinylation can be directed away from active sites or binding sites of the protein, preserving its activity. ⁸⁶

2.4. Determination of the extent of biotinylation

As seen above, many ways exist to biotinylate many different types of biomacromolecules. It is essential, once the macromolecule is biotinylated, to control the good biotinylation of the latter and determine its extent. Hereunder, some methods to do so are briefly introduced.

4'-hydroxyazobenzene-2-carboxylic acid (HABA) dye

HABA is a small dye molecule capable of binding to avidin at the biotin binding sites. The HABA-Avidin complex has a characteristic absorption at 500 nm. Visually, a change of color from yellow to dark orange is observed. Since biotin-avidin interaction is much stronger ($K_d = 1,3 \cdot 10^{-15}$ M) than HABA-avidin ($K_d = 6 \cdot 10^{-6}$ M) ¹⁰⁶, upon addition of biotin, HABA molecules are displaced by biotin molecules and the absorbance at 500 nm decreases, as represented in Figure 12. ¹⁰⁷

This colorimetric assay allows to measure the amount of biotin or biotinylated protein in a sample. This method is easy to use and is not time consuming. However, some biotinylation sites will not be able to interact with avidin due to steric hindrance and will not contribute to the signal. In most cases, the HABA detection method results in an underestimation of the extent of biotinylation of a protein. ¹⁰⁸ To circumvent this problem, it is possible to digest the biotinylated protein using an enzyme. This breaks the protein into small fragments which can therefore interact more easily with avidin if they possess a biotinylation site. Note that if the goal of the application relies on the interaction between the biotinylated protein and avidin, the HABA assay using the whole protein

could provide information on the number of accessible biotinylated sites of the modified protein.

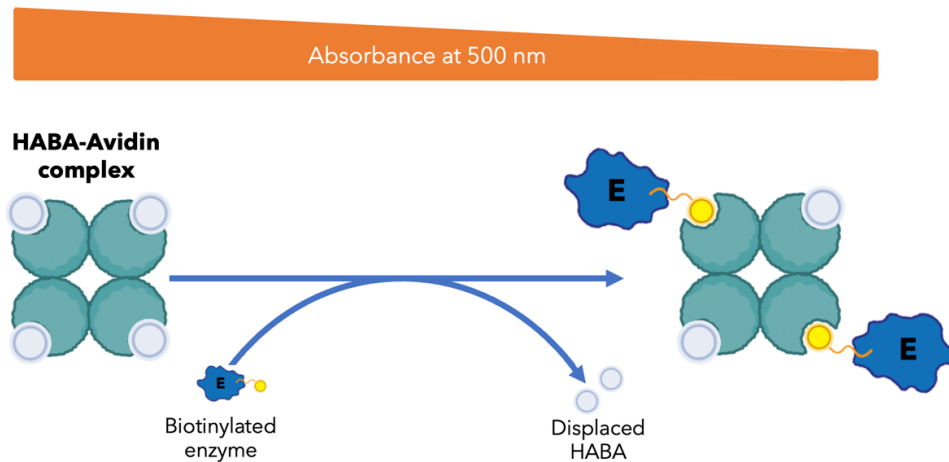


Figure 12 - Schematic representation of the HABA assay to determine the extent of biotinylation of a modified enzyme.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE can also be used to determine the extent of biotinylation of a protein. The molecular mass of biotin, or biotin reagents such as NHS-LC-biotin, is around 400 Da. Proteins and enzymes subject to biotinylation are usually much bigger (around tens of thousands Da). This big difference in molecular mass makes it difficult to visualize two different bands on the SDS-PAGE gel for non-biotinylated protein vs biotinylated protein. To overcome this issue, avidin is added to the biotinylated protein solution. If the protein is biotinylated, avidin binds to the protein and forms a bigger complex that is easily differentiated from the non-biotinylated protein. Different bands can thus be observed: one band for the non-biotinylated protein, one band for the avidin-biotinylated-protein complex and one band for avidin (if the solution is saturated in avidin). Since avidin has 4 binding sites for biotin, it is possible that several different complexes are formed with one or more biotinylated protein bound to avidin. ¹⁰⁹

Mass spectrometry

Mass spectrometry can be performed on digested peptide fragments or directly on the native form of the biotinylated protein. For the digested protein, since NHS-biotin reacts with primary amine-groups such as Lysine, the digestion of the protein using trypsin will cut the peptide fragment next to arginine residues but will not be able to cut next to Lysine residues that reacted with NHS-biotin. The peptide fragments analyzed through mass spectrometry will thus be bigger for the biotinylated protein than for the unmodified protein. When the proteins are analyzed in their native form, biotin bound to the protein

is identified by a mass shift corresponding to the mass of a biotin molecule (depending on the biotinylation reagent used, 244 to 358 Da) on the mass spectrum. Since in most cases a protein can be biotinylated more than once, a range of peaks is observed on the mass spectrum, each peak corresponding to a protein modified with a different number of biotin residues. ¹⁰⁸

Other methods

Other methods exist to determine the extent of biotinylation such as fluorescence binding assay ¹¹⁰, chromatography ^{111–113} and spectrophotometric ¹¹⁴ which will not be detailed in this work.

3. ENZYME IMMOBILIZATION FOR BIOCATALYTIC MEMBRANE APPLICATIONS

Enzymatic reactions are seen as a green and efficient method to produce and degrade molecular compounds under mild reaction conditions.¹¹⁵ Unfortunately, at the industrial scale, the unstable properties and low reusability of enzymes has greatly limited their applications.¹¹⁶ Enzyme immobilization for the development of biocatalytic materials is a promising alternative to overcome both stability and reusability limitations.^{117,118} However, in most cases, enzyme immobilization leads to a decrease in enzymatic activity.¹¹⁹ The balance between activity and stability is an important parameter to consider when immobilizing enzymes.¹²⁰

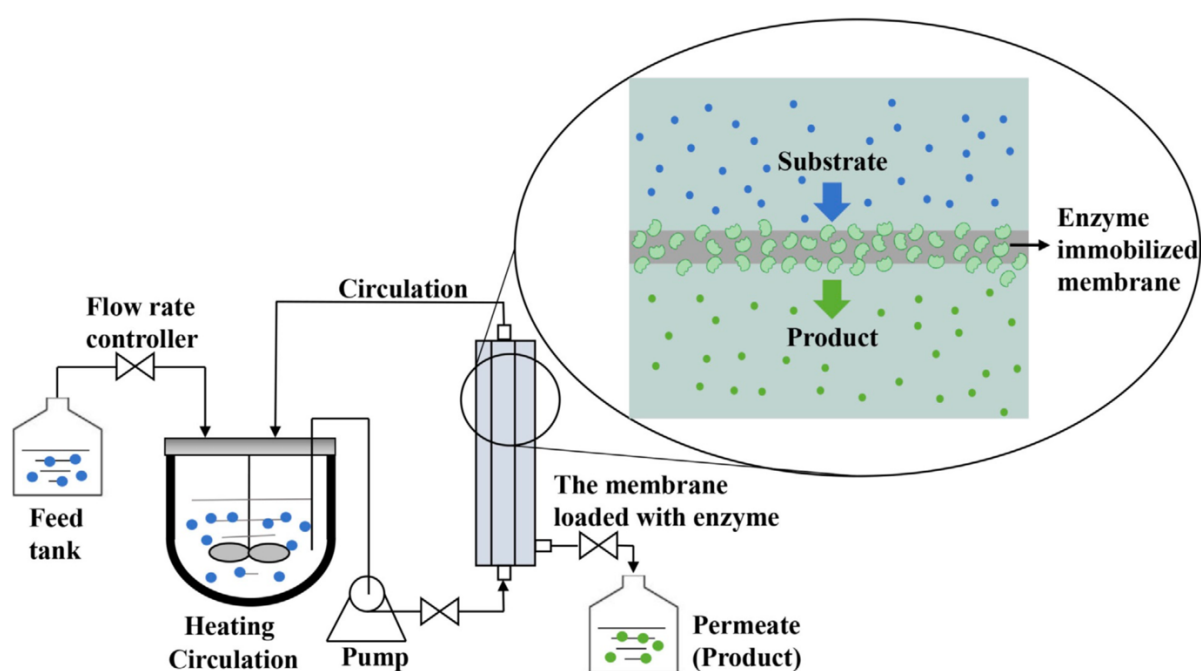


Figure 13 - Scheme of the REIM system.¹²⁰

Reactors with enzymes immobilized on membranes (REIMs) present many advantages for biocatalytic industrial applications. The membrane serves as both a selective barrier and as a support for immobilization.¹¹⁵ By passing through the membrane on which the enzymes are immobilized, the substrate is converted into the product, as shown in Figure 13. Thanks to the membrane, the products are continuously separated from the reaction system leading to a shift in the reaction equilibrium towards the product side. Moreover, the membrane presents a large surface area for reaction and immobilization of the enzymes and allows a continuous product formation.¹²⁰

REIMs present interesting systems for bioconversion. Many applications have emerged over the years in different sectors such as food, pharmaceuticals and bioremediation.

These applications can be classified into three categories: (1) degradation and synthesis of biomacromolecules, (2) degradation of organic pollutants and (3) preparation of small molecular organic compounds. ¹²⁰

3.1. Degradation and synthesis of biomacromolecules

The degradation of biomacromolecules consists mainly of the hydrolysis of proteins, oils and fats. ¹²⁰

For protein hydrolysis, trypsin can be immobilized on the membrane's surface. ¹²¹ The membrane allows the permeation of the products and retains the substrate since the latter is larger and cannot go through the pores. By controlling retention time, it is possible to control the degree of protein hydrolysis. This system presents improved efficiency compared to batch processes although enzyme inactivation and membrane fouling was observed due to shearing forces. ¹²⁰

For oil hydrolysis, lipase is immobilized on the membrane's surface. Since the substrate, oil, has limited solubility in water, the reaction is divided in two phases. ¹²² Figure 14 illustrates the continuous production of glycerol and fatty acids at the two-phase interface where the enzymes, lipase, are immobilized on the membrane. ^{123,124} An advantage of this two-phase REIM system is that it avoids emulsification. Moreover, the enzyme's stability is significantly improved compared with a free enzyme reaction.

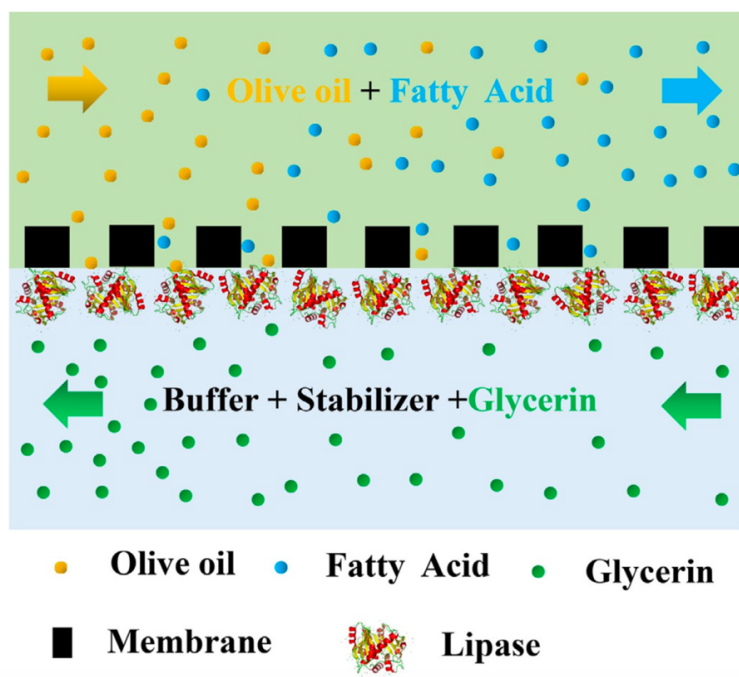


Figure 14 - **Two-phase REIM model for the continuous hydrolysis of olive oil.** Lipase is immobilized by adsorption on the membrane's surface. Olive oil is hydrolyzed at the two-phase interface to produce glycerol and fatty acids. The products are simultaneously produced and isolated. ¹²⁰

3.2. Degradation of organic pollutants

REIMs are widely used in the degradation of organic pollutants. The main enzymes used in REIMs to do so are Laccases. These oxidoreductases can transfer four electrons from four molecules of substrate to O_2 thanks to their catalytic core composed of four copper atoms, generating only water as a byproduct, as illustrated in Figure 15. These enzymes can efficiently degrade organic pollutants such as tetracycline¹²⁵, dyes¹²⁶, phenols¹²⁷ and syringaldehyde¹²⁸. These assets make it an interesting enzyme for water remediation applications.¹²⁹ However, waste-water treatment presents a big challenge at larger scale since enzymes can easily be washed away by treated effluents and membrane fouling is important.¹³⁰

Other enzymes such as esterase can be used to degrade antibiotics containing ester bonds (e.g. erythromycin).^{131,132}

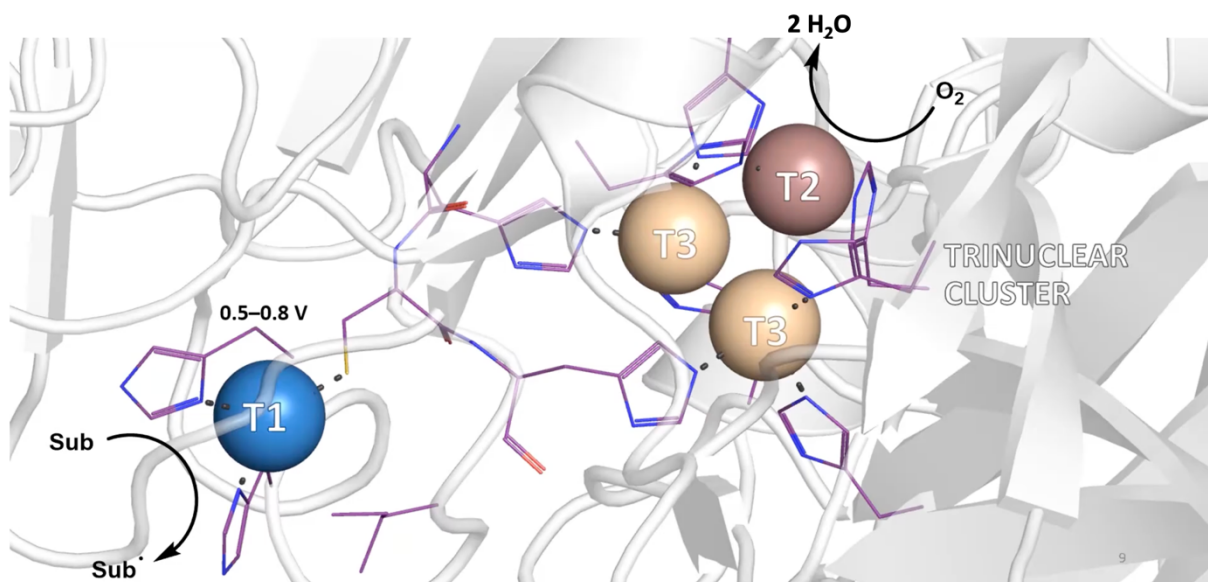


Figure 15 - **Schematic view of the catalytic core of Laccase.** T1, T2 and T3 represent the four copper atoms. T1 has the highest redox potential (0.5-0.8 V) and is the substrate oxidation site. T3 and T2 form a trinuclear cluster that reduces O_2 into H_2O . The substrate binds near T1 which extracts 1 e^- from the substrate. This e^- is relayed through protein functional groups to the trinuclear cluster. Four e^- need to accumulate at the trinuclear cluster for T3 to transfer the e^- to T2 which can only then use them to reduce O_2 into 2 molecules of H_2O .¹²⁹

Compared to free-enzyme, membrane immobilized enzymes show a significant increase in both stability and reusability. Other advantages of using REIMs for waste-water treatment applications are the high hydrolysis efficiency and the continuous operation for product degradation and separation.¹²⁰

3.3. Preparation of small molecular organic compounds

Small molecule drugs ^{133,134}, methanol synthesis ¹³⁵ as well as polymerization of small molecular sugars have been achieved using REIMs technology ¹³⁶.

Fumarase has been immobilized on asymmetrical membranes of polysulfone to produce L-malic acid. ¹³⁷ Tyrosinase was immobilized on asymmetrical tubular membranes to produce L-3,4-dihydroxyphenylalanine (L-DOPA). ¹³⁷ Finally, enzymatic deracemization of (R,S)-ibuprofen ester was made possible on REIM although product separation was difficult since product and substrate have a similar molecular weight. ¹³⁸

Enzyme immobilization on membrane using LbL assembly has proven to be a promising technique for catalytic processes comprising cascade reactions. ^{135,139} By immobilizing sequentially three enzymes, CO₂ can be converted to methanol. ¹³⁵ The three enzymes were immobilized using the LbL assembly technique on three different membranes, as shown in Figure 16. This system was compared with the co-immobilization of the three enzymes in another REIM. The sequential immobilization showed much better results in term of methanol synthesis efficiency and the ultrafiltration loss was reduced. ¹²⁰

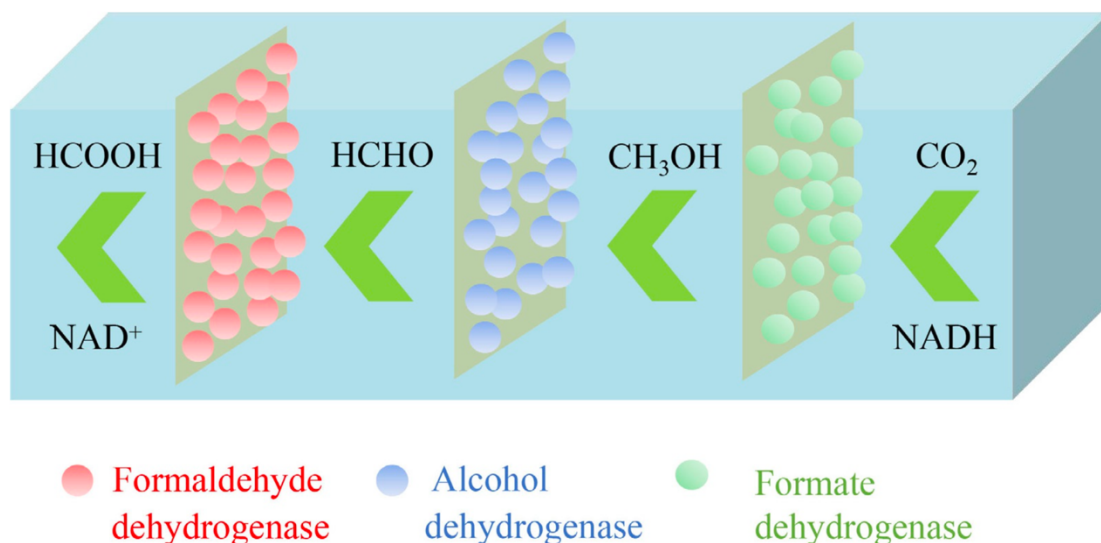


Figure 16 - **Model of the sequential immobilization by LbL of the three enzymes:** FDH (red), ADH (blue) and FaldDH (green). CO₂ is catalyzed step-by-step to synthesize formic acid. ¹²⁰

Finally, hydrolysis to form small sugar molecules or the polymerization of these small molecular sugars is possible using REIMs. ^{140,141} For example, galactosyl-oligosaccharides (GOS) are continuously synthesized by β -galactosidase immobilized on membranes. ^{142,143} The REIMs process ensures a separation and purification of GOS from the enzyme which is essential to avoid enzyme inhibition. ¹⁴⁴

3.4. Conclusion

The use of REIMs systems for degradation and synthesis of biomacromolecules, degradation of organic pollutants and synthesis of small molecule drugs has been demonstrated in many different applications. The advantages of REIMS for these processes are numerous. An increase in enzyme stability and reusability as well as the simultaneous and continuous production and separation of the products has proven useful in many applications. However, most of the applications described above were only tested at laboratory scale and membrane fouling impedes the scale up to industrial level. Moreover, the loss of enzymatic activity following enzyme immobilization adds another dimension to the scale up issue. ¹²⁰

II. OBJECTIVES AND STRATEGY

The objective of this work is to develop a versatile enzyme immobilization method using the LbL assembly technique and the specific interaction between a biotinylated enzyme and avidin protein. This innovative approach offers two significant advantages. Firstly, it enables the immobilization of almost any enzyme onto a surface, as long as it can be biotinylated. Secondly, the avidin-biotin interaction, known to be among the strongest noncovalent interactions, is expected to enhance the stability of the multilayer film.

This technique has been employed to functionalize nanoporous polycarbonate (PC) membranes to benefit from their extremely high specific surface. Glucose oxidase (GOx) was used as model enzyme. The newly developed immobilization approach relying on avidin and biotinylated GOx was compared to a classical LbL system in which GOx was immobilized using an oppositely charged PE, bPEI.

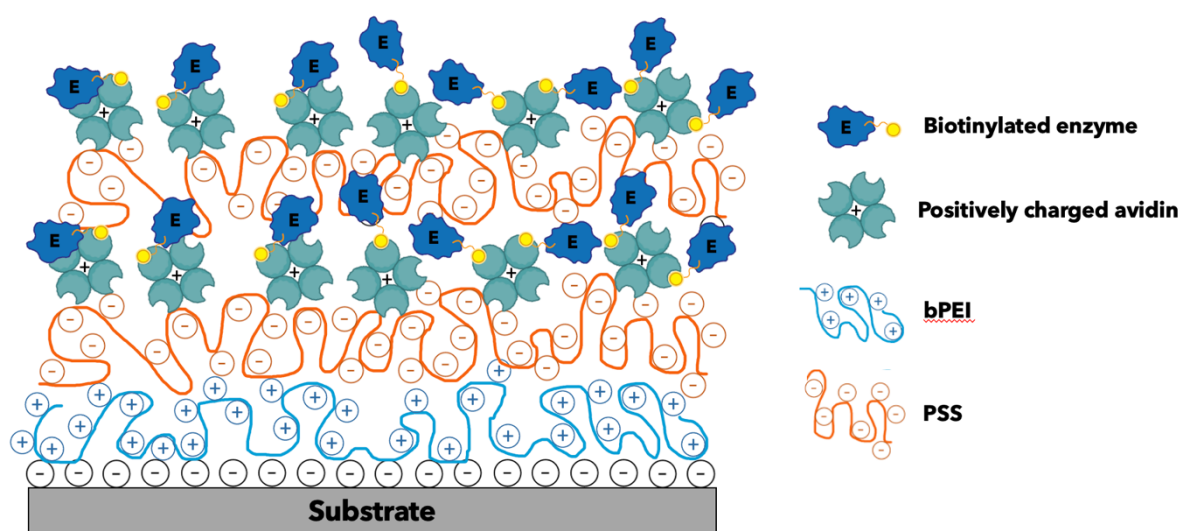


Figure 17 - **Schematic view of the enzyme immobilization using LbL assembly and biotin-avidin interaction.** First a positive PE, bPEI, is adsorbed on the negatively-charged substrate. Then a negatively-charged PE, PSS, is adsorbed onto the layer of bPEI. At pH 5, Avidin is positively charged and adsorbs onto the layer of PSS. Biotinylated enzyme binds to the immobilized avidin. To form a multilayer of PSS-Avidin-Biotinylated enzyme, the last three steps can be repeated.

The results presented in this work are divided in two sections: (1) Enzyme immobilization in the LbL film assembled on flat surfaces, (2) Enzyme immobilization in the LbL film assembled onto membranes.

In the first section, we wanted to assess the assembly of different systems on flat surface. To do so, we started by monitoring the assembly of the different materials using QCM. We then performed activity and stability comparison between different system architecture in order to select the most relevant ones. From this part, two constructions

stood out due to their higher activity and stability. We were then ready to move on to LbL film assembly onto membranes.

In the second section, we assessed and compared the efficiency of the two best LbL constructions implemented onto the membrane. To do so we developed a methodology giving us access to different information: (1) the theoretical amount of enzyme and protein immobilized onto the membrane, (2) the effective activity of the assembled membrane in static mode, (3) the amount of enzyme leached out of the membrane.

At the beginning of the project, we were using Laccase as a model enzyme. But since we were facing difficulties during the laccase biotinylation step due to purity issues, we decided to switch to a purer enzyme, GOx. This led to a part of this work in which, in parallel of the initial project, we tried different purification techniques in order to increase the purity of the initial model enzyme.

III. MATERIALS AND METHODS

1. CHEMICALS AND MATERIALS

Materials used in this work are listed in Table 1 and Table 2.

Table 1 – List of the materials and chemicals used in the experiments.

	Supplier	Reference	Comments
POLYELECTROLYTES			
PSS	Sigma-Aldrich	527483-1L	Mw ~ 70 g/mol
bPEI	Sigma-Aldrich	408727	Mw ~ 25 g/mol
ENZYMES			
Laccase (from <i>Trametes versicolor</i>)	Sigma-Aldrich	38429-10G	Mw ~ 56 kDa
GOx (from <i>Aspergillus niger</i>)	Sigma-Aldrich	G7141	Mw ~ 160 kDa
AVIDIN-BIOTIN REAGENTS			
Avidin	Thermo scientific	21128	Mw ~ 67 kDa
EZ-Link Sulfo-NHS-LC-Biotin	Thermo scientific	21335	Mw ~ 556 kDa
BUFFER			
Acetate Buffer (pH 5, 50mM)	Sodium acetate	VWR	27653.292
	Acetic acid	Sigma-Aldrich	695092
PBS (pH = 7.45)	Thermo scientific	18912-014	Tablet of 5 g
ASSAYS			
ABTS	Sigma-Aldrich	10 102 946 001	Tablet of 10 mg
GOx enzymatic assay	Megazyme	K-GLOX	
BCA	Thermo scientific	23225	
HABA	Thermo scientific	28010	
DIVERSE CHEMICALS			
H ₂ SO ₄	Fisher Scientific	S/9240/PB15	>= 95%
H ₂ O ₂	VWR	23619.297	30%
Acetone	VWR		
(NH ₄) ₂ SO ₄	Merck	539 A47317	
MOPS	Sigma-Aldrich		
OTHERS			
Dialysis cassettes	Thermo scientific	A52971	10 kDa cut-off
SDS-PAGE gel	Eurogentec	ID-PA4121-010	4-12%

Table 2 – Membranes used for the experiments.

	Supplier	Membrane diameter ϕ_m	Thickness ρ	Pore diameter ϕ_p	Pore density ε
MEMBRANE	It4ip	18 mm	25 μ m	400 nm	10 ⁸ pores/cm ²

2. METHODS

1.1. LbL assembly

LbL assembly represents a major part of the experiments carried out in this work. LbL assemblies are carried out in 96 multi-well plates (wp) for flat surface assembly and on membranes. The assembly solutions used are common to all the different LbL constructions but the order in which they are assembled varies between systems. The assembly solutions used in the different LbL constructions are the following: [PSS] = 0.5 g/L ; [bPEI] = 0.5 g/L ; [Avidin] = 0.1 g/L ; [GOx] = [GOxB] = 0.15 g/L.

The procedure for LbL assembly on flat surface in 96 wp is the following. First, the surface is functionalized with an adsorbed layer of bPEI. 100 μ L of bPEI is added in each well. After 5 min, the bPEI solution is removed and two successive washes in 100 μ L of acetate buffer are made. After the surface is functionalized, the adsorption of assembly solutions can begin. The assembly process, as illustrated in Figure 18, consists of repeating the same steps using the correct assembly solutions until the desired number of layers is obtained. The steps are as follows: (1) adding 100 μ L of assembly solution to each well using a multichannel pipette, (2) letting the solution adsorb for the required amount of time before (3) removing the assembly solution and (4) washing twice with acetate buffer. The PE adsorption step lasted 5 min while proteins (Avidin or GOx) remained 20 min in the wells. Once the films are assembled, they are stored in 100 μ L of fresh acetate buffer in the fridge at 4°C.

For the LbL assembly on membranes, the process is similar in terms of assembly solution concentration, adsorption times and washes in between depositions but differs in the manipulations. The assembly takes place in a 12 wp. Each column corresponds to a different assembly solution and each line represents a number of layer. 1 mL of assembly solution is added in each well in which the membranes are immersed. For 2- and 3-layer assemblies, the membranes are immersed a second and third time respectively in the same assembly solutions. BCA and enzymatic activity assays are carried out on assembly solutions that were collected before and after assembly. More explanations are given in 1.1 of the membrane implementation section.

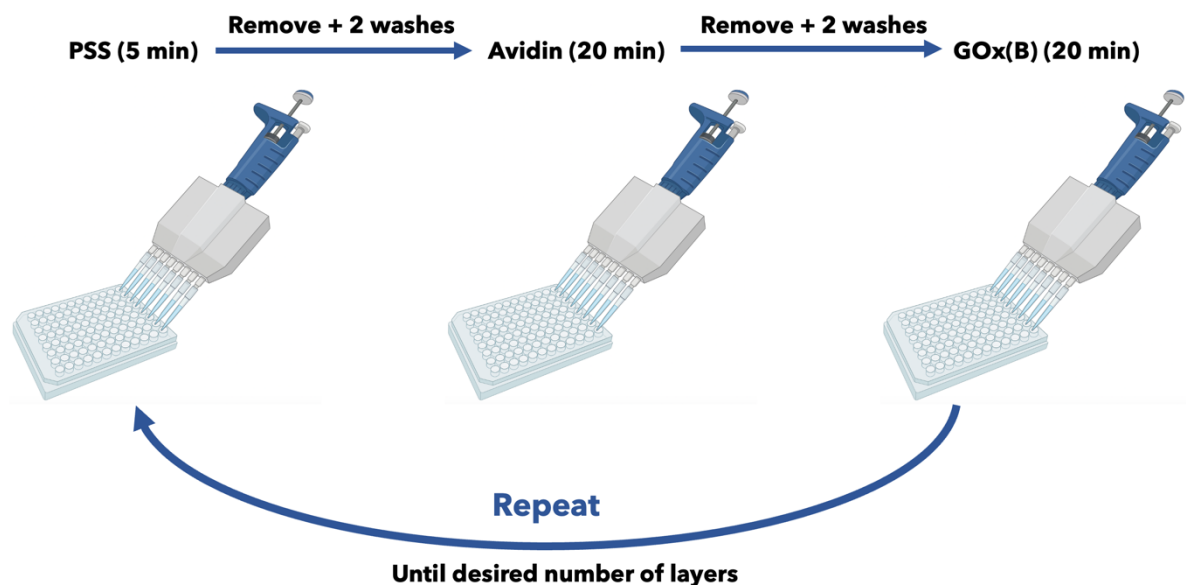


Figure 18 - **Scheme of the LbL assembly process in PS wp.** The assembly is performed by successively adding 100μL of the assembly solutions in each well, separated by two washing steps. For this construct, we started by an initial bPEI layer, then PSS was added, followed by Avidin and finally GOx(B). The process was repeated 2 or 4 times (except bPEI step) using GOx or GOx(B).

1.2. Quartz Crystal Microbalance (QCM)

QCM is based on the phenomenon of the quartz crystal's resonance frequency shifting in response to mass change occurring on its surface. When material is deposited or adsorbed onto the surface of the crystal, it alters its effective mass causing a change in its resonance frequency. This frequency change is directly proportional to the amount of mass deposited onto the crystal surface.¹⁴⁵ The Sauerbrey equation is used to calculate the amount of mass deposited onto the crystal:

$$\Delta f = -\frac{n}{C} \rho_f h_f$$

Where Δf is the measured frequency change (Hz) and ρ_f and h_f are the density and thickness of the adsorbed film. C , the mass sensitivity constant, depends solely on the fundamental resonance frequency of the quartz crystal and in our case corresponds to 19.94 ng cm⁻² Hz⁻¹. Finally, n is the overtone order and in our case, we use overtone 7. It is important to note that this equation is only valid when the deposited film is uniformly distributed, the film mass is less than the mass of the quartz crystal sensor and the adsorbed film is rigid.⁶⁹

The QCM set-up we use (Q-Sense E4 system from Q-Sense in Stockholm, Sweden), as shown in Figure 19, includes a quartz crystal sensor coated in gold (from Q-Sense, 4.95 MHz). Electrodes are connected to the sensor that apply an alternating current (AC)

voltage to the sensor and measure the resulting changes in resonant frequency. An oscillator circuit generates the AC current at the desired frequency, typically in the megahertz range. The frequency counter measures and monitors the resonant frequency of the crystal. The crystal is placed into a flow cell. The flow cell is where the interactions under study take place, the environment is well controlled and the assembly solutions pass through at a constant flow rate of 20 $\mu\text{L}/\text{min}$. Finally, a data acquisition system and a display unit allow us to record, analyze and visualize the frequency data obtained by the frequency counter. ¹⁴⁶

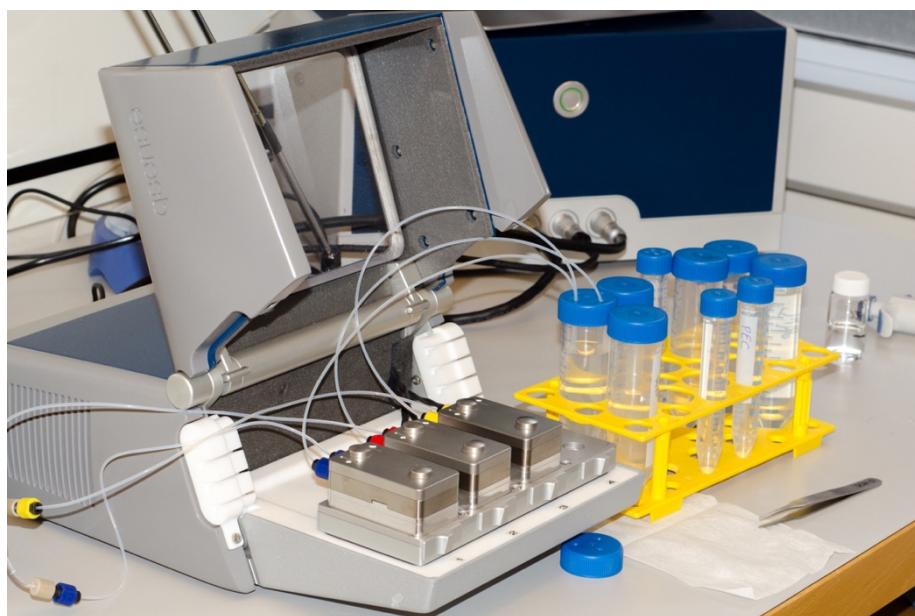


Figure 19 - Picture of a QCM set-up.

1.3. Laccase Enzymatic Assay

In 96 wp, 20 μL of the solution containing enzyme were added. Then 180 μL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) 0.36 mM (0.2 g/L) in acetate buffer 0.05 M and pH 5.0 were added to each well. The absorbance at 420 nm (A_{420}) was measured for 10 min using the microplate reader, SpectraMax iD3 from Molecular Devices. One unit of activity [U] was defined as the amount of laccase required to convert 1 μmol of ABTS per minute. The conversion between A_{420} and μmol of ABTS was performed using a calibration curve presented in Annex A.

1.4. Gox Enzymatic Assay

The GOx enzymatic activity assay kit contains all the necessary material to prepare the solutions required for the assay. These are: 200mL of POD solution and 50mL of substrate (D-Glucose) 90g/L. The solutions are prepared following the kit's instructions. POD is stored in a bottle covered with aluminum foil and stored in the fridge at 4°C. The substrate is aliquoted in 2mL Eppendorf and stored in the freezer at -23°C.

Enzymatic assays are carried out in 96 wp. 50µL of sample to analyze are added to 200µL of POD. 50µL of substrate are added to the POD-sample mixture, using a multichannel pipette, right before reading the absorbance at 510nm for 20 min. For the assays that were not performed directly in 96 wp, the proportion between the different solutions was kept constant.

To convert the absorbance into activity, we established a calibration curve using known activities of GOx. This calibration curve can be found in Annex A. We only use the linear section of the absorbance measurements over time. The slope of this linear section gives the absorbance per minute (ABS/min) which can be converted to activity using the equation obtained from the calibration curve of GOx.

1.5. BCA Assay

The Bicinchoninic Acid (BCA) assay is a common method used to quantify the concentration of protein in a sample. It relies on the reaction between the peptide bonds and Cu^{2+} ions in alkaline medium. The Cu^{2+} ions are reduced to Cu^+ ions which react with bicinchoninic acid to form a purple-colored complex.¹⁴⁷

For all the following experiments, the reaction takes place in a 96 wp. 20 µL of sample to analyze are added to 200 µL of a mixture of BCA reagent A and BCA reagent B (1:50 volume ratio of reagent B and reagent A). The plate is incubated 30 min at 60°C. After 30 min, the plate is taken out of the water bath and is left to cool down. Once the plate is back to room temperature, 200µL of each well can be transferred to a new plate and the absorbance is read at 562nm.

Each experiment has its own calibration curve made of albumin standard included in the kit. The concentrations of albumin used to do the calibration curve are 0, 0.04, 0.08, 0.12, 0.16, 0.2 g/L.

1.6. Biotinylation

To biotinylate an enzyme (see Figure 20), whether it is Laccase or GOx, we use the Sulfo-NHS-LC-Biotin reagent. The latter is added to a solution of enzyme at a molar ratio of 20:1. The biotinylation reaction consists of a nucleophilic attack of the enzyme's primary amine groups on the carbonyl group of the NHS ester. The reaction thus needs to be carried out in an appropriate, amine-free buffer. Phosphate-buffered saline (PBS) of pH 7.45 is chosen and the reaction is carried out for 30 min at room temperature. After 30 min, 3 mL of solution is placed in a dialysis cassette with a molecular cut off of 10kDa to

remove excess biotin that did not react. The cassette is then placed in a beaker filled with 1L of demineralized water. Every 2 h for 8 h, the water is replaced with fresh water. The solution is thereafter recovered and the volume of the solution is adjusted in a volumetric flask (50mL or 10mL depending on the desired concentration of biotinylated enzyme for LbL constructions). To evaluate the success and determine the extent of biotinylation, two different tests can be carried out. The methodology behind these tests is explained hereafter.

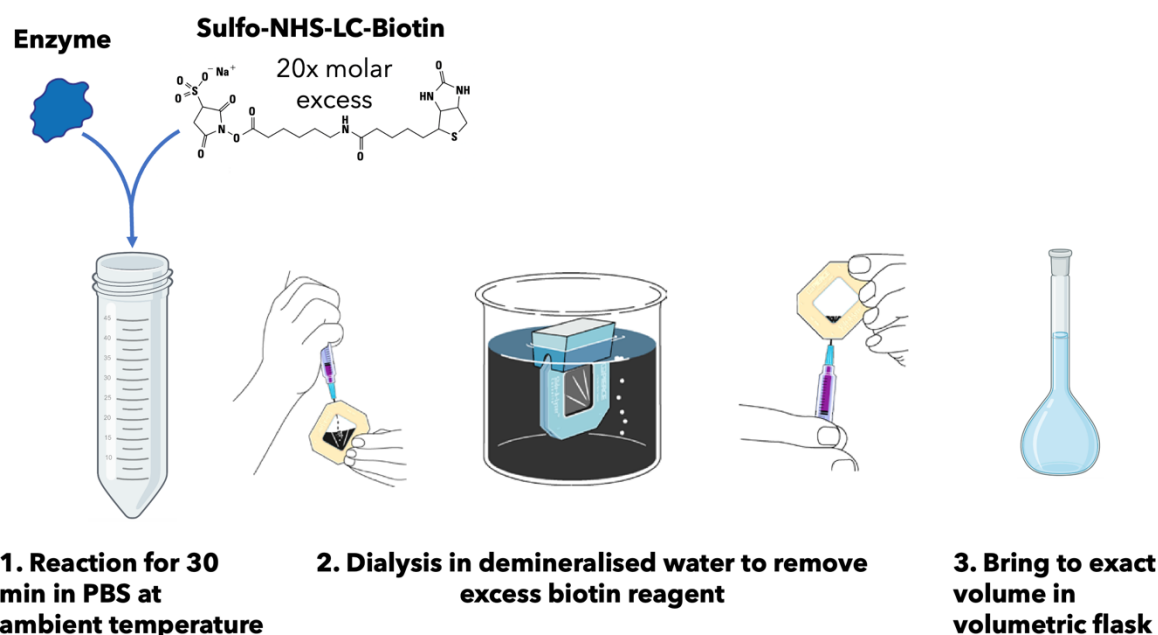


Figure 20 - **Schematic representation of the enzyme biotinylation process.** (1) The enzyme and biotinylation reagent are mixed together with a molar ratio of 1:20. The reaction takes place in PBS for 30 min at room temperature. (2) The biotinylated enzyme solution is dialyzed in demineralized water. (3) The solution is then collected and its volume is adjusted in a volumetric flask. The volume of the flask depends on the concentration of enzyme desired for the construction of LbL films.

1.7. HABA Assay

As explained in section 2.4 of the introduction, HABA can be used to assess the biotinylation degree of a protein. To do so, we started by making a calibration curve using known doses of pure biotin mixed with an HABA/Avidin solution. The HABA/Avidin complex is formed by adding HABA to Avidin following a 40:1 molar excess. This complex absorbs light at 500 nm resulting in an orange color. By adding biotin, the HABA/Avidin complex dissociates and the absorbance at 500 nm decreases. A linear relationship between A_{500} and biotin concentration in sample is observed for concentrations of biotin ranging from 0.05mM to 0.21mM (see calibration curve in Annex A). Based on the equation of this calibration curve, we can estimate the concentration of biotin in different samples of biotinylated enzyme.

1.8. SDS-PAGE

SDS-PAGE is also used to evaluate the success of enzyme biotinylation. Different samples are evaluated: (1) enzyme before biotinylation, (2) biotinylated enzyme after dialysis, (3) biotinylated enzyme mixed with avidin and (4) avidin alone. The molecular weight of a biotin moiety compared with the molecular weight (M_w) of the enzyme is too small to detect a shift on an SDS-PAGE gel. By forming complexes of avidin and biotinylated enzyme we can determine if the enzyme is biotinylated. Indeed, if the enzyme is correctly biotinylated, it will form a complex with avidin and a band will be observed on the gel at the M_w combining that of avidin and the enzyme.

First, to denature the enzymes, 10 μ L of crack buffer are added to 40 μ L of each of the four samples. The solutions are incubated for 10 min at 95°C. A precast gel is used and placed into the electrophoresis vat before filling the latter with 3-(N-morpholino)propanesulfonic acid (MOPS). Then 25 μ L of each sample is loaded onto the gel following the same order as described in the paragraph above. 6 μ L of prestained protein ladder is loaded in the first well. The samples migrate under a current of 80V for the first 15 min then under 130V for the next hour. The protein bands are revealed after immersion of the gel in Instant blue for 20 min.

IV. RESULTS AND DISCUSSION

A. ENZYME IMMOBILIZATION

1. QCM MONITORING OF AVIDIN IMMOBILIZATION

1.1. Experimental design

Three different tests were carried out for this experiment to assess the efficiency of different constructions and assembly conditions. A summary of these three experiments is presented in Figure 21.

In the first experiment, we compared three assembly systems. The first construction consists in the alternate adsorption of the polycation bPEI and Avidin in Acetate buffer, [bPEI-Avidin]. This system was chosen as a control. We don't expect a correct assembly of bPEI and Avidin since both are positively charged at pH 5. PSS was used instead of bPEI for the second system, [PSS-Avidin]. For this construction we expect a good assembly since PSS is a negatively charged PE and Avidin is highly positively charged at pH 5. The third construction is similar to the second one, but with an added layer of adsorbed bPEI in between PSS layers, [PSS-Avidin-PSS-bPEI]. Building a PSS-bPEI-PSS PE cushion in between layers of Avidin could help with charge overcompensation and charge homogeneity at the surface of the film. Charged loops of PE could also form at the surface of the film which would help with the anchorage of the next layer of Avidin. Overall, building a PE cushion in between Avidin layers could potentially help with the stability and successful assembly of the LbL film. All assembly solutions used in the above constructions are in acetate buffer.

The second experiment aimed at comparing two different assembly buffers: acetate buffer and PBS. This test was carried out for the LbL construction alternating adsorptions of PSS and Avidin layers.

For the two first tests, the concentrations of assembly solutions were kept constant ([PSS] = 0.5 g/L; [bPEI] = 0.5 g/L; [Avidin] = 0.01 g/L). The final test highlights the impact of varying assembly solution's concentration. For this test the LbL alternate adsorption of PSS (concentration = 0.05 g/L) and Avidin (concentration = 0.1 g/L) in acetate buffer is compared with the construction using concentrations of 0.5 g/L of PSS and 0.01 g/L of avidin in acetate buffer.

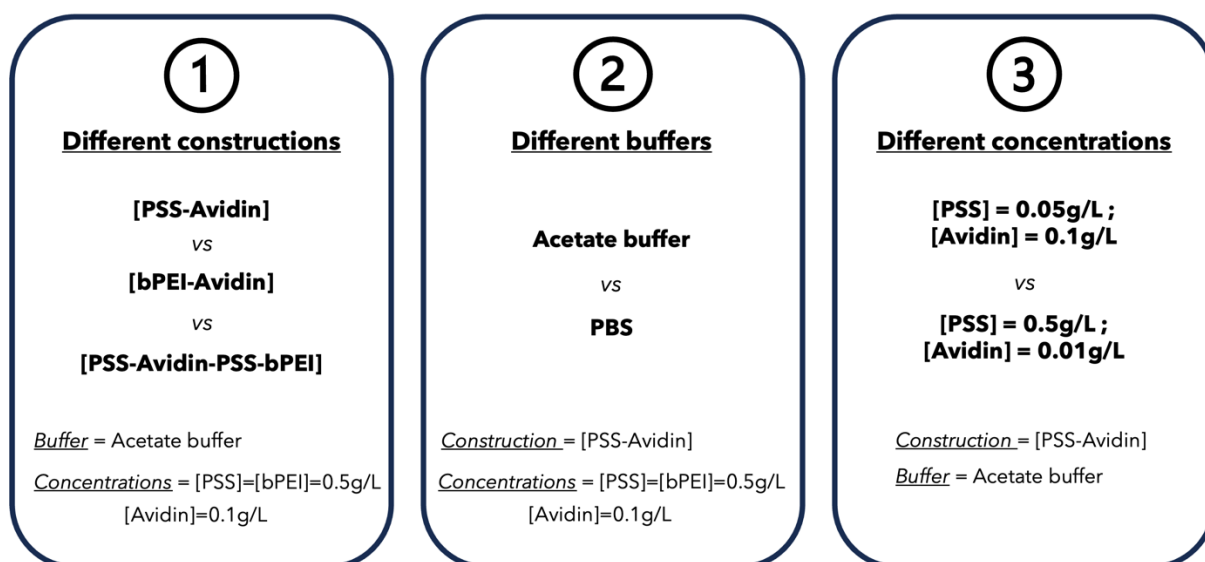


Figure 21 - **The experiment is divided in three series of tests.** The first test evaluates the efficiency of different LbL constructions. The second test compares the effect of two different buffers on the assembly of the multilayer film of [PSS-Avidin]. The third test highlights the impact of different assembly solutions concentrations. For each test the varying parameters are in bold while the parameters kept constant are in italic.

1.2. Results

System comparison

The QCM results from the LbL assembly of bPEI and Avidin can be found in Annex B. A decrease in frequency change is observed when adding bPEI onto the sensor but no change in frequency when avidin is added on top of bPEI as expected since both Avidin and bPEI are positively charged at the pH of 5. For the rest of the experiment, water passed through the QCM cell and the change in frequency increased. No significant change is observed in layer thickness.

For the [PSS-Avidin] LbL assembly, a steady decrease in Δf_7 is observed throughout the experiment, as seen on Figure 22 A. The decrease in Δf_7 is steep and short for PSS and bPEI while it is longer and more gradual for Avidin. Using the Sauerbrey equation, we can calculate Δm , the change in mass, for each layer. Since the formed layers are highly hydrated, we can make the approximation that the density is close to that of water. We can therefore estimate the layer thickness. We observe an increase of layer thickness after each assembly solution change.

A third system is tested, in which a PSS-bPEI-PSS cushion is built in between bilayers of PSS-Avidin. The results are shown in Figure 22 C and D. The same steady decrease in Δf_7 is observed at the beginning of the experiment for bPEI-PSS-Avidin as for the previous

experiment. However, when bPEI is added on top of PSS, Δf_7 increases, as highlighted in light blue in the figure. The change in frequency decreases once more when PSS is added on top of bPEI. For the estimated layer thickness, a steady increase is observed at each assembly solution change except when bPEI is added on PSS.

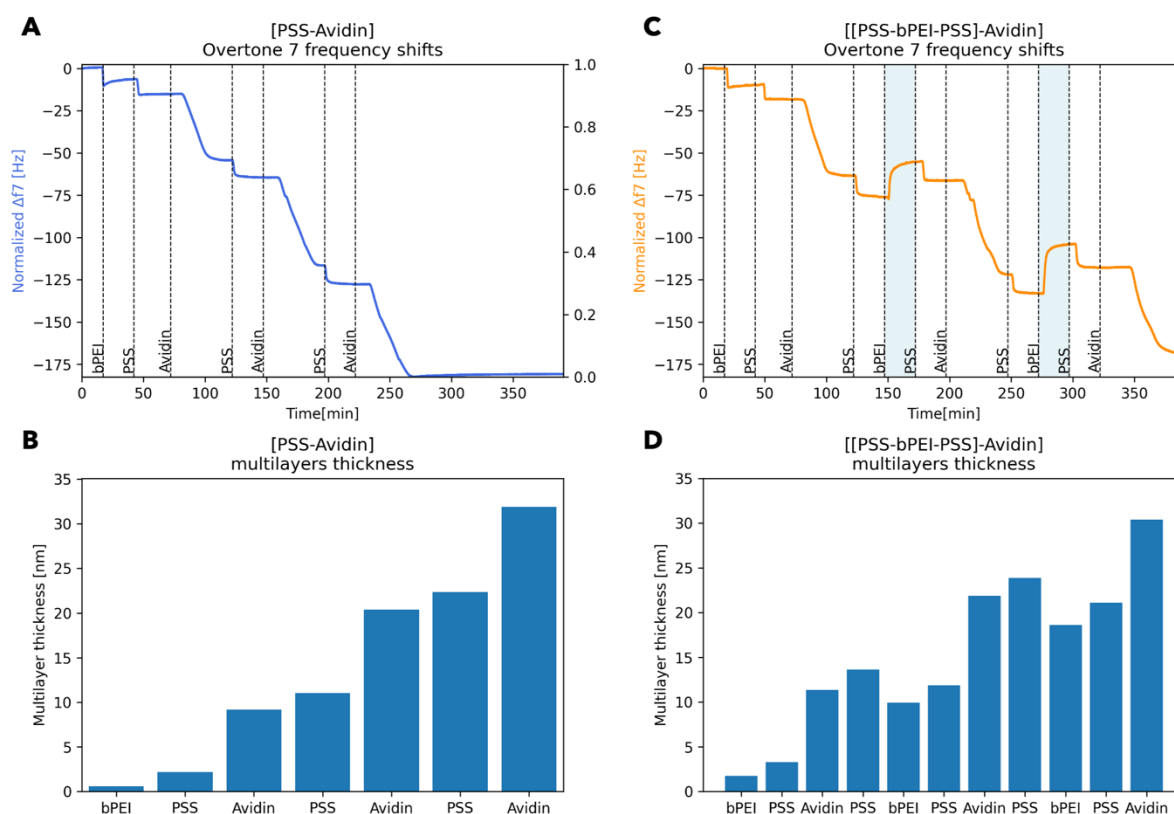


Figure 22 - Normalized frequency shift of the 7th overtone during the assembly of different construction of PSS and Avidin (A and C) and thickness after the addition of each layer calculated by Sauerbrey equation (B and D). [PSS-Avidin] constructions are on the left and [PSS-Avidin-PSS-bPEI] constructions are on the right. [PSS] = [bPEI] = 0.5 g/L; [Avidin] = 0.01 g/L.

Buffer comparison

When using PBS instead of acetate buffer for the LbL assembly of PSS and Avidin, we observe a similar decrease in Δf_7 for the first layer of PSS and avidin, as seen on Figure 23 A. Then, when PSS is added on top of Avidin, a slight decrease in Δf_7 is observed at first followed by a steep increase, as highlighted in light blue in the figure. The change in frequency stabilizes over time and once avidin is added on top of PSS, a decrease in Δf_7 is observed. The layer thickness increases steadily except when PSS is added on top of avidin. Both times, the layer thickness drops when PSS is added on Avidin.

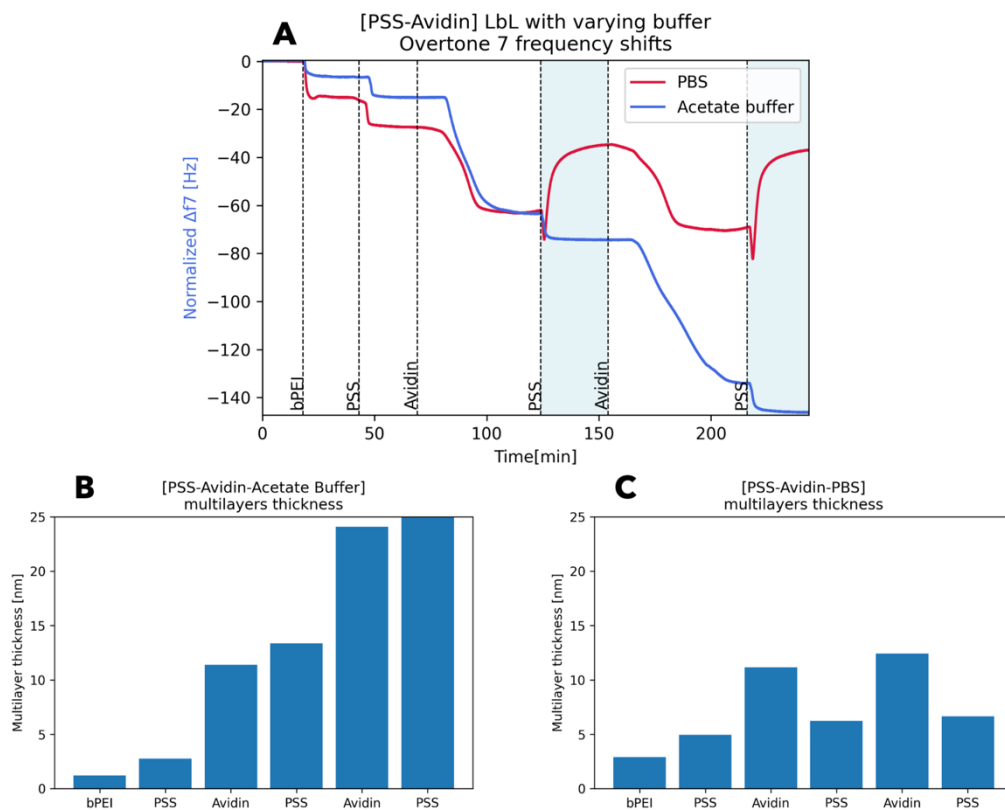


Figure 23 - **Normalized frequency shift of the 7th overtone during the assembly of [PSS-Avidin] in different buffers (A).** B and C represent the thickness after the addition of each layer calculated by Sauerbrey equation in Acetate buffer and PBS respectively. [PSS] = 0.5 g/L; [Avidin] = 0.01 g/L. Acetate Buffer is represented by the blue curve while PBS is in red.

Concentrations comparison

Figure 24 shows that when increasing the Avidin concentration and decreasing PSS concentration, no qualitative change is observed with the QCM. A steady decrease in Δf_7 is observed similar to what is observed for the LbL assembly of PSS and Avidin above. Quantitatively, the frequency change is larger than that for the experiments above, -325 Hz compared to -150 Hz respectively. This quantitative change is also observed in estimated layer thickness, 25.8 nm compared to 58.4 nm for this experiment.

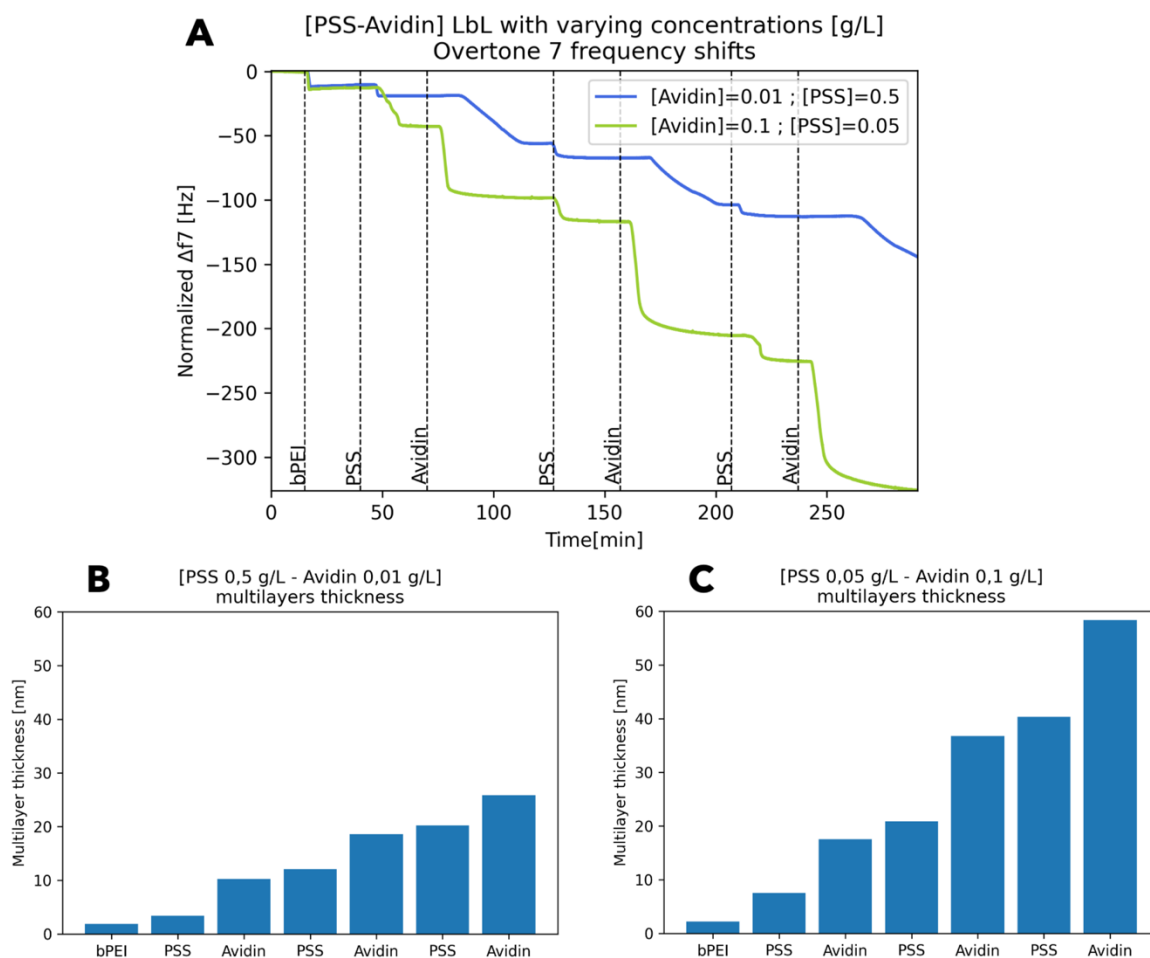


Figure 24 - **Normalized frequency shift of the 7th overtone during the assembly of [PSS-Avidin] when varying concentration of the assembly solutions (A).** B and C represent the thickness after the addition of each layer calculated by Sauerbrey equation for the different concentrations. The concentrations in B are [PSS] = 0.5 g/L and [Avidin] = 0.01 g/L. The concentrations in C are [PSS] = 0.05 g/L and [Avidin] = 0.1 g/L.

1.3. Discussion

The LbL construction resulting in the largest amount of deposited material is the alternate adsorption of PSS with Avidin in acetate buffer. The good assembly of this negative PE and the positively-charged avidin at pH 5 was expected. In the same manner, we did not expect an assembly for the bPEI-Avidin construction at pH 5 and this was confirmed by the QCM results.

The PSS Avidin assembly displayed constant decrease in Δf_7 throughout the experiment, indicating continuous deposition of assembly material onto the sensor. This was not the case for the [PSS-Avidin-PSS-bPEI] construction and for the PSS-Avidin assembly in PBS. Indeed, on Figure 22 C, we observe a sudden increase in Δf_7 when bPEI is added onto PSS. This indicates a desorption of the multilayer film. A possible explanation is that the

highly positively-charged bPEI, interacts with the highly negatively-charged PSS and desorbs the PSS loosely bound to the film. In PBS, some similar results are observed when PSS is added onto Avidin. This effect could be explained by the multivalent ions contained in PBS. The two buffers have the same concentration (50mM) but since multivalent phosphate ions are present in PBS, the ionic strength in the PBS assembly solutions is greater. This could have an effect the LbL assembly. Moreover, divalent phosphate ions could form bridging with other compounds present in the solution and disrupt the LbL assembly.

Finally, we can add that increasing the concentration of the avidin assembly solution increases the mass deposited on the assembled film. Indeed, on Figure 24, we see that the decrease in Δf_7 is greater for LbL using higher concentration of Avidin. More material is deposited onto the film and the estimated film thickness is greater. However, the gain in deposited material is not proportional to the increase in concentration. We increase the concentration of Avidin by a factor of 10 and obtain a film twice as thick. This effect is expected since above a certain concentration, the adsorption isotherm reaches a plateau and no material is further adsorbed. Therefore, increasing this much the concentration in Avidin does not seem like an optimized strategy in terms of immobilization yield.

2. BIOTINYLATION AND EXTENT OF BIOTINYLATION

2.1. Experimental design

The aim of this experiment was to biotinylate and verify the success of biotinylation of our model enzyme.

First, we biotinylated Laccase by following the protocol Biotinylation of section III. 1 mL of biotinylated Laccase solution was collected before and after dialysis to be analyzed with the HABA assay. By comparing the results obtained for the sample before and after dialysis, it is possible to determine qualitatively and quantitatively if biotin is bound to Laccase. We also performed an SDS-PAGE on the biotinylated Laccase samples as explained in **Erreur ! Source du renvoi introuvable.** of section III.

Based on the results we obtained for Laccase biotinylation, we decided to use another enzyme, GOx. We followed the same biotinylation procedure and tests as for Laccase with this new enzyme.

2.2. Results

Laccase

Results for the HABA assay conducted on laccase samples before and after dialysis are presented in Table 3. For the biotinylated Laccase sample before dialysis, the absorbance signal is out of the calibration range. Thus, the results for this sample cannot be used to quantify biotin, a dilution of the sample should have been made to obtain a signal within the calibration range. Still, we can say that the amount of biotin in this sample is higher than 0.2 mmol/L.

For the biotinylated Laccase sample after dialysis, we obtained an absorbance of 0.42 which is well in the range of the calibration curve. The concentration of biotin in the sample is 0.088 mmol/L. By multiplying this concentration with the volume of the sample, 3mL, we obtain a quantity of 0.26 μ mol of biotin in the sample.

Table 3 - Estimation of biotin concentration in LaccB sample using the HABA assay.

	LaccB before dialysis	LaccB after dialysis
Absorbance [500nm]	0.12	0.43
[Biotin] in sample [mmol/L]	> 0.2	0.088

The SDS-PAGE resulted in no signal for the different samples of Laccase, biotinylated Laccase and Avidin-biotinylated Laccase complexes.

GOx

The results of the HABA assay on biotinylated GOx before and after dialysis are shown in Table 4. The absorbance results for both GOxB before and after dialysis were in the correct range to use the HABA calibration curve. The concentration in biotin is equal to 0.19 mmol/L before dialysis and 0.05 mmol/L after dialysis. We also calculated the ratio of mol of biotin per mol of GOx which correspond to 18.16 and 6.67 before and after dialysis respectively.

Table 4 - Estimation of biotin concentration in GOxB sample using the HABA assay.

	GOxB before dialysis	GOxB after dialysis
Absorbance [500nm]	0.24	0.48
[Biotin] in sample [mmol/L]	0.19	0.05
mol Biotin per mol GOx	18.16	6.67

The SDS page, Figure 25, shows a band at approximately 80 kDa for both the GOx and GOxB samples. For the sample that contains GOxB-Avidin complexes, a band is observed in the well. For the Avidin sample the band is observed at the bottom of the gel. For the three first samples, big smears are observed on the gel.

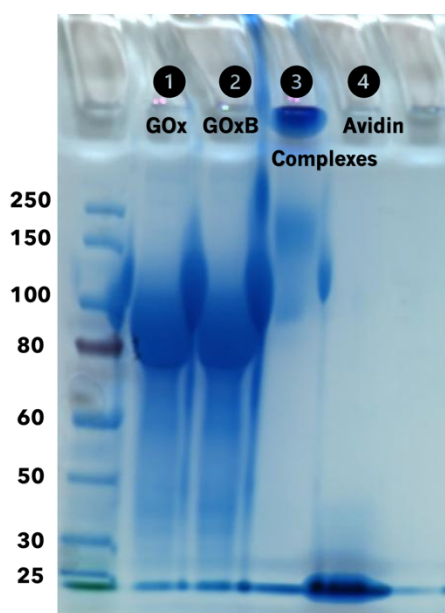


Figure 25 - **SDS page of GOx and GOxB samples.** The first well was loaded with a sample of unmodified GOx and the second with GOxB. The third well was loaded with a solution of preformed complex of Avidin-GOxB. Finally, the fourth well is loaded with avidin. The samples are denaturated using crack buffer and through heating to 98°C prior loading on the gel.

2.3. Discussion

Laccase

Even though no signal was obtained for the SDS-PAGE of Laccase samples, an interesting conclusion can be drawn. Based on the lack of signal on the SDS page and the price of the enzyme we used, we concluded that the Laccase used in our experiments has very poor purity. Indeed, we tried loading higher amounts of the Laccase samples onto the gel and still no signal was observed. Additionally, no indications on the purity of the enzyme can be found on the supplier's website. We concluded that the powder we used corresponds to a protein extract from *Trametes versicolor* containing a portion of laccase along with many other unidentified biological materials.

Great efforts to purify Laccase were undertaken. First an acetone precipitation purification was performed to remove all non-protein contaminants. In parallel, ammonium sulfate precipitation was made to separate different protein fractions. Laccase enzymatic activity assays and BCA assays provided information on the different samples obtained following purification. The results can be found in Annex D. Acetone purification resulted in a major loss, 88%, of Laccase enzymatic activity. The ammonium sulfate precipitation turned out as a cost-effective and convenient method to initially purifying the laccase sample, effectively eliminating unwanted proteins. However, a reduction of 30% in activity occurs during the purification process. Other purifying methods are available such as ion exchange chromatography or adding a HisTag to the enzyme through genetic engineering. However, these methods are time consuming and costly. Since the focus of this project did not revolve around protein purification, we decided not to go further with laccase purification and carried on our experiment using GOx, which had higher purity levels.

Results from the HABA assay with laccase samples indicate that the biotin concentration is higher before than after dialysis. Measurements on the dilution solution, post dialysis, confirmed that the dialysis worked perfectly. Consequently, all excess unreacted biotin, not bound to our enzyme, is successfully eliminated from the sample by the dialysis process. Since some biotin is still found in the sample after dialysis, we can conclude that we successfully biotinylated something in our sample. Unfortunately, this characterization technique gives no indication regarding the biotinylation target.

GOx

The HABA results for the GOxB samples before and after dialysis indicate that the biotinylation of GOx is successful. On one hand, the concentration in biotin in the sample

decreases after dialysis, indicating that excess biotin that has not reacted with the GOx is eliminated. On the other hand, biotin is still present in the GOxB sample after dialysis meaning biotin was successfully attached to GOx during the biotinylation process. The molar ratio calculated using the HABA assay on the sample before dialysis, 18.16, corresponds to the 20-molar excess in biotin we introduced for the biotinylation reaction. Once the excess biotin is removed through dialysis, we had a molar ratio of 6.67 indicating we had, on average, 6 biotins bound to one GOx.

The SDS-PAGE results show that there is a good formation of Avidin-GOxB complexes. Since no 80 kDa band can be detected when avidin is added to the sample, it seems that GOx is entirely biotinylated and that it forms complexes with avidin. The avidin-GOxB complexes did not migrate properly into the gel, the band is still in the well. When biotin is bound to avidin, the complex becomes more stable and difficult to denature. It is possible that a big network of Avidin-GOxB is formed which could explain that the proteins did not migrate properly on the gel and stayed in the well. The avidin sample alone was denaturated, in its four subunits of 16.75 kDa each, which explains the band at the bottom of the gel.

3. QCM [PSS-AVIDIN-ENZYME_B]

3.1. Experimental design

After the successful biotinylation of our model enzyme, the next step was to assess the successful immobilization of this enzyme on the previously assembled [PSS-Avidin] film. To do so, we use the QCM tool. A summary of the conducted QCM experiment is presented in Figure 26.

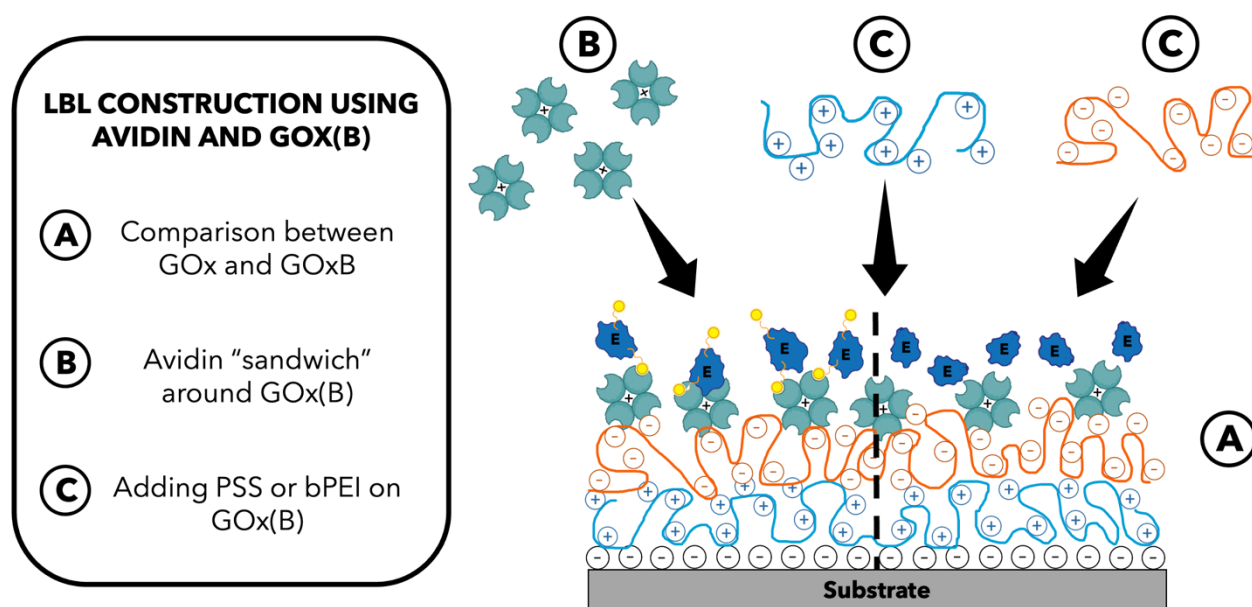


Figure 26 - **Overview of the different tests carried out to evaluate the efficiency of different LbL constructions using GOxB.** In a first place, we compare the LbL assembly when using biotinylated enzyme compared with unmodified enzyme (A). Then we compare different assemblies. Three different constructions were tested: (B) doing an avidin sandwich around GOx(B), (C) adding PSS on top of GOx(B) or adding bPEI on top of GOx(B). GOx(B) is an annotation that encompass both the use of normal and biotinylated GOx.

We compare the immobilization of GOx versus biotinylated GOx (GOxB) in the multilayer film of [PSS-Avidin]. For this part of the experiment, different tests were carried out to evaluate both the effect of biotinylating GOx and different constructions on the immobilization efficiency. Three different constructions were evaluated: (1) adding avidin on top of GOx(B) creating an avidin "sandwich" around the enzyme ([PSS-Avidin-GOx(B)-Avidin]), (2) adding PSS on top of the enzyme ([PSS-Avidin-GOx(B)-PSS]) and (3) adding bPEI on top of the enzyme ([PSS-Avidin-GOx(B)-bPEI]). For the avidin "sandwich" construction, we add Avidin on top of an enzyme layer which was already previously immobilized on avidin. The different constructions are illustrated in Figure 26 and Figure 27.

GOx(B) is an annotation that encompass both the use of normal and biotinylated GOx. All assembly solutions are in Acetate buffer and the surface of the QCM sensor is first

functionalized with a layer of adsorbed bPEI. The assembly solutions' concentrations are 0.5 g/L for PE, 0.01 g/L for Avidin and 0.1 g/L for GOx(B).

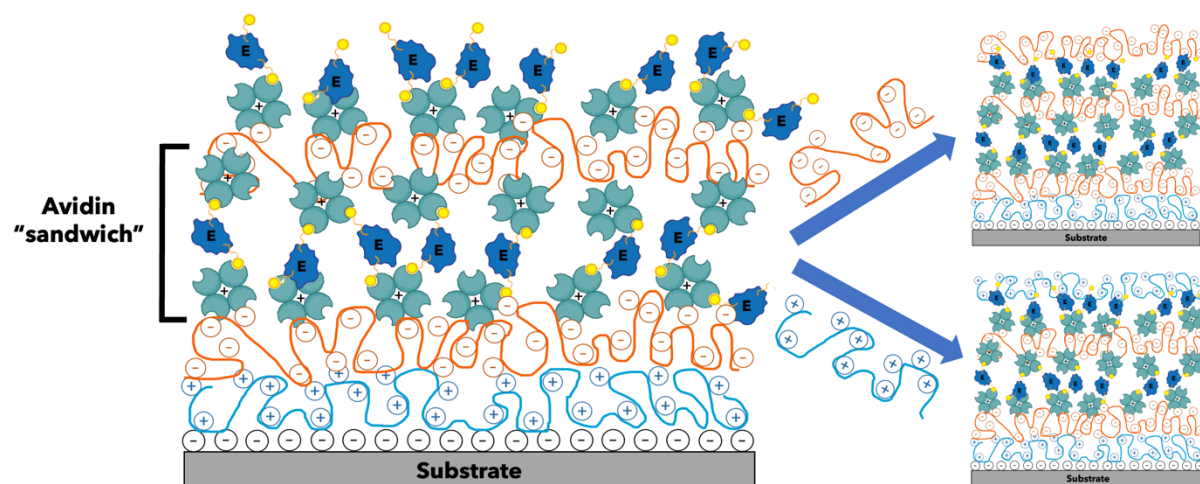


Figure 27 – **Schematic representation of the LbL assemblies performed and monitored via QCM.** The negatively-charged PE in orange is PSS and the positively-charged PE in blue is bPEI. To simplify, only Biotinylated enzymes are represented in dark blue but the constructions are tested with and without biotinylated GOx.

3.2. Results

Figure 28 regroups all the results from the experiment. Four conditions were tested with three parameters varying in each condition: the use of GOx or GOxB, the avidin “sandwich”, and the addition of PSS or bPEI on top of the last layer of GOx(B). For all four conditions, avidin was added on top of GOx(B) to make the avidin “sandwich” for the first layer. The right side of the figure regroups the results for adding bPEI onto the last layer of GOx(B) and the left side, the results for adding PSS onto GOx(B).

The decrease in Δf_7 , for the first layer of PSS-Avidin is consistent with the results from the QCM of PSS and Avidin. On Figure 28 A, we observe a steep decrease in Δf_7 , blue curve, following the addition of GOxB on top of Avidin. When adding GOx on top of avidin, the decrease in Δf_7 , red curve, is smaller.

When adding Avidin onto GOx(B) we observe almost no change in Δf_7 . However, when PSS is added onto the Avidin, the usual decrease in Δf_7 is observed. The same usual decrease in Δf_7 is also observe when Avidin is added on the PSS.

For the second layer of GOxB, the same decrease in Δf_7 as in the first layer is observed. However, for the second layer of GOx, a very soft and subtle decrease in Δf_7 is observed, much smaller than for the first layer of GOx.

Finally, by adding PSS directly on top of the GOxB or GOx, a small decrease in Δf_7 is observed, right side of the figure, corresponding to approximately - 10 Hz. Oppositely, we see a strong increase in Δf_7 , +100 Hz, when bPEI is added onto GOxB or GOx, left side of the figure.

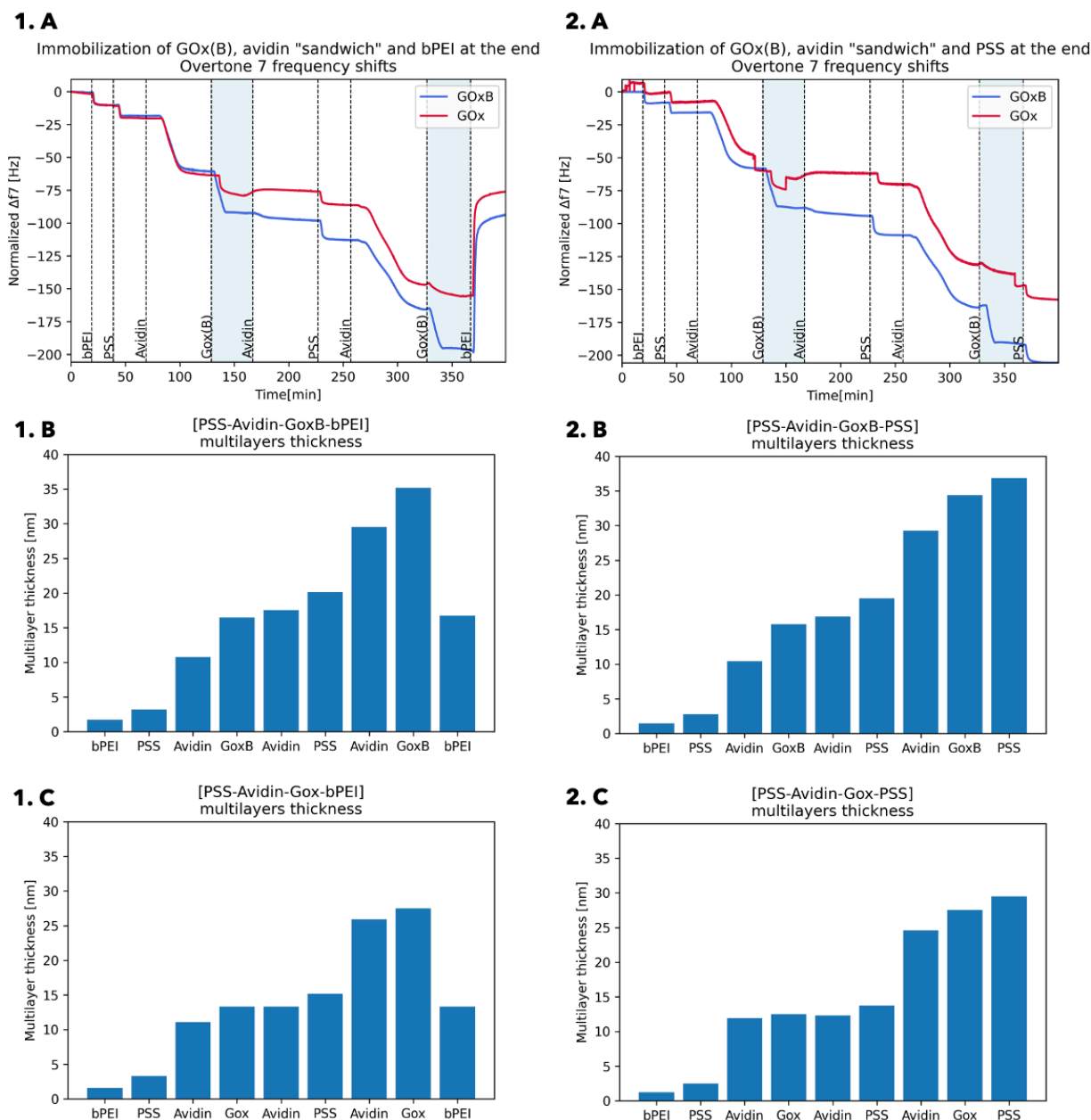


Figure 28 – Comparison between the normalized frequency shift of the 7th overtone during the assembly of [PSS-Avidin-GOx(B)-Avidin] with either PSS (right) or bPEI (left) added on top of the last layer of GOx(B). (A) are the normalized frequency shift of the 7th overtone when using GOx and GOxB, (B) represent the thickness after the addition of each layer calculated by Sauerbrey equation when using GOxB and (C) is the thickness after the addition of each layer calculated by Sauerbrey equation when using GOx.

3.3. Discussion

The first conclusion that we can draw from the above results, is that the deposition of GOxB on the LbL film of [PSS-Avidin] is much greater compared to that of GOx. The decrease in $\Delta f7$ is much greater for GOxB than for GOx even though the slopes are similar. This result clearly supports the observation that the GOx was successfully biotinylated. It also demonstrates that the interaction between avidin and biotin is a significant force driving the growth of these films.

The second conclusion is that adding Avidin onto GOxB or GOx does not lead to significant deposition of avidin onto the film. Almost no change in $\Delta f7$ is observed when adding Avidin onto GOx(B) meaning no deposition of Avidin onto the film occurs. It was described in previous studies that LbL assembly using only proteins does not lead to a successful film assembly.⁴³ For protein immobilization using the LbL technique, it is better to alternate the adsorption of protein with a linear PE. The PE interpenetrates inside the layers of the film and generates a uniform distribution of charge as well as good overcompensation of charge at the surface of the film.

Finally, based on the last part of the experiment, we observe that PSS adsorbs nicely onto GOx(B) while bPEI triggers the desorption of deposited material from the film. On Figure 28 1.A, the increase in $\Delta f7$ counterbalances the decrease in $\Delta f7$ resulting from the previous deposition of Avidin and GOx(B). The highly positively charged bPEI could interact with the PSS layer of the film leading to the desorption of layers deposited onto PSS, meaning Avidin and GOx(B) layers.

4. ASSEMBLY SYSTEM SCREENING ON FLAT SURFACE

4.1. Experimental design

Assembly

Now that the successful deposition of biotinylated GOx into the multilayer film of PSS-Avidin was demonstrated using QCM monitoring, we wanted to assess the enzymatic activity of the assembled multilayer film. Indeed, QCM measures the molecule-surface interactions and we concluded from our results that enzymes are deposited at the surface of the multilayer film. However, we had no information concerning the amount of enzyme deposited onto the film and the activity of the latter. To assess the activity and stability of the film over a period of several days, different LbL constructions were assembled directly in polystyrene (PS) wp.

A screening of different constructions was made to evaluate the activity and stability of different films. In total, five different constructions were assembled. Each construction had 2 varying parameters: the number of layers, indicated as the index n , and the use of GOx or GOxB in the construction. Each construct was performed in 5 separate wells and the experiment was replicated 3 times (Figure 29). The five constructions are: (1) [PSS-Avidin-GOx(B)] $_n$, (2) [PSS-AviGO(B)] $_n$, (3) [PSS-Avidin] $_3$ -GOx(B), (4) [PSS-AviGO(B)-PSS-bPEI] $_n$ and (5) [bPEI-GOx(B)] $_n$.

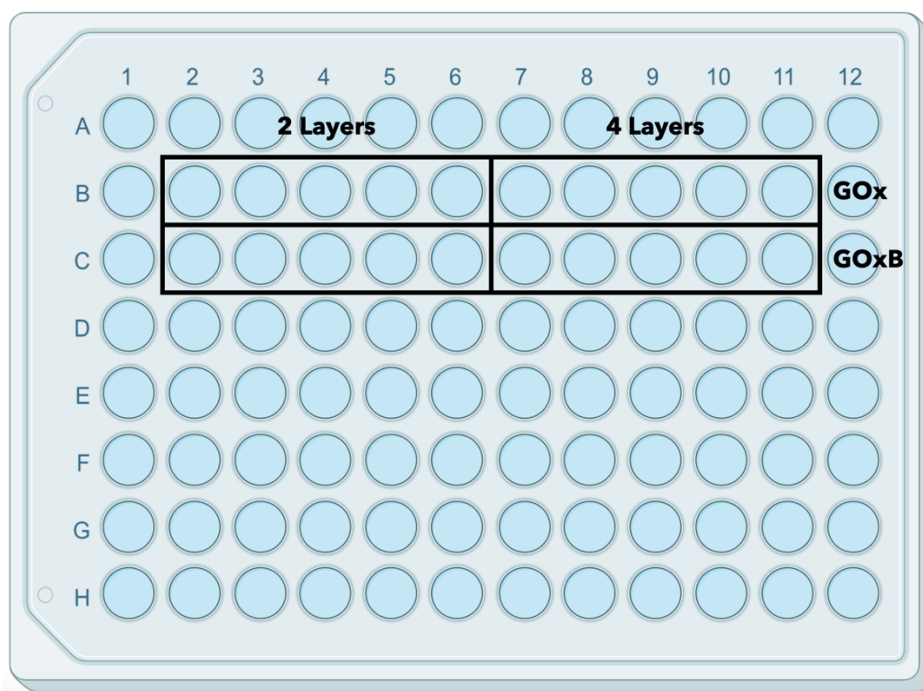


Figure 29 - **Schematic representation of the different films made for each construction.** For each construction there are 4 different conditions (2 or 4 layers and the use of GOx or GOxB), each condition is replicated five times.

The methodology behind the assembly of these multilayer films is described in 1.1 of Method in the third section of this work. Figure 30 is a schematic representation of the 5 different constructions and how they were assembled. For all construction the varying parameters were the use of GOx or GOxB and the number of layers, 2 or 4, except for construction (3) which was only built with 3 layers.

The different steps to assemble film (1) are illustrated in Figure 18 in section III. This construction was the most basic when combining the LbL of PSS-Avidin and the specific interaction between Avidin and GOxB. Construction (2) was very similar to (1) with a difference lying in the preformation of Avidin/GOx(B) complexes, AviGO(B), that are used as building blocks for the LbL assembly. The AviGO(B) complexes are formed by adding 1.675 mL of Avidin 0.125g/L to 5 mL of GOx(B) 0.15g/L. For this construction only two steps are required: PSS and AviGO(B). We wanted to highlight any difference in the activity of the film when using preformed complexes rather than adding Avidin and GOxB successively.

For construction (3) only 3 bilayer constructions were built. 10 films of 3 bilayers of PSS and Avidin were assembled. Once the three bilayers of [PSS-Avidin] are obtained, GOx and GOxB are added to adsorb on the 5 films of 3 bilayers each. This means there is only one layer of enzyme deposited on the multilayer film of [PSS-Avidin]. This construction provides information on whether it increases the activity of the surface to immobilize several layers of enzymes or if only the last immobilized enzyme layer contributes to the activity of the functionalized surface. We also asked ourselves if the biotinylated enzymes could bind to deeper layers of avidin inside the film.

Construction (4) consists in building a PSS-bPEI-PSS cushion in between each layer of AviGO(B) complexes. In the same way as in experiment 1 of this section, this PSS-bPEI-PSS cushion could help with charge overcompensation and charge homogeneity at the surface of AviGO(B) layer. This could help with the anchorage of the next layer of AviGO(B) complexes.

Finally, construction (5) relies on the traditional LbL assembly driven by the electrostatic interactions between negatively-charged GOx and positively-charged bPEI. This last construction serves as a control to be compared with the other assembly constructions.

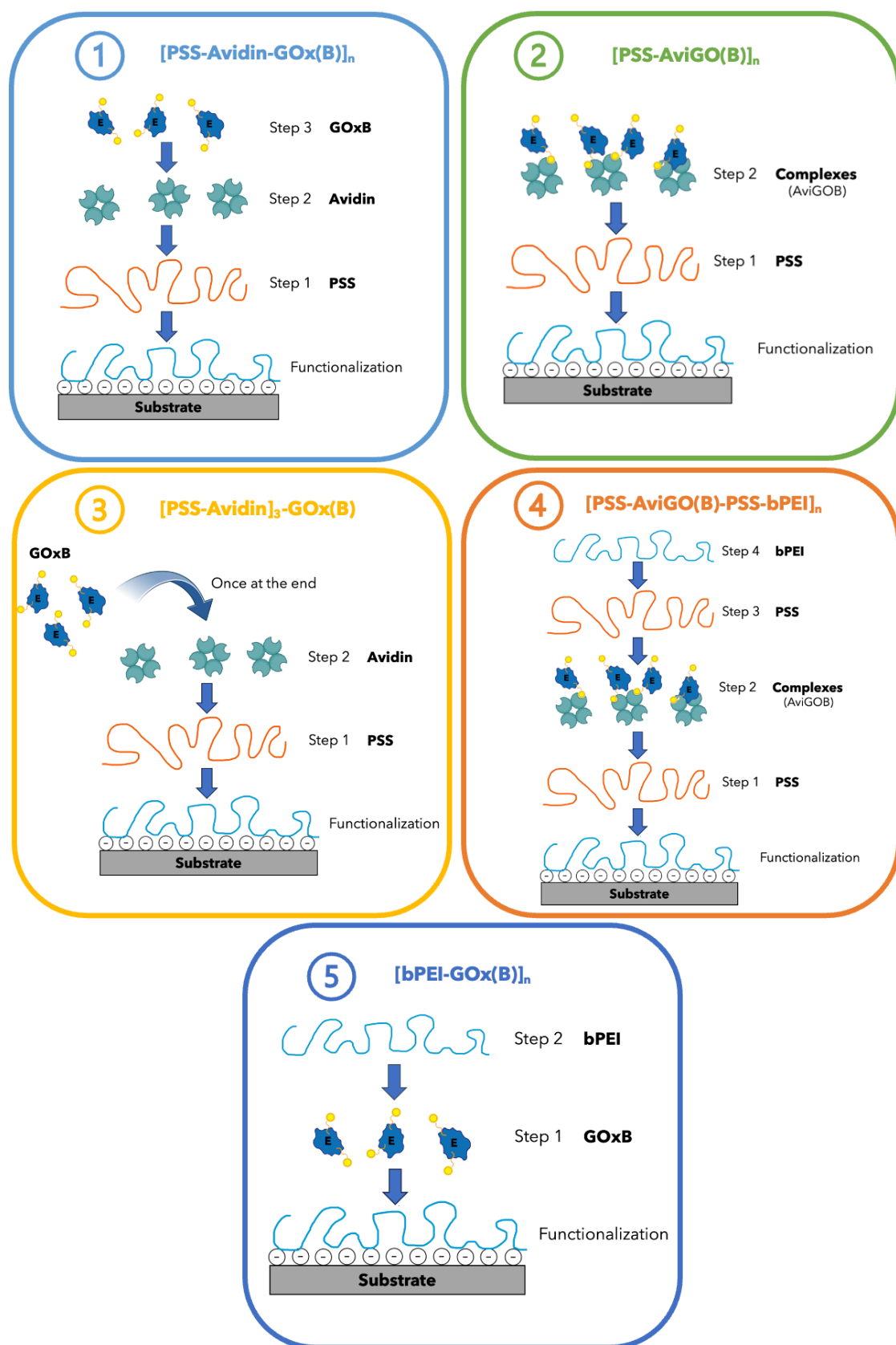


Figure 30 – **Schematic representation of the five different LbL systems.** (1) Represents the layers of [PSS-Avidin-GOx(B)]_n, (2) is [PSS-AviGO(B)]_n, (3) is [PSS-Avidin]₃-GOx(B), (4) is [PSS-AviGO(B)-PSS-bPEI]_n and (5) is [bPEI-GOx(B)]_n. To simplify, only systems using GOx(B) are represented. The steps repeated 2 and 4 times for each system except construction (3) where the steps are only repeated 3 times.

Activity and stability measurements

Once the different films were assembled, an enzymatic activity assay was carried out as described in *Gox Enzymatic Assay*. A first activity measurement was performed on day 0 of the construction. Additional activity measurements were carried out on day 1, day 3, day 6 and day 9. In between measurements, the films were stored in 100 μ L of acetate buffer in the fridge at 4°C. On the day of the measurement, the buffer was removed and replaced by 50 μ L of fresh acetate buffer for the activity measurement as instructed in *Gox Enzymatic Assay*.

4.2. Results

Comparison between systems using GOx and system using GOxB for LbL

For all the LbL constructions that have been tried, films assembled using GOxB displayed higher activity and stability over the nine days than films assembled using GOx, except for the [bPEI-GOx(B)] system obviously, since the assembly of this film did not rely on the avidin-biotin interaction. Figure 31 illustrate the effect of using GOxB versus GOx in the LbL assembly of [PSS-Avidin-GOx(B)]₄. The impact of using GOxB instead of GOx for the other LbL constructions are illustrated in Annex C.

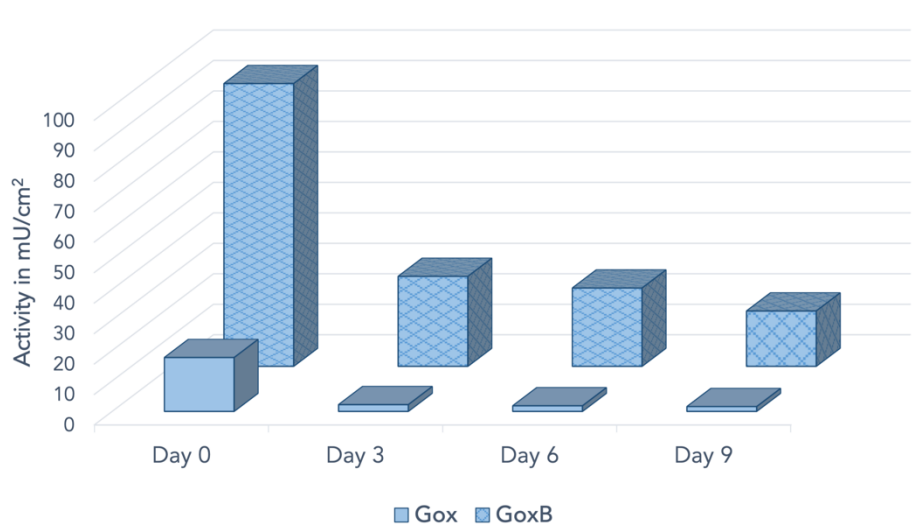


Figure 31 - Comparison of the activity and stability over 9 days of [PSS-Avidin-GOx]₄ and of [PSS-Avidin-GOxB]₄.

The following results focus on the comparison between different number of layers of LbL films assembled using GOxB.

Activity over time of [PSS-Avidin-GOxB] on flat surface

The activity of LbL films resulting from construction (1) present an activity of 68 mU/cm² and 93 mU/cm² at day 0 for 2 and 4 layers respectively. As seen on Figure 32, the activity at day 3 suffers an abrupt decline for both 2- and 4-layer constructions. Then the activity

at day 6 and day 9 undergoes a less significant decrease and stabilizes. The activity for the 4-layer film is higher than that of 2-layer films for all measurements taken over the nine days.

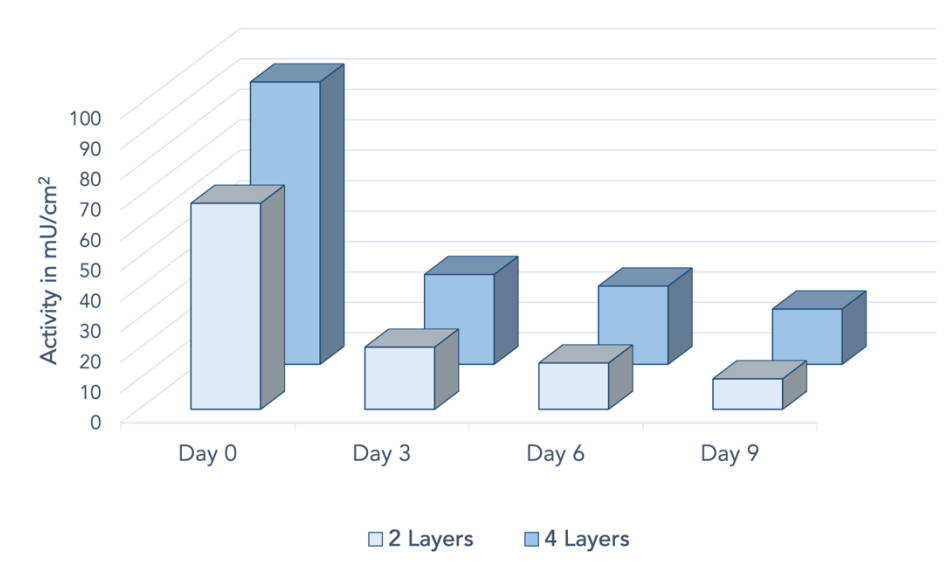


Figure 32 – Activity and stability of 2- and 4-layer films of [PSS-Avidin-GOxB].

Activity over time of [PSS-AviGOB] on flat surface

The activity and stability of LbL films assembled following construction (2) are presented in Figure 33 and Figure 34. Similarly to the activity of films for construction (1), the activity at day 0 starts high and is followed by a sudden drop at day 1. After the activity measurement at day 1, the following activity measurements at day 3, 6 and 9 suffer less significant reduction and stabilize. The activity at day 0 corresponds to 155 and 249 mU/cm² for 2- and 4-layer constructions, respectively. Moreover, the activity for 4-layer films is always higher than that of 2-layer films throughout the nine days of measurement.

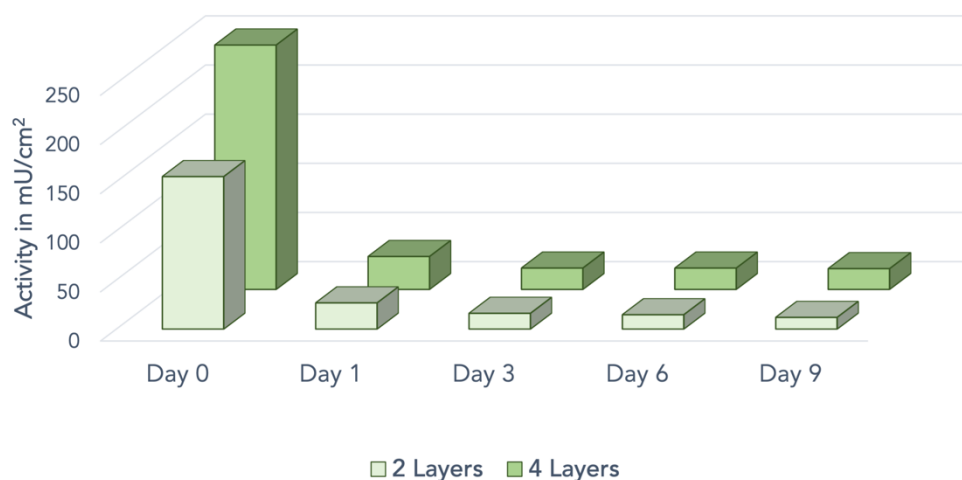


Figure 33 - Activity and stability over 9 days of 2- and 4-layer film of [PSS-AviGOB].

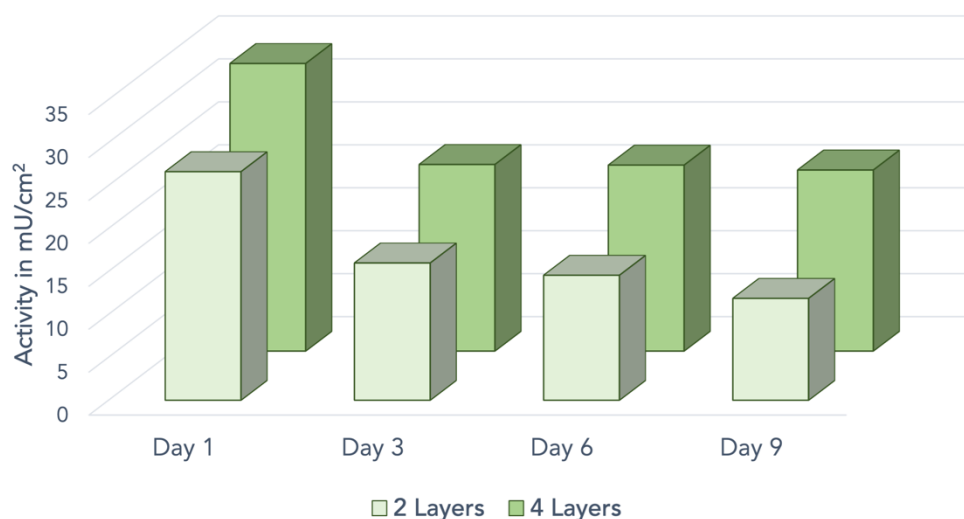


Figure 34 – Zoom on the activity and stability from Day 1 to Day 9 of a 2- and 4-layer film of [PSS-AviGOB].

Activity over time of [PSS-Avidin]-GOxB on flat surface

For the LbL construction (3), only one system was tried out: three layers of PSS-Avidin with GOxB added onto the film at the end of the construction. The activity and stability results are shown in Figure 35. The activity at day 0 is 76 mU/cm² and quickly decreases at day 1. The activity from day 1 to day 9 is stable and does not suffer a significant decline.

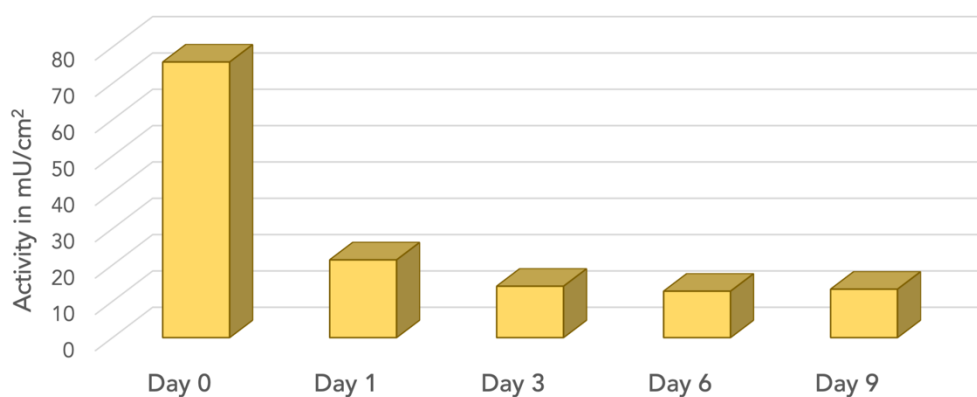


Figure 35 - Activity and stability over 9 days of a 3-layer film of [PSS-Avidin] where GOxB is added once at the end of the LbL assembly.

Activity over time of [PSS-AviGOB-PSS-bPEI] on flat surface

The activity and stability results for construction (4) are presented in Figure 36. The activity at day 0 starts high and is followed by a sudden drop at day 1. After the activity measurement at day 1, the following activity measurements at day 3, 6 and 9 suffer less significant reduction and stabilize. The activity at day 0 corresponds to 35 and 48

mU/cm² for 2- and 4-layer constructions. Moreover, the activity for 4-layer films is always higher than that of 2-layer films throughout the nine days of measurement.

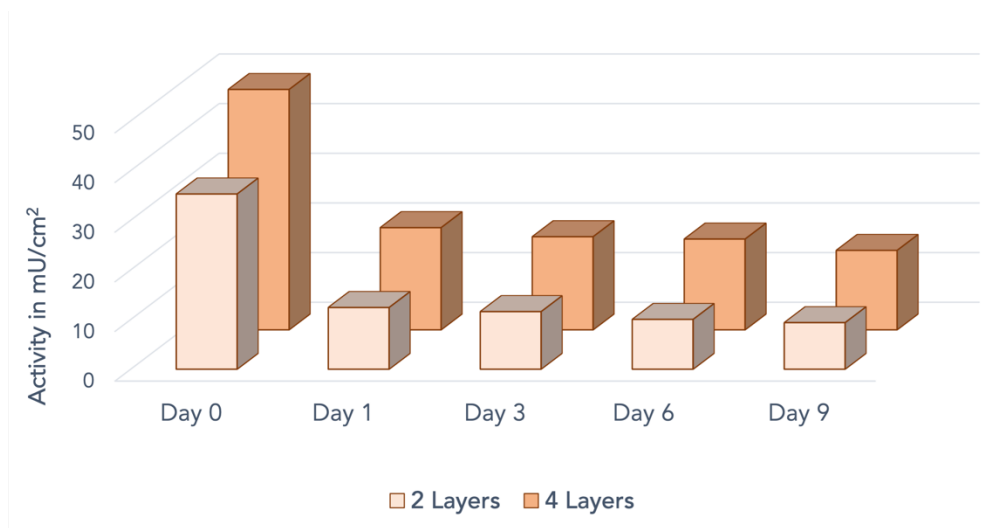


Figure 36 - Activity and stability over 9 days of a 2- and 4-layer film of [PSS-AviGOB-PSS-bPEI].

Activity over time of [bPEI-GOxB] on flat surface

The final construction has a very high activity at day 0 as can be seen on Figure 37. The drop of activity at day 1 is so important that almost 95% of the initial activity is lost after only one day. For day 3, 6 and 9 the activity continues to decrease but much more slowly as seen on Figure 38. The difference in activity between 2- and 4-layer films is less pronounced than for the constructions above even though 4-layer films still display a higher activity at all times.



Figure 37 - Activity and stability over 9 days of a 2- and 4-layer film of [bPEI-GOxB].

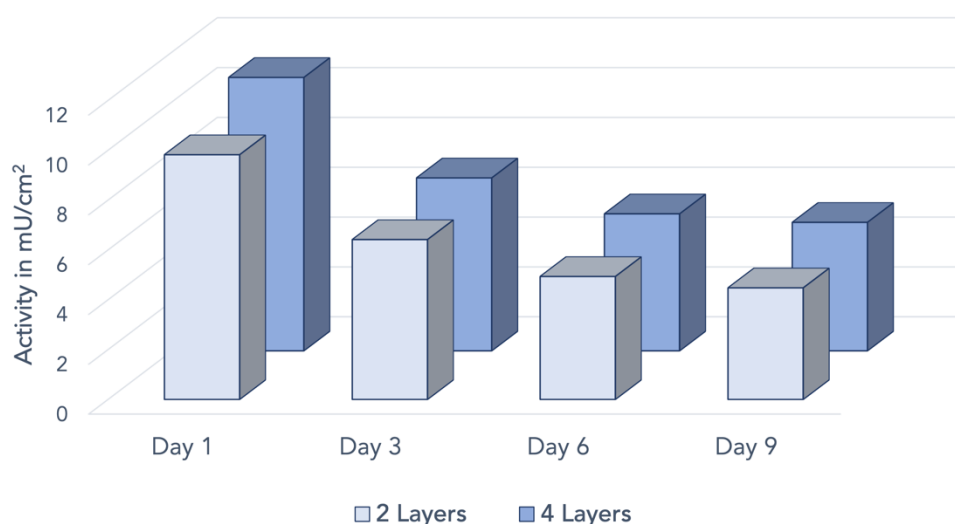


Figure 38 - Zoom on the activity and stability from Day 1 to Day 9 of a 2- and 4-layer film of [bPEI-GOxB].

4.3. Discussion

A summary of the activity at day 0 and day 9 for the different systems can be found in Table 5. Additionally, activities at day 0 and day 9 relatively to the [bPEI-GOxB] control system are presented in Table 6.

Table 5 – Table summarizing the activity at Day 0 and Day 9 of each tested LbL construction.

	Activity at Day 0 [mU/cm²]	Activity at Day 9 [mU/cm²]	% initial activity at Day 9
[bPEI-GOxB] ₂	158.2	4.5	2.8
[bPEI-GOxB] ₄	176.5	5.2	2.9
[PSS-Avidin-GOxB] ₂	67.7	10	14.8
[PSS-Avidin-GOxB] ₄	92.8	18.2	19.6
[PSS-AviGOB] ₂	155.1	11.9	7.6
[PSS-AviGOB] ₄	248.5	21.1	8.5
[PSS-Avidin] ₃ -GOxB	75.8	13.3	17.6
[PSS-AviGOB-PSS-bPEI] ₂	35.3	9.4	26.6
[PSS-AviGOB-PSS-bPEI] ₄	48.4	16	33.1

Construction (1) has an initial activity of 67.8 and 92.8 mU/cm² for 2 and 4 layers respectively which is among the highest activities at day 0 of the four systems tested. After 9 days, 14.8 and 19.6 % of this initial activity remains for 2- and 4- layer films.

The LbL assembly using AviGOB complexes displayed the highest initial activity, 155 and 248.5 mU/cm² for 2 and 4 layers, and highest activity on day 9 of all the constructions. The percentage of initial activity at day 9 is lower than for other systems but only because the initial activity started so high.

Construction (3) with GOxB added at the end of the assembly presents a low initial activity of 75.8 mU/cm². This could be explained by the fact that we only add GOxB once in the construction and even though we have multiple layers of Avidin, GOxB certainly faces diffusion limitations to access deeper layers and bind to Avidin. We could try prolonging the adsorption time for GOxB and see if it results in higher initial activity.

The initial activity for the PSS-bPEI-PSS cushion construction is the lowest of all constructions and can be explained by results previously observed of QCM analysis. In experiment 3, we tried adding bPEI in between [PSS-Avidin-GOxB-PSS] layers and we observed some decline in the mass of the film corresponding to the desorption of the previously adsorbed layer of avidin and GOxB. This could explain lower activity of the initially assembled film. However, the film seems more stable since it has the highest percentage of initial activity at day 9, 26.6 and 33.1% for 2 and 4 layers respectively.

For all the different constructions assayed in this experiment, building 4 layers instead of 2 showed more advantageous both in term of initial and final activity at day 9. The [PSS-AviGOB] construction displays the best activity results both initially and at day 9. In term of stability and of percentage of initial activity at day 9, construction (4) presents the best results but started out with lower initial activity.

When comparing the constructions (1), (2), (3) and (4) to the control bPEI-GOxB construction (5), they are all more favorable in term of stability. Indeed, the remaining activity at day 9 is always higher for the four first constructions and the percentage of initial activity at day 9 is higher than that of the control system. Conversely, the initial activity is lower compared with that of the control system, except for the initial activity of the 4-layer film of [PSS-AviGOB]. The control system starts out with a high initial activity of 176.5 mU/cm² but it very quickly drops after the first day. In comparison, the system tested relying on the Avidin-biotin interaction displayed lower initial activity for most but much better stability. The percentage of activity of the systems at day 9 compared with

the activity of the control at day 9 range from 200 to 400% for the different constructions tested.

In conclusion, the two best systems are [PSS-Avidin-GOxB] and [PSS-AviGOB]. [PSS-AviGOB]₄ is the only construction that possesses higher initial activity, 248.5 mU/cm², than the control system, 176.5 mU/cm². As seen in Table 6, it presents an initial activity of 141% of initial activity of the control and 409% of the remaining activity at day 9 of the control system. This construction presents both advantages for the initial activity and the stability of the GOx. Moreover, it presents an advantage purely in term of time efficiency since only two steps are required. The assembly process is shortened by half compared with the [PSS-Avidin-GOx(B)]_n system. This could represent an asset for the industrial scale. These two systems were selected and assembled inside nanoporous membranes for the next part of our experiments.

Table 6 - Comparison of the initial and final activity of constructions (1), (2), (3) and (4) with the [bPEI-GOxB] control system.

	Day 0 compared with control [%]	Day 9 compared with control [%]
[PSS-Avidin-GOxB] ₂	42.8	223.6
[PSS-Avidin-GOxB] ₄	52.6	352.1
[PSS-AviGOB] ₂	98.1	264.8
[PSS-AviGOB] ₄	140.8	408.7
[PSS-Avidin] ₃ -GOxB	48	297.4
[PSS-AviGOB-PSS-bPEI] ₂	22.3	209.3
[PSS-AviGOB-PSS-bPEI] ₄	27.4	310.3

B. MEMBRANE IMPLEMENTATION

1. LBL ASSEMBLY ONTO PC MEMBRANES

1.1. Experimental design

After studying the behavior of different enzyme based LbL assemblies on flat surfaces, our objective was to implement these systems onto nanoporous surfaces (Polycarbonate (PC) track-etched membranes), to benefit from the great specific surface developed by such materials. All the membranes used in this work featured the following characteristics: membrane diameter $d_m = 18\text{ mm}$, thickness $\rho = 25\text{ }\mu\text{m}$, pore diameter $d_p = 400\text{ nm}$ and pore density $\varepsilon = 10^8\text{ [pores/cm}^2\text{]}$. We estimate that the tortuosity of the pores is zero. We calculate the area developed by the membrane's surface with the following equation:

$$\text{surface of membrane} = \underbrace{2\pi r_m^2}_{\text{Surface of membrane without pores}} + \underbrace{\rho 2\pi r_m}_{\text{Surface of pores}} + \underbrace{\rho 2\pi r_p \varepsilon \pi r_m^2}_{\text{Surface of pores}}$$

The total superficial area developed by a biointerface issued from an 18 mm disc of membrane is 85 cm². Figure 39 illustrates the LbL assembly inside the nanoporous membrane.

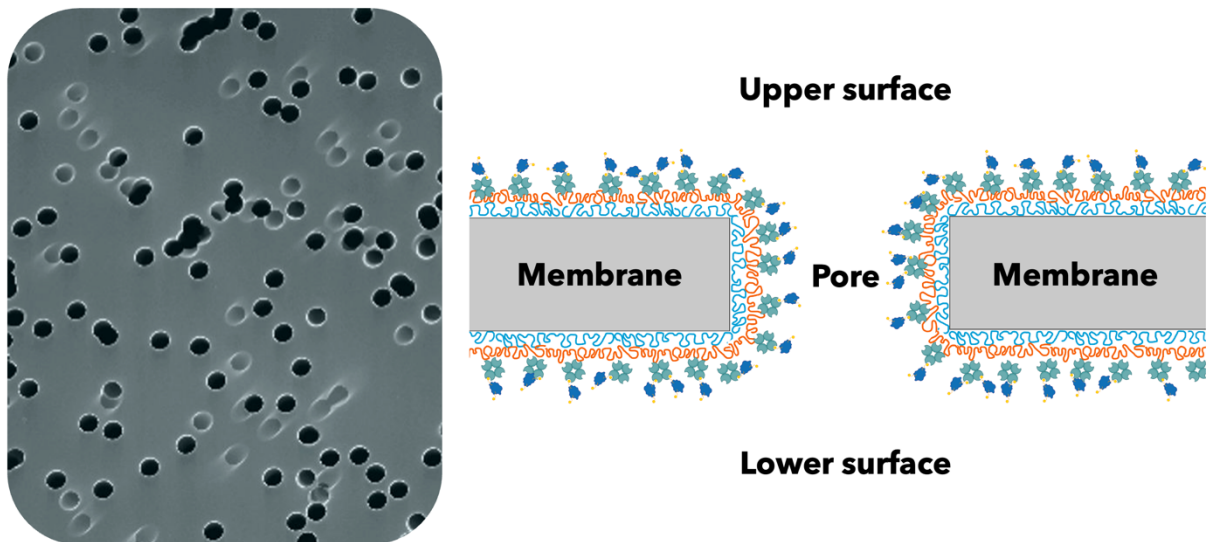


Figure 39 – SEM image of a PC membrane ¹⁴⁸ and representation of the LbL immobilization onto the nanoporous surface of the PC membranes.

Two different LbL constructions were assembled onto PC membranes: (1) the [PSS-Avidin-GOxB]_n and (2) the [PSS-AviGOB]_n. The assembly solutions were analyzed before

and after the assembly to evaluate the theoretical amount of protein and enzyme adsorbed onto the membrane. The concentration of PSS used is 0.5g/L, that of Avidin is 0.1g/L and the concentration of GOxB is 0.15g/L.

The set-up of the experiment for construction (1) is shown in Figure 40. First the membranes were immersed in 1 mL of positively-charged bPEI to functionalize their negatively charged surface. Then, we immersed the membranes in 1 mL of assembly solution and let the PE or protein adsorb onto the membranes for a certain time, 5 or 20 min respectively. Between assembly solutions, two successive washes in acetate buffer were made. We assembled 1, 2 and 3 layers of the desired construction onto three different membranes. For construction (2), we only used two assembly solutions: PSS and AviGOB complexes. The concentration of avidin remained 0.1g/L but that of GOxB was adjusted to obtain a molar ratio of avidin to GOxB of 1:4.

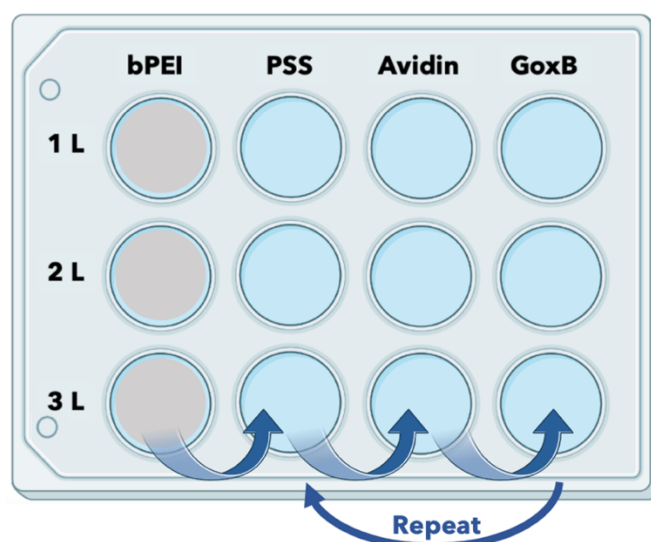


Figure 40 - **Schematic representation of the LbL assembly of 1, 2 and 3 layers of [PSS-Avidin-GOxB] onto PC membranes.** 1 mL of each assembly solution is placed into a 12 wp. The membranes are first immersed in bPEI for 5 min, then in PSS for 5 min, followed by 20 min in Avidin and finally 20 min in GOxB. Each immersion in a new assembly solution is preceded by 2 consecutive washes in 1 mL of Acetate buffer solution. For the membranes with 2 and 3 trilayers the process can be repeated, in the same assembly solutions, until the desired number of layers is obtained. Note that the bPEI step is only done once at the beginning to functionalize the surface and is not repeated in between new trilayers.

The assembly solutions were collected before and after assembly of the given systems and were used for two distinct assays: BCA (quantify the amount of avidin and GOxB immobilized) and GOx activity assay. By subtracting the protein concentration or activity of assembly solutions before adsorption onto the membrane and after adsorption, we estimated the amount of protein or enzyme theoretically immobilized onto the

membrane. The BCA and enzymatic activity assay were carried out as described in *BCA Assay* and *Gox Enzymatic Assay*.

Static enzymatic assays are also carried out on the membranes and are described in the next section.

1.2. Results

GOx enzymatic assay on assembly solutions of [PSS-Avidin-GOxB]

Figure 41 resumes the enzymatic activity of membrane assembly solutions before and after successive adsorptions of [PSS-Avidin-GOxB] onto the membranes.

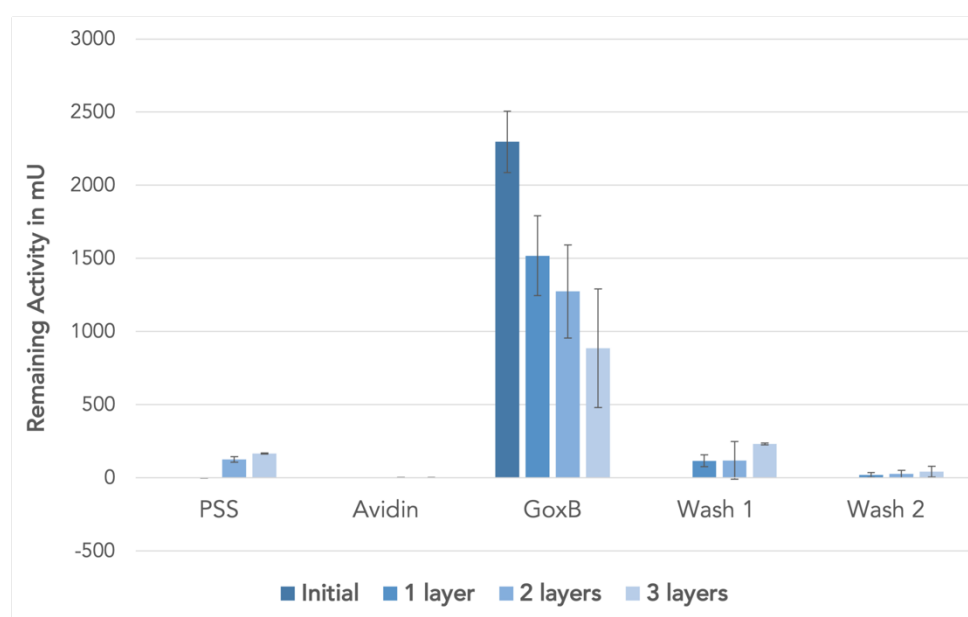


Figure 41 – **Enzymatic activity of assembly solutions before and after successive adsorptions of [PSS-Avidin-GOxB] onto the membrane.**

The activity for the initial GOxB assembly solution is 2297 mU and decreases progressively as the number of adsorbed layers increases. Initially, there is not any activity in any of the other assembly solutions but as the number of adsorbed layer increases, the activity increases slightly in wash 1, wash 2 and PSS assembly solutions.

BCA assay on assembly solutions of [PSS-Avidin-GOxB]

Figure 42 shows the protein concentration in the different assembly solutions before and after successive adsorptions onto membranes. Avidin and GOxB assembly solutions initially have high protein concentrations which then decreases progressively as the number of adsorbed layers increases. Protein concentration in Avidin assembly solution decreases more than that of GOxB assembly solutions. After 3 successive cycles of adsorption of PSS-Avidin-GOxB on the membrane, the protein concentration in avidin

assembly solution corresponds to 20.5% of the initial protein concentration while that of GOxB represents 56% of initial protein concentration. Other assembly solutions start with a protein concentration of zero which increases slightly as the number of adsorbed layer increases, especially for wash 1.

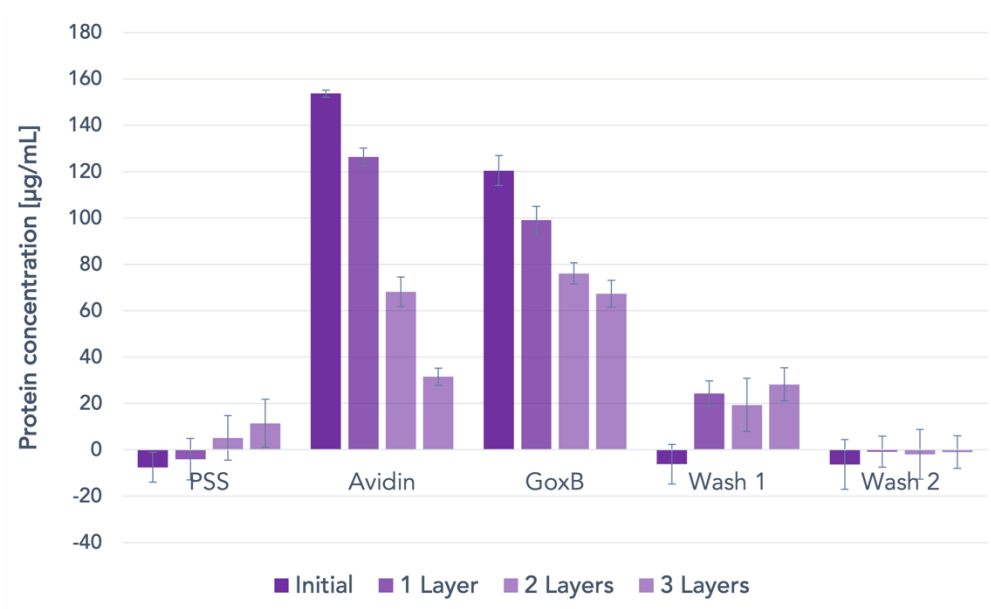


Figure 42 – **BCA assay** on assembly solutions before and after successive adsorptions of [PSS-Avidin-GOxB] onto the membranes.

GOx enzymatic assay on assembly solutions of [PSS-AviGOB]

Figure 43 regroups the results of the enzymatic activity of the assembly solutions before and after adsorption on the membrane. In this experiment, the concentration ratio between GOxB and Avidin was adjusted to have a 40:1 ratio. Initially, the activity in the complex's assembly solution is high. It decreases as the number of adsorbed layers increases. For the first adsorbed layer, the activity decreases more abruptly than for further layers. In other assembly solutions, the initial activity is zero and increases as the number of adsorbed layers increases.

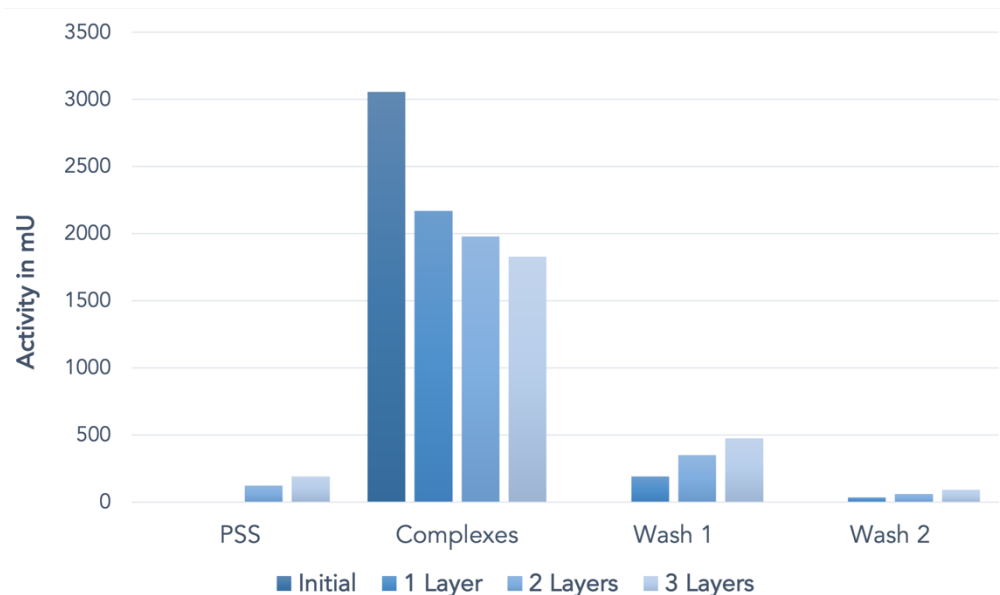


Figure 43 - Enzymatic activity of assembly solutions before and after successive adsorptions of [PSS-AviGOB] onto the membranes.

BCA assay on assembly solutions of [PSS-AviGOB]

Protein concentration of the assembly solutions before and after adsorptions onto the membranes are pictured in Figure 44. The BCA assay results are consistent with what was observed with GOx activity.

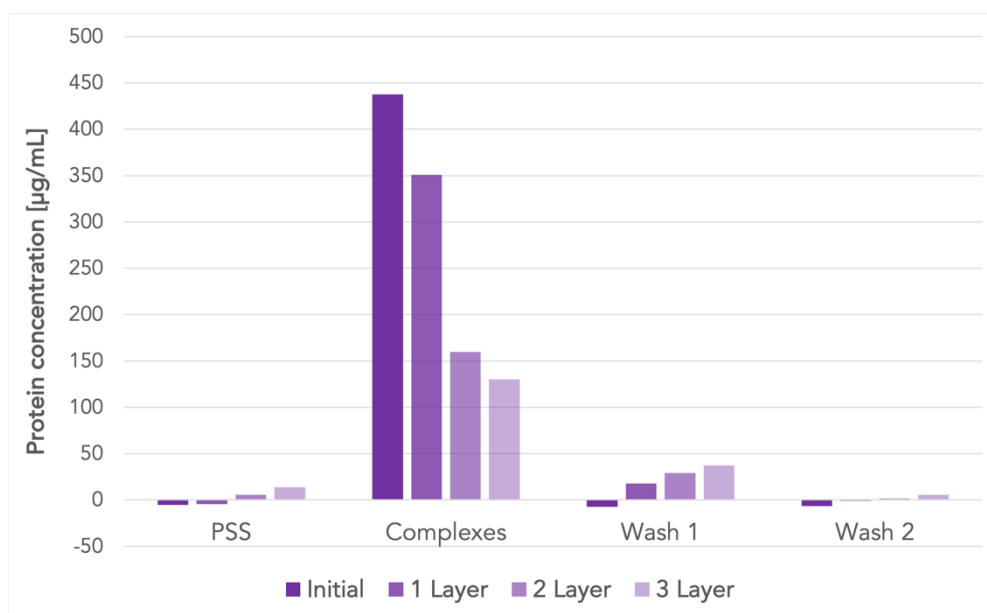


Figure 44 – BCA assay of assembly solutions before and after successive adsorptions of [PSS-AviGOB] onto the membranes. Adjusted concentration ratio.

1.3. Discussion

[PSS-Avidin-GOxB]

The results of both BCA and GOx enzymatic assays are as expected. We indeed observe a decrease in both enzyme activity and protein concentration of the assembly solutions as the number of adsorbed layers on the membrane increases. This implies that both enzymes and avidin are successfully immobilized onto the membrane. As the number of adsorbed layers of [PSS-Avidin-GOxB] increases, more material is immobilized. Additionally, the activity and protein concentration increase in other assembly solutions as the number of adsorbed layer increase. This is explained by some leaching of the loosely bound Avidin and GOxB as they're washed and immersed in new assembly solutions. The amount of enzyme theoretically immobilized at the end of the assembly can be calculated using the following equation:

$$\text{Enzyme immobilized}_{[mU]} = \text{Initially} - \text{Final} - \text{Wash 1} - \text{Wash 2} - \text{PSS} - \text{Avidin}$$

Using BCA assay results, the same equation can be used to calculate the amount of protein immobilized in mg. Protein concentrations in Avidin and GOxB assembly solutions are summed since it is impossible to differentiate which of the two is found in wash 1, 2 and PSS solutions. The results are shown in Figure 45.

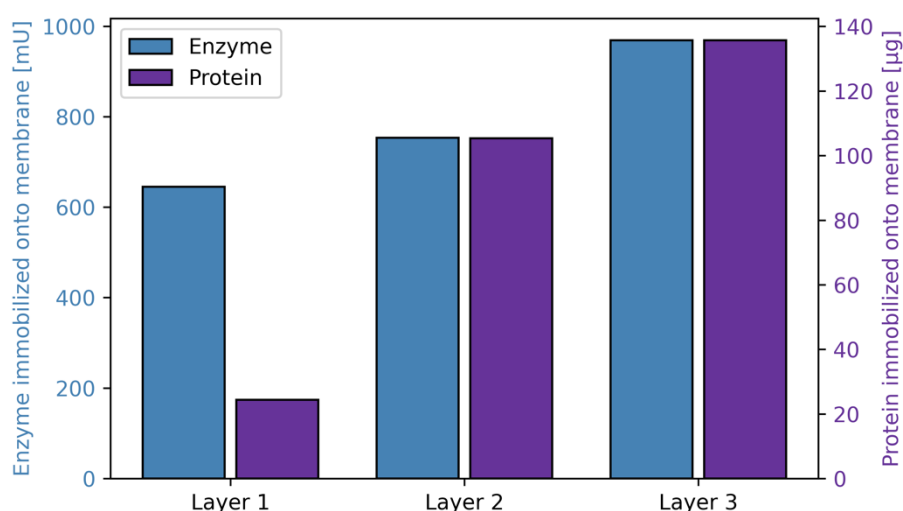


Figure 45 - Theoretical amount of enzyme and proteins immobilized onto the membrane for [PSS-Avidin-GOxB] construction.

[PSS-AviGOB]

The protein concentration in remaining assembly solutions always stayed above the threshold of 100µg/mL meaning the saturation of the membrane was reached for each layer. Both for activity and protein concentration we observe a decrease as the number

of adsorbed layer increase. These results suggest that complexes are successfully adsorbed onto the membrane. Using the same equation as described above we calculate the theoretical amount of enzyme and protein immobilized onto the membrane for each number of layers adsorbed onto the membrane as shown in Figure 46. The amount of protein immobilized increases as the number of adsorbed layer increases but it is not the case for activity. The theoretical activity of the membrane decreases as the number of adsorbed layers increases due to leaching of loosely bound complexes in the washing and PSS solutions. When subtracting the activity found in those remaining assembly solutions, the theoretical activity of the membrane decreases slightly as the number of adsorbed layer increase.

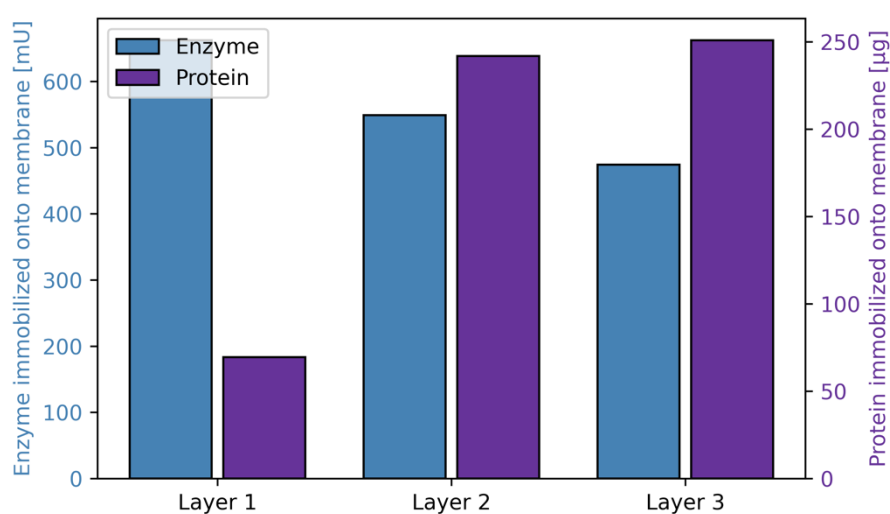


Figure 46 - Theoretical amount of enzyme and protein immobilized onto the membrane for the [PSS-AviGOB] construction.

2. STATIC ENZYMATIC ACTIVITY ASSAY

2.1. Experimental design

Having confirmed the successful LbL deposition of [PSS-Avidin-GOxB]_n and [PSS-AviGOB]_n onto PC membranes, enzymatic activity assays were carried out on the functionalized membranes to determine the effective enzymatic activity of these membranes.

The static enzymatic assay was carried out in a 12 wp. 2 mL of GOx enzymatic assay solution (1/6 D-Glucose + 1/6 Acetate buffer + 4/6 POD) was deposited in each well. Three enzymatic tests lasting 5 min each were carried out for each membrane. At first, the membranes were deposited in the first column of wells and reacted for 5 min. After five min, the membranes were removed and placed in a solution of acetate buffer. The enzymatic activity solution that reacted with the membrane was collected and transferred into a 96 wp to read the absorbance at 510nm over 5 min. Reading the absorbance over 5 min allowed to detect any potential leaching of enzyme from the membrane. The membranes were then immersed in the second column of enzymatic activity solution and the process was repeated (Figure 47). This process can be repeated three or four times to access enzymatic activity of the membrane over time.

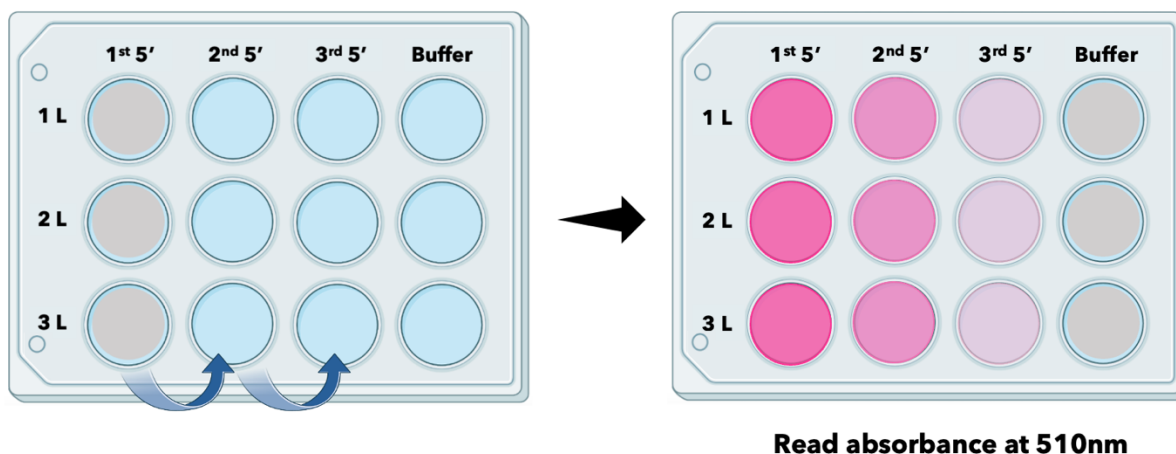


Figure 47 - **Schematic representation of the static enzymatic assay on membranes.** 2 mL of GOx activity assay solution (1/6 D-Glucose + 4/6 POD + 1/6 Acetate Buffer) were placed into each well. At first, membranes were immersed in the first column of wells for 5 min. After 5 min, the membranes were removed and placed into buffer. 3x300 μ L of solution from each well from the first column were collected and transferred to a 96 wp to read the absorbance at 510 nm. The absorbance was read over 5 min to detect leaching of the enzyme into the solution. This procedure was repeated twice to obtain the activity of the membranes over time.

2.2. Results

[PSS-Avidin-GoxB]

Figure 48 presents the activity measurements of the membrane assembled with one, two or three [PSS-Avidin-GOxB] layers. For all types of samples, the enzymatic activity is the highest for the first 5 min (1st 5') measurement, the highest activity, 100 mU, being observed for the 1-layer assembly. Then, a sudden drop of activity is observed for the second measurement. Conversely, the GOx activity between the second and third activity measurement undergoes no significant change and is stable around 30mU for all types of samples. For the second and third measurements, the enzymatic activity is quite similar for all types of membranes (1,2 and 3 layers).

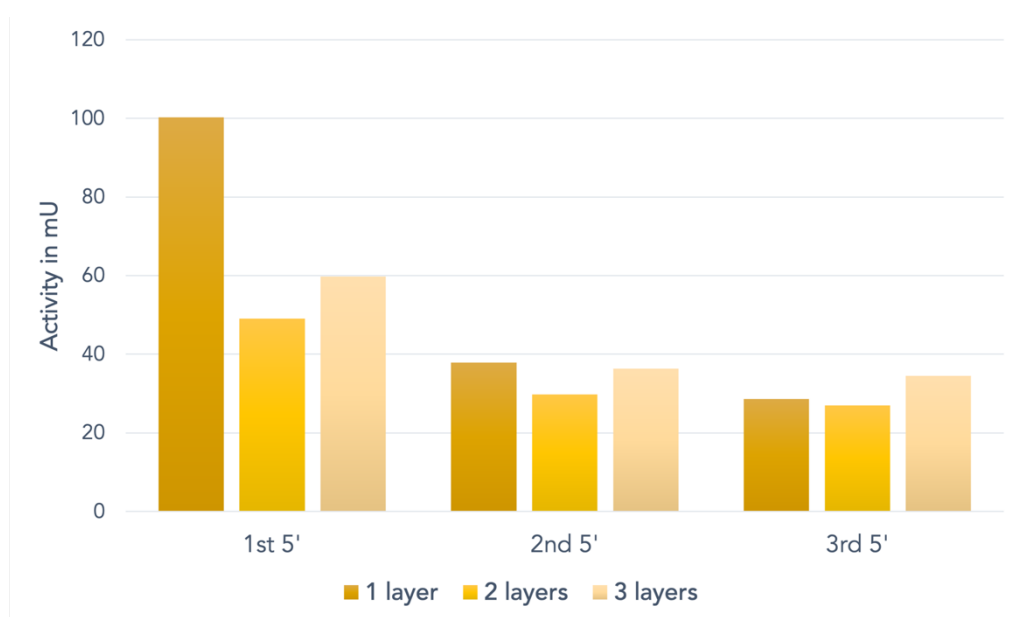


Figure 48 – Activity of GOxB immobilized on membrane by [PSS-Avidin-GOxB] LbL over 15 min.

Figure 49 resumes the activity from enzymes leached out of the membrane. For the first measurement, the highest amount of leaching is observed except for layer 1. This is because we reached the saturation limit of the absorbance for layer 1 and no further absorbance can be measured to assess leaching. The leaching then decreases for the second and third measurements and stays relatively constant between the different number of layers adsorbed onto the membrane.

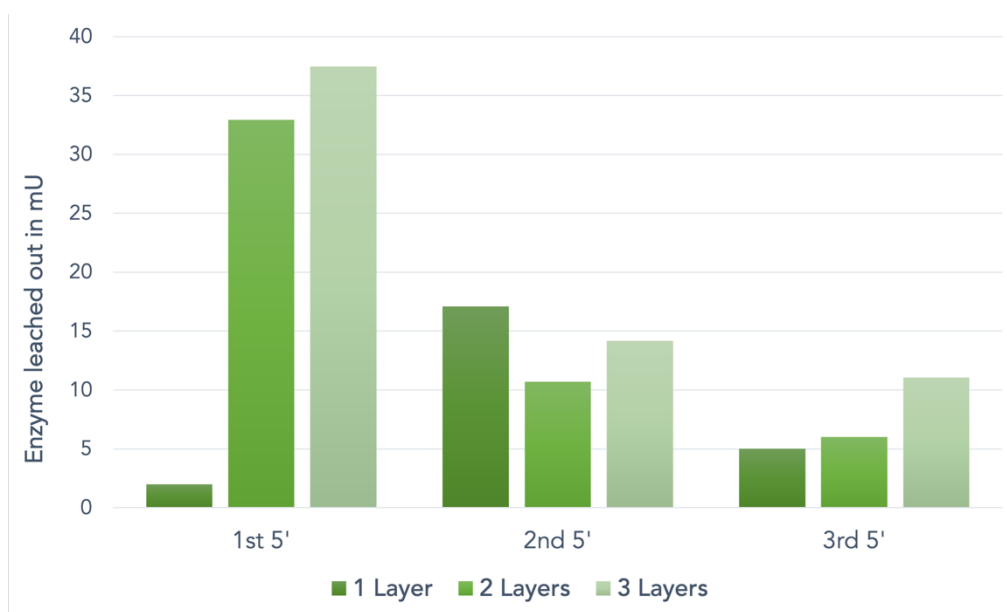


Figure 49 – Leaching of GOxB from the membrane assembled with different layers of [PSS-Avidin-GOxB] during the static activity assay.

[PSS-AviGOB]

The highest activity, 100 mU, is obtained for the first measurement and is constant when varying the number of layers adsorbed onto the membrane. In reality, the activity could be much higher than 100 mU but the maximum absorbance is reached after the 5 minute of activity assay. The activity decreases for the second measurement but remains unchanged for the following assays. The activity for 1-layer constructions for the second, third and fourth assay corresponds to 56 mU, that of 2-layer constructions, 62 mU and for 3-layer constructions, 67 mU. The activity thus increases slightly as the number of adsorbed layers on the membrane increases.

Figure 51 corresponds to the activity of enzymes leached out of the membrane following the activity assay. For the first measurement, no activity is detected from enzymes leached out of the membrane. This is because the absorbance is already at a maximum and no further absorbance can be observed when doing the 5-minute measurement to quantify leaching. Some consistency is observed for the next three measurements: the leaching is always higher for the 3-layer construction. The leaching for the three last measurements is relatively stable with a tendency to slightly decrease from one measurement to the next.

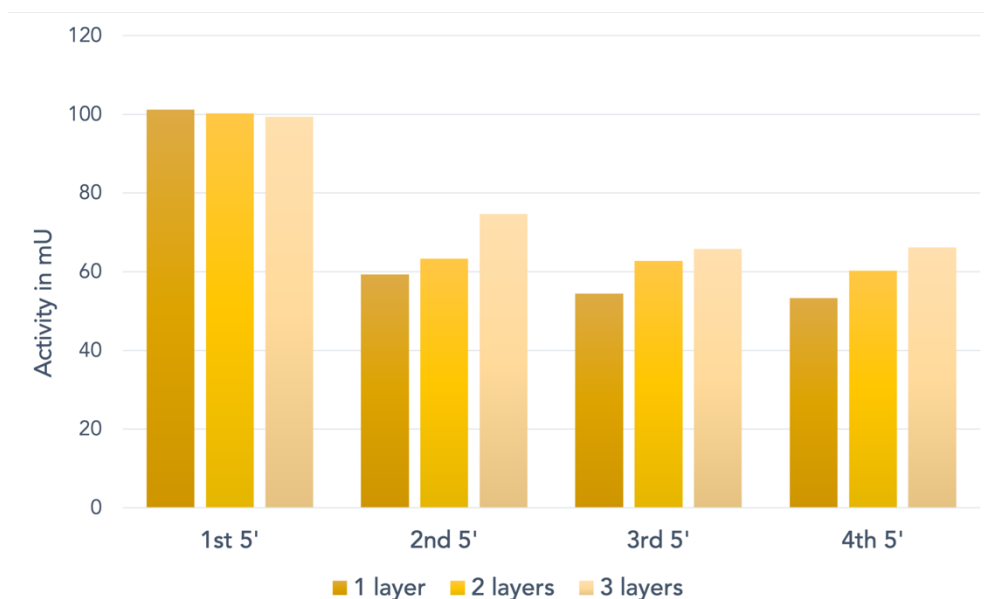


Figure 50 - Activity of GOxB immobilized on membrane by [PSS-AviGOB] LbL over 15 min.

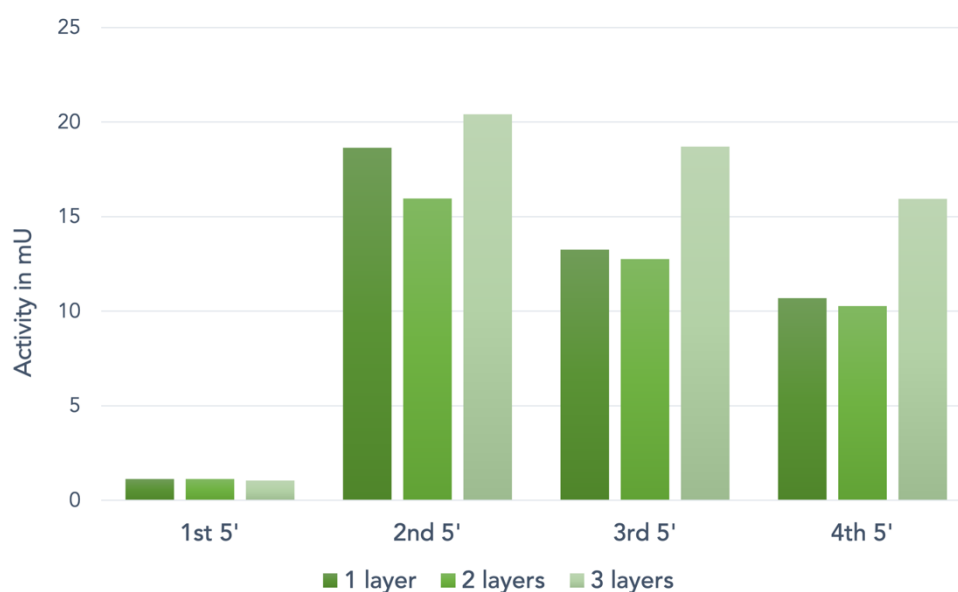


Figure 51 - Leaching of GOxB from the membrane assembled with different layers of [PSS-AviGOB] during the static activity assay.

2.3. Discussion

The static enzymatic activity assay on membrane yielded results with similar trends: a high activity on the first measurement that further decreases to reach a plateau after the second measurement. This could be explained by the enzymes leaching out of the membrane during the activity measurements. The activity of the membrane always stabilizes after the first measurement. For the [PSS-Avidin-GOxB], the activity stabilizes around values of 35 mU while the [PSS-AviGOB] construction has a stable activity of 60

mU. Similarly, leaching is high for the first measurement but then decreases and stabilizes for the following activity measurements.

For the first activity measurement, we face the issue that we reach the maximum absorbance of the enzymatic assay solution. This prevents us from measuring the activity of the membrane. To circumvent this limitation, we could do the assay in larger volumes of activity assay or use smaller membranes so that less enzyme would be immobilized onto the membrane. We could also do the assay on a shorter period than five minutes but this could cause logistical difficulties.

We can draw from the above results that no significant difference in activity is observed when the number of adsorbed layers onto the membrane increases. This could be explained by diffusion limitations of the substrate to access deeper layers of enzymes inside the film.

By dividing the activity with the surface area developed by the membrane, we obtain the activity per surface area. The surface area chosen for the membrane to do these calculations, 5.1 cm², does not include the surface developed by the pores of the membrane. This choice is justified by the fact that no decrusting of the membrane's surface was performed in between deposited layers. As a result, it is possible a crust is formed at the surface of the membrane and pore entries are blocked as illustrated in Figure 52. The enzymes immobilized inside the pores are unreachable and thus will not contribute to activity signal.

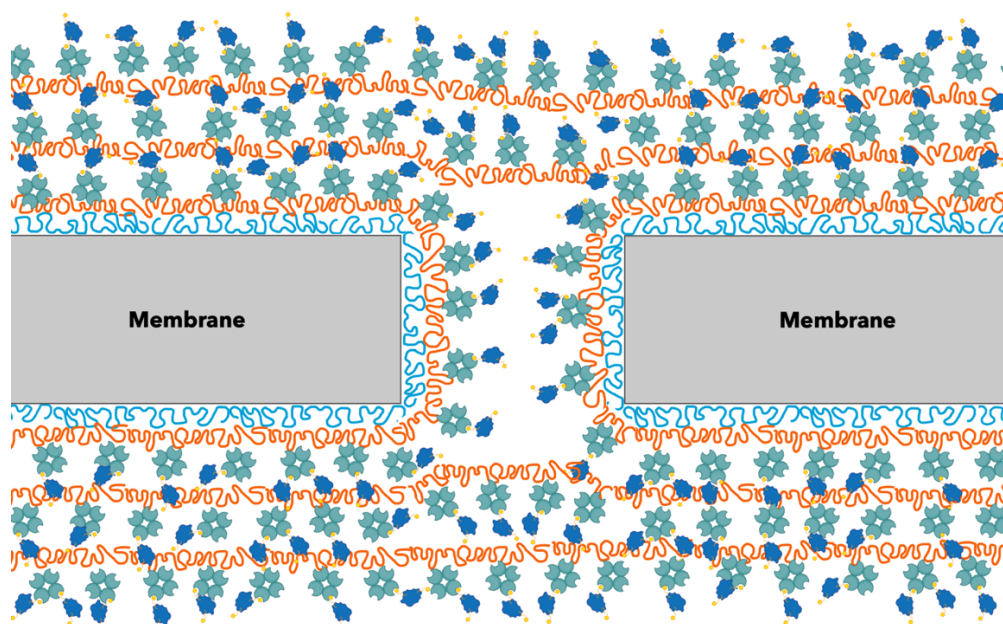


Figure 52 – **Schematic representation of the formation of a crust onto the membrane's surface following LbL assembly.** The pore entry is blocked.

These activity results are compared with the activity per surface area of the LbL assembly on flat surface, obtained in the previous experiment in section A, in Table 7. We observe that the activity per surface area obtained on membrane is lower than that obtained on flat surface. The activity per surface area for the membrane revolves around 5-20 mU/cm² while the activity for the flat surface measurements ranges from 10 to 250 mU/cm². A possible explanation is that the static activity assay on the membrane is not optimized and reaches saturation meaning we don't have access to the exact activity of the membranes.

Table 7 – Comparison of the activity in mU/cm² of the two types of LbL constructions on flat surface and on membrane.

	GOx activity in mU/cm ²			
	LbL on <i>membrane</i>		LbL on <i>flat surfaces</i>	
	1st 5'	3rd 5'	Day 0	Day 9
[PSS-AviGOB] ₁	19.83	10.67	/	/
[PSS-AviGOB] ₂	19.66	12.3	155.07	11.86
[PSS-AviGOB] ₃	19.48	12.9	/	/
[PSS-AviGOB] ₄	/	/	248.55	21.07
[PSS-Avidin-GOxB] ₁	19.66	5.61	/	/
[PSS-Avidin-GOxB] ₂	9.61	5.28	67.71	10.01
[PSS-Avidin-GOxB] ₃	11.72	6.77	/	/
[PSS-Avidin-GOxB] ₄	/	/	92.79	18.15

In conclusion, we can say that the [PSS-AviGOB] construction presents the best results in term of activity and stability. The activity per surface area is smaller on membrane when compared with our results obtained for LbL on flat surface. For a future prospect we could try assembling this construction onto smaller membranes and making the activity assay in bigger volumes to avoid reaching the saturated absorption. We could assess the real activity of the membrane and report a specific activity to be compared with other constructions. Moreover, a decrusting step could be implemented in between each deposition step to avoid blocking pore entry. In that case, the whole surface developed by the membrane could be exploited to immobilize enzyme which would contribute to the activity signal.

A preliminary test in flow-through mode was performed on membranes functionalized with [PSS-Avidin-GOxB]₂. The results of this test can be found in Annex E. This assay still needs to be optimized and more replicates need to be made for us to draw conclusions.

CONCLUSION

In conclusion, this study explored different LbL constructions for enzyme immobilization and evaluated their performance in terms of material deposition, initial activity, and stability. The alternate adsorption of PSS with Avidin in acetate buffer resulted in the highest material deposition. The deposition of GOxB on the [PSS-Avidin] film was significantly greater than that of GOx, indicating successful biotinylation and the importance of the avidin-biotin interaction. The use of previously formed AviGOB complexes for the multilayer assembly, in addition to reduce the buildup process time, lead to the formation of an active and stable system, showing better results than traditional bPEI-GOx LbL and making it the most promising construction for further experiments on membranes.

Theoretical calculations and enzymatic assays onto the membranes allowed us to assess the immobilization of enzyme and avidin onto the membrane and the activity for [PSS-Avidin-GOxB]_n and [PSS-AviGOB]_n constructions. The results showed better activity and stability for [PSS-AviGOB] constructions. No significant differences in activity and stability were observed for an increasing number of adsorbed layers. Despite these promising first results, the activity per surface area on the membrane was lower compared to the results obtained on a flat surface. These results could be due to the formation of a crust at the surface of the membrane, obstructing pore entry. Moreover, minimizing leaching remains a key challenge for achieving stable and reusable materials.

To progress further, we recommend exploring different crosslinking strategies to introduce covalent bonds within the system, aiming to enhance operational stability. Furthermore, evaluating the activity and stability of these membranes in flowthrough mode represents the logical next characterization step of this study. Finally, investigating other biotinylated enzymes as building materials is necessary to assert the versatility of this novel immobilization system.

Overall, this study provides valuable insights into optimizing enzyme immobilization and selecting appropriate LbL constructions for enhanced activity and stability.

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ANNEXES

A. CALIBRATION CURVES

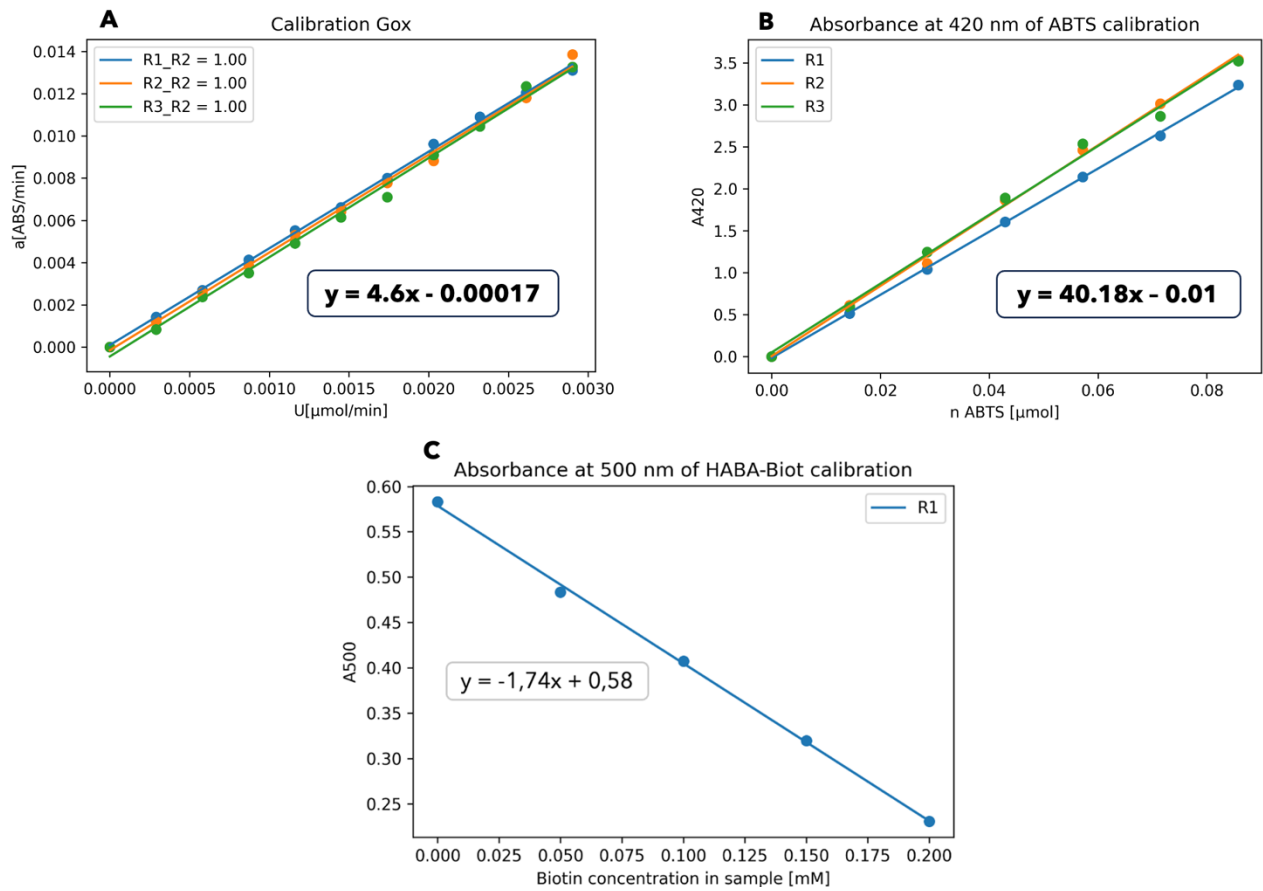


Figure 53 - Calibration curves of Laccase (A), GOx (B) and HABA (C).

B. QCM – [bPEI-AVIDIN]

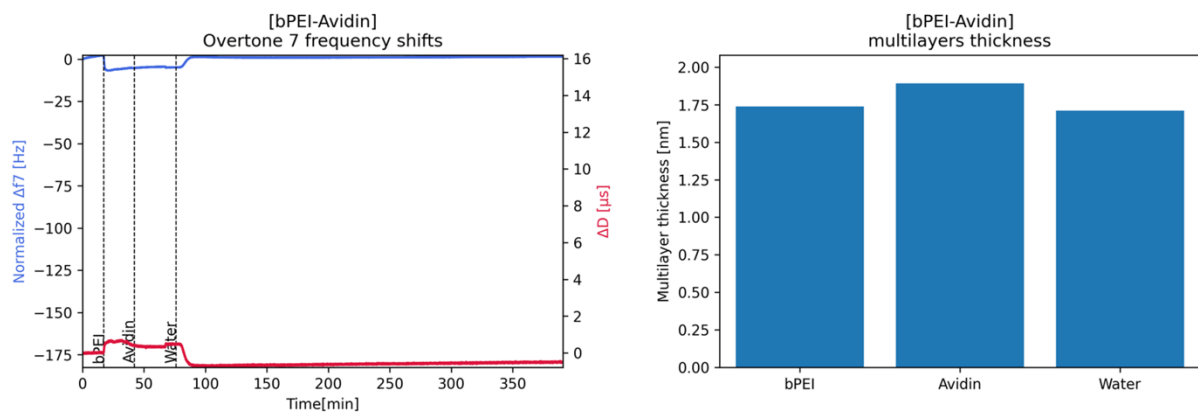


Figure 54 - Normalized frequency shift of the 7th overtone during the assembly of [bPEI-Avidin] in acetate buffer.

C. ACTIVITY AND STABILITY OVER 9 DAYS OF LBL CONSTRUCTION WITH GOX(B) ON FLAT SURFACE

1. [PSS-AviGO(B)]₃

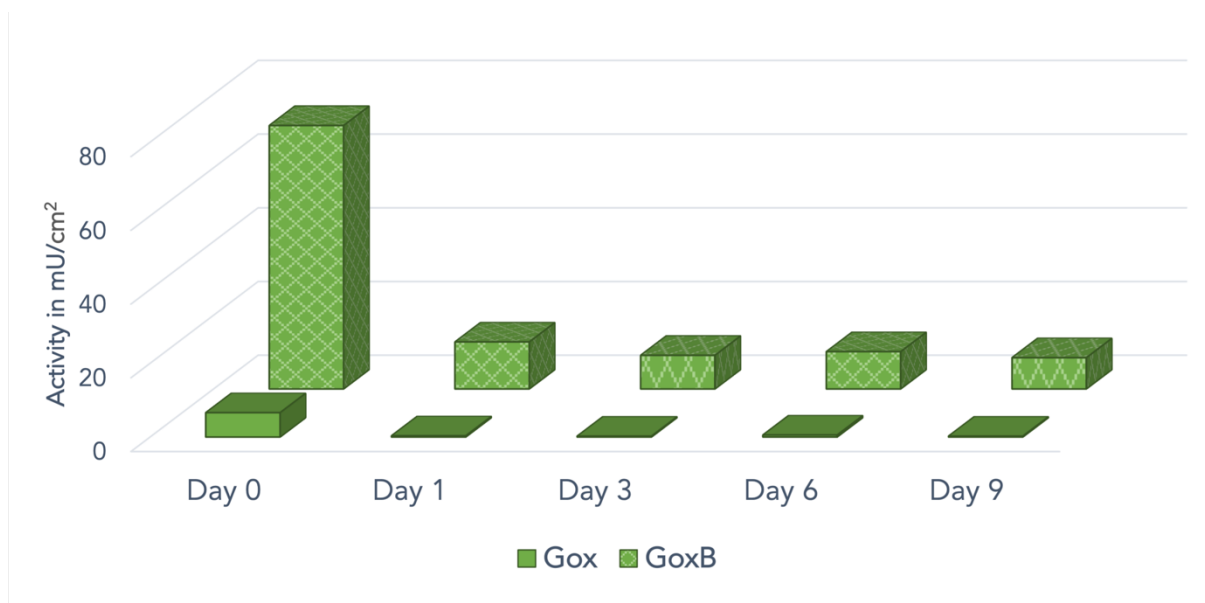


Figure 55 - Comparison of the activity and stability over 9 days of [PSS-AviGO]₃ and of [PSS-AviGOB]₃.

2. [PSS-Avidin]₃-GOx(B)

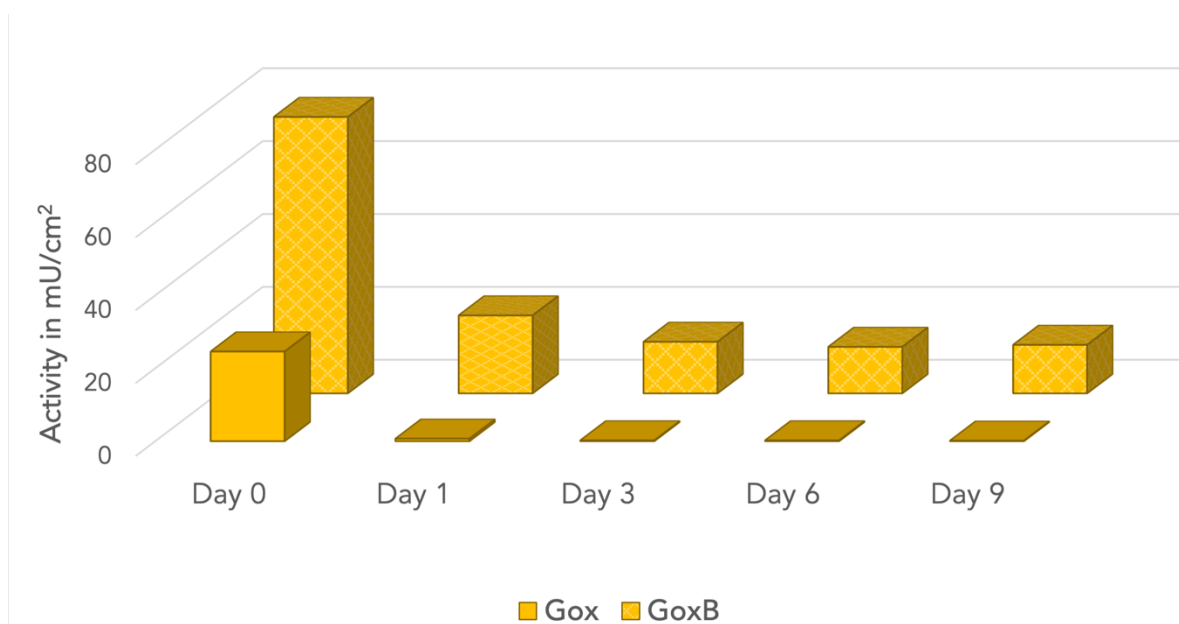


Figure 56 - Comparison of the activity and stability over 9 days of [PSS-Avidin]₃-GOx and of [PSS-Avidin]₃-GOxB.

3. [PSS-AviGO(B)-PSS-bPEI] 2 and 4 layers

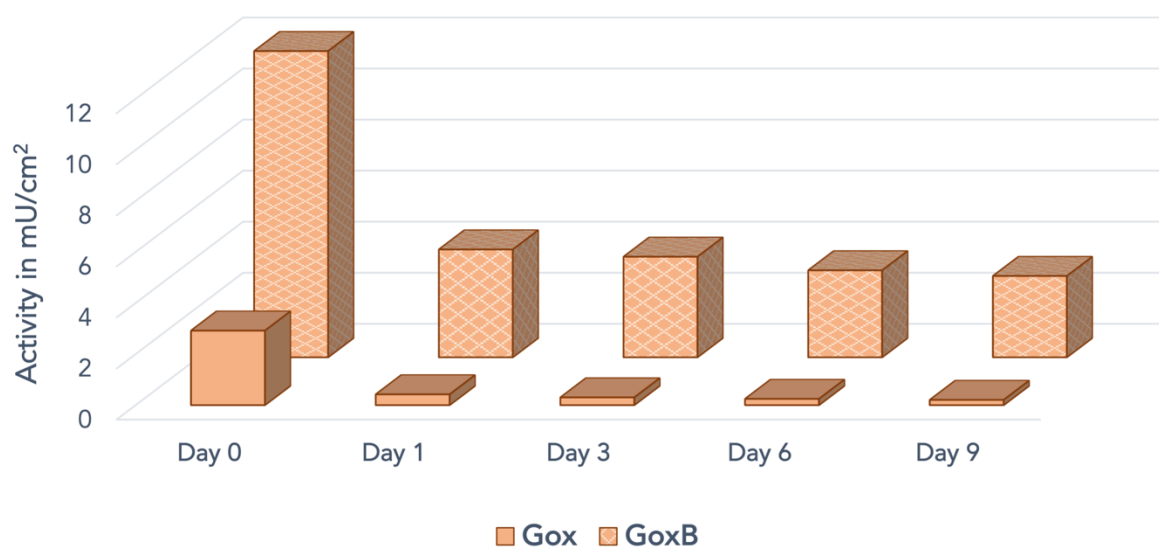


Figure 57 - Comparison of the activity and stability over 9 days of [PSS-AviGO-PSS-bPEI]₂ and of [PSS-AviGOB-PSS-bPEI]₂.

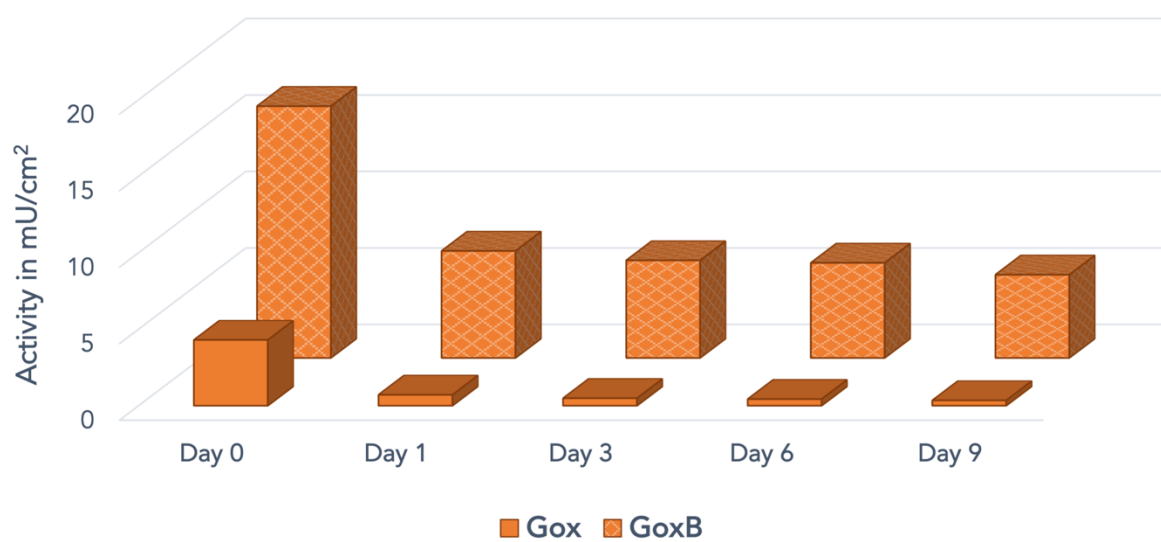


Figure 58 - Comparison of the activity and stability over 9 days of [PSS-AviGO-PSS-bPEI]₄ and of [PSS-AviGOB-PSS-bPEI]₄.

4. [bPEI-GOx(B)] 2 and 4 layers

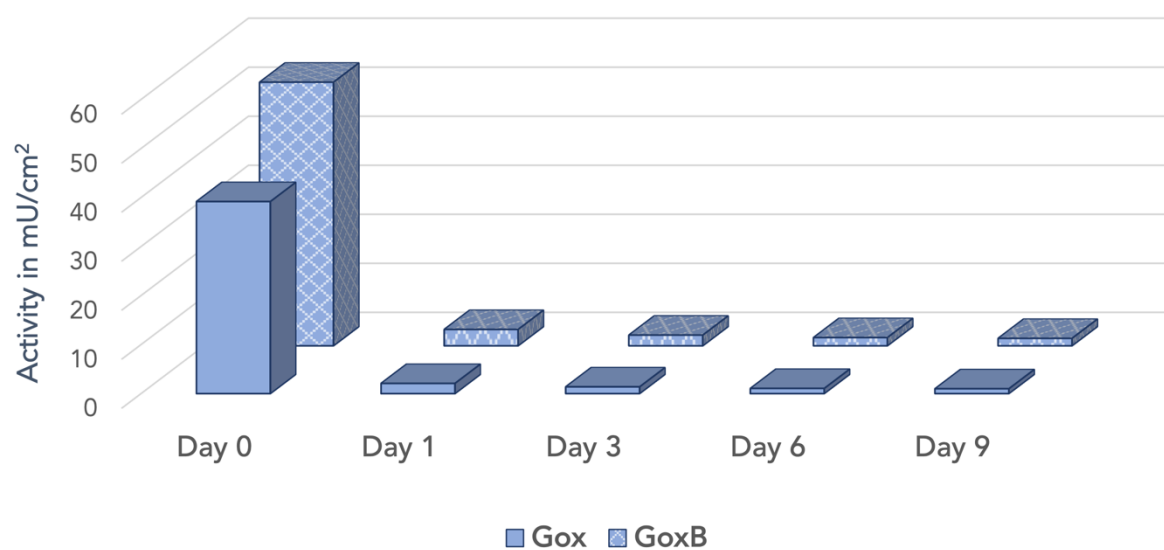


Figure 59 - Comparison of the activity and stability over 9 days of [bPEI-GOx]₂ and of [bPEI-GOxB]₂.

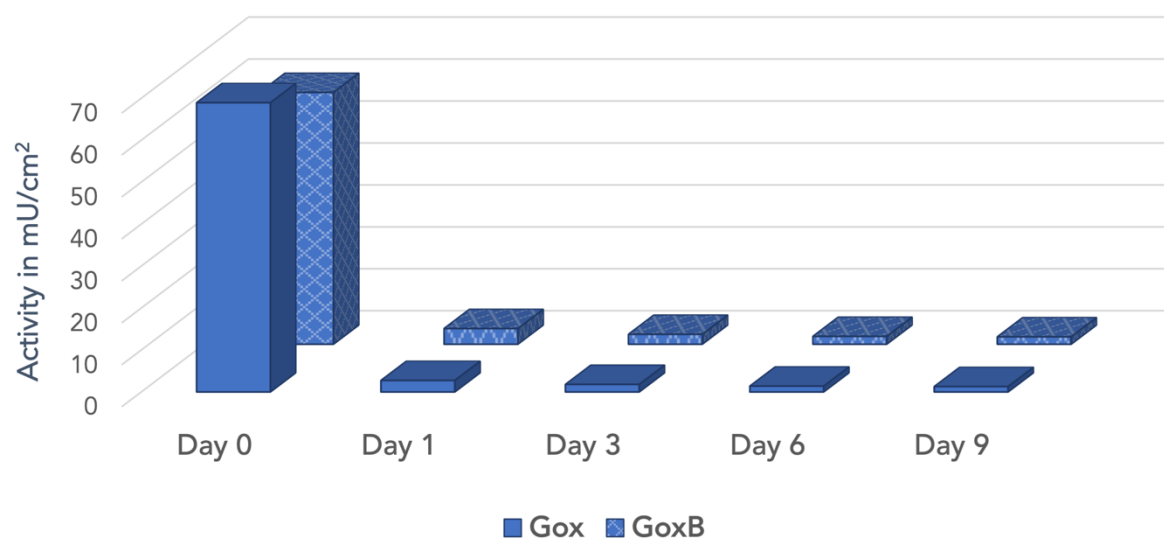


Figure 60 - Comparison of the activity and stability over 9 days of [bPEI-GOx]₄ and of [bPEI-GOxB]₄.

D. LACCASE PURIFICATION

1. Acetone precipitation

1.1. Experimental design

The idea behind this experiment is to precipitate the proteins from 10 mL of Laccase sample by successively adding acetone. By doing so, the proteins from the sample precipitate and can be separated from other impurities. 40 mL of Acetone -20°C were added to 10 mL of Laccase sample (5g/L). The solution is stored at -23°C for an hour. After centrifuging the solution at 20,000 rpm for 20 min, the supernatant can be separated from the pellet. The supernatant is kept for analysis and the pellet is resuspended in 10 mL acetate buffer before undergoing the same steps a second time. The final pellet is resuspended in 10 mL of buffer. Before analysis, the supernatants are placed in the oven at 60°C to evaporate acetone. Once all the acetone is evaporated, the volume is adjusted to 20mL in a volumetric flask. The process is schematized in Figure 61.

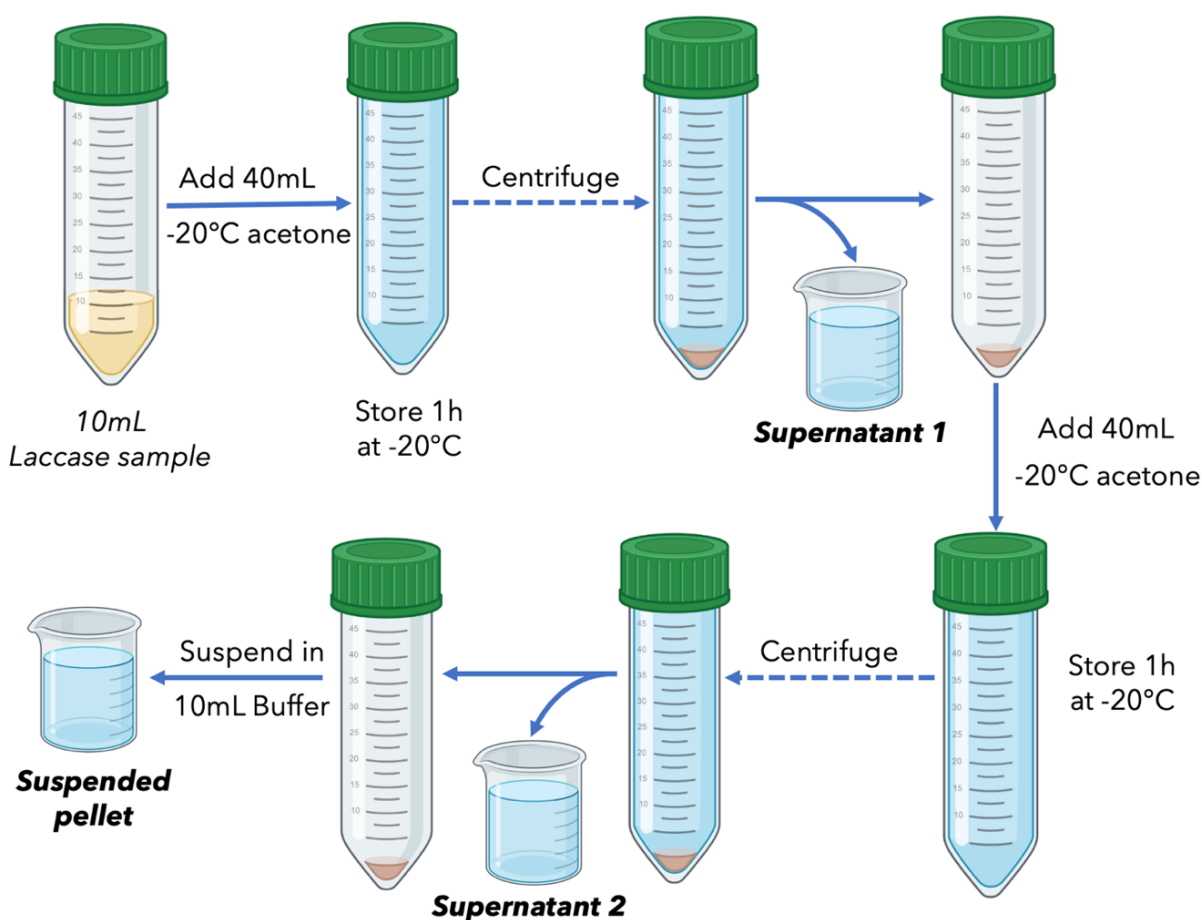


Figure 61 – Illustration of the Acetone precipitation purification process.

The final pellet and the two supernatants collected undergo both a BCA and an enzymatic activity assay such as described in *BCA Assay* and *Laccase Enzymatic Assay* of section III.

1.2. Results

Enzymatic assay

The enzymatic activities of the four analyzed samples are gathered in Table 8.

Table 8 - **Activity of Laccase sample before and after Acetone precipitation purification**

	Initial	Supernatant 1	Supernatant 2	Suspended pellet
Activity [mU]	860	0	0	120
% Initial activity	/	0	0	14

BCA Assay

Figure 62 illustrates the amount of protein in the laccase sample before purification and in the different samples after purification.

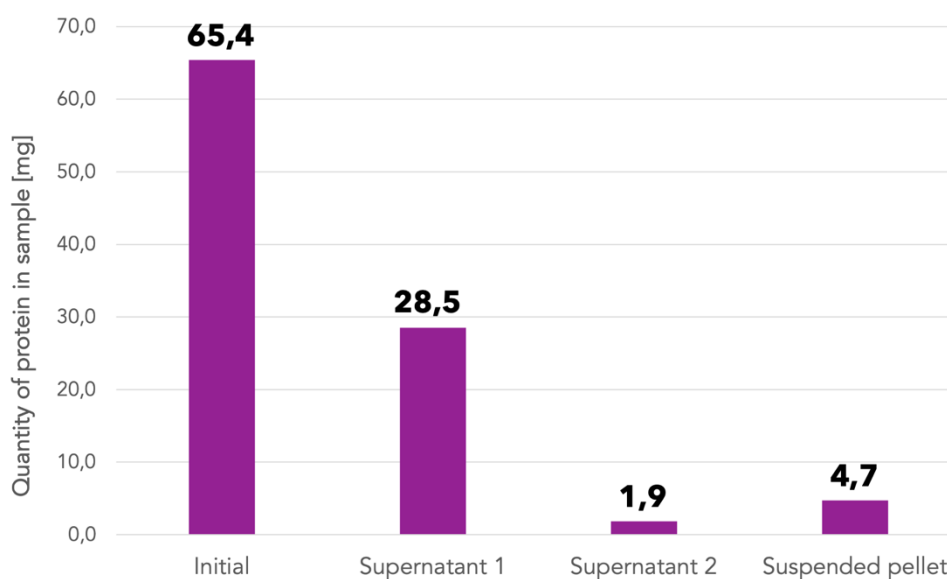


Figure 62 - **BCA assay of the initial Laccase before purification and of the three samples collected after Acetone precipitation purification.**

1.3. Discussion

The enzymatic activity assay resulted in no activity in both supernatant 1 and 2. All the remaining activity, 120 mU, after acetone precipitation purification is found in the final pellet. This remaining activity represents merely 13.95% of the initial laccase activity.

The BCA results shown in Figure 62 are startling since the sum of the protein quantity in the 3 purified samples, 35.1 mg, should be equal to the amount of protein in the initial

Laccase sample, 65.4 mg, and this is not the case. Notwithstanding, the highest amount of protein is found in supernatant 1.

In conclusion, we purify our laccase sample but at the cost of a very high loss of enzymatic activity.

2. Ammonium sulphate precipitation

2.1. Experimental design

Salting-out is the process in which, upon increasing the concentration of salts, protein solubility decreases leading to precipitation. The salts used in this process reduce the solubility of protein and enhance the stability of the native conformation. Adding these salts to the protein solution, increases the surface tension of water, resulting in increased hydrophobic interaction between protein and water. The protein attempts to minimize contact with the solvent by decreasing its surface area. Through folding and self-associating, the protein precipitates, freeing bound water and thus increasing entropy of the system.¹⁴⁹ Note that the increase in water's surface tension by salts follows the Hofmeister series (Figure 63). Since $(\text{NH}_4)_2\text{SO}_4$ has a higher solubility than any phosphate salts, it is the reagent we use to precipitate proteins in the laccase sample.

← increasing precipitation (salting-out)

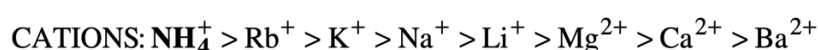


Figure 63 – Hofmeister series

The experiment consists in progressively precipitating the proteins in the 10mL of Laccase sample by reaching initially 30, then 60 and finally 90% of $(\text{NH}_4)_2\text{SO}_4$ saturation. At each step, the solution is stirred continuously at 0°C for an hour and centrifugated at 14,000 rpm for 20 min. The pellet is collected and the supernatant used for the next precipitation step. The pellets collected at each step are resuspended in 2mL of acetate buffer. The process is schematized in Figure 64.

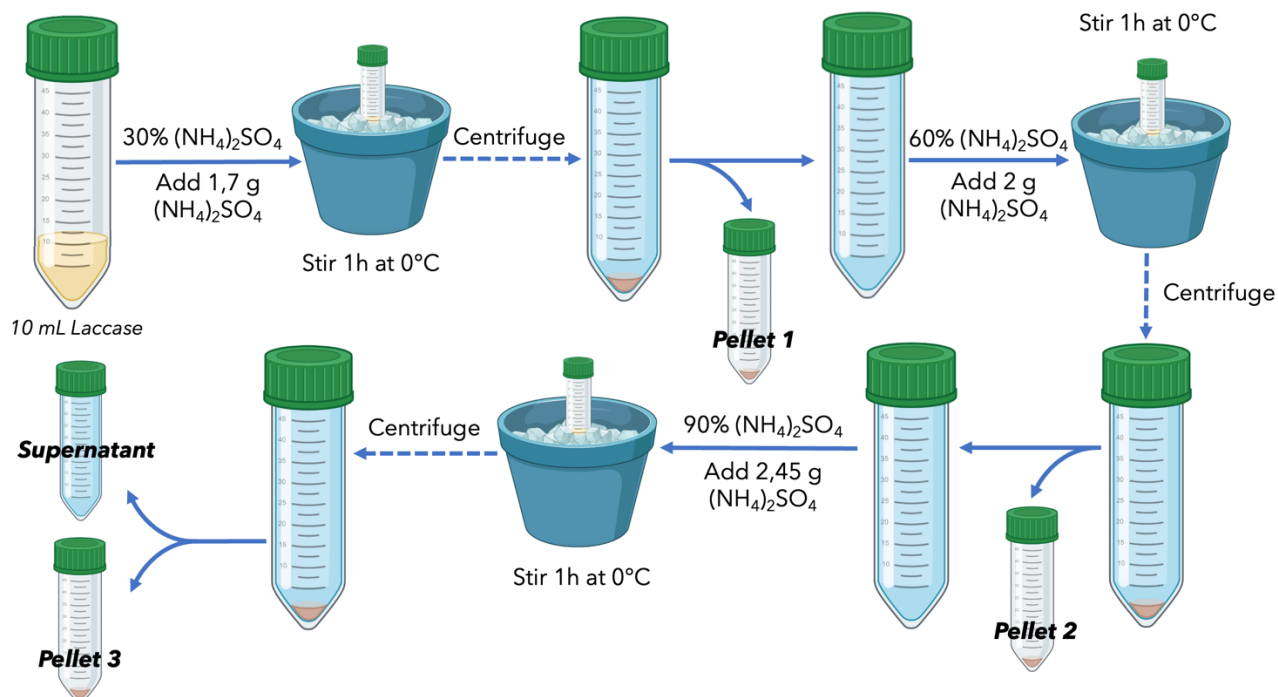


Figure 64 - Illustration of the Ammonium Sulfate Precipitation purification process.

The three pellets and the supernatant collected undergo both a BCA and an enzymatic activity assay such as described in *BCA Assay* and *Laccase Enzymatic Assay* of section III.

2.2. Results

Enzymatic assay

The enzymatic activities of the four analyzed samples are gathered in Table 9.

Table 9 - Activity of Laccase before and after Ammonium Sulfate precipitation purification

	Laccase	Pellet 1	Pellet 2	Pellet 3	Supernatant
Activity [mU]	950	14	186	356	100
% Initial activity	/	1,5	19,6	37,5	10,5
% Residual activity	/	2,1	28,4	54,3	15,2

The activity of the Laccase sample before purification was compared with the activity of each sample obtained following purification. Figure 65 illustrates the distribution of initial and residual activity according to the different samples.

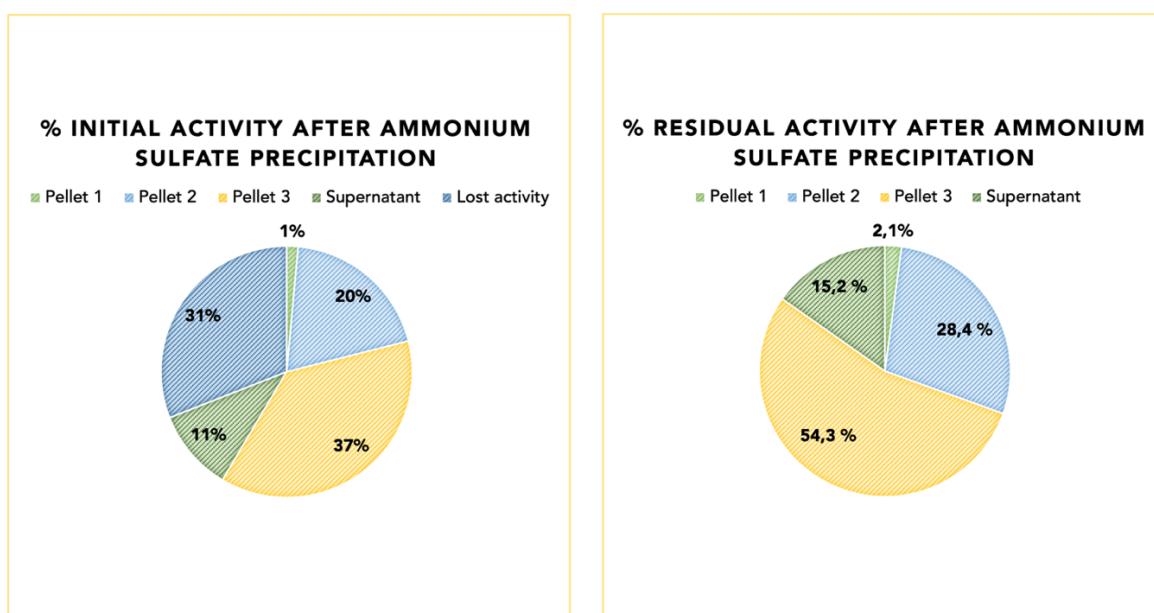


Figure 65 – Distribution of initial and residual activity according to the four samples.

BCA Assay

The amount of protein in the laccase sample before purification is compared to protein in the different samples after purification in Figure 66.

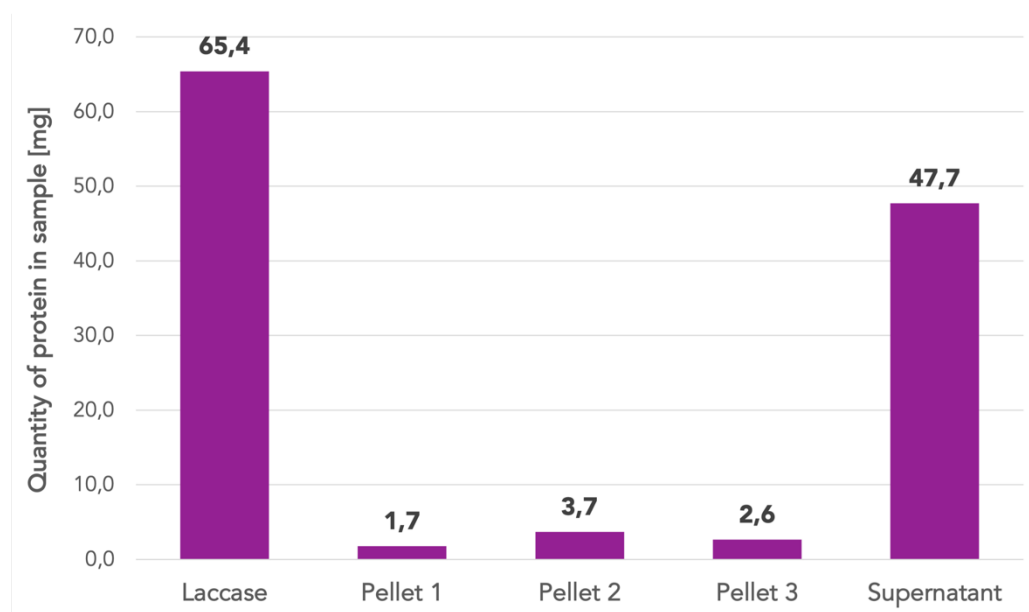


Figure 66 – BCA assay of the initial Laccase before purification and of the four samples collected after purification.

2.3. Discussion

BCA results in Figure 66 show that protein content of the sample before purification is 65.4 mg compared to 55.7 mg after purification. This difference is surprising since the BCA assay is supposed to quantify amide bond and they should be equivalent before and after purification. This difference could be due to the imprecise volumes of the samples

analyzed leading to non-negligible error on the protein amounts calculated. Nevertheless, a great majority of protein initially present in the sample can be found in the supernatant. This is an interesting observation given the Laccase activity assay revealed that the majority of laccase is found in the 3rd pellet. 31% of the initial activity of the Laccase sample is lost in the purifying process. Of the remaining activity, 54.3% is found in pellet 3 and 28.4% in pellet 2. The supernatant and pellet 1 represent less of the remaining activity.

We compare BCA results with the activity of the four different samples. The supernatant has a high amount of protein but very little activity. This suggests that we purify the Laccase sample from contaminant proteins. Furthermore, 82.7% of the remaining activity is found in pellet 2 and 3 which represent only 10% (6.3mg) of protein in the initial sample.

In conclusion, the ammonium sulfate precipitation purification process is a cheap and easy way to perform a first purification of the laccase sample. This method gets rid of a great fraction of other substances initially present in the Laccase sample. Yet only 37% of the initial activity could be recovered in the 3rd pellet, meaning purification of laccase would be at the cost of 63% of the initial activity.

E. DYNAMIC ENZYMATIC ACTIVITY ASSAY

1. Materials and Method

One of the main advantages of using membranes as solid support is to use them in flowthrough mode to maximize enzyme-substrate contact time. Therefore, this experiment constitutes a preliminary test in which a membrane functionalized with two layers of [PSS-Avidin-GOxB] was used in flowthrough mode. The system is composed of a peristaltic pump, a flow unit (from fluigent smart microfluidics) that automatically adjusts the pressure of the system to keep the flow constant and a flowthrough cell that hosts the membrane. We use the computer software OxyGEN to monitor the pressure and flow rate of the system and to record data. All the different units are connected using soft tubing.

We connect the system to 40mL substrate (45mg/mL) and we start pumping substrate in the system at 100 μ L/min. Once the substrate comes in contact with the membrane, the experiment starts. We collect the solution coming out of the system at 0, 15, 30, 45 and 60 min. These samples are collected in an Eppendorf for 1 minute to accumulate 100 μ L of solution as shown in Figure 67. These samples are diluted 10 times before

undergoing a GOx enzymatic assay. An end point measurement of absorbance at 510 nm was taken for each sample and using the equation of the calibration curve for GOx, the activity in mU/mL was measured. The activity was multiplied by the dilution factor, 10, and by the volume of sample, 0.1mL, to obtain the results shown in Figure 68.

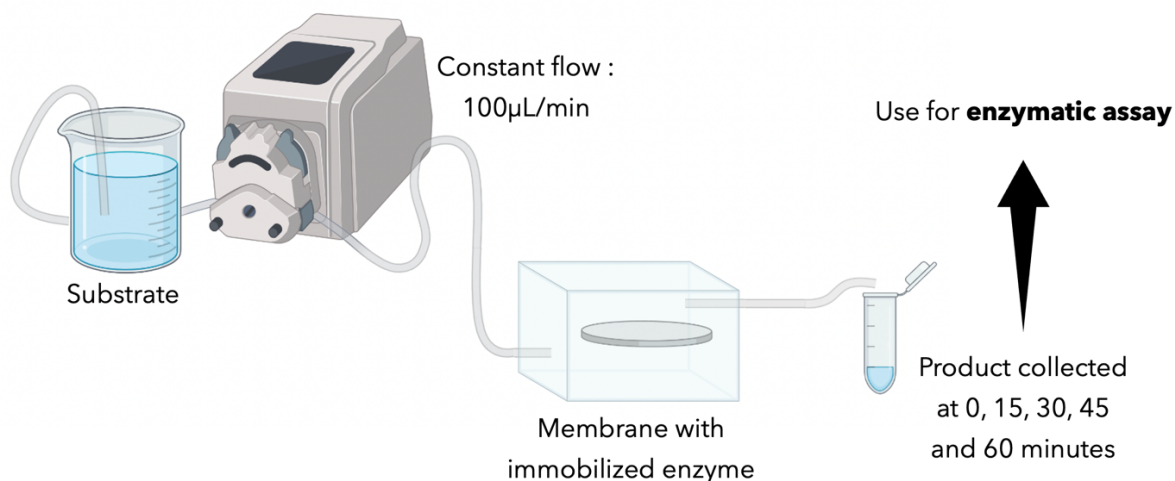


Figure 67 – Schematic representation of the dynamic enzymatic assay procedure.

2. Results

The activity results of the membrane under constant flowthrough of 100µL/min of substrate are shown in Figure 68. The activity starts high at 1350 mU/mL and decreases progressively to reach 200mU/mL at the end of the 60 min.

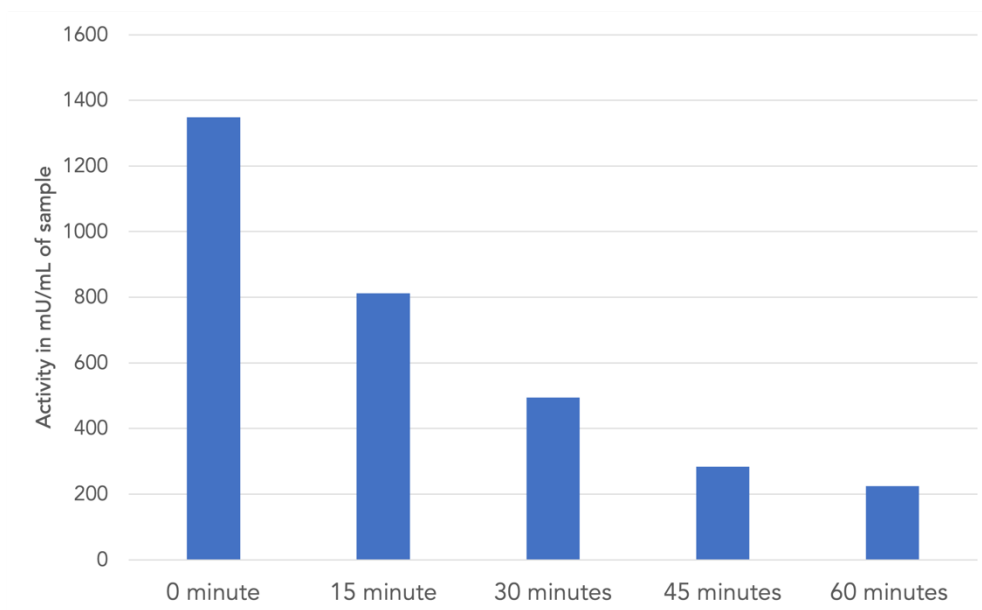


Figure 68 – Activity results for the dynamic enzymatic assay on the membrane with 2 layers of [PSS-Avidin-GOxB].

3. Discussion

The results from the dynamic assay are to be treated with caution. Firstly, the samples were collected at a specific time but the activity assay was carried out much latter (3-4 h after the samples are collected). This suggests that if some enzyme has leached out of the membrane, especially for the first min of the experiment, it could have reacted with the substrate that wasn't converted while flowing through the cell. The activity results for these samples would overshoot.

Overall, the trend is that the activity decreases over time. This result is predictable since enzymes most certainly leach out of the membrane as the substrate flows through. These results are consistent with the results from static activity measurements. Leaching of the enzymes out of the membrane seems to be the biggest issue to overcome in order to obtain stable and reusable materials. Nonetheless, this is only a preliminary test and the assay needs to be improved and optimized to free ourselves from factors of uncertainty mentioned above.

Development of an enzyme immobilization system combining LbL and Avidin-biotin interaction

By Juliette de Meeûs

This work presents a versatile method for enzyme immobilization utilizing the avidin-biotin specific interaction to immobilize a biotinylated-enzyme inside a Layer-by-Layer (LbL) film of Avidin. Nanoporous polycarbonate membranes serve as substrate to benefit from the large specific area developed by such materials.

First, the assembly of different systems on a flat surface was monitored using Quartz Crystal Microbalance measurements. Subsequently, activity and stability comparisons were conducted between different system architectures to identify the most promising approaches. Based on these evaluations, two constructions with superior activity and stability were selected for further assessment through LbL film assembly onto membranes.

In a second phase, the efficiency of the two optimal LbL constructions on nanoporous membranes was assessed and compared. A comprehensive methodology was developed to obtain multiple pieces of information, including the theoretical amount of immobilized enzyme and protein, the effective activity of the assembled membrane in static mode, and the extent of enzyme leaching from the membrane.

The results demonstrate the successful assembly of enzyme-immobilized systems on both flat surfaces and nanoporous membranes using the avidin-biotin assembly method. The comparative analysis indicates improved activity and stability compared to traditional LbL systems.

Overall, this study highlights the potential of the avidin-biotin assembly technique for enzyme immobilization on nanoporous membranes. The findings contribute to the development of innovative approaches in enzyme immobilization technology, with potential applications in various fields such as biocatalysis, biosensing, and bioseparations.