The University of South Bohemia in České Budějovice

Faculty of Science

Effects of various number of basal and covering polyelectrolyte layers on immobilized enzyme activity

Bachelor thesis

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Bachelor Thesis

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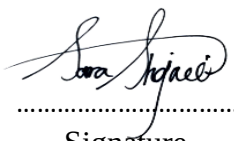
Annotation

This thesis is focused on the immobilization of a selected biomolecule, Horseradish peroxidase enzyme (HRP), into a polyelectrolyte multilayer on the glass surface in order to investigate HRP stability and activity in time and specific conditions. To accomplish this work, layer-by-layer deposition approach is employed to fabricate a multilayer of polystyrene sulfonate (PSS) and polyallylamine hydrochloride (PAH). Therefore, 0.1 µg of HRP enzyme is immobilized onto the various constructs with different number of basal and covering PSS and PAH polyelectrolyte layers. As a results, the activity of the enzyme is increased by further underlying PSS and PAH polyelectrolyte layers. The enzyme activity of the constructs which are not covered by PSS/PAH polyelectrolyte layers is diminished, whereas the enzyme activity of constructs which are covered by the PSS/PAH polyelectrolyte layers is increased.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.



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České Budějovice, 12.05.2022

Acknowledments

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1 Introduction

1.1 Enzyme immobilization technology

Enzymes are essential to life because they act as powerful and specialized catalysts. An enzyme catalyses almost every biochemical reaction. Catalysis can be stated as the stability of the transition state via tight binding to the catalyst. All known enzymes, except a few catalytic RNA molecules, are proteins. The main function of biological catalysts is to bind to a molecule or substrate in order to modify it and lower the energy required to make it react. Thus, these molecules enhance the rate of reactions by lowering the activation energy without being permanently altered or consumed by the reactions. In addition, a significant portion of the energy used to increase enzymatic rate is obtained from weak interactions, hydrogen bonds and hydrophobic and ionic interactions, between the substrate and the enzyme [1].

In 1971, at the first Enzyme Engineering Conference, the term "immobilized enzyme" was embraced [2]. This term refers to enzymes physically confined or localized in a certain limited region of space with retention of their catalytic activities, and which can be utilized repeatedly and continuously [3]. Immobilization is one of the cost-cutting methods that have been implemented to make enzyme utilization in biotechnological processes more advantageous [4].

Hereby, Immobilized enzyme technology has evolved into a diverse field of research in recent decades, with applications in clinical, industrial, and environmental samples.

In addition, Immobilized biocatalyst (not only enzymes but also other bioactive molecules) applications include the generation of useful compounds via stereo/regio specific bioconversion, the generation of energy via biological processes, the selective treatment of specific pollutants to solve environmental problems, continuous analyses of compounds with high sensitivity and specificity, and medical applications such as new types of drugs for enzyme therapy (or artificial organs as surface in order to increase biocompatibility and to prevent blood clotting) [5,6].

Furthermore, it is critical to recognize that an enzyme changes its physical and chemical properties when immobilized, depending on the method of immobilization chosen. The main components of an immobilized enzyme system are the enzyme, the support, and the mode of attachment of the enzyme to the matrix (carrier)[7]. The surface on which the enzyme is immobilized serves several important functions, including maintaining the tertiary structure of

the enzyme through the formation of electron transition complexes and the formation of hydrogen or covalent bonds with the matrix. Therefore, when immobilizing an enzyme on a surface, the key factor to consider is the proper selection of an attachment method between the reactive groups on the matrix surface and the residues outside the enzyme's substrate binding or active site [4,8].

Various methods for immobilizing biocatalysts have been devised which are widely used today, including, adsorption, covalent binding, entrapment, encapsulation, and cross-linking. These methods differ in terms of the type and character of interactions formed, as well as the form and type of support materials used. The most appropriate immobilization method and support material are massively influenced by the type and conditions of the catalytic process, as well as the type of enzyme [9,10]. An exceptionally expansive assortment of materials of different origins can be utilized as supports for protein immobilization. According to the chemical composition of these materials, support type can be classified into organic, inorganic and hybrid or composite, and the organic supports can be further classified into natural or synthetic matrices (Table 1). Moreover, the support must protect the structure of the enzyme from severe reaction conditions, thus help the immobilized enzyme to maintain high catalytic activity [7,12].

Table 1. *Classification of supports according to their chemical compositions.*

Organic	**Inorganic**
Natural polymers	***Natural minerals***
Polysaccharides: cellulose, agar Proteins: collagen, albumin Carbon	Bentonite, silica
Synthetic polymers	***Processed materials***
Polystyrene Other polymers: polyacrylate, polymethacrylates, polyacrylamide, polyamides, vinyl and allyl-polymers	Glass (non-porous and controlled pore), metals, controlled pore metal oxides

However, there are a few restrictions limitations in this area, because the matrix must not adversely affect the structure of the enzyme and disrupt the enzyme more than is needed to create stable matrix interactions [11,12]. The main properties required of support materials for effective immobilization of enzyme are summarized in Figure 1.

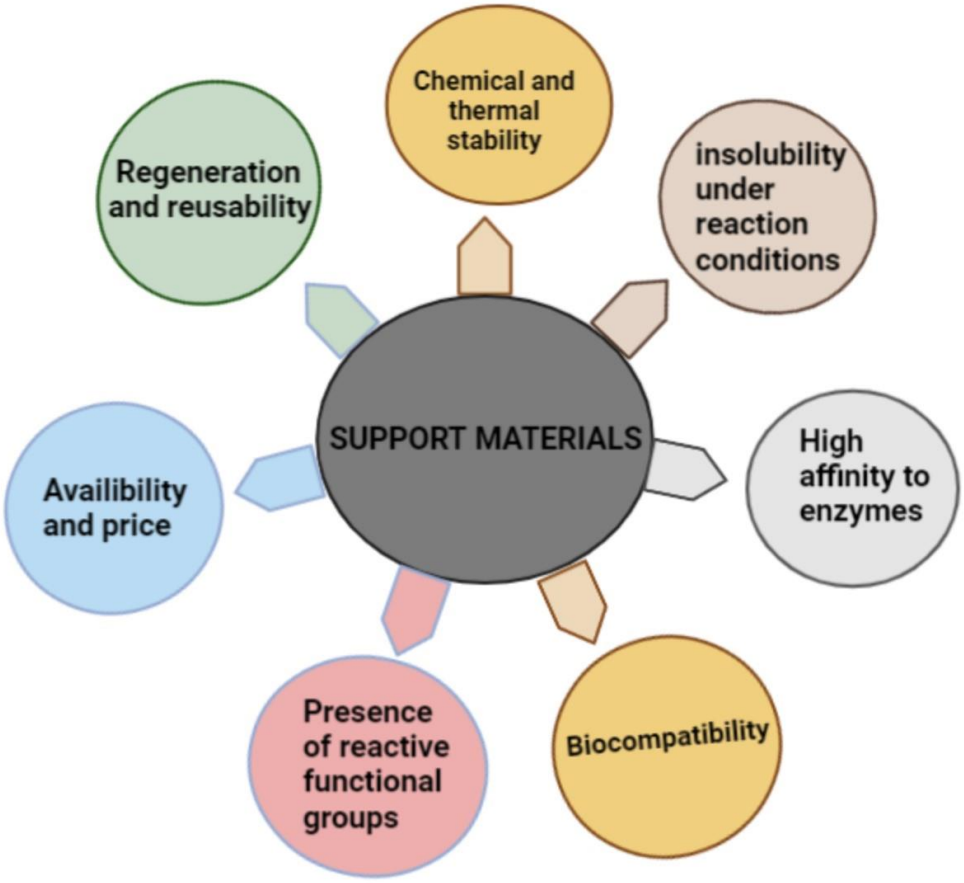

Figure 1. *main properties of support materials used for enzyme immobilization*. In general, support materials have been studied for which offer high stability, availability, relatively inexpensive, and high affinity to the bound protein *(taken from Zdarta et al., 2018 and created in BioRender.com).*

1.2 Utilization of HRP in immobilization technique

In this work, Horseradish peroxidase (HRP) was chosen as the model enzyme due to its many analytical, environmental, and clinical applications, further because it is readily available and has been broadly studied by chemical and spectroscopic methods [13]. HRP is a heme-containing glycoprotein having a single chain of 308 amino acid residues, eight oligosaccharide side chains, two calcium ions, and one porphyrin group. It catalyzes the oxidation of various organic substrates through free radical formation at the cost of hydrogen peroxidase [14].

Additionally, HRP can be conjugated to a labeled molecule that has been joined chemically. Therefore, this enzyme can determine the presence of a molecular target, by producing a colored product of the target when incubated with a suitable substrate, such as 3,3′,5,5′-Tetramethylbenzidine or TMB, hence allowing it to be detect and as well as quantified, Fig. 2 [15].

Hereby, we employed layer-by-layer assembly to immobilize HRP by constructing multilayer polyelectrolytes on the glass surface.

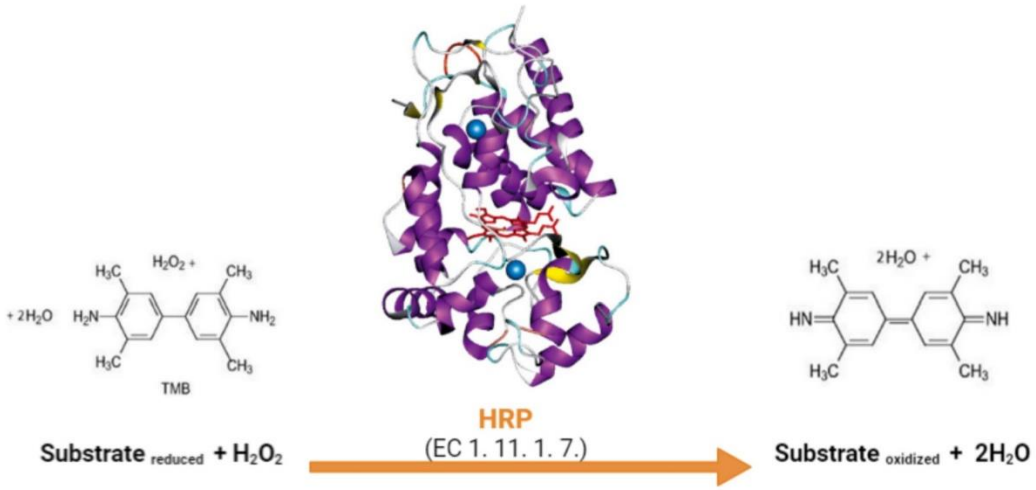

Figure 2. *Catalytic reaction of the enzyme horseradish peroxidase. The* 3,3′,5,5′-Tetramethylbenzidine (TMB) *substrate acted as hydrogen donor for the reduction of hydrogen peroxide to water by horseradish peroxidase enzymes. The results of this reaction were 3,3′,5,5′-Tetramethylbenzidine diamine, which converts the colour of the solution from white to blue, and this colour change was read on a spectrophotometer at the wavelength of 370 and 650 nm. (Created in BioRender.com)[16,17].*

1.3 Layer-by-Layer deposition approach

In 1990s, Decher and Hong pioneered the layer-by-layer (LbL) deposition approach for immobilizing biomolecules. It has become widely employed in recent years because of the benefits it provides, such as the ease of procedure, the vast range of materials that can be used, and the precise control of layer composition and thickness that it allows. This approach, as is well known, involves the alternating adsorption of polyelectrolytes with opposite charge onto conveniently modified solid surfaces via primarily electrostatic interactions [18]. Thus, protein layer-by-layer films can be assembled on different solid surfaces with precisely controllable layer thickness on the nanometer scale via alternate adsorption of proteins at proper pH and as well as oppositely charged polyions or dispersions [19]. Additionally, the LBL approach is performed in aqueous solutions under gentle conditions, which retains the activities of biomolecules [20].

According to the diversity of polyelectrolyte multilayers deposition, the functionalized surfaces with polyelectrolyte shown to be appropriate active colloidal supports for loading and releasing bioactive chemicals and contaminants. Many natural polyelectrolytes (e.g., chitosan, hyaluronic acid, etc.), have antimicrobial activity on microorganisms (e.g., *Escherichia coli* and *Staphylococcus aureus*, etc.) and their multilayer construction has found applications in the fabrication of non-adhesive or antibacterial films.

Furthermore, these structures can be acquired by LbL deposition, as well as by forming multilayers between polyelectrolytes and inorganic compounds with antibacterial activity, such as silver nanoparticles [36].

Moreover, layer-by-layer deposition is an adjustable approach for fabricating multilayers because it allows for precise control of the deposition parameters. Materials with different structures and properties can be obtained by changing the deposition conditions (pH, temperature, ionic strength) or the polyelectrolytes (molar mass, charge density) and solvent used [19].

1.4 Polyelectrolytes

Polyelectrolytes (PEs) are polymers or macromolecular compounds that contain at least 10 – 15 % functional groups or ionizable repeating groups, such as polyanions and polycations that are dissociated or dissociate in polar solution [22,23]. Depending on polyelectrolytes provenance, they can be natural, semi-synthetic, or synthetic (poly(ethyleneimine) (PEI), poly(allylamine hydrochloride) (PAH), poly(styrene sulfonate) (PSS), poly(methacrylic acid) (PMA), etc.). Additionally, PEs are classified into polyanions (polyacids, have negative charges) or polycations (polybases, have positive charges), polyampholytes (amphoteric, have the same number of positive and negative charges) based on the type of the ionic charge. Both weak and strong compounds, as well as other low molecular or macromolecular compounds, can participate in the formation of multilayers along with other low molecular or macromolecular compounds (charged/uncharged). The properties of the obtained materials depend on the nature and characteristics of the partners involved in the deposition process [19].

In this work we used poly-styrene sulfonate (PSS) negatively charged, as the polyanion and poly-allylamine hydrochloride (PAH), positive charged as the polycation. Also, poly-ethyleneimine, positively charged was used for coating of the glass surface (Figure 3).

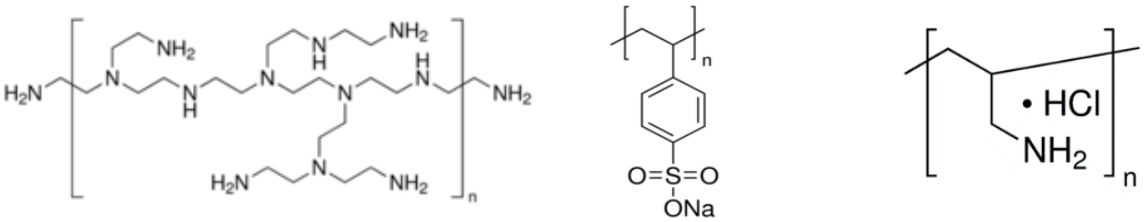

Figure 3. Polyelectrolytes used in the LbL method. *From left to right: polyethyleneimine Po lyally*

2 Aims of the thesis

The aim of this thesis was to construct the different number of basal/covering PSS/PAH polyelectrolyte layers with oppositely charged alternately on the glass surface through layer-by-layer deposition. Subsequently, immobilization of a certain amount of Horseradish peroxidase enzyme was immobilized into the prefabricated PSS/PAH layers to evaluate the effect, stability, and catalytic activity of the immobilized HRP, as well as to confirm enzyme presence.

3 Materials and methods

3.1 Immobilization of HRP into PEs using LbL method

3.1.1 First experiment

Before the immobilization of HRP, polyelectrolyte solutions need to be prepared . To start, 100 mM stock solutions of poly(ethyleneimine) (PEI), poly(allylamine hydrochloride) (PAH), poly(styrene sulfonate) (PSS) was prepared in Tris buffered saline (TBS) with a pH of 7.5 (table 2), which is a suitable buffer for HRP enzyme (table 3). Also, the enzyme stock solution with 10 mg/ml was prepared in TBS (table 3). Subsequently, polyelectrolyte solutions were filtered through a 0.22 µm filter to remove bacterial contaminations. Before layering the polyelectrolytes, a piece of glass (15 mm) was put into a well of 24-well plate. Afterwards, 250 µl of polyelectrolyte solutions were pipetted on glass slides and incubated for 15 minutes at room temperature. Between each polyelectrolyte layer, as well as enzyme layer, 250 µl buffer were added and incubated for 1 minute and removed by suction machine, as a wash step. Hence, the first experiment was done by layering polyelectrolytes on glass slides according to table 4, as shown below, 0.1 µg enzyme was layered/immobilized in a volume of 250 µl on a various number of basal and covering PSS/PAH layers and incubated for 30 minutes at room temperature. For instance, well numbers 1 and 5 (V_1 V_5) are the control sample without enzyme, V_2 & V_6 are the control with enzyme as the final layer, V_3 & V_7 are the control with two covering layers, as well as V_4 & V_8 are the control with four covering layers.

Table 2. 1x Tris-Buffered-Saline (TBS)

Reagents	[stock]	1L
Tris	20 mM	2.42 g
NaCl	500 mM	29.24 g
Deionized H_2O		~ 800 mL
pH		7.5

Table 3. Polyelectrolytes

Reagents	[100 mM]	volume
PEI	0.02 g	5 mL
PAH	0.04 g	5 mL
PSS	0.1 g	5 mL

Table 4. HRP enzyme solution

Reagents	[stock]	[final]	5ml
HRP	10 mg/mL	0.1 µg	50 µL
TBS	1x		4.95 µL
Final Vol.			5 mL

Table 5. layering polyelectrolytes and HRP on glass slides – 1st experiment

V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	Nr. Of layer
PEI	PEI	PEI	PEI	PEI	PEI	PEI	PEI	0
PSS	PSS	PSS	PSS	PSS	PSS	PSS	PSS	1
PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH	2
PSS	PSS	PSS	PSS	PSS	PSS	PSS	PSS	3
PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH	4
	HRP	HRP	HRP	PSS	PSS	PSS	PSS	5
		PAH	PAH	PAH	PAH	PAH	PAH	6
		PSS	PSS	PSS	PSS	PSS	PSS	7
			PAH	PAH	PAH	PAH	PAH	8
			PSS		HRP	HRP	HRP	9
						PAH	PAH	10
						PSS	PSS	11
							PAH	12
							PSS	13

Ultimately, after adding all the polyelectrolyte layers, 100 µl TMB (3,3′,5,5′-Tetramethylbenzidine) liquid substrate, which is a chromogenic substrate used in the staining procedure and can act as a hydrogen donor for the reduction of hydrogen peroxide to water by horseradish peroxidase, was added to all samples. Afterwards, the plate was incubated at RT for 2 hours, to develop a colour as a result of enzyme activity. Finally, 100 µl of the samples were transferred into a 96-well plate and absorbance at 650 nm was measured, which correlates with the amount of the product. The result can be seen in the results section.

3.1.2 Second experiment

The second experiment of HRP immobilization into polyelectrolyte layers by the LbL approach was performed in the same way as the first experiment but with an increase in the number of basal polyelectrolyte layers, as shown in table 6. The polyelectrolytes were layered on glass slides and 0.1 µg enzyme solution was added as a final layer on a different number of basal polyelectrolyte layers. In both experiments, the volume of every solution (buffer, polyelectrolytes, and enzyme) that were added to the wells, was 250 µl, and also with the same incubation time and temperature. Again, the enzyme activity measurement was done via microplate spectrophotometer, in the same way as the first experiment, its result is shown in the results section.

Table 6. layering polyelectrolytes and HRP on glass slides – 2nd experiment

V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	Number of layers
PEI	PEI	PEI	PEI	PEI	PEI	PEI	PEI	0
PSS	PSS	PSS	PSS	PSS	PSS	PSS	PSS	1
PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH	2
PSS	PSS	PSS	PSS	PSS	PSS	PSS	PSS	3
PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH	4
PSS	PSS	PSS	PSS	PSS	PSS	PSS	PSS	5
PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH	6
PSS	PSS	PSS	PSS	PSS	PSS	PSS	PSS	7
PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH	8
	HRP	PSS	PSS	PSS	PSS	PSS	PSS	9
		PAH	PAH	PAH	PAH	PAH	PAH	10
		PSS	PSS	PSS	PSS	PSS	PSS	11
		PAH	PAH	PAH	PAH	PAH	PAH	12
			HRP	PSS	PSS	PSS	PSS	13
				PAH	PAH	PAH	PAH	14
				PSS	PSS	PSS	PSS	15
				PAH	PAH	PAH	PAH	16
					HRP	PSS	PSS	17
						PAH	PAH	18
						PSS	PSS	19
						PAH	PAH	20
							HRP	

3.2 Verification of enzyme presence by western blot analysis

3.2.1 Sample preparation

After layering all polyelectrolytes as well as the enzyme, the buffer (TBS) was removed from all the wells by suction machine, and then 50 µl 1x loading buffer containing dithiothreitol (DTT) was added to each well. The DTT can act as reducing agent and denature protein disulfide bonds in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), therefore, to stabilize enzymes and other proteins [24]. Subsequently, the entire plate was incubated on a shaking platform for 15 minutes at 50 °C, this condition was to ensure that all proteins were released from the polyelectrolyte layers, thus accumulated in the loading buffer. Finally, the 24-well plate was removed from the incubator and the solution inside each well was transferred to the Eppendorf tubes. Then the samples were stored in the freezer.

Thereby, to confirm the presence of the enzyme, these samples containing PSS/PAH and HRP were tested by SDS-PAGE then further by Coomassie Brilliant Blue/silver staining, as well as blotting techniques. However, the obtained results were unsatisfactory, either no protein signal was visible, or it was barely visible. The problem was due to the presence of poly (allylamine hydrochloride) (PAH) and poly (styrene sulfonate) (PSS).

Because after loading samples (containing PSS/PAH and HRP) on the gel and therefore performing SDS polyacrylamide gel electrophoresis, the PSS with negatively charged and 70 kDa can affect the migration because there was plenty of negative charges for every molecule in SDS-PAGE. Additionally, PAH with positively charged and 65 kDa can bind to SDS and thus lower the amount of available SDS for proteins, as well as lower the migration.

Consequently, we decided to employ a different procedure to prepare samples.

Methanol/Chloroform protein precipitation

The purpose of this procedure was to remove the polyelectrolytes and thus obtain a sufficient amount of HRP molecule.

To begin with, 50 µl of the sample (from 3.2.1) were mixed with 50 µl deionized water in a 1.5 ml Eppendorf tubes. Then, 400 µl Methanol was added to the mixture and vortexed well, followed by 100 µl chloroform and vortexed for 1 minute. Both methanol and chloroform are protein denaturing agents, helped to removal of interfering agents (e.g., lipids or in our case polyelectrolytes) to enhance the resolution of protein on acrylamide gels. Subsequently, 300 µl ddH_2O was added to the aliquots and then was spun for 2 minutes at 14,000 g. Following the centrifugation, two phases were observed, an upper aqueous phase and a lower solvent phase. The protein was located at the interface between the phases. It may be visible as a white film, or it may not be visible at all if the protein amount is small. The upper aqueous layer was pipette away and discard. Care was taken to not disturb the interface. Again 400 µl of Methanol was added and spun for 3 minutes at 14,000 g. Finally, as much Methanol as possible was poured off from the tube without disturbing the pellet. Finally, the pellet was dissolved in 25 µl 1x loading buffer, therefore was prepared to be analyzed by SDS-PAGE as well as western blotting.

3.2.2 SDS-PAGE

SDS polyacrylamide gel electrophoresis is an important technique in many areas of molecular biology. SDS is an anionic surfactant compound that lowers surface tension, also in the case of proteins it disrupts the non-covalent bonds in protein molecules. PAGE is a biochemical technique that separates proteins based on their electrophoretic mobility through an electric field. Therefore, SDS-PAGE uses the negatively charged sodium dodecyl detergent to coat proteins with a negative charge and denaturing them, so they can be resolved on a polyacrylamide gel according to their molecular weight. An electric field is applied to the charged proteins, causing them to migrate through the matrix formed by the cross-linked acrylamide polymers. Hence, larger molecules migrate more slowly than smaller molecules.

Samples were investigated by SDS-PAGE (12% separation gel, 5% stacking gel, PanReac Applichem pre-stained protein marker), Table 7. Precast acrylamide gel was submerged in running buffer in a Bio-Rad electrophoresis unit (Table 8). Samples were loaded onto the gel next to a pre-stained protein ladder to allow the determination of the molecular weight of the proteins when detect by western blot analysis. Electrophoresis was performed at 125 V for approximately 90 minutes (until the bromophenol blue dye in the SDS-PAGE loading buffer migrated to the bottom of the gel).

Table 7. Casting of polyacrylamide gel for SDS-PAGE, volume per one gel

Reagents	12% Separation gel	5% Stacking gel
30% Acrylamide	2 mL	165 µL
4x Separation Buffer	1.25 mL	
4x Stacking Buffer		250 µL
10% APS	50 µL	10 µL
TEMED	2 µL	1 µL
ddH_2O	1.7 mL	575 µL
Final Volume	5 mL	1 mL

Table 8. 10x SDS PAGE Running buffer

Reagents	[final]	1L
Tris	250 mM	30.3 g
Glycine	1.92 M	144.4 g
SDS	1%	10 g
ddH_2O		700 mL
Final volume		1L

3.2.3 Electroblotting: Transfer of protein onto NC membrane

The principle of Western blotting relies on the specificity of binding between a molecule of interest (HRP) and a probe to detect the molecule. By using a semi-dry electroblotting apparatus, the protein from the SDS PAGE is transferred to a more stable platform in order to probe the resolved protein (HRP) with specific antibodies. First the Nitrocellulose membrane, which is hydrophobic, was activated by submerging it into small amount of blotting buffer (Table 9), then was shake for some minutes. Also, all four layers necessary for the electroblotting were equilibrated with the blotting buffer. Once the electrophoresis was finished, the gel was removed from the tank as well as between two glasses, followed by 3 minutes rinse in distilled water and finally soaking it in 20 ml blotting buffer for 15 minutes. To assemble the blotting sandwich in the apparatus, some blotting buffer was poured on the anode, where the gel was placed. Then, two pre-wetted blotting paper, transfer membrane, gel and finally two remaining blotting papers were precisely placed one over another while rolling out the air bubbles. Lastly, the cathode was placed on top of the blotting sandwich and the apparatus was closed with the lid. In this way, the negatively charged protein migrates from the gel onto the membrane due to the construction of the cathode and anode. As a last step, apparatus was placed into the Trans-Blot ® Turbo ™ transfer system and run for 30 minutes at 25 V.

Table 9. 10x Blotting buffer

Reagents	[final]	1L
Tris-HCl	480 mM	58.15 g
Glycine	390 mM	29.27 g
ddH_2O		700 mL
Final Volume		1 L

Once the transfer was completed, the gel was removed and rinsed in distilled water, followed by overnight submerging in CBB to be stained. Next day, gel was sufficiently destained and the photography was done using the Gene Sys software.

4.3.1 Immunodetection

Subsequently membrane was shaken in 50 ml PBS for 5 minutes, then immediately was immerged in 25 ml of blocking solution containing 5% dried milk in PBS-T for 1 hour. Blocking solution acts to block the membrane with abundant casein present in milk, as well as helps to prevent any nonspecific binding with the antibodies. After one hour of blocking, membrane was washed 2x in 50 ml of PBS-T and 1x in 50 ml of PBS. Subsequently, the membrane was probed withe a fresh blocking solution containing primary antibody, anti-rabbit, in a 1:2000 dilution and incubated for 1 hour at room temperature. The blocking solution was then discarded, and the membrane rinsed again with 2x PBS-T and 1x PBS washes, each with a volume of 50 ml. These washes were performed to remove any excess unbound antibodies, thereby preventing any nonspecific binding. Also, a one-hour membrane incubation with the secondary antibody was performed in the same manner as described for the first antibody. Thus, we added alkaline phosphatase conjugated anti-rabbit antibody, in a 1:1000 dilution, which recognize rabbit IgG antibody. After the probing with secondary antibody, the PBS-T/PBS washes were performed in the same manner as after the primary antibody. In order to visualize the membrane, 20 ml alkaline phosphate (AP) staining solution was poured over the membrane and developed until the HRP bands were visible. After staining any excess solution was removed and finally picture of the developed membrane was taken using the Gene Sys software.

Table 10. Phosphate-Buffered Saline, 0.1 % Tween

PBS-T Wash Buffer	**1L**
10x PBS	100 ml
Tween 20	500 µl
ddH_2O	800 ml
Final Volume	1 L

Table 11. Dilutions of used antibodies

Antibody	Dilutions
Anti-HRP	1:2000
Anti-Rabbit Alkaline Phosphate (AP)	1:1000

Table 12. AP staining solution

HRP Developing buffer	Amount
AP developing buffer	20 mL
NBT + 70% DMSO	66 µL
BCIP + 100 % DMSO	33 µL

5 Results

In the following, a layer of horseradish peroxidase enzyme was immobilized onto a various number of underlaying and overlying poly-allylamine hydrochloride & poly-styrene sulfonate polyelectrolyte layers, and this process was performed during two experiments with different constructs. Furthermore, the HRP activity was examined via spectrophotometer, hence its absorbance endpoint was measured. As a final step, the presence of the enzyme was determined through western blot analysis.

Prior to the verification examination, it was necessary to wash off poly-allylamine hydrochloride and poly-styrene sulfonate from positive constructs, at which HRP enzyme was shielded as the final layer. Thus, methanol-chloroform protein precipitation protocol was used to precipitate protein (HRP) in order to enhance the resolution of HRP on acrylamide gel, due to the effect that PSS/PAH polyelectrolyte layers have on the proper binding of HRP to SDS, thereby interfering its visualization.

However, due to the removal of PSS/PAH polyelectrolyte layers, it was essential to ensure that the obtained results from activity measurement as well as signals from SDS-PAGE are correspond to the amount of the horseradish peroxidase enzyme. Therefore, the relative quantity of protein (HRP) bands from polyacrylamide gel were measured via ImageJ software.

7.1 First experiment of Immobilized HRP

As explained in the methods, HRP enzyme was immobilized onto a sandwich of PSS/PAH polyelectrolyte layers through layer-by-layer deposition approach. This way, the enzyme was deposited as a top or final layer on a minimum of 4 to maximum of 8 underlying polyelectrolyte layers. Also, up to 4 polyelectrolyte layers were deposited on a HRP layer, to cover it. Besides that, two constructs V1 and V2 were served as negative control without enzyme addition. As shown in figure 3 (data is shown in the appendix, table 13), the positive construct V6 with eight underlying PSS/PAH displayed the highest enzyme activity absorbance, and then V8 with eight underlying and four covering PSS/PAH displayed high activity among all the positive constructs.

The negative controls V1 and V5 with only deposited polyelectrolytes are successfully shown no activity. This experiment was conducted in triplicate and results are shown as the average.

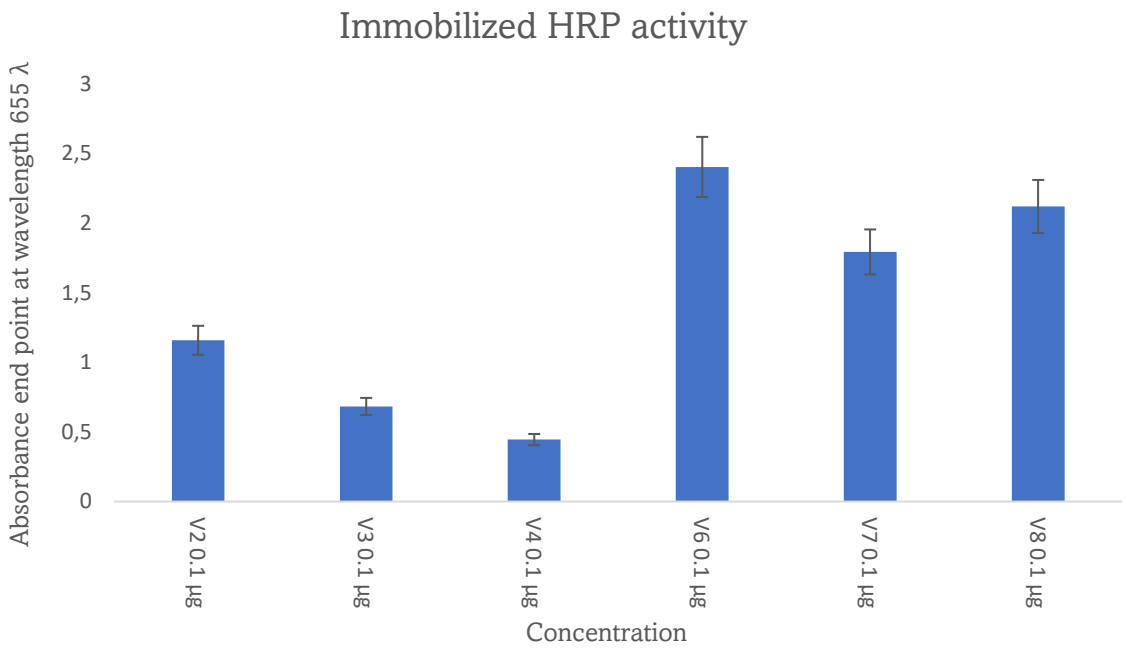

Figure 3. ***Measured absorbance endpoint of enzyme activity at wavelength of 655 via spectrophotometer.*** *A certain amount of enzyme was added in all positive constructs. V2 with 4 underlying layers, V3 with 2 covering layers, V4 with 4 covering layers, V6 with 8 underlying layers, V7 with 2 covering layers, V8 with 4 covering layers. V1 & V5 without HRP addition.*

It can be concluded that by increasing the number of basal polyelectrolyte layers, more horseradish peroxidase enzyme activity is obtained. However, the covering poly-styrene sulfonate and poly-allylamine hydrochloride polyelectrolyte layers do not significantly change the amount of enzyme activity, thus it was necessary to examine the change in the number of underlaying PSS/PAH polyelectrolyte layers.

4.2 Second experiment of Immobilized HRP

In the second experiment, the number of basal polyelectrolyte layers was increased. As it is shown in table 6, a maximum of 20 basal layers of polyanion, PSS, and polycation, PAH, were

introduced. Horseradish peroxidase enzyme was deposited on the polyelectrolyte layers as a final layer. In this experiment, there were four positive wells with the presence of HRP enzyme as the final layer, eight underlying polyelectrolyte layers (V2), twelve underlying polyelectrolytes (V4), sixteen underlying polyelectrolytes layers (V6) and twenty underlying polyelectrolyte layers (V8), respectively.

In this way, four samples were performed V1, V3, V5 and V7, one after the other without the enzyme covering layer, as the negative control. As it can be seen in figure 4 (data is shown in the appendix, table 14), sample V8 with twenty underlying polyelectrolyte layers displayed the highest amount of enzyme activity, and sample V2 with eight underlying polyelectrolyte layers displayed lowest amount of HRP activity.

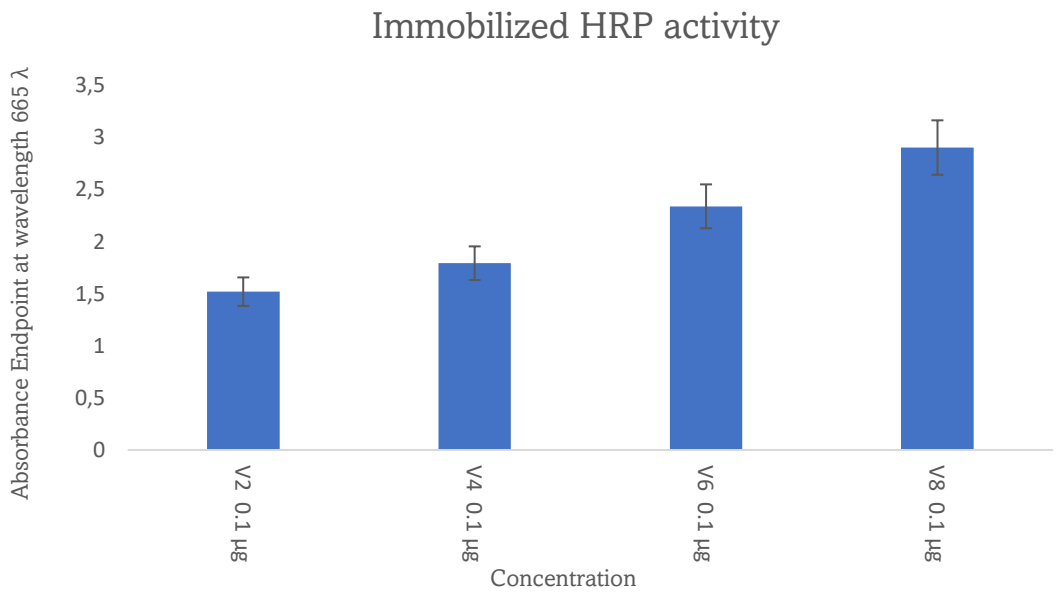

Figure 3. ***Measured absorbance endpoint of enzyme activity at wavelength of 655 via spectrophotometer.*** *A certain amount of enzyme was added in all positive constructs. V2 with 8 underlying PAH/PSS layers and HRP as the final layer, V4 with 12 underlaying PAH/PSS and HRP as the final layer, V6 with 16 underlying PAH/PSS and HRP as the final layer, V8 with 8 underlying PAH/PSS and HRP as the final layer.*

According to the above results, it can be said that the amount of enzyme activity has increased with further underlying polyelectrolytes.

4.3 Verification of enzyme presence by SDS-PAGE & Western blot analysis

4.3.1 SDS-PAGE

To verify the presence of the Horseradish peroxidase enzyme in the deposited polyelectrolyte layers via the LbL assembly, samples from the second experiment were introduced. Obtained results from spectrophotometer were subsequently verified by running 12% polyacrylamide gel electrophoresis loaded with product of 8 constructions of multilayer polyelectrolyte PSS/PAH containing enzyme. The results of immobilized enzyme via LbL assembly can be observed in constructs V2, V4, V6 and V8 (Fig. 4).

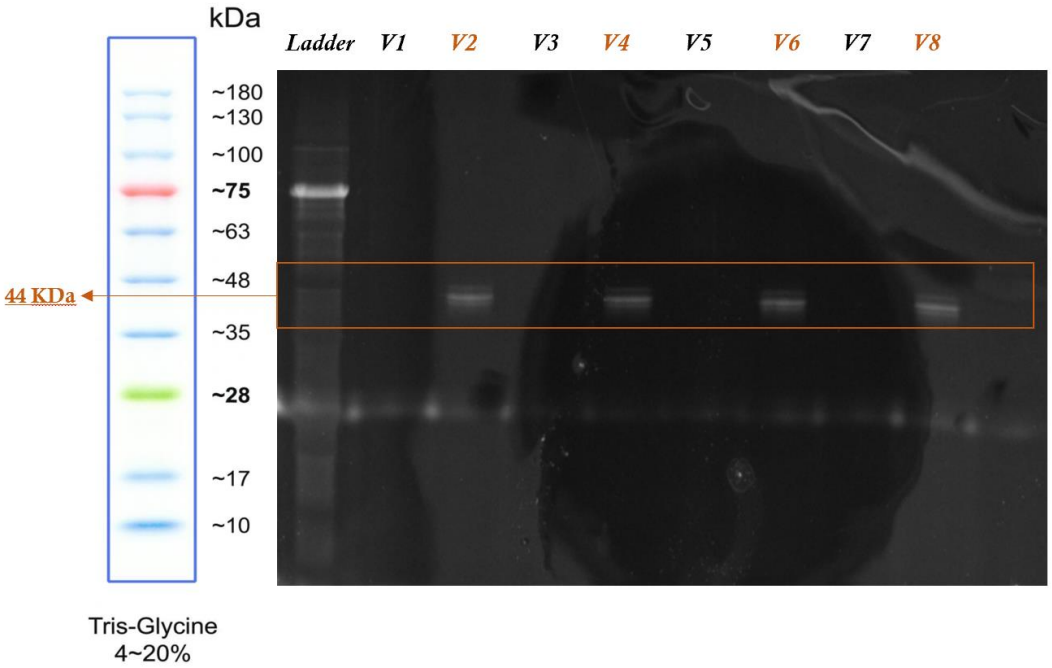

Figure 4. Stain-free detection of HRP by SDS-PAGE analysis. *12% polyacrylamide gel after loading LbL deposition of polyelectrolytes PSS/PAH layers product of HRP enzyme. V2 with 8 underlying PAH/PSS layers and HRP as the final layer, V4 with 12 underlaying PAH/PSS and HRP as the final layer, V6 with 16 underlying PAH/PSS and HRP as the final layer, V8 with 8 underlying PAH/PSS and HRP as the final layer. V1, V3, V5, V7 are shown as the negative controls. The outermost well showing the protein marker/ladder VI (10-245) prestained (PanReac Applichem).*

The positive constructs, V2, V4, V6, and V8, at which horseradish peroxidase was covered as the final layer, show pronounced bands at approximately 44 kDa, in the expected regions. The negative controls, V1, V3, V5, and V7, without enzyme covering, successfully show no bands in the gel.

4.3.2 Immunoblotting

Additionally, detection of horseradish peroxidase enzyme via immunoblotting showed a positive result. The HRP signals are visible at 44kDa, upon treatment of the blot with substrate, for all positive constructs, V2, V4, V6 and V8, respectively. This analysis showed that primary and secondary antibodies recognized the protein of interest.

Therefore, HRP identity was confirmed by its prominent signal as well as its molecular weight. The result can be observed in Fig. 5.

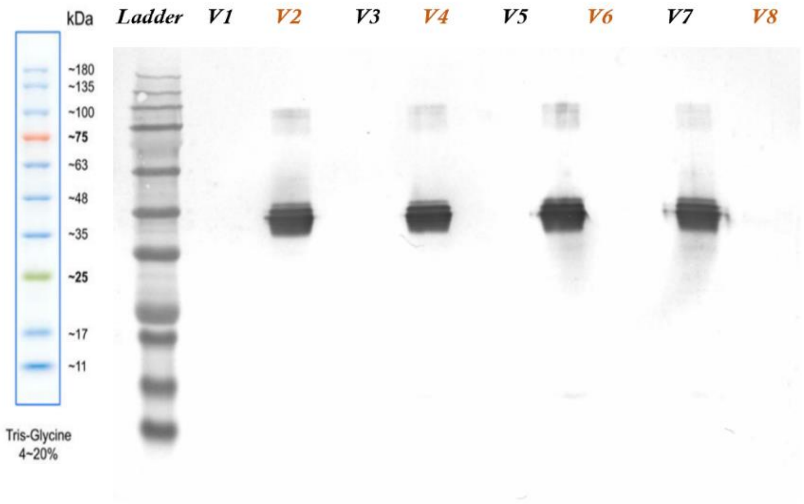

Figure 5. NC membrane after immunoblotting. *HRP detection using antibodies at positive constructs V2, V4, V6 and V8. The HRP signals are shown at approximately 44kDa. The first lane is the protein marker/ladder VI (10-245) prestained (PanReac Applichem). The constructs V1, V3, V5, V7 are shown as the negative controls, without HRP.*

4.4 Quantification of band intensity on SDS-PAGE

As a final analysis of immobilized enzyme activity, quantifying of HRP bands on SDS-PAGE from the second experiment using ImageJ software was performed [25]. In this way, the area of each peak in each of the four lanes were measured. Then, the relative density was calculated by dividing the percent by the percent value for the standard (V2). The corresponding results are shown in Table 15 (Appendix) and Figure 6. As can be seen from the below results, sample V8 obtained the highest relative density with twenty underlaying layers of poly-styrene sulfonate and poly-allylamine hydrochloride that were shielded by the horseradish peroxidase enzyme. The sample V2 with the least number of underlying PSS/PAH polyelectrolyte layers and deposited HRP as the final layer obtained the lowest amount of protein (HRP). Also, sample V4 with twelve underlying PSS/PAH and V6 with sixteen underlying PSS/PAH layers obtained a lower amount of Horseradish peroxidase enzyme compared to the sample V8, respectively.

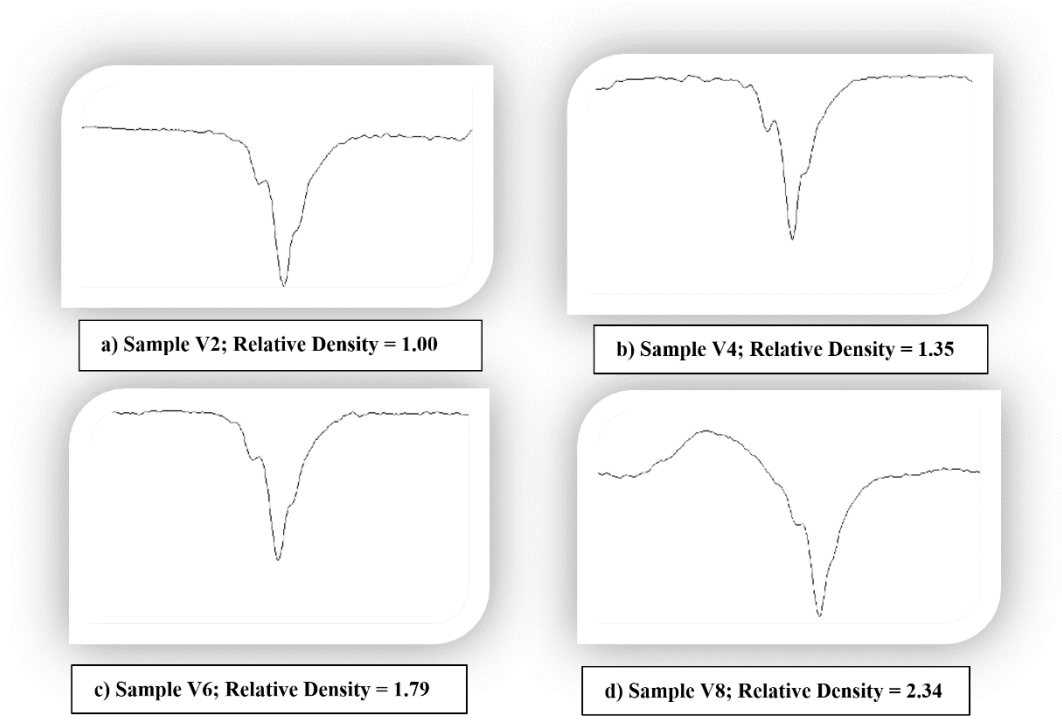

Figure 6. Peaks of each lane. *Sample V2 with 8 underlaying PSS/PAH layers covered with HRP obtained the lowest amount of protein.V8 with 20 underlaying PSS/PAH layers covered with HRP obtained the highest amount of protein.*

5 Discussion

The layer-by-layer self-assembly is a versatile technique that relies on electrostatic attraction between assembled components, or other interactions including H-bonding or polar interactions, to generate polyelectrolyte multilayers (PEMs) [26]. This build-up of polyelectrolyte layers is driven by electrostatic interactions between oppositely charged of poly-styrene sulfonate (PSS, negative charge) and poly-allylamine hydrochloride (PAH, positive charge). Thereby, LbL self-assembly is performed by coating a positive charged substrate, poly-ethyleneimine (PEI) on a glass surface followed by alternate deposition of PSS and PAH, possessing oppositely charged to form the first monolayer. After the formation of each monolayer, a washing step was applied to remove the unbound molecules as well as to prevent cross-contamination of oppositely charged polyelectrolytes. Following this, the next oppositely charged polyelectrolyte monolayer is deposited to form the second polyelectrolyte-monolayer. This procedure was repeated alternately until the desired polyelectrolyte multilayers are formed [26, 27].

In general, it can be concluded that the number of layers deposited determines whether the growth in multilayer thickness is linear or non-linear, with various factors influencing the deposition process, including ionic strength, pH, temperature, macromolecular structure, concentration, and charge density of polyelectrolytes [35]. The selection of the deposition technique depends on the materials to be constructed, the time required for the deposition, and the geometry of the substrate [36].

Enzyme involvement is now a standard procedure in a wide range of industrial applications. Researchers in a variety of fields have been pursuing enzyme immobilization for improved stability and cost effectiveness [30,31]. Immobilized enzymes can be used in a variety of solvents, and their enzymatic activity can be tweaked to provide new reactive characteristics. Immobilized enzymes are more tolerant of adverse reaction conditions such as excessive pH and high temperatures than native enzymes in aqueous media. Also, it may usually be recycled and reused while preserving their enzymatic activity, resulting in lower production costs [32]. The nature and quantity of interactions between the enzyme and the support, as well as the parameters of the carrier material, define the properties of immobilized enzymes. The stability

and kinetic properties of enzymes are frequently altered as a result of enzyme immobilization, owing to the microenvironment and alterations imposed by the supporting matrix [7].

Moreover, changes in the intrinsic activity of the immobilized enzyme or the fact that the interaction between the immobilized enzyme and the substrate occurs in a micro-environment distinct from the bulk solution can both cause changes in the characteristics. As a result, one of the most significant drawbacks of using immobilized enzymes is the loss of catalytic activity, particularly when the enzymes are operating on macromolecular substrates. Because the substrate has limited access to the enzyme's active site, the activity may be confined to the substrate's accessible surface groups only. As a result of the steric constraint, the typical pattern of products produced from the macromolecular substrate may alter [7,37]. As a general strategy for enzyme stabilization, the most common and traditional immobilization procedures can produce very quick enzyme immobilizations at neutral pH by using their amino groups. Under this condition, the amino terminal residue (pK ~ 7.5) is tens of times more reactive than lysine residues on the enzyme surface (pK ~ 10.5) [38].

Considering the variety of enzyme immobilization protocol, this should be included that a broad number of enzymes have been immobilized in polyelectrolyte multilayers, including catalase, cholesterol oxidase, glucose oxidase, lipase, lysozyme, pepsin and peroxidase [36].

Horseradish peroxidase enzyme was selected as the enzymatic model for this study because it has been subjected to numerous scientific studies until now and is ongoing [30]. The role of horseradish peroxidase in the plant and its catalytic mechanism, which has been immobilized onto various supports, has also been extensively studied [33, 34].

The main aim of this thesis was to determine the effect of different numbers of the PSS/PAH polyelectrolyte layers on amount of immobilized Horseradish peroxidase enzyme. For this purpose, by utilizing layer-by-layer deposition of poly-styrene sulfonate and poly-allylamine hydrochloride polyelectrolyte layers on the glass surface followed by immobilization of enzyme on these oppositely charged multilayers, stabilization of HRP was examined. In the first experiment, the Horseradish peroxidase enzyme was immobilized as the final layer as well

as the covering layer on different numbers of polyelectrolyte layers. Therefore, six different constructs of PSS/PAH polyelectrolyte layers were built-up on top of each other.

The outcomes of this experiment showed that deposition of PSS/PAH polyelectrolyte layers on HRP did not have a significant effect on enzyme activity. Hence, it was important to change the number of substrates (PSS/PAH underlying layers) in the second experiment, in order to determine the effect of different numbers of underlying poly-styrene sulfonate and poly-allylamine hydrochloride layers.

Thus, the second experiment was performed with a maximum of twenty and a minimum of eight underlying PSS/PAH polyelectrolyte layers. The results successfully showed that construct V8 with twenty PSS/PAH underlying layers obtained more enzymatic activity than construct V2 with eight PSS/PAH underlying layers by spectrophotometer.

According to previous studies, the molar mass of polyelectrolytes is also a parameter of interest. A recent study in 2013 by Jange *et al* has shown, the molar mass of polyelectrolytes determines the stability of the resulting multilayer, obviating the need for additional stabilization processes. A low molecular mass will increase the likelihood of polyelectrolyte desorption when it comes into touch with an oppositely charged polyelectrolyte, whereas a greater molecular mass will improve multilayer deposition and stability [39]. In systems where the LbL assembly is the result of electrostatic interactions, the concentration of the polyelectrolyte solutions must be high enough to permit the deposition of stable layers and to allow charge inversion. The lowest concentration needed to generate multilayers is determined by the properties of polyelectrolytes [40]. Also, it has been observed by Guzmán *et al*, because of the chain rearrangements of the polyelectrolytes to secure binding sites on the surface, using higher concentrations of polyelectrolytes generally results in the formation of thicker multilayers [41].

Furthermore, to confirm that the results are due to the Horseradish peroxidase enzyme in the polyelectrolyte constructs, it was necessary to perform the gel electrophoresis and further western blots as well as quantification of its signal intensity.

It has been proposed by Zhang *et al*. (2021), the immobilized enzymes can preserve their intrinsic structure and show a high activity when assembled in satisfactory conditions with the

proper polycation. Therefore, the enzymatic activity can be tuned depending on deposition conditions, e.g., nature of polyelectrolytes, pH and ionic strength of PEs solutions [28].

To prove that the future results correspond to our work, it might be of interest to use other polyelectrolytes, support surface, different pH and other charged enzyme.

In along with above lines, it can be point out that in this work we employed polyelectrolytes in order to act as a glue to adhere Horseradish peroxidase enzyme onto the supporting surface, glass, via layer-by-layer assembly technique. And the reason for choosing the polyelectrolytes is that HRP enzyme can be paired with polyelectrolytes through physical interactions without disturbing the secondary and tertiary structures [29].

6 Conclusion

These preliminary results would determine that the activity of the enzyme increased by further underlying polystyrene sulfonate/polyallylamine hydrochloride polyelectrolyte layers. This would suggest that the enzyme without covering polyelectrolyte layers is more in contact with the solution. Contrary to this, when the enzyme is covered with PSS/PAH polyelectrolyte layers, its decreases because the polyelectrolytes cover part of the enzyme that interferes its contact with the solution, thus preventing the substrate from penetrating the enzyme.

7 Appendix

7.1 Data of activity measurement - 1st & 2nd experiment

First experiment:

Table 13. Measured absorbance of enzyme activity at wavelength of 655 via spectrophotometer*. The amount of activity shown is the average of three sets of samples. V2 with 4 underlying layers, V3 with 4 underlaying and 2 covering layers, V4 with 4 underlying and 4 covering layers, V6 with 8 underlying layers, V7 with 8 underlying and 2 covering layers, V8 with 8 underlying and 4 covering layers. V1 & V5 were served as negative controls.*

V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8
0.051	1.158	0.683	0.445	0.058	2.403	1.793	2.119

Second experiment:

Table 14. Measured absorbance of enzyme activity at wavelength of 655 via spectrophotometer*. The amount of activity shown is the average of three sets of samples. V2 with 8 underlying layers, V4 with 12 underlaying PAH/PSS and HRP as the final layer, V6 with 16 underlying PAH/PSS and HRP as the final layer, V8 with 8 underlying PAH/PSS and HRP as the final layer. V1, V3, V5 & V7 were served as negative controls, no enzyme on top.*

V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8
0	1.520	0	1.793	0	2.339	0	2.903

7.3 Relative density measurement from the 2nd experiment

Table 14. Measurement of Area and Percent of each HRP-bands. *The relative density of each sample is calculated by dividing the percent by the percent value for the standard. Samples = V2 with 8 underlying PSS/PAH, V4 with 12 underlying PSS/PAH, V6 with 16 underlying PSS/PAH and V8 with 20 underlying PSS/PAH polyelectrolyte layers. Horseradish peroxidase was deposited as the final layer.*

Sample	Area	Percent	Relative Density
V2	18919.936	36.645	1.00
V4	14058.430	27.229	1.35
V6	10552.045	20.438	1.79
V8	8099.782	15.688	2.34

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