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Functionalized Membranes by Layer-By-Layer Assembly of Polyelectrolytes and In Situ Polymerization of Acrylic Acid for Applications in Enzymatic Catalysis

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Abstract

This research work was directed toward the development of highly active, stable, and reusable functionalized polymeric membrane domains for enzymatic catalysis. Functionalized membranes were created by two different approaches. In the first approach, which involved alternative attachment of cationic and anionic polyelectrolytes, functionalization was performed using a layer-by-layer (LBL) assembly technique within a nylon-based microfiltration (MF) membrane. In the second approach, a hydrophobic polyvinylidene fluoride (PVDF) MF membrane was functionalized by the in situ polymerization of acrylic acid. The enzyme, glucose oxidase (GOX), was then electrostatically immobilized inside the functionalized membrane domains to study the catalytic oxidation of glucose to gluconic acid and H_2O_2 . Characterization of the functionalized membranes, in terms of polyelectrolyte/polymer domains and permeate flux, was also conducted. The kinetics of H_2O_2 formation was discussed, along with the effects of residence time and pH on the activity of GOX. The stability and reusability of the electrostatically immobilized enzymatic system were also investigated.

1. Introduction

Traditional membranes are considered to be a separation media based solely on the size (ultrafiltration, microfiltration) or solubility (reverse osmosis, pervaporation) of the species. However, with the advancement of functionalization chemistry and the availability of novel materials, further versatility can be obtained in the form of functionalized membranes. Functionalized membranes can be tailor-made to impart the desired functionality by a variety of active groups/molecules/moieties, depending on the applications.^{1–6} This research work involves extensive studies on the development of functionalized polymeric membranes that contain electrostatically immobilized enzymes and their application in enzymatic catalysis.

Enzymatic catalysis by free enzyme possesses the advantage of higher activity; however, its use is limited, because of lower stability, inhibition from product, and a lack of reusability.⁷ On the other hand, the traditional covalently immobilized enzyme is more stable, because of

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the protective action of the solid matrix, and can easily be reused. However, its activity is significantly low,⁸ because of the conformation change of the enzyme by multipoint binding and the accessibility issues arising due to the proximity to the solid matrix. Site-directed immobilization of enzymes has been performed to restrict the conformation change and increase the accessibility.^{9,10} However, site-directed immobilization requires complicated techniques and is considerably expensive.

To improve the situation, new techniques of enzyme immobilization have been explored. Table 1 summarizes some of the recent approaches reported in the literature, along with the results of kinetic study. One approach has been to keep the mode of attachment the same (i.e., covalent) but modify the solid matrix, such as activated beads/particles,^{11–13} nanoparticles,¹⁴ membranes,^{15,16} and films.¹⁷ The other approach is the nonco-valent attachment of enzyme in the solid matrix. These include electrochemical polymerization,¹⁸ Layer-By-Layer (LBL) deposition along with gold nanoparticles,¹⁹ electrostatic immobilization on anionic polystyrene beads,²⁰ electrostatic immobilization on LBL assembled nylon membrane,²¹ and LBL assembly, along with multiwalled carbon nanotubes on gold electrodes.²²

An efficient alternative to traditional covalent immobilization of enzymes is to create a charged domain of polyelectrolytes inside a membrane pore, and then immobilize the enzyme using electrostatic interaction. The advantages of electrostatic immobilization are (i) a higher activity, close to that of free enzyme, and (ii) the stability and reusability of an immobilized enzyme. There also exists the potential of reusing the membrane matrix by detaching the enzyme, when needed, and attaching a fresh enzyme.

1.1. Layer-By-Layer (LBL) Assembly.

The alternative attachment of polycations and polyanions is a simple but robust technique to create a layered assembly in a solid support with high capacity and versatile functionality. The method was first introduced by Decher,^{23–25} based on a study by Iler²⁶ that indicated the possible existence of such a technique. It is popularly known as the layer-by-layer (LBL) technique. A detailed literature review that covered the mechanisms of layer formation, applications in various fields, and scope of future studies was recently published.²⁷ The LBL technique is used to create functional domains that contain various types of species, such as polyelectrolytes,^{21,28–31} proteins,^{32–34} dyes,^{35,36} nanoparticles,^{37–41} thiols,⁴² metal ions,^{43,44} viruses,⁴⁵ DNA,⁴⁶ and lipids.⁴⁷ It is also used in different fields of applications, such as separation,^{48–50} biosensors,^{19,22,51} and biocatalysis.^{21,52–54}

Our research group has developed polyelectrolyte domains within the membrane pores using the LBL technique.⁴ Multiple layers of charged polyelectrolytes were created inside the pores of gold-coated polycarbonate membranes for ion exclusion. The formation of an LBL assembly within the membrane pores was further extended for enzymatic catalysis.²¹ Enzymes, glucose oxidase (GOX), and alkaline phosphatase (AP) were electro-statically immobilized within polyelectrolyte domains created inside a nylon membrane by the LBL assembly. The kinetics of enzymatic catalysis was also studied. Convective flow was used for all of these studies, to enhance the mass-transfer coefficient.

1.2. Functionalized PVDF Membranes.

PVDF membranes are inherently hydrophobic in nature; however, studies have been performed to incorporate hydrophilicity into it. Polyacrylic acid (PAA), because of its hydrophilic nature and ion exchange property, has been extensively used for the formation of nanoparticles,⁵⁵ the adsorption of nonionic surfactants,⁵⁶ and the capture of metal ions⁵⁷ in the PVDF membranes. Gabriel et al.⁵⁸ reported a method to functionalize hydrophobic PVDF membranes via the in situ polymerization of acrylic acid. This technique has already been used for the formation of nanoparticles⁶ and the immobilization of Fe(II) cations⁵⁹ inside the PVDF membrane pore. Another study has also been conducted on the covalent immobilization of GOX on acrylic acid-graft-copolymerized-PVDF membrane.¹⁶ However, to the best of our knowledge, PAA-functionalized PVDF membranes have never been used for the electrostatic immobilization of GOX.

1.3. Glucose Oxidase (GOX).

In this study, GOX was used as a model enzyme and glucose was used as the substrate. GOX is a well-known enzyme from the 19th century and has been used since Müller reported its activity in *Aspergillus niger*.⁶⁰ GOX specifically catalyzes⁶¹ the oxidation of β -D-glucose to δ -gluconolactone and hydrogen peroxide in a two-step mechanism (see Figure 1). In the first step, the co-factor of the enzyme, flavin adenine dinucleotide (FAD), oxidizes glucose to δ -gluconolactone and, itself, converts to the reduced form (FADH₂). In the second step, FADH₂ is oxidized back to FAD by O₂ and O₂ itself reduces to H₂O₂. δ -Gluconolactone is an unstable compound, and it immediately forms gluconic acid. The mechanism and kinetics of glucose oxidation by GOX has been extensively investigated by several researchers^{62–64} and has been summarized in the literature.⁶⁵ The kinetics of free and immobilized GOX can be generally studied and compared using the well-known Michaelis-Menten equation, which is discussed later. The structure of GOX,^{65–67} and the factors that affect the kinetics of glucose oxidation by GOX, such as pH,^{17,68,69} temperature,¹⁷ and inhibition by the product H₂O₂,⁷⁰ has also been studied thoroughly.

1.4. Research Objectives.

The overall objective of the present study was to develop highly active, stable, and reusable GOX immobilized (electrostatically) functionalized polymeric membrane domains for the enzymatic oxidation of glucose to produce gluconic acid and H₂O₂. Two different types of domains were developed. A functionalized nylon-based membrane matrix was created by the alternative electrostatic attachment of anionic and cationic polyelectrolytes using LBL assembly technique. In another approach, a hydrophobic PVDF membrane was functionalized via the in situ polymerization of acrylic acid. Both functionalized membranes were used for the electrostatic immobilization of GOX (P₁ = 4.2). Fundamental experiments were performed to understand the kinetics of enzymatic oxidation of glucose in the aforementioned membrane domains, under convective flow conditions. The effects of pH and residence time on the activity of the enzyme were studied. The stability of the enzyme and reusability of the enzymatic system were also investigated. To the best of our knowledge, this is the first time that an attempt was made to detach and reattach the enzyme to explore the reusability of the membrane matrix.

2. Experimental Section

2.1. Materials.

The nylon-based anhydride activated micro-filtration membranes (Immunodyne ABC, pore diameter of 0.45 μm , thickness of 165 μm , 80% porosity, as supplied by the manufacturer) that were used for all the LBL experiments were purchased from the Pall Corporation. Hydrophobic PVDF membranes (pore diameter of 0.45 μm , thickness of 125 μm , 75% porosity, as supplied by the manufacturer) were purchased from the Millipore Corporation. The enzyme glucose oxidase from *Aspergillus niger* (GOX, Product No. G0543, MW 160000), β -D(+)-glucose (Product No. G5250, MW 180), and poly-L-lysine-hydrochloride (PLL, Product No. P9404, MW 105500, with the MW of the repeat units being 164.5) were purchased from Sigma. Poly(sodium 4-styrenesulfonate) (PSS, Product No. 243051, MW 70000, with the MW of the repeat units being 206), poly(allylamine-hydrochloride) (PAH, Product No. 283223, MW 56000, with the MW of the repeat units being 93.5), 15% solution of titanium oxysulfate in dilute H_2SO_4 (Product No. 495379), benzoyl peroxide (Product No. 179981), trimethylolpropane triacrylate (TMPTA, Product No. 246808), anhydrous toluene (Product No. 244511), and acrylic acid (Product No. 147230, MW 72) were purchased from Aldrich Chemical Co. The reagents for Bradford assay were purchased from Bio-Rad Laboratories. Unless noted otherwise, the remaining chemicals, including the deionized ultrafiltered water (DIUF), were purchased from Fisher Scientific.

2.2. Equipment.

Experiments were conducted in a stainless steel membrane cell from Osmonics. The effective external surface area of the membranes in this cell was 13.2 cm^2 .

2.3. Analytical Procedures.

All spectrophotometric measurements were performed using ultraviolet-visible light (UV-vis) spectrophotometry (Varian, Cary 300). All experiments were performed in triplicate, and the variations in experimental results are represented as the standard deviation.

2.3.1. Analysis of PAH and PLL.—The PLL and PAH were quantified using a total organic carbon (TOC) analyzer (Shimadzu, model TOC 5000 A), using a calibration curve in the range of 5–100 ppm. The analytical error for this analysis was 5%–10%.

2.3.2. Analysis of PSS.—The PSS in the layer was quantified by measuring the absorbance in the feed, permeate, and wash directly at 261 nm, using UV-vis spectrometry. The linear range of detection for PSS was 1–400 ppm, and the analytical error was <1%.

2.3.3. Analysis of GOX.—The amount of GOX in a solution was determined via the well-known Bradford Protein Assay,⁷¹ using a calibration curve prepared from GOX at 595 nm. The linear range of the Bradford Protein Assay was 1–24 $\mu\text{g/mL}$, and the analytical error was <1% for 2–24 $\mu\text{g/mL}$ and 1%–5% for 1–2 $\mu\text{g/mL}$ of GOX in this research work.

2.3.4. Analysis of H_2O_2 .—The H_2O_2 in solution was analyzed using a colorimetric method that was reported by Clapp et al.,⁷² using acidified titanium oxysulfate, and the

absorbance at 407 nm was measured. The linear range of calibration for H_2O_2 analysis was 0.03–3 mM (1–100 ppm), and the analytical error was <5% for 1–3 mM and 5%–10% for 0.03–1 mM.

2.4. Experimental Methods.

2.4.1. Formation of Functionalized Membranes.

2.4.1.1. Development of LBL Assembly in Anhydride Activated Nylon

Membrane.: Layers of polyelectrolytes, mainly within the membrane pores, was assembled via the alternative electrostatic attachment of cationic and anionic polyelectrolytes (see Figure 2a), under a convective flow and constant stirring conditions. A convective flow was applied to obtain the functionalization mainly within the membrane pores. Membrane pores were preferred, because of the higher pore surface area over external membrane surface area. The immobilization was nonstoichiometric, which means the charges were oversaturated with the oppositely charged polyelectrolyte in the next layer. To ensure nonstoichiometric attachment of subsequent layers, all layer formation steps were performed in the presence of a 0.25 M NaCl solution. The shielding effect of salt increases the availability of free charges of polyelectrolytes for the next layer.

To impart stability in the subsequent layers, the first layer of PLL was attached covalently in the membrane matrix via the reaction between the terminal amine group of PLL and the anhydride group of the membrane. This was done by permeating 100 mL of a 40 ppm PLL solution (4 mg or 0.039 μmol of PLL) in DIUF at 0.07 bar (1 psi) pressure and pH 9.3. The solution was recirculated once. The recirculation step also was followed for all other layer formation steps. As the first layer, PLL was preferred over PAH, because the difference of pK_a between the terminal amine group ($pK_a \sim 9$) and amine groups in the side chains ($pK_a \sim 10.5$) of PLL is known. Hence, at a working pH of 9.3, the terminal amine group is uncharged and, therefore, forms covalent attachment preferentially over the side-chain amine groups, which are positively charged at that pH. This has enhanced the probability of single-point attachment of the first layer, rather than multipoint attachment through the side-chain amine groups. The side-chain amine groups provide free positive charges available for the next layer formation step.

The second layer of PSS was attached by electrostatic interaction between the side-chain amine groups of PLL and sulfonic acid groups (pK_a 1.0) of PSS.⁷³ One hundred milliliters of the 400 ppm solution (40 mg of PSS, 0.2 mmol of negative charges based on repeat units) of PSS was permeated in DIUF at pH 6. At a working pH of 6, the net charge on amine groups of PLL is positive, whereas the net charge on sulfonic acid groups of PSS is negative. Therefore, they easily interact with each other.

The third layer of PAH was formed by the electrostatic interaction between the negatively charged sulfonic acid groups of PSS and the positively charged amine groups of PAH. PAH was attached by permeating 100 mL of a 300 ppm solution (30 mg of PAH, 0.3 mmol of negative charges, based on repeat units) in DIUF at pH 6.

Then, two more bilayers of PSS and PAH were attached in the membrane matrix to obtain PLL-(PSS-PAH)₃. The goal was to synthesize a net positively charged membrane in the

working pH (i.e., pH 6), so that GOX ($P_i = 4.2$) can be attached using its negative charges. More layers have been introduced to increase the enzyme loading and also to decrease the core leakage (see Figure 2a) of the substrate molecules.

The stability of each layer was checked by permeating solutions of varying pH (over a range of 2.7–9). The data of pure water flux, as a function of pressure, were obtained after the formation of each layer.

2.4.1.2. Functionalization of PVDF Membrane.: A method reported in the literature⁵⁸ was adopted to functionalize a hydrophobic PVDF membrane with PAA via the in situ polymerization of acrylic acid monomer. The polymerization solution consisted of 70% toluene, 30% acrylic acid, 0.5% benzoyl peroxide, and 1.2% TMPTA (by weight). Benzoyl peroxide was used as the initiator. TMPTA was used as the cross-linker to increase the stability and density of the polymer network inside the membrane matrix. The membrane was dipped into the polymerization solution for 30 s, and then it was sandwiched between two Teflon plates and clipped tightly. It was then inserted into an oven at 90 °C for 4 h under a constant N₂ environment. The membrane then was washed with a copious amount of DIUF water, under convective flow conditions, to remove the impurities. The membrane obtained at this point was functionalized with the hydrogen form (H-form) of PAA. It was converted to Na-form by permeating 200 mL of a 0.01 M NaOH solution. The Na-form resists any drastic change in pH that generally occurs in the H-form for the following ion exchange steps. Permeate flux of water at different stages of functionalization and effect of pH on water flux through PAA-functionalized PVDF (PVDF–PAA) membrane were studied.

To immobilize GOX in the PVDF–PAA membrane electro-statically, a net positively charged membrane was created by attaching a layer of PAH, using the same principle of electro-static interaction (see Figure 2b) that used earlier for the PSS–PAH layer in the LBL membrane. One hundred milliliters of a 300 ppm solution of PAH (30 mg of PAH, 0.3 mmol of negative charges, based on repeat units) in DIUF water at pH 6 was permeated through the PVDF–PAA membrane. The carboxylate anions of PAA have a pK_a of 4.7, and, at pH 6, these groups interact with positively charged amine groups of PAH to form PVDF–PAA–PAH.

2.4.2. Electrostatic Immobilization of GOX in functionalized Membranes.—

After the formation of PLL–(PSS–PAH)₃ layers, GOX was immobilized electrostatically (see Figure 2a) by permeating 100 mL of a 25 ppm solution in DIUF at pH 6. GOX was attached electrostatically in the absence of salt, so that all charges on the membrane matrix could be neutralized by it. For comparison, the covalent attachment of GOX was performed by the reaction between its amine groups and the anhydride groups in the activated nylon (Immunodyne) membrane, as reported in another study.⁷⁴ GOX was immobilized in the PVDF–PAA–PAH membrane (see Figure 2b), similar to that described for the LBL membrane. The stability of both the systems was checked frequently by analyzing the leakage of GOX in the permeate. The GOX immobilized membranes were stored at 4 °C.

2.4.3. Activity of the Electrostatically Immobilized GOX for Oxidation of

Glucose.—The activity of GOX was determined by measuring the concentration of H₂O₂

produced. All the activity measurement experiments were performed in a 50-mM sodium acetate–HCl buffer (NaOAC–HCl buffer) saturated with O₂. Oxygen is very important in the second step of the two-step reaction mechanism of the catalytic oxidation of glucose. Thus, to maintain the saturation condition of O₂ in the media throughout the reaction, the system was designed with oxygen saturated buffer and also kept under a constant pressure of air. NaOH and HCl were used to adjust the pH of the solution. β -D(+)-Glucose is referenced as glucose in the text.

2.4.3.1. Determination of Steady-State H₂O₂ Production.: The production of H₂O₂ that was due to the oxidation of glucose by GOX, in this case, was a biocatalytic reaction that was performed in a constant-flow reactor. Hence, steady-state conditions prevails. To get an idea about the time needed to reach the steady state, experiments were performed by permeating 15 and 75 mM of glucose solution (pH 5.5) at a constant flow rate through both types of GOX functionalized membranes. Permeates were collected as a function of time and analyzed for H₂O₂.

2.4.3.2. Kinetic Study.: For electrostatically immobilized GOX in an LBL membrane (LBL–GOX-convective) and a functionalized PVDF membrane (PVDF–GOX-convective), the glucose concentration was varied over a range of 0.15–75 mM (pH 5.5), while the flow rate was kept constant, so that the condition of equal residence time prevails for all the kinetic experiments. To study the kinetics of the same GOX immobilized LBL membrane in diffusive mode (LBL–GOX-soaking), experiments were performed by soaking the membrane in glucose solution and measuring the activity of GOX in reaction mixture, as a function of time. The kinetics of covalently attached GOX was also studied in convective mode and represented as covalent-GOX-convective.

2.4.3.3. Effect of Residence Time (i.e., Flow Rate).: The effect of residence time on the activity of GOX was studied by varying the permeate flow rate for a fixed concentration of glucose solution at pH 5.5. The flow rate was varied over a range of 2–25 mL/min by varying the pressure over a range of 0.34–3.4 bar (5–50 psi).

2.4.3.4. Effect of pH.: Effect of pH on the activity of GOX immobilized in an LBL membrane was studied by varying the pH over a range of 4.5–7 for a fixed concentration of glucose solution at a constant flow rate. Homogeneous phase experiments for free GOX were also performed, with the pH varying in the same range.

2.4.4. Stability of the Immobilized Enzymatic System in Functionalized Membrane.—The stability of GOX im mobilized in an LBL assembled membrane and a functionalized PVDF membrane was studied by measuring the activity after every 7 days for 28 days. The membrane was stored at 4 °C when it was not being used. A glucose solution (15 mM at pH5.5) was used for all stability studies.

2.4.5. Reusability of the Functionalized Membranes for Enzymatic System.

2.4.5.1. Reusability of the LBL Assembled Membrane for GOX

Immobilization.: Immobilized GOX, the activity of which has already been studied, was

detached from the membrane matrix by permeating 50 mL of DIUF water of pH 3 at 10–15 psi. After detachment, the pH of the solution was immediately adjusted to 5.5. The activity of any undetached GOX in the membrane matrix was determined by permeating the glucose solution at pH 5.5. The amount of detached GOX was quantified by analyzing the solution using the Bradford Assay. The activity of the detached GOX in free form was determined by homogeneous phase batch reaction at pH 5.5. The remainder of the detached GOX was then reattached in the same membrane matrix, using the principle of electrostatic interaction. The activity of the reattached GOX in the membrane was determined as done previously. The steps of detachment and reattachment were repeated. Attachment of fresh GOX in the same membrane matrix was also performed, followed by the activity study.

2.4.5.2. Reusability of Functionalized PVDF Membrane for GOX

Immobilization.: Functionalized PVDF membranes were generated by detaching the used GOX and attaching fresh GOX in the same PVDF–PAA membrane. The detachment was done by permeating DIUF water with pH 4 at 10–15 psi pressure under convective flow mode. The membrane matrix was regenerated by immobilizing fresh GOX using electrostatic interaction. The stability of the domain and the activity of fresh GOX were tested.

3. Results and Discussion

3.1. Characterization of the Functionalized Membranes.

3.1.1. LBL Assembled Nylon Membrane.—The amount of polyelectrolyte attached in each layer was determined from analytical procedures (TOC and/or UV–vis spectroscopy) and is represented in terms of repeat units in Table 2a. A single repeat unit of all the polyelectrolytes contains a unit charge. Based on that, net charges in the subsequent layers are calculated and represented in the same table. The addition of layers was always nonstoichiometric. However, it is important to note here that each layer was associated with excess net opposite charges, which has governed the electrostatic attachment of next layer. The net charge after the seventh layer of polyelectrolyte (PAH₃) was positive.

The effective cumulative thickness of the assembly (δ_n), after the addition of the n th layer, is calculated by applying Hagen-Poiseuille's equation⁷⁵ on the permeate water flow rate through the unmodified membrane (Q_{w0}) and through the membrane after the attachment of the n th layer (Q_{wn}), as shown below.

$$r_n = r_0 \left(\frac{Q_{wn}}{Q_{w0}} \right)^{0.25} \quad (1)$$

$$\delta_n = r_0 - r_n \quad (2)$$

The permeability after the attachment of the n th layer (P_n), in units of $\text{cm}^3/(\text{cm}^2 \text{ s bar})$, is calculated as follows:

$$P_n = \frac{Q_{w_n}}{A_m \Delta P} \quad (3)$$

where the Q terms represent the volumetric flow rates, r_0 is the pore radius of the unmodified membrane ($r_0 = 225 \text{ nm}$), r_n is the pore radius of the membrane after the attachment of the n th layer, A_m is the external area of membrane ($A_m = 13.2 \text{ cm}^2$), and P is the transmembrane pressure.

The calculated effective cumulative thicknesses of layers are plotted in Figure 3. As expected, cumulative thickness of layers increases as subsequent layers are assembled in the membrane pores. The final cumulative thickness of the functionalized domain after GOX immobilization was 100 nm, which corresponds to a 44% reduction in pore radius.

The inset of Figure 3 represents the decrease in water permeability of the membrane due to the decrease in pore radius with the addition of each layer. The water permeability of the nylon membrane before any functionalization was $570 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$. After the 7th layer, GOX was immobilized and the water permeability decreased to $52 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$, which is 4% of the permeability of pure nylon membrane. This shows that the addition of seven layers of polyelectrolytes and a layer of GOX affect the permeability of the membrane significantly.

3.1.2. Functionalized PVDF Membrane.—Characterization of functionalized PVDF membranes was also conducted by determining the amount of functionality incorporated into the membrane (see Table 2b). The amount of PAH and GOX immobilizations were obtained from direct analyses, whereas the amount of PAA immobilization was calculated from the PAH and GOX immobilization values and is explained later. The amount of repeat units of PAH attached in the PVDF–PAA membrane was determined to be 0.13 mmol. The cumulative thickness of the functionalized domain of PVDF–PAA–PAH–GOX membrane was calculated to be 125 nm (using the Hagen–Poiseuille equation), i.e., the effective radius of pore was 100 nm.

The effect of functionalization on the permeability of water was studied for the PVDF–PAA–PAH–GOX membrane and is represented in Figure 4 as a function of pressure at different stages of functionalization. The slope of each curve plotted in Figure 4 is the permeability of water ($\times 10^4$) through the membrane at that particular stage of functionalization. It can be observed that the water permeability decreases dramatically from the PVDF membrane before functionalization ($596 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$) to the PVDF–PAA membrane ($82 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$). This observation can be attributed to the formation of a highly dense PAA network inside the membrane pore. Permeability further decreased as the PVDF–PAA membrane was converted to the PVDF–PAA–PAH membrane (permeability) $50 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$ and PVDF–PAA–PAH–GOX membrane (permeability = $24 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$), subsequently.

The water flux behavior, as a function of pH, is demonstrated in Figure 5 for the PVDF–PAA membrane. It can be observed that, starting from pH 2.5, as the pH increases, the water flux remains constant until 4.5, and then it starts to decrease sharply. At pH 5.5, the flux reduces to 40% and at pH 7, the flux reduces to 20% of the initial value. After pH 7, again it becomes constant. This indicates that, between pH 4.5 and pH 7, some type of conformational change of PAA structure occurs. For a single layer of polyelectrolyte immobilized in a solid matrix, this type of phenomenon is called the “helix-to coil transition”.³ However, PAA, in this case, forms a random network structure inside the PVDF membrane pore, and the existence of helical or coil conformations are unlikely. It can be stated that, throughout this pH range (4.5–7), the PAA network inside the membrane undergoes “retracted-to-elongated” conformational transformation (see Figure 5). Below pH 4.5, PAA remains totally protonated and, hence, retracted. With increasing pH, it starts to deprotonate, and after pH 7, all of its acid groups become negatively charged (deprotonated) and fully elongated (repulsion of like charges to form extended configuration). This caused a substantial decrease in the membrane pore radius.

3.2. Electrostatic Immobilization of GOX.

Results obtained for electrostatic immobilization of GOX in both LBL and PVDF membranes are described below. The Bradford Assay was performed on the permeate obtained from different experiments with the GOX immobilized membranes, and they demonstrated no leakage of GOX.

3.2.1. Immobilization of GOX in LBL Assembled Nylon Membrane.—The amount of GOX immobilized electro-statically in the seven layers of polyelectrolyte assembled nylon membrane was 1.5 ± 0.2 mg ($9.37 \times 10^{-5} \pm 1.25 \times 10^{-5}$ mmol). In another set of experiments, the amount of GOX immobilized was 1.1 ± 0.1 mg for three layers of LBL assembled membrane. A comparison of the aforementioned results suggests that four additional layers did not increase the amount of immobilized GOX substantially. However, the additional layers contributed significantly in regard to reducing the core leakage of the reaction mixture through the center region of the membrane pore. The results discussed here are associated with a seven-layer membrane only. The amount of covalently immobilized GOX was 1 ± 0.1 mg (6.25×10^{-6} mmol, or 5×10^{-7} mmol/cm²). In a previous study, the amount of covalent immobilization of avidin in the same type of nylon membranes was reported to be 8×10^{-7} mmol/cm².⁷⁴ Considering the fact that GOX is bulkier than avidin, these results show that our experiments with a covalently immobilized protein in the nylon membrane are consistent.

3.2.2. Immobilization of GOX in Functionalized PVDF Membrane.—The amount of GOX attached electrostatically on the functionalized PVDF membrane was 1.8 ± 0.1 mg. This corresponds to 1.12×10^{-5} mmol GOX or 1.33×10^{-3} mmol negative charges, considering that 1 mol of GOX contains 118 mol of negative charges (calculated from the number of aspartic and glutamic acid residues present in GOX). Assuming, for the present membrane system, this is the saturation capacity of GOX attachment by electrostatic immobilization, the excess positive charges of PAH was neutralized by $0.13 - 1.33 \times 10^{-2} = 12.87 \times 10^{-2}$ mmol (9.3 mg) of PAA functionalized by in situ polymerization.

3.3. Activity Study of Electrostatically Immobilized GOX.

3.3.1. Determination of Steady-State H₂O₂ Production.—The activity of GOX was measured by determining the production of H₂O₂ (glucose + O₂ → gluconic acid + H₂O₂) as a function of time. For a fixed substrate concentration and flow rate, the concentration of H₂O₂ in the permeate reached steady state rapidly and remained almost constant with time (variation is <5%). It was also observed that the amount of H₂O₂ produced in the feed side of the membrane was <5% of that produced in the permeate after $t = 30$ min for both LBL assembled nylon and functionalized PVDF membranes. This is because the surface immobilization of GOX was insignificant, compared to pore immobilization. However, if the experiment is conducted for a long time, the production of H₂O₂ will be significant, because it accumulates on the feed side. Hence, all data were collected within the first 5–10 min of the experiments.

3.3.2. Kinetics of Glucose Oxidation.—Understanding of hydrodynamics within the reactor (membrane pores) is very important to analyze the kinetics of glucose oxidation by immobilized GOX. The hydrodynamics have an important role in determining the residence time (i.e., the ratio of the effective membrane reactor volume to the volumetric flow rate).

There are two regions inside the functionalized membrane pores; polyelectrolyte/polymer region, where GOX is immobilized, and a region void of GOX (see Figure 2). The polyelectrolyte layers were developed from the pore wall toward the pore center. The other region, which is devoid of GOX, was located near the center of the pore and known as the core region. A fraction of glucose molecules permeating through the core region never interacts with enzyme. This phenomenon is known as core leakage³ and has a significant effect on the calculations of enzyme activity.²¹ It is reasonable to consider only the volume of the membrane pore that contains the enzyme as the effective membrane reactor volume.

3.3.3. Calculation of Effective Membrane Reactor Volume.—For the LBL membrane, the thickness of layers after GOX immobilization is 100 nm, and the pore radius of the pure membrane is 225 nm. Therefore, the core region volume is given as

$$\text{core region volume} = \frac{(225 - 100)^2}{225^2} \times \text{pore volume} = 0.30 \times \text{pore volume}$$

and the effective membrane reactor volume is given as

$$0.7 \times \text{pore volume} = 0.7 \times \epsilon \times A_m \times l$$

i.e.,

$$\text{effective membrane volume} = 0.7 \times 0.8 \times 13.2 \times (165 \times 10^{-4}) = 0.122 \text{ cm}^3$$

where ϵ is the porosity of the Immunodyne membrane ($\epsilon = 0.8$), A_m the external area of membrane ($A_m = 13.2 \text{ cm}^2$), and l the length of the membrane ($l = 165 \times 10^{-4} \text{ cm}$).

Similarly, for the PVDF-PAA membrane, the effective reactor volume

$$\text{effective membrane volume} = 0.8 \times 0.75 \times 13.2 \times (125 \times 10^{-4}) = 0.1 \text{ cm}^3$$

These effective membrane reactor volumes are used to calculate the residence time. The rate of oxidation of glucose by immobilized GOX is then calculated by considering the continuously stirred tank reactor (CSTR) equation (for low conversion) as follows.

$$\text{Rate, } v = \frac{C_{\text{PH}_2\text{O}_2}}{\tau \times (\text{mass of enzyme immobilized (mg)})} \quad (4)$$

where $C_{\text{PH}_2\text{O}_2}$ is the concentration of H_2O_2 in the permeate and τ is the residence time.

Under steady-state conditions, for a fixed residence time and substrate concentration, the concentration of H_2O_2 produced in the permeate is constant, irrespective of the volume of the reaction mixture permeated.

The rate of oxidation of glucose for GOX immobilized in both the LBL (LBL-GOX-convective) and PVDF (PVDF-GOX-convective) membranes (with convective flow of reaction mixture) are obtained for different substrate concentrations using eq 4 and plotted in Figure 6. The residence time was 0.6 s (flow rate = 11 mL/min) for all convective flow experiments. As observed from the figure, at lower glucose concentrations, the rate increases very sharply with concentration, and then flattens out gradually at higher substrate concentrations. For example, the rate increases from 3 mM/(mg min) to 10 mM/(mg min) as the glucose concentration increases from 0.15 mM to 0.75 mM, whereas the rate increases only to 21 mM/(mg min) as the glucose concentration increases to 75 mM.

For comparison, the rate for the same GOX immobilized LBL membrane in soaking mode (LBL-GOX-soaking), and GOX covalently immobilized in nylon (Immunodyne) membrane (covalent-GOX-convective), are also presented in Figure 6. For a substrate concentration of 75 mM, the rates are 21, 5, and 0.1 mM/(mg min) for the LBL-GOX-convective, covalent-GOX-convective, and LBL-GOX-soaking modes, respectively. Figure 6 illustrates that the activity of electrostatically immobilized GOX with convective flow of glucose solution is superior to both covalently immobilized GOX with a convective flow of glucose and electrostatically immobilized GOX under a soaking mode of interaction with glucose. Electrostatic immobilization resists the conformation change and accessibility problems associated with covalent immobilization by keeping the enzyme away from the solid support, whereas convective flow increases the accessibility of the substrate for the enzyme, compared to the soaking mode (diffusion).

The well-known Michaelis–Menten rate equation⁷⁶ is used to describe the kinetics of glucose oxidation by immobilized GOX. The equation, which is expressed as the rate v , is defined as follows:

$$v = \frac{v_{\max} S}{K_m + S} \quad (5)$$

where S is the substrate concentration, $v_{\max}$ is the maximum possible rate, and K_m is the Michaelis–Menten constant and is defined as the substrate concentration at which the rate becomes half of $v_{\max}$. Equation 5 can be manipulated to give

$$\frac{1}{v} = \frac{1}{v_{\max}} + \frac{K_m}{v_{\max} S} \quad (6)$$

Equation 6 is used to calculate the kinetic parameters by a straight-line plot (a Lineweaver–Burke plot) of $1/v$ vs $1/S$ and then determine $v_{\max}$ and K_m from the intercept and slope, respectively.

The Michaelis–Menten rate equation is used to describe the immobilized phase reaction kinetics for the substrate concentration range of 0.75–75 mM. It has revealed some interesting facts, which are given as follows. The Lineweaver–Burke plot is constructed from the rate data and is presented in Figure 7. The figure shows that the nature of the Lineweaver–Burke plot is nonlinear for both the LBL-GOX-convective and PVDFGOX-convective modes. It can be speculated that the Michaelis–Menten rate equation ($R^2 = 0.7$) is not efficient to describe the immobilized phase reaction kinetics accurately in the entire range of substrate concentrations studied ($S = 0.75\text{--}75$ mM (i.e., $1/S = 1.33\text{--}0.013$ mM^{−1})).

To study the kinetics behavior of immobilized GOX in a specific region of substrate concentration, a Lineweaver–Burke plot is constructed for both membranes (shown as the inset in Figure 7) in the substrate concentration range of 15–75 mM ($1/S = 0.067\text{--}0.013$ mM^{−1}). For both cases, the straight- line plots fit the experimental data extremely well, as evident from the correlation values of $R^2 > 0.99$. The values of $v_{\max}$ and K_m are determined from the Lineweaver–Burke plots in this concentration range. The value of $v_{\max}$ for the LBL-GOX-convective and PVDF-GOX-convective modes are 24 and 22 mM/(mg min), respectively. The K_m value of the LBL-GOX-convective mode is 13 mM, and, for the PVDF-GOX-convective mode, it is 4.6 mM. This observation suggests that Michaelis–Menten kinetics is valid in the higher substrate concentration range of 15–75 mM for our cases of immobilized phase GOX. However, it fails to explain the rate behavior at lower ranges. It can be speculated that, at any given time, for lower substrate concentrations, the amount of immobilized enzyme per unit amount of substrate is significantly higher than that for higher substrate concentrations. This might have a role in the deviation from Michaelis–Menten kinetics.

The K_m values obtained here are significantly less than most of the reported K_m values for covalently immobilized GOX represented in Table 1. One value of particular interest in Table 1 is that reported by Ying et al. For covalently immobilized GOX in PAA grafted PVDF membrane and soaking mode oxidation of glucose, the value of K_m is 10.5. On the other hand, for our case, the K_m value is 4.6 for the PVDF-GOX-convective membrane system. Lower K_m values signify a higher affinity of the enzyme toward the substrate. This again demonstrates the advantage of electrostatic immobilization and covalent flow over covalent immobilization and diffusive flow for an enzyme-substrate system. The $v_{\max}$ values for different cases cannot be compared, because of the differences in units arising from the difference in the mode of hydrodynamics (i.e., convective and diffusive flow).

3.4. Effect of Residence Time (i.e., Permeate Flow Rate).

Effects of residence time (i.e., permeate flow rate) on conversion and rate of oxidation of glucose (production of H_2O_2) by immobilized GOX in both LBL and PVDF membranes are described by considering them as model reactors, such as plug-flow reactors (PFRs) and CSTRs. The assumption of a CSTR is valid for low conversions. A material balance is done within the membrane pore, and Michaelis-Menten reaction kinetics is incorporated to obtain a mathematical expression that predicts the exit concentration of H_2O_2 in the permeate ($C_{PH_2O_2}$).

For PFRs, the equation is

$$C_{PH_2O_2} - K_m \ln \frac{(S_0 - C_{PH_2O_2})}{S_0} - v_{\max} \times \tau \times (\text{mass of enzyme (mg)}) = 0 \quad (7a)$$

Similarly, for CSTRs, the equation is

$$C_{PH_2O_2} = v \times \tau \times (\text{mass of enzyme (mg)}) = \frac{v_{\max} S}{K_m + S} \times \tau \times (\text{mass of enzyme (mg)}) \quad (7b)$$

For different residence times and a particular concentration of substrate, the model-predicted exit concentration of H_2O_2 in the permeate ($C_{PH_2O_2}$) can be obtained using either eq 7a or eq 7b. The conversion was deliberately kept low, so that the assumption of a constant concentration of substrate remains valid. K_m and $v_{\max}$ for the immobilized GOX, determined earlier for the experiments conducted at a residence time of 0.6 s (flow rate = 11 mL/min) in the substrate concentration range of 15–75 mM, are used for calculations. The conversion is calculated as

$$\text{Conversion} = \frac{S_0 - S}{S_0} = \frac{C_{PH_2O_2}}{S_0} \quad (8)$$

where S_0 is the initial substrate concentration.

Because the conversion was very low, the predicted values from PFR and CSTR models were observed to be almost identical. Hence, for further discussions, both PFR- and CSTR-predicted values are commonly termed as “model-predicted” values.

From the model-predicted concentration of H_2O_2 in the permeate, the conversion and rate are determined using eqs 8 and 4, respectively. Both the experimentally obtained and model-predicted values for a substrate concentration of 15 mM are demonstrated in Figures 8 (LBL assembled nylon membrane) and 9 (functionalized PVDF membrane).

The conversion of glucose represents the concentration of H_2O_2 in the permeates. Figures 8 and 9 show that, as the permeate flow rate increases (i.e., the residence time decreases), the concentration of H_2O_2 in the permeate decreases and, hence, the conversion. This is because the product formation for a flow reactor is a function of residence time, unlike the rate of reaction. Figures 8 and 9 also demonstrate that the assumption of ideal reactors, such as CSTRs and PFRs, is sufficient to predict the effect of residence time on the rate and conversion of the reaction catalyzed by an immobilized enzyme in a membrane at higher flow rates. However, the ideal reactor model deviates (over predicts) at lower flow rates, because of the mass-transfer issues. At lower flow rates, mass-transfer resistance is significant and glucose molecules cannot access all the active sites of immobilized GOX in the functionalized domain of membranes. In addition, at lower flow rates, the removal of the product from the active sites of the enzyme is also retarded, because of mass-transfer resistances, thereby, further reducing the accessibility. Thus, the rate and conversion is low at lower flow rates. This observation suggests that the incorporation of a mass-transfer resistance term in the mathematical model will be useful for proper prediction.

3.5. Effect of pH.

The effect of pH on the activity of free GOX has been reported in the literature.^{13,69} It has been observed that there is a distinct maximum for the activity of free GOX at pH 5.5, and the activity decreases dramatically on either side of that pH. For example, in a study by Ozyilmaz et al.,¹³ the normalized activity (with respect to the activity at pH 5.5) for a 100 mM glucose solution was reported to be 35% at pH 4.5 and 50% at pH 7. Our study for free GOX, covering a glucose concentration range of 0.75–75 mM, also exhibited a similar trend, as shown in Figure 10. The effect of pH on the activity of the LBL-GOX system is also represented in the same figure. It can be observed that, although the activity curve goes through a maximum, the range of optimum pH is broad. In addition, the decrease in activity on either side of the optimum pH range is less than the free GOX. This proves that GOX immobilized in the membrane can be operated over a wide range of pH without sacrificing the activity significantly.

3.6. Stability.

Figure 11 demonstrates the stability of the electrostatically immobilized GOX in both the LBL assembled nylon membrane and the PVDF–PAA–PAH membrane over a period of time. It can be observed from Figure 11 that, even after 28 days, the activity of immobilized GOX reduced to only 80% of the initial activity, whereas, according to Ozyilmaz et al.,¹³ the

activity reduced to 25% after 28 days for free GOX. In another study,⁷⁷ the activity was reported to be reduced to 30% after the first 7 days. This shows that GOX immobilized within a membrane matrix is well-protected and retains its activity over a longer period of time, compared to that for free GOX.

3.7. Reusability of the Membrane Matrix by Detachment and Reattachment of GOX.

3.7.1. LBL-GOX Membrane.—The pK_a of amine groups of PAH is 9.5, and the pI of GOX is 4.2. Hence, as the pH decreases below 4.2, the negative charges on GOX are reduced and the attractive interaction with the positively charged nylon–LBL membrane decreases. It was observed, for the nylon–LBL membrane, that the maximum possible pH value at which GOX detaches from the positively charged membrane matrix is 3. The amount of detached GOX was $90\% \pm 3\%$ of the immobilized GOX. It can be hypothesized that the undetached GOX molecules were either situated in such domains of polyelectrolyte layers that they were inaccessible or they were too strongly bound to detach by changing the pH alone. The detached GOX was unaffected by the drastic change in pH environment and showed 90% activity of the fresh GOX in free form, as revealed by the homogeneous phase experiments. After the homogeneous phase activity studies, the remaining detached GOX was electrostatically reattached in the same LBL membrane matrix. The activity of the reattached GOX was $90\% \pm 5\%$ of the activity prior to the detachment. The same steps of detachment and reattachment were repeated, and similar results were obtained. GOX then was detached and fresh GOX was attached in the same membrane matrix. The attachment was 90% of the newly made seven-layer LBL membrane, because of the presence of undetached GOX (10%). The activity of the immobilized fresh GOX in the used LBL membrane was identical to the activity obtained for fresh GOX in the newly made seven-layer LBL membrane. The same trend (detachment and reattachment was 90% of the newly made LBL membrane) follows for subsequent detachment/reattachment cycles with freshly attached GOX. This study demonstrates the reusability of the LBL membrane matrix for GOX immobilization. Although the current study involves GOX only, it also can potentially be extended for other enzymes.

3.7.2. PVDF-PAA-PAH-GOX Membrane.—There was a fundamental difference in the regeneration of PVDF–PAA–PAH–GOX membrane, compared to the LBL–GOX membrane. The pK_a of amine groups of PAH is 9.5, and the pI of GOX is 4.2. Hence, to reduce the electrostatic interaction and detach GOX, the pH of the detaching solution should be less than 4.2. However, at that pH, the interaction between PAH and PAA also vanishes, because the pK_a of carboxylic acid groups of PAA is 4.7. Thus, the detaching solution dislocates the entire PAH–GOX domain from the PVDF–PAA matrix. Therefore, each time, fresh PAH and GOX were attached in the PVDF–PAA membrane. The amount and activity of fresh GOX attached in the same PVDF–PAA membrane matrix were identical to that obtained for freshly made functionalized PVDF membranes, as observed from multiple studies. This proves that the functionality within the membrane is very consistent and there is no leakage of the components.

4. Conclusions

Functionalized membranes were created by two different approaches. In one case, the alternative layer-by-layer (LBL) assembly of anionic and cationic polyelectrolytes was conducted in a nylon-based membrane. In the other case, in situ polymerization of acrylic acid was conducted in a hydrophobic polyvinylidene fluoride (PVDF) membrane. The enzyme, glucose oxidase (GOX), was then successfully immobilized inside the charged functionalized membrane domains by electrostatic interaction. The functionalized domains in both cases were stable under tested conditions. The activity of GOX was investigated for the oxidation of the substrate (glucose), to produce gluconic acid and H_2O_2 . A detailed kinetic study has been presented with electrostatically immobilized GOX and a convective flow of glucose in the LBL membrane and the polyacrylic acid–polyallylamine hydrochloride (PAA–PAH) functionalized PVDF membrane. The activity was then compared with the covalent mode of GOX immobilization and also for the soaking mode (diffusion) of substrate interaction. It was observed that electrostatic immobilization of the enzyme and pressure-induced convective flow (permeate) of the substrate is beneficial over other combinations for an enzyme–substrate system. The effect of residence time (i.e., permeate flow rate) (through pressure adjustment) and pH on the activity of GOX has also been studied. It was demonstrated that a higher flow rate of reaction mixture through the membrane exposes the maximum possible active sites of the enzyme. It was also established that GOX immobilized in the functionalized membrane has a wide range of operating pH values and was associated with higher stability, compared to free GOX. The potential of reusability of the membrane matrix was an important finding in this research. The reusability has been discussed with an extensive study of the detachment and reattachment of GOX from membrane matrix, along with the activity measurements. This technique also could easily be applied for other enzyme–substrate systems.

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Nomenclature

A_m	external surface area of membrane (cm^2)
$C_{\text{PH}_2\text{O}_2}$	concentration of H_2O_2 in the permeate (mM)
K_m	Michaelis-Menten constant (mM)
P_n	permeability through the membrane after attachment of the n th layer of polyelectrolyte ($\text{cm}^3/(\text{cm}^2 \text{ s bar})$)
Q_{W_0}	volumetric flow rate of water through the unmodified membrane (cm^3/s)

Q_{W_n}	volumetric flow rate of water through the membrane after attachment of the n th layer of polyelectrolyte (cm^3/s)
r_0	pore radius of the unmodified membrane (nm)
r_n	pore radius of the membrane after attachment of the n th layer (nm)
S	substrate concentration (mM)
S_0	initial substrate concentration (mM)
v	rate of glucose oxidation by GOX ($\text{mM}/(\text{mg min})$)
$v_{\max}$	maximum rate in Michaelis-Menten kinetics ($\text{mM}/(\text{mg min})$)

Greek Letters

δ_n	effective cumulative thickness of the n th layer of polyelectrolyte/polymer (nm)
τ	residence time (s)

Material Abbreviations

PVDF	polyvinylidene fluoride
LBL	layer-by-layer
GOX	glucose oxidase enzyme
PLL	poly-L-lysine
PAH	polyallylamine hydrochloride
PSS	polystyrene sulfonate (Na-form)
PAA	polyacrylic acid

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Figure 1. Enzymatic oxidation of the substrate (glucose) by the enzyme (glucose oxidase (GOX)), following a two-step reaction mechanism to produce gluconic acid and hydrogen peroxide.

Figure 2. Schematic of the formation of (a) layers of polyelectrolytes inside an anhydride activated nylon-based membrane using layer-by-layer (LBL) assembly, followed by electrostatic immobilization of GOX, and (b) functionalized PVDF membrane by PAA (shown here as a single chain, for illustration purposes) and electrostatic attachment of PAH, followed by electrostatic immobilization of GOX.

Figure 3. Calculated cumulative layer thickness after electrostatic immobilization inside nylon membrane pore; the Hagen Poiseuille equation was used to calculate layer thickness. Inset shows the decrease in permeability due to the formation of layers inside the membrane pores. (Conditions: pore radius of pure nylon membrane, 225 nm; water permeability of pure nylon membrane, $570 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$; permeability after GOX immobilization, $52 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s bar})$; external membrane area, 13.2 cm^2 , and membrane thickness, $165 \mu\text{m}$.)

Figure 4.

Effect of functionalization on PVDF membrane, as depicted by the curve of permeate flux versus pressure. Pore radius of the pure PVDF membrane = 225 nm, pore radius of PVDF–PAA–PAH–GOX membrane = 100 nm. Water (pH 6) permeability values for different steps of functionalized membrane are given by the slope of the curves. External membrane area = 13.2 cm², membrane thickness = 125 μm.

Figure 5. Effect of pH on permeate water flux and functionalized domain of the PVDF-PAA membrane in Na-form. PAA network inside the membrane undergoes a “retracted-to-elongated” conformational change as pH increases. Flux was normalized with maximum flux for this experiment (i.e., $330 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s})$) at pH 2.5 and a pressure of 1.38 bar. (Conditions: pore radius of the pure PVDF membrane = 225 nm, external membrane area = 13.2 cm^2 , membrane thickness = $125 \mu\text{m}$.)

Figure 6.

Rate of oxidation of glucose, as a function of the substrate concentration for different GOX immobilization experiments; the amount of GOX immobilized is 1.8, 1.5, and 1 mg for the PVDF membrane, the LBL membrane, and covalent immobilization, respectively. (Conditions: residence time for all experiments, 0.6 s; temperature, 25 °C; pH of the NaOAc–HCl buffer, 5.5.)

Figure 7.

Lineweaver–Burke plot for GOX immobilized in functionalized LBL (LBL-GOX-convective) and PVDF (PVDF-GOX-convective) membranes. Conditions: concentration of glucose solution, 0.75–75 mM ($1/S = 1.33\text{--}0.013\text{ mM}^{-1}$); amount of GOX immobilized, 1.5 and 1.8 mg for LBL and PVDF membranes, respectively; temperature, 25 °C; pH of NaOAc–HCl buffer, 5.5. Inset shows the Lineweaver–Burke plot for a substrate concentration range of 15–75 mM ($1/S = 0.067\text{--}0.013\text{ mM}^{-1}$).

Figure 8.

Effect of flow rate (i.e. residence time) on the conversion of glucose and the rate of formation of H_2O_2 for a 15 mM glucose solution fed in a LBL assembled nylon membrane. Model values are calculated considering both plug-flow reactor (PFR) and continuously stirred tank reactor (CSTR) models. Conditions: amount of GOX immobilized, 1.5 mg; volume of LBL membrane reactor, 0.122 cm^3 ; pH 5.5; temperature, 25°C ; permeate flux, $(75\text{--}265) \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s})$; and residence time, 0.35–1.24 s.

Figure 9.

Effect of flow rate (i.e. residence time) on the conversion of glucose and the rate of formation of H_2O_2 for a 15 mM glucose solution fed in a functionalized PVDF membrane. Model values are calculated considering both PFR and CSTR models. Conditions: amount of GOX immobilized, 1.8 mg; volume of PVDF membrane reactor, 0.1 cm³; pH5.5; temperature, 25 °C; and permeate flux, (60–200) 10⁻⁴ cm³/(cm²s); the residence time varied over a range of 0.37–1.25 s.

Figure 10.

Effect of pH on activity for both free GOX and GOX immobilized in an LBL assembled membrane. Activity is calculated based on the concentration of H_2O_2 in the permeate

$\left(C_{\text{PH}_2\text{O}_2}\right)$ and is normalized by dividing by the maximum activity

$\left(\text{i.e., } C_{\text{PH}_2\text{O}_2} / \left(C_{\text{PH}_2\text{O}_2}\right)_{\text{max}}\right)$. (Conditions: amount of GOX immobilized, 1.5 mg; volume of

LBL membrane reactor, 0.122 cm^3 ; permeate flux, $140 \times 10^{-4} \text{ cm}^3/(\text{cm}^2 \text{ s})$; residence time, 0.6 s; external membrane area, 13.2 cm^2 ; membrane thickness, $165 \mu\text{m}$; pH 5.5; temperature, 25°C ; and concentration of glucose fed, 0.75–75 mM.)

Figure 11.

Stability of GOX immobilized in an LBL assembled nylon membrane, as well as in a PVDF-PAA-PAH membrane based on the change in activity over a period of time. The stability is compared with that reported for free GOX.¹³ Activity is calculated based on concentration of H_2O_2 and is normalized by dividing by the activity on the first day of immobilization at pH 5.5.

Table 1.

Comparative Study of the Types of Solid Matrix Used and Kinetic Parameters Obtained for Different GOX Immobilization Techniques Reported in the Literature

reference	type of solid matrix ^a	mode of GOX immobilization	K_m (mM)	Kinetics Study of Immobilized GOX v_{max}
Ekinici et al. ¹⁸	Pt-electrode coated with copolymer of pyrrole and polystyrene	entrapment	11.8	0.04 μ mol/(min mL)
Tomotani and Vitolo ²⁰	anionic polystyrene beads + UF membrane	electrostatic	-	34×10^{-4} mmol/(min mg)
Ahuja et al. ¹¹	polystyrene beads activated with hydrazide + glutaraldehyde	site-directed	29	0.248 mmol/(min mg)
Ahuja et al. ¹¹	polystyrene beads activated with hydrazide + glutaraldehyde	covalent	39.3	0.052 mmol/(min mg)
Pandey et al. ¹⁴	gold nanoparticle + MUDA + EDC-NHS	covalent	3.74	14.2×10^{-4} mmol/(min mg)
Rauf et al. ¹⁵	CA-PMMA membrane + glutaraldehyde	covalent	41.65	1.17 mM/min
Hou et al. ¹²	PS-GMA microsphere	covalent	35.2	15.5 mM/min-mg
Ozyilmaz et al. ¹³	magnesium silicate + APTES	covalent	259	0.217 mmol/(min mg)
Ying et al. ¹⁶	grafted PAA on PVDF membrane	covalent	10.5	14×10^{-4} mmol/(min mg)
Li et al. ¹⁷	grafted AAac on polyaniline film	covalent	80.6	0.62 mM/min

^a Abbreviation legend: MUDA, mercaptoundecanoic acid; EDC, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; PMMA, polymethyl methacrylate; PS, polystyrene; GMA, glycidolmethacrylate; APTES, 3-aminopropyl trimethoxysilane; PAA, polyacrylic acid; PVDF, polyvinylidene fluoride; AAac, acrylic acid; CA, cellulose acetate.

Table 2.

Characterization of (a) LBL Assembled Nylon Membrane and (b) Functionalized PVDF Membrane by Calculating the Number of Repeat Units Immobilized in Subsequent Layers or Domains^a

layer	domain	number of repeat units immobilized ($\times 10^3$ mmol)	net charge ($\times 10^3$ mmol)
(a) LBL Assembled Nylon Membrane			
PLL		2.1	(+) 2.1
PSS ₁		6.9	(-) 4.8
PAH ₁		9.1	(+) 4.3
PSS ₂		10.4	(-) 6.1
PAH ₂		13.1	(+) 7.0
PSS ₃		8.2	(-) 1.2
PAH ₃		2.5	(+) 1.3
GOX		1.1	
(b) Functionalized PVDF Membrane			
PAA		128.7	(-) 128.7
PAH		130	(+) 1.3
GOX		1.3	

^aThe molecular weight of the repeat units of the different polyelectrolytes/polymer is as follows: PLL, 164.5; PSS, 206; PAH, 93.5; PAA, 72. External membrane area = 13.2 cm^2 , membrane thickness = $165 \text{ }\mu\text{m}$, and pH used for immobilization = 6.