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Surfaces based on amino acid functionalized polyelectrolyte films towards active surfaces for enzyme immobilization

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ABSTRACT

Surface based on polyelectrolytes functionalized with amino acids onto amino-terminated solid surfaces of silicon wafers was prepared, with the purpose of evaluate the chemical functionality of the polyelectrolyte films in adsorption and catalytic activity of an enzyme. In this work, the adsorption of the enzyme glucose 6-phosphate dehydrogenase from Leuconostoc mesenteroides (*Lm*G6PD) was studied as model. The polyelectrolytes were obtained from poly (maleic anhydride-*alt*-vinylpyrrolidone) [poly(MA-*alt*-VP)] and functionalized with amino acids of different hydropathy index: glutamine (Gln), tyrosine (Tyr) and methionine (Met). The polyelectrolytes were adsorbed onto the amino-terminated silicon wafer at pH 3.5 and 4.5 and at low and high ionic strength. At low ionic strength and pH 3.5, the largest quantity of adsorbed polyelectrolyte was on the films containing glutamine moiety as the most hydrophilic amino acid in the side chain of polymer chain (5.88 mg/m^2), whereas at high ionic strength and pH 4.5, the lowest quantity was in films containing tyrosine moiety in the side chain (1.88 mg/m^2). The films were characterized by ellipsometry, contact angle measurements and atomic force microscopy (AFM). The polyelectrolyte films showed a moderate degree of hydrophobicity, the methionine derivative being the most hydrophobic film. With the aim of evaluate the effect of the amino acid moieties on the ability of the surface to adsorb enzymes, we study the activity of the enzyme on these surfaces. We observed that the polarity of the side chain of the amino acid in the polyelectrolyte affected the quantity of *Lm*G6PD adsorbed, as well as its specific activity, showing that films prepared from poly(MA-*alt*-VP) functionalized with Met provide the best enzymatic performance. The results obtained demonstrated that the surfaces prepared from polyelectrolytes functionalized with amino acids could be an attractive and simple platform for the immobilization of enzymes, which could be of interest for biocatalysis applications.

1. Introduction

Polyelectrolytes, consisting of polymers containing ionizable groups, have generated great interest in the design of biomaterials for their application in biomedical, biotechnological, and pharmaceutical fields among others [1–6]. Such applications are based mainly on the adsorption of polyelectrolyte onto solid surfaces to obtain thin films that can modulate the chemical nature and charge of the modified surface. Polyelectrolyte adsorption onto solid surfaces is known to be strongly dependent on some factors: (i) surface properties like chemical composition, morphology, crystallinity, solubility, charge and energy (ii) polyelectrolyte properties like charge density of the polyion,

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concentration and chemical structure, and (iii) dissolution properties, like the salt concentration and pH [7–9]. In particular, polyelectrolytes functionalized with biomolecules present additional advantages to those already known associated with their chemical structure, which resembles their biological counterparts, such as proteins, polysaccharides, DNA and cells, among others. In this context, polyelectrolytes functionalized with biomolecules adsorbed onto solid surfaces can contribute to improve enzyme immobilization.

Enzyme immobilization on materials is an alternative for the efficient application of biocatalysts in food, drug and nutrition delivery technologies, as well as artificial cells, biosensors, protector coatings, chemistry and pharmaceutical [10–16]. After an enzyme is immobilized onto the surface of a material it is desirable that the enzyme retains its structure conformation and specific activity. Furthermore, modifications can be performed to the protein surface or the support surface to increase the stability of an enzyme at a material interface. [10] Fernandez-Lafuente et al. have reported a review about new immobilization protocols focusing on how immobilization can contribute to solve problems of industrial biocatalysts, such as enzyme recovery, enzyme selectivity, enzyme stability, reduction of inhibition by the medium or products, where the key to solving these problems lies in the control of the support–enzyme interaction [17].

On the other hand, Sassolas et al. reviewed different techniques for enzyme immobilization, which include classical adsorption, covalent bonds, entrapment, cross-linking or affinity, as well as the recent introduction of promising nanomaterials like conducting polymer nanowires, carbon nanotubes and nanoparticles [18]. Immobilization can also facilitate efficient enzyme recovery from the reaction media, making it possible to reuse the catalyst several times [11,19]. Immobilized enzymes offer advantages over soluble enzymes related to structural stability and enzyme catalytic activity [20]. Soluble enzymes can be immobilized on solid supports to enable their long-term operation in industrial processes [21]. It is widely accepted that the solid support must provide an inert and biocompatible environment for enzyme immobilization, which should not interfere with the native enzyme structure and its biological activity. Polyelectrolytes functionalized with amino acid moieties are a promising enhancement to biocompatibility. Since polyelectrolytes have different physicochemical features, they could be used to modulate the surface character in order to obtain better interaction with the enzyme.

Physicochemical properties like surface charge, charge density, surface free energy, and wettability have been identified as prominent factors governing enzyme adsorption [22]. Particularly, the wettability of the surface is a determining factor for enzyme adsorption since the hydrophobic/hydrophilic balance not only affects adsorption and/or adhesion, but also the formation and biological activity of the proteins [23–25]. Xu et al. determined the adhesion forces of three types of proteins, serum albumin bovine, fibrinogen and human FXII, to surfaces of low density polyethylene (LDPE) treated with glow discharge plasma to produce films of different degrees of wettability. Using atomic force microscopy, they found, a strong dependence between protein adhesion and the wettability of modified LDPE films. Using liquid atomic force microscopy, they found, a strong dependence between protein adhesion and the wettability of modified LDPE films. Liquid atomic force microscopy also has been used for studying of topography of the adsorbed layer [26]. High-density polyethylene (HDPE) films with contact angle values higher than 60–65° had higher adhesion force for the three studied proteins [24].

One enzyme that has been immobilized for different applications as biocatalyst and for biosensoring is glucose-6-phosphate dehydrogenase [27,28]. Glucose-6-phosphate dehydrogenase regulates nicotinamide adenine dinucleotide phosphate (NADPH) levels and is related to the origin and evolution of various diseases, including G6PD deficiency, type-2 diabetes, aldosterone-induced endothelial dysfunction, and cancer. Besides, immobilized G6PD is used in biosensors to determine analytes like glucose, phosphate, adenosine triphosphate, and others. The immobilization of G6PD makes efficient enzymatic assays possible due to the easy recharge and stability of the enzyme compared to when it is in solution. However, there is a need for an economical and simple method to immobilize G6PD. A variety of supports for immobilization have been reported, including nylon meshes, porous alumina membrane, glassy carbon disk electrodes, CNBr-activated sepharose, among others [28]. In most cases, immobilization involves covalent linkages between the enzyme and the supporting surface, for example by using glutaraldehyde [29] or diglycidyl ether [30]. Covalent linkage (in the form of disulfide bonds) at specific points on the surface of *Lm*G6PDH (introduced by site-directed mutagenesis) is used to control the orientation of the enzyme, revealing an impact on the resulting activity after immobilization [31]. Although direct adsorption (without involving chemical linkage) could be less laborious than covalent linkage, it has received much less attention in the case of *Lm*G6PDH. In this sense, amino acid-functionalized polyelectrolyte surfaces are excellent candidates to study G6PDH adsorption, with the purpose of evaluating the effect of different amino acid residues on the quantity of adsorbed enzyme and its activity after immobilization.

This work studies the adsorption of poly(maleic-*alt*-vinyl pyrrolidone anhydride) functionalized with amino acids onto amino-terminated solid surfaces of silicon wafers, with the purpose of evaluate the influence of the polarity of the amino acid residue of the polyelectrolyte in adsorption of *Lm*G6PDH. Thus, the final purpose of the present work is to develop a simple and low-cost adsorption platform for enzymes, that allow evaluate the efficiency of adsorption in terms of the quantity of bound enzyme and the level of retained enzymatic activity.

2. Experimental section

2.1. Materials

The following reagents inputs were acquired: Maleic anhydride, powder 95%, Aldrich (Milwaukee, WI), 1-vinyl-2-pyrrolidone, Merck, L-Glutamine ≥99% (TLC), Sigma, L-Methionine ≥98% (HPLC) (Sigma-Aldrich), L-Tyrosine ≥98% (HPLC) (Sigma-Aldrich), triethylamine (TEA), benzene, dimethyl sulfoxide (DMSO), hydrochloric acid (HCl). All the chemicals were of analytical grade and used without further purification.

2.2. Synthesis of poly (maleic anhydride-alt-vinylpyrrolidone), P(MA-alt-VP)

The copolymer poly(maleic anhydride-*alt*-vinylpyrrolidone), P(MA-*alt*-VP), was synthesized by radical polymerization in anhydrous benzene (50 mL) at 60 °C for 24 h, under N_2 atmosphere, by mixing equimolar amounts of maleic anhydride (4.9 g) and vinylpyrrolidone (5.5 g) using α, α-azobisisobutyronitrile (AIBN) (0.03 g) as an initiator.

The average molecular weight of the copolymer was obtained by capillary viscometry. The intrinsic viscosity of the polymer in solution was determined in an *Ubbelohde* viscometer at 25 ± 0.01 °C. The initial concentration of the solution was 0.02 g/cm^3 in 0.01 M HCl. The value of the intrinsic viscosity and the molecular weight of polymer were determined using an η_{sp}/c v/s *c* graph and the Mark-Houwink equation with a = 0.62 and K = 3.25×10^{-4} [32]. The viscosimetric molecular weight (M_v) of the polymers was 1.8×10^4 g/mol.

2.3. Functionalizing poly(maleic anhydride-alt-vinyl pyrrolidone) with amino acids

Poly(maleic anhydride-*alt*-vinyl pyrrolidone) or P(MA-*alt*-VP) was functionalized with glutamine (Gln), tyrosine (Tyr) and methionine (Met). To this end, P(MA-*alt*-VP) (0.5 g) was dissolved in DMSO (25 mL), and 0.3495 g of Gln, 0.5054 g of Tyr and 0.3562 g of Met were added to each reaction flask, using triethylamine (TEA, 0.3% of the reactants) as reaction catalyst at 80 °C. The obtained polymers were

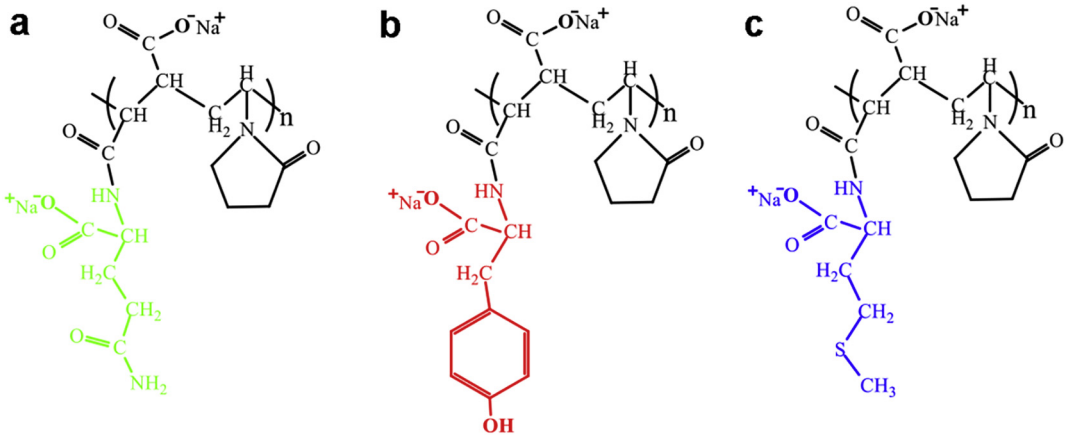


Fig. 1. Chemical structures of (a) P-Gln, (b) P-Tyr and (c) P-Met functionalized polyelectrolytes.

dried in a vacuum oven for further characterization.

2.4. Preparation of the polyelectrolyte functionalized with amino acids

To obtain the sodium salts of the polyelectrolytes shown in Fig. 1, the copolymers synthesized according to section 2.3 were dissolved in 10% $NaHCO_3$ *w/v* aqueous solution in a stoichiometric ratio of 2:1 with respect to the copolymer for ~ 10 days. Subsequently, the sodium salts were ultrafiltered and lyophilized. Polyelectrolytes functionalized with amino acids were designated as P-Gln, P-Tyr and P-Met. Fig. 1 shows the chemical structures of the polyelectrolytes.

2.5. FT-IR spectroscopy

To characterize pure P(MA-*alt*-VP) and copolymers functionalized with amino acids, KBr discs were prepared with 2 mg polymer and 100 mg of KBr. The FT-IR spectra were obtained with a Bruker Vector Model 22 at 25 °C.

2.6. Magnetic resonance of proton and carbon 13 (^{1}H NMR and ^{13}C NMR)

To study ^{1}H NMR and ^{13}C NMR, ~80 mg of P(MA-*alt*-VP) and of each copolymer functionalized with amino-acids were dissolved in DMSO-D_6 (0.5 mL). The ^{1}H NMR and ^{13}C NMR spectra were examined using an Advance-400 spectrometer at 25 °C.

2.7. Quantitative elemental analysis

Quantitative elemental compositions were determined in a CE Instruments EAGER 200 elemental analyzer. The preparation of the samples was carried out using 2 mg of sample prepared in tin crucibles, under constant flow of helium and injection of oxygen gas.

2.8. Potentiometric titrations

All these polyelectrolytes were potentiometrically titrated at 25 ± 0.1 in aqueous solution registering the solution pH with pH meter Oakton 700 equipped with a Cole-Parmer combined double junction electrode. The solution pH was registered from the basic range as aliquots of diluted HCl 0.1 M were added to these solutions.

2.9. High resolution ellipsometry

Ellipsometric measurements were taken with a monochromatic ellipsometer L116S300 STOKES model (Gaertner Scientific Corporation, USA) equipped with a He—Ne laser with a wave length of $\lambda = 632.8$ nm and an incidence angle set to 70.0°. This technique was used to measure variations in the thickness of the polyelectrolyte films adsorbed onto the amino terminated-surfaces of silicon wafers and immobilized *Lm*G6PD films.

To obtain the thickness of the adsorbed polyelectrolyte layer (d_{poly}), it was necessary to first determine the thickness of the SiO_2 layer in the air. Thus, the refractive index of Si is considered as $n = 3.88$ - i0.018 and thickness as infinite. The refractive index of the medium (air) is $n = 1.00$ and this value is constant [9,33]. The refractive index of SiO_2 is $n = 1.462$ and thus its thickness was determined. After measuring 3 or 4 samples, the average thickness of the SiO_2 was 1.5 ± 0.1 nm. The thicknesses of the modified surfaces of silicon wafer with APS were then determined. To do this, the refractive index of APS was considered as 1.424. The thickness of the layer of APS measured in air was 0.9 ± 0.1 nm. The adsorbed quantity Γ was obtained according to what was described in the literature [8].

2.10. Contact angle measurement

Contact angle measurements were taken in a home-made device equipped with a digital camera connected to a computer. The sessile water drop method was used to measure the advancing (θ_A) and receding (θ_R) angles. To carry out the analysis of the drop profile, the ImageJ software was used. For this, a drop of water of 8 μL was deposited on each surface modified. The image of the drop was recorded with the digital camera and the value of θ_A was measured. The θ_R was obtained by removing 4 μL from the original drop and measuring the angle of the remaining drop, as it has been previously reported by our research group. The hysteresis angle (Δθ) was calculated from the difference between θ_A - θ_R. The hysteresis in the contact angle stems from surface roughness or surface chemical heterogeneities. The films did not show swelling after depositing the drop, therefore it was not necessary to consider this effect in the contact angle measurements.

2.11. Atomic force microscopy (AFM)

AFM analysis was done in an AFM/SPM Controller 9500 Series System, (Keysight Technologies, CA, USA) with a 7500 scanner. The surfaces were scanned in the magnetic-AC mode (MAC-Mode® Keysight) with a scan rate of 0.3 Hz, using commercial AFM probes (Olympus). Height AFM images were collected in air conditions at room temperature (23–25 °C). Once the incubation was complete, the surfaces were removed from the polyelectrolyte solutions, rinsed, and dried with N_2 stream. The AFM measurements were registered immediately.

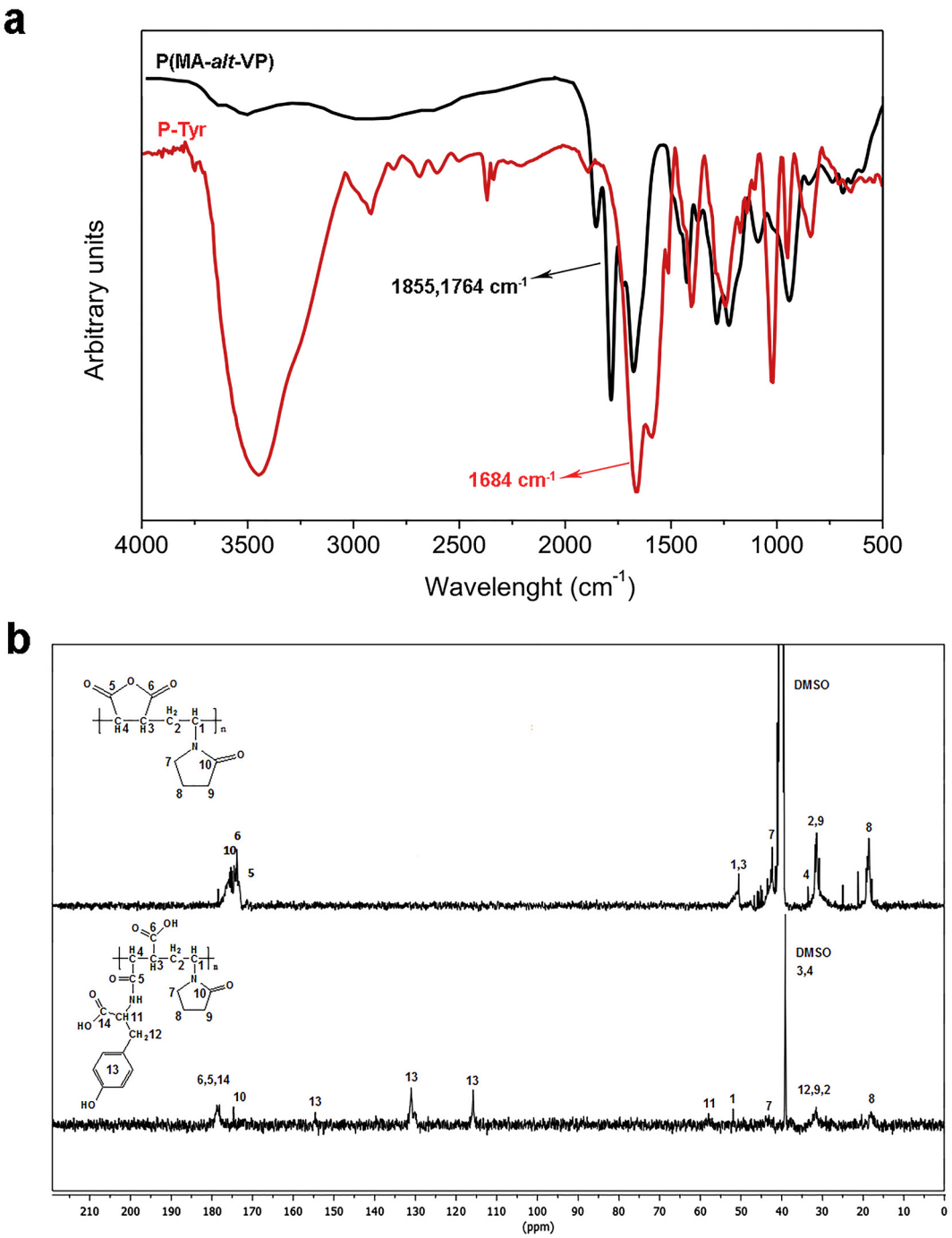


Fig. 2. (a) FT-IR and (b) ^{13}C NMR spectra of: P(MA-*alt*-VP) and P-Tyr.

2.12. Adsorption of polyelectrolytes functionalized with amino-acids onto amino terminated surfaces

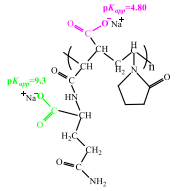
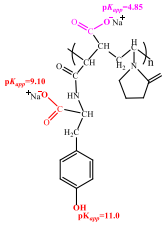
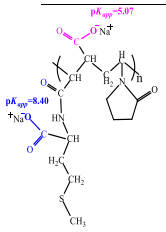
To study the adsorption of polyelectrolytes functionalized with Gln, Met and Tyr residues onto amino-terminated surfaces, solutions of the functionalized polyelectrolytes were prepared in deionized water containing 0.001 or 0.1 mol/L NaCl. The pH of these solutions was adjusted by adding diluted HCl to pH 3.5 and 4.5. The concentration range of the polyelectrolyte solutions ranged from 0.001 to 1.0 g/L.

Silicon wafers (Si/SiO_2) ([100], p-doped, $R = 0.01\Omega cm$) were obtained from Silicon Quest, USA. The substrates have a native oxide layer approximately 2 nm thick. The silicon wafers were washed and modified following a standard procedure [33]. The substrates were then functionalized with 3-aminopropyltrimethoxysilane (APS) to obtain the amino-terminated surface [33]. The amino-terminated surfaces were inmersed in solutions of each polyelectrolyte at different concentrations for 3 h. After, the films of adsorbed polyelectrolytes were dried with N_2 for characterization. The amount of adsorbed polyelectrolyte was determined by ellipsometry.

2.13. Purification of recombinant LmG6PD

The wild type gene sequence of *Lm*G6PDH (GenBank accession number M64446.1) was obtained from the plasmid pET25-*Lm*G6PDH, kindly donated by Dr. Michael Cosgrove (Syracuse University, Syracuse, NY, USA), and subcloned in plasmid pTEV [34] to facilitate over

Table 1
The pK*app,* contact angle of advance [θ_a] and hysteresis [$\Delta\theta_H$] of P-Gln, P-Tyr and P-Met and (M_v 1.8 × 10^4 g/mol, C = 0.6 g/L), adsorbed on the hydrophilic surface (APS) at 0.1 mol/L and 0.001 mol/L NaCl to pH 3.5 and pH 4.5.

Polyelectrolyte	pH 3.5		pH 4.5		NaCl (mol/L)
	θ_A (°)	$\Delta\theta_H$ (°)	θ_A (°)	$\Delta\theta_H$ (°)	
	39 ± 2	26 ± 2	39 ± 5	19 ± 1	0.1
	42 ± 2	24 ± 6	46 ± 7	21 ± 6	0.001
	52 ± 3	26± 2	56 ± 1	25 ± 2	0.1
	57 ± 4	18 ± 2	54 ± 2	24 ± 2	0.001
	71 ± 4	38 ± 4	59 ± 2	22 ± 4	0.1
	55 ± 3	29 ± 2	40 ± 4	31 ± 2	0.001

expression and His-tag-based purification of the enzyme. This construction (termed pET-TEV-zwfLm) was transformed in the *E. coli BL21* (DE3) strain. The cells were cultured in the presence of tetracycline (20 mg/mL) and kanamycin (50 mg/mL). The enzyme *Lm*G6PDH was produced and purified as described by Olavarría et al., except for the following modifications: [35] (i) The cellular pellet harvested after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG), used as inductor of the process of targeted protein expression in *E. coli* BL21(DE3) and phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor that reacts with serine residues of serine-proteases. Thus, IPTG was resuspended in 50 mM of cold Tris (Merck) at pH 8.2, 10 mM of $MgCl_2$ (Merck), 1 mM of PMSF (Calbiochem), 500 mM of NaCl (Calbiochem), and 40 mM of imidazole (Calbiochem), prior to sonication. (ii) The cellular extract was centrifuged for 30 min at 18,000 × *g* and 4 °C, and the supernatant was inoculated directly in the HisTrap HP (GE Healthcare) column. (iii) A solution containing the eluted enzyme and the TEV protease in a ratio of 50:1 (*w*/w) was incubated for 4 h at 25 °C.

2.14. Enzyme activity of LmG6PD and immobilization on amino-terminated surfaces modified with the polyelectrolytes, P-Gln, P-Tyr and P-Met

*Lm*G6PD was adsorbed onto silicon wafers modified with the functionalized polyelectrolyte by immersing the plaque (1 cm × 1 cm) in an enzyme solution containing 0.025 mg/mL of the enzyme in buffer Tris-HCl pH 8.2, 50 mM, 10 mM $MgCl_2$, which was incubated for 16 h at room temperature. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Measurements were performed in triplicate. The quantity of immobilized enzyme was calculated based on the difference between the initial concentration and concentrations at given intervals. Specific activity was also quantified at the same intervals as an indicator of enzyme stability under incubation.

*Lm*G6PD enzyme activity was measured in a reaction mixture containing Tris-HCl pH 8.2, 50 mM, 10 mM $MgCl_2$, 1 mM NAD^+ and G6P 2 mM at 25 °C. Substrates NAD^+ and G6P were neutralized and titrated prior to using them. The time course of product (NADH) formation was followed by increased absorbance at 340 nm, measured in a Biotek Synergy 2 UV–visible, using 96-well plates (Nunc, model 269620). We used a molar extinction coefficient of 5186 M^{-1} cm^{-1} for NADH, as determined for our experimental conditions, to obtain the μmol of NADH formed per minute per mg of enzyme.

After 16 h of incubation, the plaques were thoroughly washed with Tris-HCl buffer 50 mM, $MgCl_2$ 10 mM, pH 8.2, to remove the unadsorbed *Lm*G6PD. Immobilized *Lm*G6PD activity was determined in 2 mL of the reaction mixture, as described above. After initiating the reaction by immersing the plaque, samples of 100 μL were taken at different intervals, and the absorbance spectrum was recorded. The net absorbance at 340 nm (after subtracting the absorbance at 600 nm, *i.e.* the baseline of the spectrum) was used to calculate the quantity of NADH produced between each interval.

3. Results and discussion

3.1. Characterization of P-Gln, P-Tyr and P-Met

P(MA-*alt*-VP) and P(MA-*alt*-VP) functionalized with Gln, Tyr and Met residues were characterized by IR spectroscopy. The analysis of the FT-IR spectra was carried out according to the standard method mentioned in the experimental part. Fig. 2a shows the characteristic bands of the carbonyl groups of the maleic anhydride monomer units of P(MA-*alt*-VP) at 1855 and 1764 cm^{-1}. The disappearance of these bands and the appearance of a band around ~1684 cm^{-1}, characteristic of the amide carbonyl group in the side chain of the functionalized copolymer, can also be observed. A band is present in both spectra at 1665 cm^{-1}, characteristic of γ-lactam group of the vinyl pyrrolidone monomer unit. A broad band appears at ~3500 cm^{-1}, which corresponds to the hydroxyl of the carboxylic group in the tyrosine functionalized copolymer.

The ^{13}C NMR spectrum of the copolymer P(MA-*alt*-VP) (Fig. 2b) shows bands at 172.0–173.1 ppm of the carbonyl groups of maleic anhydride. In the spectrum for the functionalized copolymer, P-Tyr, the characteristic bands of the carbonyl group of carboxylic acid and amide carbonyl in this copolymer are observed at 180.0 and 174.0 ppm, respectively. Similar behavior was observed for the other studied polyelectrolytes. (See supporting information). The functionalization of P(MA-*alt*-PV) was determined by quantitative elemental analysis, obtaining values of 81%, 88%, and 73% for P-Gln, P-Met and P-Tyr, respectively. The percentages of C, H, and N obtained for the determination of the functionalization of the polyelectrolytes are summarized in Table 1 of supplementary material.

3.2. Adsorption of P-Gln, P-Tyr and P-Met onto amino-terminated surfaces

Charged functional groups have been reported to regulate the specificity of the modified solid substrate by means of electrostatic interactions. Interactions like hydrophobic, H-bonding, and van der Waals dispersion forces can also play an important role in modifying solid surfaces with polyelectrolytes [8]. As noted above, polyelectrolyte adsorption depends strongly on pH and salt concentration [9]. Therefore, in order to establish the kind of interaction that governs adsorption between the polyelectrolytes P-Gln, P-Tyr and P-Gln and the amino-terminated surface and to study the immobilization of the enzyme on the surfaces modified with polyelectrolytes, an adsorption behavior study of these systems was performed using two different salt concentrations (0.001 M and 0.1 M NaCl), and at pH values, pH 3.5 and 4.5, (for both salt concentration). This study characterized the type of surface in which the immobilization of the biomolecule takes place.

The isotherms show that in most cases, the quantity of polyelectrolyte adsorbed (Γ) varies with the salt concentration added, reaching

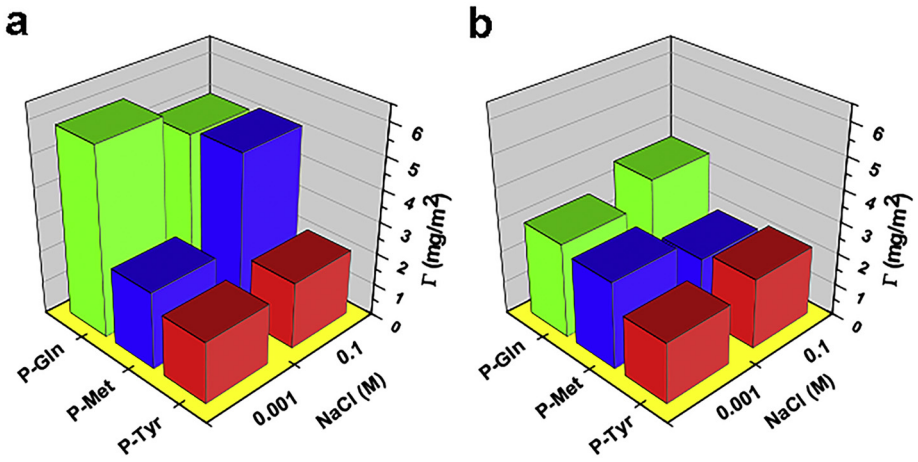


Fig. 3. Amount of adsorbed polyelectrolyte in plateau ($\Gamma_{plateau}$) determined for P-Gln, P-Tyr and P-Met, with 0.001 and 0.1 mol/L NaCl and at pH (a) 3.5 and (b) 4.5.

an adsorption plateau ($\Gamma_{plateau}$) between 0.05 and 0.1 g/L of polyelectrolyte (See supporting information). The adsorption of these systems is mainly determined by the chemical nature of the side group of the polyelectrolyte. To better understand the effect of the pH of the solution on polyelectrolyte adsorption, the apparent pK_a (pK_{app}) values of these systems were determined (see structures on the Table 1).

The $\Gamma_{plateau}$ values in Fig. 3 show that the quantity of P-Gln adsorbed increases as pH decreases, with the largest quantity adsorbed with the lower quantity of salt added (0.001 M NaCl) and a pH of 3.5. At this pH, the structure of P-Gln contains more –COOH than $–COO^-$ groups, because its pK_{app} value is 9.3 for the α-carboxylate group belonging to the amino acid moieties of P-Gln, and 4.8 the value corresponding to the carboxylate group of the polyelectrolyte backbone. These groups interact with the highly charged amino-terminated surface by H-bonding and electrostatic interactions, respectively (pK_a 3.7) [36]. Therefore, at pH 4 the amino-terminated surface is partially deprotonated and polyelectrolyte carboxylated groups are partially protonated, therefore it is expected that electrostatic interactions and H-bonding are favored. The quantity of P-Gln increases at pH 3.5 as the quantity of salt added decreases, due to a lower shielding effect by salt counterions of the charged segments in the polymer chain. Chains of P-Gln adopt a more extended form due to the higher level of electrostatic repulsion between charged groups, which form small loops in order to minimize the repulsion. This leads to the adsorption of P-Gln mainly through electrostatic interactions between charged segments of polyelectrolyte and the amino-terminated surface.

In the case of P-Met the amount adsorbed is greater at high ionic strength. This behavior can be explained due to the conformation that the polymer adopts in the solution, that is to say in solution this polymer is like a compact ball where the ionizable groups are screened favoring an adsorption governed by hydrophobic interactions. The behaviors described above are in accordance with the regimes proposed by Van de Steeg [37] who describes the effects of salt concentration, segment charge of the polymer chain and surface charge on the adsorption of polyelectrolytes onto solid surfaces. In the case of P-Tyr, the ionic strength does not show an effect on the adsorption behavior. This could be determined by the hydrophilic/hydrophobic balance of its structure.

In contrast, at pH 4.5 the quantity of P-Gln adsorbed increases with increased ionic strength of the solution. This adsorption behavior is mainly governed by hydrophobic interactions between the polyelectrolyte and the surface. By increasing the salt concentration of the solution, the strong repulsion between charged polyelectrolyte segments is shielded by increasing the distance between their charged segments and the polyelectrolyte adopts a coiled shape that favors the formation of inter- or intramolecular aggregates, so that the P-Gln behaves like an uncharged polymer. Similar behavior has been found in the adsorption of polyelectrolytes containing aryl-alkyl groups in the side chain onto modified solid surfaces [8].

The quantity of P-Tyr adsorbed was on average 2.02 ± 0.05 mg/m^2, independent of the quantity of salt added or the pH level of the solution. The adsorption of this system onto the amino-terminated surface is probably due mainly to hydrophobic interactions of the phenyl group and electrostatic interactions between carboxylate groups in the polymer chain and amino-terminated. Therefore, the amphiphilic nature of P-Tyr is determined by the hydrophobic/hydrophilic balance, which affects adsorption onto the amino-terminated surfaces independent of the pK_{app} values of the polyelectrolyte, which were similar to those of P-Gln.

The behavior of P-Met is different from that of P-Gln and P-Tyr. The quantity of adsorbed P-Met increases with the decreasing pH of the solution with more added salt, and the quantity does not vary significantly at low ionic strength with respect to pH. This could be explained by the hydrophobic nature of the amino acid residue of methionine in the side chain. The presence of the -CH_2- groups of methionine may increase the flexibility of the polyelectrolyte side chain, allowing methionine residue to be exposed to the solution, favoring H-bonding, van der Waals and hydrophobic interactions with the amino-terminated surface. The effect of alkyl groups in the side chain of the polymer in adsorption has been reported previously [8].

We also determined the adsorption constants (K_{ads}) which are summarized in Table 3 (Supplementary materials), these values are in a range between 9.0×10^4 L/mol and 1.8×10^6 L/mol which reflect a high affinity of P-Gln, P-Met and P-Tyr with the amino-terminal surface. The values of K_{ads} are related to the values of amount of adsorbed polyelectrolyte in plateau (Fig. 3) obtained in the adsorption experiments, in fact the higher affinity shown by P-Gln at pH 3.5 and 0.001 M NaCl is reflected in the higher value of adsorbed amount found for this polyelectrolyte in those same conditions. On the other hand, the K_{ads} values found for P-Tyr do not vary for the different pH and salt conditions, which is also evidenced in the values of adsorbed amount in the plateau shown in Fig. 3. The K_{ads} values for P-Met show an intermediate affinity between P-Gln and P-Tyr. Therefore, it is important to mention that the adsorption process not only depends on the intermolecular interactions between the polyelectrolyte and the surface, but also the entropic contribution is of great importance [6], due to the release of the counterions to the bulk, in this case counterion Na^+ present in the polyelectrolytes and also due to the cooperative effect between the first polyelectrolyte chains adsorbed on the following polyelectrolyte chains.

3.3. Wettability of the P-Gln, P-Tyr and P-Met films

The contact angle values are shown in Table 1. The values of contact angle (θ_a) obtained for a drop of water on films of P-Gln, P-Tyr and P-Met and adsorbed on an amino-terminated surface at pH 3.5, changed between 71 ± 4° and 39 ± 2°, with high added salt and 57 ± 4° and 42 ± 2°, with low added salt. At pH 4.5, the values of the contact angle (θ_a) ranged between 59 ± 2° and 39 ± 5° with high added salt and between 54 ± 2° and 40 ± 4° with low added salt. The P-Gln film showed greater wettability than the P-Tyr and P-Met films with both quantities of added salt and pH levels, indicating that this polyelectrolyte is adsorbed exposing their -NH_2 groups to the air.

Advance angle values (θ_a) show that the hydrophobicity levels of the P-Gln, P-Tyr and P-Met films were higher than those of the APS monolayer ($\theta_a = 22°$) [36]. However, these values are more hydrophilic than the θ_a values of polyelectrolyte films derived from poly(maleic anhydride-*alt*-styrene) containing aryl-alkyl groups in the side chain. The reported θ_a values vary between 73 ± 4° and 69 ± 2° at low ionic strength, and between 70 ± 4° and 63 ± 3° at high ionic strength [8].

In the present work, low molecular weight polyelectrolytes (M_v of the polymers was 1.8×10^4 g/mol) (see section 2.2) were used to increase the mobility of the main polymer chain, which resulted in a larger fraction of functional groups available to interact with the amino-terminated surface. It has been reported that polymers of relatively low molecular weight possess high chain mobility, which is facilitated by increasing chain ends. This allows for more interaction with the amino-terminated surface, while subsequent wetting provides many contact points between the polymer and the amino-terminated surface [38].

The θ_a values obtained for P-Gln and P-Tyr and films adsorbed at pH 3.5, that is, the polyelectrolytes that contain polar groups in their side chain have θ_a values similar to those obtained at pH 4.5, at both salt concentrations. P-Gln and P-Tyr are mainly adsorbed onto the amino-terminated surfaces exposing the –COOH and –OH groups to the air. Due to their pK_{app} values (Table 3), these groups can form hydrogen bonds with the drop of water deposited onto the film, obtaining more hydrophilic θ_a values. Zhang et al. found that the presence of −COOH and nitrile groups that participate in hydrogen bonding with water improve surface hydrophilicity [38]. The θ_a value of the P-Met film varied significantly in function of pH, at low and high added salt.

At pH 3.5 and high ionic strength, P-Met adopt loop- and tail-like shapes. In this conformation it is probable that the amino acid residue of known hydrophobic character left exposed to the air providing a more hydrophobic character to the surface.

Finally, high hysteresis values of the P-Gln, P-Tyr and P-Met films adsorbed onto amino-terminated surfaces reflect the high degree of chemical heterogeneity of these films, especially the P-Met film, probably due to the presence of hydrophilic –COOH groups and the predominance of the hydrophobic alkyl groups of the side chain of this polyelectrolyte.

The different functional groups in the structures of the studied polyelectrolytes allow us to obtain nanostructured films for enzyme immobilization.

3.4. Morphology of the polyelectrolyte films

The morphology and roughness of polyelectrolyte films adsorbed onto amino-terminated surfaces were assessed by AFM. Fig. 4 shows AFM images of P-Gln, P-Tyr and P-Met films, adsorbed onto an amino-terminated surface at 0.001 and 0.1 mol/L of NaCl at pH 3.5. The morphological distribution of the polyelectrolyte films was generally homogenous, although there was a slight increase in roughness with a higher salt concentration, which is probably due to short-range interactions among the hydrophobic groups in the polyelectrolyte side chain. This can result in the formation of small islands (Fig. 4). This behavior is reflected in the higher RMS values of the P-Gln, P-Tyr and P-Met films. The P-Met film had the highest roughness value, which agrees well with the highest hysteresis value found by contact angle for this film. Similar behavior has been observed with polyethylenimine and poly(isobutylene-*alt*-maleic anhydride) films on amino-terminated surfaces [22].

3.5. Immobilization of Glucose-6-phosphatedehydrogenase adsorbed onto polyelectrolyte films

Once the films were obtained from polyelectrolytes modified with amino acid, the effect of the amino acid moiety on the ability of the surface to adsorb *Lm*G6PDH was evaluated. After characterizing the surface properties of the films, we continued only with the films prepared at pH 4.5 and NaCl 0.1 mol/L, since they had similar $\Gamma_{plateau}$ values, and hysteresis behavior, as well as homogeneous topography according to the RMS values.

A slice from each polyelectrolyte film adsorbed onto amino-terminated surfaces was incubated in a solution of 0.025 mg/mL of the *Lm*G6PDH. In all cases, the greater the time of adsorption of the enzyme (Fig. 5a), the greater the amount of enzyme, until a plateau was reached at 4 h. Interestingly, the final amount of adsorbed *Lm*G6PDH was affected by the nature of the amino acid residue in the polyelectrolyte. After 16 h of incubation, the P-Gln film adsorbed 3.9 μg, P-Met film 1.8 μg and P-Tyr film 2.9 μg of enzyme. These results indicate that the nature of the amino acid residue in the film controls the quantity of adsorbed enzyme, with a more a positive effect going from non-polar to polar side chain. In addition, there could be an effect of the topography of the films on the adsorption, it is probable that less rough surfaces leave functional groups more exposed to establish interactions with the enzyme, which contributes to increase the amount of adsorbed enzyme, this is related to the hysteresis and RMS values found for the films of the obtained polyelectrolytes at pH 4.5 and NaCl 0.1 mol/L, in particular for the P-Gln and P-Tyr film. Contrariwise, for rough surfaces as in this case the P-Met film obtained (Fig. 5c), could present interstices of smaller size than the enzyme, becoming regions where the enzyme cannot bind. Globular structures can be observed in the image of the polyelectrolytes films (Fig. 5e–g) after 16 h of immersion in a solution of 0.025 mg/mL *Lm*G6PD, probably due to the presence of the adsorbed enzyme, these structures show an increase in the roughness of the films of the polyelectrolytes with adsorbed enzyme in relation to its counterpart without adsorbed enzyme. It should be noted that, these polyelectrolytes are able to immobilize *Lm*G6PDH at a significantly lower enzyme concentration than is used to immobilize other enzymes. For instance, lysozyme was adsorbed at 1 mg/mL [39] and the horse radish peroxidase at concentrations between 12.5 and 300 mg/mL [40].

3.6. Activity of Glucose-6-phosphatedehydrogenase adsorbed onto polyelectrolyte films

The specific activity of the enzyme in solution was measured. This result showed that the specific activity of the enzyme in solution was stable throughout the incubation period (550 μmol NADH $min^{-1} mg^{-1}$), indicating that the enzyme did not change during the process. We also assessed the enzymatic activity of the *Lm*G6PDH adsorbed on polyelectrolyte films. Fig. 6 shows NADH production per reaction over time, starting with the immersion of the film in the reaction mixture. In all cases, a given quantity of NADH was detected after the reaction dead-time. The dead-time quantity was lowest with P-Gln, but then the NADH concentration increased steadily. The highest dead-time quantity was with P-Tyr, but the rate of increase after that was lower than that of P-Gln. Most notably, the initial quantity of NADH with P-Met was low, but increased rapidly up to 30 s, when a more gradual slope was established. Thus, comparing the initial velocity in the case of P-Gln to those in P-Tyr and P-Met, the most active condition is obtained by the most hydrophobic film of polyelectrolyte surface. This response is supported by the adsorption behavior

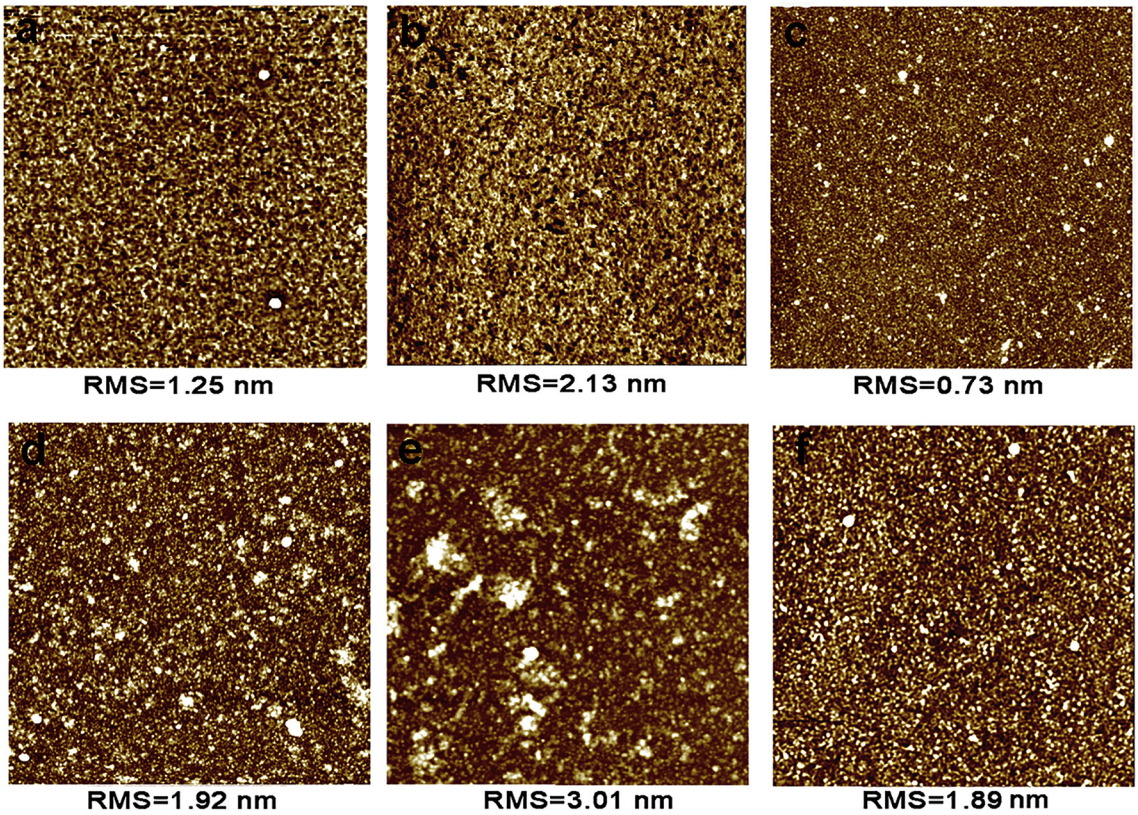


Fig. 4. AFM images of (a) P-Gln, (b) P-Met and (c) P-Tyr (M_v 1.8 x 10^4g/mol, C = 0.6 g/L), adsorbed onto an amino-terminated surface of 0.001 mol/L (above) and 0.1 mol/L (below) of NaCl at pH 3.5. Images (2.0 × 2.0 μm).

described above where the adsorption of P-Met at pH 4.5 and 0.1 mol/L NaCl on amino-terminal surfaces suggests an exposure of the amino acid residue of methionine oriented towards the air and therefore a surface of hydrophobic character. For this case, it is expected that the presence of the -CH_2- group in the residue provides flexibility to the surface which would allow preserve the conformation of the active site of the enzyme. The presence of a rapid phase is somewhat reminiscent of the pre-steady-state kinetics of soluble enzymes, perhaps related to restraints in the structural dynamics of the enzyme imposed by the environment at their adhesion points in the polyelectrolyte surface.

Taking the rate of product formation (Fig. 6) for *Lm*G6PDH adsorbed onto the silicon wafer with pre-adsorbed P-Met film, normalized by the amount of bound enzyme, the resulting value (50 μmol NADH min^{-1} mg^{-1}) is approximately ten times as low as the specific activity of the soluble enzyme (see above). This difference could be due to substrate accessibility to the active site, which could be limited for some enzymes in a population of randomly bound enzymes. It is probably that the increased roughness of the P-Met surface could affect the accessibility of substrate to the active site.

Nonpolar interactions have proved to be favorable with respect to interaction with immobilized enzymes. Silva et al. concluded that hydrophobic interactions between surface patches in lysozyme and the alkyl side groups in a polycation-possessing aliphatic chains of 5 carbon atoms drove adsorption in their system [39]. They also provided evidence for interactions of apolar side chains in polycations with hydrophobic clefts in bovine serum albumin or β-lactoglobulin.

Other authors have studied the Glucose-6-phosphate dehydrogenase as a model system of coenzyme requiring enzyme. Glucose-6-phosphate dehydrogenase immobilized on alumina particles was coupled with the soluble form of bound NADP in a fluidized bed type of reactor. [41].

On the other hand, Simon et al. found that the oriented immobilization of *Lm*G6PDH by disulfide bond formation (using engineered mutant forms containing cysteine residues located at specific points in the surface) was important in order to retain 50% of the specific activity of the soluble form [31] In the present case, the hydrophobic P-Met film allowed the non-oriented immobilization of the enzyme, with a resulting specific activity lower than but still comparable to that observed by Simon et al. This indicates that the polyelectrolyte films functionalized with amino acids, presented here as *ad-hoc* surfaces for the adsorption of enzymes, can retrieve activity values comparable with adsorption systems based in the orientation of the immobilized enzyme.

Taken together, our results allow to present surfaces prepared from functionalized polyelectrolytes as a simple platform, easy to prepare, without the need for covalent binding. Further studies are needed using amino acid-functionalized polyelectrolytes to address the effect of other amino acid residues and mixtures of them, as well as the stability, desorption and recycling of the bound enzyme. In this way, these films really represent a platform for enzyme immobilization that could be used to evaluate the effect of any amino acid residue on the catalytic performance of bound enzymes.

4. Conclusion

The adsorption behavior of polyelectrolytes onto an amino-terminated surface depends on the chemical nature of these systems, their ionic strength and the pH level of the solution. The polyelectrolyte films exhibited a moderate degree of hydrophobicity, which depended on the hydrophobic/hydrophilic balance of the amino acid residues exposed to the air. The polyelectrolyte films showed a high level of hysteresis due to the high degree of chemical heterogeneity of the polyelectrolyte film adsorbed onto the amino-terminated surfaces. AFM images showed homogeneous morphological distribution of the polyelectrolyte adsorbed on the amino-terminated surfaces, with the formation of small islands as ionic strength increased. Globular structures were observed in the image of the polyelectrolytes films with *Lm*G6PD adsorbed, these

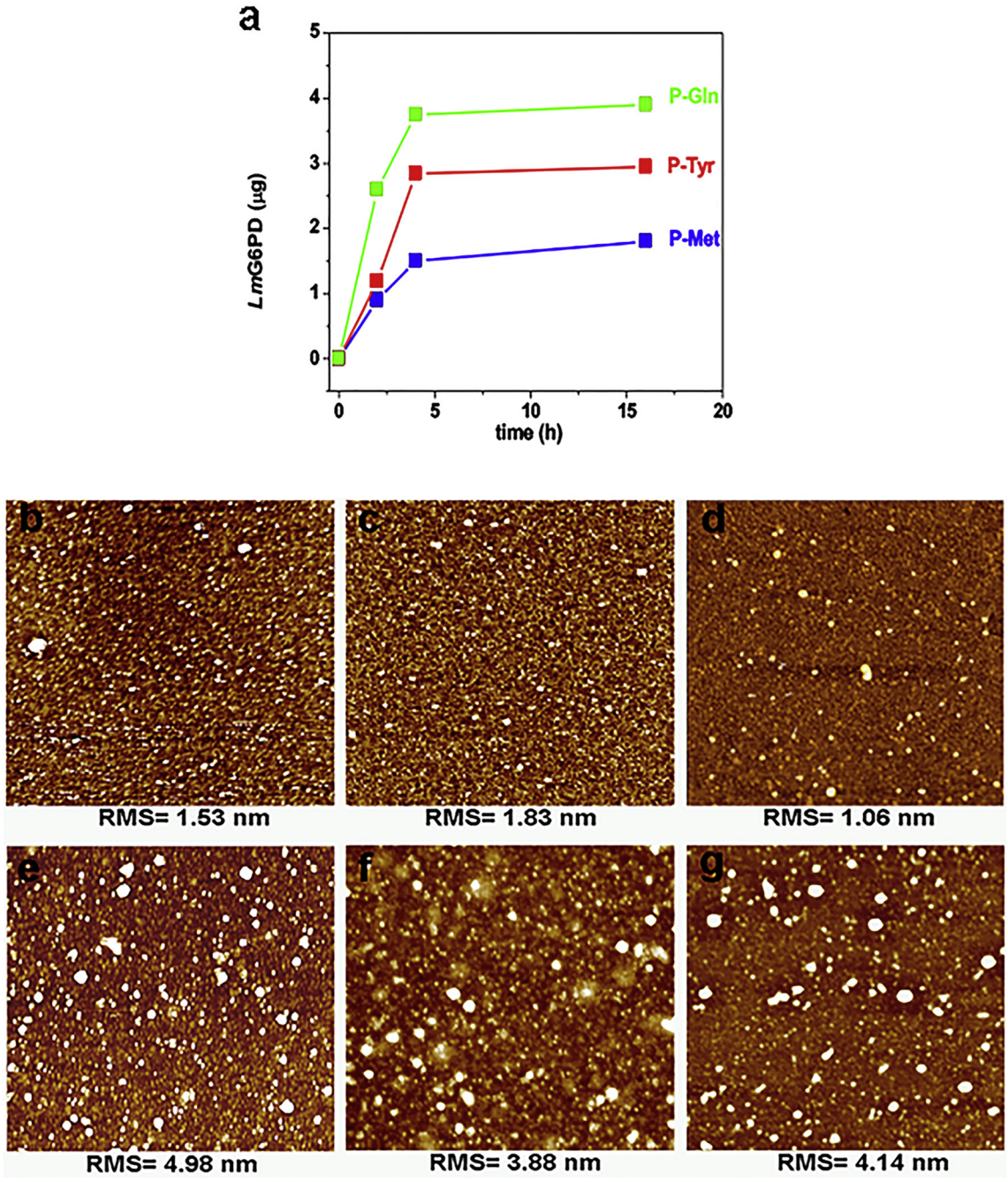


Fig. 5. (a) *Lm*G6PDH adsorption onto P-Gln, P-Met P-Tyr and films over time. The process was initiated by immersing the surface in a 0.025 mg/mL solution of *Lm*G6PD. AFM image of (b) P-Gln, (c) P-Met and (d) P-Tyr films obtained in 0.1 M NaCl and pH 4.5 conditions. AFM images of the *Lm*G6PD enzyme adsorbed onto (e) P-Gln, (f) P-Met and (g) P-Tyr films. Images (2.0 × 2.0 μm).

structures showed an increase in the roughness of these films in relation to polyelectrolytes films, corroborating the adsorption of the enzyme.

The specific activity of the adsorbed enzyme was higher on the P-Met film, independent of the quantity of enzyme adsorbed. Thus, the hydrophobic character of this polyelectrolyte proves to be more favorable with respect to the specific activity of the immobilized species. For P-Met it is expected that the presence of the -CH_2- group in the residue provides flexibility to the surface which would allow preserve the conformation of the active site of the enzyme.

In conclusion, films obtained from polyelectrolytes functionalized with amino-acids are presented as a simple and low-cost platform for the adsorption of the enzyme glucose 6-phosphate dehydrogenase, maintaining its properties, as well as, its enzymatic activity. We believe that this platform could be useful for immobilization of other enzymes and interesting for biocatalysis applications.

Author contributions

The manuscript was written with contributions by all the authors, all of whom have given approval to the final version of the manuscript. Ximena Briones, Valeria Villalobos, Yves Queneau, Caroline Silva Danna, Rodrigo Muñoz, Hernán E. Ríos, Jorge Pavez, Maritza Páez, Ricardo Cabrera, Laura Tamayo and Marcela D. Urzúa contributed equally.

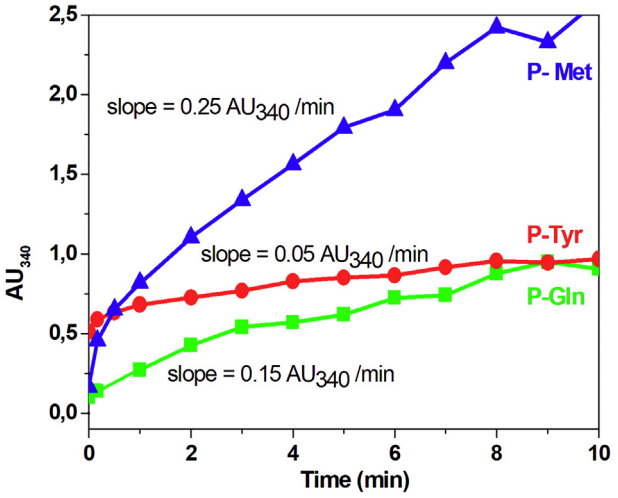


Fig. 6. NADH production by *Lm*G6PD adsorbed on the surface of silicon wafers modified with polyelectrolytes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2019.109938.

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