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Surface coverage dictates the surface bio-activity of D-amino acid oxidase

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ABSTRACT

This work presents a systematic study on the relationship between the adsorption mechanism and the surface bio-activity of D-amino acid oxidase (*pk*DAAO). This rational approach is based on measuring the characteristic filling and relaxation times under different experimental conditions. With such a goal, real-time adsorption–desorption experiments at different degrees of surface coverage were performed tuning the electrostatic and hydrophobic interactions by changing the pH condition for the adsorption and the substrate properties (silica or gold). Surface bio-activity was measured *in situ* by amperometry using the bio-functional surface as the working electrode and *ex situ* by spectrophotometry. On both solid substrates, *pk*DAAO adsorption is a transport-controlled process, even under unfavorable electrostatic interactions (charged protein and substrate with the same sign) due to the high percentage of basic amino acids in the enzyme. On silica, the relaxation step is electrostatic in nature and occurs in the same time-scale as filling the surface when the substrate and the enzyme are oppositely charged at low surface coverage. Under unfavorable electrostatic conditions, the relaxation (if any) occurs at long time. Accordingly, the bio-activity of the native *pk*DAAO is preserved at any surface coverage. On gold, this step is driven by hydrophobic interactions (pH-independent) and the surface bio-activity is highly dependent on the degree of surface coverage. Under these conditions, the surface bio-activity is preserved only at high surfaces coverage. Our results clearly indicate that *pk*DAAO bio-functionalized surfaces cannot be coupled to amperometry because the analyte interferes the electrochemical signal. However, this simple bio-functionalized strategy can be joined to other detection methods.

1. Introduction

The adsorption of proteins on solid substrates is of primary importance in biomedical applications ranging from biosensors to tissue engineering [1]. For instance, protein–substrate interactions give a real insight into the design of highly specific bio-functional surfaces and the development of new biomaterials. The key issue behind these applications is to control the strong perturbation produced by the solid substrate on the surface bio-activity. Although this topic has been extensively explored [2–4] the relationship between the adsorption mechanism and the surface bio-activity is still not well understood. This limitation is mostly due to the extra complexity provided by the lateral interactions upon crowding the solid substrate with protein molecules [5,6].

Understanding the protein adsorption mechanism comprises the study of several stages, from the transport of the molecule toward the substrate up to the final protein conformation, which ultimately dictates the surface bio-activity [7]. After the first protein attachment step, the molecules optimize their interaction with the substrate, leading to relaxation with some degree of spreading. The extent of the protein spreading depends on the ratio between the rates of these two steps (*i.e.* filling the surface and protein relaxation). Experimentally, this ratio is evaluated from the characteristic filling (τ_f) and relaxation (τ_r) times which can be calculated from real-time adsorption profiles [8]. A limiting value of this ratio is given by $\tau_f \gg \tau_r$, where all the protein molecules have the same relaxed conformation in steady state because a quick relaxation occurs after the attachment. This relaxation step strongly affects the protein conformation of all the adsorbed molecules and the degree of surface coverage does not depend on the filling rate. In

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the other limit ($\tau_f \ll \tau_r$), the relaxation step is too slow and the adsorbed proteins do not change their conformation upon adsorption. In this case, the degree of surface coverage is also independent of the filling rate because the substrate crowding is so fast that prevents protein–substrate optimization. Finally, when both steps are competitive ($\tau_f \sim \tau_r$), the protein spreading is correlated with the surface coverage because the relaxation of the molecules strongly depends on the available surface sites [7,9].

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the conversion of D-amino acids to α-keto acids in the presence of O_2 to yield H_2O_2. DAAO is strictly stereospecific and oxidizes a variety of D-amino acids which have been recently linked to aging and pathological conditions such as schizophrenia, epilepsy, Alzheimer's disease, and renal disease [10–14]. Therefore, DAAO bio-functionalized surfaces have been used as the first step toward biosensor development to specifically detect D-amino acids [15–18]. Since H_2O_2 is one of the enzymatic products, the bio-recognition event has been mostly detected by electrochemical methods [19,20]. In fact, several articles have reported the performance of DAAO-based biosensors using this enzyme from different sources, diverse solid substrates, and immobilization strategies [13,15,17,18]. Most of these works are mainly devoted to the analytical response of the designed biosensors with little attention regarding the interactions between the enzyme and the substrates and, more importantly, the consequences of the surface perturbation on the bio-activity of adsorbed DAAO. Furthermore, DAAO has also been used for biocatalytic applications, particularly in the two-step conversion of the natural antibiotic cephalosporin C to 7-amino cephalosporanic acid [21,22]. Although this process represents the most important industrial use of DAAO, the enzyme–support interactions and their effect on the bio-activity have not been entirely described.

The purpose of this work is to systematically study the relationship between the adsorption mechanism and the surface bio-activity of *pk*DAAO (commercially available from pig kidney). This rational approach to correlate the adsorption mechanism and surface bio-activity implies measuring τ_f and τ_r under different conditions, as previously indicated. With such a goal, real-time adsorption–desorption experiments at different degrees of surface coverage were performed tuning the electrostatic and hydrophobic interactions by changing the pH of the adsorption and the substrate properties (silica or gold). Surface bio-activity was measured *in situ* by amperometry using the bio-functional surface as the working electrode and *ex situ* by spectrophotometry to evaluate the extent of the relaxation step.

2. Experimental

2.1. Chemicals and solutions

All reagents were of analytical grade and were used without further purification: *pk*DAAO (Sigma), D-alanina (Fluka), H_2O_2, $KMnO_4$ (Cicarelli), KH_2PO_4, K_2HPO_4, $K_4P_2O_7$, $HClO_4$, NaOH, $KClO_4$ and KOH (Baker), $Na_2C_2O_4$ (Riedel-de-Haën), and $KClO_4$ (Erba). Aqueous solutions were prepared by using 18 MΩ cm^{-1} resistance water (Milli-Q, Millipore, Billerica, MA). H_2O_2 concentration was determined by titration with $KMnO_4$ which was standardized against $Na_2C_2O_4$. 5 mM buffer solutions (PB) were prepared by dissolving the desired amount of KH_2PO_4, K_2HPO_4, $K_4P_2O_7$ in water or 100 mM $KClO_4$ (PBS) and adjusting the pH with either 2 M KOH or 2 M $HClO_4$ to reach pH 5.0, 7.0 or 8.5. The pH measurements were performed with a combined glass electrode and a digital pH meter (Orion 420A+, Thermo, Waltham, MA). Unless noted, all experiments were performed at room temperature (26 ± 2 °C).

2.2. Substrates

The sorbent properties were studied using two different substrates: silica (Si/SiO_2) and gold (Si/SiO_2/Au), both prepared on silicon wafers (100 mm, Silicon Valley Microelectronics, Inc., Santa Clara, CA). In order to obtain a silica layer of about 100 nm thick (essential for obtaining a high sensitivity in reflectometry experiments [23]) the wafers were oxidized at 1000 °C for 1 h (thickness was verified by ellipsometry) and cut in strips (1 cm × 4 cm) following the crystallographic plane of silicon (1 0 0). Prior to each adsorption experiment, Si/SiO_2 strips were cleaned with boiling piranha solution (2:1 H_2SO_4:H_2O_2) and rinsed thoroughly with deionized water. (*Caution! Piranha solution is a powerful oxidizing agent that reacts violently with organic compounds; it should be handled with extreme care*). Si/SiO_2/Au substrates were prepared by sputtering (SPI #12162-AB) gold on the oxidized wafer strips up to reaching a 10 nm layer (thickness was verified by ellipsometry) and used without any further treatment. This thickness did not substantially modify the optical behavior of the 100 nm SiO_2 layer [24].

2.3. pkDAAO adsorption mechanism

Real-time adsorption–desorption experiments were performed in a reflectometer (AKZO Research Laboratories, Arnhem), equipped with a stagnation point flow cell as described elsewhere [25,26] using Si/SiO_2 and Si/SiO_2/Au substrates. Briefly, from 0 to 200 s, only PB was introduced into the cell and a stable baseline was obtained. Then (from 200 to 2700 s), the flow was switched from PB to a *pk*DAAO solution (prepared in the same buffer). Next (between 2700 and 3500 s), the flow was switched back to the initial PB solution in order to analyze the desorption process by dilution. Desorption was also induced by applying a constant potential (potentiostat CHI760C; CH Instruments, Inc.). This desorption was induced on the Si/SiO_2/Au substrates (working electrode) by using a platinum wire (counter electrode) and a $Ag/AgCl/KCl_{sat}$ (reference electrode) placed directly in the reflectometer cell from 3500 to 4000 s. The adsorption–desorption experiments were conducted with *pk*DAAO at different concentrations (ranging from 0.001 to 0.100 mg mL^{-1}) and pH values (5.0, 7.0 and 8.5).

As described elsewhere [27], to calculate the sensitivity factor (Q-factor) that provides the proportionality constant between the measured signal and the adsorbed amount, the substrate was modeled as a Si substrate (refraction index of 3.80) with a 100 nm SiO_2 layer (refraction index of 1.46) and a 10 nm Au layer (refraction index of 0.10) immersed in aqueous solution (refraction index of 1.333) and the increment in the refraction index with the protein concentration (dn/dc) was considered to be 0.18 [28]. The calculated Q-factors resulted in 30 ± 5 mg m^{-2} for Si/SiO_2 and 110 ± 5 mg m^{-2} for Si/SiO_2/Au.

The protein transport toward the substrate was well controlled by the stagnation point flow of the reflectometer setup, so the supply rate depended on the geometry of the cell, the flow rate, the diffusion coefficient of the macromolecule (D), and its concentration in solution (C_p) [29]. Hence, for a particular cell setup and protein molecules, the supply rate was directly proportional to C_p. The proportionality coefficient provided the transport constant ($k_{tr} = 5 \times 10^{-6}$ m s^{-1}) of the protein ($D = 6 \times 10^{-11}$ m^2 s^{-1}) at the specified cell setup.

2.4. Enzyme bio-activity

2.4.1. pkDAAO in solution

Electrochemical experiments in solution were carried out by using two amperometric methods to determine the H_2O_2 concentration produced by the enzymatic reaction in the presence

of D-alanine: (1) batch experiments: a potentiostat CHI101 (CH Instruments, Inc.) was employed with a three electrodes cell (8.5 mL) containing a gold working electrode (geometric surface area 0.12 cm^2), a reference electrode (Ag/AgCl/KCl_{sat}) and a platinum wire as counter electrode. (2) Flow injection analysis (FIA): a peristaltic pump (Gilson Miniplus 3), adjusted to 1.0 mL min^{-1} flow rate and a injection valve (Rheodyne Type 7125) with a 20 μL sample injection loop were used together with an electrochemical flow cell consisting of glassy carbon disk electrode (3 mm diameter MF-2012 BAS), a Ag/AgCl/KCl_{sat} reference electrode and a stainless steel block as counter electrode, with a 51 μm TG-2M Teflon cell gasket (MF-1046 Bioanalytical Systems, BAS), and an LC-4C amperometric detector (BAS).

Amperometric measurements were conducted in a stirred PBS solution (used as the electrolyte to enhance the electrochemical signal) by applying a potential (H_2O_2 oxidation) of 450 mV (pH 8.5), 550 mV (pH 7.0), or 650 mV (pH 5.0) and allowing the transient currents to decay to a steady-state value prior to the addition of the analyte and subsequent current monitoring. The analyte was a 100 μM H_2O_2 solution (calibration curve), 100 μM–65 mM D-alanine solution (blank) or the enzymatic reaction mixture prepared in PB. This reaction mixture was prepared by adding D-alanine (ranging from 100 μM to 65 mM) solutions to a fixed concentration *pk*DAAO (1 μg mL^{-1}, 5 μg mL^{-1} or 10 μg mL^{-1}) solution. 100 μL aliquots of this mixture were added to the electrochemical cell at different stages of the enzymatic reaction. Calibration curves were performed before and after each activity experiment to check the reproducibility and the electrode stability. The analyte addition (H_2O_2, D-alanine, or the enzymatic reaction mixture) was performed in triplicate.

2.4.2. Adsorbed pkDAAO

Surface bio-activity experiments were performed by applying two methods: (1) batch amperometry as previously described using *pk*DAAO bio-functionalized gold strip (1.5 cm × 0.5 cm) as working electrode. Blank experiments were conducted by adding D-alanine to denaturalized *pk*DAAO bio-functionalized gold electrodes. (2) Spectrophotometry assay: based on a modified version of a previously reported method [30] for the reaction of D-alanine to the corresponding α-keto acid and H_2O_2. The produced α-keto acid then reacts with hydrazine to produce hydrazone, which is determined spectrophotometrically at 445 nm [30]. The assays were performed either by using a solution of *pk*DAAO or by dipping *pk*DAAO bio-functionalized Si/SiO_2 or Si/SiO_2/Au wafers (strips of 1.5 cm × 0.5 cm) in the adsorption solution at the selected conditions (pH and protein concentration) for 1.5 h. Afterwards the bio-functional surface was rinsed with PB in order to remove loosely bound protein and immersed in the reaction solution (300 μL D-alanine 60 mM in PB) and incubated for 72 h. Finally, 300 μL of 2,4-dinitrophenyl hydrazine was added, followed by 600 μL of concentrated NaOH. The absorbance of each solution was measured at 445 nm after the reaction was completed, and the activity was calculated and contrasted against the activity of *pk*DAAO in solution. Surface area was calculated as the geometric area of the wafer in contact with the reaction solution.

3. Results and discussion

3.1. pkDAAO adsorption mechanism

It is well-known that the adsorption–desorption process depends, among other variables, on the electrostatic interactions between the proteins and the solid substrate [8,31]. In order to investigate the effect of these interactions, the adsorption process was measured above, at, and below the isoelectric point (IEP) of the enzyme (pH 7.0) [32,33]. At these three pH values, Si/SiO_2 substrate (PZC = 2.0) is negatively charged while Si/SiO_2/Au is only negative at pH higher than 4.8 [34]. These substrates show markedly different hydrophobicities as well: Si/SiO_2/Au is more hydrophobic than Si/SiO_2 [35,36]. Fig. 1 shows a typical adsorption–desorption kinetics profile, adsorbed amount (Γ) as a function of the adsorption time, of *pk*DAAO on Si/SiO_2/Au at pH 7.0. Similar behaviors were observed under all studied conditions of pH and *pk*DAAO concentrations on both Si/SiO_2 and Si/SiO_2/Au substrates. The measured stable ($t < 200$) baseline was followed by a significant increase in Γ, observed in a short period of time. In fact, steady state conditions were reached in less than 40 min in all the studied conditions. Γ did not significantly change with the addition of buffer solution or upon applying a constant potential (supporting information, Table SI 1). The low desorption percentages pointed to strong and stable interactions between *pk*DAAO and both substrates after reaching steady state conditions. It is important to note that H_2O_2 oxidation potential did not disrupt the adsorbed molecules indicating that amperometry was well-suited to determine the surface bioactivity.

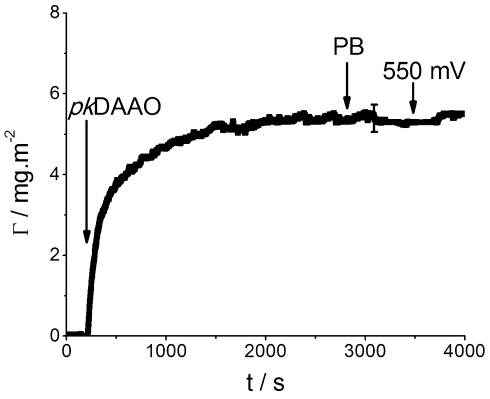

Fig. 1. Adsorbed amount (Γ) as a function of time during the adsorption–desorption process of *pk*DAAO (0.01 mg mL^{-1}) on Si/SiO_2/Au in PB at pH 7.0. The arrows indicate the addition of PB or 550 mV *vs.* Ag/AgCl/KCl_{sat} applied potential. The error bar represents the standard deviations of three repeats.

The profiles Γ–t are very useful to study the different steps involved in the overall protein adsorption process in order to evaluate the rate determining step and the extent of the relaxation process [7]. The initial slopes of the profiles provide with the initial adsorption rate (v_a) which is associated with the rate determining step of the adsorption process. The adsorption kinetic constant (k_a) was calculated from the slope of Fig. 2 that displays v_a as a function of the protein concentration (C_{pkDAAO}). The linear behavior up to 0.1 mg mL^{-1} indicated that the usual surface-limiting effect observed with attachment-controlled processes may occur at higher protein concentrations [25]. Furthermore, within experimental error, k_a values were not significantly different for the substrates and studied pH or to the value of k_{tr}. Therefore, the adsorption process of *pk*DAAO was transport-controlled with an average k_a value of $(2 \pm 1) \times 10^{-6}$ m s^{-1} for all the studied conditions. This behavior has not generally been observed under electrostatic repulsion between the adsorbed enzymes and the solid substrate [8,25,31] like *pk*DAAO at pH 8.5. For example, k_a values of BSA on SiO_2 were significantly different than k_{tr} (between 4 and 80 times lower, depending on the pH and ionic strength) [8,25], clearly indicating an adsorption barrier or, for that matter, a surface-controlled process. However, this behavior has been observed when proteins are adsorbed through their oppositely charged residues on repelling solid substrate [4,37–41]. Due to their complex structure, the surface of the enzymes exhibits domains, positively or negatively charged, whose positions depend on the

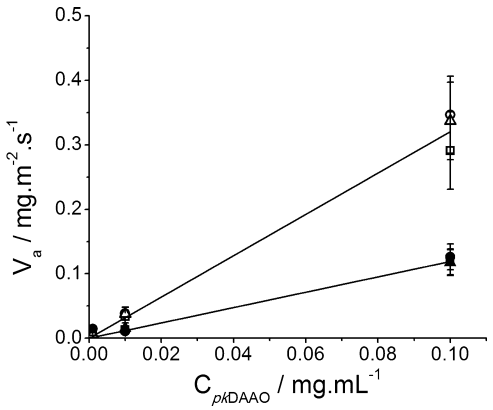

Fig. 2. Initial adsorption rate (v_a) as a function of the protein concentration on Si/SiO_2 (solid symbols) and Si/SiO_2/Au (open symbols) at three pH values: 5.0 (triangles), 7.0 (circles) and 8.5 (squares). Error bars denote standard deviations of three repeats.

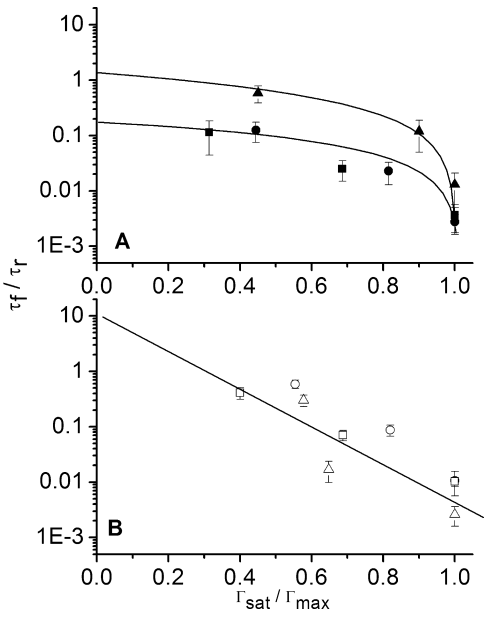

Fig. 3. The ratio between the filling (τ_f) and relaxation (τ_r) times as a function of the degree of surface coverage ($\Gamma_{sat}/\Gamma_{max}$) on Si/SiO_2 (A) and Si/SiO_2/Au (B) at three pH values: 5.0 (triangles), 7.0 (circles) and 8.5 (squares). Error bars denote standard deviations of three repeats. Lines only point out data trend.

local composition of the amino acids [4,42,43]. The primary structure of *pk*DAAO shows that 45% of the charged amino acids are basic with pK_a values higher than 10 (lysine and arginine) [34]. Therefore, the side chains of these amino acids were positively charged in all the tested pH conditions. The transport-controlled adsorption of *pk*DAAO suggested that the enzyme was adsorbed on Si/SiO_2 and Si/SiO_2/Au exposing these positively charged residues to the solid substrate even at pH values higher than the IEP.

As indicated in the introduction, a simple way to evaluate the relaxation step is to determine the relationship between the characteristic filling (τ_f) and relaxation (τ_r) times [8]. When the adsorption process is transport-controlled, τ_f can be calculated as the ratio between the saturation adsorbed amount (Γ_{sat}) and v_a [37], that represents an average filling rate because protein molecules continuously attach and relax on the sorbent substrate. The characteristic τ_r was estimated by extrapolating to zero Γ_{sat}; in such a condition, a protein molecule has enough room to optimize its interaction without any neighboring molecule hampering the protein spreading [9]. Table 1 displays the average Γ_{sat} values of three independent measurements and the extrapolated τ_r values from the corresponding τ_f *vs.* Γ_{sat} curves (supplementary information Fig. SI 1). These results clearly indicated that Γ_{sat} values were always higher for Si/SiO_2/Au than for Si/SiO_2 with a slight pH effect on both substrates. Furthermore, τ_f strongly depended on Γ_{sat} when *pk*DAAO was adsorbed on both substrates at the studied pH values.

Recalling that the adsorbed amounts on both substrates were different, Γ_{sat} values were normalized by the maximum adsorbed amount (Γ_{max}) measured at any given condition to provide with a closer idea of the actual degree of surface coverage. Fig. 3 shows the dependence of the ratio τ_f/τ_r on the degree of surface coverage ($\Gamma_{sat}/\Gamma_{max}$) on Si/SiO_2 (A) and Si/SiO_2/Au (B) at the three studied pH values. The ratio τ_f/τ_r decreased as $\Gamma_{sat}/\Gamma_{max}$ increased indicating that the degree of surface coverage had an effect on the extent of the relaxation step. This effect was less pronounced on Si/SiO_2 substrate at pH 7.0 and 8.5 ($\tau_f/\tau_r < 1$ at any given coverage). On the other hand, this ratio approached 1 (or even exceeded it) at low surface coverage on Si/SiO_2 at pH 5.0 and at the three pH values on Si/SiO_2/Au substrate. This last behavior indicated that the enzyme attachment and relaxation were competitive steps. Moreover, at very low coverage the relaxation may be faster than filling the surface (*i.e.* every attached enzyme relaxed on the surface when it had enough room on the substrate). It is important to note that τ_r showed the lowest value at pH 5.0 on Si/SiO_2 *i.e.* the relaxation rate was the fastest.

Besides the first attractive electrostatic interaction which leads to a transport-controlled adsorption process, the hydrophobic character of Si/SiO_2/Au induced the spreading of *pk*DAAO to exhibit the nonpolar residues toward the surface. This relaxation step is driven by the amount of water molecules removed from the substrate upon the enzyme unfolding. This relaxation step is also responsible of the high affinity between the enzyme and the solid substrate observed in steady state conditions at low *pk*DAAO concentrations (Table 1). In fact, these Γ_{sat} values depend on the balance between the available surface sites and the effective area occupied by each adsorbed (relaxed) molecule. At high *pk*DAAO

Table 1
Saturation adsorbed amount (Γ_{sat}) and relaxation time (τ_r) as a function of pH and *pk*DAAO concentrations ([*pk*DAAO]) on Si/SiO_2 and Si/SiO_2/Au. Error bars denote standard deviations of three repeats.

pH	[*pk*DAAO] (mg mL^{-1})	Γ_{sat} (mg m^{-2}) Si/SiO_2	Γ_{sat} (mg m^{-2}) Si/SiO_2/Au	τ_r (s) Si/SiO_2	τ_r (s) Si/SiO_2/Au
5.0	0.100	2.1 ± 0.3	7.1 ± 0.6	10^2	10^3
	0.010	1.8 ± 0.3	4.6 ± 0.6		
	0.001	0.9 ± 0.3	4.1 ± 0.1		
7.0	0.100	2.7 ± 0.4	8.3 ± 0.1	10^3	10^3
	0.010	2.2 ± 0.4	6.8 ± 0.7		
	0.001	1.2 ± 0.2	4.6 ± 0.3		
8.5	0.100	3.5 ± 0.5	8.0 ± 0.5	10^3	10^3
	0.010	2.4 ± 0.4	5.5 ± 0.4		
	0.001	1.1 ± 0.1	3.2 ± 0.1		

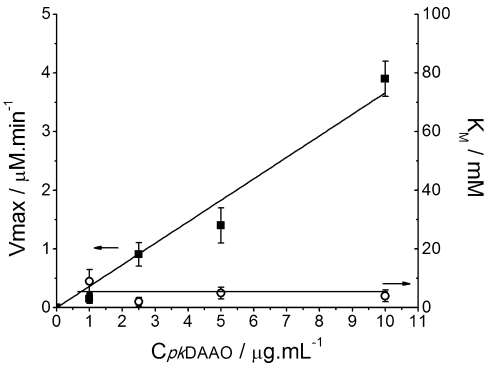

Fig. 4. Maximum enzyme rate (V_{max}) as a function of *pk*DAAO concentration (C_{pk}DAAO) in PB at pH 8.5. Applied potential: 450 mV *vs.* Ag/AgCl/KCl_{sat}. Error bars denote standard deviations of three repeats.

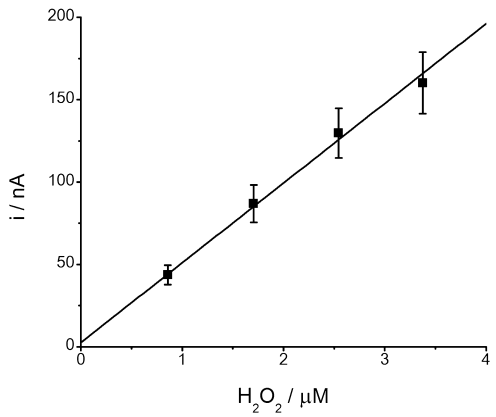

Fig. 5. Calibration curve. Amperometric response (i) as a function of H_2O_2 concentration in PB at pH 8.5 to measure the *pk*DAAO surface activity. Applied potential: 450 mV *vs.* Ag/AgCl/KCl_{sat}. Working electrode: *pk*DAAO bio-functionalized Au. Counter electrode: Pt. Error bars denote standard deviations of three repeats.

concentrations, surface crowding and lateral interactions between adsorbed enzymes restrict the relaxation extent and the value of Γ_{sat}. High affinity adsorption isotherms together with a higher percentage of relaxed molecules at low degree of surface coverage were also observed when *pk*DAAO was adsorbed on Carbon nanotubes [33].

On the other hand, hydrophobic contribution (if any) is less significant than electrostatic interactions on the hydrophilic Si/SiO_2 substrate. Hence, the relaxation step at pH 5.0 is not hydrophobic in nature but it is controlled by the electrostatic interactions between the positive enzyme residues and the negatively charged solid substrate. *pk*DAAO at pH 5.0 has a high positive charge (+7.2 relative to the pI) [32,33] which enhances the contact points between the enzyme and the oppositely charged surface sites in shorter time than hydrophobic interactions. This high positive charge also inhibits the values of Γ_{sat} due to the electrostatic repulsion between adsorbed enzymes. At the other two pH values, the relaxation step is less important on Si/SiO_2 than at pH 5.0 because the positive charge of *pk*DAAO decreases with a concomitant reduced possibility of increasing the electrostatically favorable contact points between the enzyme and the substrate. It is worth mentioning that the adsorption process is practically independent of these pH values because the reduction of the positive charge from pH 7.0 to 8.5 of the protein is compensated by increasing the negative charge of the solid substrate.

3.2. Enzyme bio-activity

Prior to evaluate the surface bio-activity, the kinetic parameters that describe the *pk*DAAO activity in solution were determined amperometrically (batch and FIA) using D-alanine as the enzyme substrate and the produced H_2O_2 as the analyte. The calibration curves and the analytical parameters to quantify H_2O_2 are given as supplementary information (Fig. SI 2–4 and Table SI 2) together with the *pk*DAAO kinetic curves (Fig. SI 5). It is important to note that the addition of D-alanine also caused a change in the current intensity which was constant for all the studied concentrations and negligible at H_2O_2 concentrations higher than 10 μM (supplementary information Fig. SI 2). In any case, this interference was also considered to calculate the H_2O_2 concentration produced during the enzymatic reaction. Fig. 4 shows the maximum reaction rate (V_{max}) and the Michaelis constant (K_M) as a function of *pk*DAAO concentration (from 1 μg mL^{-1} to 10 μg mL^{-1}) at pH 8.5. As expected from the simple Michaelis–Menten model [44], V_{max} was directly proportional to the enzyme concentration and K_M remained invariant (within experimental error) with the enzyme concentration (5 ± 3 mM). At the same *pk*DAAO concentration, K_M values were of the same order while V_{max} was two orders of magnitude lower at pH 7.0. Finally, at pH 5.0 no activity could be measured from the amperometry experiments. This behavior agrees with reported results showing that D-amino acids with deprotonated amino groups are the best substrates for DAAO [5,45] and that pH 8.5 is optimum to measure DAAO enzymatic activity. It is important to note that these experiments also indicate that *pk*DAAO is a viable bio-recognition element of D-amino acids because it exhibits an amperometrically detectable bio-activity at concentrations as low as those expected on a bio-functional surface.

Surface bio-activity was measured at pH 8.5 using *pk*DAAO bio-functionalized gold substrates prepared at Γ_{max} as working electrodes. Fig. 5 shows a typical H_2O_2 calibration curves determined from the current intensity *vs.* time profile (supplementary information Fig. SI 6A). According to the calibration curve, H_2O_2 concentration as low as 1 μM (corresponding to current intensities to 40 nA) could be detected with the *pk*DAAO bio-functionalized working electrode. On the other hand, the change in the current intensity due to the addition of D-alanine to the electrochemical cell was comparable to the signal produced by H_2O_2 (supplementary information Fig. SI 6B). In fact, as previously mentioned, D-alanine interferes the H_2O_2 amperometric signal at low concentration. Consequently, even when *pk*DAAO bio-functionalized gold is well suited to be used as a working electrode to measure the change in current intensity associated with H_2O_2, the produced concentration by the enzymatic reaction is too low to overcome the D-alanine interference, even at the maximum *pk*DAAO adsorbed amount.

The enzymatic activity of *pk*DAAO either in solution or adsorbed on Si/SiO_2 and Si/SiO_2/Au was also determined spectrophotometrically. Fig. 6 shows the ratio between the enzyme activity in solution and the surface bio-activity as a function of the degree of surface coverage ($\Gamma_{sat}/\Gamma_{max}$) at pH 7.0 (A) and 8.5 (B) on both substrates. On Si/SiO_2 this ratio was close to one at both pH values at all degrees of surface coverage indicating that the surface bio-activity was the same as observed in solution with native *pk*DAAO. On the other hand, this ratio strongly depended on the surface coverage when *pk*DAAO was adsorbed on Si/SiO_2/Au: almost no surface bio-activity was detected at low coverage while it almost approached the activity of native *pk*DAAO upon crowding the sorbent substrate. In line with the proposed adsorption mechanism, the bio-activity of the native enzyme is preserved on the hydrophilic Si/SiO_2 substrate because the relaxation step is too slow (compared to the filling rate) at pH 8.5 and 7.0 to promote *pk*DAAO spreading. This correlation between the adsorption mechanism and the surface bio-activity is also clear on the hydrophobic Si/SiO_2/Au substrate which strongly induces the enzyme spreading at low degree of surface coverage. It has been widely demonstrated [3,46,47]

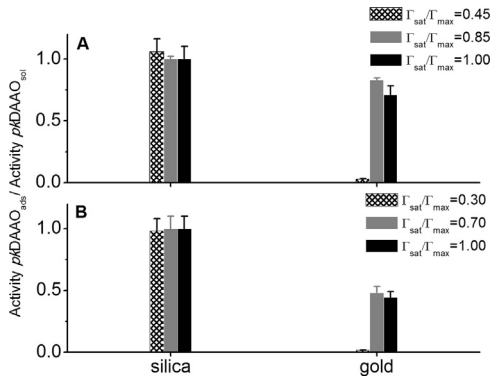

Fig. 6. Surface bio-activity expressed as the relationship between the enzymatic activity of adsorbed and native *pk*DAAO (*pk*DAAO$_{ads}$/*pk*DAAO$_{sol}$) for both solid substrate at different degree of surface coverage ($\Gamma_{sat}/\Gamma_{max}$) at two pH values: 7.0 (A) and pH 8.5 (B). Error bars denote standard deviations of three repeats.

that hydrophobic interactions disrupt the native structure of the enzyme, which in most cases leads to loss of bio-activity. An orientation mismatch may be discarded due to the strong dependence of the surface bio-activity concomitantly with the ratio between the filling and relaxation rates on the surface coverage. It is important to note that although the relative surface bio-activity was higher at pH 7.0, *pk*DAAO activity in solution was lower than observed at pH 8.5. Therefore, this pH value remains the optimum to measure the enzyme activity of *pk*DAAO in solution and adsorbed to solid substrates. Finally, these results clearly indicate that *pk*DAAO bio-functionalized surfaces cannot be coupled to the commonly used amperometry technique because D-alanine interference increases the detection limit of the method. However, this simple bio-functionalized strategy on both hydrophilic and hydrophobic substrates can be joined to other detection methods.

4. Conclusions

The adsorption of *pk*DAAO on silica and gold solid substrates is a transport-controlled process, even under unfavorable electrostatic interactions due to the high percentage of basic amino acids in the enzyme. On the other hand, the extent of the relaxation step depends on the surface properties. On silica, this step is electrostatic in nature and takes place in the same time-scale as filling the surface when the substrate and the enzyme are oppositely charged (pH 5.0) at low degree of surface coverage. Under unfavorable electrostatic conditions (pH 7.0 and 8.5), the relaxation (if any) occurs at long time. Accordingly, the bio-activity of the native *pk*DAAO is preserved at any degree of surface coverage. On gold, this step is driven by hydrophobic interactions (pH-independent) and the surface bio-activity is highly dependent on the degree of surface coverage. Under these conditions, the native bio-activity is preserved only at high degree of surfaces coverage. Consequently, the amount of adsorbed *pk*DAAO mostly controls the surface bio-activity and, hence the bio-recognition capabilities of the bio-functional surface.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2014.02.050.

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