Langmuir and Langmuir−Blodgett Films of Organophosphorus Acid Anhydrolase

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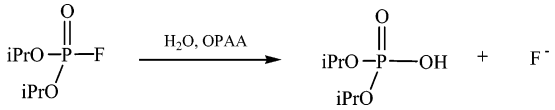
In this paper, we describe the preparation and characterization of Langmuir and Langmuir−Blodgett (LB) monolayers of the enzyme organophosphorus acid anhydrolase (OPAA). Langmuir films of OPAA were characterized on different subphases, such as phosphate, ammonium carbonate, and bis-tris-propane buffers. Monolayers at the air−water interface were characterized by measuring the surface pressure and surface potential−area isotherms. In situ UV−vis absorption spectra were also recorded from the Langmuir monolayers. The enzyme activity at the air−water interface was tested by the addition of diisopropylfluorophosphate (DFP) to the subphase. LB films of OPAA were transferred to mica substrates to be studied by atomic force microscopy. Finally, a one-layer LB film of OPAA labeled with a fluorescent probe, fluorescein isothiocyanate (FITC), was deposited onto a quartz slide to be tested as sensor for DFP. The clear, pronounced response and the stability of the LB film as a DFP sensor show the potential of this system as a biosensor.

1. Introduction

The potential use of enzymes for the decontamination of chemical warfare agents has been a subject of study for over 50 years. An enzyme-based decontamination system not only provides the rapid removal of toxic agents but is also environmentally safe and noncorrosive in nature. Despite the urge for more efficient detection systems, the fundamentals of the mechanisms involved in biotransformation of toxic compounds are still under discussion.

In 1946, Mazur[1] first reported a rabbit tissue enzyme with the ability to hydrolyze an organophosphorus compound, diisopropylfluorophosphate (DFP). Since then, the use of enzyme-based systems as toxic organophosphate (OP) decontaminants has been extensively investigated.[2−5] Over the years, a diverse number of neurotoxic OP compounds have been used as agricultural pesticides or chemical nerve warfare agents. In the past decade, two bacterial enzyme systems, organophosphorus hydrolase (OPH: EC 3.1.8.1) and organophosphorus acid anhydrolase (EC 3.1.8.2), have been identified and characterized for use in the detoxification of a variety of neurotoxins, including pesticides, G-type and V-type chemical nerve agents.[6,7] Organophosphorus hydrolase was well characterized as a metalloenzyme that contains one or two cobalt ions.[8,9] This enzyme has been shown to be effective in degrading a range of organophosphate esters such as paraoxon, parathion, as well as warfare GB and VX.[6] OPAA was characterized to be a prolidase (X-Pro dipeptidase) and the genes encoding this enzyme were cloned and sequenced from two species of *Alteromonas*.[9] Besides cleaving the C−N bond of X-Pro dipeptides, it catalyzes the hydrolysis of a wide range of OPs with P−F, P−O, P−CN, and P−S bonds.[10,11]. As an example, the enzymatic reaction catalyzed by OPAA is shown below.



The mechanism of action of proteins and enzymes is one of the most challenging issues in the field of biochemistry. Technological advances in genetic engineering and new physicochemical tools accelerated the understanding of the mechanism of enzymatic actions, and helped in the rapid isolation and purification of new compounds. Other studies also helped to understand the behavior of proteins in solution, their surface adsorption, and diffusion through the medium.[12−15]

The fact that proteins are water-soluble macromolecules is not an impediment for them to form monolayers at air−water interface. Since MacRitchie's[14] pioneering studies on this subject, much has been done and understood about this phenomenon. According to the theory, a high degree of insolubility of water-soluble molecules is not required for them to form a monolayer. It is only necessary that the individual monomer units have a finite free energy of adsorption from the bulk solution to the surface for a monolayer to be stable. One can point out the primary need of having a suitable electrolytic medium to attain conditions of the metastable equilibrium.[12] As a powerful tool, the Langmuir film technique is being used to investigate macromolecules at the air−water interface. In this technique,

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monolayers at the air−water interface may be studied in situ using surface pressure and surface potential−area measurements. The use of in situ spectroscopy and microscopy broadened enormously the understanding and usefulness of this technique. The possibility to obtain well-ordered films using movable barriers may lead to an artificially organized film, which is amenable to be transferred to a solid surface and characterized for potential applications. As an example, the behavior of acetylcholinesterase (AChE) at the air−water interface was investigated by in situ FT-IR, which revealed the integrity of the secondary structure of this enzyme.[16] AChE is well-known as a component in the impulse transmission in the nervous system. Its behavior was also investigated at the air−water interface in the presence of OP compounds. Based on the principle of AChE inhibition by OP compounds, the usage of a LB film of AChE on the design of a biosensor was proposed.[17] Different approaches of enzyme-based biosensors have been reported in the literature, regarding the immobilization techniques and the transducer systems. The importance of the detection of toxic materials in the environment, such as OP compounds, the components of several pesticides,[18−27] has been discussed and evaluated.

The purpose of this study is to investigate the surface chemistry of OPAA at the air−water interface and its interactions with organophosphorus compounds. We have used Langmuir and LB techniques to evaluate such interactions for its potential application in an enzyme-based DFP sensor.

2. Materials and Methods

2.1. Enzyme Solution. The enzyme OPAA (EC 3.1.8.2; MW 58.5 kD) was prepared from *Alteromonas* strain JD6.5 as reported elsewhere.[28,29] The spreading solvent for the enzyme was the same buffer used as the subphase in each experiment. Different buffers were prepared with different compositions and pH. Phosphate buffer (pH = 7.5) was prepared with KH_2PO_4 (Sigma-Aldrich, St. Louis, MO) 0.1 M and NaOH 0.1 M; carbonate buffer (pH = 8.5) was prepared with 0.5% ammonium carbonate; BTP buffer (pH = 7.2) was prepared with bis-tris-propane 10 mM, $MnCl_2$ 0.05 mM, and ditiotreitol (DTT) 0.1 mM. The typical enzyme concentration used was 1 mg mL^{-1}. DFP and paraoxon were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Methods. Surface pressure measurements were performed in two different troughs, Kibron μS-minitrough (Helsinki, Finland) and KSV 2000 (Helsinki, Finland). UV−vis in situ measurement was performed in the KSV trough, which is fitted with a quartz window at its center. The UV−vis spectrometer (Hewlett-Packard) was slid over the trough by a rail conveniently set to the experiment. Water used in the preparation of all solutions was collected from a Modulab System 2020 with resistivity of 18.2 MΩ cm, pH 5.5−6.0, and surface tension of 72.6 mN m^{-1}. All of the monolayer experiments were performed at a temperature of 20.0 ± 0.5 °C.

To use OPAA as an optical sensor for DFP, we labeled the enzyme with fluorescein isothiocyanate (FITC), OPAA/FITC (3:1, w/w). The excess of the fluorophore guarantees the efficiency of the labeling process. To separate the unreacted FITC, we used the dialysis membrane method (Spectra/Por Biotech DispoDialyzers, M_w = 8000, Rancho Dominguez, CA). Fluorescence measurements were performed with a Spex Fluorolog(1680) spectrofluorimeter.

OPAA monolayers were transferred as LB films onto hydrophobic quartz substrates. The preparation of a hydrophobic layer was performed in two steps: i. the preparation of the hydrophilic surfaces using the RCA method[30] and ii. the silanization of the dried surface with octadecyltrichorosilane (OTS), 10% in toluene. One monolayer of OPAA was deposited onto quartz slides with downward strokes. The surface pressure for deposition was 20 mN m^{-1}, and the deposition speed was 1.2 mm min^{-1}.

Atomic force microscopy (AFM) images were obtained in a Pico-SPM microscope from Molecular Imaging, Inc. (Phoenix, AZ). LB films of OPAA were scanned in air with a head scanner S and using AFM in tapping mode (Mac mode). Integrated silicon tips and cantilevers of nominal spring constants were used, and the force was maintained minimal during the scanning measurements. The low level of force was required to prevent the tip from penetrating the surface (soft film). The scan was performed at either 256 or 512 points per traverse, with the same number of traverse steps, forming a two-dimensional image 256 × 256 or 512 × 512 pixels.

3. Results and Discussion

One of the main concerns when using the Langmuir films technique to study biological systems is the water solubility of these materials, because the traditional subphase employed is an aqueous solution. Studies of protein films have been long reported, but the relationship between the spreading and the denaturation of the proteins still remains unclear.[14] Different approaches to obtaining a monolayer have been made by considering details such as spreading procedure, volume spread, equilibrium time, and the presence of charge in the biomacromolecules.[15] On spreading the enzyme from a diluted aqueous solution, the formation of the film at the air−water interface can be regarded as an adsorption phenomenon, similar to that formed from a solution with sufficient area for the biomacromolecules to migrate from the bulk to the surface. In the experiments reported here, we also observed several characteristics pointed out to be common to protein surface films. The OPAA enzyme used in this report has an optimal activity in the pH range from 6 to 10.[28] We used buffer solutions as a subphase and also added salt to the subphase to decrease the enzyme solubility. The area per molecule was calculated assuming that only the enzyme molecules were contributing to the formation of the film, despite the presence of impurities that might be dissolved in the bulk.

3.1. Phosphate and Ammonium Buffers. Using KCl-free phosphate and ammonium carbonate buffers as subphase, pH = 7.5 and 8.5, respectively, no reproducible results were obtained, possibly due to the solubilization of enzyme molecules in the bulk subphase. Stable monolayers were

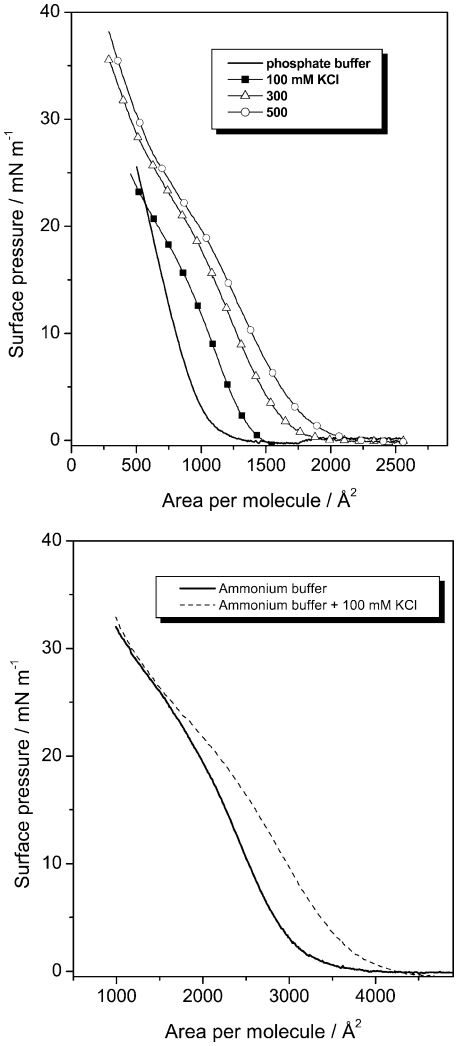

Figure 1. Surface pressure−area isotherms of OPAA. (a) Phosphate buffer, pH 7.5, at different KCl concentrations (100, 300, and 500 mM); (b) ammonium carbonate buffer, pH = 8.5, at KCl concentration of 100 mM.

formed, otherwise, when KCl was added to the subphase. The addition of salt in the subphase was tested in order to decrease the solubility of the enzyme monolayer and promote a better spreading condition. From Figure 1, parts a and b, three experimental evidences might be outlined. First, we observe that higher salt concentrations added in the subphase (buffers pH = 7.5 and 8.5) result in higher values for limiting molecular area. Although the shape of the isotherms is very similar for these two buffers, a large difference in the area per molecule at any surface pressure can be observed. For a phosphate buffer, the lifting points at nil surface pressure for each $\pi-A$ curve are 1330, 1450, 1900, and 2060 Å^2 per molecule for KCl concentrations of 0, 100, 300, and 500 mM, respectively. By using the ammonium buffer, the lifting points of KCl-free and KCl 100 mM isotherms appear at 2900 and 4000 Å^2 per molecule, respectively. The minimum molecular area for phosphate buffer is in the range of 1900−1100 Å^2, and from 4100 to 3500 for the ammonium buffer subphase. Second, as the amount of salt increases, the isotherms tend to show a kink point in the slope at approximately 15 mN m^{-1}, which was not observed for those in the KCl-free bulk subphase. The change in the slope can

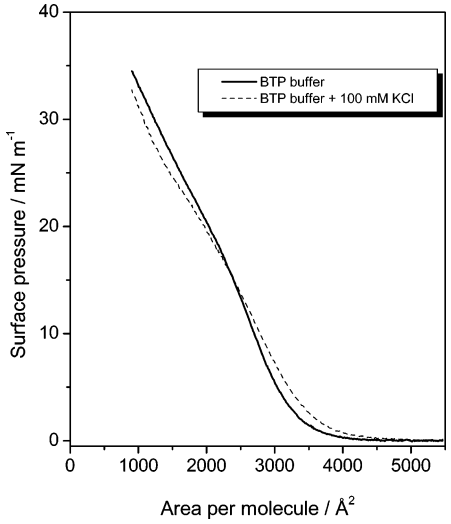

Figure 2. Surface pressure−area isotherms of OPAA on a BTP buffer subphase, pH = 7.2, in the absence and presence of KCl 100 mM.

be interpreted as a change in conformation of the enzyme. Third, as expected, no sharp change in the slope that could be considered a collapse is observed. For the phosphate buffer, KCl-free and KCl 100 mM subphase, the isotherms reach the highest surface pressures at 25 mN m^{-1}, whereas those with KCl concentration of 300 and 500 mM go up to ∼38 mN m^{-1}. Stability measurements (not shown here) were performed in cycles of compression/decompression, giving no hysteresis up to the pressure of 20 mN m^{-1}.

In situ UV−vis absorption spectra were measured at different surface pressures from a film spread on a subphase with phosphate buffer (not shown here). The band with its maximum at 202 nm (attributed to $\pi-\pi^*$ transitions) increases with the increasing surface pressure. A bathochromic shift of the absorption maximum (5−8 nm) of the monolayer, compared to that in solution, suggests a low degree of aggregation of the enzyme molecules at the air−water interface.

3.2. Bis-tris-propane Buffer. The conditions for BTP buffer experiments were similar to those previously reported in the study of OPAA activity on DFP.[29] In Figure 2, we show the surface pressure−area isotherms for the monolayers formed on the subphases with and without KCl. The salt effect here is much less pronounced when compared to that with the phosphate buffer. One possible explanation is that the ionic strength of the KCl-free buffer is already sufficient for the enzyme molecules to assume an extended and stable conformation without the addition of KCl. The limiting molecular area is approximately 2800 Å^2 molecule^{-1}, indicating changes in the conformation of the enzyme molecules in comparison to the phosphate buffer. The stability of OPAA monolayer on BTP buffer was confirmed by measuring the decrease of area per molecule with time at constant surface pressure of 15 mN m^{-1} (Figure 3). A decrease of less than 15% in the area over a period of 80 min was observed.

In Figure 4, the surface pressure and surface potential−area isotherms were recorded simultaneously. The surface potential starts increasing at a larger area per molecule (5000

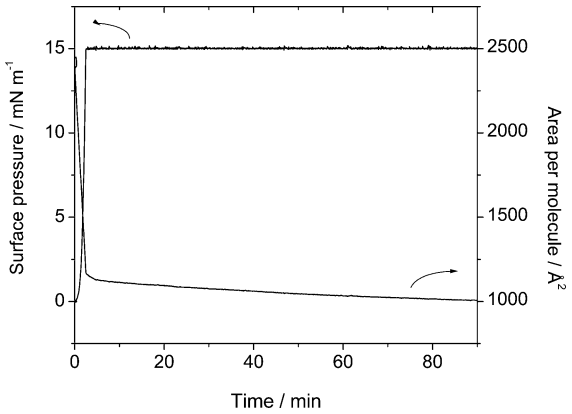

Figure 3. Change in area per molecule of OPAA monolayer at constant surface pressure of 15 mN m^{-1} on BTP buffer subphase, pH = 7.2.

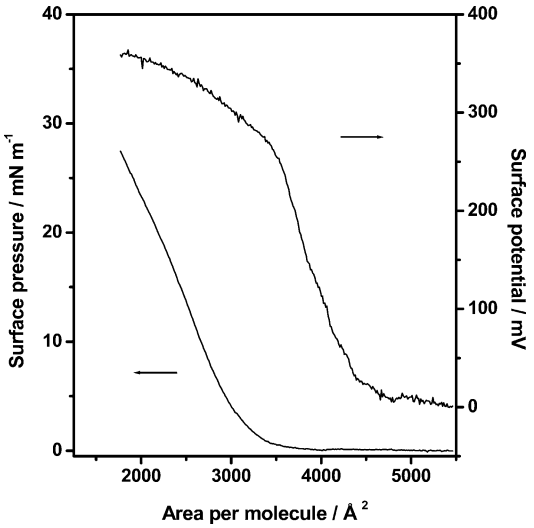

Figure 4. Surface pressure and surface potential-area isotherms of OPAA on BTP buffer subphase, pH = 7.2.

Å^2 molecule^{-1}) than the surface pressure (3500 Å^2 molecule^{-1}), as seen for most Langmuir films, even though the enzyme monolayer cannot be modeled as a typical amphiphile. There is no saturation, indicating that the monolayer did not achieve any phase in which the dipole moments of its polar groups were ordered.

The measurement of UV−vis absorption in situ in BTP buffer is shown in Figure 5. The absorbance increases with the surface pressure indicating the low degree of aggregation of the enzyme at the air−water interface. The $\pi-\pi^*$ band at 202 nm appears at the same wavelength as for the phosphate buffer. The band with its maximum at 280 nm suggests the absorption of tryptophan residues. Based on this assumption, it was possible to test the intrinsic fluorescence for this enzyme at the wavelength of ∼280 nm, as it will be described in the next section.

3.3. Activity Measurements. *3.3.1. Langmuir Monolayers.* The activity of the enzyme against paraoxon was examined by a surface pressure−area isotherm and absorption spectroscopy. As expected, it was difficult to detect the low activity of OPAA against paraoxon. As known from the literature, OPAA has higher selectivity against nonaromatic organophosphorus compounds such as DFP.[25,28] In Figure 6, the effect of DFP in the subphase on the surface pressure−

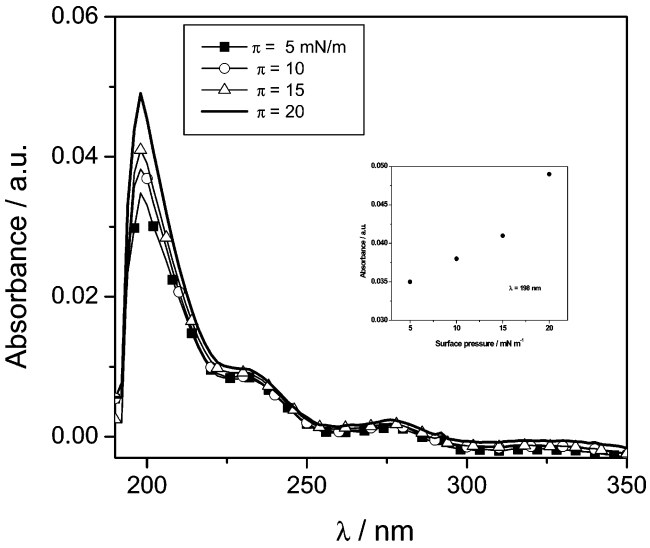

Figure 5. Absorption spectra of OPAA monolayer on BTP buffer subphase, pH = 7.2.

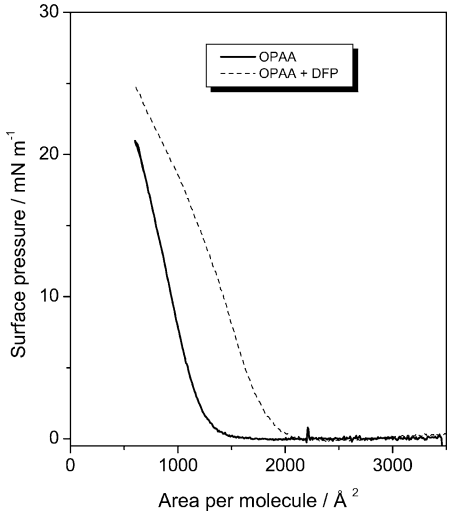

Figure 6. Surface pressure−area isotherms of OPAA on a BTP buffer subphase in absence and presence of DFP 10^{-4} M, pH = 7.2.

area isotherm is presented. The mean molecular area increases significantly when DFP (10^{-4}M) is present, indicating the unfolding of enzyme molecules in the catalytic process. Because DFP is not an amphiphile molecule, we would not expect any change in the isotherm of OPAA because of an adsorption of DFP on the enzyme monolayer. This leads to the conclusion that there is reaction between the enzyme and DFP, where DFP is hydrolyzed releasing fluorine ions. The activity was also tested in solution by measuring fluorescence at ∼348 nm when excited at 285 nm. As we can see in Figure 7, the addition of DFP (∼10^{-4} M) to the enzyme solution (10^{-5} M) causes a reduction in the fluorescence intensity even after 1 min. After 30 min, the fluorescence intensity decreased drastically, suggesting that OPAA reacts with DFP. The enzyme changes its conformation in a way that metal ions and histidine groups could be rearranged to favor the catalytic reaction on DFP, consequently interfering with the conjugation of the tryptophan moieties. The fact that DFP reacts with the enzyme rules out the possibility of a simple adsorption of DFP onto the enzyme monolayer.

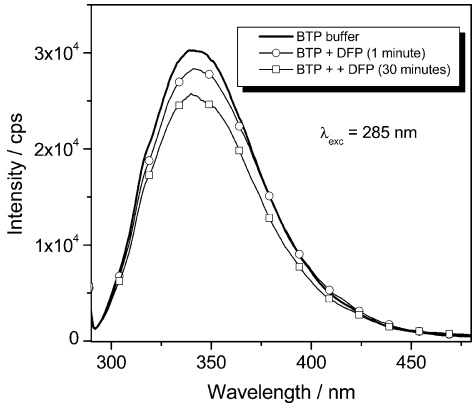

Figure 7. Emission spectra of OPAA (10^{-5}M) in BTP buffer in the presence of DFP 10^{-4} M, pH = 7.2 (λ_{exc} = 285 nm).

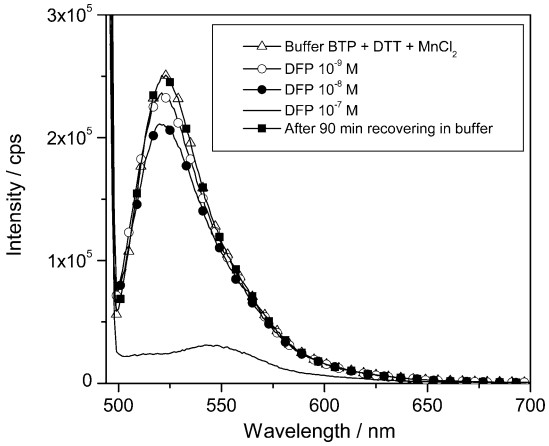

Figure 8. Emission spectra of OPAA-FITC labeled LB film. Quenching effect of fluorescence in the presence of DFP and total recovery after washing out the film with buffer solution.

3.3.2. Langmuir-Blodgett Monolayers. We have tested the fluorescence quenching of a one-layer LB film of the complex OPAA/FITC in the presence of DFP solution. The as-deposited LB film was tested inside the cuvette first with buffer and subsequently by adding aliquots of DFP. The emission spectra were taken immediately after the addition of DFP to the cuvette. Figure 8 shows the decrease of the emission intensity of the LB film as DFP concentration was increased from 10^{-9} to 10^{-8} M. The total quenching was achieved at a DFP concentration of 10^{-7} M. After the addition of the last aliquot of DFP, the slide was washed out and kept in a buffer solution for 90 min, and the fluorescence was fully recovered. This cycle was repeated five times with reproducibility of nearly 100%. The LB film was kept in a buffer solution at room temperature. The emission quenching was interpreted as a self-quenching process of the fluorophore moiety coupled to the enzyme. When a hydrophobic slide is used for the LB deposition, the hydrophilic sites of OPAA are exposed to the environment, as well as the FITC residues. By exposing the film to DFP, the enzyme rearranges to prepare the active sites to catalyze the hydrolysis of DFP. This mobility brings the residues together in a 3D conformation that in the final step might be causing a self-quenching of FITC. The recovering phenomenon can be explained by the fact that when the system is free of DFP and placed in buffer, the enzyme returns to the initial conformation in the LB film.

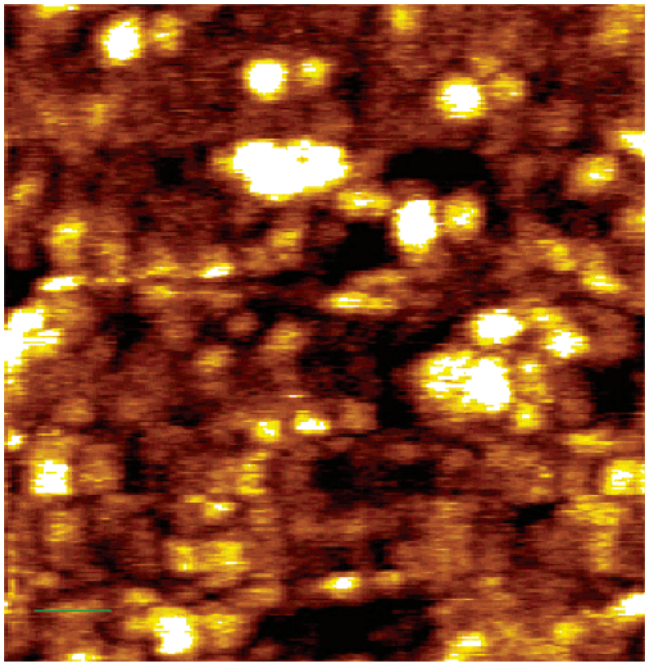

Figure 9. Atomic force microscopy image of a one-layer LB film of OPAA deposited on mica from BTP buffer, pH = 7.2. Scanned area: 300 nm × 300 nm.

3.4. Atomic Force Microscopy. Langmuir−Blodgett films were deposited onto freshly cleaved mica slides in order to perform atomic force microscopic measurements. The subphase used was BTP buffer. Figure 9 shows the image of a pure film of enzyme transferred at a surface pressure of 15 mN m^{-1} using a deposition speed of 1.5 mm min^{-1}. It can be noticed that the shape of the enzyme molecules tends to be spherical. Size measurements indicate that OPAA can exist in a monomeric form (the radius of the small particles we observed was, on average, 10 nm) and as dimers (the size of the large particles we observed averaged 25 nm). The most abundant population here is the monomer. OPAA LB films were also prepared in the subphase containing DFP 10^{-5} M (Figure 10). It can be visualized that the particles have a defined shape (ellipsoidal), although the dimensions of these particles, 37 nm × 16 nm, are larger than the OPAA dimer. The difference in the size of the particles in the presence and the absence of DFP are in agreement with the surface pressure−area isotherm, where we can observe a larger area per molecule with DFP in the subphase. Despite the differences, in both conditions, a uniform distribution of the molecules along the surface is observed, indicating the high transferability of the Langmuir film to the solid substrate.

4. Conclusion

We have presented the behavior of OPAA at the air−water interface as a first step to understand its adsorption mechanism and consequently to extend it to design a prototype of a biosensor. The presence of ions in the subphase was essential to obtain reproducible results. The data show that even the addition of a small amount of KCl to the buffer

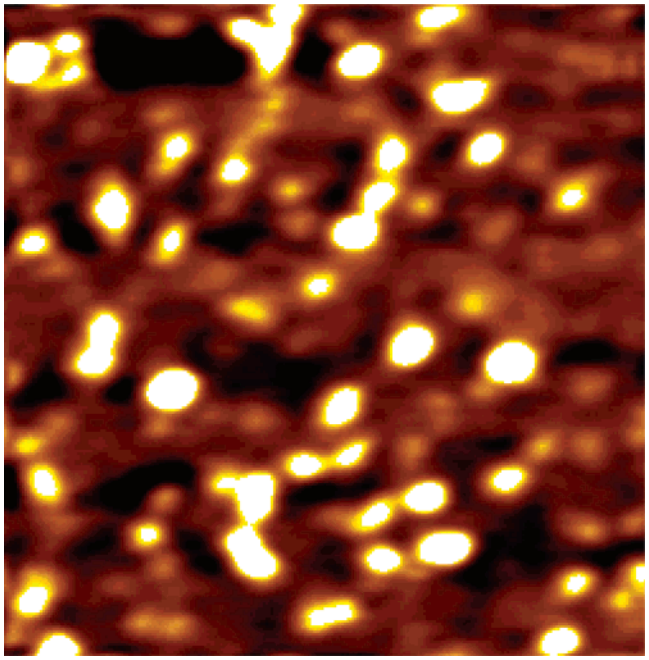

Figure 10. Atomic force microscopy image of a one-layer LB film of OPAA deposited on mica from BTP buffer, pH = 7.2, in the presence of DFP 10^{-5} M. Scanned area: 300 nm × 300 nm.

drastically changes the adsorption process of the enzyme at the interface, i.e., its solubility. The activity of Langmuir monolayers of OPAA against DFP was detected by surface pressure−area isotherms. As significant outcome was that the one-layer LB film of OPAA/FITC deposited onto a quartz slide resulted in a very sensitive and reversible system, which is relevant to a practical application of OPAA-based biosensors.

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