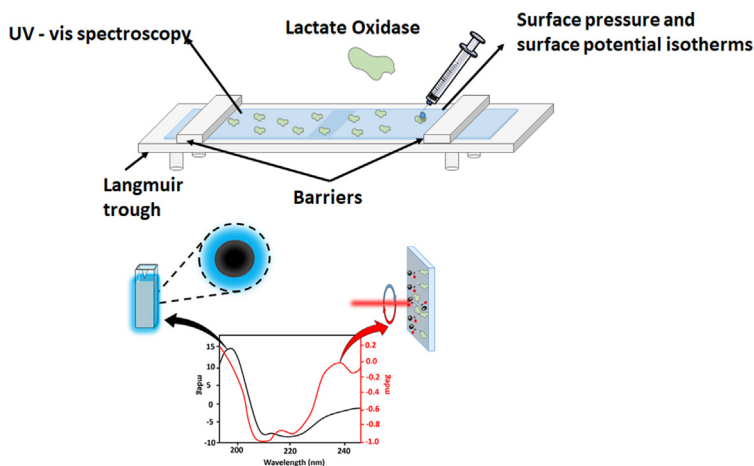


Regular Article

Interfacial behavior of Lactate Oxidase at Air-Subphase interface

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GRAPHICAL ABSTRACT



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ABSTRACT

This article investigates the main aspects of the surface chemistry properties of the lactate oxidase (LacOx) enzyme monolayer at the air-subphase interface. Surface chemistry study determined the important properties like the surface packing and stability of the formed layer, whereas the spectroscopic experiments provided information regarding its secondary structure conformation of the enzyme. We have demonstrated that the LacOx in the monolayer form remained active for extended time period. In accordance to the data obtained from the isotherm it was also found that LacOx forms a stable monolayer that does not aggregate at the air-subphase interface. The stability of the monolayer at the air-subphase interface was studied by using compression-decompression cycles which revealed the stability with no significant evidence of aggregates or irreversible domains. This was further confirmed by UV-vis absorption and fluorescence measurements. Spectra from circular dichroism (CD) showed that the LB film retains the characteristic of an α -helix conformation.

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1. Introduction

Enzymes are proteins that act as biological catalysts [1]. They are responsible for the metabolism of all living organisms [2]. In the conversion of reactants to products, enzymes increase the rate of biochemical reactions by lowering the activation energy so that the biological processes occur at a perceptible rate [3]. Particularly, the Lactate Oxidase (LacOx) is a Flavin mononucleotide-dependent alpha hydroxyl acid oxidizing enzyme. It employs Flavin mononucleotide as a cofactor [4]. The enzyme belongs to the family of oxidoreductases, specifically those acting on single donors with O_2 as oxidant and incorporation of two atoms of oxygen into the substrate (oxygenases) [5]. LacOx enzymes appear in viruses and cellular organisms [6]. The enzyme catalyzes the oxidation of L-lactate to pyruvate in the presence of dissolved oxygen and forms hydrogen peroxide [7]. They are used in biosensors and *in vitro* tests in order to detect lactate in the blood [8–9]. They can also be modified to suppress the tumor [10].

As an example of an important enzyme, its structure, function, and action mechanism of are well studied [11–12]. However, the study of the interaction of this important enzyme with different types of interfaces is a subject with its own importance inside the area of surface chemistry, especially when it comes to the development of sensing devices [13–14]. In this context, the Langmuir technique may be considered as an effective way for the production of monomolecular films at the air-subphase interface with high control over its structure and composition [15–18]. The Langmuir monolayer technique provides the understanding of the surface chemistry behavior of this important enzyme in a model systems representing vaguely cell membrane environments, to prove the activity of the deposited enzyme in a form of the monolayer for a possible microfluidics device development, and to prove existence of catalytic activity and stability of monolayer for biosensing applications [19]. Moreover, important information about the surface packing, phase transitions, dipole orientation and stability of molecular layers can also be obtained from this technique [20].

To the best of our knowledge the surface chemistry properties of LacOx enzyme has never been reported. In this work, we explored the surface chemistry properties and activity of LacOx monolayer on NaCl subphase. Further, we exploited the Langmuir monolayer film technique to assimilate basic interfacial properties and to scrutinize its behavior at the air-subphase interface at different surface pressures that happen to different states, ranging from the gaseous phase to the liquid compressed phase. We employed classical surface pressure experiments in order to access LacOx surface packing and compressibility. Investigations over the stability and surface dipole orientation of LacOx enzyme molecules were accessed via compression-decompression cycles, stability curves, surface pressure and surface potential experiments. Apart from this, we also employed *in situ* UV-vis, *in situ* fluorescence, circular dichroism (CD) to reveal the important information about the LacOx floating monolayers, secondary structure and homogeneity. To determine the change in the secondary structure of enzyme, Langmuir Blodgett (LB) film was analyzed *ex situ* using circular dichroism.

2. Experimental section

2.1. Materials

Lactate Oxidase from *Aerococcus viridans* (LacOx) was obtained from Sigma Aldrich with molecular weight 80 kDa as informed by the fabricant. The aqueous solution utilized in all experiments was obtained from Millipore Direct-Q 3 (type 1) purifying system

(resistivity 18.2 MΩ.cm), a surface tension of 72.1 mN m⁻¹, at pH = 6.0 at 20.0 ± 0.5 °C. NaCl (0.5 mol L⁻¹) subphase was prepared using molecular biology grade ≥ 99.5%, purchased from MP Biomedicals, LLC.

2.2. Equipment

All experiments were performed in a class 1,000 clean room at 20.0 °C and humidity (about 50%) maintained constantly. A Kibron μ-trough (Kibron Inc.) with area of 124.5 cm² was used for the studies of surface pressure-area (π-A) isotherms, surface potential-area (ΔV-A) isotherms, compression-decompression cycles and stability. A Kelvin probe consisting of a capacitor-like system was used to measure the surface potential at air-subphase interface. The vibrating plate was adjusted around 1 mm above the surface of Langmuir monolayer to measure the surface potential. Monolayer compression was carried out at a barrier speed of 10 mm min⁻¹ and the experiment was conducted with a Wilhelmy plate method, provided by Kibron. A KSV mini-trough (KSV Instrument Ltd., Helsinki, Finland) having an area of 225 cm² was used for the *in situ* experiments (UV-vis and Fluorescence) in the air-subphase interface. The *in situ* UV-vis absorption spectra of the LacOx monolayer was taken with an equipment model 8452 A HP spectrometer fixed on a rail closed to the KSV mini-trough whereas *in situ* fluorescence spectra were obtained using Horiba Jobin Yvon Fluorolog connected with KSV mini-trough via optical fiber.

Circular dichroism (CD) spectra were obtained using a JASCO J-810 spectropolarimeter. For enzyme aqueous solution, the spectrum was obtained at 1.0 mg mL⁻¹ using a 1 cm optical path length quartz cell. Langmuir-Blodgett (LB) technique was used to transfer LacOx monolayer onto quartz slide. LB film was analyzed *ex-situ* using circular dichroism spectrophotometer.

LacOx aqueous solution at 4.12 × 10⁻⁶ mol L⁻¹ was characterized by UV-vis absorption using a Cary 100 UV-vis spectrophotometer, Agilent Technologies. Luminescent emission spectrum of enzyme aqueous medium at 2.10 × 10⁻⁶ mol L⁻¹ was measured by a Horiba Jobin Yvon Fluorolog using an excitation and emission bandwidth both at 5 nm.

2.3. Langmuir monolayer

The LacOx solution was prepared in ultrapure water at the concentration of 4.12 × 10⁻⁶ mol L⁻¹. Monolayers were spread onto NaCl 0.5 mol L⁻¹ subphases. After 15 min time for equilibrium the Langmuir monolayer was compressed at the rate of 100 Å² molecule⁻¹ min⁻¹. These experiments were repeated at least three times to ensure reproducibility. Stability tests were performed for the Langmuir films at 6, 12 and 19 mN m⁻¹ while the time evolution of the area per molecule was recorded. Successive cycles of compression-decompression (three cycles) of the Langmuir films were carried out with reproducibility.

2.4. Langmuir Blodgett (LB) film preparation

8-Layers LB films were produced by transferring the enzyme monolayer at air-subphase interface onto quartz slide for CD analysis. Langmuir monolayer isotherm was compressed until the pressure reached 12 mN m⁻¹ and this pressure was kept constant. The typical dipping speed was 5 mm min⁻¹, with 30 min elapsing time between the first and the second layer and 5 min for the subsequent layers when the substrate was out of the NaCl aqueous subphase. The transfer ratio (TR: area of the quartz slide to the area of the film transferred) was about 1.0.

3. Result and discussion

3.1. Surface pressure and surface Potential-Area isotherm

The Langmuir monolayer behavior of the aqueous LacOx ($4.12 \times 10^{-6} \text{ mol L}^{-1}$) on the surface of 0.5 mol L^{-1} NaCl was investigated. LacOx enzyme is water soluble, therefore, to induce its adsorption onto the surface, subphase of salt is necessary. Fig. 1A shows the surface pressure and surface potential-area isotherms of pure enzyme. In this figure, the area per molecule at the condensed phase, extrapolated to zero surface pressure from the high-pressure part of the curve, is approx. $650 \text{ \AA}^2 \text{ molecule}^{-1}$ for the LacOx monolayer. This result was compared with X-ray diffraction study of LacOx enzyme by Umena et al [12]. The study depicts the lattice parameters (unit cell) $a = b = 192.6 \text{ \AA}$ and $c = 200.3 \text{ \AA}$ of LacOx enzyme. Crystallography study can show the structure of enzyme and how enzyme molecules pack together in a crystal structure but the geometrical detail of the enzyme such as interactions among the enzyme and subphase cannot be described by the method. In the Langmuir monolayer of the enzyme, the whole enzyme structure may not be accounted while determining its surface area as some portion of the enzyme may be inside the subphase (hydrophilic part) and some portion of enzyme may be projecting above the subphase (hydrophobic part). The mean

molecular area of LacOx ($650 \text{ \AA}^2 \text{ molecule}^{-1}$) that we have obtained actually accounts the area of the enzyme at the air-subphase interface.

When compression of the Langmuir monolayer starts, the surface potential-area increases due to the charges present on the enzyme. The surface potential increases until 200 mV in the liquid condensed phase (approx. $475 \text{ \AA}^2 \text{ molecule}^{-1}$) under the vibrating electrode. After liquid condensed phase, the enzyme molecules are packed tightly such that it behaves as a solid phase. At this value, due to short distance between molecules some of dipole-dipole interaction get canceled out which ultimately results in a steady value in the surface potential. Fig. 1B shows a schematic representation of the LacOx monolayer isotherm technique and Fig. 1C shows an image of the biologic unit of the LacOx.

Fig. 2 shows the adsorption kinetics for the LacOx. We found that most of the enzyme adsorption occurs in the first 1300 s, with a maximum surface pressure at 8 mN.m^{-1} . In this figure, it is also possible to observe that the enzyme is surface active.

3.2. Compression-Decompression cycles

Following the adsorption profile in Fig. 2, further surface chemistry experiments were performed within a 15 min lag time after spreading for LacOx adsorption equilibrium. Firstly, stability tests

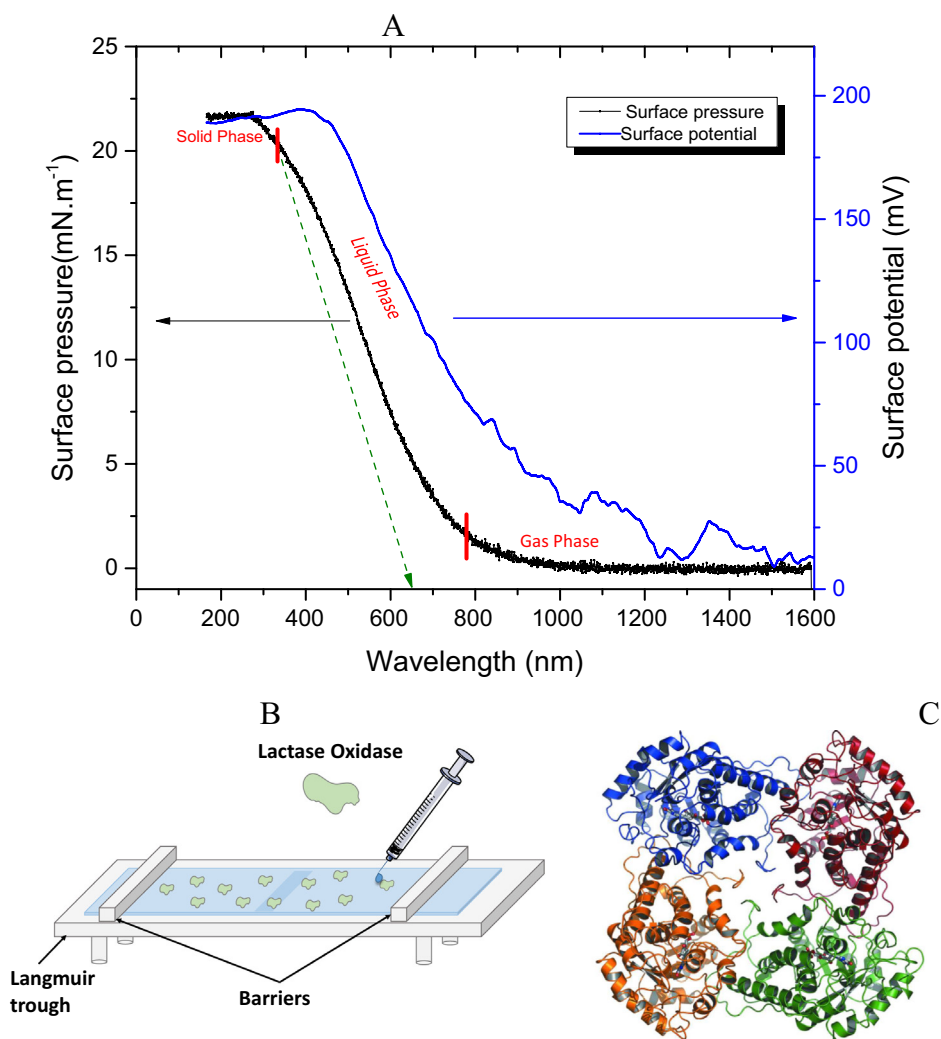


Fig. 1. A) Surface pressure (black) and surface potential (blue) isotherms for the LacOx ($4.12 \times 10^{-6} \text{ mol L}^{-1}$) Langmuir monolayer at air-subphase (NaCl 0.5 M ; pH 6.0). B) Schematic representation of the LacOx Langmuir monolayer isotherm technique. C) Image of the biologic unit of the Lactate oxidase [21]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

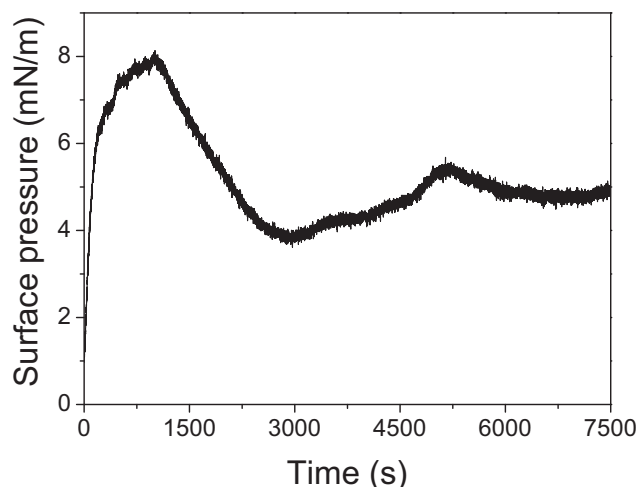


Fig. 2. Lactate Oxidase (LacOx) adsorption kinetics at air-subphase (NaCl 0.5 M; pH 6.0) for a 3000 mm² surface area.

(results not shown) were performed for the LacOx Langmuir monolayer films. The surface pressure was kept at 6, 12 and 19 mN m⁻¹ while the time evolution of the area per molecule was recorded, respectively. In the gaseous phase, for 6 mN m⁻¹, for a period of 80 min, the area per molecule decreased 20%. This large reduction of the area per molecule is due to the conformation variation in the gaseous phase. In the same time period for 12 and 19 mN m⁻¹, in liquid and solid phase respectively, the variation is about 3%. This shows that there is a slight conformation change of the enzyme on the subphase at these surface pressure during 80 min.

Fig. 3 exhibits the successive cycles of compression-decompression of the Langmuir films carried out at 6, 12 and 19 mN m⁻¹. From this figure, it was found that change in the hysteresis was almost 4.5%. At lower surface pressure (6 mN m⁻¹), the change of conformation of the enzyme is similar to 12 and 19 mN m⁻¹. After 3 cycles the film reached stability, with the pressure-area isotherms being the same in consecutive runs. This result demonstrates that the loss of enzyme due to solubilization in water is negligible because we don't have a large change in the hysteresis after three cycles of compression-decompression.

3.3. *In situ* UV-VIS spectrum of Langmuir monolayer

Fig. 4A shows the UV-vis absorption spectra for LacOx in aqueous solution 4.12×10^{-6} mol L⁻¹. The absorbance peak at 280 nm is a characteristic band of the tryptophan group present in the enzyme [22]. The broad peak in Fig. 4B at 230 nm is referred to $n \rightarrow \pi^*$ transition of the peptide bonds present in the enzyme [23,24]. Further experiments were performed to study the *in situ* UV-vis spectra of the Langmuir monolayer compressed at different surface pressure as shown in Fig. 4B. The absorbance spectra at the monolayer of the enzyme show two bands at 207 nm and 230 nm which is attributed to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, respectively. The inset of the Fig. 4B, shows the absorbances values at wavelength, $\lambda = 230$ nm versus surface pressures. A linear relationship suggests that during the compression the number of molecules per unit area increased as the surface pressure was increased. This observation gives a strong evidence that the Langmuir monolayer of the LacOx enzyme presents a good homogeneity at the air-subphase interface.

3.4. *In situ* fluorescence spectrum of Langmuir monolayer

From *in situ* UV-vis spectroscopy, we observed the homogeneity and the stability of the LacOx Langmuir monolayer. To confirm

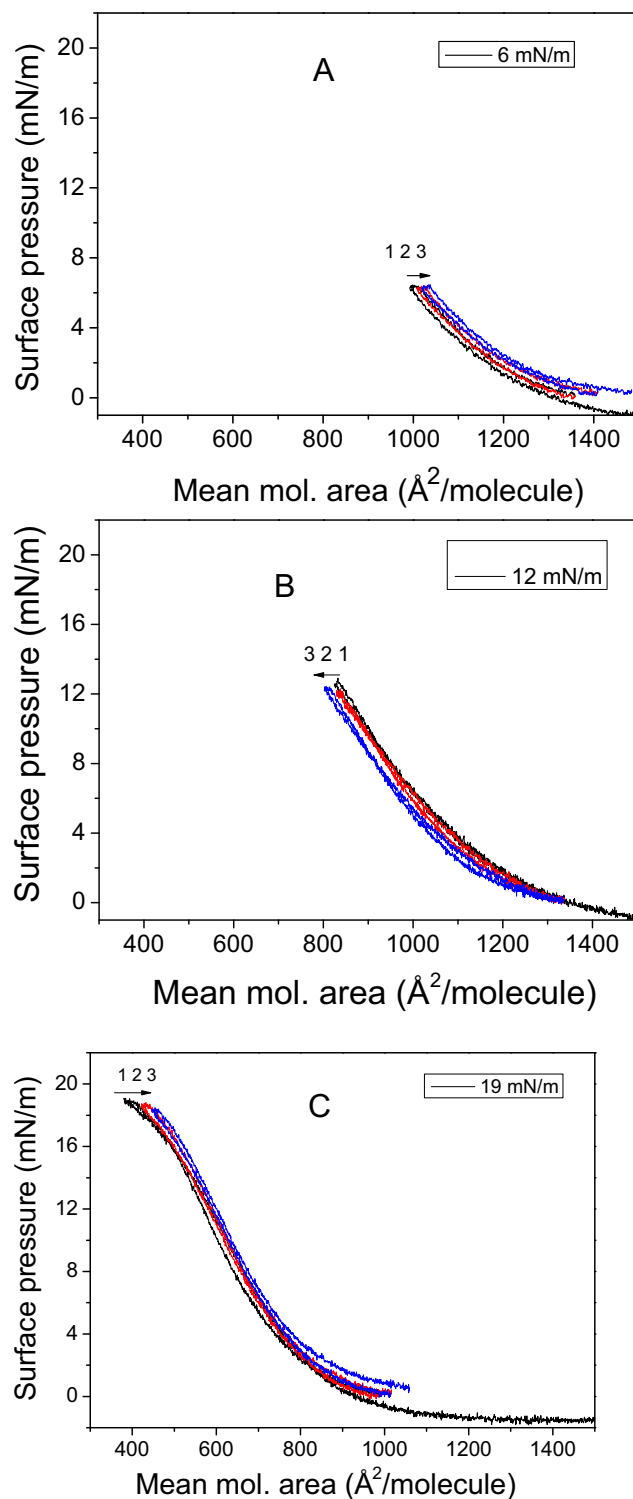


Fig. 3. Compression-decompression cycles of the Langmuir monolayer at air-subphase (NaCl 0.5 M; pH 6.0), maintained at the surface pressure of (A) 6; (B) 12 and (C) 19 mN m⁻¹.

this observation, we performed *in situ* fluorescence spectroscopy of the Langmuir monolayer as shown in Fig. 5. Similar to the UV-vis observation, we obtained a linear relationship by plotting intensity at 360 nm versus surface pressure. This finding ruled out the hypothesis of loss of the enzyme in the subphase during the formation of the Langmuir monolayer. Furthermore, this observation signifies a homogeneous increase in the surface concentration of

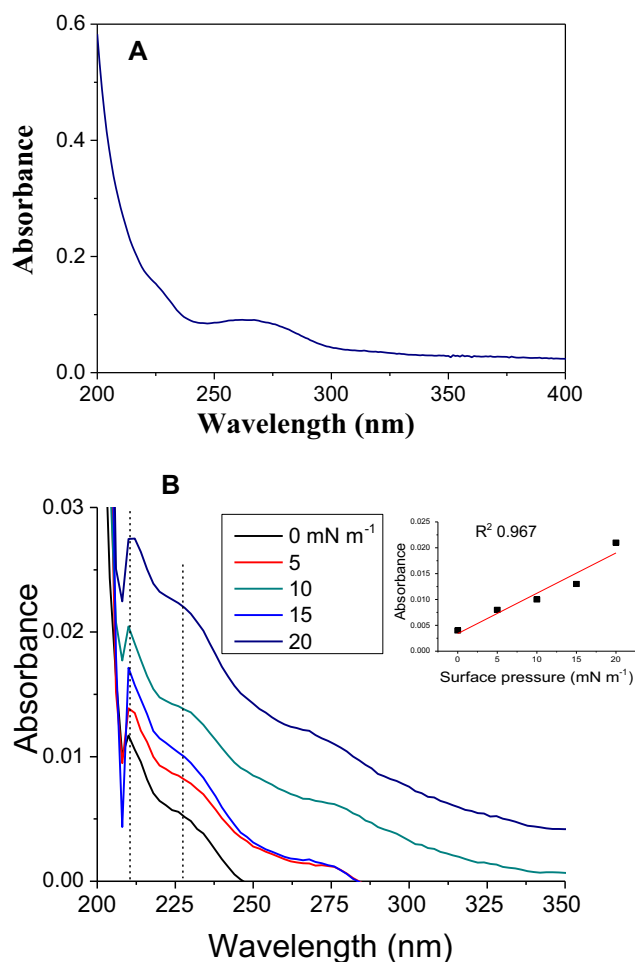


Fig. 4. UV-vis absorption spectra of the LacOx for solution of $4.12 \times 10^{-6} \text{ mol L}^{-1}$. Inset: zoomed region of the band 280 nm (A). Langmuir monolayer at air-subphase NaCl 0.5 mol L^{-1} (B). Inset: Plot of absorbance at 230 nm in the different surface pressures.

tryptophan groups upon compression. Although there are low number of tryptophan residues presented by the film, the high fluorescence quantum yield of the tryptophan amino acid seemed to play a critical role in its interfacial detection. Although there was no absorbance band for tryptophan residues in *in situ* UV-vis spectroscopy, the *in situ* fluorescence of the interfacial film presented

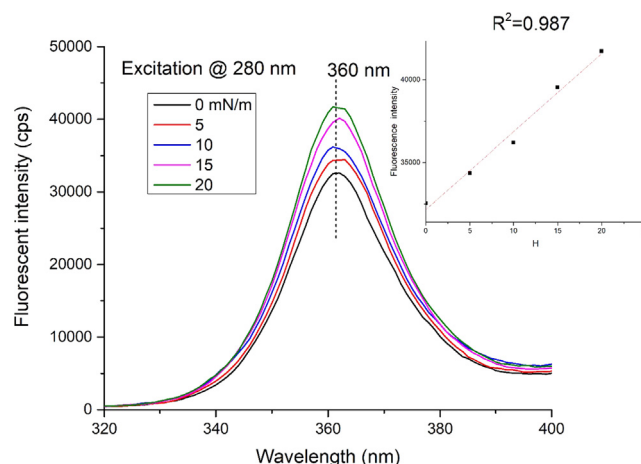


Fig. 5. *In situ* fluorescence spectrum of the Lactate Oxidase (LacOx) Langmuir monolayer at different surface pressures.

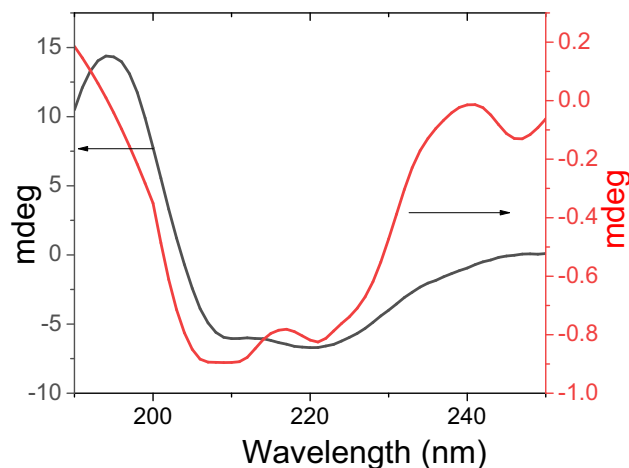


Fig. 6. Circular dichroism spectra of the Lactate Oxidase (LacOx) in solution (black curve) and 16 layers LB film (red curve) onto quartz substrate deposited at 12 mN m^{-1} . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

emission bands for the amino acid at 360 nm when excited at 280 nm. This band is due to tryptophan chromophores present in the enzyme [25].

3.5. Circular dichroism

Since we were interested to know the change in secondary structure of the LacOx enzyme at the air-subphase interface, we conducted the circular dichroism (CD) experiment. As it was not possible to directly measure the CD spectra at the air-subphase interface with our equipment, the Langmuir monolayer of LacOx was transferred to a solid quartz support via Langmuir-Blodgett (LB) technique. The LB technique is crucial in the transfer of the Langmuir monolayer film at a given surface pressure via immersion and withdrawal of a solid support into or from the aqueous subphase. Fig. 6 shows the CD spectra of LacOx solution (black curve) and Langmuir Blodgett (LB) films (red curve) transferred at 12 mN.m^{-1} surface pressure. Both the spectra exhibited two minima at around 208 and 219 nm, which is a characteristic of an α -helix conformation. For the solution, a positive band at 190 nm is characteristic of an α -helix conformation [26]. The CD spectrum of LB film shows that the structure of enzyme is preserved. The negative bands are attributed to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, respectively at 208 and 219 nm [27]. The change in the CD spectrum of LB films compared to the solution may be associated to the scattering of circularly polarized light due to the refractive index of quartz slide.

The results show that the secondary structure has been conserved in the Langmuir-Blodgett (LB) films. This confirms the stability of the LB films to be used in the biosensing devices.

4. Conclusion

We demonstrated the use of Langmuir monolayer technique to form stable monolayer of the LacOx at air-aqueous salt subphase. Since the enzyme is soluble in water, and *salting out* effect plays a major role, the presence of NaCl in the subphase is essential to form stable monolayer. This was confirmed by the compression-decompression cycles when observed mainly in the condensed liquid phase at 19 mN m^{-1} . The *in situ* UV-vis and fluorescence spectra of the monolayer compressed at different surface pressure showed a linear relationship during the compression suggesting that the Langmuir monolayer presented a good homogeneity. Spectra from CD showed that the LB film keep the characteristic

of an α -helix conformation. In general, we believe that the present work may support the understanding on the generation and characterization of enzymes forming Langmuir monolayers and Langmuir-Blodgett films in biosensing. For example, this work may be important in the development of LacOx immobilized electrodes for the detection of Lactic acid as nanostructured biosensors.

5. Author contribution statement

The conceptualization of the work was brought by M. Ferreira, S. K. Sharma, and R. M. Leblanc. Data curation was performed by M. Ferreira, S. K. Sharma and S. Paudyal. Formal analysis and funding acquisition was done by R. M. Leblanc. Investigation and methodology development was accomplished by R. M. Leblanc, M. Ferreira and S. K. Sharma. R. M. Leblanc executed the project administration, provided resources and supervised the work. Data validation and visualization was carried out by S. K. Sharma and R. M. Leblanc. M. Ferreira, S. K. Sharma, and S. Paudyal used the software for data analysis. M. Ferreira and S. K. Sharma wrote the original draft. Revision and editing was performed by R. M. Leblanc. All authors discussed the results and contributed to the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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