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Nanoarchitectonic approaches for measuring the catalytic behavior of a membrane anchored enzyme. From Langmuir-Blodgett to a novel Langmuir-Schaefer based nanofilm building device

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Highlights

Monolayers from bovine erythrocyte membranes (BEM) were transferred to alkylated glass surfaces.

Langmuir-Schaefer (LS) and Langmuir-Blodgett (LB) transference methods were compared.

LS_{BEM} preserved the activity of the acetylcholinesterase (AChE) located in BEM

LS_{BEM} allowed a one-step full kinetic analysis of AChE catalytic activity.

LS_{BEM} stand for a prototype of optical biosensor for monoterpene pesticides.

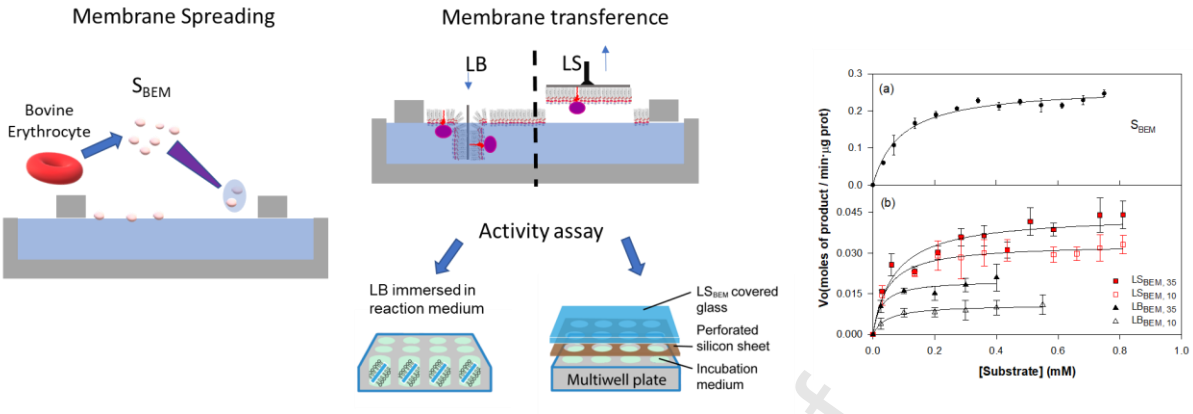
Abstract

Self-organized lipid monolayers at the air-water interface (Langmuir films, LF) are commonly used for measuring the catalytic properties of membrane-bound enzymes. This methodology allows to provide a consistent flat topography molecular density, packing defects and thickness. The aim of the present work was to show the methodological advantages of using the horizontal transfer method (Langmuir-Schaefer) with respect to the vertical transfer method (Langmuir-Blodgett) when mounting a device to measure catalytic activity of membrane enzymes. Based on the results obtained we can conclude that it is possible to prepare stable Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) films from Bovine Erythrocyte Membranes (BEM) preserving the catalytic activity of its native Acetylcholinesterase (BEA). In comparison, the LS films showed V_{max} values more similar to the enzyme present in the vesicles of natural membranes. In addition, it was much easier to produce large amounts of transferred areas with the horizontal transfer methodology. It was possible to decrease the time required to mount an assay with numerous activity points such as building activity curves as a function of substrate concentration. The present results show that LS_{BEM} provides a proof of concept for the development of biosensors based on transferred purified membrane for the screening of new products acting on an enzyme embedded on its natural milieu. In the case of BEA, the application of these enzymatic sensors could have medical interest, providing drug screening tools for the treatment of Alzheimer's disease.

Keywords

Erythrocyte acetylcholinesterase; Erythrocyte ghost membranes, Langmuir-Blodgett films; Langmuir-Schaefer films; biosensor proof of principle.

Graphical Abstract



1. INTRODUCTION

A main objective in our laboratory has been to measure the catalytic properties of membrane-bound enzymes. The use of self-organized lipid monolayers at the air-water interface (Langmuir films, LF) comprise model membranes that provide a consistent flat topography and allow dissecting the effects of variables that are mutually affected, such as curvature, molecular density, packing defects and thickness. LF can also be formulated to include a simulated glycocalyx with the inclusion of lipopolymers and to control the degree of molecular crowding at the membrane-aqueous interface by tuning the molecular packing[1-5]. They can also be transferred to solid substrates, maintaining the organization of the floating monolayers, to obtain Langmuir-Blodgett films (LB). LB constitute a useful tool to investigate the effects of membrane composition, topography and dynamics on the activity of enzymes and neurotransmitter receptors [2, 6-10] and also to develop a variety of 2D nanoarchitectured materials [11-13].

However, understanding the behavior of complex systems requires, at some point, the restoration of the elements that allow a more direct approach to the real system. The use of enzymes in their native molecular environment for activity determinations brings us closer to their actual catalytic mechanism. Consequently, an intermediate situation between the natural membrane and the models with pure lipids, could be LB composed of natural membranes [2, 8, 9]. A further advantage of this approach is that it avoids previous purifications steps of the protein of interest.

In a previous work, we have measured the activity of a GPI-anchored enzyme, the Bovine Erythrocyte Acetylcholinesterase (BEA) from Bovine Erythrocyte Membranes (BEM), that were successfully transferred to alkylated glasses [14]. The GPI anchor combines with the carboxyl-terminal of the protein, which locates close to the membrane surface embedded in the glycocalyx [15, 16]. So, the activity and conformation of the protein can be coupled to the membrane dynamics [17-19] and thus may affect the access of ligands to their binding sites in the protein [20]. We demonstrated the transmission of information between the membrane and the environment comprised by the subphase immediately below the membrane where anchored proteins are hosted, as reflected by the membrane packing-induced modulation of BEA catalytic activity [14].

The use of the LB membrane model provided fruitful and quite reproducible results [2, 9, 14, 21, 22]. However, kinetic and screening type experiments require preparing a huge number of individual samples so that to cover the whole spectrum of conditions to be analyzed with their replicates, becoming very time consuming. In the present work we have focused on optimizing the film transfer process, since all the necessary steps to measure the activity are very laborious.

An alternative to the LB vertical deposition is the Langmuir-Schaefer (LS) technique. This methodology, also called horizontal deposition, consists of transferring a lipid monolayer by stamping the substrate with a parallel orientation to the air-water interface at a low speed. The substrate is then lifted up until it is separated from the water. Recent works have used the LS technique to study protein

films [23], biophysical properties of phospholipid monolayers [24-26] as well as their interactions with drugs [10, 27]. Although the LS methodology is often used in combination with LB vertical deposition [24, 28], when the number of publications mentioning each of these techniques is analyzed over the last 20 years, it can be observed that LS is gaining relevance over LB, probably due to a greater simplicity of this deposition procedure (Fig. 1).

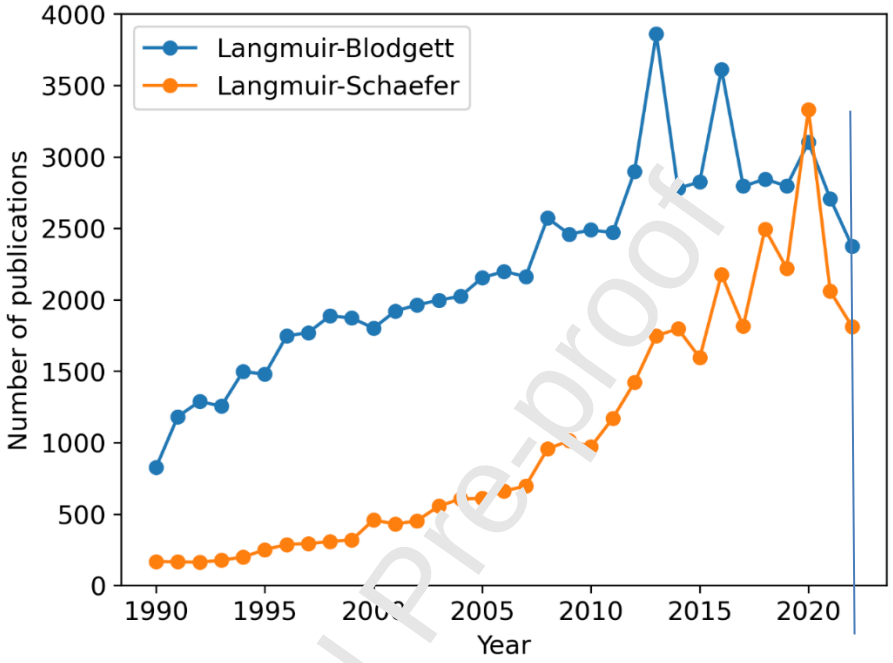


Fig. 1. Number of publications by year mentioning Langmuir-Blodgett (blue) and Langmuir-Schaefer (orange) techniques, from 1990 to 2022. The data were extracted from Dimensions (https://app.dimensions.ai/discover/publication).

In the present work we prepared LF by the spreading of BEM membranes (LF_{BEM}) at the air-water interface. Subsequently, LF were horizontally transferred at a constant lateral pressure to alkylated glasses to obtain BEM Langmuir-Schaefer films (LS_{BEM}). The topography of LS_{BEM} films and the catalytic activity of BEA-contained films were characterized. The catalytic efficiency of BEA in LS_{BEM} was compared with that in LB_{BEM}. The use of larger glass pieces as substrate in the case of LS_{BEM} allowed us to build a cassette with a multiwell culture plate. This arrangement allowed us to measure the activity in all the wells available in the plate with one LS_{BEM} coming from a single monolayer.

2. MATERIALS AND METHODS

2.1 Bovine erythrocytic membranes (BEM)

BEM was purified from blood of healthy cattle extracted by venipuncture and preserved at 4°C in extraction bags containing monohydrate dextrose and sodium citrate as anticoagulant. The procedure for obtaining the purified membranes was the same as that described before [14].

2.2 Langmuir-films from bovine erythrocyte membranes (LF_{BEM})

In this work we will use the terms Langmuir films and monolayers interchangeably. LF were prepared and monitored as described previously [2, 9, 14]. Briefly, an aqueous dispersion of BEM (S_{BEM}) was spread over an air-phosphate saline buffer (PBS, 100 mM pH 7.4 phosphate buffer with 145 mM NaCl) interface placed in a Teflon trough. The absence of surface-active compounds in pure solvents and in the subphase solution was checked before each run. S_{BEM} (20µl with 0.53 mg/ml protein concentration) was spread with a syringe directly at the interface at ~10 µl/min rate. Monolayers were lead ~15 min to stabilize before starting the compression. The compression rate was 7.5 cm^2/min beginning with an initial total trough area of 242.25 cm^2. Surface Pressure-Area (π-A) and Surface Potential-Area (ΔV-A) isotherms were recorded simultaneously. A detailed analysis of LF_{BEM} stability and thermodynamic behavior was done as described previously [14].

2.3 Langmuir-Blodgett Films (LB_{BEM}) and Langmuir-Schaefer film (LS_{BEM}) from BEM

Through π-A isotherm analysis, the presence of a single 2D transition of ~12 mN/m was determined (see section 3.1 below). Then, two different π were selected for LF preparation and transference, 10 mN/m ($LB_{BEM,10}$, $LS_{BEM,10}$) and 35 mN/m ($LB_{BEM,35}$, $LS_{BEM,35}$) which were below and above the transition, respectively. LB_{BEM} and LS_{BEM} were prepared by the transference of LF onto chemically modified glasses (12 mm diameter round microscopy coverslips for LB, and square 6 x 8 x 0.3 cm (wide, length and thickness) glasses for LS). All glasses had been previously submitted to a surface modification consistent on a chemical reaction between the glass silicate groups and the trichlorosilane group attached to a 18-carbon chain [29]. This procedure renders the glass surface hydrophobic, which is ideal for the adhesion of the transferred monolayer through the hydrophobic face of the molecules composing the LF.

2.3.1 Preparation of hydrophobic supports

The first step consists of the sonication of the glasses in the presence of hot detergent for 30 minutes. After rinsing in distilled water, they are placed in sulfochromic solution for 15 min. and then rinsed again with distilled water. Subsequently, the coverslips are sonicated for 15 minutes in a 3 mM Na (OH) solution, rinsed with double distilled water and placed in an oven at 140 ºC for approximately 30 min. Once cold, they are immersed in 0.1% V/V Octadecyl-Trichloro-Silane (OTS)

in toluene, for 1 hour with constant stirring. After silanization, the glasses are subsequently rinsed in chloroform three times for 5 minutes. The effectiveness of the hydrophobicity achieved was qualitatively evaluated by observing the contact angle between a drop of water and the glass surface.

LB films were built as previously reported [14] and here the procedure is addressed in full detail in Fig.2. A special trough was designed (500 ml of subphase volume) with a 4 cm depth that allow a multiwell plate to get fully submerged (Fig. 2a). The transference process begins after π stabilization of the LF (Fig.2b) through the perpendicular immersion of the alkylated glass in the subphase at 1 mm/min rate (Fig.2c). The entire transfer process occurs at a constant π, which entails a constant loss of monolayer area equivalent to the monolayer area transferred from the air-water interface to the glass. At least 8 LB_{BEM} films (i.e. 8 alkylated coverslips coated with one layer the transferred LF) could be obtained from a single monolayer, which were individually received in a well of a 24-well plate previously immersed in the sub-phase (Fig.2c). Once the 8 LB_{BEM} films have been transferred, a re-expansion of the trough barriers is carried out and more membrane is spread without changing the subphase (Fig.2d). This procedure allows us to prepare a total of 16 glasses in a single multiwell plate (Fig.2c-2d loop). Then, the remaining LF was eliminated from the interface by aspiration to obtain a clean air-aqueous interface (Fig.2e) and the original aqueous subphase was exchanged several times with the enzymatic assay buffer to eliminate all membrane debris dispersed in the subphase that might have been detached from the glass (Fig.2e; note that the color used to represent the liquid was change to highlight its replacement). Samples were stored at 4°C until further use (not more than 48 hours later).

The alkylated glass used for LS films had the exact size to cover half of an entire standard cell culture plate. For the LF preparation and film transference a standard flat Teflon trough (150 ml subphase volume) with an initial area of 242.25 cm^2 (Fig.2a') was used. The transference began after the π stabilization of the LF (Fig.2b'). The alkylated glass plate, hanging from a motorized arm holding a suction cup connected to a vacuum pump, approached parallel to the monolayer surface until adhering to it (see Fig.2c'). The glass was maintained in this position for 5 minutes allowing the transferred film to stabilize. Later, the glass was raised until it detached from the monolayer (Fig.2d') and the LS coved glass was stored in an environment with a temperature set at 4°C and controlled humidity.

2.4 Topographic characterization of Langmuir films

The two-dimensional structures of LF, LB and LS films were studied by epifluorescence microscopy. A KSV Minisystems surface barostat was mounted on the stage of a Nikon Eclipse TE2000-U (Tokyo, Japan) microscope, with a color video camera Nikon DS-5 M with a supported resolution up to 2,560–

1,920 pix (Capture) and a long distance 20X objective. BEM were doped with 0.5 mol% of 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide (DiI-C18).

After acquisition, images were processed and analyzed similarly as in [10]. Briefly, RGB images were transformed to a greyscale, 8-bit version, their background was subtracted with a rolling ball radius of 70 pixels to eliminate de excessive brightness of some probe aggregates. Lastly, three greyscale ranges were established and their area measured. A range with the lower grey values (dark grey area, DGA), a second range with intermediate grey values (light grey area, LGA) and, finally a range with the highest grey values (bright grey area, BGA). Triplicate images were used for each measurement.

2.5 Determination of BEA enzymatic activity

The catalytic activity of BEA was measured according to the spectrophotometric method of Ellman et al [30]. Briefly, the hydrolysis of the artificial substrate analog acetylthiocholine catalyzed by BEA in phosphate buffer 0.1 M, pH 8 at 37 ºC, to produce thiocholine and acetate was coupled to the reaction of the thiocholine with the dithiobisnitrobenzoate ion (DTNB). The final product has an extinction molar coefficient at 412 nm $\boldsymbol{\varepsilon}_{412}$ = 13.600 M^{-1} cm^{-1}.

The incubation system for S_{BEM} consisted of 3 μl of DTNB (2mM), 40 μl of S_{BEM} sample (0.28 mg/ml protein), in the presence of the substrate, in a final volume of 220 μl whose absorbance was read in a 96-well culture plate. The same final volume was used in the case of LS_{BEM} with the difference that the enzyme source was the transferred monolayer.

In LB_{BEM} films, maintaining the same proportion of the previous incubation medium the glasses were immersed in 2200 µl final volume to ensure the glass is completely submerged in the reaction medium in a 24 wells culture-plate. The buffer excess inside the well was removed by aspiration using a homemade device that allowed the same volume to be left in all wells (Fig 2f). All these procedures ensure that there is no rearrangement of the transferred monolayer by contact with the air. The absorbance measurement was carried out directly on the culture plate without removing the LB films (Fig. 2g).

To measure the activity in LS_{BEM} films, a cassette-type assembly such as that outlined in Fig. 2e' was mounted. With the multiwell plate at the bottom, the LS_{BEM} film at the top and with the perforated silicon sheet in the middle the whole arrangement was pressed with binder clips to avoid liquid leakage (Fig.2e'-f'). Then the whole system is turned 180 ° and in this way the incubation medium is put in contact with the monolayer and the reaction begins in a thermostated stove (Fig. 2f'). The reaction stops when the system is flipped to the original position and the absorbance is measured immediately (Fig. 2g').

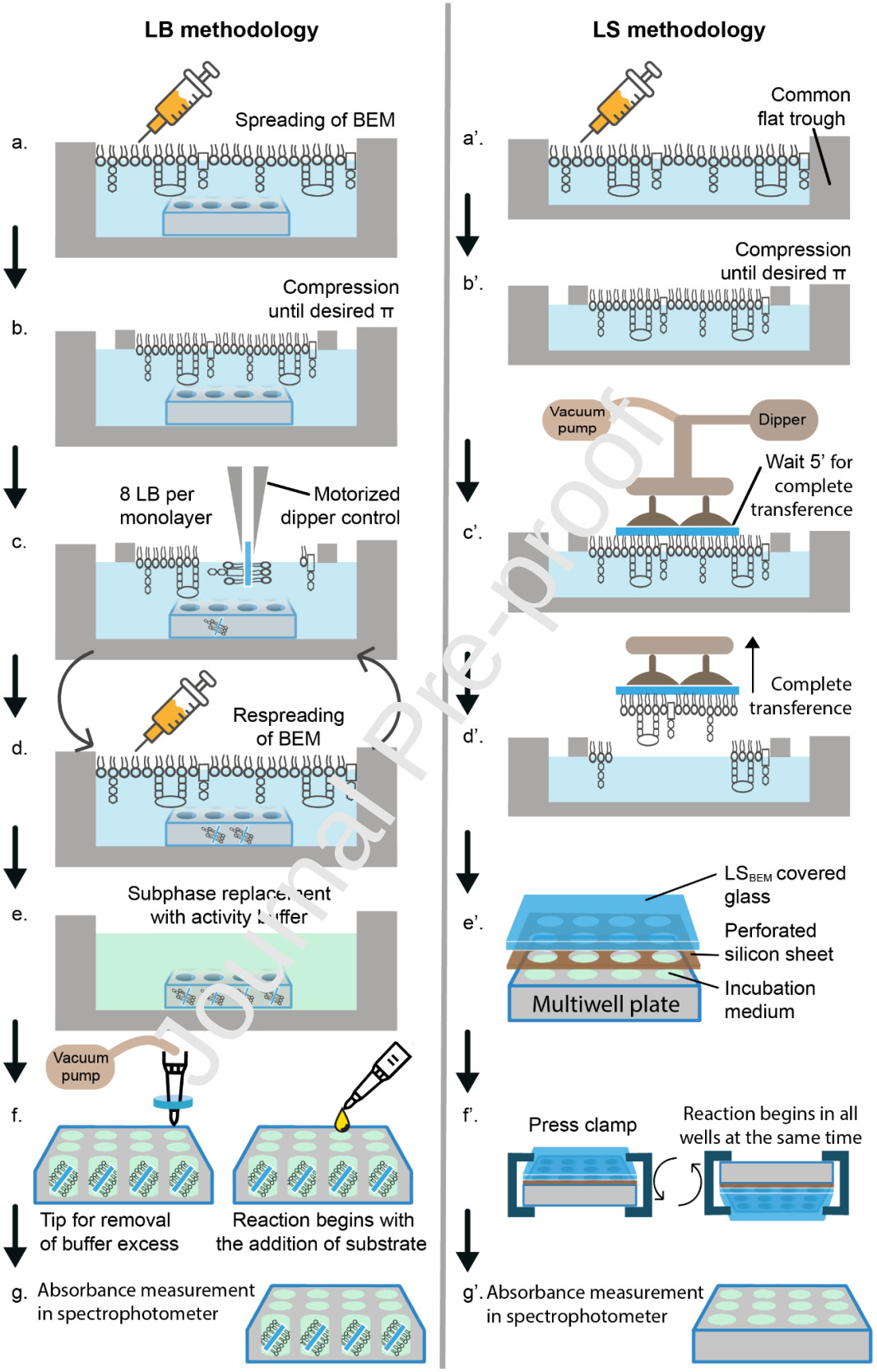


Fig. 2. Schematic representation of BEA activity measurement systems based on LS or LB films. Note that the color used to represent the liquid changes from light blue (d and d') to light green (e and e') to highlight the replacement of the initial aqueous subphase for the buffer used to carry out the enzymatic reaction.

The assays were performed as independent triplicates for each substrate concentration point. The blanks were filled with alkylated glasses without transferred monolayer. The absence of non-enzymatic hydrolysis due to the presence of silanized glass was verified.

V_{max} (maximal reaction rate) and K_M (Michaelis-Menten constant) parameters resulted from the Michaelis–Menten (Eq.4) fitted to the plot of the initial reaction rate (V_0) vs. the substrate (S) concentration.

$$V_0 = \frac{V_{max} \cdot S}{K_M + S} \qquad [4]$$

The activity of BEA in LB_{BEM} and LS_{BEM} was tested at a single protein concentration since the amount of BEA in these samples is related to the BEM present in the LF transferred to the alkylated glass.

2.6 Protein quantitation

Proteins were quantified by the Lowry method [31] modified by Markwell et al. [32] by the addition of SDS in the EDTA-Cu^{2+} reagent. 100 µl of BEM suspensions were added to 1 ml of the reaction mixture (0.3% w/v SDS in 0.63 mM EDTA Cu^{2} Reagent A). In the case of LB transferred films [1], the coverslips were firstly cut in small pieces, washed with Reagent A and sonicated for 15 min at 80 W and 40 kHz frequency at room temperature. In all cases, samples were incubated for 30 min at room temperature. In the case of LS, wells filled with Reagent A were contacted with the transferred glass in the same way that activity is measured, and the system were stirred for 30 min. Finally, the Folin–Cicalteau reagent was added and the absorbance at 750 nm was measured after another 1 h incubation period. A glass slide was separated from each transferred monolayer to determine the proteins concentration. Knowing the amount of area transferred, a ratio of mg of protein per mm^2 was established and this value was used to establish the amount of protein that carried out the activity in each well.

2.7 Statistical Calculations

The least squares method was applied to fit functions through a nonlinear regression analysis. Student's t test was applied to compare individual averages. The propagation error method was used to evaluate the error associated to variables calculated from other ones determined experimentally. ANOVA and Tukey post-hoc tests were applied for comparisons with Sigma Plot 12.5 software.

3. RESULTS AND DISCUSSION

3.1 Langmuir films from bovine erythrocytic membranes (LF_{BEM}).

A detailed analysis of the chemical composition of BEM membranes as well as the rheology of LF_{BEM} prepared over a water subphase, was done previously [14]. In the present work we used a buffered saline solution, (PBS buffer pH 7.4) as a subphase. This change was intended to preserve the structure of AchE as much as possible.

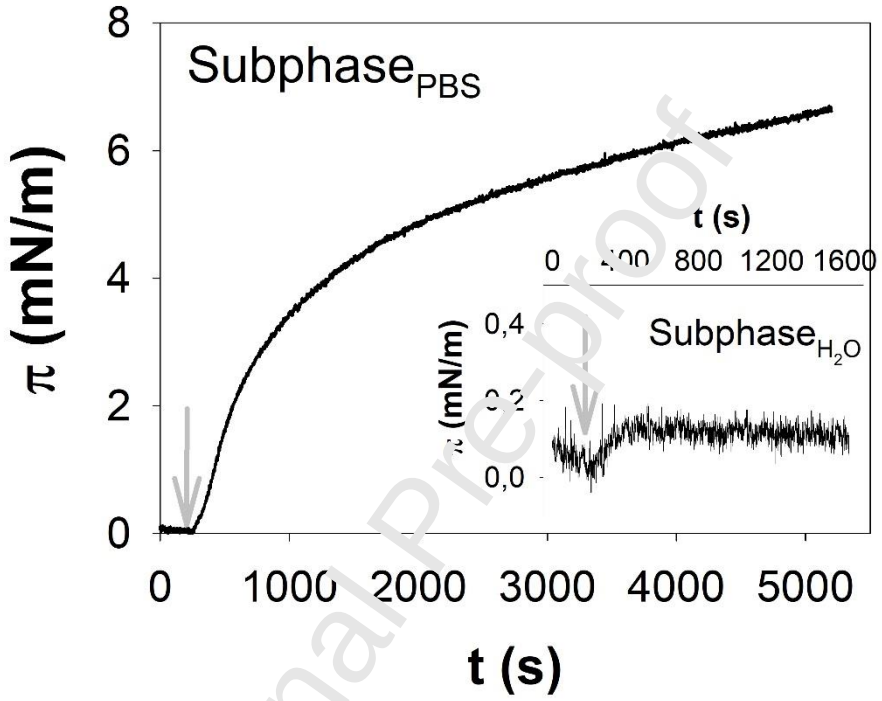


Fig. 3: Spreading kinetics of BEM vesicles over the air-aqueous interface. The main panel shows the spreading behavior of BEM on an air-buffer interface. The inset shows the spreading behavior over a water subphase**.** The amount of BEM vesicles dispersion (20µl with 0.53 mg/ml protein concentration) spread was the same in each case.

As depicted in Fig. 3, compared to the water interface, the spreading kinetics of BEM changes remarkably when it is deposited over a buffered subphase. The main differences were the faster and significantly higher increase in π observed after the membrane deposition (gray arrows in the main panel and inset). Furthermore, when it is dispersed over water, (Fig 3, inset) more membrane was required to reach the same surface pressure.

Stable monolayers were formed from the spreading of S_{BEM} at the air-buffer interface. The compression π-A isotherms and the calculated compressional modulus (K) vs. A are shown in Fig. 4.

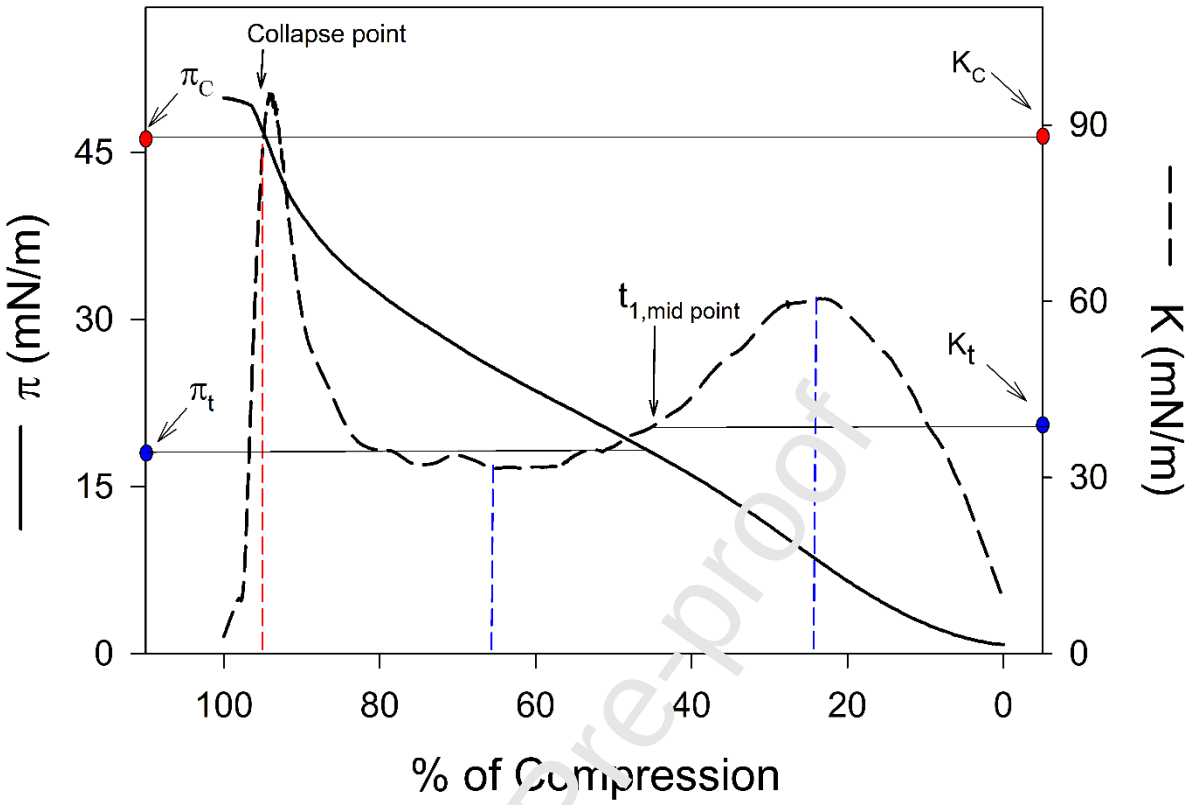


Fig. 4. Surface pressure (π) and compressional modulus (K) vs. % of compression isotherms of BEM at the air-buffer interface. the surface pressure value at the midpoint of the 2D-transition t1 and πc the collapse point identified from the evolution of the K-% compression isotherm also shown. Temperature 25 °C.

Along the isothermal compression, the BEM monolayer reached a molecular coherence at the lift-off point, marked by the departure of π from zero, and the closest molecular packing at the collapse point (π_C). In the π-A isotherm, one two-dimensional (2D) transition was observed (marked as t_1), in contrast to the three transitions found in the previous work with a pure aqueous subphase [14]. The transition was clearly evidenced as an inflexion point in the K-A isotherms, beginning at a maximum and continuing with an abrupt slope decrease in the K-A plot. The π_t value pointed to in Fig.4 correspond to the mid-transition point. The values found for these two parameters were listed in **Table 1.** In the previous work mentioned before, we proposed, as in other works, that the transitions could be due to the reorganization at the interfacial level of the proteins[14, 33-35]. This could be the explanation why here BEM showed a more compact isotherm.

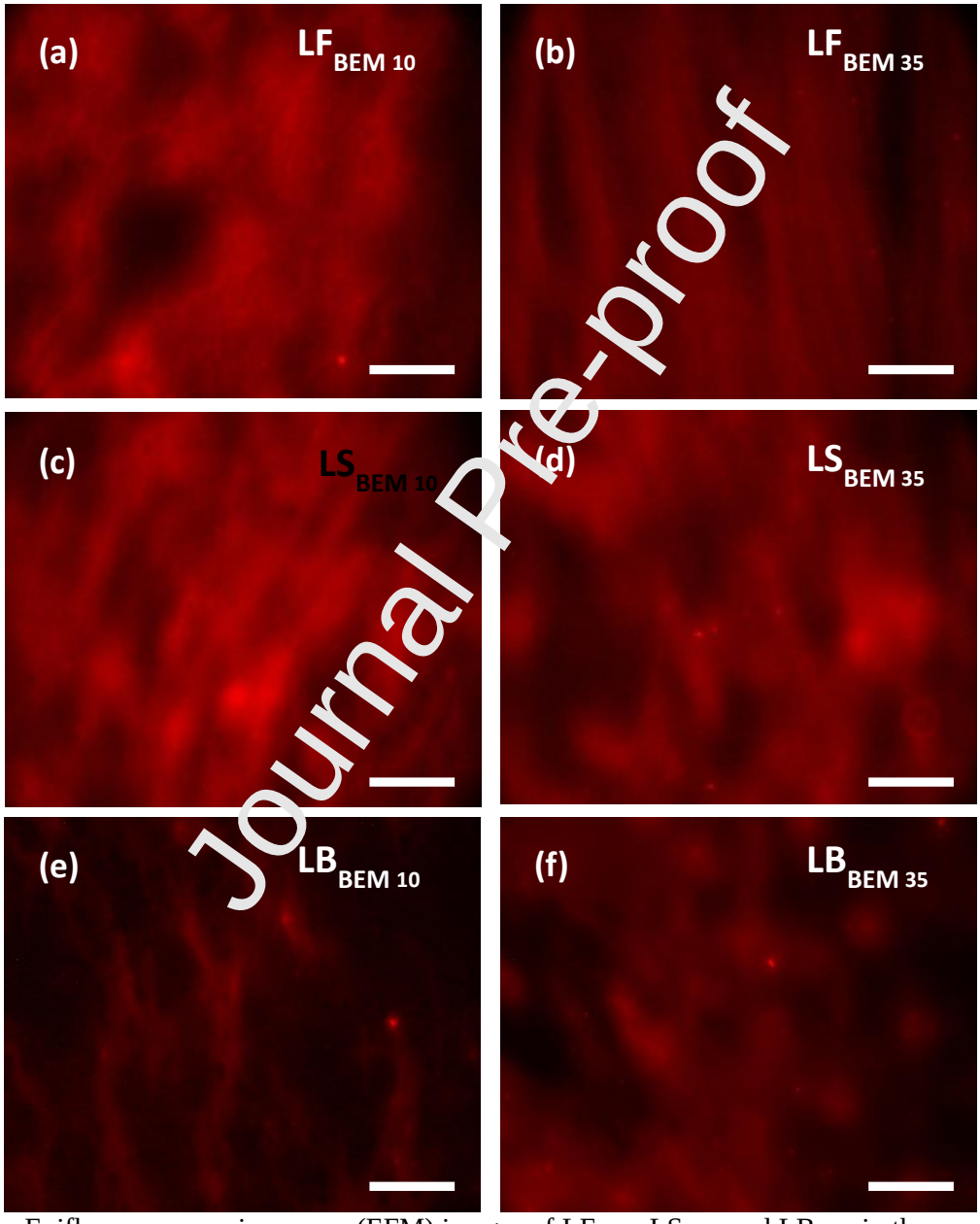
Table 1 Molecular parameters derived from π-A, and K-A compression isotherms.

Significant points	π (mN/m)	K (mN/m)
Transition midpoint	18.1	38.65
Collapse point	46.3	88.2

The presence of this transition evidenced the complex nature of a LF_{BEM} which comes from a biomembrane composed approximately of 50% protein by weight. The values of K at the π_c and at the transition midpoint (π_t), indicate that BEM monolayers, at the temperature of the present assays, exhibits a liquid expanded behavior throughout the whole isotherm [3[illegible]37].

Fig. 5: Epifluorescence microscopy (EFM) of BEM Films. Micrographs of Langmuir films at the air-PBS interface compressed at 10 mN/m (A) and 35 mN/m (B) are shown. LS films transferred at 10 mN/m (C) and 35 mN/m (D) as well as LB films transferred at 10 mN/m (E) and at 35 mN/m (F) are also shown. The fluorescent dye DiIC18 was present at 1 mol% in relation to phospholipids. White bars represent 100 μm.

3.2.2 Topographic characterization of LF_{BEM}, LB_{BEM} and LS_{BEM} films obtained from the monomolecular layers of BEM.



Epifluorescence microscopy (EFM) images of LF_{BEM}, LS_{BEM} and LB_{BEM} in the presence of the fluorescent probe Dil-C18 were recorded at two different π, 10 mN/m and 35 mN /m. These surface pressures were chosen to be comparable to those chosen in a previous work [14] (Fig.5). In LF_{BEM} it is possible to distinguish areas of the micrographs that exhibit the highest fluorescence intensity, which

could be assumed as a liquid-ordered (Lo) phase (Fig. 5a-b). The marked heterogeneity observed in the topography of the interface is related to the large number and diversity of lipids and proteins coming from the natural membrane. It is known that the fluorescent probe DiI-C18 is preferentially partitioned into the most ordered phases [63].

The horizontal transfer of LF_{BEM} to the alkylated glass (Fig.5c and 5d) led to LS_{BEM} whereas the vertical transfer of LF_{BEM} led to LB_{BEM} (Fig.5e and 5f). LB_{BEM} at both lateral pressures showed several darker areas that were not present in LF_{BEM} which probably shows non-transferred areas. However, within all the differences both $LB_{BEM,35}$ and $LS_{BEM,35}$ are very similar to $LF_{BEM,35}$, showing that both transference systems (LB and LS) are able to reproduce the original structure of the floating monolayer (LF). A similar behavior has already been observed with LB films from other natural membranes [9]

To make a quantitative characterization of the influence of the transfer process on the morphology of the LF_{BEM}, the epifluorescence micrographs were analyzed as described in Materials and Methods. It was possible to distinguish three gray levels in the micrographs, as previously found by other authors in DiIC18-labeled natural membranes [10]. These three kinds of grey areas (GA) are related to different membrane environments of the probe. The first range included the darker grey values which consisted of areas from which the probe was mostly excluded (DGA). The second range that included lighter grey values representing areas in which the probe was properly spread (LGA). The last range contained brighter well-defined structures probably associated with protein aggregates which may favor a local probe accumulation (BGA) [10].

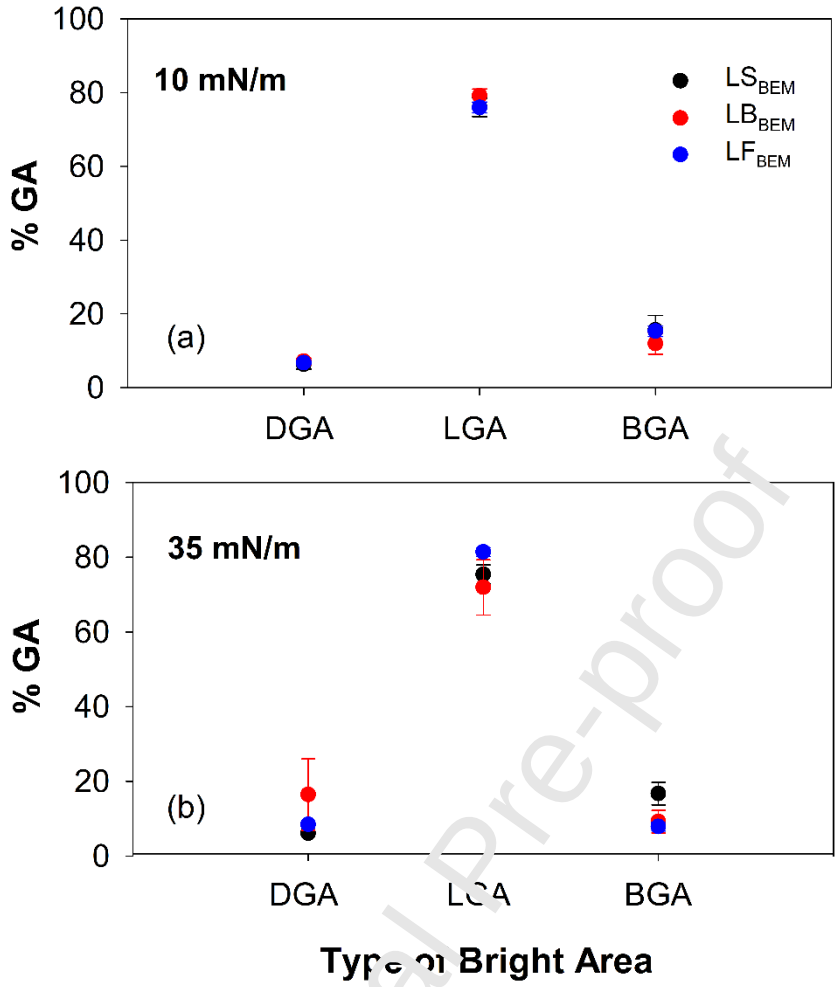


Fig. 6. Percentual composition of different gray areas (% GA). Epifluorescence microscopy data obtained from the floating Langmuir Film at the air buffer interface (LF_{BEM}), and from the films transferred by Langmuir Schaefer (LS_{BEM}) or Langmuir Blodgett (LB_{BEM}) techniques. Three different gray areas were identified in the films named dark gray area (DGA), light gray area (LGA) and bright gray area (BGA). Films obtained at π of 10 mN/m (a) and of 35 mN/m (b).

In figure 6 we can see that at both lateral pressures, 10 and 35 mN/m, the proportions of the different gray areas were not altered during the transfer process. That is clear when compared the superposition of blue dots (LF_{BEM}) with red (LB_{BEM}) and black (LS_{BEM}) dots. The different proportions found in GA observed in LF_{BEM} apparently did not change with the increase of π. This confirms what we previously discuss that neither of the two transference techniques applied affect the structure of the original floating monolayer.

3.4 Kinetics of BEA catalyzed acetylthiocholine hydrolysis

In the experimental conditions of this work, the different sources of enzyme in which BEA is found are: membrane suspension (S_{BEM}), $LB_{BEM,10}$, $LB_{BEM,35}$, $LS_{BEM,10}$ and $LS_{BEM,35}$ (Fig.7). In all the conditions tested, BEA exhibited a michaelian (hyperbolic) behavior. Therefore, the kinetic

parameters K_M and V_{max} could be calculated by fitting the Michaelis-Menten equation (Eq.2) to the experimental data.

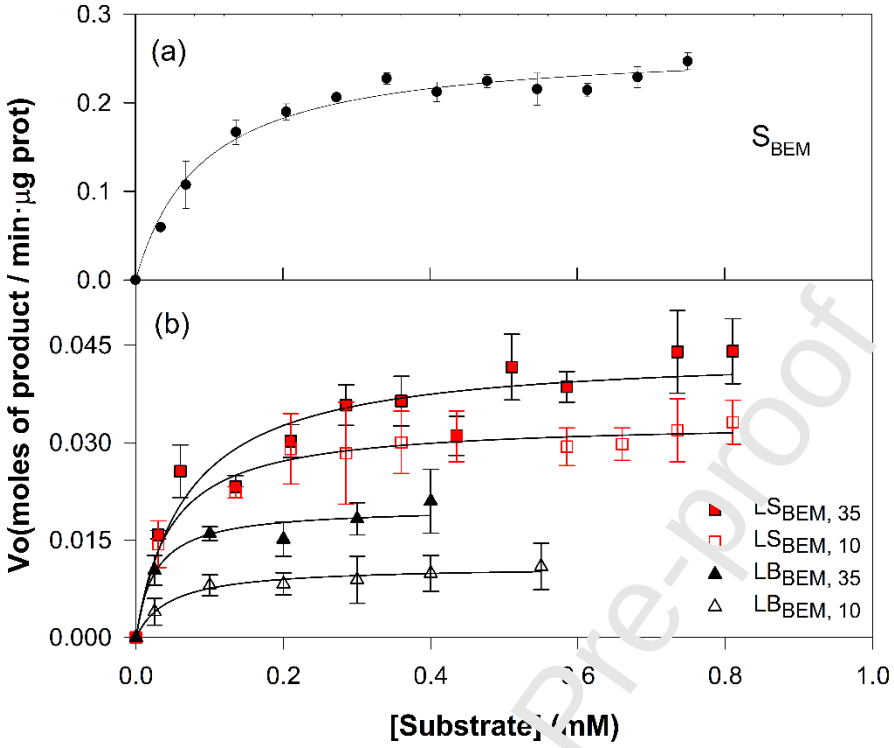


Fig. 7. BEA catalytic activity vs. substrate (acetylthiocholine) concentration. BEA was present in a suspension of BEM vesicles (S_{BEM}) (a) or in BEM films transferred by the Langmuir-Blodgett (LB) or the Langmuir-Shaefer (LS) methods (b), at 10 or 35 mN/m (LB_{BEA10}; LB_{BEA35}; LS_{BEA10} and LS_{BEA35}). A Michaelis-Menten model was fitted to the experimental data. Points represent de mean of at least triplicates.

It is important to note that the specific activity of BEA drops once it is transferred to a solid support. The magnitude of this decrease in activity is clearly appreciable in Fig. 7. If compared with the membrane suspension, the drop in enzyme activity once BEM is transferred to LB and LS films, could be explained due to the restriction of the enzyme in terms of translational movements [38]. In previous works we have also observed a drop in the activity of transferred enzymes and we have attributed it to the reduced dimensionality of the environment [2].

3.4.1 V_{max} and K_M values in Langmuir films transferred to alkylated glasses

In **Table 3** the comparative effects of the different types of transfer processes on the kinetic parameters K_M and V_{max} of BEA are shown. In the case of K_M, slight differences are observed between the transferred films and the membranes in suspension. Accordingly, no significant differences were found in the statistical test, showing that the affinity of the enzyme for the substrate was not affected by the transference process.

In a previous work [14], it was also shown that the V_{max} measured with LB_{BEM} were considerably lower than that obtained with S_{BEM}. This behavior is also observed in the present work in the case of LS_{BEM} films. This result can be explained by several factors. Firstly, the exposure of BEA to the air-water interface could have caused an unfolding or other structural change in the protein, leading to an inactivation of the enzymatic activity at a certain proportion of all active BEA molecules. Likewise, a selective transfer of proteins from the Langmuir film to the solid support could have occurred, with the loss of BEA with respect to other proteins. In a previous work we performed electrophoretic profiles showing that the protein composition of samples at the air-water interface (LF_{BEM}) differed from that of S_{BEM}, although no significant changes were observed in the region corresponding to proteins bands with molecular weights similar to that of BEA [14]. This would confirm the fact that BEA, because of its amphipathic nature, has a favorable tendency to remain at the air-water interface, and therefore to be transferred to the solid support, but in a conformational or environmental state with a lower catalytic activity. However, the membranes transferred by the Langmuir-Schaefer technique showed better performance than the LB films at 35 mN/m (**Table 3).**

Regarding the molecular packing degree in the LB_{BEM} films, it was observed that the V_{max} is higher in the $LB_{BEM,35}$ than in the $LB_{BEM,10.}$ This trend is also observed for $LS_{BEM,\ 35}$ and $LS_{BEM,\ 10}$. This difference could be due to the fact that a π = 35 mN/m the film is close to the equilibrium spreading pressure of a bilayer [39]. Therefore, at this surface pressure, the enzyme would be in a condition that favors its native structure. However, for another GPI enzyme it has been observed that when the enzyme activity was measured directly at the air-water interface [40] at different packing levels, the activity increased as long as the surface pressure increased since a higher number of enzyme molecules per area is present when the film is compressed (in our case the activity has been normalized by the amount of protein transferred). But, after the monolayer suffers a transition from the liquid-expanded phase to a liquid-condensed phase (second-order transition), the enzyme activity decreased. As a result, a direct relationship between the surface compressional modulus and the enzyme activity could be observed [41]. The above may be our case because the selection of two different π for LF preparation and transference, 10 mN/m and 35 mN/m, was based on the fact that they were below and above the main transition observed in Langmuir isotherm (Fig. 4).

Table 3 Kinetic parameters of acetylcholine hydrolysis catalyzed by BEA in BEM suspensions and in LB_{BEM} films

Enzyme source	V_{max} (nmoles PNP/min·µg prot)	K_M (mM)	n	Catalytic efficiency (V_{max}/K_M) (nmoles /min·µg prot.mM)
S_{BEM}	0.265 ± 0.008^{a}	0.090 ± 0.012^{a}	11	2.94
$LB_{MEB.10}$	0.011 ± 0.001^{b}	0.054 ± 0.011^{a}	5	0.24
$LB_{MEB.35}$	0.020 ± 0.001^{b}	0.033 ± 0.011^{a}	5	0.77
$LS_{MEB.10}$	$0.033 \pm 0.001^{b,c}$	0.055 ± 0.011^{a}	9	0.73
$LS_{MEB.35}$	0.044 ± 0.003^{c}	0.070 ± 0.024^{a}	10	0.63

Numbers correspond to the mean ± s.e.m (d.f.) of *n* experiments. The incubation time was 15 min for S_{BEM} and 90 min for LB_{BEM} films. Experiments were performed at 37 ºC. The Michaelis-Menten model (Eq.2) was fitted to the experimental data. BEM, bovine erythrocyte membranes; S_{BEM}, BEM suspension; $LB_{BEM.10}$ and $LB_{BEM.35}$, Langmuir-Blodgett films of BEM packed at 10 mN/m and 35 mN/m; . $LS_{BEM.10}$ and $LS_{BEM.35}$, Langmuir-Schaefer film of BEM packed at 10 mN/m and 35 mN/m, respectively. Different superscript letters indicate statistical significance for ANOVA and Tukey post-hoc tests.

With respect to catalytic efficiency, it was observed that S_{BEM} showed the highest value (2.94 nmol/min.µg prot. mM). The transferred enzyme showed a considerable drop in catalytic efficiency, mainly due to the loss of activity (or amount of enzyme) reflected in the V_{max}. However, taking S_{BEM} as a reference and considering both the LB and LS films, the transference at 35 mN/m improved the activity recovery if compared with that obtained at 10 mN/m. In addition, the kinetic parameters (K_M and V_{max}) exhibited by $LS_{BEM,35}$ were the closest to $S_{BEM.}$ The catalytic efficiency of LB_{35} was a slightly higher than LS_{35} due to the lower value the of the apparent K_M in LB_{35}, but the difference between K_MLB_{35} and K_MLS_{35} was not statistically significant even when compared with K_MS_{BEM} (see Table 3).

CONCLUSIONS

The aim of the present work was to improve the efficiency and practicality of a film-based sensor to measure spectrophotometrically the activity and determine the kinetic parameters of membrane-bound enzymes. For this, we designed a devise that allow the measurement of 96 data in one step instead of measuring one at a time. This is based on the methodological advantages and higher quality of using the horizontal transfer method (Langmuir-Schaefer) with respect to the vertical transfer method (Langmuir-Blodget) of a Langmuir film at the air-water interface to a solid alkylated substrate.

Based on the results obtained we can conclude that it is possible to prepare stable Langmuir-Blodgett and Langmuir-Schaefer films from BEM preserving the catalytic activity of BEA. In

comparison, the LS films showed V_{max} values more similar to the enzyme present in the vesicles of natural membranes. In addition, it was much easier to produce large amounts of transferred areas with the horizontal transfer methodology. It was possible to decrease the time required to mount an assay with numerous activity points, as those necessary to build an activity vs. substrate concentration plot.

Beyond the contribution to the understanding of BEA activity modulation, the present results show that LS_{BEM} provides a proof of concept for the development of biosensors for the screening of new green pesticides acting through BEA interaction. Also, the application of this enzymatic sensors could have medical interest, making drug screening for the treatment of Alzheimer's disease. The main treatments for this disease are aimed at inhibiting acetylcholinesterase in order to increase acetylcholine levels in the brain and improving the cognitive functions of patients [42]. There is evidence that acetylcholinesterase promotes the assembly of amyloid fibers and that it can be inhibited by drugs that bind to the peripheral site of the enzyme [43].

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Declaration of interests

☐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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