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Improvement of the amperometric response to L-lactate by using a cationic bioinspired thymine polycation in a bioelectrode with immobilized lactate oxidase

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ABSTRACT

We report the electroanalytical properties of an amperometric bioelectrode containing L-lactate oxidase (LOx) immobilized on glassy carbon electrode with a hydrogel film composed of laponite and different amounts of a novel bioinspired polycation obtained by copolymerization of 4-vinylbenzyl thymine (VBT) and 4-vinylbenzyl triethylammonium chloride (VBA) in a molar ratio 1:4, respectively. The electrochemical behavior of the redox couple probe $[Fe(CN)_6]^{3-/4-}$ of these VBT–VBA bioelectrodes was compared with that observed for a bioelectrode containing the classical polycation polydiallyldimethylammonium chloride (PDDA). The best response was obtained for a bioelectrode containing a VBT–VBA/laponite mass ratio double than the cationic exchange capacity of the clay, demonstrating that under this condition the polycation induces an optimal microenvironment in the interlamellar space of the clay, both for the position and the functionality of LOx.

The VBT–VBA bioelectrode displayed a very high sensitivity $(7.2 \pm 0.2) \times 10^2\ \mu A\ mM^{-1}\ cm^{-2}$, a short time response (<5 s), a wide linear response range (e.g. 0.01–1.0 mM of L-lactate) and an excellent stability over a storage period of 60 days, when sensing L-lactate. The analytical response of the bioelectrode was tested in real food samples, e.g. milk, white wine, and beer, as well as during milk fermentation at 37 °C. No effect of molecular interferences in the food matrices was detected, and the quantification of L-lactate was in complete agreement with standard assays reported values.

Current results indicate that polycations containing the multifunctional green monomer VBT have high potential for their use in hydrogel film formation producing more responsive and stable electrochemical biosensors.

1. Introduction

In several food and beverages the L-lactate is produced by microbiological fermentation of lactose (dairy products) or malolactic fermentation (alcoholic beverages) [1,2], being the source of a strong influence on sour flavor, freshness, and storage quality of dairy products, sausages, and wines [3]. Therefore, L-lactate is a key analyte to be quantified in food industry that in general it is carried out by separation-based techniques, such as liquid chromatography [4] and capillary electrophoresis [5]. These techniques, although highly accurate and sensitive, require time-consuming sample preparations besides that are expensive and not always easily available. Therefore, the development of direct non-invasive methods for quantification of L-lactate in food matrices is a highly required issue in the food industry. In this sense, the application of bioelectrodes with immobilized redox enzymes, such as L-lactate oxidase (LOx), has been an interesting approach for a fast and accurate determination of L-lactate [6–8]. They are based in the catalytic oxidation of L-lactate by LOx, using either natural occurring mediators (M) (such as molecular oxygen, O_2) or artificial mediators (e.g. ferrocene methanol, FcMe), Eqs. (1) and (2), and with the electroanalytical signal given by the anodic reaction of M_{red}.

$$\text{L-lactate} + (\text{LOx})_{ox} \rightarrow \text{pyruvate} + (\text{LOx})_{red} \quad (1)$$

$$(\text{LOx})_{red} + M_{ox} \rightarrow (\text{LOx})_{ox} + M_{red} \quad (2)$$

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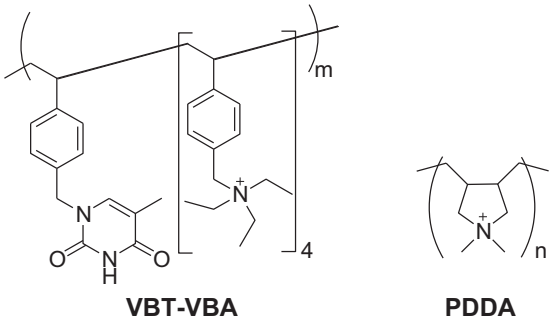
The key step for a successful preparation of a bioelectrode is the methodology for enzyme immobilization, assuring low detection limits, large sensitivity and selectivity.

In the case of LOx, several methods for its immobilization have been used, including covalent binding to gold surfaces [9] or to electropolymerized copolymer films [10]; in screen-printed graphite composite with Teflon® [11] or coated with Nafion® [12], by adsorption on composite films of platinum nanoparticles and multi-walled carbon nanotubes [13], entrapped in polymeric films [14], and also embedded in laponite-polycation based hydrogels [7,8], etc. Among the different enzymatic immobilization methods, the use of hydrogel-modified electrodes (HME) is a reproducible and simple one-step procedure that involves the deposition of an aqueous mixture of clay, enzyme, and polycation on the electrode surface. After air-drying of this mixture and its successively swelling into the aqueous electrolytic medium, an adherent film with the intact enzyme entrapped is obtained [7,8,15]. In particular, the use of synthetic layered silicate laponite combined with suitable polycation provides host matrices with high hydrophilic character, which are appropriate for enzyme immobilization [15].

For an optimal HME performance, besides sensitivity and extensive linear detection range, another relevant issue for an ideal bioelectrode is the time-dependent stability and reproducibility. These issues are highly related with the hydrogel mechanical properties given by the nature and structure of the polycation. Through the identification of abundant, well-designed green mechanisms and inspirational resources synthesized by Nature, novel materials have been developed. The study of bioinspired functional polymers containing reactive moieties had grown enormously in last few years as a consequence of the environmental and toxicological problems linked to the synthesis and non-degradability of traditional plastics [16]. During the last decade, extensive research has particularly focused on the design of synthetic polymers containing nucleic acid bases, which are appealing due to their capacity to combine the advantages of synthetic functional polymers, while simultaneously exhibiting interesting supramolecular properties found in Nature.

A novel highly multifunctional green monomer containing 4-vinylbenzyl thymine (VBT) has been developed [17]. The synthesis of polymers containing VBT acquired particular significance and had opened the possibility of producing a wide variety of environmentally benign copolymers. Since the VBT homopolymer is water insoluble due to strong intermolecular interactions, the copolymerization with charged functional groups, such as 4-vinylbenzyl triethylammonium chloride (VBA), produces a variety of water-soluble polycations with different VBT–VBA ratio. Furthermore, their exposure to low levels of UV light ($\lambda \approx 280$ nm) induces the photo-dimerization reaction between adjacent thymine moieties generating covalent cyclobutane dimers, as it happens with the thymine base units of DNA [18], and resulting in the crosslinking of polymer chains [19–21]. Therefore, the adaptability of VBT–VBA copolymers makes these materials very attractive due to their photo-reactivity, solubility and noncovalent interactions, allowing for the fine-tuning for a specific target application, including antibacterial-coated surfaces [22], hair care products [23], printed circuit boards and photo-imaging systems [24], as well as controlled release systems for pharmaceutical and agricultural use [25,26]. However, the application of these copolymers for enzyme immobilization is still almost unexplored [24].

Recently, we reported the preparation and functionality of laponite based HME using different polycations such as a synthetic organosilasesquioxane [7] and natural occurring chitosan [8] for immobilization of LOx on a glassy carbon electrode (GCE) for L-lactate quantification. In the present work, we extended the study on this type of LOx containing bioelectrodes focusing on the use of the bioinspired water-soluble polycation $\{[(VBT)(VBA)_4]Cl_4\}_{40}$ (labeled as VBT–VBA, Scheme 1) for the formation of laponite hydrogels on GCE and evaluating its behavior in both model and real food samples. As compared with HME made with other polycations, including the well-known polydiallyldimethylammonium chloride (PDDA), the present results indicate that the use of VBT–VBA significantly improves the amperometric response and stability of the bioelectrode.



Scheme 1. Structures of VBT–VBA and PDDA polycations.

2. Materials and methods

2.1. Materials

Laponite RD, a synthetic hectorite (monovalent cation exchange capacity, *c.e.c.* = 0.74 meq g^{-1}) was obtained from Laportes Industries (Detroit, EEUU). Lactate oxidase (LOx, EC 232-841-6 from *Pediococcus* species) lyophilized powder containing 20 units mg^{-1} solid, reagent grade L-(+)-glucose, L-(+) tartaric dipotassium salt, succinic acid (99%), citric acid, D-fructose, DL-malic acid, polydiallyldimethyl-ammonium chloride (PDDA, MW = 100–200 kDa), ferrocene methanol (FcMe), potassium ferrocyanide and ferricyanide $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 99% and L-(+)-lactic acid lithium salt 97% were purchased from Sigma–Aldrich SA (Buenos Aires, Argentina). Buffer phosphate sodium salts NaH_2PO_4 and Na_2HPO_4 were reagent grade from J.T. Baker (Mexico D.F., Mexico). Organic solvents such as acetic acid, formic acid, methanol and ethanol were of analytical grade (Cicarrelli SRL, Buenos Aires, Argentina). Compressed ultrapure nitrogen was purchased from Indura SRL (S.M. de Tucumán, Argentina). All solutions were prepared with triply distilled water. Working electrodes were prepared with glassy carbon disks CHI104 from CH Instruments (Texas, USA).

2.2. Copolymer synthesis and characterization of $\{[(VBT)(VBA)_4]Cl_4\}_{40}$ (VBT–VBA)

All reagents were purchased in the purest available form and were used as received. Sodium hydroxide, isopropanol, acetone, dichloromethane and hexane were purchased from Merk or Cicarelli of Argentina; 4-vinylbenzyl chloride, 2,2′-azobis-2-methylpropionitrile, triethylamine, 2,6-di-tert-butyl-4-methylphenol and thymine were purchased from Sigma Aldrich of Argentina. Vinylbenzylthymine (VBT) was synthesized from vinylbenzyl chloride and thymine, while vinylbenzyltriethylammonium (VBA) was synthesized from vinylbenzyl chloride and triethylamine as described previously [18]. Based on ^{1}H NMR spectra and melting point results, the monomeric products were deemed pure enough for the synthesis of the copolymer. The copolymerization was performed in a round bottom flask by a free radical process with a VBT (0.0413 M) and VBA (0.1647 M) mixture in isopropanol (20.23 M) as solvent using and keeping a co-monomer molar ratio 1:4 to produce the water-soluble polycation. The VBT–VBA mixture

was stirred and heated to 65 °C until the solution was clear. Afterwards, 2,2′-azobis-2-methylpropionitrile (AIBN) (0.0031 M) was added to the flask under inert N_2 atmospheric conditions and the solution was kept at 65 °C and stirred for 16–20 h. Subsequently, the reaction mixture was removed from heat, cooled to room temperature and rotary evaporated to concentrate to about 50%. The resulting solution was slowly poured into a beaker containing acetone and stirred until a white powder precipitate crashed out of the acetone/isopropanol mixture. The powder was filtered twice, washed with cold acetone and allowed to dry in the vacuum oven. To verify the absence of unreacted monomers, the precipitated polymer was analyzed by ^{1}H NMR spectroscopy (Bruker 300 MHz) and the typical vinyl group signal at chemical shifts between 5 and 6 ppm was not observed in the spectra indicating complete copolymerization.

2.3. Conditioning and preparation of the bioelectrode

Prior to each experiment, the glassy carbon electrodes (GCE) were polished sequentially with alumina powder (Buehler, USA) of decreasing particle size, e.g. 1.0, 0.3, and 0.05 μm, copiously rinsed with ultra-pure water and sonicated for 1 min between polish stages. A colloidal suspension of laponite was prepared by dispersing 2 g L^{-1} of the clay in water overnight and with continuous stirring. The hydrogel-modified GCE without enzyme (HME) were fabricated depositing 20 μL of a mixture containing 30 μg of laponite and different amounts of polycations, Scheme 1 (e.g. for VBT–VBA 0, 10, 15 and 20 μg and for PDDA 15 μg), and subsequently air-dried at 4 °C. For the preparation of enzymatic hydrogel modified electrodes (EHME) with lactate oxidase (LOx), 20 μg of the enzyme were also incorporated into the mixture with laponite and VBT–VBA, and afterwards treated as previously. In this case, the total volume deposited on the electrode surface was 25 μL.

In all cases, after drying the modified electrodes were immersed for 45 min in phosphate buffer (0.1 M pH 7.0) for swelling. When not in use, the electrodes were stored at 4 °C in phosphate buffer solution.

2.4. Electrochemical measurements

Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and chronoamperometry (CA) studies were carried out with an Autolab (Eco-Chemie, Utrecht, Netherlands) equipped with a PGSTAT 30 potentiostat, with the software package GPES and FRA 4.9. When necessary, convective conditions were maintained with a magnetic stirrer. Experiments were performed in a three-compartment electrochemical cell with standard taper joints thus all compartments could be hermetically sealed with Teflon® adapters [7,8]. Working electrodes were prepared on glassy carbon (GC) disks, with geometric area of 0.036 and 0.027 cm^2 as determined by CA technique using the Cotrell equation for the diffusional current as function of time, and a diffusion coefficient of 7.3×10^{-6} cm^2 s^{-1} for the redox probe [27,28]. A large-area platinum wire was used as a counter electrode. The conductivity properties of the hydrogel films were characterized by EIS in aqueous solutions containing 2.5 mM $K_3[Fe(CN)_6]$ + 2.5 mM $K_4[Fe(CN)_6]$ and 0.1 M KCl. The sine wave potential amplitude applied was 5 mV, at a bias potential of 180 mV and frequency range 0.05 Hz–10 kHz. The electroanalytical properties of the bioelectrodes were study by CV and CA using 0.1 M phosphate buffer at pH 7.0 as background electrolyte solution. FcMe (0.2 mM) was used as artificial mediator, and L-lactate was added to the background electrolyte in the chosen concentrations. The potentials were measured against a reference electrode Ag|AgCl|Cl^- (3 M), and all measurements were performed at room temperature (25° C) in deoxygenated solutions as a result of N_2-bubbling for at least 30 min prior measurements. CV experiments were done at potential sweep rates, ν, in the range of $10 \leq \nu\,(\mathrm{mV\,s^{-1}}) \leq 300$, while CA experiments were performed at 400 mV to guarantee complete oxidation of FcMe.

2.5. L-Lactate determinations in food and beverages

Typically, aliquots of real samples (100 μL of beer, 25 μL of wine, or 5 μL of yogurt) were added to 5 mL of 0.1 M pH 7.0 phosphate buffer containing 0.2 mM FcMe. L-Lactate concentration was determined using both standard addition and calibration methods [9]. In the case of the standard addition, aliquots of 10 μL of 0.025 M L-lactate were added to the electrolytic cell containing the real sample. A calibration plot was performed with control stock solutions of L-lactate in the concentration range of 0.03–0.30 mM.

Fermentation model studies of milk were performed by dissolution of 10 mL of a fresh commercial yogurt into 250 mL of pasteurized bovine milk. The starting mixture was incubated in 2 mL Eppendorf tubes at 37 °C with continuous shaking using a Thermomixer Comfort (Eppendorf, USA). Aliquots of 100 μL of the mixture were taken at times along the fermentation process up to 360 min. The lactic acid content was measured likewise as described for the real samples.

In order to validate the amperometric determinations, a standard spectrophotometric method based on the absorbance at 340 nm of NADH formed in the presence of L-lactate by L-lactate dehydrogenase (LDH) was also used (Boehringer Mannheim/R-Biopharm, Cat. No. 10139084035). Absorption spectra were registered with a diode-array Hewlett Packard 8453 UV-visible spectrophotometer (Palo Alto, CA, USA).

All L-lactate determinations were performed by triplicate, and the results are reported with the respective standard deviation.

3. Results and discussion

3.1. Electrochemical behavior of hydrogel modified electrodes (HME)

Fig. 1 shows the Nyquist impedance plots obtained by electrochemical impedance spectroscopy (EIS) for the redox couple $[Fe(CN)_6]^{3-/4-}$ for several electrodes. In all cases (bare GCE or HME), the impedance curves showed the semicircle shape at high frequency regime followed by a linear response in the low frequency range. These results indicate that regardless of the surface condition (e.g. without or with hydrogel), the impedance response of the redox couple is controlled by combined kinetic and diffusion processes as a function of the frequency regime. Due to the presence of the hydrogel film on the electrode surface, its linear region of the Nyquist impedance plots showed ≈3–5% smaller slope than the bare GCE.

Satisfactory fittings of the experimental data (solid lines in Fig. 1) were obtained using the Autolab FRA 4.9® software by non-linear least squares method with the equivalent circuit indicated in Fig. 1, where R_s represents the ohmic resistance of the electrolyte solution measured between the working and reference electrodes; Q_{dl} is the double layer capacitance (in this case a constant phase element due to the non-ideal behavior of the double layer); R_{ct} is the charge transfer resistance of the redox probe represented by the diameter of the impedance curve; and finally the Warburg impedance (Z_w) that reflects the influence of the electroactive species mass transport from the bulk solution to the interface [29]. Table 1 collects the fitting parameters of the impedance curves. It can be observed that the R_s value was almost constant among the different electrodes, as it was expected for the same bulk properties of the solution independently of the composition of the electrode interface. On the other hand, the charge-transfer resistance R_{ct} was governed by

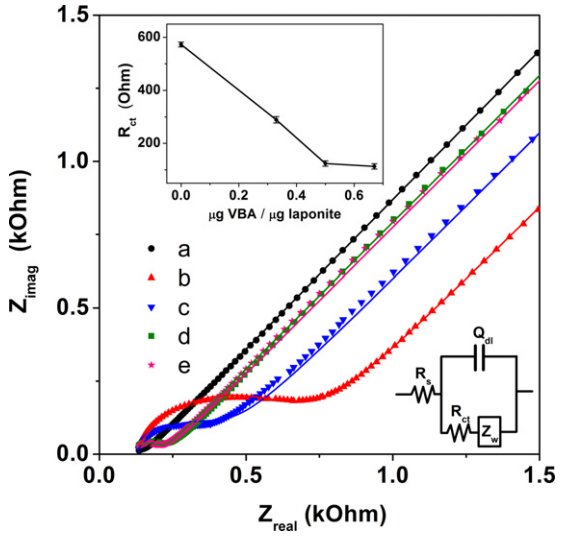


Fig. 1. Nyquist impedance plots of glassy carbon electrodes modified by laponite/VBT–VBA based hydrogels: (a) bare electrode; (b) with 30 µg laponite; (c) with 30 µg laponite and 10 µg VBT–VBA; (d) with 30 µg laponite and 15 µg VBT–VBA, and (e) with 30 µg laponite and 20 µg VBT–VBA; in the presence of 5 mM $[Fe(CN)_6]^{4-/3-}$ in 0.1 M KCl. The biased potential was 180 mV (vs. Ag/AgCl), sine wave potential amplitude 5 mV and frequency range between 0.05 Hz and 10 kHz. Upper inset: charge transfer resistant (R_{ct}) values as a function of the VBT–VBA/laponite mass ratio in the hydrogel film of the modified electrode. Lower inset: equivalent electrical circuit used for the fitting of the impedance Nyquist plots.

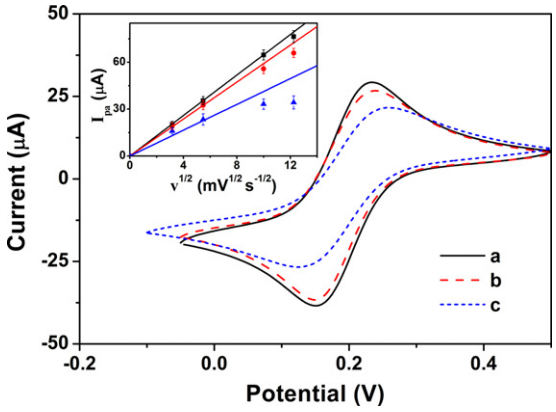


Fig. 2. Cyclic voltammograms of 5 mM $[Fe(CN)_6]^{4-/3-}$ in 0.1 M KCl solution at scan rate 30 mV s^{-1} of: (a) bare glassy carbon electrode; and hydrogel modified electrodes (HME) with: (b) 30 µg laponite + 15 µg VBT–VBA; and (c) 30 µg laponite + 15 µg PDDA. Inset: anodic peak current as function of the potential scan rate square root for three electrodes.

the hydrogel composition, indicating changes in both dielectric and insulating features of the electrolyte/electrode interface [30].

The largest R_{ct} value was observed for the HME containing only laponite. However, the progressive addition of polycations to the hydrogel reduces R_{ct}, as shown in the inset of Fig. 1 for the HME with VBT–VBA. A similar effect on R_{ct} was previously also observed for GCE with laponite hydrogels modified with the natural chitosan polycation [8]. For the polycation/clay mass ratio of 0.5, the reduction effect of R_{ct} was larger for the HME with VBT–VBA than with PDDA. Under these conditions, the electrical charge ratio between the polycation and laponite was ≈2 for VBT–VBA and ≈4 for PDDA, indicating a complete excess of positive charges in the hydrogel network. Therefore, the decrease of R_{ct} value could arise from electrostatic attraction of the redox probe $[Fe(CN)_6]^{3-/4-}$, given that the incorporation of polycations increases the excess of positive charges into the laponite film. However, since the same mass amount of PDDA produces double of positive charges than VBT–VBA, a large reduction of R_{ct} should be observed for PDDA as well. Therefore, extra-electrostatic effects should play a role in the electro-kinetic properties of these HME. It has been mentioned, that the addition of polycations produces a pillar effect of the laponite platelets allowing a better reticulation of the hydrogel network with a higher mesoporosity [15].

Under our experimental conditions, the impedance curves were obtained with a small sinusoidal amplitude perturbation of ±5 mV. Therefore, the redox couple remains near the equilibrium conditions, corresponding to the linear portion of the i–η Tafel's relationship. In that case, it is possible to obtain the time constant (τ_{ct}) of the electron-transfer reaction between the redox couple and the electrode using Eq. (3) [31]:

$$\tau_{ct} = R_{ct} Q_{dl} \quad (3)$$

Table 1 also presents the calculated τ_{ct} values for all the electrodes showing, in comparison with the CG bare electrode, that the τ_{ct} of the HME containing only laponite was strongly increased because of the lower charge transport properties of the clay [32]. However, the addition of increasing amounts of VBT–VBA polycations in the HME decreases τ_{ct} up to a value closer to that observed for the bare electrode. In contrast, for the HME with the same amount of PDDA (15 µg), the τ_{ct} value was closer to the value of the electrode containing only laponite. These results suggest that VBT–VBA improves the physical diffusion in the channels of the HME, probably by a better swelling and/or lamellar-like structure of the clay [32].

In order to confirm this effect, the reversibility of the redox-couple reaction was analyzed by CV experiments, Fig. 2. Interestingly, the voltammogram of the HME containing VBT–VBA showed the same potential peak separation than the bare electrode, e.g. $\Delta E = E_c - E_a = 78 \pm 1$ mV, indicating that the presence of VBT–VBA in the hydrogel does not modify the reversibility of the process. Instead, in the presence of PDDA a larger $\Delta E = 126 \pm 1$ mV value was observed, pointing out that the process becomes quasi-reversible. Furthermore, the comparison of the initial linear slopes (s_0) of the plot peak current (I_p) vs. square root of the potential sweep rate ($v^{1/2}$) for the HME with the bare GCE allows the estimation of the percentage of redox couple permeability, e.g. $\%P = (s_0^{HME}/s_0^{GCE}) \times 100$ [33], Table 1. As it was observed in the impedance experiments (see above), the presence of polycations in

Table 1
Electrochemical properties of glassy carbon electrodes (GCE) modified with hydrogels made with 30 µg of laponite R.D. and different amounts of VBT–VBA or PDDA polycations in 5 mM $[Fe(CN)_6]^{4-/3-}$ and 0.1 M KCl aqueous solutions.

Hydrogel composition	R_s (Ω)	Q_{dl} (µF)	R_{ct} (kΩ)	Z_w (mΩ)	τ_{ct} (µs)	%P[a]
Bare GCE	137 ± 5	0.069 ± 0.004	0.049 ± 0.003	0.55 ± 0.02	3.4 ± 0.3	100
+30 µg laponite	135 ± 7	0.13 ± 0.02	0.573 ± 0.009	0.68 ± 0.02	76 ± 4	48 ± 1
+30 µg laponite + 10 µg VBT–VBA	129 ± 4	0.12 ± 0.02	0.29 ± 0.01	0.71 ± 0.02	36 ± 6	55 ± 1
+30 µg laponite + 15 µg VBT–VBA	131 ± 8	0.071 ± 0.006	0.124 ± 0.006	0.86 ± 0.02	8.8 ± 0.8	91 ± 2
+30 µg laponite + 20 µg VBT–VBA	134 ± 5	0.041 ± 0.002	0.113 ± 0.008	0.91 ± 0.04	4.6 ± 0.4	96 ± 1
+30 µg laponite + 15 µg PDDA	132 ± 9	0.36 ± 0.01	0.27 ± 0.02	0.43 ± 0.05	98 ± 4	69 ± 1

[a] Permeability defined as: $\%P = (s_0^{HME}/s_0^{GCE}) \times 100$.

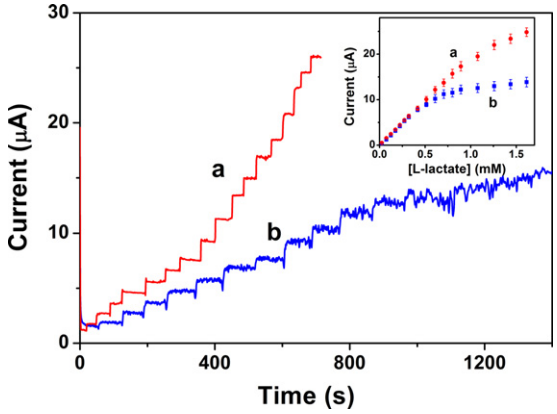


Fig. 3. Current–time response at 400 mV of hydrogel modified bioelectrode containing 20 μg of lactate oxidase (LOx) and 30 μg of laponite with (a) 15 μg of VBT–VBA and (b) 15 μg of PDDA, observed after successive additions of L-lactate aliquots to 0.1 M phosphate buffer pH 7.0 with 0.2 mM FcMe. Inset: corresponding calibration plot of current as function of L-lactate concentration. Regression equations: (a) $y = (5 \pm 3) \times 10^{-5}\ \mu A + (19.7 \pm 0.5)\ \mu A\ M^{-1} \times (R^2 = 0.999)$, (b) $y = (1.1 \pm 0.7) \times 10^{-4}\ \mu A + (18.1 \pm 0.7)\ \mu A\ M^{-1} \times (R^2 = 0.998)$.

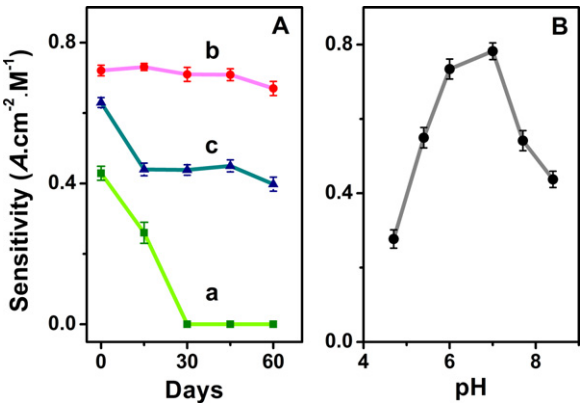


Fig. 4. (A) Stability curves (sensitivity vs days) of the hydrogel modified bioelectrode composed by 30 μg of laponite, 20 μg of LOx and (a) 10, (b) 15 or (c) 20 μg of VBT–VBA. (B) pH effect on the sensitivity of the bioelectrode composed by 30 μg laponite, 20 μg LOx and 15 μg VBT–VBA.

the hydrogel composition improves the %*P* value compared to the hydrogel containing only laponite, being VBT–VBA more efficient than PDDA.

These results confirm the role of the polycation nature on the structure and/or electrokinetic properties of the hydrogel films over the electrode surface [34]. The size and hydrophobic effects of the polycation on the laponite multi-lamellar structures was investigated for a series of PDDA derivatives with varying hydrophilic/hydrophobic balance and bulkiness of the cationic group [35], and it was shown that more bulky and hydrophobic PDDA derivatives could be better accommodated between the rigid exfoliated aluminosilicate platelets without disturbing the laponite lamellar-like structure. Ellipsometry and X-ray reflectivity experiments showed that hydrophobic and bulky substitution in the polycations favored the formation of thicker films, due to more coiled conformation of the polycation [35]. This could be the case of VBT–VBA, which contains more bulky and hydrophobic moieties than PDDA, Scheme 1.

3.2. Electrochemical behavior of enzymatic HME (EHME)

The incorporation of 20 μg LOx into the laponite-polycation hydrogels increases the R_{ct} value for the discharge of the redox couple $[Fe(CN)_6]^{3-/4-}$, e.g. $169 \pm 4\ \Omega$. This result indicates the successful immobilization of the enzyme into the hydrogel structure with a consequential interference on the electron-transfer process of the redox probe [36].

Fig. 3 shows the plots of the current–time response with the step-wise increment of L-lactate concentration of two modified bioelectrodes with hydrogels containing the same amount (15 μg) of VBT–VBA or PDDA, respectively.

A less noisy response was observed for the EHME containing VBT–VBA than for PDDA. The inset of Fig. 3 shows the respective current–concentration calibration curves, which initial slopes represent the sensitivity (*s*) of the bioelectrode, Table 2. The detection limit of both EHME was calculated using the criterion of $3 \times$ S.D./*s* where S.D. is the standard deviation of the average current obtained after ten measurements ($n = 10$) of 1×10^{-4} M L-lactate [7,37], and for the EHME with VBT–VBA the detection limit was almost 3 times lower than for the bioelectrode with PDDA, Table 2. A similar sensitivity ($\approx 20\ \mu A\ mM^{-1}$) was observed for both EHME, but that with VBT–VBA showed faster time response and two-times linear range than for the bioelectrode with PDDA.

For the sake of comparison, the sensitivity of bioelectrodes with LOx immobilized by sol–gel matrices were smaller, i.e. $6.4\ \mu A\ mM^{-1}$ [13] and $12\ \mu A\ mM^{-1}$ [14], with linear ranges of 0.2–2.0 mM and 0.05–0.60 mM, respectively.

In the case of EHME containing different polycations and the same amount of LOx (20 μg) a better comparison can be performed by correcting the observed sensitivity (*s*) by the electrode area *A* (see Section 2.4). Thus, the corrected sensitivity was 720, 500, and $330\ \mu A\ mM^{-1}\ cm^{-2}$ for EHME with VBT–VBA, PDDA, and chitosan [8], respectively. The comparison of the electroanalytical properties of the different EHME in Table 2 indicates that the bioelectrode with VBT–VBA presents the best behavior of the EHME series.

Considering this fact, the sensitivity of the bioelectrode with different amounts of VBT–VBA was analyzed during 60 days by chronoamperometric (CA) experiments, Fig. 4a. The highest sensitivity and stability over the time was observed for the EHME containing 15 μg of VBT–VBA. This result indicates that under this hydrogel composition no significant structural changes and/or leaching of the immobilized enzyme were happening. Instead, the lowest initial amperometric response to the L-lactate concentration was observed for the EHME containing 10 μg of VBT–VBA. In addition, the amperometric signal dropped with the storage and completely lost after 30 days. The EHME containing 20 μg of VBT–VBA, despite of its highest permeability and lowest R_{ct} (Table 1), showed an initial loss of sensitivity to reach a plateau with almost half of the amperometric response than the EHME with 15 μg VBT–VBA. Since the isoelectric point (p*I*) of the isolated LOx is 4.6 [38], the enzyme is negatively charge at the working pH. The effectiveness of the enzyme entrapped depends on the hydrogel texture, mesoporosity and electrical charge. Therefore, despite of the improvement of the electrokinetic properties of the EHME with 20 μg of VBT–VBA, the larger excess of positive charges in the hydrogel film induces stronger electrostatic interactions with the enzyme, which can lead to structural changes of the enzyme, and reducing the bioelectrode sensitivity.

Therefore, the balance between the polycation bulkiness and charge density is critical for the tuning of the hydrogel film electrokinetic properties, as well as the stability and functionality of the enzyme.

The pH dependence of the sensitivity of the bioelectrode containing 15 μg VBT–VBA was also investigated over the range pH 4.7–8.4, Fig. 4b. A maximum response was observed at pH 7.0, as typically reported for other O_2-requiring native and immobilized enzymes [14,39,40]. Therefore, the sensitivity profile of the EHME is modulated by the intrinsic pH dependence of the LOx activity. This result also suggests that the laponite hydrogel formed with 15 μg

Table 2
Electroanalytical properties of glassy carbon electrodes containing immobilized Lactate oxidase (LOx) onto laponite hydrogels with different polycations.

Polycation (laponite/polycation mass ratio)	Detection limit (μM)	Linear range (mM)	Sensitivity (μA mM^{-1})	Time response (s)	Ref.
Oligosilasesquioxane (1)[a]	1.0 ± 0.1	0.003–0.300	26.0 ± 0.8	10 ± 1	[7]
Chitosan (0.5)[b]	3.8 ± 0.2	0.01–0.07	11.5 ± 0.3	5 ± 1	[8]
VBT–VBA (2)[b]	3.4 ± 0.1	0.01–1.0	19.7 ± 0.5	5 ± 1	This work
PDDA (2)[b]	10 ± 1	0.03–0.50	18.1 ± 0.7	10 ± 1	This work

Enzyme mass immobilized: (a) 40 μg and (b) 20 μg.

of VBT–VBA does not modify substantially the enzyme structure and its functionality.

3.3. Evaluation of molecular interferences to the VBT–VBA bioelectrode response

Citric, tartaric, acetic, malic, formic and succinic acids as well as fructose and glucose have been reported as interferences in wine and beer analysis with bioelectrodes [9,11]. In addition, dairy products analysis also involves the study of glucose, plus citric, acetic and ascorbic acids as possible interferences [9]. Methanol and ethanol are obstacles as well, since ethanol is the main component of wine and methanol is an undesired fermentation subproduct [41]. In the case of the VBT–VBA bioelectrode, only ascorbic acid was electroactive among the interferences mentioned above at the working potential of 400 mV for the CA experiments. Therefore, the electroanalytical response of the bioelectrode to AA was evaluated (data not shown), obtaining a sensitivity of 12 μA mM^{-1} and detection limit of 83 mM, a value two-order of magnitude larger than the highest concentration levels of AA in milk, e.g. ≈0.11 mM [42]. For yogurt samples, a similar or even lower concentration of AA can be expected. Therefore, the AA interference in the real samples for the measuring of L-lactate with EHME containing VBT–VBA can be neglected.

3.4. L-Lactate determination in food and beverages samples

Considering that the EHME composed by 15 μg VBT–VBA, 30 μg of laponite, and 20 μg of LOx showed the best electroanalytical properties, in order to explore a technological use of this electrode its performance for the determination of L-lactate in several commercial yogurt, white wine and beer samples was evaluated, Table 3. The real samples were only diluted with phosphate buffer solutions in order to fit into the linear range response of the bioelectrode. Control experiments recorded with HME with identical polycation/clay composition but without LOx showed no changes in the stationary currents for FcMe oxidation after the addition of either L-lactate aliquots or real samples (data not shown). No significant differences were found between the standard addition and calibration plot methods, pointing out that the matrix effects are not important [9]. Furthermore, according to a *t*-test at the 95% confidence level, no significant difference for L-lactate determination between the EHME and the spectrophotometric standard was observed, Table 3.

Table 3
L-Lactate concentrations (mM) in commercial samples of yogurt, white wine, and beer determined using glassy carbon electrodes containing immobilized lactate oxidase (LOx) by laponite/VBT–VBA hydrogel and by a standard colorimetric commercial kit.

Sample	Bioelectrode (standard addition method)	Bioelectrode (calibration plot)	Spectrophotometry assay
White wine	14 ± 2	12 ± 1	13 ± 1
Yogurt	37 ± 5	34 ± 2	41 ± 7
Beer	4.2 ± 0.2	3.8 ± 0.3	4.8 ± 0.9

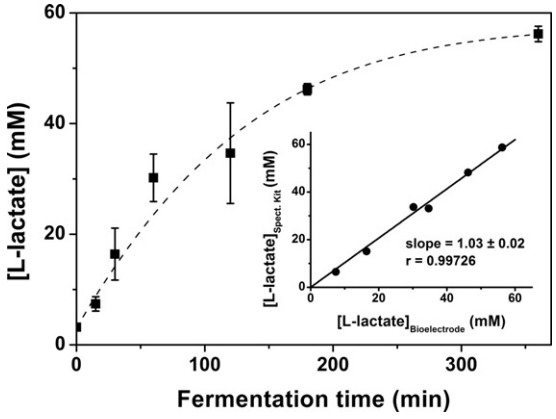


Fig. 5. Development of L-lactate during milk fermentation at 37 °C determined with the hydrogel modified bioelectrode made with 30 μg laponite, 20 μg LOx and 15 μg VBT–VBA. Inset: correlation of the L-lactate concentration determined with the standard spectrophotometric kit (Boehringer Mannheim/R-Biopharm) and the bioelectrode with VBT–VBA.

Therefore, it can be claimed that the bioelectrode with VBT–VBA can be successfully used to determine L-lactate both in alcoholic beverages and dairy products.

3.5. Monitoring of L-lactate in a fermentation process

Finally, the L-lactate production by fermentation in yogurt preparation at 37 °C was also analyzed using the bioelectrode with 15 μg of VBT–VBA and compared with the spectrophotometric standard assay as function of the fermentation time. Fig. 5 shows the continuous increase of L-lactate concentration monitored with the bioelectrode. As it is shown in the inset of Fig. 5, the substrate concentration determined by the use of the bioelectrode was in accordance with the calculated from the spectrophotometric standard assay (within 2% of confidence), validating the performance and versatility of the VBT–VBA based bioelectrode.

4. Conclusions

The present results demonstrate the suitability of the bioinspired water-soluble polycation VBT–VBA for the preparation of laponite hydrogels for LOx immobilization on GCE, compared with other polycations such as chitosan and oligosilasesquioxane [7,8]. The HME with VBT–VBA showed more remarkable electrokinetic properties than for that with PDDA, probably due to most favorable hydrophilic/hydrophobic and electrical charge balances, given by the laponite/VBT–VBA ratio. The properties enhancement is also retained in the presence of LOx, indicating that VBT–VBA provides an optimal inter-lamellar space and environment for the enzyme fitting into the laponite hydrogel film. Thus, the better amperometric response and operational stability for sensing of L-lactate after 60 days were obtained for a bioelectrode with a VBT–VBA/laponite mass ratio corresponding to two-time the cation exchange capacity of the clay. Furthermore, this bioelectrode resulted very useful

for the selective determination of L-lactate in food samples such as white wine and fermented milk without sample pretreatment.

Therefore, the use of novel and bioinspired polyelectrolytes such as thymine based copolymers can result in promising developments of biosensor devices.

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