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A highly sensitive and stable glucose biosensor using thymine-based polycations into laponite hydrogel films

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A series of glucose bioelectrodes were prepared by glucose oxidase (GOx) immobilization into laponite hydrogel films containing DNA bioinspired polycations made of vinylbenzyl thymine (VBT) and vinylbenzyl triethylammonium chloride (VBA) with general formulae $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ with $m=0$, 1 and $n=2$, 4, 8, deposited onto glassy carbon electrode. The bioelectrodes were characterized by chronoamperometry, cyclic voltammetry and electrochemical impedance spectroscopy. Results indicated that the electrochemical properties of the laponite hydrogel films were largely improved by the incorporation of thymine-based polycations, being proportional to the positive charge density of the polycation molecule. After incorporation of glucose oxidase, the sensitivity of the bioelectrode to glucose increased with the positive charge density of the polycation. Additionally, the presence of the vinylbenzyl thymine moiety played a role in the long-term stability and reproducibility of the bioelectrode signal. As a consequence, the $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ was the most appropriate polycation for bioelectrode preparation and glucose sensing, with a specific sensitivity of $s_e = 176\ \mathrm{mA\ mmol^{-1}\ L\ cm^{-2}\ U^{-1}}$, almost two-order of magnitude larger than other laponite immobilized GOx bioelectrodes reported elsewhere. These features were confirmed by testing the bioelectrode for a selective determination of glucose in powder milk and blood serum samples without interference of either ascorbic or uric acids under the experimental conditions.

The present study demonstrates the suitability of DNA bioinspired water-soluble polycations $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ for enzyme immobilization like GOx into laponite hydrogels, and the preparation of highly sensitive and stable bioelectrodes on glassy carbon surface.

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1. Introduction

Glucose is the most abundant monosaccharide in nature, and its catabolism represents the primary source of energy synthesis in cells. Moreover, it is the principal constituent of structural and energetic biopolymers, such as cellulose, starch and glycogen. In wellbeing, the detection of glucose in the blood stream has greatest implications in the control of diabetes disease. On the other hand, in food industry the glucose content is associated to storage time, fermentation process and final quality control, consequently its determination is routinely performed in wine [1,2], juice [3], honey [4], and milk [5] samples. For those reasons, an accurate quantification of glucose with a quick and easy method has driven the search for new procedures of analysis. In this regard, electrochemical methods have shown to be very suitable and have been significantly developed in recent years, especially those involving the use of biosensors [6] which offer outstanding features such as specificity, portability, quickness and comparative low cost [7]. In particular, the glucose biosensors have been widely studied and developed [8]. However, scientific advances have not been accompanied by corresponding technological improvements, and their application in health and general industry areas is very limited. A major cause of this problem is the necessity to use non expensive and environmentally friendly materials. Furthermore, one-step enzyme immobilization methods are preferred, using a fixative material that provides both efficient enzyme functioning and permeability of the substrate. Among several immobilization methods, the enzyme fixation using

Abbreviations: (VBT), Vinylbenzyl thymine; (VBA), Vinylbenzyl triethylammonium chloride; (FcMe), Ferrocene methanol; (AA), Ascorbic acid; (UA), Uric acid; (GCE), Glassy carbon electrode; (HME), Hydrogel-modified glassy carbon electrode without enzyme

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hydrogel films made of natural or artificial clays, seems to be a very suitable method that satisfies most of the above mentioned requirements [9–11]. Laponite RD is an artificial clay with a negatively charged surface and a disk shape of nanoscopic dimensions (1 nm width × 25 nm diameter). Laponite has been extensively used in combination with polycations for the formation of hydrogel films on a suitable electrode and entrapping an active enzyme for bioelectrochemical sensing [11,12]. Due to the poor conductivity of the anionic clay, the incorporation of polycation molecules into the hydrogel film improves both the mechanical stability and the electrical conductivity of the interface, besides retaining the active enzyme [11–13]. Therefore, the design of innovative polycation molecules [14] becomes a relevant issue in the development of efficient and robust bioelectrodes.

In order to develop new benign materials, different natural mechanisms have been inspected identifying processes potentially adaptable to synthetic systems. Thymine-based polymers are artificial macromolecules inspired on DNA, which recently have gained much attention due to their photo-sensitivity and molecular recognition patterns [15–18]. Extending the knowledge achieved from the study of the natural phenomena related to thymine reactivity and intermolecular interactions has generated a diapason of potential applications [19–22] ranging from photoresists materials [23,24] and organic-inorganic hybrid devices [16], to antibacterial coatings [25], drug delivery systems [17,26–28], recyclable plastics [29] and biosensors [12]. On the other hand, the increasing attention given to the environmental and toxicological dilemmas linked to commercial materials, situates the thymine based polymeric systems as a Green Chemistry platform [30]. From an intellectual standpoint, thymine polymeric systems offer an approach to physiologically relevant processes, whereas offering commercially applicable opportunities in materials science. This synergy is fairly unique in scientific attempt and will make available new understandings and materials which have not yet been imagined.

In the present work, our previous studies on laponite hydrogels stabilized by thymine-based polycations [12] were extended, focusing mostly about the influence of the hydrophilic–hydrophobic balance on the electroanalytical properties of the bioelectrode. A series of polycations made of vinylbenzyl thymine (VBT) and vinylbenzyl triethylammonium chloride (VBA) with general formulae $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ with $m=0$, 1 and $n=2$, 4, 8, were used in combination with laponite for the immobilization of glucose oxidase (GOx) onto glassy carbon electrode (GCE). The enzymatic reaction was conducted using the natural mediator of the enzyme (O_2) and also an artificial mediator, ferrocene methanol (FcMe), allowing the electrochemical detection of glucose at two ranges of very different working potentials. The electrochemical properties of the bioelectrodes were characterized by chronoamperometry (CA), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). Finally, the analytical performance of the most sensitive and stable bioelectrode of the series was evaluated for glucose determination in powder milk and serum blood samples. The potential interference effect of ascorbic and uric acids was also analyzed.

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, USA). Glucose Oxidase (GOx, EC 1.1.3.4 from *Apergillus Niger* type II) lyophilized powder containing 17300 units g^{-1} solid. Reagent grade L-(+)-glucose, ferrocene methanol (FcMe), ascorbic acid (AA), uric acid (UA), potassium ferrocyanide and ferricyanide $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 99% were purchased from Sigma-Aldrich SA (Buenos Aires, Argentina). Reagent grade buffer phosphate sodium salts NaH_2PO_4 and Na_2HPO_4 were obtained from J.T. Baker (Mexico D.F., Mexico). Water-soluble polystyrene copolymers of vinylbenzyl thymine (VBT) and vinylbenzyl triethylammonium chloride (VBA) were synthetized as described before [31,32], obtaining polycations of general formulae $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ with $m=0$, 1 and $n=2$, 4, 8. Solutions were prepared with triply distilled water. Compressed ultrapure nitrogen was purchased from Indura SRL (S.M.de Tucumán, Argentina). Working electrodes were prepared with glassy carbon disks CHI104 from CH Instruments (Texas, USA).

2.2. Bioelectrode conditioning and preparation

Prior to each experiment, the glassy carbon electrodes (GCE) were polished sequentially with alumina powder of decreasing particle size, e.g. 1.0, 0.3, and 0.05 µm (Buehler, USA), copiously rinsed with ultra-pure water and sonicated for 1 min between polishing steps. Afterward, the GCE was immersed in acid piranha solution [3:1 mixture of concentrated sulfuric acid (H_2SO_4) with hydrogen peroxide (H_2O_2, 98%)] for 2 min following by a last sonication for 1 min. 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) was prepared by deposition of 22 µL of stock solution mixture containing 30 µg of laponite and 15 µg of different thymine-based polycations, e.g. $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$, and subsequently air-dried at 4 °C. Bioelectrodes were prepared by adding 20 µg of glucose oxidase (GOx) into the mixture of clay and polycation, and placing between 17–32 µL of the prepared mixture on the GCE surface. Afterwards, the bioelectrodes were dried as described above. In all cases, after drying the modified electrodes were immersed for 45 min in phosphate buffer (0.1 mol L^{-1} pH 7.0) for swelling. The electrodes were stored at ~8 °C in phosphate buffer solution when not in use.

2.3. 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, and analyzed with the software package GPES and FRA 4.9 [12]. Experiments were performed in a three-compartment electrochemical cell with standard taper joints; as a consequence all compartments could be hermetically sealed with Teflon® adapters. Working electrodes were prepared on glassy carbon disks of 0.07 cm^2. 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 mmol L^{-1} $K_3[Fe(CN)_6]$ + 2.5 mmol L^{-1} $K_4[Fe(CN)_6]$ and 0.1 mol L^{-1} KCl. The sine wave potential amplitude applied was 10 mV at a bias potential of 200 mV within 0.05 Hz–10 KHz frequency range. The electroanalytical properties of the bioelectrodes were studied by CV and CA. The potentials were measured against a calomel reference electrode Ag|AgCl|Cl^-(3 mol L^{-1}), and all measurements were performed at 25 °C.

A 0.1 mol L^{-1} phosphate buffer solution at pH 7.0 was used as background electrolyte, with 0.2 mmol L^{-1} FcMe as artificial mediator of the enzymatic reaction. Glucose aliquots of chosen concentrations were added between measurements. In this case, solutions were deoxygenated by controlled N_2-bubbling during 30 min preceding the measurements and the CA experiments were performed at 400 mV. Additionally, CA experiments were carried out in air-saturated phosphate buffer solutions at a fixed

potential of −700 mV, with dissolved O_2 as enzymatic mediator and with glucose aliquots added in the desired concentrations. In both cases, CA experiments were performed under convective conditions using magnetic stirring.

2.4. Glucose determination in powder milk and serum blood samples

In powder milk samples, glucose quantification was carried out by CA with FcMe under the experimental conditions described above. On the other hand, glucose in serum blood samples was determined through an O_2-mediated enzymatic reaction, due to a possible interference of coexisting electroactive species such as ascorbic and uric acids.

Milk samples were prepared according to the specifications provided by the supplier (La Serenisima®, Argentina). Briefly, 2 g of powder milk were mixed with 80 mL of distilled water in a test tube, thoroughly shaken until the powder was completely dissolved, and adding water to a final 100 mL volume. Milk aliquots of 9 μL were added to 10 mL of 0.1 mol L^{-1} pH 7.0 phosphate buffer+0.2 mmol L^{-1} FcMe. Glucose concentration was determined using a calibration plot. The stationary current obtained for 0.1 mmol L^{-1} Glucose was compared with the current recorded for the sample. Sample analysis was carried out for 5 consecutive days using the same electrode, as part of an inter-day validation. Every day a new sample solution was prepared.

In the case of blood samples, 250 μL of the serum obtained after coagulation (15 min at 37 °C) and centrifugation (7000 rmp during 10 min) were placed in 10 mL of phosphate buffer. The quantification of the substrate was carried out using a calibration plot, as described above. Results were validated by comparison with a standard spectrophotometric method (Stanbio Glucose LiquiColor® Reagent Cat. N°1071) based on the reaction of H_2O_2 with phenol and 4-aminoantipyrine in the presence of peroxidase. This reaction forms a quinone derivative having a typical absorbance at 505 nm that was measured with an Agilent 8453 diode array spectrometer (Palo Alto, CA, USA). The inter-day validation for the serum samples was carried out using the same electrode on days 1 and 4.

All determinations were performed by triplicate, and the average results are reported with proper standard deviations.

3. Results and discussion

3.1. Polycation composition effect on the electrochemical behavior of hydrogel modified electrodes (HME)

Fig. 1A shows the cyclic voltammograms for 1 mmol L^{-1} $Fe(CN)_6^{4-}$ prepared in 0.1 mol L^{-1} KCl obtained with the bare glassy carbon electrodes (GCE) and laponite-hydrogel modified electrodes (HME) with and without various thymine-based polycations $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$. The influence of hydrogel composition on the potential peak separation ΔE_p and the corresponding anodic ($i_{p,a}$) and cathodic ($i_{p,c}$) peak current values was evaluated. A classical reversible behavior for the redox couple on the bare GCE was observed, since $\Delta E_p = 68 \pm 2$ mV and $i_{p,a} \approx i_{p,c}$ [33]. By deposition of 30 μg of laponite on the electrode surface, the ΔE_p was increased up to ≈ 356 ± 9 mV together with a large decreases of both $i_{p,a}$ and $i_{p,c}$ values. This behavior suggests that the redox kinetics at the electrode surface was significantly modified as a result of the semiconductor nature of the clay and neat electrostatic repulsion effects between the negative nanosurface of laponite and the redox probe. Nevertheless, this effect was strongly reverted with the addition of 15 μg of $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ polycations into the laponite matrix of the HME, being $\Delta E_p = 98 \pm 6$ mV, accompanied by a large increase of the peak currents. In fact, the

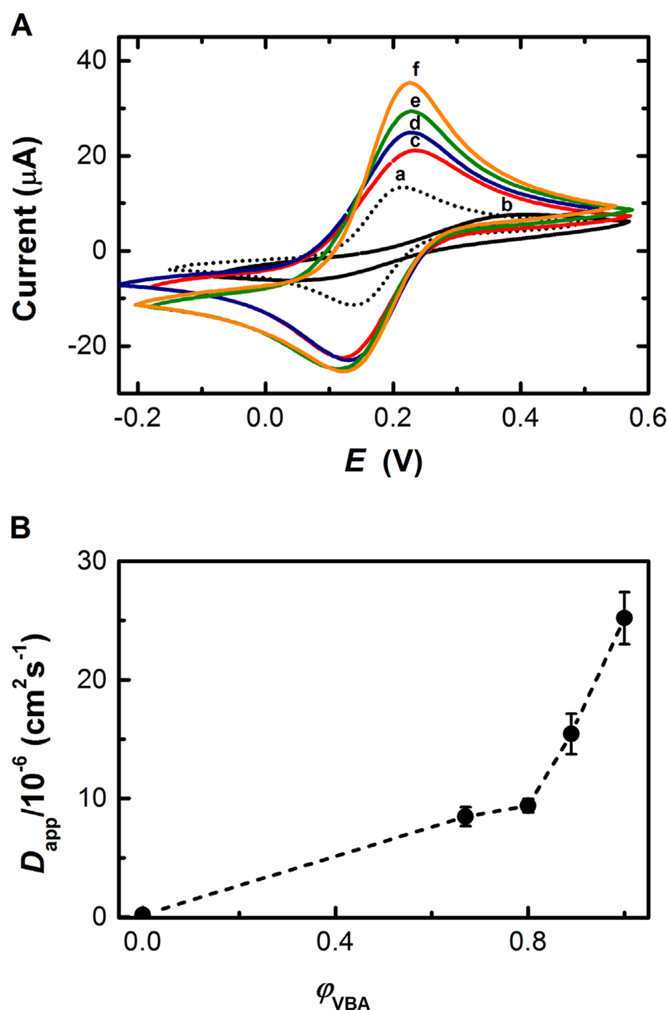


Fig. 1. (A) Cyclic voltammograms of 1 mmol L^{-1} $Fe(CN)_6^{4-}$ in 0.1 mol L^{-1} KCl monitored at 100 mV s^{-1} for: (a) Bare GCE; (b) HME with laponite; (c) HME with laponite and $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$: $m=1$ and $n=2$; (d) $m=1$ and $n=4$; (e) $m=1$ and $n=8$; and (f) $m=0$ and $n=1$. (B) Apparent diffusion coefficient (D_{app}) as function of the positive charge density fraction of the polycation $\varphi_{VBA} = n/(n+m)$.

latter effect was proportional to the increment of positive charges into the copolymeric unit of the polycation (Fig. 1A). Since the redox kinetics at the GCE was significantly modified only when the electrode was coated by hydrogel film made of neat laponite, the anodic peak current increase observed in the presence of polycations can be due to larger diffusion of $Fe(CN)_6^{4-}$ inside the hydrogel films. By using the Randles–Sevcik equation (1) [34], the apparent diffusion coefficient D_{app} ([=] cm^2 s^{-1}) of $Fe(CN)_6^{4-}$ was calculated.

$$i_p = 2.7 \times 10^5 n^{3/2} A D_{app}{}^{1/2} \nu^{1/2} c \quad (1)$$

In Eq. (1) n is the number of exchange electrons, A is the area of the electrode (cm^2), ν is the scan rate (V s^{-1}) and c is the concentration of the electroactive specie (mol cm^{-3}). In all cases, cyclic voltammograms were performed at ν values ranging between 10 and 300 mV s^{-1}. Subsequently $i_{p,a}$ was plotted versus $\nu^{1/2}$ yielding a linear relationship, and D_{app} was calculated from the slope (Fig. S1, Supplementary Information). Fig. 1B shows the variation of D_{app} as a function of $\varphi_{VBA} = n/(n+m)$, which is the fraction of positively charged comonomer VBA into the polycation copolymer unit $[(VBT)_m(VBA)_n]^{n+}$. Except for the HME containing purely laponite, the D_{app} values were larger than in aqueous media, *e.g.* $D_w \sim 7 \times 10^{-6}$ cm^2 s^{-1} [35], as a result of the incorporation of polycations into the laponite matrix that results in

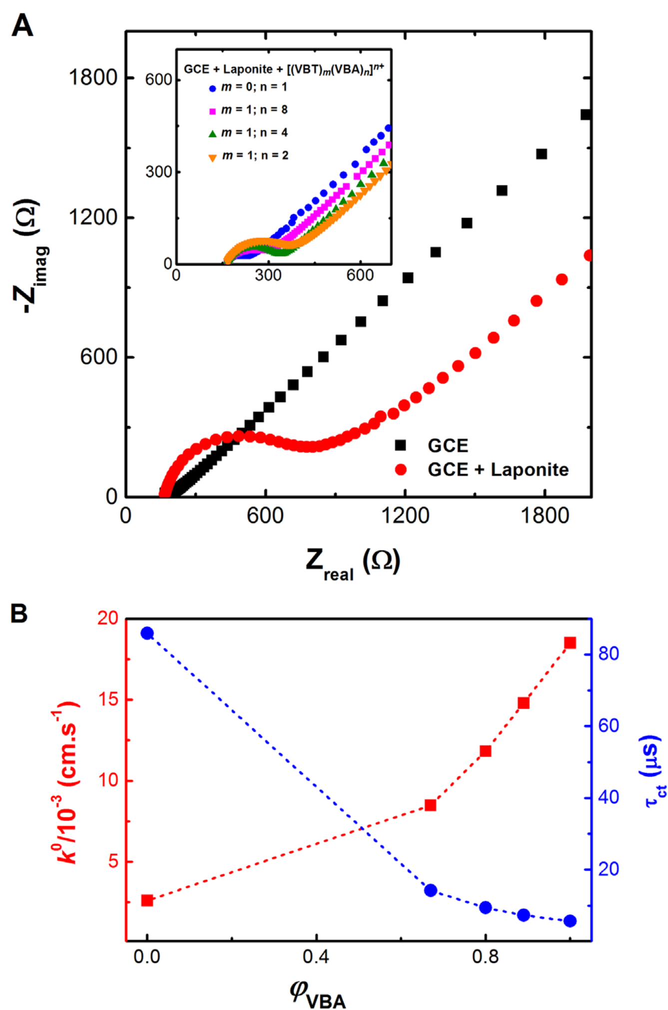


Fig. 2. (A) Nyquist plots for the bare GCE and different HME in the presence of 5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ in 0.1 mol L^{-1} KCl. The sine wave potential amplitude applied was 10 mV, at a continuous potential of 200 mV and frequency range 0.05 Hz–10 KHz. (B) Heterogeneous rate constant $k°$ (squares) and time constant τ_{ct} (circles) of the electron-transfer reaction as a function of φ_{VBA}.

hydrogels films with net positive charge density, increasing the electrostatic interactions with the negative redox probe. In fact, this effect is remarkable larger for HME containing polycations with $n \geq 4$ and for the polycation made with merely VBA (i.e. $\varphi_{VBA}=1$). This result suggests that the pendant VBT moiety could be responsible of hydrophobic effects affecting the diffusion of the redox probe.

Fig. 2A displays the Nyquist's impedance plots for the redox couple $[Fe(CN)_6]^{3-/4-}$ obtained by electrochemical impedance spectroscopy (EIS) for HME with different $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ polycations. In all cases, the impedance curves showed a semi-circle shape at high frequency regime followed by a linear dependence in the low frequency range. These results indicate that regardless on the presence of hydrogel film on the surface of GCE, the impedance response of the redox couple is controlled by combined kinetic and diffusion processes as a function of the frequency regime. Satisfactory fittings of the experimental data (Fig. S2, Supplementary Information) were obtained by non-linear least squares method with the Randles equivalent circuit (Fig. S2 and S3 of Supplementary Information), and all recovered data were collected in Table S1 (Supplementary Information). The EIS performed at small sinusoidal perturbation amplitudes provides an excellent method to evaluate the kinetics parameters without mass-transport complications, given that the technique allows the separation in frequency between both kinetic and mass-transport processes. The diameter of the semi-circle in the Nyquist´s plots is associated to the rate of electron-transfer process as a charge-transfer resistance R_{ct}. From the inset of Fig. 2A it can be observed that this parameter is largely dependent on the hydrogel composition, suggesting changes in both dielectric and insulating features of the electrolyte/electrode interface [36]. Under these experimental conditions and assuming a single electron transfer kinetics concurrently with equal concentration of reactants and products ($c_{ox}=c_{red}=c$), the R_{ct} is given by Eq. (2) [33]:

$$R_{ct} = RT/F^2Ak^0c \tag{2}$$

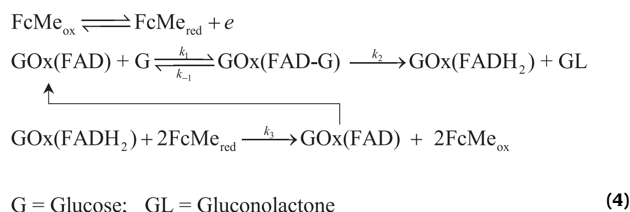
where R is the universal constant of ideal gases, T is the temperature in Kelvin, F is the Faraday constant, A is the total electrode area, and $k°$ is the standard heterogeneous rate constant. For the bare GCE, a $k°=3.7\times10^{-2}$ cm s^{-1} was obtained. When the laponite layer is placed onto the glassy carbon surface, $k°$ significantly decreased to 2.6×10^{-3} cm s^{-1}, but then again for the HME containing $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ polycations as well, the $k°$ value increases with φ_{VBA} (Fig. 2C). The observed values fell within the expected typical range for vitreous carbon surfaces [37]. The opposite behavior was observed for the time constant (τ_{ct}) of the electron-transfer reaction as calculated with Eq. (3) [38], where Q_{dl} is the double layer capacitance value obtained from the fitting of the EIS curves with the Randles circuit model (Table S1, Supplementary Information).

$$\tau_{ct} = R_{ct}Q_{dl} \tag{3}$$

Considering the cation exchange capacity (c.e.c.) of laponite [39] and the amount of clay and polycation mixed in the hydrogel preparations, the HME containing $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ has an excess of positive charges, ratifying that the improvements on the conductive and electrochemical properties are associated with this effect. The mentioned event plays a relevant role in both, the immobilization and the electrochemical performance of negatively charged redox enzymes at neutral pH in modified laponite matrixes [12,40].

3.2. Electrochemical behavior of glucose oxidase bioelectrodes

The influence of $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ polycations on the catalytic response of different bioelectrodes containing glucose oxidase (GOx) was studied by CV experiments through scan rates between 10 and 300 mV s^{-1} with 0.2 mmol L^{-1} FcMe as enzymatic mediator in N_2-saturated 0.1 mol L^{-1} phosphate buffer (pH 7.0) solutions. In absence of glucose, reversible redox waves of FcMe were observed, with both $i_{p,a}$ and $i_{p,c}$ showing a linear relationship with $v^{1/2}$, as it was expected for a diffusion controlled process (Fig. S4, Supplementary Information). On the other hand, CV experiments after addition of 200 mmol L^{-1} glucose showed a well-defined sigmoidal catalytic behavior for all bioelectrodes (Fig. 3), as a result of GOx catalytic oxidation of glucose according to the set of Eqs (4).



G = Glucose; GL = Gluconolactone (4)

The catalytic current (i_{cat}) of each bioelectrode in presence of glucose was calculated by subtracting the anodic current at 400 mV without the substrate, and it can be observed that increases with the amount of positive charges of the $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ polycation. In agreement to Eqs (4), the catalytic

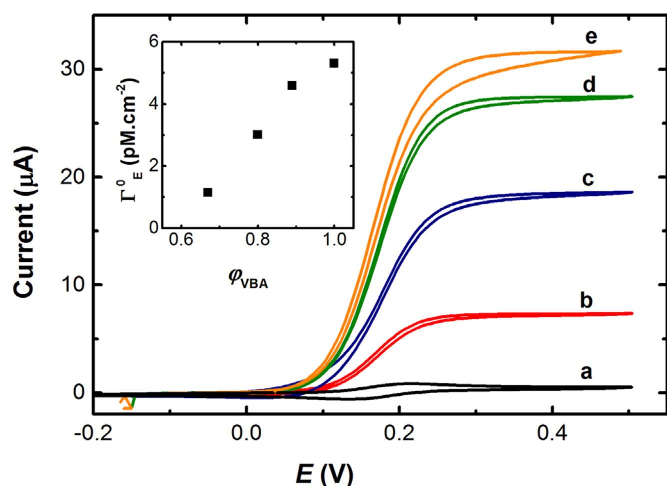


Fig. 3. $i-E$ profiles performed at 10 mV s^{-1} on the different bioelectrodes in 0.1 mol L^{-1} phosphate buffer pH 7.0+0.2 mmol L^{-1} FcMe, without (curve a) and with (curves b–e) 200 mmol L^{-1} glucose. Gel composition: 30 μg laponite+20 μg GOx+15 μg $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$: (b) $m=1$, $n=2$; (c) $m=1$, $n=4$; (d) $m=1$, $n=8$; and (e) $m=0$, $n=1$. Inset: Surface concentration of active enzyme (Γ^0_E) as function of φ_{VBA}.

current (i_{cat}) can be expressed as [41]:

$$i_{cat}^{-1} = \left(2FA\Gamma^0_E\right)^{-1}\left[\left(k_3[\text{FcMe}]\right)^{-1} + k_2^{-1} + \left(k_{red}[\text{G}]\right)^{-1}\right] \quad (5)$$

where F is the Faraday constant, A the area of the electrode, Γ^0_E the surface concentration of active enzyme, [FcMe] and [G] the concentration of mediator and glucose, respectively, and $k_{red}=[k_1k_2/(k_{-1}+k_2)]$. Γ^0_E can be estimated for systems that do not have mass transport limitations, such as the present case, by using the reported values of $k_2=700$ s^{-1}, $k_3=1.2\times10^7$ mol^{-1} L s^{-1} and $k_{red}=1.1\times10^4$ mol^{-1} L s^{-1} [41]. The calculated Γ^0_E values increased between 1.1 and 5.3 pmol L^{-1} cm^{-2} as the modified hydrogel becomes more positively charged (inset of Fig. 3). These Γ^0_E values are comparable to that of $\Gamma^0_E=$ 1.7 pmol L^{-1} cm^{-2} calculated for a close-packed GOx monolayer adsorbed onto antigen-modified GCE surface [41]. At neutral pH, GOx is negatively charged ($pI=4.2$) [9], and therefore the electrostatic attractive interactions with the positively charged hydrogel film favor a larger incorporation of GOx molecules. By considering the sponge-like structure of laponite-based hydrogel films, 3D entrapments of GOx molecules into the mesopores spaces of the modified hydrogel films can be expected.

Fig. 4A shows the chronoamperometry (CA) response with the step increments of 0.2 mmol L^{-1} glucose concentration obtained immediately after preparation of the bioelectrodes with different polycations. The corresponding current-concentration calibration curves are presented in Fig. S5 of the Supplementary Information, and the bioelectrode sensitivity (s) was obtained from the initial slope of those curves. The bioelectrode sensitivity as a function of the storage time at ~8 °C is presented in Fig. 4B. The initial sensitivity was higher for the bioelectrode containing the polycation made with pure VBA, and it decreased as the VBT/VBA ratio in the copolymer increased, e.g. $[VBA] > [(VBT)(VBA)_8]^{8+} > [(VBT)(VBA)_4]^{4+} > [(VBT)(VBA)_2]^{2+}$. However, after 30 days of storage and re-using, the sensitivity of the bioelectrodes made with the polycations [VBA] and $[(VBT)(VBA)_2]^{2+}$ decreased up to 66% and 47% of their initial values, respectively, while for the those containing $[(VBT)(VBA)_4]^{4+}$ and $[(VBT)(VBA)_8]^{8+}$ the sensitivity was very stable (Fig. 4B and Table 1). This fact suggests the presence of a hydrophilic-hydrophobic balance induced by the electrostatic attractions between the polycations and the anionic clay, together by π-stacking and hydrogen bonding interactions of both VBT and VBA moieties. This fine balance contributes to the stabilization of

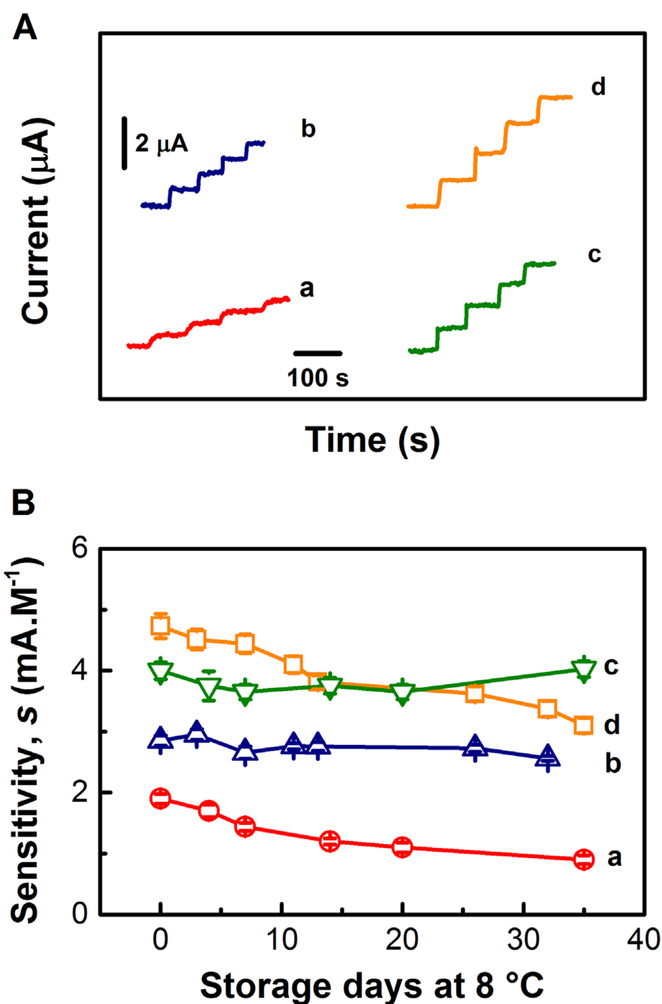


Fig. 4. (A) Current–time response at 400 mV of the different bioelectrodes containing 30 μg laponite+20 μg GOx+15 μg $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$: (a) $m=1$, $n=2$; (b) $m=1$, $n=4$; (c) $m=1$, $n=8$; and (d) $m=0$, $n=1$. Consecutive additions of 0.2 mmol L^{-1} glucose to 0.1 mol L^{-1} phosphate buffer pH 7.0 with 0.2 mmol L^{-1} FcMe. (B) Stability curves (sensitivity vs days). References as in (A).

the immobilization matrix and the conservation of the catalytic properties of the enzyme. The electroanalytical properties of the bioelectrodes immediately after preparation are presented in Table 1. The detection limit (DL) for each bioelectrode was calculated using the criterion of 3 × SD/s where SD is the standard deviation of the background current and s the sensitivity [42].

From the data presented in Table 1, it can be concluded that the best electroanalytical performance was obtained for the bioelectrode containing $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ polycation, with the lower time response, larger linear range and time-extended sensitivity (*i.e.* stability). Therefore, this bioelectrode was used for the analytical applications, as described in the following section. Table 2 compares the electroanalytical performance of the bioelectrode containing $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ with others described in the literature using GOx immobilized with laponite-based hydrogels with different additives. All bioelectrodes showed similar electroanalytical properties, e.g. linear range, response time and detection limit. However, when the specific sensitivity (s_e) is compared, calculated dividing the observed sensitivity (s) by the electrode area and the enzymatic units used, it can be observed that the bioelectrode containing $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ stands out with s_e values one- and two-orders of magnitude larger than the reported ones. This enhancement of sensitivity is very suitable for electroanalytical applications since a lower enzyme amount is required for similar

Table 1
Electroanalytical properties of glassy carbon electrodes (GCE) containing immobilized Glucose Oxidase (GOx) onto laponite hydrogels having vinylbenzyl thymine- vinylbenzyl triethylammonium chloride polycations $[(VBT)(VBA)_n]^{n+}$.

Polycation	Initial sensitivity (mA mol^{-1} L)	EHME Stability (%)	Response time (s)	Linear range (m mol L^{-1})	Detection limit (μ mol L^{-1})
$[(VBT)(VBA)_2]^{2+}_{\approx 25}$	1.9 ± 0.1	47	17 ± 3	0.2–2	60
$[(VBT)(VBA)_4]^{4+}_{\approx 25}$	3.0 ± 0.2	90	3 ± 1	0.06–2	18
$[(VBT)(VBA)_8]^{8+}_{\approx 25}$	4.1 ± 0.2	100	3 ± 1	0.03–2	9
$[(VBA)]^{+}_{\approx 25}$	4.7 ± 0.3	66	8 ± 2	0.03–2	13

responses.

The variation of the sensitivity of the bioelectrode as function of the polycation mass added and the pH was studied for the bioelectrode with $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ (Fig. 5). The best enzymatic response was obtained for the hydrogel film prepared using a laponite/polycation mass ratio ≈ 2, as it was observed for the immobilization of lactate oxidase with hydrogel films made with laponite and $[(VBT)(VBA)_4]^{4+}_{\approx 25}$ [12]. Consequently, it can be assumed that this hydrogel composition allows an optimal balance for the accommodation of the polycation molecules within the mesopores generated by the laponite in the hydrogel matrix. The pH dependence of the sensitivity of the bioelectrode over the pH range 5.0–11.0 showed a maximum around neutral pH (solid circles of Fig. 5). This bell-shaped dependence of the bioelectrode sensitivity is parallel to the intrinsic-pH dependence of enzyme activity of GOx, confirming that the hydrogel film formed with laponite and $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ does not substantially modify the GOx structure and its functionality.

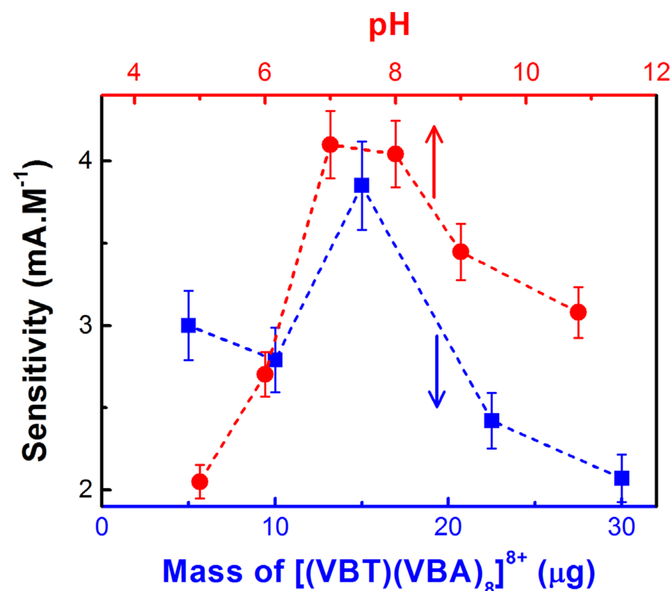
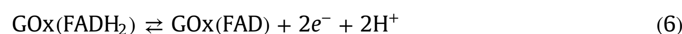


Fig. 5. (■) Influence of the $[(VBT)_1(VBA)_8]^{8+}_{\approx 25}$ mass on the sensitivity of a bioelectrode composed in addition by 30 μg laponite and 20 μg GOx (●) pH effect for the given composition with 15 μg of $[(VBT)(VBA)_8]^{8+}_{\approx 25}$.

3.3. Electrocatalysis of the O_2 reduction reaction

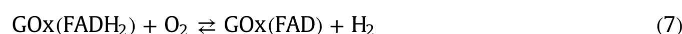
Under aerobic conditions it is possible to produce direct glucose oxidation with GOx immobilized in laponite matrices onto GCE without using electron mediators [43]. Fig. 6A shows the cyclic voltammograms obtained with the bioelectrode made with laponite and $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ in 0.1 mol L^{-1} phosphate buffer at pH 7.0 under different conditions.

In absence of both, dissolved O_2 (N_2-purged solutions) and artificial mediator (Fig. 6A, curve a) the couple of redox peaks for the direct electron transfer of the GOx was observed, according to Eq. (6):



$$GOx(FADH_2) \rightleftarrows GOx(FAD) + 2e^- + 2H^+ \quad (6)$$

In presence of dissolved O_2 (air-saturated solutions) a large cathodic current was observed at potentials < -0.5 V (Fig. 6A, curve b), which was strongly decreased after addition of 5 mmol L^{-1} glucose (Fig. 6A, curve c). This behavior can be explained in terms of the electrocatalysis of the reduced form of the GOx, GOx($FADH_2$), in the presence of dissolved O_2, Eq. (7) [44,45].



$$GOx(FADH_2) + O_2 \rightleftarrows GOx(FAD) + H_2 \quad (7)$$

At the same time, in the presence of glucose its oxidation is catalyzed by the oxidized form of GOx, *i.e.* GOx(FAD) Eq. (8) [46].

$$GOx(FAD) + Glucose \rightarrow GOx(FADH_2) + Gluconolactone \quad (8)$$

Fig. 6B shows the amperometric response of the bioelectrode after consecutive additions of 0.2 mmol L^{-1} of glucose into air-saturated solutions, whereas Fig. 6C exhibits the corresponding calibration plot. From those data a sensitivity of 3.7 mA mol^{-1} L, detection limit of 40 μmol L^{-1} and linear range up to 1.5 mmol L^{-1} were obtained. Under these conditions, the sensitivity and the linear range where similar to those observed for the same bioelectrode using the enzymatic mediator FcMe (Table 1). However, in absence of mediator the detection limit was reduced to about 5 times.

Finally, Table 3 compares the electroanalytical properties of the present bioelectrode with others using GOx immobilized into different nanocomposite matrices under aerobic conditions [47–49]. It can be observed that the bioelectrode made with

Table 2
Comparison of electroanalytical properties of glassy carbon electrodes (GCE) containing immobilized Glucose oxidase (GOx) onto hydrogel films made of laponite hydrogels with different additives.

Laponite + additive hidrogel matrix	Amount of enzyme (μg)	Linear range (mmol L^{-1})	Response Time (s)	Detection limit (μmol L^{-1})	Specific sensitivity (mA mmol^{-1} L cm^{-2} U^{-1})	Ref.
Laponite alone	33	< 2	< 10	–	4.5	[52]
+ glutaraldehyde	33	< 4	< 10	–	15	[52]
+ polyalkoxysilane	23	–	–	–	18.3	[39]
+ Chitosan	10	< 0.05	< 5	0.6	32	[53]
+ $[(VBT)(VBA)_8]^{8+}_{\approx 25}$	20	< 2	< 4	9	176 ± 9	This work

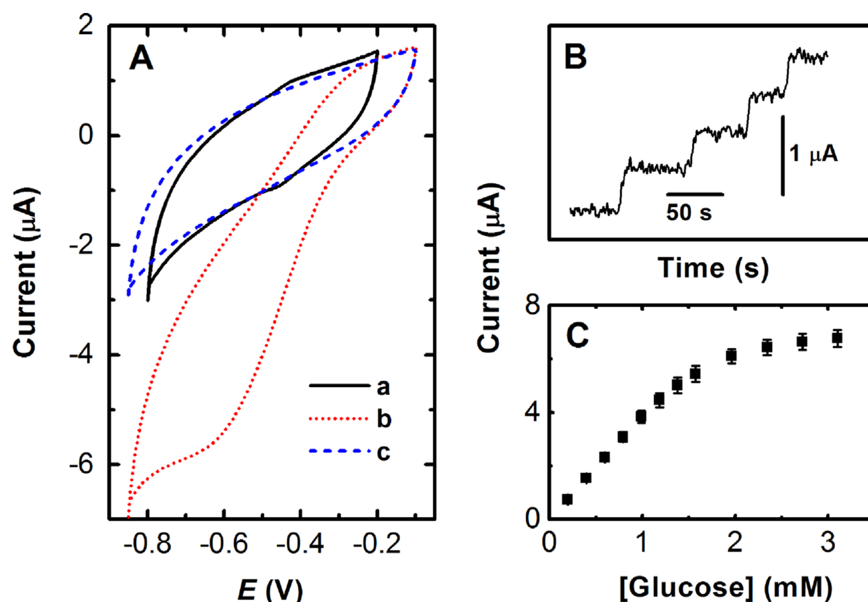


Fig. 6. (A) Voltammograms performed on a bioelectrode with 30 μg laponite, 20 μg GOx and 15 μg $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ in 0.1 mol L^{-1} phosphate buffer pH=7 for: (a) saturated nitrogen solution, (b) aerated solution, and (c) aerated solution+5 mmol L^{-1} glucose. Scan rate: 100 mV s^{-1}. (B) Current–time response at −700 mV for consecutive additions of 0.2 mmol L^{-1} glucose. (C) Calibration curve corresponding to Fig. 6B.

laponite and $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ shows one-order of magnitude larger sensitivity per electrode area than other reported bioelectrodes, confirming a superior analytical performance even under aerobic conditions and in the absence of artificial mediator.

3.4. *Glucose determination in powder milk and blood serum samples and potential effect of molecular interferents*

Given the remarkable electroanalytical performance of the bioelectrode made with laponite and $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ for glucose determination, this bioelectrode was tested in real samples of commercial powder milk and blood serum. In all cases, the real samples were diluted with phosphate buffer solutions in order to fit into the linear range response of the bioelectrode (Tables 2 and 3). Control experiments recorded using HME with identical laponite/polycation composition and without GOx showed no changes in the stationary currents for FcMe oxidation after the addition of either glucose aliquots or real samples (data not shown). As it was described in the experimental section, the glucose analysis in powder milk samples of La Serenisima® was performed during a period of 5 days using the same electrode. The obtained values for the five consecutive determinations were 2.01 ± 0.09, 1.99 ± 0.09, 1.98 ± 0.09, 2.04 ± 0.08, 2.07 ± 0.09 g L^{-1}. According to a *t*-test at 95% confidence level, these values agree with the manufacturer reported value, e.g. 2 g L^{-1}. For the serum blood samples, the glucose content tested with the same bioelectrode on days 1 and 4 were 1.03 ± 0.08 and 1.09 ± 0.08 g L^{-1}, respectively, very similar to the value of 1.1 ± 0.1 g L^{-1} obtained for the same samples with the colorimetric method (see Experimental section).

Electroanalysis of glucose in real samples can involve the evaluation of both ascorbic (AA) and/or uric acids (UA) as potential interferents [50,51]. Hence, the contribution of AA and UA to the analytical current with the present bioelectrode was evaluated. The electro-oxidation of AA studied at +400 mV with FcMe as enzymatic mediator yielded a sensitivity of 0.53 μA mmol^{-1} L and a detection limit of 0.16 mmol L^{-1} (Fig. S6 of Supplementary Information). This detection limit is comparable to the maximum concentration of AA found in fresh milk, e.g. 0.11 mmol L^{-1} [50]. Therefore, in a 1/1000 sample dilution, as typically used in electroanalysis of milk, the final AA concentration into the electrochemical cell should not interfere in the glucose quantification of milk samples.

The potential interference of AA and UA was also investigated at −700 mV by amperometry under aerobic conditions. Each substance was added to the buffer solution containing 1 mmol L^{-1} glucose with interferent/glucose molar ratio of 1/10, 1/5 and 1/3. In all cases, the amperometric signal of glucose was not modified by the presence of either AA or UA, even under concentrations that exceed the maximum physiological concentrations of theses interferents in human blood [51].

4. Conclusions

The present study demonstrates the suitability of DNA bioinspired water-soluble polycations $[(VBT)_m(VBA)_n]^{n+}_{\approx 25}$ for enzyme

Table 3
Comparison of electroanalytical properties of glassy carbon electrodes (GCE) containing Glucose Oxidase (GOx) immobilized with different nanocomposites under aerobic conditions.

Matrix	Linear range (mmol L^{-1})	Response time (s)	Detection limit (μmol L^{-1})	Sensitivity/Area (mA mmol^{-1} L cm^{-2})	Ref.
Laponite	0.02–1.9	8	10	4.8	[43]
Mesocellular silica-carbon nanocomposite	0.05–5	4	35	–	[48]
TiO_2-nafion	0.15–1.2	–	–	4.3	[47]
Chitosan-SWCNT	0.6–2.8	–	100	3.3	[53]
Laponite + $[(VBT)(VBA)_8]^{8+}_{\approx 25}$	0.1–1.6	10	40	52.8	This work

immobilization like GOx into laponite hydrogels for the preparation of highly sensitive and stable bioelectrodes on glassy carbon surface. At neutral pH, the bioelectrode sensitivity (mA mol^{-1} L) was proportional to the number of positive charges into the copolymeric unit $[(VBT)_m(VBA)_n]^{n+}$ favoring the progressive incorporation of the negatively charged GOx. In addition, the insertion of the thymine moiety in the copolymer plays a role in the hydrophilic–hydrophobic balance essential for the stability of the laponite-based hydrogel film and consequently a long-term GOx activity. Both factors make $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ the most suitable polycation for the preparation of laponite hydrogel films for GOx immobilization, as shown by the large electroanalytical response combined with a long-term stability. Furthermore, this bioelectrode showed similar electroanalytical performance with both FeMe and O_2 as artificial and natural enzymatic mediator, respectively.

Comparing the specific sensitivity s_e ([=] mA $mmol^{-1}$ L cm^{-2} U^{-1}) for several bioelectrodes of GOx immobilized by laponite films and stabilized with different additives, demonstrated the significant advantage of the bioelectrode containing $[(VBT)(VBA)_8]^{8+}_{\approx 25}$ for electroanalytical determination of glucose, probably by combination of several suitable factors such as, optimal enzyme conformation into the hydrogel matrix, good conductivity properties and permeability to the glucose substrate. These features were confirmed by testing the bioelectrode for a selective determination of glucose in powder milk and blood serum samples without interference of either ascorbic or uric acids under the experimental conditions. In summary, the present work establishes that vinylbenzyl thymine-based polycations are promising materials for the development of highly sensitive and selective biosensor devices.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.12.046.

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