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Rapid optimization of a lactate biosensor design using soft probes scanning electrochemical microscopy

Medeya M. Pribil [a], Fernando Cortés-Salazar [b], Egor A. Andreyev [a], Andreas Lesch [b], Elena E. Karyakina [a], Oleg G. Voronin [a], Hubert H. Girault [b,*], Arkady A. Karyakin [a,*]

[a] Chemistry faculty of M.V. Lomonosov Moscow State University, Moscow 119899, Russia
[b] Laboratoire d'Electrochimie Physique et Analytique, Ecole Polytechnique Fédérale de Lausanne, Switzerland

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ABSTRACT

We report the mapping of biocatalytically active surfaces, particularly on an express search for optimal immobilization conditions of the enzyme lactate oxidase by means of scanning electrochemical microscopy (SECM). With this aim, soft stylus SECM probes containing a carbon paste ultramicroelectrode were modified with Prussian Blue yielding reproducible hydrogen peroxide (H_2O_2) sensors with a sensitivity of 1.6 ± 0.5 A M^{-1} cm^{-2} for screening applications. The ultramicroelectrode response was stable under harsh conditions of 1 mM H_2O_2 during the first hour, while the response decay during the second hour was less than 4% providing sensor suitability for long-term experiments. SECM imaging in contact mode of different lactate oxidase spots containing membranes allowed for a straightforward optimization of the enzyme immobilization conditions on rough screen-printed carbon paste substrates. The resulting lactate biosensor was characterized by improved analytical performance characteristics: a four times enhanced sensitivity (up to 0.3 A M^{-1} cm^{-2}) in comparison to previous reports and a remarkably increased operational stability.

1. Introduction

Scanning electrochemical microscopy (SECM), introduced in the family of scanning probe microscope techniques in 1989 [1,2], became a powerful imaging tool since the mapping of local chemical and electrochemical reactivity at several interfaces with a micro- or nanoscopic resolution is achieved. SECM experiments are based on a probe that scans laterally (x, y direction) and in close proximity over a sample to sense the consumption, release or recycling of redox active species [1,2]. Besides bare amperometric ultramicroelectrodes (UMEs), modified electrodes [3–5], miniaturized biosensors [6,7] and potentiometric probes [8,9] have been also employed as SECM probes. Due to the versatility of SECM, biological samples [10,11], corrosion processes [12,13] and human fingerprints [14,15] have been studied. Moreover, SECM has demonstrated its ability to screen combinatorial catalyst libraries [16,17] where the catalytically most active composition of a mixture of several materials can be identified in a single experiment and under identical experimental conditions. In a similar manner, SECM can also be applied for the elaboration and optimization of biosensors, which represent by definition immobilized biorecognition elements [18–20]. Hence, the immobilization of biomolecules is one of the key aspects in biosensorics and comprises nowadays the largest family of tools for chemical analysis.

Lactate is an important metabolite considered as a marker for glycolysis, the anaerobic metabolism causing death of tissues, and represents a relevant target for biosensing. Indeed, the so-called "lactate threshold" indicates the physical training level of a sportsman. Starting from the early 80s lactate oxidase became the terminal enzyme for lactate biosensors [21–24]. Although this enzyme is among the less stable oxidases, it became possible to form on its basis stable and active membranes by using a previously reported immobilization protocol from water–organic mixtures with a high content of organic solvents [25]. Recently, Parra *et al.* reported a sensitive and very stable lactate oxidase sensor based on the direct immobilization of lactate oxidase by drop casting over a bare glassy carbon or highly oriented pyrolytic graphite (HOPG) electrode and the recycling of ferrocene methanol as the redox mediator [26]. Despite of the obtained long-term stability (*i.e.* one month), SECM studies of such biosensor demonstrated an inhomogeneous immobilized enzyme containing film and, consequently, an inhomogeneous catalytic activity mainly due to the applied immobilization protocol [26]. Those results emphasize

* Corresponding authors. Tel.: +41 21 693 3145; fax: +41 21 693 3667 (H.H. Girault). Tel.: +7 495 939 4605; fax: +7 495 939 4675 (A.A. Karyakin).
E-mail addresses: hubert.girault@epfl.ch (H.H. Girault), aak@analyt.chem.msu.ru (A.A. Karyakin).

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the advantages of SECM for surface reactivity characterization and also the importance of the immobilization strategy. Furthermore, a SECM probe modified with lactate oxidase prepared either by drop casting or electropolymerization has been reported also for the scanning of cancer cells [27]. The detection strategy relied on the oxidation of H_2O_2, produced during the enzymatic reaction between lactic acid and the immobilized enzyme, at a Pt ultramicroelectrode. Approach curves using the enzymatically-modified ultramicroelectrodes were performed and employed for the vertical positioning of the SECM probe. This process is always a challenge when using functionalized electrodes whose response depends mainly on the presence of specific chemical species present at very low concentrations.

The most progressive way for monitoring lactate oxidase activity is to detect electrochemically hydrogen peroxide (H_2O_2), the side product of the catalysed reaction [28]. As a transducer for H_2O_2, Prussian Blue is known to be the most advantageous electrocatalyst. Compared to pure platinum, which is the most widely used electrode material in this respect, Prussian Blue modified electrodes show (i) a three orders of magnitude higher activity for H_2O_2 reduction and oxidation in neutral media and (ii) a three orders of magnitude higher selectivity for H_2O_2 reduction in the presence of oxygen [29]. The latter allows low-potential H_2O_2 detection by its reduction with advantageous analytical characteristics [30,31,29,32,33]. Moreover, Prussian Blue based nanoelectrode arrays [34] have shown a prolonged linear calibration range over 7 orders of magnitude of analyte concentration [35].

For screening lactate biosensor libraries by SECM, Prussian Blue modified ultramicroelectrodes are suitable to detect locally and in a reliable way the production of H_2O_2. However, electrode miniaturization poses also problems in electrochemical analysis. Electrode covering films become less stable as the disk curvature radius is decreased, most probably due to the increase in surface tension. This provides a search for SECM probe materials with the highest possible adhesion for the modifying films. Voronin *et al.* reported the immobilization of stable Prussian Blue films over Au microelectrodes previously modified with a platinum–carbon composite material by using focused ion beam [36]. Despite SECM experiments for the detection of H_2O_2 were successful by using such type of probes, the highly specialized fabrication methodology of the intermediate Pt/C layer represents a limitation for its widespread use. Clausmeyer *et al.* presented recently a carbon-based nanoelectrode for H_2O_2 detection where a Prussian Blue film was hosted inside a previously etched nanocavity in order to provide a more mechanically stable environment. With this aim a nanopipette was filled with a butane/propane gas mixture and subjected to pyrolysis forming a carbon nanoelectrode, which is further anodically etched in a basic pH solution [37].

Recently, a new type of scanning sensor, the so-called "soft stylus probe", has been introduced as SECM probe operable in contact mode and with the aim to extend the applicability of SECM to rough, tilted, large or initially dry samples [38–41]. For these probes, a thin polymeric film is employed as support of a carbon ink track deposited in a microchannel prepared by UV laser ablation and enclosed by a thin insulating film of Parylene C. The active electrode area is simply exposed by laser-assisted or razor blade cutting. The main advantage of using soft stylus probes is their ability to bend when brought into mechanical contact with the substrate and to slide during lateral scanning providing a nearly constant working distance. In this way, these flexible probes accommodate the topography of the sample when scanning in contact mode. Thus not only a higher sensitivity, but also a topographic artefact-free response can be recorded when scanning substrates of complex surface features. Moreover, no contamination or damage of both the probe and the sample have been observed, even when brushing over soft samples such as self-assembled monolayers [42]. Finally, since carbon electrodes are among the most suitable support materials for transition metal hexacyanoferrates due to the possible complexation of metal atoms with the oxidized carbon surface, functionalizing the soft stylus probes with a catalytic layer of Prussian Blue represents a valuable further development of the soft stylus probes.

Herein, we report on the rapid screening of various immobilizing matrix compositions for lactate oxidase biosensors by means of SECM in contact mode with soft stylus probes functionalized with a catalytically active Prussian Blue film. SECM in contact mode was required for avoiding topographic artefacts from the rough and tilted carbon paste substrate employed for the deposition of lactate oxidase. For instance, the soft probes allowed a convenient probe approaching, *i.e.* the identification of the working distance by the mechanical probe body-sample contact rather than relying only on the enzymatic response at the modified ultramicroelectrode. Moreover, it enabled the simultaneous study of different enzyme immobilizing matrixes based on γ-aminopropylsiloxane sol under identical experimental conditions with the aim to identify the optimal immobilization strategy. Based on the SECM studies, the superior performance of the optimized composite membrane has been confirmed by investigations with the resulting lactate biosensor. As a result, lactate biosensors with highly enhanced sensitivity and stability have been elaborated.

2. Experimental

2.1. Materials

Experiments were carried out with Milli-Q water from a Millipore Milli-Q system. All inorganic salts, γ-aminopropylsiloxane (γ-$NH_2PrSi(OEt)_3$), organic solvents, and hydrogen peroxide (30 vol%) were obtained at the highest purity from Reachim (Moscow, Russia) and used as received. Sodium lactate, 40%, was purchased from ICN Biochemicals (Aurora, USA). Lactate oxidase (EC 1.1.3.2) from Pediococcus sp. (lyophilized powder, activity 72 IU) was purchased from Sorachim, Switzerland.

Planar three-electrode hydrogen peroxide sensors (*i.e.* Prussian Blue modified carbon paste working electrode, carbon paste counter electrode and Ag/AgCl paste reference electrode) were purchased from Rusens LTD (Moscow, Russia). Sensor performance characteristics in batch-regime mode showed a sensitivity of 1 ± 0.1 A M^{-1} cm^{-2} and a lower detection limit of $5 \cdot 10^{-7}$ M.

2.2. Instrumentation and methods

The cyclic voltammetry and chronoamperometry experiments were conducted in a three electrode setup with an Ag|AgCl|0.1 M KCl reference electrode and a platinum counter electrode using either a CHI842B bipotentiostat in combination with a Faraday cage and a preamplifier from CH Instruments or a PalmSens electrochemical interface connected to an IBM PC. The thickness of the γ-aminopropylsiloxane membrane was determined with a surface profilometer Talystep (Taylor-Hobson) and a laser scanning microscope (VK 8700, Keyence).

SECM experiments were performed using the SECMx software [43] and a custom built SECM setup, which is composed of a three axes Märzhäuser positioning system (Märzhäuser, Wetzlar, Germany), an electronic tilt table (Zaber Technologies, Vancouver, Canada) and a bipotentiostat CompactStat (Ivium Technologies, Eindhoven, The Netherlands). For SECM experiments, a Pt wire was employed as a counter electrode, a Ag wire as a quasi-reference electrode (QRE) and a soft stylus as the working electrode.

A soft stylus probe for SECM measurements was made into 100 μm thick polyethylene terephthalate sheets (Dupont, DE) by

UV photoablation through a metallic mask using a 193 nm ArF excimer laser beam (Lambda Physik, Göttingen, Germany, fluence = 0.2 J, frequency = 50 Hz) as reported elsewhere [38]. Carbon ink (Electrador, England) was manually applied into the microchannels to create a carbon ink track and was subsequently cured at 80 °C for 1 h. Then, a 5 μm thick Parylene C coating was deposited using a Parylene C depositing system (Comelec SA, La Chaux-de-Fonds, Switzerland) covering and sealing the electrode track. The active ultramicroelectrode area was exposed by razor blade cutting and the resulting ultramicroelectrode area was approximately 300 μm^2.

The deposition of Prussian Blue on the soft stylus carbon ultramicroelectrode was carried out by cyclic voltammetry applying 5–7 scans (scan range 0.4–0.75 V) with a scan rate of 20 mV/s in a solution containing 4 mM $FeCl_3$ and 4 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl and 0.1 M HCl. After deposition of Prussian Blue, the modified carbon ultramicroelectrodes were rinsed with distilled water and activated by applying 15–18 cycles in the supporting electrolyte (0.1 M KCl and 0.1 M HCl) from −0.05 to +0.35 V at a scan rate of 40 mV/s. Finally, the modified ultramicroelectrodes were rinsed with Milli-Q water and annealed at 100 °C for 1 h [31,44,36].

Lactate oxidase containing membranes were made by suspending an aqueous enzyme solution (10 mg/mL) in isopropanol containing γ-aminopropylsiloxane (1–5 vol%). Final concentrations in the water–isopropanol mixture were: lactate oxidase 1 mg/mL, γ-aminopropylsiloxane 0.1–3 vol%, water 10 vol%. The mixture (0.1 μL) was drop casted onto a rough screen-printed carbon paste electrode without any Prussian Blue film (Rusens LTD, Moscow, Russia) and dried in a refrigerator (4 °C) during 1 h.

Hydrogen peroxide detection was carried out at a potential of 0 V (*vs* Ag|AgCl|0.1 M KCl), allowing H_2O_2 reduction on Prussian Blue modified electrodes, *i.e.* the biosensors as well as the SECM probes. The carrier solution in the experiments was a 50 mM phosphate buffer (pH 6.0) containing 0.1 M KCl as supporting electrolyte. Finally, SECM experiments we performed by bringing the probe in mechanical contact with the sample through an approach curve over the lactate oxidase spot. To assure a contact mode operation during all SECM experiments an h_P distance equal to −100 μm was employed in almost all cases. h_P is defined as the difference between the attachment point of the probe to the positioning system (h_A) and the length of the unbent probe (l_T) (*i.e.* $h_P = h_A - l_T$). h_P represents the working distance given by the positioning system that in contrast to the commonly used working distance d, can take negative values describing further probe-sample contact and not the penetration of the sample. On the contrary when the probe touches the substrate the probe bends and slides, which leads to a small and positive d defined by the thickness of the polymeric film (t_L) that encloses the carbon track and the angle (α) between the cross sectional plane of the probe and the sample surface. The relation between h_P and d can be thus defined by $d = h_P + t_L \sin(\alpha)$ in a contact less mode and $d = t_L \times \sin(\alpha)$ in a contact mode. Since the soft stylus probe is always scanned by placing the side of the thin Parylene C layer (*i.e.* 5 μm thick) in contact with the sample substrate, a small and almost constant working distance is achieved. In fact, as the probe is approached to the surface with a predefined inclination angle (β) equal to 70°, at the point of contact with the sample the working distance is equal to 1.7 μm. A more detailed explanation of h_P and its relationship with d can be found in the supporting information SI-1. Forward line scans during SECM imaging were performed in contact mode, while a lift-off (LO) routine [38] was used in-between reverse scans and horizontal probe movements in order to control precisely the bending direction and bending degree of the probe. All data were processed by using MIRA software [45]. It is important to notice that abrasion of the Parylene C layer might occur during long periods of SECM imaging in contact mode. However as reported previously, no drastic changes in the recorded current due to removal of the Parylene C layer or deactivation of the soft stylus probes have been observed in SECM imaging experiments lasting even 18 h [46]. Furthermore, before each experiment a fresh and clean electrode cross section including the working electrode area and the Parylene C coating is always regenerated by razor blade cutting.

3. Results and discussion

3.1. Soft stylus probes as hydrogen peroxide sensors

Soft stylus probes functionalized with Prussian Blue were employed for the localized detection of H_2O_2 generated from different immobilized lactate oxidase spots. The electrochemical deposition of ferric hexacyanoferrate by cyclic voltammetry provided a highly regular Prussian Blue coating over the carbon ultramicroelectrodes with peak currents in the range of 8–12 mA cm^{-2} after electrochemically induced activation in the supporting electrolyte solution (see Fig. 1a).

The inset in Fig 1a shows consecutive cyclic voltammetries for the deposition of PB with a considerable slope most likely due to the inherent resistance of the carbon paste employed for the fabrication of the microelectrodes (*i.e.* 12.8 kΩ) [38]. The latter could also be generated by the ions intercalation inside the crystal for charge compensation during the Berlin Green|Prussian Blue redox reaction (with redox potential between 0.75 and 0.8 V), which could explain the fact that the CV presented in Fig 1a does not show such slope. Analytical performance characteristics of the Prussian Blue modified soft stylus probes have been investigated in batch regime upon stirring. Fig. 1b displays the corresponding calibration curve, where a linear trend between the H_2O_2 concentration and the modified ultramicroelectrode response extends over almost three orders of magnitude, allowing the H_2O_2 detection from $1 \cdot 10^{-5}$ to $1 \cdot 10^{-2}$ M. The sensitivity of the micro-sensors determined as the slope of the calibration graph is of 1.6 ± 0.5 A M^{-1} cm^{-2}, which is in good agreement with results obtained for Prussian Blue modified gold ultramicroelectrodes that were reported earlier [44,36]. The rather high detection limit at such a high sensitivity is due to the noise coming from the continuous stirring of the solution. The latter obviously does not deteriorate SECM experiments that are performed under conditions in which the mass transport is controlled only by diffusion.

A crucial parameter for sensor applications, in particular for SECM experiments, is the sensor stability. We have chosen carbon paste as the electrode support material in order to improve the hexacyanoferrate stability. Indeed, even under harsh conditions of 1 mM H_2O_2 (inactivation rate of Prussian Blue is known to be dependent on H_2O_2 concentration [47] in batch regime upon stirring the sensor retains its initial response during the first hour. The activity decay after the second hour is in the frame of 4%. Moreover, we note that even after 17 h of continuous operation under 1 mM of H_2O_2 the sensor retained more than 60% of its initial activity (Fig. 1c). Since in average the SECM probe is exposed to much lower H_2O_2 concentrations during the scanning of immobilized lactate oxidase spots, the soft stylus probe based sensor is suitable for long-term SECM experiments of several hours if required (*vide infra*).

3.2. SECM imaging of enzymatic spots

The detection of H_2O_2 by SECM probes has been already studied by using bare Pt ultramicroelectrodes or Prussian Blue modified ultramicroelectrodes supported on complex structures such as Au/Pt/C [27,48,36]. Soft stylus probes offer a more stable and a simpler fabrication protocol of H_2O_2 microsensors based on

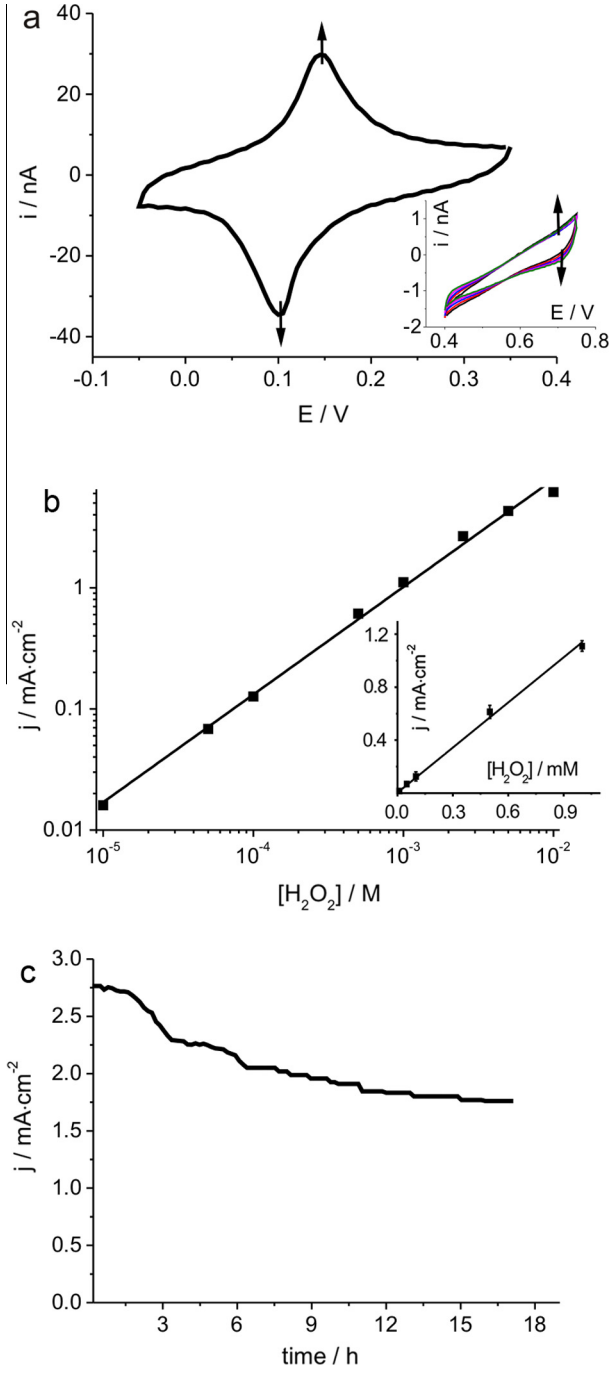

Fig. 1. (a) Activation cyclic voltammogram of Prussian Blue deposited on a soft stylus microelectrode in 0.1 M KCl and 0.1 M HCl. Scan rate of 40 mV/s. The inset shows the deposition of Prussian Blue, 5–7 scans in a solution of 4 mM $FeCl_3$, 4 mM $K_3[Fe(CN)_6]$, 0.1 M KCl and 0.1 M HCl. Scan rate of 20 mV/s. The arrows show increasing number of cycles. (b) Calibration plot for H_2O_2 detection in batch conditions upon stirring (500 rpm) with Prussian Blue modified ultramicroelectrode recorded at 0 V. (c) Stability test of the Prussian Blue modified soft stylus microelectrode in presence of 1 mM H_2O_2 after 17 h of continuous measuring in batch conditions upon stirring (500 rpm). Potential probe = 0 V in 50 mM phosphate buffer, pH 6.0 with 0.1 M KCl. For all experiments: Ag/AgCl as reference electrode and a platinum wire as counter electrode.

Prussian Blue catalytic films for SECM studies. Moreover, in SECM experiments it is crucial to position precisely the probe close to the substrate, which is typically performed by applying approach curves. However, when employing functionalized probes that only detect a single target that is not homogeneously distributed in the system or that is present in a low concentration, approach curves for probe positioning are cumbersome. In the present work, approach curves over the lactate oxidase spots were performed by applying a potential equal to 0 V *vs* Ag/AgCl for the reduction of H_2O_2 (see Fig. 2a). As expected, when the probe was translated closer to the substrate an increase on the cathodic current was observed due to the higher concentration of H_2O_2 in the proximity of the lactate oxidase spot. When the probe body gets in mechanical contact with the substrate a clear change on the current trend is observed allowing the unequivocally determination of the substrate position and therefore the positioning of the probe. Please note, that the soft probe is approached under an inclination angle. Thus, the polymeric probe body is in mechanical contact with the substrate while the active electrode area is not.

During the approach curves, the soft probe is allowed to get in physical contact with the sample surface and to bend upon further movement in *z* direction of the positioning system (*i.e.* towards the substrate, negative values of h_P). Thanks to the flexibility and softness of the probe, no contamination or damage was observed in the sample or the probe during approach curves or further SECM imaging experiments. The latter is not surprising, since it has been recently demonstrated that soft stylus probes can be employed for the scanning and precise chemical modification in contact mode of self-assembled monolayers without introducing any mechanical damage or contamination of the sample [42]. To assure a contact operation mode during SECM imaging experiments on the rough substrate, an h_P value equal to −100 μm was fixed before each experiment (*vide supra*) and during all forward scans.

The possibility to map lactate oxidase containing membranes by means of SECM in contact mode was demonstrated by performing SECM *x*-line scans over γ-aminopropylsiloxane membranes deposited on rough carbon paste substrates in the presence and absence of lactate oxidase (Fig. 2b). Indeed an active lactate oxidase immobilized spot described by a cathodic current resulting from the reduction of the enzymatically produced H_2O_2 was observed when lactate oxidase was contained in the deposited membrane. The SECM line scan over the carbon substrate without lactate oxidase shows a negligible variation on the current profile, which taking into account the roughness and topographic features of the substrate corroborates the capabilities of soft stylus probes to extract valuable information without the influence of topographic artefacts (*i.e.* different in heights of 18 μm, mountain shape structures, see supporting information SI-2). The latter is further confirmed by the clear visualization of an immobilized lactate oxidase spot by SECM imaging in contact mode (Fig. 2c, please notice that the current axis has been inverted for an easier appreciation).

As reported previously [25], lactate oxidase can be successfully immobilized into the gel of siloxanes from a water–isopropanol mixture with a 90 vol% content of organic solvent. The optimal siloxane was γ-aminopropylsiloxane in a concentration of 0.3 vol% in the casting solution. Despite this particular concentration seemed to be optimal and its slight increase causes the decrease in sensitivity of the resulting sensor (previously tested concentrations were: 0.1–0.5 vol% of γ-aminopropylsiloxane, phenylsiloxane and vinylsiloxane) [49], we decided to vary the concentration of the herein used γ-aminopropylsiloxane in a wider range in order to determine an improved optimal value. With this aim, SECM imaging of a biosensor library was performed in order to enable a rapid and clear comparison of several immobilization conditions for lactate oxidase–siloxane membranes.

Fig. 3 displays the SECM image of four different spots of lactate oxidase containing membranes of γ-aminopropylsiloxane sol. The enzyme concentration in the casting mixture was always 1 mg/mL. In this particular example, siloxane contents (in vol%) of 0.3, 0.9, 1.5 and 2.8, respectively, have been chosen as well as a lactate concentration of 50 mM for the SECM experiments. Surprisingly,

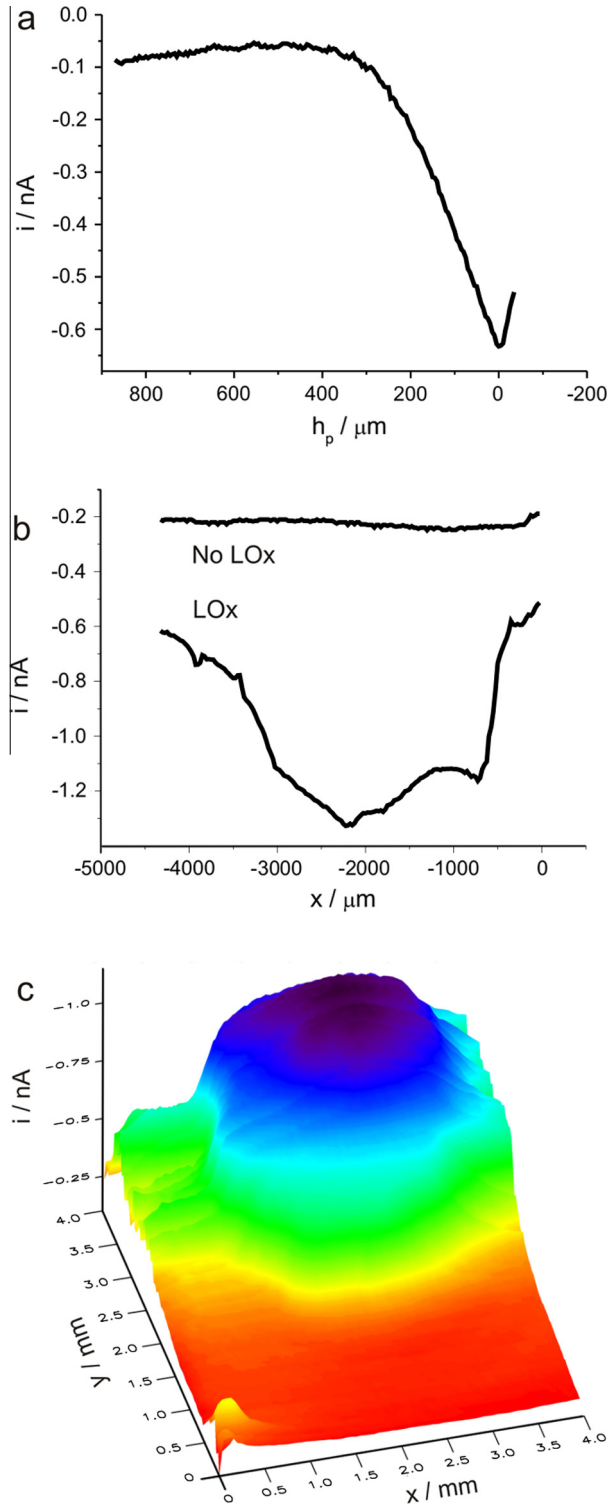

Fig. 2. (a) Experimental approach curve in a solution of lactate 50 mM by using a Prussian Blue modified soft stylus. Translation rate = 1 µm/s, step size = 0.5 µm. (b) SECM *x*-line scan in the presence and absence of lactate oxidase (LO_x) in the γ-aminopropylsiloxane membrane. Concentration of lactate = 50 mM; translation rate = 50 µm/s, step size = 50 µm, probe position (h_P) = −100 µm. (c) SECM image of an immobilized lactate oxidase spot (membrane of 1.5 vol% of γ-aminopropylsiloxane). Lactate 50 mM; translation rate = 50 µm/s, step size = 50 µm, probe position (h_P) = −100 µm. For all experiments: probe potential = 0 V *vs* Ag-QRE; electrolyte solution: 50 mM phosphate buffer, pH 6.0 with 0.1 M KCl. SECM imaging time was equal to 4 h.

the spot corresponding to the mixture with 0.3 vol% γ-aminopropylsiloxane content generates the lowest detected levels of

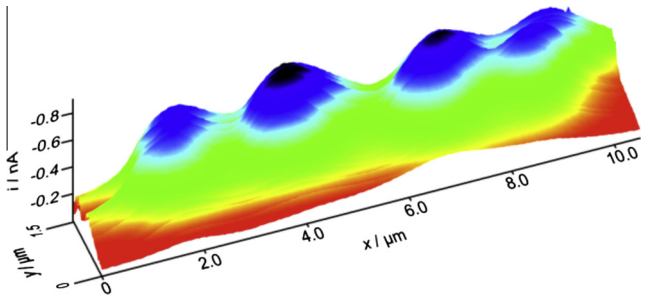

Fig. 3. SECM image of four lactate oxidase containing membrane spots in 50 mM lactate; translation rate = 50 µm/s, step size = 50 µm, probe position (h_P) = −100 µm, potential probe = 0 V *vs* Ag-QRE. From left to right γ-aminopropylsiloxane content in the casting mixture were (in vol%): 2.8, 1.5, 0.9 and 0.3. Electrolyte solution: 50 mM phosphate buffer, pH 6.0 with 0.1 M KCl. SECM imaging time was equal to 4 h.

H_2O_2 as determined by the lower current recorded over this region (see Fig. 3). Much higher enzyme activity has been recorded for the spots corresponding to the casting mixtures containing 0.9–1.5 vol% of siloxane – a region that we have never considered before. A further increase in the siloxane concentration in the casting mixture causes a decrease of the immobilized enzyme activity.

The densities of the resulting enzyme containing siloxane membranes were compared by calculating the relative densities considering both the amount of siloxane in the casting mixture and the volume of the resulting membrane. Three lactate oxidase spots were immobilized with a different composition of γ-aminopropylsiloxane, such as (in vol%): 0.3, 1.5 and 2.8 on a polyethylene terephthalate substrate. The membrane thickness was estimated by averaging at least 10 profilometer scans (see Section 2 and supporting information SI-2). Then, the areas occupied by the membrane were measured and the volumes of the membranes were calculated (Table 1).

As expected, the density of the enzymatic membrane increases as the siloxane content in the casting mixture is increased. Membranes resulted from the casting solution containing 1.5 vol% and 2.8 vol% γ-aminopropylsiloxane showed respectively 1.1 and 1.3 times higher relative densities than those corresponding to the 0.3 vol% siloxane. Hence, the increase in the activity of the immobilized enzyme can be explained by the creation of a thicker, more regular matrix for enzyme immobilization that provides a better fixation and stability to lactate oxidase on the electrode surface. However, further enhancement of the membrane density results in a decrease of activity, most probably due to diffusional constraints in the too dense and thick membrane. The latter exemplifies the need and importance of the optimization of enzyme-immobilized sensors by rapid and reliable tools such as SECM.

3.3. Analytical performances of the lactate biosensor

Lactate biosensors were prepared on the basis of a three-electrode screen printed planar design modified with Prussian Blue for the *in situ* detection of H_2O_2, as described elsewhere [49]. Lactate oxidase containing membranes were formed onto the top of the Prussian Blue modified working electrodes. Fig. 4 displays the obtained sensitivity of different lactate biosensors determined as the slope (in the low concentration range) of the corresponding calibration graph as a function of the γ-aminopropylsiloxane content in the casting mixture. The sensitivity of all biosensors was normalized by the one obtained at 0.3 vol% γ-aminopropylsiloxane, which corresponds to the previously "optimized" value [49]. As it can be seen in Fig. 4, an increase of the siloxane content from 0.3 to 1.5 vol% resulted in a four times improved sensitivity. Hence, the data obtained from the SECM imaging are in a good agreement

Table 1
Volume of the membranes deposited on a PET substrate and resulting relative densities with an applied sample volume of 2 μL.

Membrane composition	Siloxane content/vol%		
	0.3	1.5	2.8
Membrane occupied area/mm^2	11.9	18.1	16.6
Membrane thickness average/μm	1.7	5.0	9.0
Volume of the membrane/mm^3	0.02	0.09	0.15
Relative density to the 0.3 vol% siloxane membrane	1.0	1.1	1.3

with the analytical performance characteristics of the corresponding biosensor. Further increase of the membrane density causes a decrease of the biosensor sensitivity as also observed by SECM. It is important to notice that the experiments performed by using a sensing microelectrode as working electrode such as in SECM and by using the biosensor itself as the working electrode differ in respect to the location where H_2O_2 is detected (*i.e.* at the scanning microelectrode or at the substrate electrode). Thus as discussed by Burchardt *et al.*, different mass transport limitations can occur generating even discrepancies on the kinetic information collected by both strategies [50]. In the present study, similar data trends have been observed between SECM and biosensors experiments, most likely due to the fact that the possible differences in mass transport limitations might have partially been overcome by the close working distance for SECM experiments (*ca.* 1.7 μm; *wide supra*) and perhaps a less limited H_2O_2 transport through the achieved lactate oxidase containing membrane. Moreover, it is important to highlight that the result obtained in Fig. 4 demanded not only a long experimental time, but also a considerable consumption of chemicals and resources. Preparation of one sensor takes about 3 h and making the calibration curve for this biosensor takes about an hour. Thus, one full set of experiments corresponding to one point in Fig. 4 takes approximately 4 h and each tested concentration shown in Fig. 4 was repeated at least ten times (for an approximately total time of 760 h), since the obtained results present a high data dispersion due to their dependence on the hydrodynamic conditions in which the experiments are performed. Noise reduction could be achieved by working in a continuous flow system or by stopping the stirring conditions, however it will increase considerably the experimental time and chemicals consumption or will not provide a fast mass transport regime as it is required for high sensitivity, respectively. In contrast the SECM image shown in Fig. 3 provides similar information to the one obtained in Fig. 4, but with a single experiment that took in total only 8 h (*i.e.* 4 h for the sample and sensor preparation and mounting into the SECM setup, and 4 h for SECM imaging). Indeed, since more densified sample arrays can be prepared, equal or higher number of parameters can be tested in a single experiment that will still last for the same short period of time (*i.e.* 8 h, if all sample spots are prepared simultaneously and covering the same scanned area). Moreover, SECM experiments avoid any influence from hydrodynamic alterations, as only mass transport by diffusion is allowed during the SECM experiment.

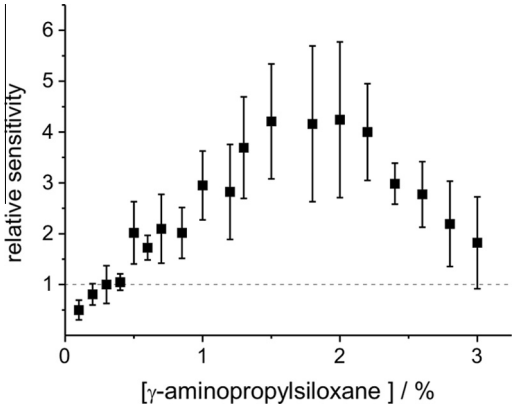

Fig. 4. Relative sensitivity of the lactate biosensor as a function of γ-aminopropylsiloxane content (in vol%) in the casting mixture; 50 mM phosphate buffer with 0.1 M KCl, pH = 6.0 in batch condition upon stirring (500 rpm).

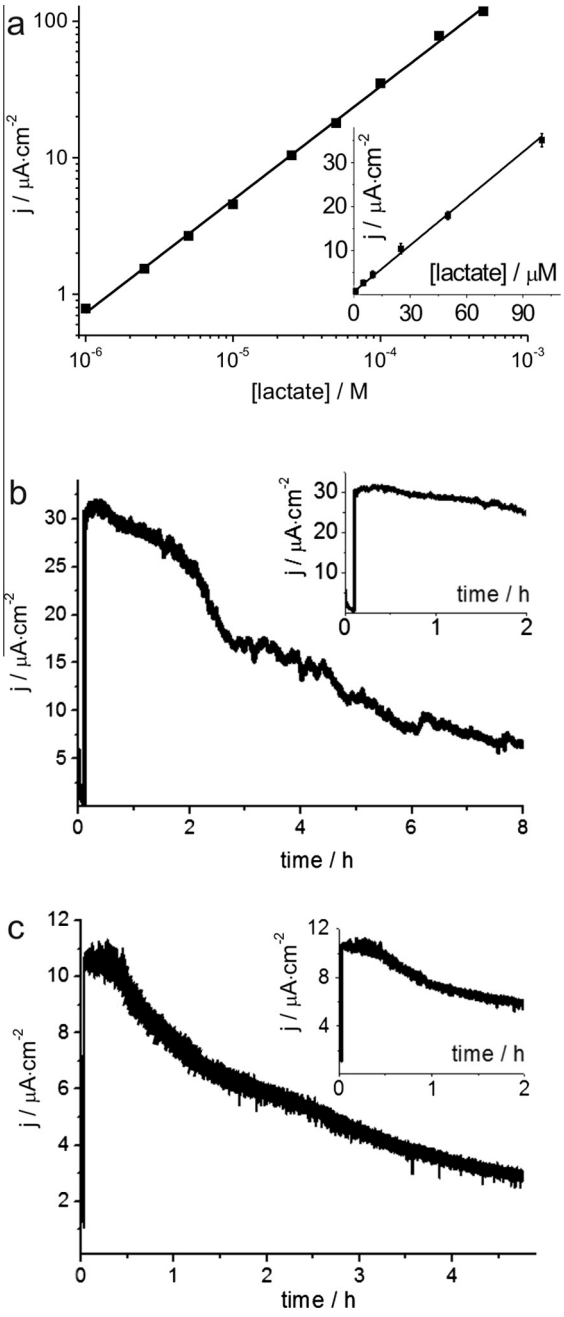

Fig. 5. (a) Calibration plot of the lactate biosensor corresponding to 1.5 vol% of γ-aminopropylsiloxane content in the casting mixture; batch mode upon stirring (500 rpm). (b) Stability test of the optimized (the composition of the immobilization matrix = 1.5 vol% of siloxane) lactate biosensor after more than 4 h of operation where the half activation time is observed. (c) Stability test of the non-optimized (0.3 vol% of siloxane) lactate biosensors after more than 2 h of operation where the half activation time is observed. For all experiments: 0 V *vs* Ag/AgCl; 50 mM phosphate buffer with 0.1 M KCl, pH 6.0, stability test was carried out in a $2.5 \cdot 10^{-4}$ M lactate solution.

The calibration graph of the lactate biosensor made on the basis of 1.5 vol% γ-aminopropylsiloxane in batch mode is displayed in Fig. 5a. As it can be seen, the linear calibration range extends over

almost three orders of magnitude of lactate concentrations: from $1 \cdot 10^{-6}$ to $5 \cdot 10^{-4}$ M. Lactate detection is possible up to $5 \cdot 10^{-3}$ M concentration. The sensitivity determined as the slope of the calibration graph at the lower concentration limit is >0.3 A M^{-1} cm^{-2}, which is in the present conditions just three times less, that the sensitivity of the H_2O_2 transducer used (*vide supra*).

A crucial characteristic considering further applications is the stability of the presented biosensor. The increased density of the enzyme containing membrane is expected to improve the operational stability. Indeed, the half inactivation time under continuous operation in $2.5 \cdot 10^{-4}$ M lactate containing solution increases twice (from 2 to 4 h) for the biosensor made using 1.5 vol% γ-aminopropylsiloxane in casting solution in comparison to the one on the basis of 0.3 vol% siloxane (see Fig. 5b and c, respectively). Hence, mapping enzyme containing membranes with SECM allows an express search of the optimal immobilization parameters without the need of the construction of several sensors that are not easily tested under identical conditions. The resulting biosensors are characterized by both four times improved sensitivity and two times prolonged half inactivation time.

4. Conclusions

Herein, we extended the application of soft contact mode SECM probes to map (bio)catalytically active surfaces with the aim to optimize lactate biosensors. Soft stylus probes were successfully functionalized for the first time with highly active and stable catalytic Prussian Blue films for the detection of H_2O_2. The use of a carbon ink based ultramicroelectrode as support for the Prussian Blue deposition resulted in a highly operational and stable ferric hexacyanoferrate sensing layer allowing long-term SECM imaging. Moreover, SECM in contact mode showed to be beneficial for the scanning of rough and tilted samples allowing the extraction of relevant information without topographic interferences and without damaging the membrane structure. Thanks to the demonstrated SECM screening experiments of different enzymatic immobilization conditions a lactate biosensor with remarkably improved analytical performance characteristics, *i.e.* sensitivity and operational stability, has been achieved. The use of SECM for screening enzyme containing membrane libraries with the exploration of optimal immobilization conditions could provide the elaboration of various advanced biosensors.

Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data include topographic, laser scanning and scanning electron microscopy images of the rough carbon paste substrate employed for the immobilization of lactate oxidase, deposited lactate oxidase membranes over PET and immobilized lactate oxidase membranes over carbon paste substrates, respectively.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jelechem.2014.08.014.

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