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# Improved reuse and storage performances at room temperature of a new environmentally friendly lactate oxidase biosensor prepared by ambient electrospray immobilization†

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A new, environmentally friendly lactate oxidase (LOX) based biosensor for lactate detection, with unprecedented reuse and storage capabilities at room temperature, has been manufactured using the ambient electrospray deposition (ESD) technique. This technology allows for an efficient, green and easy ambient soft-landing immobilization of the LOX enzyme on a cheap commercial screen-printed Prussian blue/carbon electrode (PB/C-SPE), employing sustainable chemistry. This study shows how ESD can confer the biosensor the ability to be stored at ambient pressure and temperature for long periods without compromising the enzymatic activity. The fabricated biosensor shows a storage capability for up to 90 days, without any particular care under storage conditions, and a reuse performance for up to 24 measurements on both the electrode just prepared and on a three-months-old electrode. The LOX-based biosensor has been tested for lactate detection in the linear range of 0.1–1 mM with a limit of detection of  $0.07 \pm 0.02$  mM and does not show any memory effects. The absence of an entrapment matrix as well as any additional hazardous chemicals during the immobilization phase makes the process competitive in terms of environmental sustainability and toxicity. Moreover, the application of a new electrospray deposition cycle on the used biosensors makes the biosensors work again with performances comparable to those of freshly made ones. This demonstrates that the technique is excellent for recycling and eliminates the waste of disposable devices.

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## 1 Introduction

As stated by Yáñez-Sedeño *et al.*<sup>1</sup> “electrochemical biosensors are analytical tools fully integrated into the principle of green chemistry.<sup>2,3</sup> They allow the development of analytical methodologies for real-time detection and in situ monitoring and management, avoiding the formation of hazardous substances due to the direct analysis of samples with no need for previous treatment.<sup>4</sup> Moreover the need of minimum environmental impact has driven

the use of eco-friendly solvents, reagents, and materials.” In accordance with the green chemistry philosophy,<sup>5</sup> in this work we show the fabrication of an electrochemical biosensor which follows the principles of green chemistry throughout the entire life cycle of the product, starting from manufacturing to the use, reuse and finally recycling. Hereinafter, we show both the production of an electrochemical biosensor for the detection of lactic acid and the use of the ambient electrospray deposition (ESD) technique for the immobilization phase, which is able to give the biosensor unprecedented storage and reuse capabilities, thus reducing the consumption of disposable devices.

Biosensors for lactate detection are attracting increasing interest in clinical diagnosis to reduce morbidity and mortality because they provide a rapid point of care and allow serial measurements of early symptoms of disorders such as intra-abdominal sepsis. Lactate concentration is commonly used as a key parameter to assess patients' health conditions and in ongoing surveillance in surgery.<sup>6,7</sup> In addition to those in the biomedical field, lactate biosensors find also extensive application in the food industry,<sup>8,9</sup> for fermentation analysis as an indicator of food quality, shock/trauma,<sup>10</sup> and sports medicine.<sup>11</sup>

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When the energy demand from the tissues cannot be satisfied by aerobic respiration, there will be an increase in the concentration of lactate from the anaerobic metabolism, which if not adequately eliminated can cause an increase in the concentration of lactic acid with consequent lactic acidosis.<sup>12</sup> During exercise, the blood lactate level is used as an indicator of the athlete's physical readiness as elevated blood lactate levels can lead to a decrease in the pH level in the blood resulting in fatigue. Lactate detection also plays an important role in the food industry, particularly in the analysis of fermentation products such as fermented milk products, wine, cured meats, and fish. In these products, the presence of lactate is associated with bacterial fermentation; therefore, it is used as an indicator of the freshness and quality of food.<sup>13</sup>

The methods currently used for the detection of lactate in the biomedical field are not fast enough requiring the presence of highly specialized personnel as they are mainly based on venous or arterial blood sampling and subsequent spectrophotometric analysis.<sup>14</sup> Therefore, there is a high demand for developing new accurate and sensitive devices for fast screening of different samples. Biosensors are ideal tools since they allow rapid "point of care" and serial measurements at the bedside during hospitalization without the need for special skills or complex equipment.<sup>15</sup>

To date, biosensors for lactate detection have been produced using various supports including conductive or non-conductive polymer matrices,<sup>16–18</sup> membranes,<sup>19</sup> transparent gel matrices,<sup>20</sup> screen-printed electrodes,<sup>21–23</sup> hydrogel supports,<sup>24</sup> and nanoparticles.<sup>25,26</sup>

In many biosensors manufactured for lactic acid detection, the most commonly used biological recognition element is the L-lactate oxidase (LOX) enzyme because of the simplicity of the enzymatic reaction, which makes the sensor design considerably simpler. LOX is a flavoprotein that can be obtained from different bacterial sources like *Pediococcus*, *Aerococcus viridans*, and *Mycobacterium smegmatis*. It catalyses the oxidation of L-lactate to pyruvate in the presence of dissolved oxygen and forms hydrogen peroxide, which is electrochemically active and can be either reduced or oxidized to give a current proportional to the L-lactate concentration.<sup>27,28</sup>

In the fabrication of an enzyme-based biosensor, immobilization of the enzyme is the fundamental step for a successful device. The main objective is to maintain the biostability of the enzyme and at the same time ensure that the efficiency of its enzymatic reactions is preserved. To this purpose, the enzyme must be immobilized in its active form that has to be maintained over time, giving the biosensor stability in terms of reusability and long-term storage, combined with linearity, high sensitivity, and selectivity towards the chosen analyte. All known methods used to immobilize both the enzyme moiety and other components,<sup>29</sup> currently include physical adsorption,<sup>30</sup> membrane confinement,<sup>31</sup> covalent bonding,<sup>32</sup> cross-linking,<sup>33</sup> electrical polymerization,<sup>34</sup> and finally monolayer formation by self-assembly,<sup>35,36</sup> and all of these have their pros and cons.<sup>37</sup> More in detail, many of the methods involve the use of membranes and hazardous

chemicals together with a complex and most often not eco-sustainable synthesis process.

Moreover, when an enzyme is immobilized on the surface of the transducer, it may trap the redox center inside its active site, which, however, is at a certain distance from the electrode surface. This prevents rapid electron transfer to the electrode surface associated with the redox reaction. To overcome this problem, a mediator is used. This artificial electroactive species functions as an electron transfer agent, such as a shuttle, between the enzyme and the electrode.<sup>38</sup> To ensure efficient electron transfer, the mediator must remain immobilized near the electrode. Another problem that plagues any biosensor employing an oxidase enzyme as a recognition element is that the direct detection of hydrogen peroxide, whose concentration is directly proportional to the concentration of the enzymatic substrate (*i.e.* L-lactic acid in our case), is only possible at high potentials. At these high potential values, the presence of easily oxidizable compounds such as ascorbate, bilirubin, urate, *etc.* can easily interfere with the measurement, being oxidized at the electrode together with hydrogen peroxide.<sup>39</sup> To avoid this problem, the most used mediator to lower the potential and allow for selective detection of hydrogen peroxide is Prussian blue.<sup>40–45</sup> This compound has wide use in different fields as in medicine for its biocompatibility and low toxicity because of the strong bond between the cyanide and iron ion.<sup>46–49</sup>

In this work, we present a new Prussian blue/L-lactate oxidase-based biosensor manufactured using the ESD technique to perform soft-landing immobilization of the enzyme.<sup>50,51</sup> The ESD is a one-step immobilization technique which does not need any matrix synthesis and the use of aggressive chemical agents while remaining competitive with the main immobilization methods used so far. We demonstrate how the application of the ESD technique on an enzyme commonly known to be unstable when stored under dry conditions at room temperature and pressure<sup>52</sup> can guarantee improved performances of the biosensor in terms of storage and prolonged reuse over time never achieved before by any other immobilization techniques. It must be highlighted that since LOX is an unstable enzyme, an adequate immobilization technique is fundamental to build a robust sensing device.<sup>53</sup> Moreover, the absence of polymeric matrices, hazardous chemicals, and nanomaterials during the immobilization phase, the storage at room temperature without the need for a low temperature, the prolonged reuse of the device, the possibility of recycling the sensor even one year after its last use, making it performant again, and finally the use of Prussian blue as a mediator make both the process and the final product environmentally friendly and sustainable.

## 2 Materials and methods

### 2.1 Chemicals and instrumentation

L-Lactate oxidase (LOX) from *Aerococcus viridans* (EC.1.1.3.2, activity: 54 U mg<sup>-1</sup>), potassium phosphate dibasic (K<sub>2</sub>HPO<sub>4</sub>),



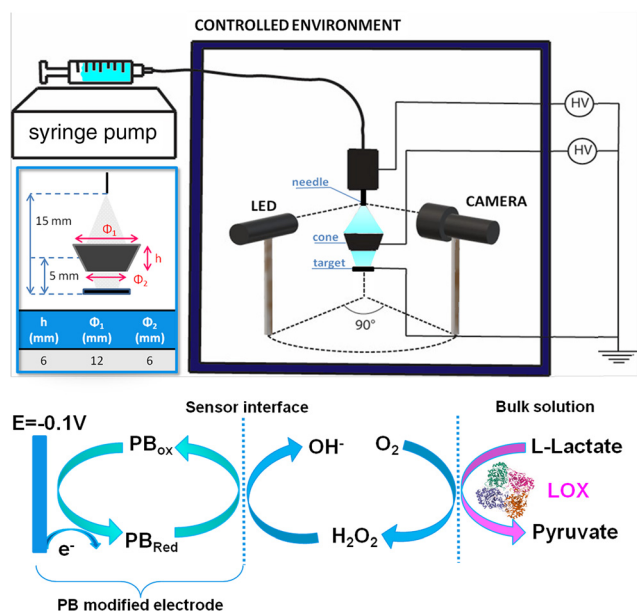
potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ), sodium chloride ( $\text{NaCl}$ ), potassium chloride ( $\text{KCl}$ ) and pure water for chromatography (LC-MS grade) were purchased from Sigma Aldrich (Merck Group). Isopropanol and L-lactic acid were purchased from Carlo Erba (Dasit Group). All the reagents were used as provided by the companies.

L-Lactic acid was used in a 12 mM solution with 0.1 M phosphate-buffered saline (PBS) at pH 7 for amperometric measurements. The screen-printed electrodes used for the deposition of LOX were the Metrohm DropSens screen-printed Prussian blue/carbon electrodes DRP-710 (PB/C-SPE) with a Prussian blue/carbon working electrode (4 mm diameter), carbon counter electrodes and a silver reference electrode. The electrochemical measurements were performed using a portable potentiostat PalmSens@4 (Palm Instruments, The Netherlands), connected to a personal computer. The images of the deposit on the PB/C-SPE were acquired using a Malvern Panalytical Morphologi 3-ID. The amount of LOX deposited was quantified using a custom quartz crystal microbalance (QCM) manufactured at the Institute of Atmospheric Pollution Research (IIA) of the CNR, Research Area of Rome 1 in Montelibretti (Rome, Italy).<sup>54,55</sup> The custom ESD setup is shown in Fig. 1, in which the counter electrode (target) at the ground is replaced by a PB/C-SPE. A schematic of the redox reaction that occurs at the electrode surface during the amperometric measurements is shown in the bottom panel of Fig. 1. The electrospray ionization source with the use of high voltage, which can trigger ion-/radical-molecule reactions,<sup>56–58</sup> and the entire set-up have already been described in our pre-

vious work<sup>50</sup> and will be only briefly summarized here. It consists of a Pump 11 Elite infusion (Harvard Apparatus) equipped with a Hamilton syringe (250  $\mu\text{L}$  total volume), which is connected to a silica capillary (300  $\mu\text{m}$  internal diameter) ending with a steel needle (100  $\mu\text{m}$  inner diameter), where a high voltage is applied. Between the needle and the target, a focusing electrode (cone) has been added. The relative distance between the needle, the cone and the PB/C-SPE together with the size of the cone is depicted in the inset of Fig. 1. The alignment between the spray needle, the focusing cone electrode, and the target is a crucial parameter in the deposition. Moreover, the distance between these three elements can be influenced by the composition of the sprayed solution. The voltages applied to the needle and the cone are  $\sim 5$  kV and 2 kV respectively. When a high voltage is applied at the needle, the charges on the liquid surface at the end of the needle repel each other and expand at the solution/gas interface into a Taylor cone where the electrostatic force is counterbalanced by the surface tension of the liquid. When the surface tension cannot stand the charges anymore, a Coulomb explosion creates a spray of charged droplets, whose size decreases as the solvent evaporates to form a gas of molecular ions. In this way, a spray consisting of charged LOX is created and can reach the surface of the SPE working electrode to cover it, by discharging. Compared to other immobilization techniques the energy consumption necessary for the ESD process is strictly connected to the energy demand of the syringe pump and the power supplies for the needle and the cone. The technique completely avoids waste of material and energy consumption related to the chemical synthesis of matrices or other types of nanomaterials instead widely used in the fabrication of biosensors (see Table 2). Moreover, avoiding intermediate steps, materials, and low temperatures in the fabrication and storage of a biosensor has major advantages in reducing the energy consumption, pollution and waste at the industrial scale up.

## 2.2 LOX preparation for electrospray immobilisation

The stock solution of LOX was prepared by dissolving Sigma-Merck-lyophilized LOX in 1 mL of ultra-pure water for chromatography (LC-MS grade) to a final concentration of 2  $\mu\text{g mL}^{-1}$  and added to Eppendorf tubes maintained at  $-18^\circ\text{C}$ . For the preparation of the working solution used for ESD spray, the stock solution was diluted to 1  $\mu\text{g mL}^{-1}$  at 10% of isopropanol in water. This procedure avoids temperature degradation of the sample, and all tests use a fresh solution of equal concentration. The ESD process is carried out by spraying the working LOX solution (1  $\mu\text{g mL}^{-1}$ ) at a flow rate of 1  $\mu\text{L min}^{-1}$ , for 40 minutes in order to deposit about 2 Units of enzyme on the surface of the biosensor. To quantify the enzyme amount actually immobilized after the entire spray session, depositions under the same conditions were carried out directly on the resonator of a quartz microbalance, set in place of the PB/C-SPE. All the details of these measurements are described in the ESI.†



**Fig. 1** Schematic of the ESD setup (top right panel) and enlarged scheme of the deposition region (top left panel) where the dimensions and the relative distance between the needle, the cone and the PB/C-SPE are depicted. Schematic of the redox reactions that occur at the electrode surface during the amperometric measurements (bottom panel).

### 2.3 Electrochemical characterization of the electrospray deposited LOX on Prussian blue screen-printed electrodes (e-LOX/PB/C-SPE)

Electrochemical experiments were carried out at room temperature by amperometric analysis with an applied potential of  $-0.10$  V vs. the Ag/AgCl reference electrode.<sup>59</sup> The study of the LOX activity *versus* L-lactic acid was performed in a total volume of  $100\ \mu\text{L}$  of  $0.1$  M PBS buffer at pH 7, recording the current signals every  $0.5$  s. The fabricated biosensor must catalyze the oxidation of the substrate L-lactate into pyruvate. This reaction occurs in the presence of dissolved oxygen that oxidizes LOX by producing hydrogen peroxide which in turn is reduced by Prussian blue, which is finally reduced at the electrode surface (see Fig. 1 bottom panel) at  $-0.1$  V. The current generated, as a result of the reduction, is proportional to the concentration of L-lactate in the sample.

## 3 Results and discussion

### 3.1 Microscopic analysis of the e-LOX/PB/C-SPE

The morphology of the working electrode of the PB/C-SPE was observed using the Malvern Panalytical Morphologi 3-ID microscope before and after the L-lactate oxidase enzyme immobilization by ESD (Fig. 2). Fig. 2a and b show a magnification of the central part of the working electrode before and after the deposition respectively, while Fig. 2c and d show its right edge. From these images, it appears that the adopted working conditions and the deposition time ensure a surface coverage of the working electrode quite homogeneous and optimally centred with a quite sharp edge. According to the calibration performed on deposition carried out under the same conditions on a quartz microbalance (see the ESI†), the amount of LOX units actually deposited on the working electrode was found to be  $2.24 \pm 0.20$  U. This demonstrates that the totality of the sprayed LOX reaches the electrode surface

and that no material is lost on the cone as observed in a previous work.<sup>50</sup>

### 3.2 Electrochemical characterization of the e-LOX/PB/C-SPE

The e-LOX/PB/C-SPE works with the highest LOX activity at pH = 7 and with an applied potential of  $-0.10$  V.<sup>59</sup> The analytical performances of e-LOX/PB/C-SPEs, including the range of linear response, the detection limit, the working/storage stability and the carryover effect, were evaluated under these fixed optimal conditions. These parameters strictly depend on the immobilization method as well as on the use of screen printed electrodes and Prussian blue as the electrochemical mediator. The storage stability is strongly connected to the immobilization process, which must keep the enzyme in its folded structure and ensures that the enzyme does not denature over time. Furthermore, efficient immobilization prevents enzyme leaching after several consecutive washes and measurements ensuring good working stability of the device.

**3.2.1 Analytical features.** The detection capability of the e-LOX/PB/C-SPEs has been tested towards L-lactic acid. The amperometric measurements have been performed by dropping  $100\ \mu\text{L}$  of  $0.1$  M PBS buffer at pH 7 on the screen-printed electrochemical cell and adding increasing concentrations of L-lactic acid in the range from  $0.1$  to  $1$  mM. The signal has been recorded every  $0.5$  s and it appears to increase linearly with the L-lactic acid concentration (Fig. 3a). The measurement in Fig. 3a was repeated three times on electrodes produced in different batches, and for each case, the reading of steady current after L-lactic acid addition was background subtracted. The average value of the current and the standard deviation of these measurements are reported vs. the L-lactic acid concentration in Fig. 3b. The calibration curve using the linear regression given by  $y\ (\mu\text{A}) = 2.40(\pm 0.17)\ (\mu\text{A}\ \text{mM}^{-1}) \times (\text{mM}) - 0.04(\pm 0.09)\ (\mu\text{A})$ , with an  $R^2 = 0.98$ , is shown in Fig. 3b. The calibration curve returns a limit of detection (LOD), equal to  $0.07 \pm 0.02$  mM, defined as  $3.3\sigma/S$ , where  $\sigma$  is the standard deviation of the amperometric signals for three different measurements at  $0.1$  mM concentration on the same electrode, and  $S$  is the slope of the calibration curve.<sup>60</sup>

**3.2.2 Working and storage stability studies.** Improving the operational stability without loss of sensitivity over a long period of time is one of the most important issues in develop-

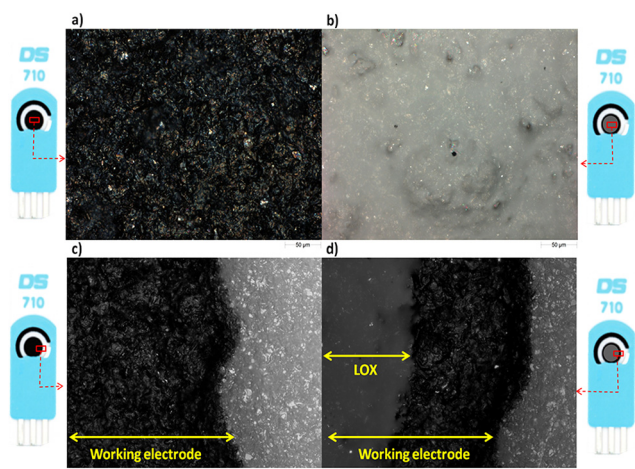


Fig. 2 Images (magnification 20x) of the central and right edge part of the pristine (a and c) and modified (b and d) PB/C-SPE working electrode.

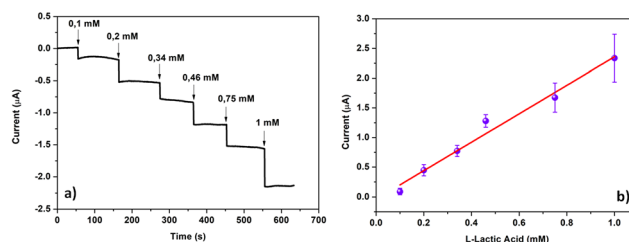


Fig. 3 (a) e-LOX/PB/C-SPE chronoamperogram at an applied potential of  $-0.1$  V, showing the addition of increasing amounts of L-lactate and (b) the corresponding calibration plot. The number of repetitions is  $n = 3$ . Measurement volume:  $100\ \mu\text{L}$ ,  $0.1$  M PBS buffer at pH 7.





ing a commercial biosensor. Operational stability generally depends on the type of bio-receptor used for the detection of L-lactic acid, the storage conditions and the methods used for the immobilization of the bio-receptor on the transducer.<sup>61</sup> In a previous work,<sup>50</sup> we have shown how the application of ESD for the immobilization of an enzyme, such as laccase,<sup>62</sup> gives the biosensor unprecedented storage and reuse capability.

Reuse or working stability refers to the number of times a sensor can be operated and is ready for the next reading. In Fig. 4 we show the results in terms of working stability (a and c) and storage stability (b) obtained for the e-LOX/PB/C-SPEs fabricated with ESD. In the case of the working stability, a comparison with the sensor fabricated using the drop-casting methodology is also shown.

The working stability has been investigated by repeated amperometric measurements on the same electrode, alternating washes with 0.1 M PBS buffer at pH 7 and tests in the presence of 0.46 mM L-lactic acid, to observe if enzyme leaching occurs. The test was conducted on a batch of three electrodes just modified *via* ESD (Fig. 4a) showing a near 100% retainment of the activity up to 24 consecutive measurements within the statistical error. To highlight the performance of the e-LOX/PB/C-SPE sensors, we measured the working stability of electrodes modified by drop-casting with the same quantities of the LOX enzyme. The measurements are performed on a batch of four electrodes modified using the drop-casting technique. The results show that the stability *versus* repeated washes is dramatically worse in the case of drop-casting SPEs, which lose 74% of activity already after only 3 consecutive washes and 90% after 15 washes. The gradual decrease of the

current signal may be ascribed to a weaker anchoring of the enzyme in the deposition by drop-casting. The leaching of the enzyme is immediate and evident already on the third measurement on the drop-casting SPEs, while the ESD sensors are stable and can be reused with the same performances for repeated measurements. The excellent results in terms of working stability confirm those already achieved in the case of laccase<sup>50</sup> and seem to be independent of the type of immobilized enzyme, but rather a property of the ESD immobilization technique.

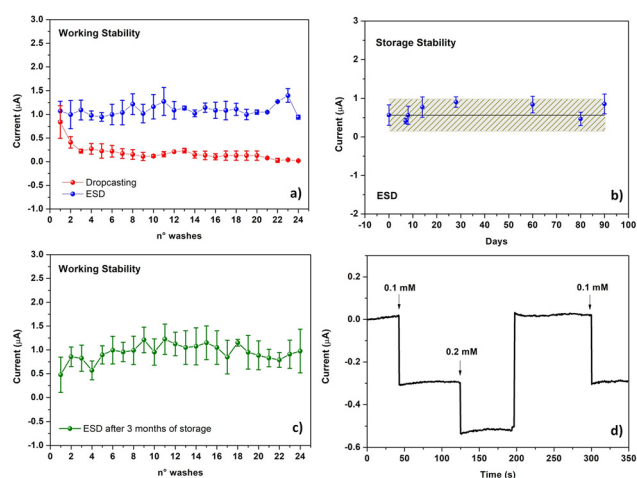
To evaluate the storage stability, e-LOX/PB/C-SPEs deposited from different batches of preparation were preserved in the dark at room temperature and ambient pressure and tested after a time variable from a few days to 3 months, with a solution of 0.46 mM L-lactic acid in a 0.1 M PBS buffer at pH 7 (Fig. 4b). Each measurement was repeated at least three times. The results in Fig. 4b display excellent storage stability for up to 90 days, achieved without any particular care in the storage of the electrodes. These results are consistent with those already obtained for laccase in our previous works,<sup>50,51</sup> but are even more striking if we consider that laccase is known to be a resistant enzyme<sup>62</sup> while lactate oxidase is considered an unstable one.<sup>52</sup> Even in the case of long-term stability, these results seem not to depend on the type of enzyme deposited but rather on the ESD immobilization technique used.

To highlight the long-term storage property of the biosensor, the same working stability tests carried out on ESD just modified electrodes (Fig. 4a) were also performed on a batch of three electrodes left in storage for three months (Fig. 4c). In this second case, the electrodes were modified by electrosprayed lactate oxidase and put in storage for up to three months in the dark at room temperature and pressure. After this period, the operational stability has been tested by repeated 24 amperometric measurements on the same electrode alternating washes with buffer.

The ESD results in Fig. 4a and c show a near 100% retainment of the activity up to 24 consecutive measurements within the statistical error both in the case of newly made electrodes and in the case of three months old electrodes. In the latter case, the first four measurements show a slightly lower value than the average probably because the dry enzyme immobilized and left in storage for three months needs a certain period of time to fully regain its catalytic capacity.

It must be emphasized that the working capacities have not been tested beyond the 24th wash, which does not show any significant signal decrease. This bodes well that the maintenance of the activity can last well beyond the 24th wash as well as it can exceed three months of storage.

Finally, to test the possibility of recycling the biosensors, a further immobilization cycle was carried out on already used sensors. A batch of three new electrodes have been electrosprayed with LOX, tested against 0.46 mM L-lactic acid (named just made in Table 1), then put in storage for 24 h, subjected to another immobilisation cycle and tested again (24 h storage 1st measure in Table 1). Moreover a wash has been performed to test the leaching of the enzyme (24 h storage 1st wash in



**Fig. 4** (a) Working stability of the e-LOX/PB/C-SPE freshly made by ESD (blue dots) and by drop-casting (red dots). (b) Storage stability of the e-LOX/PB/C-SPE. (c) Working stability of the e-LOX/PB/C-SPE tested after 3 months of storage (green dots). (d) Chronoamperogram of the e-LOX/PB/C-SPE recorded for sequential additions of L-lactic acid at different concentrations. For all the measurements, the applied potential is  $-0.1$  V. The measurement volume is  $100\ \mu\text{L}$  of  $0.1$  M PBS buffer at pH 7. For the measurements in (a–c) the concentration of L-lactic acid tested is  $0.46$  mM.



**Table 1** Measurement of the current obtained after the addition of 0.46 mM L-lactic acid on biosensors just made, subjected to another process of LOX deposition after 24 h of storage and after 1 year of storage.  $N = 3$ . On the 24 h old and 1 year old used biosensors, a wash measurement has been performed

	Just made	24 h storage		1 year storage	
		1st measure	1st wash	1st measure	1st wash
Current ( $\mu\text{A}$ )	$1.16 \pm 0.13$	$1.58 \pm 0.06$	$1.56 \pm 0.34$	$1.67 \pm 0.06$	$1.39 \pm 0.11$

Table 1) The same treatment was carried out on another batch of three sensors which have been stored for one year after their first use (1 year storage in Table 1). The results show that a new cycle of ESD makes the sensor work again with slightly higher performance than the electrode just made even after a year of their last use and no leaching has been found (see 1st wash for 24 h and 1-year-old electrodes in Table 1) suggesting prolonged reuse of these reconditioned biosensors in the same way as those freshly made.

The purpose of making another deposition of LOX on a one-year-old electrode was to demonstrate that ESD is able to restore the sensor to factory performance as if it had just been manufactured and that the fabrication process complies with the important and fundamental aspects of recycling in order to reduce pollution from disposable devices. A company producing a sensor by ESD may withdraw the product even after a

year of its last use and submit it to another ESD process, putting it back on the market with comparable performances. This will avoid disposal of the sensors and an increase in pollution. Furthermore, the absence of metal nanoparticles or nanostructures as well as any kind of polymeric matrix on the substrate, composed solely of graphite and Prussian blue, makes the device more prone to a green disposal with respect to the others.

**3.2.3 Study of the carry-over effect.** To test the possibility of using the same biosensor to analyze different samples consecutively, the carry-over/memory effect was investigated using the same biosensor for the sequential analysis of the following L-lactic acid concentrations: 0.1 mM, 0.2 mM and 0.1 mM again. At time zero 100  $\mu\text{L}$  of 0.1 M PBS buffer has been added to measure the background signal, and after about 50 seconds and 125 seconds, 0.1 mM and 0.2 mM solution of L-lactic acid, respectively, have been added. In order to test the memory effect of the biosensors, after 200 seconds the 0.2 mM solution has been removed and 100  $\mu\text{L}$  of PBS buffer has been added again. As shown in Fig. 4d, the background signal is restored to the original level as if the sensor had not been affected by the previous measurements. The final addition of 0.1 mM returns the previously measured value within the uncertainty of the measurement. This is the definitive proof that no carry-over/memory effect exists.<sup>68,69</sup>

**3.2.4 Comparison of biosensors.** Table 2 lists LOX-based biosensors that show good storage performance obtained under different experimental immobilization methods and working electrode materials, which were compared in terms of

**Table 2** Comparison of LOX-amperometric biosensors fabricated using different immobilisation methods and working electrode materials

LOX origin; the amount used	Immobilization method	Immobilization matrix; working electrode material	LOD	Linearity	Storage stability (days)	Storage conditions	Ref.
<i>Pediococcus</i> sp.; 5.5 U	Cross-linking	Polyvinyl alcohol (PVA) matrix; platinized graphite electrode	0.01 mM	$2 \times 10^{-5}$ – $4 \times 10^{-3}$ M	80	In phosphate buffer (pH 7.0) at 5 °C or dry in phosphate salt at room temperature or dry in a phosphate-sodium azide salt mixture at room temperature	63
NR; 0.2 U	Entrapment	Mucin/albumin hydrogel matrix/Nafion polymer; Pt electrode	0.8 $\mu\text{M}$	2–1000 $\mu\text{M}$	150	Rinsed with buffer and stored in the fridge at 4 °C	64
Microorganism; 0.93 U	Entrapment	Nafion/cobalt phthalocyanine/polyvinyl alcohol/screen-printed carbon electrode	18.3 $\mu\text{M}$	18.3 $\mu\text{M}$ –1.5 mM	270	Stored desiccated at 4 °C	65
NR; 5 U	Adsorption	Carboxymethyl cellulose (CMC); carbon screen printing ink electrode	1 mM	1–50 mM	300 or 7	Storage at –30 °C (stability 300 days). Storage at room temperature (stability 7 days)	66
<i>Pediococcus</i> sp.; NR	Adsorption	(TBABr)-modified Nafion binder; nitrogen-doped CNT/GCE	$4.1 \pm 1.6$ $\mu\text{M}$	14–325 $\mu\text{M}$	90	Stored in SPB sodium phosphate buffer at 4 °C	67
<i>Aerococcus viridans</i> ; 2.24 $\pm$ 0.20 U	ESD	No matrix; Prussian blue/carbon electrode	$0.07 \pm 0.02$ mM	0.1–1 mM	90	In the dark at ambient pressure and temperature	Current work



the LOD, linear range of response, storage stability, storage conditions, and immobilization matrix. It can be immediately observed that the most noticeable performance of the present e-LOX/PB/C-SPEs lies in good storage stabilities under cheap and handy room pressure and temperature conditions. The other biosensors exhibit the same or higher storage capabilities, but under conditions that require low temperatures (4 °C, 5 °C or even −30 °C) in most cases combined with the presence of buffer solutions. Moreover, all the biosensors listed in Table 2 require the presence of a matrix in the immobilization process and despite this, if left at room temperature they lose their efficiency already after 7 days. The newly fabricated biosensor has also a good LOD and linearity considered in line with the average detection limit of lactic acid in competitive biosensors.

We want to underline the green aspect of the ESD technique considering the only use of water (90%) and isopropanol (10%) in the spray solution. To highlight the topic, Table S1 in the ESI† groups all the main hazardous chemicals used in the literature for the manufacturing of the biosensors listed in Table 2. Table S1† lists the hazardous chemicals identified according to Regulation (EC) No. 1272/2008 and the GHS classification as well as the nomenclature, for some of them, identified by the CHEM21 selection guide.<sup>70</sup> All the sensors listed in Table 2 require the use of chemical substances which are harmful to both humans and the environment. The main damage to the environment is highlighted in red in Table S1.† The isopropanol and water solvents used in the present work are considered recommended by the CHEM21 selection guide and are highlighted in green, together with Prussian blue which is not recognized as harmful according to Regulation (EC) No. 1272/2008. For the above considerations, the new device can be considered a promising low cost and greener device compared to the others mentioned, for a rapid quite efficient and handy screening of lactic acid.

## 4 Conclusions

The results of this work demonstrate that electrospray deposition (ESD) is an effective, low-cost, efficient, and successful alternative green and sustainable technique for the construction of an electrochemical amperometric biosensor, through the direct soft landing immobilization of the LOX enzyme on a surface of a low-cost commercial PB/C-SPE. The e-LOX/PB/C-SPEs fabricated have been tested toward L-lactic acid detection showing a LOD ( $0.07 \pm 0.02$  mM) and a range of linearity (0.1–1 mM) comparable to those of other biosensors reported in the literature. On the other hand, the e-LOX/PB/C-SPE biosensor shows no memory effect and an excellent anchoring of the bio-receptor on the surface of the transducer as proved by its working stability for 24 consecutive measurements on the same electrode just fabricated as well as on a three months old electrode. Another outstanding result concerns the tests of working stability performed on reconditioned one year and 24 h old electrodes to demonstrate the recycling nature of the newly fabricated biosensor. The ESD sensor, after being used,

can be left in air for 24 hours or one year and, after reconditioning, gives a signal of current which is slightly higher than the sensor just made, but which can be reused with the same performances after proper calibration. The test of washing on these sensors shows the maintenance of performance without leaching of the enzyme. This demonstrates that ESD is able to restore the sensor to factory performance as if it had just been manufactured and that the fabrication process complies with the important and fundamental aspect of recycling, therefore reducing the pollution from disposable devices.

It has to be outlined that the excellent storage performance for up to 90 days was achieved here for the first time without any particular care in the storage of the biosensor, which has been stored in the dark at ambient pressure and temperature without the need for a fridge or buffer solutions. These conditions reduce energy waste due to the use of refrigerators and facilitate their sorting and transport. This result has to be emphasized as it was obtained using an enzyme such as LOX commonly known to be an unstable enzyme at room temperature and pressure. The good performances achieved in terms of working and storage stabilities are to be attributed to the ESD technique, which does not use any additional chemical agent (apart from a small amount of isopropanol in water) or polymeric matrices for immobilization. Moreover, thanks to the gentle ionization and deposition, the ESD technique is able to lay the enzyme in its optimal conformation maintaining its catalytic activities unaltered. The ESD immobilization technique used in this work is a one-step, environmentally friendly method, which allows reducing production costs for commercial application and creating an eco-designed device. The mere presence of carbon and Prussian blue on the electrochemical cell of the SPE and the absence of other hazardous chemical compounds in the design and manufacturing make the biosensor more suitable for green disposal. The present results together with the previous ones achieved on a laccase-based biosensor<sup>50</sup> prove that the ESD technique can find interesting and successful applications in the construction of commercial biosensors to be used in the biotechnology and bioengineering fields.

## Author contributions

M. C. C.: conceptualization, investigation, data curation, formal analysis, methodology, writing – original draft, and writing – review and editing. V. S.: investigation, review and editing. E. T.: investigation, review and editing. J. C.: methodology, review and editing. M. P.: investigation, review and editing. V. F.: review and editing. M. T. G.: conceptualization, review and editing. L. A.: funding acquisition, review and editing. D. R.: investigation, review and editing. A. C.: supervision, funding acquisition, methodology, data curation, review and editing.

## Conflicts of interest

There are no conflicts to declare.



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