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Continuous capillary-flow sensing of glucose and lactate in sweat with an electrochemical sensor based on functionalized graphene oxide

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ABSTRACT

We describe an electrochemical device for the simultaneous monitoring of glucose and lactate in sweat, based on enzymatic sensors exploiting capillary flow to induce continuous, stable sensing. The enzymes, namely glucose oxidase and lactate oxidase, were anchored to a graphene oxide and chitosan composite (GO-Ch) of original synthesis, to achieve stable deposition of the bioreceptors on the electrochemical platform. We tested both biosensors on a realistic device architecture: they were embedded in a nitrocellulose strip, to exploit capillary force to induce a continuous flux of sweat on the sensor platform, ensuring the constant renewal of sample. We could achieve good sensitivity at potentials close to zero by using Prussian Blue as redox mediator, thus avoiding interference from other chemical species present in the complex matrix. The sensing signal was stable and linear over two hours in a concentration range of glucose and lactate between the limit of quantification (32 and 68 nM, respectively) and the upper limit of linearity (3.8 and 50.0 mM, respectively). The device is simple, robust, stable, and can be easily worn without the direct contact of the active part with the skin, making it suitable for simultaneous monitoring of glucose and lactate in human sweat.

1. Introduction

Wearable biosensors are receiving increasing attention from scientific and medical communities since they constitute non-invasive and cheap devices to monitor, also by remote, parameters of primary importance for human health or fitness goals: different physical and chemical sensors can be employed, allowing personalized analyses or training programmes. Scientific literature has already reported several examples of physical wearable sensors to aid the identification of severe diseases at an early stage [1]: accelerometers, pressure sensors, ECG-Holter monitors that can be applied for the diagnosis of Parkinson disease [2] multiple sclerosis [3], epilepsy [4], sedentary behaviour [5].

Wearable chemical sensors, instead, aim at the continuous monitoring of selected molecules from human fluids, such as sweat, saliva, tears, and interstitial fluids; among different transduction methods, the most consolidated approach is the electrochemical one, exploiting different techniques, namely potentiometry, voltammetry, chronoamperometry and electrochemical impedance spectroscopy [6]. Analysis of the human sweat is often preferred, since it enables non-invasive monitoring of many parameters at the same time, e.g., electrolytes, glucose, lactate, ethanol. Devices proposed so far include wristbands, tattoos, and patches [7,8]. If on the one hand they allow an easy detection of these chemical parameters, a still open problem is how to achieve a continuous flow of sweat on the sensor platform in order to induce a punctual definition of the physiological status of the person under monitoring; only a few devices proposed so far, in fact, include a capillary fluidic system, which can collect higher and more homogeneous amounts of sweat to correctly wet all the electrodes, independently of the individual perspiration rate [9,10]. This, to our opinion, is a critical aspect which should be evaluated in the realization of a feasible device, since the lack of a continuous flow system could accumulate the analytes in the close proximity of the electrode surface, consequently compromising the accuracy of the detection.

In the development of a wearable chemical sensor, the choice of the most proper sensing material is of outmost importance. Glucose and lactate are normally detected by an enzymatic reaction, so that the

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chosen material should allow a stable anchoring of the bioreceptors and possess a great biocompatibility to improve the analytical performance of the resulting device. Graphene-based materials, especially graphene oxide (GO), have been widely employed in the development of physical [11] and chemical [12–14] sensors: in particular, GO possesses a high number of oxidized moieties well exposed to the surrounding environment, suitable to provide a stable anchoring site for the covalent bonding of many (bio)molecules [15,16] and to affect the electrochemical properties of the coating: as an example, alcoholic moieties were reported to activate electrocatalysis towards NADH oxidation [17].

In a previous work, we covalently functionalized GO with a natural cationic polysaccharide, namely chitosan (GO-Ch) [18], to obtain an efficient amperometric sensor for glucose detection. Chitosan and its composites, in fact, have been widely exploited in drug and gene delivery or in tissue engineering thanks to its properties, such as high biocompatibility, biodegradability, hydrophilicity, mechanical strength, good processability, and anti-bacterial properties. Spectroscopic investigations performed on GO-Ch, with respect to the chitosan and GO blend, demonstrated that the covalent modification induced a significant number of amine moieties to be present on the electrode surface to stably anchor a biological recognition element; at the same time, the chemical functionalisation induces a partial reduction of GO, thus ultimately increasing the electrical conductivity of the material [18]. Therefore, GO-Ch can be employed without any preliminary reduction and it is an excellent platform for the development of biosensors, in terms of stability of the biological recognition element.

The preliminary results on GO-Ch-based electrodes rely on oxygen detection, i.e., in a configuration typical of first generation amperometric sensors [19]. Oxygen detection is a consolidated technology but is not viable for the development of wearable sensors working in real matrices, since it requires a stable amount of oxygen dissolved in the analysed solution; this can be certainly achieved in a laboratory with model solutions, but in the case of a human fluid, oxygen content can fluctuate according to physiologic or external factors, changing the sensor response and linearity [20]. Therefore, to use the biosensor directly inside the complex matrix, the involvement of a redox mediator is often the preferred choice. Prussian blue (PB) is still today one of the most used redox active species used to such a purpose [8,21–23], since it induces the electrocatalytic reduction of hydrogen peroxide produced by the enzymatic reaction at potential values close to 0.0 V, whereas direct reduction of hydrogen peroxide without any redox mediator occurs at potential values overlapping oxygen reduction ($E < -0.30$ V). Direct oxidation of hydrogen peroxide is also possible, but it occurs concurrently with the oxidation of many other species present in biological fluids, namely ascorbic and uric acids, proteins, and metabolites. Therefore, the use of PB improves sensitivity and selectivity of the amperometric detection [24,25]. The electrochemical deposition of PB has several advantages over a manual functionalization via drop-casting, e.g., it allows to achieve a stable and reproducible layer, is simpler and less time-consuming [26–28].

In the scientific literature there are already some examples for the simultaneous detection of glucose and lactate in sweat on the same platform [29–32], but in most cases a linear response is achieved over tight concentration range and measurement time. Indeed, the linear range sought for glucose in sweat is between 0.01 and 2.5 mM, while for lactate concentration is wider, from 3.0 up to 50.0 mM [33,34]; moreover, a linear-concentration response should be stable for over thirty minutes, to employ the biosensors in real applications for the continuous monitoring of sweat.

In this work, we propose an enzymatic biosensor based on GO-Ch and electrodeposited PB for the simultaneous detection of glucose and lactate directly in a continuous flow of a complex matrix resembling human sweat. Stable anchoring of the enzyme is achieved thanks to the use of a tailor-made GO-Ch nanomaterial, as demonstrated by strenuous tests in a flow of artificial sweat: the analytical performance of the developed biosensors is defined by Flow Injection Analysis (FIA) to better simulate the real flow of sweat. However, to achieve a truly wearable biosensor without any complicated external device, a driving force to ensure the continuity of sweat transport is required: as a proof of concept for the realization of instrumental working conditions very close to the actual application, the device was integrated in capillary flow system activated by a nitrocellulose strip to achieve the collection and delivery of sweat on the sensor platform.

2. Materials and methods

2.1. Reagents and solutions

D-glucose was purchased anhydrous from Zeus Chemicals; $FeCl_3$ and $K_3[Fe(CN)_6]$ were acquired from Carl Roth; sodium lactate, glutaraldehyde (25 wt%), Nafion (5 wt%) and Glucose Oxidase (GOx) (from Aspergillus Niger, type VII, lyophilized powder, >100 U mg^{-1}) were purchased from Sigma-Aldrich. Lactate Oxidase (LOx) (from microorganism, lyophilized powder, >80 U mg^{-1}) was purchased from Sorachim. GO-Ch was synthesized according to our previous work [18] and 1.0 g L^{-1} homogeneous dispersions of GO-Ch in deionized water were obtained by one-hour sonication in an ultrasonic bath (Bandelin Sonorex, 80 W); the vessel was placed in a bath of water and ice renewed every ten minutes to avoid excessive heating of the solution, which may modify functionalized GO foils in an irreproducible manner; the so-obtained GO-Ch suspensions have a spontaneous pH around 6. Artificial sweat solutions were prepared according to ISO 3160-2:2018 (pH 4.7) using deionized water (18 MΩ cm resistivity) and were stored at +4 °C. All the electrochemical analyses were performed at room temperature and in solutions in equilibrium with the atmosphere, i.e. in the presence of O_2.

2.2. Apparatus and instrumental analyses

Scanning Electron Microscopy (SEM) analysis was performed directly on the modified electrodes with a SEM-FEG Nova NanoSEM 450, Fei Company. All the electrochemical measurements were performed with an Autolab PGSTAT12 potentiostat/galvanostat (Metrohm-Ecochemie) on DRP-110 Screen Printed Electrodes (SPEs) acquired from Metrohm-DropSens: they consisted of a 0.126 cm^2 graphite working electrode, a graphite auxiliary electrode, and an Ag^0 pseudo-reference electrode. Dual SPEs (DRP-x1110, Metrohm-DropSens) consisted of two 0.063 cm^2 elliptical graphite working electrodes, a graphite auxiliary electrode, and an Ag^0 pseudo-reference electrode. Electrochemical measurements on dual electrodes were performed with a μStat 400 portable bi-potentiostat/galvanostat (Metrohm-DropSens). When working in artificial sweat, the potential of the pseudo-reference electrode was fixed due to the presence of chloride ions.

2.3. Biosensor preparation

SPEs were first modified with PB; parameters for the electrodeposition of PB were optimized in previous works [35,36]: 70 μL of a freshly prepared solution containing 2 mM $K_3[Fe(CN)_6]$, 2 mM $FeCl_3$, 10 mM HCl and 0.1 M KCl were placed on a new SPE; a constant cathodic current of -40 μA cm^{-2} was applied for 60 s and 180 s for glucose and lactate detection, respectively.

Glucose biosensors were prepared by drop-casting 5.0 μL of 1.0 g L^{-1} GO-Ch dispersion on the PB modified surface. Stable deposition of the enzyme was achieved [37] by slow evaporation of 5.0 μL of glutaraldehyde solution (1 % in deionized water) on the GO-Ch coating for one hour, to allow cross-linking and covalent bonding to the nanomaterial. Then, 6.7 μL of 10 g L^{-1} GOx dispersion in 0.1 M phosphate buffer at pH 7.00 (PBS), were drop-casted on the electrode surface and left drying at +4 °C.

For lactate biosensors, 10 μL of 20 g L^{-1} LOx dispersion in 1.0 g L^{-1} GO-Ch were drop-casted on the electrode surface and left drying at +4

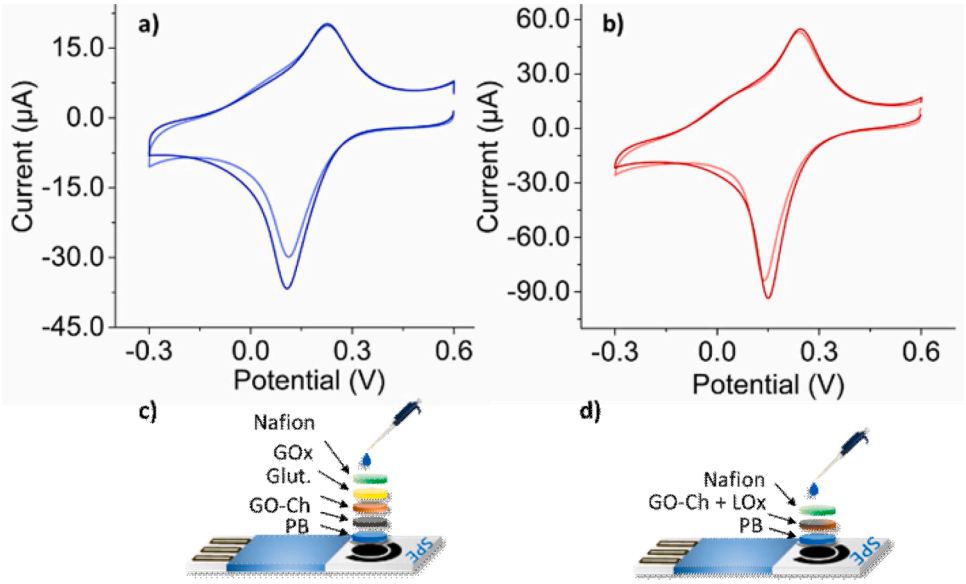

Fig. 1. Typical CV responses in the presence of *(a)* 5.0 mM glucose (blue) and *(b)* 10.0 mM lactate (red) in comparison to the relevant blank signal in artificial sweat (lighter lines). The relevant deposition steps for the two biosensors are reported in *(c)* and *(d)*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

°C.To achieve a more stable anchoring of the coating, 2.5 μL of a 1 % pH-neutralized Nafion solution were drop-casted on the electrode surface of both biosensors. The obtained devices were left drying at +4 °C overnight before the use.

The volume of all the solutions employed in the functionalization of dual SPEs were calculated according to the different geometric area of these electrodes, to achieve similar volume/surface ratio.

2.4. Electrochemical tests

Cyclic voltammetry (CV) responses were carried out by performing four consecutive potential scans between +0.60 V and −0.30 V, at 0.02 V s^{-1} potential scan rate in artificial sweat; the response at the steady state is reported for each CV measurement. The analytical performance of the biosensors was defined by flow injection analysis (FIA), mimicking the flux of sweat on the human skin: in this technique a constant flux of artificial sweat was kept at 1.0 mL min^{-1} flow rate using a Minipuls 3 (Gilson) peristaltic pump and a constant potential of 0.0 V was applied while injecting through a Rheodyne valve 100 μL of either glucose or lactate solutions at concentration levels ranging between 0.03 and 10 mM and between 0.1 and 50 mM, respectively. Solutions at different analyte concentration levels were analysed randomly to highlight the possible occurrence of memory effects. Each solution was injected at least three times on the same SPE to test the sensor repeatability at different concentration levels. This procedure was repeated at least on three SPEs obtained in the same conditions to test the sensor reproducibility. Both repeatability and reproducibility were expressed in terms of Relative Standard Deviation (RSD%). Limit of detection (LOD) and limit of quantification (LOQ) were calculated from the signal in the absence of analyte, as three and ten times the standard deviation of the noise, respectively. Limit of linearity (LOL) was calculated from the trend of the current responses obtained for the two analytes.

2.5. Capillary flow tests

Capillary flow tests were performed both on SPEs and on dual electrodes, by mimicking a working device in direct contact with a human body fluid. The device built exploited the capillary flow activated by a nitrocellulose strip generally used for lateral flow (70CNPH, MDI), to renew the solution in contact with the biosensor. The strip was alternatively immersed in a reservoir only containing the blank solution, i.e. artificial sweat, and the same solution also containing the analytes at different concentration levels. Capillarity of the paper allowed the analysed solutions to reach the electrode surface and to achieve a continuous flow of solution on the SPE thanks to an adsorbing pad placed at the end of the strip. In feasible real applications, the reservoir is not present, but the paper strip is directly in contact with the skin which will act as a continuous, even if variable, natural source of sweat.

3. Results and discussion

3.1. Electrochemical behaviour of PB-based biosensors

In our previous work we demonstrated that the formation of covalent bonds significantly improves the stability of enzyme anchoring and the analytical performance for glucose detection [18]. As already stated in the Introduction section, the performance of the GO-Ch modified electrodes was tested by monitoring the amount of oxygen consumed by the enzymatic reaction in an ideal solution, namely PBS; this detection method allowed us to directly determine the performance of the material in comparison to unfunctionalized GO and a GO and Ch blend, but it is not suitable for real applications of the device. Aiming at developing the sensor towards working conditions closer to an actual application in contact with the skin, we introduced a redox mediator, namely PB, and we tested the device in a synthetic matrix mimicking the human sweat. PB allows the reduction of H_2O_2 produced by the enzymatic reaction at a low potential value, thus increasing the selectivity of the device. PB was deposited using an electrochemical approach: a constant current density was applied for 60 s and 180 s in the case of glucose and lactate biosensors, respectively, in order to make the devices sensitive to these analytes in a different linearity range [8] (see hereafter). The use of a galvanostatic deposition, which at difference from the potentiostatic technique implies the deposition of the film at a constant rate, resulted in a uniform coating on the electrode surface, as observed from SEM images at low magnification collected in different positions of the electrode surface (Fig. S1). The morphology of carbon-based SPEs was minimally affected by the deposition of PB, with no particular feature observed for 60 s deposition time (Fig. S2b), while cubic crystalline aggregates could be observed for longer (180 s) deposition times (Fig. S2c) [27,38]. Electrochemical responses obtained on PB-modified

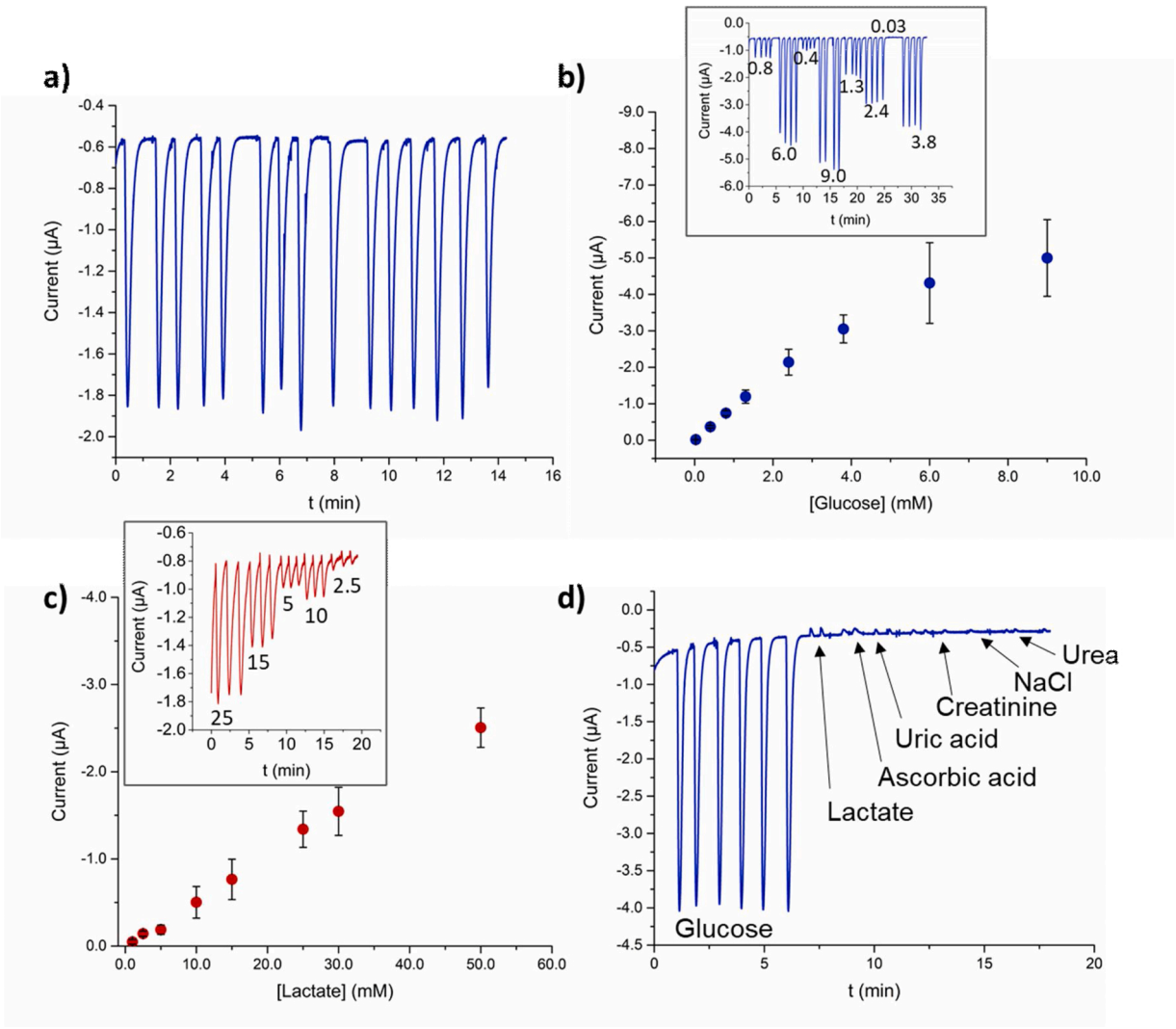

Fig. 2. *(a)* Amperometric response registered at 0.0 V in FIA for fifteen consecutive injections of 1.3 mM glucose solution. Current values obtained for *(b)* glucose and *(c)* lactate detection; the insets report the relevant amperometric signals recorded for four consecutive injections of glucose solutions (0.03–9.0 mM) and three consecutive injections of lactate solutions (2.5–25 mM), respectively. (d) Selectivity tests on the glucose biosensor; injected interferents are reported in the picture.

electrodes (Fig. S3), both obtained for 60 and 180 s, demonstrated that the deposit is highly stable and reproducible, since well overlapping CV traces are collected for consecutive cycles and for a great number of SPEs tested. The two deposition times employed for glucose and lactate sensors allow the PB layers to have a different thickness, assuring the biosensors the required linear ranges (see hereafter). However, the formation of a thicker layer does not affect the resistance to charge transfer, as confirmed by the relevant values of ΔE, which are not significantly different each other (Table S1). All these tests were performed in artificial sweat, since the PB coating is stable at pH values below 6 [26]. A comparison with biosensors obtained by chemical synthesis of PB is provided in Section S2 of ESI, to highlight the better performance of devices obtained following an electrochemical approach.

The deposition of GO-Ch and enzyme coatings on the electrode completely changes the morphological structure of the surface (Section S3 of ESI), however still resulting in a well homogenous structure that evidences a complete coverage of the underlying PB deposit. SEM images on the glucose biosensor (Fig. S5a) evidence a very flat surface, quite similar to different GO-based coatings [17]. A more corrugated structure is observed in the case of lactate biosensor (Fig. S5b): GO-Ch and LOx were mixed before the deposition on the electrode surface since the amount of enzyme required in this case is higher.

To demonstrate the effectiveness of the devices obtained in the detection of the two analytes, exploratory CV responses in absence and in presence of either glucose or lactate are reported in Fig. 1, together with a schematic representation of the sensor assembly steps in the two cases. As observed, the reduction peak of PB centred at +0.13 V increases in intensity in the presence of the analyte due to the electrochemical reduction of H_2O_2 produced by GOx and LOx, mediated by the Fe(III) complex. As expected, the sensitivity acquired by voltammetric analysis is quite low.

3.2. *Definition of the electrochemical performance of the biosensors in FIA*

The analytical performance of glucose and lactate biosensors were defined by FIA to simulate the flux of sweat on the skin in well-defined experimental conditions. Repeatability of the response, mainly ascribable to the stability of the enzyme anchoring under continuous flow of the solution, was first tested by making consecutive injections of 1.3 mM glucose on the same biosensor (Fig. 2a); on the basis of this test, we calculated a RSD of 5.9 %, which confirmed the high stability of the enzyme anchoring on the GO-Ch film already stated in our previous work [18]. The analysis was then repeated by testing solutions of glucose at different concentration (from 0.03 mM to 9.0 mM) in a

Table 1
Analytical performance of the biosensors when employing FIA and a capillary flow device for the detection of glucose and lactate.

	FIA		Capillary flow device	
	Glucose	*Lactate*	*Glucose*	*Lactate*
Sensitivity (μA mM^{-1} cm^{-2})	8.20	0.39	0.93	0.57
LOD (nM)	6.7	28	9.6	20
LOQ (nM)	22	93	32	68
LOL (mM)	3.8	50.0	3.5	50.0
RSD_{slope} (%)	8.5	9.0	7.6	8.6
Tested time (min)	35	25	140	100
Electrodes tested	5	4	3	4

randomized manner (Fig. 2b) to rule out possible memory effects. Similar tests were repeated for the detection of lactate, using a second biosensor, containing the enzyme LOx. As already stated in the Introduction section, an optimal lactate biosensor should work in a wider linear range, achieved by the deposition of a higher amount of PB. At difference with the previous biosensor described, LOx was mixed with GO-Ch due to the low stability of the higher amount of enzyme required in this case. GO-Ch, thanks to its mild acidic spontaneous pH and to the biocompatible nature of chitosan ramifications [39], allows a good stability of LOx by the simple mix of the two substances. Results obtained for lactate detection in FIA, once more randomizing the analysis of solutions at different concentration, are reported in Fig. 2c.

Amperometric responses obtained at the two single biosensors (Fig. 2b and c) highlight the high repeatability of the signal obtained at each glucose and lactate solution tested: calculated RSD always resulted below 10 %, a value which is well acceptable for commercial SPEs. In addition, the baseline is stable for a remarkable amount of time (over 30 min) indicating that the biosensors are potentially suitable for the continuous monitoring of these biomarkers in sweat. This confirms the excellent stability of enzyme anchoring, guaranteed by GO-Ch, and the lack of any memory effect due to the analysis of solutions at higher concentration.

Figures of merits for glucose and lactate detection, extracted from the amperometric responses recorded from various devices obtained in similar conditions, are summarized in Table 1 (left columns).

Both biosensors showed good sensitivity, as well as LOD and LOL values required for the analysis of glucose and lactate in sweat. In addition, the satisfactory reproducibility of the sensor response, quantified as the RSD of the calibration slope (RSD_{slope}), is the necessary condition to assure the accurate detection of these biomarkers in sweat by disposable biosensors.

The performance of the devices proposed in this work were compared to similar biosensors for glucose and lactate reported by scientific literature, tested using FIA and other amperometric techniques (Table 2). As observed, the sensitivity of the biosensors obtained in this

Table 2
Comparison of analytical performance for glucose and lactate detection obtained in other scientific works through amperometric techniques.

System	Detection technique	Potential studied (V)	Solvent	Sensitivity (μA mM^{-1} cm^{-2})	Linear range (mM)	Ref.
Glucose detection:						
SPE/PB/GO-Ch/GOx	FIA	0.0 V	Artificial sweat (pH 4.7)	8.20	0.02–3.8	This work
Pt/GOx immobilized on polymer/silica gel support	FIA	+0.60	PBS (pH 5.8)	4.52	0.01–20	[41]
SPE/Graphene/CtCDH/GOx	FIA	+0.15	PBS (pH 7.4)	0.51	0.5–5	[31]
GC/ GOx + poly(m-PD/Res)	FIA	+0.60	PBS (pH 7.3)	2.97	0.1–50	[42]
SPE/GO-Ch/GOx	FIA	−0.40	PBS (pH 7.0)	7.61	0.5–3.5	[18]
Au/CNT/b2LOxS/PEI-PEGDGE/CA	Chronoamp.	+0.15	Artificial sweat (pH 5.4)	0.41	0.5–20.0	[43]
GC/rGO-AuNPs/PB/GOx-chitosan	Chronoamp.	+0.10	PBS (pH 7.4)	84.0	0.013–2.0	[44]
Porous carbon/PB/GOx-chitosan	CV	−0.05	PBS (pH 6.5)	219	0.03–0.4	[45]
GC/nanoCh, PB, graphene /AuNPs/ Concanavalin A/GOx	Chronoamp.	−0.20	PBS (pH 6.0)	58.7	0.003–3.2	[46]
GC/nanoCh + graphene /GOx	CV	−0.50	PBS (pH 7.4)	37.9	0.08–12.0	[47]
ITO/sPEEK-functionalized graphene/AuNPs/ chitosan	CV	+0.10	PBS (pH 7.0)	6.51	0.5–22.2	[48]
GC/rGO/Chitosan-GOx	Chronoamp.	−0.45	PBS (pH 7.3)	6.82	0.02–3.2	[49]
GC/ferrocene-branched sol-gel/chitosan/GO/ GOx	Chronoamp.	+0.40	PBS (pH 7.0)	19.5	0.02–5.39	[50]
GC/GO/chitosan/ZrO_2/GOx	Chronoamp.	−0.40	PBS (pH 7.4)	7.6	0.2–1.6	[51]
GC/GO/PB/GOx/Chitosan	Chronoamp.	+0.10	PBS (pH 6.0)	15.3	0.1–13.5	[52]
Lactate detection:						
SPE/PB/LOx + GO-Ch	FIA	0.0	Artificial sweat (pH 4.7)	0.39	1.0–50.0	This work
SPE/Graphene/PB/PVA-SbQ-LOx	FIA	−0.10	PBS (pH 7.4)	1.64	0.25–5.0	[31]
Au/CNT/b2LOxS/PEI-PEGDGE/CA	Chronoamp.	+0.15	Artificial sweat (pH 5.4)	0.56	0.1–5.0	[43]
Chitosan/PVI-Os/CNT/LOx	Chronoamp.	+0.30	PBS (pH 7.0)	19.7	0.005–0.8	[53]
Laponite/chitosan hydrogel/LOx	Chronoamp.	+0.40	PBS (pH 7.0)	326	0.01–0.7	[54]
Printed AgNP/BSA-LOx	Chronoamp.	+0.65	PBS (pH 7.0)	0.26	1.0–20.0	[55]
G-paper/MoS_2/Cu/LOx	Chronoamp.	−0.25	PBS (pH 7.4)	83.0	0.01–18.4	[56]
SPE/rGO-3,4DHS /LOx	Chronoamp.	+0.10	PBS (pH 7.0)	0.59	0.01–0.80	[57]
SPE/rGO/K_3[Fe(CN)$_6$]/LOx	Chronoamp.	+0.15	PBS (pH 7.4)	42.4	0.5–15.0	[58]
GC/CNT/LOx/Nafion	Chronoamp.	+0.21	PBS (pH 7.0)	40.0	0.01–0.33	[59]
N-graphene/$Ti_3C_2T_x$/PB/LOx	Chronoamp.	−0.10	Artificial sweat (pH 6.2)	21.6	0–20.0	[60]
Pt/rGO/CNT/AuNP/LOx	Chronoamp.	+0.20	PBS (pH 7.5)	35.3	0.05–100	[61]

Acronyms: CtCDH: cellobiose dehydrogenase from the ascomycete Corynascus thermophilus; poly(m-PD/Res): poly(1,3-phenylenediamine/resorcinol); GC: glassy carbon; NPs: nanoparticles; nanoCh: nanocomposite of chitosan; ITO: indium tin oxide; sPEEK: sulfunated PEEK; PVA-SbQ: poly(vinyl alcohol) bearing styrylpyridinium groups; PVI: polyvinilimidazole; CNT: carbon nanotubes; b2LOxS: DET-type engineered LOx; PEGDGE: poly(ethylene glycol) diglycidyl ether; CA: cellulose acetate; BSA: bovine serum albumine; G-paper: graphene paper; 3,4DHS: *N*,*N*'-Bis(3,4-dihydroxybenzylidene); N-graphene: *N*-doped graphene.

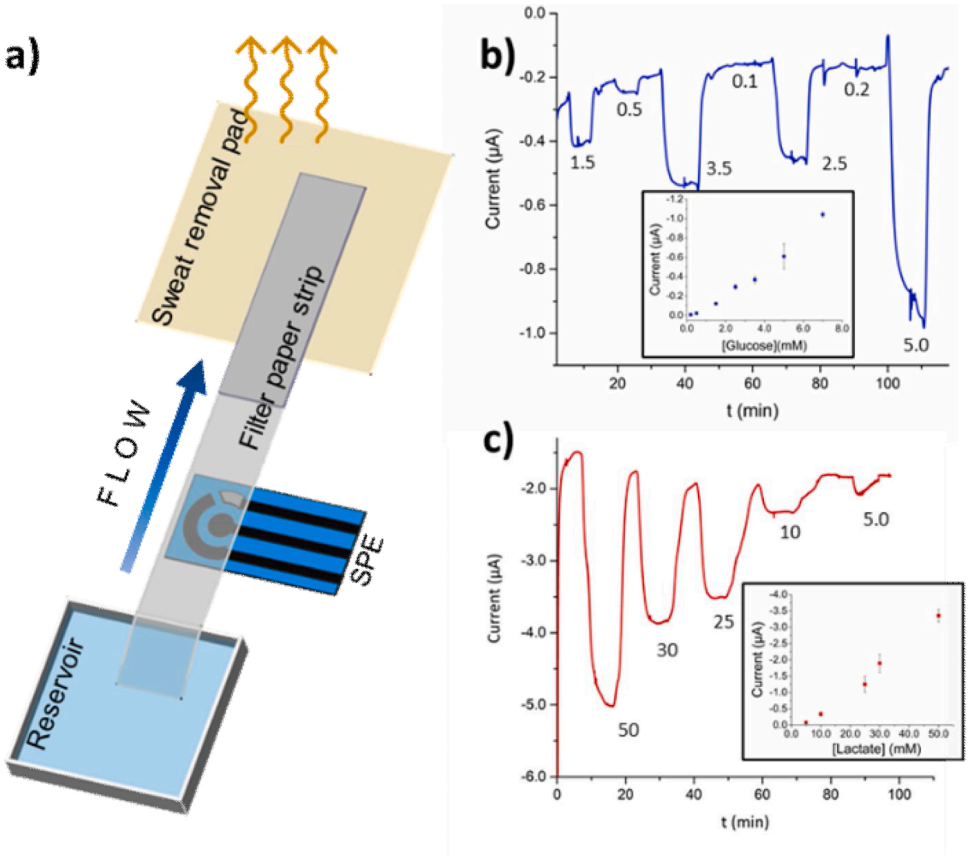

Fig. 3. *(a)* Setup employed to obtain a capillary flow of artificial sweat on each of the two biosensors, and relevant amperometric response obtained in absence and in presence of *(b)* glucose and *(c)* lactate at different concentration levels (in mM). The insets of *(b)* and *(c)* report the relevant calibration plots together with standard deviation calculated for three and four different biosensors, respectively.

work is higher than similar reported systems for glucose detection through FIA, but slightly worse than those developed for lactate detection. However, it is difficult to compare the biosensors reported in the literature with those described in this work, as the sensing strategies adopted are quite different from each other: several biosensors show a much greater sensitivity than ours, thanks to the use of metal nanoparticles or stabilizers. However, they were employed at a much higher potential value, where several species present in biological fluids can be oxidized. In addition, the analytical performance of many devices reported in the literature was measured in ideal conditions, i.e. in PBS close to neutral pH, and not using the severe working conditions employed in this work: when working at acidic pH values, the enzymatic activity decreases [40], thus decreasing the sensitivity of the biosensor. The great majority of lactate biosensors show a very high sensitivity, but the linear range is very tight and not suited for applications in sweat, which requires a wide detection range up to 30 or even 50 mM. In view of all these considerations, we consider the coatings here developed well suitable for the use in wearable sensors for glucose and lactate from human sweat.

3.3. Interference studies

We studied the selectivity of the biosensors described in the previous section by considering a wide range of possible interferents, at concentration values even higher than those generally found in human sweat [33]. In particular, typical interferents in sweat include ascorbic acid (tested at 0.5 mM) and uric acid (tested at 10 mM), as they are species which can be easily oxidized. Creatinine (tested at 2 mM) and urea (tested at 50 mM), which are the waste products of muscle and protein metabolism, respectively, were also tested, as their concentration in sweat is not negligible. Then, NaCl (tested at 20 mM) was also tested, since it is the main ionic constituent of human perspiration and it may affect the signal baseline; this species was also used to test the influence of other electrolytes normally present in sweat, e.g. K^+ and Ca^{2+}, in the electrochemical response. Finally, cross-contamination was checked by detecting glucose at high concentration with a lactate biosensor and vice versa, 3 mM and 50 mM respectively. Selectivity tests were performed in FIA at 0.0 V on both glucose (Fig. 2d) and lactate biosensors. The selected interfering species cannot be detected by the biosensors: both ascorbic and uric acids show a negligible oxidation response which does not significantly affect the electrochemical response, whereas all the other species only induce minor deviations of the baseline, which results undisrupted.

3.4. Results on capillary flow

To better simulate the real working conditions of a wearable device in contact with the skin, the obtained biosensors were tested employing a simple but challenging method, briefly schematized in Fig. 3a. The results obtained for glucose and lactate detection with the two biosensors are shown in Fig. 3b and c, respectively.

Repeatability of the baseline, corresponding to the signal obtained in the absence of analyte, is high (RSD% = 7.0 % and 9.8 % for glucose and lactate detection, respectively) for both biosensors, despite the low pH value of artificial sweat. The analytical performance obtained for glucose and lactate detection employing this setup is reported in Table 1 (columns on the right).

As expected, the sensitivity for glucose detection employing the capillary flow device is lower than that obtained using FIA, due to the lower flow rate wetting the electrode. Surprisingly, for lactate detection

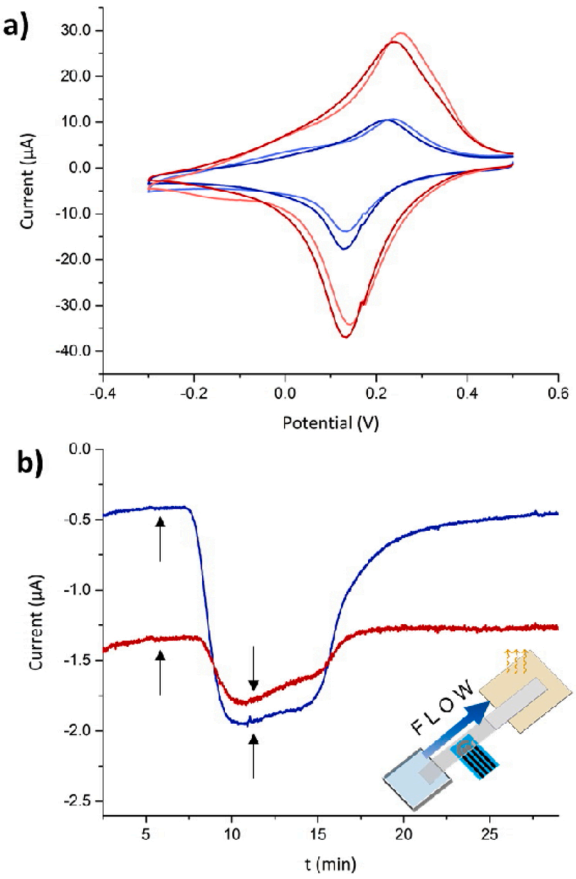

Fig. 4. *(a)* CV responses obtained simultaneously on modified dual SPEs in artificial sweat in absence and in presence of 2.5 mM glucose and 20 mM lactate; blue and red lines refer to the signals obtained at glucose and lactate biosensor, respectively, whereas the lighter lines are the relevant blank signals; *(b)* amperometric responses obtained at 0.0 V under a capillary flow of artificial sweat in absence and in presence of 1.5 mM glucose and 50 mM lactate (arrows indicate the change of the solution in the reservoir); blue and red lines refer to the signals obtained at glucose and lactate biosensor, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the sensitivity obtained in capillary flow analysis is higher than the one reported using FIA detection. This can be ascribed to the slower kinetics of this enzymatic reaction, which requires longer times to produce a high amount of H_2O_2. A numeric evidence of this statement could not be provided: in scientific literature, kinetics parameters for enzymatic reactions are generally given for enzymes in solution and their immobilization on a substrate strongly affects them [62]. Retention of the analytes on the electrode surface, due to the possible low permeability on the paper strip, can be excluded, as in the two biosensors a plateau is reached both in the presence and in absence of the analyte after a few minutes and the baseline is quickly restored in the absence of analyte.

Even though the device employed is simple, the response obtained for both biosensors is robust: linearity is maintained over a long period of time (*ca.* 2 h) on randomly analysed solutions. These results demonstrate the possibility of employing a GO-Ch-based biosensor for glucose and lactate detection on a device directly in contact with the skin, which exploits a capillary flow of real matrices to monitor a training athlete with a continuous sampling. The considerable time span required for the measures is within the average sport training time of a human user.

3.5. Simultaneous detection on the same device

The electrodes with the two enzymatic functionalization described in previous sections were deposited directly on the same device (dual SPEs), to highlight the possibility of the simultaneous in-line detection of both lactate and glucose.

CV responses in Fig. 4a demonstrate the different functionalization of the two working electrodes present in the same device, with PB coatings at different thickness, as well as the efficiency of the device in detecting both glucose and lactate in sweat; as usual, the sensitivity induced by CV detection is very low. To test the effectiveness of the device in the simultaneous analysis of the two analytes on a capillary flow of artificial sweat, the dual electrode system was included in a flux system similar to that previously described for the two singular electrodes and shown in the inset of Fig. 4b. The relevant amperometric responses simultaneously collected for the detection of glucose and lactate demonstrated that both baselines are stable for an extended interval time (over 30 min), before and after the addition of analyte to the matrix: RSD of the signal, calculated by sampling the current values registered in absence and in presence of analyte for several minutes, was in any case well below 5 %. Moreover, over 90 % of the starting signal in absence of the analyte can be restored after the detection of lactate and glucose, confirming that the paper-based fluidic system induces an effective flux of artificial sweat, avoiding retention of analytes. This is a demonstration of the possibility of employing the developed biosensors in direct contact with skin, to achieve a continuous in-line monitoring of sweat.

4. Conclusions

In summary, we demonstrated the efficiency of two biosensors based on GO-Ch and PB for the continuous monitoring of two health-related biomarkers, namely glucose and lactate, in sweat. The employed functionalization methods allow a stable anchoring of the biomolecules on GO-Ch, that is the required condition for the use of these devices in this specific application. The obtained biosensors are characterized by good sensitivity within the linear ranges suited for these analytes in a real matrix and the proper reproducibility necessary for disposable sensing systems. We also demonstrated the possibility of employing a GO-Ch-based biosensor for glucose and lactate detection on a device directly in contact with the skin, exploiting a capillary flow on paper.

CRediT authorship contribution statement

Fabrizio Poletti: Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. **Barbara Zanfrognini:** Formal analysis, Investigation, Methodology, Visualization, Writing - review & editing. **Laura Favaretto:** Investigation, Resources. **Vanesa Quintano:** Validation, Writing - review & editing. **Jinhua Sun:** Validation, Writing - review & editing. **Emanuele Treossi:** Project administration, Resources. **Manuela Melucci:** Supervision. **Vincenzo Palermo:** Conceptualization, Funding acquisition, Writing - review & editing. **Chiara Zanardi:** Conceptualization, Data curation, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vincenzo Palermo reports financial support was provided by European Union. Vincenzo Palermo reports financial support was provided by Swedish Research Council. Vincenzo Palermo reports a relationship with European Union that includes: funding grants.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2021.130253.

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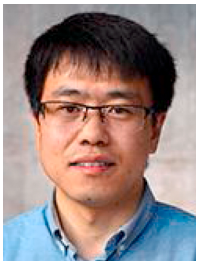

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