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Dual Biosensor for Simultaneous Monitoring of Lactate and Glucose Based on Thin-layer Flow Cell Screen-printed Electrode

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Abstract: A simple and robust monitoring system for lactate and glucose detection is presented. The system is based on a suitable integrated screen-printed electrode in thin layer flow cell design (TLFCL) to perform flow injection analysis. The TLFCL is consisted of a two working and one counter electrodes made of carbon and a silver pseudoreference electrode. Both working electrodes (WE) are first modified by graphene nanomaterials. Glucose sensor based on cellobiose dehydrogenase from the ascomycete *Corynascus thermophilus* (*Ct*CDH) and lactate sensor based on lactate oxidase (LOx) are made on the first and second WE respectively. Both enzymes are entrapped in a photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinium groups (PVA-SbQ). The electrocatalytic signal of both sensors did not show any cross-talking, whereas the linear quantification ranges of both glucose and lactate sensors are large enough for wine monitoring.

Keywords: Screen-Printed electrode · Thin layer flow cell design · Amperometric flow-injection measurement · Simultaneous monitoring · *Ct*CDH · LOx

1 Introduction

Screen-printed electrodes (SPE) have been widely used and customized for electro-chemical application in analytical chemistry. Main advantages of SPE are related to the small device comparing to the classical electrochemical cell which allow the miniaturisation of device and make it easily portable with opportunity of customisation and producing in large scale according to the application with low cost manufacturing [1–5]. In addition, disposable and ready to use SPE are a good alternative to solve polishing and pre-treating of conventional electrodes surface as well as laborious maintenance activities for the reference electrode. Taking into account SPE advantages and the need to establish fast analysis with automation of the measurements, several electrochemical enzymatic sensors coupled to flow injection analysis (FIA) have been reported [6–9]. The main importance of FIA was related to a continuous flow carrier stream over the electrode surface which plays a key role for cleaning purposes avoiding memory effect between consecutive injections. FIA cells for SPE are usually wall-jet type which can be aggressive for the biological recognition element immobilized on the surface of the electrode and then limit the operational stability of the system. In order to simplify and decrease this side effect, screen-printed electrode integrated in one channel flow-cell (TLFCL, Thin layer flow-cell) was developed. In the new flow-cell the injection is done through an "in-line luer injection port" where the sample volume can be easily controlled by the operator through a Hamilton syringe. The one channel flow-cell is attached and directly changeable with electrode. The channel slide is transparent which allows to monitor the flow of solution into the electrode and controlling the presence of air bubbles in the device (Figure 1). In this study, new TLFCL electrode containing two graphene modified working electrodes was developed. Based on this transducer, an amperometric flow injection dual-biosensor, based on *Ct*CDH and LOx immobilized in PVA-SbQ, for simultaneous monitoring of glucose and lactate in wine was studied. As it is very well known, the quantification of glucose and lactate is of great importance in wine control, especially to determine quality, flavour, fermentation and stability of wine [10,11]. Amperometric flow-injection measurement for ■separately (separate)■ detection of lactate and glucose in wine were developed using graphite-teflon electrode with convenient enzyme and ferrocene mediator [12,13]. The combination of both sensors in flow system using glucose oxidase and lactate oxidase for glucose and lactate detection respectively can produce a cross talking effect due to the diffusion of hy-

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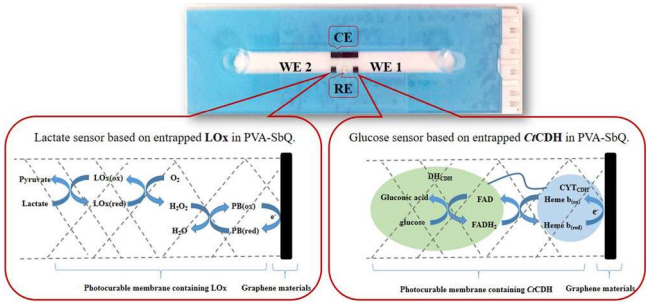

Fig. 1. Dual biosensor for lactate and glucose using modified TLFCL1110 with 2 enzymatic sensors: WE1: glucose sensor based on *Ct*CDH with DET process, WE2: lactate sensor based on LOx with mediated electroanalytical process using PB layer for hydrogen peroxide detection. The electrode area = 2 mm^2. (Abreviation: CE: counter electrode/RE: pseudoreference electrode. LOx: lacate oxidase/PB: Prussian blue/DH_{CDH}: Dehydrogenase domain containing FAD cofactor/CYT_{CDH}: Cytochrome domain containing Heme *b* cofactor).

drogen peroxide between two working electrodes [14]. ■There for (Therefore?)■ the aim of this work was to develop a dual biosensor for simultaneous monitoring of glucose and lactate based on two different systems, *Ct*CDH with direct electron transfer (DET) and LOx with Prussian Blue (PB) mediator, in order to decrease cross-talking possibility and high working and storage stability.

2 Experimental

2.1 Reagents

Recombinant cellobiose dehydrogenase from *Corynascus thermophilus* (*Ct*CDH, volumetric activity with cytochrome *c* at pH 7.5 = 33.5 U ml^{-1}, protein concentration = 25 mg ml^{-1}) was used for electrode modification. *Ct*CDH was recombinantly expressed in *Pichia pastoris*. The production and purification of the enzyme are described elsewhere [15]. The homogeneous preparation of the enzyme was stored in 50 mM acetate buffer pH 5.5 at −80°. Lactate oxidase from Pediococcus sp. (L0638), sodium lactate, glucose, potassium hexacyanoferrate(III), and all salts for making phosphate-buffered saline (PBS) used in this work were purchased from Sigma-Aldrich (Madrid, Spain). Poly(vinyl alcohol) bearing styrylpyridinium groups (PVA-SbQ) was provided by Polyscience (Warrington, USA). All solutions were prepared using Milli-Q water. The graphene solution for electrodes modification (DRP-GPHSOL) was made by DropSens.

2.2 Materials and Instrumentations

All experiments were performed with thin layer flow cell screen printed electrodes (DRP-TLFCL1110, denoted TLFCL1110) fabricated by Dropsens. This electrode consists of two working and a counter electrode made of carbon and a silver pseudoreference electrode printed on the ceramic substrate. A transparent slide is mounted over the screen-printed platform delimiting a flow channel with a height of 0.4 mm (max volume 100 uL). The general dimensions of this device are 80.5x 25.4 × 1 mm and each working electrode has an area of 2 mm^2 (Figure 1).

Figure 2 shows the FIA system ■using (used)■ in this work. The TLFCl1110 electrode was located into a holder (DRR-TLFCL-HOLDER) and connected to a potentiostat/galvanostat μStat400, interfaced to an Intel(R) Core (TM)2 Duo CPU computer system and controlled by DropView 8400 2.1 software, using a connector fabricated

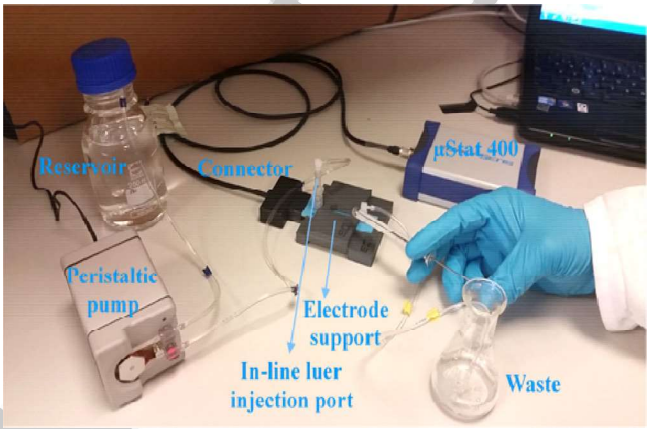

Fig. 2. Miniaturized FIA system with TLFCL1110 screen-printed electrode.

by Dropsens (DRP-CAC-TLFCL). A peristaltic pump INSTECH (Model P720, USA) is connected to the electrode using flow fittings (DRP-TLFCL-FLOWFITTING) ■which allows the change in the easy way the electrode■. The injection is carried out using a Hamilton syringe through an in-line luer injection port (DRP-TLFCL-INLINEPORT) connected to the one of the hole of the TLFCL cover.

2.3 Electrode Modification

The working electrodes were previously modified with 2 μl of graphene solution to improve the electron transfer process between immobilized enzymes and electrode surface (graphene modified TLFCL1110 was denoted TLFCL1110GPH). ■After (After that?)■ electrochemical modification of second graphene electrode with potassium hexacyanoferrate (III) was carried out. For this, an electrodeposit layer of PB onto the surface graphene electrode was carried out using Turner method [16]. The working electrode was cycled 15 times between 0.0 and +2.5 V at a scan rate of 0.2 V/s in a solution of 0.1 M potassium hexacyanoferrate (III) dissolved in Milli-Q water. The electrolyte was not stirred during the modification procedure, so a blue/black precipitate appeared to form around the working electrode whilst the bulk solution re-

mained yellow. The modified electrodes were washed in water until the initial pale blue colour was no longer observed. The electrodes were stored dry at room temperature until their use.

*Ct*CDH and LOx immobilization: the immobilization of enzymes in the photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinium groups (PVA-SbQ) was carried out following the method developed by Noguer *et al.* [17]. PVA-SbQ was diluted in Milli-Q water (20% w/v) and mixed with enzymatic solution (*Ct*CDH or LOx) in a ratio 50:50% (v/v). The mixture was vortex-mixed and briefly centrifuged to eliminate the foam. A volume of 5 µL of PVA-*Ct*CDH and PVA-LOx was then spread by drop-casting onto the first and the second working electrode of TLFCL1110GPH, respectively. The electrodes were exposed to neon light (15 W) for 4 h at 4° to allow the photo-polymerisation between styrylpyridinium groups. The amount of *Ct*CDH and LOx finally entrapped in the sensing layer was calculated to be 54 mU and 250 mU per electrode, respectively.

2.4 Electrochemical Measurements

To obtain the analytical signal, an aliquot of 60 µL of glucose and/or lactate solution was injected through the in-line luer injection port using Hamilton syringe. The chronoamperogram was recorded applying a detection potential of +0.15 V and −0.1 V (vs. an Ag pseudoreference electrode) for glucose and lactate sensors respectively. A 0.01 M PBS buffer solution (pH 7.4) was employed as flow carrier at a flow-rate of 1 ml min^{-1}.

In case of wine test, the sample was first diluted 80 times in order to be into the calibration ranges of glucose and lactate. A 60 µL of diluted wine was then injected and the concentration of glucose and lactate were calculated by correlation with the corresponding calibration curve.

3 Results and Discussion

3.1 Principle of Glucose and Lactate Sensors

Figure 1 shows the mechanistic of each enzyme with his appropriate analyte. First, both working electrodes of TLFCL1110 were modified with graphene nanomaterials to obtain TLFCL1110GPH. Recently we have proved that graphene increases the electroactive area and electronic communication between enzymes and surface electrode (results under review).

CDH consists of a dehydrogenase domain (DH_{CDH}) containing flavin adenine dinucleotide (FAD) associated cofactors, and a cytochrome domain (CYT_{CDH}) with heam *b* cofactors. The surface-exposed haem *b* of the CYT_{CDH} makes it possible to deliver the electrons from the DH_{CDH} to the CYT_{CDH} and from the CYT_{CDH} to a polarized electrode surface via interdomain electron transfer (IET) and subsequently DET [18,19]. The mechanism involves oxidation of the glucose in a 2 e^-, 2H^+ reaction at the DH_{CDH} via formation of the gluconic acid and the fully reduced FAD. Then, the DH_{CDH} sequentially delivers the electrons to the CYT_{CDH} via IET, followed by shuttling the electrons to the electrode (DET) acting as an electron acceptor, and the response was registered. Cyclic voltammetry to demonstrate the DET mechanism was provided (Figure S2) and the results were adequate respect to previous study by Gorton *et al.* [20,21].

For *Ct*CDH on the WE1 the electrocatalytic oxidation of glucose is done under an adequate applied potential (+0.15 V). For LOx on the WE2 the electrocatalytic oxidation of lactate produces hydrogen peroxide which is systematically reduced to water with oxidation of PB. $PB_{(ox)}$ is then reduced on surface electrode acting as an electron donor (−0.1 V), and the response is registered. Both enzymes have been entrapped in photocrosslinkable PVA-SbQ 10%. The effect of the PVA concentration on the current response was previously studied and we have proved that a high concentration of the polymer increases the strength of enzyme entrapment, but at the same time decreases the access of substrate to the enzyme and decreases the current density due to a restriction of domain movement which is responsible for electron transfer under these conditions (results not show).

For the lactate sensor, an electrodeposit layer of PB onto the surface graphene electrode was perfomed using Turner method [16]. A typical cyclic voltamogramm of this modified electrode shows one redox couple with stable behaviour and formal redox potential of $E^{\circ\prime} = +0.086$ V (Figure 3). It is observed that a potential of −0.1 V is enough to achieve the total reduction of the PB_{ox} generated during the enzymatic reaction. The sensitivity of PB layer to hydrogen peroxide in FIA system reveales a low limit of quantification of 50 µM (Figure S1).

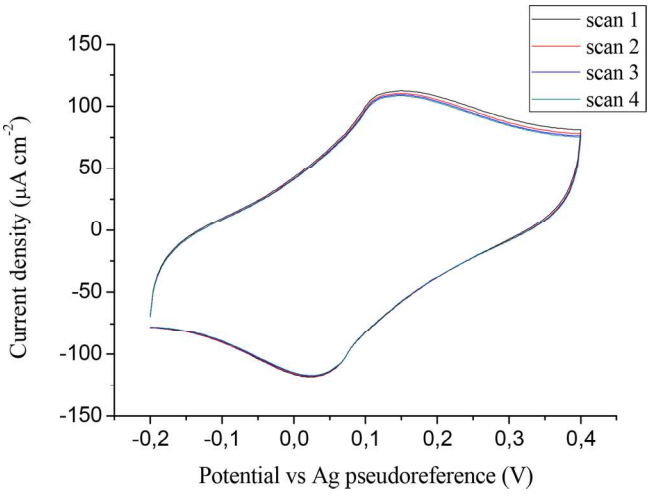

Fig. 3. Cyclic voltamogramms TLFCL1110GPH-Prussian Blue modified electrode measurement in 0.1 M KCl, scan rate 0.05 V/s.

3.2 Cross-talking Behaviour and Sensors Calibration

The enzymatic oxidation of the lactate with LOx produces hydrogen peroxide within the surface of the sensors. Whereas the oxidation of glucose with CDH is not dependent of oxygen reduction and the electrons were directly transferred to surface electrode acting as electron acceptor. As two enzymes have two different electrocatalytic mechanisms a cross-talking is not expected. The cross-talking behaviour of the system was investigated. The solutions were injected directly through the in-line luer port with a Hamilton syringe. The flow rate was adjusted to 1 mL min^{-1}. In a first experiment the response of the glucose sensor to lactate concentration changes was examined. A solution with the highest lactate concentrations (5 mM) but without glucose were injected (Figure 4A). Similarly, in a second experiment the cross-talking response of the lactate sensor on highest glucose concentration (5 mM) was investigated (Figure 4B). The flow was directed from the glucose sensor to the lactate sensor. In both cases the cross-talking between glucose and lactate sensors was not found. Then the simultaneous monitoring for both analytes was perfomred without interference of electrocatalytic response of each enzyme (Figure 4C). The calibration curves for lactate and glucose were obtained simultaneously with the flow-through TLFCL1110GPH sensor chip. The measurements were performed with calibration solutions containing different amounts of lactate and glucose. Table 1 shows the main analytical characteristics of the dual biosensor for the two analytes. The linear measurement ranges of both glucose and lactate sensors can be large enough for practical application (Table 1). The sensitivities were 0.51 μA mM^{-1} cm^{-2} for glucose and −1.6 μA mM^{-1} cm^{-2} for lactate (Figure 5). The developed dual biosensors exhibit a good storage stability without decrease in analytical response after 3 months storage at 4 °C.

Finally, the developed dual sensor was tested for analyzing a Sherry wine (Canasta) which is a fortified wine produced from white grapes that are grown near the town of Jerez de la Frontera in Andalucia, Spain. The diluted sample was then spiked with known concentration of glucose and lactate and tested under the same procedure as described before. The concentration of glucose and lactate were calculated by correlation with the corresponding calibration curve. The comparison of the results obtained using spiked and nonspiked wine demonstrated the absence of matrix effect in glucose and lactate determination (Table 2). The values of recovery tests were acceptable and in good agreement with data from HPLC

Table 1. Analytical characteristics of the dual biosensor.

Analyte	Calibration plot	Linear range (mM)	R^2	% RSD*
Glucose	y = 0.51x + 0.47	0.5–5	0.991	3 %
Lactate	y = −1.64x − 0.34	0.25–5	0.995	3 %

* Calculated for a concentration of glucose and lactate of 2 mM (n = 3)

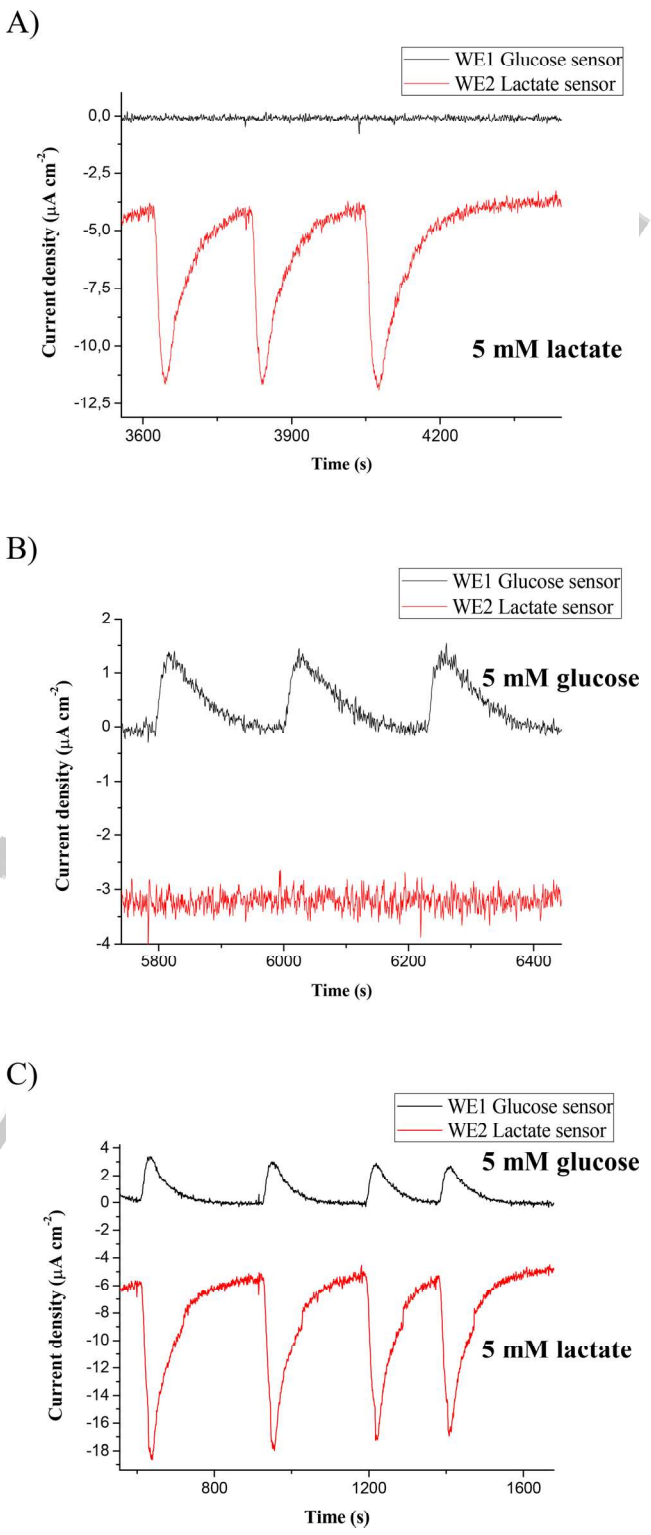

Fig. 4. Test of cross-talking behaviour of (A) the response of the glucose sensor (black colour) on 5 mM lactate solution, (B) the response of the lactate sensor (red colour) on 5 mM glucose solution. (C) Simultaneous response of the two sensors to glucose and lactate solution. For lactate sensor $E_{det} = -0.1$ V and for glucose sensor $E_{det} = +0.15$ V *vs* Ag pseudoreference, flow rate 1 mL min^{-1}, volume injection 60 μL. The electrode area = 2 mm^2.

Table 2. Determination of glucose and lactate in spiked and unspiked Sherry wine.

	Glucose (mM)			Lactate (mM)		
Sample	Added	Found	Recovery (%)	Added	Found	Recovery (%)
Canasta	–	160 ± 2	–	–	20 ± 1.3	–
	80	244 ± 3	103	120	141 ± 1.3	105

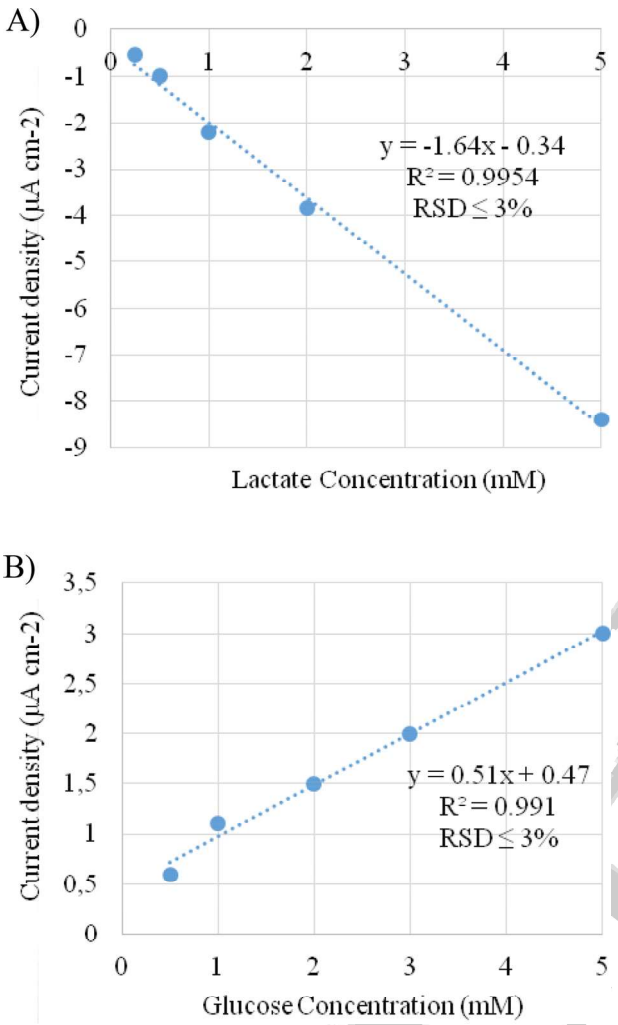

Fig. 5. A) Calibration curve of lactate (RSD < 3%) working potential of −0.1 V versus Ag pseudoreference. B) Calibration curve of glucose (RSD < 3%) working potential of +0.15 V versus Ag pseudoreference. Experiments were performed in 0.01 M PBS (pH 7.4) with a flow rate of 1 ml min^{-1}, injection volume 60 µL.

analysis for the fortified wine realized by Shkotova *et al.* (2016) [22].

4 Conclusions

A new FIA system adapted for multi-enzyme sensors based on screen-printed electrodes integrated in one channel disposable thin layer transparent flow-cell was developed. This system was successfully applied for the first time to study the simultaneous detection of glucose and lactate using *Ct*CDH and LOx respectively. The low detection potentials for glucose sensor (+0.15 V) and lactate sensor (-0.1 V) avoid the interference from other electrochemically active species which can be due to their presence in some real samples such as ascorbic acid and uric acid (Figure S3). *Ct*CDH and LOx were successfully immobilized in PVA-SbQ, which provides a long operational and storage stability (3 months). The new concept was tested with real samples of Sherry wine and provides a good correlation without matrix effect.

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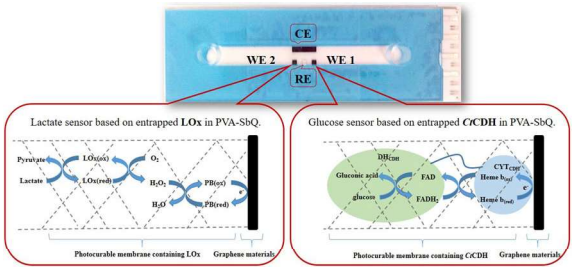

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