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Original article

Fabrication of L-lactate biosensor based on redox species mediated lactate oxidase using micro-device

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ABSTRACT

A sensitive L-lactate biosensor based on redox species ($K_3Fe(CN)_6$) mediated lactate oxidase (LOx) has been fabricated on N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) activated Thioglycolic acid (TGA) self-assembled monolayer (SAM) on micro-device, where $K_3Fe(CN)_6$ and TGA-SAM are used as a mediator and an enzyme immobilization matrix, respectively. The lactate biosensor displayed a low detection limit (DL, 0.299 nM), a wide linear dynamic range (LDR, 0.1 nM to 1.0 mM), good linearity (R=0.9887), and higher sensitivity (0.1242 $\mu A \mu M^{-1} cm^{-2}$), and small sample volume (70.0 µl) as well as good stability and reproducibility. The Au/TGA system exemplified a simple and effective approach to the assimilation of LOx and electrodes, which can provide analytical access to a large group of enzymes for wide range of bio-medical applications in health care fields.

1. Introduction

The development of biosensors has been the subject of considerable interest in recent years [1-5] based on selective enzymes immobilization on unique and specific matrixes [6-9]. Such modifications and devices offer great promise for biomolecules detection with features that include high sensitivity, inherent miniaturization, low cost, high selectivity, low sample volume, independence of sample turbidity or optical path length, minimal power demands, and high compatibility with advance micro-fabrication technologies [10-13]. The development of electrochemical biosensors for sensitive and selective detection of bio-molecules commonly requires innovative approaches that couple different modification/amplification processes [14-17]. Reliable measurement of L-lactate is of great interest in clinical chemistry, the dairy and wine industry, biotechnology, or sport medicine. In general, blood lactate levels are indicative of various pathological states, including shock, respiratory insufficiencies, and heart and liver diseases. Determination of L-lactate is based on spectrophotometry [18], but this method involves complicated procedures and the cost is high because expensive enzyme must be used in each assay [19]. Biosensors have been considered to be among the devices most suitable for biochemical analysis due to their good selectivity, fast response, miniature size, and reproducible results. Up to now, many studies dealing with lactate electrochemical biosensors have been reported. [20-24].

L-Lactate is constantly produced from pyruvate by LOx or dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise. L-Lactate concentration plays an important role in clinical diagnostics, medicine validation, and food analysis. In last decade, various approaches based on LOx or LDH have been developed for the detection of lactate. They employ different methods to attach enzymes on the sensing layer, including adsorption [25], cross-linking [26], covalent attachment [27, 28], conducting polymer entrapment [29, 30], and confinement in sol-gel matrix [31]. Flow injection analysis [32, 33] and mediator-based lactate biosensors [34] have also been developed. Biochemical sensors based on semiconductor field-effect, such as Ion Sensitive Field Effect Transistor (ISFETs) or light addressable potentiometric sensors (LAPS), have been also extensively studied in recent years. The ISFET has several diadvantages, such as sizes, response time, low output impedance and higher cost by mass production of the devices [35]. Besides, LAPS offers the possibility to select the point of measurement by addressing active sites on a completely unstructured surface simply by illuminating the region of interest [36], so that surface potentials can be measured in a spatially resolved manner by scanning the light-pointer across the surface of the device. In addition, the third type of a semiconductor field-effect sensor so-called electrolyte insulator semiconductor (EIS) or electrolyte membrane insulator semiconductor (EMIS) capacitive sensor is very attractive for many applications [37]. In fact, it combines some advantages and disadvantages of the LAPS (complete flatness, simple layout, easy and high cost fabrication)

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and the ISFET (absence of a light source in contrast to LAPS, possibility of miniaturization). Ion- or analyte-sensitive capacitive EMIS sensors are often constructed from a pH sensitive EIS structure by coupling the gate with different chemical or biological recognition elements. The coupling is made using an additional organic layer of the linking molecules or by depositing an additional membrane containing chemical or biological recognition elements (e.g. ionophores or enzymes). Nevertheless, the influence of this membrane on impedance is not always negligible resulting in unusual capacitancevoltage curves and consequently in inaccurate measurements [38]. Recent reports [39] showed that the use of the nanostructured electrode surfaces can improve the performance of electrochemical determinations thanks to an improved signal to noise current ratios with respect to macroelectrodes. Nanostructuring the electrode surface is particularly interesting for biosensor development [40], for the measurement of analytes with (high) charge transfer rate constants and for solving adsorption related problems. With respect to the latter issue, it was shown that operating in much diluted solutions of the analytes it was possible to obtain diffusion-controlled responses even for molecules which can adsorb on electrode surfaces [41] or cytochrome c [42]. The biosensor performance has been significantly enhanced by introduction of nanomaterials in the sensing layer such as CNTs [43], Nanowires [44] and nanoparticles [45]. However, to construct a nanostructured composite with different components for high stability and good performance is a very challenging state-of-art, which needs to engineer the nanostructure for required chemical and physical properties, functionalization, and efficient surface immobilization.

Here, a lactate biosensor is fabricated based on LOx on TGA SAMs by peptide conjugations. The characteristics of the biosensor to the detection of L-lactate are presented using redox species mediated LOx. We report a simple and sensitive electrochemical sensor for L-lactate detection based on redox species mediated LOx attached on sensing area of micro-devices through covalent bond conjugation to the TGA monolayer. At first, the sensor chips are constructed on Si wafer. Then the electrodes and passivation layers are deposited and patterned using conventional photolithographic techniques. After packaging the sensor chip, TGA SAM is fabricated on micro-device by chemical adsorption techniques, carboxyl group of which is activated by EDC, followed by immobilization of LOx. Lactate concentration was measured from the oxidation current of redox mediator ($K_3Fe(CN)_6$) upon addition lactate solution in every CV-cycles. Analytical performances of the sensor in terms of the dynamic linear range, sensitivity, and reproducibility are investigated.

2. Materials and Methods

2.1. Chemical reagents

Lactate oxidase (from Pediococcus sp., ≧ 20 units/mg) was purchased from Sigma. Thioglycolic acid and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich. All other chemicals were analytical grade and used without further purification. Phosphate buffers (PBS) of 0.2M NaH_2PO_4 and 0.2M Na_2HPO_4 (0.1M PBS, pH 7.4) were used. All aqueous solutions were prepared with distilled water, which was obtained from a water purifying apparatus (12.0 MΩcm) (AQUA MEDIA).

2.2. Apparatus

The electrochemical experiments were performed using a votammetric analyzer (CV-50W, BAS) connected with newly constructed micro-device. The set-up system of CV-devices is presented in the Figure 1A. The expanded-view of micro-device is shown in Figure 1B. All electrochemical measurements were carried out on electrochemical micro-device (5mm x 5mm). The sensing area of smart micro-device is 0.0805cm^2. All chemical investigations were carried out with enzyme-modified device composed as working, Pt layer as counter, and an Ag/AgCl (saturated KCl) as a reference electrode. Cyclic voltammograms (CVs) were recorded at LOx/TGA/Au electrode from -0.1 to +0.5 V versus Ag/AgCl in a 0.1M PBS (pH 6.4) at 0.1V/s scan rate.

Figure 1. Schematic photograph of the CV50W and micor-device set-up system for the detection of L-lactate.

2.3. Construction of Micro-devices

Electrochemical chips were fabricated by conventional photolithographic technique. Electrodes and passivation layers are fabricated on Si wafer followed by dicing and packaging.

2.4.Wafer Preparation

N-doped Si wafers are prepared. The size, resistivity, and orientation are 4.0 inch, 3 to 6 Ohm/cm, and (100), respectively. Wafers are cleaned as follows: DHW ($HF:H2_0$ = 1:50, 20sec), Aqua Regia (HCl : HNO_3 = 3:1, 10min.), APM ($NH_4OH:H_2O_2:H_2O$ = 1:1:6), HPM ($HCl:H_2O_2:H_2O$ = 1:1:6, 10min.), DHF (20sec.). Wafers are overflowed by extra-pure water for more than 5.0 min after each step. In this step, all contaminations on the surface and native SiO_2 layer are removed.

2.5. Oxidation

At first, the wet oxidation (H_2 250 l/hr, and O_2 250 l/hr, for 4 hours) is processed, and then secondly dry oxidation (O_2 250 l/hr 10 min.) is executed. Wafers are annealed in the condition of N_2 250l/hr, 10min. All processes are under at 1000°C. The thickness of SiO_2 layer is about 7,500 A°. (Oxidization furnace was made by Koyo Lindberg).

2.6. Fabrication of aluminum layer

Aluminum is sputtered by using ANELVA C-7250 with Al-1% Si target. RF Power and Ar gas pressure are set to 1.0 kW and 0.5 Pa (50 sccm), respectively. Then the Photolithograph processes are processed as follows: baking at 160 °C 5 min. OAP coating (500 rpm for 5 s, 5000 rpm for 20 s.) by spinner (1H-360S MIKASA), baking 110 °C for 60 s. Resist (OFPR8600/52cP) coating (500 rpm for 5 s., 5,000 rpm for 20 s.), baking 110 °C for 90 s., exposure [NSR-TFT1, Nikon, g-ray (436nm), 225 msec.], development by NMD-3 (Kanto Chemicals. Co.), 65 s. rinsed by distilled Ion Water (DIW) for 1.0 min. and baking at 140 °C for 5 min. Al is etched by etching solution (H_3PO_4: CH_3COOH: HNO_3 = 250:20:3) for 4 to 6 min at 50.0 °C. Resist is removed by plasma etching instrument (PX-250M, SAMCO). Then wafers are cleaned by the following process: Acetone for 5 min, Methanol for 1 min and then plasma cleaned for 5 min.

2.7. Fabrication of SiN layer

SiN layer is deposited by Chemical Vapor Deposition (CVD) technique with following conditions. 10% SiH_4/H_2 117 sccm, NH_3 6 sccm, N2 183 sccm, RF 100 W, Pressure 100 Pa, at 300 °C 27 min. by CVD instrument (PD220M SAMCO). Then photolithographic process, which is already explained in the previous section. Areas for pad electrodes are etched by Reactive Ion Etching (L-451D-L ANELVA). Finally residual resist layer is removed by plasma ashing (PX-250M SAMCO).

2.8. Fabrication of Pt layer

Photolithographic process is executed. Two kinds of resists are used (LOR30B and OFPR8600/52cP) for lift-off patterning. TiN is sputtered by C-7250 (ANELVA) with the following conditions: Ar gas pressure 0.5 Pa, RF 0.5 kW, stage rotation ON. Continuously, Ti is sputtered with the condition of Ar gas pressure 0.2 Pa, RF 0.2 kW, stage rotation ON. TiN and Ti layers are used as adhesive. Then Pt is sputtered by SP150-HTS (HiTeck Co.) with the condition of 0.5 Pa 4x15 min. (60 min. in total) RF power 50 W. PT is patterned by lift-off technique, in which wafers are immersed into the remover PG (U.S.), and then washed with IPA.

2.9. Fabrication of Au layer

Photolithographic process is executed. Ti is sputtered by ANELVA C-7250 as a binding layer, and then Au is evaporated by deposition apparatus (L-420-FHL, ANELVA). Finally, Au layer is patterned by lift-off process.

2.10. Formation of Palylene layer

Palylene passivation layer is formed for the protection of the chip from water. Photolithographic process is executed for pad protection, which is explained prevously. Then palylene dimer is evaporated by deposition apparatus (PDS 2010, SCS). Photolithography process is executed again for patterning. Palylene layer is patterned by etching (PX-250M, SAMCO). Finally, unnecessary resists are removed by acetone, and wafer is cleaned by IPA.

2.11. Dicing of the device

Resist (OFPR8600/52cP) is coated on a whole surface of the wafer for protection during dicing process. Si wafer is diced into pieces of 5.0 mm square by dicing apparatus (A-WD-10A, Tokyo Seimitsu Co. Japan) and stored into the desiccators.

2.12. Die and wiring of device

Resist on device surface is removed by acetone and cleaned with IPA. The backside of the chip is roughed by a sheet of sandpaper for better adhesion and electrical stability. The chip is die-bonded to the 80-pin 17-mm-square package (QC-080324-WZ Kyocera co.) by silver paste and package is dried in a drying oven (009G KDF) for 90 min at 100°C. Pads on device are connected to the package through gold wire with Bonding machine (7700D West Bond). Finally, Si-based adhesive (TSE387W, MPMJ Co.) is put on the periphery of the device to protect pads and gold wire from sample solution. Adhesive is dried at room temperature for 24 hours.

2.13. Preparation of micro-device

The semiconductor micro-devices were made on Si wafer. Al was sputtered to fabricate as wiring and bonding pads. Pt/Ti/TiN was sputtered on thermal oxide of silicon and patterned by photolithography to fabricate counter electrode (CE). Ti/TiN layers were used for better adhesion. Au/Ti were sputtered and lithographed, which made circular working electrode (WE) with a diameter of 1.6mm in the center of the chip. After electrodes fabrication, palylene layer was fabricated by evaporation method as a passivation layer. The wafer was diced to 5.0 mm square devices. This device was bonded to a package by silver paste. Aluminum pads were connected to the package by gold wire. Finally, adhesive (Araldite, epoxide-based adhesive, Hantsman, Japan) was put on the periphery of the device, which prevents target solution from contacting pads (Figure 2A). The expanded view of device-center (sensing area) is shown in the (Figure 2B). A cross section of the sensor device is shown in (Figure 2C). The function of each layer is listed in Table 1.

Table 1. Function and thickness of every layer on the micro-device

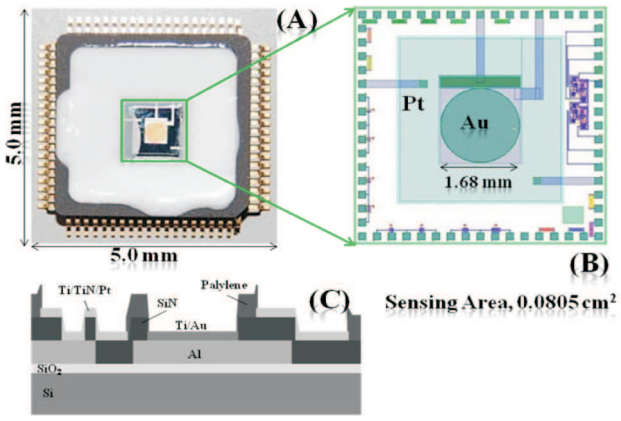
Material	Function	Thickness (µm)
Si	Wafer material	500.0
SiO_2	Insulation	0.40
Al	Electric wiring	1.0
SiN	Protection/separation	1.0
Ti/TiN	Binding	0.15
Ti	Binding	0.10
Pt	Counter Electrode	0.25
Au	Working Electrode	0.30
Palylene	Passivation/protection	1.0

3. Results and Discussions

Figure 3 outlines the biosensing protocol using the LOx/TGA/Aumodified micro-device. It is used for covalent bond formation to immobilize the LOx enzyme on the TGA SAM via peptide conjugation in presence of activating agent (EDC). First, the self-assembled monolayer of TGA is formed by dropping the TGA solution onto the sensing area of device for 8 hours. Then LOx enzyme is immobilized on the TGA SAM by the amide bond formation between the terminal-unbound carboxylic acids groups on the TGA film and the amine groups of the enzyme.

For the stable attachment of LOx onto TGA SAM, the biochip was kept for 24 hours into the refrigerator at 4.0°C. The enzymatic reactions involved in the biosensing system for the detection of lactate are as follows (see Figure 2):

Figure 2. Schematic diagram of Smart micro-device. (A) Camera view of biodevice, (B) Magnified view with scale of core area, and (C) Cross-section view.



$$\text{Lactate} + O_2 \xrightarrow{LOx} \text{Pyruvate} + H_2O_2 \quad (1)$$

$$H_2O_2 \longrightarrow 1/_2\, O_2 + 1/_2\, H_2O_2 + 2e^- \quad (2)$$

Reaction (1) is lactate depended. On the micro-device, lactate is oxidized to form pyruvate and H_2O_2. Then H_2O_2 is auto-dissociation to produce the current (Reaction 2). This oxidation current is directly proportional to the lactate concentration in the solution.

3.1. Preparation of TGA SAM

Successful fabrication of sensitive lactate sensor using LOx on the sensing area of micro-device has been demonstrated based on TGA-SAMs. Conventional electrochemical method, CV is the most versatile electroanalytical technique for the study of electroactive species, and is widely used in industrial applications and academic or biochemical or biomedical research laboratories. Successful fabrication of sensitive lactate sensor using LOx on the sensing area of micro-device has been demonstrated based on TGA-SAMs. Conventional electrochemical method, CV is the most versatile electroanalytical technique for the study of electroactive species, and is widely used in industrial applications and academic or biochemical or biomedical research laboratories. CV is also an important technique to evaluate the blocking property of the monolayer-coated electrodes using diffusion controlled redox couples. Here, the device surface was cleaned by H_2SO_4:H_2O_2 (3:1) solution and washed with pure water, then dried by N_2. TGA was dissolved in ethanol to make 10.0 mM solution. TGA solution was dropped on a sensimg area of chip, and then kept wet for 8 hours at room temperature. Figure 3 shows the CV of un-modified and TGA SAM-modified device electrodes in 5.0 mM $K_3Fe(CN)_6$ with 0.1 M PBS as the supporting electrolyte at a potential scan rate at 0.1 V/s. It can be seen from the Figure 3, that the bare device electrode (black line) shows a reversible voltammogram for the redox couple indicating that the electron transfer reaction is completely diffusion controlled. In contrast, the absence of any peak formation in the CVs of the TGA monolayer-modified electrodes shows that the redox reaction is inhibited or totally blocked. The CVs for TGA indicate a good blocking behavior for the electron transfer reaction, which means that a highly ordered, compact monolayer is formed on the Au surface of the biochips [Figure 4].

Figure 3. Fabrication scheme of Lactate sensor on smart biodevices

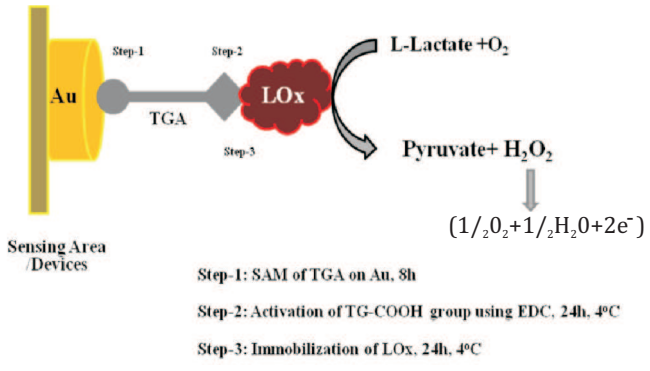


Figure 4. Cyclic voltammogram in 5.0 mM potassium ferrocyanide with 0.1 M PBS as supporting electrolyte at a potential scan rate at 0.1 V/s for bare (black line) and TGA SAM (blue line) on Au surface of micro-device.

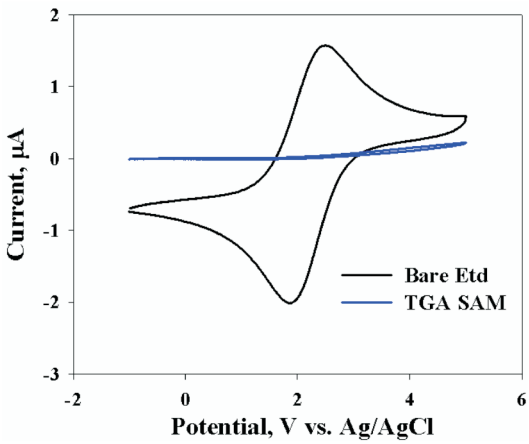
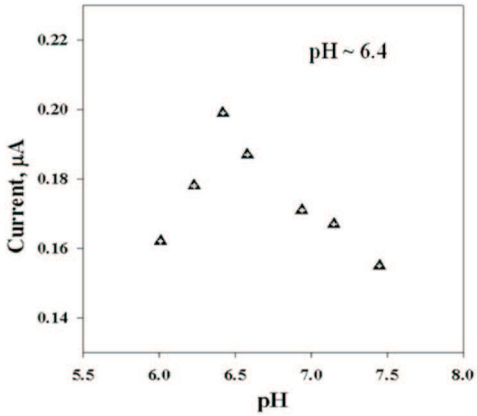


Figure 5. Effect of the sensing solution pH on the oxidation peak for 0.1mM lactate obtained with LOx/TGA/Au on the micro-devices.



3.2. LOx Immobilization

Lactate oxidase was immobilized onto the TGA SAM surface by peptide conjugation. First 10.0 mM EDC in 0.1 M PBS was put on the device and device was kept still at 4.0 °C in the refrigerator to activate carboxylic group of TGA for 24 hours. Then EDC-treated electrode was washed gently with 0.1 M PBS to remove excess EDC. Then 50.0 UN/ml LOx solution was put on the sensing are of micro-device and incubated in the refrigerator at 4.0 °C for 24 hours. LOx was successfully immobilized onto TGA SAM via covalent bond.

3.3. Optimization of pH

The experimental conditions affecting the performances (detection limit, sensitivity, response time etc) of the sensor were optimized in term of pH and shown in Figure 5. The pH of the buffer shows a strong effect on the activity of the biosensing layer on the device. The effect of the pH of the buffer on the current responses was studied over the pH range of 6.0 to 7.5. Figure 5 shows the CV peak currents obtained at different pH values for 0.1 mM lactate in 0.1 M PBS system. The peak height increased from pH 6.3 to 6.4 and then decreased above pH 6.4. The oxidation peak current decrease above pH 6.4 might have been due to the poor enzyme activity at higher pH. Therefore, the pH of the PBS system was preset at 4.4 throughout the experiments.

3.4. Calibration plot and lactate response

Cyclic voltammetric study was achieved to confirm the detection of lactate concentration. 70.0 uL of each sample solution with mediator was dropped on the sensing area of micro-device and measured the sensing oxidation current in manifestation of mediator. Figure 6A shows a typical cyclic voltammogram (current-voltage) plot for the addition of varying amounts of lactate in a 0.1M PBS (pH 6.4). The currents increased gradually with the increasing the concentration of lactate (0.1 nM to 10.0 mM) to a stable and saturated value. The LOx/TGA/Au modified bio-device electrode achieved steady state currents versus lactate concentration with in 10 sec. The increase of oxidation current was observed. This was because LOx oxidized lactate, and the oxidation current change lead to the negative current increased.

Figure 6B shows the calibration plots for the lactate obtained from the current-voltage responses with fabricated biochips. Under the optimized conditions, the steady-state currents showed a linear relationship with the lactate concentration in the range from 0.1 nM to 1.0 mM, which is shown in the inset Figure 6B. The linear dependence of the lactate concentration yielded with a correlation coefficient of 0.9887, which is shown in Figure 6B (inset). The detection limit for lactate detection was executed to be approximately 0.299 nM, based on signal to noise close to 3(S/N=3). This detection limit is much lower than the detection limits obtained in the previously reported lactate biosensors, which is shown in the Table 2 [46-50]. The sensitivity is calculated from the slope of 0.299 nM, based on signal to noise ratio close to 3 (S/N=3). the calibration plot is 0.1242 $\mu A \mu M^{-1} cm^{-2}$.

Figure 6(A) Variation of lactate concentration in presence of mediator, (B) Calibration plot and LDR (inset).

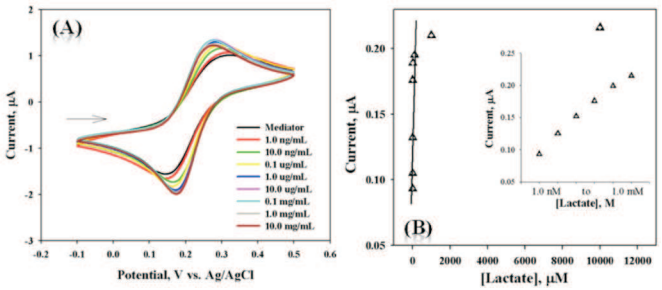


Table 2. Comparison of the performance of some lactate biosensors constructed based on different modification on electrode surfaces.

Sensors	DL (nM)	Dynamic Range (M)	Sensitivity $A\mu M^{-1}cm^{-2}$)	Type	References
Chip-Au/TGA/LOx	0.299	1.0 nM~1.0mM	0.1224	Chip	Present report
PAA/LDH	5.0×10^{2}	0.5µM ~ 6.0mM	0.682×10^{-3}	Chip	Patel et al. [46]
pTTCA/MWNT/ LDH/NAD^{+}	1.0×10^{3}	5.0 ~ 90.0µM	1.06×10^{-2}	Cell	Rahman et al. [47]
Nafion/LOx	1.0×10^{2}	0.2 ~ 10.0µM	-	Chip	Lupu et al. [48]
SPE/LDH	1.0×103	5.0 ~ 500.0µM	0.34×10^{-2}	Chip	Avramescu et al. [49]
SPE/LOx	2.9×10^{5}	1.0 ~ 6mM	0.363×10^{-6}	Chip	Rawson et al. [50]

A series of five successive measurements of 0.1mM lactate in 0.1M PBS yielded a good reproducibility signal at LOx/TGA/Au sensor. The sensor-to-sensor and run-to-run reproducibility for 0.1mM L-lactate detection were obtained. To examine the long-term storage stabilities, the response for the LOx/TGA/Au sensor was examined with the respect to the storage time. After each experiment, the sensor was washed with the buffer solution and stored in 0.1M PBS at 4.0 °C until use. The long-term storage stability of the sensor device was tested every three days. The sensitivity retained almost of initial sensitivity up to three weeks. After that, the response was gradually decreased, possibly due to the loss of the enzyme activity. The above results clearly displayed that the micro-device lactate sensor can be used for a three weeks without any significant loss in sensitivity.

The selectivity (interference effect) of the LOx/TGA/Au smart sensor was evaluated by cyclic voltametrically in the presence of other electroactive compounds such as glucose, uric acid, glutamate, and acetylcholine etc. No current response was observed when 0.1mM glucose, uric acids, glutamate, and acetylcholine were introduced into the 0.1M PBS buffer. But when 0.1mM lactate solution was added to the electrolyte solution, a clear oxidation response was executed with mediator current, indicating the selective detection of lactate with the LOx/TGA/Au sensing layer. Importantly, at this concentration level, glucose, uric acid, glutamate, and acetylcholine have no interference for 0.1mM lactate detection. Thus, the selectivity of the LOx/TGA/Au smart sensor is acceptable for lactate detection in the presence of the common interfering compounds in normal physiological levels.

4. Conclusions

Successful fabrication of sensitive redox L-lactate biosensor based redox species mediated and immobilization of LOx on the 5.0 mm square sensor mirco-device has been demonstrated. Sensor devices were constructed using photolithographic technique, which was possible to low-detect target solution of tiny volume. The fabricated biosensor exhibited a low-detection limit with satisfactory stability, a large linear dynamic range, and higher sensitivity, and repeatability. The simple fabrication method of the biosensor has many advantages such as ease of fabrication, enhanced electrocatalysis, and efficiently preserving the activity of biomolecules. It would have potential applications in lactate determination in health care biomedical fields.

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