Highly Sensitive Lactic Acid Biosensors Based on Photoresist Derived Carbon

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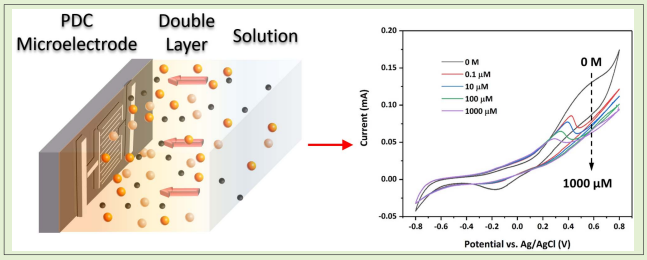
***Abstract*—In this study, a highly sensitive electrochemical capacitive lactic acid enzymatic sensor based on a carbon-microelectromechanical system (C-MEMS) is developed. The sensing platform of the biosensor, which is photoresist-derived carbon (PDC) microelectrode, was synthesized by pyrolysis of photo-patterned photoresist polymer in oxygen-free and high-temperature conditions. The surfaces of the microelectrodes were functionalized by the oxygen-plasma pretreatment technique to form –COOH functional groups. The lactate oxidase enzyme was immobilized on the carboxylic group covered surfaces of the microelectrodes. The sensor demonstrated the detection of lactic acid over a wide dynamic range of 0.1-5000 μM with a low detection limit of 1.45 μM (S/N=3). The sensitivity found to be 33.48 mF.cm^{-2}. (Log (M))$^{-1}$. This mediator-free lactic acid sensor exhibited 80.07% accuracy in the presence of uric and ascorbic acid and only a 2.35% decrease of measured capacitance in 20 days, which implies good selectivity and high stability. The presented results show that the C-MEMS based lactic acid biosensor is a promising contender for the highly demanding point of care health monitoring applications.**



***Index Terms*—C-MEMS, lactic acid, lactate-oxidase, electrochemical.**

I. Introduction

LACTIC acid with the chemical formula of $C_3H_6O_3$ is a product of anaerobic glycolysis generated in muscles and liver due to insufficient supply of O_2 in blood [3]. The level of LA in human blood can change from 0.5 – 1 mM for healthy persons up to >4 mM [4], [5] under the influence of different diseases and conditions, such as cancer in the metastatic spread of cancer cells in stage II or stage III [8], [9], antitumoral treatments. [6], cardiovascular diseases (ischemia, hypoxemia, and anemia) [10], and head trauma [11]. This feature can be employed as an essential complementary indicator for different cancer types and metastatic levels [8], [12]. In the cases of brain stroke and head trauma, increasing LA concentration from the cut off level of 2.35 mM in mildly injured to >4mM in severely injured conditions, can be used to determine the severity of the case [11], [13]. Considering its significance in various diseases and conditions, fast and accurate detection of the LA level can be useful for their early diagnosis as well as monitoring their progress and severity.

Carbon-microelectromechanical systems (C-MEMS) are composed of well-defined carbon micropatterns in which carbon (such as glassy carbon) is synthesized through pyrolysis of micropatterned photoresist polymer in an oxygen-free environment at high temperatures [14], [15]. Photoresist-derived carbon (PDC) possesses various remarkable properties of carbon-based materials such as high biocompatibility, wide electrochemical window, low absorption of non-specific biomolecules, and scalability [16]. Additionally, PDC exhibits excellent electrical conductivity, as well as low background capacitance, better tolerance toward bio-fouling than gold, glass, and silicon substrates, and higher stability when exposed to different physical/chemical treatments [17]. The surface of PDC can be functionalized efficiently via various chemical, physical, or electrochemical procedures [18], [19]. Moreover, utilizing high surface area C-MEMS in miniaturized electrochemical devices circumvents the significant drawbacks associated with commercialized screen-printed carbon electrodes such as low resolution, low aspect ratio, and limitations for further miniaturization [16], [20]

Various studies have been conducted to develop non-invasive electrochemical biosensors based on carbon materials for real-time measuring of LA concentration. For example,

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Luo *et al.* developed a wearable cotton fabric biosensor for amperometric monitoring of LA concentration in sweat with a detection range of 0.5-1.5 mM [2]. The electrochemical sensor developed by Lamas-Ardisana *et al.* for measuring LA in human sweat based on screen-printed carbon electrodes functionalized with platinum nanoparticle has presented a linear range of 25–1500 μM [21]. However, these non-invasive electrochemical sensors have narrow dynamic ranges compared to the actual LA concentrations in sweat and saliva. Therefore, developing highly feasible LA sensors with high sensitivity and sufficiently broad dynamic range to meet the demands for medicine and personal health monitoring is highly significant.

In this work, we developed an electrochemical enzymatic LA sensing platform based on interdigitated PDC microelectrode arrays and using the lactic oxidase (LOx) enzyme as a catalyst for the oxidation of LA. The PDC microelectrodes were pre-treated with oxygen-plasma to form –COOH functional groups on their surfaces, through which LOx enzymes were immobilized directly on them. The scanning electron microscope (SEM) and Fourier-transform infrared spectroscopy (FTIR) were used to study the morphology and surface characteristics of the fabricated electrodes. Cyclic voltammetry (CV) was used to analyze the performance of the sensor. The results showed that the developed PDC-based LA sensors are highly sensitive, selective, and stable, and have a sufficiently broad dynamic range compared to previously reported carbon-based LA sensors. Furthermore, the C-MEMS technique is both feasible and amenable to mass production, thus making PDC-based LA sensors highly promising for point-of-care health monitoring applications.

II. Materials and Methods

A. Fabrication of Interdigitated Photoresist Derived Carbon MicroElectrodes

The PDC microelectrodes were fabricated by a standard C-MEMS process [14], [22]. They consist of fingers with 100μm width and 100 μm distance between each finger (total of 25 fingers in 0.25cm^2 area). The C-MEMS process is schematically illustrated in Fig. 1A (1-3). A 4-inch silicon dioxide covered silicon wafer was cleaned by acetone and methanol followed by a 20 min bake in the oven at 200°C to evaporate any solvent and moisture. NANO^{TM} SU-8 25 negative photoresist (Microchem., USA) was spin-coated using photoresist spinner (Headway Research^{TM}) at 500 rpm for 12 s and then 3000 rpm for the 30 s to get a uniform photoresist layer with a thickness of approximately 25 μm. The photoresist was baked at 65°C for 3 min and 95°C for 8 min to evaporate any solvents. The photoresist was optically patterned using the OAI model 800 contact aligner (light intensity, 13.2 mW.cm^{-2}) for 22 s to crosslink polymer chains in the photoresist. Post-expose bake was conducted at temperatures of 65°C for 1 min and 95°C for 3 min. The photo-patterned sample was developed using NANO^{TM} SU-8 25 negative photoresist developer (Microchem., USA). The carbonization process of the photo-patterned photoresist was performed in a Lindberg tube furnace via 200 mL.min^{-1} flow of forming gas (95% N_2+ 5% H_2). The samples were heated from room temperature to 350°C with the ramp of 3°C.min^{-1} with a dwell time of 30 min continued by ramping to 900°C with the same rate and hold time of 60 min. The samples were cooled down to room temperature with the ramp of 5°C.min^{-1}.

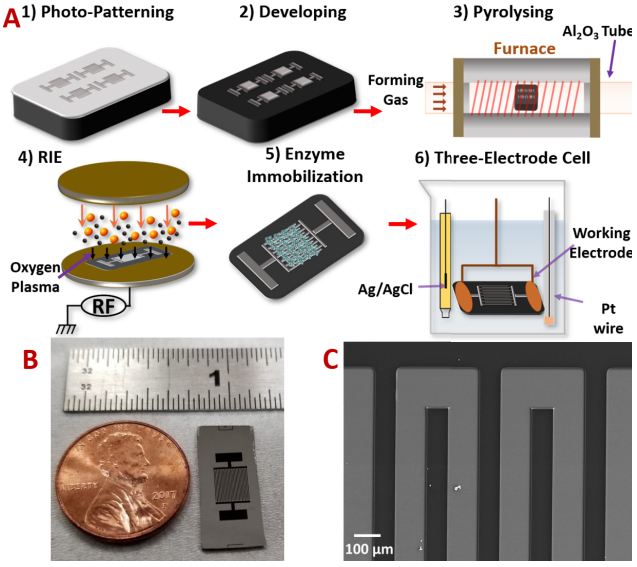

Fig. 1. A) Schematic of the fabrication process of the C-MEMS based LA sensor and electrochemical test cell. B) Image of the PDC_{RIE} chip next to a one-cent coin. C) SEM picture of the PDC_{RIE}.

B. Oxygen-Plasma Treatment

The PDC surface was treated with oxygen-plasma using the MARCH CS-1217 RIE system, which has a parallel-plate reactor equipped with 13.65 MHz RF source, as illustrated in Fig. 1A(4). The gas line for oxygen was utterly evacuated before the process. The etching parameters were: O_2 gas flow rate of 60 mL.min^{-1}, the RF power of 100 W, the chamber pressure of 400 mTorr, and the etching duration of 3 - 10 minutes. This process has no adverse effect on silicon dioxide underneath [23].

C. Enzyme Immobilization

As shown in Fig. 1A (5), firstly, disodium phosphate salt (Na_2HPO_4, Sigma Aldrich, USA) was used to adjust the pH of the phosphate buffer solution (PBS, Sigma Aldrich, USA) to pH 6.6. The stock solution of 10 mg.mL^{-1} LOx enzyme (Mybiosource, USA) was prepared in PBS (0.1 M, pH 7.4). LOx enzymes were immobilized on the surfaces of functionalized PDC microelectrodes with the amounts ranging from 5 μg to 40 μg confined on the 0.25 cm^2 working area, which followed by drying at 4°C in the refrigerator overnight. The devices were washed thoroughly with PBS for 10 minutes to wash away the unbounded enzymes. The prepared electrodes were stored in the refrigerator at 4°C when they were not used. The optimum amount of utilized enzymes was determined by measuring the responses of the functionalized PDC microelectrodes with various amounts of immobilized LOx enzyme to 500 μM LA. The response reached saturation at 25 μg of immobilized LOx enzymes; hence, 25 μg was chosen as the optimum amount.

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D. Sensor Characterization

The electrochemical characterization was carried out using a Bio-Logic versatile multichannel potentiostat (VMP3) at room temperature. For CV studies, the three electrode cell shown in Fig. 1A (6) with the PDC microelectrodes, platinum wire, and Ag/AgCl were used as the working electrodes, counter electrode, and reference electrode, respectively. The working electrodes were tested between potentials of −0.8 V and 0.8 V (vs. Ag/AgCl) in which 3mL aqueous electrolytes of 0 - 15 mM LA in 0.1M PBS (0.1 M, pH 6.6) were used. The pH of the PBS was chosen as 6.6 based on the fact that the pH of human sweat is variable between 4.0 and 7.0, with the mean value of 6.3 [24], [25]. In order to define the optimum number of CV cycles, CV tests were conducted for 20 continuous cycles. The obtained curves after the cycle of n=4, repeated with a small deviation (STDEV=7.3311 μA). Hence, n=4 was chosen for further measurements. A series of CV tests of the sensing electrodes were conducted with the scan rates of 10, 20, 40, 60, 80, and 100 mV.s^{-1} in an aqueous electrolyte of 15 mM LA in 0.1M PBS (pH 6.6). Moreover, series of CV tests of the sensing electrodes were conducted in a 3 mL aqueous electrolyte of 500 μM LA with 9.92 mg.mL^{-1} uric acid (Sigma Aldrich, USA) and 1.76 mg.mL^{-1} ascorbic acid (Sigma Aldrich, USA). FTIR analysis was carried out using a JASCO FTIR 4100 equipped with an attenuated total reflectance (ATR) accessory to verify –COOH carboxylic group and other functional groups on the sensor surfaces. The morphology of the fabricated microelectrodes was studied using an SEM (JEOL SEM 6330F, Peabody, MA, USA) in the secondary electron imaging mode.

III. Results and Discussions

A. Surface Morphology and FTIR Characterization

A summary of PDC microelectrode fabrication and sensor development is illustrated in Fig. 1A. The photolithography and carbonization parameters were optimized based on the well documented C-MEMS procedures in previous studies [15]–[19]. The optimum exposure time in oxygen-plasma treatment was found to be 7 minutes without breaking or detaching the PDC micro-fingers. LOx enzyme was directly immobilized on the activated surfaces of the PDC microelectrodes without utilizing commonly applied crosslinks such as EDC [(1-ethyl-3-(3-dimethylamino) propyl carbodiimide, hydrochloride]. It is worth mentioning that in order to minimize the possible side reactions, all areas of the working electrode and connection wires were isolated utilizing bee wax, and only 5mm×5mm window was kept open. Fig. 1B shows a picture of an oxygen-plasma treated PDC microelectrode chip (designated as PDC_{RIE}) and Fig. 1C shows the SEM picture of PDC_{RIE}.

The FTIR spectroscopy was used to track the formation of carboxylic groups and enzymes' immobilization on the surfaces of the working electrodes. As shown in Fig. 2, the FTIR spectra of all three samples showed two peaks between 2800 - 3000 cm^{-1} and two peaks between 2290 - 2400 cm^{-1}, which are attributed to OH and sp C-H stretching, and sp C≡C bending, respectively [26]. Furthermore, PDC_{RIE} and PDC microelectrodes functionalized through oxygen-plasma

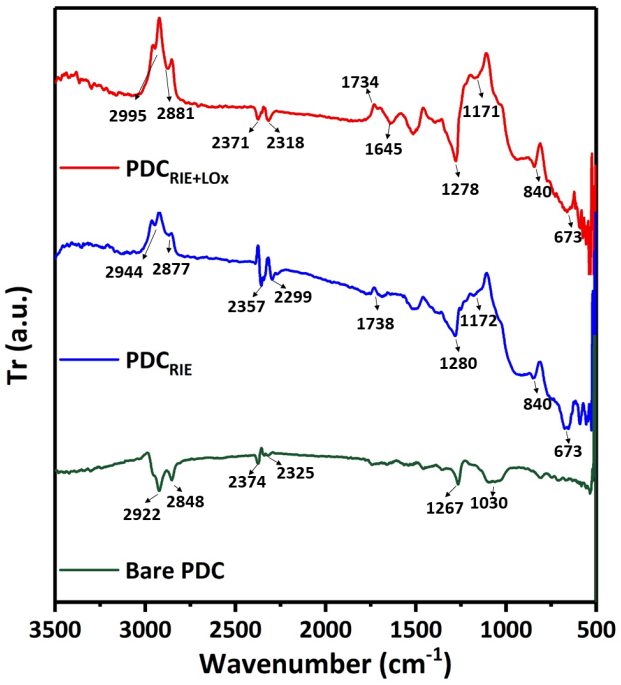

Fig. 2. FTIR spectrum of PDC microelectrodes.

treatment and having immobilized LOx enzyme (designated as $PDC_{RIE+LOx}$) show two peaks at 673 cm^{-1} and 840 cm^{-1} which are attributed to sp^2 C-H bending [26]. The spectrum of the bare PDC shows two peaks at 1030 and 1267 cm^{-1}, which are assigned to alkoxy C-O and acyl C-O, respectively [27]. These peaks are the result of native oxidation of carbon in contact with air. The FTIR of PDC_{RIE} and $PDC_{RIE+LOx}$ exhibited the same peaks around 1172 and 1287 cm^{-1}, respectively. The peak at 1738 cm^{-1} in the PDC_{RIE} spectrum and 1734 in the $PDC_{RIE+LOx}$ spectrum are attributed to C=O stretching vibrations, which indicate that the PDC microelectrodes were successfully functionalized with carboxylic groups. The FTIR spectrum for $PDC_{RIE+LOx}$ shows an apparent peak at 1645 cm^{-1} which is related to amide bond and represents covalent bonding of enzymes on the surfaces of the PDC microelectrodes [27]. The FTIR results suggest that the oxygen-plasma treatment was a simple yet effective means for generating reactive surfaces with the high possibility of covalent bonding of LOx enzymes on PDC surfaces.

B. CV Characterization of the PDC Microelectrodes

LOx is an FMN (Flavin mononucleotide)-dependent alpha hydroxyl acid oxidizing enzyme, which catalyzes the aerobic oxidation of LA to pyruvate and releases H_2O_2 [28]. Oxidation of LA is followed by releasing H_2O_2 (eq. 1). Although there are vast studies on LA reactions, the precise information of the H_2O_2 reduction reaction on the surface of carbon electrodes is absent [29], [30]. In this study, equation 2 is considered for H_2O_2 reduction [29], [30]. Fig. 3A shows the CV responses with a scan rate of 20 mV.s^{-1} (vs. Ag/AgCl) for the bare PDC, PDC_{LOx}, and $PDC_{RIE+LOx}$ in the 500 μM LA solutions. The CV response of the bare PDC shows no apparent oxidation peak, whereas the PDC_{LOx} has an oxidation peak at

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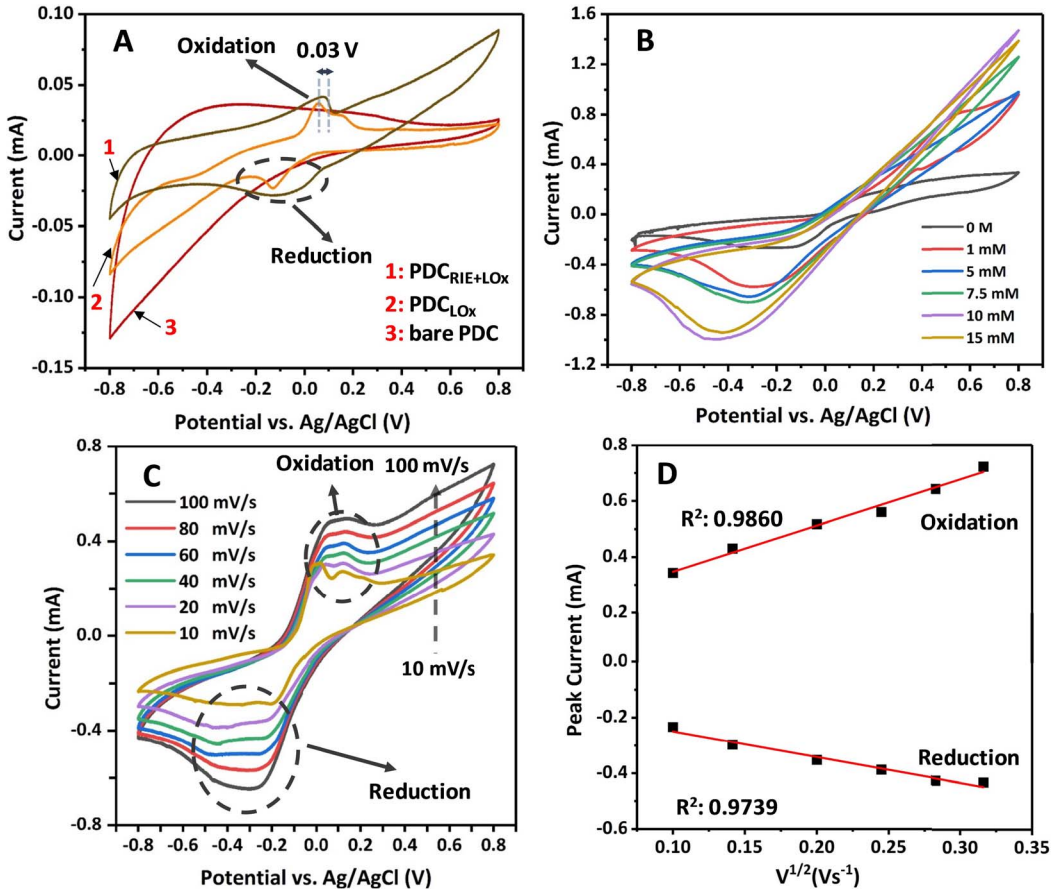

Fig. 3. (A) CV response of the bare PDC, *PDC$_{LOx}$*, and PDC$_{RIE+LOx}$ in 500 μM LA solution. B) CV response of the bare PDC to different H_2O_2 concentrations. C) CV responses and D) correlation of peak currents to the square root of scan rate of PDC$_{RIE+LOx}$ in a 15 mM LA solution and different scan rates of 10 - 100 mV.s^{-1} (vs Ag/AgCl).

0.05 V/0.028 mA, and the PDC$_{RIE+LOx}$ exhibits an oxidation peak at 0.08 V/0.042 mA. The appeared oxidation peaks are related to the oxidation reaction of LA molecules catalyzed by the LOx (eq. 1) and they are in good agreement with previous studies on electrochemical LA sensors [31], [32] while the reduction peaks are related to the reduction of H_2O_2 (eq. 2).

$$\underset{(Lactic\ Acid)}{CH_3CH(OH)CO_2^- + O_2} \xrightarrow{LOx} \underset{(Pyruvate)}{CH_3COCOO^- + H_2O_2} \quad (1)$$

$$2H^+ + H_2O_2 + 2e^- \longrightarrow 2H_2O \quad (2)$$

The enhanced current response of PDC$_{RIE+LOx}$ to LA compared to PDC$_{LOx}$ can be explained by the promoted immobilization of LOx enzymes onto the PDC microelectrodes surfaces by introducing carboxylic groups to the surface. In order to confirm the origin of observed peaks in CV results, the response of bare PDC microelectrode to H_2O_2 as a product of the LA oxidation reaction was studied. The results presented in Fig. 3B shows that only the reduction reaction takes place in the presence of H_2O_2, and the reduction peak current increases by increasing the concentration of H_2O_2. The absence of oxidation peaks and a proportional increase in reduction current indicate that the observed oxidation and reduction peaks in Fig. 3A are associated with LA oxidation (eq. 1) and H_2O_2 reduction (Eq. 2), respectively. The CV curves of PDC$_{RIE+LOx}$ in a 15 mM LA solution with various scan rates ranging from 10 - 100 mV.s^{-1} (vs. Ag/AgCl) are presented in Fig. 3C. The oxidation and reduction peak currents increase with the increasing scan rates. The relations between the square root of the scan rates and the oxidation/reduction peak currents are shown in Fig. 3D. The peak currents are linearly proportional to the square root of the scan rates up 100 mV.s^{-1} with the correlation coefficient (R^2) of 0.9860 and 0.9739 for oxidation and reduction, respectively. The linearity indicates that the process involves diffusion of LA, which is highly important for enzymatic electrochemical sensors [33].

C. Sensing Characterization of PDC Microelectrodes

The CV results of the PDC$_{RIE+LOx}$ in the 0 - 1000 μM LA solutions are represented in Fig. 4A. The oxidation peaks of 0.2 - 0.35 V (vs. Ag/AgCl) were observed by introducing the LA to the solution. The observed shift in the oxidation peaks by increasing LA concentration can be explained by the increased amount of oxygen uptake (eq. 1) [34]. The areal capacitances were calculated from the CV curves to study the sensing performance of the PDC$_{RIE+LOx}$ using the equation (3) [35]:

$$C = \frac{1}{2As\Delta V}\int IdV \quad (3)$$

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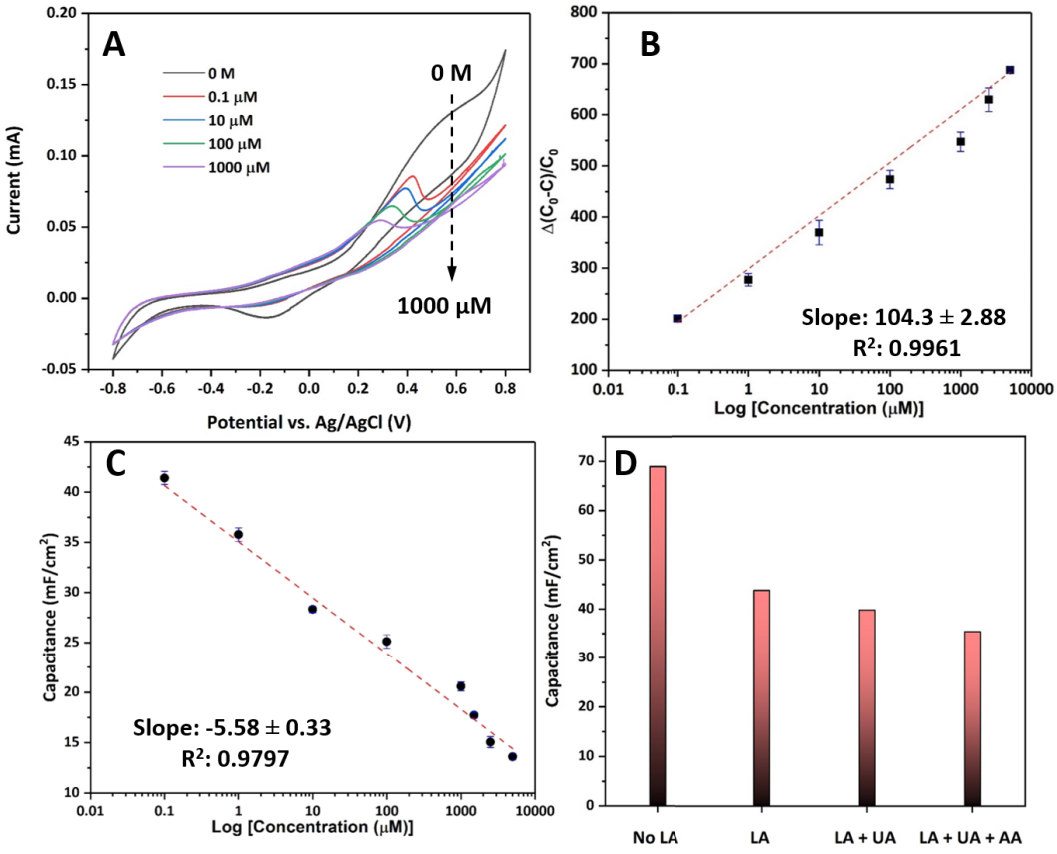

Fig. 4. A) CV curves of $PDC_{RIE+LOx}$ in 0, 0.1, 10, 100, and 1000 μM LA solutions. B) Calibration plot of normalized areal capacitances calculated from CV curves in 0.1-5000 μM LA solutions. C) Calibration plot of absolute areal capacitances calculated from CV curves in 0.1-5000 μM LA solutions. D) The response of $PDC_{RIE+LOx}$ microelectrode to 500 μM LA and with 9.92 mg.mL-1 uric acid (UA) and 1.76 mg.mL-1 ascorbic acid (AA).

where A is the electrode area, s is the scan rate, ΔV is the voltage window, I is current, and V is the voltage. Fig. 4B shows the calibration plot of normalized areal capacitances [$\Delta C_{normalized} = \Delta(C_0\text{-}C_i)/C_0$] for LA sensing in 0.1 - 5000 μM LA solutions, where each point is an average value of C (μF.cm^{-2}) from responses of optimized PDC sensor with repeated measurements of N=4. The area under the CV curves decreased substantially upon increasing the LA concentrations. The decrease in capacitance (charge density) suggests that the LA oxidation products are adsorbed to the PDC microelectrode surface and displace the double layer. The normalized areal capacitance showed a semi-logarithmic relationship with the LA concentrations ranging from 0.1 - 5000 μM. The linear dependence of the output capacitance on the logarithm of LA concentration has a slope of 104.3 $\pm$ 2.88 μF.cm^{-2}.(Log (μM))$^{-1}$ and the correlation coefficient (R^2) of 0.9961. The sensitivity of $PDC_{RIE+LOx}$ to LA can be estimated from the slope of the calibration curve of absolute capacitances (Fig. 4C) as 33. 48 mF.cm^{-2}.(Log(M))$^{-1}$. The dependence of capacitance to the logarithm of LA can be estimated as following (Eq. 4),

$$C = 35.07 + 33.48\ Log(M)^{-1} \tag{4}$$

where C is areal capacitance (mF.cm^{-2}) and M is the concentration (mol.L^{-1}) of LA.

The limit of detection (LoD) for PDC microelectrodes was calculated as 1.45 μM based on linear regression (Eq. 5) [36],

$$LoD = \frac{3S}{b} \tag{5}$$

where S is the standard deviation of the blank response (STDEV = 1.81 mF.cm^{-2}, N = 8) and b is the slope of the calibration curve ($b = -5.5815$ mF.cm^{-2}.(Log (M))$^{-1}$) of absolute areal capacitance.

D. Selectivity and Stability of PDC Microelectrodes

The selectivity of the $PDC_{RIE+Lox}$ was studied by recording the response of the sensor to LA in the presence of uric acid (UA) and ascorbic acid (AA) in the system. The amounts of these interference agents were chosen to be 9.92 mg.mL^{-1} for UA and 1.76 mg.mL^{-1} for AA based on a suggested recipe for artificial human sweat [37]. Fig. 4D shows the areal capacitance of $PDC_{RIE+Lox}$ before adding LA, in the presence of pure 500 μM LA, 500 μM LA with uric acid, and 500 μM LA with uric acid and ascorbic respectively. The response of the sensor to LA in the presence of uric acid and ascorbic acid showed 80.07% of the initial signal, which indicates good selectivity. The operational stability of the $PDC_{RIE+LOx}$ was evaluated by recording the CV response to 500 μM LA every 2 days over 20 days. The results revealed a 2.35% decrease of the capacitance in 20 days,

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TABLE I
COMPARISON OF RECENT ELECTROCHEMICAL CARBON-BASED ENZYMATIC LA SENSORS

SUBSTRATE	ACTIVE ELECTRODE	MEASUREMENT TECHNIQUE	LINEAR RESPONSE RANGE	DETECTION LIMIT	STABILITY	REF
hydrophilic cloth	screen printed carbon electrode	electrochemiluminescence	0.05 - 2.5 mM	0.035 mM	96.4% after 18 days	[1]
cotton fabric	hand printed carbon graphite	amperometry	0.05 – 1.5 mM	5.0 µM	N/A	[2]
screen printed carbon	reduced graphene oxide with Au nanoparticles	amperometry	10 µM – 5 mM	0.13 µM	N/A	[5, 6]
pencil graphite electrode	multi walled carbon nanotubes/copper nanoparticles/polyaniline	amperometry	1.0 µM – 2.5 mM	0.25 µM	60% after 180 days	[7]
SiO_2/Si	interdigitated PDC micro-fingers	capacitance measurement	100.0 nM - 5.0 mM	1.45 µM	97.65% after 20 days	current study

implying sufficiently well long lifetime and stability of the sensor. Table I compares the developed sensor in the current study with some of the recently reported carbon-based LA electrochemical sensors. The obtained detection limit in this work is sufficiently low in comparison to the results reported in the literature. Additionally, the developed sensor provided a wide linear response range, which adequately covers the target ranges of LA concentrations in healthy humans as well as most diseases and head trauma. The linear response range is wider than other carbon-based LA electrochemical sensors. Moreover, the sensor exhibited better stability and selectivity compared to most reports.

The developed PDC based enzymatic sensors can be further enhanced to be more feasible for clinical applications, which could be an interesting topic for future studies. For instance, the performance of the purposed sensor can be improved by studying the effect of the ionic strength of LA and its oxidation products on the sensor's response and activity of LOx enzymes. Various optical-based techniques can be used for studying enzyme activity, such as spectrophotometric assays, fluorometric, and chemiluminescent [38], [39]. Moreover, the developed enzymatic LA sensors can be integrated with other MEMS devices such as lab-on-disk designed by Dr. Madou group for point-of-care applications which is based on a compact disk (CD) centrifugal platform [40]. Furthermore, PDC microelectrodes can be adopted for detecting multiple targets in one sensing unit, which is commonly referred to as multiplexing [41]. Multiplexing the C-MEMS based biosensors can be achieved by minor modifications of the PDC microelectrodes fabrication and functionalization process.

IV. CONCLUSION

In this work, an electrochemical enzymatic lactic acid sensor based on photoresist-derived carbon microelectrode is successfully demonstrated. The oxygen-plasma treatment of photoresist derived carbon microelectrodes enhanced the immobilization of lactate oxidase enzymes on the surfaces of the electrodes through the covalent bonding of enzymes and -COOH groups. Furthermore, the oxygen-plasma treated photoresist derived carbon microelectrodes exhibited higher current generated during the oxidation of lactic acid in the electrolyte. The sensor yielded an extremely low detection limit of 1.45 μM, high sensitivity of 33.48 mF.cm^{-2}.(Log (M))$^{-1}$, and a relatively wide dynamic detection range of 0.1 - 5000 μM. The highly sensitive, selective, and stable LA sensor indicates that the C-MEMS platform is very promising for developing fast, precise, and biocompatible sensors for non-invasive detection of lactic acid.

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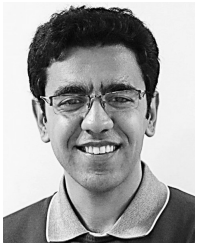

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