Flexible Electrochemical Biosensors based on Graphene nanowalls for the Real-time Measurement of Lactate

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Qianwei Chen,[1,2†] Tai Sun,[2†*] Xuefen Song,[2] QincuiRan,[2] Chongsheng Yu,[2] Jun Yang,[2] Hua Feng,[1*] Leyong Yu,[2] and Dapeng Wei[2*]

† These authors contributed equally to this work and should be considered as co-first authors.

[1]Department of Neurosurgery, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China

[2]Chongqing Key Laboratory of Multi-scale Manufacturing Technology, Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, Chongqing 400714, PR China

E-mail: dpwei@cigit.ac.cn; suntai @cigit.ac.cn; fenghua8888@vip.163.com

Abstract

We demonstrated a flexible lactate detection biosensor based on L-lactate oxidase immobilized by chitosan film cross-linked with glutaraldehyde on the surface of graphene nanowalls (GNWs) electrode. The oxygen-plasma technique was developed to enhance the wettability of GNWs, and the sensors exhibited stronger oxidation response on the concentration of lactate. First, in order to eliminate the interference from other substances, biosensors were primarily tested in deionized water and displayed good electrochemical reversibility at different scan rates (20 mV/s to 100 mV/s), a large index range (1.0 μM to 10.0 mM) and a low detection limit (1.0 μM) for lactate. Next, these sensors were further examined in phosphate buffer solution for mimicking the human body fluids, and still exhibited high sensitivity, stability and flexibility. These results exhibit that the GNWs-based lactate biosensorspossess important potential for the application in clinical analysis, sports medicine and the food industry.

Keywords:Graphene nanowalls, Biosensor, Oxygen-plasma, Lactate oxidase, Lactate.

1. Introduction

Lactic acid, as a product of anaerobic glycolysis of glucose, is the accumulation in the case of hypoxia and fatigue. Lactate content of body fluids and tissues can effectively reflect whether local tissue is under hypoxic conditions.[1]Therefore, fast, easy and accurate lactate detection is important to diseases diagnose and health management, such as myocardial ischemia,[2] brain injury[3] and respiratory failure.[4] At present, the most common lactate detectors are invasive, and need an expensive and complex analysis system.[5] Moreover, the repeated drawings not only cause sustained injury, but fail to monitor the lactic acid in real time. Recent studies have reported several wearable electrochemical lactate biosensors. For example, Thomas et al. developed an amperometric lactate sensor on a contact lens for a minimally invasive detection of lactate in the tears.[6]Jia et al. reported a tattoo-based electrochemical biosensor for noninvasive real-time monitoring of lactate in human sweat.[7] Park et al. designed a mouthguard-based biosensor for salivary lactate monitoring via integrating a printable enzymatic electrode onto the mouthguard.[8] These lactate biosensors could precisely and continuously detect the lactate level of human body fluids, such as saliva, sweat, and tears, in a non-invasive way. However, the fabrication processes are usually complicated, expensive and high in technical requirements. Furthermore, the low accuracy results in failing to precisely reflect changes in lactic acid levels. Thus, it is urgent to develop new lactic acid detection technology to meet the real application in physical health monitoring.

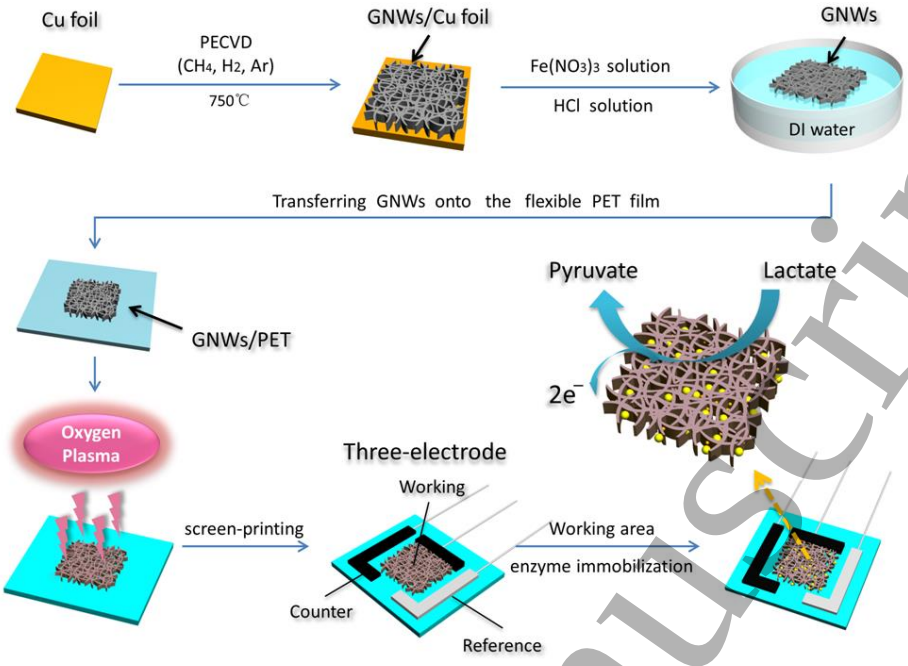

Figure 1. Schematic illustration of the preparation of plasma/GNWs/LOx/PET lactate biosensor.

Over the past decade, graphene has attracted the interest in various scientific fields due to its outstanding physiochemical and electronic properties.[9] Particularly, the extraordinary electrical conductivity, excellent mechanical strength, high thermal conductivity, flexibility and biocompatibility render it a promising material for next-generation sensors.[10-13] A variety of graphene-based biosensors have been reported for the detection of biological molecules, including DNA, protein and biological small molecules.[14-16]Labroo et al fabricated a flexible graphene-based lactate biosensor via transferring graphene to a flexible substrate, patterning with electrodes, and immobilizing a specific enzyme for lactate on graphene.[17] Follow on, the lactate biosensor was successfully developed using a multi-layered graphene.[18] However, in some cases, graphene micro-sheets may suffer from aggregation or re-stacking in macroscopic scale, which may reduce effective accessible surface areas and limit the performance of sensors.[19]To overcome the above mentioned issues, lots of strategies have been developed to achieve 3Dassembled macroscopic structures. Graphene nanowalls (GNWs) are networks of "graphitic" sheets that are typically standing vertically on a substrate.[20]Compared with 2D graphene films, these wall-like 3D structures could not only provide super-large surface area for enzyme loading, but also could work as an eminent electrode network.[21-22] Moreover, the GNWs exhibit an excellent flexibility, due to its vertically aligned graphene nanowalls structure.[23-24] These advantages enable GNWs as highly sensitive, flexible and stable lactate responsive materials.

Here, we developed an electrochemical lactate biosensor based on high-quality GNWs. Firstly, the Plasma Enhanced Chemical Vapor Deposition (PECVD) and O2-plasma techniques were employed for thegrowth and modification of GNWs, respectively. The chitosan-glutaraldehyde crosslinking method was adopted to immobilize a specific enzyme for lactate on GNWs. Then, the SEM-EDS/Mapping techniques were used to determine the optimized time for oxygen plasma treatment on GNWs. Additionally, due to the large specific surface area and outstanding 3D-conductive networks of GNWs, the as-prepared O_2-plasma/GNWs/Lox/PET flexible electrode presentselectrochemical characteristics of sensors were examined by an electrochemical analyzer, which exhibited superior performance for lactate detection with a low detection limit, a wide detection range, high sensitivity and flexibility, good stability and selectivity.We also compare our actual obtained values to these obtained by similar electrochemical biosensing detectors at Table S1 in the supporting information. These results exhibit that the GNWs-based lactate biosensors possess important potential for the application in a variety of fields such as wearable electronics, real-time diagnosis, physical training evaluation of athletes, and even environment health monitoring.

2. Experimental

2.1 Reagents and Instrumentations. L-Lactate oxidase (LOx) (activity: 100 U mg^{-1}) was purchased from Sigma Aldrich (St. Louis, MO). L-lactic acid, uric acid (UA), L-ascorbic acid (AA), chitosan, Glutaraldehyde solution(8%), bovine serum albumin (BSA), acetic acid, potassium phosphate dibasic (Na_2HPO_4) and potassium phosphate monobasic (NaH_2PO_4) were obtained from Sigma Aldrich (St. Louis, MO). All reagents were used without further purification. Ultrapure water (18.2 MΩ cm) was employed in all of experiments.The GNWs were treated with oxygen plas-

ma to improve their wettability by the PLASMA cleaner (PDC-32G).Surface morphology of GNWs was analyzed by scanning electron microscopy (JEOL JSM-7800F), operated at 10kV. The GNWs was also characterized using Raman spectrometer (Renishaw inVia Reflex). Electrochemical characterization was performed at room temperature using an electrochemical analyzer (CHI 760E).

2.2 Preparation of GNWs. Based on our previous work[25], the GNWs film was grown on the surface of a copper (Cu) foil (25μm thick, Alfa Aesar) via radio frequency (RF) plasma enhanced chemical vapor deposition (PECVD). First of all, before the GNWs growth, the Cu foils were annealed at 750 °Cin H_2 for 60 min, in order to improve the quality of the GNWs film. Then, PECVD synthesis was performed using CH_4 and H_2 in a vacuum chamber at temperature of 750 °C under 50Pa. During the growth of GNWs, the gas-flow rate of H_2: CH_4 was kept at 1:1 (8 sccm: 8 sccm) under a 200 mw RF for 1 hour. Thus, the initial GNWs film with special interlaced 3D conductive network was produced on copper foils.

2.3 Fabrication and integration of lactate biosensor. Fig. 1 indicates the preparation processes of the flexible electrochemical biosensors based on GNWs for the real-time measurement of lactate.First, the GNWs/Cu foil was treated with 0.2M $Fe(NO_3)_3$ solution overnight, followed by a 5% HCl solution to completely remove the Cu foil and the Fe nanoparticles. Next, the floating GNWs film was cleaned in deionized (DI) water for several times to remove the residual, and then was transferred onto a flexible PET substrate carefully. Before electrodes printed, the GNWs/PET was treated with oxygen plasma to improve their wettability. The treatment settings were: O_2 flow of 10 sccm, treatment pressure of 0.2 mbar, RF power of 50 W, and treatment time of 3 min.[26] Afterwards, conductive carbon ink was printed firstly as counter electrodes. The second layer, serving as the reference electrode, was printed using Ag/AgCl conductive ink. The third insulating layer was printed using the DuPont 5036 Dielectric paste. Following each printing step, the printed pattern was annealed at 85 ° C for 15 min.

After the three electrodes printing, the working electrode was modified with the functional layer.[27] First, 5 μL Lox solution of 50 mg mL^{-1} (with the BSA stabilizer of 15 mg mL^{-1}) was spread over the GNWs and dried at room temperature. Next, this surface was covered with 5 μ L chitosan solution (1 wt % prepared in 0.8 wt % acetic acid). Then, 5μL glutaraldehyde of 0.025% (m/v) was covered onto the chitosan covered electrode and dried for an hour at room temperature. Finally, the sensor was immersed in a 0.1M phosphate buffer solution (PBS) for 20 min in order to remove the excess non-bound enzyme from the electrode surface. The sensor was stored in 0.1M PBS at 4 °C.

2.4 Electrochemical characterization. In order to eliminate the interference from other substances, the property of sensor was tested in deionized water. Firstly, three samples of four different groups were measured in electrochemical characterization. The four groups of samples including GNWs group (normal GNWs only), GNWs + Plasma group (O_2-plasma treated GNWs), GNWs + Lox group (normal GNWs covered with Lox), GNWs + Plasma + Lox group (O_2-plasma treated GNWs covered with Lox) were prepared for a cyclic voltammetry test with a scan rate of 100 mV/s from −1.0 to 1.0 V in 10 mM lactate. Next, a series of cyclic voltammetry tests of GNWs + Plasma + Lox groups were conducted with different scan speeds from 20mV/s – 100mV/s, using a potential step to +0.3 V (vs Ag/AgCl) for 60s. Follow on, the amperometric response was recorded in a variety of magnitude concentration of lactate solution respectively, under a +3 V (vs Ag/AgCl) potential and a 100mV/s scan rat for 60s.

3. Results and Discussion

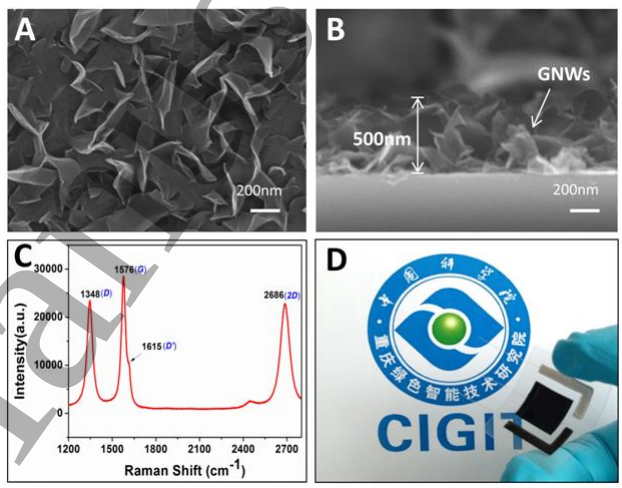

Figure2. (A) Typical SEM image of GNWs. (B) Cross-sectional SEM view of GNWS. (C) Raman spectrum of GNWs. (D) A flexible GNWs/PET-based original device.

Fig. 2A displays typical morphology of GNWs, which reveals that the entire surface is fully covered by continuous free-standing graphene nanosheets. The corresponding cross-sectional image of GNWs in Fig. 2B shows that the height of GNWs is approximately 500nm. It is clear that these free-standing graphene nanosheets were connected with each other to form a three-dimensional (3D) structure, which could provide a large loading area for lactate oxidase.[23-24] Meanwhile, the synthesized GNWs could also act as a 3D-conductive network for faster electronic transmission in the process of lactate detection.[21-22] In addition, Raman spectrum was employed to evaluate the quality of the GNWs, as shown in Figure 2C. It is observed that a strong D band peak at 1348 cm^{-1} and D' band peak at 1615 cm^{-1}, suggesting that lots of edges and defects exist in graphene nanosheets.[28] It has been found that the graphene edges of zig-zag or armchair types would result in high electrocatalytic activity toward several biological molecules.[29] Moreover, the defect sites at their basal planes could also play a similar role to graphene edges.[30] Thus, a lot of edges and defects on GNWs may jointly give this sensor with highly electroactive and fast kinetics for biological molecules. After printing three electrodes on

GNWs/PET substrate, flexible electrochemical devices could be fabricated, as shown in Fig. 2D.

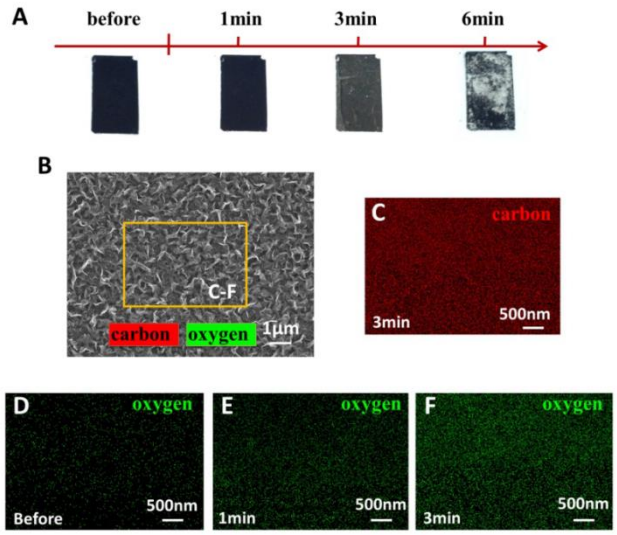

Figure3. The treatment process of GNWs by O_2-plasma. (A) The optical imagesof GNWs after the O_2-plasma treatment. (B)The SEM image of GNWs area for mapping test, marked by a square frame, to analyze the elementcontent of GNWs after O_2-plasma treatment. (C)The Mapping of carbon element after 3 min. (D-F)The mappings of oxygen element after a 0, 1, 3min O_2-plasma process.

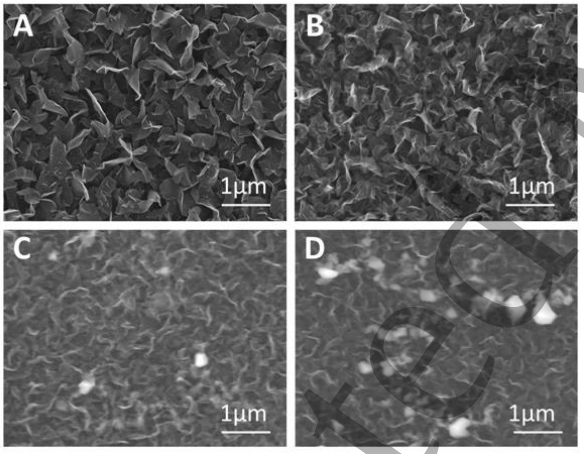

Figure 4. The SEM images of four groups of GNWs materials: (A) The non-treatment group. (B) The 3min O_2-plasma treatment group. (C) The only enzyme immobilization treatment group. (D) The 3min O_2-plasma treatment plus enzyme immobilization group.

In order to improve the enzyme immobilization ability, we adopted the oxygen plasma technology to increase the wettability of GNWs surface[26]. The GNWs samples were processed with a consecutive multiple exposures to O_2-plasma. As shown in Fig. 3A, the structures of GNWs did not suffer an obvious damage after the treatment for 1 or 3 minutes but for 6 minutes. Then, in order to compare the different efficiencies of O_2-plasma treatment time between 1 minutes and 3 minutes, we evaluated the chemical compositions of oxygen element of GNWs samples using EDS mapping technology. In Fig. 3B, the area of GNWs, marked by a square frame, was selected for the EDS and Mapping analysis. The mapping image of carbon element on GNWs surface is shown in Fig. 3C. In fact, the experimental results that the mapping of carbon element after 0, 1, 3minutes oxygen plasma are same. Meanwhile, both EDS (in supporting information) and Mapping analysis (in Fig. 3D-F) show the obvious increase of oxygen element on GNWs surface after O_2-plasma treatment, and more oxygen content with the treatment of 3 minutes than 1 minute, suggesting that the 3-minute O_2-plasma exposure was optimal for GNWs modification. Enzyme immobilization ability on GNWs was evaluated in Fig. 4. It is found that GNWs would suffer no significant damage after 3 min O_2-plasma treatment. More importantly, the plasma-treated GNWs (in Fig. 4D) display more enzyme immobilization than that of GNWs without plasma treatment (in Fig. 4C). The reason might be that the O_2-plasma process would provide more surface functional sites to connect with enzyme.

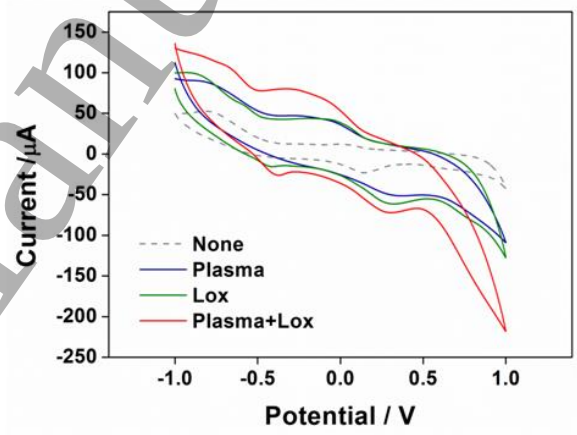

Figure 5. Cyclic voltammograms of GNWs/PET based electrode, plasma/GNWs/PET based electrode, GNWs/Lox/PET based electrode, Plasma/GNWs/Lox/PET based electrode in 10mM L-lactate deionized water solution. Lox represents Lactate oxidative enzyme.

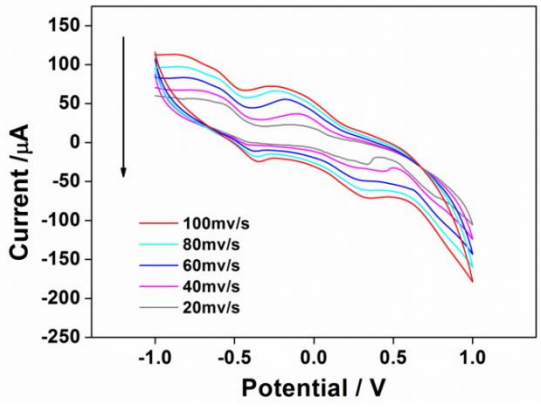

Figure 6. Cyclic voltammograms of optimized sensor in 10mM L-lactate deionized water solution with different scan rates (20-100mV/s). $E_{applied}$ = +0.35 V (vs Ag/AgCl).

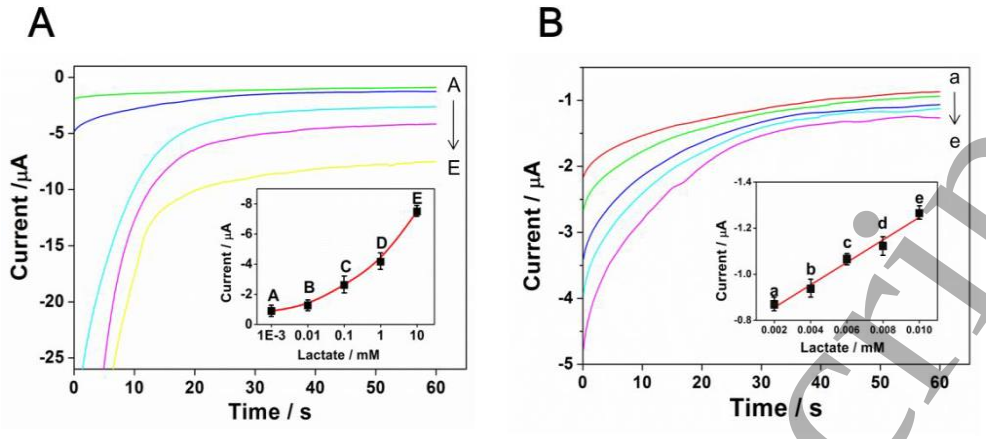

Figure 7. Amperometric responses to different concentrations of lactate (A) From 1μM to 10mM and (B) From 0.002mM to 0.01mM. All experiments were performed with $E_{applied}$=+0.35 V (vs. Ag/AgCl) and a current sampling time of 60 s. The inset is the corresponding calibration curve.

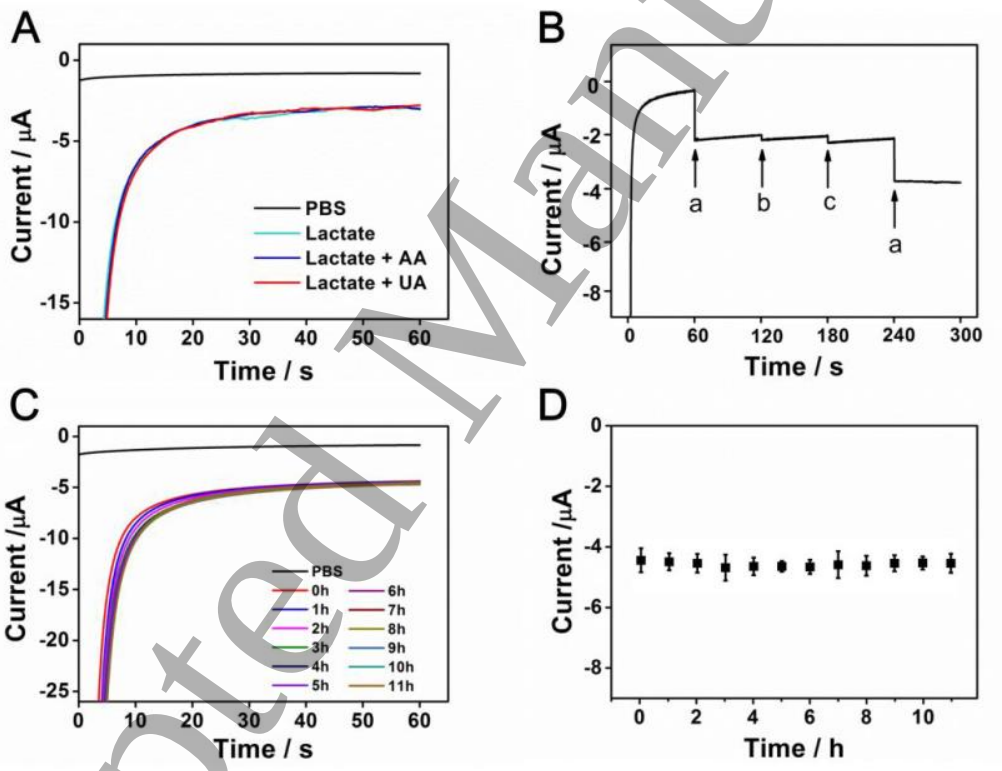

Figure 8.(A) Selectivity of the biosensor. Response to 4mM lactate in 0.1M PBS in the presence of electroactive physiological interferents. AA indicates ascorbic acid (10μM), UA indicates uric acid (59 μM). $E_{applied}$=+0.35 V (vs. Ag/AgCl). (B) Dynamic selectivity study: Response to (a) 4 mM lactate, (b) 10 μM ascorbic acid, (c) 59 μM uric acid at +0.35 V (vs Ag/AgCl). (C) Stability of the response to 8 mM lactate at 1 h intervals over a 11 h period. (D) The corresponding current-time plot of the chronoamperometric response.

Fig. 5 shows the cyclic voltammograms of the pristine and modified GNWs electrodes in 10 mM L-lactate solution. Two electrochemical reactions occurred on the electrodes.[5] The biochemical recognition was catalyzed by Lox:

$$\text{lactete} + O2 \rightarrow \text{pyruvate} + H2O2 \qquad (1)$$

The signal was generated by the transduction of the biochemical recognition to current via the following electrochemical redox reaction:

$$H2O2 \rightarrow 2H^{+} + O2 + 2e^{-} \qquad (2)$$

The pristine GNWs electrode shows no observable response, whereas three other modified GNWs electrodes

exhibit obvious oxidation and deoxidization peaks. The plasma/GNWs electrodes caused high current response, which may be attributed to the increased oxygenic groups of GNWs by O_2-plasma. The plasma combined with enzyme immobilization electrodes displayed more remarkable redox peaks, compared with the only enzyme immobilization treated electrodes. These results revealed that plasma assisted interface engineering would effectively enhance the current response to lactate, which might be explained by that plasma promoted more immobilization of enzyme onto the surface of GNWs, as shown in Fig 4C and D.

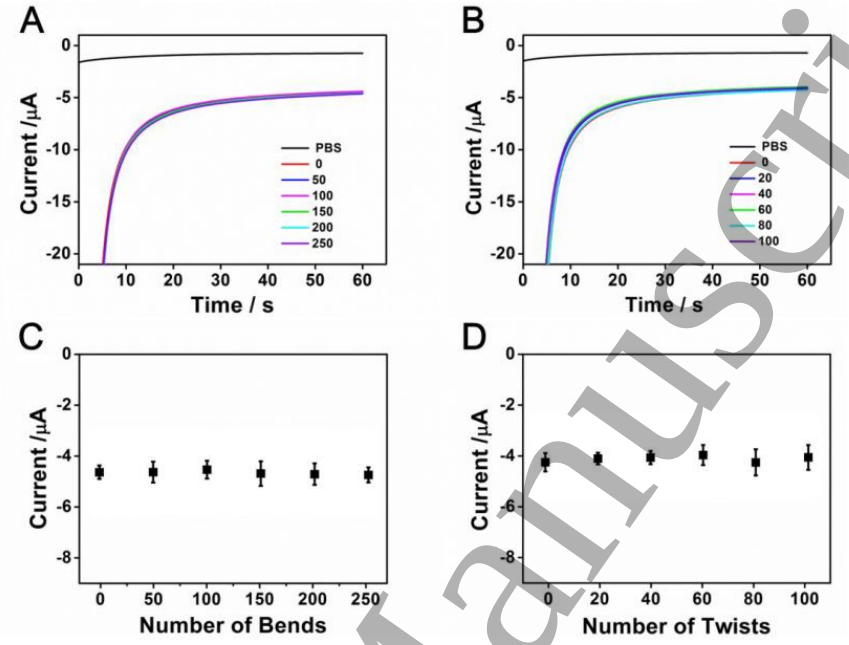

Figure 9. Electrochemical response of optimized GNWs lactate biosensor undergoing repeated bending (A) and twisting (B) to 8 mM lactate at +0.35 V (vs Ag/AgCl), and their normalized current (C) and (D), respectively.

In addition, typical cyclic voltammograms of our optimal biosensors in 10mM L-lactate deionized water solution with different scan rates are shown in Figure 6. The peak potential is independent on the scan rate in the range from 20 to 100 mV/ s, which implies that such lactate biosensor has good electrochemical reversibility. The dynamic sensor response as function of L-lactate concentration was subsequently investigated. Figure 7A shows chronoamperograms obtained with the optimized sensor in 0.001- 10M lactate deionized water solution. The GNWs sensor displays well-defined lactate concentration dependence, with a logistic increasing curve response from the 1μM to 10 mM and a linear response from 0.002mM to 0.01mM (in Fig. 7A, B). Importantly, this biosensor displays a higher sensitivity toward lactate than the previous reports, [17-18][31-32] which may be attributed to that the GNWs provided large specific surface area for enzyme loading and excellent 3D-conductive network for charge conduction. Furthermore, the O_2-plasma technique further enhanced the quantity of enzyme on GNWs.

The specificityof the sensor was evaluated in lactate solution, which contained ascorbic acid and uric acid to mimic human sweat.[33] Figure 8A displays the similar chronoamperometric response to 4mM of lactate in both presence and absence of such physiological concentrations of uric acid (59μM) and ascorbic acid (10μM). Furthermore, as shown in Fig. 8B, the biosensor responded favorably to 4 mM lactate, while the selected interfering agents induced responses were negligible. These results reflect the highly special recognition of this GNWs-based sensor. The stability of the lactate sensor was further examined using 8mM lactate solution in 0.1M PBS (pH7.0). The stability was evaluated according to the variance of current response over a continuous 11 h period, which was recorded every 1 hour. Figure 8C and D show current responses and the corresponding repetitive chronoamperograms, which reveal a high stability over the 11 hours monitoring.

Furthermore, we tested the flexibility of sensor in 8mM lactate solution (0.1M PBS, pH7.0), as shown in Figure 9. The response of the biosensor after bending (Fig. 9A) and twisting (Fig. 9B) remain highly uniform, and the corresponding normalized data (Fig. 9C and D) are highly stable with anrelative standard deviation (RSD) of 1.31% and 1.49%, respectively. These results indicate a highly stable current response over the entire 250 times bending and 100 times twisting, suggesting that the biosensor could be applied in human epidermis directly. The well-performed flexibility of sensor can be attributed to the excellent flexibility of GNWs material and PET substrate.

4. Conclusions

In summary, we developed a GNWs-based electrochemical biosensing technique for high-sensitive lactate detection, which included plasma-enhanced CVD

for GNWs growth, oxygen plasma assisted interface engineering and enzyme immobilization method using chitosan film cross-linked with glutaraldehyde. Compared with GNWs/LOx/PET electrode and O_2-Plasma/GNWs/PET electrode, the catalytic current for lactate oxidation at the O_2-Plasma/GNWs/LOx/PET electrode was significantly increased. Due to the large specific surface area and outstanding 3D-conductive networks of GNWs, the as-prepared O_2-plasma/GNWs/Lox/PET flexible electrode presents good sensitivity, specificity, stability, and excellent flexibility. Such flexible GNWs-based biosensor exhibits its potential in a variety of fields such as wearable electronics, real-time diagnosis, physical training evaluation of athletes, and even environment health monitoring.

Acknowledgements

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