Volume 14, Issue 2, Page 188-194, 2025 https://doi.org/10.46810/tdfd.1626235 Research Article

BİNGÖL UNIVERSITY

Türk Doğa ve Fen Dergisi
Turkish Journal of Nature and Science
www.dergipark.gov.tr/tdfd

Use of L-Lactate Dehydrogenase Immobilized on Carboxylated Multiwalled Carbon Nanotubes/Polyaniline/Pencil Graphite Electrode as a Lactate Biosensor

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(Received: 24.01.2025, Accepted: 07.05.2025, Online Publication: 27.06.2025)

Keywords
Biosensor, Lactate dehydrogenase, Pencil graphite electrode, Carbon nanotubes

Abstract: In this study, L-Lactate dehydrogenase (L-LDH) was covalently immobilized on carboxylated multiwalled carbon nanotubes (cMWCNT)/polyaniline (PANI)/pencil graphite electrode (PGE). LDH/cMWCNT/PANI/PGE was used as a lactate biosensor. Electrochemical polymerization of PANI was carried out using a three-electrode cell technique via cyclic voltammetry (CV). The characterization of LDH/cMWCNT/PANI/PGE electrode was achieved using electrochemical and scanning electron microscopy (SEM) techniques. The effect of pH and lactate concentration (with and without NAD^+) on biosensor was assessed. The optimal pH was determined as 7.0. The current densities tend to increase with increasing of lactate concentration in NAD^+ containing solution during the forward potential scan. These current values were obtained as 0.026 and 0.038 mA cm^{-2} for 0.166 and 1.331 mM lactate solution with NAD^+ as a cofactor, respectively at +0.2 V. As a result, LDH/cMWCNT/PANI/PGE biosensor showed good bio-electrocatalytic activity.

Karboksillenmiş Çok Duvarlı Karbon Nanotüpler/Polianilin/Kalem Grafit Elektrot Üzerine İmmobilize Edilen L-Laktat Dehidrojenazın Laktat Biyosensörü Olarak Kullanımı

Anahtar Kelimeler
Biyosensör, Laktat dehidrojenaz, Kalem grafit elektrot, Karbon nanotüpler

Öz: Bu çalışmada, L-Laktat dehidrojenaz (L-LDH), karboksillenmiş çok duvarlı karbon nanotüpler (cMWCNT)/polianilin (PANI)/kalem grafit elektrot (PGE) üzerinde kovalent olarak immobilize edilmiştir. LDH/cMWCNT/PANI/ laktat biyosensörü olarak kullanılmıştır. PANI'nin elektrokimyasal polimerizasyonu, dönüşümlü voltametri (CV) ile üç elektrot tekniği kullanılarak gerçekleştirildi. LDH/cMWCNT/PANI/PGE elektrotunun karakterizasyonu, elektrokimyasal ve taramalı elektron mikroskobu (SEM) teknikleriyle gerçekleştirilmiştir. pH ve laktat derişiminin (NAD^+ ile ve olmadan) biyosensör üzerine etkileri araştırılmıştır. Optimum pH 7,0 olarak belirlendi. Akım yoğunlukları, ileri potansiyel taraması boyunca NAD^+ içeren çözeltideki laktat derişiminin artmasıyla birlikte artma eğilimindedir. +0,2 V' ta akım değerleri, kofaktör olarak NAD^+ ile 0,166 ve 1,331 mM laktat çözeltisi için sırasıyla 0,026 ve 0,038 mA cm^{-2} olarak belirlenmiştir. Sonuç olarak, LDH/cMWCNT/PANI/PGE biyosensörü iyi bir biyo-elektrokatalitik aktivite göstermiştir.

1. INTRODUCTION

Lactate is an important metabolite, being formed in all tissues, such as the skeletal brain, muscle, red blood cells, and kidneys under anaerobic conditions [1]. The determination of lactate level is also a crucial indicator for clinical diagnostics, fermentation, food analysis and sports medicine [2,3]. Blood lactate concentration is one of the important parameters in predicting some diseases such as multiple organ failure. Lactate balance is related to acid–base homeostasis, which its accumulation leads to lactic acidosis. Lactic acidosis causes some discomfort such as muscle cramps, body weakness and diarrhea [4,5].

To date, various non-enzymatic methods have been used for the determination of lactate, such as colorimetric method [6], spectrophotometric and titrimetric techniques [7], liquid chromatography [8,9], voltammetric [10], and proton nuclear resonance methods [11]. L-lactate dehydrogenase (L-LDH) is a usually used enzyme in the design of L-lactate biosensors for the determination of lactate because of their simple sensor design and comparatively effective enzymatic detection. This enzyme works in the presence of an oxidized/reduced form of nicotinamide adenine dinucleotide (NADH or NAD) that is electrochemically detected using an appropriate detector [12]. L-LDH has a high catalytic activity in the conversion of lactate to pyruvate and NAD^+ to its reduced form NADH (Fig. 1).

There is a need for a requirement that acts as a shuttle for its electrons and electrode installation. For this, NAD/NADH is used as a mediator because NADH can provide high sensitivity to measure the amount of lactate. LDH immobilization on the electrode surface is also necessary to increase the performances of the electrochemical detectors, mainly their sensitivity and performance. Therefore, there is an increasing momentum for the immobilization of L-LDH as well as selection of electrode materials to obtain robust lactate biosensor. Different electrodes have been used to determine the lactate, such as gold electrodes, platinum electrodes, and carbon-based electrodes [13,14]. Carbon-based electrodes are preferred because of their wide usage range, low cost, and inertness [15]. Additionally, carbon electrodes have more advanced electrochemical properties than noble metals. They cause the oxidation/reduction of both of organic and biological molecules in aqueous and non-aqueous reaction mediums. Some materials can easily crack and fall from the surface of the electrode, and this situation leads to the loss of enzymes.

Pencil graphite electrodes have attracted much attention because their preparation and surface modification are easier than other carbon-based electrodes. They are preferred for immobilizing of L-LDH or binding nanoparticles to form reliable modified biosensors when conducting polymers like polyaniline are used [16]. Among the advantages of coating the electrode surface with PANI film are the controllable oxidative polymerization properties, such as superior environmental stability, anti-interference properties, thickness, and permeability. Investigation of excellent biosensor designs using PANI together with various different nanomaterials (carbon nanotubes (CNT), graphene (GR), platinum nanoparticles (PtNPs), gold nanoparticles (AuNPs), etc.) has popularized the use of these materials to obtain wide-ranging biosensors [17-19].

Recently, the use of CNTs has appeared as a versatile tool to provide important supports for the stabilization of enzymes [20-22]. Nanoparticles strongly affect the mechanical properties of the materials such as stiffness and elasticity. Furthermore, they offer biocompatible environment for enzyme immobilization. CNTs have attracted enormous interest because of their unique structural, small size, large surface area, and mechanical and electronic properties [23-25]. Modified electrodes with CNTs have most frequently been used for electro-analytical purposes to improve many biosensors [26-28]. Multi-walled carbon nanotubes are regarded as electrical conductors with highly effective properties as electrodes for enzyme biosensors [29].

This study describes the development of an L-LDH biosensor modified with PANI and cMWCNT on PGE. The surface morphology of LDH/cMWCNT/PANI/PGE electrode was achieved via SEM techniques. The optimal pH value was found as 7.0. The influence of lactate concentration (in the absence and presence of NAD^+) was also studied on biosensor properties. The current values increased in NAD^+ containing environment with increasing of lactate concentration during the potential scan (from -0.2 to 0.6 V).

2. MATERIAL AND METHOD

2.1. Materials

L-lactate dehydrogenase (from porcine), sodium L-lactate (98%), and NAD^+ (nicotinamide adenine dinucleotide disodium hydrate), and carboxylic acid-functionalized multi-walled carbon nanotubes (MCNT-COOH) were purchased from Merck. All other chemicals were used without further purification. Pencil lead (2B) was purchased from the local market.

2.2. Activation of Carboxylated Multiwalled Carbon Nanotubes

MWCNT-COOH was activated according to our previous publication [30]. Briefly, 1.0 mL of MES buffer (50 mM pH 6.1) and 4.0 mL of 5% N-hydroxysuccinimide (NHS) solution were treated with 1.0 g of MWCNT and stirred for 10 min at 25 °C. Subsequently, 1.2 mL of 1% fresh N-(3-Dimethylaminopropyl)-N′-ethyl carbodiimide (EDC) solution was added. The mixture was stirred for 2 hours at 25 °C. Then, the supports obtained were filtered under vacuum and washed with distilled water. Finally, the activated supports were kept at 5 °C overnight.

2.3. Preparation of L-Lactate dehydrogenase/Carboxylated Multiwalled Carbon Nanotubes/Polyaniline/Pencil Graphite Electrode

The biosensor preparation process is schematically presented in Fig. 2. In this study, PANI was electrochemically synthesized on PGE in 0.1 M HCl solution containing 0.1 M aniline with electrochemical analyzer. A conventional three-electrode setup was used, employing PGE as the working electrode and a platinum sheet (2 cm^2) as the counter electrode where an Ag/AgCl (3 M KCl) was the reference. All the potentials were reported with respect to the reference electrode. CV method was utilized for the electrochemical polymerization of aniline. Applied potential range was between -1.0 and +1.0 V with 100 mV s^{-1} scan rate. After that, activated cMWCNT was adsorbed on the working electrode by the dripping and drying method. In the last step, 5 mL of L-LDH solution (1.0 mg mL^{-1}) was dripped on the electrode surface. L-LDH was immobilized and dried at 4 °C for 6 h.

2.4. Electrochemical Measurements

An electrochemical analyzer (CHI660C) was used to perform all electrochemical measurements. A lactate biosensor was fabricated by connecting of LDH/cMWCNT/PANI/PGE as the working electrode. In addition, platinum sheet (2 cm^2) and Ag/AgCl (3 M KCl) was utilized to be counter, and reference electrodes, respectively. Cyclic voltammograms of the LDH/cMWCNT/PANI/PGE electrode was recorded in buffer solution with and without NAD^+ containing lactate (0.166 – 1.331 mM) from -0.2 and +0.6 V with 50 mVs^{-1} scan rate.

2.5. Surface Morphology of the Materials

Surface morphology of the different electrodes was examined by field emission scanning electron microscopy (FE-SEM, Zeiss, Supra 55).

3. RESULTS AND DISCUSSION

In this study, pencil leads (pencil graphite electrode) were preferred for the modified LDH biosensor because of their good electrical conductivity, low cost, and easy availability. When compared to many other electrodes, the regeneration of the surface has an important effect for subsequent analysis since it may easily cause a change in the electrochemical reaction of the molecule, and in the surface properties of electrode. The regeneration of the electrode surface may change the selectivity and sensitivity of the lactate measurements. In the literature, Purushothama et al. developed a simple electrochemical sensor for investigation of chlorpromazine using pencil leads and they reported good electrocatalytic activity [31]. Batra et al. designed LDH/GrONPs/PGE based on immobilization of LDH on graphene oxide nanoparticles-PGE and characterized the surface of the electrode by SEM [32].

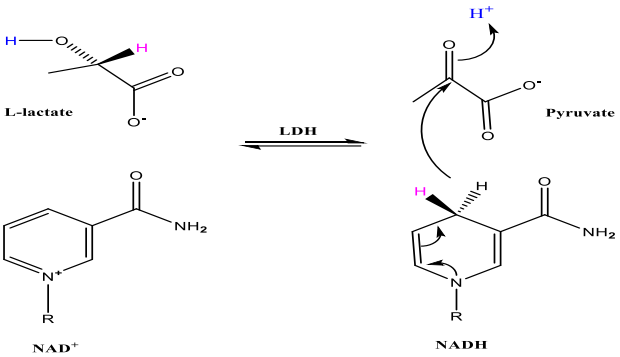

Figure 1. Schematic representation of L-LDH catalytic activity.

Fig. 2. shows the schematic demonstration of construction of enzyme working electrode based on the covalent immobilization of L-LDH on cMWCNT/PANI/PGE. One of the most significant aspects in the development of the LDH biosensor is the immobilization of LDH on the surface of the PGE. First, the PGE surface was successfully coated with PANI film using the electropolymerization method before L-LDH was immobilized, as shown in Fig. 3.

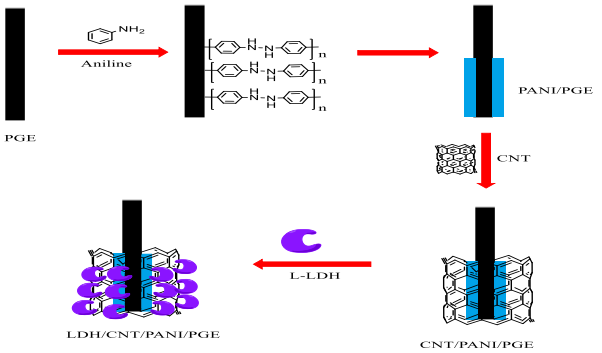

Figure 2. Schematic representation of steps and chemical reaction involved in the preparation of LDH/cMWCNT/PANI/PGE.

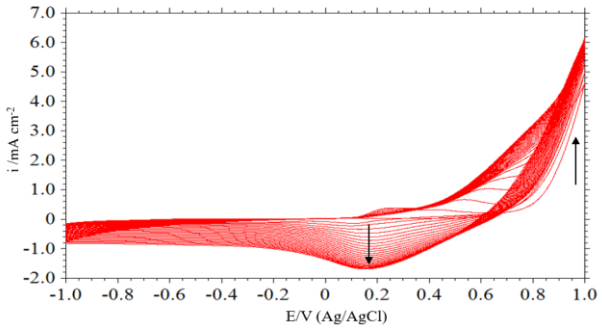

Figure 3. Electropolymerization of PANI on PGE with 100 mV s^{-1} scan rate.

The film thickness can be controlled for PANI-coated PGE using CV. PANI is used here as a good electron transfer provider because of its good reduction and oxidation properties between the reaction site and the PGE surface and biomolecules. cMWCNT is generally used as electron tunneling center to increase the electrocatalytic behavior of PANI. Thus, synergistic enhancement between PANI and cMWCNT can improve the biosensor property by facilitating the electron transfer rate. As a result, PANI offers an efficient material for

novel electrochemical L-LDH biosensors with cMWCNT.

Recently, a lot of enzyme biosensors based on nanoparticles or nanotubes have been developed to increase the performance of enzyme-based biosensors [33-35]. Zhang et.al reported that direct electrochemical oxidation of NADH at classic electrodes had large overpotential (>1 V), and performance (stability, sensitivity etc.) of electrochemical determination of NADH have been inadequate in the samples [36]. Therefore, carbon nanotubes (CNTs)-based electrodes are used to significantly decrease overpotential for electrochemical oxidation of NADH [37-39]. CNTs have been offered in development of sensors design thanks to electrical properties, chemical stability, and high surface-to-volume ratio [40-43]. In the literature, Luo et.al reported that effect of PANI/CNT for HRP biosensor and enhanced stability and eight times more sensitivity [44]. Granot et al. reported that charge transport property of CNT/PANI hybride system as 3.5 times higher than without CNT biosensor system for glucose measurements [45]. The surface morphology of different electrodes was investigated by SEM. Figs. 4a and b shows SEM images of bare PGE. SEM image of PANI/PGE shows that PGE covered with PANI layer as given in Figs. 4c and d. The binding of cMWCNT onto PANI/PGE was depicted in Figs. 4e and f. SEM image for LDH/cMWNT/PANI/PGE in Figs. 4g and h shows different morphology from cMWCNT/PANI/PGE. The obtained image indicates that LDH successfully immobilized to the surface of the cMWCNT/PANI/PGE. The step by step modification of PGE can be seen clearly from these presented SEM images.

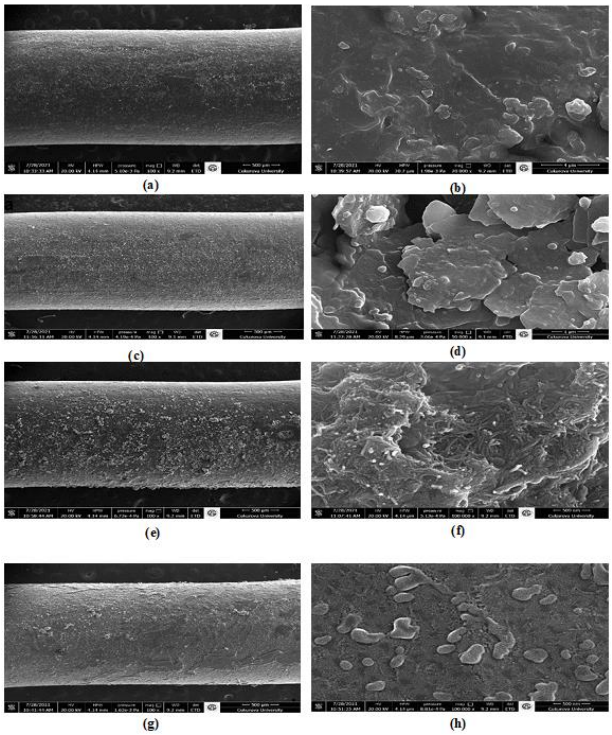

Figure 4. SEM images of materials: PGE a (100x) and b (20000x), PANI/PGE c (100x) and d (50000x), cMWCNT/PANI/PGE e (100x) and f (100000x), LDH/cMWCNT/PANI/PGE g (100x) and h (100000x).

L-LDH enzyme activity depends on the working pH. LDH activity of the biosensor at different pH values is not the same; the structural differences of the enzyme at different conditions are associated with changes in the surface regions of the LDH molecule. A plot of relative activity on the LDH/cMWNT/PANI/PGE with different pH values (5.0-9.0) in buffer solution was determined spectrometrically, as shown in Fig. 5. Different pH buffer solutions showed a strong impact on activity of biosensing layers. The relative activity increases with increasing pH from 4.0 to 7.0. The relative activity reaches a maximum value at pH 7.0. The activity decreases with an increase in pH above 7.0. This situation is raleted to NAD^+ which is unstable in alkali medium. Thus, pH 7.0 is the optimal pH for the lactate biosensor. This range of values was reported as optimal pH range for this enzyme, which is between 6.0 and 8.0 [38,46]. Istrate et al. reported that an increase in LDH activity was obtained in pH range 2.0-7.5 and maximum value was 7.5 for GA-LDH/AuNPs-ERGO-PAH/SPE [38]. Rahman et al. prepared pTTCA/MWNT/LDH/NAD^+ as a modified electrode for lactate determination and characterized the surface modification of the designed electrode by SEM. In their study, LDH and NAD^+ were covalently immobilized on the surface, and determined as 7.0 effect of pH on the current responses [39].

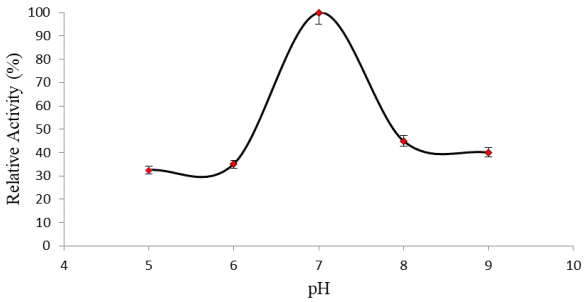

Figure 5. The effect of pH value on LDH/cMWCNT/PANI/PGE.

NAD^+/NADH coenzymes behave to be mediators to shuttle electrons between electrode and enzyme. Nevertheless, the difficulty in incorporating the coenzymes into the biosensor, as well as its regeneration (high oxidation potential) frequently poses problems. Therefore, L-LDH has attracted more attention for the production of amperometric lactate sensors [47].

The obtained CV results for the LDH/cMWCNT/PANI/PGE electrode were given in Figs. 6a and b in buffer solutions without and with NAD^+ containing lactate solution (0.166 – 1.331 mM), respectively. The applied potential range was from -0.2 to +0.6 V with 50 mV s^{-1} scan rate. As seen from Fig. 6a, current densities from -0.2 to +0.6 V potential scan decreased generally with increasing of lactate concentration. In this forward potential scan, current values were found as 0.034, 0.027, 0.030 and 0.024 mA cm^{-2} at +0.2 V for lactate solutions from 0.166 to 1.331 mM without NAD^+, respectively. On the other hand, these current densities were determined as 0.026, 0.031, 0.037 and 0.038 mA cm^{-2} at +0.2 V for from 0.166 to 1.331 mM lactate solutions with NAD^+, respectively. Thus, current values have a tendency to increase in NAD^+ containing

solution with increasing of lactate concentration at the forward potential scan. These results show that NAD^+ as a cofactor has an important effect on the increase of the current densities after 0.332 mM lactate solution.

In the literature, Tian et al., [43] was produced a biosensor with the immobilization of lactate oxidase and horseradish peroxidase on flower-like $NiCo_2O_4$ microspheres coupled with single-walled carbon nanotubes (SWCNTs). It was found that synergistic effect between SWCNTs and $NiCo_2O_4$ increase the conductivity and the active surface area of the material. Chronoamperometry method was used for the electrode at different lactate concentrations. The results showed that current response increases with increasing of the lactate concentration.

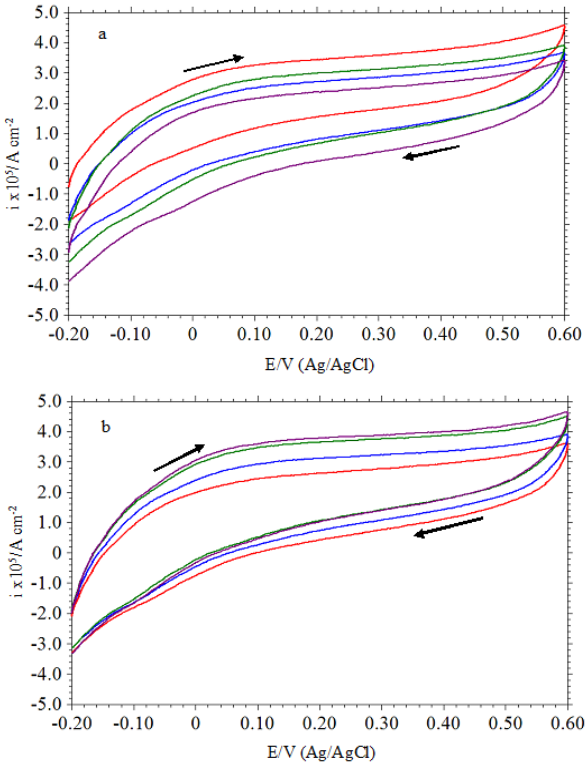

Figure 6. CV results of biosensor without (a) and with (b) NAD^+; 0.166: —, 0.332: —, 0.998: —, 1.331 mM: — containing lactate solution.

4. CONCLUSIONS

LDH/cMWCNT/PANI/PGE electrode showed a good electrocatalytic property to the oxidation of lactate when the cMWCNT was used for the design of the biosensor. The formation of cMWCNT/PANI composite improves the electrical and mechanical properties of the conductive polymer material. Furthermore, NAD^+ is not need to measure low lactate concentrations. However, NAD^+ as a mediator is required at high lactate concentrations. The current densities increased with the increasing of lactate concentration in NAD^+ containing solution during the forward potential scan. These current densities at +0.2 V were found as 0.026 and 0.038 mA cm^{-2} for 0.166 and 1.331 mM lactate solution with NAD^+, respectively. Consequently, LDH/cMWCNT/PANI/PGE biosensor exhibited good bio-electrocatalytic activity.

Acknowledgements

The authors would like to thank Cukurova University Scientific Research Project Unit for supporting the study (Project Number: FBA-2020-13283).

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