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A lactate biosensor based on lactate dehydrogenase/nictotinamide adenine dinucleotide (oxidized form) immobilized on a conducting polymer/multiwall carbon nanotube composite film

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ABSTRACT

An amperometric lactate biosensor was developed based on a conducting polymer, poly-5,2′-5′,2″-terthiophene-3′-carboxylic acid (pTTCA), and multiwall carbon nanotube (MWNT) composite on a gold electrode. Lactate dehydrogenase (LDH) and the oxidized form of nicotinamide adenine dinucleotide (NAD^+) were subsequently immobilized onto the pTTCA/MWNT composite film. The modified electrode was characterized by quartz crystal microbalance (QCM), scanning electron microscopy (SEM), and electrochemical experiments. The detection signal was amplified by the pTTCA/MWNT assembly onto which a sufficient amount of enzyme was immobilized and stabilized by the covalent bond formation between the amine groups of enzyme and the carboxylic acid groups of the pTTCA/MWNT film. Experimental parameters affecting the sensor responses, such as applied potential, pH, and temperature, were assessed and optimized. Analytical performances and dynamic ranges of the sensor were determined, and the results showed that the sensitivity, stability, and reproducibility of the sensor improved significantly using pTTCA/MWNT composite film. The calibration plot was linear ($r^2 = 0.9995$) over the range of 5 to 90 μM. The sensitivity was approximately 0.0106 μA/μM, with a detection limit of 1 μM, based on a signal/noise ratio of 3. The applicability of the sensor for the analysis of L-lactate concentration in commercial milk and human serum samples was demonstrated successfully.

The development of biosensors has been the subject of considerable interest. Such devices offer great promise for biomolecule detection with features that include high sensitivity, inherent miniaturization, low cost, independence of sample turbidity or optical path length, minimal power demands, and high compatibility with modern microfabrication technologies [1]. The development of electrochemical biosensors for sensitive and selective detection of biomolecules commonly requires innovative approaches that couple different modification/amplification processes [2–5]. Here a simple, selective, and sensitive biosensor for L-lactate detection is proposed.

L-Lactate is constantly produced from pyruvate by lactate dehydrogenase (LDH)[2] 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. For example, the concentration of L-lactate in blood is a fundamental parameter for the prevention and diagnosis of a number of clinical disorders such as hypoxia [6], some acute heart diseases, and drug toxicity tests. The most common analytical method for determination of lactate concentration is high-performance liquid chromatography (HPLC) [7] with either ultraviolet (UV)–spectrophotometric or refractive index detection. However, this method is time-consuming because of the requirement for pretreatment and the complicated procedure involved. Therefore, the demand for a sensitive, simple, and accurate method has arisen.

Over the past few years, various approaches based on lactate oxidase or LDH have been developed for the detection of lactate. They employ different methods to attach enzymes on the sensing layer, including adsorption [8], cross-linking [9], covalent attachment [10,11], conducting polymer entrapment [12,13], and confinement in sol-gel matrix [14]. Flow injection analysis [15,16] and mediator-based lactate biosensors [17] have also been developed.

In the LDH-based amperometric biosensor, LDH catalyzes the oxidation of lactate to pyruvate in the presence of the oxidized form of nicotinamide adenine dinucleotide (NAD^+). This catalytic reaction detects amperometrically with the sensing layer. However, the LDH-based biosensors have some common drawbacks, including the fact

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[2] *Abbreviations used:* LDH, lactate dehydrogenase; HPLC, high-performance liquid chromatography; UV, ultraviolet; NAD^+, oxidized form of nicotinamide adenine dinucleotide; NADH, reduced form of nicotinamide adenine dinucleotide; CNT, carbon nanotube; Au, gold; pTTCA, poly-5,2′-5′,2″-terthiophene-3′-carboxylic acid; MWNT, multiwall carbon nanotube; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; PB, phosphate buffer; SEM, scanning electron microscopy; QCM, quartz crystal microbalance; TBAP, tetrabutylammonium perchlorate; CV, cyclic voltammogram; PBS, phosphate-buffered saline; RSD, relative standard deviation.

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that they are unstable and that the electrochemical oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) commonly occurs at high potentials, which can allow interference from other electroactive compounds usually present in real samples. Moreover, the intermediates produced during the oxidation reaction can lead to electrode fouling. Therefore, there is a demand for the development of a biosensor that is stable, reproducible, and sensitive for lactate detection in real samples. More recently, Cui and coworkers proposed a stable and sensitive biosensor system based on a positively charged polymer and carbon nanotube (CNT) nanocomposite on a gold (Au) electrode [18].

CNT [19] has received a great deal of attention as an electrode material because it has good electrocatalytic properties [20,21]. CNT can display metallic, semiconducting, and superconducting electron transport and possesses a hollow core suitable for storing guest molecules. These unique properties of CNT make it extremely attractive for chemical sensors, particularly for electrochemical detection of biological compounds [22,23]. Such potential applications would benefit greatly from the ability of CNT to promote the electron transfer reactions of important biomolecules, including cytochrome *c* [24], NADH [25], catecholamine neurotransmitters [26], and ascorbic acid [27].

Conducting polymer has received considerable attention as an immobilizing matrix for biomolecules in biosensor systems [28,29]. Carboxylic acid group functionalized conductive polymer, poly-5,2′-5′,2″-terthiophene-3′-carboxylic acid (pTTCA), has advantages to immobilize biomolecules through covalent bond formation and, consequently, offers stable and sensitive biomolecule detection [30–32]. Recently, the formation of polymer/CNT composites has been explored for possible improvement in the electrical and mechanical properties of polymers [33]. CNT-based nanocomposites usually exhibit a higher conductivity than materials loaded with other fillers because of the high intrinsic conductivity and the high aspect ratio of CNT [34]. Thus, cconductive polymer/CNT composite film can be a useful platform for immobilizing biomolecules to enhance sensor performances. Such a composite-modified electrode combines the ability of CNT and conductive polymer to promote electron transfer reactions with the advantages of entrapped electrode materials. To our knowledge, pTTCA/CNT composite film has not yet been reported for biosensor applications.

Here we report a simple and sensitive electrochemical method for lactate detection based on pTTCA/multiwall carbon nanotube (MWNT) composite film coimmobilized with LDH and NAD^+ on an Au substrate. Initially, a pTTCA/MWNT composite film was made on the electrode surface. Thereafter, the LDH and NAD^+ were immobilized sequentially on the pTTCA/MWNT film, followed by an *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) activation step. Experimental parameters affecting the sensor responses, such as applied potential, pH, and temperature, were assessed and optimized. Analytical performances of the sensor in terms of the dynamic linear range, sensitivity, selectivity, reproducibility, and stability of the electrode were determined. Finally, the applicability of the sensor for the analysis of lactate concentration in real samples was demonstrated.

Materials and methods

Materials

L-Lactate dehydrogenase (811 U/mg, rabbit muscle), sodium L-lactate (98%), and NAD^+ (disodium hydrate) were purchased from Sigma. MWNT (4–12 nm diameter, 99%) was purchased from Iljin Nanotech. EDC and *N*-hydroxysuccinimide (NHS) were purchased from Sigma. MWNT was purified by refluxing in 3 M HNO_3 for 3 days. After precipitation and centrifugation, the MWNT was washed with deionized water at least three times until a neutral pH value was reached. Finally, the solid sample was vacuum-dried for approximately 4 h and stored at room temperature until use. A terthiophene monomer bearing a carboxylic acid group, TTCA, was synthesized and characterized according to our previously reported procedure [35,36]. All other chemicals were of extra pure analytical grade and used without further purification. Phosphate buffers (PBs) of 0.1 mM NaH_2PO_4 and 0.1 mM Na_2HPO_4 (0.1 mM PB, pH 6.8 or 7.0) were used. All aqueous solutions were prepared with doubly distilled water, which was obtained from a Milli-Q water purifying system (18 MΩ cm). The commercial milk samples were collected from a local Korean market. Human serum samples were purchased from Sigma–Aldrich.

Apparatus

The electrochemical experiments were performed using a KST-P1 potentiostat/galvanostat (Kosentech and BAS CV-50 W Systems). All electrochemical measurements were carried out in a 5-ml cell containing a modified Au working electrode (3 mm diameter), an Ag/AgCl reference electrode, and a platinum wire counter electrode. Scanning electron microscopy (SEM) images were obtained with a Cambridge Stereoscan 240 (KBSI). An Au working electrode (0.196 cm^2 area, 9 MHz, AT-cut quartz crystal) was used for quartz crystal microbalance (QCM) experiments.

Functionalization of MWNT

The carboxylic acid functionalization of MWNT was carried out by acid treatment [37]. Briefly, 100 mg of purified MWNT was refluxed in 35 ml of a mixture of concentrated HNO_3 and H_2SO_4 (1:3, v/v) for 13 h. The contents were allowed to cool, filtered, and then washed extensively with deionized water until no further change in the filtrate pH was observed. After drying at 80 °C under vacuum overnight, a black powder was obtained. Then 9.82 mg of this black CNT was added to 3.5 ml of distilled water, and the stock solution with a concentration of 2.8 mg/ml was obtained by dispersing with the aid of ultrasonic agitation. The buffer containing a definite amount of black CNT (10 mg/4 ml) was prepared with the aid of ultrasonic agitation, and then the solution was filtered through 0.45-μm syringe filters and dried overnight. The functionalized MWNT was stored until use.

Preparation of the pTTCA/MWNT film onto Au electrode

Prior to its modification, the Au electrode was polished with 0.3- and 0.05-μm alumina slurries and rinsed with doubly distilled water. The purified MWNT (0.1%) was mixed in 1.0 mM TTCA monomer solution in 0.1 M tetrabutylammonium perchlorate (TBAP)/CH_2Cl_2 by ultrasonication for 8 h. The MWNT comprising conducting polymer layer onto an Au electrode was obtained through the electropolymerization reaction of MWNT/TTCA mixture solution in 0.1-M TBAP/CH_2Cl_2 by cycling the potential between 0 and 1.6 V at a scan rate of 0.1 V/s. After that, the electrode was washed with CH_2Cl_2 to remove the excess monomer.

Immobilization of LDH/NAD^+ onto the pTTCA/MWNT film

The LDH and NAD^+ were immobilized onto the pTTCA/MWNT surfaces using the procedure developed previously for bienzyme immobilization onto the pTTCA-modified electrode [38]. First, the pTTCA/MWNT-modified electrode was immersed for 6 h in a 0.1-M PB (pH 7.0) containing a mixed solution of 10 mM EDC and 10 mM NHS to activate the unbound and free carboxylic groups of pTTCA/MWNT composite layer. Then EDC-treated electrode was washed thoroughly three times with a 0.1-M PB (pH 6.8) to remove excess EDC and NHS and was incubated for 3 h in a 0.1-M PB

(pH 7.0) containing 1 mg/ml solution of LDH at 4 °C. NAD^+ was then successively immobilized onto the pTTCA/MWNT/LDH-modified layer by incubating the electrode for 3 h in a 0.1-M PB (pH 6.4) containing 10 mM NAD^+ solution at 4 °C (Scheme 1, stated as pTTCA/MWNT/LDH/NAD^+ electrode). The polyTTCA electrode was modified with LDH and NAD^+ in a similar way to that described above for pTTCA/MWNT electrode except that polyTTCA was substituted as the initial sensing layer (stated as pTTCA/LDH/NAD^+ electrode).

Electrochemical measurements

Cyclic voltammograms (CVs) were recorded at pTTCA/MWNT/LDH/NAD^+ electrode from –0.2 to +0.7 V versus Ag/AgCl in a 0.1-M PB (pH 6.8). Chronoamperometric experiments were carried out by applying the potential of 0.3 V. A freshly prepared 0.1-M PB (pH 6.8) was placed into the cell, and the steady-state current on the first lactate addition was monitored with the pTTCA/MWNT/LDH/NAD^+ sensor, at the optimal pH and temperature, with stirring. Chronoamperometric experiments were continued with the successive addition into the cell of varying amounts of lactate.

Results and discussion

Scheme 1 outlines the biosensing protocol using the pTTCA/MWNT/LDH/NAD^+-modified electrode. We used covalent bond formation to immobilize the enzyme and NAD^+ on the electrode surfaces. First, the conducting polymer/MWNT layer was formed by the potential cycling method. Thereafter, LDH was immobilized on the pTTCA/MWNT surfaces by the amide bond formation between the unbound carboxylic acid groups on the film and the amine groups of the enzyme. After this stable attachment of LDH, NAD^+ was immobilized at unreacted carboxylic acid groups on the polymer/MWNT layer by immersing the pTTCA/MWNT/LDH-modified electrode in an NAD^+ solution. The enzymatic reactions involved in the biosensing system are as follows (see Scheme 1B):

$$\text{Lactate} + NAD^+(\text{LDH}) \rightarrow \text{Pyruvate} + \text{NADH} + H^+ \quad (1)$$

$$\text{NADH} \rightarrow NAD^+ + H^+ + 2e^-. \quad (2)$$

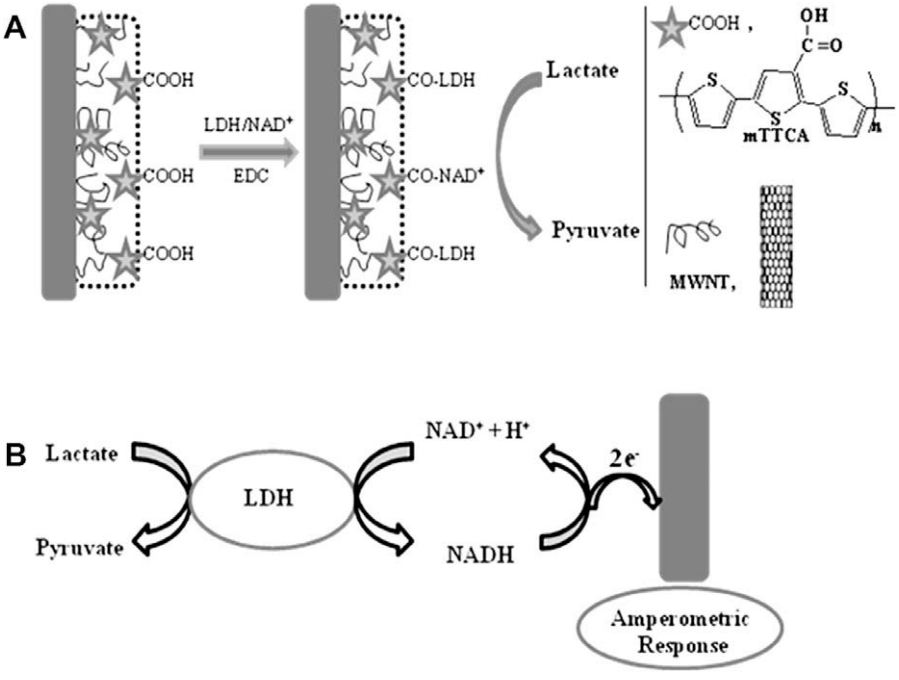
Reaction (1) is lactate dependent. On the electrode surface, NADH is oxidized (Reaction 2) and the oxidation current is directly proportional to the lactate concentration in the solution.

Characterization of the polymer/MWNT-modified electrode

The pTTCA/MWNT layer was formed by the electropolymerization of TTCA/MWNT solution in 0.1 M TBAP/CH_2Cl_2 (not shown). For comparison, the pTTCA layer was formed on an Au electrode through the electropolymerization reaction of TTCA solution in 0.1 M TBAP/CH_2Cl_2 by cycling the potential between 0 and 1.6 V at a scan rate of 0.1 V/s, following a previous report [2]. During the first anodic scan of TTCA monomer, the CV exhibited one oxidation peak at approximately 1.3 V due to the TTCA oxidation. A polymer reduction peak appeared at approximately 1.1 V in the reverse cathodic scan. The peak currents at approximately 1.1 and 1.3 V increased as the potential cycle numbers increased. For the pTTCA/MWNT layer, in the forward anodic scan, the monomer oxidation peak appeared at approximately 1.4 V, and in the reverse cathodic scan, a new peak appeared at approximately 0.6 V along with the polymer reduction peak, and it shifted approximately 0.3 V from 1.1 to 0.8 V. With increasing potential cycle numbers, the peak currents at approximately 1.4, 0.8, and 0.6 increased, indicating the formation of the pTTCA/MWNT layer on the electrode surfaces. During the formation of the pTTCA/MWNT layer, higher peak currents for all peaks were observed compared with those obtained for the pTTCA layer. This was due to the presence of the MWNT with TTCA in the composite solution.

Determination of the amount of immobilized LDH and NAD^+

QCM studies were carried out to determine the amount of LDH and NAD^+ on the pTTCA/MWNT-modified electrode. During the immobilization of LDH, the frequency decreased and attained a steady state after 2 h (Fig. 1, curve a). This indicates that the immobilization of LDH onto the polyTTCA layer was completed within 2 h at room temperature. Therefore, we immobilized LDH for the



Scheme 1. (A) Schematic view for the fabrication of pTTCA/MWNT/LDH/NAD^+-modified electrode. (B) Mechanism of lactate oxidation in the presence of LDH/NAD^+.

first 1 h (Fig. 1, curve b), and immediately after immobilizing LDH the electrode was used to immobilize NAD^+ onto it for another 1 h (Fig. 1, curve c), followed by three washings with phosphate-buffered saline (PBS). After the immobilization of LDH for 1 h onto the pTTCA/MWNT layer, the frequency change was 0.66 kHz and the mass change was determined using the equation reported previously [36]. The amount of LDH immobilized on the pTTCA/MWNT/LDH electrode after 1 h was 0.725 ± 0.08 μg. For 1 h NAD^+ immobilization on the electrode, the frequency change was 0.27 kHz. The amount of NAD^+ was determined to be 0.31 ± 0.06 μg. The numbers of molecules per area for LDH onto the pTTCA/MWNT layer and NAD^+ onto the pTTCA/MWNT/LDH layer were estimated to be $1.51 \pm 0.6 \times 10^{-11}$ mol cm^{-2} and $3.0021 \pm 0.41 \times 10^{-9}$ mol cm^{-2}, respectively.

Surface characterization using SEM

The morphology of the modified electrodes was characterized by SEM. Fig. 2 shows SEM images of the gold substrate coated with pTTCA, pTTCA/MWNT, and pTTCA/MWNT/LDH. The SEM image of pTTCA/MWNT film (Fig. 2B) onto the Au electrode shows the presence of some extra black lines (indicated with arrow) that were not observed in the SEM image for the pTTCA layer on the same substrate (Fig. 2A). The presence of these lines is due to the attachment of MWNT on the pTTCA layer. The SEM image for pTTCA/MWNT/LDH (Fig. 2C) also shows a very different morphology from that obtained for the pTTCA/MWNT layer, indicating that LDH successfully immobilized to the surface of the pTTCA/MWNT layer.

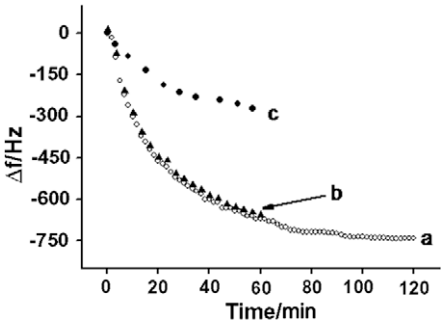


Fig. 1. Frequency shifts obtained from QCM experiments during immobilization of LDH and NAD^+. A 2-h continuous immobilization of LDH onto the pTTCA/MWNT layer (a), the first 1-h immobilization of LDH onto the pTTCA/MWNT layer (b), and the second 1-h immobilization of NAD^+ onto the same electrode (c) are shown.

Detection of lactate with the pTTCA/MWNT/LDH/NAD^+- and pTTCA/LDH/NAD^+-modified electrodes

Fig. 3 shows the CVs recorded for the pTTCA/MWNT/LDH (dotted curve) and pTTCA/MWNT/LDH/NAD^+-modified (solid curve) electrodes in a 0.1-M PB (pH 6.8) containing 5.0 μM lactate solution. No peak was observed when the CV was recorded with the pTTCA/MWNT/LDH for 10 μM lactate solution due to the absence of the cofactor NAD^+ on the sensing layer. An anodic peak was observed at approximately 0.3 V versus Ag/AgCl for 10 μM lactate solution, and this was due to the oxidation of lactate in the presence of NAD^+ on the sensing layer. The catalytic oxidation current at approximately 0.3 was found to be increased on increasing the lactate concentration in the detection buffer solution.

LDH and NAD^+ can be directly attached onto the polyTTCA layer (without the MWNT layer) by amide bond formation between the carboxylic groups of polymer and the amine groups of the protein. Thus, we compared the sensitivity of the polyTTCA/MWNT/LDH/NAD^+- and polyTTCA/LDH/NAD^+-modified electrodes (not shown) in a 0.1-M PB (pH 6.8) containing 10 μM lactate solution. No catalytic reduction was observed with the polyTTCA/LDH/NAD^+-modified electrodes, possibly due to the insufficient sensitivity of the sensing layer when 10 μM lactate solution was used in the detection buffer. However, when 100 μM lactate solution was present in the detection buffer, a four times lower magnitude of peak current at approximately 0.3 mV was observed. A more than 40-fold (peak height ratio × concentration factor = 4×10) sensitivity enhancement

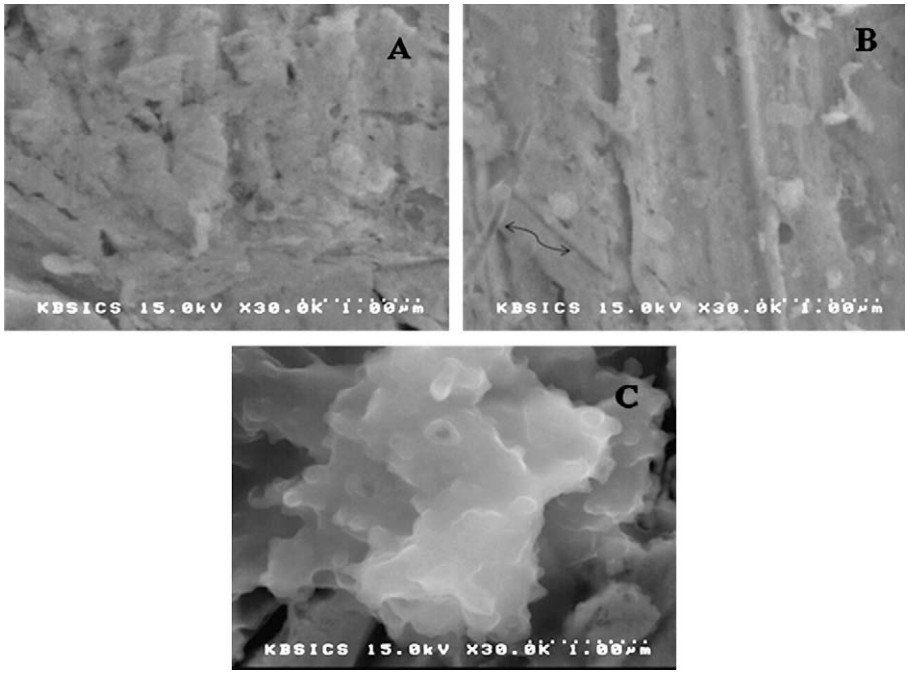


Fig. 2. SEM images of pTTCA (A), pTTCA/MWNT (B), and pTTCA/MWNT/LDH (C) on Au substrates.

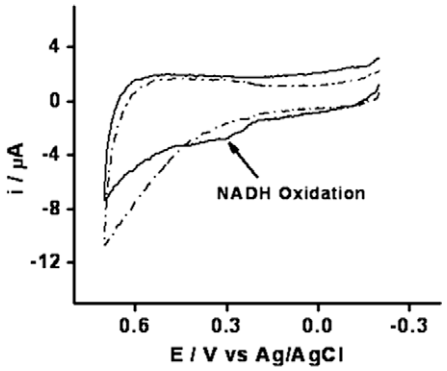


Fig. 3. CVs recorded for 5 mM lactate solution in 0.1 mM PB (pH 6.8) with the sensor in the absence (dotted line) and presence (solid line) of NAD^+ in the biosensing layer. The scan rate was 0.1 V/s versus Ag/AgCl.

was obtained using a polyTTCA/MWNT/LDH/NAD^+-modified electrode. The enhancement of the signal is due to both the catalytic activity and larger surface area provided by the pTTCA/MWNT layer.

Optimization of experimental conditions

The experimental conditions affecting the sensitivity of the sensor were optimized in terms of pH, temperature, and applied potential, and they are shown in Fig. 3. The pH of the buffer shows a strong effect on the activity of the biosensing layers. The effect of the pH of the buffer on the current responses was studied over the pH range of 5.0 to 8.0. Fig. 4A shows the CV peak currents obtained at different pH values for 10 μM lactate in 0.1 mM PB. The peak height increased from pH 4.0 to 6.8 and then decreased above pH 6.8. The oxidation peak current decrease above pH 6.8 might have been due to the poor enzyme activity at higher pH. Therefore, PB at pH 6.8 was used for all experiments.

The effect of the applied potential on the amperometric current response was also studied with the pTTCA/MWNT/LDH/NAD^+-modified electrode. The CV current response for 10 μM lactate in 0.1 mM PB increased as the applied potential increased from 0.0 to 0.3 V. The maximum response was attained at 0.3 V, and the application of the more positive potential up to 0.6 did not improve the current response (Fig. 4B). Thus, 0.3 V was chosen as the optimal potential for amperometric detection of lactate.

The effect of the temperature on the sensor response was studied. Fig. 4C shows the temperature dependence of the current response for 10 μM lactate in 0.1 mM PB (pH 6.8). The peak current gradually increased as the temperature increased from 15 to 30 °C and then began to decrease as the temperature increased above 35 °C. Above 40 °C, the peak current decreased nearly 50% because of the thermal inactivation of enzymes on the electrode surfaces. For practical convenience, all experiments were carried out at 30 °C.

Interference effect

The selectivity of the pTTCA/MWNT/LDH/NAD^+ biosensor was evaluated amperometrically in the presence of other electroactive compounds such as glutamate, ascorbic acid, glucose, uric acid, dopamine, and acetaminophen. No amperometric response was observed when 0.5 mM glutamate, ascorbic acid, glucose, uric acid, dopamine, and acetaminophen were introduced into 0.1 mM PB (Fig. 5). When 5 μM lactate solution was added to the electrolyte solution, a clear oxidation response was observed, indicating the selective detection of lactate with the pTTCA/MWNT/LDH/NAD^+ sensing layer. Importantly, at this concentration level, glutamate, ascorbic acid, glucose, uric acid, dopamine, and acetaminophen have no interference for 5 μM lactate detection. Thus, the selectivity of the pTTCA/MWNT/LDH/NAD^+ biosensor is acceptable for lactate detection in the presence of the common interfering compounds in normal physiological concentrations.

Calibration curve and amperometric response

Fig. 6 shows a typical current–time plot for the addition of varying amounts of lactate in a 0.1-M PB (pH 6.8). The currents increased steeply to a stable value as soon as the potential was applied. The pTTCA/MWNT/LDH/NAD^+ electrode achieved 95% of steady state currents within 10 s. The inset in Fig. 6 shows the calibration plots for the lactate determination obtained from the cur-

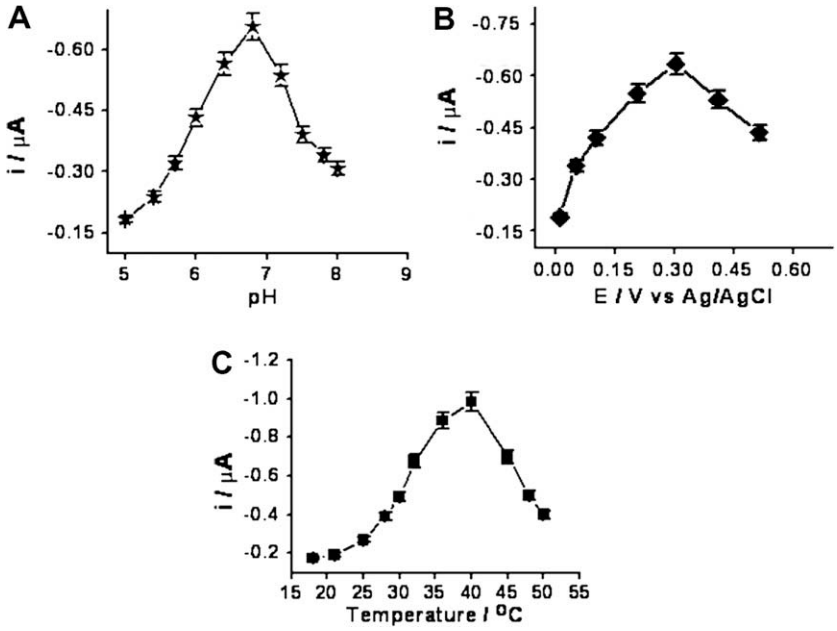


Fig. 4. Effects of the sensing solution pH (A), applied potential (B), and temperature (C) on the oxidation peak current for 10 μM lactate obtained with the pTTCA/MWNT/LDH/NAD^+ sensor in 0.1 mM PB solution from the CV experiments. The scan rate was 0.1 V/s versus Ag/AgCl. Data obtained were the averages of four measurements ($n = 4$).

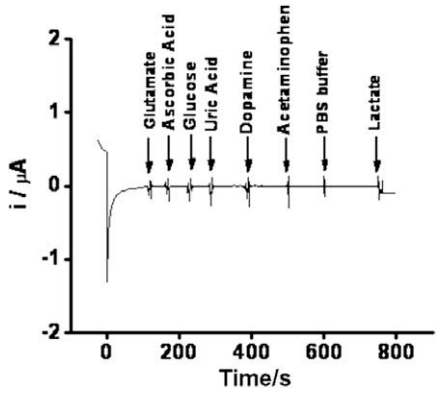


Fig. 5. Amperometric detection of 0.5 mM glutamate, ascorbic acid, β-glucose, uric acid, acetaminophen, and 5 μM lactate with the pTTCA/MWNT/LDH/NAD$^+$ sensor. The amperometric potential was 0.3 V/s versus Ag/AgCl.

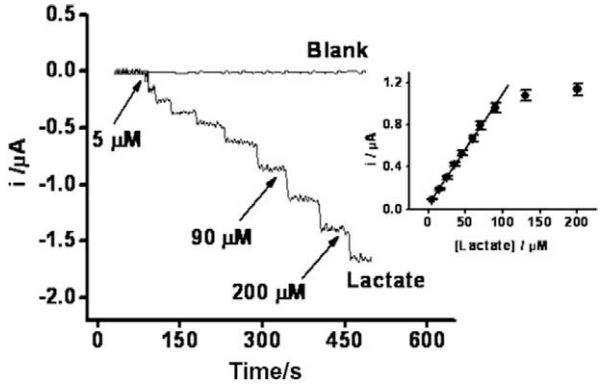


Fig. 6. Current–time curve obtained with a pTTCA/MWNT/LDH/NAD$^+$ sensor. Inset: Calibration plot for a pTTCA/MWNT/LDH/NAD$^+$ sensor.

rent responses with pTTCA/MWNT/LDH/NAD$^+$ electrode. Under the optimized conditions, the steady-state currents showed a linear relationship with the lactate concentration in the range from 5 to 90 μM. The linear dependence of the lactate concentration yielded the regression equations of i_p (μA) = (0.036 ± 0.005) + (0.0106 ± 0.00028) [*C*] (μM), with a correlation coefficient of 0.9995. The detection limit for lactate detection was determined to be approximately 1 μM, based on three times measurement for the standard deviation of the blank noise (95% confidence level, $k = 3$, $n = 5$). This detection limit is much lower than the detection limits obtained in previously reported lactate biosensors [39–41] and is comparable to the value obtained in the Au nanoparticle-based amperometric lactate sensor [42].

Reproducibility and long-term stability of the biosensor

A series of five successive measurements of 5 μM lactate in 0.1 mM PB yielded a good reproducible signal at pTTCA/MWNT/LDH/NAD$^+$ sensor with a relative standard deviation (RSD) of 4.3%. The sensor-to-sensor and run-to-run reproducibility for 0.5 μM lactate detection were found to be 1.7 and 1.1%, respectively. To examine the long-term storage stabilities, the response for the pTTCA/MWNT/LDH/NAD$^+$ sensor was examined with respect to the storage time (not shown). After each experiment, the sensor was washed with the buffer solution and stored in 0.1 mM PBS at 4 °C. The long-term storage stability of the sensor was tested every 5 days. The sensitivity retained 98% of initial sensitivity up to 30 days. After 30 days, the response gradually decreased, possibly due to the loss of the enzyme activity. The above results clearly suggested that the sensor can be used for 1 month without any significant loss in sensitivity.

Real samples analysis

The proposed lactate biosensor was tested to detect the lactate concentration in commercial milk and human serum samples. The real samples were diluted 1000 times with 0.1 mM PB before performing amperometric experiments. The standard addition method was followed, and the lactate concentration in real samples was determined from standard addition curves. The lactate concentrations in the serum and milk samples were found to be 0.22 ± 0.01 and 0.18 ± 0.006 mM, respectively. The reproducibility in current response for the real samples analysis was 5.8% ($n = 4$). The lactate concentrations measured in the real samples were compared with those obtained by a biochemical analyzer (Shimadzu 7200), where the lactate concentrations were found to be 0.24 ± 0.01 and 0.174 ± 0.01 mM for serum and milk samples, respectively. This result demonstrated that the current lactate sensor can be used for monitoring lactate in serum and milk samples.

Conclusion

A new biosensor for lactate detection has been developed based on a carboxylic acid-functionalized conductive polymer/CNT composite film onto which LDH and NAD$^+$ were coimmobilized. The sensor was characterized by SEM, QCM, and electrochemical experiments, and it showed excellent electrocatalytic oxidation response at approximately 0.3 V versus Ag/AgCl. The pTTCA/MWNT composite allowed enormous active sites onto the biosensing layer and was used to complete immobilization of sufficient amount of LDH through the covalent bond formation, resulting in a sensitive and stable biosensor system. It also avoids the use of NAD$^+$ in the solution phase for lactate detection. The performance of the proposed sensor is excellent in terms of sensitivity, selectivity, response time, and reproducibility. The developed sensor was applied to detect lactate concentration in human serum and commercial milk, and satisfactory results were obtained.

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