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Novel L-lactic acid biosensors based on conducting polypyrrole-block copolymer nanoparticles†

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The development of advanced nanomaterials for the highly efficient electrical detection of biological species has attracted great attention. Here, novel polypyrrole-Pluronic F127 nanoparticles (PPy-F127 NPs) with conducting and biocompatibility properties were synthesized and used to construct a L-lactic acid biosensor that could be applied in biochemical assays. The PPy-F127 NPs were characterized by transmission electron microscopy (TEM), elemental analysis and UV-vis spectroscopy. Lactate oxidase (LOx) structure variation on the PPy-F127 NPs was investigated by circular dichroism (CD). The cyclic voltammetric results indicated that LOx immobilized on the PPy-F127 NPs exhibited direct electron transfer reaction with a formal potential value ($E^{0\prime}$) of 0.154 V *vs.* SCE. Moreover, the biosensor had good electrocatalytic activity toward L-lactic acid with a wide linear range (0.015–37.5 mM) and a low detection limit of 0.0088 mM. The regression equation was I (μA) = 0.02353c (mM) + 1.4135 (R^2 = 0.9939). The L-lactic acid biosensor had a good anti-interference property towards uric acid (UA), ascorbic acid (AA), glucose and cysteine. The idea and method provide a promising platform for the rapid development of biosensors that can be used in the detection of biological species.

1. Introduction

L-Lactic acid, one of the two stereoisomers of lactic acid, exists widely in the metabolins of humans, animals and microorganisms. L-lactic acid level is usually related to freshness, stability and the storage quality of foods, and can be used as an indicator of fermentative processes in the food industry. The determination of the L-lactic acid level in tissues is essential for the evaluation of meat quality,[1,2] and it can be employed to distinguish potential Pale Soft Exudative (PSE) muscle from Dark Firm Dry (DFD) and normal muscles.[3] Traditional methods for the quantitative detection of L-lactic acid concentration are mainly based on spectrophotometry,[4–6] and comprise complex procedures (*e.g.* reagent preparation and sample pre-treatment), take a long time, and require expensive instrumentation and trained operators.[7,8] Therefore, great attention has been paid to designing a simple, rapid and sensitive device for L-lactic acid detection. In the last decade, electrochemical biosensors have been increasingly applied to L-lactic acid determination because of their comparatively low cost, ease of operation, excellent sensitivity and good selectivity.[9–11]

Analysts are always enthusiastic about finding new materials with good properties to improve the behavior of biosensors. As is well known, conducting polymers have been the subject of much interest, not only from a fundamental scientific perspective but also from a practical point of view, such as in various functional applications, including solar cells, separation membranes, molecular electronic devices and biosensing devices. Among this class of applications, biosensors are at the forefront because of the conducting polymer's inherent charge transport properties and biocompatibility.[12–16]

In recent years, the application of novel nanomaterials in bioassay has become a hot topic, and many research papers have been published.[17–24] The dimensions of nanomaterials are similar to those of biomolecules such as enzymes. Nanomaterials provide an ideal remedy to the contradictory issues for the optimization of immobilized enzymes, and incur minimum diffusion limitations, and offer the maximum surface area per unit mass and highly effective enzyme loading.[25] It is believed that conducting polymer nanomaterials offer great promise for novel applications in biosensors, and will be widely employed in the construction of sensing devices when their physicochemical properties are modified for more and more specific needs.[26]

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In this paper, polypyrrole-Pluronic F127 nanoparticles (PPy-F127 NPs), a novel conducting polymer nanomaterial, were designed and synthesized. Furthermore, lactate oxidase (LOx) and the PPy-F127 NPs were immobilized on the surface of a glass carbon electrode (GCE) to construct a novel enzyme biosensor. These novel nanomaterials were employed in the design of biosensors, which were further used in biochemical assays of L-lactic acid in pig muscle samples.

Why did we choose Pluronic F127 (triblock copolymer $PEO_{106}PPO_{70}PEO_{106}$, F127) as the doped agent for the PPy nanoparticles? Firstly, it is difficult to obtain PPy nanoparticles with the desired small size and good dispersion in the absence of F127. F127 can be used as a template and dispersant for preparing nanoparticles with special shapes.[27,28] Secondly, as is well known, F127 has a good biocompatibility. Thus, the LOx immobilized on PPy-F127 NPs could retain their secondary structure and biological activity in the following electrochemical experiments. Next, we wondered if it was possible that we could take advantage of the cooperation effects, which mixed good biocompatibility with the electronic conductive property of PPy-F127 NPs, to design and prepare a novel L-lactic acid biosensor that could be used in the detection of L-lactic acid. This is the subject of this research work. More details about the preparation process and the electrochemical behaviors of the proposed novel L-lactic acid biosensor are presented. The results indicated that the modified electrode has a wide linear range and a low detection limit. Furthermore, the L-lactic acid biosensor could be used to determine L-lactic acid in a real sample analysis without interference.

2. Experiments

2.1. Materials

F127, L-lactic acid, Nafion solution (5 wt%) and LOx were purchased from Sigma-Aldrich Co. (USA); pyrrole (99%) was received from ACROS Organics Co. Ltd. (USA); and ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was purchased from Aladdin Chemistry Co. Ltd. (China). All the above reagents were used without further purification. Phosphate-buffered saline 0.1 M, pH 7.4 (PBS) was prepared by mixing a stock standard solution of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4. All the other chemicals were of analytical grade and were used as received. All the solutions were prepared with doubly distilled water.

2.2. Preparation of PPy-F127 NPs

The process for preparing PPy-F127 NPs was as follows: 1.5 g F127 was dissolved in 30 mL water, and 0.90 g $FeCl_3 \cdot 6H_2O$ was added into the F127 aqueous solution under stirring until it was dissolved completely. Afterwards, 100 μL of pyrrole agent was added slowly into the above solution in a 0–5 °C ice water bath. In the process, $FeCl_3 \cdot 6H_2O$ and pyrrole were used as the oxidant and reductant, respectively. F127 acted as a dispersant and dopant in the synthesis process of the PPy-F127 NPs.[17,27,28] The colour of solution quickly turned to black, and then the mixed solution was further stirred for 6 h to obtain the PPy-F127 NPs. The product was dialyzed with Spectra/Por CE (MWCO = 14 400) in water for three days to thoroughly remove iron ions and excessive F127. The resulting PPy-F127 NPs were used for characterization and detection.

2.3. Characterization

A transmission electron microscopy (TEM) image was obtained using an interface high-resolution transmission electron microscopy (HITACHI H-7650, Japan). The size and zeta potential (ζ) of PPy-F127 NPs were detected using a Nano ZS90 Zetasizer (Malvern Instruments Ltd., UK). All the measurements were made in the automatic mode and the data were analysed using the software supplied by the manufacturer. The existence of F127, PPy and PPy-F127 NPs were measured using a Vario EL III elemental analyzer (Elementar Analysen System GmbH, Germany) and a UV-vis spectrophotometer (Cary 50 Conc, Australia).

2.4. Characterization of the LOx/(PPy-F127) bioconjugates

The circular dichroism (CD) spectra were collected from 190 to 280 nm at 1.0 nm intervals on an Applied Photophysics Chriascan circular dichroism spectrometer using a quartz cell with a path length of 1 cm at room temperature. All the measurements were performed in triplicate and the results were expressed as millidegrees (mdeg).

2.5. Construction of Nafion/LOx/(PPy-F127)/GCE and its measurement

GCE (diameter 3.0 mm) was successively polished to a mirror using 0.3 and 0.05 μm alumina slurry, and then the electrode was washed thoroughly with ethanol and water and dried at room temperature. Afterwards, 5.0 μL of PPy-F127 NPs was successively dropped onto the electrode surface and dried in air. The ζ-potential of the PPy-F127 NPs was 34.2 ± 0.61 mV (Fig. S1†), and this positive potential was ascribed to PPy. Then, the pretreated GCE was cast with 5.0 μL of 200 U/L LOx solution in 0.1 M PBS (pH = 7.4), followed by drying at ambient temperature. Thus, the LOx was combined with PPy-F127 NPs by electrostatic absorption. Subsequently, 5 μL of 0.5% Nafion was dropped on the LOx/(PPy-F127)/GCE and dried in the dark at room temperature for 15 min to form a protective film, and a Nafion/LOx/(PPy-F127) modified electrode was thus obtained. The other electrodes used as contrast experiments were prepared by the same modified method. When not in use, the electrodes were stored at 4 °C in a refrigerator.

2.6. Biochemical assays in muscles

L-Lactic acid was extracted according to the method described by Immonen *et al.* with some modification.[29] In brief, 1 g pig muscle was homogenized in 20 mL ice-cold doubly distilled water with an Ultra Turrax (T25, IKA, Germany), followed by dilution to a volume of 25 mL with water. 10 mL of the homogenate was centrifuged for 15 min at 15 000*g* in 4 °C (Allegra 64R, Beckman, USA). The supernatant was collected and heated at 95 °C for 15 min, followed by centrifugation for 5

min at 10 000*g* in 4 °C, and the supernatant was collected and diluted with buffer for the biochemical assay.

All amperometric measurements were carried out on a CHI 760D electrochemical workstation (Shanghai, China). A three-electrode cell was used with the modified GCE as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum electrode as the counter electrode. All the potentials were measured *versus* the SCE, and all the experiments were carried out at room temperature. The cyclic voltammogram (CV) experiments were performed in 0.10 M PBS (pH = 7.4) in the potential between −0.4 and 0.6 V, whereas the parameters of the differential pulse voltammetry (DPV) were a pulse amplitude of 50 mV, pulse width of 50 ms and a voltage range from −0.4 to 0.6 V.

The control experiment employed to detect L-lactic acid by high performance liquid chromatography (HPLC) was performed according to the literature with some modifications.[30] For the determination of L-lactic acid, a C18 (250 mm × 4.6 mm) column (Xbridge, Ireland) at 30 °C was used. The mobile phase was 10 mM $NH_4H_2PO_4$–methanol (99 : 1), pH 2.2, at a flow rate of 1 mL min^{-1}. The samples and standards (20 μL) were injected using an auto-injector. L-lactic acid was detected with a Waters 2998 PDA detector at 210 nm. The L-lactic acid standards were diluted in doubly distilled water. All the solutions were stored at room temperature. The chromatographic conditions for L-lactic acid were chosen in terms of peak shape, chromatographic analysis time, selectivity and resolution.

3. Results and discussion

3.1. Characterization of PPy-F127 NPs

In the construction of biosensors with nanomaterials, the biocompatibility and conductivity of the nanoparticles are the most important factors. Moreover, nanoparticles for future applications should have good biocompatibility and conductivity as well. F127 has been widely used in biomedical materials, and its good biocompatibility has also been confirmed by many researchers.[31,32] F127 plays an important role in the synthesis process of the PPy-F127 NPs. From the inset of Fig. 1, when the synthesis process proceeds without the addition of F127 (bottle a), PPy was clearly precipitated. However, PPy-F127 NPs were dispersed in aqueous solution excellently, which could be attributed to the fact that F127 acted as a dispersant and dopant (bottle b).[17,27,28,33] Moreover, due to the characteristics of conductive polymers, their applications as conducting polymers in biosensors have recently aroused much interest.[12,34,35] PPy is a representative of conducting polymers; thus, the PPy-F127 NPs were expected to present all the advantageous properties of these two kinds of materials.

The morphology of the PPy-F127 NPs is depicted in Fig. 1. As shown in Fig. 1, PPy-F127 NPs were formed, with an average particle size of about 120 nm, which is in agreement with the particle size distribution given by a Zetasizer Nano ZS90 dynamic light scattering all for three analyses (193.4 ± 3.93 nm, Fig. S2†).

The nitrogen contents of F127, PPy and PPy-F127 NPs were detected by elemental analysis. The nitrogen contents in PPy and PPy-F127 NPs were 11.26% and 0.613%, respectively. It is known that there is no elemental nitrogen in F127. Such results indicated that PPy was combined with F127 as expected.

UV-vis absorption spectra were used to confirm the binding function between F127 and PPy. As shown in Fig. 1B, F127 showed no absorption peak from 300 to 700 nm (curve a), whereas a characteristic absorption of PPy appeared at 480 nm in curve b (arrow). Such differences indicated that PPy was successfully bound to F127, which is in good agreement with other studies.[36–38]

3.2. The influence of PPy-F127 NPs on the LOx structure as evaluated by CD spectroscopy

The maintenance of enzyme activity on the supporting materials is crucial in the biosensor designs, because the secondary conformational variations of the enzyme can markedly affect its activity. In this paper, CD was utilized to investigate the secondary conformation variations of the polypeptide chain of LOx on the PPy-F127 NPs.[39,40] Fig. 2 presents the CD spectra of LOx before and after the addition of PPy-F127 NPs in aqueous solution. The spectrum (curve a in Fig. 2) clearly shows the characteristic peaks at 195 nm and 204 nm, originating from the folded secondary structure of the enzyme.[41] Upon the addition of PPy-F127 NPs, the characteristic peaks were similar

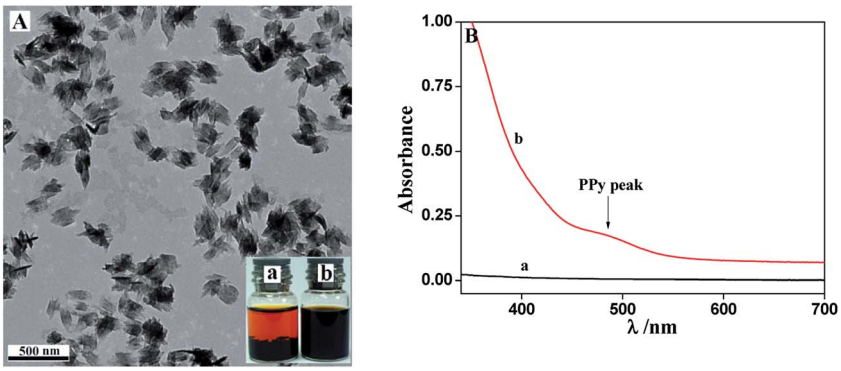

Fig. 1 Characterization of PPy-F127 NPs. (A) TEM image of the PPy-F127 NPs, inset: (bottle a) PPy in the absence of F127, and (bottle b) PPy-F127 NPs. (B) UV-vis adsorption spectra of (a) F127 aqueous solution, and (b) PPy-F127 NPs.

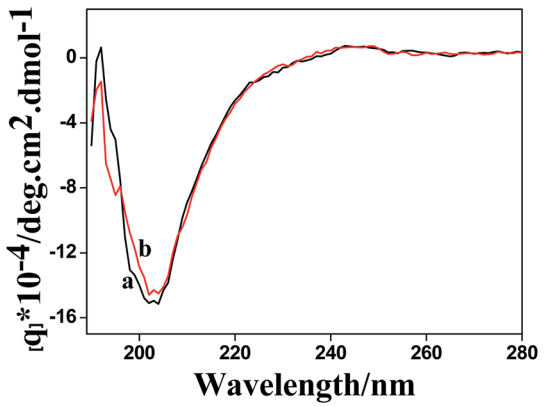

Fig. 2 CD spectra of (a) pure LOx and (b) LOx/(PPy-F127) in 0.1 M PBS (pH = 7.4) in the wavelength region of 190–280 nm.

to the pure enzyme in both peak intensity and peak position (curve b). The results exhibited that PPy-F127 NPs indeed had good biocompatibility. Thus, the LOx immobilized on PPy-F127 NPs could retain their secondary structure and biological activity in the following electrochemical experiments.

3.3. Direct electron transfer reactivity of the Nafion/LOx/(PPy-F127)/GCE

In order to investigate the electrochemical properties of Nafion/LOx/(PPy-F127)/GCE, different modified electrodes were recorded by CV. No voltammetric signal was observed at the bare electrode (curve a, Fig. 3) and at the PPy-F127 film modified electrode (curve b, Fig. 3). Moreover, the background current of (PPy-F127)/GCE was much higher than that of the bare electrode because of the good conductivity of PPy. However, Nafion/LOx/(PPy-F127)/GCE (curve c, Fig. 3) showed a pair of well-defined and nearly symmetrical redox peaks with anodic and cathodic peak potentials at 0.206 and 0.102 V (*vs.* SCE), respectively. When L-lactic acid was added into the substrate solution, the current signals of the redox peaks at the Nafion/LOx/(PPy-F127) film increased (curve d, Fig. 3), which suggests an obvious electrocatalysis of Nafion/LOx/(PPy-F127)/GCE to L-lactic acid. The good electrochemical response of Nafion/LOx/(PPy-F127)/GCE indicated that PPy-F127 NPs play an important role in facilitating the electron exchange between the electroactive centers of LOx and GCE. In addition, the PPy-F127 NPs provide a mild environment; thus, the bioactivity of LOx can be retained.

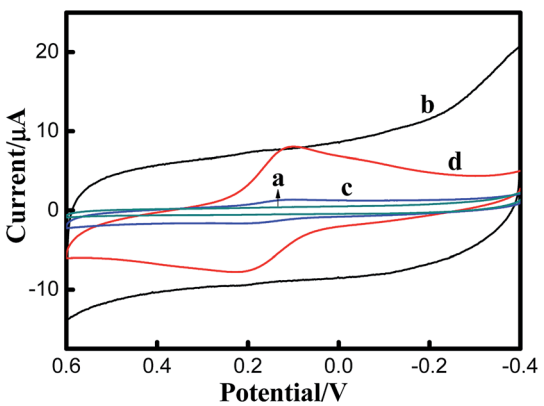

Fig. 3 CVs of (a) bare electrode, (b) PPy-F127 NPs film modified electrode, (c) Nafion/LOx/(PPy-F127)/GCE in 0.1 M PBS (pH = 7.4), and (d) Nafion/LOx/(PPy-F127)/GCE in 0.1 M PBS (pH = 7.4) with a concentration of L-lactic acid of 10 mM. Scan rate: 100 mV s^{-1}.

For further characterization of the different modified electrodes, electrochemical impedance spectroscopy (EIS) was used in the frequency range from 0.1 Hz to 10 kHz at a formal potential value ($E^{0\prime}$) of 0.154 V *vs.* SCE. As is well known, the semi-circle diameter in EIS equals the interface electron-transfer resistance (R_{et}), which controls the electron-transfer kinetics of the redox probe at the electrode interface. Fig. S3† shows the Nyquist diagrams of different electrodes in 10 mM $[Fe(CN)_6]^{3-/4-}$(1 : 1) solution containing 0.1 M KCl. Curve a in Fig. S3† shows the electrochemical impedance spectrum of the bare GCE, indicating a very low electron transfer resistance to the redox-probe dissolved in the electrolyte solution. The (PPy-F127)/GCE decreased the R_{et} tremendously (curve b), because PPy can improve the conductivity of the GCE and facilitate the electron transfer between the solution and electrode interface. A bigger well-defined semi-circle at high frequency regions was observed for LOx/(PPy-F127)/GCE (curve c) compared with (PPy-F127)/GCE (curve b), indicating that the non-conductivity of LOx inhibited the electron transfer of the redox probe of $[Fe(CN)_6]^{3-/4-}$ to the electrode surface to some degree. The results demonstrated that LOx and PPy-F127 NPs had been successfully immobilized on the electrode surface.

3.4. Electrocatalysis of Nafion/LOx/(PPy-F127)/GCE to L-lactic acid

Calibration studies were performed with the fully developed Nafion/LOx/(PPy-F127)/GCE biosensor ranging from 0.015 to 37.5 mM. A new biosensor was used to obtain each measurement, and this was performed in triplicate for each L-lactic acid concentration. Fig. 4 displays the typical DPV curves of the Nafion/LOx/(PPy-F127)/GCE on exposure to various concentrations of standard L-lactic acid solutions. The resulting calibration plot (the inset of Fig. 4) exhibits an apparent Michaelis–Menten kinetics with a linear range up to 37.5 mM. The linear regression equation was I (μA) = 0.02353c (mM) + 1.4135 (R^2 = 0.9939), where I is the current and c is the L-lactic acid concentration. The experimental limit of detection (LOD) of the biosensor was measured to be 0.0088 mM based on a signal-to-noise ratio of 3. These performance characteristics are superior to the published results obtained from other enzyme immobilization studies, which tended to have reduced linear ranges[42–45] and/or poor detection limits[44,46] compared with our biosensor.

3.5. Interference study

To evaluate the anti-interference of the constructed Nafion/LOx/(PPy-F127)/GCE biosensor, several possible interfering biomolecules, such as uric acid (UA), ascorbic acid (AA), glucose and cysteine, were examined.[47] The current response to 50 mM UA, 50 mM AA, 50 mM glucose, 50 mM cysteine and 5 mM L-lactic

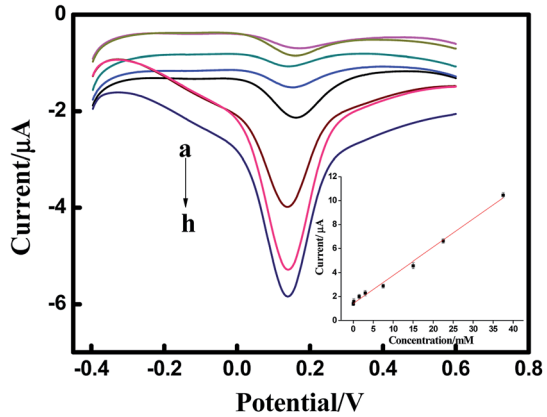

Fig. 4 DPVs obtained at Nafion/LOx/(PPy-F127)/GCE in 0.1 M PBS (pH = 7.4) with a concentration of L-lactic acid (from a to h) of 0.015, 0.15, 1.5, 3, 7.5, 15, 22.5, 37.5 mM. The parameters were as follows: pulse amplitude of 50 mV, pulse width of 50 ms, and voltage range from −0.4 to 0.6 V. The inset: calibration curve for L-lactic acid obtained at the Nafion/LOx/(PPy-F127)/GCE biosensor.

acid are shown in Fig. 5. It can be seen that these interferences, even at higher concentrations, did not cause an obvious interference in the determination of L-lactic acid. The result indicates that the anti-interference of the Nafion/LOx/(PPy-F127)/GCE biosensor is acceptable, indicating its promising application for the detection of L-lactic acid in real sample analysis.

3.6. Stability and reproducibility of Nafion/LOx/(PPy-F127)/GCE

The reproducibility and repeatability of the developed biosensor were determined. In this paper, a series of 10 biosensors were prepared in the same way and a relative standard deviation (R.S.D.) of 5.9% was obtained towards 5 mM L-lactic acid, indicating the reliability of the method. A set of 10 different amperometric measurements for 5 mM L-lactic acid with a single biosensor yielded a R.S.D. of 4.3%.

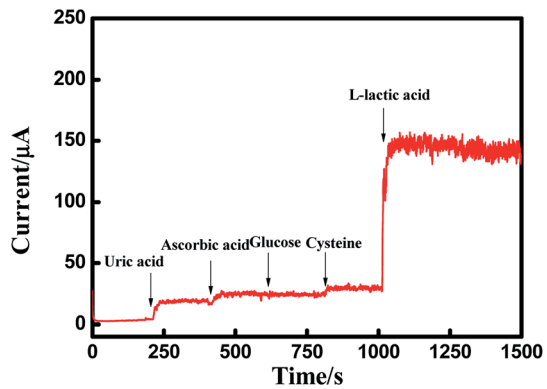

Fig. 5 Amperometric responses of the biosensor upon the addition of 50 mM UA, 50 mM AA, 50 mM glucose, 50 mM cysteine and 5 mM L-lactic acid in 0.1 M PBS (pH = 7.4). The biosensor was biased on the potential of 0.154 V *vs.* SCE.

Table 1 Comparison of the two methods obtained using practical samples

Samples	Muscle-1	Muscle-2	Muscle-3	Muscle-4
HPLC (mM)	105.8 ± 5.8	95.2 ± 2.5	93.0 ± 3.9	101.6 ± 4.2
Biosensors (mM)	107.1 ± 3.2	94.8 ± 1.3	92.5 ± 2.8	102.3 ± 4.3

The stability of the L-lactic acid biosensor was explored. The proposed biosensor was stored in air under ambient conditions. The response to 5 mM L-lactic acid was tested each week, and after 15 days of storage, the response of the biosensor only decreased 4.2% compared to the initial response, which shows its long-term stability.

3.7. Real sample analysis

To illustrate the feasibility of the Nafion/LOx/(PPy-F127)/GCE in practical analysis, it was applied to detect L-lactic acid in the analysis of practical samples. 500 μL of the extract was added in 4.5 mL of 0.1 M PBS, and the current response was recorded. To investigate the reliability of the Nafion/LOx/(PPy-F127)/GCE biosensor for real samples, four samples were assayed using the present biosensor and HPLC was used as a reference method (Table 1). It was shown that the values measured by the proposed biosensor were consistent with the data determined by HPLC. The results showed the applicability of the biosensor for the determination of the concentration of L-lactic acid in practical samples.

4. Conclusion

Developments in polymer nanocomposites have considerable effects on biochemical assays. In this paper, novel PPy-F127 NPs possessing conductivity and biocompatibility were synthesized and used to construct a L-lactic acid biosensor that could be applied in the analysis of practical samples. The L-lactic acid biosensor exhibited a low detection limit of 0.0088 mM, a linear calibration plot was obtained in the wide concentration range from 0.015 to 37.5 mM, and good anti-interference properties. The results showed that the Nafion/LOx/(PPy-F127)/GCE biosensor exhibit good electrochemical behavior that was attributed to the conducting PPy and biocompatible F127. This method possesses great potential, especially for the detection and evaluation of meat quality. Furthermore, the significant advance we propose in the fabrication of biosensors using nanostructured conducting polymers and a biocompatible technique will be investigated by more in-depth research in near future.

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