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Novel L-lactic Acid Biosensors Based on Conducting Polypyrrole-Block Copolymer Nanoparticles

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Abstract

The development of advanced nanomaterials for the highly efficient electrical detection of biological species has received great attention. Here, the novel polypyrrole-Pluronic F127 nanoparticles (PPy-F127 NPs) with conducting and biocompatibility were synthesized and used to construct 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 ($E^{0'}$) 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) = 0.02353 c (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 will provide a promising platform for the rapid development of biosensors that can be used in the detection of biological species.

Keywords: Polypyrrole-Pluronic F127 nanoparticles, Biochemical assays, Biosensors, Lactate oxidase

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 storage quality of foods, and can be used as an indicator of fermentative processes in food industry. The determination of L-lactic acid level in tissues is essential for the evaluation of meat quality,[1,2] and can be employed to distinguish potential Pale Soft Exudative (PSE) muscle from Dark Firm

Dry (DFD) and normal muscles.[3] The traditional methods for quantitative detection of L-lactic acid concentration are mainly based on spectrophotometry,[4-6] which comprise complex procedures (e.g. reagent preparation and sample pre-treatment), consume a long time, require expensive instrumentation and trained operators.[7,8] Therefore, great attention has been attracted to design a simple, rapid and sensitive device for L-lactic acid detection. In the last decade, electrochemical biosensor has been increasingly applied to L-lactic acid determination because of its 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 we know, conducting polymers have been the subject of much interest, not only from a fundamental scientific interest but also from a practical point of view, such as various functional applications including solar cells, separation membrane, molecular electronic devices and biosensing devices. Among the class of applications, biosensors take up the running due to conducting polymer's inherent charge transport properties and biocompatibility.[12-16]

In recent years, the applications of novel nanomaterials in bioassay seem to be hot topics, 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 used in the optimization of immobilized enzymes, and bring about minimum diffusion limitations, maximum surface area per unit mass and highly effective enzyme loading.[25] It is believed that

conducting polymer nanomaterials offer a great possibility for novel applications in biosensors, and will be widely employed in the construction of sensing devices when their physico-chemical properties are modified for more and more particular need.[26]

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 glass carbon electrode (GCE) to construct a novel enzyme biosensor. These novel nanomaterials were employed in the design of biosensors, which was further used in biochemical assays of L-lactic acid in pig muscle samples.

Why we choose Pluronic F127 (triblock copolymer $PEO_{106}PPO_{70}PEO_{106}$, F127) as doped agent for PPy nanoparticles? Firstly, it is difficult to obtain PPy nanoparticles with small size and good dispersion in the absence of F127. F127 can be used as template and dispersant for preparing nanoparticles with special shapes.[27,28] Secondly, as we know, F127 has good biocompatibility. Thus, the LOx immobilized on PPy-F127 NPs can be retained the secondary structure and biological activity in the following electrochemical experiments. Is it possible that we take advantage of the cooperation effects which mixed good biocompatibility with electronic conductive property of PPy-F127 NPs to design and prepare a novel L-lactic acid biosensor which can be used in the detection of L-lactic acid? It is the subject of this research work. More details about the preparation process and electrochemical behaviors of the novel L-lactic acid biosensor we proposed were presented. The results indicated that the modified electrode have a wide linear range and a lower detection limit.

Meanwhile, the L-lactic acid biosensor can be used to determine L-lactic acid in 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), 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 stock standard solution of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4. All other chemicals were of analytical grade and were used as received. All solutions were prepared with double-distilled water.

2.2. Preparation of PPy-F127 NPs

The process of preparation of 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. After that, 100 μL of pyrrole agent was added slowly into the above solution under 0~5 °C ice water bath condition. In the process, $FeCl_3 \cdot 6H_2O$ and pyrrole were used as oxidant and reductant, respectively. F127 acted as dispersant and dopant in the synthesis process of the PPy-F127 NPs.[17,27,28] The colour of solution quickly turned into black, 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 = 14400) in water for three days to thoroughly remove iron ion and excessive F127. The resulting PPy-F127 NPs were used for characterization and detection.

2.3. Characterization

Transmission electron microscopy (TEM) image was obtained by 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 measurements were made in automatic mode and the data were analysed using the software supplied by the manufacturer. The existences of F127, PPy and PPy-F127 NPs were measured using Vario EL III elemental analyzer (Elementar Analysen System GmbH, Germany) and UV-*vis* spectrophotometer (Cary 50 Conc, Australia).

2.4. Characterization of 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 done in triplicate and the results were expressed as millidegrees (mdeg).

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

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 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 Nafion/LOx/(PPy-F127) modified electrode was 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 ºC 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 double-distilled water with an Ultra Turrax (T25, IKA, Germany), followed by dilution to volume of 25 mL with water. 10 mL of the homogenate was centrifuged for 15 min at 15000 *g* in 4 ℃ (Allegra 64R, Beckman, USA). The supernatant was collected and heated at 95 ℃ for 15 min, followed by centrifugation for 5 min at 10000 *g* in 4 ℃, 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 potentials were measured versus the SCE, and all 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, while the parameters of differential pulse voltammetry (DPV) were pulse amplitude of 50 mV, pulse width of 50 ms and 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 modification.[30] For the determination of L-lactic acid, a C18 (250 mm × 4.6 mm) column (Xbridge, Ireland) at 30 ºC 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. 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 double-distilled water. All 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, biocompatibility and conductivity of the nanoparticles are the most important factors. Nanoparticles with application future should be of good biocompatibility and conductivity as well. F127

has been widely used in biomedical material, 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 insert of Fig. 1, when the synthesis process without addition of F127 (bottle a), PPy was precipitated obviously. However, PPy-F127 NPs were dispersed in aqoeous solution excellently, which should be attributed to the fact that F127 acted as dispersant and dopant (bottle b).[17,27,28,33] Moreover, due to the characteristics of conductive polymer, their applications of 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 was depicted in Fig. 1. As shown in Fig. 1, the PPy-F127 NPs were formed and the average particle size was 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 no nitrogen element in F127. Such results indicated that PPy has been 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), while a characteristic absorption of PPy appeared at 480 nm in

curve b (arrow). Such difference indicated that PPy was successfully bound to F127, which was in good agreement with other studies.[36-38]

3.2. The influence of PPy-F127 NPs on LOx structure 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 affect its activity markedly. 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 presented 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 showed 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 to the pure enzyme in both peak intensity and peak position (curve b). The results exhibited that PPy-F127 NPs indeed have good biocompatibility. Thus, the LOx immobilized on PPy-F127 NPs can be retained the 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 bare electrode (curve a, Fig. 3) and PPy-F127 film modified electrode (curve b, Fig. 3). Meanwhile, the background current of (PPy-F127)/GCE was much higher than that of bare electrode due to the good

conductivity of PPy. However, the Nafion/LOx/(PPy-F127)/GCE (curve c, Fig. 3) showed a pair of well defined and nearly symmetrical redox peaks with the anodic and cathodic peak potentials at 0.206 and 0.102 V (vs. SCE), respectively. When L-lactic acid was added into substrate solution, the current signals of the redox peaks at Nafion/LOx/(PPy-F127) film increased (curve d, Fig. 3), suggesting an obvious electrocatalysis of Nafion/LOx/(PPy-F127)/GCE to L-lactic acid. The good electrochemical response of Nafion/LOx/(PPy-F127)GCE indicated PPy-F127 NPs play an important role in facilitating the electron exchange between the electroactive center of LOx and GCE. In addition, the PPy-F127 NPs provide a mild environment so that the bioactivity of LOx can be retained.

For further characterization of the different modified electrodes, the electrochemical impedance spectroscopy (EIS) was used in the frequency range from 0.1 Hz-10 kHz at a formal potential value ($E^{0'}$) of 0.154 V vs. SCE. As we know, 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 showed 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 showed the electrochemical impedance spectrum of the bare GCE, implying 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 facilitates the electron transfer between solution and electrode interface. A bigger well-defined semi-circle at high frequency regions was

observed at LOx/(PPy-F127)/GCE (curve c) compared with the (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 have 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 done in triplicate for each L-lactic acid concentration. Fig. 4 displayed 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) exhibited an apparent Michaelis-Menten kinetics with a linear ranging up to 37.5 mM. The linear regression equation was I (μA) = 0.02353 c (mM) ＋ 1.4135 (R^2 = 0.9939), where I is 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-noise ratio of 3. These performance characteristics are superior to 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,

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 acid was shown in Fig. 5. It could be seen that these interferences even at higher concentration did not cause obvious interference in the determination of L-lactic acid. The result implied that the anti-interference of the Nafion/LOx/(PPy-F127)/GCE biosensor is acceptable, indicating the promising application to 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%.

The stability of the L-lactic acid biosensor was explored. The proposed biosensor was stored in air at ambient conditions. The response to 5 mM L-lactic acid was tested each week, after 15 days of storage, and 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 is 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 well consistent with the data determined by HPLC. The results showed the applicability of the biosensor for determination of the concentration of L-lactic acid in practical samples.

4. Conclusion

The development in polymer nanocomposites has considerable effects on biochemical assays. In this paper, the novel PPy-F127 NPs with conductivity and biocompatibility were synthesized and used to construct the L-lactic acid biosensor that can 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 0.015-37.5 mM, and good anti-interference property. The results showed the Nafion/LOx/(PPy-F127)/GCE biosensor exhibit good electrochemical behavior that attributed to conducting PPy and biocompatible F127. This method proposes a great potential especially for detection and evalution of meat quality. Furthermore, the significant advance we proposed in the fabrications of biosensors using nano-structured conducting polymers and biocompatible technique will be investigated by more in-depth research in near future.

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Appendix A. Supplementary material

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Legends for the figures:

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.

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 the concentration of L-lactic acid 10 mM. Scan rate: 100 mV/s.

Fig. 4. DPVs obtained at Nafion/LOx/(PPy-F127)/GCE in 0.1 M PBS (pH=7.4) with the concentration of L-lactic acid (from a to h) 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 insert: calibration curve for L-lactic acid obtained at the Nafion/LOx/(PPy-F127)/GCE biosensor.

Fig. 5. Amperometric responses of the biosensor upon additions 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 two methods obtained in practical samples.

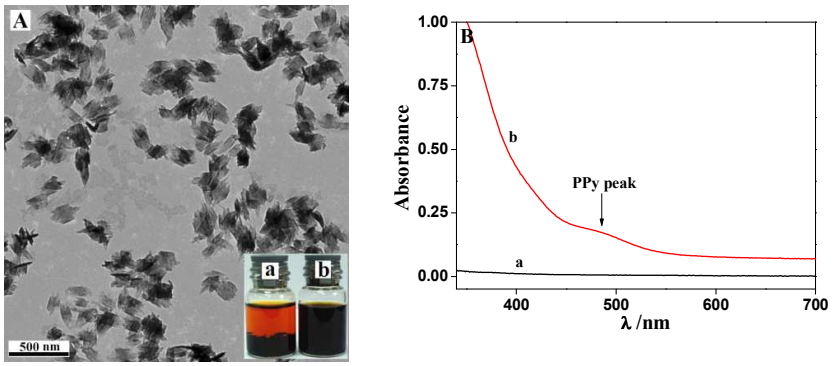


Fig. 1. Characterization of PPy-F127 NPs. (A) TEM image of the PPy-F127 NPs, inset: (bottle a) PPy in the absence of F127, and (bottle b) PPy-F127 NPs. (B) UV-*vis* adsorption spectra of (a) F127 aqueous solution, and (b) PPy-F127 NPs.

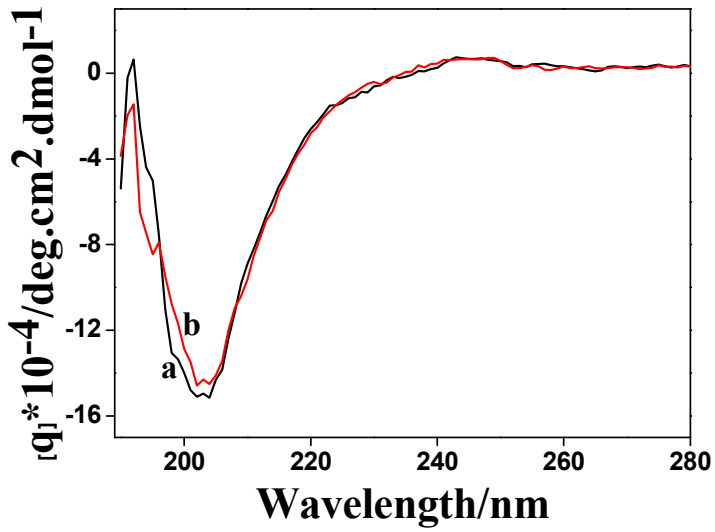
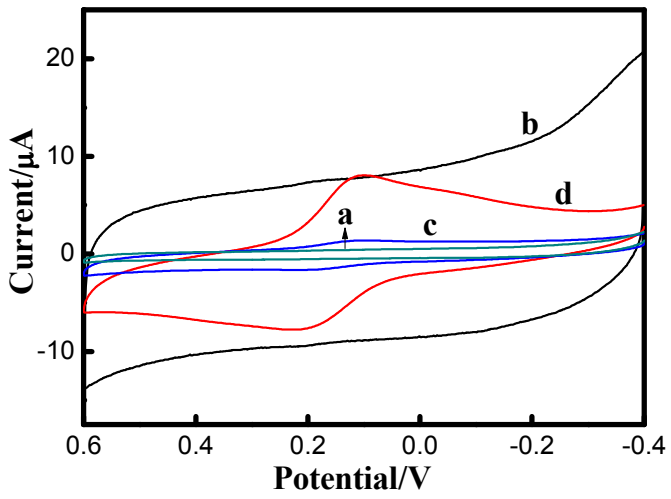


Fig. 2. CD spectra of (a) pure LOx and (b) LOx/(PPy-F127) in 0.1 M PBS (pH=7.4) in the wavelength region of 190-280 nm.



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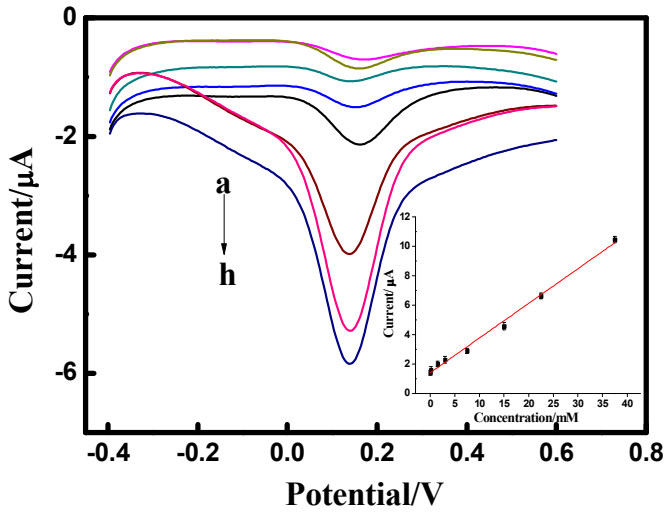

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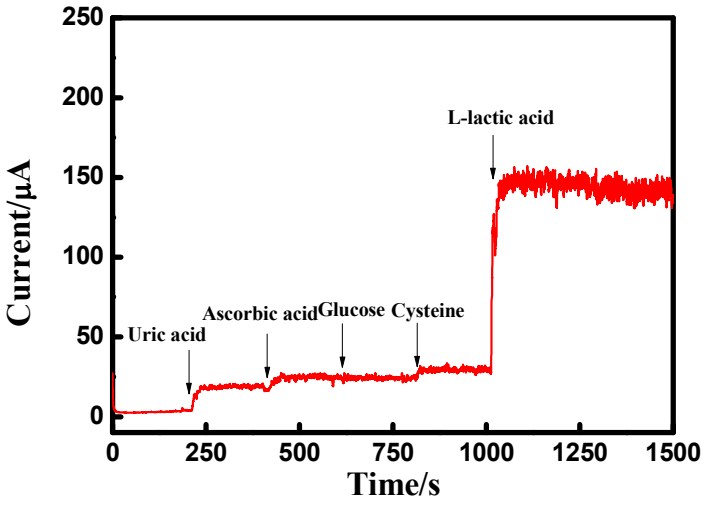

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Table 1. Comparison of two methods obtained in practical samples.

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