Sensitive Detection of Serum Creatinine Based on β-Cyclodextrin-Ferrocenylmethanol Modified Screen-printed Electrode

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Ferrocenylmethanol (Fc-OH) is included in β-cyclodextrin (β-CD) to form the β-CD-Fc-OH complex by host-guest supramolecular interaction. β-CD dissociates from the β-CD-Fc-OH complex due to the conversion of Fc-OH to Fc^+-OH under a stimulus of oxidant. In our study, Fc-OH is oxidized after a series of enzymatic reactions of creatinine, which blocks the other means for oxidation of Fc-OH. And the background noise is reduced for testing for serum creatinine (sCr). The chronoamperometry signal for creatinine (with a constant potential –0.3 V *vs.* Ag/AgCl) increases linearly in the 1 – 1000 μM range, with a limit of detection as low as 0.5 μM. The amperometric potential of –0.3 V greatly prevents the interference of various redox substances in serum. The biosensor was used to test 120 clinical specimens and the results showed a linear correlation with the biochemical analyzer (R^2 = 0.9885). The biosensor could be applied to clinical trials and offers good prospects for clinical sCr detection.

Keywords Ferrocenylmethanol, β-cyclodextrin, biosensor, serum creatinine, screen-printed electrode

(Received January 20, 2019; Accepted April 15, 2019; Advance Publication Released Online by J-STAGE April 26, 2019)

Introduction

Cyclodextrin (CD) is used in many different fields of analytical chemistry because it tends to form reversible clathrates and selectively recognizes analytes. This feature shows that cyclodextrin can play a role in sensitivity and selectivity improvement. It is usually used as the host of various guests, such as ferrocene (Fc),[1,2] adamantane,[3,4] and azobenzene[5,6] to form a superamphiphilic complex by geometric compatibility and hydrophobic interactions.[7,8] Li *et al.*[9] reported the reversible control of electron transmission through nanopore-tethered Fc based on the complexation with β-CD. However, most of the β-CD dissociates from the β-CD-Fc complex due to the conversion of Fc to Fc^+. In Basit's work,[10] Fc is oxidized to carry a charge upon application of +0.4 V, leading to the released β-CD diffusing to the lower leaflet of the lipid bilayer. Fc and its derivatives like ferrocenylmethanol (Fc-OH) have become popular as electronic mediators for electrochemical biosensors.[11] As is well known, β-CD can increase the solubility and stability of Fc to prevent Fc loss.[12,13] But there are few reports on the application of β-CD-Fc-OH in the detection of creatinine.

Serum creatinine (sCr) as an endogenous filtration scavenger is filtered only through the glomerulus and not reabsorbed by kidney tubules. Therefore, it is recommended to calculate the estimated glomerular filtration rate (eGFR).[14] And eGFR is considered to be a simple, rapid and reliable indicator in evaluating renal function. Furthermore, sCr is an integral part of screening for chronic kidney disease (CKD).[15] Currently, the common methods for detecting sCr include the Jaffe method, the compensated Jaffe method, the liquid method of enzyme and the dry chemical test.[16] However, the Jaffe method's poor anti-interference sometimes results in clinical misdiagnosis.[17,18] A large biochemical instrument is required by the enzyme liquid method and detection time is also long. The dry chemical test method has a large CV value when sCr concentration is low.[16] At present, the electrochemical biosensor has great potential as a detection instrument in clinical practice, and there are many reports for sCr detection.[19–21] Thompson *et al.*[22] designed an ammonia gas electrode combined with creatinine deaminase (E.C.3.5.4.21) to detect creatinine. Then Kubo *et al.*[23] produced a Clark oxygen electrode also consisting of creatinine deaminase and immobilizing nitrifying bacteria, which was applied to the amperometric determination of creatinine. Tsuchida *et al.*[24] first designed a three-enzyme method for less interference, on which subsequent amperometric creatinine biosensors rely.[25–29] The three-enzyme sequence consisting of creatininase (CRN, EC 3.5.2.10), creatinase (CR, EC 3.5.3.3) and sarcosine oxidase (SOX, EC 1.5.3.1), catalyzes the conversion of creatinine through creatine and sarcosine to glycine, formaldehyde and hydrogen peroxide (H_2O_2) as described in the following reaction sequence:

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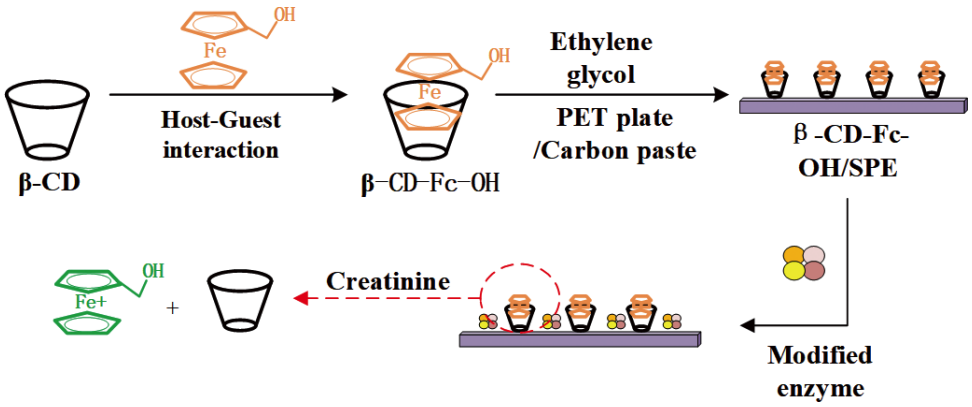

Fig. 1 Schematic diagram for the formation of the β-CD-Fc-OH/SPE.

$$\text{Creatinine} + H_2O \xrightarrow{CRN} \text{Creatine} \quad (1)$$

$$\text{Creatine} + H_2O \xrightarrow{CR} \text{Sarcosine urea} \quad (2)$$

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{SOX} \text{Glycine} + CH_2O + H_2O_2 \quad (3)$$

The direct electrooxidation of H_2O_2 requires a high working potential (about +0.7 V *vs.* Ag/AgCl), and the amperometric detection of H_2O_2 is usually accompanied by serious interference from oxidizable metabolites such as ascorbic acid, uric acid and ascorbic acid. Usually, ferrocene and horseradish peroxidase (HRP) were both introduced to minimize such interference. Kinoshita *et al.*[30] designed a sensor with peroxidase entrapped on a Fc-embedded carbon paste, which decreased the potential to 0.1 V. The sensor of Serafín *et al.*[31] just needed 0.0 V to work with HRP and ferrocene. Harada and colleague prepared and studied the cyclodextrin-ferrocene inclusion complexes.[32] However, there are not many reports about the electrochemical application of inclusion complexes.[12]

Screen-printed electrodes (SPE) are suitable for the manufacture of highly efficient, versatile, low-cost miniature sensors that provide highly sensitive and repeatable results in biochemical testing. Also, screen-printed technology has become a preferred method for mass-produced biosensors for point-of-care testing (POCT).[33–36] In this study, β-CD-Fc-OH dissolved with ethylene glycol was mixed into carbon paste and printed on PET plates to form preliminary electrodes. And the creatininase, creatine oxidase, sarcosine oxidase and HRP were modified on preliminary electrodes to form ultimate electrodes (Fig. 1). The electrochemical behaviors of electrodes were investigated and used to detect the sCr in clinical trials.

Experimental and Methods

Reagents and chemicals

Ferrocenylmethanol, tetrahydrofuran and dimethyl sulfoxide (DMSO) were purchased from traditional Chinese medicine suppliers. β-Cyclodextrin, ascorbic acid (AA), and reduced glutathione (GSH) were acquired from Aladdin Co., Ltd. Creatinine standard solution was purchased from Beijing Tanmao Quality Testing of China. Creatinine hydrolase, creatine oxidase, sarcosine oxidase, and horseradish peroxidase (HRP) were from Toyobo in Japan. Na_2HPO_3–KH_2PO_3 was purchased from Qitai Biotechnology Co., Ltd. (Hangzhou, China). Sarcosine (sa), lipid, uric acid (UA), bilirubin, and acetaminophen (AP) were from Sigma-Aldrich (St. Louis, USA). Conductive carbon paste and insulating ink were purchased from Jujo Chemical Co., Ltd. in Japan.

Preparation of β-CD-Fc-OH compound

The preparation process for the β-CD-Fc-OH compound was as follows: 0.567 g β-CD was prepared, and deionized water was used as a solvent to prepare a 0.5 mmol solution.[32] Fc-OH was weighed to 0.432 g, the amount of which was 2 mmol. According to the molar ratios of β-CD to Fc-OH (nCD/nFc) of 1:2, 1:1, 2:1, 3:1 and 4:1, orange fine needle-like Fc-OH was dissolved by a little dimethyl sulfoxide (DMSO) and added to a β-CD solution at 60°C. The solution was shaken for 6 h in a constant temperature shaker (TS-100B, Shanghai Jiecheng of China), then returned to room temperature. It was then placed in a refrigerator and kept at 4°C overnight. The obtained yellow crystal precipitate was washed three times with water to remove excess β-CD, and the free Fc-OH was removed by washing with tetrahydrofuran. The supernatant of centrifugation after washing changed from yellow to colorless, indicating that the free Fc-OH was removed completely. The precipitate was dried in a vacuum oven (2XZ (S)-2, Shanghai Deying Vacuum Equipment Co., Ltd. of China).

Preparation of β-CD-Fc-OH/SPE and Fc-OH/SPE

First, 2.6 g β-CD-Fc-OH and 0.5 g Fc-OH were respectively dissolved in 2.4 and 4.5 g of ethylene glycol. Then, the solution was added to 10 g carbon paste to disperse by stirring at 2000 – 5000 rpm. After that, the mixture was deposited on PET to form specific regions with a working electrode and a counter electrode. The reference electrode was Ag/AgCl. The reaction zone was constructed by printing insulating ink on the working and reference electrodes. After the PET plate was dried, the enzyme solution was printed. On the basis of the literature,[37] a white material mainly including carboxymethyl cellulose and polyvinyl pyrrolidone was prepared. Next, 400 U/mg creatinine hydrolase, 350 U/mg creatine oxidase, 300 U/mg sarcosine oxidase, and 400 U/mg HRP were added to the white material and stirred well. After waiting for 15 min, an enzyme paste was obtained. The humidity was controlled at about 90% with an enzyme printing machine to print the enzyme layer, and the enzyme layer was fixed in the reaction area. A hydrophilic membrane was used to cover over the reaction area in order to prevent the enzyme from being contaminated and becoming damp. Finally, a slitting mill was used to cut the prepared modified SPE, which was packed into a plastic box with desiccant. The thickness of the SPE we prepared was 50 µm.

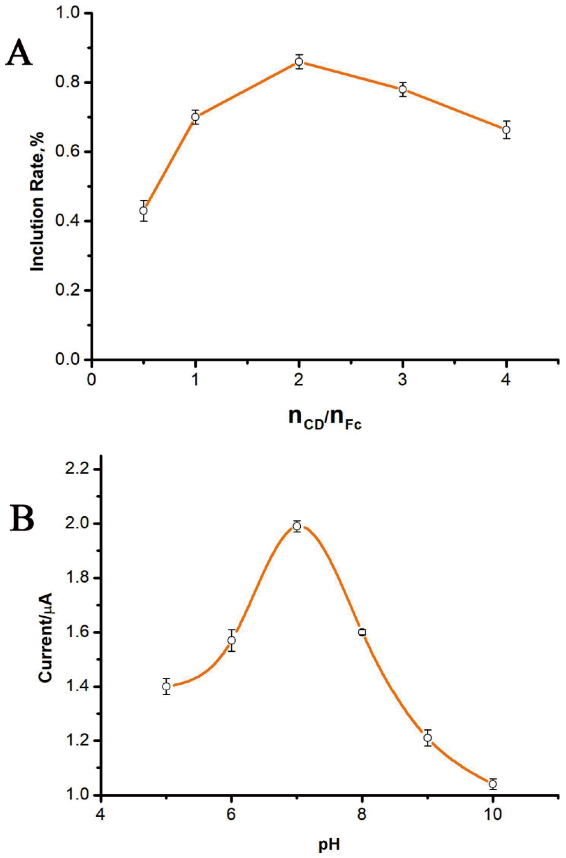

Fig. 2 (A) Comparison among the n_{CD}/n_{Fc} ratios of 1:2, 1:1, 2:1, 3:1, 4:1 for inclusion efficiency. (B) Oxidation current of the β-CD-Fc-OH/SPE in buffers at pH 5, 6, 7, 8, 9, 10. The error bars stand for RSD value (n = 3).

Electrochemical performance test of modified electrode

The electrochemical characterization of the β-CD-Fc-OH/SPE and Fc-OH/SPE was carried out by cyclic voltammetry. The working electrode, reference electrode and counter electrode were respectively connected by a CHI760D electrochemical workstation (Shanghai Chenhua Instrument Co., Ltd. of China). The scanning potential was from +0.4 to 0 V with scan rate of 0.1 V/s. A volume of 8 μL of pH 7.0 1/15 M Na_2HPO_3–KH_2PO_3 buffer was added to the electrode to detect the redox potential and oxidation current. Multiple cycles were carried out, and the final stable CV cycle containing redox peaks was selected for observation and comparison.

Sensitivity comparison between β-CD-Fc-OH/SPE and Fc-OH/SPE

Creatinine standard solutions of 100 and 500 μM were both detected by the β-CD-Fc-OH/SPE and Fc-OH/SPE with cyclic voltammetry. The size of the reductive current increment implied the level of sensitivity.

The β-CD-Fc-OH/SPE and Fc-OH/SPE were prepared without hydrophilic membrane in order to add 100 μM creatinine continuously. At this time, chronoamperometry was applied to test creatinine and we added the 100 μM creatinine every 10 s at a constant voltage of –0.3 or 0.1 V (*vs.* Ag/AgCl).

Testing of clinical specimens

From December 2017 to April 2018, the serum of 60 healthy subjects and 60 patients with nephropathy were collected from the Third Xiangya Hospital of Changsha, Hunan. The serum was stored in a refrigerator at –80°C. The practices used in this study were in line with the Declaration of Helsinki and an ethical review. We obtained the informed consent of the patients in advance. Clinical specimens were separately measured by the sensor and biochemical analyzer. The sensor adopted the chronoamperometry to obtain the concentration under the constant voltage of –0.3 V (*vs.* Ag/AgCl). At the time, some of the samples were also used to evaluate the recovery rate and stability.

Results and Discussion

Evaluation of optimal inclusion efficiency and pH

Compounds were prepared for several about four different n_{CD}/n_{Fc} ratios, 1:2, 1:1, 2:1, 3:1 and 4:1. The formed inclusion compound was weighed, and the amount of Fc-OH included by β-CD was calculated based on both relative molecular masses. The quantitative ratio of Fc-OH after and before inclusion was the inclusion ratio. As shown in Fig. 2A, the amount of inclusion compound prepared by a ratio of 2:1 was the highest, and the inclusion efficiency was the best. Therefore, the inclusion compound used in the subsequent experiments was prepared in 2:1 ratio. The results of this study differ from the results described in Ref. 32, which may be due to the differences in experimental procedures. In this experiment, Fc-OH was dissolved in DMSO, then added into a β-CD solution. But in the previous study, Fc-OH crystals were directly incorporated into the β-CD solution, and the stirring method or speed were also different.

The β-CD-Fc-OH/SPE was detected in buffers with diverse pH by cyclic voltammetry. The results (Fig. 2B) showed the peak of oxidation current was the highest at the pH of 7.0. Therefore, pH 7.0 was taken as the optimum pH value for our experiment. This seems to indicate the sensor can be adaptable to detection in body fluids.

Characterization of β-CD-Fc-OH/SPE and Fc-OH/SPE

Cyclic voltammetry of the bare SPE, Fc-OH/SPE and β-CD-Fc-OH/SPE were performed in phosphate buffer at pH 7.0 (Fig. 3). Compared with the bare electrode, both modified electrodes presented obvious redox peaks. The β-CD-Fc-OH/SPE had a lower peak current than the Fc-OH/SPE, and the oxidation peak was found to be positively shifted. These results agree with parts of work of Juan and colleagues.[38] In theory, a positive shift of oxidation peak indicates the electrode is more difficult to oxidize.[39] Therefore, it is recognized that β-CD-Fc-OH is more stable than Fc-OH. And β-CD-Fc-OH is more difficult to be oxidized by potential. The oxidation peak voltage (E_{pa}) and reduction peak voltage (E_{pc}) of Fc-OH/SPE were 0.172 V (*vs.* Ag/AgCl) and 0.111 V. ΔE_p was 0.061 V, which meant the whole process was reversible. E'_{pa} and E'_{pc} of the β-CD-Fc-OH/SPE were 0.271 and 0.156 V. $\Delta E'_p$ was 0.115 V, which meant the whole process was not reversible enough. The reason might be that the reduction of Fc^+-OH was easier than oxidation of Fc-OH because of the inclusion effect. According to a comparison of Figs. 3(b) and 3(c), β-CD-Fc-OH was synthesized successfully.

Sensitivity difference between β-CD-Fc-OH/SPE and Fc-OH/SPE

Firstly, the cyclic voltammetry curves of the β-CD-Fc-OH/SPE and Fc-OH/SPE were measured with 100 and 500 μM standard creatinine concentration. In the experiment,

the β-CD-Fc-OH was oxidized by enzymatic reaction of creatinine. So, we set up a reductive scan first to reduce the Fc^{+}-OH obtained by HRP^{+} oxidation. The reduction current obtained at this time represents the true concentration of creatinine. An oxidative scan was then performed. As shown in Figs. 4A and 4B, both SPEs exhibited an obvious reduction peak. This phenomenon signified β-CD-Fc-OH and Fc-OH have been oxidized by the enzymatic reaction of creatinine. The SPE reduced Fc^{+}-OH to form a reduction current. But the oxidation peaks were both lower than the reduction peaks, especially for the β-CD-Fc-OH/SPE, which meant β-CD-Fc-OH was more difficult to be oxidized by potential. This is similar to the results of Zhang's study.[12] In addition, comparing Figs. 4A and 4B, the β-CD-Fc-OH/SPE is more susceptible than the Fc-OH/SPE. This is assumed because the spacing of reduction peak current of ab in Fig. 4(A) was bigger than in Fig. 4(B). There was still a small oxidation peak for Fc-OH/SPE, indicating that Fc-OH was more easily oxidized by voltage instead of HRP^{+} when it was not included by β-CD. So the β-CD-Fc-OH/SPE is more suitable than the Fc-OH/SPE to detect creatinine concentration. The sensor is consistent with Dong's study,[13] which improved the sensitivity of detecting H_2O_2 by introducing β-CD.

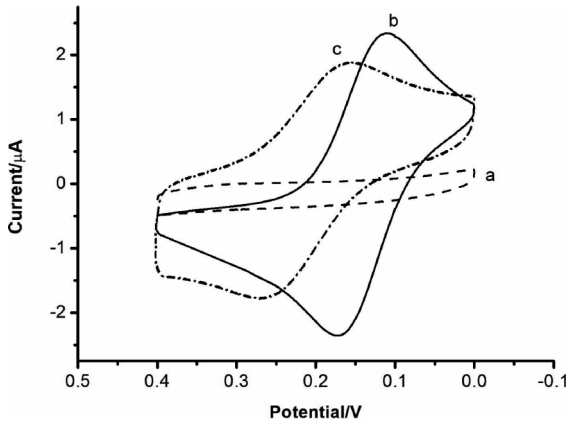

Fig. 3 Cyclic voltammetry of the bare SPE (a), Fc-OH/SPE (b) and β-CD-Fc-OH/SPE (c) in phosphate buffer of pH 7.0 (0.1 V/s).

Secondly, verify the difference of sensitivity from another perspective between the β-CD-Fc-OH/SPE and Fc-OH/SPE. We chose the voltage of –0.3 V as a constant potential of chronoamperometry for the β-CD-Fc-OH/SPE. In the determination of actual serum by electrochemical method, electrochemical substances are often interfered by other redox substances, such as uric acid (UA), ascorbic acid (AA), *etc.* These substances usually have similar redox potentials with electronic mediators, so the interference of UA and AA on the determination of creatinine by the sensor was investigated. When the potential is greater than 0.1 V, the electrochemical response signals of UA and AA in the sensor increase with the increase of the potential. This phenomenon denotes that UA and AA react directly on the surface of the electrode. When the potential is –0.3 - 0.1 V, the electrochemical response signal is basically unchanged. Under –0.3 V, the electrochemical response signal increases with the increase of potential, which represents that the redox reaction of creatinine is incomplete at low voltage. The voltage of –0.3 V in the β-CD-Fc-OH/SPE is lower than for many other amperometric sensors.[30,31] Also, we investigated the direct reduction of H_2O_2 at –0.3 V. When

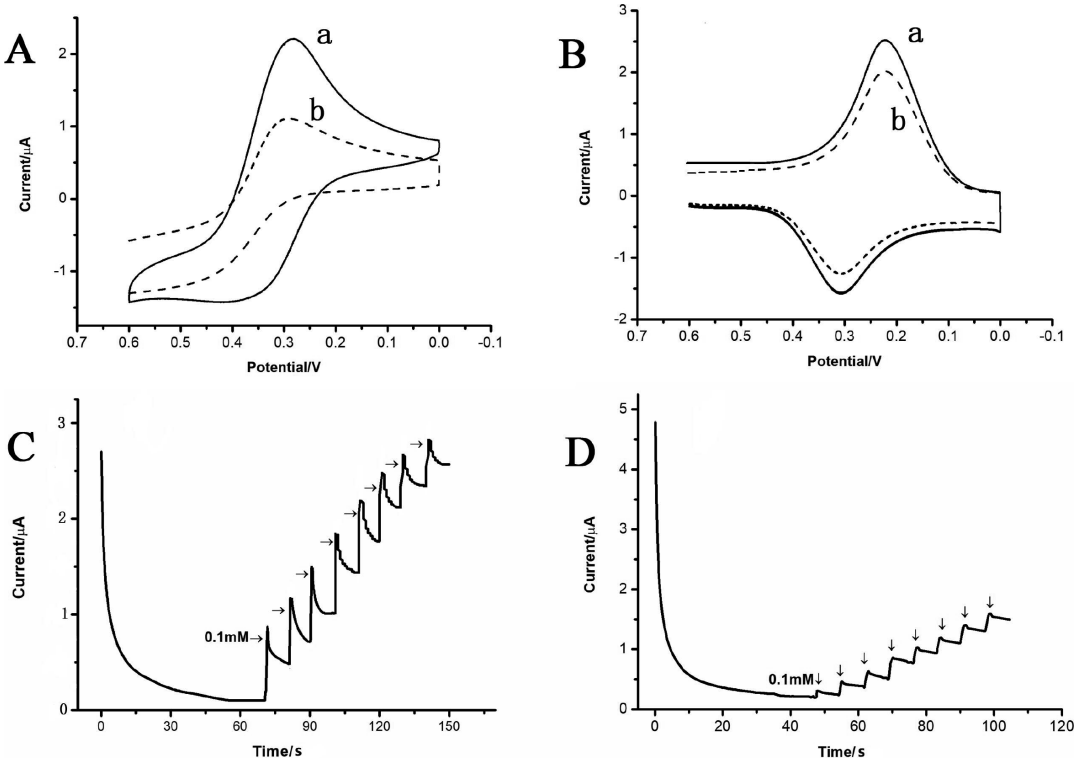

Fig. 4 (A) Cyclic voltammetry of the β-CD-Fc-OH/SPE at 100 μM (b) and 500 μM (a) creatinine (0.1 V/s). (B) Cyclic voltammetry of the Fc-OH/SPE at 100 μM (b) and 500 μM (a) creatinine (0.1 V/s). (C) Chronoamperometry of the β-CD-Fc-OH/SPE added 100 μM creatinine every 10 s at –0.3 V. (D) Chronoamperometry of the Fc-OH/SPE added 100 μM creatinine every 10 s at 0.1 V.

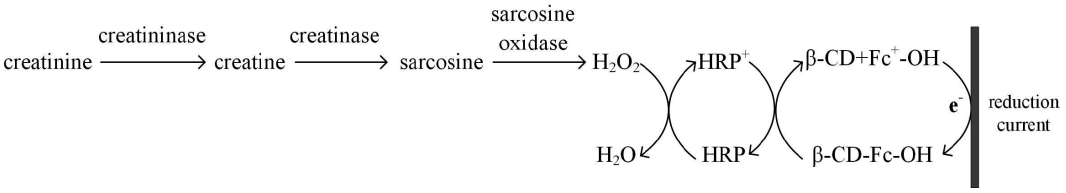

Fig. 5 The reaction principle of creatinine, the principle of β-CD-Fc-OH approaching the enzyme activity center and the reduction current detected by electrodes.

100 µM H_2O_2 was added to the β-CD-Fc-OH/SPE, the reduction current increased significantly. This was mainly because H_2O_2 was reduced to H_2O under the catalysis of HRP. The electrochemical behavior of hydrogen peroxide solution is consistent with the hydrogen peroxide produced by the enzymatic reaction of creatinine on a screen printed electrode, both of which ultimately form an obvious reduction current. Based on Ref. 37, we chose 0.1 V as a testing potential of chronoamperometry for the Fc-OH/SPE. As shown in Fig. 4, it was found that the current became stable when the reaction was carried out for 60 s (Fig. 4C) and 40 s (Fig. 4D). On this basis, 100 µM creatinine was added to the electrode every 10 s, so a gradient curve was obtained, as shown in Figs. 4C and 4D. The appearance of a gradient curve meant that as the creatinine concentration continued to increase, the electrode exhibited good current response. Comparing Fig. 4C with Fig. 4D, the current increased by 0.24 µA for every addition of 100 µM creatinine on the β-CD-Fc-OH/SPE, but there was only an increment of 0.1 µA on the Fc-OH/SPE. These results indicate the β-CD-Fc-OH/SPE can improve the sensitivity of sCr detection compared with the Fc-OH/SPE.

As shown in Fig. 5, the process of converting creatinine to H_2O_2 by a series of enzyme-catalyzed reactions is demonstrated. The process by which β-CD-Fc-OH was oxidized is also shown. H_2O_2 is generated after three enzymatic reactions and then reduced to H_2O by HRP. HRP was also changed to HRP^+ in the previous reaction with H_2O_2. The inclusion compound β-CD-Fc-OH is highly hydrophilic, which ensures sufficient mobility of the mediator.[12] So β-CD-Fc-OH is more accessible to HRP^+ than the hydrophobic Fc-OH, and receives electrons transmitted by HRP^+ easily.

Minimum detection limit and linear range of creatinine

To evaluate the performance of the β-CD-Fc-OH/SPE for detecting creatinine, a calibration curve was prepared (Fig. 6B). The concentrations were 1, 50, 250, 500, 750, and 1000 µM, and the current value was measured by chronoamperometry at –0.3 V (Fig. 6A). As shown in Fig. 6A, the current tended to be stable at 60 s. The response time is less than 98 s of Shin's designed sensor.[40] The current value at 60 s was used to draw scatter plots with the corresponding concentration, and regression analysis was performed. The regression equation is $i = 0.0468 + 0.0013[Cr]$ ($R^2 = 0.9928$). The linear range of 1 - 1000 µM in our study is wider than 3.2 - 320 µM of GH Hsiue's research, in which an amperometric creatinine biosensor was fabricated from the covered platinum/silver electrode with an immobilized multienzyme membrane.[41] The sampling volume of the β-CD-Fc-OH/SPE in our study was 8 µL, which is lower than 30 µL of CD Rasmussen's membrane-based potentiometric biosensor.[42] We calculated three times the value of background noise with the matrix blank, and the corresponding creatinine concentration was the limit of detection. The calculated limit of detection was 0.5 µM, which is significantly lower than that of Chen.[37] Soldatkin and co-workers prepared a sensitive biosensor based on ion sensitive field-effect transistors with a limit of detection of about 10 µM.[43] In summary, the limit of detection of the biosensor was measured to be 0.5 µM and the time of detection was 60 s. The volume of the loading sample was about 8 µL. The linear range was 1 - 1000 µM in our study.

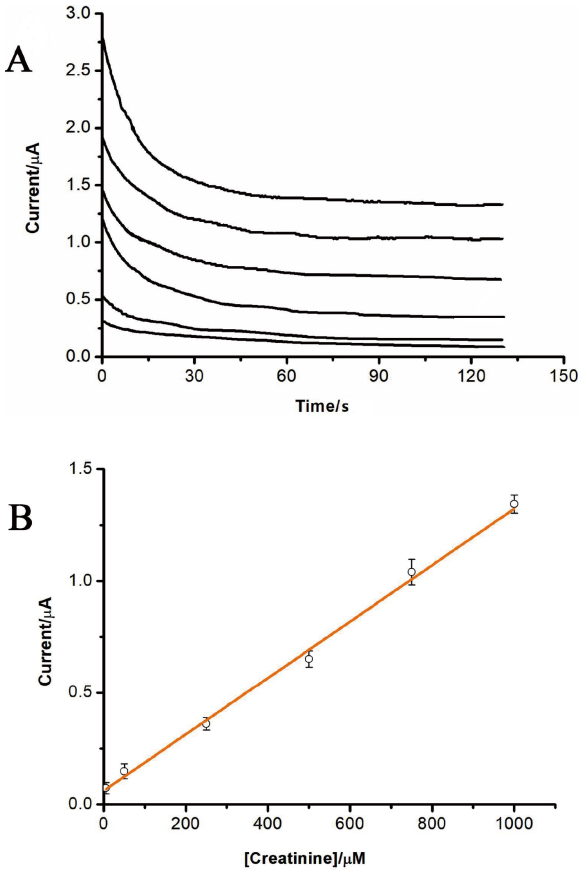

Fig. 6 (A) Chronoamperometry of the β-CD-Fc-OH/SPE for gradient creatinine of 1, 50, 250, 500, 1000 µM at –0.3 V. (B) Calibration curve of chronoamperometry for the β-CD-Fc-OH/SPE. The error bars stand for RSD value ($n = 3$).

Assessment of recovery rate

Different concentrations of standard creatinine solution were added to 55, 200, and 400 µM creatinine serum. Chronoamperometry was used to test the total concentration. The calculated recovery rate ranged from 90 to 110% (Table 1).

Table 1 Recovery rate experiment of serum creatinine

Background concentration of sCr/μM	Added concentration/ μM	Measured value/ μM	Recovery rate, %
55	50	55.3	95
	75	69.9	93
	100	71.8	108
200	150	190	92
	200	212.8	94
	250	212.3	106
400	300	333.3	105
	400	416.7	96
	500	483.9	93

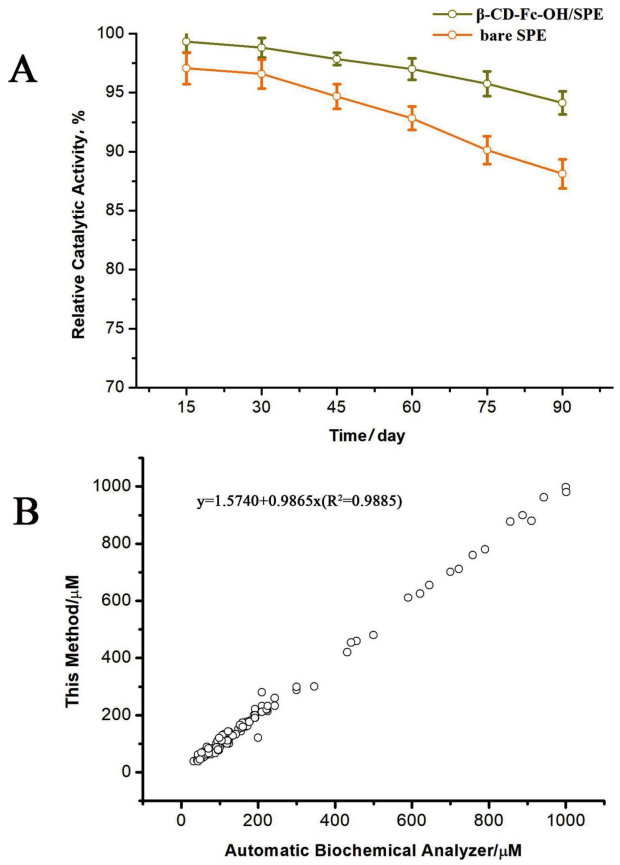

Fig. 7 (A) Stability of the β-CD-Fc-OH/SPE and bare SPE (n = 3). (B) Scatter diagram for creatinine value detected by the β-CD-Fc-OH/SPE and automatic biochemical analyzer.

Estimation of stability

The relative enzyme activity of the β-CD-Fc-OH/SPE and bare SPE were measured at 15, 30, 45, 60, 75, and 90 days, respectively. Chronoamperometry was applied to detect the catalytic activity. Relative catalytic activity is the percentage of the later enzyme activity to initial enzyme activity (Fig. 7A). Using a Wilcoxon rank sum test, W was 21, and Z was 2.097. P was about 0.031, so the β-CD-Fc-OH/SPE and bare SPE are significantly different at a test level of 0.05. The sensor exhibited good stability.

Assessment of clinical specimens test

A total of 120 serum samples were detected by biochemical analyzer and the β-CD-Fc-OH/SPE, and the samples were from 60 healthy subjects and 60 nephropathic patients (Fig. 7B). The regression equation ($y = 1.5740 + 0.9865x$ ($R^2 = 0.9885$)) was obtained by regression analysis, using the rank sum test of the paired samples, $S = 41$, $Z = 1.421$, $P = 0.155$. At the statistical level of 0.05, there was no significant difference between the two methods. The sensor based on chronoamperometry has the clinical adaptability to detect serum creatinine.

Conclusions

Our study reduces the applied potential of amperometric creatinine biosensor to –0.3 V, and greatly prevents the interference of various redox substances in serum. By introducing β-CD, the background noise of Fc-OH oxidized by voltage is eased, so the sensitivity of creatinine detection is improved. The limited of detection was as low as 0.5 μM with a sampling of 8 μL, which provides potential for miniaturization of a creatinine detection sensor for clinical application.

Acknowledgements

This work was supported by the Natural Science Fund of Hunan Province (10JJ5010) and the Science and Technology Plan Project of Hunan Provence (S2014S2033).

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