Sensitive Detection of Serum Creatinine Based on β-Cyclodextrin-Ferrocenymethanol Modified Screen-Printed Electrode

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Abstract

Ferrocenylmethanol (Fc-OH) is included in β-cyclodextrin (β-CD) to form β-CD-Fc-OH complex by host-guest supramolecular interaction. β-CD dissociates from 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 way for oxidation of Fc-OH. And the background noise is reduced for testing 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. 120 clinical specimens were tested by the biosensor and the results showed a linear correlation with biochemical analyzer (\(R^2=0.9885\)). The biosensor could be applied to clinical trials and has a good prospect in clinical sCr detection.

**Keywords:** Ferrocenylmethanol; β-cyclodextrin; biosensor; serum creatinine; screen-printed electrode
Introduction

Cyclodextrin (CD) is used in many different fields of analytical chemistry because it tends to form reversible clathrates and selectively recognize 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)\(^1\), adamantane\(^3\), azobenzene\(^5\) to form superamphiphilic complex by geometric compatibility and hydrophobic interactions.\(^7,8\) L. Feng et al.\(^9\) reported the reversible control of electron transmission through nanopore-tethered Fc based on the complexation with β-CD. However, most of β-CD dissociates from the β-CD-Fc complex due to the conversion of Fc to Fc\(^+\). In H. Basit's work\(^10\), Fc is oxidized to carry 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 losing.\(^12,13\) But there are few researches on the application of β-CD-Fc-OH in 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 Jaffé method, the compensated Jaffé method, the liquid method of enzyme and the dry chemical test.\(^16\) However, the Jaffé method’s poor anti-interference sometimes results in clinical misdiagnosis.\(^17,18\) A large biochemical instrument was required by the enzyme liquid method with a long detection time. The dry chemical test method has a large CV value when sCr concentration is low.\(^16\) At present, electrochemical biosensor has great potential as a detection
instrument in clinical practice, and there are many reports for sCr detection. Rechnitz et al. designed an ammonia gas electrode combined with creatinine deaminase (E.C.3.5.4.21) to detect creatinine. Then Kubo et al. 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. first designed three-enzyme method for less interference, on which subsequent amperometric creatinine biosensors rely. 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:

\[
\text{creatine} + H_2O \xrightarrow{\text{CRN}} \text{creatine} \quad (1)
\]

\[
\text{creatine} + H_2O \xrightarrow{\text{CR}} \text{sarcosine} + \text{urea} \quad (2)
\]

\[
\text{sarcosine} + H_2O + O_2 \xrightarrow{\text{SOX}} \text{glycine} + \text{CH}_2O + H_2O_2 \quad (3)
\]

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) both were introduced to solve these interference. Kinoshita et al. designed a sensor with peroxidase entrapped on a Fe-embedded carbon paste, which decreased the potential to 0.1 V. The sensor of Serafín et al. just needed 0.0 V to work with HRP and ferrocene. Harada and colleague firstly prepared and studied the cyclodextrin–ferrocene inclusion complexes. However, there are not many reports about the electrochemical application of inclusion complexes.

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 test (POCT). 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. β-cyclodextrin, ascorbic acid (AA), and reduced glutathione (GSH) were applied by Aladdin Co., Ltd. Creatinine standard solution were purchased in Beijing Tanmao Quality Testing of China. Creatinine hydrolase, creatine oxidase, sarcosine oxidase, and horseradish peroxidase (HRP) were from Toyobo in Japan. Na₂HPO₃·KH₂PO₃ was purchased from Qitai Biotechnology Co., Ltd. (Hangzhou, China). Sarcosine (sa), lipid, uric acid (UA), bilirubin, and acetaminophen (AP) were applied by Sigma-Aldrich (St. Louis, USA). Conductive carbon paste and insulating ink were purchased from Ten Companies in Japan.

**Preparation of β-CD-Fc-OH compound**

The prepared process of β-CD-Fc-OH compound was specifically as follows: 0.567 g β-CD was prepared, and deionized water was used as a solvent to prepare a 0.5 mmol solution. Weigh 0.432 g Fc-OH the amount of which was 2 mmol. According to the molar ratio of β-CD to Fc-OH (nCD/nFc) of 1:2, 1:1, 2:1, 3:1 and 4:1, the orange fine needle-like Fc-OH dissolved by a little dimethyl sulfoxide (DMSO) was added to a β-CD solution at 60°C. Shake for 6 hours in a constant temperature shaker (TS-100B, Shanghai Jiecheng of China). And then, take them
out and return to room temperature. At last place in a refrigerator at 4℃ overnight. The obtained yellow crystal precipitate was washed 3 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 changing from yellow to colorless indicated that the free Fc-OH was removed completely. The precipitate was dried in a vacuum oven (2XZ (S)-2, Shanghai Deying of China).

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

2.6 g β-CD-Fc-OH and 0.5 g Fc-OH were respectively dissolved in 2.4 g 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, the white material mainly including carboxymethyl cellulose and polyvinyl pyrrolidone was prepared. 400 U/mg creatinine hydrolase, 350 U/mg creatine oxidase, 300 U/mg sarcosine oxidase, 400 U/mg HRP were added to the white material to stir well, and waited for 15 min to obtain enzyme paste. Control the humidity at about 90% with an enzyme printing machine to print the enzyme layer, and fix the enzyme layer in the reaction area. Hydrophilic membrane was covered over the reaction area in order to prevent the enzyme from being contaminated and damp. Finally, slitting mill was used to cut the prepared modified SPE, which was packed into a plastic box with desiccant. The thickness of SPE we prepared is 50 μm.

**Electrochemical Performance Test of Modified Electrode**

The electrochemical characterization of 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 CHI760D electrochemical workstation (Shanghai Chenhua Instrument Co., Ltd. of China). The scanning potential was from +0.4 V to 0 V with scan rate of 0.1 V/s. Add 8 μL of pH 7.0 1/15 M Na$_2$HPO$_4$-KH$_2$PO$_4$ buffer 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.

*The Sensitivity Comparison between β-CD-Fc-OH/SPE and Fc-OH/SPE*

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

Prepare the β-CD-Fc-OH/SPE and Fc-OH/SPE 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 V or 0.1 V (vs. Ag/AgCl).

*Testing of Clinical Specimens*

From December 2017 to April 2018, 60 healthy subjects and 60 patients with nephropathy were collected from the Third Xiangya Hospital of Changsha, Hunan. Their serum was collected and stored in a refrigerator at -80 °C. This study is consistent with Declaration of Helsinki and ethical review. We have got the informed consent from 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

Preparing compound about four different $n_{\text{CD}}$/n$_{\text{Fc}}$ ratios of 1:2, 1:1, 2:1, 3:1, 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 masse. 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 2:1 was the highest, and the inclusion efficiency was the best. Therefore, the inclusion compound used in the subsequent study was prepared in 2:1 ratio. The results of this study differ from the results described in reference 32, which may be due to the differences in experimental procedures. In this experiment, Fc-OH was dissolved in DMSO then to be added into a β-CD solution. But the literature directly incorporated Fc-OH crystals into β-CD solution, and the stirring method or speed were also different.

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 as the pH was 7.0. Therefore, pH 7.0 was taken as the optimum pH value for our experiment. It seems to indicate the sensor can be adaptable to the detection of body fluid.

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

Cyclic voltammetry of 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. β-CD-Fc-OH/SPE had lower peak current than Fc-OH/SPE, and the oxidation peak was found to be positively shifted. These results agree with parts of work of M. Juan and colleagues. In theory, a positive shift of oxidation peak indicates the electrode is
more difficult to oxidize. 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. Δ$E_p$ was 0.061 V, which meant the whole process was reversible. $E'_{pa}$ and $E'_{pc}$ of β-CD-Fc-OH/SPE were 0.271 V and 0.156 V. Δ$E'p$ 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 inclusion effect. According to the comparison of the Fig. 3(b) and (c), β-CD-Fc-OH were synthesized successfully.

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

Firstly, the cyclic voltammetry curves of 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 Fig. 4A and B, both SPEs exhibited an obvious reduction peak. This phenomenon signified β-CD-Fc-OH and Fc-OH have been oxidized by enzymatic reaction of creatinine. The SPE reduced Fc$^+$-OH to form reduction current. But the oxidation peaks were both lower than reduction peaks, especially for 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 G. Zhang's study. In addition, comparing Fig. 4A with B, β-CD-Fc-OH/SPE is more susceptible to the Fc-OH/SPE. Because the spacing of reduction peak current of ab in Fig. 4A was bigger than Fig. 4B. There was still a small oxidation peak in Fc-OH/SPE, indicating that Fc-OH was more easily oxidized by voltage instead of HRP$^+$ when it wasn’t included by β-CD. So β-CD-Fc-OH/SPE is more suitable than Fc-OH/SPE to detect concentration of creatinine. The sensor is consistent with W. Dong’s
study\textsuperscript{13}, which improved the sensitivity of detecting H\textsubscript{2}O\textsubscript{2} by introducing \( \beta \)-CD.

Secondly, verify the difference of sensitivity from another perspective between \( \beta \)-CD-Fc-OH/SPE and Fc-OH/SPE. We chose the voltage of -0.3 V as a constant potential of chronoamperometry for \( \beta \)-CD-Fc-OH/SPE. Because 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 V—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 \( \beta \)-CD-Fc-OH/SPE is lower than the many other amperometric sensor\textsuperscript{30,31}. Also, we investigated the direct reduction of H\textsubscript{2}O\textsubscript{2} at -0.3 V. When 100 \( \mu \)M H\textsubscript{2}O\textsubscript{2} was added to \( \beta \)-CD-Fc-OH/SPE, the reduction current increased significantly. Mainly because H\textsubscript{2}O\textsubscript{2} was reduced to H\textsubscript{2}O 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 screen printed electrode, both of which ultimately form a obvious reduction current. According to the literature\textsuperscript{37}, we chose 0.1 V as a testing potential of chronoamperometry for Fc-OH/SPE. In Fig. 4, it was found that the current became stable when the reaction was carried out for 60 s (Fig. 4C) and 40s (Fig. 4D). On this basis, 100 \( \mu \)M creatinine was added to the electrode every 10 s, so a gradient curve was shown in the Fig. 4C and 4D. Appearance of gradient meant that as the creatinine concentration continued to increase, the electrode had a good current response.
Comparing Fig. 4C with Fig. 4D, the current increased by 0.24 μA for every adding 100 μM creatinine on β-CD-Fc-OH/SPE, but there was only increment of 0.1 μA on Fc-OH/SPE. These results indicate the β-CD-Fc-OH/SPE can improve the sensitivity of sCr detection compared with Fc-OH/SPE.

As shown in Fig. 5, the process that creatinine was converted to H$_2$O$_2$ by a series of enzyme-catalyzed reaction is demonstrated. The process by which β-CD-Fc-OH was oxidized is also shown. H$_2$O$_2$ has been generated after three enzymatic reaction and then reduced to H$_2$O by HRP. HRP was also changed to HRP$^+$ in the previous reaction with H$_2$O$_2$. The inclusion compound β-CD-Fc-OH is highly hydrophilic, which ensures sufficient mobility of mediator. 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 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 JH Shin’s designed sensor. 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 Hsiu’s research, which fabricated an amperometric creatinine biosensor from the covered platinum/silver electrode with an immobilized multienzyme membrane. The sampling volume of β-CD-Fc-OH/SPE in our study is 8 μL, which is lower than 30 μL of CD Rasmussen’s membrane-based potentiometric biosensor. We calculated 3 times value of background noise with the matrix blank, and the corresponding creatinine concentration was the
limit of detection. The calculated limit of detection is 0.5 μM, which is significantly lower than that of P. Chen.\textsuperscript{37} A.P. Soldatkin and co-workers prepared a sensitive biosensor based on ion sensitive field-effect transistors with limit of detection about 10 μM.\textsuperscript{43} In summary, the Limit of Detection of the biosensor is measured to be 0.5 μM and the time of detection is 60 s. The quality of loading sample is about 8 μL. The linear range is 1-1000 μM in our study.

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).

Estimation of Stability

The relative enzyme activity of 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 β-CD-Fc-OH/SPE and bare SPE are significantly different at a test level of 0.05. The sensor has a good stability.

Assessment of Clinical Specimens Test

120 serum samples were detected by biochemical analyzer and β-CD-Fc-OH/SPE including 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 method. The sensor based on chronoamperometry has a
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 Fe-OH oxidized by voltage is eased, so the sensitivity of creatinine detection is improved. The limited of detection is as low as 0.5 μM with 8 μL sampling, which provides potential for miniaturization of creatinine detected sensor in clinical application.

Acknowledgments

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References


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**Figure Captions**

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

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

Fig. 3  Cyclic voltammetry of 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).

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

Fig. 6  (A) Chronoamperometry of 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 β-CD-Fc-OH/SPE. The error bars stand for RSD value (n=3)

Fig. 7  (A) Current of 400 µM Creatinine added different interfering substances (n=3). (B) Scatter diagram for creatinine value detected by β-CD-Fc-OH/SPE and automatic biochemical analyzer
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Fig. 3 Cyclic voltammetry of 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).
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