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Short Communication

Electrochemical Quantification of CK-MB in Serum Based on Immunoassay

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Creatine kinase isoenzyme (CK-MB) is a cardiac biomarker for post-PCI recovery evaluation. This work describes a novel biosensor concept that can be used to quantify enzyme levels where the substrate is immobilized directly on the glassy carbon electrode (GCE). The phosphorylated form of creatine (PC) was immobilized on the electrode with cysteamine (Cys) by a self-assembling monolayer technique. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) studies were used to follow the chemical modification process. The proposed electrochemical sensor could detect MB-CK at a range from 0.1 to 2000 ng/mL. The detection limit was determined to be 0.04 ng/mL.

Keywords: Creatine kinase-MB; Immunoassay; Serum; Electrochemical determination; Creatine kinase isoenzyme

1. INTRODUCTION

Cardiovascular medicine not only embraces new technologies, but also puts them into practical use according to appropriate guidelines [1-3], whereas this is not the case with cardiac troponin. On the other hand, cTn has been distorted due to the improper use cTn in many fields, leading to confusion in the area of post-PCI biomarker increases [4], as in the case recently reported in JACC [5]. In this report, the areas of cardiac injury putatively related to the procedure were visualized by MR imaging post-PCI. Increases in CK-MB were more frequently found to be associated with findings of delayed hyperenhancement compared with increases in cTn, considering the sensitive cTn detection and the

recommended cut-off values used in their study [6-8]. These results suggest that it is essential to develop a specific, sensitive, and easily applied technique for the determination of CK-MB.

CK-MB can be detected by immunoinhibitory measurements performed at 37 °C using the *N*-acetylcysteine-activated configuration to assess CK-MB activity [9]. This configuration has been shown to be highly stable, cost-effective, and it has been proposed as a product by many companies [10-16]. Unfortunately, this approach suffers some inherent drawbacks, including a high incidence of false results and susceptibility to interference. Wang and co-workers [17] proposed a chemiluminescence method involving magnetic separation for the determination of CK-MB. Magnetic beads functionalized with anti-FITC antibodies, and anti-CK-MB antibodies labelled with ABEI and FITC were applied to produce a CK-MB complex after magnetic separation, with a chemiluminescence intensity readout signal. A low LOD was obtained at 0.296 ng/mL. In addition, Garay and co-workers [18] presented a quantitative method to detect CK-MB in untreated serum specimens using an electrochemical immunosensor. The differential cell of the SPR instrument affords the possibility of simultaneous electrochemical detection [19-23].

The present work reports on the development of a GCE-based electrochemical immunosensor. Enzyme attachment was achieved by assembling a glutaraldehyde (GA) layer. Then, Pcrea was covalently linked over the Au-SPE film, which was pre-treated with Cys. Several electrochemical measurements were performed to characterize the final biosensor. As shown by its application for the detection of biological specimens, the biosensor developed here has the potential to be used in screening detection, clinical diagnosis, and biomedical research.

2. EXPERIMENTS

2.1. Chemicals

Potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$) and potassium hexacyanoferrate II-3-hydrate ($K_4[Fe(CN)_6].{}_3H_2O$) were provided by Riedel Haen. *N*-Hydroxysuccinimide (NHS), myoglobin (Myo), bovine serum albumin (BSA), cysteamine (Cys), creatine kinase (CK-MB), and troponin T (TnT) were provided by Fluka. Glutaraldehyde (GA, 25% aqueous solution) and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDAC) were provided by Sigma-Aldrich Chemical Co. Creatine monohydrate (Crea) and creatine phosphate (Pcrea) were obtained from Applichem. The stock solutions of K_2HPO_4 and KH_2PO_4 were mixed together to yield the PBS (0.1 M) working solution. The PBS (0.1 M) adjustment was carried out using H_3PO_4 and NaOH (0.1 M), and 0.1 M Tris–HCl containing 0.05% (w/v) Tween-20 was used as the wash buffer.

2.2. Apparatus

A CHI 660C electrochemical workstation (Chenhua, Shanghai, China) was used for the electrochemical experiments in a three-electrode configuration. The working, reference, and auxiliary electrodes were a bare or modified GCE, a saturated calomel reference electrode (SCE), and a

platinum wire, with the potentials applied against the reference electrode. The EIS measurement was carried out using a multi-impedance test system, with a frequency range of 10 kHz - 10 mHz and an AC amplitude of 10 mV. The potential applied to the CV measurement ranged from -0.8 to 0.8 V. A DPV measurement was also carried out with a step potential and pulse amplitude of 2 mV and 50 mV, respectively. We also applied a 50 ms pulse, a scan rate of 10 mV/s, and a sampling time of 20 ms, along with a 100-ms pulse interval.

2.3. Electrochemical immunosensor fabrication

The bare GCE (diameter: 3 mm) was successively polished using alumina powder (0.3 and 0.05 μm) before the preparation of the immunosensor. Then, the as-prepared sensor was thoroughly rinsed with double-distilled water and absolute alcohol between each polishing step under an ultrasonic bath. This was followed by incubating the GCE surface in a GA solution (1%) and Cys (25 mM) for 60 min each (denoted as GA-Cys/GCE), immersing the as-prepared electrode in 1 mM of activated Pcrea (CP) prepared in PBS for 60 min (denoted as GA-Cys/CP/GCE). Afterwards, this electrode was incubated in a mixture of 25 mm of NHS and 50 mM of EDA for another 60 min at ambient temperature (denoted as GA-Cys/CP/CK-MB/GCE).

2.4 CK-MB activity analyses

For comparison, an immunohistochemical staining method was used for CK-MB analysis. Protein concentrations were detected with a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Using a Hitachi 7600 automatic biochemical analyser (Tokyo, Japan), plasma and myocardial CK-MB activity was determined by inhibition kinetics (Maker Science & Technology, Ltd., Sichuan, China).

3. RESULTS AND DISCUSSION

Electrochemical biosensors enable the content of a biological sample to be determined based on the direct conversion of a biological event to an electronic signal, provided that these are setup properly and reproducibly. Several sensing concepts have been developed in recent decades [24] involving the immobilization of organic films on a suitable support followed by electrical modifications at the solid-state probe. Here, CV and EIS studies were performed to follow the electrical behaviour of the sensor surface. The electrical performance of the electrode preparation was studied using CV and EIS measurements. As shown in the CV curves of Fig. 1, the peak-to-peak potential separation was higher during the modification of GA-Cys and Pcrea than that of the redox probe in the bare GCE, ascribed to the slow kinetics resulting from the elevated electron transfer resistance (R_{et}). Simultaneously, a decrease in the peak current was observed during the electrode preparation.

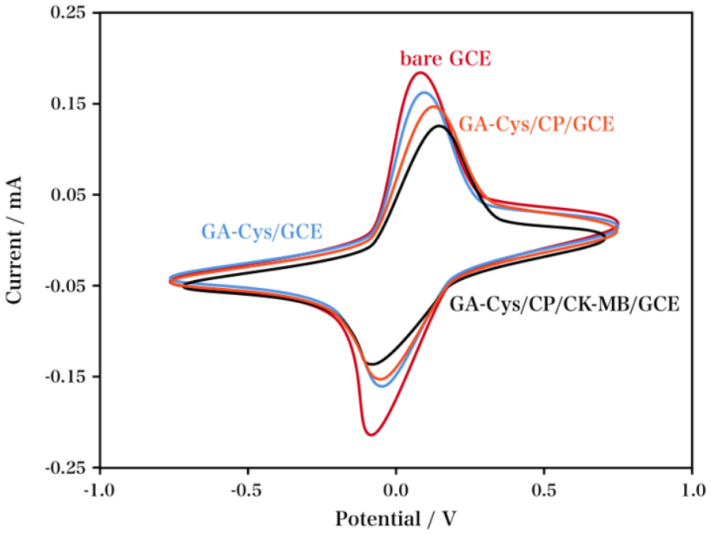

Figure 1. Electrochemical CVs of the bare GCE, GA-Cys/GCE and GA-Cys/CP/GCE and GA-Cys/CP/CK-MB/GCE in the presence of $[Fe(CN)_6]^{3-/4-}$ in pH 7.4 PBS buffer.

The EIS technique is a useful method for the quantitative and qualitative measurement of electrochemical processes at the solution-electrode interface, and it has been acknowledged to be significant for the assessment of immunosensor behaviour. The response of the developed configuration to the small amplitude AC signal at varying frequencies was also monitored by the EIS method. In addition, the EIS technique could provide data about the reaction rates at the solution-electrode interface through an 'equivalent circuit' (an assembly of electrical circuit elements) that modelled the physicoelectric features of the solution/electrode, although the chemical bonds or intermediates could not be identified by this method. This report proposed the experimentally fitted Randles equivalent circuit model containing 4 elements, including R_s, Z_w, C_{dl}, and R_{et}, representing the ohmic resistance of the electrolyte solution, the Warburg impedance, the interfacial double layer capacitance (between a solution and an electrode), and the electron-transfer resistance. Herein, Z_w denoted the diffusion of ions from the bulk electrolyte to the electrode interface, and C_{dl} suggested the surface condition of the electrode. The classical capacitance was replaced with a constant phase element (CPE) to modify the equivalent circuit model described above, where the heterogeneity or roughness of the electrode surface was incorporated with the Helmholtz double layer. The CPE was also parallel with both Z_w and R_{et}, and all of these were arranged in series with R_s. The CPE related impedance is described by the following equation:

$$Z_{CPE}(\omega) = 1/Y_0(j\omega)^n$$

where Y_0 represents a constant, j represents an imaginary number, ω represents the angular frequency, and n represents the CPE exponent, which can be used as a gauge of the heterogeneity and offers detailed information on the degree of surface inhomogeneity. Based on the value of n, CPE can denote resistance, capacitance (n = 1, Y_0 = C), inductance (n = −1) or Warburg element (n = 0.5). The values of the exponent n were approximately 1, suggesting that the modification layer had minimal defects. CPE was observed to resemble a pseudo capacitor in the present study. The impedance performance of the GCEs was represented by the Nyquist plots (the real part of the

impedance Z' *against* the imaginary part $-Z''$), which normally exhibited two main segments, including a semicircle at high frequencies (R_{et}: semicircle diameter) and a straight region at lower frequencies. The former segment suggested a Faradaic electron transfer process, whereas the latter indicated the diffusion-limited transport of the redox species from the electrolyte to the electrode interface. The fitted circuit model was convincing, as confirmed by the small values of χ^2 on the order of 10^{-4}.

The Nyquist plots were shown for these different modified GCEs (Fig. 2), and the insets showed the equivalent circuits. The GA-Cys modified GCE showed a higher R_{et} (99.5 Ω cm^2) than the bare GCE (82.40 Ω cm^2), which suggested that the charge transfer rate at the interface of electrode surface was decreased. When the Y_0 value was relatively high (5.22 μF/cm), R_{et} was decreased, since the anionic probe $[Fe(CN)_6]^{3-/4-}$ concentration was increased at the interface between the solution and the electrode due to its strong affinity to the polycationic layer of GA. After the CP was covalently attached, an apparent increase in R_{et} up to 111.4 Ω/cm^2 was observed, along with a decrease in Y_0 to 2.78 μF/cm^2. GA-Cys/CP/GCE attached to CK-MB was then used for the EIS measurement. The attachment of Cys increased the electron transfer resistance, resulting in an increase in the semicircular section of the Nyquist plot [25]. A sharp increase in R_{et} up to 186.6 Ω/cm^2 was observed, suggesting that an additional barrier was formed for access of the redox probe to the modified GCE after the MB-CK was successfully linked.

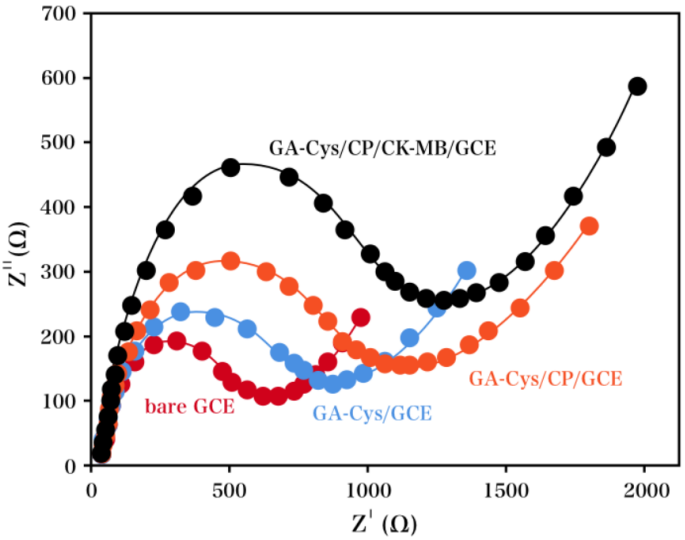

Figure 2. Nyquist plots of the bare GCE, GA-Cys modified GCE, GA-Cys/CP modified GCE and GA-Cys/CP/CK-MB modified GCE in the presence of $[Fe(CN)_6]^{3-/4-}$].

Based on previous reports [26, 27], the development of electrochemical sensors should consider the preparation time to ensure suitability for practical applications. In our case, a 60-min incubation period can be used for clinical applications. One of the most significant factors affecting the enzyme-catalysed reaction was the substrate solution pH (7.0 - 10). The recorded response was highest at a pH of 8.5, as shown in Fig. 3. Therefore, the pH was optimized at 8.5 based on results suggesting maximal CP activity.

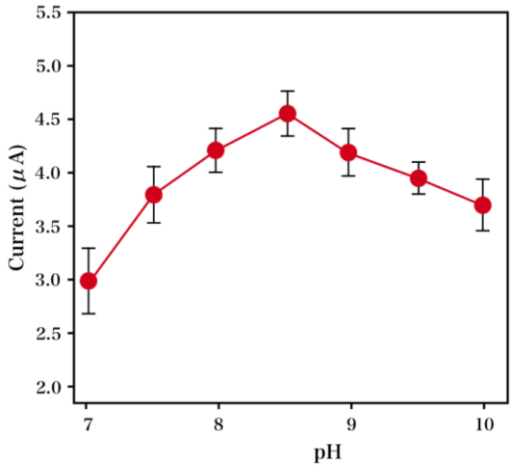

Figure 3. Influence of peak current on the pH of the detection solution.

The analytical behaviour of the GA-Cys/CP/GCE based immunosensor as a detection probe was investigated using GA-Cys/CP/GCE bound by a MB-CK detection probe for comparison. Immunosensors from the same batch were used with different detection probes and varying MB-CK levels (Fig. 4). The control immunosensor was subjected to the whole detection process, resulting in no apparent peaks in the detection solution. The CP modified GCE showed a DPV signal of 6.85 μA. However, when the detection probe was GA-Cys/CP modified GCE, the DPV signal increased sharply up to 15.55 μA. This increase confirmed the enhancement of the signal intensity of the GA-Cys/CP/GCE sensor developed here, promoting the sensor sensitivity towards the determination of MB-CK. A proportional decrease in the DPV peak current was observed with the logarithmic increase in the concentration of MB-CK under optimized parameters.

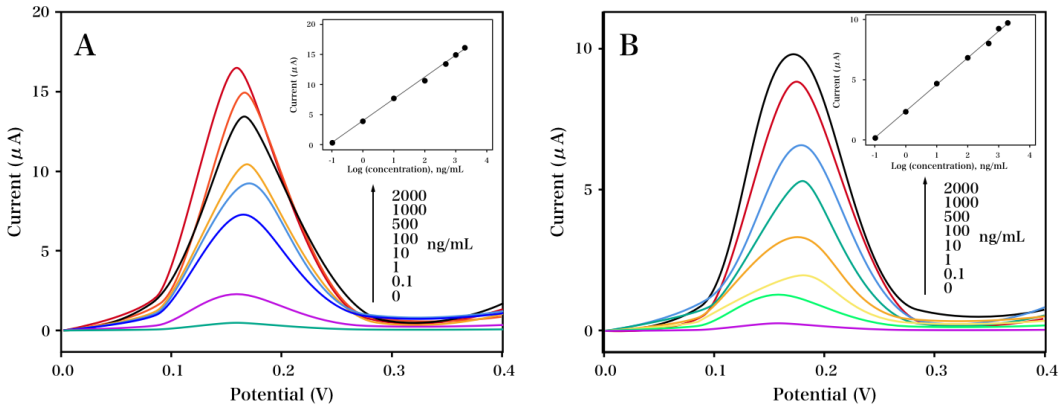

Figure 4. Characteristic DPV curves of GA-Cys/CP modified GCE and CP modified GCE in the presence of 0, 0.1, 1, 10, 100, 500, 1000, 2000 ng/mL MB-CK. Inset: Calibration curves of MB-CK concentrations against peak current.

The linear response ranged from 0.1 ng/mL to 2 μg/mL. The excellent selectivity of the proposed immunosensor can be ascribed to the high specific surface area of the GA-Cys/CP as well, providing a platform for MB-CK loading [28]. The correlation coefficient and LOD were 0.987 and 0.04 ng/mL, respectively. Comparing the analytical performance with other sensors, the immunosensor developed here showed favourable advantages including high sensitivity and the capacity to detect biomarkers at high concentrations. These results suggest that our proposed method could be used for the direct quantification of the target protein over a wide range of concentration in complex clinical serum samples. Table 1 presents a comparison of sensitivity results between the GA-Cys/CP modified GCE and other methods.

Table 1. Comparison of the present GA-Cys/CP/GCE with other MB-CK determination methods.

Electrode	Linear detection range	Detection limit	Reference
Surface plasmon resonance aided electrochemical immunosensor	30 to 300 ng/mL	13 ng/mL	[18]
Magnetic separation integrated with chemiluminescence	0 to 500 ng/mL	0.0296 ng/mL	[17]
Cys/Au/SPE	50 to 480 ng/mL	1 ng/mL	[29]
GA-Cys/CP/GCE	0.1 to 2000 ng/mL	0.04 ng/mL	This work

Our immunosensor and the traditional immunohistochemical staining technique were used to analyse the 5 serum specimens to confirm the performance of the proposed immunoassay, with the results displayed in Table 2. The relative standard deviation was below 4.07%, suggesting good agreement between the results obtained using these two methods.

Table 2. Determination of MB-CK in clinical serum samples.

Sample	Our immunosensor (ng/mL)	Immunohistochemical staining method (ng/mL)	RSD (%)
1	204.1	202.3	3.57
2	255.4	249.3	4.05
3	605.5	600.4	2.66
4	788.5	788.9	4.07
5	988.4	990.6	1.45

4. CONCLUSIONS

This study reports the determination of trace levels of MB-CK using an electrochemical immunosensor. Compared with the group without sample pretreatment, the group with the enzyme showed elevated selectivity. The modified GA-Cys contributed to the improvement of the

immunosensor developed here, including the selectivity, rapidity, and sensitivity. The LOD was obtained as 0.04 ng/mL, meeting the clinical requirements for early diagnostic tests.

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