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MCM-41 mesoporous material modified carbon paste electrode for the determination of cardiac troponin I by anodic stripping voltammetry

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Abstract

An anodic stripping voltammetric method for the determination of cardiac troponin I (cTnI) at a MCM-41 mesoporous material modified carbon paste electrode (MCM-MCPE) was investigated. The test was based on the dual monoclonal antibody "sandwich" principle using colloidal gold as a labeled substrate. Four main steps were carried out to obtain the analytical signal, i.e. electrode preparation, immunoreaction, silver enhancement, and anodic stripping voltammetric detection. The anodic stripping peak current increased linearly with the concentration of cTnI over the range of 0.8–5.0 ng/ml. A detection limit of 0.5 ng/ml was obtained. The established method was applied to detect cTnI in acute myocardial infarction (AMI) samples using routine enzyme-linked immunoadsorbent assay (ELISA) for comparison analysis, and good results were obtained.

Keywords: Cardiac troponin I; Mesoporous materials; MCM-41; Carbon paste electrode; Anodic stripping voltammetry

1. Introduction

It is a long history to immobilize proteins (enzymes) on solid inorganic materials [1,2]. One of the most widely used methods is to encapsulate them inside sol–gel-derived materials [3]. However, due to the small pore size, most studies showed lower specific activity than that of the free one in solution [4]. MCM-41 mesoporous material is a new kind of molecular sieves, the synthesis of which was first reported by researchers of the Mobil Oil Corporation [5,6]. The unusual properties such as large pore size, uniform pore structure, high surface areas, and high loading capacity of MCM-41 have attracted increasing attention in the immobilization of larger biomolecules [7].

Recently, electrochemical methods based on the specific reaction of antibody and antigen with electrochemical transduction have received considerable attention in clinical diagnosis [8–11]. Most of these methods have been commonly achieved in connection to electroactive indicators or enzyme tags [12–14]. Nanoparticle tracers are the possible substitute for electrical detection. Metal nanoparticles, especially gold nanoparticles (nano-gold), possess many unique physical and chemical properties, and therefore, have been intensively studied in analytical chemistry [15–18]. For example, due to that nano-gold particles can strongly interact with biomaterials, they have been used in non-enzyme immunoassays [19–23].

Cardiac troponin I (cTnI) is a subunit of cardiac troponin complex, which is comprised of troponin I, troponin T and troponin C. The molecular weight of cTnI is 22.5 kDa. Following the myocardial damage, the troponin complex is broken up and the individual protein components are released into the bloodstream [24]. Cardiac troponin I has high tissue specificity because of its structural difference from the corresponding skeletal isoforms in its amino acid composition. This distinction allows the two forms of troponin I to be dis-

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tinguished immunologically and thereby ensures an accurate test assay [25–27]. Therefore, cTnI in the serum of patients has been considered as the "gold standard" for diagnosis of myocardial injury [28–30]. And many methods have been developed for detecting cTnI [31–37].

The aim of the present study is to establish a novel electrochemical immunoassay of cardiac troponin I. This assay combines the concepts of the dual monoclonal antibody "sandwich" principle, the nanoparticle-promoted precipitation of silver, the modified carbon paste electrode, and the anodic stripping voltammetry.

2. Experimental

2.1. Reagents and apparatus

cTnI from human myocardium muscle and two monoclonal anti-human cTnI antibodies used in the assay were obtained from Research Institute of Cardiovascular Disease of First Affiliated Hospital of Nanjing Medical University. Colloidal gold was purchased from Sino-American Biotechnology Company (China). Other reagents were commercially available and were all of analytical grade. Deionized water was used in all experiments.

The colloidal gold-labeled detection antibody (cAu–IgG_2) conjugates were centrifuged at a BECKMAN Avanti^{TM} J-25 Centrifuge. Transmission electron micrographs were recorded with a Hitachi 8100 transmission electron microscope (TEM). All electrochemical experiments were carried out on a CHI600A electrochemical workstation (CH Instruments, Inc., USA) with a platinum wire used as the counter electrode and a Ag/AgCl electrode as the reference. The enzyme-linked immunoadsorbent assay (ELISA) comparison analysis was performed on a EL_X808_{IU} Ultra microplate reader (Bio-tek Instruments, Inc. USA).

2.2. Synthesis and characterization of MCM-41 mesoporous material

The MCM-41-type mesoporous material was synthesized in ethylenediamine aqueous solution medium referred to the procedure described by Yang et al. using cetyltrimethylammonium bromide (CTAB) as templates [38]. The physical properties of MCM-41 mesoporous material were characterized using transmission electron microscopy, X-ray powder diffraction, and nitrogen physisorption isotherm. The structural data are summarized in Table 1.

2.3. Preparation of the modified carbon paste electrode

Glass tubes (4 mm inner diameter) were served as the electrode body. Electrical contact was made with a copper wire through the center of the tube. Unmodified carbon paste was prepared by adding 0.5 g of paraffin oil to 2.0 g of high purity graphite powder. Modified carbon pastes were prepared by substituting corresponding amounts of the graphite powder (1.0%, 2.0%, 5.0%, 10.0%, and 20.0%, w/w) with MCM-41 mesoporous material and then adding the paraffin oil and thoroughly hand-mixing in a mortar with a pestle. The pastes were packed into the hole of glass tube and the surface was polished on a sheet of graph paper while a slight manual pressure was applied to the piston.

Table 1
Pore characterization of MCM-41 mesoporous material

Surface area, BET (m^2/g)	961.9
Pore volume (cm^3/g)	0.75
d_{100}-Spacing (Å)	38.4
a_0[a] (Å)	44.3
Pore size[b] (Å)	31.0

[a] $a_0 = 2d_{100}/\sqrt{3}$.
[b] Calculated from N_2 adsorption isotherms.

2.4. Preparation and characterization of the colloidal gold-labeled detection antibody

Both solutions of colloidal gold and detection antibody (IgG_2) were adjusted to pH 8.2 before the label procedure. To 50 ml of colloidal gold solution, 1.13 mg of IgG_2 was added dropwise under constant stirring at room temperature. After the addition of an appropriate concentration of bovine serum albumin (BSA), 15 min incubation under constant stirring was required. The mix was centrifuged in the cold at 300 × *g* for 20 min and the pellet was discarded. The supernatant was then centrifuged in the cold at 10,000 × *g* for 1 h and the pellet of the colloidal gold–IgG_2 complex was resuspended in 5 ml of 0.05 M Tris(hydroxymethyl)aminomethane (Tris)–C1 buffer solution (TB, pH 8.2) containing 0.1% BSA. Finally, the mix was centrifuged in the cold at 300 × *g* for 20 min, and the supernatant was harvested and stored in a refrigerator for further use. The cAu–IgG_2 conjugates could be preserved for at least 6 months under this condition.

TEM technique was used to analyze the size and structure of the unlabeled and labeled colloidal gold (Fig. 1). A brief description of the procedure was as follows. Gold particle samples were prepared by dropping 10 μl of nanoparticle solutions onto a formvar membrane coated on a copper grid.

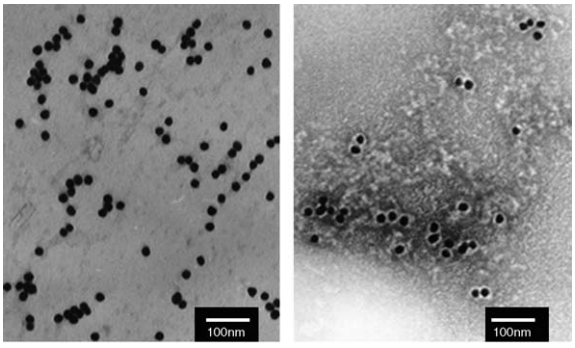

Fig. 1. TEM images of (A) unlabeled and (B) labeled colloidal gold after uranium acetate negative staining.

Any remained solution was wicked away, and the grids were dried for 30 min under ambient conditions. After uranium acetate negative staining, the grids were subsequently imaged using the transmission electron microscope. A halo layer surrounding the colloidal gold particle could be seen from the TEM image of the protein labeled gold colloidal particles (Fig. 1B), while that kind of halo was not observed from the images of the unlabeled particles (Fig. 1A).

2.5. Procedures

The measurement cycle consisted of four steps, as described below: (1) the electrode surface was activated by immersing it in a 0.1 M H_2SO_4 stirred solution with successive cycling between +1.50 V and −0.50 V until the based line was stable; (2) the capture antibody (IgG_1) was immobilized onto the MCM-41 mesoporous material modified carbon paste electrode (MCM-MCPE) surface by immersing the pretreated MCM-MCPE in a solution containing IgG_1 for 30 min. The electrode was then blocked with 2% gelatin, followed by immersion in the cTnI sample solution to immunoreact for 30 min. Then the electrode was allowed to react with cAu–IgG_2 by incubating it in 1 ml of gold particles solution (3.7×10^{11} gold particles per ml) for 30 min. Note that after each step mentioned above, a subsequent washing of the electrode surface in 0.05 M TB of pH 8.2 was needed. This step was called immunoreaction; (3) after the immunoreaction, the electrode surface was immersed in a silver amplifying solution containing 1.0×10^{-5} M silver nitrate and 2.0×10^{-4} M hydroquinone for 10 min of silver deposition. This step was called silver enhancement; (4) finally, the voltammetric detection was performed. The working electrode was washed with water and introduced into the separate voltammetric cell containing only a supporting electrolyte (0.10 M HNO_3), while anodic stripping voltammograms (ASV) were scanned from 0.20 V to 0.40 V at a scan rate of 100 mV s^{-1}. The anodic stripping peak at about 0.27 V was recorded.

The protocol format was outlined in Fig. 2.

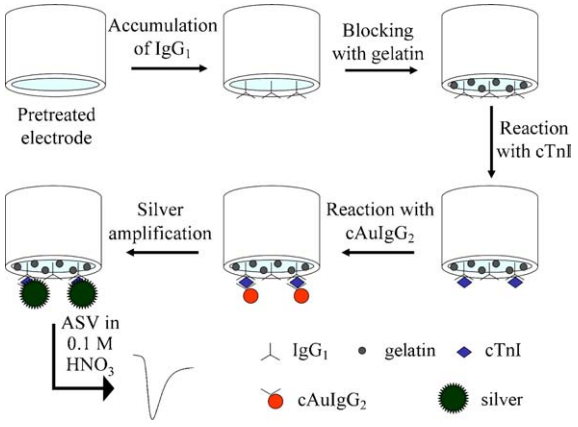

Fig. 2. Protocol format of analytical procedure.

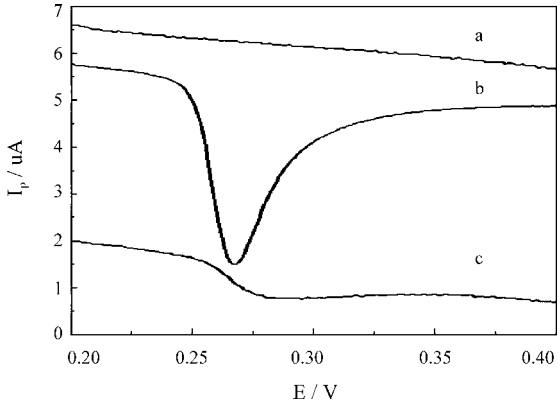

Fig. 3. Anodic stripping curve of MCM-41 modified CPE be processed according to the protocol in Fig. 2: (a) without and (b) with 1 ng/ml cTnI in the serum, and (c) unmodified CPE be processed according to the protocol in Fig. 2 with 1 ng/ml cTnI in the serum.

3. Results and discussion

3.1. Analytical signal of cTnI

Anodic stripping voltammograms (ASVs) were obtained with MCM-41 modified CPE in normal serum (Fig. 3, curve a) and the serum with a cTnI concentration of 1 ng/ml (Fig. 3, curve b). No peak was found in curve a while there was a sensitive anodic stripping peak at about 0.27 V in curve b. Anodic stripping voltammogram was also recorded in the serum containing 1 ng/ml cTnI with unmodified CPE (Fig. 3, curve c). The anodic stripping peak at about 0.27 V was also found, but it was much lower (about 1/6) than that in curve b. It suggested that IgG_1 can be immobilized more efficiently on MCM-MCPE than on pure CPE due to the large pore size of MCM-41 mesoporous material. Whereas no current peak in the potential range used was found when no silver enhancement was applied to following the immunoreaction step. Further experiments also showed that no anodic stripping signal was obtained when the MCM-MCPE was processed as showed in Fig. 2 except the reaction with cAu–IgG_2. A conclusion could be drawn that cTnI in the serum can react with the IgG_1 at the electrode surface and then react with cAu–IgG_2 during the immunoreaction period, and finally the immunogold caused catalytic deposition of silver on gold nanoparticle during the silver enhancement step. The anodic peak at about 0.27 V corresponds to the oxidation of silver atoms and its magnitude reflected indirectly the amount of cTnI.

The silver enhancement method was first described by Holgate et al. [39] and Danscher and Norgaard [40]. At the beginning of the silver enhancement process, the gold particle catalyses the reduction of silver ions onto its surface in the presence of the reducing agent (hydroquinonine). The silver layer itself then catalyses the reduction of more silver ions, consequently causing further deposition of metallic silver. The exact mechanism for the silver enhancement process has not been conclusively established, but it is believed that the

gold nanoparticles selectively accept an electron from the reducing agent (hydroquinonine); the reduced nanoparticle then passes the electron to a silver(I) ion which is reduced to a silver(0) atom and deposited. The process is effective for gold nanoparticles both because their redox potentials are more closely matched to those of hydroquinonine and the silver ions, and because of their large surface area which means they can efficiently react with species in solution [41].

3.2. Optimization of analytical parameters

To maximize the sensitivity and shorten the assay time, factors affecting the amplitude and shape of the anodic stripping peak were optimized.

In view of the significant influence by the content of the modifier on the performance of MCM-MCPE, electrodes prepared with pure carbon paste and different percentages of modified carbon pastes (1.0%, 2.0%, 5.0%, 10.0%, and 20.0%) were tested for their electrochemical and adsorptive behavior. The best results were obtained when the MCM-41 composition in the paste was 5.0–10.0%. Higher content (≥20%) of MCM-41 significantly increased the noise current (Fig. 4), which is presumably due to the reduction of conductive area (carbon particles) at the electrode surface. The effect of paraffin oil ratio in the paste was investigated as well. Too high oil contents would increase the hydrophobic character of the electrode surface, limiting somewhat the immobilization of IgG_1 onto the MCM-MCPE, but, however, too low oil contents resulted in rather poor reproducibility due to the lack in compaction of the electrode materials. The MCM-MCPE had a good reproducibility and lower background current when the graphite to paraffin oil ratio was 4:1. Hence electrodes containing 5.0% MCM-41 (20% paraffin oil) were employed in all subsequent experiments.

Solutions with different pH value (typically 7.4, 8.2, and 9.0) of Tris–HCl buffer were examined as supporting buffer solutions of the immunogold. The highest signal to noise ratio was obtained when the pH 8.2 buffer solution was used. It is most probably due to the fact that the stability of cAu–IgG_2 conjugates is partly depended on pH. The most suitable pH should be close to the isoelectric point (p*I*) of IgG (p*I* approximate to 7–9). When the solution pH is too lower than p*I* the protein is positively charged, which leads to form unstable aggregates owing to the electrostatic affinity interaction between IgG_2 and colloidal gold. In a solution possessing pH too higher than p*I*, however, both the IgG_2 and colloidal gold are negatively charged, the cAu–IgG_2 conjugates are also unstable as a result of the mutual repulsion between them. Therefore, a pH 8.2 Tris–HCl buffer solution was used from the preparation of cAu–IgG_2 conjugates to the immunoreaction step.

Fig. 5 illustrates the influence of the silver enhancement time on the ratio of signal to noise of the system in a silver deposition solution containing 1.0×10^{-5} M silver nitrate and 2.0×10^{-4} M hydroquinone. In serum containing 1 ng/ml cTnI, the anodic peak current increased nearly linearly with increasing silver enhancement time (□), indicating that the longer the silver enhancement time, the more the silver atoms deposited onto the electrode surface by nano-gold catalytic reduction, which might conduce the higher sensitivity. When there is no cTnI in the serum, the peak current was found nearly zero at shorter silver enhancement time, and then increased rapidly with longer silver enhancement period (●). It could be interpreted by the fact that the silver reduction process was very slow in this silver deposition solution, while the reduction process could be greatly accelerated by nano-gold catalysis. Longer silver enhancement time could conduce more silver atoms to be catalytic reduced by nano-gold particle on the electrode surface, and therefore, a higher anodic peak current signal was obtained. But, however, it could also induce the spontaneous deposition of silver onto other components of the electrode surface, which might cause a false positive signal. Therefore, from the experiment results we come to the conclusion that 10 min of silver amplification time could offer the best trade-off between high sensitivity and selectivity.

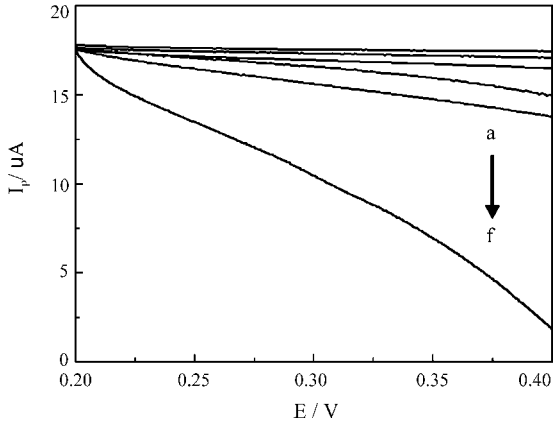

Fig. 4. Anodic stripping curves of negative serum at (a) pure CPE and MCM-MCPE with MCM-41 percentage (b) 1.0%, (c) 2.0%, (d) 5.0%, (e) 10.0%, and (f) 20.0%.

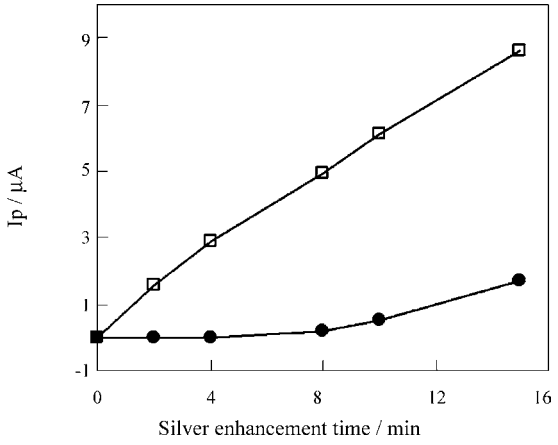

Fig. 5. Effect of silver enhancement time on the peak current: (□) with 1 ng/ml cTnI, and (●) without cTnI in the serum.

The most suitable initial scan potential was also tested. Considering that silver presents a reduction peak at about 0.03 V in 0.10 M HNO_3 at MCM-MCPE, an initial potential of 0.2 V during the anodic scanning was selected. This strategy could maximally avoid the possible electrochemical deposition of Ag^+ on the electrode surface. Therefore, a scan range from 0.20 V to 0.40 V was selected to reduce the undesirable background signal.

The relationship between the anodic stripping voltammetric peak current and the concentration of capture antibody was investigated in the range of 0.01–1.0 mg/ml. It was found that the maximum and constant peak currents could be obtained when the concentration of IgG_1 was higher than 0.10 mg/ml, and therefore, a concentration of 0.20 mg/ml IgG_1 was selected.

The peak current increased with increasing IgG_1 immobilization time as well as the cTnI reaction time initially. Experiment results showed that 30 min in each of these two steps were sufficient.

Moreover, the influence of the concentration of cAu–IgG_2 on the ratio of signal to noise of the system was studied. The non-specific adsorption of immunogold onto the electrode surface increased rapidly when a high concentration of cAu–IgG_2 was used, which would lead to a high background peak current, and therefore, a false positive signal. However, the signal was dramatically decreased with a too low concentration of cAu–IgG_2 due to the insufficiency of immunogold for the immunoreaction and the subsequent silver enhancement. An immunogold solution containing 3.7×10^{11} gold particles per ml was selected accordingly. Meanwhile, taking into account of sensitivity and selectivity, an immunoreaction time of 30 min was sufficient.

Solutions of bovine serum albumin, low-fat dry milk, and gelatin were tested as the blocking agent to reduce the non-specific adsorption of cTnI and cAu–IgG_2. It was found that gelatin was the best choice for the lowest background current. When the concentration of gelatin was 2%, the highest signal to noise ratio was reached. A 2% gelatin solution was selected as the blocking reagent accordingly and the blocking time was 30 min at room temperature.

3.3. Analytical performance

Using the protocol described in the experimental section, it is feasible to quantify cTnI. The anodic stripping curves of different concentrations of cTnI were listed in Fig. 6. A linear relationship between the anodic stripping peak current of silver and the concentration of cTnI from 0.8 ng/ml to 5.0 ng/ml was acquired. The calibration equation was found to be the following: I_p (μA) = 0.6 + 2.1C (ng/ml) with $R^2 = 0.98$. A detection limit of 0.5 ng/ml of cTnI was obtained. The serum cTnI in several patients with AMI were determined using the established method and routine ELISA simultaneously. The sera were diluted with TB before the measurement. Typical results were summarized in Table 2.

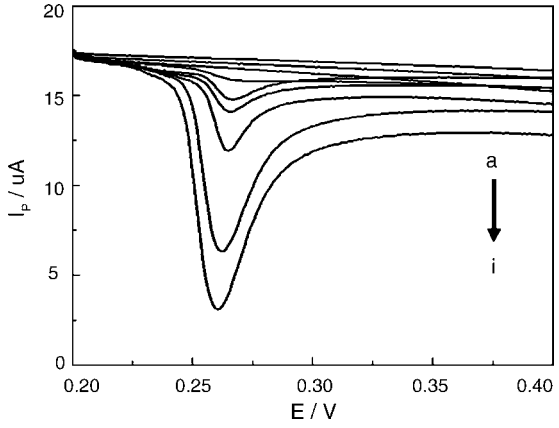

Fig. 6. Anodic stripping curves of different concentrations of cTnI. (a) 0 ng/ml, (b) 0.1 ng/ml, (c) 0.2 ng/ml, (d) 0.5 ng/ml, (e) 0.8 ng/ml, (f) 1.0 ng/ml, (g) 2.0 ng/ml, (h) 5.0 ng/ml, and (i) 10.0 ng/ml.

Table 2
Determination results of cTnI in AMI serum samples

Sample No.	This method (ng/ml)	ELISA (ng/ml)
1	4.53	4.16
2	1.79	1.96
3	0.85	0.77

The results from this method showed good agreement with those from ELISA.

4. Conclusions

In this paper, the MCM-41 mesoporous material was demonstrated to be a promising material for the adsorption of proteins (antibody). This excellent property allows a novel electrochemical immunoassay for antigen (cTnI) at the MCM-41 modified carbon paste modified electrode, combining the concepts of the dual monoclonal antibody "sandwich" principle, the silver enhancement on the nano-gold particle, and the chemically modified electrode. The established method worked well when determining cTnI in AMI serum samples and showed good accordance with those detected by routine ELISA.

Acknowledgments

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