Biosensors and Bioelectronics 56 (2014) 258–263

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A highly sensitive immunosensor for calmodulin assay based on enhanced biocatalyzed precipitation adopting a dual-layered enzyme strategy

Ying Fu [a], Kai Liu [a], Qianqian Sun [a], Bin Lin [a], Danqin Lu [a], Zhiai Xu [a,*], Chen Hu [a], Guangjian Fan [b], Shengping Zhang [b], Chuangui Wang [b], Wen Zhang [a,*]

[a] Department of Chemistry, East China Normal University, Shanghai 200062, People's Republic of China
[b] Institute of Biomedical Sciences, College of Life Science, East China Normal University, Shanghai 200062, People's Republic of China

ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
4 January 2014
Accepted 17 January 2014
Available online 24 January 2014

Keywords:
Immunosensor
Calmodulin
Biocatalyzed precipitation
Cancer

ABSTRACT

Calmodulin (CaM) is a ubiquitous protein in eukaryotic cells, and it plays an important role in cancer progression. In this paper, a highly sensitive immunosensor adopting a dual-layered enzyme strategy was proposed for electrochemical detection of CaM. This immunosensor was constructed by introducing honeycomb-like mesoporous carbon (HMPC) as a sensor platform to sequentially immobilize antibody (Ab_1), CaM and a multi-functionalized label. The label (HRP-PAupc-Ab_1) was synthesized by covalently binding Ab_1 and horseradish peroxidase (HRP) to poly(acrylic acid)-functionalized Au popcorn (PAupc) nanoparticles. A novel dual-layered enzyme strategy was employed by incubating HRP-secondary antibody (HRP-Ab_2) onto the label surface and the enhanced biocatalyzed precipitation was therefore induced. This immunosensor exhibited satisfactory analytical performances for CaM detection with a linear response ranging from 5.0 pg mL^{-1} to 100 ng mL^{-1} and a detection limit of 1.5 pg mL^{-1}. The immunosensor has also been successfully applied to the CaM analysis in two cancer cells (HepG2 and MCF-7) with high sensitivity, which has shown great potency for cancer study.

1. Introduction

Calmodulin (CaM) is an important protein that regulates many cellular processes, including metabolism, cell cycle, apoptosis and cell communication (Ciccone et al., 2008; Klein et al., 2013; Lu et al., 2011; Zemlyanskikh and Kofanova, 2006; Zhou et al., 2013). More importantly, it plays a critical role in the regulation of cancer-related cellular activities (Le Bihan et al., 1998). Many investigations have indicated that there is a close relationship between CaM expression and cancer cell progression (Jacobs et al., 2000; Rasmusen and Means, 1990). For example, the concentration of CaM appears to correlate positively with the growth rate of liver cancer cells. Furthermore, the CaM expression in a breast cancer cell line has involved in the control of cell proliferation (Coticchia et al., 2009; Das and Sharma, 2005; Gallo et al., 2008; Singh et al., 2010). Therefore, development of sensitive methods for CaM detection is significant to understand its role in cancer cell progression and to provide tools for clinic diagnosis and treatment.

Up to now, Western blotting (Michelhaugh and Gnegy, 2000), radioimmunoassay (Muthukumar et al., 1985), and phosphodiesterase assay (Liu et al., 1996) have been applied in CaM assay. Compared with these methods which are often time-consuming and laborious, electrochemical immunosensor opens a promising avenue for the rapid and cost-effective determination of biological analytes (Liu and Ma, 2013; Singh et al., 2013). Although it has not been applied to CaM analysis yet, electrochemical immunosensor appears to be a suitable method inspired by the successful applications for many other important proteins (An et al., 2012). These applications often employ enzymatic reactions to impart proteins with detectable electrochemical signal output such as multiple enzyme probe (Mani et al., 2009), biocatalyzed precipitation (Alfonta et al., 2001; Fortin et al., 2006), catalytic chemical process (Das et al., 2006), and redox cycling (Akanda et al., 2012). Among these reactions, biocatalyzed precipitation has attracted rising interests owing to its quick response, simple operation process and high sensitivity. The quantitative analysis was realized by monitoring the extent of electrode insulation after a precipitation reaction was catalyzed by the carried enzyme (Alfonta et al., 2000; Patolsky et al., 2003, 1999). Unfortunately, most of these typical immunosensors cannot fulfill the CaM expression evaluation in cancer cells due to their limited sensitivity. Take antibiotin IgG antibody determination as an example, biotin molecule was

* Corresponding authors. Tel.: +86 21 62232801; fax: +86 21 62232627.
E-mail addresses: zaxu@chem.ecnu.edu.cn (Z. Xu), wzhang@chem.ecnu.edu.cn (W. Zhang).

0956-5663/$ - see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2014.01.027

conjugated with single layer of HRP molecules (single-layered enzyme strategy) which obtained a dynamic detection range from 0.1 μg mL^{-1} to 100 μg mL^{-1} (Yoon et al., 2002). However, the concentrations of CaM in cancer cells are much below 1.0 ng mL^{-1} (Persechini and Cronk, 1999), which is beyond the detection range of these immunosensors and requires an effective amplification strategy to enhance the sensitivity of the immunosensor based on biocatalyzed precipitation.

In this paper, we report an electrochemical immunosensor for highly sensitive detection of CaM based on enhanced biocatalyzed precipitation adopting a dual-layered enzyme strategy. The immunosensor was constructed by using honeycomb-like mesoporous carbon (HMPC) as the sensor platform for CaM loading, not only because of the excellent conductivity, but also due to its superiorities of good mechanical stability and large pore volume (Zhao et al., 2013; Zolfaghari et al., 2013). A multi-functionalized label (HRP-PAupc-Ab_1) was synthesized by covalently binding antibody (Ab_1) and horseradish peroxidase (HRP) to poly(acrylic acid)-functionalized Au popcorn (PAupc) nanoparticles, which has been applied to recognize the loaded CaM, to anchor a large amounts of HRP as the first enzyme layer and catalyze the precipitation reaction. Horseradish peroxidase-secondary antibody (HRP-Ab_2) was further incubated on the label surface as the second enzyme layer to induce the enhanced biocatalyzed precipitation. The developed immunosensor exhibited the best performance in comparison with immunosensors based on single-layered enzyme strategy: a wider linear calibration (ranging from 5.0 pg mL^{-1} to 100 ng mL^{-1}) and a lower detection limit (1.5 pg mL^{-1}). Furthermore, the immunosensor has been successfully applied to the CaM analysis in different cancer cell lines, which has displayed great potency of the immunosensor for studying the relationship between CaM expression and various cancer cellular activities.

2. Experimental section

2.1. Materials

Calmodulin (CaM), cethyltrimethylammonium bromide (CTAB), ascorbic acid, poly(acrylic acid) (PAA, M_w 1800), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Horseradish peroxidase (HRP), 4-chloro-1-naphthol (CN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiol-terminated poly(ethylene glycol) (CH_3O-PEG-SH, M_w 3000) was obtained from Yare Biotechnology Co., Ltd. (Shanghai, China). Mouse polyclonal anti-calmodulin (Ab_1) was purchased from Zhenjiang Hope Biotechnology Co., Ltd. (Zhenjiang, China). Horseradish peroxidase-labeled goat anti-mouse IgG (HRP-Ab_2) was supplied by Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). HMPC was provided by Shanghai Key Laboratory of Green Chemistry and Chemical Processes, East China Normal University (Shanghai, China). Chitosan and other chemical reagents were purchased from Chemical Reagents Co., Ltd. (Shanghai, China). 10 mM phosphate buffer saline (PBS) solutions of pH 7.4 were used in all experiments. Any other solutions were prepared by MilliQ water (18 MΩ cm, Millipore).

2.2. Apparatus

Electrochemical measurements were performed with a CHI 660C workstation (Chenhua Corp., Shanghai, China) with a conventional three-electrode configuration comprised of a glassy carbon working electrode (GCE), a platinum wire auxiliary electrode, and a saturated calomel reference electrode (SCE). Cyclic voltammetry (CV) was carried out at a scan rate of 100 mV s^{-1}. Electrochemical impedance measurement (EIS) was performed with an Autolab PGSTAT12 (Ecochemie, BV, The Netherlands) controlled by GPES 4.9 and FRA 4.9 software using an alternating current voltage of 5.0 mV, within the frequency range of 0.01 Hz–100 kHz. The UV–vis absorption spectra were obtained from Cary 50 UV–vis spectrophotometer (Agilent Technologies, Palo Alto, CA). The images of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were collected from JEM-2100 (JEOL Ltd., Tokyo, Japan) and S-4800 (Hitachi Co., Ltd., Tokyo, Japan), respectively. Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet Nexus 670 instrument (Thermo Nicolet Co., USA) in KBr pellet form.

2.3. Cell culture and lysis

The cell lines (HepG2 and MCF-7) were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). They were grown in RPMI-1640 medium with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. At the logarithmic growth phase, cells were trypsinized and washed twice with PBS. After removal of the final wash solution from the cells, 1 mL RIPA lysis buffer was added and incubated on ice or in a refrigerator (2 °C to 8 °C) for 5 min. The residual cells must be rapidly removed by a cell scraper and transferred to a tube on ice. The lysate was then clarified by centrifugation at 8000 rpm for 10 min at 4 °C to pellet the cell debris. Finally, the supernatant containing the soluble proteins were transferred to a tube on ice for electrochemical detection.

2.4. Preparation of Aupc nanoparticles

In a 20 mL mixed solution containing 2.5×10^{-4} M $HAuCl_4 \cdot 3H_2O$ and 10^{-4} M trisodium citrate, ice-cooled $NaBH_4$ (60 μL, 0.1 M) was injected into the mixture under vigorous stirring. The solution turned pink immediately and became red after it was kept in dark overnight. Growth solution was further prepared for the seed-mediated growth procedure. After dissolving 0.05 g of CTAB in 46.88 mL water, 2 mL of 0.01 M $HAuCl_4 \cdot 3H_2O$ and 0.3 mL of 0.01 M $AgNO_3$ were injected under constant stirring, respectively. The solution turned from yellow to colorless upon reacting with 0.32 mL of 0.1 M ascorbic acid, followed by rapid injection of 0.5 mL of Au seeds under stirring for 2 min. The color changed into blue within 2 min. Then the solution was washed three times and stored at 4 °C.

2.5. Synthesis of Au nanoparticles (AuNPs)

AuNPs were prepared by using the procedures reported previously (Deng et al., 2013). In brief, 500 mL of 1 mM $HAuCl_4$ was brought to a rolling boil with vigorous stirring. After rapid addition of sodium citrate (50 mL, 38.8 mM) to the vortex, the solution changed its color from pale yellow to burgundy and the AuNPs were obtained.

2.6. Fabrication of PAupc nanoparticles

The Aupc nanoparticles were first synthesized by a seed-mediated growth approach (Lu et al., 2010). 1 mL of 10 mg mL^{-1} CH_3O-PEG-SH solution was sonicated for 10 min and treated with 10 μL of 0.1 M $NaBH_4$ under sonication for another 15 min to reduce dimerized CH_3O-PEG-SH. After that, 1 mL Aupc nanoparticles was added and reacted for 6 h. The resulting PEGylated Aupc nanoparticles were collected by centrifugation at 8000 rpm for 10 min and resuspended in 1 mL H_2O. 200 μL of 10 mg mL^{-1} PAA in 10 mM NaCl solution and 100 μL of 10 mM NaCl solution were injected into the PEGylated Aupc nanoparticles simultaneously. After being stirried for 30 min and washed for three times, the PAupc nanoparticles were obtained.