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Peptide-based electrochemical approach for apoptosis evaluation

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ABSTRACT

This paper reports a strategy to assemble apoptotic cells on a solid surface using a peptide as the recognition element. And a peptide-based electrochemical biosensor to directly evaluate apoptosis is described for the first time. The peptide modified on an electrode is designed to contain the sequence that can recognize externalized phosphatidylserine on apoptotic cells, and can then capture the cells onto the electrode surface. In the electrochemical system, the immobilized cells can not only provide significant steric hindrance for electron transfer, but also shield the positive charges of the peptide that can attract negatively charged electrochemical probes. Therefore, the obtained electrochemical signals drop significantly after the incubation of apoptotic cells, which can be used to reveal the apoptosis level. The experimental results of this approach are well in line with other standard methods. Moreover, this electrochemical method is simple, cost-effective, convenient, sensitive, and holds great potential toward apoptosis evaluation, therapeutic effect assessment and deeper cellular biological studies.

1. Introduction

Distinguished from necrosis, apoptosis stands for programmed cell death, which plays critical roles in a wide range of physiological processes like proper development of immune system (Hirose and Horvitz, 2013; Kale">Thompson, 1995). A timely monitoring of apoptosis can effectively enhance early diagnosis and continuous evaluation of the efficacy of drugs (Bhattacharjee, 2008; Y.">Zhao et al., 2001).

Since cells undergoing apoptosis may have well-defined morphological changes and some typical biochemical events, different strategies can been developed for apoptosis characterization, such as externalized phosphatidylserine (PS) measurement (L. Zhang et al., 2013), cysteine-dependent, aspartate-specific proteinases (caspases) activity assay (Chen et al., 2009), capacitance sensor (Lee et al., 2009), DNA fragmentation assay (Jiang et al., 2013) and so on. The observation of morphological change of apoptotic cells by electron microscopy or fluorescence microscopy is considered to be the gold standard for apoptosis characterization. However, it requires professional skills and redundant sample preparation procedures. Moreover, quantitative analysis cannot be achieved, which may limit its application to a great extent. Several alternative strategies have been investigated. For example, caspases, a family of proteases closely related to cellular apoptosis, are usually detected and identified as markers of apoptosis (Zhang et al., 2011). Nevertheless, in many cases the analysis is performed in cell lysates system, in which abundant non-target proteins and other complex contents may cause certain interference for accurate detection of caspases. Another universal indicator for apoptosis characterization refers to PS translocation from inner layer of the cell membrane to outer layer (van den Eijnde et al., 1998). In most PS measurement-based detection methods, annexin V, a 36 kDa weighted protein is usually employed to recognize externalized PS in the presence of Ca^{2+} (Liu et al., 2009). So far, annexin V-based flow cytometric assay has now being widely used in the detection of apoptosis. However, this method has certain defects such as high cost and inconvenient operation. Therefore, there is an ongoing need to develop advanced methods for apoptosis evaluation.

Recently, optimized peptides for PS targeting based on the original sequence of PS-binding site in PS decarboxylase have now being exploited to replace annexin V (Burtea et al., 2009; Stace and Ktistakis, 2006). The advantages are as follows. First, peptides are less expensive than the reagents, mainly the fluorescent ones, used for annexin V-based methods. Second, the specific binding of peptides is more convenient without the requirement of Ca^{2+}. Third, the lower molecular weight of peptides can increase the

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binding efficacy with PS. Moreover, in some solid phase reaction based methods, peptides have higher stability and the succinct structures can accelerate the self-assembly on the solid surface, while annexin V is difficult to retain its native structure and biological functions without certain treatment.

It is well-known that electrochemical techniques exhibit attractive merits in terms of inherent simplicity, low cost, fast-response, high sensitivity and are convenient for analysis purposes (Qiu et al., 2011). In recent years, due to the development of the functionalization of electrode surface, electrochemical techniques have been widely used to detect various analysts at extremely low concentrations (Inoue et al., 2010; Miao et al., 2013; Miodek et al., 2013; Zhang et al., 2008). In this work, we have designed a PS-specific peptide for electrochemical evaluation of apoptosis. Peptide bridge possesses excellent electric conductivity (Xiao et al., 2008). Compared with high molecular weighted annexin V that may hinder electric transfer rate, peptide is more suitable for PS recognition in electrochemical systems. This biosensor does not need any fluorescent materials, complicated procedures or expensive instruments, which may offer great promise for cost-effective, simple, convenient and sensitive analysis of apoptosis.

2. Experimental

2.1. Materials and chemicals

Two peptides, FNFRLKAGAKIRFGRGC and AFGNRGRAAKNFHARGC were manufactured and purified by China peptides Co., Ltd. (Shanghai, China). The underlined part could recognize PS with high affinity (Xiong et al., 2011). MCF-7 cells (ATCC) were graciously provided by Soochow University (Suzhou, China). Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Engineering Material Co., Ltd. (Hangzhou, China). DMEM was from Gibco (Gaithersburg, USA). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), mercaptohexanol (MCH) and Hoechst 33342 were purchased from Sigma (USA). Propidium iodide (PI) was from Shanghai Sangon Biological Technology & Services Co., Ltd. (Shanghai, China). H_2O_2 was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All the other chemicals were of analytical grade and used as received. Peptide immobilization buffer was 20 mM HEPES with 10 mM TCEP (pH 6.2). Double-distilled water used in this work was purified with a Milli-Q purification system (Barnstead, USA) to a specific resistance of 18 MΩ cm.

2.2. Cell culture and apoptosis induction

In this work, MCF-7 cells were chosen as a model and were maintained in DMEM medium containing 10% (v/v) fetal bovine serum at 37 °C in 5% CO_2 atmosphere. Experiments were conducted during the logarithmic growth phase of the cells. After reaching 80% of confluence, the cells were washed with phosphate buffered saline (PBS) and then exposed to DMEM medium containing 0, 25, 50, 80, 100 μM H_2O_2 for 30 min. Subsequently, cells were washed, detached and resuspended in PBS with the final concentration of 10^6 cells/mL. The cells were kept in ice before further experiments.

2.3. Preparation of peptide modified electrode

The substrate gold electrode (2 mm diameter) was firstly immersed in piranha solution (98% H_2SO_4:30% H_2O_2=3:1) for about 5 min (*Caution: Piranha solution dangerously attacks organic matter!*). After carefully rinsed with double-distilled water, it was polished with P5000 silicon carbide paper and then 1, 0.3, 0.05 μm alumina slurry, respectively. Next, sonication procedures were carried out for 5 min in ethanol and water. Afterward, the electrode was dipped in 50% HNO_3 for 30 min, and then electrochemically cleaned with 0.5 M H_2SO_4. Subsequently, the cleaned electrode was dried with nitrogen before its incubation with 100 μM peptide for 16 h. The pretreated electrode was further dipped in 1 mM MCH for 30 min to obtain well aligned monolayer (Miao et al., 2009).

2.4. Electrochemical measurements

Electrochemical measurements were performed on an electrochemical analyzer (CHI660C, CH Instruments) with a three-electrode system. Before the experiment, the peptide modified electrode was incubated with H_2O_2-treated cells for 1 h, which was then used as the working electrode. A platinum wire electrode served as the auxiliary electrode, while a saturated calomel electrode (SCE) was used as the reference electrode. The buffer solution for electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and differential pulse voltammetry (DPV) was 5 mM $Fe(CN)_6^{3-/4-}$ with 1 M KNO_3. Experimental parameters were as follows: for EIS, bias potential (0.204 V versus SCE), amplitude (5 mV), frequency range (1–100,000 Hz); for CV, scan range (−0.3–0.6 V), scan rate (0.1 V/s); for DPV, scan range (0.6 V–0.3 V), pulse amplitude (50 mV).

2.5. Apoptosis assessment by fluorescent and colorimetric assays

To verify the reliability of the proposed electrochemical approach, some standard methods such as fluorescent and colorimetric assays were carried out for comparision.

H_2O_2-induced apoptosis of MCF-7 cells could be assessed by Hoechst/PI staining fluorescent images. Briefly, cells were washed and cultured in 1 mL PBS containing 10 ng Hoechst 33342 and 10 ng PI for 20 min at 4 °C in dark. Cells undergo apoptosis (stained by Hoechst 33342) and necrosis (stained by both Hoechst 33342 and PI) were respectively viewed under a fluorescence microscope (Axio observer A1, Zeiss, Germany).

To further check the apoptosis level induced by different concentrations of H_2O_2, caspase-3 activity was measured using a colorimetric activity assay kit (C1115, Beyotime Institute of Biotechnology, Nantong, China). Briefly, the treated cells were collected, homogenized in lysis buffer and incubated on ice for 15 min. Afterward, the lysates were centrifuged at 2000 rpm at 4 °C for 15 min. The protein contents were determined using the Bradford method. The cell lysates were then mixed with the reaction buffer containing 0.2 mM Ac-DEVD-*p*NA. After incubation at 37 °C for 2 h, the mixtures were measured at 405 nm on a Synergy HT multifunction microplate reader (BioTek Instruments, Inc., USA).

3. Results and discussion

3.1. Sensing mechanism

Scheme 1 outlines the principle of the sensing mechanism of this peptide-based electrochemical approach for apoptosis evaluation. First, a smart peptide for PS recognition was designed and immobilized on a gold electrode surface via the interaction between its cysteine end and the gold (Miao et al., 2012). Then, MCH was used to backfill the electrode to prevent any nonspecific adsorption and make the peptide more accessible to PS. Subsequently, the modified electrode is incubated in H_2O_2-treated cells to capture apoptotic cells through the specific recognition of peptide and externalized PS on cell membrane. Before the capture reaction, the positively charged peptide can attract the negatively charged electrochemical species, $Fe(CN)_6^{3-/4-}$, which contributes to