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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.

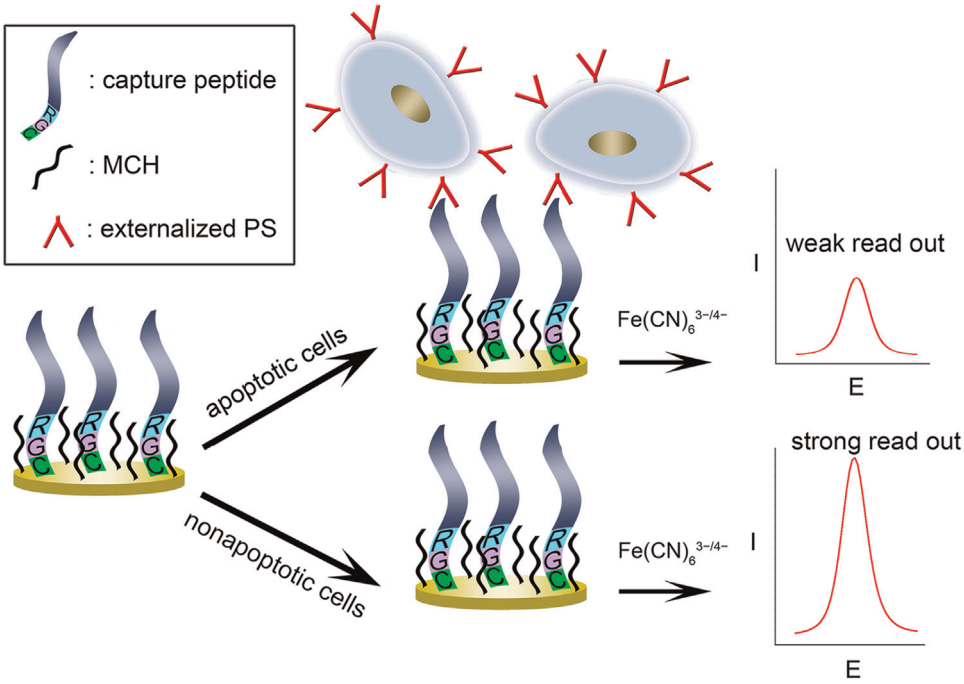
H_2O_2-induced apoptosis of MCF-7 cells could be assessed by Hoechst/PI staining fluorescent images. Briefly, cells were washed and cultured in 1 mL PBS containing 10 ng Hoechst 33342 and 10 ng PI for 20 min at 4 °C in dark. Cells undergo apoptosis (stained by Hoechst 33342) and necrosis (stained by 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



Scheme 1. Schematic representation of the peptide-based electrochemical approach for apoptosis evaluation.

strong electrochemical signals. After the capture reaction, apoptotic cells on electrode surface can increase the interfacial charge transfer resistance and can further block the positive charges of the peptide, which leads to the significant signal fall. From the distinguishable electrochemical readouts, different apoptosis levels can be evaluated.

3.2. Characterization of the modified gold electrode

EIS has been obtained to characterize the surface alteration of the electrode during each modification procedures. Impedance spectra are consisted of a linear portion at lower frequencies corresponding to diffusion and a semicircle portion at higher frequencies which reflect the electron transfer limited process. As shown in Fig. 1A, no obvious semicircle domain of impedance spectrum is observed on bare electrode (curve a), and a tiny one appears on the peptide modified electrode (curve b) due to the balance of the steric hindrance and positive charges to attract $Fe(CN)_6^{3-/4-}$. Curve c with larger semicircle domain indicates that the incubation of nonapoptotic cells may cause certain nonspecific adsorption on the peptide modified electrode. Specific interaction occurring between the peptide and externalized PS on apoptotic cells contributes to a much larger impedance spectrum (curve d). From a to d, the R_{ct} are 78.8 Ω, 392.5 Ω, 845.4 Ω and 1208.0 Ω, respectively. The EIS results have confirmed well our proposed sensing mechanism. Moreover, the successful immobilization of apoptotic cells on the gold surface has been further verified by scanning electron microscope images (Fig. S1).

The electrochemical properties of the modified electrode is then studied by CV, which is a powerful and commonly used electrochemical tool. Curve a in Fig. 1B is a typical CV of bare gold electrode with a pair of well-defined redox peak. As displayed in Fig. 1B, after the modification of peptide and subsequent MCH, the current peak drops. Another tiny decrease of current peak is observed after the incubation of nonapoptotic cells, demonstrating that nonspecific adsorption exists but is indistinctive. In the case of apoptotic cells, the CV current peak drops to a large extent, which confirms effective interfacial charge transfer resistance from captured apoptotic cells on the electrode.

3.3. Electrochemical detection of apoptosis

To validate the electrochemical approach for the detection of apoptosis, we have then conducted DPV, a more sensitive electrochemical technique (Tootoonchi et al., 2013), to obtain electrochemical signals. The top curve in Fig. 2A depicts a voltammetric wave of the peptide modified electrode. The peak current is very high since the positively charged electrode surface can effectively attract electrochemical species. The other curves represent the DPVs of the modified electrodes incubated with MCF-7 cells treated with H_2O_2 of various concentrations. Fig. 2B is the calibration plot of the decreased DPV peak current with the concentration of H_2O_2. The experimental results reveal that larger amount of H_2O_2 induces more apoptotic cells, which cause larger interfacial charge transfer resistance and lower DPV peak. Note that the signal intensity is linearly related to the concentration of H_2O_2 with the regression equation being $y=0.671+0.127x$, where y is the decreased peak current, $\times 10^{-6}$ A; x is H_2O_2 concentration, μM; $n=3$; $R^2=0.998$. This biosensor can distinguish H_2O_2 (as low as 3.0 μM)-induced apoptosis from nonapoptotic cells ($S/N=3$).

DPV has also been carried out on the electrode modified by control peptide without PS-specific sequence. Fig. 3 shows the comparision of the DPV performances. The nearly unchanged peak currents demonstrate that control peptide cannot capture apoptotic cells to block the electron transfer.

3.4. Fluorescent and colorimetric assays of H_2O_2-induced apoptosis

To check the reliability and practicality of the proposed electrochemical approach, H_2O_2-induced apoptosis has also been checked by some standard methods for From fluorescence microscopy, it is observed that untreated cells cannot be stained by Hoechst 33342

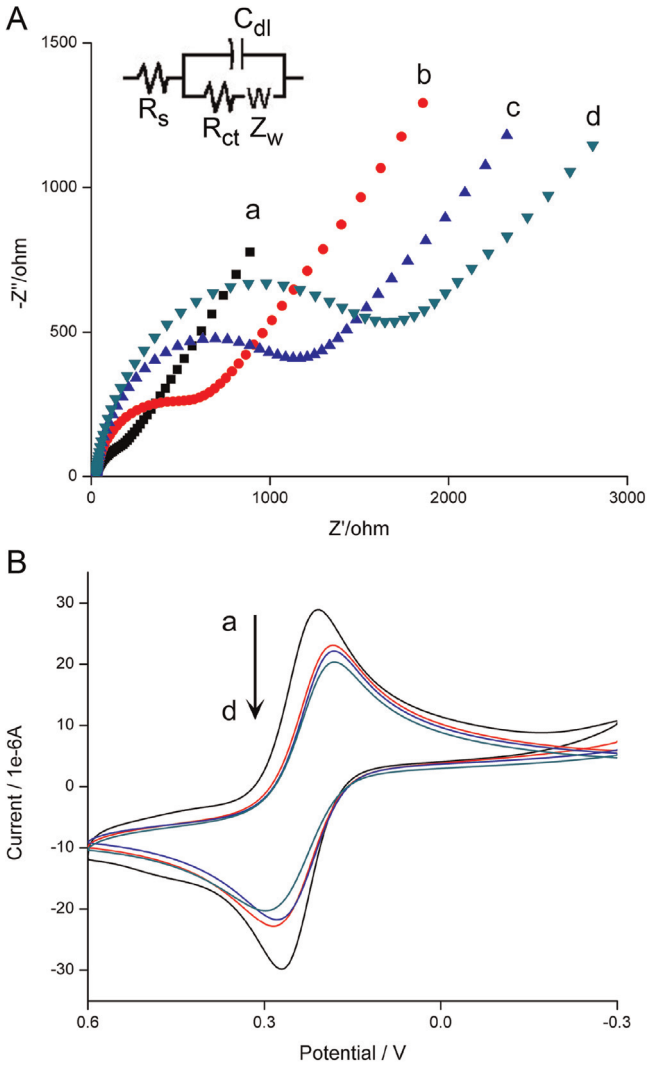


Fig. 1. (A) Electrochemical impedance spectra and (B) Cyclic voltammograms obtained for $Fe(CN)_6^{3-/4-}$ (5 mM) containing KNO_3 (1 M) at (a) bare electrode, (b) peptide modified electrode, after further incubation with (c) nonapoptotic and (d) apoptotic cells.

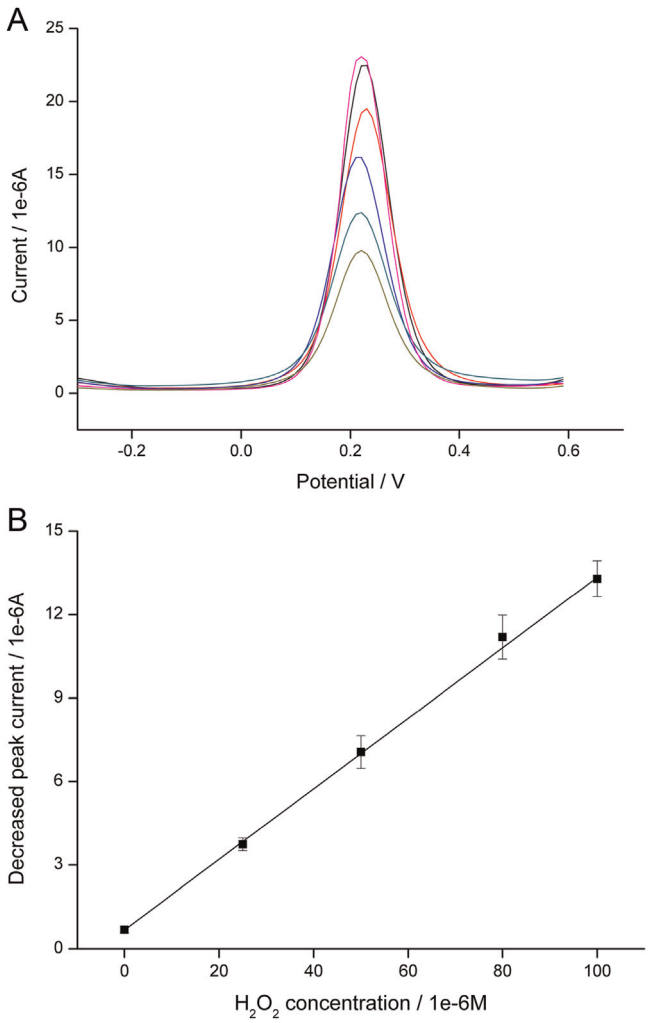


Fig. 2. (A) Differential pulse voltammograms for apoptosis evaluation. The top line is the case of peptide modified electrode. The other curves are the cases after incubated with cells which are previously treated with H_2O_2 (0, 25, 50, 80, 100 μM from top to bottom). (B) Calibration plot of the decreased DPV peak current with the concentration of H_2O_2. Error bars represent standard deviations of three independent measurements.

and PI. After a treatment of 50 μM H_2O_2, over half of the cells exhibit a condensed chromatin and are stained by Hoechst 33342, while they are seldom stained by PI, which are distinct characteristics of apoptosis (Fig. 4). Experimental results of caspase-3 assay suggest that the enzyme activity increases with more H_2O_2 added in a dose dependent manner which correlated well with both a previous report and the proposed electrochemical approach (Fig. 5) (Liu et al., 2013).

4. Conclusion

In summary, we have proposed a strategy to assemble apoptotic cells on a solid surface based on a designed peptide, which can be further used for electrochemical evaluation of apoptosis. Current methods for the detection of apoptosis may lack sufficient sensitivity or require expensive instruments and complicated procedures. This proposed biosensor does not need any cell pretreatment like fluorescent staining and the detection can be completed in about 1 h. By combining the advantages of electrochemical techniques and peptide-based recognition process, the biosensor offers attractive merits in inherent simplicity,

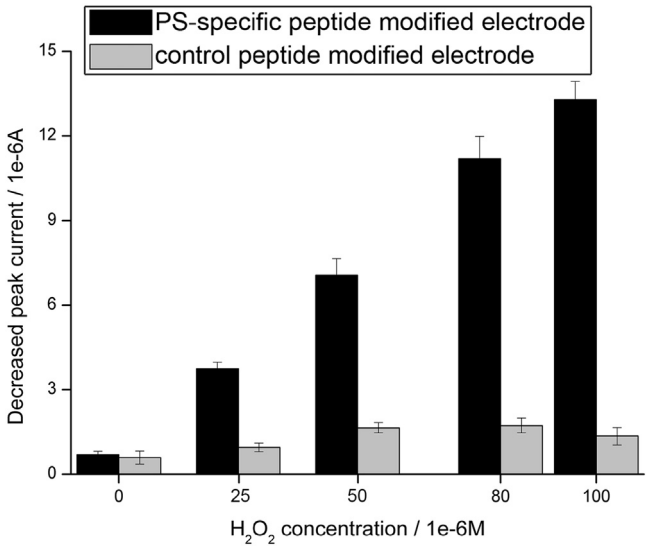


Fig. 3. Histogram of the decreased peak current versus H_2O_2 concentration. Black columns are the cases of PS-specific peptide modified electrode, while gray columns are for control peptide modified electrode.

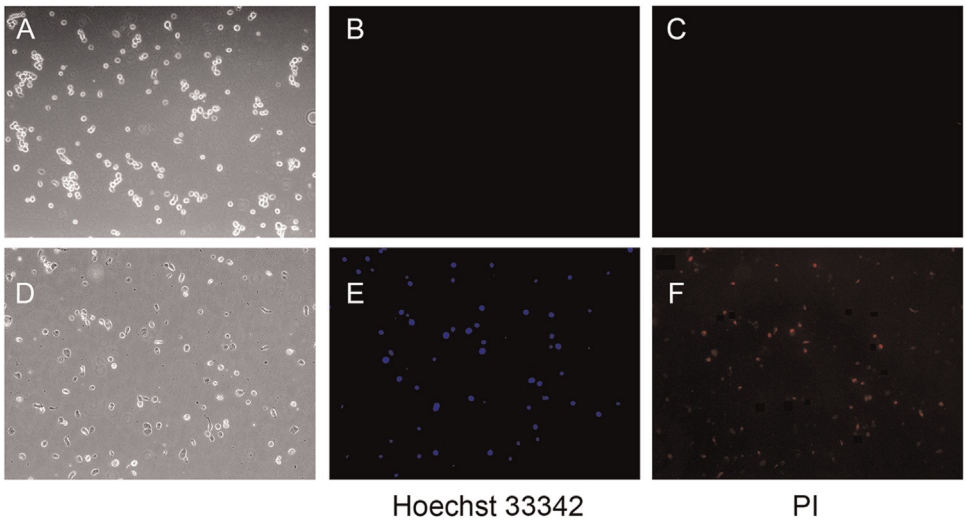


Fig. 4. Hoechst 33342 and PI staining in (A-C) nonapoptotic and (D-F) apoptotic MCF-7 cells. The bright blue color from Hoechst 33342 stands for apoptosis (B, E), while the red color from PI stands for necrosis (C, F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

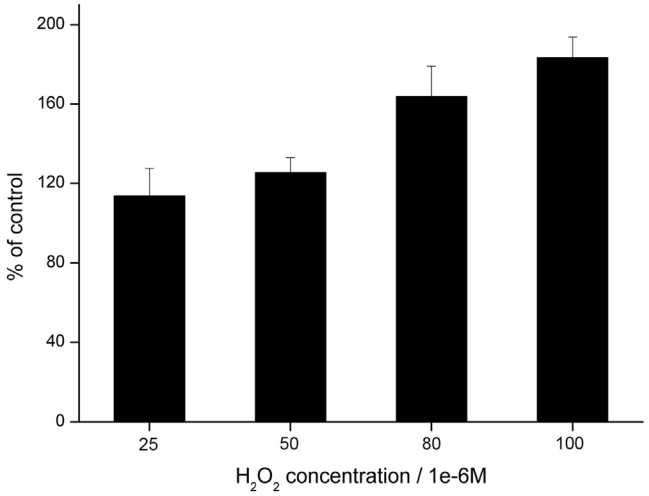


Fig. 5. Caspase-3 assay of cell lysates which are previously treated with H_2O_2. The vertical coordinate stands for the caspase-3 activity of the cells versus that without H_2O_2 treatment.

convenient operations, low cost and high sensitivity. Therefore, it may have potential applications towards apoptosis evaluation, therapeutic effect assessment and deeper cellular biological studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.06.035.

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