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# Peptide and carbon nanotubes assisted detection of apoptosis by square wave voltammetry

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ABSTRACT

Apoptosis is a significant process in embryogenesis, tissue homeostasis and development of immune system. Apoptosis evaluation is not only a hot topic for biologists, but also one of the most important tasks of toxicology. Moreover, a timely monitoring of apoptosis can effectively assist early diagnosis of related diseases and continuous evaluation of the efficacy of drugs. Therefore, developing advanced methods for apoptosis evaluation has attracted more and more attention. In this work, we have proposed a novel electrochemical approach for apoptosis evaluation. Specifically designed peptide is immobilized on a gold electrode as the recognition element, which can bind to the exposed membrane phosphatidylserine of apoptotic cells with high affinity. This process may hinder the interaction between peptide and carbon nanotubes/methylene blue and results in great decline of electrochemical signals. By studying the relationship between the detected electrochemical signal and cell concentration, a sensitive apoptosis detection method can be achieved. Experimental results demonstrate the method great utility for sensitive, convenient and inexpensive quantification of apoptotic cells.



## 1. Introduction

Apoptosis, also named as programmed cell death, is a highly regulated physiologic process in response to a stress, which is significant in embryogenesis, tissue homeostasis and development of immune system [1]. In contrast to necrosis, a form of cell injury that results in the premature death of cells, the morphology of apoptosis is crucial for not only numerous physiological processes like plasma membrane blebbing, chromatin condensation, DNA fragmentation, but also pathogenesis of a wide variety of diseases including autoimmune diseases, acquired immunodeficiency syndrome (AIDS) and tumor proliferation [2,3]. Therefore, timely evaluation of apoptosis can be utilized for early diagnosis and effective therapeutic intervention [4–6].

Current apoptosis detection strategies are based on monitoring morphological changes and certain typical biochemical events during apoptosis [7,8]. For example, loss of plasma membrane asymmetry and attachment, plasma membrane blebbing, condensation of the cytoplasm and nucleus and internucleosomal cleavage of DNA may all assist the determination of apoptosis. Various techniques can be employed, such as electron microscopy [9], fluorescence microscopy [10], flow cytometry [11] and microfluidic device [12]. Nevertheless, some methods may require complicated and laborious processes to maintain the expected sensitivity and selectivity, which may hinder their wide applications; some may involve the use of sophisticated and expensive equipments, which can only be manipulated by specially trained operators.

In this contribution, we have developed a promising electrochemical approach for the apoptosis evaluation by detecting the representative phenomenon of phosphatidylserine (PS) translocation from the inner to the outer leaflet of the plasma membrane [13]. Electrochemical techniques may endow the assay many merits like high sensitivity, low cost and fast response [14–16]. Unlike traditional PS measurement by using annexin V [17,18], a specifically designed peptide from the original sequence of PS-binding site in PS decarboxylase is used as an efficient

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recognition element towards apoptotic cells with high affinity [19]. Moreover, to acquire a high sensitivity, signal amplification strategies are always needed [20,21]. Herein, single walled carbon nanotubes (SWNT) with broad access areas are employed to carry multiple methylene blue (MB) molecules by electrostatic interaction and π–π stacking. Additionally, the materials also have excellent electrical conductivity, which further enhance the signal intensity [22–25].

## 2. Experimental

### 2.1. Materials and chemicals

4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES), mercaptohexanol (MCH), trisodium citrate, tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), MB were purchased from Sigma (USA). Carboxyl SWNT (XFS07) was obtained from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). Peptide was synthesized and purified by China Peptides Co., Ltd. (Shanghai, China) with the sequence of FNFRLKAGAKIRFGRGC. HeLa cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. (Shanghai, China). Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Gaithersburg, USA). Fetal bovine serum was from Hangzhou Sijiqing Biological Engineering Material Co., Ltd. (Hangzhou, China). Cell apoptosis and necrosis assay kit was from Beyotime Biotechnology (Nantong, China). Other reagents were of analytical grade and were used as received. Double-distilled water used to prepare all solutions was purified with a Millipore system under 18 MΩ cm resistivity.

### 2.2. Gold electrode treatment

Before peptide modification, the substrate gold electrode was incubated with piranha solution (98% $H_2SO_4$: 30% $H_2O_2$ = 3:1) for 5 min (*Caution: Piranha solution was highly corrosive and reacts violently with organic matter!*). Subsequently, it was rinsed with double-distilled water and carefully polished to a mirror-like surface with P3000 silicon carbide paper and alumina slurries (1, 0.3, 0.05 μm), respectively. After that, it was cleaned by ultrasonication for 5 min in both ethanol and double-distilled water. Then, it was electrochemically treated with 0.5 M $H_2SO_4$ to remove any remaining material. Afterward, the electrode was dried with nitrogen for further modification. The pretreated electrode was further incubated with 100 μM peptide solution (20 mM HEPES, 10 mM TCEP, pH 7.0) at room temperature for 16 h. Then, it was soaked in 1 mM MCH for 30 min to resist nonspecific binding on the electrode surface [26]. After thoroughly rinsed with double-distilled water and dried with nitrogen, the peptide modified electrode was ready for the following experiments.

### 2.3. Cell culture and apoptosis induction

HeLa cells were cultured in DMEM medium with 10% (v/v) fetal bovine serum at 37 °C in a humidified incubator (5% $CO_2$: 95% air). After reaching a confluence of 80%, the cells were washed with phosphate buffered saline (PBS) and detached by trypsin. After collected by centrifugation at 1000 rpm for 10 min, the cells were diluted to a certain concentration and were treated with Apopida at 37 °C for 16 h, using a cell apoptosis and necrosis assay kit according to the manufacturer's procedures. Cell status was then analyzed by flow cytometry and fluorescence microscope.

### 2.4. Assembly of apoptotic cells onto the electrode

The peptide modified electrode was immersed in 300 μL apoptotic cell suspension with different dilutions at 37 °C. The peptide could bind to the apoptotic cells and located the cells on the electrode surface. After 0.5 h incubation, the electrode was carefully rinsed with double-distilled water to remove nonspecific adsorbed cells. Then, the modified electrode was treated with 50 μL SWNT (1 mg/mL). After that, it was soaked in 0.5 mM MB for 30 min.

### 2.5. Electrochemical measurements

Electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV) experiments were carried out on a CHI 660D electrochemical workstation (CH Instruments, China) at room temperature. Three electrode system was employed, consisted of the saturated calomel reference electrode (SCE), the platinum auxiliary electrode and peptide modified gold electrode as the working electrode. Buffer for EIS experiments was 5 mM $[Fe(CN)_6]^{3-/4-}$ with 1 mM KCl. SWV experiments were carried out in 20 mM Tris–HCl (pH 7.5) upon modulation amplitude of 25 mV, frequency of 90 Hz and step potential of 4 mV [27].

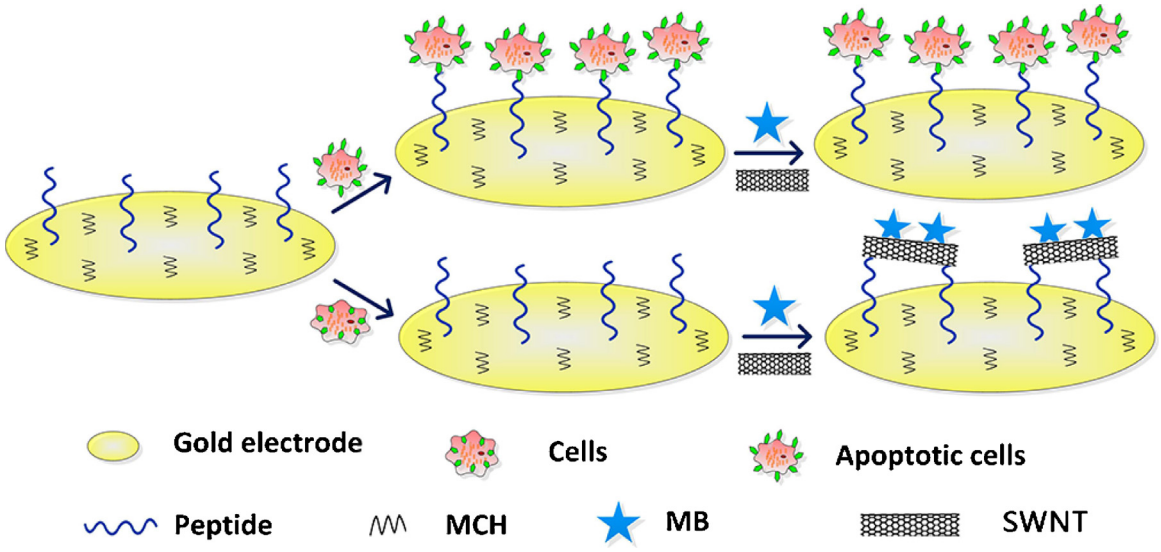


**Fig. 1.** Illustration of the apoptosis evaluation approach coupling peptide recognition and SWNT-assisted signal amplification.