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Short communication

An Annexin V-based biosensor for quantitatively detecting early apoptotic cells

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ABSTRACT

In this paper, we reported that a novel biosensor was developed to detect early apoptotic cells by the specific interaction between Annexin V and phosphatidylserine based on electrochemical impedance. Annexin V was immobilized on a self-assembled layer of gold nanoparticles, which allowed stable and high loading of Annexin V on the electrode surface, offering the possibility of sensitivity enhancement. Early apoptotic cells showed an increased exposition of phosphatidylserine on the cell membrane caused by physiological and pathological response reaction, leading to a strong interaction between the apoptotic cells and the electrode surface, which could be probed by electrochemical impedance spectroscopy. As examined using a model system of cells integrated by phosphatidylserine-modified liposome and a real one of early apoptotic cell induced by 5-fluorouracil, this biosensor demonstrated the great potential for rapid detection of cell apoptosis and drug screening. The results agreed well with those obtained using fluorescence microscopy and flow cytometry.

1. Introduction

Apoptosis is a form of programmed cell death in multicellular organisms and an essential biological process occurred in tissue homeostasis. It involves a series of biochemical events leading to a characteristic cell morphology, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation, and finally cell death (Kerr et al., 1972). This process can be influenced by a wide variety of regulatory stimuli (Thompson, 1995). In pathological physiology, the measurement of apoptotic activity may greatly enhance possibilities for staging the disease, and equally importantly, apoptosis may serve as an early indicator for the effect of therapeutic interventions. For example, in anti-tumor therapies, since the unrestrained division of tumor cells is related to insufficient apoptosis, the induction of apoptosis by cytotoxic drugs results in an arrest of tumor proliferation (Brown and Attardi, 2005). One of the plasma membrane alterations of the early apoptotic cells is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS (Meers and Mealy, 1993), therefore this protein can be used as a sensitive probe for PS exposure upon the cell membrane. PS externalization is a relatively early event in apoptosis and occurs before plasma membrane integrity (Kerr et al., 1972). Since 1994, Annexin V has been used in the detection of PS expression on B cells undergoing apoptosis with flow cytometry (Koopman et al., 1994). Flow cytometry-based fluorescence labeling Annexin V has become one of the most commonly used methods for detecting apoptosis (Vermes et al., 1995; Aubry et al., 1999; Fischer et al., 2002; Numnuam et al., 2006). However, there exist some technical problems for fluorescent labeling technology. For example, chemical cross-linking reaction influences the activity of protein (Janne, 2004). In addition, the detection of early apoptotic cells requires the immediate and sensitive strategies without time-consuming fixation (Schutte et al., 1998). It will provide a useful tool for probing the apoptosis of cells to develop a labeling-free, simple, rapid and immediate detection method.

It is well-known that electrochemical method is simple, rapid, sensitive and convenient for detection, and especially in recent years, due to the rapid development of the functionalization of the electrode surface, electrochemical method has been widely used for detecting various biological targets, such as proteins (Lin et al., 2008), DNA (Sassolas et al., 2008), bacteria (Maalouf et al., 2007), cells (Liu et al., 2007) and some biological processes, such as ion-channel behavior of Annexin V in phosphatidylcholine bilayer membranes (Huang et al., 2008). For the determination of cells,

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extensive effort has been made on cell activities, like cell division (Kintzios et al., 2006), cell proliferation (Guo et al., 2008), apoptosis (Xiao et al., 2008), but there have been few reports concerning the development of electrochemical sensors for the quantitative detection of early apoptotic cells.

In this paper, we described for the first time that an Annexin V-modified biosensor was developed for quantitatively detecting early apoptotic cells. The biosensor was constructed by assembling gold nanoparticles on a 1,6-hexanedithiol self-assembled monolayer-modified gold electrode, then followed by the adsorption of Annexin V on the nanoparticles layer. Using impedance spectroscopy, this modified electrode offered an extremely sensitive biosensor for rapid determination of apoptotic cells. To demonstrate the feasibility of the method, a model system of cells integrated by PS-modified liposome and a real one of early apoptotic cells induced by 5-fluorouracil (5-Fu) were constructed and investigated using this biosensor.

2. Experimental

2.1. Apparatus and reagents

2.1.1. Reagents

Chloroauric acid ($HAuCl_4$) was obtained from Shanghai Jinshan Chemicals Co. Annexin V and 1,6-hexanedithiol were purchased from Sigma. HEPES was obtained from BBI (Canada). Annexin V-FITC staining kit was from Invitrogen (USA). All other chemicals were of analytical grade. The solutions were prepared with the water purified by a Milli-Q purification system (Millipore, USA), in which the specific resistance was greater than 18 MΩ cm, and stored in refrigerator at 4 °C HEPES buffer (pH 7.4) was prepared with 10 mM HEPES, 150 mM NaCl. HEPES buffer containing 5.0 mM $[Fe(CN)_6]^{4-/3-}$was used for impedance measurements. Gold nanoparticles (NPs) were prepared as described previously (Chen et al., 2007).

2.1.2. Apparatus

The detections with flow cytometry and fluorescence microscopy were done by using the Annexin V-FITC staining kit following the manufacturer's instruction. Briefly, after treated with Annexin V-FITC, the cells were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, New York, USA) and fluorescence microscopy (NIKON TE2000, Japan).

2.2. Preparation of the modified electrode

The modification of gold electrode was performed as described previously (Szymańska et al., 2007). The process containing three steps was shown in Fig. 1. In brief, after polished sufficiently with 0.5 μm alumina powder, the gold electrode was rinsed with ethanol for three times and taken turns to immerse in 1 mM 1,6-hexanedithiol (Fig. 1a) and 10 nM gold nanoparticles (Fig. 1b) to allow the formation of self-assembly monolayer. Then the gold NPs modified electrode was co-incubated with 0.2 μg/μl Annexin V for 1 h at 37 °C (Fig. 1c), followed by rinsing with HEPES buffer for 5 min. The Annexin V-gold NPs modified electrode (AME) biosensor was prepared. The AME biosensor was kept in HEPES buffer at 4 °C before use.

2.3. Cell culture and early apoptosis induced by 5-Fu

Jurkat cells was obtained from cell bank of Xiangya Central Experiment Laboratory of Central South University of PR China and cultured in RPMI 1640 (GIBICO) medium supplemented with 15% fetal calf serum (Ivitrogen), 2 mmol/l glutamine, 100 U/ml penicillin and 100 U/ml gentamicin. Cells were incubated at 37 °C in humidified air with 5% CO_2 and kept in logarithmic phase by routine passage every 2–3 days. Before use, cells were spun down and resuspended in HEPES buffer. Early apoptosis could be induced by incubating with 25 μg/ml 5-Fu 24 h while the cells were cultured, and the normal culture cells were as control.

2.4. Construction of the model of early apoptotic cells by liposome

Chloroform solutions of lipids (PS:PC:PE = 1:1:8) were dried under a stream of nitrogen and followed by the removal of the solvent under a high vacuum for 2 h. The lipids were hydrated in the HEPES buffer. The collected suspension of multilamellar vesicles

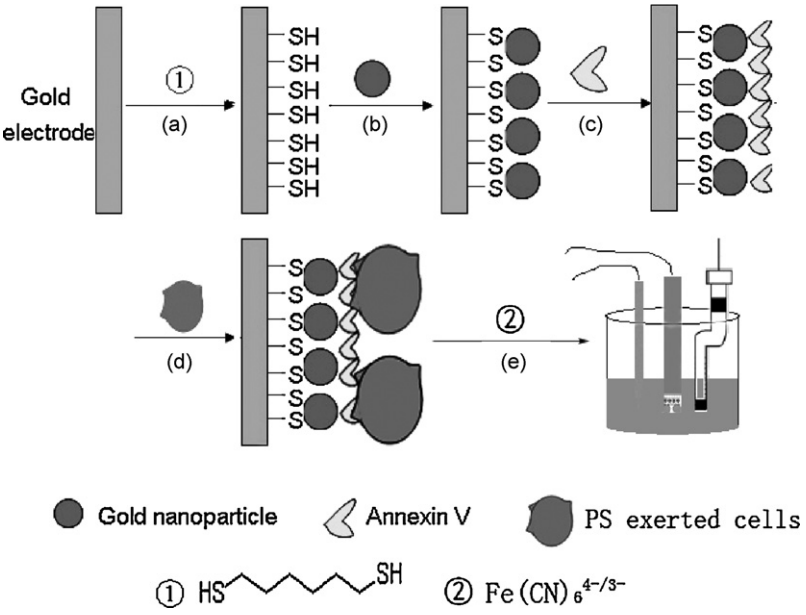

Fig. 1. Schematic illustration of the stepwise self-assembly fabrication process: formation of (a) 1,6-hexanedithiol layer; (b) Au-NPs layer; (c) Annexin V layer; and the detection steps: (d) incubation with cells and then (e) detection in $[Fe(CN)_6]^{4-/3-}$ by EIS.

was sonicated for 30 s and subsequently extruded 10 times through a 220-nm pore-size polycarbonate filter. The size distribution of the liposome was determined by photon correlation spectroscopy using a Zeta-Sizer 3000, Malvern Instruments (Grovewood Lane, UK).

Jurkat T cells were washed twice and resuspended in a cold HEPES buffer. Liposome containing PS was added at a series of concentration to a cell suspension (1×10^6 of cells) in 1 ml of HEPES buffer. The mixture was then incubated for 1 h at 37 °C. The cells were collected by centrifuge and kept in HEPES buffer at 37 °C until detection.

2.5. Impedance spectroscopy measurements

Electrochemical experiments were performed with a CHI760c electrochemical workstation (Shanghai Chenhua Instruments, Shanghai) and a three-electrode system. The modified Au electrode was used as the working electrode. A saturated calomel electrode

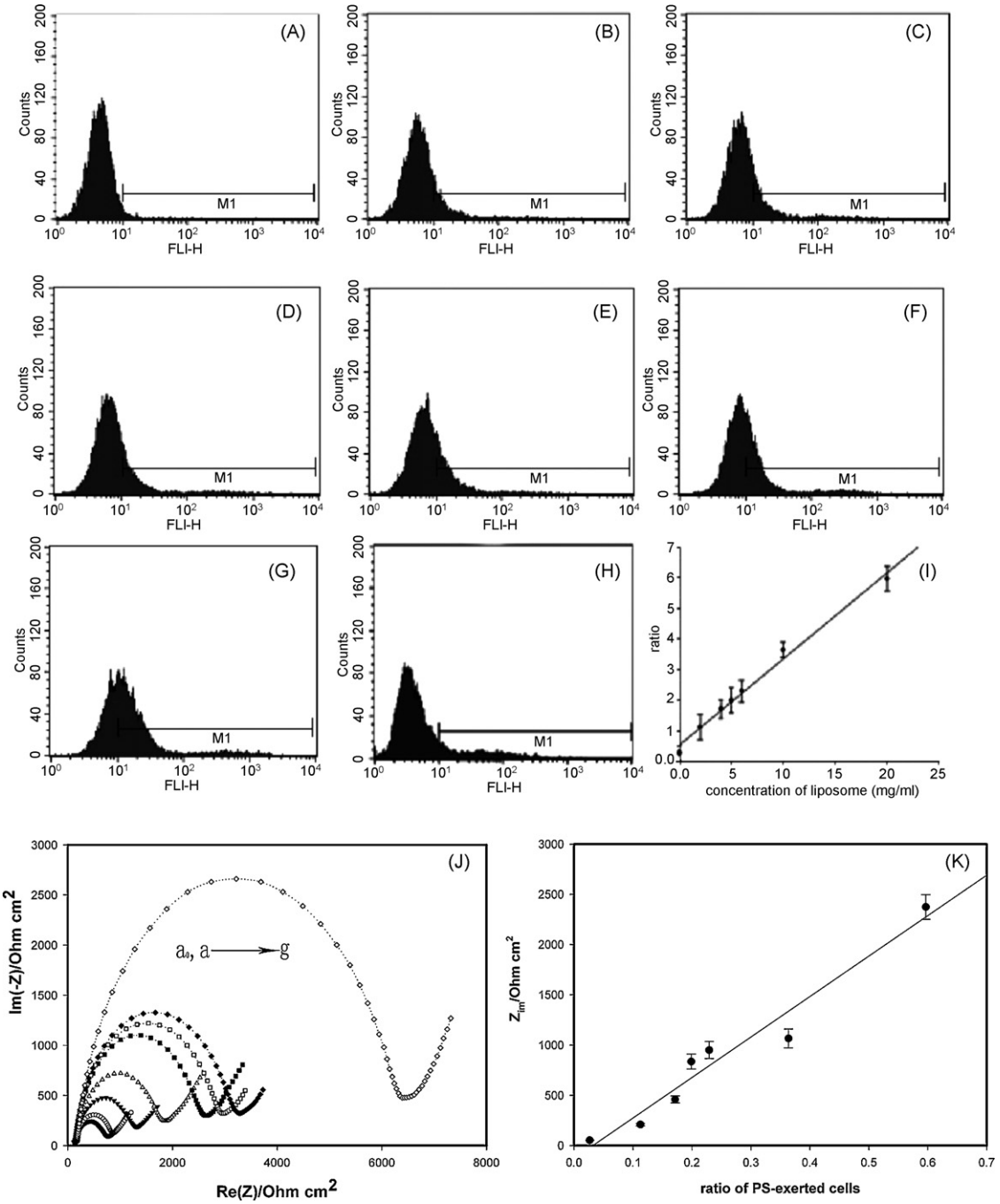

Fig. 2. AME biosensor was trained by the model system of apoptotic cells. A–H were histogram statistics of Annexin V-FITC flow cytometry of PS exerted on Jurkat cells membrane, which A–G were integrated by liposome from 0 to 2 mg/ml with 10^6 cells, H was cultured with 25 μg/ml 5-Fu for 24 h. I was the calibration curve ($Y = 0.0544 + 0.2804C$, $R = 0.9951$) between the ratios of the model system of apoptotic cells vs. the concentration of liposome. J was the Nyquist plane impedance spectra obtained for the AME biosensor before (a_0) and after (a–g) incubated with the model of apoptotic cells (A–G), K was the calibration curve ($Y = -127.83 + 4027.26X$, $R = 0.9769$) represented as impedance vs. the ratios of apoptotic cells.

was served as the reference electrode, and a platinum wire auxiliary electrode was as the counter electrode. EC MFC application was used for collecting and calculating data and ZSimpWin electrochemical software was used for data analysis. The collected cells were diluted to 10,000 cells/ml in HEPES buffer containing Ca^{2+}, and incubated with AME biosensor with constant stirring, and then the AME biosensor was measured by EIS after it washed three times in HEPES buffer. In CV potential were cycled from +0.6 to −0.2 V with scan rate 100 mV s^{-1}. The EIS measurements were recorded within the frequency range from 0.1 Hz to 100 kHz at the formal potential of the redox couple $[Fe(CN)_6]^{4-/3-}$ (0.24 V) with ac amplitude of 5 mV.

3. Results and discussion

3.1. Characterization of modified electrode

Cyclic voltammetry and EIS were recorded to monitor each immobilization and binding step (as shown in Fig. S1). We observed that cyclic voltammograms (Fig. S1B) and Nyquist plane impedance spectra (Fig. S1A) changed substantially after the self-assembly of 1,6-hexanedithiol (dash–dot–dot line), AuNPs (solid line) and Annexin V (dotted line). This was due to the fact that 1,6-hexanedithiol and Annexin V were electrically insulator, while the AuNPs provided the necessary conduction pathways and promoted the electron transfer between the redox marker and the electrode surface (Lu et al., 2002; Yang and Zhang, 2004). When the AME electrode was incubated with cells including apoptotic cells, it led to the further increase in the diameter of semicircle of Nyquist plane impedance spectra (Fig. S1C, medium–medium line), but further decrease in the CV current (Fig. S1D). In contrast, the impedance and CV current did not yield significant changes when the biosensor interacted with control cells (Fig. S1C and D, solid line). This was because that the apoptotic cells could specifically adsorb on the electrode, and then weaken the electron transfer between the electrode and solution.

3.2. Investigation with a model system of apoptotic cells

A model of apoptotic cells was constructed through the method of cells integrated by liposome (Fadok et al., 1992). The construction was consisted of two parts: synthesis of liposome, and incubation of Jurkat cells and liposome.

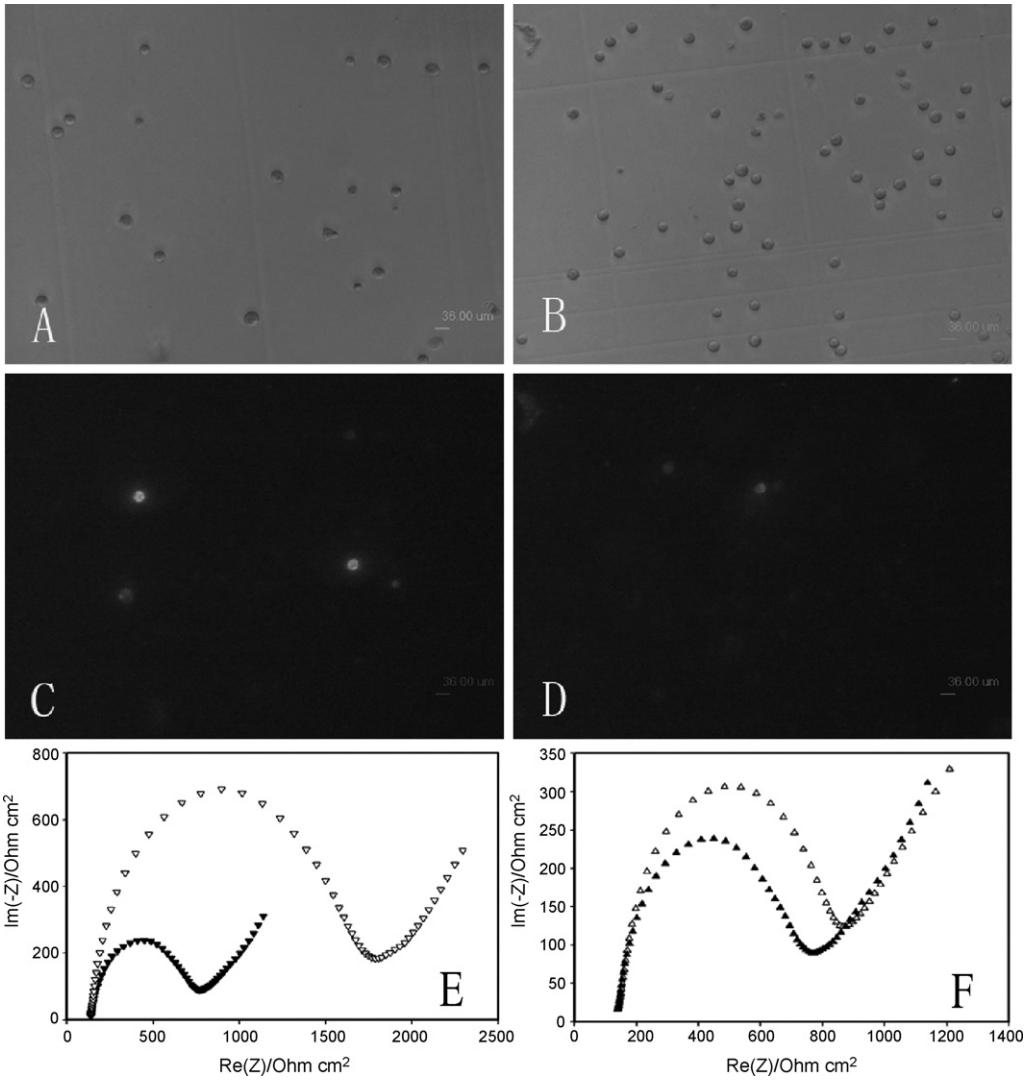

Fig. 3. Determination of AME biosensor used for early apoptotic cells induced by 5-Fu. A–D showed the photomicrographs of the detection of PS exposed on cells membrane by Annexin V-FITC. A and C: cultured with 5-Fu ((A) phase-contrast; (C) fluorescence image; 100×); B and D: control ((B) phase-contrast; (D) fluorescence image; 100×). E and F were the Nyquist plane impedance spectra obtained for the AME biosensor before ((F, ▼) control; (E, ▲) 5-Fu) and after ((F, ▽) control; (E, △) 5-Fu) incubated with cells.

The liposome synthesized by the ultrasound dispersion contained 10% PS (the data not shown) and were approximately 100 nm in the average size with PdI of 0.231. Annexin V-FITC could recognize the integrated cells with PS exposed on the membrane, thus making the cells display green fluorescence under the excitation of blue light. As shown in Fig. S2, we observed that the integrated cells displayed green fluorescence (red arrowhead), but the unintegrated cells were blank (black arrowhead). The Annexin V-FITC/PI assay (as shown in Fig. S4C) indicated that the integrate process did not influence the activity of cells.

The number of cells with surface-exposed PS was related to the concentration of liposome, which could confirm the results obtained by flow cytometry. As shown in Fig. 2, histogram statistics of A–G showed the results of model Jurkat cells obtained via integration with 10^6 cells by 0–2 mg/ml PS-modified liposome. The ratios of the cells with fluorescence changed with the increase of liposome concentration, and a linear correlation was obtained between these two variables with a regression equation $Y = 0.0544 + 0.2804C$ (The correlation coefficient was 0.9951), as shown in Fig. 2I. It indicated that the integration method was successful to make cells with PS-exposed membrane, which might be used for the model of early apoptotic cells.

Before the biosensor was used to detect the cells based on EIS, the detection conditions were optimized. Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for PS. Its binding capacity with PS depended upon the concentration of calcium ion, the temperature and the reaction time. As shown in Fig. S3, the detection system with 5 μM Ca^{2+} reaction 1 h at 37 °C gave the largest response. Therefore such conditions were used in the detection of apoptotic cells throughout the study.

Fig. 2J depicted the impedance responses of the biosensor to model apoptotic cells in different concentrations. The electrochemical impedance was observed to gradually increase with the increasing ratio of apoptotic cells (as shown in Fig. 2J, a–g). Obviously, the PS-exerted cells would be absorbed on the biosensor interface, making the electron transfer retarded. As more cells were absorbed, the higher electrochemical impedance was expected. Furthermore, as shown in Fig. 2K, ΔZ_{im} was linearly correlated to the ratio of apoptotic cells. The regression equation was $Y = -127.8 + 4027X$ with a correlation coefficient of 0.9769.

3.3. Determination of apoptosis induced with 5-Fu

Fig. 3 described the detection of the apoptotic cells induced by the typical anti-cancer drug 5-Fu (Gruber et al., 2004) for 24 h. As shown in Fig. 3E, ΔZ_{im} was about 696.6 Ω, which was much greater than that without induction by 5-Fu (control, Fig. 3F). According to the above-mentioned regression equation relating ΔZ_{im} to the ratio of apoptotic cells, the ratio of apoptotic cells was estimated to be 14.37%. As shown in fluorescence images (Fig. 3A–D), compared with the control experiment, the total number of cells decreased, but the ratio of apoptotic cells (white bright spots on behalf of green fluorescence as shown in Fig. 3C) increased to approximately 14%. The ratio of apoptotic cells detected by flow cytometry was 14.14% (Fig. 2H). As shown in Fig. S4A and B, it can be sure that the apoptotic cells were in early apoptosis stage. These results obtained with flow cytometry and fluorescence microscopy were well consistent with that estimated by the biosensor, indicating that the AME could detect the ratio of apoptotic cells accurately. Because the detection procedure with AME biosensor was much more simple and cost-efficient and, required only approximately 10^4 cells to accomplish the assay, only 1% of the number of the cells needed for flow cytometry, the biosensor reported in this paper was expected to have great potential for trace screening, and medical filtration, especially for large throughput screening.

4. Conclusion

A novel simple and feasible electrochemistry impedance biosensor was described for detecting early apoptosis. The biosensor applied a self-assembled layer of gold nanoparticles for stable immobilization of Annexin V for selective recognition of the apoptotic cells. The performance of the biosensor was validated using a model of early apoptotic cells by integrating normal cells with PS-modified liposome and a real system of early apoptosis induced by 5-Fu. The detection results were in good agreement with those obtained by the costly fluorescence microscopy or flow cytometry. The AME biosensor possessed many salient advantages such as cost-efficiency and simplicity. These advantages would make AME biosensor have a wide range of applications in apoptosis studies and drug filtration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.07.040.

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