Facile and quantitative electrochemical detection of yeast cell apoptosis

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An electrochemical method based on square wave anodic stripping voltammetry (SWASV) was developed to detect the apoptosis of yeast cells conveniently and quantitatively through the high affinity between Cu²⁺ and phosphatidylserine (PS) translocated from the inner to the outer plasma membrane of the apoptotic cells. The combination of negatively charged PS and Cu²⁺ could decrease the electrochemical response of Cu²⁺ on the electrode. The results showed that the apoptotic rates of cells could be detected quantitatively through the variations of peak currents of Cu²⁺ by SWASV, and agreed well with those obtained through traditional flow cytometry detection. This work thus may provide a novel, simple, immediate and accurate detection method for cell apoptosis.

Apoptosis is a vital cellular process of programmed cell death and it is critically involved in both normal development and the pathogenesis of a wide variety of diseases2,3. It is also a morphologically distinct form of cell death that is crucial for embryogenesis, tissue homeostasis, and disease control in multicellular organisms. The process of apoptosis can be influenced by a diverse range of either endogenous or exogenous stimuli4–6, trigger different intracellular signal transduction pathways7–9.

Detection of apoptosis is of great importance in many areas of biological research. It could be considered as an indicator for the effect of therapeutic interventions and disease progression10,11. Some characteristic cell morphology changes (such as cell shrinkage, cell cycle changes, and nuclear fragmentation) and apoptotic markers (such as chromatin condensation, DNA fragmentation, phosphatidylserine (PS) externalization, and cytochrome c release from mitochondria, etc.) are routine detection items in cells to identify the apoptotic processes2,3. Several methods have been developed for apoptosis detection9, such as electron microscopy13, spectroscopic techniques14,15, flow cytometry14,16,17, TUNEL assay18,19, single-molecule spectroscopy20, and microfluidic devices21,22, etc. However, these methods are usually time-consuming and require sophisticated instrumentation and technical expertise. It is necessary to search for a rapid, sensitive and simple method to detect apoptosis. Electrochemical method, as a simple and convenient technique, could be a promising approach compared with those methods. In recent years, detection of cell apoptosis by some electrochemical methods have been reported23–26. Based on the specific interaction between Annexin V and PS14, electrochemical detection of externalized PS in the early apoptotic process is mostly employed. Liu et al. prepared an Annexin V and polyethylennimine (PEI) comodified electrode to electrochemically detect PS exposing to the outer leaflet of the plasma membrane23. However, Annexin V is rather expensive and those electrochemical methods mainly focus qualitative detection. Therefore, it is important to develop an quantitative electrochemical detection method with low cost and convenience.

It is known that Cu²⁺ forms a 1:2 complex with PS in solution containing water, methanol and chloroform27, and the interaction between PS and Cu²⁺ could be detected28. Therefore, based on such characteristic, an electrochemical method was herein developed through square wave anodic stripping voltammetry (SWASV) to detect cell apoptosis in Cu²⁺ solution. When apoptotic cells were added into Cu²⁺ solution, the exposed PS could combine Cu²⁺, and the SWASV peak current of Cu²⁺ was influenced. In light of the current variation, detection of cell apoptosis becomes available.

In the present study, we described for the first time that apoptosis of yeast cells were quantitatively detected using SWASV method. Saccharomyces cerevisiae (S. cerevisiae) was selected as an in vivo model system to study the apoptotic cell on biochemical process29. At the same time, traditional flow cytometry assay was carried out and proved the reliability of the above electrochemical method. Therefore, detection of apoptosis based on SWASV response of Cu²⁺ may offer a sensitive and feasible method to determine apoptotic cells conveniently, inexpensively and quantitatively.
Results

First, to detect PS sensitively by electrochemical method, 1,10-diaminodecane (DAD) was used to modify glassy carbon electrode (GCE). As shown in Figure S1, after a bare GCE was immersed into DAD ethanol solution, there was an obvious anodic peak shown in the first cycle (line 1), and the anodic peak disappeared at the second cycle (line 2), which indicated the DAD was formed on the electrode surface and the film blocked further oxidation of DAD30. The electrochemical impedance spectra of the electrodes were also obtained (see Figure S2). To obtain the largest response of detection, a series of experimental conditions containing pH, deposition time, supporting electrolytes, and deposition potential were optimized (see Figure S3). The detection system with pH of 8.2, deposition time of 120 s, 0.1 M Tris-HCl buffer solution and deposition potential of −0.6 V were chosen as the optimal parameters of SWASV throughout the study.

Standard PS (5 mg) was added into 10 mL of 0.1 M Tris-HCl buffer solution making a standard PS solution of 0.5 mg/mL, and each of 20 μL of PS solution was added into 0.6 ppm Cu²⁺ solution. Figure 1a showed the SWASV responses of the DAD/GC electrode to successive addition of standard PS solution. Anodic stripping peak appeared at approximately −0.21 V. It could be seen PS specifically combined Cu²⁺ in solution, consequently the Cu²⁺ peak currents were decreased compared with PS free system, and diminished linearly with the addition of PS ranging from 0 to 0.55 ppm (Figure 1b). The results demonstrated that the varying amounts of PS molecules could be detected by SWASV.

Since the discovery of yeast apoptosis in 199729, several studies had shown that apoptosis might also occur in unicellular organisms besides mammalian cells. H₂O₂ can induce apoptosis in S. cerevisiae as well as in mammalian cells31,32. As shown in Figure S4, in our study, after treated with 1 mM H₂O₂ for 200 min, S. cerevisiae cells viability evaluated by colony formation decreased to approximately 30%, and even lower with higher concentrations of H₂O₂ (3 mM and 5 mM). Obviously, H₂O₂ caused apoptotic cell death in a concentration-dependent manner. The result was consistent with previous findings, and this apoptotic cell death appeared as the exposure of PS29,31,33.

Figure 2a presented the SWASV electrochemical responses of Cu²⁺ before and after successive addition of H₂O₂ treated yeast cell solution in 0.1 M Tris-HCl solution (pH 8.2). The highest peak current was obtained when Cu²⁺ in pure Tris-HCl solution, and then the peak currents decreased gradually with successive addition of 20 μL of 1 mM H₂O₂ treated apoptotic yeast cells solution. For comparison, normal yeast cells were added into Cu²⁺ solution, and the peak currents showed no obvious change (Figure 2b), since there were no PS molecules on the outer membrane of the nonapoptotic cells.

Figure 3 depicted the classical flow cytometric analysis of PS externalization by FITC-Annexin V/PI costained S. cerevisiae cells after a 200 min treatment with ethanol (Figure 3a) and different

Figure 1 | (a) SWASV responses of Cu²⁺ with successive addition of standard PS over a concentration range of 0 to 0.55 ppm in 0.1 M Tris-HCl buffer solution (pH 8.2). (b) Dependence of peak current on the concentration of PS. Deposition potential, −0.6 V; deposition time, 120 s.  

Figure 2 | SWASV electrochemical responses of Cu²⁺ of actual cells. (a) With successive addition of 20 μL of 1 mM H₂O₂ treated apoptotic yeast cell solution. (b) With addition of normal yeast cell in 0.1 M Tris-HCl solution (pH 8.2). SWASV conditions: frequency, 15 Hz; potential step, 4 mV; pulse amplitude, 25 mV.
concentration of \( \text{H}_2\text{O}_2 \) (Figure 3b–d). In each diagram, two separated regions (“negative” and “positive”) could be seen. The peak area of “positive” region represented the number of AV \( 1 \) cells, namely apoptotic cells, while that of the “negative” region denoted the viable or necrotic cells. The results indicated that the ratio of apoptotic cells treated with ethanol, 1 mM, 3 mM and 5 mM \( \text{H}_2\text{O}_2 \) were 9.52%, 26.87%, 36.64% and 55.05%, respectively.

The apoptosis of the treated yeast cells were also detected through SWASV as shown in Figure 4. The peak current of electrochemical response decreased 1.4 \( \mu \text{A} \) with addition of 20 \( \mu \text{L} \) of ethanol treated cells solution (Figure 4a), 4.8 \( \mu \text{A} \) with 20 \( \mu \text{L} \) of 1 mM \( \text{H}_2\text{O}_2 \) treated cells solution (Figure 4b), 7.6 \( \mu \text{A} \) with 20 \( \mu \text{L} \) of 3 mM \( \text{H}_2\text{O}_2 \) treated cells solution (Figure 4c), and 10.3 \( \mu \text{A} \) with 20 \( \mu \text{L} \) of 5 mM \( \text{H}_2\text{O}_2 \) treated cells solution (Figure 4d), respectively. When the ratio of peak current decrease was set as the quantitative analysis index of the ratio of apoptotic cells, the ratios in four treatments were 6.67%, 22.86%, 36.19% and 49.05%, respectively.

**Discussion**

Orij et al. reported that \( \text{S. cerevisiae} \)’s physiological pH range was 7.2–7.5, which was in response to the nutrient availability and respiratory chain activity\(^{34}\). PS lipids are found in a wide variety of cell types, and it is an important signaling molecule in clotting, apoptosis, and embryonic development\(^{35}\). PS bears a negative charge at physiological pH. Monson et al. found that PS could reversibly bind \( \text{Cu}^{2+} \) with extremely high affinity in a pH-dependent fashion\(^{28}\). The pH value used in our study was 8.2, which was not far away from yeast physiological pH and thus scarcely affected yeast apoptosis.

Therefore, on the basis of the specific interaction between \( \text{Cu}^{2+} \) and PS, electrochemical detection of apoptosis becomes available.

Previous investigation showed that 1 ~ 3 mM Cu\(^{2+}\) might be beneficial for cell growth after 24 h of incubation, whereas the extensive apoptosis occurred at 6 mM Cu\(^{2+}\), and apoptosis was largely replaced by necrosis at 10 mM Cu\(^{2+}\)\(^{36}\). Therefore, low concentration of Cu\(^{2+}\) in our study (less than 1 mM, actually 0.6 ppm) and rapidly detection could make the effect of Cu\(^{2+}\) on cell growth ignored.

To confirm the cell apoptosis could be detected by SWASV method, influence of PS alone on Cu\(^{2+}\) needs to be first tested through SWASV. With the modified DAD/GC electrode, the Cu\(^{2+}\) peak currents in solution and additional standard PS content showed a linear correlation relationship (Figure 1). It was obvious that the amounts of Cu\(^{2+}\) deposited on the electrode decreased because of binding with PS.

In actual cells, PS molecules are normally located on the inner membrane. The normal yeast cells had scarcely any externalized PS molecules, thus would not affect the SWASV responses of Cu\(^{2+}\) (Figure 2b). PS can be externalized in the early stage of the apoptosis process\(^{16}\). Once PS exposed to the extracellular environment, it could interact with some cations. In the present study, \( \text{H}_2\text{O}_2 \) induced apoptosis in \( \text{S. cerevisiae} \), and released PS in the test solution can result in the change of CVs of the electrochemical response of Cu\(^{2+}\) (Figure 2a). Therefore, we could get to know whether the test cells are apoptotic cells, since apoptotic cells will make an obvious CV change, while healthy or necrotic cells will not.

In order to evaluate the feasibility and accuracy of SWASV method in quantitative detection and analysis of apoptotic process, yeast cells
treated with ethanol and H$_2$O$_2$ were separately detected by flow cytometry (Figure 3) and SWASV (Figure 4). Because the number of cells with surface-exposed PS was related to the signal response of detection, the same amount of apoptotic yeast cells (1 $\times$ $10^4$ S. cerevisiae cells) were detected in the present study by two technologies, and the ratios of the apoptotic cells were both analyzed.

Obviously, compared with the control experiment, as the degree of cell apoptosis increased, more exposed PS molecules interacted with Cu$^{2+}$ in solutions and caused the greater decrease of Cu$^{2+}$ peak current. The results obtained with SWASV were well consistent with that from flow cytometry, indicating this electrochemical technique could detect the ratio of apoptotic cells accurately. The values from SWASV were slightly lower than that from flow cytometric detection, this might be because the electrochemical detection is rapid and immediate and does not need the time-consuming stain process, whereas stains probably influence cell apoptosis at a certain degree.

In conclusion, we developed a novel and simple strategy to detect yeast apoptosis quantitatively by an electrochemical method, SWASV, instead of the usage of expensive fluorescent labeled protein, Annexin V. The declines of peak currents were in response to the high affinity between Cu$^{2+}$ and PS externalization from apoptotic cells. Experimental results showed that the SWASV responses diminished with addition of PS continuously, and the results were also well consistent with that obtained from flow cytometry. SWASV detection procedure could not only easily perform and quantitatively reflect the ratio of apoptotic cells, but also accurately represent the instantaneous situation of cell apoptosis. To a certain extent, it has some advantages over the traditional methods. With cost-efficiency, simplicity and accuracy, this electrochemical technique may have great potentials for detecting cell apoptosis, not only in vitro, but also in vivo systems.

**Methods**

**Materials.** S. cerevisiae strain 1812 (MATa) was purchased from China Center of Industrial Culture Collection (Beijing, China). PS was purchased from Sigma-Aldrich Company (St. Louis, USA). FITC Annexin V Apoptosis Detection Kit I (556547) was purchased from BD Pharmingen Company (San Diego, USA). Stock solutions of H$_2$O$_2$ were used at a concentration of 250 mM in 100% ethanol and stored at 4°C before assays. 1,10-Diaminodecane (DAD) was obtained from Aike Chemical Reagent Company (Chengdu, China). Other chemicals were of analytical grade. All solutions were prepared with deionized water purified by a Milli-Q purification system (Millipore, USA), and stored in a refrigerator at 4°C.

**Yeast cell culture and apoptosis induced by H$_2$O$_2$.** Yeast cells were grown at 30°C in 30 mL of YPD medium (1% yeast extract, 2% glucose, 2% peptone). For each experiment, the cultures were inoculated with an overnight culture (exponential phase) to an OD$_{600}$ of 0.1 (approximately 1 $\times$ $10^6$ cells/mL). To induce yeast apoptosis, three samples (each 1 mL of exponential yeast cells in a 2 mL tube) were separately incubated with 20 $\mu$L of H$_2$O$_2$ dissolved in ethanol with different concentrations (1 mM, 3 mM, and 5 mM) at 30°C for 200 min, and a sample with pure ethanol treatment was set as a control. After 200 min, cells were harvested at 6000 rpm for 3 min.

**Yeast cell survival assay.** Cell survival was determined by plating aliquots of cell suspensions diluted in saline (1 : 1000) onto YPD agar plates with an YLN-50 colony counter (YLN Electromechanical Technology Research Institute, China). For calculation of death rates, the control was set to 100% survival under the same condition. Colony-forming units were determined after incubating the plates for 48 h at 30°C. At least three replicates of viability tests were performed.

**Apoptosis assay by flow cytometry.** Before electrochemical detection was conducted, traditional Annexin V/PI costaining on apoptotic yeast cells was performed. Spheroplasts of cells induced by different concentration of H$_2$O$_2$ were stained with FITC Annexin V Apoptosis Detection Kit I (556547). The cell treatments are same as Figure 3.
FITC-labeled Annexin V and PI using a FITC-Annexin V apoptosis detection kit as described in the technical data sheet. After treated with Annexin V-FITC, 1 × 10^6 cells were analyzed with a FACS Calibur flow cytometry system (BD Biosciences Co., USA) accompanied with Cell Quest Pro software.

Preparation of DAD modified glassy carbon electrode. To detect Cu^{2+} sensitively, DAD was used to modify glassy carbon electrode (GCE). Prior to modification, the GCE was carefully polished with 1, 0.3, 0.05 μm alumina slurry, cleaned with ethanol and deionized water and dried in nitrogen. Afterwards, the GCE was immersed into 8 mM DAD ethanol solution containing 0.1 M LiClO_4 and the potential was applied from 0 to 1.6 V for two times at a scan rate of 15 mV s^{-1}, then a DAD modified GCE was obtained.

Detection of Cu^{2+} and apoptosis by SWASV. Detection of Cu^{2+} was carried out by SWASV (frequency = 15 Hz, sensitivity = 4 mV, amplitude = 25 mV) in Tris-HCl buffer solution (0.1 M, pH 8.2). As for apoptosis detection, 20 μL of H_2O_2 treated yeast solution (approximately 1 × 10^6 cells) was added into Cu^{2+} solution and stirred for 2 min, and then the electrochemical responses of the DAD/GC electrode were recorded. The experiments were performed at least in triplicate.

Electrochemical apparatus. Electrochemical experiments were recorded using a CHI 660D computer-controlled electrochemical workstation (CH Instruments, USA) with a standard three-electrode system. The DAD modified GCE served as a working electrode; a platinum wire was used as a counter-electrode with a saturated Ag/AgCl electrode completing the cell assembly. A pH meter (Mettler Toledo FE20, Switzerland) was used for measuring pH.

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Author contributions
Z.W. and X.X. conceived the research. Q.Y. performed the yeast apoptosis experiments. S.X. performed the electrochemical experiments. D.C. gave constructive comments on the manuscript. All authors contributed to writing of the manuscript.

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