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Facile and quantitative electrochemical detection of yeast cell apoptosis

SUBJECT AREAS:
ELECTROCHEMISTRY
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27 December 2013Accepted
24 February 2014Published
14 March 2014Correspondence and
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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 Cu^{2+} and phosphatidylserine (PS) translocated from the inner to the outer plasma membrane of the apoptotic cells. The combination of negatively charged PS and Cu^{2+} could decrease the electrochemical response of Cu^{2+} on the electrode. The results showed that the apoptotic rates of cells could be detected quantitatively through the variations of peak currents of Cu^{2+} 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 diseases^{1,2}. 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 stimuli^{3–6}, trigger different intracellular signal transduction pathways^{7–9}.

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 progression^{10,11}. 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 processes^{2,12}. Several methods have been developed for apoptosis detection⁹, such as electron microscopy¹³, spectroscopic techniques^{14,15}, flow cytometry^{14,16,17}, TUNEL assay^{18,19}, single-molecule spectroscopy²⁰, and microfluidic devices^{21,22}, 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 reported^{23–26}. 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 polyethylenimine (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 a quantitative electrochemical detection method with low cost and convenience.

It is known that Cu^{2+} forms a 1 : 2 complex with PS in solution containing water, methanol and chloroform²⁷, and the interaction between PS and Cu^{2+} 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 Cu^{2+} solution. When apoptotic cells were added into Cu^{2+} solution, the exposed PS could combine Cu^{2+} , and the SWASV peak current of Cu^{2+} 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 Cu^{2+} may offer a sensitive and feasible method to determine apoptotic cells conveniently, inexpensively and quantitatively.

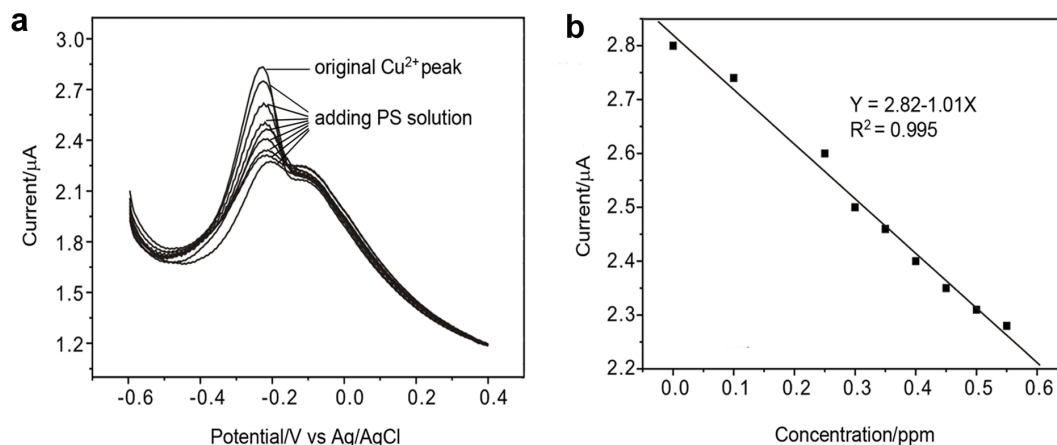


Figure 1 | (a) SWASV responses of Cu^{2+} with successive addition of standard PS over a concentration range of 0 to 0.55 ppm in 0.1 M Tris-HCl buffer solution (pH 8.2). (b) Dependence of peak current on the concentration of PS. Deposition potential, -0.6 V; deposition time, 120 s.

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 as supporting electrolyte 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 Cu^{2+} 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 Cu^{2+} in solution, consequently the Cu^{2+} 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. H_2O_2 can induce apoptosis in *S. cerevisiae* as well as in mammalian cells^{31,32}. As shown in Figure S4, in our study, after treated with 1 mM H_2O_2 for 200 min, *S. cerevisiae* cells viability evaluated by colony formation decreased to approximately 30%, and even lower with higher concentrations of H_2O_2 (3 mM and 5 mM). Obviously, H_2O_2 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 PS^{29,31,33}.

Figure 2a presented the SWASV electrochemical responses of Cu^{2+} before and after successive addition of H_2O_2 treated yeast cell solution in 0.1 M Tris-HCl solution (pH 8.2). The highest peak current was obtained when Cu^{2+} in pure Tris-HCl solution, and then the peak currents decreased gradually with successive addition of 20 μL of 1 mM H_2O_2 treated apoptotic yeast cells solution. For comparison, normal yeast cells were added into Cu^{2+} 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

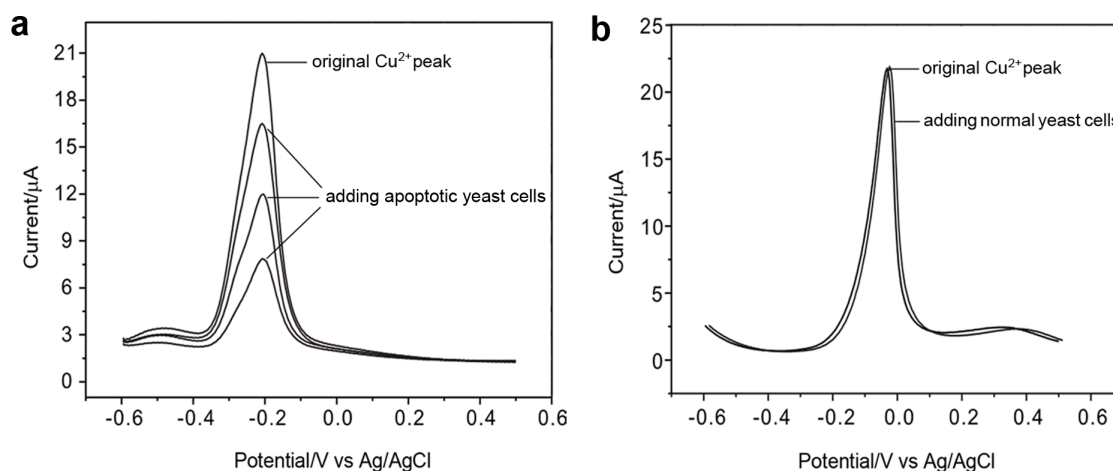


Figure 2 | SWASV electrochemical responses of Cu^{2+} of actual cells. (a) With successive addition of 20 μL of 1 mM H_2O_2 treated apoptotic yeast cell solution. (b) With addition of normal yeast cell in 0.1 M Tris-HCl solution (pH 8.2). SWASV conditions: frequency, 15 Hz; potential step, 4 mV; pulse amplitude, 25 mV.

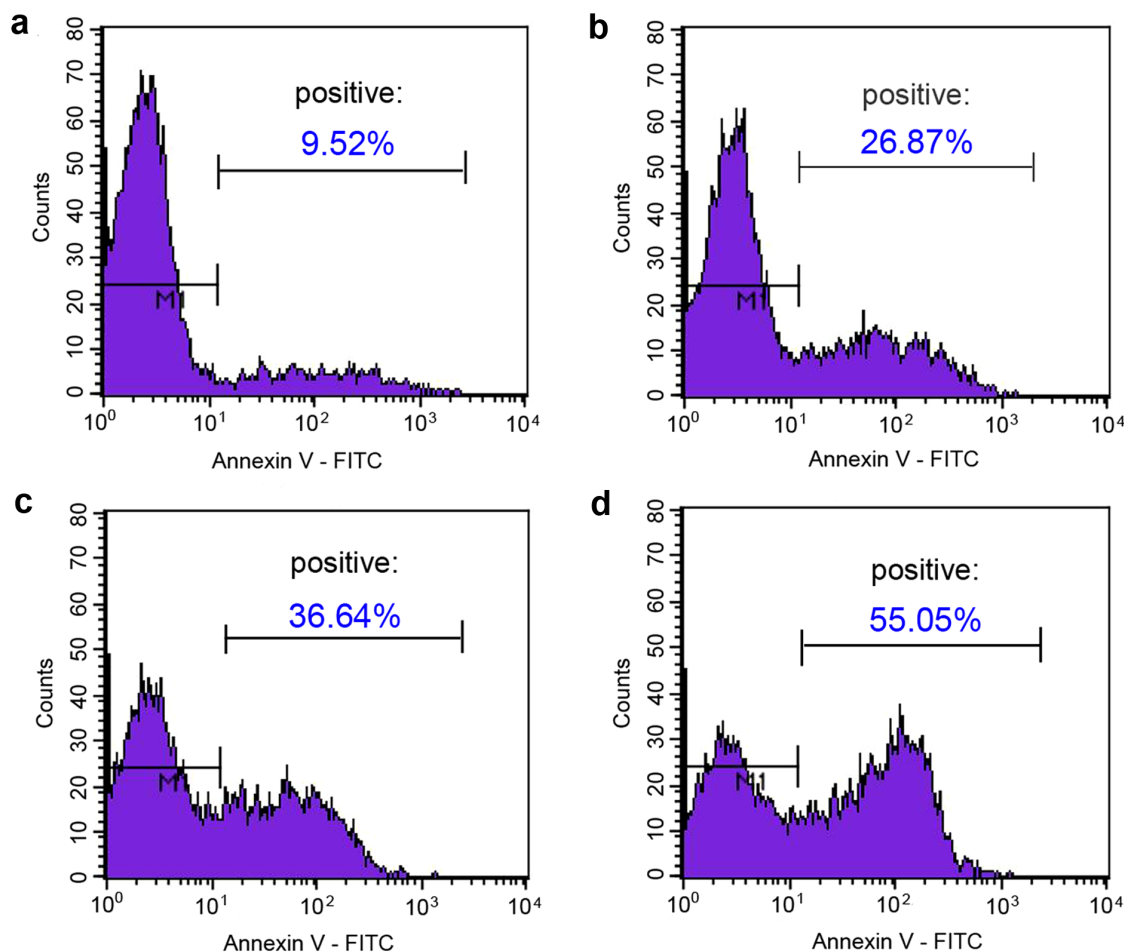


Figure 3 | Flow cytometric analysis of PS externalization by FITC-Annexin V/PI costained *S. cerevisiae* cells after a 200 min treatment with (a) ethanol, (b) 1 mM H₂O₂, (c) 3 mM H₂O₂, and (d) 5 mM H₂O₂.

concentration of H₂O₂ (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⁺ 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 H₂O₂ 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 μA with addition of 20 μL of ethanol treated cells solution (Figure 4a), 4.8 μA with 20 μL of 1 mM H₂O₂ treated cells solution (Figure 4b), 7.6 μA with 20 μL of 3 mM H₂O₂ treated cells solution (Figure 4c), and 10.3 μA with 20 μL of 5 mM H₂O₂ 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 *S. cerevisiae*'s physiological pH range was 7.2–7.5, which was in response to the nutrient availability and respiratory chain activity³⁴. PS lipids are found in a wide variety of cell types, and it is an important signaling molecule in clotting, apoptosis, and embryonic development³⁵. PS bears a negative charge at physiological pH. Monson et al. found that PS could reversibly bind Cu²⁺ with extremely high affinity in a pH-dependent fashion²⁸. 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 Cu²⁺ and PS, electrochemical detection of apoptosis becomes available.

Previous investigation showed that 1 ~ 3 mM Cu²⁺ might be beneficial for cell growth after 24 h of incubation, whereas the extensive apoptosis occurred at 6 mM Cu²⁺, and apoptosis was largely replaced by necrosis at 10 mM Cu²⁺³⁶. Therefore, low concentration of Cu²⁺ in our study (less than 1 mM, actually 0.6 ppm) and rapidly detection could make the effect of Cu²⁺ on cell growth ignored.

To confirm the cell apoptosis could be detected by SWASV method, influence of PS alone on Cu²⁺ needs to be first tested through SWASV. With the modified DAD/GC electrode, the Cu²⁺ peak currents in solution and additional standard PS content showed a linear correlation relationship (Figure 1). It was obvious that the amounts of Cu²⁺ 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 Cu²⁺ (Figure 2b). PS can be externalized in the early stage of the apoptosis process¹⁶. Once PS exposed to the extracellular environment, it could interact with some cations. In the present study, H₂O₂ induced apoptosis in *S. cerevisiae*, and released PS in the test solution can result in the change of CVs of the electrochemical response of Cu²⁺ (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

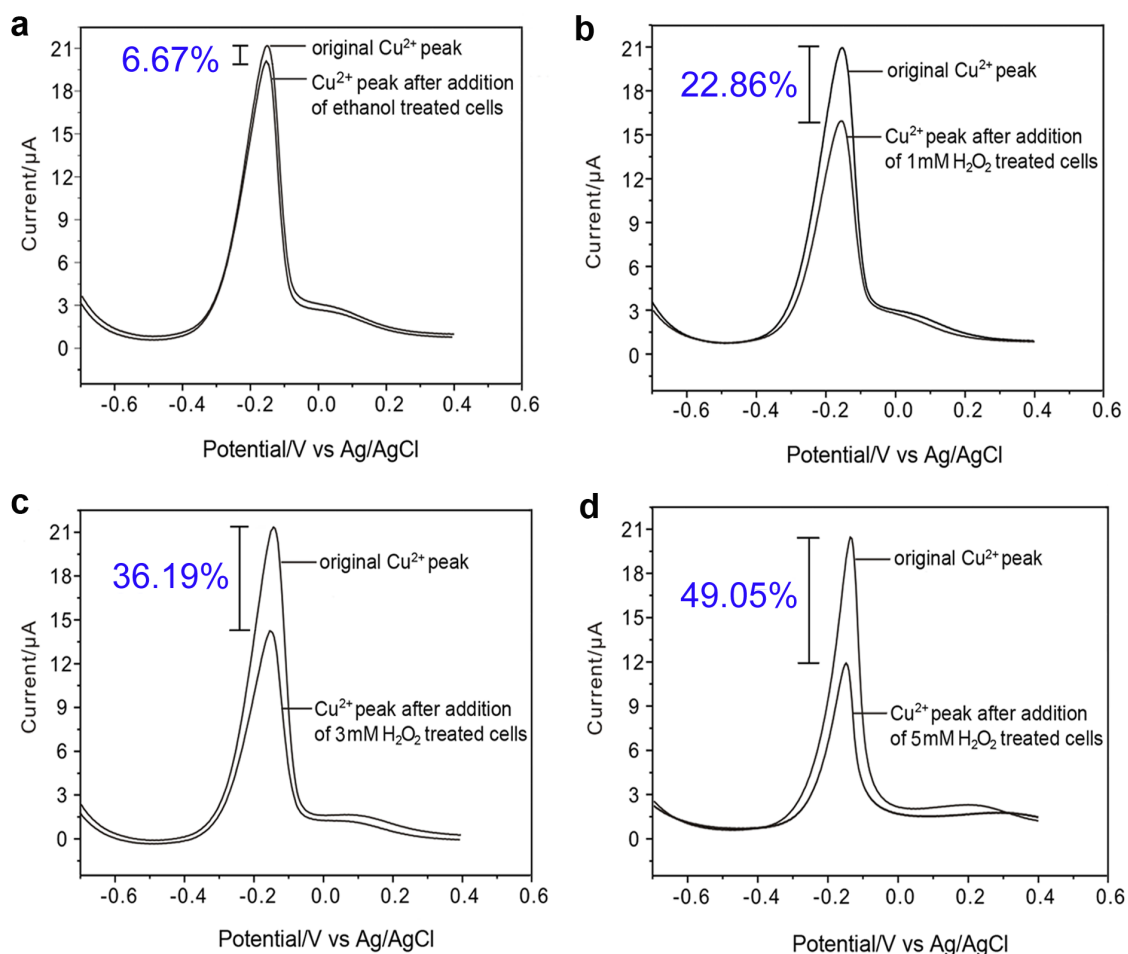


Figure 4 | SWASV responses of Cu^{2+} of apoptotic *S. cerevisiae* cells in 0.1 M Tris-HCl solution (pH 8.2). The cell treatments are same as Figure 3.

treated with ethanol and H_2O_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, same amount of apoptotic yeast cells (1×10^4 *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 immediately and does not need the time-consuming stain process, whereas stains probably influence cell apoptosis at a certain degree^{9,24}.

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_2O_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_2O_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 1×10^6 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 μL of H_2O_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 staining on apoptotic yeast cells was performed. Spheroplasts of cells induced by different concentration of H_2O_2 were stained with



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, 1×10^4 cells were analyzed with a FACSCalibur 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 1×10^4 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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Acknowledgments

The authors acknowledge financial support from the National Natural Science Foundation of China (No. 10975154 and No. 21072002).

Author contributions

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

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yue, Q.L., Xiong, S.Q., Cai, D.Q., Wu, Z.Y. & Zhang, X. Facile and quantitative electrochemical detection of yeast cell apoptosis. *Sci. Rep.* **4**, 4373; DOI:10.1038/srep04373 (2014).



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