Direct Electrochemiluminescence Imaging of a Single Cell on a Chitosan Film Modified Electrode

Gen Liu,†‡ Cheng Ma,‡ Bao-Kang Jin,*† Zixuan Chen,*‡ and Jun-Jie Zhu*‡

†College of Chemistry and Chemical Engineering, Anhui University, Hefei, Anhui 230601, China
‡State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, 210023, China

ABSTRACT: Single-cell imaging is essential for elucidating the biological mechanism of cell function because it accurately reveals the heterogeneity among cells. The electrochemiluminescence (ECL) microscopy technique has been considered a powerful tool to study cells because of its high throughput and zero cellular background light. However, since cells are immobilized on the electrode surface, the steric hindrance and the insulation from the cells make it difficult to obtain a luminous cell ECL image. To solve this problem, direct ECL imaging of a single cell was investigated and achieved on chitosan and nano-TiO₂ modified fluoride-doped tin oxide conductive glass (FTO/TiO₂/CS). The permeable chitosan film is not only favorable for cell immobilization but also increases the space between the bottom of cells and the electrode; thus, more ECL reagent can exist below the cells compared with the cells on a bare electrode, which guarantees the high sensitivity of quantitative analysis. The modification of nano-TiO₂ strengthens the ECL visual signal in luminol solution and effectively improves the signal-to-noise ratio. The light intensity is correlated with the \( \text{H}_2\text{O}_2 \) concentration on FTO/TiO₂/CS, which can be applied to analyze the \( \text{H}_2\text{O}_2 \) released from cells at the single-cell level. As far as we know, this is the first work to achieve cell ECL imaging without the steric hindrance effect of the cell, and it expands the applications of a modified electrode in visualization study.

Electrochemiluminescence (ECL), also called electrogenerated chemiluminescence, involves the process where species generated at the electrode surface undergo high-energy charge-transfer reactions to form excited states that emit light. ECL was first reported by Dufford et al. in 1927 and in recent years has become a widely used analytical technique in a diversity of fields including bioanalysis, nanomaterials, and light-emitting devices. Though ECL instruments based on a photomultiplier (PMT) detector have high sensitivity for detecting the optical signal from the whole electrode, the fine details of the electrode are unclear because they have no spatial resolution. Fortunately, ECL imaging provides a high temporal—spatial resolution approach, and much work so far has focused on the visualized ECL analysis. For example, Xu et al. successfully established a method for visualizing latent fingerprints on the electrode surface. Wilson et al. reported the generation of ECL at single gold nanowire electrodes. A three-dimensional ECL imaging approach was developed by Sojic’s group and applied to study the ECL mechanistic route.

Single-cell analysis can offer significant information regarding the cellular heterogeneity and the expression protein on the cell surface. The imaging of a single cell is an effective means to obtain insight into the processes that occur between drugs and cells, and it can help us to intensively understand cell-to-cell communication. Because of the lack of an external light source, ECL microscopy for single-cell imaging possesses zero background and a high signal-to-noise ratio compared with photoluminescence imaging. Unlike the works of determining cells using electrochemical or ECL methods, which rely on the information on cell groups, ECL imaging can offer visual details of a single cell. Zhou et al. reported indirect ECL imaging of a single cell for the analysis of active cell membrane cholesterol. Recently, Sojic’s group realized the first spatially resolved ECL imaging of membrane proteins of a single cell. However, the cells on the electrode surface unavoidably block the electron transfer and hinder the diffusion of the ECL reagent, which leads to dark contrast in the cell regions or only ECL emission at the cell edges. It is a significant challenge to overcome this negative effect when we investigate cells using the ECL imaging technique.

\( \text{H}_2\text{O}_2 \) is one of the reactive oxygen species (ROS) that are associated with the regulation of cellular processes in vivo. \( \text{H}_2\text{O}_2 \) at an appropriate level is vital for cells to maintain normal functions, and the excessive \( \text{H}_2\text{O}_2 \) usually induces biological damages, causing cancer, aging, diabetes, and so on. Therefore, the development of a sensitive and specific method for the detection of \( \text{H}_2\text{O}_2 \) is of great significance.

Received: January 13, 2018
Accepted: March 6, 2018
Published: March 6, 2018
for reliable detection of H2O2 concentration under physiological conditions is of great importance for early cancer screening and diagnosis.

Chitosan is one deacetylated form of a natural polymer derived from various sources (insects, crustaceans, the cell walls of fungi and yeast, etc.).18,19 Due to its good biocompatibility, some cells can adhere and proliferate on chitosan or its composites.19—21 In addition, as the most popular binder, a chitosan-modified electrode has been widely applied in electrochemical analysis.22—24 By virtue of these merits, we developed a direct ECL microscopy strategy of single cells on a chitosan film modified electrode. The cervical cancer cells (HeLa) could be cultured and stably immobilized on the chitosan film. The cells were elevated by the film and did not touch the electrode surface, so the steric hindrance effect of cells was obviously reduced. The ECL reagent in the bulk solution could pass around the cell, enter into the chitosan film, and finally realize ECL on the electrode surface. Upon being stimulated by N-formylmethionyl-leucyl-phenylalanine (fMLP), the cells secreted H2O2 immediately, and then a bright image was observed in the cell region because of the classic ECL reaction between luminol and H2O2. Furthermore, we found that the nano-TiO2 modified FTO electrode could obviously amplify the ECL signal and offer high signal-to-noise ratio due to the electrocatalysis of TiO2 in the ECL reaction. FTO/TiO2/CS showed sensitive responses to H2O2 and demonstrated the heterogeneity of H2O2 released from individual cells. This work provides a new and simple ECL strategy to visually study cells at the single-cell level.

■ EXPERIMENTAL SECTION

Materials and Reagents. All reagents were used without further purification, and deionized distilled water was used throughout the experiments. Fluoride-doped tin oxide conductive glass (FTO, sheet resistance ≤10 Ω/sq) was purchased from Guangdong Huanan Xiangcheng Technology Co., Ltd. (China). TiO2 powder (P25) was obtained from Degussa Co. (Germany). 8-Amino-5-chloro-7-phenylpyrido[3,4-d]-pyridazine-1,4(2H,3H)-dione (L012, a luminol analogue) was bought from Wako Chemical U.S.A., Inc. (Richmond, VA). Chitosan (CS, 80—95% deacetylation) was obtained from SinopharmChemicalReagentCo.,Ltd.(China).N-Formylmethionyl-leucyl-phenylalanine (fMLP) and diphenyleneiodonium (DPI) were purchased from Aladdin (U.S.A.), and they were dissolved in dimethyl sulfoxide (DMSO). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) was obtained from Nanjing KeyGen Biotech. Co., Ltd. (China). H2O2 was purchased from Shanghai Ling Feng Chemical Reagent Co., Ltd. (China). All solutions were prepared using ultrapure water (18.2 MΩ cm resistivity).

Apparatus. The ECL imaging setup, as demonstrated in Figure 1, was assembled with two major apparatuses. A CHI 660D electrochemical workstation (CH Instruments Co., China) was the signal actuating device to generate and record electrochemical responses. An electron multiplying CCD (EM-CCD) (Evolve, Photometrics, Scientifica, U.S.A.) was the signal collecting device to output images. The optical path system was built with a water immersion objective (60X, NA 1.1, Olympus, Japan), a convex lens, a reflector, and tubes. The ECL imaging test must be operated in a shielding room.

A conventional three-electrode system was used for all electrochemical experiments, which consisted of an FTO glass or a modified electrode as the working electrode, a Ag/AgCl (saturated KCl) as the reference electrode, and a platinum wire as the auxiliary electrode.

Scanning electron microscopy (SEM) tests were performed on a Hitachi S-4800 scanning electron microscope (Hitachi Co., Japan) and a Shimadzu SSX-S550 (Shimadzu Co., Japan).

Cell Culture and Immobilization on the Electrode. The reagents for the cells experiments were all obtained from Nanjing KeyGen Biotech. Co., Ltd. (China). The cervical cancer cells (HeLa) and human breast adenocarcinoma cells (MCF-7) were cultured with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 4.5 g/L of glucose, 0.584 g/L of l-glutamine, 3.7 g/L of sodium bicarbonate, 0.11 g/L of sodium pyruvate, 80 U/mL of penicillin, and 80 mg/mL of streptomycin. The cells were proliferated and formed as a monolayer in an incubator in a humidified air atmosphere (relative humidity of 95%) with 5% CO2 at 37 °C.

The cells in the exponential growth phase were digested for 10 min by trypsin (0.1%, m/v), and then separated from the cell culture medium. Centrifuging at 1000 rpm for 5 min was essential to gather the cells after washing thrice with a sterile phosphate buffer solution (PBS, 10 mM, pH 7.4). The cell precipitate was then resuspended in 2 mL of DMEM.

Prior to seeding cells, FTO slices were successively cleaned in an ultrasonic bath with NaOH solution, ethanol, and ultrapure water for 5 min, and were then dried with nitrogen flow. TiO2 aqueous dispersion was cast onto the FTO surface with an area of 1.5 × 1.5 cm2 and dried in air. The chitosan solution was prepared by dissolving it into 1.0% (v/v) acetic acid aqueous solution. This obtained solution was cast on FTO/TiO2 and dried in air. After forming chitosan film, the electrode (FTO/TiO2/CS) was washed with 0.1 M sodium hydroxide solution for a given time to neutralize the excess acetic acid. After that, FTO/TiO2/CS was gently washed in ultrapure water and dried in air again. Finally, HeLa cells were seeded on the FTO/TiO2/CS surface and cultured in an incubator for 8 h, which achieved the immobilization of cells. Before measurement, the electrode with the cells was washed with PBS two times to wash out the unattached cells. For comparison, the bare FTO and the chitosan-modified electrode (FTO/CS) were prepared and used to immobilize cells under the same conditions.

■ RESULTS AND DISCUSSION

Reducing the Steric Hindrance Effect of Cells. HeLa cells were immobilized on a bare FTO and used for ECL imaging in a solution of 10 mM PBS (pH 7.4) and 400 μM L012. MTT experiments confirm that 400 μM L012 has low
cytotoxicity (Supporting Information Figure S1). To our knowledge, cells can release a greater deal of H$_2$O$_2$ within a short time when simulated by fMLP,$^{25}$ and H$_2$O$_2$ can enhance the ECL of luminol. Hence, fMLP was adopted to trigger the cell ECL signal in the L012 solution. The ECL images before the addition of fMLP (100 ng/mL) and after the addition of fMLP were collected and are shown in Figure 2, parts A2 and A3, respectively. The adhered cells exert an undesirable effect on the diffusion of L012 and the charge transfer, which leads to the dark ECL imaging of cells, which is consistent with a previous report.$^{14}$

To overcome the steric hindrance effect of cells on the electrode, a bigger space below the cells and the liquid exchange is needed. As a permeable and biocompatible polymer, chitosan is a suitable biomaterial candidate to modify the electrode. Chitosan film was applied to increase the space between the bottom of the cells and the electrode surface. Then, 100 $\mu$L of chitosan solution with concentrations of 0.5%, 1%, 2%, 3%, and 4% (w/v) was cast on the FTO ($1.5 \times 1.5$ cm$^2$). After air drying, alkaline washing, water washing, and air drying again, the chitosan films were peeled off from the electrode carefully. The chitosan film has a homogeneous and dense structure, as shown in the SEM images (Figure 3). The thicknesses of the dry films prepared from 0.5%, 1%, 2%, 3%, and 4% (w/v) chitosan solutions are $\sim$1.8, 3.9, 8.3, 11.9, and 16.1 $\mu$m, respectively. Thus, the thickness of the chitosan film prepared from 0.1% (w/v) chitosan solution can be estimated to be $\sim$0.5 $\mu$m. In addition, the dark-field imaging demonstrates that the thickness of the chitosan film changes little in the aqueous solution (Supporting Information Figure S2 and Table S1).

Following the same imaging procedure, the FTO/CS/cell modified by 0.1% (w/v) chitosan ($\sim$0.5 $\mu$m) also gives dark ECL imaging of cells (Figure 2, parts B$_2$ and B$_3$) because the chitosan film is too thin to effectively eliminate the steric hindrance effect of cells. Interestingly, for the FTO/CS/cell modified by 0.5% (w/v) chitosan ($\sim$1.8 $\mu$m), the brightness of the immobilized cells is directly observed after fMLP stimulation (Figure 2C$_3$), and no dark cell imaging appears before the addition of fMLP (Figure 2C$_2$), suggesting the steric hindrance effect of the cell has been eliminated. The ECL reagent L012 in the bulk solution passes around the cell, enters

Figure 2. Images of HeLa cells on bare FTO (A), FTO/CS modified by 0.1% (B), 0.5% (C), 1.0% (D), and 2.0% (E) (w/v) chitosan, and FTO/TiO$_2$/CS modified by 0.5% chitosan (F). First row (A$_1$, B$_1$, C$_1$, D$_1$, E$_1$, and F$_1$): bright-field images. Second row (A$_2$, B$_2$, C$_2$, D$_2$, E$_2$, and F$_2$): ECL images before the addition of fMLP. Third row (A$_3$, B$_3$, C$_3$, D$_3$, E$_3$, and F$_3$): ECL images after the addition of fMLP. The L012 concentration was 400 $\mu$M, and the exposure time was 10 s. The pixel size of all images was 0.32 $\mu$m.

Figure 3. SEM images of the cross section of chitosan films prepared from 0.5% (A), 1% (B), 2% (C), 3% (D), and 4% (E) (w/v) chitosan solution.
into the chitosan film, and finally realizes ECL in the space between cells and electrode. With continuing increase of the thickness (the thickness of the chitosan film is the distance between the bottom of the cells and the FTO surface), the FTO/CS/cell modified by 1.0% (w/v) chitosan (∼3.9 μm) shows similar results (Figure 2, parts D2 and D3), except for the low luminosity. Nevertheless, with further increase in the thickness of the chitosan film, for the FTO/CS/cell modified by 2.0% (w/v) chitosan (∼8.3 μm), it is difficult to see the cell ECL image after the addition of fMLP (Figure 2E3), indicating the brightness can be reduced when exceeding the optimal chitosan thickness.

Without fMLP stimulation (Figure 4A), the background is brighter than the cell for chitosan concentration lower than 0.5%, and the light intensity of the background is approximately equal to the cell from 0.5% to 2%. However, after fMLP stimulation, the cell is brighter than the background for 0.5% and 1.0% (Figure 4B), particularly for 0.5%, indicating that a chitosan film with an appropriate thickness is beneficial to realize the direct ECL imaging of cells. For the cells adhered on the chitosan film, the film raises the cells and increases the space between the bottom of the cells and the electrode, which allows more L012 remaining in the space and guarantees the constant concentration of ECL reagent below the cells. It is crucial to accurately analyze the H2O2 released from the cells. In fact, chitosan film is also a poor diffusion barrier, and a thick film has a negative impact on mass transfer, which can weaken the luminescence. Therefore, a chitosan film with an appropriate thickness can reduce or offset the steric hindrance effect from cells in the process of the ECL reaction. The sufficient L012 and the fast substance exchange below the cells not only support the available brightness but also ensure a sensitive and quantitative analysis in our cell ECL imaging. Herein, the electrode modified by 0.5% (w/v) chitosan (∼1.8 μm) was chosen in the following work, unless stated otherwise.

Recently, some reports have indicated that TiO2 nanoparticles can greatly intensify the ECL of luminol, since the excitons of luminol and the reactive oxygen species are easily created on the TiO2 surface.26,27 Moreover, nano-TiO2 has already proven to be of low toxicity.28,29 Therefore, nano-TiO2 was used to enhance the ECL intensity of L012. The optimization of TiO2 modification and the electric parameters of chronoamperometry (CA) are clarified in the Supporting Information (Figures S3 and S4). Under the optimal conditions, FTO/TiO2/CS (Figure 5B2) exhibits more obvious cell ECL imaging compared with FTO/CS (Figure 5A2).
light intensities of the background and the cells on FTO/TiO2/CS are both higher than those on FTO/CS, as demonstrated in Figure 5, parts A3 and B3. Moreover, the signal-to-noise ratio (S/N) is improved obviously on FTO/TiO2/CS (S/N = 5.5:1) compared with FTO/CS (S/N = 3.2:1), which is ascribed to Figure 5, parts A3 and B3. Furthermore, the signal-to-noise ratio of CS are both higher than those on FTO/CS, as demonstrated in Figure 5, parts A3 and B3. Moreover, the signal-to-noise ratio (S/N) is improved obviously on FTO/TiO2/CS (S/N = 5.5:1) compared with FTO/CS (S/N = 3.2:1), which is ascribed to Figure 5, parts A3 and B3. Moreover, the signal-to-noise ratio (S/N) is improved obviously on FTO/TiO2/CS (S/N = 5.5:1) compared with FTO/CS (S/N = 3.2:1), which is ascribed to the electrocatalysis of nano-TiO2.

Considering that the location of the cell is uncertain on FTO/TiO2/CS, we randomly chose six areas with a size of 30 × 30 μm2 (the cell size) and recorded the luminescence. The light intensity fluctuated within a narrow range with a relative standard deviation (RSD) of 5.3%. This low RSD supported the uniform modification of chitosan and TiO2 and guaranteed the analysis accuracy of cells. FTO/TiO2/CS was a practical ECL electrode in the visualization study, especially in cell imaging. More cells were stimulated by fMLP and tested on FTO/TiO2/CS, and the RSD of the light intensity of cells was 30.6% (from 15 cells), indicating the heterogeneity of H2O2 released from a single cell. Furthermore, the detachment and the morphological change of the cells were not observed after ECL imaging, suggesting that the ECL process has a negligible influence on the cells (Supporting Information Figure S5). In addition, the influence of DMSO (the solvent of fMLP) on ECL imaging was eliminated because it does not cause cells to produce H2O2 (Supporting Information Figure S6).

Detection of H2O2 Released from a Single Cell.
According to the previous discussion, the steric hindrance of cells on the bare electrode results in the low light intensity in the cell region relative to the background. Increasing the distance between the cell and the FTO via a chitosan film can overcome this negative effect. Moreover, the transparent cells cannot block the light emitted from the FTO surface. Herein, various concentrations of H2O2 were measured on the FTO/TiO2/CS/cell in 400 μM L012 solution containing PBS (10 mM, pH 7.4). With the increase in H2O2 concentration (Supporting Information Figure S7), the light intensity becomes higher and has a linear correlation: \( I = 35.93C + 3645, r = 0.9781 \), from 20 to 350 μM (n = 5); the detection limit is 10 μM. However, a nonlinear relationship appears above 350 μM H2O2, which is probably caused by the deficiency of L012. Besides, the FTO/TiO2/CS/cell has lower detection limit and higher sensitivity than the FTO/CS/cell and FTO/cell (Supporting Information Table S2).

To our knowledge, the H2O2 released from cells is at a micromole level under exogenous stimulation.14,30 After fMLP stimulation, the average H2O2 concentration released for a single cell is ∼45 μM (RSD = 38.9%, n = 25), as shown in Figure 6, parts A and C. In this study, diphenyleneiodonium (DPI, an NADPH oxidase inhibitor) was applied to inhibit the production of fMLP-stimulated H2O2. DPI (30 μg/mL) was incubated with the cells for 30 min. Then, fMLP was added and the ECL image was collected immediately. As shown in Figure 6B2, the luminescence of HeLa cells with DPI pretreatment obviously decreases compared with those without DPI inhibition. A single cell secretes ∼15 μM H2O2 on average. The RSD of 67.1% (n = 25) illustrates that there are distinct individual differences in H2O2 secretion at the single-cell level, which reveals the superiority of single-cell ECL microscopy. The similar experiments were carried out on MCF-7 cells (Supporting Information Figure S8). The average H2O2 concentration is ∼17 μM (RSD = 70.2%) for DPI-pretreated cells and ∼50 μM (RSD = 40.6%) for non-DPI-pretreated cells, respectively. The cells with DPI treatment secrete H2O2 less than those without DPI treatment, indicating that ECL imaging is a simple and effective technique to evaluate drug effects, especially promotion and inhibition effects from exogenous substrates.

§ CONCLUSION
In summary, we successfully prepared an FTO/TiO2/CS electrode for direct ECL imaging of single cells. This ECL imaging method overcame the steric hindrance effect caused by cells by virtue of a permeable chitosan film, and it was successfully used to evaluate the drug effect at the single-cell level. As a binder with good biocompatibility, chitosan shows potential for applying the classic and resourceful ECL detection strategy to ECL imaging, especially cell imaging. Expanding the space between the cells and the electrode is favorable for the ECL visualization study of cells. Hence, modifying electrodes with materials containing an open structure or good permeability, such as gelatin and agarose, is worth considering. Apart from H2O2, other intracellular substances or exocytosis including NO, H2S, glucose, lactic acid, etc. are also expected to realize ECL imaging by a similar method. Therefore, based on this high-throughput ECL imaging system, more visible information regarding cells will be explored and presented in the future.

§ ASSOCIATED CONTENT
$ Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b00194.
MTT assay, monitoring thickness, optimization of measurement conditions, effect of DMSO, determination of H₂O₂ at different electrodes (PDF)

■ AUTHOR INFORMATION

Corresponding Authors
*E-mail: bkjinfh@aliyun.com.
*E-mail: chenzixuan@nju.edu.cn.
*E-mail: jjzhu@nju.edu.cn.

Gen Liu: 0000-0001-6749-8091
Cheng Ma: 0000-0001-5729-8483
Zixuan Chen: 0000-0002-3017-1121
Jun-Jie Zhu: 0000-0002-8201-1285

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
This research is supported by the National Natural Science Foundation of China (Grant Nos. 21335004, 21427807, 21605081, and 21375001).

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