Wireless Electrochemical Visualization of Intracellular Antigens in Single Cells

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Electrochemical microscopy has been developed in the past decades for imaging biomolecules in single cells; however, electrochemical visualization of an intracellular protein in one cell is difficult. Herein, for the first time, we have developed a model whereby the protein, KDM1/LSD1 antigen, in the nucleus of a single MCF 7 cell could be visualized using wireless bipolar electrochemiluminescence (BPE-ECL). Submicron-sized, single-walled carbon nanotubes were linked with anti-KDM1/LSD1 antibodies and loaded into the cells for the recognition of the corresponding KDM1/LSD1 antigen. With a low electric field of 1000 V/cm, L012, a luminol analog, was oxidized electrochemically at one end of the nanotube that emitted light for the wireless visualization of its location. The significant drop in the applied voltage permitted the observation of apparent ECL emission inside the nucleus of a single cell, supporting the electrochemical imaging of intracellular KDM1/LSD1 antigen at a single-cell level. This work solves a long-standing task in the field of electroanalysis for intracellular wireless visualization, which should advance the development of electrochemistry in single-cell analysis.

Keywords: bipolar electrochemiluminescence, electrochemical visualization, intracellular proteins, single-cell, carbon nanotubes

Introduction

The specific detection of proteins inside single cells is significant for the elucidation of molecular mechanisms in cell biology and the diagnosis of diseases.1–3 Fluorescence imaging is a popular approach for the visual detection of proteins at the plasma membrane and inside the living cells.4–7 However, the presence of background fluorescence emission affects the quantitative analysis of these proteins. Alternatively, as a near-zero background optical technique, electrochemiluminescence (ECL) is widely used for sensitive and quantitative detection of various antigens in serum and cells.8–12 In this process, the luminophore (e.g., Ru(bpy)32+) is linked to the antibody that binds specifically to the corresponding antigen to form an immune complex.13 Under a specific voltage, the luminophore near the electrode surface is excited to generate intermediates, which further react with the co-reactant to emit light.14 By recording the ECL illumination, the established ECL microscopy achieves visualization of the antigens in the plasma membrane of single cells. Despite the tremendous development of ECL
imaging, direct contact of the cells with the electrode is required; this leads to challenges in visualizing intracellular proteins in single cells. Therefore, it is imperative to develop a wireless electrochemical strategy to visualize proteins for this purpose, which has been a long-lasting task in the field of electroanalysis.

Bipolar electrochemistry (BPE) is a well-established wireless approach that generates polarization at a conductive object in a sufficiently high electric field. Accordingly, this phenomenon induces electrochemical reactions at two ends of the object without connection with a voltage generator. When the luminophore-labeled cells are cultured with the conductive object and introduced into the BPE system, the ECL reaction from the luminophore occurs at one end of the object to emit light. Recently, BPE-ECL has been used successfully to analyze biomolecules and surface proteins of a cell population. Unlike the traditional electrochemical system that needs the wired electrode for voltage introduction, this BPE mode is suitable for multiplex cellular analysis without the design of an electrode array. Moreover, the avoidance of contact between the cell and wire electrodes offers the feasibility of electrochemical detection of molecules inside the cells. Despite the feasibility of the principle, the realization of BPE-ECL for intracellular analysis in a single cell is still challenging.

Technically, to achieve BPE analysis in one cell, the conductive object must be at submicron size to enable loading into the cells. For a typical BPE configuration, the polarization potential difference at the bipolar electrode ($\Delta V_{BE}$) is directly related to the potential difference applied between the feeder electrodes ($\Delta V_{EF}$) and the resultant electric field strength ($\Delta V_{EF}/d$, where $d$ is the distance between both feeder electrodes), as described by eq 1:

$$\Delta V_{BE} = \frac{\Delta V_{EF}}{d} L$$

where $L$ is the length of the conducting object. Assuming 500 nm long nanotubes are used as the bipolar electrodes, the minimal voltages to induce the oxidation of luminol and the reduction of oxygen at two ends of the object are $\sim 0.4$ and $\sim 0.1$ V (vs Ag/AgCl), respectively. If the distance ($d$) between the two feeder electrodes is 1 cm, a voltage of $\sim 10$ kV (i.e., the order of tens of kV) must be applied between the two feeder electrodes to induce ECL reactions on the nanotubes. The application of such a high voltage is not easy to operate. Meanwhile, this high voltage leads to vigorous water electrolysis, forming gas bubbles in the cellular medium that affects the imaging process. To prevent the generation of gas bubbles, an agarose-based hydrogel with an electrolyte solution has been added into a capillary in previous reports to induce BPE-ECL at micron-sized multilayered carbon nanotubes (MWCNTs).

The porous structure of hydrogel inhibits the formation of bubbles at the feeder electrode. Consequently, spatially resolved ECL emission is imaged at individual MWCNTs; thus, providing a strategy for the wireless ECL visualization of a tiny emitter.

In this work, the wireless electrochemical visualization of proteins inside a single cell was achieved initially using BPE-ECL. Nanometer-sized single-walled carbon nanotubes (SWCNTs) were chosen as the ECL emitters linked to an antibody (e.g., anti-KDM1/LSD1 protein antibody), which could recognize the corresponding model antigen (KDM1/LSD1 antigen) in the nucleus of the cell. Since SWCNTs have good cellular permeability and biocompatibility, the SWCNTs-antibody complex could be loaded into the cells to bind intracellular antigens, as illustrated in Figure 1. To avoid bubbles during the BPE-ECL process, the cells were fixed with 4% paraformaldehyde, permeabilized by Triton X-100, and embedded into the hydrogel. In this design, the voltage drop could be spatially confined at the micropores of the hydrogel that retain the cells. The locally enhanced polarization of a bipolar electrode has been proved to happen under an extremely low voltage, which is more than two orders of magnitude lower than that in classic bipolar configurations. Thus, a relatively low voltage is sufficient to initiate ECL reaction at individual nanotubes inside the cells. Using L012 (a luminol analog with a strong ECL emission at a low voltage) as the luminophore, bright ECL at the nanotubes inside the cells were observed, exhibiting the electrochemical image of an intracellular protein in single cells. The image solved a long-term challenge associated with the electrochemical visualization of intracellular proteins in one cell, which should advance the development of single-cell electrochemistry.

**Results and Discussion**

For the initial visualization of single SWCNTs using BPE-ECL, agarose hydrogel with SWCNTs-COOH (0.005 wt %), LO12 (1 mM), and 10 mM phosphate-buffered saline (PBS; pH 7.4) were placed in the capillary (i.e., 0.6 mm). The characterized dimension of individual SWCNTs-COOH was $\sim 500$ nm in length and $\sim 10$ nm in width using transmission electron microscopy (TEM; Supporting Information Figure S1a). Upon forming the gel inside the capillary, scanning electron microscopy (SEM; Supporting Information Figure S1b) image exhibited a porous structure with a pore size of $\sim 70$ μm, suitable to retain one cell. The conductivity of gel containing PBS is measured by positioning two silver wires (distance: 1 cm) into the gel. The current in the gel was estimated to be 1.2 µA under a voltage of 1 V, comparable with the value (1.34 µA) recorded in the aqueous PBS solution. This result supported the concept that the porous gel should not affect the electrochemical behavior of LO12. We characterized the minimal voltage required for the electrochemical reactions at the SWCNTs employing cyclic voltammetry and ECL measurement of LO12 at SWCNTs-COOH-modified electrode. The current and ECL trace in Figure 2a...
demonstrated that the starting potential for the oxidation of L012 to emit the ECL light is ∼0.4 V (vs Ag/AgCl). Meanwhile, the reduction of dissolved oxygen occurs at ∼−0.1 V (vs Ag/AgCl). Thus, a minimum voltage of 0.5 V should be present in the electrolyte near the two ends of the nanotube to induce the luminol oxidation and reduction of dissolved oxygen (eqs 2 and 3).

\[
\text{Anodic pole: } \quad L^{-} + O_{2}^{-} \rightarrow LO_{2}^{-} \rightarrow AP^{2-} \rightarrow AP^{2-} + h\nu_{ECL} \quad (2)
\]

\[
\text{Cathodic pole: } \quad H_{2}O + O_{2} + 2e^{-} \rightarrow HO_{2}^{-} + OH^{-} \quad (3)
\]

where L is L012, and AP^{2-} is the aminophthalate-type anion generated from the oxidation and radiative relaxation of L012.

Considering the length (∼500 nm) for individual SWCNTs, an electric field as high as 10 kV/cm should be applied at the two feeder electrodes with a 1 cm distance to initiate the oxidation of L012 at individual SWCNTs. Interestingly, when the electric field of 1 kV/cm was applied, distinct ECL spots were observed in the capillary (Figure 2b). With the gradual increase in the electric field from 1 to 1.2 kV/cm, the ECL intensities of these spots became stronger (Supporting Information Figure S2). The dependence of ECL intensity on the applied electric field suggested the occurrence of ECL reactions at SWCNTs. The size of each ECL spot was measured to be between 0.5 and 1 μm that matched the dimension of one nanotube. A control experiment was conducted in the capillary without SWCNTs, and no ECL spots are

Figure 1 | The schematic BPE-ECL wireless setup for electrochemical visualization of KDM1/LSD1 antigen at the nucleus inside the cell. The feeder electrodes are graphite electrodes. The cells are positioned in the agarose hydrogel containing L012 and PBS located between two feeder electrodes. The amplified part shows the ECL reaction of L012 at single SWCNTs labeled at the nucleus through the antibody-antigen immune-complex. The ECL emission (h\nu) happens when a voltage is applied at the feeder electrodes.

Figure 2 | (a) Cyclic voltammetry (red line) and ECL measurement (black line) from L012 at SWCNTs-COOH-modified electrode. The solution is 10 mM PBS (pH 7.4) containing 1 mM L012. The scan rate is 10 mV/s. (b) ECL image of SWCNTs in the hydrogel inside the capillary. The gel includes SWCNTs-COOH (0.005 wt %), L012 (1 mM) and 10 mM PBS (pH 7.4). The exposure time is 30 s.

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observed. Collectively, these results suggested that the ECL spots observed in the capillary originated from the SWCNTs. The significant decrease in the electric field required for the ECL reaction should be ascribed to the confined voltage drop in the micropores of the gel. The spatially resolved observation of ECL from individual SWCNTs using BPE-ECL demonstrated that the SWCNTs could behave as an ECL tag at the antibody to visualize cellular antigens.

Traditionally, high ionic strength is known to favor the ECL reaction for enhanced ECL intensity. However, with the open BPE-ECL process, an increase in the solution conductivity decreased the applied voltage at the nanotubes, reducing the ECL intensity. Consequently, the ECL intensity became weak. To obtain the optimized ionic strength, the agarose hydrogel with SWCNTs-COOH, L012, and varying concentrations (1, 5, 10, 50, and 100 mM) of PBS were placed in the capillary. After applying an electric field of 1 kV/cm, ECL spots were observed in the capillary (Supporting Information Figure S3). The highest ECL intensity was profound from SWCNTs at 10 mM PBS concentration, as revealed in the above experiments.

The initial application of the nanotubes for single-cell BPE-ECL imaging was to attempt to visualize antigens at the cellular membrane. Anticarcinoembryonic antigen-antibody (anti-CEA Ab) was chemically linked to SWCNTs and incubated with the fixed MCF7 cells with a high expression of membrane CEA antigen. After the cells were retained in agarose hydrogels with L012, the voltage of 1000 V was applied on the two feeder electrodes, and the ECL imaging was recorded simultaneously. Visible ECL emission was observed from the whole surface of the individual cells, which had a good overlap with the bright-field cell imaging (Figures 3a–3c). A negative control experiment was conducted using Hela cells that do not express CEA antigen at the cellular membrane. Hela cells were incubated with anti-CEA antibody/SWCNTs, and a negligible ECL signal was observed from the cells (Supporting Information Figure S4). This result confirmed that specific binding between anti-CEA antibody/SWCNTs and CEA must be present to provide visible ECL emission. To further exclude possible nonspecific adsorption of SWCNTs on the cells, the cells were incubated with SWCNTs without an antibody, and images were collected under the electric field.

We observed almost no ECL emission from the cells (Supporting Information Figure S5), confirming negligible adsorption of SWCNTs on the cells. The successful visualization of ECL from the surface of single MCF7 cells confirmed that this BPE-ECL approach could be used for the wireless visualization of membrane proteins in single cells.

The aim of this work was to image intracellular proteins using single cells. This was conducted by visualizing a nuclear marker, lysine-specific demethylase 1 (also called KDM1/LSD1), inside the cells. High KDM1/LSD1 expression blocks cellular differentiation and confers a poor prognosis in acute myeloid leukemia. According to various reports, the SWCNTs-antibody complex could enter the living cells via endocytosis with minor cytotoxicity. The complexes escape the endolysosomal pathway to enter the cytosol, where they interact with the corresponding antigen at the nucleus. Experimentally, the living cells were initially coincubated with the anti-KDM1/LSD1 antibody modified SWCNTs and then fixed in paraformaldehyde. Subsequently, the cells were permeabilized by Triton X-100 to allow penetration of L012 and PBS. To ensure the transportation of the nanotubes inside the cells, a fluorescence tag of Alexa Fluor®488 was linked to the antibody for fluorescence tracking. The fluorescence image (Supporting Information Figure S6) showed evident fluorescence in the nucleus, which suggested that the antibody-modified SWCNTs penetrated the cells and bound specifically with the KDM1/LSD1 antigen in the nucleus. No fluorescence was observed in the surrounding cytoplasm, indicating that all of the added nanotubes bound specifically within the nucleus. According to the correlation between the fluorescence intensity and the concentration of Alexa Fluor® 488, the amount of luminophores in one cell was determined to be $3.6 \times 10^{-10}$ mol (Supporting Information Figure S7). Considering ~4000 carboxyl groups existed in one nanotube and assuming each carboxyl group was linked with one Alexa Fluor® 488 molecule, $9.0 \times 10^{-23}$ mol of SWCNTs were

Figure 3 | (a) Bright-field, (b) ECL, and (c) overlapping images of MCF7 cells labeled with anti-CEA antibody/SWCNTs. The plasma membrane of each cell in the overlapping image (c) is lined in white. The exposure time for ECL imaging is 30 s.

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calculated. Therefore, the number of the luminophore-labeled SWCNTs in one cell was estimated to be \(\sim 56\).

Then, SWCNTs-antibody without the fluorescence tag was loaded into the cells for the ECL imaging. The bright-field and ECL images of the cells in the gel are recorded in Figures 4a and 4b. The overlapping image (Figure 4c) of the bright-field and ECL images illustrated that the ECL emission was concentrated near the nucleus of the cells. The pattern of ECL inside the cell was significantly different from the ECL distribution for the visualization membrane protein (Figure 3c). Coupled with the fluorescence characterization regarding the location of antibody-modified SWCNTs, the spatially resolved ECL emission reflected information about KDM1/LSD1 antigen in the nucleus. To further support the special recognition and ECL visualization of proteins in the cell nucleus, a control experiment was conducted by culturing the cells with pure SWCNTs without the modified antibody. From the ECL image (Supporting Information Figure S8), a weak ECL emission was distributed throughout the cell without specificity for any cellular compartment. Altogether, these phenomena indicated that the antibody-modified nanotubes could effectively bind with the target protein at a specific cellular compartment for the wireless electrochemical visualization inside the cells.

**Conclusion**

In summary, the electrochemical visualization of intracellular protein at single cells is realized using a wireless BPE-ECL approach. SWCNTs were employed as the sub-micron carrier to bring the antibody for the recognition of intracellular antigen. Meanwhile, the BPE phenomenon occurred at the SWCNTs to induce luminol ECL illumination to visualize the antigens. Our strategy successfully solves the problem commonly found in electroanalysis that requires contact with an electrode (or electrode array) with a cell; thus, it uncovers a field for single-cell electrochemical analysis. Moreover, since the ECL analysis yielded a near-zero background emission, this strategy is feasible for the quantitative measurement of intracellular proteins in single cells, thereby providing more information for biological studies.

**Supporting Information**

Supporting Information is available and includes the experimental procedures, characterization, fluorescence images, and more ECL images.

**Conflict of Interest**

The authors declare no conflict of interest.

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