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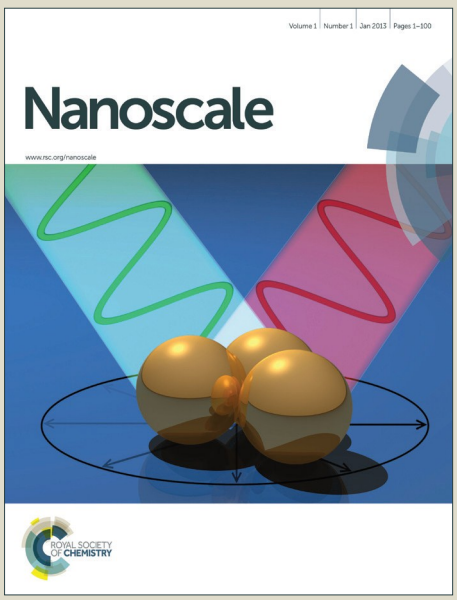

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Electro-triggering and electrochemical monitoring of dopamine exocytosis from a single cell by ultrathin Au nanowire electrodes based on Au nanowire

Received 00th September 2015,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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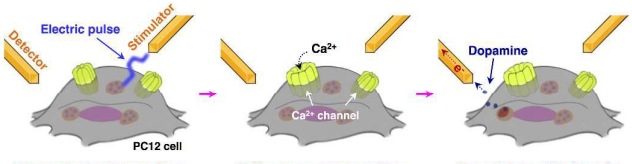
A sophisticated set of Au nanowire (NW) stimulator − Au NW detector system is developed for electrical cell stimulation and electrochemical analysis of subsequent exocytosis with very high spatial resolution. Dopamine release from a rat pheochromocytoma cell is more stimulated by a more negative voltage pulse. This system could help to improve the therapeutic efficacy of electrotherapies by providing valuable information on their healing mechanism.

Cell communication by delivering signaling molecules is essential for biological viability.[1,2] Malfunction in such communication may cause serious diseases including Parkinson's disease, Alzheimer's disease, cancer, and schizophrenia. While electrotherapies such as deep brain stimulation have been effectively employed to cure such diseases,[3,4] their healing mechanisms have not been fully elucidated yet.[5-7] Detailed single-cell level studies of the biological responses to electrical stimulation could help us to understand such mechanisms and improve therapeutic efficacy.[8-12]

The spatial precision of electrical cell stimulation has been improved by the technical advancement in miniaturizing electrodes. At an early stage, electric field was applied between two macroscopic electrodes, stimulating a population of cells lying between them. Reducing the size of electrodes down to a nm-size can enhance the precision of stimulation point and thus could optimize the efficiency of the stimulating electrodes.[13] Here, we employed ultrathin Au nanowire (NW) electrodes based on Au nanowire (NW) having a diameter of 100 ~ 200 nm to stimulate a rat pheochromocytoma (PC12) cell, generally used as a model in neuroscience,[14,15] with voltage pulses, and observed subsequent release of signaling molecules (dopamine) in real time by a detector composed of another Au NW electrode (Scheme 1). Precise control of the position of Au NW electrodes in nm scale helped us to find that the active release zones are sparsely distributed over the cell membrane. The ultrathin Au NW electrode based on Au NW having such high spatial precision could allow us to figure out in detail how each synapse of a single neuron that possesses many heterogeneous synapses responds differently to the electrical stimulation by detecting the dopamine exocytosis from each synapse.[16-19]

With this well-controlled experiment, we observed that the PC12 cell released dopamine more frequently when the applied voltage was set more negatively. Furthermore, we observed that such release was inhibited by Cd^{2+}, a Ca^{2+} channel blocker. This indicates that the opening of Ca^{2+} channels by electrical stimulation triggers the dopamine exocytosis. The sophisticated set of Au NW stimulator − Au NW detector system for the electrical cell stimulation and the detection of subsequent cellular responses would help to reveal the working mechanism of electrotherapies, widely employed to treat diverse diseases in cardiac, muscular, urinary as well as neural systems, leading to improved therapeutic efficacy and reduced side effects.

Ultrathin nanoscale stimulator and detector made of Au NWs were synthesized by using vapor transport method as we previously reported (see detail in Supporting Supplementary Information).[20] Although Au NWs are extremely thin with a diameter of 100 ~ 200 nm, their strong light scattering allows optical observation. A Au NW electrode was fabricated by



Scheme 1. Schematic illustration representing the electrical stimulation of a PC12 cell with a voltage pulse using a stimulating Au NW electrode (stimulator), leading to the opening of Ca^{2+} channels and the Ca^{2+} influx into the cell. The subsequent dopamine exocytosis is electrochemically detected by a recording Au NW electrode (detector).

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†Electronic Supplementary Information (ESI) available.
See DOI: 10.1039/x0xx00000x
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picking up one of the Au NWs vertically grown on a sapphire substrate with a macroscopic tungsten tip and completely insulating the tungsten part of the combined nanowire-tip with UV-curable polymer and nail varnish (see detail in Supporting Supplementary Information).[21,22] The scanning electron microscope (SEM) image of the Au NW attached on a tungsten tip is shown in Fig. 1a, which was taken before insulating the tungsten tip to avoid surface charging.

In this Au NW electrodes-based platform, each electrical stimulation or electrochemical recording part was connected to its own independently operating electrochemical workstation. The stimulating Au NW electrode (the right one in Fig. 1b-d) approaches a PC12 cell within a few μm by using a micromanipulator under optical monitoring with a saturated calomel electrode (SCE) put as a reference electrode to constitute 2-electrode system. We observed that the gap between the cell and the stimulating Au NW electrode did not significantly affect the electrically triggered exocytosis in the range from ~20 μm to ~5 μm (see Supplementary Information). Generally, 3-electrode system is preferred since it controls the potential application more accurately; therefore, the recording part was made of a 3-electrode system. When 3-electrode configuration was employed for the stimulation, however, the amperometric trace measured by a recording Au NW electrode was severely interfered. This interference was not observed when 2-electrode configuration was employed for stimulation. We also confirmed that the 2-electrode configuration did not significantly affect the electrochemical behavior of a Au NW electrode (see Supplementary Information, Fig. S1†). Electrical stimulation was done by applying voltage pulses of – 0.3, – 0.1, 0.1, and 0.3 V with 1 s pulse duration to the Au NW electrode, respectively. The range of pulse voltage was set not to exceed the onset of preoxidation of Au (ca. 0.35 V) and the onset of oxygen reduction reaction (ca. – 0.3 V, see Fig. S32†). Another Au NW electrode (the left one in Fig. 1b-d), working as an electrochemical recorder, approaches more closely to the same cell within 1 μm (Fig. 1b to c): as closely as possible to minimize the quantity of the released dopamine that significantly diffuses out and, consequently, is not detected by the recording Au NW electrode.[23] This recording Au NW electrode was used as a working electrode. Ag/AgCl electrode and Pt wire were put in the same solution as a reference and a counter electrode, respectively, to constitute a 3-electrode system (Fig. S1†). The monitoring of dopamine exocytosis was achieved by amperometry, measuring the current as a function of time. Since most part of PC12 cell surface is inactive for dopamine exocytosis and only several localized zones, active release zones, are in charge of dopamine exocytosis,[23] we sought out such zones by finely maneuvering the recording Au NW electrode (Fig. 1c and d).

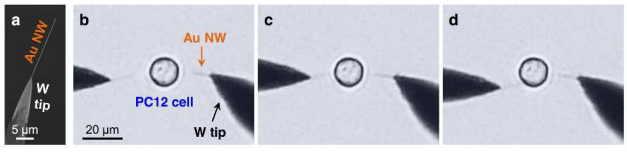

Fig. 1 (a) SEM image of a Au NW electrode before insulating the tungsten (W) tip. (b-d) Optical image showing the procedure for the electrical stimulation of a single PC12 cell using a Au NW electrode (right) and the simultaneous monitoring of dopamine exocytosis using another Au NW electrode (left). The stimulating electrode was placed near the cell and then the recording electrode was approached to the cell (b to c). The recording electrode can locate active release zones by looking over the surface of a cell (c and d).

Prior to triggering and monitoring dopamine exocytosis, we examined the electrochemical characteristics and the dopamine detection ability of Au NW electrodes. Cyclic voltammogram was obtained from a Au NW electrode in a phosphate buffer (pH 7.4) solution under 3-electrode configuration, in which single peaks for Au oxidation and reduction were observed at ~ 0.9 and ~ 0.4 V, respectively (Fig. 2a). This indicates that the Au NW is enclosed by a well-defined single-crystalline surface and in good contact with the tungsten tip. Tungsten oxidation, which has strong oxidation peaks at 0.2 and 0.6 V during anodic scan and 0.4 and 0.2 V during cathodic scan (Fig. S23†), was not observed, guaranteeing the complete insulation of the tungsten tip. Therefore, undesirable reaction or noise from tungsten surface was avoided.

Next, we examined whether Au NW electrodes were able to quantitatively detect dopamine. Dopamine oxidation was measured with increasing dopamine concentration from 0 to 250 μM (Fig. 2b). The concentration range was set around 190 μM from the following approximation; the dopamine concentration that a Au NW electrode would feel right after exocytosis was roughly calculated to be ~190 μM by simply considering that the electrode was positioned within the cubical space (volume: 1 μm^3) right in front of the release zone and all the released dopamine (average quantity at a single exocytotic event: ~190 zeptomole[24]) was in that space before significantly diffusing away from the release zone. Potential was scanned up to 0.7 V to avoid Au oxidation reaction that occurs at over 0.75 V. Dopamine started to be oxidized from 0.1 V and the oxidation current gradually increased up to 0.7 V (Fig. 2b), at which the current intensity linearly increased with increasing dopamine concentration (Fig. 2c). These results indicate that quantitative measurement of dopamine is possible with these Au NW electrodes (see Supplementary Information). For all of the following amperometric monitoring of dopamine exocytosis, recording Au NW electrodes were polarized at 0.7 V so that the dopamine arriving at the Au NW electrode could be immediately oxidized resulting in current spikes.

For the study of cellular exocytosis, it is practically

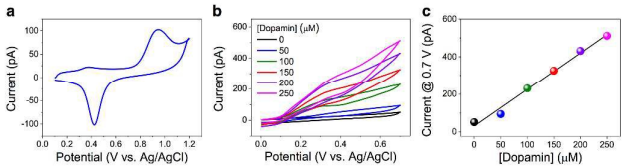

Fig. 2 Electrochemical characterization of a Au nanowire electrode. (a) Au oxidation (~ 0.9 V) and reduction (~ 0.4 V) measured in a 10 mM phosphate buffer solution (scan rate: 100 mV/s). No reaction related to tungsten is observed, indicating the complete insulation of the tungsten part. The onset potential of Au oxidation is ~ 0.75 V. (b) Dopamine oxidation measured in a 10 mM phosphate buffered saline solution with increasing dopamine concentration from 0 to 250 μM (scan rate: 50 mV/s). (c) Current intensity at 0.7 V in (b) as a function of dopamine concentration fitted with the linear equation.

important to accurately find the active release zones.[25] We applied voltage pulses to a PC12 cell using a stimulating Au NW electrode and observed dopamine release at several different sites of the cell with a recording Au NW electrode. We observed that while most of the monitored sites were inactive to dopamine exocytosis, activity was found at some zones,[23] where we analyzed the effects of electrical stimulation.

To investigate how the voltage of electric pulses affects dopamine exocytosis, we stimulated cells with different voltage pulses at an interval of 60 s. When we applied – 0.3, – 0.1, 0.1 and 0.3 V pulses in this order to a cell, an active release zone (zone A, Fig. 3a) had 2, 1, 1, and 0 current spikes, respectively. This means that the PC12 cell tended to release dopamine more frequently as the pulse voltage was set more negative. It might be, however, simply due to the accumulation of electrical stimulations or the depletion of intracellular dopamine as time passed. To clarify the effect of pulse voltage, pulses at four different voltages were applied to the same cell oppositely to the above (i.e., 0.3, 0.1, – 0.1 and – 0.3 V) and to another cell having an active release zone B in the same order as above (i.e., – 0.3, – 0.1, 0.1 and 0.3 V). In both cases, more negative pulse induced more dopamine signals (Fig. 3b and c). After we applied voltage pulses having the height from – 0.3 to 0.3 V or from 0.3 to – 0.3 V to five cells, we analyzed the total number of current peaks appeared. The most peaks appeared at – 0.3 V (n = 8) and the least peaks appeared at 0.3 V (n = 3) (Fig. 3d). Taken together, these results indicate that cells are likely to release dopamine more frequently by the application of more negative pulse regardless of the sequence of applied pulses, the accumulation of stimulation, the time-dependent depletion of intracellular dopamine, or cell-to-cell variation.

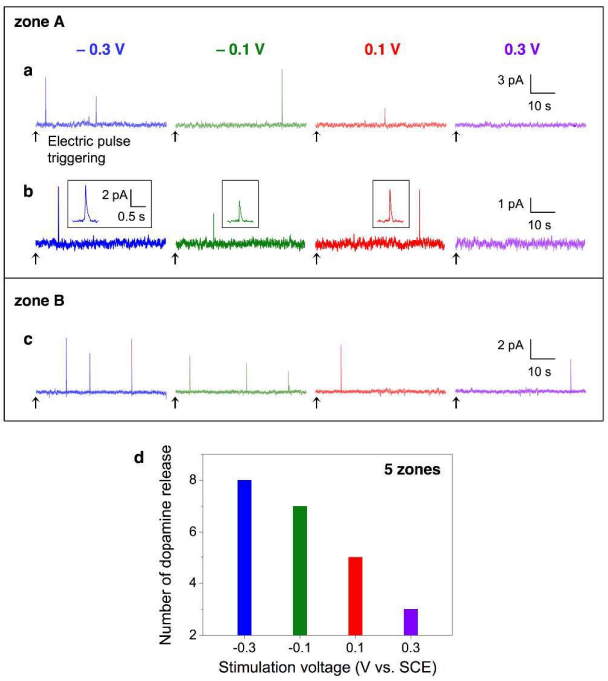

Fig. 3 (a) Amperometric traces measured from an active release zone of a PC12 cell (zone A) upon the application of – 0.3, – 0.1, 0.1, and 0.3 V pulses (pulse width : 1 s) in this order with an interval of 60 s. All traces were plotted right after the cease of voltage application (as indicated by arrows). Current spikes indicate dopamine exocytosis. (b) Amperometric traces measured from zone A upon the application of 0.3, 0.1, – 0.1, and – 0.3 V (pulse width: 1 s) in this order with an interval of 60 s. Amperometric spikes are magnified in the insets. (c) Amperometric traces measured from an active release zone of another PC12 cell (zone B) upon the application of – 0.3, – 0.1, 0.1, and 0.3 V (pulse width : 1 s) with an interval of 60 s. (d) The number of dopamine signals measured from 5 cells (5 zones) upon electrical stimulation of various pulse voltages. Each cell was stimulated by a set of 1-s voltage pulses sequenced from – 0.3 V to 0.3 V or from 0.3 V to – 0.3 V with an interval of 60 s. More negative voltage pulse induced more frequent dopamine release regardless of the applied pulse sequence or cell-to-cell variation.

For the detailed information of the amperometric spikes measured by Au NW electrodes, we calculated their average height, area (charge) and half-width from the spikes shown in Fig. 3, which are 4.46×10^{-12} A, 2.71×10^{-13} C, and 46 ms, respectively. These statistical information showed no significant tendency as a function of the stimulating voltage (see Supplementary Information). The spikes have similar shape as shown in the insets of Fig. 3b. From the charge value (Q), the number of released dopamine molecules (N) per exocytosis event was calculated to be 842,800 using Faraday's law (Q = nNF, where n is the number of electrons involved in the electrochemical reaction (2 for dopamine oxidation) and F is the Faraday's constant (96,485 C/mol)). This is in the same order of the reported values such as 114,300,[24] but higher than them to some extent. Compared to the reported amperometric spikes measured by carbon electrodes, the spikes we recorded have similar peak height and larger half-width, resulting in the higher charge value. This might be due to the different material characteristics of the electrode as well as different instrumental setup.

The electric stimulation via the Au NW electrode seems to trigger dopamine exocytosis by opening the Ca^{2+} channels. For exocytosis to occur, the concentration of intracellular Ca^{2+} should be elevated, which can be caused by the release of Ca^{2+} from intracellular Ca^{2+} stores or the influx of extracellular Ca^{2+}. Extracellular Ca^{2+} enters the cell when the Ca^{2+} channels located at cell membrane open. We examined if the electrical

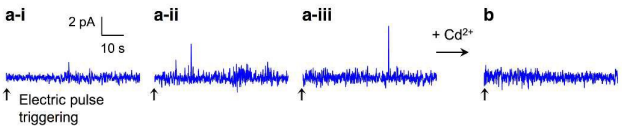

Fig. 4 Representative amperometric traces showing the effect of Ca^{2+} channel blocker (Cd^{2+}) on the electrically triggered dopamine release. Amperometric traces were measured from an active release zone of a PC12 cell (zone C) upon the application of – 0.3 V pulses (pulse width: 1 s) before (a-i,ii,iii) and after (b) adding Cd^{2+} into the extracellular solution. The concentration of Cd^{2+} in the extracellular solution was 100 μM. In the absence of Cd^{2+}, electric pulse was applied for three times and each pulse induced dopamine release within 60 s after the cease of voltage application (as indicated by arrows). In the presence of Cd^{2+}, no dopamine release was observed for 60 s after electrical stimulation. This indicates the electrical triggering of dopamine release is dependent on the opening of Ca^{2+} channel.

stimulation via Au NW electrodes lead to the opening of the Ca^{2+} channels of PC12 cells. First, we found an active release zone (zone C) where dopamine was released by each of three − 0.3 V pulses (Fig. 4a). Thereafter, Cd^{2+}, a Ca^{2+} channel blocker, was added to the extracellular solution[26] and the same cell was stimulated by a − 0.3 V pulse. Dopamine signal was no more observed at the zone C (Fig. 4b). The same result was obtained from three independent experiments, suggesting that dopamine exocytosis by electrical stimulation through Au NW electrodes is related to the opening of Ca^{2+} channels (Scheme 1). Considering that (1) Ca^{2+} channels are opened by the depolarization of cell membrane, (2) when external electrical field is applied to a cell, the side facing the negative electrode is transiently depolarized while the side facing the positive electrode is hyperpolarized,[27] (3) such effects of external electrical field on the membrane potential are reinforced as the distance between the electrode and the cell is reduced,[28] and (4) the stimulating Au NW electrode is positioned more closely to the cell than the counter electrode, it is conceived that the portion of depolarization over hyperpolarization is increased when the pulse voltage applied to the stimulating Au NW electrode gets more negative, which would result in more dopamine exocytosis.

Conclusions

In conclusion, we stimulated a single PC12 cell with voltage pulses and electrochemically monitored the subsequent dopamine exocytosis using a set of Au NW electrode − Au NW electrode. The ultrathin Au NW electrode based on Au NW with diameters of 100 ~ 200 nm can electrically stimulate a single cell and at the same time can monitor dopamine exocytosis at localized regions of cell surface where dopamine is actively released, enabling precise studies on cell communication. We observed that dopamine release was promoted as the pulse voltage was negatively tuned from 0.3 V to − 0.3 V and such exocytosis is attributed to the opening of Ca^{2+} channel by the electrical stimulation.

Au NW electrodes would establish new strategies for investigating the effects of electrical stimulation on diverse cellular phenomena as well as exocytosis. We expect that Au NW electrodes could contribute to improving the therapeutic efficacy of electrotherapies used for treating paralyzed muscles, cardiac arrhythmia or overactive bladder by providing more detailed and invaluable information on their working mechanism.

Acknowledgements

The work of B.K. was supported by the NRF of Korea funded by MSIP (NRF-2012M3A2A1051686, NRF-2013R1A2A2A01069073). The work of S.Y.L. was supported by the Intelligent Synthetic Biology Center through the Global Frontier Project (2011-0031963) of the Ministry of Science, ICT & Future Planning through the NRF of Korea.

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