SPATIAL RESOLUTION OF EXOCYTOSIS ACROSS A SINGLE CELL BY A MICROWELL-BASED INDIVIDUALLY ADDRESSABLE THIN FILM ULTRA-MICROELECTRODE ARRAY

Jun Wang1,2, Raphaël Trouillon1, Johan Dunevall2, Andrew G. Ewing1,2

1Department of Chemistry and Molecular Biology, University of Gothenburg, SWEDEN and
2Department of Chemical and Biological Engineering, Chalmers University of Technology, SWEDEN

ABSTRACT

We report the fabrication and characterization of microwell-based individually addressable ultra-microelectrode arrays (MEAs) and their application to spatially and temporally resolved detection of neurotransmitter release across a single pheochromocytoma (PC12) cell. The MEAs consist of sixteen 4-µm-width square microelectrodes, or twenty-five 3-µm-width square microelectrodes, or thirty-six 2-µm-width square microelectrodes. Each MEA is tightly defined in a 30×30 µm square area, which is further encased inside a 40×40 µm SU-8 microwell. We demonstrate the excellent stability and reproducibility of these microelectrodes by using cyclic voltammetry and we have performed recording of spatially resolved measurement of single cell exocytosis with multiple ultra-microelectrodes in 2-µm resolution.

KEYWORDS: Microwell-based ultra-microelectrode array, spatial and temporal resolution, single cell exocytosis

INTRODUCTION

The key dynamic event in neuronal communication is the release of transmitter molecules through the process called exocytosis. Amperometry utilizing carbon-fiber microelectrodes has provided significant quantitative and kinetic information to various cellular or molecular mechanisms of exocytosis. Carbon-fiber microelectrode arrays (MEAs) or thin film metal MEAs are the most common methods for single cell study, and can be used to provide insights about the spatial variability of biological events [1, 2]. One of the unique properties of MEA-based methods is that the spatial heterogeneity of these exocytotic events can be directly observed at the single cell level. For example, exocytotic activity has been found to be heterogeneous at the surface of a single cell [3], resulting in hotspots where neurotransmitters are released more frequently. This subcellular heterogeneity across a single cell thus led to the design of devices capable of resolving the spatial variation of exocytosis across a single cell. However, those carbon-fiber based MEAs are mostly used to collect vesicular release information from the apical pole of single cell. Conversely, in order to detect the cell heterogeneity from basal side of a single cell, Micro-Electro-Mechanical Systems (MEMS) techniques that involve the use of the of photolithography, thin film metal deposition, and reactive ion etching have been the most common methods used to fabricate individually addressable MEAs for single cell experiments. However, few papers have described MEAs with individual electrodes smaller than 5 µm, the typical size of the carbon fiber used for single cell analysis. Furthermore, most of these papers reported single cell trapping or detection at a single electrode. The development of MEAs with electrodes small enough to allow quantitative measurement of released molecules from exocytotic hot spots distributed across the surface of a single cell would be very attractive for amperometric measurements. We recently reported the fabrication of thin-film ultra-microelectrode (microelectrode size from 4 µm to 2 µm) arrays to image the exocytotic release of dopamine from cell clusters [4].

In this paper, we present the fabrication and characterization of 40 µm × 40 µm size microwells containing up to 36 closely packed individually addressable and small platinum microelectrodes. The microwell-based MEAs have compatible sizes with individual neuronal or neuron-like cells. Effective targeting and culture of a single cell in the microwell is achieved by combining cell-sized microwell trap and micropipette picking techniques. The surface of the microelectrodes in the MEA was coated with collagen IV to promote cell adhesion. Steady state voltammetry has been applied to study the activity of these microelectrodes. We also describe differential electrochemical detection of exocytosis at a single PC12 cells cultured on top of 8 microelectrodes. In this case, a spatial resolution for dynamic electrochemical measurements near 2 micrometers was achieved.

EXPERIMENTAL

MEAs were fabricated on a 4-inch Borofloat glass wafer by use of photolithography, thin-film metal deposition, and reactive-ion etching techniques. Figure 1a-f shows the schematic process for fabrication of individually addressable and insulated MEAs. Here we emphasize the fabrication of the microwell on top of the MEA. Glass wafer with 5-nm Ti/45-nm Pt MEAs was coated with SU-8 2035 and patterned on top of MEAs by UV lithography with a mask showing the microwell design. SU-8 on top of the MEAs was developed with SU-8 developer and formed a 40×40 µm size microwell (Figure 1g and h). To create the big sample well for cell culture on the wafer, a polydimethylsiloxane (PDMS) well was prepared and bonded on the top surface of the glass wafer. Electrical contact was achieved by using silver paste 4922N (Dupont) to connect the connection pad to the socket connectors (ELFA), then the MEAs were placed in an oven at 100°C overnight to ensure proper connection between the connection pads and the socket connectors. The final microwell-based MEA device is shown in Figure 1i.
Mouse collagen IV (BD Biosciences, Bedford, MA BD chemicals, stock solution 1 mg/mL) was diluted in sterile water to desired concentrations (1 µg/mL, except where specifically mentioned in the text). A 2 mL volume of this collagen IV solution was used to coat the 6-cm² surface area of the PDMS well and incubated for 8 h. The device was then washed three times with sterile PBS buffer.

Cells were then deposited by adding 2 mL of PC12 cell suspension (about 10⁴ cells/mL). After loading cells on the PDMS chamber, we used a micropipette to pick an individual cell and put it into the microwell. Then the device was placed in a sterile incubator for cell culture. For stimulated exocytosis experiments, PC12 cells were grown in the well on the MEAs for 1-2 days before the experiment and the cell media was replaced every day.

Cell experiments using microwell-based MEAs. Electrochemical recordings of exocytotic events from single PC12 cells were performed on an inverted microscope (IX71, Olympus) using a Triton™48-channel patch clamp amplifier (Tecella, Foothill Ranch, CA) placed in a Faraday cage, and connected in the two-electrode mode. The output was filtered at 2.2 kHz using a Bessel filter and digitized at 5 kHz. Before experiments, the cells were rinsed three times with physiological saline at 37°C. The experiments were performed in physiological saline at 37°C. A glass micropipette containing a high K⁺ (100 mM KCl) stimulating solution was positioned at ~20 µm from the PC12 cells on top of the MEA to stimulate exocytosis. Each cell was stimulated for 25 s with 20-psi pulses, every 40 s or 50 s through the micropipette coupled to a microinjection system (Picospritzer II, General Valve Corporation, Fairfield, NJ). A constant potential (800 mV) was applied to each ultra-microelectrode with respect to a single Ag/AgCl reference electrode placed in the cell bathing solution. All cell experiments were performed at 37±1°C.

RESULTS AND DISCUSSION

Optical images of the three kinds of microwell-based MEAs (i.e. containing 16, 25, and 36 ultra-microelectrodes) are shown in Figures 2a, b and c, respectively. The corresponding recessed square ultra-microelectrodes in the 16, 25, and 36 microelectrodes arrays have different sizes (4, 3, 2 µm, respectively). The whole MEA is tightly defined in a 30×30 µm square area, which is potentially useful to measure exocytosis across single cell or closely spaced clusters of single cells. Each type of MEA was further defined in a 40×40 µm SU-8 microwell, which can be used to trap a single cell on top of multiple electrodes. Figures 2d, e and f show examples of single cells successfully trapped in the microwells for different kinds of MEAs. Since single cells are targeted to electrodes without nearby extraneous cells, individual cell responses can be unambiguously recorded. Vigorous solution exchange can be carried out without displacing cells from the electrodes. The device can be cleaned and reused multiple times without significant degradation of performance.

The imaging ability of the electrochemical array is demonstrated in Figure 3. A single cell is trapped and cultured on MEAs containing 36 microelectrodes (Figure 3a). This single cell covered about 8 electrodes, each electrode was identified with a specific number, and these notations were conserved for the rest of the study (Figure 3b). Electrodes 1-8 are those covered by a single cell, and exocytotic events are observed at these electrodes upon stimulation (Figure 3c). Each current transient corresponds to the electrochemical oxidation of dopamine molecules released from a single intracellular vesicle at the cell surface. Noticeable in Figure 3c is the subcellular heterogeneity observed in single-cell exocytosis. The location of ‘hot’ release spots has been found to vary across the surface of a single cell. Here, the electrodes showing the highest release frequency are also the ones located at the places of cell covering all part of electrodes (electrodes 1, 2, 4, 5). Less release events were measured at electrodes 3 and 6 and no release events were detected by electrodes 7 and 8, which are only partially covered by the cell. This might demonstrate that incomplete cell adhesion over the electrode can affect the detection threshold.
CONCLUSION

We have fabricated microwell-based thin film MEAs that are applicable for spatially probing chemical changes in tight spaces, such as studying exocytosis from different regions of the cell surface. Optical microscopic characterization showed that these arrays were on the order of 30 µm and were geometrically well defined. An effective cell-targeting microwell-based MEA device has been developed suitable for high-throughput amperometric measurement of quantal exocytosis from individual cells without the need for microfluidic forces to be applied to the cell. A 6 by 6 MEA was used for simultaneous electrochemical monitoring of exocytotic events from different surface regions of a single PC12 cell. Subcellular heterogeneity in exocytosis was shown with 2 µm spatial resolution. These results show that microelectrode arrays are suitable for electrochemical imaging of fast exocytotic events at single cells. This device, with small MEA area (30×30 µm), is potentially useful to spatially measure exocytosis and drug effects across single cells or clusters of single cells. It is also suitable for analytical electrochemistry in cell culture and parallel recording from multiple cells at the single-cell level.

REFERENCES


CONTACT
*Andrew Ewing, andrew.e@chalmers.se