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Preferential cell attachment to nitrogen-doped diamond-like carbon (DLC:N) for the measurement of quantal exocytosis

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ABSTRACT

Electrochemical measurement of transmitter or hormone release from individual cells on microchips has applications both in basic science and drug screening. High-resolution measurement of quantal exocytosis requires the working electrode to be small (cell-sized) and located in immediate proximity to the cell. We examined the ability of candidate electrode materials to promote the attachment of two hormone-secreting cell types as a mechanism for targeting cells for to recording electrodes with high precision. We found that nitrogen-doped diamond-like carbon (DLC:N) promoted cell attachment relative to other materials tested in the rank order of DLC:N > In_2O_3/SnO_2 (ITO), Pt > Au. In addition, we found that treating candidate electrode materials with polylysine did not increase attachment of chromaffin cells to DLC:N, but promoted cell attachment to the other tested materials. We found that hormone-secreting cells did not attach readily to Teflon AF as a potential insulating material, and demonstrated that patterning of Teflon AF leads to selective cell targeting to DLC:N "docking sites". These results will guide the design of the next generation of biochips for automated and high-throughput measurement of quantal exocytosis.

1. Introduction

The development of high-throughput assays for probing the function of individual cells is an important research priority. Single-cell assays are particularly informative because they can capture the stimulus–response relationship much more precisely than assays from cell populations where cells often respond in an heterogeneous and asynchronous manner (see Ref. [1] for a review). A popular single-cell assay of transmitter release is carbon-fiber amperometry, whereby a carbon-fiber electrochemical electrode measures the current that accompanies oxidation of electroactive substances released from the cell (reviewed in Ref. [2]). Cells release transmitter in discrete packets, a process called "quantal exocytosis", as intracellular vesicles fuse with the cell membrane and release their contents. If the electrode is placed immediately adjacent to a cell, then spikes of current resulting from release from individual vesicles can be detected. The time course of these spikes in current gives detailed information about the process of quantal exocytosis, this has contributed greatly to our understanding of this important biological process [3]. Carbon-fiber amperometry is fairly labor-intensive, however, because it requires manually positioning microelectrodes to cells under a microscope.

Our group and others are developing microchip devices to assay quantal exocytosis that offer the potential for high throughput in order to increase the pace of basic research and enable screening of drugs that affect exocytosis [4–9]. In order to resolve quantal exocytosis, the size of the working area of the electrode must be small (~cell sized) and the cells must be positioned immediately adjacent to the working electrochemical electrode. Various approaches based on microfluidics have been used to target cells to specified regions on a microchip ([8,10–12] and our own unpublished results), but this form of positioning may introduce forces on the cell membrane that can affect exocytosis. Approaches based on micropatterning cell-attachment molecules have also been used to

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pattern cells on microchips, but these methods are not always appropriate for aligning cells in registry with electrodes (e.g., "microcontact printing" [13]) or may passivate electrodes (e.g., self-assembled monolayers on gold [14]).

We are pursuing a material-based "self alignment" approach with the goal that cells attach to electrochemical electrodes following some time in culture, yet adhere much less readily to the material used to insulate inactive areas of the chip. Cell attachment to artificial substrates has been extensively studied (see Ref. [15] for a review), but there are significant differences in cell-attachment properties among different cell types and culture conditions. Therefore, it is essential to study cell attachment with the neuroendocrine cell types and culture conditions that are specific for our exocytosis application. We studied attachment of primary bovine adrenal chromaffin cells as the most widely used cell type for electrochemical studies of quantal exocytosis as well as the murine INS-1 insulin-secreting cell line. The cell types were chosen to be complementary in their origin (primary versus propagated cell line), source animal (cattle versus mouse) and secreted hormones (catecholamines versus insulin). We chose nitrogen-doped diamond-like carbon (DLC:N), In_2O_3/SnO_2 (ITO), platinum and gold, as potential electrochemical electrode materials, since each of these materials have recently been used to measure quantal exocytosis [4,5,16,17]. We also studied cell attachment to Teflon AF as an excellent insulating material to prevent cell attachment to inactive areas of the chip so that electrochemical signals only originate from the individual cell bound to each of the electrode "docking sites".

2. Materials and methods

2.1. Deposition of potential electrode materials

All conductive materials were deposited using a magnetron sputtering system (ATC2000, AJA International Inc., North Scituate, MA, USA). Microscope glass slides (25 mm × 75 mm), purchased from Fisher Scientific (Waltham, MA, USA) were used as substrates for all depositions. The slides were cleaned by soaking in a 3:1 mixture of sulphuric acid and hydrogen peroxide (piranha solution) for 10 min at room temperature followed by rinsing in purified water (Millipore, Billerica, MA) and then dried with a nitrogen stream. The slides were then taped onto a silicon wafer for deposition by magnetron sputtering. Prior to film deposition, the substrates were also sputter cleaned by biasing the substrate holder for about 1–2 min at a pressure of 4 m Torr and a RF power of 40 W. The chamber base pressure was about 5×10^{-7} Torr. Gas flow was regulated by mass-flow controllers.

ITO was deposited using an ITO target (In_2O_3/SnO_2, with 10% SnO_2 by weight, 75 mm diameter, 3 mm thick, 99.99% purity, Williams Advanced Materials Inc.). An RF power supply was used at a radio frequency of 13.56 MHz and a power of 180 W. The 20 sccm argon gas flow was regulated by a mass-flow controller and maintained at a working pressure of 4 m Torr. The substrate temperature was maintained at 50 °C and the deposition time was 20 min to result in a 100 nm-thick ITO film.

DLC:N films were deposited on top of ITO films to obtain the surface properties of DLC:N together with the high conductivity and transparency of the underlying ITO. The nitrogen is incorporated in DLC to reduce the ohmic resistance and increase its suitability for use as an electrochemical electrode. A graphite sputter target (Williams Advanced Materials Inc., Buffalo, NY, USA) with 99.99% purity, 75 mm in diameter and 3 mm thick was the carbon source. Sputtering of the two films was sequential without breaking the vacuum using a multi-target source with independent power supplies. For depositing DLC, the power supply was switched to a DC source of 400 W while maintaining the same temperature of 50 °C. Gas flow rates were 15 sccm for Ar and 5 sccm for N_2 and a pressure of 2 m Torr was maintained for the deposition of DLC:N. Deposition time of 15 min resulted in a 25 nm-thick DLC:N film. The thickness of the films was measured using a profiler (Alpha step 200, Tencor, San Jose, CA, USA).

In order to test the nitrogen content of our DLC:N films we deposited films of 100 nm thickness both on glass and Si substrates to ensure that the measurement was independent of the substrate used. The nitrogen and carbon contents were measured using SEM–EDS (scanning electron microscope–energy dispersive spectroscopy). Measurements were compared with standards with predetermined elemental content. The measurements were repeated three or more times on each substrate. The nitrogen content of the film was found to be 30 At.%.

Platinum of thickness 100 nm was deposited by supplying an RF power of 90 W to a platinum target. The argon flow rate was 20 sccm under chamber pressure of 4 m Torr and the deposition was carried out at ambient temperature. Gold deposition requires an adhesion layer of titanium. This base layer of 50 nm was deposited by supplying RF power of 100 W to a titanium target, with an argon flow rate of 30 sccm, a chamber pressure of 4 m Torr and the deposition was carried out at ambient temperature. Gold deposition was done without breaking the vacuum at the same chamber pressure, substrate temperature and argon flow rate by switching the RF power supply of 100 W to the gold target. The thickness of the gold film was 100 nm.

2.2. Deposition and patterning of Teflon AF films

Substrates were prepared by spin coating 5% FSM 660 (3M, St. Paul, MN, USA) as an adhesion layer at 3500 rpm for 30 s on glass slides followed by baking at 100 °C for 10 min. 2% Teflon AF (DuPont, Wilmington, DE, USA) in FC-75 (3 M) solution was then spun on it at 3000 rpm for 30 s followed by baking in three steps: 115 °C for 15 min, 230 °C for 15 min and 300 °C for 1 h.

For patterning Teflon AF, coated substrates were spin coated at 3000 rpm with S 1813 (Rohm and Haas, USA) photoresist for 30 s followed by a bake of 1 min. This process was repeated another time to cover any pinholes that remained on the substrate after the first coat of photoresist. Transparency masks (Output City, OR, USA) were used to pattern the Teflon-coated substrate using mask alligner (OAI Model 200 IR) with UV exposure of 14 s. The photoresist was developed with MF 321 (Rohm and Haas, USA) developer for 1 min 40 s, washed with DI water and blow dried with air to remove the moisture. The openings on this patterned substrate were created using PECVD (Precision 5000 mark II Applied materials) etch. It was etched using a flow rate of 40 sccm for CF_4 and 30 sccm for CHF_3 and creating a plasma at 1000 W and 250 m Torr. The etching time depended on the thickness of the Teflon AF film. For a 2% film the average thickness was 250 nm. The final step was to perform lift-off of S1813 photoresist using acetone during sonication for ~1 min. The device obtained was washed with ethanol and DI water and blow dried.

2.3. Substrate and gasket processing

All substrates were taken out of a storage desiccator and cut into 4 pieces (25 mm × 18 mm) using a diamond knife. Each sample was rinsed meticulously in steps of acetone, 90% ethanol and 18.2 MΩ Millipore water, and then blown dry. For polylysine coating, 5 mg poly-D-lysine hydrobromide (Sigma, St. Louis, MO, USA) was dissolved in 25 ml of purified water. Substrates to be coated were dipped in poly-D-lysine (PDL) solution for 20–30 min before being washed with purified water on a rocking shaker set at 100 rocks/min for 5 min. Further washing was done on a 3D-rotator set at 60 rpm for 2 min. The substrates were left to dry in the laminar flow hood.

Gaskets were made of poly(dimethyl siloxane) (PDMS, Dow Corning Corp., Midland, MI, USA). The curing agent was mixed with the monomer in a ratio of 1:3, degassed in a vacuum to remove bubbles, and poured onto a Petri dish. The mixture was then baked in an oven at 60 °C for 45 min to make a ~3 mm–thick slab. The slab was cut into gaskets or wells by using a pair of hollow punches with diameters of 5 and 12 mm to yield several gaskets with an opening diameter of 5 mm. Samples and PDMS gaskets were sterilized with UV light for 1 h. Two gaskets are sealed to each sample using vacuum grease (Dow Corning) applied with a cotton swab.

2.4. Contact angle measurements

The sessile drop method was used for contact angle analysis using deionized water. The sample was mounted on an X–Y stage illuminated by a lamp. A droplet of deionized water (5 μl, measured using a pipette) was formed at the end of the pipette tip and lowered onto the surface of the sample, and the pipette withdrawn when the drop detached. Images were recorded by a digital video camera (Sony DFW SX-900). The images were analyzed using NIH ImageJ software. Three different areas were measured for each sample. All measurements were performed at ambient temperature.

2.5. Cell isolation and culture

2.5.1. Bovine chromaffin cell culture

Chromaffin cells were harvested from bovine adrenal glands as recently described [18]. Following purification, the cell density was measured using a hemocytometer and the cell suspension was then diluted to a final concentration of 10^6 cells/ml in culture medium (Dulbecco's modified eagles medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin). The cell suspension was maintained in a plastic centrifuge tube on an ice bath until the substrates were ready for plating. Cells were mixed with a pipette before plating onto the substrates within several hours after completing the isolation.

2.5.2. INS-1 cell line culture

The INS-1 cell line was established from cells isolated from an X-ray-induced rat transplantable insulinoma. These cells were a kind gift from C. Wollheim, University of Geneva, Switzerland, and were maintained as described in Ref. [19]. In short, these cells were maintained in culture media consisting of RPMI 1640 medium