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Encapsulated Electrodes for Microchip Devices: Microarrays and Platinized Electrodes for Signal Enhancement

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Abstract

In this paper, we present two new methodologies of improving the performance of microchip-based electrochemical detection in microfluidic devices. The first part describes the fabrication and characterization of epoxy-embedded gold microelectrode arrays that are evenly spaced and easily modified. Electrodepositions using a gold plating solution can be performed on the electrodes to result in a 3-dimensional pillar array that, when used with microchip-based flow injection analysis, leads to an 8-fold increase in signal (when compared to a single electrode), with the limit of detection (LOD) for catechol being 4 nM. For detecting analytically challenging molecules such as nitric oxide (NO), platinization of electrodes is commonly used to increase the sensitivity. It is shown here that microchip devices containing either the pillar arrays or more traditional glassy carbon electrodes can be modified with platinum black for NO detection. In the case of using glassy carbon electrodes for NO detection, integration of the resulting platinized electrode with microchip-based flow analysis resulted in a 10 times signal increase relative to use of a bare glassy carbon electrode. In addition, it is demonstrated that these electrodes can be coated with Nafion to impart selectivity towards NO over interfering species such as nitrite. The LOD for NO when using the platinum black/Nafion-coated glassy carbon electrode was 9 nM. These electrodes can also be embedded in a polystyrene substrate, with the applicability of these sensitive and selective electrodes being demonstrated by monitoring the ATP-mediated release of NO from endothelial cells immobilized in a microfluidic network without any adhesion factor.

Introduction

Microchip-based systems have gained much attention as analytical tools due to their ability to use small sample volumes [1], integrate multiple processes [2-4], and perform fast/high-throughput analysis [5, 6]. The small channel dimensions and sample volumes dictate that the use of a sensitive detection technique. The most popular detection mode is laser induced fluorescence (LIF), which can achieve low limits of detection (nM is routine) [7, 8]. Disadvantages of the LIF approach include the requirement for derivatization with a fluorophore for most analytes and the fact that only a select number of wavelengths can be used for excitation. Another major detection technique that has been utilized for microchip devices is electrochemistry. Many biologically significant compounds (such as catecholamine neuro-transmitters and nitric oxide) can be detected sensitively and selectively without derivatization so that close to real-time analysis is possible [9, 10]. While there are numerous advantages to electrochemical detection in microfluidic devices, in general, the limit of detection (LOD) is not as low as when LIF detection is utilized. To address this limitation, we have recently introduced the use of epoxy-embedded single electrodes that can be made in a 3-dimensional manner so that it protrudes into the

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microfluidic network. With this approach, the LOD for microchip-based flow injection analysis of catechol was found to be 20 nM [11].

One possible way to further increase the sensitivity of microchip-based electrochemical analysis is through the use of an electrode array. Various electrode microarrays have been implemented into microfluidic networks. The first category is known as dual arrays, which are composed of two comb-type electrode arrangement adjacent to one another and individually addressed [12-14]. In this microarray, one electrode (generator) is held at an oxidative potential and the second electrode (collector) is held at a reductive potential [15]. One of the attractive aspects of these dual arrays is their ability to enhance the redox currents for the reversible species due to redox cycling occurring between the adjacent electrodes. The other type of microarray is a band of electrodes that are optimally spaced and held at the same potential. The main advantage of these arrays, as shown in several papers from Amatore's group, is that signal is enhanced relative to a single electrode of the same overall electrode size due to fresh analyte diffusing to each successive electrode [16-19]. This concept has been demonstrated by our group for in-channel detection, after an electrophoretic separation, using an array of carbon ink electrodes [3]. Others have utilized sputtered, patterned (*via* photolithography) 3-dimensional microarrays for post-channel detection (outside of the fluidic network) with microchip electrophoresis [20] and multiplexed, protein sensor arrays (not involving microfluidics) [21]. One of the goals of this work was to develop 3-dimensional electrode arrays that can easily be integrated within a fluidic network.

Another manner in which microelectrodes can be modified for signal enhancement is through platinization, which is a process in which platinum black (Pt-black) particles are electrochemically deposited on the electrode surface, resulting in an increased electrode roughness and active surface area [22-24]. Several different groups have demonstrated the use of platinum black microelectrodes to increase the electrode surface area and act as a catalyst to increase the electron transfer rate, with the resulting signal being increased up to 10 times for the detection of nitric oxide (NO), as compared to the use of unmodified electrodes [25-27].

In this work, we explore the use of microarrays and platinization for signal enhancement in microfluidic devices. We first demonstrate a methodology of fabricating epoxy-embedded gold microelectrode arrays that are evenly spaced, easily modified, polishable, and result in a significant signal enhancement, as compared to a single electrode, when used with microchip-based flow analysis. Electrodepositions using a gold plating solution can be performed on the embedded array to result in a 3-dimensional electrode array that, when used with microchip-based flow injection analysis, leads to a LOD of 4 nM for catechol. For sensitive and selective detection of NO, commonly used glassy carbon electrodes were modified with Pt-black and coated with Nafion (for nitrite exclusion), with the resulting signal for NO detection being enhanced 10 times compared to the use of an un-modified electrode. Finally, these electrodes were embedded in polystyrene, with this substrate enabling the immobilization of endothelial cells without an adhesion factor. The resulting device was used to directly monitor the ATP-mediated release of NO from an endothelial cell layer that was immobilized in the microfluidic network.

Materials and Methods

Materials

The following chemicals and materials were used as received: Nano SU-8 developer, SU-8 50 (Microchem, Newton, MA, USA); catechol, potassium dicyanoaurate (I), sodium carbonate, sodium phosphate monobasic, chloroplatinic acid hydrate, lead (II) acetate

trihydrate, Hanks balanced salt solution, and potassium nitrate (Sigma Aldrich, St. Louis, MO, USA); Armstrong C-7 resin, Activator A, and Sylgard 184 (Ellsworth Adhesives, Germantown, WI, USA); 1 mm glassy carbon rod and gold gauze (100 mesh woven from 0.064 mm dia wire, Alfa Aesar, Ward Hill, MA, USA); soldering wire and heat shrink tubes (Radioshack); isopropanol and acetone (Fisher Scientific, Springfield, NJ, USA); colloidal silver (Ted Pella, Redding, CA, USA); electrode polishing pads (Allied High Tech Products, Inc., Rancho Dominguez, CA, USA); disposable aluminum dishes (Sigma Aldrich, St. Louis, MO, USA); Diethyl NONOate (Cayman Chemical, Ann Arbor, MI, USA); Nitric oxide (NO) tank (99.5%) (Airgas Inc., Radnor, PA, USA); polystyrene (PS) powder (Goodfellow Cambridge Ltd., Huntingdon, England).

Fabrication

The fabrication of single epoxy-embedded electrodes has been previously reported [11]. A similar procedure was used here, with the fabrication of the epoxy-embedded gold array electrode base, a grid with 100 μm mesh woven with 65 μm diameter wire was used. The gold grid (4 wires in width) was cut with a razor blade to the desired length (5 mm) and affixed (soldered or connected with colloidal silver) to a copper extending wire to provide the electrical connection. Heat shrink tubing was used to insulate the connection. The electrode was inserted into a 70 mL disposable aluminum dish (6.7 cm in diameter and 1.6 cm in depth) that had a designated hole punched with a 20 gauge Luer stub adapter where the electrode sits. Following the assembly of the mold and electrode, a thoroughly mixed combination of 57.5 g Armstrong C-7 adhesive (resin) and 4.8 mL Armstrong Activator A was poured into the mold and left to cure overnight. The same procedure was used for polystyrene bases with glassy carbon electrodes (1 mm diameter, 7 mm in length glassy carbon rod), except polystyrene powder was poured around the electrode mold and melted at 270°C (on a hot plate) [28]. The aluminum mold was removed and either the epoxy or polystyrene was shaped by wet polishing, as previously reported [28].

Microchip-based Flow Injection Analysis

Negative masters for the PDMS structures were fabricated as previously described [7, 9] using SU-8 50 photoresist for PDMS-based flow injection channels. The structure heights were measured with a profilometer (Dektak³ ST, Veeco Instruments, Woodbury, NY, USA). The setup used for microchip-based flow injection analysis has been previously reported [29-31]. In studies involving electrode characterization, a straight 20:1 PDMS channel (200 μm width, 100 μm height, and 3 cm length) was used. Cell studies utilized a 20:1 PDMS channel (700 μm width, 350 μm height, 1.5 cm in length). In all cases, the PDMS flow channels were reversibly sealed on the epoxy surface over the electrode. A hole punch was used to make the outlet reservoir and the inlet hole was created using a 20 gauge luer stub adapter (Becton Dickinson and Co., Sparks, MD). For all straight channel studies, a buffer flow stream was continuously pumped at 3.0 $\mu\text{L}/\text{min}$ to the flow channel *via* a 500 μL syringe (SGE Analytical Science) and a syringe pump (Harvard 11 Plus, Harvard Apparatus, Holliston, MA, USA). The syringe was connected to a 75 μm i.d. capillary tubing using a finger tight PEEK fitting and a luer adapter (Upchurch Scientific, Oak Harbor, WA, USA). The same connectors and a 75 μm i.d. capillary fitted with a 794 μm o.d. microtight sleeve (Upchurch Scientific, Oak Harbor, WA, USA) were used to transition from a 4-port rotary injection valve (Vici Rotor, Valco Instruments, Houston, TX) to the microchip. The four-port injection valve enabled reproducible 200 nL injections to the PDMS-based flow channel. Amperometric detection was done with a 3-electrode system using a CH Instruments potentiostat (Austin, TX, USA). The working electrode was the gold array, gold pillar array, gold pillar array/Pt-black, or glassy carbon/Pt-black electrode. A platinum wire was used as the auxiliary electrode and Ag/AgCl was the reference electrode; both were

placed in the outlet reservoir. For both catechol and NO a working electrode potential of +0.9 V (vs. Ag/AgCl) was utilized.

For flow studies where the Pt-black deposition process was optimized, NO stock solutions were made daily by adding diethylamine NONOate (DEANO) to deoxygenated buffer (10 mM phosphate, 7.4 pH) under a sealed volumetric flask that had been previously evacuated of oxygen. DEANO (2 min $\frac{1}{2}$ half-life) was allowed to completely dissociate for 10 minutes at 37°C [32]. For all other NO studies, a NO standard stock solution (1.9 mM) was prepared by first deoxygenating Hanks buffer salt solution with Ar for 30 min, then saturating the solution with pure NO gas (99.5%) for 30 min [27]. The NO gas was purified before use by passing it through a column packed with KOH pellets to remove trace NO degradation products. Individual samples were made in deoxygenated volumetric flasks (sealed with scuba seal) and deoxygenated buffer.

Electrode Modification

Electrodepositions were used to create gold pillar electrodes that protrude into the channel. Electrodepositions were carried out by filling a 200 μ L PDMS reservoir with 50 mM dicyanoaurate (I) and 0.1 M Na₂CO₃ solution and applying a potential of -1.2 V to the gold electrode array (vs. Ag/AgCl). The current produced during the electrodeposition was monitored using a CH Instruments potentiostat. For all detection arrays, depositions were allowed for 30 minutes to produce 20 μ m pillars (in height). For the preparation of platinum black electrodes (gold pillar array and glassy carbon), a PDMS reservoir was filled with 3.5% chloroplatinic acid (w/v) and 0.005% lead (II) acetate trihydrate. Electrode plating was achieved by cycling the potential from +0.6 to 0.35 V (vs Ag/AgCl) at a scan rate of 20 mV/s using CH instruments potentiostat. Nafion coatings over the Pt-black electrodes were accomplished by sealing a large (1 mm wide $\times$ 350 μ m tall $\times$ 2 mm in length) PDMS flow channel over the electrode. A 0.5% Nafion solution (made from a 5% solution of commercially available Nafion, Sigma Aldrich, St. Louis, MO) was pipetted into the reservoirs and left to dry on the electrodes overnight.

Endothelial cell culture and channel immobilization

Bovine pulmonary artery endothelial cells (bPAECs) were purchased frozen (Lonza, Walkersville, MD, USA). The cell vial was thawed to room temperature and added to a T-25 tissue culture flask containing 5 mL of endothelial growth media (EGM) that had been warmed to 37 °C. The EGM consists of a low glucose (5.5 mM) Dulbecco's Modified Eagles Medium (DMEM, MIDSCI, St. Louis, MO, USA) supplemented with 2.5% v/v adult bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 7.5% fetal bovine serum (Lonza, Walkersville, MD, USA), penicillin, streptomycin, and amphotericin B (MIDSCI, St. Louis, MO USA). The bPAECs were allowed to grow in a humidified incubator at 37 °C and 5% CO₂ until they were determined as confluent by optical microscopy. Media was changed the day after plating and then every 2 days thereafter. The bPAECs were subcultured when the cells reached >80% confluence as visualized by optical microscopy.

To prepare PDMS channels and the polystyrene device for cell immobilization, both were plasma treated for one 1 minute in a plasma cleaner (PDC-32G, Harrick Plasma, Ithaca, NY). The electrode in the PS base was covered with PDMS to prevent any damage to the Nafion membrane. After treatment, the PDMS channel was irreversibly sealed over the GC/Pt-black/0.5% Nafion electrode. In order to immobilize bPAECs into the channels of the microfluidic device, a confluent T-25 flask of bPAECs was washed with 5 mL of HEPES and then treated with 2 mL of 0.25% trypsin/EDTA, which was then neutralized with 5 mL of neutralizing trypsin reagent. The cell suspension was removed from the flask and centrifuged at 1500 g for 5 min. The supernatant was removed and the pellet was re-

suspended in 400 μL of equilibrated media. From this concentrated cell solution, 100 μL was introduced (via the outlet reservoir) to the plasma treated PDMS flow channel. The microchip was incubated for 2 hours at 37 $^{\circ}\text{C}$ and 5% CO_2 humidity, after which time the cells adhered to the microchip.

For analyzing NO release from the cells, the microchip-based flow injection analysis system was interfaced via a capillary to the microchip device (as described above), using a HBSS buffer flow rate of 3 $\mu\text{L}/\text{min}$. Detection of NO from these cells was accomplished by injecting a plug of ATP stimulant (100 μM) through the buffer channel while continuously monitoring the current produced at the GC/Pt-black/0.5% Nafion electrode. For imaging of the cells, acridine orange, a cell nucleus stain, was used to visualize the cells after the flow analysis studies were complete. A 100 $\mu\text{g}/\text{mL}$ of acridine orange solution was pipetted into the outlet reservoir and aspirated through the microchannel, after which the chip was incubated for 5 minutes [33]. Later, the dye was washed by pulling HBSS buffer through the channel, followed by imaging of the cells on a fluorescent microscope.

Imaging

Color images, with the exception of the image shown in Figure 1C, were captured with an upright Olympus, BX51 microscope equipped with an Infinity3 camera (Hirschfeld Instruments, Inc). Black and white non-fluorescent images were obtained from a stereoscope (Olympus SXZ12) operating in bright field mode using a Sony 3CCD color camera (Leeds Precision Instruments, Minneapolis, MN, USA). Fluorescent imaging was accomplished with fluorescein (Sigma Aldrich, St. Louis, MO) and an upright fluorescence microscope (Olympus EX 60) equipped with a 100 W Hg Arc lamp and a cooled 12-bit monochrome Qicam Fast digital CCD camera (QImaging, Montreal, Canada). Images were captured with StreamPix Digital Video Recording software (Norpix, Montreal, Canada) and Image Pro express software (Media Cybernetics, Silver Spring, MD) was used to measure channel dimensions. Confocal images of the gold pillar array electrodes were imaged with a Keyence VK-9170 Violet Laser Scanning Confocal Microscope (Keyence Corp). The accompanying VK Viewer software allowed collection of color 3-D image data over the entire electrode surface as well as a line scan to obtain a profile of the entire electrode. The SEM Image (Figure 1E) was obtained from an FEI Inspect F50 SEM with Schottky Field Emission as electron source. The secondary electron (SE) detector mode was used. The electrode was encapsulated in the epoxy base (10 mm dia.) and sputtered with a thin layer of gold and placed on the SEM stage for imaging.

Results and discussion

In this work, we introduce two new methodologies of performing electrochemical detection within microchip devices that enables nM detection limits for microchip-based flow injection analysis. One is based on a microelectrode array and the other on platinized electrodes. Previously, our lab and others have used thin-layer electrodes on glass substrates that were fabricated with sputter coating and photolithography [29, 34-36]. While thin layer electrodes offer many advantages and are relatively well-established, their fabrication is costly, time consuming, the electrodes are of relatively small surface area, and when used for detecting discrete plugs in flow analysis or electrophoretic separations, the LOD is typically in the low μM or high nM regime. As an alternative approach, we have recently presented the development of epoxy-embedded electrodes that offer many advantages over the use of thin-layer electrodes in that any electrode material, with any dimension and/or configuration, can be embedded into an epoxy base and polished for use in either flow injection analysis or microchip-based electrophoresis [11, 37]. One of the biggest advantages of epoxy-embedded electrodes is that the connections come from the bottom of the epoxy. This allows for simple reservoir-based electrodepositions to create 3-dimensional

pillar electrodes. In our previous work, we showed the creation, integration, and characterization of a single pillar electrode ($\sim 30\ \mu\text{m}$ in height) for microfluidic devices [11]. Analogs to LIF detection where the laser spot is directed through the entire channel, pillar electrodes access more molecules (as compared to planar electrodes) by protruding into the fluidic channel without increasing band broadening from increasing the detection zone size. While impressive detection limits (20 nM for catechol) were reached with the single pillar electrode; lower limits of detection are desired for physiologically relevant cellular release studies. Earlier work by Amatore's group has shown that an optimally spaced array of electrodes, as opposed to use of a large single electrode where analyte may become depleted at the electrode surface, can offer significant signal enhancement by allowing fresh analyte to diffuse to the surface of each electrode in the array [16-18]. In this work, we show the development and characterization of an electrode array that can also be made in 3-dimensions for significant signal enhancement. The other approach that was explored for signal enhancement involves platinization with Pt/black. We show that either the gold array or more traditional glassy carbon electrodes can be modified with Pt/black and used for the sensitive and selective detection of NO, including the direct detection of NO release from endothelial cells.

Fabrication and characterization of gold array electrodes—Previously, we utilized a single wire (25 μm diameter) and electrodeposition to create a 3-dimensional pillar electrode [11]. To build upon this, an array approach with multiple, evenly spaced electrodes that could be held at the same potential was desired. However, the challenging nature of physically aligning multiple 25 μm wires in this manner led to the use of a mesh (grid) electrode material to fabricate arrays of desired number of electrodes with even spacing. These mesh electrode metals are commonly used as counter electrodes in macro flow cells and for SEM support specimen grids. The mesh is industrially made with even grid spacing, varying wire diameters, and in gold, platinum, and other metal composition. For these studies, a gold 100 grid mesh with a 65 μm wire diameter and 200 μm spacing between each electrode was used for detection electrodes. The length of the array can vary, since the 25 $\times$ 25 mm mesh (shown in Figure 1A) is physically cut with a razor blade to desired electrode length and affixed to an extending wire. The number of electrodes in the array was set to 4 electrodes (Figure 1B and C). Later, the electrodes were assembled into an epoxy base mold and a mixture of epoxy was poured and left to cure overnight. The newly epoxy-embedded array electrodes were shaped and finely polished by wet polishing. Figure 1B shows a top-view micrograph of the epoxy-embedded array with 4 electrodes.

Electrodepositions can be used to modify an electrode surface to become more sensitive or selective toward an electro-active species. To create an array of 3-dimensional pillar electrodes; electrodepositions were carried out with a dicyanoaurate solution by applying a reductive potential to the gold electrode array [38]. The deposition time was optimized to 30 minutes to result in arrays that had $\sim 20\ \mu\text{m}$ height pillars, with each pillar being $\sim 85\ \mu\text{m}$ diameter (initial wire diameter 65 μm). The confocal image in Figure 1D shows the uniformity of the depositions and the spacing of the array. The complex and relatively rough structure of the high surface area pillar is captured by the SEM image shown in Figure 1E. The 20 μm pillar array did not cause leakage or flow resistance when sealed within a PDMS channel (200 μm width $\times$ 100 μm height).

Earlier work has demonstrated that an array of electrodes is a possible way to increase signal in microfluidics [3, 16]. When integrating these microarray electrodes with microchip-based flow injection analysis a similar performance was seen, with a 4-fold increase in signal for a 100 μM catechol sample when using a flat (polished) array of 4 evenly spaced (200 μm space) gold electrodes (60 μm diameter), as compared to use of a single 60 μm diameter electrode (Figure 2). An even greater 8-fold increase in signal was observed when using the

3-dimensional pillar array (20 μm height), as compared to the single electrode. It should be noted that the somewhat tailing peak shape seen in the data is typical of flow injection analysis [39]. Calibration curves for 1-100 μM catechol were determined for both arrays (flat and pillar). The pillar array offered higher sensitivity (0.091 nA/ μM) than the flat array (0.053 nA/ μM), while both had a linear correlation with the concentration (r^2 of 0.999 and 0.993 respectively). When utilized with microchip-based flow injection analysis, the limit of detection for catechol was found to be 4 nM. It is clear that pillar array electrodes offer significant improvements in both the sensitivity and LOD, as compared to similarly fabricated single electrodes.

Gold pillar array electrodes modified with Pt/black—The widespread interest in detection of NO (nitric oxide) and due to its diverse biological roles has generated a significant demand for analytical techniques capable of its measurement and quantification [26, 40, 41]. However, such measurements can be difficult due to NO's widely ranging concentrations and stability. It is well known that in the human body, the effect of NO is dependent on its concentration, varying anywhere from sub-nanomolar to micromolar levels [41]. Furthermore, NO has a short half-life in *vivo* (typically < 10 s) due to its fast reactivity with oxygen, free radicals, thiols, and hemes [41-43]. An analytical tool to effectively detect NO demands a wide dynamic range, sufficient sensitivity, and fast response time. The method must also be selective toward NO over interfering species that arise from complex biological samples and NO derivatives. Electrochemistry offers many of these requirements for near real-time detection, with simple modifications to the electrode enabling sensitive and selective NO detection [26, 40].

To make an electrode more sensitive for NO, many groups have used platinized electrodes [25, 27, 42]. In this process a chloroplatinic acid solution (containing a small amount of lead acetate) is used to electrochemically deposit black particles of platinum onto the working electrode. The primary benefit of platinized electrodes is the increased surface area, with a rough initial surface being key to Pt-black adhesion [24]. Many different techniques like thermal etching, sandblasting, and etching with aqua regia have been previously used to increase the roughness of the initial electrode [23]. To our advantage, pillar arrays already exhibit this characteristic due to the electrodeposition of gold (see Figure 1E for a detailed image of the surface area). The number of deposition cycles needed for optimal NO signal enhancement was first investigated. Repeated injections of a 70 μM NO solution (made from a NONOate salt) were made over a bare pillar array (4 electrodes with 20 μm tall pillars), a pillar array with 1 deposition cycle of Pt-black, 2 cycles of Pt-black, and 3 cycles of Pt-black. Figure 3A illustrates the bare gold pillar array along with the different Pt-black surface modifications. As the number of deposition cycles increased, the electrode became more black and powdery. As reported earlier, this over growth of platinum black disturbed the uniformity and adhesion of the platinum black particles [24]. We found (Figure 3B) that one deposition cycle gave the highest signal improvement; a 17 fold increase over an array that did not have any Pt-black (bare array). Two or three deposition cycles had a 7 and 8 times (respectively) signal increase over the bare pillar array, but were lower than the 1 cycle deposition. For subsequent studies, a 1 cycle Pt-black deposition process was utilized. When using with microchip-based flow analysis and standards derived from NO gas, the gold pillar array modified with Pt-black showed an LOD of 5 nM. Clearly, the combination of the array approach and the Pt-black modification result in impressive detection limits for NO.

Glassy carbon electrode modified with Pt/black for NO detection—Glassy carbon is a commonly used electrode material, especially in LC flow cells. Also carbon is a common electrode material for Pt/black depositions and NO studies [24, 25, 42]. Since it is more widely used than gold (for NO detection) and easier to align with the fluidic network

(important for cell studies), the final goal of this work was to demonstrate the ability of glassy carbon to be modified with platinum black and investigate the use of these electrodes for NO release from endothelial cells in a microfluidic device. Glassy carbon was electrochemically modified with Pt-black as previously described with the pillar arrays. One cycle Pt-black deposition was also optimum for the 1 mm glassy carbon (see Figure 4A). To illustrate the surface modifications on the glassy carbon electrode, the micrograph in Figure 4A shows half of the 1 mm glassy carbon electrode electroplated with one cycle of Pt-black catalyst. The image also shows the Nafion channel coating on the entire electrode (introduced *via* a PDMS-based flow channel, discussed below). To demonstrate the signal enhancement of Pt-black depositions on glassy carbon, repeated injections of a 70 μM NO solution were analyzed over a bare glassy carbon and on a Pt-black modified glassy carbon. As shown in Figure 4B, a 10 time signal increase was observed for the Pt-black modified glassy carbon electrode (average peak height = 334.0 ± 1.5 nA, $n = 3$) relative to a bare glassy carbon electrode (average peak height = 33.2 ± 0.7 nA, $n = 3$). Meyerhoff and others have previously reported similar values in signal enhancement (8-13 times signal amplification) when using Pt-black electrodes for NO detection in studies not involving microfluidic devices [25].

One common interferent in the electrochemical analysis of NO is nitrite (NO_2^-), which is oxidized at similar potentials to NO. To minimize this interference, electrodes are commonly coated with perm-selective membrane such as Nafion to selectively permit neutral or positive small molecules to the electrode [44, 45]. While this layer does minimize the transport of negatively charged species to the electrode, in a flow system the membrane thickness also limits the transport of all molecules to the electrode surface. To investigate the use of Nafion for selectivity, the glassy carbon electrodes were electroplated with Pt-black, coated with a 0.5% Nafion solution (via a microfluidic channel as described in the experimental section, see Figure 4A), and used for microchip-based flow injection analysis of NO. Significant exclusion of NO_2^- (70 μM) was achieved (average peak height = 11.9 ± 0.3 nA, $n = 3$) compared to signal obtained from equimolar injections of NO (average peak height = 133.2 ± 5.2 nA, $n = 3$, see Figure 4C). A NO calibration curve for this electrode demonstrated a linear correlation ($r^2 = 0.993$) over a wide range of concentrations (500 nM - 95 μM). The LOD for NO on this Pt-black electrode (coated with Nafion) was 9 nM. This is a significant improvement from the previously reported LOD of 3 μM for NO detection with a carbon ink microelectrode [36]. These experiments show that these Pt-black/Nafion glassy carbon electrodes are sensitive, selective, have a wide dynamic range (which is important for NO detection), and provide < 10 nM detection limits for NO.

Cell experiments were conducted using a glassy carbon electrode embedded into a polystyrene (PS) base, since we have previously shown that PS-based devices are more biologically compatible and promote cell adhesion, as compared to PDMS devices [28, 46]. Immobilization of bovine pulmonary artery endothelial cells (bPAEC) in PDMS channels that were sealed over the PS base was achieved by treating the channels and PS substrate in an oxygen plasma cleaner. This oxidized the surface of the channels and the substrate, resulting in an irreversible seal. Previously, immobilization in PDMS channels was achieved by coating the channels with fibronectin (an adhesion protein) [47]. As shown in Figure 5A, with the PS-based device, cell adhesion and the resulting confluency without any adhesion factor are impressive.

To demonstrate the ability of Pt-black/Nafion glassy carbon electrodes to detect cell-derived NO, a 100 μM ATP standard was periodically injected (*via* an off-chip injector) into a microchannel containing bPAECs. ATP stimulates the release of NO from bPAECs after binding to $\text{P}_{2\text{y}}$ purinergic receptors [48]. The results of such experiments are shown in Figure 5B, with the cell-derived NO signals being compared to the injection of NO

standards (95 μM). Control studies including injecting buffer over the cells and injecting ATP into a channel that did not contain cells are also demonstrated (see overlay plot, Figure 5B). For the ATP control, all experimental procedures (including incubation) were followed except adding cells to the channel. Analysis of ATP-derived cell release from 6 different fluidic devices yielded an average NO release of $4.6 \pm 1.4 \mu\text{M}$, with the error being expressed as the pooled standard deviation (for each chip, $n = 3$). Taking into account the cell density of each channel, the average concentration of NO released per cell was found to be $\sim 530 \text{ pM}$. Figure 5C depicts the notable adhesion of stained bPAECs to PS device after 2 hours of continuous flow analysis.

Conclusion

In this work, we presented two new methodologies of improving the performance of microchip-based electrochemical detection in microfluidic devices, one involving the use of 3-dimensional microelectrode arrays and the other based upon platinized electrodes. With both approaches, low nM detection limits were possible, even for analytically challenging molecules such as nitric oxide. Finally, the ability of microchip-based flow injection analysis with Pt-black/Nafion-coated glassy carbon electrodes to detect nitric oxide was demonstrated, with this experimental setup being used to directly monitor the ATP-mediated NO release from endothelial cells immobilized in a microfluidic network on a polystyrene base. The pillar electrodes could be used for signal enhancement in other areas such as neurotransmitter analysis [49, 50], while the Pt-black electrodes may be useful for measuring NO in a variety of matrices. Future work will focus on using these pillar electrodes to create planar membranes that, along with these Pt-black electrodes, can be used to study the interplay between red blood cells and endothelial cells in near real-time.

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References

1. Li, MW.; Bowen, AL.; Batz, NG.; Martin, RS. Lab on a Chip Technology, Part II: Fluid Control and Manipulation. Herold, KE.; Rasooly, A., editors. Caister Academic Press; Norfolk, UK: 2009. p. 385-403.
2. Genes LI, Tolan NV, Hulvey MK, Martin RS, Spence DM. Lab Chip. 2007; 7:1256-1259. [PubMed: 17896007]
3. Mecker LC, Martin RS. Anal. Chem. 2008; 80:9257-9264. [PubMed: 19551945]
4. Noblitt SD, Lewis GS, Liu Y, Hering SV, Collett JL, Henry CS. Anal. Chem. 2009; 81:10029-10037. [PubMed: 19904999]
5. Halpin ST, Spence DM. Anal. Chem. 2010; 82:7492-7497. [PubMed: 20681630]
6. Jacobson SC, Culbertson CT, Daler JE, Ramsey JM. Anal. Chem. 1998; 70:3476-3480.
7. Duffy DC, McDonald JC, Schueller OJA, Whitesides GM. Anal. Chem. 1998; 70:4974-4984. [PubMed: 21644679]
8. Ocvirk G, Tang T, Jed Harrison D. Analyst. 1998; 123:1429-1434.
9. Martin RS, Gawron AJ, Lunte SM, Henry CS. Anal. Chem. 2000; 72:3196-3202. [PubMed: 10939387]
10. Pasas, SA.; Fogarty, BA.; Huynh, BH.; Lacher, NA.; Carlson, B.; Martin, RS.; Vandever, WR., IV; Lunte, SM. Separation Methods in Microanalytical Systems. Kutter, JP.; Fintschenko, Y., editors. CRC Press; Boca Raton: 2006.
11. Selimovic A, Johnson AS, Kiss IZ, Martin RS. Electrophoresis. 2011; 32:822-831. [PubMed: 21413031]

12. Martin RS, Gawron JA, Lunte MS. *Anal. Chem.* 2000; 72:3196–3202. [PubMed: 10939387]
13. Mecker LC, Martin RS. *Electrophoresis.* 2006; 27:5032–5042. [PubMed: 17096314]
14. Holcomb RE, Kraly JR, Henry CS. *Analyst.* 2009; 134:486–492. [PubMed: 19238284]
15. Atsushi A, Tomokazu M, Isamu E. *Anal. Chem.* 1990; 62:2206–2210.
16. Amatore C, Da Mota N, Sella C, Thouin L. *Anal. Chem.* 2010; 82:2434–2440. [PubMed: 20184349]
17. Amatore C, Klymenko OV, Oleinick AI, Svir I. *Anal. Chem.* 2009; 81:7667–7676. [PubMed: 19697937]
18. Amatore C, Da Mota N, Lemmer C, Pebay C, Sella C, Thouin L. *Anal. Chem.* 2008; 80:9483–9490. [PubMed: 19007242]
19. Amatore C, Da Mota N, Sella C, Thouin L. *Anal. Chem.* 2008; 80:4976–4985. [PubMed: 18470995]
20. Carroll S, Baldwin RP. *Anal. Chem.* 2010; 82:878–885. [PubMed: 20067252]
21. Das J, Kelley SO. *Anal. Chem.* 2011; 83:1167–1172. [PubMed: 21244005]
22. Feltham AM, Spiro M. *Chemical Reviews.* 1970; 71:177–193.
23. Ilic B, Czaplewski D, Neuzil P, Stanczyk T, Blough J, Maclay GJ. *J. Mater. Sci.* 2000; 35:3447–3457.
24. Sherstyuk OV, Pron'kin SN, Chuvilin AL, Salanov AN, Savinova ER, Tsirlina GA, Petrii OA. *Russ. J. Electrochem.* 2000; 36:741–751.
25. Lee Y, Oh BK, Meyerhoff ME. *Anal. Chem.* 2004; 76:536–544. [PubMed: 14750844]
26. Privett BJ, Shin JH, Schoenfish MH. *Chem Soc Rev.* 2010; 39:1925–1935. [PubMed: 20502795]
27. Shin JH, Privett BJ, Kita JM, Wightman RM, Schoenfish MH. *Anal. Chem.* 2008; 80:6850–6859. [PubMed: 18714964]
28. Johnson A, Anderson K, Halpin ST, Kirkpatrick DC, Spence DM, Martin RS. *Analyst.* 2013; 138:129–136. [PubMed: 23120747]
29. Batz NG, Martin RS. *Analyst.* 2009; 34:372–379. [PubMed: 19173065]
30. Hulvey MK, Martin RS. *Chem. Educator.* 2004; 9:1–7.
31. Kovarik ML, Torrence NJ, Spence DM, Martin RS. *Analyst.* 2004; 129:400–405. [PubMed: 15116230]
32. Keefer, LK.; Nims, RW.; Davies, KM.; Wink, DA. *Methods in enzymology Part A: Sources and detection of NO; NO synthase.* Parker, L., editor. Academic Press; Berkeley, CA: 1996. p. 281–293.
33. Baptiste CD, Hartwick TEA, Jollimore ABC, Baldrige HW, Seigle MG, Kelly EMM. *Mol. Pharmacol.* 2004; 66:111–1122.
34. Madou, MJ. *Fundamentals of Microfabrication: The Science of Miniaturization.* CRC Press; Boca Raton, FL: 2002.
35. Mecker LC, Martin RS. *Electrophoresis.* 2006; 27:5031–5042.
36. Hulvey MK, Martin RS. *Anal. Bioanal. Chem.* 2009; 393:599–605. [PubMed: 18989663]
37. Johnson AS, Selimovic A, Martin RS. *Electrophoresis.* 2011; 32:3121–3128. [PubMed: 22038707]
38. Shulga OV, Zhou D, Demchenko AV, Stine KJ. *Analyst.* 2008; 133:319–322. [PubMed: 18299744]
39. Ruzicka, J.; Hansen, EH. *Flow Injection Analysis.* John Wiley and Sons; New York: 1988.
40. Malinski, T.; Mesáros, S.; Tomboulían, P. *Nitric Oxide Part A Sources and Detection of NO; NO Synthase.* Packer, L., editor. Academic Press, Inc.; London: 1996. p. 58–69.
41. Pacher P, Beckman JS, Liaudet L. *Physiol. Rev.* 2007; 87:315–424. [PubMed: 17237348]
42. Amatore, C.; Arbault, S. *Electrochemical Methods for Neuroscience.* Michael, AC.; Borland, LM., editors. CRC Press; Boca Raton: 2007.
43. Lancaster, RJ. *Nitric Oxide Part A Sources and Detection of NO; NO synthase.* Packer, L., editor. Academic Press; Berkeley, CA: 1996. p. 31–50.
44. Kristensen WE, Kuhr GW, Wightman RM. *Anal. Chem.* 1987; 59:1752–1757. [PubMed: 3631500]
45. Zimmerman BJ, Wightman RM. *Anal. Chem.* 1991; 63:28–33.

46. Johnson AS, Selimovic A, Martin RS. *Anal Bioanal Chem*. 2013
47. Spence DM, Torrence NJ, Kovarik ML, Martin RS. *Analyst*. 2004; 129:995–1000. [PubMed: 15508026]
48. Ellsworth ML, Forrester T, Ellis CG, Dietrich HH. *Am J Physiol*. 1995; 269:H2155–2161. [PubMed: 8594927]
49. Li MW, Spence DM, Martin RS. *Electroanalysis*. 2005; 17:1171–1180.
50. Bowen AL, Martin RS. *Electrophoresis*. 2010; 31:2534–2540. [PubMed: 20665914]

Figure 1. Gold pillar arrays as detection electrodes. (A) Micrograph of gold mesh, 65 μm diameter with 200 μm spacing before it is embedded (B) Micrograph of an epoxy-embedded 4 electrode flat array; (C) Micrograph of 200 μm wide PDMS-based microchannel sealed over pillar array. (D) Confocal image of the 20 μm (in height) pillar array. (E) SEM image of a single gold pillar (20 μm in height).

Figure 2. Comparison of electrode response for a single bare gold electrode, a flat array of 4 electrodes, and a pillar array (4 electrodes) with repeated injections of 100 μM catechol samples. For bar graphs, the peak height shown is an average of 3 catechol injections.

Figure 3. Pillar arrays with Pt-black depositions. **(A)** Micrographs of bare array and various deposition cycles of Pt-black on the array. **(B)** Bar graph comparing the signals of 3 injections of 70 μM NO (from NONOate salt) on bare array, 1,2, and 3 Pt-black deposition cycles. Error bars are the standard deviation.

Figure 4.

(A) Micrograph of a Nafion-coated 1 mm glassy carbon (GC) electrode, with only part of the electrode intentionally modified with Pt-black. (B) i) Amperograms of 3 NO injections (70 μ M) over GC bare (black) and GC/Pt-black (green). ii) Bar graph demonstrating the signal enhancement resulting from Pt-black deposition. (C) Bar graph comparing the signal for NO & NO₂⁻ on a GC electrode modified with Pt-black and coated with Nafion.

Figure 5. In-channel bPAEC NO detection. (A) Micrograph of immobilized endothelial cells in a PDMS channel before analysis. (B) Amperogram overlay of NO standard, ATP-stimulated NO cell release, buffer and ATP blanks. (C) Micrograph of endothelial cells in channel after 2 hrs. of analysis.