Published in final edited form as:

Analyst. 2014 November 21; 139(22): 5686–5694. doi:10.1039/c4an01062k.

Microfluidic Device with Tunable Post Arrays and Integrated Electrodes for Studying Cellular Release

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Abstract

In this paper, we describe the development of a planar, pillar array device that can be used to image either side of a tunable membrane, as well as sample and detect small molecules in a cell-free region of the microchip. The pores are created by sealing two parallel PDMS microchannels (a cell channel and a collector channel) over a gold pillar array (5 or 10 μm in height), with the device being characterized and optimized for small molecule cross-over while excluding a flowing cell line (here, red blood cells, RBCs). The device was characterized in terms of the flow rate dependence of cross-over of analyte and cell exclusion as well as the ability to perform amperometric detection of catechol and nitric oxide (NO) as they cross-over into the collector channel. Using catechol as the test analyte, the limits of detection (LOD) of the cross-over for the 10 μm and 5 μm pillar array heights were shown to be 50 nM and 106 nM, respectively. Detection of NO was made possible with a glassy carbon detection electrode (housed in the collector channel) modified with Pt-black and Nafion, to enhance sensitivity and selectivity, respectively. Reproducible cross-over of NO as a function of concentration resulted in a linear correlation ($r^2 = 0.995$, 7.6 μM - 190 μM), with an LOD for NO of 230 nM on the glassy carbon-Pt-black-0.05% Nafion electrode. The applicability of the device was demonstrated by measuring the NO released from hypoxic RBCs, with the device allowing the released NO to cross-over into a cell free channel where it was detected in close to real-time. This type of device is an attractive alternative to the use of 3-dimensional devices with polycarbonate membranes, as either side of the membrane can be imaged and facile integration of electrochemical detection is possible.

Introduction

Microfluidic devices are now widely accepted analytical tools, having matured from their initial characterization work,^{1,2} to integrated microfluidic devices,^{3–5} and, more recently, even more impressive devices that allow incorporation of biological entities into the chip format with the ability to stimulate and analyze their releasates with various detection schemes.^{6–8} Some of these fully integrated microchip systems have extended into multidimensional (3-D) analysis schemes with complex solution handling, thus placing demands on the device design and functionality.^{9,10}

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Related to the work presented here, multiple-layer PDMS (poly-dimethylsiloxane) microchips with polycarbonate membranes sandwiched between two channel networks have been used for a variety of applications. These stacked, 3-D PDMS microfluidic designs utilize polycarbonate membranes with various pore sizes (commercially available from 10 nm to 2 μ m in diameter) that allow the passage of molecules and ions from one channel to another. Initial work with these devices investigated differential analyte transport (based on pore size and charge) across the membrane.¹¹ Additionally, it was shown that electrokinetic control can be used to select a plug of analyte from a separation in the bottom fluidic layer and transfer the plug into the top layer for further electrophoretic separation.^{10, 12} It was also demonstrated that if the pore size is less than 50 nm, pH gradients can be established and maintained on either side of the membrane.¹³ Later, membranes with larger pores (1.0 μ m) were applied in cell studies and enabled immobilization of endothelial cells in a reservoir on top of the membrane and introduction of a stream of flowing red blood cells (RBCs) in the bottom flow channel layer.¹⁴ The membrane allowed the passage of small molecules (ATP) released by RBCs from the bottom fluidic layer through the membrane to interact with endothelial cells immobilized on the membrane to result in NO production. The NO was fluorescently detected (after a 30 minute derivatization period) upon reaction with a DAF-FM solution that resided over the immobilized cell layer.¹⁴ A similar approach was taken with electrodes on either side of the membrane to measure the transendothelial electrical resistance (TEER, a common method used to determine cell confluency) in cells cultured on chip.^{15, 16} The advantage of these membranes is that they are commercially available, the pore size can be selected depending on size of the analyte being studied, and their placement within the fluidic device is relatively simple.

However, there are limitations associated with the use of polycarbonate membranes in microfluidic devices. For example, many membranes are non-transparent, resulting in the inability to image the bottom fluidic layer and making cell imaging somewhat challenging. Difficulties can also arise in the adhesion between the membrane and the two PDMS layers when constructing multidimensional chips, often requiring irreversible sealing.¹⁷ In addition, when co-culture cell communication studies are desired, where a cell line is in the bottom fluidic layer and a different cell layer is on the membrane in a reservoir, direct cell-to-cell contact is not possible due to the membrane being in between the cell lines.¹⁴

One way to approach some of the shortcomings of 3-dimensional chip designs that use a polycarbonate membrane is by utilizing a design where the fluidic networks and the sampling membrane are in the same dimensional plane. Previously, we have developed microfluidic devices for directly detecting NO released from endothelial cells upon stimulation with ATP,^{18, 19} including a recent report where the immobilized cells are in the same channel as the detection electrode,²⁰ however, the direct contact of the cells can cause the electrode to be coated and passivated. Here, we describe a PDMS chip design that has parallel flow channels bridged by a tunable pillar array posts. One flow channel (cell channel) contains analytes/cells that are injected/flowing and the other is the analysis channel (collector channel) containing the detector. A planar membrane between the channels is developed using a pillar array approach, where a gold electrode array is used as the base for a gold deposition to make 3-dimensional pillars that, after sealing against the fluidic network, protrude into the PDMS chip creating pores in between the parallel flow

channels. The pore size is tunable *via* the electrochemical deposition rate and grid spacing. Small molecule sampling and larger particle (cells) exclusion is controlled by height of the pillar array and fluid manipulation in the parallel flow channels. With this parallel flow design, the measurement step of the analysis can be employed in the collector channel and away from complex matrix in the cell channel while still enabling close to real-time (and direct) detection. In addition, both fluidic networks and the array posts can be simultaneously imaged. Importantly, the device can be disassembled, cleaned, reassembled, and reused multiple times without sacrificing the pillar array.

In this work, we show the development of the parallel channel and planar pillar array membrane device. Different pillar array heights and flow parameters were investigated for the optimal small molecule cross-over while excluding a flow cell line (RBCs). Detection electrodes were integrated for electrochemical detection of analytes that cross through the membrane. For sensitive and selective detection of NO, a glassy carbon detection electrode modified with Pt-black and Nafion was utilized. Finally, the release of NO from hypoxic RBCs was used to demonstrate the ability of the device to exclude the cells, while sampling NO release through the planar pillar array membrane followed by detection at a glassy carbon/Pt-black/Nafion electrode in the collector channel.

Experimental

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 (HBSS), sodium dodecyl sulfate, and potassium nitrate (Sigma Aldrich, St. Louis, MO, USA); 1 mm glassy carbon rod (Alfa Aesar, Ward Hill, MA, USA); Au, 3 mm 200 mesh regular grid (SPI supplies, West Chester, PA, USA); soldering wire, extending wire and heat shrink tubes (RadioShack); 70 mL disposable aluminum dish, 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); Permatex 5 minute epoxy (ITW Performance Polymers, IL, 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 polystyrene-embedded electrodes has been previously reported.²¹ For the fabrication of the polystyrene device containing an embedded gold array and glassy carbon detection electrode, a gauze with 75 μm parallel spacing between 30 μm diameter gold wire (13 wires total) and 1 mm diameter glassy carbon rod (7 mm) were cut with a razor blade to desired lengths and affixed with colloidal silver to a copper extending wire to provide the electrical connection. Heat shrink tubing was used to insulate the connection. Both the gold array and glassy carbon electrode were inserted into a 70 mL disposable aluminum dish (6.7 cm in diameter and 1.6 cm in depth) that had designated holes punched with a 20 gauge luer stub adapter. Following the assembly of the mold and electrodes,

polystyrene powder (250 μm particle size) was poured into the electrode mold and melted at 270°C (on a hot plate) for approximately 4 hours.²¹ After the polystyrene powder melted, it was left to cool slowly (~20 minutes). The aluminum mold was removed and the polystyrene was shaped by wet polishing, as previously reported.²¹

Flow Injection Analysis

Negative masters for the PDMS structures were fabricated as previously described^{22,23} 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.²⁴ A 10:1 (elastomer:curing agent) PDMS parallel chip design (400 μm width, 140 μm height, 3 cm in length, and 50 μm spacing between each channel) was used for membrane studies. A hole punch (5 mm dia) was used to make the outlet reservoir, and the inlet hole was created using a 20 gauge luer stub adapter (Beckton Dickinson and Co., Sparks, MD). PDMS flow channels were reversibly sealed on the polystyrene surface over the membrane array and detection electrode. It should be noted that using a 20:1 (elastomer:curing agent) PDMS microchip led to more adherent devices that would seal in between the pillars, resulting in no cross-over (closed pores). For all studies except for flow rate optimization studies, to the cell channel, a buffer (HBSS) flow stream was continuously pumped at 3.0 $\mu\text{L}/\text{min}$ *via* a 500 μL syringe (SGE Analytical Science Austin, TX, USA) and a syringe pump (Harvard 11 Plus, Harvard Apparatus, Holliston, MA, USA). The collector channel had a flow stream of buffer (1 mM SDS in HBSS) at 0.5 $\mu\text{L}/\text{min}$. Both channels were interfaced with a syringe pump *via* a 75 μm i.d. capillary tube (360 μm o.d.) using a finger tight PEEK fitting and a luer adapter (Upchurch Scientific, Oak Harbor, WA, USA). The end of each capillary was fitted with a 794 μm (o.d.) microtight sleeve (Upchurch Scientific, Oak Harbor, WA, USA) that was inserted into the microchip. For studies utilizing discrete injections (200 nL), a 4-port rotary injection valve (Vici Rotor, Valco Instruments, Houston, TX) was placed between the pump and cell channel. Amperometric detection was implemented as a 3-electrode system using a potentiostat (812B, CH Instruments, Austin, TX, USA). Either the glassy carbon or glassy carbon/Pt-black was the working electrode and was housed in the collector channel. Platinum wire was used as the auxiliary electrode, and Ag/AgCl was the reference electrode and was placed in the reservoir at the end of the collector channel. A working electrode potential of +0.9 V (vs. Ag/AgCl) was utilized.

For all NO studies, a standard stock solution (1.9 mM) was prepared daily by deoxygenating HBSS with Ar for 30 min, then saturating the solution with pure NO gas (99.5%) for 30 min.²⁵ The NO gas was purified before use by passing through a column packed with KOH pellets to remove trace NO degradation products. Individual samples were made in deoxygenated volumetric flasks (sealed with suba seal) and deoxygenated buffer. Use of air-tight syringes and capillaries limit oxygen contact to the sample.

Electrode Modification

Electrodepositions were used to create gold pillar electrodes that protrude into the PDMS to create pores between the 2 channels. Electrodepositions were carried out by filling a 200 μL PDMS reservoir (sealed over the embedded array) with 50 mM dicyanoaurate (I) and 0.1 M

Na₂CO₃ solution and applying a potential of −1.2 V (vs. Ag/AgCl) to the gold electrode array. For the membrane array studies, the deposition rate was ~1 μm/minute so that a five minute deposition produced 5 μm pillars (in height). For the preparation of Pt-black glassy carbon electrodes, a PDMS reservoir was filled with 3.5% chloroplatinic acid (w/v) and 0.005% lead (II) acetate trihydrate solution over the glassy carbon electrode. Electroplating was achieved by cycling the potential from +0.6 to −0.35 V (vs Ag/AgCl) at a scan rate of 20 mV/s. Nafion coatings over the Pt-black glassy carbon electrodes were accomplished by sealing a large (1 mm, in width, ×350 μm tall ×2 mm, in length) PDMS flow channel over the electrode. A 0.05% Nafion solution (diluted with IPA and made from a 5% solution of Nafion, Sigma Aldrich, St. Louis, MO) was pipetted into the reservoirs and left to dry on the electrodes overnight. The PDMS channel was later removed and the parallel PDMS chip was sealed over the pillar array membrane and glassy carbon/Pt-black/0.05% Nafion electrode.

Red Blood Cell Derived NO Release

The RBCs used in the studies (obtained from healthy humans) were isolated and donated by Dr. Randy Sprague's laboratory in the Department of Pharmacological and Physiological Sciences at the Saint Louis University Medical School, as previously reported.²⁶ RBCs were diluted to 7% hematocrit with HBSS buffer. For characterization experiments where RBCs were pumped in the cell channel, the cells (in HBSS) were loaded into the syringe pump and continuously pumped to the microchip at 3.0 μL/min. In the collection channel, the same cell buffer with addition of 1 mM SDS was pumped at 0.5 μL/min.

For the NO release studies, normoxic samples were prepared as 7% RBCs in HBSS. Hypoxic 7% RBC samples were prepared by adding oxyrase (Oxyrase, Inc., Mansfield, OH, USA), a commercially available product that contains an oxygen-consuming enzyme, in a 1/10 ratio to HBSS. Specifically, 200 μL of oxyrase broth (time for complete oxygen removal 6 min at pH 6.8 – 8.4) was added to 2 mL of HBSS. An aliquot of RBCs (170 μL) was then added to the oxyrase/buffer mixture and allowed to incubate at room temperature for 30 minutes to allow the enzyme to fully deoxygenate the RBC sample. It has been previously shown that this 30 minute incubation period reduces the dissolved oxygen in a buffer to approximately 3% of the saturated concentration.²⁷ For inhibition studies with L-N^G-Nitroarginine methyl ester hydrochloride (L-NAME, Sigma-Aldrich), the RBC samples were incubated in 100 μM L-NAME at room temperature for 1 hour prior to the addition of oxyrase.

Imaging

Bright field images of RBCs and fluorescent (with fluorescein, Sigma Aldrich, St. Louis, MO) imaging were accomplished with 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), while Image Pro express software (Media Cybernetics, Silver Spring, MD) was used to measure channel dimensions. The confocal image of the gold pillar array electrode was 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.

Results

Pillar Array as Planar Membrane

Cell-to-cell communication is of great interest to many researchers.^{14, 19, 27–29} Several previously developed chip designs for cell studies have involved the use of a polycarbonate membrane to separate two cell lines, one in the bottom fluidic layer (RBCs) and another (endothelial cells) immobilized on the polycarbonate membrane in another layer (either in a channel or reservoir).^{14, 16, 30, 31} Detection has been normally accomplished by using spectroscopic techniques such as chemiluminescence (for ATP released from RBCs)³² or fluorescence (for intracellular NO after reaction with DAF-FM).¹⁴ While this method allows sampling of molecules released from the bottom layer (in these examples, ATP released from RBCs) to diffuse through the polycarbonate membrane and bind to a receptor on the immobilized cell line (endothelial cells) it does not allow for direct cell-to-cell contact. One other disadvantage of the polycarbonate membrane approach, as mentioned previously, is the difficulty of imaging the lower fluidic channel (under the non-transparent membrane).

One possible way to improve such *in vivo* mimics is to utilize a planar approach, where imaging can be performed on either side of a membrane in a manner where electrodes can also be incorporated in the device for direct detection of electrochemically active analytes of interest. Figure 1 shows the chip design used here to circumvent these drawbacks, where a planar membrane is made from a pillar microelectrode array so that small molecules can pass between the membrane pores into the collector channel (that contains a detection electrode), while flowing cells are excluded and remain in the cell channel. In addition, the entire interface (and both flow layers) can easily be imaged due to its planar nature. In Figure 1, a depiction of a parallel chip design with a cell channel and a collector channel is shown. The dimensions of the channels have been optimized to 400 μm width and 140 μm height with a 50 μm spacing in between them; when sealed against a flat substrate (no pillar), no cross-over of cells or molecules is observed (Figure 2).

In order to sample small molecules from the cell channel into the collector channel for analysis, a planar approach was employed. To develop a planar membrane in between the cell channel and the collector channel, a gold pillar array (shown in Figure 1 as a schematic) and Figure 2 (as a confocal image) was fabricated by encapsulating a 3 mm (in length), gold grid (200 mesh) in a polystyrene base. The grids used here were gold wires (30 μm diameter) with a 75 μm spacing. Together with the array, a 1 mm glassy carbon electrode is embedded to allow electrochemical detection in the collector channel (Figure 1Aii). The pillars were formed as described above by electrodepositing gold (from a gold cyanide solution) onto the gold grid. Following the fabrication of the PDMS chip and the pillar-array-glassy-carbon polystyrene substrate, the PDMS microchannels (400 $\times$ 400 μm cross-section separated by 50 μm) were reversibly sealed over the pillar array and detection electrode, aligning the chip so that the space in between the channels is bridged over the pillar array and the collector channel lays over the detection electrode as illustrated in Figure 1Bi. When sealed over the array, a planar membrane was formed between the 2 channels

where the pore size was based upon the pillar height and spacing. The advantage of this approach, as opposed to the use of a polycarbonate membrane sandwiched between a channel and reservoir, is that the collector channel (see Figure 1Bii for flow and 1Biii for cross-sectional depiction) continuously collects and detects analytes that diffuse across the pillar-based membrane while both channels can be imaged. Also, since the PDMS parallel microchip is reversibly sealed over the pillar array and the glassy carbon detection electrode, it can be easily disassembled, cleaned, electrodes polished, and the polystyrene base can be reused multiple times.

Pillar Array Height and Flow Rate Characterization

With this type of device, the cross-over of small molecules is dependent on the pillar height, pillar spacing, and the flow rate of both the cell channel and collector channel. This pressure and diffusion-driven small molecule cross-over was first optimized by varying the pillar height using selected channel flow rates (3 $\mu\text{L}/\text{min}$ to the cell channel and a variable flow rate of either 1, 0.5, or 0.05 $\mu\text{L}/\text{min}$ to the collector channel). The pillar height can be tuned by varying the electrodeposition time, with the deposition rate in terms of height being ~ 1 $\mu\text{m}/\text{minute}$. A variety of pillar heights were investigated, with 5 μm and 10 μm pillar arrays selected for in-depth characterization. Initially, a 500 μM fluorescein sample serving as the small molecule and visual indicator was used to image the cross-over at the channel/membrane interface. A 7% hematocrit of (~ 7 μm diameter) RBCs were used as the flowing cell line. A systematic study of pillar array heights and flow rate dependence for small molecule cross-over are shown in the supporting information (Figures S.1 and S.2), where fluorescein cross-over and the exclusion of RBCs (7% hematocrit) from the collector channel at varying flow rates is given. As can be noted from the micrographs, an increased amount of fluorescein cross-over is seen as the flow rate on the collector channel is decreased (while the flow rate on the cell channel kept constant at 3 $\mu\text{L}/\text{min}$). With the 5 μm pillar array height (Figure S.1), the pores are small enough so that RBCs are restricted from crossing into the collector channel even at very low flow rates being applied to the channel (0.05 $\mu\text{L}/\text{min}$). However, with the 10 μm pillar array height (Figure S.2), the pores are larger and at flow rates of 3 $\mu\text{L}/\text{min}$ in the cell channel and 0.05 $\mu\text{L}/\text{min}$ in the collector channel, the RBCs can cross-over into the collector channel. For both pillar array heights (5 μm and 10 μm), flow rates of 3 $\mu\text{L}/\text{min}$ in the cell channel and 0.5 $\mu\text{L}/\text{min}$ in the collector channel flow rates were subsequently used, due to these flow rates leading to significant small molecule cross-over but exclusion of cells from the collector channel. It is important to note that the addition of 1 mM SDS into the HBSS buffer to the collector channel was the key for stable and reproducible cross-over because of its ability to provide a charged and dynamic hydrophilic coating throughout the PDMS microchannel.^{33,34} Importantly, the SDS does not cross-over into the cell channel where it could cause cell lysis (see Figure S3 in supplemental information). Figure 2C demonstrates that the cross-over of fluorescein and exclusion of RBCs is possible with the 5 μm pillar height and the optimized flow rates (3 $\mu\text{L}/\text{min}$ cell channel and 0.5 $\mu\text{L}/\text{min}$ collector channel). Control chips designs were fabricated to show that the PDMS space and pillar array posts are the crucial aspects of the chip design. Shown in Figure 2Biii, the parallel channels are designed the same way, except the channels are not separated (the two flow streams merge into 1 larger channel). As can be seen, the flow profile of RBCs merges at the fork where the flow goes to both the electrode

and the waste reservoir. This verifies that cells cannot be separated from the detection electrode while sampling small molecules such as fluorescein without the use of the separated PDMS channels and pillar array membrane, and that the separation is not due to the flow rates being used.

Electrochemical Cross-Over Characterization

Electrochemistry was also used to characterize the transport across the planar membrane using catechol as the test analyte, and both 5 μm and 10 μm pillar heights. For these studies, a 200 nL plug of sample was injected *via* an off-chip 4-port injector to the cell channel and the cross-over from the cell channel to the collector channel was detected at the glassy carbon electrode in the collector channel. The results in Figure 3A show that a 100 μM catechol sample has a 10-fold (9.17 ± 0.11 nA) greater molecule cross-over at the 10 μm pillar array height when compared to the 5 μm height pillar array membrane (0.94 ± 0.04 nA). Using catechol as the test analyte, the limits of detection (LOD) for the 10 μm and 5 μm pillar array height were found to be 50 nM and 105 nM, respectively. Since the 5 μm pillar array demonstrated satisfactory detection limits and showed no cell cross-over at these flow rates (see previous section), a 5 μm pillar height was used for all further studies. The cross-over with the 5 μm pillar array also showed linear correlation ($r^2 = 0.993$) with the change of concentration for the sample analyte catechol (1–100 μM).

To determine the percent of small molecules that cross-over to the collector channel, the total amount of (catechol) that can be detected (without crossing over) was determined by inserting the capillary leading to the 4-port injection valve into the collector channel (where the detection electrode lies), and the flow rate on the cell channel was adjusted so that no cross-over occurred. In this case, the entire catechol stream was directed over the electrode (4 $\mu\text{L}/\text{min}$ applied to the cell channel and 3 $\mu\text{L}/\text{min}$ to the collector channel). The amount that crossed over was then determined by adjusting the capillaries and flow rates back to normal (capillary from 4-port injector to cell channel, with a 3 $\mu\text{L}/\text{min}$ flow rate for cell channel and a 0.5 $\mu\text{L}/\text{min}$ flow rate for collector channel). Due to the different linear velocities used in each experiment, peak area was used calibration. It was determined that 14% of the analyte (200 μM catechol) plug crosses over to the collector channel (0.24 ± 0.03 μC) from the total plug injected (1.77 ± 0.04 μC).

While the pillar array serves as the membrane in these studies, it can also be used as the detection electrode. Having the detection source (pillar array) right where the injection plug would cross-over has the benefit of minimal diffusion and greater electrode surface area with at least 3 dimensional (both sides of the pillar) detection ability. This is shown with the arrays higher sensitivity (0.013 nA/ μM) when compared to the downstream glassy carbon detection electrode (0.003 nA/ μM) (demonstrated in the calibration curve in Figure 3B). Shown in Figure 3B is a comparison between the membrane array electrode and the glassy carbon electrode response to a wide range of concentrations To prevent electrode passivation (in case the pillar array would be in contact with cells) and to impart higher sensitivity and selectivity with various electrode modifications (more details bellow), the glassy carbon electrode was chosen as the ideal detection electrode and used for all other studies.

Nitric Oxide Detection

Nitric oxide is an analytically challenging molecule to measure with its short life-time, highly reactive nature (fast degradation), and multiple analyte interferences.³⁵ Nitric oxide has a wide range of concentrations (low nanomolar – micromolar) in the human body, thus a method that can detect NO in close to real-time, with sensitivity and selectivity, and over a wide dynamic range is desired. With the goal of detecting NO release from cells, the microchip device proposed in these studies was electrochemically characterized and optimized for NO detection. As with our previous work for NO detection,²⁰ the glassy carbon electrode is first modified with Pt-black catalyst for highly sensitive NO detection (see experimental section for details).³⁶ Initially, a NO cross-over reproducibility study was carried out with the 5 μm pillar array height. In Figure 4A, the reproducibility of NO cross-over is shown with 15 injections of a 500 μM NO solution, with detection at the Pt-black modified glassy carbon electrode (7.2 % RSD).

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 a perm-selective membrane such as Nafion to selectively permit neutral or positive small molecules to the electrode.^{37,38} To investigate the use of Nafion for selectivity, the Pt-black glassy carbon electrodes were coated with a 0.05% Nafion solution (*via* a microfluidic channel as described in the experimental section) and used for microchip-based flow injection analysis of NO. Significant exclusion of NO_2^- (95 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 4B). The amperogram in Figure 4C demonstrates the raw data for a NO calibration curve over the glassy carbon-Pt-black-0.05% Nafion electrode with a linear correlation ($r^2 = 0.995$) over a wide range of concentrations (7.6 μM - 190 μM). The LOD for NO on this Pt-black electrode (coated with Nafion) was 230 nM.

Nitric Oxide Release From Hypoxic RBCs

Several reports in the literature have shown that RBCs play a major role in vasoactivity.^{39–42} Under hypoxic conditions, RBCs not only release ATP, a recognized stimulus of NO production in various cell types, but also release NO directly.^{27,40,43} To demonstrate the applicability of the planar membrane device to sample small molecules released from cells for detection in the cell-free collector channel, hypoxic RBCs were pumped in the cell channel and NO release was detected at the glassy carbon-Pt-black-0.05% Nafion electrode. Initially, normoxic 7% RBC samples were prepared in HBSS buffer and a 200 nL sample plug was injected *via* a 4-port injector to the chip (in the cell channel). A detectable signal was not observed, as represented in the amperogram in Figure 5A, and thus is expressed as the device LOD on the bar graph in Figure 5B. Subsequently, 7% RBC samples were prepared and incubated with a oxyrase/buffer solution that has been previously used to make RBC suspensions hypoxic.²⁷ After the 7% hematocrit RBC sample was incubated with the enzyme solution, they were injected into the cell channel, and their NO release was detected at the glassy carbon-Pt-black-0.05% Nafion electrode. The hypoxic 7% RBCs released 16.5 ± 1.5 μM NO, with the error being expressed as pooled standard deviation (3 donors, each donor run in triplicate). This value is almost double previously reported data of hypoxic

RBC-NO release using the aforementioned sandwich fluidic device containing a polycarbonate membrane to allow sampling and using fluorescence detection (after derivatization with DAF-FM).²⁷ The values can be reasoned in that we achieve close to real-time detection, where with fluorescence detection, the incubation period (30 min) and multidimensional chip design may lead to NO degradation or possibly absorption of NO into the PDMS and/or polycarbonate membrane. NO production is inhibited with L-NAME, a competitive inhibitor of nitric oxide synthase (NOS).³¹ Significant NO inhibition was seen in the control studies (Figure 5), with $9.2 \pm 1.3 \mu\text{M}$ NO (standard deviation pooled, 3 donors, for each donor $n = 3$) being detected from the L-NAME incubated RBC sample.³¹ The results of these studies are compiled in Figure 5, with Figure 5A being the overlay of raw data for 7% RBC, inhibited NO release (7% RBC-L-NAME-oxyrase), and stimulated NO release (7% RBC-oxyrase) and Figure 5B a bar graph of the compiled data (each bar = RBCs from 3 different donors), with the cell-derived NO signal being compared to injections of NO standard (95 μM). There is a statistical difference between the stimulated and inhibited NO release at the 95% confidence level ($p\text{-value} = 0.0014$).

Conclusion

In this work, we presented the development of a planar membrane microfluidic device for NO sampling in a cell-free detection region. The device was characterized and optimized for small molecule cross-over through the tunable pores created by sealing two parallel PDMS microchannels over the pillar array. Characterization of flow rates for the cross-over when using 5 μm pillar array heights were optimized to be 3 $\mu\text{L}/\text{min}$ on the cell channel and 0.5 $\mu\text{L}/\text{min}$ on the collector channel. It was also shown that a glassy carbon detection electrode in the collector channel can be used to detect catechol and, after modifying the electrode with Pt-black catalyst and a Nafion membrane, NO. Importantly, the applicability of the device is demonstrated by detecting NO release from hypoxic RBCs in a cell free detection region. In the future, the parallel, planar membrane device has broad bioanalytical potential with the possibility of creating an *in vitro* vascular mimic by allowing endothelial cell immobilization in the cell channel, while RBCs transverse through the channel (allowing direct cell-to-cell contact) and their cellular communication being monitored in the collector channel electrochemically. Because this device allows for continuous pumping and ability to always sample fresh analyte into the collector channel, embedding a capillary⁴⁴ in the collector channel could lead to coupling with other off-chip detection modes such as mass spectrometry. These studies will be the subject of future reports.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Support from the National Institute of General Medical Sciences (Award Number R15GM084470-03) for A.S. and R.S.M. is acknowledged, as is support from the National Institute of Biomedical Imaging and Bioengineering (Award Number R21EB016379-01) for J.L.E. and D.M.S.

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Figure 1.

Schematic of the formation of the planar pillar array membrane. **Ai)** Parallel PDMS chip design with a cell channel and a collector channel; **Aii)** Polystyrene-embedded gold pillar array and glassy carbon electrode; **Bi)** The assembled chip device with the PDMS chip over the polystyrene base; forming the planar pillar array membrane between two PDMS channels; **Bii)** Flow profile schematic at the membrane/channel interface; **Biii)** Cross-sectional view at the membrane/channel interface.

Figure 2.

A) Microchip schematic that depicts the use of a gold pillar array as a membrane between 2 PDMS channels. **B)** Confocal micrograph of the gold pillar array. **C) i)** Micrograph that shows the ability of the gold membrane array to allow the passage of small molecules such as fluorescein; **ii)** Micrograph where a 7% hematocrit of RBCs are pumped through the cell channel. As can be seen, no RBCs are found in the collector channel; **iii)** Control experiment where the channels are not separated and there is no pillar array membrane. This demonstrates that the pillar array does exclude RBCs even with flow rates used to force small molecules to cross the array.

Figure 3. Characterization of small molecule cross-over. **A)** 100 μM catechol cross-over with 5 μm and 10 μm high pillar array membrane. **B)** Catechol calibration curve overlay of glassy carbon (down-stream in collector channel) as the detection electrode versus the membrane array as the detection electrode.

Figure 4.

A) Reproducible cross-over of 500 μM NO on 1 mm glassy carbon electrode. **B)** Bar graph demonstrating the exclusion of nitrite (95 μM) when compared to injections of NO (95 μM) on a glassy carbon/Pt-black/0.05%Nafion electrode. **C)** NO calibration (7.6-190 μM) amperogram on glassy carbon electrode modified with Pt-black and 0.05% Nafion.

Figure 5. NO release from RBCs. **A)** Amperogram overlay of normoxic 7% RBCs (expressed as LOD), and hypoxic RBCs NO release (RBCs treated with Oxyrase) and RBCs inhibited NO release (cells pre-treated with L-NAME); **B)** Bar graph of compiled data from 3 RBC donors (each done in triplicate). Error bars are pooled standard deviations. * denotes statistical difference at 95% confidence level.