MICROMECHANICAL STIMULATION CHIPS FOR STUDYING MECHANOTRANSDUCTION IN MICTURITION

K. Svennersten[1], A. Maziz[2], K. Hallén-Grufman[1], and E.W.H. Jager[2]
[1] Dept. of Molecular Medicine and Surgery, Karolinska Institute, Stockholm, SWEDEN
[2] Dept. of Physics, Chemistry and Biology, Linköping University, SWEDEN

ABSTRACT

We have developed a micromechanical stimulation chip that can apply physiologically relevant mechanical stimuli to single cells to study mechanosensitive cells in the urinary tract. The chips comprise arrays of microactuators based on the electroactive polymer polypyrrole (PPy). PPy offers unique possibilities and is a good candidate to provide such physiological mechanical stimulation, since it is driven at low voltages, is biocompatible, and can be microfabricated. The PPy microactuators can provide mechanical stimulation at different strains and/or strain rates to single cells or clusters of cells, including controls, all integrated on one single chip, without the need to pre-prepare the cells. The chips allow for *in situ* stimulation during live imaging studies. The use of these devices will increase experimental quality and reduce the number of biological samples. These unique tools fill an important gap in presently available tools, since the chips provide array-based stimulation patterns and are easily integrated in existing cell biology equipment. These chips will generate a leap forward in our understanding of the mechanisms involved in mechanotransduction in cells that may lead to break-throughs, for instance in therapies for urinary incontinence.

KEYWORDS

Mechanobiology, mechanostimulation chip, polymer microactuator, electroactive polymer, urinary tract, purinergic receptors.

INTRODUCTION

Mechanotransduction in the urinary tract

In 2018, 3.1 billion people are expected to be affected by dysfunction of the lower urinary tract worldwide. [1] Central to the understanding of lower urinary tract dysfunction is the cellular mechanotransduction involved in bladder filling and voiding. The bladder is highly dependent on the ability to sense and process mechanical inputs, illustrated by the regulated filling and voiding of the bladder. However, the mechanisms by which the bladder integrates mechanical inputs, such as intravesicular pressure, remain unknown. The purinergic signalling system is suggested to be a major component of the cellular mechanotransduction involved in bladder function. This mechanotransduction is mediated by release of the ATP molecule. There are two principally different classes of receptors for ATP, P2Y which are G-protein coupled and P2X which are ion channels. Recently, the expression of purinergic receptors has been mapped in different regions and cells, such as the ICC-like cells, in the bladder. [2] Activation of these receptors leads, via a series of events, to an increase in intracellular [Ca^{2+}] which in turn regulates many cellular processes. The increase in [Ca^{2+}] can be measured in real time using Ca^{2+} binding dyes.

To date no tools exist that satisfactorily mimic *in vitro* the dynamic micromechanical events initiated *e.g.* by an emerging inflammatory process or a growing tumour mass in the urinary tract of the patient. There is a need for tools to study these events on a single cell level or in a small population of cells. Current technologies can either stimulate large tissue sections [3], provide non-relevant stimulation like poking with an AFM [4] or cannot provide arrays with individually controllable actuators [5].

Urinary incontinence, overactive bladder, and bladder cancer are common bladder diseases where mechanotransduction is a significant factor, often causing patients tremendous discomfort while seriously impacting the quality of life. There is great clinical value in learning about the mechanisms involved in mechanotransduction in the lower urinary tract.

EXPERIMENTAL

Mechanostimulation chip

The electroactive polymer PPy changes volume upon electrochemical oxidation or reduction by applying a low potential (≤ 1 V). The volume change is predominantly caused by the insertion or ejection of ions and solvent into the polymer matrix and is used to build polymer actuators in different modes. [6, 7] PPy doped with the anion dodecylebenzenesolfonate, PPy(DBS), has a large perpendicular expansion. [8, 9] We utilise this large perpendicular expansion as the actuating principle to engineer biochips (Figure 1) that provide a stretching stimulation to the urothelial cells. [10] The chips are fabricated using standard photolithography based microfabrication methods. We started with a Si wafer that we thermally oxidised to create an insulating substrate. Next, we thermally evaporated 100 nm Au with a 5 nm Cr adhesion layer. We spincoated, exposed and developed SU8 2010 according to the standard processing. Next we applied a photoresist layer with pattern with openings where the PPy was to be synthesised. We electrosynthesised PPy(DBS) in these openings from a aqueous 0.1M NaDBS, 0.1M pyrrole monomer solution at 0.57 V vs Ag/AgCl using a 3-electrode set-up.

Figure 1: Mechanostimulation chip 2 x 2.5 cm^2 comprising two actuator areas (A) and integrated three control areas (C1-C3). The actuator area comprises an array of 100 µm wide PPy actuators and 100 µm wide SU8 lines.

We designed the microactuators as alternating patterned lines, 100 µm wide and 10 µm thick, of PPy(DBS) and the passive polymer SU8. Upon application of a potential, the PPy expands vertically stretching cells that are situated along the borders, *i.e.* that adhere to both the PPy and SU8, see Figure 2. Cells that are located on only PPy or SU8 are not mechanically stimulated.

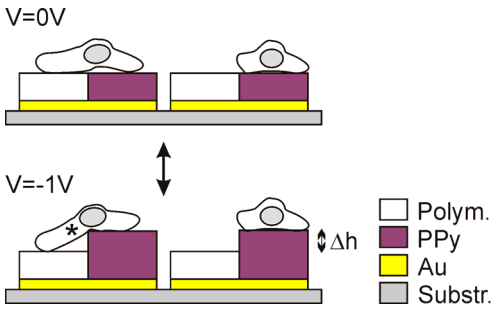

Figure 2: Principle of the cell stretching by the PPy actuators. Upon application of a potential, the PPy actuator will expand vertically and thus stretch cells that are situated along the borders, i.e. that adhere to both the PPy and the passive polymer SU8 (A in Figure 1). Cells that are located on only PPy or the passive polymer SU8 are not mechanically stimulated (C2 in Figure 1). Areas C1 and C3 are passive, non-addressed control areas to investigate surface and topography effects.

The biochips comprise two actuator areas A and an active control area C2 that is a large PPy actuator that provides only a lifting motion to the cells. It further comprises two passive control areas C1 and C3 to investigate the effects of topography and surface properties, respectively. This design allows us to do all experiments, including the controls, on one single chip thus increasing experimental quality as cells from the same passage are used simultaneously.

Cell culture

T24 human bladder carcinoma cells (ATCC nr: HTB-4) were propagated according to supplier's recommendations in RPMI-1640 (Sigma) supplemented with fetal bovine serum (10%), L-glutamine (0.3 g L^{-1}), and penicillin or streptomycin (100 U mL^{-1}; 100 µg mL^{-1}). Cells were seeded on microactuator chips placed in 30 mm cell culture dishes (Sarstedt). Cells were incubated in a humidified 37 °C, 5% CO_2 cell incubator.

Cell stimulation

To perform mechanical stimulation the chip comprising the cells was mounted in a 60 mm cell culture dish and prewarmed (37 °C) RPMI without supplement was added. The mechanical stimulation chips were operated using a Gamry potentiostat with Gamry PHE200 software. For Ca^{2+} imaging a 300 s stimulation of −1.0 V was followed by a period of 300 s at 0.0 V against the Ag/AgCl reference electrode. For immunostaining and SEM experiments cells were immediately fixed after 300 s stimulation of −1.0 V.

Ca^{2+} imaging

Loading of cells with Fura-2 was performed during 40 min incubation at 37 °C in RPMI with 2 µM Fura-2-AM. Samples were mounted on a Nikon upright Eclipse 80i microscope with a CFI Fluor DLL 40X dip down objective for ratiometric Ca^{2+} imaging 8 (Figure 3). Excitation at 340 and 380 nm was achieved with a DeltaRAM illuminator and a DeltaRAM-V monochromator with a computer controlled SC-500 shutter controller. Emission (510 nm) was collected with a Photometrics Coolsnap CCD camera. Data were analysed using Image J (U. S. National Institutes of Health).

Immunostaining and analysis

After stimulation, the cells were fixed in 3.7% formaline. The actin filaments of the cells on the mechanical stimulation chip were stained with FITC-phalloidin (Sigma). The nuclei were stained with Hoechst 33258 (Molecular Probes). The cells were then incubated with primary antibodies raised in rabbit against P2X2, P2X3, P2X7, P2Y2, P2Y4, and P2Y6 respectively (Santa Cruz Biotechnology) diluted 1:50 in PBS containing 0.05% Triton X-100 and 0.2% BSA over-night in a moist chamber at room temperature.

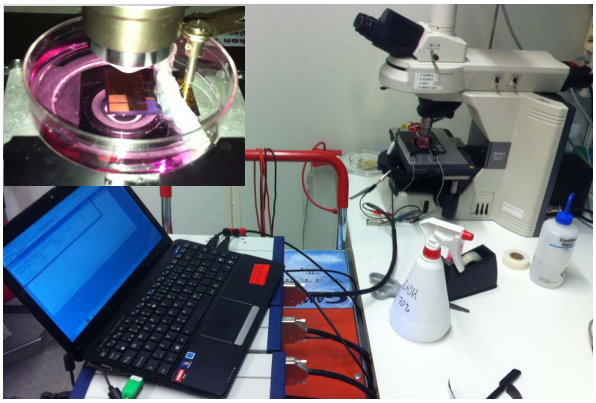

Figure 3: Fluorescence microscopy is used to follow intra-

cellular Ca^{2+} signalling in real time. Inset: the mechano-stimulation chip with the seeded cells is mounted in a standard cell culture dish under the objective of the microscope and simply addressed using an external PC controlled power source.

RESULTS

Cell viability of T24 urothelial cells

We seeded urothelial cells (T24) on the chips and cultured them for 24 h in RPMI medium. Cells adhered to and proliferated on the microchip surface. Figure 4 shows that cells spread on the surface and developed actin fibres indicating firm adhesion to the surface. Cell nuclei in different stages of the cell cycle where found while no necrotic or apoptotic cell nuclei were found, indicating proliferating cells.

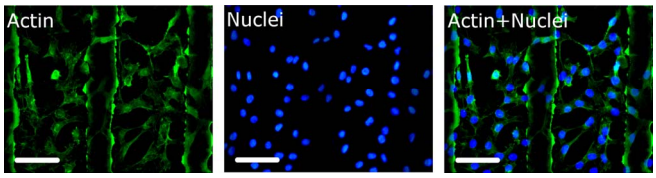

Figure 4: T24 Urothelial cells proliferate on PPy actuators. FITC-phalloidin staining of actin (green) and Hoechst staining of nuclei (blue). Scale bar = 100µm

Purinergic signalling

We used antibodies raised against P2X2, P2X3, P2X7, P2Y2, P2Y4, and P2Y6 to investigate if these receptors where expressed in the T24 cells. Figure 5 shows expression of these receptors in T24 cells. To see if these receptors were functional, we stimulated cells loaded with the Ca^{2+} sensitive dye fura-2 with ATP. ATP gave a rapid increase in the intra-cellular [Ca^{2+}] as can be seen in Figure 6.

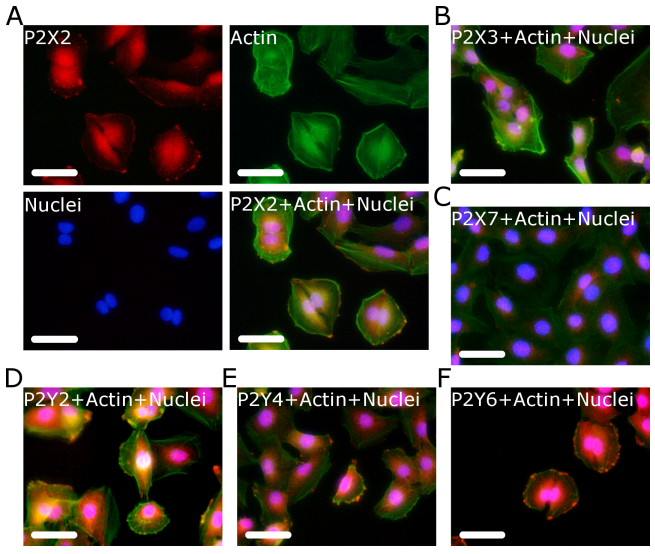

Figure 5: P2 receptors in T24 cells A) P2X2, B) P2X3, C) P2X7, D) P2Y2, E) P2Y4, F) P2Y6. For A) the three different stainings are shown individually and merged. For B)-F) only the merged images are shown. Scale bar=50 µm.

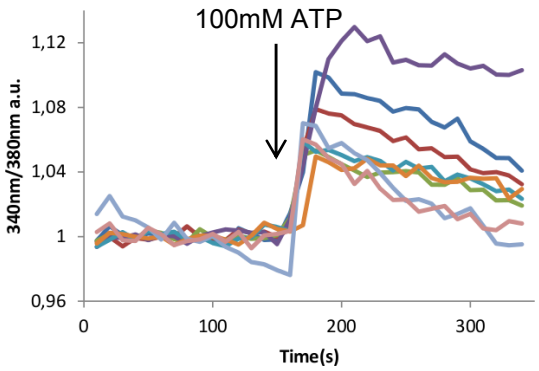

Figure 6: Live Ca^{2+} signalling of ATP-stimulated urothelial cells. At t=150s 100mM of ATP is added. Each line is the response of a single cell.

Mechanical stimulation of T24 urothelial cells

Applying a small negative potential of -1V will induced a volume change of the PPy. This will generate a step height of 1-3 µm (indicated by Δh in Figure 2) between the PPy and SU8 that will stretch the adhered cells. The full expansion takes ~90s. Following the previously describe protocol, T24 cells were seeded on the chip and cultured overnight. Next, the T24 cells were stimulated for 5 mins at -1V in order to achieve a full expansion and allow the cells to respond. It showed that the urothelial cells also proliferated well after this stimulation indicating that it did not cause any negative effects such as detachment of cells or cells entering necrosis.

Finally, we investigated the mechanotransduction of the cells. Using the live-imaging set-up as explained in Figure 3 we can follow the intra-cellular [Ca^{2+}] increase as a response of the cells to mechanical stimulation in real-time. As can be seen in Figure 7 upon the mechanical stimulation the cells expressed an increased intracellular Ca^{2+} concentration which followed the mechanical stimulation pattern, indicating that the mechanical stimulation did trigger intracellular Ca^{2+}-signalling pathways.

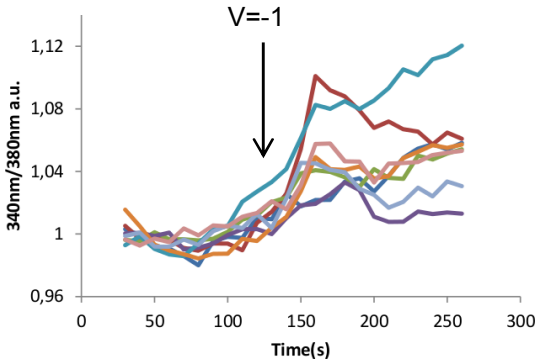

Figure 7: Live Ca^{2+} signalling of mechanostimulated urothelial cells on the PPy actuators. At t=130s ($V_{applied}$=-

1V) the actuators were activated, stretching the cells which results in an increased Ca^{2+} response. Each line is the response of a single cell.

DISCUSSION AND CONCLUSION

The developed mechanostimulation chip comprising PPy microactuators can provide mechanical stimulation to single cells or clusters of cells, including controls, all integrated on one single device, thus increasing experimental quality. Cells can be cultured on the chips without any need to prepare them. As shown in Figure 7, the biochips allow for *in situ* stimulation for instance, during live imaging studies, and can be easily integrated in existing equipment (see Figure 3), filling an important gap in presently available tools.

We are currently developing the second generation biochips that will include addressable, and thus individual controllable microactuators, in order to provide mechanical stimulation at different strains and/or strain rates simultaneously to the cells on the chip. This will generate more insights in the mechanisms of the mechanoreceptors.

In this paper we present initial results on the mechano-response of urothelial cells. We have shown that the T24 cells are viable on our devices and do respond with an increase of intra-cellular Ca^{2+} to a micro-mechanical stimulation. We are now investigating the exact signalling pathways of the urothelial cells that are triggered by this mechanical, basal stretching from the microactuators.

The fact that ATP can function as a mechano-transduction signalling molecule is a rather new concept. The idea is that upon mechanical stimulation ATP is released from the cells and activates purinergic receptors via auto- and paracrine signalling. There are two functionally different classes of ATP receptors: the ionotropic P2X receptors and the metabotropic P2Y receptors. Here, we have shown that at least three different subclasses from each class are expressed on the T24 urothelial cells. However which one of these receptors contributes the most to the response we see to both ATP and mechanical stimulation remains to be investigated in more detail. Also, a cell line from a solid urothelial tumour, as used in this preliminary study, is disparate from the physiology of the healthy human urinary bladder. Therefore we will complement our studies with human tissue samples and endeavour to identify the mechanosensitve cells and receptors in the bladder tissue.

Our studies will increase our understanding of the mechanisms involved in mechanotransduction in cells in general and urothelial cells specifically. This may lead to new therapies or treatments for urinary incontinence. For instance, results from our studies may have rapid clinical impact since selective antagonists against a variety of P2 receptors are available.

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CONTACT

* E. W. H. Jager, tel: +46-13-281246; edwin.jager@liu.se