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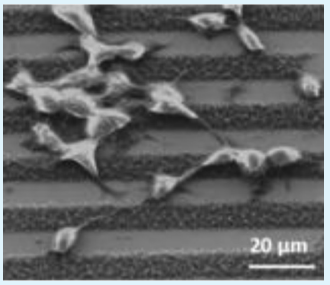
# Combined Cell Culture-Biosensing Platform Using Vertically Aligned Patterned Peptide Nanofibers for Cellular Studies

Mehmet B. Taskin,[†] Luigi Sasso,[‡] Maria Dimaki,[†] Winnie E. Svendsen,[†] and Jaime Castillo-León*[,†]

[†]Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsted Plads 345B. 2800 Kgs. Lyngby, Denmark

[‡]School of Biological Sciences, University of Canterbury, Christchurch, New Zealand

**ABSTRACT:** This Article presents the development of a combined cell culture−biosensing platform using vertically aligned self-assembled peptide nanofibers. Peptide nanofibers were patterned on a microchip containing gold microelectrodes to provide the cells with a 3D environment enabling them to grow and proliferate. Gold microelectrodes were functionalized with conductive polymers for the electrochemical detection of dopamine released from PC12 cells. The combined cell culture−biosensing platform assured a close proximity of the release site, the cells and the active surface of the sensor, thereby rendering it possible to avoid a loss of sensitivity because of the diffusion of the sample. The obtained results showed that the peptide nanofibers were suitable as a cell culturing substrate for PC12 cells. The peptide nanofibers could be employed as an alternative biological material to increase the adherence properties of PC12 cells. Dopamine was amperometrically detected at a value of 168 fmole.




**KEYWORDS:** *self-assembly, peptide nanofibrils, electrochemical biosensor, cell culture, dopamine, PC12 cells*

## INTRODUCTION

In vitro detection of analytes has allowed researchers to make groundbreaking discoveries in fields like biology, drug discovery, and medicine. In vitro analysis offers the advantage of mimicking in vivo situations, thus enabling the recreation of conditions close to the ones occurring inside the body even though they will never be exactly the same. For this, the collaborative efforts between researchers in fields like physics, chemistry and biology contribute to the development of micro- and nanofabrication techniques and play an important role in the development of new materials for the fabrication of lab-on-a-chip devices and micro-total-analysis systems with which cells and tissues can be studied.[1] These microdevices offer a physiological microenvironment that resembles the in vivo situation and at the same time provides an option for measuring compounds secreted from the biological samples after their stimulation with drug compounds, toxins, or electrical stimulus. An example of these kinds of systems is the development of artificial organs like the kidney-on-a-chip system. It was developed by growing kidney epithelial cells in a microfluidic device and enables to test different medicines to evaluate the response of the cells to renal toxins.[2]

Microdevices of this kind require a cellular platform, where cells can grow and replicate in a favorable environment. Traditional methods used to grow cells, for example, culture flasks, do not resemble the in vivo situation. Cells in the human body grow in a complex 3D environment. Several studies have suggested that 3D porous nanotopographies mimicking physiological conditions offer a more favorable environment for the growth of cells and tissues used in biomedical studies such as signal transduction, cell migration, drug assays, or regenerative medicine, among others.[3−5] Nonbiological nanostructures, such as epitaxial gallium phosphide nanowire arrays or carbon nanotubes, were used to grow cells in cellular force, cell survival, and cell renewal studies.[6−10] These 3D nanostructures showed better results in terms of cell adhesion and survival rate as compared with flat surfaces.

Silicon nanostructures have been used to explore cell attachment and spreading behavior.[11−13] In a different study, platinum nanopillars were used to study cell migration, rendering it possible to pin the position of neurons in a noninvasive way.[14] The fabrication of these 3D nanostructures requires the use of specialized equipment or clean-room conditions increasing the fabrication cost and time. Another limitation is the toxic nature of the substrate materials used in their fabrication as in the case of the gallium phosphate nanowires.[15,16]

Self-assembled peptides are biological building blocks able to self-organize into a variety of nanostructures.[17,18] Diphenylalanine is a short dipeptide that can be use for the synthesis of self-assembled structures, such as nanotubes or nanofibers under very mild conditions.[19] These structures have been structurally, electrically and chemically characterized.[20−24] The application of these biological nanostructures for applications, such as the development of biosensors, the fabrication of new nanostructures, and cell culture has been described.[17,18,25−30]

Our group recently reported on the growth of HeLa and PC12 cells in vertically aligned self-assembled peptide nanofibers.[1,30] Here, we went even further by designing a combined cell culture-biosensing platform where self-assembled nanofibers were patterned next to a traditionally used functionalized gold transducer for the detection of dopamine from PC12 cells.

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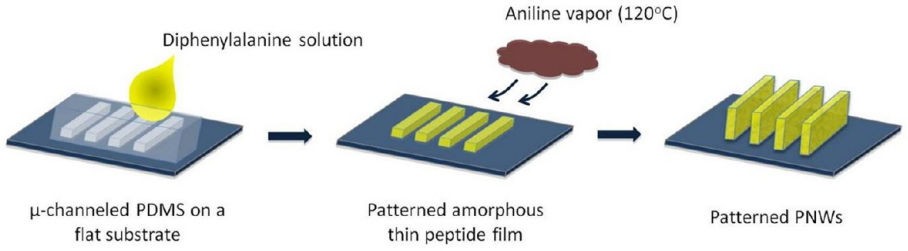
**Scheme 1. Patterning of Vertically Assembled Peptide Nanofibers Using a PDMS Microstructure**



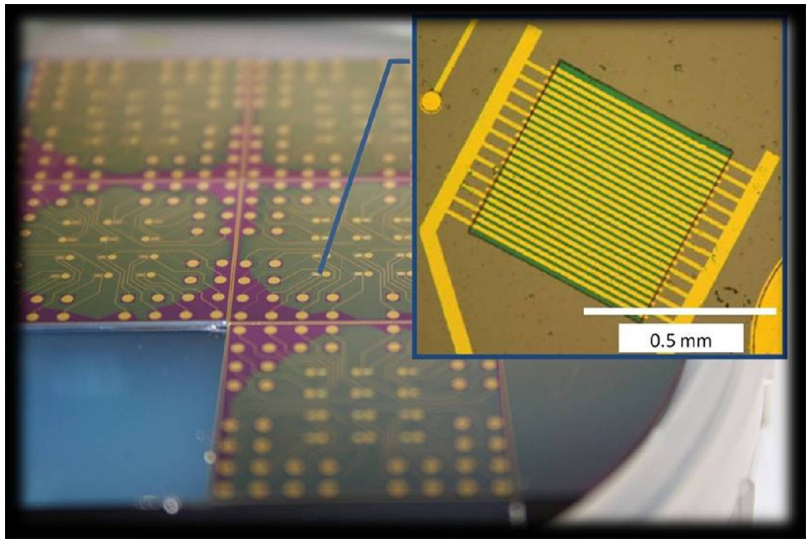




**Figure 1.** Image of the set of fabricated interdigitated gold electrodes used in this study. Inset: Individual set of interdigitated working electrodes and counter electrode.

The obtained results confirmed the developed platform as an alternative to traditionally employed cell culture methods with the advantage of incorporating the sensing feature allowing us to detect the neurotransmitter dopamine that is released from PC12 cells.

## MATERIALS AND METHODS

**Growth and Patterning of Vertically Aligned Peptide Nanofibrils.** Peptide nanofibrils were fabricated according to a method described elsewhere.[31] The freshly prepared 50 mg/mL diphenylalanine solution in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) was dropped onto the substrate next to the gold electrodes. Once it was dry, the resultant film was moved to a vacuum desiccator where it was aged under an aniline vapor environment in the absence of water vapor during 10 h at 120 °C.

For the patterning of vertically aligned peptide nanofibrils (VAPNFs) lines, a soft-lithography method using a microchanneled polydimethylsiloxane (PDMS) stamp was employed. The PDMS stamp was located on top of the gold electrode chip, and the microchannels were placed in between the gold electrodes to allow the peptide solution to be sucked inside by capillarity. Subsequently, the peptide nanofibrils were aged under an aniline vapor as previously described, Scheme 1.

**Cell Culturing.** The cell-based experiments were conducted using rat pheochromocytoma (PC12) cells from Deutsche Sammlung von Microorganismen and Zellkulturen GmbH (Braunschweig, Germany). Initially, PC12 cells were grown for 48 h in PEI-coated (2-h coating at room temperature using sterile filtered 50 μg/mL PEI diluted in PBS followed by two rinsing cycles with PBS) T25 culture flasks from Nunc A/S (Roskilde, Denmark). A Dulbecco's Modified Eagle medium/Ham's Nutrient Mixture F12 supplemented with 15% fetal bovine serum, 1.5% horse serum, 100 Units/ml penicilin, 100 μg/mL penicillin/streptomycin, and 25 mM HEPES was used as cell culture medium. After one day of incubation, the cell culture medium was changed with a differentiating medium containing DMEM/F12 supplemented with 0.5% HS, 0.5% FBS, 100 μg/mL penicillin/streptomycin, 25 mM HEPES, and 0.1 μg/mL Nerve Growth Factor (NGF). Twenty-four hours prior to seeding the cells onto an electrode chip, the growth medium was changed for the differentiation medium to initiate the cellular response to NGF.

Sterilization of all microfabricated cell culture substrates and materials was done by immersing them in 0.5 M NaOH for 30 min followed by three rinsing cycles with PBS. Electrode chips were coated with laminin (20 μg/mL laminin diluted in PBS) in a Petri dish for 2 h to ensure cellular adhesion. The adsorption of the laminin, and hence adhesion of the cells, only in the central part of an electrode chip where the electrode sets are located, was achieved using a hydrophobic pen from Dako Denmark A/S (Glostrup, Denmark).

The predifferentiated PC12 cells were rinsed with PBS and trypsinized for 3 min followed by a 5-min centrifugation cycle at 850 rpm at 20 °C. The cell pellet was resuspended in the differentiation medium and the cells were seeded onto the coated electrode chips with a surface density of $10^5$ cells/cm$^2$. All cell culturing and differentiation was performed in an incubator at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air.

SEM imaging of the differentiated cells was done using an FEI Nova 600 NanoSEM system after fixation with 2% glutaraldehyde solution diluted in PBS for 1 h followed by rinsing with PBS (two cycles of 15 min) and cell culture-tested water (two cycles of 5 min). As a final step, a dehydration process was carried out by immersing the samples in ethanol solutions with increased concentration of 60−70−80−90−100 vol/vol %, respectively, for 5 min and followed by air dehydration. After the fixation process was completed, the samples were taken to the SEM for imaging under low vacuum.

Cell counting on the microchips was carried out using a Zeiss Axio Imager M1 microscope equipped with an AxioCam MRc5 computer-controlled CCD camera (Carl Zeiss AG, Göttingen, Germany).

**Gold Electrode Preparation.** The microchips having 12-electrode sets, each comprising an interdigitated electrode (IDE) (see Figure 1), were fabricated using standard cleanroom-based micromachining techniques. A 670-nm layer of thermal oxide ($SiO_2$) was grown on a silicon wafer (one side polished) in a drive-in furnace at 1050 °C in the presence of water vapor (wet growth). The gold structures, that is, electrodes, leads and contact pads, were photolithographically defined in positive photoresist (AZ 5214E from MicroChemicals GmbH, Ulm, Germany) using an image reversal process, followed by metal deposition (10-nm thick Ti adhesion layer and 150-nm thick gold layer) through electron beam evaporation and lift-off in acetone. Prior to the metal deposition, the $SiO_2$ was etched for 100 s in the areas having opened resists. This was done using a buffered HF solution and resulted in the formation of ∼150-nm deep isotropic undercuts[32] that eliminated the formation of lift-off ears at the edges of the metal structures.

The nonactive gold areas (i.e., the leads connecting the electrodes to the contact pads) were passivated with 500-nm thick silicon nitride deposited using plasma-enhanced chemical vapor deposition. A second photolithography step coupled with reactive ion etching of the silicon nitride was used to expose the active electrode areas and contact pads. Removal of the final photoresist was achieved by ultrasonication in acetone followed by intermediate rinsing with ethanol and final rinsing with deionized water.

**Gold Electrode Functionalization.** For the electropolymerization and electrode characterization, the electrode chips were placed in a homemade holder. The holder formed a 500 μL vial on top of the electrode chip to facilitate liquid handling during the experiments. Before use, the electrode chips were cleaned for 10 min in a solution containing 50 mM KOH and 25% $H_2O_2$, followed by a potential sweep on the IDE working electrodes (WE) from −200 to −1200 mV in 50 mM KOH at 50 mV/s to remove the gold oxides formed during the chemical cleaning.[33]

The polypyrrole (PPy) electrode modification was achieved by potentiostatic electropolymerization (10 s at 700 mV). The IDEs of the microchip were used as WEs together with the counter electrode from each respective electrode set. An external Ag/AgCl was used as the reference electrode. The electrochemical behavior of the PPy-modified electrodes was characterized by cyclic voltammetry in a potential window from 0 to 700 mV at various sweep rates in a 10-mM $K_3[Fe(CN)_6]$ solution versus Ag/AgCl.

**Exocytosis Measurements.** After 4 days of differentiation, the differentiation medium was replaced by the L-DOPA medium and the cells were kept in the incubator for one hour to increase the dopamine load in the vesicles.[34] Before conducting the exocytosis measurements, each electrode chip with the differentiated PC12 cells was placed in a homemade holder to facilitate contact between the individual electrode sets and the potentiostat. The open vial in the holder facilitated the addition of the necessary buffer solutions during the exocytosis measurements.

The medium was immediately replaced by 160 μL of the low $K^+$ buffer to record a baseline for the measurements. Each array of interdigitated WEs was poised at 400 mV vs Ag/AgCl RE adjacent to the array. After a stable baseline had been recorded, exocytosis was triggered by pipetting 80 μL of the high $K^+$ buffer directly into the vial of the holder to raise the $K^+$ concentration to 150 mM.[35] The current peaks corresponding to the oxidation of the dopamine released by the cells were obtained shortly after triggering the exocytosis. All the amperometric recordings were done at room temperature (∼22 °C) using an Ag/AgCl reference electrode.

## RESULTS AND DISCUSSION

**Self-Assembled Peptide Nanofiber Patterning on the Microchip Surface.** PNFs were patterned in-between the gold interdigitated electrodes to act as a cell culture platform for the growth of the PC12 cells. As described before, the patterning involved the use of a PDMS stamp containing microchannels used to define the location were the peptide solution would be deposited. Once the solution filled the PDMS microchannels by capillarity, stripes of the peptide solution were defined on the surface of the interdigitated electrode chip. Figure 1 displays an image of the chip used in this study.

The solution was dried at room temperature in a desiccator with a water-free environment. Once the solution was dry, the aging process using aniline at 120 °C was initiated. As a result, VAPNFs were observed on defined stripes on the chip. The patterning and aging process are presented in Scheme 1.

Vertically aligned PNFs stripes with a separation of approximately 30 μm were obtained. The VAPNFs displayed a diameter between 150 and 300 nm and 4−8 μm in height. A more detailed characterization of the VAPNFs was already presented by Sasso and co-workers.[30] Figure 2 shows a SEM micrograph of well-defined stripes of vertically aligned PNFs patterned on the chip.

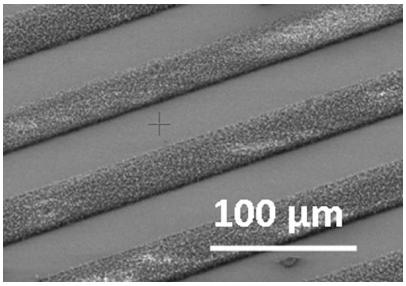



**Figure 2.** SEM image of patterned aligned self-assembled peptide nanofibers with a separation of 30 μm.

**Growth of PC12 Cells on Top of Vertically Aligned Patterned Peptide Nanofibers.** To probe the possibility of using VAPNFs as a cell culture platform, PC12 cells were seeded on four different surfaces (i.e., bare gold, laminin coated gold, VAPNFs, and laminin-coated VAPNFs) and a cell growth study was performed.

For the cell growth study, an optical microscope was used to count cells after 48 and 96 h of culture. Several areas of the samples were scanned and an average number of observed cells were used to plot the cell growth.

Figure 3 displays the results obtained in the growth study. It can be seen that the cell growth with the VAPNFs is comparable to that of laminin-coated surfaces, a growth enhancer protein commonly use in cell culture platforms.[36] However, the results obtained when using only VAPNFs as the cell culture platforms also showed an improvement when compared with bare gold or a laminin-coated surface. This suggested that these nanostructures were good candidates for the application in question. The reason behind this result had to do with the formation of a 3D nanostructure that offered the cell a more favorable environment mimicking physiological conditions as previously reported in several studies.[3,37,38]

Figure 4 shows SEM images of PC12 cells seeded on patterned stripes of VAPNFs separated by 10 μm. It is clear that some of the cells adhered to the nanofibers and connected with the next stripe showing the typical cell adhesion, differentiation morphology and confluency expected of PC12 cells. Such a cell culture platform configuration next to a set of gold electrodes is optimal for accommodating the axon-like

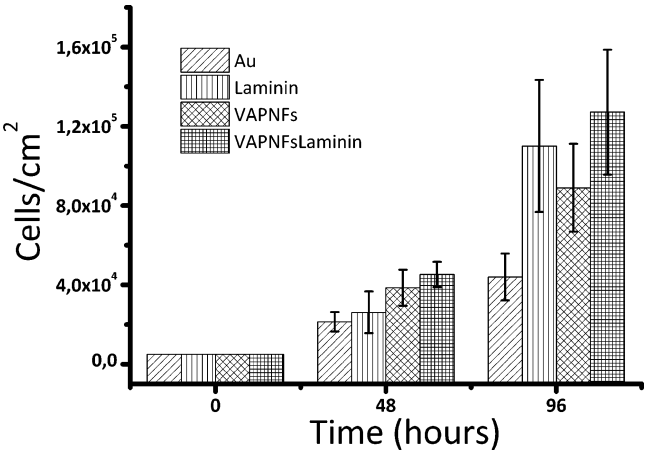



**Figure 3.** Comparative PC12 cell growth study showing cells present on four different surfaces: bare gold (Au), laminin-coated gold, VAPNFs, and laminin-coated VAPNFs. The error bars reflect the standard deviations of the measurements.

outgrowths when monitoring release of neurotransmitters from a whole population of differentiating PC12 cells.

**Electrochemical Detection of Dopamine from PC12 Seeded on Vertically Aligned Peptide Nanofibers Using Gold-Modified Microelectrodes.** For this study, we used PPy-functionalized gold microelectrodes, a well know and characterized electrochemical sensor for the detection of dopamine.[39] The electrochemical response of the gold microelectrodes covered with PPy film was evaluated by cyclic voltammetry.

Figure 5 shows voltammograms obtained for several potential sweep rates where the current peaks reflected the oxidation and reduction of polypyrrole.[40] The heights of the current peaks were found to be proportional to the square root of the potential sweep rates, showing signs of reversibility. The goal of this study was not the electrochemical characterization of the PPy-functionalized gold electrodes but the integration of a cell culture platform using VAPNFs. Consequently, values of parameters such as the detection limit and linear range are not presented here. These parameters have been extensively characterized and presented for this type of sensor in previous studies.[39,41,42]

Figure 6 depicts typical amperometric-current time traces generated on IDEs during a dopamine release measurement. As expected, the transfer of electrons as a result of the dopamine oxidation generated an increase of the electrochemical current.

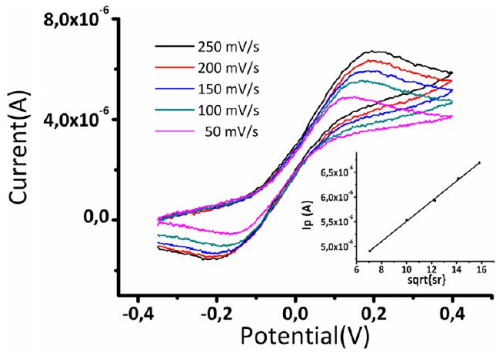



**Figure 5.** Cyclic voltammograms of PPy-modified gold microelectrodes in 10 mM $K_3[Fe(CN)_6]$ versus an Ag/AgCl reference electrode at differential potential scan rates. Inset: Current peak heights versus square root of the sweep rates.

Amperometry is widely used to study the release of neurotransmitters from different cell types.[43,44] The sensitivity of this electrochemical technique renders possible the quantification of the neurotransmitter molecules released by integrating the area of the curve corresponding to the current increase because of the dopamine release (the charge Q yielding the amount of molecules released during each release event).[43] For this, the number of dopamine molecules, $N$, is calculated as $N = Q/(nF)$, where $F$ is Faraday's constant (96 480 C/mol) and $n$ is the number of electrons donated by each oxidized molecule, which for dopamine equals two.[45,46]

In the case of the event displayed in Figure 6, the calculation of the amount of neurotransmitter released after triggering PC12 cells using KCl resulted in a value of 168 fmole of dopamine. As a control experiment, the same amount of KCl was injected in a set of electrodes in the absence of PC12 cells (inset of Figure 6) and gave rise to a neglected current increase.

## CONCLUSIONS

A combined cell culture–biosensing platform using vertically aligned peptide nanofibers was presented and used for the detection of dopamine released from PC12 cells upon chemical triggering. The platform was compared with various surfaces used in cell culture and displayed positive results. It was thus suggested that they be used to promote the growth of cells in biosensing platforms, thereby reducing the distance between the place of release and the detecting surface. A future step will include the functionalization of the VAPNFs with compounds such as fluorescent molecules or drugs to influence the behavior

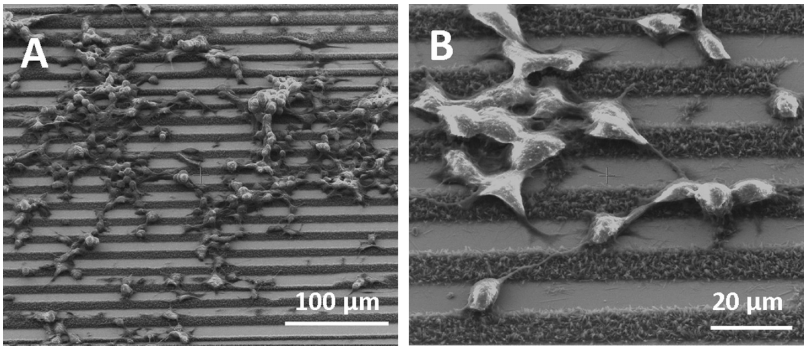



**Figure 4.** (A) SEM micrograph of PC12 cells cultured on laminin-coated VAPNFs stripes separated by 10 $\mu$m. (B) Zoom image of PC12 cells cultured on VAPNFs stripes separated by 10 $\mu$m displayed PC12 cells growing across patterned nanofibers lines.

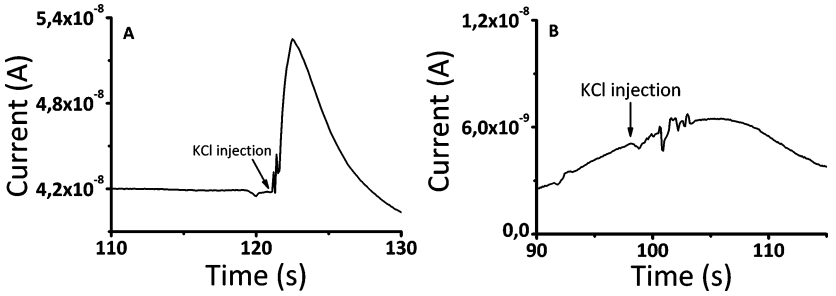



**Figure 6.** Typical amperometric current−time traces, corresponding to (A) the dopamine release from differentiating PC12 cells after triggering with KCl and (B) control experiment, injection of KCl in the absence of PC12 cells.

of the seeded cells in order to study the corresponding effect on them.

## AUTHOR INFORMATION

### Corresponding Author

*Tel: +45-4525-6837. Fax: +45 4588 7762. E-mail: jaic@nanotech.dtu.dk.

### Notes

The authors declare no competing financial interest.

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