# Interactions between cells of nervous tissue and nanostructured implant surfaces

Theses of the PhD dissertation

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2018.

# 1. Abstract

Neural interface technologies, including the development of recording and stimulating electrodes are highly investigated fields of biomedical engineering. Implanted devices aim to treat patients with serious neurological disorders as Parkinson's disease [1], essential tremor [2] or dystonia [3] which are non-responsive to drug treatments. Brain prostheses can be used also as parts of brain machine interfaces (BMI) for patients with various disabilities [4], [5]. Recording action potentials from individual neurons is impossible with non-invasive electrodes, like EEG, because the neuronal spiking is lost by averaging and filtering across the scalp [6]. The lifetime of invasive recording devices such as microfabricated neural probes is, however, limited by the foreign body response (FBR) of the central nervous system [7]–[9].

FBR results in a neuronal cell loss and the formation of a glial scar, which insulates neurons from the recording sites electrically, which corresponds to the reduction of the Signal-to-Noise Ratio (SNR) of signal acquisition, thus impairing the functionality of the device [10]. Moreover, reactive astrocytes release proinflammatory and neurotoxic factors that lead to neuronal death and degeneration, and inhibit axonal regrowth and regeneration [10]. The immune response around the neural implant can modify the appropriate interpretation of *in vivo* recordings [8], since it leads to reduced sensitivity, stability and very often to device failure [11].

As cells in their native microenvironment interact with 3D nanoscale structures of the extracellular matrix [12], topographical modification of implant surfaces may provide an alternative solution to the negative tissue response. Imitating the structure of the native environment affects the attachment and behaviour of neurons and glial cells [13].

In the presented PhD theses, I investigated a maskless cryogenic nanostructuring method, the black silicon method [14] and its possible application for tuning the topography of brain implant surfaces. The advantage of the fabrication scheme is that it can be integrated into the fabrication process of the MEMS microelectrodes. Nanostructured, metallised contact surfaces provide a higher specific surface area and therefore a better SNR.

First, I discuss the effect of the fabrication parameters for producing surface morphology composed of black silicon regions. Next, I move on to the description and explanation of the results regarding the *in vitro* and *in vivo* investigations on the interactions of the fabricated surfaces with living cells and neural tissue. *In vitro* studies, the adhesive behaviour of NE4C neural stem cells, BV2 immortalised microglia cells and primary astroglia cells are investigated on flat and nanostructured silicon and platinum surfaces. In the chronic *in vivo* study, the effects of the micro- and nanostructured surface topography and surface chemistry were investigated on the development of the glial scar and on the number of surviving neurons in the vicinity of the implant.

My results suggest that the presented nanostructuring method may provide more reliable neural electrodes for long-term applications, however, it should definitely be confirmed by further studies focusing on the long-term quality of *in vivo* signals in the vicinity of the device.

# 2. Methods

## 2.1. Nanostructuring and surface characterisation [I]

- Black poly-silicon nanostructures (BPS) were formed in single-crystalline silicon and in LPCVD deposited poly-silicon thin films by deep reactive ion etching (DRIE).
  Etching parameters were varied as it is shown in Table 1.

| Sample | Temperature of polySi deposition | $O_2$ flow [sccm] | Temperature of etching [°C] | $P_{RF}$ [W] | Loading [%] |
|---|---|---|---|---|---|
| polySi A<br>polySi B | 630°C<br>610°C | 10, 15 | -110, -100, -90 | 2, 3 | 10, 90 |
| c-Si | - | | | | |

**Table 1. Etching parameters used in this work. Relevant common etching parameters were: $SF_6$ flow= 40 sccm; Pressure in the etching chamber: 40 mTorr; Etching time=1 min; $P_{ICP}$= 700 W**

- Morphology of the resultant thin film was analysed by scanning electron microscopy using a LEO XB1540. Average pillar height and pitch density, normalised to 1 $\mu m^2$ were investigated.
- 15 nm Ti and 100 nm Pt was sputtered onto the fabricated surfaces and electrochemical measurements (cyclic voltammetry – CV and electrochemical impedance spectroscopy – EIS) were carried out to determine the change in the specific surface area [15] and in the impedance value with respect to that of the flat Pt surfaces. Both CV and EIS measurements were performed in a three electrode system using an Ag/AgCl reference electrode and a counter electrode of platinum wire.

- All the sample fabrication and characterisation presented in this work were carried out in the Centre for Energy Research, Institute of Technical Physics and Materials Science (EK MFA), Hungarian Academy of Sciences (HAS). Dr. Attila Tóth, dr. Levente Illés and András Straszner helped a lot in SEM imaging. Ágoston Horváth worked on the electrochemical measurements.

## 2.2. Neuroectodermal stem cell and immortalised microglial cell culturing and investigations [II]

- Test chips for *in vitro* cell culturing were prepared with smooth and nanostructured poly-Si surfaces. Besides non-coated samples, Pt-coated surfaces were also prepared. Small (3.2 mm x 3.2 mm) chips were engineered with a single type of surface, while the large chips (7.1 mm x 7.1 mm) carried different surfaces arranged in various grids or stand-alone square/cross

patterns (Fig 1). Glass surfaces were used as references. Test chips were cleaned in 70 % ethanol for 20 min, and then sterilized with dry heat.

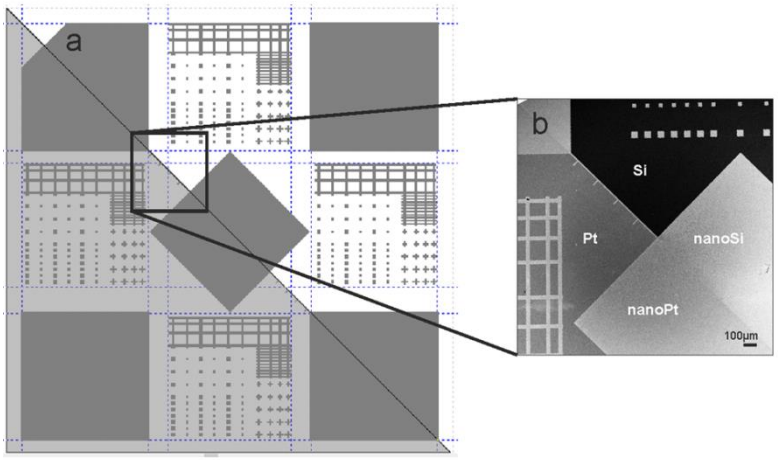


**Figure 1. Pattern of the 7.1 mm x 7.1 mm *in vitro* test chips**

- GFP-NE-4C (ATCC CRL-2936) and BV2 cells were seeded onto the engineered surfaces and were maintained in MEM-FCS (Minimum Essential Medium (MEM; Sigma-Aldrich Corp., USA) supplemented with 10 % fetal calf serum (FCS; Gibco-BRL-Life Technologies, UK), 4 mM glutamine (Sigma-Aldrich Corp., USA) and 40 mg/ml gentamycin (Chinoin Private Co., Hungary), at 37 $^{o}$C in 5 % $CO_2$ and 95 % air atmosphere for 4h or 24h. After the incubation periods photometric assays and fluorescent microscopic investigations were carried out on the cultures.
- For the microscopic investigations, cultures were fixed in 4% (w/v in PBS) paraformaldehyde. After fixation, the cultures were washed with PBS and mounted on microscope slides with DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich Corp., USA) containing Mowiol (Mowiol 4.88; Polysciences Inc., Germany). The preparations were investigated with fluorescence microscopes: a Zeiss Axio Vert.A1 with Zeiss Zen Blue edition software and a Zeiss Axiovert 200 M with AxioVision 4.8 software (Jena, Germany). Based on the images, cell shapes and arrangements were investigated.
- The viability of GFP-NE-4C and BV2 cells attached for 4 and 24 hours to various surfaces was determined by MTT-reduction assays [16]. 10 µl MTT (3-[4,5-dimethylthiazol-2yl]-2-5-diphenyl-tetrazolium bromide (Sigma-Aldrich Corp., USA) was added and after incubation the formed formazan crystals together with the cell material were dissolved in acidified (0.08N HCl) iso-propyl alcohol. The formazan content of the solutions was determined by light absorption (optical density; OD) at 570 nm measuring and 630 nm reference wavelengths in a TECAN F50 plate reader (Männedorf, Switzerland).

- The number of NE-4C and BV2 cells attached to the surfaces at the end of the 4- or 24-hour incubations was determined by fluorometric assays on cell nuclei. Cultures were fixed with 4% (w/v) paraformaldehyde in PBS and fixed cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich Corp., USA). Intensity of the emitted light (at 460 nm) was determined using a TECAN SPARK 20 plate reader.
- For the investigation of the differentiation of the stem cells on the different surfaces, the MEM-FCS medium was supplemented with $10^{-6}$ M all-trans retinoic acid (RA). After 24 hours, the medium was changed to serum-free neuronal DMEM-F12 ITS medium and cells were let to differentiate for 9 days. Differentiated cultures were fixed in 4% (w/v in PBS) paraformaldehyde and neurons were visualized by staining for neuron-specific (IIIβ-)tubulin. Samples were then mounted onto microscopy slides with DAPI containing Mowiol® (Mowiol 4.88; Polysciences Inc., Germany). Samples were investigated qualitatively with fluorescence microscopes.
- Culturing and fixing the GFP NE 4C and the BV2 cells and the MTT photometric assays were carried out in the laboratories of the Institute of Experimental Medicine, HAS, under the supervision of dr. Emília Madarász, by Tímea Kőhidi, Judit Pomothy and Attila Jády. The fluorometric assays of cell nuclei were carried out in PPCU Faculty of Information Technology and Bionics.

## 2.3. Primary astroglial cell culturing and investigations [III]

- Primary astrocytes were prepared postnatally from 1-4-day-old CD1 wild type mouse pups. Cells were seeded onto test chips shown on Fig1. Cultures were maintained in HDMEM (Sigma) with 10% FCS (Gibco), 2 mM glutamine (Sigma), 40 ug/ml gentamicin (Hungaropharma, Budapest, Hungary) and 2.5 ug/ml amphotericin B (Sigma) and were kept at 37°C in a 5% $CO_2$ atmosphere. Cultures were fixed after 24h or 48h.
- Cells were fixed with PBS with 4% PFA (TAAB), and immunostained for GFAP (anti-GFAP; 1:1000; mouse; monoclonal; Sigma and anti-mouse-Alexa488; 1:500;Molecular Probes) and for actin cytoskeleton (Alexa546-conjugated phalloidin; 1:300; Molecular Probes). DAPI in Mowiol 4.88® (Polysciences, Hamburg, Germany) was employed to visualize nuclei. Samples were investigated by a Zeiss Axio Observer Z1 inverted fluorescence microscope equipped with a Zeiss Colibri white LED illumination system. Images were captured by an AxioCamMR3 camera using AxioVision software.
- Whole-chip images were acquired by a mosaic-type image stitching technique using 10x magnification to analyse the density of nuclei on different surfaces based on DAPI staining within a given ROI.
- Morphology of cells was investigated qualitatively by three channel fluorescent microscopy using 20x and 40x magnification.

- Cells for scanning electronmicroscopic (SEM) investigations were fixed with 2.5% glutaraldehyde (Sigma) and 5% saccharose in 0.1 M cacodylate buffer and dehydrated using increasing concentrations of ethanol (50%, 60%, 75%, 90%, 100%), and were afterwards placed into amyl-acetate. Dried samples were sputter coated with 20nm gold for scanning electron microscopy. Samples were investigated in a LEO XB1540 SEM.
- Isolating, culturing and fixing of the primer astroglial cells were carried out by Hanna Liliom, in Department of Physiology and Neurobiology, Eötvös Loránd University (ELTE), under the supervision of dr. Katalin Schlett. Bence Csernyus worked on the fluorescent microscopic imaging in the Laboratories of ELTE. dr. Péter Lőw worked on fixing the cultures for SEM and the SEM imaging was carried out in EK MFA.

## 2.4. *In vivo* investigation of micro- and nanostructured implant surfaces [IV]

- Two types of Si microprobes, with a rectangular cross section of 180 μm × 380 μm and a length of 11 mm were fabricated, with both nanostructured and flat front surfaces (Fig2). Nanostructuring was carried out by the black silicon method (see 2.1.).

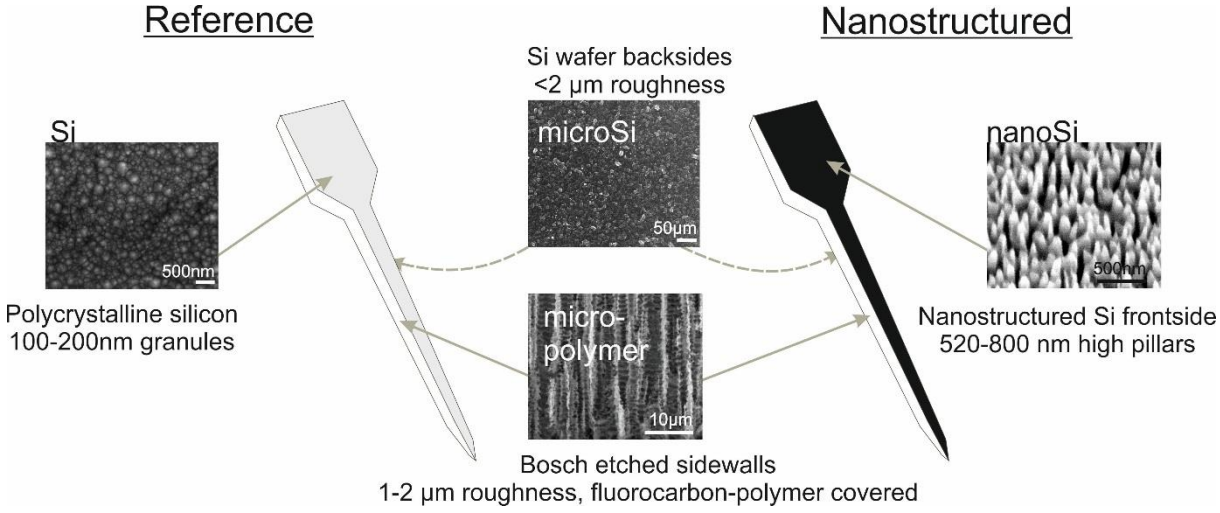


**Figure 2. Two types of implants with engineered surface topographies used in this work**

- *In vivo* studies were performed on three Wistar rats, with a total number of 12 implants (6 nanostructured Si, 6 reference probes). Animal care and experiments were carried out in compliance with Animal Care Regulations of the Institute of Cognitive Neuroscience and Psychology of the Hungarian Academy of Sciences and order 243/1988 of the Hungarian Government, which is an adaptation of directive 86/609/EGK of the European Committee Council. The study was approved by the Institutional Animal Care and Use Committee of the Research Centre for Natural Sciences, Hungarian Academy of Sciences. Eight weeks after the implantation, the rats were perfused and 60 μm thick horizontal sections were cut from the

brains with a Leica 1200S Vibratome. For the visualization of neurons and glial cells, a monoclonal mouse antibody against neuronal nuclei (NeuN, Millipore, clone A60, 1:2000) and a monoclonal mouse antibody against glial fibrillary acidic protein (GFAP, Millipore, clone GA5, 1:2000) was used. Optical microscopic images were taken of the slices using a Zeiss AxioScope.A1 microscope and a Jai GO-5000M-PGE digital camera at 10-fold magnification using a Zeiss EC Epiplan 10x/0.2 HD objective.

- Images were segmented using an ImageJ macro into specified regions of interest (ROIs) based on the work of Azemi *et al.* (Figure 3).

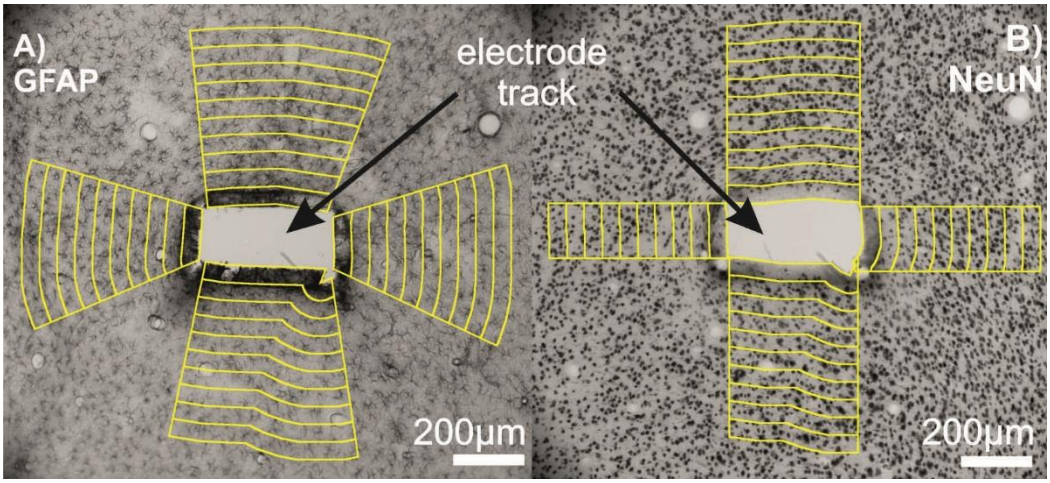


**Figure 3. GFAP (A) and NeuN (B) stained slices with the defined ROIs around the implantation track.**

- The extent of gliosis was determined on GFAP stained sections based on an average pixel intensity calculated in each ROI as a function of the distance from the implantation track. Neuronal cell loss was quantified using NeuN stained sections. Stained nuclei were counted in each ROI manually, and then the average neurons/mm$^2$ was estimated. Measures were normalised to the average pixel intensity or to the cell density of the 400–500 µm zone on the images of both the GFAP and the NeuN stained slices, respectively. Statistical analysis was performed using IBM SPSS Statistics 22 software.
- All the *in vivo* investigations were carried out in the Institute of Cognitive Neuroscience and Physiology HAS, with the Group of Comparative Psychophysiology, under the supervision of dr. István Ulbert. Surgery was done by dr. Gergely Márton and the immunohistochemical staining was done by dr. Kinga Kocsis. Bálint Kováts-Megyesi worked on the microscopic imaging and image analysis in the laboratories of EK MFA.

# 3. Novel scientific results

## 3.1. Surface modification by black silicon nanostructuring

Nanostructured surfaces were formed by deep reactive ion etching (DRIE), which can be integrated into the MEMS fabrication process flow of the implantable electrodes. Many parameters of the etching process affect the morphology of the formed surface. The nanostructured and platinum coated surfaces have higher specific surface area compared to flat Pt surfaces, therefore they provide lower impedance, and better signal-to-noise ratio may be achieved by their application in signal recording. In my experiments, I was seeking answers to the following questions:

- How do the parameters of the fabrication process: polycrystalline Si deposition temperature, $O_2$ flow, etching temperature, RF power, and loading affect the surface morphology (pillar height and density) of the nanostructures?
- How does the specific surface area of the nanostructured, platinum coated electrodes increase compared to that of the flat Pt electrode?

**THESES**

**I. I have shown that it is feasible to develop nanostructures from a polycrystalline silicon seed layer by a dry etching technique in a controlled manner and it can be integrated into the fabrication process of the MEMS based brain implants. [I]**

**I.a.** I have concluded that the average pillar height and pillar density of the black-Si nanostructures can be controlled by controlling the DRIE etching parameters. Using the investigated parameters *(T: -90 °C,-100 °C, -110 °C, $O_2$ flow:10 sccm, 15 sccm, RF power: 2 W,3 W, 1 min etching time)* the pillar height is between 690 nm±76 nm and 1973 nm±131 nm, and the pillar density is between 7.6±2.5 pillar/μm$^2$ and 22.5±5.9 pillar/μm$^2$ in the case of single-crystalline silicon. In the case of 1 μm thick polycrystalline silicon (polySi) seed layer, the pillar height is between 355 nm±6 nm and 783 nm±116 nm and the pillar density in between 9.4±2.2 pillar/μm$^2$ and 71.7±14 pillar/μm$^2$.

In the case of the polycrystalline silicon samples the morphology-change of the nanostructures when changing the $O_2$ flow remarkably depends on the polySi deposition temperature. By changing the deposition temperature form 610 °C to 630 °C and by decreasing the $O_2$ flow there is a decrease in pillar density and an increase in pillar height in both samples. However the samples made of polySi deposited on 630 °C are much less sensitive to the $O_2$ flow change.

**I.b.** I have proven that metallised *(15 nm Ti, 100 nm Pt)* black polysilicon layers can be integrated in the process flow of a basic biopotential sensor, and contributes to the increase in the specific surface area up to 44x compared to that of the flat Pt reference.

## 3.2. Interaction between immortalised cell lines and nanostructured surfaces

In *in vitro* studies, the effect of nanostructuring was investigated on two commercially used neural implant materials: silicon and platinum. The adhesion, survival and arrangement of neural stem cells (NE4C) and microglial cells (BV2) were investigated and compared to nanostructured and flat Si and Pt surfaces using cell viability assays and fluorescent microscopy image analysis. I was seeking answers to the following questions:

- Do nanofabricated surfaces support the initial attachment of neural stem cells, and immortalized microglial cells?
- How is the neuronal differentiation of stem cells influenced by the investigated surfaces?
- Can the differentiated cells attach to and survive on the investigated surfaces?

**THESES**

**II. I have shown that the black-silicon and platinum coated black-silicon surfaces influence the attachment and viability of various cells with a nervous tissue origin in different ways. [II]**

**II.a.** I have proven that the investigated surfaces *(flat polycrystalline Si and polycrystalline Si nanostructures: 70-100 pillar/$\mu m^2$randomly spread over the surface, 600 nm average height, without bioactive coating and the same topographies coated by 15 nm Ti and 30 nm Pt)* have no primary toxic effects on the NE-4C and the BV2 cell lines.

**II.b.** I have concluded that during 4 h culturing, more NE-4C cells attach to and are viable on nanostructured and flat Si surfaces compared to Pt coated surfaces at a p=0.05 significance level. Cell counts decreased drastically over 24 h culturing which indicates a weak attachment of the cells to the surfaces. The lowest rate of cell count decrease could be seen on nanostructured Si surfaces.

**II.c.** I have shown that remarkably more viable immortalised microglial cell (BV2 cell line) could be found on the flat Pt surface compared to the other investigated surfaces over 4 h and 24 h culturing periods.

**II.d.** I have proven experimentally that NE-4C neuroectodermal stem cells can differentiate into neurons by retinoic acid induction in 9 days culturing period on all the investigated surfaces.

## 3.3. Behaviour of the primer astroglial cells on nanostructured Si and Pt coated surfaces

Primary mouse astroglial cells were cultured on the above described fabricated surfaces with 24 h and 48 h culturing periods. Wide field microscopy, immunocytochemical staining of the nuclei and the actin system, and scanning electron microscopy were used to characterize the attachment, spreading and proliferation of the cells. In the experiments, I was seeking answers to the following questions:

- Do the surface material and topography of the investigated surfaces influence the number of astroglial cells attached to the surfaces over 24h and 48h periods?
- How viable are the cells on the silicon and platinum surfaces based on fluorescent and SEM microscopic studies?
- Does the surface nanostructuring influence the attachment and viability of the primary astroglial cells?

**THESES**

**III. I have shown experimentally that primer mouse astroglial cells show significant differences in their preference to surfaces based on the surface material but not on the structure. [III]**

**III.a.** I have concluded that the significantly more primer mouse astroglial cell attach to the platinum surfaces than to the silicon surfaces at a p=0.05 significance level, regardless of nanostructuring. The morphology of the cytoskeleton and the surface of the cytoplasm under SEM suggest that cells weakly attach to Si surfaces and often have apoptotic signs. In contrast, cells on Pt surfaces show healthy cytoskeletal and cytoplasm surface morphology.

**III.b.** Based on the SEM investigation of cell cytoplasm surfaces, I have concluded that the primary mouse astroglial cells prefer flat *(100-200 nm grain size)* surfaces over nanostructured *(70-100 pillar/$\mu m^2$ randomly spread over the surface, 600 nm average height)* surfaces, they attach weakly to the latter and try to avoid them.

## 3.4. *In vivo* investigations of micro and nanostructured implant surfaces

Bioactive properties of implanted black polySi nanostructured surfaces were investigated and compared to microstructured Si surfaces in eight week- long *in vivo* experiments. Glial encapsulation and local neuronal cell loss were characterised using GFAP and NeuN immunostaining respectively, followed by a systematic image analysis. In my research, I was seeking answers to the following questions:

- Do the different implant surface materials and topographies influence the severity of gliosis?
- Do the different implant surface materials and topographies influence the number of surviving neural cells in the vicinity of the implanted device?

**THESES**

**IV. I have proven that in the vicinity of an implanted device having sidewalls with different surface chemistries and morphologies, there is a significant difference in the extent of GFAP staining and in the number of surviving neurons.**

**IV.a.** I have shown that regarding the extent of GFAP staining there is no significant difference in the closest vicinity *(50 μm)* of the different implant surfaces *(nanostructured polycrystalline silicon: 70-100 pillar/μm$^2$randomly spread over the surface, 600 nm average height –* ***nanoSi****; microstructured Si with a polymer coating –* ***micro-polymer****; polycrystalline silicon with a 100-200 nm grain size –* ***Si*** *and non-polished silicon with a >2 μm surface roughness-* ***microSi***). However, at a 50 μm-400 μm distance from the implantation track, the GFAP staining is stronger next to the micro-polymer surface compared to the microSi and nanoSi surfaces at a p=0.05 significance level.

I have proven that the number of surviving neurons at a 50 μm distance from the implant adjacent to nanoSi surfaces was higher than that close to the micro-polymer and the Si surfaces, and the number of surviving neurons close to the microSi surfaces was also significantly higher compared to the micro-polymer surfaces at a p=0.05 significance level.

# 4. Possible applications

Based on my findings, NE4C neuroectodermal stem cells are able to differentiate into neurons under appropriate conditions, and the formed neurons develop basic networks. In contrast, the developed „tissue model" cannot be kept on platinum surfaces as the adhesion between cells is much stronger than between cells and the artificial surface. The platinum preference of BV2 microglial and primer astroglial cells also suggest that platinum is not the best surface in terms of long-term biocompatibility. We could also see (although qualitatively) that the platinum preference of these cells is hindered by surface nanostructuring. From *in vivo* measurements, I concluded that more viable neurons can be counted adjacent to the nanostructured Si surface compared to flat or microstructured Si surfaces, which implies that the nanostructuring of the implant surface may be beneficial. Platinum is a commonly used material for neural implants, as it is non-toxic, and has relatively low impedance at frequencies relevant in brain signal recording. The increase of the specific surface area due to nanostructuring may partly compensate the above described negative effects. It should be noted that on the implant surface most used, only the recording sites are covered by platinum, and the rest of the surfaces are composed of either silicon or dielectric materials, which cover significantly larger areas. Nanostructuring these areas by the described method may promote neural cell viability near the implant.

To confirm the conclusion that an implant with the above described modified surface may lead to improved long-term recordings, a chronic *in vivo* neural recording should be carried out in the near future.

# 5. Publications

## 5.1. Publications related to the theses

[I] Z Fekete, Á Horváth, Zs Bérces, A Pongrácz, Black poly-silicon: a nanostructured seed layer for sensor applications, ***SENSORS AND ACTUATORS A-PHYSICAL*** **216:** pp. 277-286. (2014)

[II] Zs Bérces, Á Horváth, A Jády, A Pongrácz, E Madarász, Z Fekete, Neural Cell Response to Nanostructured Biosensor Surfaces, ***PROCEDIA ENGINEERING*** **87:** pp. 971-974. (2014)

[III] Bérces Z, Csernyus B, Liliom H, Schlett K, Pinke D, Low P, Horváth Á, Fekete Z, Pongrácz A, Attachment of Primary Mouse Astroglial Cells on Neural Implant Surfaces, ***PROCEDIA ENGINEERING 168*****:** pp. 172-175. (2015)

[IV] Zs Bérces, K Tóth, G Márton, I Pál, B Kováts-Megyesi, Z Fekete, I Ulbert, A Pongrácz, Neurobiochemical changes in the vicinity of a nanostructured neural implant, ***SCIENTIFIC REPORTS*** **6:** Paper 35944. (2016)

## 5.2. Other publications

### 5.2.1. Journal papers

A Pongrácz, Z Fekete, G Márton, Zs Bérces, I Ulbert, P Fürjes, Deep-brain silicon multielectrodes for simultaneous neural recording and drug delivery, **SENSORS AND ACTUATORS B: CHEMICAL**, Volume 189, pp. 97–105. (2013)

Fekete Z, Pálfi E, Márton G, Handbauer M, Bérces Zs, Ulbert I, Pongrácz A, Négyessy L Combined *in vivo* recording of neural signals and iontophoretic injection of pathway tracers using a hollow silicon microelectrode, **SENSORS AND ACTUATORS B: CHEMICAL,** Volume 236: pp. 815-824. (2016)

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Z. Fekete, E. Pálfi, M. Handbauer, A. Pongrácz, Zs. Bérces, L. Négyessy, *In vivo* iontophoretic BDA injection through a buried microfluidic channel of a neural multielectrode, **Procedia Engineering**, Volume 120, pp. 464-467, (2015)

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Zs Sztyéhlikné Bérces, Investigation of neural stem cells' response to nanostructured biosensor surfaces, **PhD Proceedings Annual Issues of the Doctoral School, Faculty of Information**

**Technology and Bionics, Pázmány Péter Catholic University**, G. Prószéky, P. Szolgay, Eds. Budapest: Pázmány University ePress, pp 93– 96. (2015)

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