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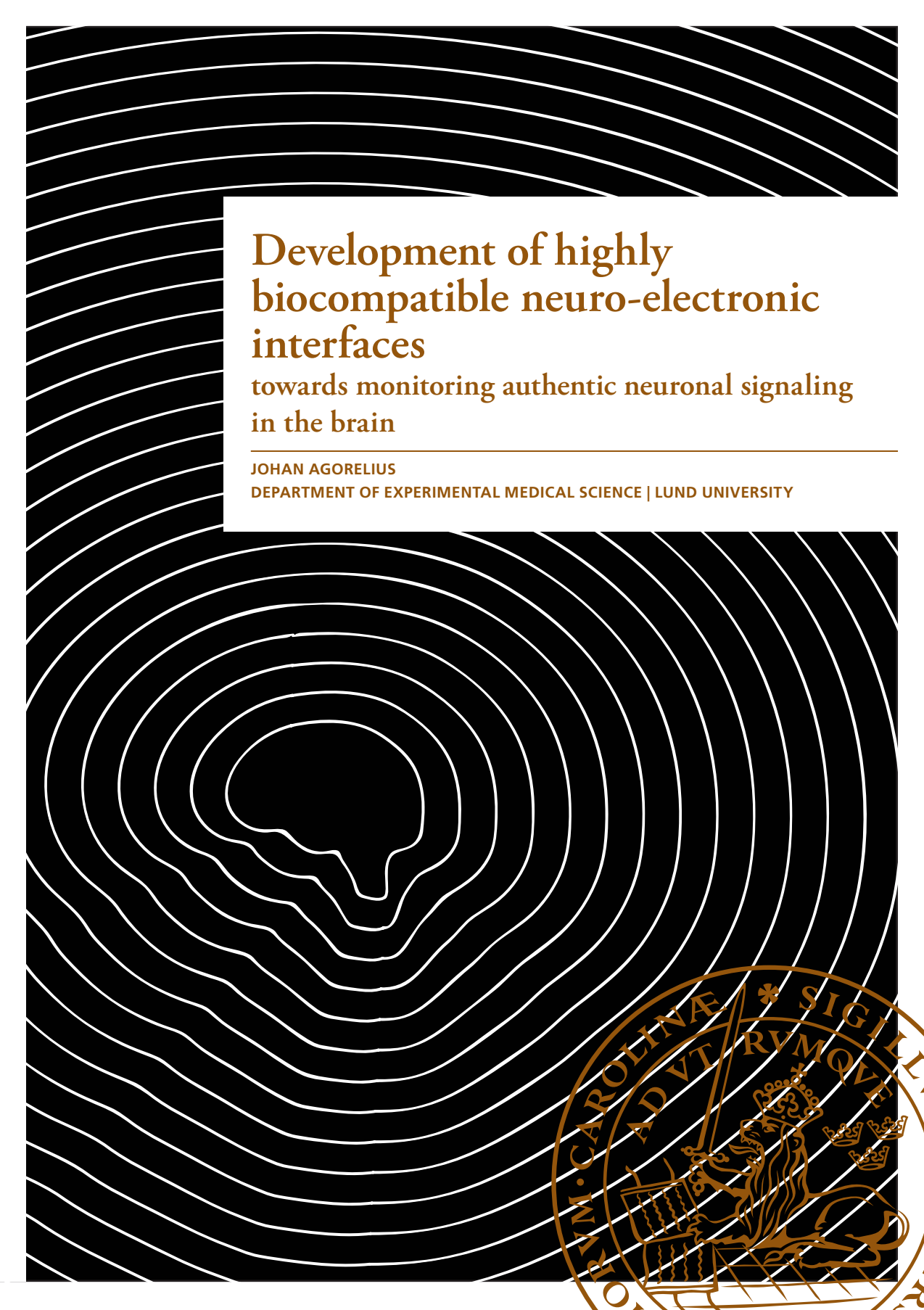
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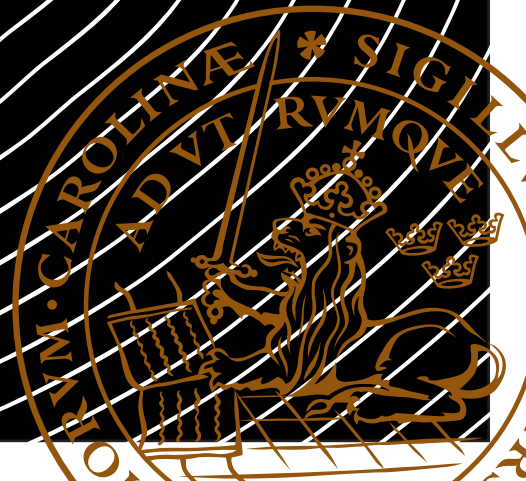


Development of highly biocompatible neuro-electronic interfaces

towards monitoring authentic neuronal signaling
in the brain

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DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY



Development of highly biocompatible neuro-electronic interfaces

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Johan Agorelius



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DOCTORAL DISSERTATION

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<p>Abstract</p> <p>Background: To understand how the neuronal circuits in the brain process information there is a need for novel neuro-electronic interfaces that can interact chronically with brain tissue with minimal disturbance of the physiological conditions in the tissue, in awake and freely moving animals. For this, there is a need for implantable neuro-electronic interfaces that are mechanically compliant with the tissue and that can remain positionally stable with respect to the neurons, despite the continuous micromotions in the brain. To reach this goal it is also important to be able to conduct a detailed analysis of the tissue reactions in the juxtapositional tissue around the implant as well as to incorporate additional strategies such as adding tissue modifying drugs to the implant.</p> <p>Aim: To this end, two different types of implantable neuro-electronic interfaces, addressing the issue of mechanical compliance with two different approaches, as well as a novel method of sustained drug delivery from the neural implants were designed, manufactured and evaluated in vivo.</p> <p>Method: First, arrays of thin gold leads, flexible in 3D, were cut from a 4 µm thin gold sheet, insulated with a thin layer of Parylene-C (4 µm) and then embedded and structurally locked in a stiff gelatin matrix that dissolves after implantation. These arrays were implanted in rats and evaluated electrophysiologically for 3 weeks. Second, a novel tube-like electrode with an opening on the side, comprising a conducting lead embedded in glucose enveloped by a thin layer of Parylene-C, was developed. After implantation the glucose in this protoelectrode dissolves transforming the protoelectrode into a highly flexible and low density electrode inside the tissue. Such tube electrodes were implanted in rats and evaluated by means of immunofluorescence microscopy after 6 weeks. Further, minocycline loaded nanoparticles were embedded into a gelatin matrix surrounding neural implants and the tissue reactions were evaluated in genetically modified mice exhibiting fluorescent microglia by means of immunofluorescence microscopy 3 and 7 days after implantation.</p> <p>Results: The developed 3D arrays were found to be implantable with preserved conformation and electrophysiological recordings showed relatively stable recordings, with spike amplitudes over 400 µV. The tube electrode proved to be sliceable in the brain without dislocating, making it possible to analyze the tissue right outside the recording site, showing minute glia reactions and no significant loss of neurons as compared to baseline tissue, even in the inner most zone (0-20 µm). The minocycline loaded nanoparticles were successfully incorporated in gelatin-coatings of neural implants, and histological analysis showed a significant attenuation of glia reactions.</p> <p>Conclusion: Two new types of mechanically compliant neuro-electronic interfaces and implantation methods, as well as a compatible embedding method of local delivery of drug content, has been successfully developed and evaluated, showing very promising biocompatibility and stability in the tissue.</p>		
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“As long as our brain is a mystery, the universe,
the reflection of the structure of the brain,
will also be a mystery.”

Santiago Ramón y Cajal

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Abbreviations

BCI	Brain-computer interface
BMI	Brain-machine interface
CD68	Cluster of differentiation 68
DAPI	4',6-diamidino-2-phenylindole
DBS	Deep brain stimulation
EEG	Electroencephalographic
ECoG	Electrocorticographic
FDA	Food and drug administration
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
ISI	Inter spike interval
IQR	Inter quartile range
NeuN	Neuronal nuclei
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCB	Printed circuit board
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PLGA	Poly(lactic-co-glycolic acid)
PMMA	Polymethylmethacrylate
ROI	Region of interest
SEM	Scanning electron microscopy
SNR	Signal to noise ratio

Populärvetenskaplig sammanfattning

Sedan det för ett par tusen år sedan föreslogs att det var hjärnan snarare än hjärtat som var centrum för vårt mentala liv, så har detta mjuka, pulserande organ fascinerat och gäckat filosofer och vetenskapsmän. Idag vet vi mer om den mänskliga hjärnan än vi någonsin gjort. Vi vet att den består av över hundra miljarder nervceller och att dessa kommunicerar med varandra med hjälp av elektricitet och vi vet allt mer om hur information bearbetas och behandlas i hjärnan, men mest av allt vet vi kanske hur lite vi egentligen vet om hur hjärnan fungerar. På så sätt är detta ”organ” kanske en större gåta idag än det någonsin varit.

Hjärnan kan undersökas på en mängd olika sätt, allt från analyser av hur blodflödet förflyttas mellan olika delar och detaljerade anatomiska studier, till beteendexperiment och terapisofter. Men, ett allt viktigare verktyg för att undersöka hur hjärnan fungerar på cellnivå, är att läsa av den elektriska aktiviteten hos enskilda nervceller och undersöka hur denna aktivitet hänger ihop med beteenden och upplevelser. Eftersom nervcellerna i hjärnan ligger tätt och dessutom är väldigt små, så måste ytterst små elektroder implanteras i hjärnan så att de hamnar tillräckligt nära för att känna av nervcellernas enskilda elektriska signaler. Sådana mikroelektroder har revolutionerat inte bara förståelsen av hjärnan, utan även gett upphov till behandling av en rad neurologiska sjukdomar. Parkinson-patienter kan genom en knapptryckning stimulera skadade delar i sin hjärna och bli av med merparten av sina rörelsehinder och förlamade människor kan styra robotarmar eller datorer med blotta tanken.

Traditionellt har dessa elektroder varit gjorda av styva material som kisel eller metall. Hjärnan, å andra sidan, är mycket mjuk och dessutom rör den sig hela tiden inuti kraniet, till exempel på grund av hjärtslag och pulserande blodådror, huvudrörelser och andning. Problemet som uppstår när en styv elektrod implanteras i den mjuka rörliga hjärnan är att elektroden rör sig i relation till vävnaden, och därmed skaver mot och skadar den känsliga vävnaden. Detta leder efter hand till att elektroderna kapslas in av hjärnans immunförsvar och förlorar kontakt med omkringliggande nervceller. Viktig information för att förstå vad som händer i gränssnittet mellan hjärna och elektrod kan fås genom att analysera vävnaden runt elektroden, men eftersom styva elektroder måste tas ut ur hjärnan innan vävnaden kan analyseras, så har det varit svårt att se exakt vad som faktiskt äger rum i vävnaden precis intill elektroden.

Så, trots de stora framgångarna med mikroelektroder, både vad gäller behandling av sjukdomstillstånd och undersökandet av hjärnans funktion, finns det stora begränsningar med dagens teknik. Eftersom vävnaden runt elektroden skadas kan inte längre signalerna som registreras från nervcellerna anses komma ifrån en normalt fungerande hjärna, något som är oerhört viktigt om man vill studera hur en hjärna fungerar i sitt normaltillstånd. Dessutom slutar elektroderna att fungera efter

ett tag vilket försvårar långtidsstudier och kan leda till att elektroderna måste tas ut ur patienter. Eftersom elektroderna inte sitter still i hjärnan, så kan de inte heller registrera från samma nervcell över tid, vilket gör det svårt att studera förloppet för neurodegenerativa sjukdomar så som Parkinsons och Alzheimers sjukdom, eller formeringen av minne och inlärning, något som är centrala delar av hjärnans funktionalitet.

Om det skulle vara möjligt för en elektrod att istället följa med hjärnans rörelser och sitta så stabilt i vävnaden att de kunde registrera från samma nervcell över tid, utan att skada vävnaden och utan att kapslas in av immunförsvaret, så skulle detta alltså kunna revolutionera inte bara vår förståelse av hjärnan, utan dessutom ge betydligt effektivare behandlingsmetoder mot en rad olika neurologiska och psykiatriska sjukdomar, så som Parkinsons sjukdom, epilepsi, depression, MS och kronisk smärta.

Med bakgrund av detta har mycket forskning och utvecklingsarbete gjorts för att utveckla nya typer av elektroder som är mjukare och flexiblare än de som vanligtvis använts. Framförallt har detta gjorts genom att göra elektroderna mycket tunna och ersätta styva material med mjukare. Sådana supertunna elektroder har mycket riktigt visat sig ge upphov till mindre vävnadsreaktioner i hjärnan, men det finns fortfarande flera utmaningar som denna strategi inte helt har löst. Till exempel, så verkar det inte som om de sitter helt förankrade i vävnaden och styrkan på de signaler de läser av tyder på att det inte finns fungerande nervceller alldeles intill elektroderna. De går inte heller att göra vävnadssnitt i hjärnan utan att elektroderna flyttar på sig, vilket försvårar analysen av vävnaden alldeles intill elektroderna.

Forskningen vid Neuronano Research Center (NRC) vid Lunds Universitet har under en längre tid fokuserat på att förstå och kartlägga de underliggande problemen kopplat till instabilitet och vävnadsskador runt neurala elektroder. Denna forskning har tidigare visat att flexibiliteten hos elektroderna är viktig, men också att densiteten är viktig och att storleken i sig självt inte är lika viktig som ofta antagits. Dessutom har det utvecklats en metod där elektroder bäddas in i hårt men upplösningsbart gelatin, ett material som visat sig minska vävnadsreaktionerna och öka nervcellsöverlevnaden runt elektroderna.

En utmaning för alla typer av flexibla elektroder är själva implanteringen. Eftersom de är alldeles för mjuka för att penetrera hjärnans vävnad, krävs någon form av hjälpmedel. För supertunna elektroder används ofta en styv guide klistrad till elektroden och på senare tid har konstruktioner utvecklats där en styvare nål används för att ”sy” in elektroden i vävnaden. Detta kan öka den initiala skadan och dessutom leda till att elektroden hamnar snett i vävnaden, vilket kan bli ett problem om man vill ha kontroll över exakt var elektroderna sitter och vilka områden de registrerar ifrån, något som är viktigt för de flesta applikationer.

För att komma runt dessa problem och på allvar inkorporera det vi hittills vet om vad som krävs för att elektroder ska kunna sitta stabilt nog i vävnaden utan att skada

den, presenteras i den här avhandlingen två nya typer av neurala elektroder som adresserar dessa problem från olika håll, samt en metod för att bekläda sådana elektroder med läkemedel som kan minska vävnadens reaktion mot implantatet.

Först och främst har en array av ultratunna guldelektroder utvecklats, som förutom att vara flexibel i två dimensioner, även har en följsam upphängning som gör att den är rörlig i alla tre dimensioner. Dessa elektroder kunde implanteras i hjärnan genom att bäddas in i vävnadsvänligt gelatin, som är styvt nog att penetrera hjärnvävnaden, men som sedan löser upp sig efter implantering och lämnar kvar de flexibla elektroderna i vävnaden.

Den andra elektroden är en tubliknande konstruktion med ett tunt polymerskal som är fyllt med glukos. Konstruktionen är tillräckligt styv för att elektroden ska kunna implanteras i hjärnan, men efter att glukoset löst upp sig blir den både flexibel och får en densitet nära hjärnans egen. Denna konstruktion har även den stora fördelen att möjliggöra histologiska vävnadssnitt i hjärnvävnaden utan att den rör sig, vilket gör att vävnaden närmast elektroden kan analyseras i detalj.

Slutligen har även en ny metod utvecklats för att belägga elektroder med läkemedel (i det här fallet Minocyklin, ett antibiotikum, som har visat sig dämpa hjärnans vävnadsreaktioner). Genom att ladda biovänliga nanopartiklar (som bryts ner långsamt i vävnaden) med Minocyklin och sedan bädda in dessa partiklar i en gelatinbeklädnad runt elektroden, kan frisläppningen av läkemedlet göras lokalt runt elektroden, samt utdraget över tid.

Både de nya typerna av flexibla elektroder samt elektroder beklädda med läkemedel, har sedan implanterats i försöksdjur (råttor och möss), och därefter har vävnaden utvärderats dels genom att undersöka hur cellerna ser ut, dels genom att läsa av nervcellernas signaler när djuren är vakna och fritt rörliga.

Resultaten från det första arbetet i avhandlingen visar att den tunna elektrod-arrayen, förutom att kunna implanteras utan att ändra konformation, även kan läsa av den elektriska aktiviteten från enskilda nervceller under de tre veckor som experimenten pågick. Styrkan på de inspelade signalerna indikerar att det i vissa fall ligger nervceller mycket nära elektroderna. Vidare analys av signalerna visar att stabiliteten är så god att det går att följa samma nervcell under en enskild inspelning och ger även goda indikationer på att det går att följa samma nervcell upp till en vecka i vakna fritt rörliga djur. Dessutom förbättras kvalitén av signalerna över tid vilket tyder på att nervcellerna kommer närmare elektroderna.

Analysen av vävnaden runt de tub-liknande elektroderna (det andra arbetet i avhandlingen) visade att det var mycket lite vävnadsreaktion runt implantatet, och ännu viktigare, att det i princip inte fanns någon mätbar minskning av nervceller precis intill elektroden. Dessa fynd bekräftade därmed grundantagandet att flexibla elektroder med vävnadsanpassad densitet och hög flexibilitet borde vara mycket bättre för vävnaden.

I det tredje arbetet undersöktes om nanopartiklarn med Minocyklin kunde minska immunförsvarets reaktion i vävnaden runt elektroder och det visade sig att reaktionen var betydligt mindre än runt elektroder utan läkemedel, vilket indikerar att läkemedlet levererades som det skulle till vävnaden runt elektroden.

Tillsammans visar dessa resultat på att det är möjligt att implantera mycket flexibla elektroder på vävnadsvänliga vis utan att de böjer av eller ändrar konformation, och att den stabilitet de uppvisar i vävnaden är lovande. De visar också att det faktiskt är möjligt att ha en tubliknande elektrod implanterad i hjärnvävnaden utan att det blir någon synlig påverkan på de intilliggande nervcellerna.

Dessa resultat indikerar att vi är på god väg att utveckla en teknologi som kan läsa av aktiviteten hos enskilda nervceller över lång tid i en vaken hjärna utan större påverkan av vävnaden och därigenom öppna upp för möjligheten att lyssna på autentiska signaler från hjärnan, något som äger en enorm potential att revolutionera behandlingen av en rad olika sjukdomstillstånd, samt öka vår förståelse av hur hjärnan fungerar.

Papers included in this thesis

- I. J. Agorelius, F. Tsanakalis, A. Friberg, P. T. Thorbergsson, L. M. E. Pettersson, and J. Schouenborg. 2015. *An array of highly flexible electrodes with a tailored configuration locked by gelatin during implantation – initial evaluation in cortex cerebri of awake rats*. *Frontiers of Neuroscience*, vol. 9, no. SEP, 2015, doi: 10.3389/fnins.2015.00331
- II. J. Agorelius, L. Gällentoft, L. M. E. Pettersson, C. Eriksson, A. Retelund and J. Schouenborg. 2020. *A flexible and density-matched novel microelectrode construct minimize long-term glial reactions and prevent loss of nearby neurons*. Manuscript, ready for submission 2021.
- III. A. D. Holmkvist, J. Agorelius, M. Forni, U. J. Nilsson, C. E. Linsmeier, and J. Schouenborg. 2020. *Local delivery of minocycline-loaded PLGA nanoparticles from gelatin-coated neural implants attenuates acute brain tissue responses in mice*. *Journal of Nanobiotechnology*, 2020, doi: 10.1186/s12951-020-0585-9.

Introduction

Neuro-electronic interfaces

Definition

A neuro-electronic interface, also called neural interface, brain-machine interface (BMI) or brain-computer interface (BCI), is a technology for establishing a direct communication pathway between a brain and an external device. These interfaces can be either non-invasive, such as electroencephalographic (EEG) electrodes placed on the scalp or semi-invasive such as electrocorticographic (ECoG) electrodes surgically placed on top of the brain surface. Both these methods, however, give limited spatial resolution, as the electrical signal generated mainly by individual neurons throughout the superficial layers of the cortex cerebri dilutes and mixes while spreading to the brain surface¹. To get greater specificity and/or reach deeper targets of the brain for stimulating specific group of neurons or record localized activity such as local field potentials and/or extracellular action potentials from single neurons, the interface needs to be implanted into the brain tissue and get into close proximity to individual neurons. Such neural implants has proven to be a very powerful tool both for clinical applications²⁻⁴ and neuroscience⁵⁻⁷. However, as will be discussed throughout this thesis, important challenges remain before this technology can close in on its true potential.

A brief history

The concept of neuro-electronic interfaces can be traced back to the late 18th century, and Luigi Galvani's legendary experiments with stimulating dead frog nerves to induce muscle contractions⁸, something that triggered another Italian scientist Alessandro Volta, to start experimenting with stimulating his own senses with electricity. Volta discovered that electrical stimulation created the sensation of light when applied to his eyes and the perception of sound when applied to his ears⁹, revealing that biological tissue can be electrically active.

This line of research trying to understand the electrical properties of nervous tissue has been continued ever since, leading up to hallmark experiments such as the

intracellular recording of an action potential by Hodgkin and Huxley in 1939¹⁰, earning the famous duo the 1963 Nobel prize.

Since then implantable neuro-electronic interfaces has been an important tool for neuroscientists trying to map and understand the basic functioning of the brain⁵ as well as for clinical applications, for example letting tetraplegic patients control neuroprosthetic robotic limbs and/or computer interfaces^{2,4} or for deep brain stimulation (DBS), alleviating parkinsonian symptoms³.

State of the art

Since the emergence of standardized implantable microelectrodes in the 1950s and the state of the art technologies emerging in the late 80s, the most well-known being the Utah Array¹¹ and the Michigan probe¹², not much has actually changed in the underlying concept and design, except the possibility of fitting more channels on the same probe. Even if the field is now progressing towards the use of more compliant materials with reduced footprints (as will be discussed in greater details further on) the standard electrodes used in many of the BMI applications and neurophysiological studies are still based on metallic wires^{6,13,14} or silicon probes¹⁵⁻¹⁷. One important reason why these electrodes are stiff, are so they can penetrate the brain tissue and be implanted to intended target without deviating. However, the stiffness also comes with a number of serious problems, most importantly relating to the mechanical mismatch between electrode and brain tissue resulting in tissue trauma and instability of recordings¹⁸⁻²⁰.

As an effect, the progress of neuroscientific research and clinical relevance of neuro-electronic interfaces has increasingly been hampered by the poor reliability and questionable validity of the signals provided from chronic electrodes^{21,22}. Optimally, these interfaces should be able to record and follow single neurons over the course of a human lifetime^{23,24} without affecting the physiological conditions in the surrounding tissue²⁵. Only then can we hope for the recorded neuronal signaling to be authentic, i.e. representing what normally occurs in an intact brain. For this to be possible, there is a need for shift of technology.

In order to grasp what would be required from such technology, it is important to understand what happens in the brain after an interface has been implanted.

Tissue trauma

The more or less stereotypic tissue response, occurring despite type of implant, method of surgery and implantation or species used, have so far been described in numerous studies ¹⁹, with a growing consensus of what takes place. The trauma to the tissue caused by an implanted neuro-electronic interface, is usually divided into an acute and chronic trauma.

Acute trauma

The very act of implanting a probe into the brain causes an acute mechanical trauma, typically by causing the rupture of blood vessels and disrupting the blood-brain barrier, as well as tissue dimpling, compression and cell ruptures ^{18,22,26,27}. This tissue damage in turn activates an inflammatory response in the brain, most obviously detected by activation and migration of glial cells towards the implant ^{18,28–30}. Although an acute implantation trauma might to a certain degree be unavoidable as the brain tissue needs to be penetrated in order to get the implant into contact with intended target neurons, the method of implantation do affect the severity of this trauma. For example, it is generally considered that a fine tip decrease dimpling and vessel dragging ^{27,31}. When it comes to speed of insertion, the situation seems more complicated, as on the one hand it has been shown that straight sharp devices should preferably be inserted fast ²⁷, minimizing the dragging of tissue. But on the other hand it seems that fast insertion speeds can lead to increased traumatic injuries ²², and that a slow insertion instead would allow for the tissue to relax and accommodate compression forces with less shock to the tissue ²⁹. However, there are also indications that a slow insertion speed before penetration of the pia and/or dura causes dimpling and compression of the tissue ²². This suggests that a faster speed before penetration of the meninges and then a slower insertion speed down to intended target would be optimal, although more data is needed to conclude this. Further, it is also not uncommon that the implant themselves suffer mechanical failure during the implantation ³² or after ³³ indicating that the method and details of surgery and implantation, is in any case of importance for the acute trauma.

The importance of the acute tissue trauma, in relation to the chronic sustained trauma, has been debated ¹⁸. However there are data showing that a stab wound to the tissue can give long lasting effects (> 1 year) in brain tissue ^{34,35}, and that after the removal of an implant there still remain glia activation and neurodegeneration ^{22,36}, suggesting that minimizing the acute tissue trauma would be preferable.

Chronic trauma and micromotions

Another aspect, where there at least lately, seems to be more of a consensus^{18,37}, is the importance of the sustained tissue trauma around an implanted neuro-electronic interface, and that this is connected to the continuous micromotions between brain tissue and implant^{28,38-40}. To understand what happens at the interface between an implant and brain tissue, it is important to consider the mechanical properties of the brain itself. The elastic modulus, (i.e. a materials resistance to elastic deformation under stress) of brain tissue is on the order of 1 kPa¹⁸. The brain is located inside the cavity of the skull, where it is suspended in cerebrospinal fluid. As an effect relative movements occur between the brain and the skull, induced mainly by respiration, heart beat and body movements^{40,41}, which in rats, can be in the order of at least 30-40 μm ³⁹. Beside these relatively large movements, there are also smaller but significant micromotions inside the brain itself caused by propagating waves of blood inside the vessels⁴².

Considering then that a silicon electrode has an elastic modulus in the range of at least a 100 GPa⁴³, and a tungsten wire even higher than that⁴⁴, and that these electrodes, in order to transduce the electrical signals from the neurons to amplifiers, needs to be tethered to the skull, after being implanted into the soft brain tissue. The majority of relative movements between the skull and the brain, will thus translate to the tissue/electrode interface, causing the stiff implant to move in relation to the tissue around it. The result of these micromotions are unstable recordings, i.e. recording from different neurons over time, as well as a continued tearing of the tissue triggering an activation of glial cells^{30,36,45-48} and likely loss of neurons in the vicinity^{29,36,41,49}. It has been argued that many problems relating to such micromotions is connected to the flexibility of the implant^{28,43,50}, and the tethering mode⁴⁷, and in 2015, it was also shown that a flexible probe induce less tissue reactions as compared to an identical but non-flexible probe³⁸, and that there is a correlation between tissue responses and quality of recordings⁵¹.

To better understand the connection between the tissue response and the deterioration of electrode functionality, and how to solve this problem, it is necessary to take a closer look at the underlying dynamics of the foreign body response, and especially the glia activation and so called glia scarring.

Foreign body response

The ultimate failure of neuro-electronic interfaces is most obviously observed in the brain tissue by the lack of neurons in the vicinity of the implant, the very cell types that the implant is meant to record from. However, an equally visible effect in the tissue is that of the aggregation and activation of nearby glia cells. The most

commonly recognizable glia cells involved in this so called foreign body response, are astrocytes and microglia ⁵².

Astrocytes

Astrocytes, making up about half of the glia cells, are star-like cells with long ranging extensions forming an interconnected network throughout the brain. Astrocytes are believed to be involved in a large number of processes, such as giving cues to neurons during development, support neuronal circuits mechanically, buffer neurotransmitters and nutrients, modifying the electrical activity of neurons ^{52,54} and even being involved in mechanisms such as plasticity and memory formation ⁵⁵. Astrocytes can be activated by mechanical trauma to the brain tissue, resulting in an activated reactive phenotype with among other traits, increased migration and proliferation and the upregulation of glial fibrillary acid protein (GFAP) ⁵⁶, a cell marker for astrocytes that is especially associated to astrocytic activation. There is evidence that activated astrocytes can prevent toxic glutamate elevations in the brain by taking up extracellular glutamate ⁵⁷ and protect neurons from oxidative stress by elimination of free radicals ⁵⁸. But there is also evidence, that under some circumstance such as excitatory crisis, the glutamate uptake can be reversed and contribute to neural damage ⁵⁹.

Microglia

Microglia are versatile, motile cells that are spread throughout the brain, where they mostly reside in a so called “resting state”, remaining positionally stable while actively monitoring the tissue via their branched and motile processes. Primarily they act as cytotoxic cells killing pathogens or as phagocytes to degrade debris after injury or normal cell turnover ^{52,60}, but they have also been shown to for example interact with dendritic spines and engulfing synapses during learning ⁶¹. Microglia can also secrete neurotrophic factors ⁶² as well as neurotoxic factors ⁶³, and thus as the astrocytes, may also have detrimental as well as protective effects on neurons in the vicinity. In fact they are involved in a wide range of signalling pathways, making it difficult to fully map their impact on the foreign body response ²⁸. When microglia becomes activated they upregulate the expression of a transmembrane glycoprotein called CD68, which can be detected using ED1 antibodies, commonly used to identify and quantify the level of microglial activation ⁶⁴.

Time course

The time course of the foreign body response around a brain implant, as mostly studied in rats, is pretty consistent. Minutes after tissue damage in the brain tissue

of surrounding microglia, within approximately 100 - 150 μm , can be seen extend their processes towards the site of injury and within half an hour the activated microglia starts encapsulating the implant/foreign body with lamellipodia⁶⁵. In less than 12 hours after implantation, microglia starts moving their cell bodies towards the site of injury^{65,66} and within 24 hours proliferating microglia and/or macrophages with amoeboid shapes can be seen surrounding the implant^{67,68}. Besides the activation of resident microglia, monocytes-derived macrophages can also be recruited from the blood stream⁶⁹, however these are in most aspects indistinguishable from resident activated microglia and both are stained by ED1.

The next phase of the foreign body response involves astrocytes. Upregulation of GFAP in astrocytes surrounding tissue damage can be seen as early as 12 hours post injury, but typically peaking after 6-7 days⁷⁰. Whereas the acute tissue response to an injury eventually will decline, the presence of an implant in the tissue, sustains the tissue response. Typically, within the first week, astrocytes up to several 100 micrometres around an implanted probe are activated. Around 2-3 weeks after implantation the astrocytes have started forming a compact sheet outside the activated microglia around the probe^{22,36,68} and after 5-6 weeks they have formed an even denser layer, a so called glia scar^{36,68,71}. It has been suggested that this layer is formed to encapsulate the site of injury and to protect the blood-brain barrier and to prevent lymphocyte infiltration⁷². Even if the time-course of these events are pretty consistent, the extent of the glia scarring vary greatly, not only between different types of implants, but also within the same type of implant^{73,74}. This suggest that other factors, for example connected to the implantation procedures is also of great importance.

Significance of the foreign body response

In parallel to the glia scarring, as discussed, there is also a loss of neurons around the implant^{36,49}. It has been suggested that it is the infiltration of glia cells that increase the distance between implant and nearby neurons^{36,75}, hindering diffusion and increasing impedance^{45,74}, or that the glia scar inhibit/repel neural processes from the electrode⁷⁶. As discussed above it is also known that activated microglia and astrocytes can act both neuroprotective and neurotoxic, and so the exact mechanisms and roles of astrocytes and microglia in the loss of neurons are not fully understood. On top of that, it should be mentioned, that even if the most obvious and described cell types involved in the tissue response around an implant, are microglia and astrocytes, many other cell types are likely also involved, such as fibroblasts³⁶, pericytes²² and precursor oligodendrocytes⁷⁷, all interacting with each other in a myriad of processes and signaling pathways.

In the end, even if the complete mechanisms of the interplay between implant, glial cells, neurons and other cell types are far from being fully mapped or understood, the fact remains; there is a lack of recordable neurons in the close vicinity of the

implant. This leads to impeded recording quality and eventual failure of the implant. Further, even during the functional window, the brain tissue cannot be considered as behaving under normal physiological conditions, which is an obvious problem, not the least for researchers trying to understand the neural dynamical processing of information in a normally functioning brain.

Analysing the tissue in the vicinity of the probe

To really understand what takes place in the tissue at the very interface of the neural probe, a much more detailed analysis of the juxtapositional tissue, than what is typically possible today, would be essential. Stiff neuro-electronic interfaces, for example, has to be completely pulled out before any tissue analysis can be done, ripping out and/or rearranging the tissue most interesting for analysis^{36,40,48}. Even if some recent very thin polymer probes^{78,79}, can indeed be sectioned while resident in the tissue, these probes are reported to get pulled out or dislocated while slicing the tissue, making a detailed and accurate analysis of the tissue at the recording sites after sectioning, impossible.

Attempted solutions and remaining problems

Flexible probes

As discussed above, many of the known problems with state of the art neural implants are likely related to the mechanical mismatch between the brain tissue and the implant, in combination with the tethering giving rise to micromotions.

An obvious way to test this hypothesis is to build probes with softer materials and with smaller footprints. Advances in material chemistry and micro/nano fabrications technologies has made it possible to build fine featured probes in polymers⁸⁰, which have elastic modulus in the range of 1 MPA to 5 GPa, and therefore induces less stress in the tissue than does more rigid materials such as silicon or tungsten⁸¹. This has given rise to the development of flexible neuro-electronic interfaces, where the conducting leads are made of thin metallic films inside a polymer substrate^{18,78,79,82-85}.

Often, however, as these probes are thin and flat, the flexibility is only considerably improved in one or two dimensions, even if strategies with tailoring the shape by reducing the effective length for improvements in flexibility^{86,87}, the use open structures^{46,88,89} for better tissue integration or the incorporation of protruding anchors in order to further attenuate micromotions⁹⁰⁻⁹², has been tried out. More recently there has also been progresses with extreme miniaturizations with probe-

thicknesses down to single micrometres in the smallest dimension^{79,82,93}. Indeed several of these polymer based probes shows evidence of reduced tissue scarring^{18,50,78,79,86}. However, preliminary results from these probes, especially with respect to amplitudes of the single unit recordings^{78,79,82,93}, indicates that there is still a lack of recordable neurons in very close vicinity of these implants, suggesting that just making the probes thinner and smaller does not completely solve the situation. In addition, the long term stability in the brain tissue of the this kinds of polymer implants, has been questioned, for example in relation to swelling of the polymers, delamination as well as the brittleness of thin metal films^{22,94,95}.

Implantation Strategies

To implant highly flexible probes that themselves cannot be implanted without structural support, several different strategies have been adopted, usually encompassing either (1) the usage of stiff guiding rods, (2) materials with adaptable flexibility, and (3) bioresorbable coatings.

Stiff guiding rods

The earliest implantations of flexible probes were made possible by gluing the probe to a stiff silicon guide with dissolvable polyethylene glycol (PEG) that dissolves after implantation, and then to withdraw the guide⁹⁶. This technique has been repeatedly used since then^{86,97}, and was recently refined by minimizing the guiding rod diameter down to 7 μm ⁹³. Other methods of attaching the guiding-rod involves using electrostatic forces⁹⁸, gluing with caramelized sucrose⁹⁹ or sticking the guiding rod into a small hole in the end of the probe, “sewing” it into the brain^{78,79,82}. In some cases the probe has been designed in such a manner that the guiding rod can be placed inside the probe itself^{100,101}, or in the opposite manner, the probe has been implanted/injected through a larger cannula^{88,102}. Even if this can be an efficient method for implanting flexible probes, there are reportedly issues with the retraction of the guiding rod, displacing the probe^{18,97,101}, making precise positioning problematic. Also, as the retraction force has been shown to be of similar magnitude as the implantation force, acute mechanical stress to the tissue is likely increased^{103,104}.

Materials with adaptable flexibility

Another approach for getting flexible implants into the brain without additional aid, has been to use polymers that can change flexibility, being stiff before implantation but gradually becoming more flexible after implantation, in response to the soaking^{94,105,106}, temperature shift^{107–109}, or controlled by chemical triggers¹¹⁰. While interesting, the efficiency of these methods remains to be seen, as the flexibility change is either relatively small or the materials have not yet efficiently been utilized into fully functional neural electrodes evaluated in vivo.

Bioresorbable materials

A highly promising, and nowadays relatively widespread strategy for implanting flexible probes, as reviewed by Lecomte et al ¹⁸, is to embed the probe into a matrix of bioresorbable materials that together with the electrodes is stiff enough for implantation when dry, but that softens and dissolves after implantation, leaving the flexible probe in the tissue. Several materials have been tested out, including gelatine, that was developed in our lab ¹¹¹, silk ^{92,112,113}, carboxymethyl cellulose ¹¹⁴, maltos ¹¹⁵, polyethylene-glycol (PEG) ¹¹³, collagen ¹¹⁶, sucrose ¹¹⁷, maltose ¹¹⁵ and poly(lactic-co-glycolic acid) (PLGA) ¹¹⁸, among others.

Some of these materials, such as gelatin and silk that are degradable biopolymers that when first coming into contact with the warm aqueous environment of the brain, takes up liquid and swells, becoming soft, and then dissolves and/or degrades by enzymatic reactions, leaving lower molecular weight products that are removed by metabolic processes ¹⁸. In the case of gelatine there are endogenous enzymes such as gelatinases, that can break down the dissolved constituents ²⁶. The speed of these processes depends heavily on water uptake capability, hydrophilic affinity and molecular weight ¹⁸, and the behaviour of the biopolymer can vary distinctly between in vitro and in vivo settings. For example it has been observed that carboxymethylcellulose that dissolves completely within 20 minutes in vitro, remained in vivo rat brains for several days ¹¹⁴. This illustrates that it is inherently hard to predict the kinetics of bioresorbable materials ¹⁸ in vivo. Biopolymers such as gelatine, chitosan or collagen tends to induce less inflammatory response than does synthesized polymers ¹¹⁹, such as PEG, and as has been shown in our lab, gelatine can in fact mitigate the inflammatory response on its own ^{38,111} and enhance repair of the blood-brain barrier ²⁶, making it a very potent candidate for a biofriendly implantation vehicle for flexible neural electrodes.

Local drug delivery

An additional strategy, beyond tailoring the mechanical properties of the neuro-electronic interface itself, has been to pharmaceutically manipulate the brain tissue surrounding the interface. The most commonly used compounds tried out so far is the steroid dexamethasone ¹²⁰ or the antibiotic minocycline ¹²¹, shown to inhibit microglia activation ^{122,123} and to be neuroprotective ¹²¹.

A variety of methods for adding local drug delivery capabilities to neural probes has been suggested, for example by incorporating a microfluidic channel into the neural probe ¹²⁴⁻¹²⁶ making it possible to inject drugs through the probe. An inherent problem with this approach being that of an increased footprint of the neural probe as well as the requirement of an injection mechanism potentially inducing tissue damages upon injection. Another approach has been to embed the drugs into a degradable coating around the probe ^{113,127,128}, so that it is released after

implantation. However, most of the drug content will be released within a time window coupled to the swelling and dissolution of the coating material. To get around this, the controlled release from a conducting polymer using electrical stimulation ¹²⁹, has instead been suggested, as well as first loading the drug content into biofriendly ¹³⁰ PLGA nanoparticles ^{127,131}, that sustains the release of the drug over time. To conclude the efficiency of these strategies, it would be desirable at this point to study how a highly localised release of a tissue modifying drug, such as minocycline, around a neural probe with a prolonged release-curve, would affect the microglia and astrocytic reactions as well as the neuronal survival, over time.

Next generation of neuro-electronic interfaces

Considering the highly complex milieu of the brain and complex dynamics at the tissue/implant interface, it might not be surprising that not any one single approach (such as making ultrathin flexible polymer-probes) has yet completely solved the problem of creating a truly biocompatible neuro-electronic interface. This may be due to, the multidimensionality of the problem where any single cause can lead to failure in this aspect. Thus, the next generation of neuro-electronic interfaces will likely need to incorporate a number of features rather than any single one, such as tissue matching mechanical properties and density as well as a method of tissue friendly implantation and the potential additions of protective pharmaceuticals.

Aims

The overall aim of this thesis can be summarized in the pursue towards a truly biocompatible neuro-electronic interface that can remain chronically stable and functional in the brain without damaging the neural tissue around it. This gets down to the following specific aims:

- I. The design and construction of two new types of flexible neuro-electronic interfaces, incorporating identified key parameters for true biocompatibility as well as the possibility for evaluating the tissue in the close vicinity of the interface.
- II. The development and evaluation of implantation methods for these interfaces without distortion of the implants and with minimal insertion trauma.
- III. The evaluation of these implants in vivo by assessing the surrounding tissue response with histological methods, and the quality of electrophysiological recordings over time.
- IV. The evaluation of the effect on brain tissue surrounding neural implants utilizing a new method of local drug delivery from the implants themselves.

Methods & Development

The methodology, when developing new pieces of technology, especially in a highly interdisciplinary field such as brain machine interfaces, is inherently a combination of using and adapting more or less established methods as well as developing new methods. In this thesis the method development has been a heavy part, deeply intertwined with experiments performed for evaluation. Typically, the results gained from pilot experiments, both in vitro and in vivo, has guided the direction of method and technology development, in turn giving rise to new experimental data, and so on. In this iterative process the continuous refinement of technology and methodology has gone hand in hand with a better understanding of the biological system that it is applied to. Therefore, this method description is also to some part an account of the developmental process during the project.

Development of Neuro-electronic interfaces

In the follow section, the development and manufacturing process of two novel types of mechanically compliant neuro-electronic interfaces, as well as methods for implantation and a method for drug coating neural implants, will be described.

Material considerations

For all neuro-electronic interfaces mentioned in this thesis work, the insulating polymer of choice was Parylene-C, both because of its high standard of biocompatibility and excellent barrier properties, but also as it is FDA (U.S. Food and Drug Administration) approved for use in humans, making a future transition to clinical applications easier. Another important reason for choosing Parylene-C is its compatibility with vacuum deposition at room temperature, thus not ruining temperature sensitive organic materials. This deposition method provides for a highly flexible approach, where the Parylene-C forms a tight seal around the substrate, void of layers that can delaminate. The choice of metal was gold, mainly because of its biocompatibility, corrosion resistance, relative softness and future possibilities for surface modifications based on golds ability to interact with sulphur¹³².

A thin electrode array flexible in three dimensions

In Paper I, we developed a so called 3D-array of gold electrodes flexible in all three dimensions, including a flexible tethering and with protruding recording sites for better anchorage inside the tissue. The method of production, by laser milling of thin gold sheets (4 μm thick gold foil 23 $\frac{3}{4}$ karat) makes it easy to implement new layouts and therefore to tailor the design for different applications and/or targets. In fact, over ten different layouts were developed, manufactured and evaluated before settling on the final design, as presented in Paper I.

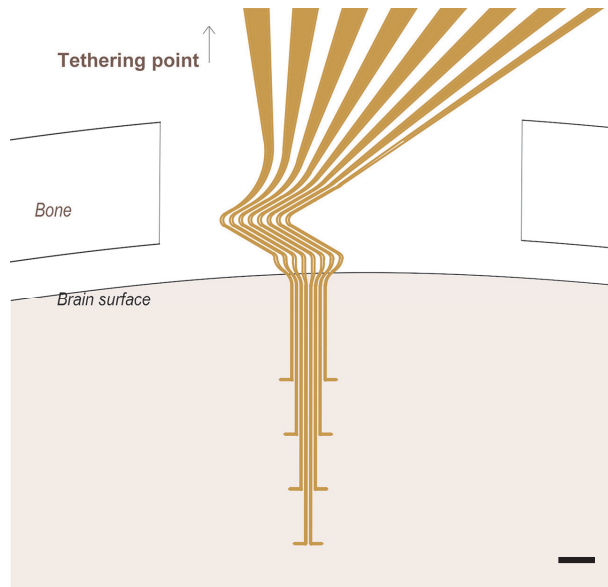


Figure 1. Schematic drawing of the electrode array, showing the flexible suspension between brain and the tethering part and the protruding recording sites. Scale bar 250 μm . From Paper I.

Manufacturing of the 3D-array

The 3D-array, consists of eight, wavy gold electrodes each individually separated and flexible/extendable in three dimensions, each equipped with a distal protruding branch from where neuronal recordings can be made (Figure 1). The dimensions and the shape of this array was designed to allow recordings from different layers of the rat somatosensory cortex, corresponding to the depths of 1800, 1400, 1000 and 600 μm below the surface of the brain.

In short (more details can be found in Paper I), the layout was designed in AutoCAD and converted into a laser milling scheme, transferred to the Laser Milling System (Laser mill 50, standard micro-milling system). To make the array, a thin gold sheet was attached to a glass slide with Polyethylene-glycol (PEG-3000) in order both to flatten and support the fragile gold sheet during subsequent handling. The shape of

the 3D-array was then milled from the gold sheet using pulsed laser light (50 Hz, 532 nm, 10 J/cm²), resulting in 8 individually separated gold leads, 4 µm thick and about 10 µm wide. After milling, the glass holding the array was mounted with silicon (Elastosil A07) to a metal frame, and the individual channels of the array were manually soldered to a printed circuit board (PCB). The soldering points were insulated by applying a water-resistant acrylic coating (HumiSeal) over the leads and the connectors on the PCB.

The metal frame containing the 3D-array was immersed in 70% ethanol, dissolving the PEG and making it possible to manually slide the glass away from the electrode array. As the electrodes are extremely delicate, a continuous challenge was to keep the array intact and to avoid the individual channels to adhere to each other. For this reason, different support strategies had to be developed for the different steps in the manufacturing process, and during the course of the project several methods were tested and evaluated. For example, insulating the whole glass slide with a sheet of Parylene-C and then aligning and re-milling the shape of the array once again before parylenization of the other side. However, in addition to being cumbersome and time-consuming, this process increased the width of the individual channels due to the need for dimensional margins during the second laser milling. In the end, a more efficient method proved to be the use of several holding points in the initial milling scheme, in effect connecting the individual channels by bridges, which could later be removed.

After release of the array from the glass slide, it was coated with 2 µm of Parylene-C and after this the holding points could be cut away by means of laser milling. The 2 µm Parylene-C layer proved enough to support the conformation of the array and to avoid short circuits, but in order to seal the exposed gold where the holding points had been cut, a second layer of 2 µm of Parylene-C was applied to the array.

To construct the active recording sites, the gold was exposed at the tip of each channel by selectively photo-ablating the Parylene-C coating (25 Hz, 355 nm, 1.4 J/cm²), up to around 10 µm. To serve as ground wire, a silver wire (150 µm in diameter) was soldered to the assigned animal ground channel at the PCB, and finally the array was removed from the metallic frame.

Embedding of the electrode array

Very flexible electrode arrays, like the 3D-array, are too soft to be implanted into the brain on their own (in fact the 3D-array bends even upon attempting to penetrate a water surface). One strategy to overcome this issue, whilst avoiding additional support such as guiding rods or cannulas, is to embed the flexible array into a biocompatible gelatine based mix, which is hard enough to penetrate the brain when dry, but which softens and dissolves after implantation, leaving the flexible electrode array in the tissue.

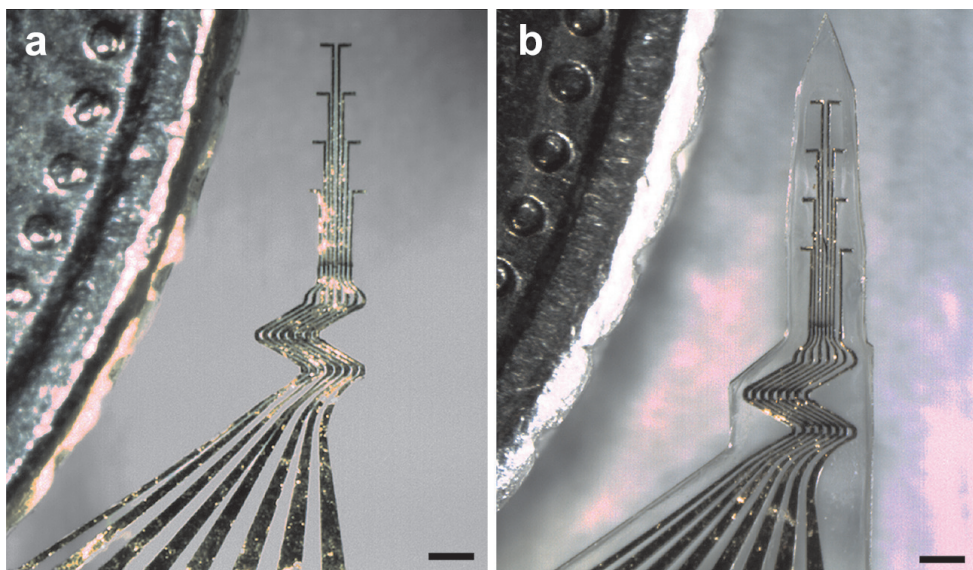


Figure 2. Photographs (by light microscopy) of a cut out electrode array next to a five penny coin, a) before being embedded, and, b) after being embedded into a gelatine matrix. Showing the retaining of the relation and shape of the individually separated channels. Scale bar 250 μm .

Several different methods for embedding and implanting the 3D-array were evaluated during the course of this project, for example embedding the array into a whole sheet of gelatine and cutting out the shape of the implant by means of laser milling. Even if this method gives good control of thickness and the two-dimensional shape of the construct, the tip was not sharp in the 3rd dimension, and was therefore prone to cause dimpling of the brain surface before penetrating it. Other methods such as dip-coating or drop coating (basically moving a droplet of dissolved gelatine over the probe) were also evaluated, sometimes giving good results, but making it hard to reproduce the exact same shape and dimensions of the embedding. In order for the gelatine embedded construct to reliably penetrate the brain surface, while not being too large causing dimpling, but still allow for a slow and smooth implantation without conformational changes of the delicate array, a high degree of control of both dimensions and the 3D-shape of the embedding proved crucial. In the end, the best method turned out to be micro-molding, in effect giving full control over the shape and size of the implant. To accomplish this, custom made molds with sharp tips were manufactured in polymethylmethacrylate (PMMA). Four different molds were produced, with the same width (400 μm at its widest) but with different thicknesses, (75 μm , 100 μm , 125 μm and 200 μm) in order to try out the minimum thickness of the embedding required for implantation. Both 75 μm and 100 μm were generally too weak to reliably penetrate the pia of the brain or without deformation during implantation (leading to brain dimpling or failure to penetrate the surface all together), and 200 μm although strong enough for

penetration, caused unnecessary large footprint and dimpling of the brain surface. The 125 μm thick probes, however, proved sufficiently stiff for reliable implantation (mean buckling force=0.373 N, SD=0.18 N, n=5), without visible dimpling of the brain surface, and (as shown later on) with retained conformation of the electrode arrays after implantation.

The material in the gelatine matrix was constituted not only of gelatine (gelatine-B 27,5%) and water (Millipore 64,2%), but also of PEG 400 (6,9%) in order to minimize shrinkage and deformation of the array conformation during drying, as well as glycerol (1,4%) serving as a plasticizer to reduce the brittleness of the matrix composition.

Basically, (more detail in Paper I), the gelatine embedding was prepared by continuous mixing of the substances during heating up to 70°C, and then slowly injecting the mix into the mold, already holding the electrode array. The mix was left to dry inside the mold in 21% humidity for 24 h, and another 24 h with the top lid of the mold removed, before the probe was dry enough to be detached. The water content in the dried implants was estimated by weighing the probe before and after drying and was found to be below 4%. Finally, probes were dip-coated twice in 5% Kollicoat™ (polyvinyl alcohol/polyethylene) and ethanol, forming a thin film, that dissolves in contact with environments of pH > 5.5¹³³ in order to further delay the water uptake into the embedding¹³⁴ and avoid premature swelling and softening of the implant during insertion of the electrode array to the intended target.

A low density and flexible tube-like electrode

In paper II, a tube-like electrode that consists of an outer Parylene-C shell holding an internal gold sputtered polyester wire surrounded by dry glucose that is hard enough to penetrate the brain tissue, was developed. After dissolution of the glucose core inside the target tissue, the construction becomes both very flexible and with a density close to that of the brain tissue itself. The conducting lead is protected inside the tube, and the electrical contact with the tissue outside is via the liquid interface at the orifice. This design does not only provide a flexible and low density electrode, but also enables sectioning of the electrode inside the tissue without dislocation, allowing for a detailed analysis of the tissue in direct vicinity of the tube interface as well as further out.

Manufacturing of the tube electrode

A low-density conducting core was manufactured by sputtering a polyester fibre (25 μm), with a thin (50 nm) layer of gold. This conducting core was coated with glucose by electrospinning and then the whole construct was insulated with Parylene-C. Finally, an opening in the insulation was made (using focused laser) in order to enable communication between the implant and neural tissue.

Different materials were evaluated to serve as the dissolvable core inside the tube, including gelatine as it has proved beneficial for the tissue in previous studies. However, the swelling of gelatine tended to induce cracks and stretchmarks in the Parylene-C coating, thus invalidating this choice. Another candidate, glucose, which serves as natural fuel of neurons proved to be a promising candidate. Initial tests showed that the glucose core provide enough strength for implantation when dry, but then dissolved quickly, without swelling and causing damage or deformation to the tube.

Several approaches for coating the conducting core with glucose were evaluated, including dip coating in either melted glucose or glucose solutions of different concentrations, crystallization of glucose around the core as well as micro-molding. In the end, however, the method of electrospinning was chosen, mainly because of the high yield (many tubes could be coated at the same time), and the relatively uniform coating thickness, which after a selection process could provide tubes of similar dimensions. The glucose applied in this way also proved to form a relative solid structure, with densities ($1.57 \pm 0.2 \text{ g/cm}^3$) close to that of the crystalized powder glucose (1.56 g/cm^3).

More details on the electrospinning can be found in Paper II, but in brief a glucose-based solution was ejected from a syringe (anode) to the conducting core (cathode), with a voltage of 20 kV applied in between, resulting in the wires being coated with a layer of 20-30 μm of glucose after approximately 40 minutes.

After electrospinning, the wires were insulated with a layer of Parylene-C (2 μm), manually cut to a length of 3 mm and an angle of around 45° , and then coated with one more layer of Parylene-C (2 μm), to seal the tips. This resulted in tubes with a mean diameter of $73 \mu\text{m} \pm 9.7 \mu\text{m}$.

The orifice ($\sim 20 \times 60 \mu\text{m}$) in the coating (serving both as recording site and dissolution channel for the glucose) was made around 1100 μm from the proximal end, by photo ablation (355 nm, 1.4 J/cm^2). Separate in vitro tests (see characterization methods below) showed that the glucose in a 3 mm tube, dissolved from an orifice of this size, in less than 2 hours.

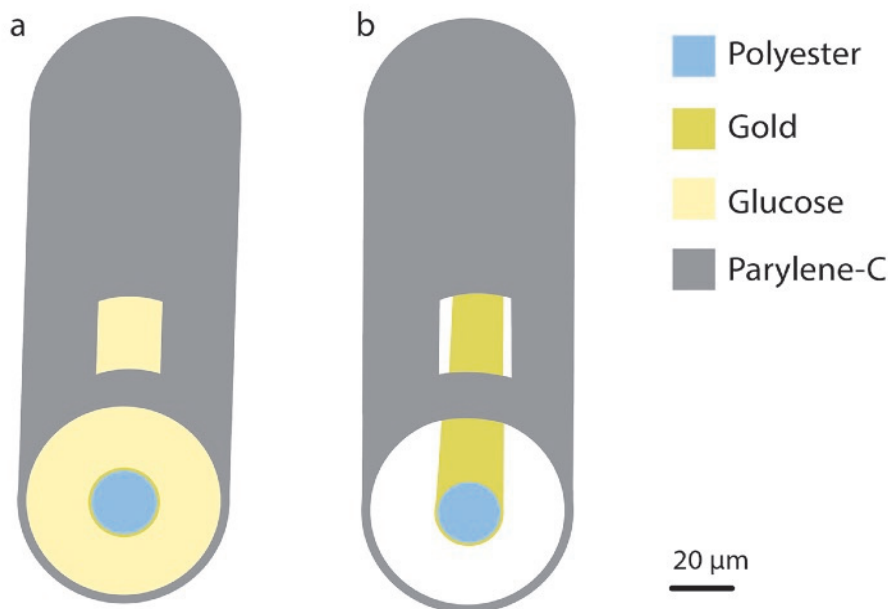


Figure 3. Schematic drawing of a tube electrode, a) before dissolution of the glucose core (indicated as light yellow), and b) after the dissolution, leaving the Parylene-C (gray) tube hollow, exposing the conducting lead inside, as constituted by a polyester fiber (blue) sputtered with 50 nm of gold (yellow). Scale bar 20 μm . Adapted from Paper II.

Coating of the tube-electrodes

Although the tubes had enough strength when dry to be implanted without any additional support, they were coated with a thin layer of gelatine in order to provide additional strength for minimizing potential deviation during implantation, and as it has been shown that gelatine coating can alleviate the tissue reaction surrounding a neural implant^{38,111}. The gelatine (type A, 300 Bloom strength) was prepared as a low concentration (10 wt%) solution, that was heated to 60°C under magnetic stirring, and then drop coated onto the electrode. Basically, a drop of the solution was ejected from a syringe and slid along the tubes to form a thin ($9,7 \mu\text{m} \pm 3,3 \mu\text{m}$, $n=16$) layer. Before implantation, the tubes were attached proximally to a stainless-steel needle (200 μm) with a drop of gelatine serving as a holding point to the micromanipulator used during implantation. In this manner, the gelatine holding point could be dissolved after the tube had been implanted to the intended depth, and the tube could be released from the micromanipulator without ever touching it.

Drug coated implants

In Paper III, an additional strategy for pharmacological modification of the tissue response was adopted. Basically, a new method for local and sustained drug release

surrounding neural implants was developed. The delay of the release was controlled via the use of degradable nanoparticles loaded with drug content, and the location of the release was controlled by embedding the particles in the dissolvable gelatine coating surrounding the implant.

Drug loaded nanoparticles

The nanoparticles, made of the biocompatible and biodegradable polymer PLGA¹³⁰, loaded with minocycline (an antibiotic known for inhibiting the microglia reactivity¹²³ were prepared by a so called single oil-in-water technique previously described¹³¹. The resulting nanoparticles from this process were 220 ± 6 nm in size, with a drug content of $1.12 \pm 0.01\%$, and with an in vitro release where there is an initial burst of 20% of the drug content during the first day, followed by a prolonged release during the next 30 days.

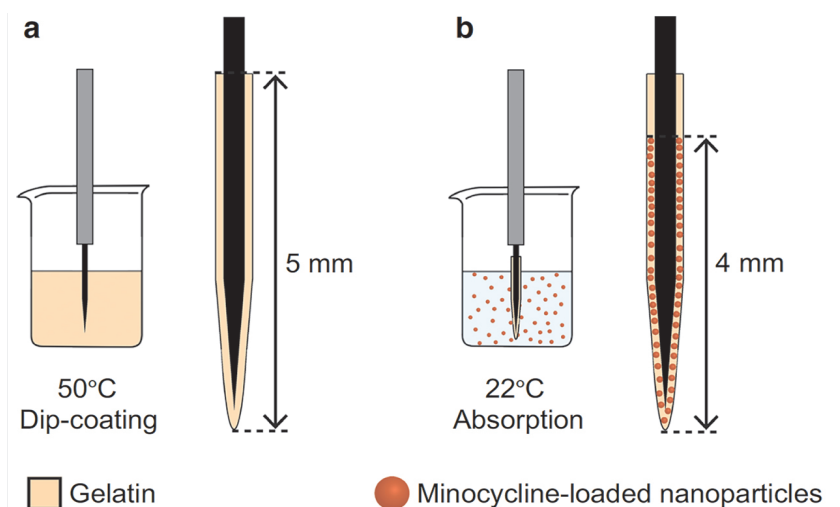


Figure 4. Schematic illustration of the method of a) first dip coating the implant in gelatine to form the coating, and then b) the immersion of the coated implant into a solution of nanoparticles, resulting in an coating that has absorbed the nanoparticles. From Paper III.

Coating of implants

The implants used for drug coating, was intentionally made stiff, in order to produce an initial stab-wound trauma and elicit a measurable tissue response. Stainless steel needles (diameter= 100µm) were insulated with 4 µm Parylene-C and coated with gelatine. A 30 wt% gelatine solution was prepared by mixing gelatine (porcine, type A, 300 Bloom) in artificial cerebrospinal fluid, and then heated to 60 °C during magnetic stirring for 1 hour. The implants were dip coated in the gelatine mixture with a retraction speed of 600 µm/s and dried at room temperature (RT) in the dark together with silica gel beads (less than 1% humidity). This gave rather uniform coatings of 4.8 ± 0.9 µm. By immersing the dry gelatine coated implants into a room

temperature water based solution of nanoparticles containing the drug (minocycline), waiting for the gelatine to swell and then removing the implant, the nanoparticles were absorbed into the gelatine coat. After drying, the thickness of the gelatine coat was almost doubled (9.1 ± 1.2 nm, $n=7$), indicating trapped nanoparticles inside the gelatine coat.

Characterization of drug content

In order to estimate the drug content of the coated implants, fluorescently labelled particles were manufactured. Details are described in Paper III, but in short, a fluorescent dye (Alexa Fluor 568) was conjugated to the PLGA that was used for making the nanoparticles, resulting in particles with a diameter of 150 ± 2 nm. Implants were then coated in the same manner as above, and imaged with confocal microscopy (Zeiss LSM 510), showing a relatively uniform distribution of particles in the gelatine coating. The increase in thickness of the gelatine coating was similar between fluorescently labelled particles and drug loaded particles (almost doubling), indicating that the loading efficiency was at least not radically different, further suggesting that the distribution of the drug loaded nanoparticles, although a bit larger than the fluorescent particles, was likely also rather uniform.

As the concentration of nanoparticles in the solution used for immersion was known, it was possible to estimate the drug content of the implants by measuring the diameter of the swelled gelatine by optical microscopy, in the end giving an estimate of about 1 μ g of nanoparticles (34 ng minocycline) for each implant.

Characterization methods

The neuro-electronic interfaces developed, were characterized by a number of methods to make sure they held up to mechanical, chemical and electrical standards suitable for functional neuro-electronic interfaces.

Impedance measurements

The impedance at 1 KHz, was measured in 0,9 % saline solution, using a 3-cell system (Gamry G300) to characterize the electrical impedance of neural electrodes, making sure they were suitable for single neuron recordings, and to determine the amount of deinsulation (3D-array), and size of orifice (Tube electrode).

Compression test

For evaluating the mechanical compliance of the neuro-electronic interfaces, compression tests were performed (Zwick GmbH & Co. KG). The force was measured during compression of the probe, and the flexibility was estimated by means of the buckling force, represented by the maximum force applied before the probe bends or breaks.

SEM-imaging

All neuro-electronic interfaces were imaged with Scanning Electron Microscopy (SEM), both for ensuring the integrity of the Parylene-C coating during manufacturing and after bending and/or soaking tests, but also for confirming the size of the deinsulated area at the active recording sites.

Glucose density

To evaluate the density of the glucose (applied via electrospinning) inside the tube, quantification of the glucose content was made by dissolving open tubes in 0,9% saline at 37°C, and then measure the glucose concentration with a glucose-meter (Contour next link 24). This way the glucose density could be calculated, as the volume and the concentration of the dissolved glucose, as well as the original volume of the dissolved tube were known.

Glucose dissolution

To measure the dissolution-rate of the glucose content, tubes with an orifice of 20x60 µm were inserted into a 36°C agarose mix (0.2% agarose in saline 0,09%), and imaged with optical microscopy, with intervals of 1 min for up to 2 hours. In this way, as the border between dissolved glucose and undissolved glucose is visible, the dissolution time can be measured.

Tissue analysis

Different aspects of the neural implants and the implantation strategies, such as the electrode conformation in the brain after implantation or the tissue reaction surrounding the implants, were evaluated by analysing the tissue. Either, by the clarification of the brain tissue, revealing the conformation of electrode while still in the tissue, or by slicing and staining the brain by means of immunohistochemistry in order to visualize specific cell types.

Tissue clarification

To examine the conformation of the delicate 3D-array inside the brain, thick sections of tissue with the undisturbed 3D-array still in situ, were clarified. The method is described in Paper I, but in brief, after tissue fixation with PFA (including transcardial perfusion), the rat heads with the electrode and connector still in-situ, were snap frozen in isopentane on dry ice. This was done in order to fixate the arrays position and conformation in the tissue during subsequent handling. While frozen, the top part of the skull and upper part of the electrodes were sawed off with a high speed circular diamond-saw. Then the deeply frozen head was cut coronally in ~5

mm thick slices with a meat cutter in such a manner that one of the slices contained the whole electrode array.

To make the tissue slices containing the array transparent, they were rinsed in phosphate buffered saline (PBS), dehydrated through increasing concentrations of ethanol (50, 70, 99.5%), and then clarified by immersion in increasing concentrations (50, 70, 100%) of methyl salicylate in ethanol, at least 5 h in each immersion. After this, the tissue was transparent enough to clearly visualize and image the conformation of the electrode array with optical microscopy, and to compare this with the conformation of the electrode array in images taken before implantation.

Immunohistology

To evaluate the biocompatibility of the neural implants, their effects on the surrounding tissue, was analysed by means of immunohistochemistry. By staining brain tissue slices, with antibodies associated with specific cell types and then connect these to fluorescent markers of different colour, it is possible to identify and image different cell types. In this case the most interesting cell types were neurons as well as microglia and astrocytes, which are important parts of the foreign body response.

Tissue preparation

Before immunostaining can take place, the tissue needs to be fixed to preserve cells and tissue components by cross-linking proteins. This was done through the process of transcardial perfusion. The animals were deeply anaesthetized with pentobarbital, followed by transcardial perfusion with saline to clear away blood, and thereafter with paraformaldehyde (PFA, 4%) in phosphate buffer, followed by brain dissection and post fixation into PFA overnight. After cryoprotection by immersion into sucrose (20%) until equilibrated, brains were frozen and stored at -80°C. Brains containing tube electrodes were frozen with the tube still in situ, whereas stiffer implants required explantation. The frozen brains were horizontally sectioned (16 µm) in a cryostat and mounted onto glass slides and stored at -80°C, until staining.

Immunohistochemistry

After thawing and rinsing (3xPBS) the frozen brain sections were blocked with either goat or donkey serum in PBS, in order to prevent unspecific binding and triton X-100 for permeabilizing the cell (1h, RT). Subsequently, sections were incubated over night at RT with primary antibodies for the specific cell types; Neurons (NeuN), Astrocytes (GFAP), microglia (Iba1) and activated microglia (ED1/CD68), and rinsed again before being incubated with secondary antibodies conjugated to fluorescent dyes (Alexa Fluor 488, 594 or 647) to visualize the cell specific labelling, and DAPI to visualise all cell nuclei. Finally, slides were rinsed, cover

slipped (using polyvinyl alcohol mounting media), and stored at 4°C until evaluation and image analysis.

Imaging and quantification.

Stained tissue sections, from relevant depths of the cortex, were imaged under a fluorescence microscope (Nikon eclipse 80i microscope) using a 10X or a 20X objective and photographed using a Nikon DS-R1 camera mounted on the microscope, or for more detailed imaging, a multiphoton laser scanning microscope (Zeiss LSM 710 NLO, MaiTaiT) with a 20X objective.

The stained cell types; neurons (NeuN), astrocytes (GFAP), and microglia (ED1/Iba1) as well as all cell nuclei (DAPI) were quantified within different regions of interest (ROIs) with a set radius measured from the border of the implants. For the tube electrode, the ROIs were 0-9, 0-20, 0-50, 20-50 and 50-100 μm , around the tube, both at the level of the tube orifice and along the shaft, as well as a similarly sized area in a peripheral area of the section to serve as baseline for the neuronal density (NeuN). For the drug loaded implants, the ROIs used were 0-50 μm and 50-100 μm .

Both the total number of cells (DAPI) and neurons (NeuN), were quantified in the regions of interest, by manually counting the stained cells. Only cells staining for NeuN with a DAPI positive cell nuclei were counted. Quantification of GFAP, ED1 and Iba1 positive cells, i.e. cells having a morphology with overlapping network-forming processes, was performed by measuring the fluorescent area fraction corresponding to the relevant colour/ wavelength of the fluorescently conjugated antibody, within the ROI's.

Statistical Analysis

For the drug loaded implants, groups were compared using Mann–Whitney test, and for the tube electrode, a non-parametric Kruskal Wallis test with Dunn's multiple comparison test was used for analysing ED1, GFAP, NeuN and DAPI and Friedman's test with Dunn's multiple comparison test for the Iba1, ED1 and ED1/Iba1 comparison at different depths around tubes with an orifice. In all cases, p-values less than 0.05 were considered significant.

Electrophysiology and data analysis

The most direct method of evaluating the performance of neuro-electronic interfaces, is to conduct in vivo electrophysiological recordings in awake and behaving animals, and then evaluate the quality of these recordings. This was done both by looking at basic metrics such as noise level and signal to noise ratios (SNRs) over time, as well as perform estimations of the single unit stability over

time, something that would give indications of the mechanical stability of the probe in the tissue.

Electrophysiological recordings

All in vivo electrophysiological recordings were performed inside a Faraday cage (to minimize electromagnetic disturbances). Rats with an implanted 3D-electrode array were placed inside a recording cage and the electrodes were connected to a Plexon data acquisition system (OmniPlex, Plexon Inc) with a 20x head stage. The cables were connected via a commutator (Plexon), to prevent cable entanglement and enable unhindered movements of the awake animals. Neuronal activity was recorded 2-5 times a week for up to 3 weeks (sampled at 16 bit, 40 kHz per channel, bandpass filtered 0.25 – 8 kHz). In some cases, to further verify the biological nature of recorded single units, tactile stimulations of the contralateral hind paw was trigger from a Master 8 stimulator, at least 150 event per session, and the events were recorded together with the neural recordings.

Data analysis

To remove arbitrary human errors, all data processing was automated and made by in-house Matlab scripts, based on already established and published methods for spike sorting and validation ¹³⁵.

The main concern for evaluation of flexible neuro-electronic interfaces, is to assess not only the basic functionality of the interface, but also to try to estimate the relative stability in the neural tissue. Thus, the analysis was focused on sorting out single neuronal activity, more specifically, single units or spikes (the extracellular representation of the individual action potentials). This requires that the spikes are filtered out from the raw data and then sorted to separate spikes originating from different neurons from each other. This is especially important when the neuro-electronic interface is biocompatible and flexible, as it is potentially in relatively close proximity to the neuronal tissue picking up activity from several neurons at the same time. During this process, reliable unit verification is important to reduce the probability of false positives of single units, contaminating the analysis.

Spike sorting and validation

In short, (details in paper I), the low frequency content (< 300 Hz) was filtered away, the noise level was estimated, and then raw spikes were picked out from a simple amplitude threshold ($-4 \times \text{noise}$). These raw spikes were then sorted based on their waveshape features, by means of principal component analysis (PCA) and cluster analysis. Outliers and spikes with more than 0.5 % inter spike intervals (ISIs) shorter

than 1 ms (indicating that spikes are too close to each other in time to represent true neurons that have a refractory period), were removed. Further validation of the spikes was done by removing spikes that didn't reflect known aspects of the action potential¹³⁶ and thus likely represented artefacts. Finally, the signal to noise ratio was calculated for each spike by dividing the peak-to-peak amplitude with the signal noise.

Performance over time

The recording performance over time, was evaluated first of all on general metrics, such as the noise levels, SNR of valid single units, yield (number of valid single units) and the absolute amplitudes of single units and how they change over time. This gives a rough picture of the overall stability. However, to further evaluate the main issue, the capacity to record from the same neuron over time, the analysis was expanded to also include waveform cross-correlations between single units on the same channel but on different days of recording.

Animals and surgery

Animals

In the experiments using rats (the 3D electrode array and the tube electrode), female Sprague-Dawley rats (Taconic) were used. In the mouse experiments (local drug delivery implants), transgenic mice (male and female; B6129P-Cx3cr1, Jackson laboratories, USA) expressing green fluorescent protein (GFP) in brain microglia were used. All animals had free access to food and water and were being kept in a 12-h light and dark cycle with a constant temperature of 21°C and 65% humidity. Their weight was monitored, making sure they followed a normal weight curve. Approval was obtained in advance from the Malmö/Lund Animal Ethics Committee for all Animal Experiments, and all experiments follows the regulations of this approval.

Anaesthesia

For implantations of the 3D-electrode array (rats) and implants for local drug delivery (mice), anaesthesia was induced by placing the animals in a chamber of 2% isoflurane (Isoba®vet., Apoteksbolaget, Sweden) with 40% oxygen and 60% nitrous oxide. For implantations of the tube electrode (rats), animals were anaesthetized by intraperitoneal (i.p.), injection of a mixture of fentanyl (0.3 mg/kg

body weight) and Domitor vet (medetomidine hydrochloride, 0.3 mg/kg body weight).

Surgery and craniotomy

After induction of anaesthesia, the surgical area of the animals head was shaved and they were attached to a stereotactic frame (KOPF instruments, USA) under a stereomicroscope (Leica Microsystems, Germany), and placed on a heating pad with continuous monitoring and feedback of the body temperature. The eyes were shielded with a wet (physiological sterile NaCl) compress, which was continually refreshed during surgery. The scalp was disinfected and the animal received local subcutaneous injection of xylocaine/adrenaline prior to a midline incision being made in the scalp. Connective tissue attached to the skull was removed and the skin deflected using forceps. Under stereotactic control, the craniotomies (ranging in number, size and shape for different preparations) were carefully drilled through the bone of the skull (with a high speed drill, Dest 300 IN, model MM 323IN, Silfradent, Italy). For rats being implanted with the 3D electrodes, three small custom-made titanium screws with a length of 1900 μm were screwed $\sim 570 \mu\text{m}$ into the bone of the skull to serve as anchorage for the electrode and dental cement.

Implantation

In rats, the dura mater was incised and deflected before the implantation, but in mice the dura was left intact as it is much thinner and can be easily penetrated by a microelectrode. All implantations were made by attaching the implant to a hydraulic micromanipulator (KOPF instruments, USA), and then carefully, under stereotactic control insert the implant into the brain tissue to the intended target depth with controlled speed. All implantations were made, using a burst mode in steps of 50 μm for the initial 100-150 μm at a burst speed of 2 mm/s, both to minimize the dimpling of the brain surface and to limit the time that the implants were exposed to fluids on the brain surface. After bursting through the pia mater (rats) and the dura mater and the pia mater (mice), the implants were being slowly implanted with speeds of 50 $\mu\text{m/s}$ for the tube and 3D electrodes (rats), and 500 $\mu\text{m/s}$ for the drug loaded implants (mice).

For in vivo recordings (the 3D electrode array), the animal ground wire was circled around one of the screws and then inserted under the bone in a small craniotomy on the contralateral side of the skull. A piece of collagen biomatrix (TissuDura, Baxter International Inc., USA) was positioned in the hole on top of the animal ground wire and the wire was attached with dental cement (RelyX Unicem, Elipar, 3M ESPE).

After implantation, animals were given postoperative analgesia, the craniotomy was sealed using dental cement and the skin was closed using surgical clips. In the case of Domitor sedation, animals were injected with an antidote to release the anaesthesia, and were supervised during awakening in their home cage.

Results & Comments

In this section, the mechanically compliant probes as well as the drug coating method that was developed as described in the Methods & development section, were evaluated *in vivo*, by being implanted into the brains of rats or mice. The evaluation was then done either by analysing the tissue response (mainly the activation of glia cells and the neuronal densities around the implants) or by performing electrophysiological recordings of single neurons in the brain and analysing the data.

An array of ultrathin electrodes flexible in 3D

In line with the aims of this thesis, a neuro-electronic interface in the form of an array of thin electrodes, flexible in all three dimensions, and which could be implanted with retained conformation, was developed.

The very thin and individually separated gold leads, renders flexibility in two dimensions. The addition of a z-shaped suspension in the parts of the array located between the brain surface and the tethering point to the skull, adds flexibility in the dorso-ventral direction, resulting in an array with flexibility in all three dimensions. The reason for physical separation of the channels, beyond improving the flexibility for each lead, was to also allow for better tissue integration between the physical channels, potentially improving both electrode anchorage and diffusion in the tissue. For additional anchorage and flexibility, each channel was designed with a perpendicular, 100 μm long tip, holding the active recording sites. In the current design, the recording sites were positioned to correspond to cortical layers of the rat brain.

Implantation

To test the hypothesis, that thin electrodes flexible in all three dimensions, would result in a high positional stability in the tissue and thus give rise to stable recordings from single neurons, the arrays were implanted and evaluated in awake and behaving rats. In order to implant such a flexible electrode array in the brain, and to do so with minimal insertion trauma as well as without disturbing the shape or the

relative positions between the single electrodes, the whole array was embedded into a biofriendly gelatine-based matrix, stiff enough for implantation when dry, but that softens and dissolves after implantation. With an embedding thickness of 125 μm , the probe could be implanted to the intended target depth of 1,8 mm into the rat brain ($n=15$), without visible dimpling or bleeding of the brain surface. After implantation the gelatine was readily dissolved, leaving the flexible array inside the brain. Tissue clarification of the brains with the 3D-electrode arrays still in situ, 3 weeks after implantation, showed that the conformation was kept intact (Figure 5).

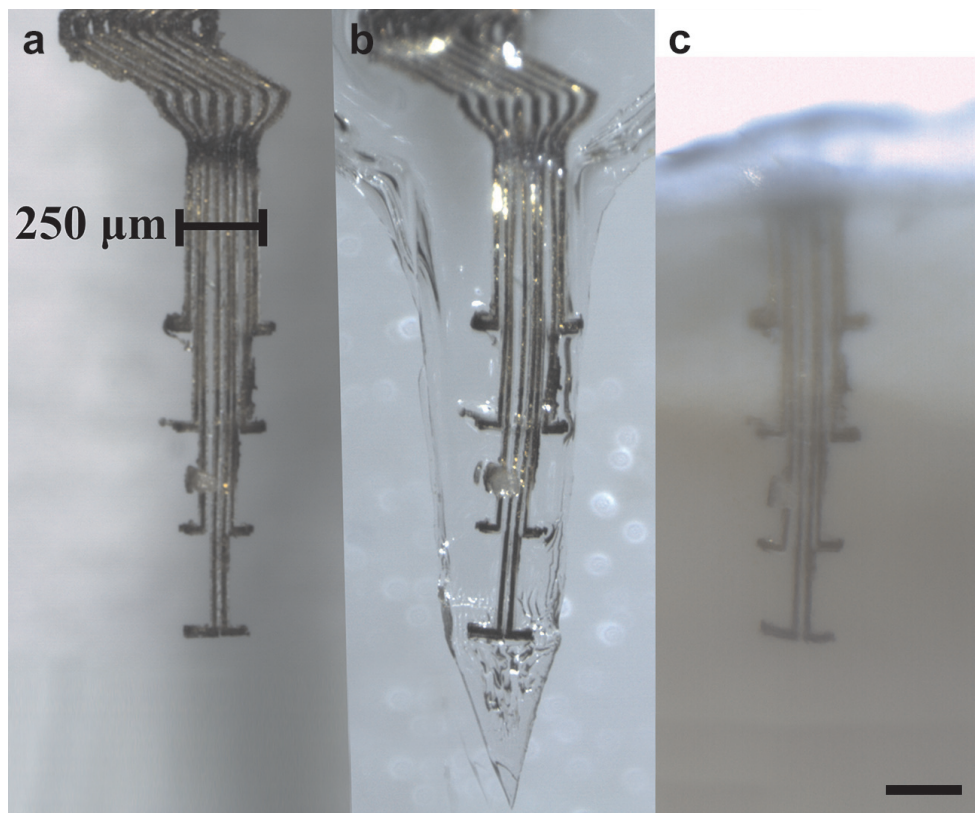


Figure 5. Electrode array, a) before embedding, b) after embedding into the gelatine matrix, and c) inside clarified tissue after 3 weeks of implantation. Showing a well preserved conformation between all steps, especially between pre implantation embedding and after being implanted into the brain. Scale bar 250 μm . From Paper I.

Electrophysiological evaluation

For proof of concept, in vivo electrophysiological recordings, in awake and behaving animals, were conducted 2-5 times a week for 3 weeks. Recordings showed generally good signal qualities throughout the whole experiment and it was clear that the 3D electrode array could readily pick up single-unit neuronal activity (spikes) on most of the channels (87,5%), with units of a higher signal to noise ratio (SNR) > 4 on 29,2% of the channels.

Short term stability

Single units, during individual recording sessions (up to 50 min), showed stable amplitudes and waveforms (Figure 6). As the amplitude of a recorded spike correlates to the distance between the neuron and the recording site of the electrode the positional stability within the recording sessions was assessed by looking at the amplitude variation of the same unit (as grouped by Principal Component Analysis) over time. To reduce the influence of noise, the variation was defined as the standard deviation of a one second moving average of the spike amplitude. The analysis showed a median variation of 8.65% (IQR=6.21%) across all identified single units. This indicates that the 3D-array, although not being completely still in the tissue, in any case remain stable enough to record from the same neuron within time periods that would involve the most commonly recurring types of micromotions caused for example by, breathing, normal head movements and vascular pulsation.

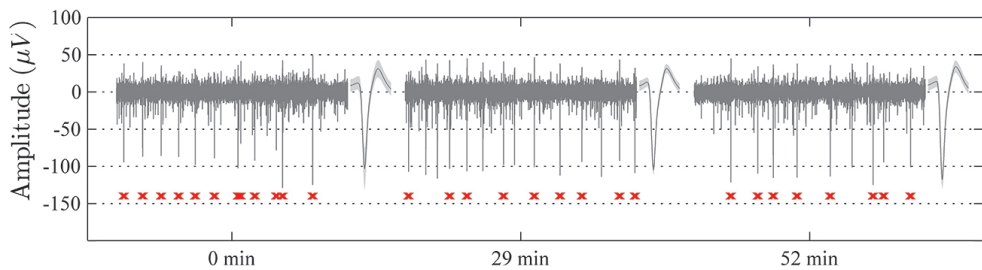


Figure 6. One second samples of recordings, in the beginning (0 min), middle (29 min) and end (52 min) of an hour long recording from one channel of the electrode array. Spike times are marked by the red cross on the timeline and the unit waveform (mean waveform \pm standard deviation) of the highest SNR unit is shown to the right of the sample. In this case the units are highly correlated (waveform correlation > 0.99) between the samples. From Paper I.

Long term stability

To assess the potential for long term stability, the units with the highest amplitudes (> 150 μ V) were selected, as these are assumed to be closer to the electrode and thus have a higher susceptibility to relative movements. Such high amplitude units that originated from the same channel and that had very high waveform correlation (> 0.99) between different recording sessions, were selected. In this case, these units, were assumed to represent the same neuron (although without other metrics such as

firing statistic or longer continuous recordings, it can't be entirely excluded that they are generated from different neurons). In total, 8 such highly correlated high amplitude units were identified, remaining stable from 1 up to 7 days, indicating that the 3D-array could in some circumstances remain stable enough to record from the same neuron up to a week, even when this neuron was very close to the electrode site.

Examination of the general recording metrics over time, shows that even if there was a slight increase in median noise levels during the time period (4.3–5.9 μV) the mean SNR and yield of units with high SNR also increases over time, indicating an improved situation rather than a deterioration. This suggests an ongoing integration between electrodes and tissue, in effect decreasing the distance between electrode recording sites and recordable neurons. Occasionally it was also possible to record units with exceptionally high amplitudes (up to 424 μV), suggesting that the electrode sites were in very close proximity to recordable neurons.

In total, these results, suggest that even if the 3D-array is not completely still in the tissue, it can remain very close to recordable units, and remain stable enough to follow the same neuron within a recording session and most likely also over longer periods of time.

A flexible and low-density tube electrode

Another approach towards a mechanically compliant neuro-electronic interface, resulted in the design of tube-like electrode with a stiff glucose core that dissolves after implantation, leaving only a hollow tube of thin Parylene-C in the tissue. This construct was assumed to be able to accommodate both for micromotions from tissue movements due to its flexibility, as well as accelerating forces like head movements, due to the tissue matched density. Further, the hollow tube construct was hypothesized to be sliceable inside the tissue without dislocation and thus allow for a detailed analysis of the inner zone of the tissue juxtapositional to the implant.

Implantation and sectioning

Tube electrodes (both gelatine-coated and un-coated) were implanted without any visible dimpling of the brain surface or bending of the probes, showing that the core of the tubes, while the glucose is dry, is stiff enough for reliable implantation without any additional aid. Stiffer and heavier tungsten electrodes of similar dimensions, insulated with Parylene-C and coated with gelatine in the same manner as the tube electrodes, were implanted contralaterally as controls.

The analysis of the brain tissue (after 6 weeks), showed that the tubes indeed could be sectioned in the tissue without visible dislocation or deformation (Figure 7). This made it possible to conduct a detailed analysis of the tissue in the inner zone (0-9 μm) from the border of the tube edge, both at the recording sites and along the shaft.

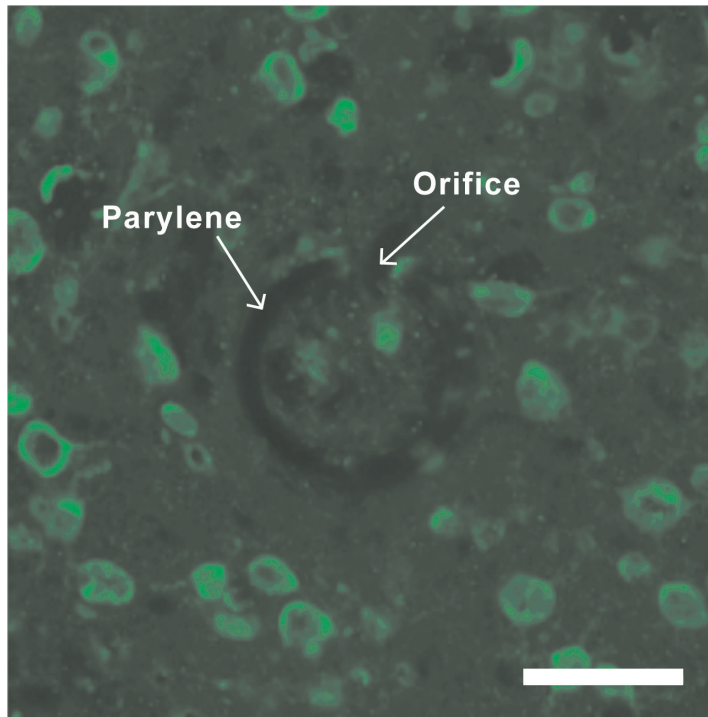


Figure 7. Confocal image of a brain slice with neurons stained in green (NeuN), showing the Parylene-C sheet of the tube electrode intact in the tissue slice, as well as the break in the Parylene-C, depicting the recording site. Scale bar 50 μm . From Paper II.

Tissue reactions

Generally, the foreign body response as measured by activation of microglia (ED1) and astrocytes (GFAP) was minute around the tube electrode, and significantly lower compared to the tungsten control, indicating substantial beneficial effects of the density regulation and flexibility of the probe. Moreover, there were no significant decline in neuronal densities (NeuN) at all surrounding the tube, when compared to the situation in baseline tissue (tissue located further away from the implantation site) (Figure 8). As a comparison, the neuronal density in the inner region (0-20 μm) around the tungsten control was only around 70 % of that of baseline tissue, indicating a substantial loss of neurons (despite the control being both untethered and gelatine-coated). However, as stiff probes such as the tungsten

control have to be explanted before sectioning^{36,40}, pulling out and/or rearranging the tissue in the inner zones⁴⁸. This was evident also in this study, by the non-circularity of the voids left in the tissue after removal of the tungsten implants. Thus, the most accurate and interesting comparison is that between the tissue around the tube, and unaffected reference baseline tissue.

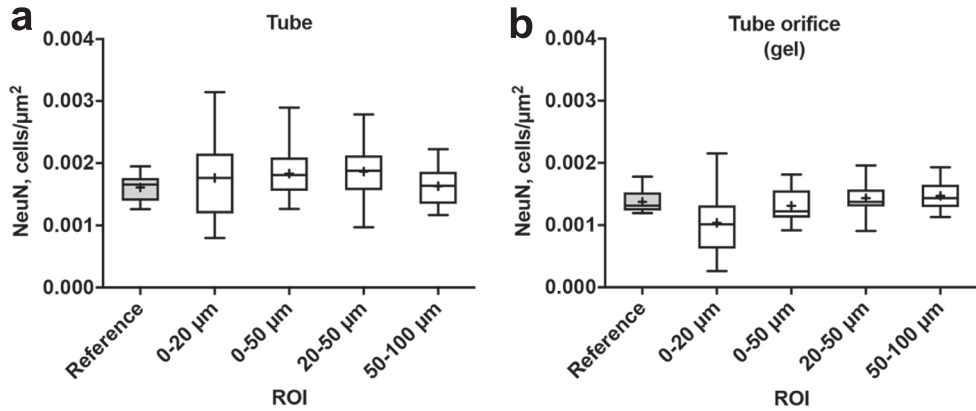


Figure 8. The neuronal densities (NeuN) normalised to reference baseline density (taken from reference tissue way from the implant), in ROIs of 0-50 μm , 50-100 μm , 0-20 μm , and 20-50 μm , around the a) tube shaft (coated with gelatine), and b) around the orifice of the tube (also coated with gelatine), showing no significant differences between the ROIs nor in comparison to the reference tissue. Boxplots indicate the 25th and 75th percentile, whiskers show min and max, line in box indicate median and plus (+) indicate mean. From Paper II.

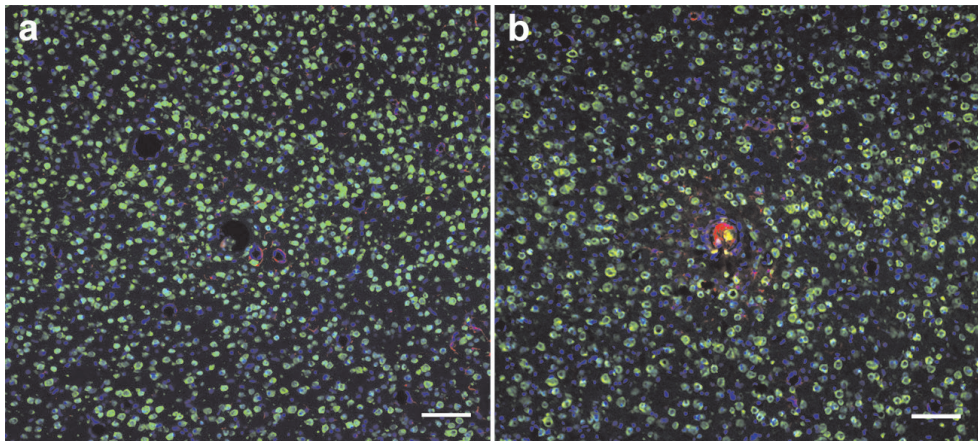


Figure 9. Representative fluorescent images (6 weeks after implantation), with neuronal density (NeuN) in green, astrocytes (GFAP) in (red) and all cell nuclei (DAPI) in blue. In a) a slice with the tube shank, and b) a slice with the tube orifice, showing GFAP positive staining inside the tube. Scale bar 50 μm . Adapted from Paper II.

As the orifice could be identified in the tissue sections as a visible break in the Parylene-C layer (Figure 7), the tissue outside the tube shank and outside the recording site could be compared to each other. Generally, very small tissue reactions were seen also outside the tube orifice, and there were no significant difference of activated microglia or neuronal density between the tube orifice and the tube shank. There were however signs of ED1 positive and GFAP positive staining (sometimes also NeuN) inside the tube (Figure 9), potentially suggesting migration of glial cells into the tube. Although this migration was not enough to clog the tube orifice, suggesting an open electrical interface to the neural tissue. The only significant difference between the tube orifice and tube shank was a slight increase in astrocytic reactions (GFAP) outside the tube orifice (50 – 100 μm).

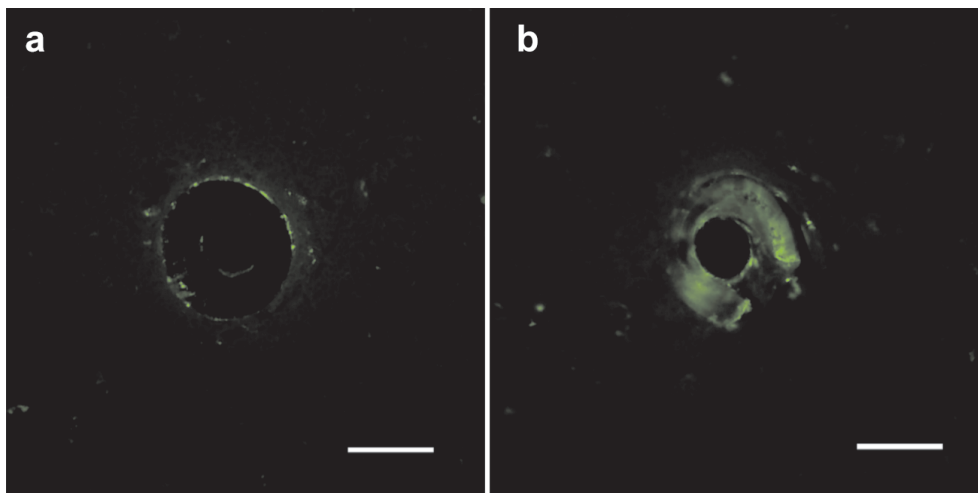


Figure 10. Representative fluorescent images (6 weeks after implantation), with activated microglia (ED1) in green. In a) a slice with the tube shank, and b) a slice with the tube orifice, showing very minute microglia reactions. Scale bar 50 μm .

When comparing the gelatine coated tubes with the non-coated tubes, there were no significant differences in any of the regions of interest, which is in contrast to what has been seen in previous studies where gelatine was shown to attenuate glia activation^{38,137}. Conceivably, this lack of effect of gelatine, is due to the already very low tissue responses to the implanted tubes.

Together, these results show a detailed analysis of the tissue in the innermost zone around electrodes, comparing the shank of the probe to the recording sites. The results of this analysis indicate that it is actually possible to design and implant a neural probe, such as the tube construct, into the brain with very small foreign body response and, more importantly, with normal neuronal densities right outside the probe itself.

Drug coated implants

In parallel with the development of mechanically compliant probes for reducing the tissue damage and the foreign body response, an additional approach was examined. Namely, the usage of the neural probe coating as a vehicle for local and sustained drug delivery. In this initial study, minocycline, known to inhibit microglia activation and having neuroprotective properties^{138,139}, was delivered via this route, to evaluate if this could have effects on the foreign body response and neuronal survival around a neural implant.

Previous work has developed and evaluated the process on how to encapsulate minocycline in nanoparticles¹³¹, for a sustained released (> 30 days) of the drug. Here, a method for embedding such nanoparticles into the gelatine coating of neural implants, to achieve local release, allowing for radically lower dosages than traditionally used, was developed and the effects on neural tissue was evaluated in vivo in mice.

Effects on the brain tissue in vivo

Implants, coated with gelatine containing minocycline loaded nanoparticles, were implanted into mice brains together with implants coated with only gelatine (as controls), and the tissue was analysed at two different time points (3 and 7 days post implantation).

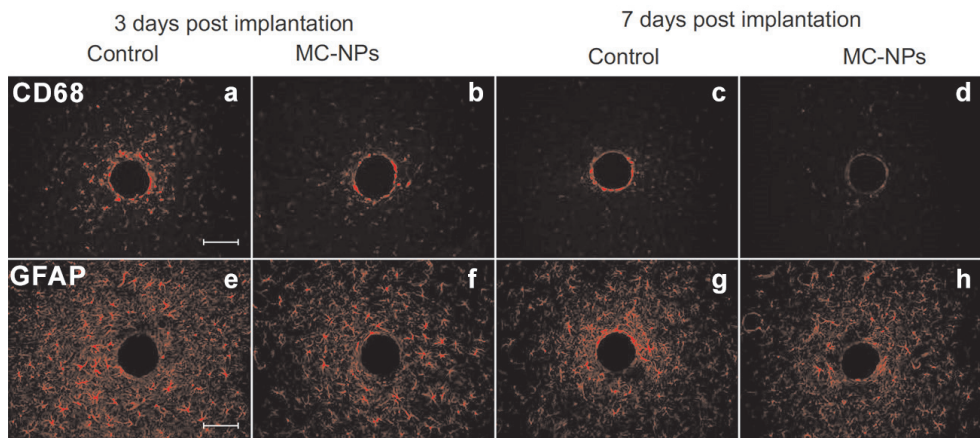


Figure 11. Representative immunofluorescent images of tissue reaction around gelatine coated control implants (Control), and implants coated with gelatine and minocycline loaded nanoparticles (MC-NPs), 3 and 7 days post implantation. (a-d) showing microglia activation (CD68) and (e-h) showing astrocytic activation (GFAP). Scale bar 100 μ m. Adapted from Paper III.

At 3 days after implantation, there was a clear reduction in activated microglia (CD68) around the implant coated with minocycline loaded nanoparticles, as compared to the implant devoid of nanoparticles (Figure 12a and 11a-b). This indicates that the minocycline (known to inhibit microglia activation¹²³) was successfully delivered to the tissue in sufficient dosage to reduce the early microglia activation normally associated with neural tissue damage. At 7 days after implantation, the microglia activation had diminished in both groups, but it was still significantly lower surrounding the drug loaded implant (Figure 11a and 12b), suggesting a sustained effect of the minocycline release.

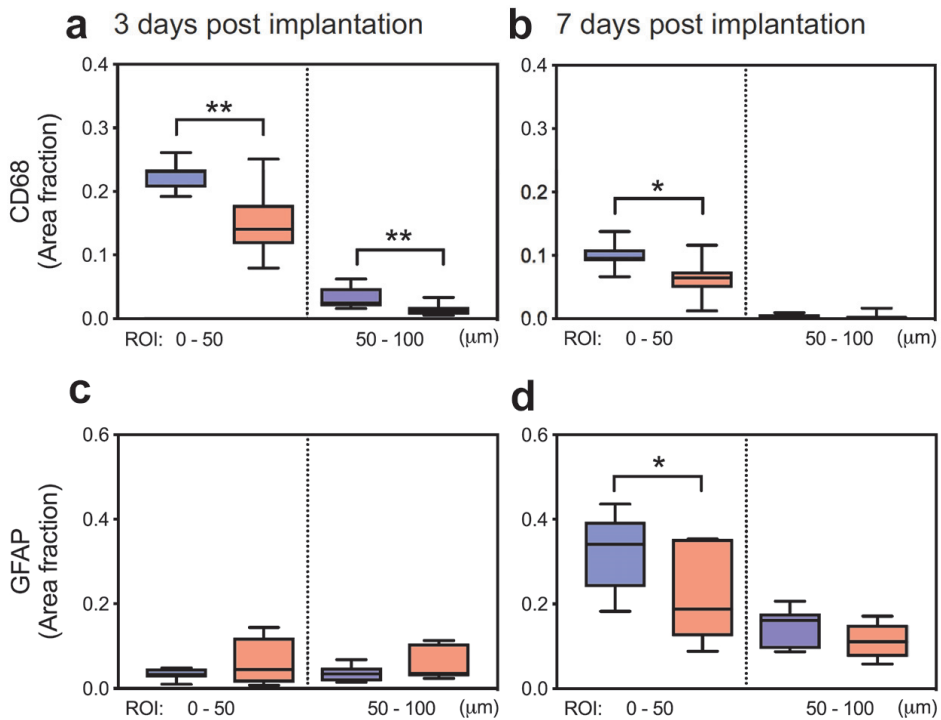


Figure 12. Comparisons of foreign body response between gelatine coated control implants (blue), and implants coated with gelatine with minocycline loaded nanoparticles (red), in ROIs 0-50 μm and 50 -100 μm. (a-b) shows level of activation of microglia (CD68) and (c-d) level of astrocytic activation (GFAP), both represented as the fluorescent area fraction. The boxplots represents first and third quartile with median as horizontal lines, and min and max values as whiskers. Statistical significance, is indicated by * ($p < 0.05$) or ** ($p < 0.01$). From Paper III.

Further, there were no difference in astrocytic response (GFAP), between the groups at 3 days post implantation (Figure 12c). However, 7 days after implantation (Figure 12d), the astrocytic response was significantly lower in the inner region around implants coated with minocycline loaded nanoparticles, as compared to the control (although the total astrocytic response had increased in both groups). This suggests

a delayed but indirect effect also on the astrocytic response by the minocycline, which is in line with previous studies suggesting that activated microglia can induce astrocytic activation ²².

Despite the effect on glial cell, the neuronal densities were not significantly different between the two groups at either time-point. This indicates that at least the minocycline loaded particles do not have a neurotoxic effect, but also that the reduction of glia activation did not have effect on the neuronal densities under these circumstances and time points.

Discussion & Conclusions

In this thesis, the challenge of designing a truly biocompatible neuro-electronic interface that can remain stable in the tissue without effecting the physiology of the brain, has been addressed, and a number of different strategies has been proposed. These strategies connect both to the mechanical compliance of the probe itself, the method of implantation, the tissue analysis after implantation and of local drug delivery surrounding the probes. Two novel types of compliant neural probes, as well as a new method for drug delivery, were designed, manufactured, characterized and evaluated in awake and behaving animals *in vivo*.

The results presented here show that a novel type of flexible and density regulated neuro-electronic interface, transiently stiffened by a dissolving core (allowing support during implantation), can almost totally avoid triggering the foreign body reaction and neuronal degradation in the tissue surrounding the implant, as well as enable a detailed analysis of the tissue juxtapositional to the contact of the electrodes. It was further demonstrated that an electrode array, highly flexible in three dimensions, can be implanted with preserved conformation, and that this array of microelectrodes yields very high quality recordings that remains stable despite being obtained in freely moving animals. Finally, it was shown that a new method for delivering drug content for local and sustained release, around neural brain implants, could be used to diminish the foreign body reaction.

The foreign body reaction

An overarching challenge in the field of neuro-electronic interfaces, has long been the deterioration of electrode performance, as well as the neuronal degradation and foreign body response in the tissue surrounding implants^{22,140,141}. We know from previous studies, that both the flexibility³⁸ and the specific weight⁴⁸ of the neural probe are important properties effecting these conditions. Here, we show that it is possible for a flexible and density regulated tube-like probe to almost abolish the foreign body response and to preserve normal neuronal densities around the probe. Comparably, a stiff and heavy probe of the same dimension and surface structure, induced an approximate 30% reduction in neuronal density in the vicinity. The tube-like construct is implantable, without any additional aids, thanks to the inner stiff glucose core that dissolves inside the brain. After dissolution of the glucose core,

only a thin polymer sheet is left in contact with the tissue, resulting in a probe with both low density and high flexibility. It was hypothesized that such a probe would move with the brain-tissue, rather than in relation to it. In fact, the minimal tissue reactions seen around the tube shank, together with a small upregulation of astrocytes right outside the tube orifice, as well as glia cells growing into the orifice, gives strong indications that the tube construct has remained stably positioned inside the tissue. Although recent progress with extreme miniaturization of ultra-thin polymer based microelectrodes^{78,79,82,93}, has showed reductions in foreign body response and neuronal degradation^{78,79}, they have also shows indications of instability in the tissue, described as migration between neurons and electrode⁷⁸.

Ultimately, it is the presence of neurons, in close proximity to the electrode recording site, that is the most important observation in the tissue around the tube electrode. The interactions between microglia, astrocytes, neurons and other cell types *in vivo* are highly complex, and much remains unknown about how these cells influence each other. For example, both microglia and astrocytes are known to be able to exert neurotoxic effects, as well as neuroprotective effects under different circumstances^{58,59,62,63}. One possibility is that a minute glia response (as observed outside the tube orifice) could be beneficial, as long as it does not grow dense or large enough to interfere with the viability of neurons. In this case, as there were no signs of neuronal degradation, the small glia reactions found seem to not affect the neuronal population in a negative way. Further, there were no significant differences, either in neuronal density or glia activation, between gelatin coated and non-coated probes (in contrast to what has been shown previously with regards to gelatin^{38,111}), most likely due to the already very small tissue effects seen here.

Tissue analysis of the inner zone of the implant

Another challenge, impeding proper analysis of the tissue immediately surrounding neural probes, is connected to the fact that a rigorous quantifiable analysis of the tissue generally requires tissue sectioning and staining. Currently, stiff probes must be pulled out from the brain^{30,36,40,142}, dragging adherent tissue along⁴⁸, before the brain tissue can be sectioned. In the case of ultrathin probes that are thin and soft enough to be sectioned while remaining inside the tissue these are likely to dislocate and can even be pulled out during slicing^{78,79,93}. For example, we could not slice the thin 3D-array in the tissue without some displacement of the electrodes. By contrast, the tube-construct can be sliced in frozen tissue without visible dislocation, most likely because the liquid inside of the tube, is frozen together with the surrounding tissue, which sufficiently fixates the polymer sheet before slicing. This, allowed a proper analysis of the tissue slices including the inner zone (0-9 μm) of tissue around

the implant shaft as well as the recording sites. Notably, the recording site was identifiable in tissue sections as a small opening in the Parylene-C coating. This made it possible to compare glia activation and neuronal density in the tissue outside the recording site to that of the rest of the probe, giving an indication of a slightly increased tissue reaction outside the orifice. Moreover, analysis of the innermost zone (0-9 μm) revealed that, especially in relation to the tube orifice, there is a thin ring of microglial cells, possibly also some astrocytes, more or less adhering to the Parylene-C sheet as well as migrating into the tube. However, it appears that the glia cells do not span or clog the orifice, thus the conducting lead inside is in fluidic contact with the neural tissue outside. To what extent traditionally designed recording sites, with exposed metallic surfaces in direct contact with the tissue, would have been covered by glia cells during this highly stable situation in the tissue, can of course only be speculated about. However, the typically observed increase in impedance over time^{22,143} of implanted neuro-electronic interfaces, as well as observations of glia cells adhering to explanted probes⁴⁸, suggest that such electrode contacts (positioned at the surface of the probes) often are covered by cells. Further, the possibility to perform a detailed analysis of the tissue outside the tube electrodes, enables correlation of histological analysis of single neurons outside the recording site, with qualities of electrophysiological recordings. This in turn opens up for improved identification and classification of single neurons potentially giving valuable information about the underlying functioning of the brain as well as for pathological conditions.

Electrophysiological recording stability

Neuro-electronic interfaces, cannot be said to have reached optimal clinical and scientific potential, until they are able to remain stable, i.e. follow the movements of the brain well enough to record from the same neurons over long periods of time²⁵. Here, we present a thin electrode array, flexible in all three dimensions in contrast to most flexible probes which are straight and therefore mainly flexible in one or two dimensions^{18–20,79,99,144}. Our results show that this array could record single units with very high amplitudes (up to 424 μV), higher than what is typically seen for chronic electrodes in awake and behaving animals, even for the more recently developed ultrathin polymer based electrodes^{79,82}. This suggests the presence of viable neurons very close to the electrode, given that the registered amplitudes decline rapidly with distance from neurons. The absence of single units with such high amplitudes, when using many of the ultrathin flexible probes developed elsewhere, can at this point only be speculated about, but it could be connected to the potential instability in the tissue as discussed above. The short term stability of single unit amplitudes in recordings using the 3D-array, indicates sufficient positional stability for recording from the same neurons at least within recording

sessions. The long-term stability, measured by the longevity of highly correlated units (>0.99) with high amplitudes ($> 150 \mu\text{V}$), indicates that the array can also remain sufficiently stable for recording the same units up to a week. However, the fact that these units did not remain equally correlated for the full 3 week period, and that there were small amplitude fluctuations during single recording sessions, suggest that the array would likely benefit from even further increases in flexibility. In fact, ongoing work has already shown that the 3D-array can be made considerably thinner and smaller (at least down to $\frac{1}{4}$ of the present cross section) using the same manufacturing method as described in this thesis. Worth noting however, is that already with the current design, there was an increase in signal to noise ratio over the 3 week experimental period, suggesting an ongoing integration of tissue and electrodes, indicating that the stability is actually improving over time rather than the opposite. This will, however, require longer lasting studies to determine. Further, to verify that the same neuron is in fact followed over time, future studies should also include other metrics such as firing statistics of single units connected to behaviour and/or longer continuous recordings.

Implanting delicate electrodes with retained conformation

As neural electrodes become thinner and more mechanically compliant, the challenge of implantation into the brain also becomes greater. This is especially true for delicate arrays of thin electrodes, with a conformation tailored for certain targets and/or applications. Here we present an embedding method with a gelatine-mix (previously shown to reduce the foreign body response around implants^{38,111}) that can provide sufficient structural support for implanting delicate electrode arrays, without changing conformation of the individual electrodes. This can be compared to a commonly adapted strategy of implanting ultrathin probes with the aid of a stiff guiding rod^{78,82}, which subsequently has to be explanted, potentially displacing the probes in the tissue as well as increasing the acute implantation tissue trauma¹⁸. For single neuronal recordings to be of high scientific and/or clinical relevance, it is of utmost importance that the tissue remains undisturbed, but also that the electrode array reaches the intended target with high accuracy and with retained conformation. In other words, the array needs to preserve its shape and relation between individual electrodes during the whole implantation procedure, as well as after, to know where in the brain the signals originate from.

Drug release

As an additional strategy to reduce the tissue reactions around neuro-electronic interfaces, a new method of coating neural implants with drug content was developed. By loading the drug into degradable nanoparticles¹³¹ that are incorporated into the gelatine coating of an implant, a local and sustained release was achieved (> 30 days in vitro). This approach allows precise delivery with dosages substantially lower than what systemic administration requires. The results presented here shows that the usage of the drug minocycline, could decrease the glia activation around neural implants without affecting the neuronal density. This could be an interesting complement in the pursuit towards a truly biocompatible neuro-electronic interface, for example in connection with the tube-electrode. It may be worth repeating that, although the neuronal density surrounding the tube-electrode was not statistically different from normal tissue, there were indications of slight tissue effects just outside the orifice of the tube, which might or might not be of importance. Also, even if the tube orifice didn't seem to be clogged by the migrating glia cells, it is still not known to what degree this migration could affect functionality over time. In case it proves a problem, the addition of drug release from the orifice of the tube, could be an interesting strategy for potentially clearing the tube/tube orifice of activated glia cells. In fact, the very design of the tube, with a dissolvable core and orifice, makes it highly suitable for loading with drug content for local release at the orifice. However, it is not known to what degree the activation of glial cells is actually benign rather than malign for the neuronal tissue. In fact, the modification of glia activation by the minocycline did not show any effects on neuronal survival, possibly because there were no or very little neuronal degradation around the implants, and/or the glia reactions might not have detrimental effects on neuronal survival under these circumstances and time points. In relation to this, pharmaceutical manipulation of the foreign body response can be questioned as a strategy for improving the biocompatibility of an implant. Nevertheless, the use of this highly localized and sustained drug delivery holds potential also for other applications, e.g. for locally targeted pharmaceutical treatment or very precise delivery of viral vectors for optogenetics.

Future perspectives

How to define a truly biocompatible neuro-electronic interface is not obvious, and there has already been many success stories using neuro-electronic interfaces, both for clinical treatment^{3,4} and for gaining information regarding brain functions^{5,6}. However, as has been touched upon several times throughout this thesis, there has been holdbacks to the progress connected to the instability and signal deterioration of current neuro-electronic interfaces^{21,22}. It is only when we can record single

neuronal activity from the same neurons over time, without disturbing the tissue, that we can accurately determine the modulation of this activity over time, and assume that the activity reflects a normally functioning brain, rather than that of damaged neural tissue. If possible, this would give the opportunity to, for example, follow the progression of neurodegenerative disorders such as Alzheimer's or Parkinson's disease, or other debilitating conditions such as epilepsy, depression or chronic pain, or for gaining a greater understanding of memory formation and learning, something that would give invaluable information aiding in the quest for understanding the brain. Further, if adapting this technology for stimulation, it could endow radically increased efficiency, specificity and longevity of treatments using deep brain stimulation, e.g. during Parkinson's disease, multiple sclerosis, depression or epilepsy, and diminish or even abolish adverse side effects, as well as the need to replace the technology during the course of a human lifetime. The full potential of such truly biocompatible neuro-electronic interfaces, remains to be seen, but as history has shown many times, scientific progress is deeply intertwined with the advent of new tools ¹⁴⁵, making previously impossible observations possible and thus laying the foundation for new paradigms within science.

Conclusions

The results presented in this thesis, suggest that the construction of truly biocompatible neuro-electronic interfaces, might not be too far away. For example it can already be speculated that the combination of a flexible and density regulated tube-like electrode, or array, together with a highly flexible tethering point, would have the potential to remain stable in the brain without disturbing the tissue and to record from the same neurons over time. In addition, such a probe would also allow for a detailed in situ tissue analysis, making it possible to correlate histological observations, even in the area immediately outside the electrode site, with electrophysiological recordings. Long-term studies, including e.g. continuous long-term recordings, are however necessary to confirm this potential. However, when realized, such a neuro-electronic interface would overcome the limitations of currently existing technology and have the potential to radically improve clinical treatments, as well as becoming an unparalleled tool for probing the mysteries of the brain.

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