

Electroporation with PEDOT: PSS Coated Microelectrodes

Gerrit Jan Dijk

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N°d'ordre NNT : 2021LYSEM020

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Electroporation with PEDOT:PSS Coated Microelectrodes

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'I am among those who think that science has great beauty'

- Marie Curie

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Acknowledgements

In one of his books, Stephen Hawking points out that we are privileged to be alive and live at this exact moment in time. 'Never has humanity known this much', he says. Regardless all the knowledge, being alive is remarkable. We are part of a marvelous world and science helps us to appreciate some of its magnificence. And I feel blessed, because I am among those who think that science has great beauty.

So what do you do with that passion? After looking back at the age of 35, my career path has been everything but a straight line. I have always had a passion for research and education, however, it took me quite some time to end up in academia. Now I found a place to share my enthusiasm and inspire others, and I'm very curious what the future holds.

Jolien and me arrived in the south of France in 2015. Jolien started her PhD and I tried to find a job in industry. After 1.5 year my job situation was pretty hopeless. A solution was offered by Jolien's professor, George, who welcomed me in his department and introduced me to world of organic bioelectronics. A year later, when Jolien just finished her PhD, I started mine. It's the grandeur of live, keep going and somehow wonderful things happen.

The PhD journey I embarked upon was wonderful, I really liked it. However, it seems inevitable to not get lost during your PhD. Research is challenging and unpredictable. While pushing the boundaries of our knowledge, we address places where no one has ever been before. Throughout my PhD, I met quite some students that told me academia is hard and stressful. Students burn-out, get depressed and leave science. For me this is unacceptable, and I hope that one day we can bring back joy and passion to the world of academia. Nonetheless, there will always be tough moments, this thesis has been no exception. Some of our hypotheses turned out to be fully wrong and in my second year I was pretty lost. But it was at that moment that we discovered new things and started to build the story of this thesis. This is the essence of research, we go out there with an unwavering plan, only to find something completely unexpected that can be used to make this world better place.

With that, I would like to thank the people who were part of my life during these past 3 years.

Rod, head of the expandaBELs, thank you very much for this great PhD! I had such a good time and your guidance and endless support have been a key element to its success. You always believed in me, trusted me and respected me. You listened and shared your wisdom. You always choose the person before work. And somehow, you manage to remove all the barriers between professors and students. Those are important boundary conditions for a learning environment. Thank you, and Denise and Gaby, for all the karaoke's and great times we had at your place. Throughout my PhD, you suffered severe personal circumstances which impacted your life profoundly, but it seems it made you stronger than ever before. I wish you health, happiness and all the best for the future!

At this moment BEL consists of Rod, David, Esma. Charles, Martin, Attila, Aimie, Marie, Remy, Hajar, Marina, Romanos and Rita and the interns Camille, Nathan and Yujia. I guess some would also include Bastien and Severine. It has been a great pleasure to share the 'troissieme etage' with you. David, thanks for being so steady, helpful and pragmatic. Students love you, and for a good reason. Esma, you are the constant factor in BEL. Thanks for all the help and chitchats over last years. Charles, gutentag, guinness, goethe! I admire how open you

are to feedback, always willing to learn and make things better. I cherish our friendship and enjoyed the countless beers we drunk and patisserie we ate. Martin, thank you very, very (!) much for the countless discussions we had, you helped me transform from being ignorant to an electrochemistry rookie. Whenever talking to you, I feel I'm in the company of a sincere man, I feel at ease. Thanks for being so kind. Attila! I could say so many things, but above all you're a great person. Kind, reliable and always striving to do experiments properly. I'm very glad we got to work together in the last year of my PhD, I learned a lot in the basement in Marseille. Aimie, despite your rough journey, you are the shining star of the department. You were a great office buddy, you're so funny. I admire your passion and how you're able to be nice to others when you're having a really bad day. Marie, fellow-Rod-PhD-student, they should never let you go, you are motor of BEL in all aspects. It's amazing how you combine hard work with so much fun. Your energy and involvement in Fidelglio gave the project a direct impulse. Keep stimulated! Remy, I believe you to be an excellent PhD candidate, it's impressive to see how fast you pick up new knowledge. I'm happy to have found a fellow fabrication enthusiast in you, keep the fab in BEL a live! Hajar, mommy, I'm so happy I got to know you. Being 30+ united us, we understand each other and I loved discussing the important matters of life with you. I'm looking forward to seeing Kiana grown up, if she looks anything like you, she'll be lovely. Marina, you're my definition of a kind person. Wherever you go evil disappears. Your dedication to work and kindness will open many doors for you, I'm sure. Romanos, how is it possible that you are always this calm and peaceful? That is probably why you integrated in BEL so easily. I needed a biologist to test my impedance sensors, and there you were, thanks for all the help. Rita, you made it easy when all my old office buddies were gone. I love talking to you, thank you for listening to all my endless stories. I wish you a happy PhD, without stress and worries. Hubert, you're on a sabbatical at the moment. I hope you get better soon!

There are quite some people who left BEL during my PhD. Mary, thanks for all the fun and beer brewing. Gaurav, I cherish our shared passion for physics and friendship. Alex, thanks for being such a sincere person and an exemplary scientist. Chris, thanks for all the fun we had. Vincenzo, Viviana, Donata thank you for your friendship and the precious moments. Ian, thank you for all your help and friendship. Being a fellow Dutchy helps, but you're simply a great person to hang out with. We are quite different, but had so much to talk about. You have great humor and I'm happy you found a 'home'. Janja, your visit to BEL was an absolute pleasure. You're knowledgeable, skillful and cheerful, I enjoyed our discussions very much and hope we stay in touch. Diana, it was so great to meet you at EBTT, and even better when you visited our lab. You bring so much positivism. You make this world a better place.

One of the great things about my PhD is that I got to work with a company, Panaxium. It helped to have colleagues around who strive towards transforming basic research to the clinic. I'm especially grateful for the tremendous freedom they gave me during this project. The Panaxium colleagues helped me with literature, fabrication processes, development of ideas, connecting Fildelgio probes, proofreading manuscripts, countless discussions and so much more. A big, big thanks to Brad, Pierre, Carolyn, Aaron, Sylvain, Olivier, Karima, Loig, Bastien, Marcel, Marie, Bahar, Alwin, Jean and SiMing.

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Because of covid I didn't travel as much as I wanted. However, I was privileged to meet some of the giants in the field of electroporation. Damijan and Lluis thank you for the EBTT workshop, the visits, the discussions, your patience when I asked so many questions, explaining me why we use 8 pulses for ECT and making sure I got diabetic approved desserts.

Life is nothing without family and friends. Although they probably didn't add to much scientifically, it goes without saying I needed them the most. Therefore, a massive, big thanks to all my friends! Life is nothing without you. Eddie, we have known each other since forever. You're the best friend a person can ask for. I love to see you do 'Rekkie&Bankie'. You believe in change, do what you love, crucial ingredients for a happy life. Thanks to all those other 'Hardenbergers', the people I met in Eindhoven and all the ex-Pushphairanen en ex-Isisianen that are still part of my life. Thanks to all of you who came to Aix and visit us. You made our stay in Aix so much better. The impressive size of the guestbook and the fact that we needed a second one, says it all. Thanks to my 'Groupie' friends in Aix. Isabel, Gilles, Chris, Carlijne, Alexandra and Yuri, thanks for all the drinks, food and activities.

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My lovely family. I'm so proud of each and every one of you. We are with so many, we're all so different, but then again, we are almost the same. I always smile when I get to tell people I have 2 parents, 6 siblings and 7 nephew and nieces. But it is not the numbers that count, what matters most, is that it is you who are my family. Mom and dad, everything I do started with you. You raised me and always encouraged me to explore life, find answers and enjoy. But also to reflect, be satisfied and be moderate. I'm a clear reflection of you both, you made me who I am. Sonja, Janine, Rutger, Linda, Berne, Xander, David, Ruben and Yvonne. I could write a book about each of you. You're all examples I look up to. Through good and bad times, you have all accomplished so much. You motivate me and make me laugh. Emmely, Louise, Julia, Annelin, Manoah, Rosalie and Gijs, you are the best nephews and nieces ever. I'm looking forward sharing more lifetime memories with you!

My dearest Jolien. Thank you for being so incredibly awesome! You're among the strongest persons I know. I deeply admire your attitude in life. You love and hate. You laugh and cry. You play and relax. You are an example to live up to. The last couple of years were not easy on you and it is in such difficult times that one gets to really know a person. All I can say is that I love you even more now. Thank you for accepting me the way I am, that can't be easy. Thank you for being there for me, always. Thank you for exploring the Provence with me. Thank you for exploring life with me. Above all, thank you for showing me the beauty of life that is not based on math, logic or reason. Because that's the part where it's all about.

Curriculum Vitae

Profile

Passionate physicist. Switched from industry to academia. Common to international environments, adapts easily. Excellent team player with clear communication. Can lead, take initiative and make decisions.

Positivist • Organized • Dedicated • Accurate • Hands-on • Social • Feedback & Progress

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PhD Microelectronics (Bioelectronics)	2021
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Technical University Eindhoven, Eindhoven, The Netherlands	
Specialization: Optics and Plasma Physics	
BsC Engineering Physics	2007
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Experience

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Department of Bioelectronics, supervisor Prof. G.G. Malliaras	
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• Support system integration at customer site • Organize knowledge transfer

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Scientific Contributions

Publications

Influence of PEDOT:PSS Coating Thickness on the Performance of Stimulation Electrodes. **G. Dijk**, H. J. Ruigrok, and R. P. O'Connor, Adv. Mater. Interfaces, 2020.

PEDOT:PSS Coating of Stimulation Electrodes Attenuates Irreversible Electrochemical Events and Prevents Cell Electropermeablization. **G. Dijk**, H. J. Ruigrok, and R. P. O'Connor, Adv. Mater. Interfaces. *Sumitted*.

Electroporation microchip with integrated conducting polymer electrodes for highly sensitive impedance monitoring. **G. Dijk**, R. Poulkouras, and R. P. O'Connor. *In preparation.*

Electroporation as a tool for opening of the blood-brain barrier in vivo.

A.Kaszas, G. Dijk, and R. P. O'Connor. In preparation.

Transparent PEDOT:PSS multielectrodes for combined electrophysiology and imaging in vitro and in vivo.

G. Dijk, A. Kaszas, J. Pas and R. P. O'Connor. In preparation.

PEDOT:PSS coating prevents Pt dissolution and improves cell viability for neuromodulation applications.

G. Dijk, J. Pas and R. P. O'Connor. In preparation

Integrating flexible electronics for delivery of pulsed electric fields in a vascularized 3D cultured glioblastoma model.

M. Lefevre, G. Dijk, A. Kaszas, M. Baca, D. Moreau, and R. P. O'Connor. npj Flexible Electronics. Submitted.

 Ca^{2+} roles in electroration-induced changes of cancer cell physiology: from membrane repair to cell death.

D. Navickaitė, P. Ruzgys, M. Maciulevičius, G. Dijk, R. P. O'Connor, and S. Šatkauskas. Bioelectrochemistry. *Submitted.*

Conferences and Workshops

2018 BioEM, International conference of bioelectromagnetics. June 24-29, 2018, Portorož, Slovenia.

EBBT, International workshop on electroporation-based technologies and treatments. November 11-17, 2018, Ljubljana, Slovenia.

2019 NANS, Annual conference of the north american neuromodulation society. January 17-20, 2019, Las Vegas, United States of America.

3rd WC electroporation, World congress on electroporation and pulsed electric fields. September 3-6, 2019, Toulouse, France

Awards

1st Place Student Poster Award, BioEM conference 2018, Portorož, Slovenia

Abstract

The field of electropermeabilization, or electroporation, is advancing and has found successful biomedical applications such as electrochemotherapy, tissue ablation, gene therapy and transdermal drug delivery. Despite significant progress in the last decades, a better understanding of underlying mechanisms and advancement in technology are needed to improve outcomes. In this thesis, the performance of the conducting polymer PEDOT:PSS as an electrode coating material is explored in the context of electropermeabilization. Microfabricated PEDOT:PSS coated electrodes are investigated, both, to deliver stimulation pulses and to monitor changes in impedance as a consequence of cell electropermeabilization. The results can be summarized in three parts.

Firstly, increased PEDOT:PSS coating thickness improves the polymer-electrolyte interaction due to a higher electrode capacitance. Thicker coatings exhibit higher stimulation thresholds before electrode failures such as delamination is observed. This emphasizes the importance of PEDOT:PSS coating thickness as a design parameter for optimized performance and increased stability of stimulation electrodes.

Secondly, electrical stimulation with PEDOT:PSS coated electrodes strongly reduces irreversible electrochemical reactions at the electrode interface that otherwise lead to changes in pH and the generation of reactive oxygen species. These findings indicate that charging in PEDOT:PSS is dominated by reversible processes, also at voltage beyond the water window. In addition, coated electrodes induce nearly no electropermeabilization at voltages that greatly exceed the threshold for uncoated electrodes. The absence of Faradaic reactions in combination with marginal electropermeabilization reveals that electrochemical events at the electrode surface play an important role in the cellular response. Moreover, PEDOT:PSS separates the delivery of electric pulses from chemical changes which paves the way for future experiments that could lead to a better understanding of underlying mechanisms.

Thirdly, an array of PEDOT:PSS coated sensing electrodes is integrated with a pair of uncoated stimulation electrodes. Reduced impedance of the sensing electrodes due to the PEDOT:PSS coating significantly improves the sensitivity of impedance monitoring during the growth of adherent cells and treatment with electrical pulses. Such microscale electrodes enable accurate assessment of changes in cellular impedance with high spatial resolution which can be used to study electroporation dynamics and monitor treatment progression.

Résumé

L'électroperméabilisation, ou électroporation, a été utilisée avec succès dans des applications biomédicales telles que l'électrochimiothérapie, l'ablation tissulaire, la thérapie génique et la libération transdermique de médicaments. En dépit des progrès significatifs de ces dernières décennies, une meilleure compréhension des mécanismes sous-jacents ainsi que de nouveaux développements technologiques pourraient améliorer les résultats obtenus. Dans cette thèse, les performances du polymère conducteur PEDOT:PSS, utilisé comme revêtement pour les électrodes, sont étudiées dans le cadre de l'électroperméabilisation. L'utilisation des microélectrodes recouvertes de PEDOT:PSS a été investiguée pour, d'une part, délivrer des impulsions de stimulation, et d'autre part, surveiller les changements d'impédance résultants de l'électroperméabilisation de la cellule. Les résultats obtenus peuvent être classés en trois catégories.

Premièrement, l'augmentation de l'épaisseur du revêtement de PEDOT:PSS améliore l'interaction polymère/électrolyte grâce à une capacité d'électrode plus élevée et montre également des seuils de stimulation plus élevés avant la défaillance des électrodes, principalement due à une délamination du film de PEDOT:PSS. Ce résultat montre l'importance d'utiliser un revêtement de PEDOT:PSS pour optimiser les performances et augmenter la stabilité des électrodes de stimulation.

Deuxièmement, la stimulation électrique avec les électrodes recouvertes de PEDOT:PSS réduit considérablement les réactions électrochimiques irréversibles à l'interface avec l'électrode qui autrement produisent une modification du pH et des espèces réactives de l'oxygène. En outre, les électrodes recouvertes de PEDOT:PSS ne montrent quasiment pas d'électroperméabilisation pour des tensions qui excèdent largement le seuil pour des électrodes nues. Ces résultats montrent que charger le PEDOT:PSS est dominé par des processus réversibles. De plus, l'absence de réactions faradiques combinée avec une électroperméabilisation marginale indique que les processus électrochimiques à la surface de l'électrode jouent un rôle important dans la réponse cellulaire.

Troisièmement, pour surveiller l'évolution d'un traitement par électroperméabilisation, une matrice de microélectrodes recouvertes de PEDOT:PSS a été intégrée avec des électrodes de stimulation interdigitées et nues. L'impédance réduite des électrodes recouvertes de PEDOT:PSS améliore grandement la surveillance des changements d'impédance des cellules lors de l'électroperméabilisation. Ces électrodes microscopiques conduisent à des mesures précises de changement d'impédance cellulaire avec une haute résolution spatiale, permettant ainsi une meilleure surveillance de la progression du traitement.

1. Organic Bioelectronics for Electrical Treatment of Cancer

Despite major scientific advances, adequate and successful cancer therapies remain a huge challenge. In the fight against this devastating disease there is a need for alternative interventions and new technologies. Electrical pulses delivered by flexible, conformal devices with unique mechanical and bio-electrical properties might enable new approaches. This thesis presents the first steps that were taken to investigate devices based on organic materials for electrical treatment of cancer by means of electropermeablization.

1.1 Electrical stimulation for cancer applications

Cancer is devastating and the fight against this terrible disease remains a societal challenge. While prevalence and mortality of many other diseases decreases, cancer is becoming the leading cause of death, eclipsing cardiovascular diseases. New technological approaches and therapeutic interventions are therefore constantly in demand.

Primary focus of this thesis is glioblastoma, the most common and lethal type of brain cancer. In the United States, more than 10 000 new cases are reported annually. Glioblastoma is incurable and almost invariably leads to death. Aggressive treatments such as tumor resection, chemo- and radiotherapy might benefit the patient, however, the average survival time for patients is less than 15 months. Due to a high degree of tumor infiltration in the surrounding tissue and the complex brain micro environment (Figure 1.1), resection is not curative and tumor cells inevitably remain leading to disease progression or recurrence (I).



Figure 1.1 | The complexity of glioblastoma. (A) Tumor environment where glioblastoma cells interact with different brain cells such as neurons, astrocytes and microglia. (B) Glioblastoma infiltration through migration along perivascular spaces and white matter tracts initiating secondary tumors that often settle centimeters from the original tumor. Images taken from references (2, 3).

Scientific advancements and better understanding of the glioblastoma biology has led to a variety of novel therapeutic approaches, however, many have provided limited or no therapeutical efficacy in human trials. One particular approach that holds great potential, but has yet to be exploited for glioblastoma, is based on pulsed electrical stimulation.

Pulsed electric fields (PEFs) are among the most innovative approaches to cancer treatment. Electric fields can be highly targeted and treatments are without side effects enabling therapies with significant advantages. Much of understanding of electric field effects on cells comes from the discovery of electroporation by Neumann and Rosenheck (4), who first showed that the application of pulsed electric fields to cells changed the permeability of their membranes allowing external molecules to enter (Figure 1.2). This effect was used to transfer genes into cells in vitro (5), and later, also tissue in vivo (6). Electroporation was also found to be a useful adjunct to enhance the delivery of chemotherapy to cancer cells in vitro (7) and in vivo (8), an approach know as electrochemotherapy (ECT). In addition to cell membrane permeabilization, PEFs alters the blood flow near the electrodes due to shape changes of vascular endothelial cells and vasoconstriction. This 'vascular lock' destroys the micro environment of the tumor and prolongs entrapment of the drug within. A third effect is the activation of the immune cells which is believed to be related to antigen shedding after electrochemotherapy from the

destroyed cells. After the first clinical trials in the early 1990s (9), ECT is now widely used in more than 60 cancer treatment centres in the world (10).

The electroporation effect of an electric field on a cell depends on the pulse length, amplitude and the number of pulses. Applications that include molecule delivery into the cell require electroporation to be reversible meaning a transient permeabilization after which the cell recovers. Typical stimulation consists of 8 square wave, monophasic pulses of 100 μ s with an amplitude of 100-1000 V. Irreversible electroporation results in cell death and generally involves more pulses of higher amplitudes (*11*).



Figure 1.2 | Electroporation for cancer treatment. (A) Cell membrane consisting of a bilayer of lipids and the formation of pores due to pulsed electric fields. (B) Reversible and irreversible electroporation as a function of pulse duration and amplitude. C) Electrochemotherapy increases cellular uptake of chemotherapeutic drugs (red particles) leading to cell death. (D) Survival of glioblastoma in rats that are treated with only Cisplatin and a combination of Cisplatin and electroporation. Images taken from references (11-13).

In the context of glioblastoma, electrical stimulation has shown great potential. In vitro, PEFs applied to human brain cancer cells causes membrane permeabilization and breakdown of the microtubule network, which could lead to loss of mitochondrial viability and apoptosis (14). In vivo, changes in tumor blood flow were demonstrated, leading to disruption of the tumor vascular perfusion that could be applied to sabotage and damage the solid tumor environment (15). PEFs have also shown to transiently disrupt the blood-brain barrier (BBB) with relatively low voltages such that no cell electroporation was observed (16). Such a mechanism could be applied to deliver chemo drugs to the tumor environment, which in normal circumstances is not possible since the BBB tightly regulates the transport of molecules between blood vessels and the brain. Finally, Sharabi et al. showed that a combination of electroporation and Cisplatin in glioblastoma rats reduces tumor growth and increases survival (13). Radial electric field were generated by a needle point source placed within the tumor mass. Electroporation enhanced the

uptake of chemo drugs and at larger distances from the electrode disruption of the BBB was observed that could serve to address zones with infiltrated cells.

Despite the promising advances described above, complete destruction of glioblastoma tumors remains unresolved. Treatment of glioblastoma has proven to be complex and extremely challenging. New methods are thus needed to further our understanding of underlying mechanisms and to develop better tools to fight this disease. This thesis investigates whether organic bioelectronics might hold some answers.

1.2 Organic bioelectronics

Bioelectronic devices offer tremendous opportunities to communicate with biological systems which are exploited in a wide variety of fundamental research, diagnostics and therapies. Readout of biological signals has greatly enhanced our understanding of signaling processes and is commonly used to monitor vital functions such as heartbeat and glucose concentration. Interventions based on electrical stimulation include clinical applications such as deep brain stimulation for movement disorders (17), pacemakers for arrythmia (18), and cochlear implants for deafness (19), as well as recent advances such as deep brain stimulation for traumatic brain injuries (20), and schizophrenia (21).

Despite numerous successful applications, interfacing electronics with biology is challenging because of their fundamental different nature. Electronics generally consists of static and rigid materials that communicates via holes and electrons, whereas the human body is dynamic, soft and signaling takes place via ions and specific transmitters. For instance, the large difference in Young modulus can hinder smooth integration of electronics with the body causing a foreign body response where the body is fighting non-compatible objects (22).

The inherent mismatch of conventional electronics with biological environments gave rise to a field that took the name organic bioelectronics. Electronics based on organic materials has enabled displays and photovoltaics that found applications in flexible, ultrathin and light weighted consumer electronics (23). In the last decades such materials have also been exploited for biological interfaces and has reached a point of proven record with great promise for future biomedical applications.

As shown in Figure 1.3, organic bioelectronics takes advantages of unique materials that are soft, flexible, conformable, stretchable and bioresorbable (24-27). These properties result in devices that interact with biological systems for both wearable and implantable devices in an effort to make the interfaces as seamless as possible. The marvelous ability of organic materials to optimize performance by smooth integration of device and tissue is beautifully demonstrated with a stretchable vagus nerve neuromodulator (Figure 1.3A). Conventional, rigid implants wrapped around the nerve of an infant impedes growth of the nerve leading to additional surgeries. By employing viscoplastic materials with specific engineered properties, a device can be made that can be stretched and maintains its shape after force is released while minimally affecting the electrical performance (28).



Figure 1.3 | The marvelous world of organic bioelectronics. (A) Viscoplastic neuromodulators can stretch, deform and adapts to nerve growth. (B) Conformal electrode arrays conform to the brain curvature, however, device thickness determines the degree of brain depression. (C) Pliable electroporation patch for plasmid DNA transfection in tumors. (D) Structure of the conducting polymer PEDOT:PSS. (E) Flexible and transparent PEDOT:PSS sensors. Images taken from references (28-32).

For optimal integration and performance of electronics with the biological milieu, the polymeric materials can be processed with thin film fabrication techniques to manufacture flexible and high resolution devices (33). The thickness of such devices can be precisely tuned to the needed level of conformability for implantation. Such adequate integration of the device with the tissue curvature is essential to maintain healthy tissue at the implant site and substantially increases device the electrode-tissue interaction improving performance (29).Moreover. photolithography establishes a patterning technique that offers nearly unlimited, high resolution design opportunities. Micro and macro features of arbitrary shape, size and numbers can be easily adapted according to specific needs which makes this a powerful tool to produce and test a variety of device architectures. Photolithographically patterned, closely spaced electrodes on a thin parylene C substrate are pliable and can be wrapped tightly around curved and irregular surfaces. Such devices have shown to be efficient for the delivery of nucleic acids (DNA and siRNA) into tissue by means of electroporation (30).

Generally, bioelectronic devices include metal electrodes such as platinum (alloys), gold, titanium nitride or iridium oxide (34). However, in the last decades conducting polymers have advanced rapidly as valuable materials for interfacing with biological environments (35). These unique materials combine the mechanical, soft properties of polymers with electronic

conduction. Moreover, they support ion transport throughout the material, a crucial property that allows ion exchange between the biological environments and the bulk of the material. Currently, PEDOT:PSS is the established champion material due to its high conductivity, stability and commercial availability (36-39). Its ease of processing and the ability to synthetically modify mechanical, electrical and optical properties have made it the material of choice for various organic electronic applications. Coating metallic electrodes with PEDOT:PSS improves the electrode-electrolyte coupling due to its large capacitance which is essential for efficient signal transduction (40). Thin PEDOT:PSS films are transparent in the visible spectrum which is applied in optoelectronic devices such as organic photovoltaics, but has yet to be fully exploited in bioelectronic devices (41). In the last decades, the unique properties of PEDOT:PSS have initiated an explosion in the development of wearable and implantable devices ranging from sensors for electrophysiology, metabolism and pressure to actuators and scaffolds.

The application of organic materials for bioelectronic devices is expected to expand much further. Development of new materials and devices is needed to further enhance seamless integration with biology and more investigation is required on the fundamentals of the biotic-abiotic interface. Additional studies are needed to determine the stimulation performance of PEDOT:PSS, especially regarding electroporation-related therapies for cancer.

1.3 Thesis objective and outline

This thesis explores organic materials to develop devices for the delivery of bioelectric therapeutics, or so-called electroceuticals, for the treatment of cancer. The start of such a project is rather ambitious and it helps to narrow the scope. The first step, to which this thesis is dedicated, is formulated by the following question:

Is PEDOT:PSS beneficial for electroporation-based applications?

The following points provide some guidelines to which this thesis is focused:

- Bioelectronic devices for treatment of glioblastoma. Therefore, in vitro validation was performed with U-87 human glioblastoma and blood-brain barrier cells.
- Devices. a) Design: the experimental setups often contained imaging equipment that necessitated a transparent device architecture. Planar, interdigitated electrodes were used which allowed imaging between the electrode fingers. b) Fabrication: manufacturing required dedicated processes that were adapted to organic materials. c) Characterisation: device properties typically involved voltage-current relations, stability of the stimulation electrodes and the electrochemical performance. Finally, the devices were tested in vitro. At the moment of writing, in vivo experiments in mouse models were ongoing.
- Stimulation and sensing performance of electrodes, where a major part of this thesis investigates the difference between PEDOT:PSS coated and uncoated electrodes.

The outline of this thesis is as follows. In chapter 2, the properties of the conducting polymer PEDOT:PSS are discussed, including a short review of stimulation applications. In chapter 3, the influence of PEDOT:PSS coating thickness on the electrode performance is examined, as well as the electrode stability for electroporation relevant voltages. Chapter 4 compares the stimulation performance of PEDOT:PSS coated electrodes with uncoated electrodes. The

charge injection mechanism, electrochemical events at the electrode interface and the electropermeabilization performance of both type of electrodes is investigated. Chapter 5 presents a multi-layered electroporation device with an integrated, sensing electrode array for impedance assessment of adherent cells. The sensing capabilities of PEDOT:PSS coated electrodes is investigated during cell growth and treatment with electrical pulses. Finally, chapter 6 summarizes the findings of this thesis.

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2. The Conducting Polymer PEDOT:PSS

The conducting polymer PEDOT:PSS is a valuable material for bioelectronic devices. Its unique properties such as mixed ionic and electronic conduction, volumetric capacitance and stability depend on the material structure which can be tuned with additives. Despite beneficial electrochemical properties, few reports exist on the electrical stimulation properties of PEDOT:PSS, establishing a research item that most certainly deserves more attention.

2.1 Conducting polymers for bioelectronics

The discovery of conducting polymers (CPs) is considered a ground-breaking accomplishment since it fundamentally changed our view of polymers from insulating to electrically functional materials. Their success is marked by commercialization of the material for antistatic coatings and more recent applications such as organic solar cells, thin film transistors and organic light-emitting diodes (1). In the context of bioelectronics, CPs have proven to be valuable additions or replacements of conventional metallic and semiconductor materials. The tremendous progress includes one of the first CP coated electrodes in 2003, (2) a study on the human brain in 2015 with CP coated electrodes (3) and unpreceded electrophysiological recordings with CP containing transistors in 2020 (4).

The success of conducting polymers in the field of bioelectronics arises from the excellent match between the material properties and the requirements for biotic/abiotic interfaces (5). CPs are inherently soft which enables manufacturing on flexible substrates for seamless integration with biology and exhibit beneficial electrochemical properties that greatly enhances the coupling between electronics and the biological milieux. Besides, CPs can be synthetically modified, blended with other materials such as hydrogels, loaded with drug molecules and their surface can be functionalized which offers a tremendous degree of versatility that is not feasible with conventional metallic and semiconductor materials (6).

The polythiophene PEDOT:PSS (poly(3,4-ethylenedioxythiophene)-polystyrene sulfonate) has been particularly successful due to its stability in common electrolytes, commercial availability and ease of processing for instance from a dispersion. PEDOT:PSS has been applied in wearable devices (7), neural probes for electrophoretic drug delivery (8) and as an electrode coating to record and evoke electrophysiological signals in vitro (9) and in vivo (10). One of the crucial advantages of PEDOT:PSS is its ability to efficiently transport and couple electronic as well as ionic charges (11). Besides, the combination of its high electronic conductivity and optically transparency makes it suitable to replace ITO in transparent electrode applications. This chapter provides an overview of the charge transport, commonly used additives and an overview of stimulation characteristics of PEDOT:PSS electrodes.

2.2 Organic mixed ionic-electronic conduction

The blend of PEDOT and PSS forms a rather complex structured material in which electronic transport, ionic transport and electronic-ionic coupling can occur (11). These processes take place in the bulk of the material and therefore strongly depend on its structure. In a dispersion, PEDOT is believed to template along the PSS chain which together form gellike particles surrounded by a PSS rich shell (Figure 2.1A). The resulting film can be categorized as heterogeneous with a high level of disorder and impure phases containing PEDOT:PSS rich and PSS rich phases governed by van der Waals interactions (12).



Figure 2.1 | Structural and transport properties of PEDOT:PSS. (A) Multiple PEDOT molecules couple along the PSS chain to form gel-like particles that results in a disordered film morphology with PEDOT:PSS rich and PSS-rich domains. (B) Mixed ionic-electronic conduction enhances electrolyte-electrode coupling. An ion that migrates into the polymer (1) is stabilized by an electronic charge (2) that transports via the polymer chains (3) and transfers to the metal electrode (4). Images taken from references (11, 12).

Electronic transport is enabled by weakly bound electrons that are a result of conjugation. The polythiophene PEDOT is a conjugated polymer meaning it contains overlapping p-orbitals. Conventionally, this is represented by alternating single and multiple bonds. The electrons of these overlapping orbitals do not belong to a single bond and are delocalized which allow them to be transported through the conjugated system. This property makes PEDOT an intrinsically conducting polymer, i.e. an organic, carbon-based material that conducts holes.

The molecular orbitals of the conducting polymer are linear combinations of the atomic orbitals which causes a splitting of energy levels. The resulting energy gap is called the band gap. PEDOT is a p-type semiconductor in which holes are the main charge carriers. It has a moderate band gap, low redox potential and is optically transparent in its conducting state.

Along the π -conjugated PEDOT molecules, transport occurs through delocalized orbitals and between molecules that have sufficient overlap to allow so-called hopping. Disorder in the polymer structure limits the overlap of orbitals and generally reduces the electrical conductivity (11). Tighter stacking of the polymer generates order which enhances hopping and increases the electronic conductivity. PEDOT:PSS conductivity above 4000 S/cm are achieved by post-treatment of the material with strong acids which induces structural rearrangement resulting in highly ordered, dense PEDOT networks (13).

A major disadvantage of PEDOT is the poor solubility, however, this is greatly improved with the present of a polyanion such as polystyrene sulfonate (PSS). Together they form a polymer dispersion of positively charged PEDOT and negatively charged PSS, which acts as a counterion.

Ionic transport arises from the ability of PEDOT:PSS to absorb liquid electrolytes (14). Swelling of the material establishes a porous structure through which ions can migrate. This represents one of the key advantages of CPs since it enables ionic exchange between the electronic material and biological environment (5). Organic materials that conduct electrons, as well as ions, have been termed organic mixed ionic-electronic conductors (11).

Ionic-electronic interactions take place when an electrode material is brought in contact with an electrolyte (Figure 2.1B). This interaction can be modulated by applying an external potential upon which two mechanisms might take place: capacitive coupling by means of an electrostatic, double layer and Faradaic processes that involve charge transfer across the interface (15, 16). Both mechanisms require charge balance such that an injected ion is stabilized by a charge carrier that is supplied via the electronic conduction of the polymer.

Whether charge transfer occurs between the coupled ion and the polymer molecule remains controversial and confusion exists about how to best describe the processes that take place (11). PEDOT:PSS has shown Faradaic side reactions which involves the production of H₂O₂ (17, 18). However, ideal capacitive behavior were observed by measurements such as electrochemical impedance spectroscopy (19) and moving front (14). Moreover, ion penetration enables charge storage within the bulk of the material which gives rise to a volumetric capacitance which scales with the thickness of the material (20).

2.3 Performance enhancing additives

To enhance processing and optimize the material properties, PEDOT:PSS dispersions are commonly supplemented with solvents, surfactants and cross-linkers which will be discussed here.

For optimal performance of bioelectronic devices a subtle trade-off in the material structure is required to balance the electronic and ionic conductivity. Supplementing the PEDOT:PSS dispersion with solvents like ethylene glycol (EG), glycerol or dimethyl sulfoxide (DMSO) greatly improves the electronic conductivity from typical values of 1S/cm to 800 S/cm (21). Figure 2.2A shows that 5% of added solvent is needed to achieve such notable change, whereas >5% does not further increase the conductivity significantly. The improved electronic conductivity is a result of tighter molecular packing, and larger, denser and purer PEDOT:PSS rich domains. However, ionic transport is believed to take place in the PSS rich domains such that the increased heterogeneous morphology reduces the ion mobility. Therefore, the change in material structure has opposite effects regarding electronic vs ionic conductivity and will ultimately determine the device performance (12).



Figure 2.2 | Enhanced PEDOT:PSS performance by additives. (A) Solvents DMSO, EG and glycerol greatly enhance the electronic conductivity. The surfactant DBSA is primarily added for wetting, but also increases the conductivity. (B) GOPS mediated cross-linking improves material stability in aqueous environments through GOPS-PSS, GOPS-GOPS and GOPS-substrate bonds. Images taken from references (21, 22).

Film processing with PEDOT:PSS dispersions can be facilitated by the addition of dodecyl benzene sulfonic acid (DBSA). This surfactant acts as a wetting agent to improve deposition by means of spin casting and enhances the electronic conductivity (21). Addition of >0.5 % DBSA causes phase separation in the dispersion which leads to poor deposition and film quality. Typically, a minimum amount of DBSA is added to achieve sufficient wetting and further enhancement of electronic conductivity is obtained with the above-mentioned solvents

Applications in bioelectronics require material integrity in aqueous environments. Mechanical stability and adhesion to underlying substrates is commonly achieved by addition of a cross-linking agent such as (3-glycidyloxypropyl)trimethoxysilane (GOPS). As shown in Figure 2.2B, improved stability is achieved via GOPS-PSS, GOPS-GOPS and GOPS-substrate bonds which prevent dissolution and delamination (22). Addition of GOPS reduces the electronic and ionic conductivity of the material and should therefore be kept to a minimum (14, 23).

2.4 PEDOT:PSS coated electrodes for stimulation applications

There exists an extensive amount of literature on PEDOT:PSS coated electrodes. Reports on the sensing ability of micro- and macroelectrodes are abundant, however, there are few studies investigating the stimulation performance. Studies primarily focus on applications for neural interfaces. Here, a short overview is presented which includes electrochemical properties, addresses electrode stability and describes the influence of electrode size and PEDOT:PSS thickness. Also, topics that require further investigation are identified.

Primary advantage of coating stimulation electrodes with PEDOT:PSS arises from its mixed ionic-electronic conduction (11). Ion migration in the PEDOT:PSS matrix enables 3D electrostatic coupling of ions with the electronic material. This increases the electrode capacitance tremendously (up to 2-3 orders of magnitude) compared to a smooth metallic electrode that exhibits relatively low capacitance due to limited surface area that is accessible to ions (16). The amount of charge that can be stored within PEDOT:PSS depends on the volume of material such that for a given electrode surface area the material thickness determines the capacitance. This dependency can be observed in the cyclic voltammograms where thicker coatings show a larger surface area indicating a larger capacitance (Figure 2.3A). Optimizing device performance by tuning the film thickness have been reported for organic electrochemical transistors (20) and low frequency (<1Hz) stimulation electrodes (24), but remains to be investigated for pulsed electrical stimulation.

The increased electrode capacitance benefits three correlated electrochemical performance properties that are explored in various studies: it lowers the impedance, increases the charge injection limit and reduces the potential excursion of the electrode when a current pulse is applied (Figure 2.3B and C) (25-28). Typically, the charge injection of PEDOT:PSS lies in the range of 2-3 mC cm⁻² which is comparable to iridium oxide (25, 27, 28). Titanium nitride has a charge injection of 0.2-0.9 mC cm⁻² (29, 30), platinum 0.8 mC cm⁻² and gold 0.2 mC cm⁻² (28). The high charge injection and the associated reduced electrode potential is suggested to limit undesired electrochemical side effects and is therefore an important performance parameter with respect to safe stimulation. However, literature seems to lack verification of this hypothesis with characterization of possible generation of harmful species and the effect on cells and tissue.



Figure 2.3 | Stimulation performance of PEDOT:PSS coated electrodes. (A) Increased currents in the cyclic voltammogram for increasing film thickness demonstrates the volumetric dependency of the capacitance. (B) Reduced potential excursions for PEDOT:PSS coated electrodes for 10, 20 and 30 μ A. (C) Increased charge injection capacitance of coated platinum and gold electrodes as a function of electrode diameter. (D) Cracking and delamination of PEDOT:PSS coatings after 2 weeks of stimulation with biphasic pulse current at 0.35 mC/cm2 and 50Hz. (E) Change in potential excursion after various amount of pulses indicating material degradation (current pulses 100 μ A, 200 μ s and 25 Hz). Images taken from references (24, 25, 27, 28, 31).

Adequate stimulation electrode performance requires stability which is one of the main concerns with respect to long-term applications with PEDOT:PSS. Figure 2.3D and E show cracking and delamination of the materials after 7 days of stimulation and the increase of the potential excursion after prolonged pulsing, respectively (25, 31). Other studies have shown changes in impedance and charge injection after prolonged stimulation with 0.1-1 ms pulses of 100 μ A – 5 mA that could be acceptable for applications up to 7 weeks (27, 32). Therefore, electrode stability remains a point of attention where the influence of coating thickness and higher voltages or currents on electrode integrity is yet to be explored.

Finally, the enormous parameter space of stimulation electrodes has to be taken into account. Electrode size, electrode configuration and pulse parameters influence key metrics such as current injection limit, charge injection capacity and power consumption. Especially the pulse parameter space is large, involving the waveform, amplitude, duration and frequency.

2.5 Conclusion

PEDOT:PSS has a proven record for bioelectronic applications. The inherent nature and versality of the conducting polymer makes it an excellent candidate for interfacing with biological systems. The blend of PEDOT and PSS forms a disordered material with impure phases. Electronic transport is mediated by weakly bound electrons as a result of conjugation, whereas ionic transport arises from the porous structure through which ions can migrate. Additives such as glycerol, DBSA and GOPS are used to enhance electronic conductivity, wettability and mechanical stability. These supplements affect the material structure and properties and should be well balanced to obtain optimal material performance. PEDOT:PSS

offers advantages regarding charge injection, however the limited material stability should be taken into account. Findings in literature are primarily based on electrochemical characterizations performed in saline solutions without cells or tissue and at relatively low amplitudes suited for neuromodulation applications. However, its performance in actual biological relevant environments and its behavior at higher stimulation amplitudes remains to be investigated.

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3. Influence of PEDOT:PSS Coating Stimulation Thickness on the Performance of Electrodes

PEDOT:PSS is a conducting polymer commonly used to coat the electrodes of biomedical devices to reduce impedance and improve charge injection. The stimulation performance of such electrodes depends on coating thickness due to the volumetric properties of the conducting polymer. Here, the influence of PEDOT:PSS coating thickness on impedance, charge injection, and electrode stability is examined. The results show that stacking spin-coated layers is a reliable method to control coating thickness without introducing electronic or ionic blocking elements in between layers. The electrode capacitance scales linearly with the coating thickness which reduces the impedance and enhances the ability to inject charge. Thicker coatings can endure more stimulation pulses and withstand higher voltages without loss of performance, thus enhancing the lifetime of the electrode. These findings provide guidance in the development of organic bioelectronic stimulation electrodes for optimum performance and longevity.

3.1 PEDOT:PSS coating thickness matters

Stimulation electrodes are used for a wide variety of biological applications. Electrical stimulation can be applied to, both, excitable and non-excitable cells and tissue and is becoming increasingly important for acute and chronic in vivo treatments as well as for in vitro platforms. Examples include cardiac pacemakers for arrhythmia (1, 2), deep brain stimulators for movement disorders (3, 4), spinal cord stimulators for chronic pain (5), cochlear implants for deafness (6), cardiac ablation (7), plate and needle electrodes for electrochemotherapy and irreversible electroporation (8-12) and microchips structures for gene electrotransfer (13-15). The remarkable possibilities of stimulation electrodes brings an ever-expanding interest in materials and devices that requires optimal integration of electronics with biology and demands better understanding of underlying mechanisms.

Stimulation electrode devices require efficacy, tissue safety, and electrode integrity where material selection and manufacturing play an essential role. Commonly used materials include platinum (Pt) and platinum iridium (PtIr) which are considered biocompatible and chemically inert (16, 17), porous titanium nitride (TiN) and iridium oxide (IrOx) to increase the charge injection capacity (18, 19) and common metals such as stainless steel and aluminium (20). A particularly interesting coating material is the organic conducting polymer poly(3,4-ethylenedioxythiophene) doped with polystyrene sulfonate (PEDOT:PSS). As discussed in chapter 2, the success of PEDOT:PSS arises from the unique mechanical and electrochemical material properties, as well as the stability, ease of processing and commercial availability (21–24).

A preeminent aspect of PEDOT:PSS for biological applications is the mixed conductivity of both electrons and ions which makes it an excellent candidate to transduce conventional electronic signals (electrons) into biological signals (ions) (25). The mixed conductivity originates from the ability of PEDOT:PSS to absorb water and uptake dissolved ions, meaning that the entire bulk of the material participates in the electron-ion interaction giving rise to a volumetric capacitance (26). Therefore, the stimulation performance of PEDOT:PSS coated electrodes is expected to depend on the coating thickness.

Indeed, electrochemical impedance decreases and the charge storage capacitance increases as a function of coating thickness (27, 28). However, charge injection capacities are usually calculated based on relatively slow cyclic voltammetry (CV) scan rates, while actual stimulation is often done on much shorter times scales where access to all the available charge is limited by the material morphology (29). Measurements of the actual amount of injected charge upon pulses for various PEDOT:PSS coating thicknesses would give more insight in the real performance of such electrodes.

Furthermore, PEDOT:PSS coating thickness affects the material stability. Coatings are reported to crack and delaminate upon stimulation pulses and the coating thickness seems to influence mechanical stability (27). The authors suggest stress imposed on the coating materials as a possible failure mechanism, however, only two different thicknesses were compared. Coating adhesion and stability is a major concern regarding the use of PEDOT:PSS. Strategies to improve coating durability include adhesion promotors, such as IrOx (30), surface functionalization with (EDOT-NH2) (31) and surface roughening (32). Other works have studied the longterm performance of PEDOT:PSS coated electrodes (23, 33, 34), but did not study the influence of coating thickness. A systematic and detailed analysis of the influence of coating thickness could provide more insight on the stability of PEDOT:PSS coatings.

In this study, the influence of PEDOT:PSS electrode coating thickness on the stimulation performance is investigated by employing unipolar voltage pulses in the range of clinically relevant pulse widths. The electrodes are electrochemically characterized and the charge injection is determined for several pulse parameters. Furthermore, the impact of coating thickness on electrode stability is examined for repetitive and single pulses of various amplitudes.

3.2 Device and fabrication

The performance of PEDOT:PSS coatings was studied with a planar pair of interdigitated electrodes fabricated on a glass substrate (Figure 3.1). The electrodes have a width and spacing of 50 μ m and cover a total surface area of $3.5 \times 4.5 \text{ mm}^2$. The gold electrodes with a thickness of 200 nm were coated with various PEDOT:PSS thicknesses ranging from 60 to 1180 nm. Interdigitated electrode geometry was chosen to deliver homogenous electric fields across the device. Similar designs are used by others to evoke uniform cell response for stimulation and electroporation applications (*14*, *35*).



Figure 3.1 | Interdigitated electrodes. (A) 4 pairs of electrodes on a glass substrate including a glass well to confine the electrolyte. (B) Electrodes with a width a spacing of 50 μ m covering a total area of 3.5 x 4.5 mm.

The fabrication process is shown in Figure 3.2 and is an adapted version of a previously published protocol (*36*). After cleaning the glass substrates, thermally deposited gold was patterned by lift-off. Chromium was used as an adhesion promotor between gold and the glass. PEDOT:PSS was patterned through a mechanical lift-off or so-called "peel-off". Chemical vapor deposited parylene C was patterned by reactive ion etching and removed after PEDOT:PSS spin coating and annealing. The PEDOT:PSS dispersion consisted of a mixture of Heraeus Clevios PH1000, glycerol, dodecyl benzene sulfonic acid, and (3-glycidyloxypropyl)trimethoxysilane. Various PEDOT:PSS thicknesses were obtained by variable spin speeds and deposition of multiple layers.


Figure 3.2 | Fabrication scheme. Patterning of gold electrodes with a lift-off process and patterning of PEDOT:PSS with a sacrificial parylene C layer.

3.3 Electrochemical characterisation

 $\mathbf{P}_{\mathrm{EDOT:PSS}}$ coatings on plain substrates were studied first to determine the thickness and electrical conductivity. Figure 3.3A shows the thickness and electrical conductivity of the PEDOT:PSS film as a function of spin speed is shown. The thickness shows an inverse proportionality with the spin speed and varies from 325 to 60 nm for 500 to 5000 RPM, respectively. A spin speed below 500 RPM resulted in nonuniform films and spin speeds higher than 5000 RPM are not expected to produce much thinner films. The electrical conductivity (σ) was calculated as $\sigma = 1/(Rs \times t)$, where Rs is the sheet resistance measured with a four-point method and t is the thickness. The conductivity is independent of the spin speed with a value of about 360 S cm⁻¹ which is similar to previous findings (37). This independence suggests that possible changes in the film morphology and/or structure of the polymer chains, due to larger centrifugal forces, has no effect on the electrical conductivity. In Figure 3.3B the thickness and electrical conductivity is shown as a function of the number of layers (all layers are spun at 1500 RPM). The thickness of a single layer is 120 nm and the correlation is linear such that the total thickness equals $t_{total} = 120 \text{ nm} \times \text{number of layers}$. The electrical conductivity is around 360 S cm⁻¹ which equals the conductivity of single layers. The conductivity is independent of the number of layers which indicates there is no electrical barrier between the different layers. Similar relations between PEDOT:PSS spin coating process, film thickness, and electrical conductivity have been reported before, however, no cross-linker was used and comparison is not trustworthy since dependencies rely on dispersion composition (38).



Figure 3.3 | Various PEDOT:PSS thickness and electrical conductivity. (A) various spin speeds and (B) multiple layers (all layers spun at 1500 RPM).

Although plain substrates were used to determine the electrical conductivity, the actual device is obtained through a patterning process that includes the etching of the electrode pattern into a 3 μ m thick sacrificial parylene C layer. Spin coating PEDOT:PSS on top of this topology makes the thickness in the trenches higher compared to spin coating the solution onto a plain substrate. Figure 3.3B shows that for an actual device the thickness still linearly depends on the number of layers, but instead of 120 nm, the individual layers have a thickness of 131 nm and thus t_{total} = 131 nm × number of layers.

Electrochemical characterization of the electrodes was performed in phosphate buffered saline (PBS) solution with a two-electrode setup where the cathode and anode of the interdigitated electrodes were the working (WE) and counter (CE) electrode, respectively. The magnitude of the electrochemical impedance spectra (EIS) and the cyclic voltammograms (CV) for various coating thicknesses, as well as for an uncoated pair of electrodes, are shown in Figure 3.4. The impedance exhibits the typical shape of PEDOT:PSS coated electrodes which can be described with a capacitive dominated region at low frequencies and a resistive dominated region at high frequencies. The two regimes are separated by the cut-off frequency (f_c). Increasing the thickness of the PEDOT:PSS coating lowers f_c and decreases the impedance for frequencies $< f_c$, which can be related to an increase in capacitance. The CV was performed with a scan rate of 1 V s⁻¹ and the voltage was swept between -0.7 and 0.8 V. This window was determined to be the "water window" including a small margin. Voltages outside this range resulted in a drastic increase in current. The voltammograms have a rectangular shape without noticeable redox peaks suggesting double-layer charging and the absence of electrochemical reactions. Thicker coatings show larger currents indicating an increase in capacitance.



Figure 3.4 | EIS and CV for various PEDOT:PSS thicknesses. (A) Magnitude of the electrochemical impedance as a function of frequency. (B) Cyclic voltammogram obtained with a scan speed of 1 V s^{-1} .

For a better understanding of the nature of the electrode–electrolyte interface, the capacitance (C) of the electrodes was determined by modelling the EIS with the equivalent $R_s(R_e||C_e)$ circuit shown in Figure 3.5A. The fitted parameters are listed in table 1. Additionally, the charge storage capacity (CSC) was calculated from the CV by integrating the current over a scan from -0.7 to 0.8 V. As shown in Figure 3.5B, the capacitance and CSC depend linearly on the coating thickness. This reveals that the entire bulk of the PEDOT:PSS film is involved in the electrode–electrolyte interaction and that there is no barrier between the stacked PEDOT:PSS layers that hinders ion diffusion in the film, confirming an intact volumetric capacitance. Boehler et al., reported similar electrochemical characterization of electrodes where the PEDOT:PSS capacitance scales linear with the coating thickness (28). However, electrochemical deposited PEDOT:PSS was studied which is likely to have a different morphology since no solvents and cross-linkers aided the formation of the PEDOT:PSS film. Such structural changes of the material might change ionic and electronic transport phenomena in the bulk of the material. Nonetheless, volumetric capacitance was reported which is in agreement with the findings reported here.



Figure 3.5 | Volumetric capacitance of PEDOT:PSS. (A) $R_s(R_e||C_e)$ equivalent circuit. (B) Capacitance and charge storage capacitance as function of PEDOT:PSS coating thickness.

<u>Thickness (</u> nm)	<u>R</u> ε (Ω)	<u>R_e</u> (ΜΩ)	C _e (μF)	τ (μs)
110	11,2	4,9	9,4	106
160	11,1	3,8	13,0	144
285	11,3	2,4	21,9	247
390	10,7	1,6	31,1	333
520	10,8	0,8	40,0	432
650	10,7	0,9	50,0	535
920	11	0,6	65,7	723
1180	10,7	0,5	83,6	895
0	15,3	15,2	0,3	5

Table 1. Fitted parameters of the $R_s(R_e||C_e)$ circuit.

Although the capacitance scales linearly with the coating thickness, it is important to realize that the impedance does not. In Figure 3.6, the normalized impedance is shown as a function of coating thickness for 10 Hz, 1 kHz and 10 kHz. For 10 Hz, the correlation is nonlinear and the impedance saturates, but still decreases for a thickness >1 μ m. For 1 kHz the saturation occurs around 650 nm and the 10 kHz impedance is independent of the thickness. This is an inherent property of a R_s(R_e||C_e) circuit, where for high frequencies the capacitor is in "shorted state" such that the impedance equals R_s. A higher capacitance shifts the capacitive dominated region

to lower frequencies such that the impedance at those frequencies converge to R_s . Indeed, the impedance for the frequencies in the capacitive region (10 Hz and 1 kHz) decreases and converges to R_s .



Figure 3.6 | Normalized impedance. as a function of coating thickness for 10 Hz, 1 kHz, and 10 kHz.

The EIS and CV provide means to determine the capacitance and the amount of charge that can be stored, however, the actual injected charge must be determined with current measurements. In Figure 3.7A and B, the current for various coating thicknesses is shown for a unipolar 1 V pulse of 100 μ s and 1 ms, respectively. For reference, the current of an uncoated device is shown as well. All currents resemble ideal capacitor behavior, where the initial current has its maximum value I₀ followed by an exponential decay according to I = I₀ × e^{-t/r}. Due to the larger capacitance, thicker coatings have larger time constants ($\tau = \text{Rs} \times \text{Ce}$) which results in a slower decay of the current. Time constants calculated by fitting the EIS measurements with a R_s(R_e||C_e) circuit (Table 1) are in agreement with the measured currents in Figure 3.7B. The linear correlation of the response time with thickness, as shown in Figure 3.7C, confirms full access to all the available charge for pulsed stimulation applications and validates the stacking of PEDOT:PSS layers as a fabrication method to obtain various coating thicknesses.



Figure 3.7 | Current response for unipolar, 1 V pulses for various coating thicknesses. (A) Current for 100 μ s pulses. (B) Current for 1 ms pulses. Solid lines represent measurements and dashed lines show the modelled current with the time constant determined with the R_s(R_e||C_e) equivalent circuit. (C) Time constants and (D) normalized charge injection as a function of coating thickness.

The charge injection was determined by integrating the current over time and is shown in Table 2 for various coating thicknesses. Figure 3.7D shows the normalized charge injection as a function of coating thickness. The normalization was performed with respect to the charge injection for a 110 nm coating which was the thinnest coating that we studied here. For 100 μ s pulses, the charge injection saturates around 650 nm and the charge injection is 1.6 times larger than for 110 nm coating. For 1 ms pulses the normalized charge injection is 5 times larger for 1180 nm coating and the graph did not saturate yet. Increasing the thickness further would further increase charge injection, however, it is expected to saturate at some point.

Table	1.	Cha	arge inje	ection (μC) for	variou	s thickr	nesses,	voltages	and p	ulse lengths.
			110 nm	160 nm	285 nm	390 nm	520 nm	650 nm	920 nm	1180 nn	uncoated
100u	S	1V	4,8	5,7	6,4	7,1	7,2	7,6	7,8	7,9	0,4
		5V	27,4	31,1	34,0	37,2	38,1	39,4	41,1	41,0	15,6
]	10V	58,3	61,4	65,6	72,1	74,8	75,5	76,2	80,6	49,6
_1ms	5	1V	10,5	13,1	21,1	28,2	32,7	38,5	43,5	50,6	1,4

Note that the current of the 100 μ s pulses are identical to the first part of the current for the 1 ms pulses for each thickness. The response time remains the same, but the charging of the polymer is stopped at different times. The thinnest coating charges fast ($\tau = 106 \mu$ s) and at the end of the 1 ms pulse less than 3% of the initial current is left such that increasing the pulse length beyond this value will not increase the charge injection much further. For a 1180 nm coating with

 $\tau = 895 \,\mu s$ the capacitor charging is still ongoing after 1 ms hence still injecting charge (current is 30% of the initial current). Consequently, to fully exploit the superior PEDOT:PSS capacitance in terms of charge injection, longer pulses are more beneficial for voltage driven stimulations.

Figure 3.8A and B show the current for 100 μ s pulses for various coating thicknesses for 5 and 10 V, respectively. The magnitude of the initial currents I₀ scales with the voltage according to I₀ = V/R_s. For 5 V the current of the coated electrodes is still transient, whereas the uncoated electrodes show a constant current. Steady state currents indicate Faradaic currents which are to be expected since these potentials exceed the water window. However, the current of PEDOT:PSS coated electrodes remain transient suggesting capacitive charging. For pulses of 10 V constant currents were observed for the 110 and 160 nm coating, but for the >650 nm coatings no steady state currents were observed, and the currents remained transient. In Figure 3.8C, the normalized charge injection for 1, 5, and 10 V is shown. The charge injection for an optimum charge delivery. For higher voltages the gain in charge injection by making the coating thicker becomes less pronounced which can be related to the change in current from transient to constant.



Figure 3.8 | Current response for unipolar, 100 µs pulses for various coating thicknesses. (A) Current for 5 V. (B) Current for 10 V. Current for 1 ms pulses. (C) Normalized charge injection for as a function of thickness for 1, 5, and 10 V.

To obtain electropermeablization of cells and tissue it is crucial to deliver a sufficiently strong electric field and the required applied potentials depend on the electrode architecture. For microchips consisting of interdigitated electrodes with spacings of 100-500 μ m, 20-60 V is need to obtain optimum electropermeabilization (14, 39). Therefore, Faradaic processes typically dominate the current injection and steady state currents are to be expected. More details regarding transient and steady state currents, and the effect on electropermeabilization will be discussed in chapter 4. In the context of neurostimulation applications, Faradaic currents should be avoided to prevent electrode degradation and the production of harmful species for cells and tissue. The transient current for thicker coatings suggests capacitive charging and therefore safer charge injection.

3.4 Influence of PEDOT:PSS thickness on stability

Besides the charge injection, a critical parameter for stimulation electrodes is their stability upon application of pulses. The influence of coating thickness on electrode stability was determined by exposing electrodes with various coating thicknesses to a series of 100 μ s pulses at 10 V with a repetition frequency of 10 Hz. Every 3000 pulses the current and EIS were measured and the experiment was stopped after 21 000 pulses.



Figure 3.9 | Electrode stability for repetitive 10 V, 100 μ s, 10 Hz pulses. Change in A) impedance and B) current for a 390 nm PEDOT:PSS coating during prolonged application of pulses. Impedance and current were measured after every 3000 pulses. C) Normalized 10 kHz impedance and D) normalized charge injection as a function of number of pulses for various coating thicknesses.

In Figure 3.9A and B, the gradual increase of the impedance and decrease in current after each 3000 pulses for a 390 nm and 920 nm coating are shown. The increase of impedance and decrease of current indicates a change in the electrochemical electrode-electrolyte interaction and a loss in performance. The degree of loss in performance, however, depends on the coating thickness which is shown in Figure 3.9C and D, where the normalized 10 kHz impedance and charge injection as a function of number of pulses are shown for various coating thicknesses. The impedance of coatings <650 nm gradually increases, whereas for coatings >650 nm the impedance remains near its initial value. Similarly, the charge injection decreases for the thinner coatings, whereas it remains 90% of its initial value for coatings >650 nm.

Figure 3.10 shows scanning electron microscopy (SEM) micrographs which were taken after the experiments. The gradual decrease in electrode degradation with increasing coating thickness was evident. Severe damage was observed for the 115 and 285 nm coatings in the form of coating wrinkling, cracking, and delamination, exposing the underlying gold layer. The 390 nm coating suffered the same degradation to a lesser extent. The 650 nm coating wrinkled, but did not crack or delaminate and the 920 nm coating showed almost no visible damage. These visual observations are consistent with the analysis of the EIS and charge injection in Figure 3.9 and reveal that thicker coatings increase the electrode stability.



Figure 3.10 | SEM images after 21 000 pulses of 10 V for various coating thicknesses.

Note that this minimum thickness is application dependent. For lower voltages or when less pulses are required, a thinner coating might be sufficient. Figure 3.11 shows the charge injection as a function of number of pulses for 5 V pulses.



Figure 3.11 | Electrode stability for repetitive 5 V, 100 µs, 10 Hz pulses. Normalized charge injection as a function of number of pulses for various coating thicknesses.

Depending on the application targeted, various voltages might be applied, and it is important to study the effect of different voltage amplitude on the electrodes. To examine how the coating thickness affects the maximum voltage an electrode can withstand, single pulses were applied while gradually increasing the voltage in steps of 2 V. The EIS was measured after each pulse. Figure 3.12A shows how the EIS gradually increases around the 1 kHz frequency (105 nm coating) indicating first signs of electrode damage. The gradual increase of the normalized 1 kHz impedance for 105, 235, 500, and 900 nm coatings are shown in Figure 3.12B. Values are the average of 3 devices and error bars represent the standard deviation. The failure voltage was defined as the voltage where the 1 kHz impedance increased by 10 % or more from its initial value and is shown as a function of coating thickness in Figure 3.12C. The failure voltage for a 900 nm coating was more than 2.5 times higher than a 105 nm coating demonstrating the improved capability of thicker coatings to withstand higher voltages. However, the dependency is nonlinear and seems to saturate suggesting there is a maximum failure voltage that cannot be further increased. These results can be related to Figure 3.9-3.11 where endurance of the electrodes was improved by increasing the coating thickness. Figure 3.12D and E show a tilted and cross sectional SEM image of the 235 nm coated electrode after the experiment. Without cracking, the PEDOT:PSS film detached from the gold, bended and wrinkled. Interestingly, this happened without decreasing the thickness which implies there was no stretching but rather a permanent increase in volume. The observations from the SEM micrographs suggest that electrode degradation starts with detachment and wrinkling of the PEDOT:PSS followed by

cracking and full delamination. This damage to the film could be caused by mechanical stress due to expansion and shrinkage of the PEDOT:PSS layer. Ion migration in and out of the polymer changes its volume, which results in a stress gradient between the PEDOT:PSS and gold layers, a mechanism that is exploited in PEDOT-based actuators (40).



Figure $3.12 \mid$ Electrode stability for single 100 µs pulses. a) Electrochemical impedance spectra before and after pulses of various voltages for a 105 nm coating. b) Normalized 1 kHz impedance as a function of voltage for various coating thicknesses. c) Failure voltage as a function of thickness. For b) and c), values are the average of 3 devices and error bars indicate the standard deviation d) Tilted and e) cross sectional SEM of a typical damaged electrode (coating thickness: 235 nm).

To determine whether it is the voltage or the current that degrades the electrodes, the single pulse experiments were repeated with a lower conductive electrolyte to lower the currents. Failure of the coating for undiluted PBS occurred at 14 V, whereas for diluted PBS it occurred at 22 V. The results in Figure 3.13 suggests that it is not the magnitude of the voltage or current that determines the failure threshold, but the shape of the current. Increasing the voltage changes the shape from a transient to a constant current. This gradual change in shape occurs at different voltages for the two electrolytes with different conductivity, however, the failure threshold is reached where the shape of the currents match (Figure 3.13E). Similar reasoning holds for the results on repetitive pulses, where for 10 V the thick coatings have transient currents and do not degrade, while the thin coatings with constant currents do.



Figure 3.13 | Stability for single pulses in lower conductive media. Coating thickness is 105nm. PBS was diluted with de-ionized water in a 1:2 ratio. A) Normalized 1kHz impedance as a function voltage. B) Normalized 1kHz impedance as a function current. C) Current for different voltages for diluted media. D) Current for different voltages for undiluted media. E) Normalized current for undiluted 14 V pulse and diluted 22 V pulse. The shape of the current is similar indicating a similar (faradaic) nature of the current.

Constant currents suggest Faradaic currents that are established by electrochemical reactions between the electrode and electrolyte. In case of irreversible reactions this possibly leads to deterioration of the electrode material, which was indeed observed for thin coatings. Irreversible electrochemical reactions for PEDOT films in aqueous solutions starts at 0.8 V versus KCl-saturated calomel electrode and increases for higher voltages (*41*). This so-called over-oxidation of the polymer causes the formation of side groups, such as SO2 and carbonyl and ultimately leads to the cleavage of the C-C bonds that result in changes in the morphology and crevices in the film which decreases the electrical conductivity. However, these characterizations were done employing CVs with moderate scan rates, whereas our experiments were performed with relatively fast pulses. Thicker coatings show no constant currents, which could indicate the absence of irreversible faradaic currents, hence less electrode deterioration. The correlation between the shape of the (Faradaic) currents and the electrochemical events at the electrochemical events at the electrochemical deterioration are two likely mechanism that causes PEDOT:PSS coated electrodes to degrade.

3.5 Conclusion

I his work demonstrated the fabrication of electrodes with various PEDOT:PSS coating thickness by varying spin speed or stacking multiple layers and studied the influence on stimulation performance. Electrical conductivity was found to be independent of thickness and EIS and CV confirmed ionic bulk properties for a thickness up to 1180 nm. These findings reveal unhindered electronic and ionic transport without blocking elements between layers. Thicker coatings reduce the impedance and increase the charge injection. Improved charge injection becomes more profound for longer pulses, but less profound for higher voltages,

which indicates that the full charge injection capabilities of PEDOT:PSS are achieved in slow applications with moderate voltages. Furthermore, the coating thickness influences the stability. Thicker electrode coatings can endure more pulses and higher voltages before losing their charge injection capability. This is particularly important for chronic applications where longterm stability is required and applications that require high voltages such as electroporation. Improved durability of thicker coatings was linked to the possible absence of irreversible electrochemical processes that could deteriorate the material.

Figure 3.14 | Illustration of the performance parameters as a function of coating thickness. The electrode stability, charge injection and impedance are determined by the PEDOT:PSS thickness which has to be optimized for each specific application.

Finally, performance parameters such as impedance, charge injection and stability saturate as a function of coating thickness indicating an optimum thickness that has to be determined for each specific application (Figure 3.14). The optimal coating thickness for 100 μ s pulses was found to be around 600 nm. This coating thickness can endure 21 000 pulses of 10V with >90 % charge injection remaining and nearly no visual damage, and a failure voltage of 30 V at which the very first indications of damage were observed.

3.6 Materials and methods

Device Fabrication: The fabrication of the devices is an adapted version of a previously published protocol (*36*) and is shown in Figure 3.2. Glass slides were cleaned by sonication in solvents followed by an oxygen plasma treatment. Metallic electrodes were obtained by lift-off with photolithographically patterned AZ nLOF 2070 (SUSS MJB4 contact aligner) and deposition of 10 nm chromium and 180 nm gold (BOC Edwards thermal evaporator). PEDOT:PSS coatings were patterned and deposited as follows. After deposition of a 3 μ m sacrificial parylene C layer (SCS labcoater 2) a positive photoresist AZ9260 was spin-coated and photolithographically patterned. The parylene C was etched (Oxford 80 Plasmalab) followed by stripping the remaining photoresist. The PEDOT:PSS mixture as described below was spin-coated, different devices having different amount of layers. After annealing (140 °C bake for 1 h), devices were immersed in deionized water and the sacrificial parylene C layer was peeled-off.

Multiple PEDOT:PSS Layers: A mixture of Heraeus Clevios PH1000, glycerol, dodecyl benzene sulfonic acid, and (3-glycidyloxypropyl) trimethoxysilane (42) was spin-coated, baked at 110 °C and cooled down before spin-coating the next layer. This was repeated until the

desired number of layers was attained. The final substrate was baked at 140 °C for 1 h and rinsed with deionized water.

Thickness measurement: PEDOT:PSS thickness on plain substrates was measured by carefully scratching the film with a plastic tip to create a step that was measured with stylus profilometry (Bruker Dektak). For the electrodes the gold thickness was measured after the lift-off and the gold + PEDOT:PSS thickness was measured on the final device. Subtracting the two measures gives the PEDOT:PSS thickness of the electrode.

Electrical Conductivity: The sheet resistance (R_s) of PEDOT:PSS films was measured using a 4-point probe method with 4 metallic, spring loaded, rounded electrodes and a Keithley 2450 source meter with a custom programmed script. The electrical conductivity (σ) was calculated with $\sigma = 1/(Rs \times t)$, where t is the film thickness.

Electrochemical Characterization: EIS and CV were performed with an Autolab PGSTAT128N in a 2-electrode setup. One of the interdigitated electrodes functioned as the working electrode and the other as the counter electrode. PBS was used as an electrolyte. For EIS, a sinusoidal voltage of 10 mV was applied between 1 and 100 000 Hz. For CV the voltage ranged from -0.7 to 0.8 V, the scan rate was 1 V s⁻¹ with steps of 2.44 mV. Three scans were used to stabilize the shape of the voltammogram.

Pulse experiments: An Avtech AV-1011-C pulse generator was used to apply voltage pulses to the electrodes. Current and voltage were measured employing a digital multimeter from National Instruments. LabView was used for the data acquisition.

Stability Measurements: PBS was used as an electrolyte. Single or repetitive pulses (10 Hz) were applied and after each pulse or time period the electrolyte was refreshed and EIS and/or current was measured.

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4. Exploring the Electrochemistry and the Electropermeabilization Performance PEDOT:PSS Coated Stimulation Electrodes

Electropermeabilization occurs when cells are exposed to sufficiently high pulsed electric fields and this phenomenon is now an important technique to facilitate the transmembrane transport of molecules. The application of high amplitude, electric pulses can initiate electrochemical events at the electrode-electrolyte interface that alter the extracellular environment. Such processes strongly depend on the electrode material and the effect on cell electropermeabilization is not fully understood yet. Coating electrodes with the conducting polymer PEDOT:PSS enhances the charge injection capacity, however, the electrochemistry and electropermeabilization performance at higher voltages is unknown. Here, we show that electrical stimulation with PEDOT:PSS coated electrodes strongly reduces irreversible electrochemical reactions that otherwise lead to changes in pH and the generation of reactive oxygen species (ROS). Coated electrodes exhibited a slightly higher stimulation threshold, however, little electroporation was observed at voltages that greatly exceed the threshold for uncoated electrodes. Our findings indicate that electrically induced, extracellular chemical changes represent an important mechanism in the cellular electropermeabilization response. Moreover, PEDOT:PSS coated electrodes dissociate the delivery of electric pulses from chemical changes which paves the way for future experiments that allow separation of these events which might lead to a better understanding of the underlying mechanisms.

4.1 The role of oxidative species on electropermeablization

Exposure of cells and tissue to sufficiently strong pulsed electric fields (PEFs) induces a transient increase in membrane permeability known as electropermeabilization. This allows external compounds, that otherwise cannot penetrate the membrane, to enter the cells (1, 2). Molecular delivery by means of electropermeabilization, also known as electroporation, is used to electrotransfer genes (3, 4) and cytotoxic drugs which enables successful treatment of cancerous tumors (5-7). Moreover, pulses with higher amplitude and longer duration lead to irreversible electroporation and direct cell death which can be used for nonthermal ablation of tissue and tumors (8, 9).

Despite decades of investigation and successful applications, the underlying mechanisms that cause the increased permeability of the cell membrane are not fully understood. The formation of aqueous pores in the cell membrane throughout application of the pulse is widely acknowledged, however, simulations (10, 11) do not support the long-lasting permeabilization that is found in experiments (12, 13). Chemical changes to the lipid bilayer and embedded proteins have been hypothesized as an additional mechanism that could explain such difference in timescales (14). Oxidative stress caused by reactive oxygen species (ROS) can initiate chemical reactions such as peroxidation of the membrane lipids which affect the cell membrane integrity (15). PEF induced generation of ROS and lipid peroxidation correlates with the intensity, duration and number of pulses and increased generation of ROS is associated with higher electroporation efficiencies (16-20).

Electrically induced generation of ROS occurs within cells as well as at the electrode site (17). At sufficiently high voltages, metallic electrodes induce Faradaic currents that are established by electrochemical reactions at the electrode-electrolyte interface. Besides ROS, such irreversible Faradic processes might result in pH changing species, formation of gas, chloride oxidation products and electrode dissolution (21–24). The nature and produced species of these reactions strongly depend on the electrode material. Aluminium and stainless steel as well as metals which are generally considered inert and chemically stable such as platinum, platinum alloys and gold, support electrochemical reactions resulting in a change in the chemical composition of the electrolyte (25–28).

A particularly interesting material that has yet to be investigated for electropermeablization is the conducting polymer PEDOT:PSS (poly(3,4-ethylenedioxythiophene) doped with polystyrene sulfonate). As discussed in chapter 2, PEDOT:PSS has emerged as a popular material for biomedical devices due to its unique mechanical and electrochemical properties, as well as the ease of processing, stability and commercial availability (29-31). PEDOT:PSS coated electrodes exhibit a large charge injection capacity due to the mixed ionic-electronic conduction which benefits neurostimulation applications (32). Despite its proven record for biomedical applications, the electrochemistry of PEDOT:PSS remains controversial and is yet to be fully understood (33, 34). At relatively low voltages (<1V), PEDOT:PSS exhibits ideal capacitive behavior without inducing redox reactions as shown by electrochemical impedance spectroscopy (35), 'moving front' (36), volumetric capacitance (37) and modelling that match cyclic voltammograms obtained from experiments (38). On the other hand, PEDOT:PSS is commonly described as pseudocapacitive where charge is stored reversibly through fast oxidation and reduction reactions at the electrode surface (39) and the polymer has been shown to efficiently reduce oxygen to produce hydrogen peroxide (H_2O_2) , indicating the presence of irreversible Faradaic processes (40, 41). Despite considerable progress in the understanding of PEDOT:PSS and its coupling with electrolytes, the stimulation performance and possible generation of ROS at higher voltages has yet to be explored.

In this study, we characterized and compared the electropermeablization performance of PEDOT:PSS coated and uncoated gold electrodes. The charge injection mechanism was studied by analyzing the current response for conventional electropermeablization pulse parameters: constant voltage, monophasic and 100 μ s pulse width. pH indicators and a fluorescent ROS indicator are used to evaluate irreversible Faradaic reactions and the generation of species that change the chemical composition of the electrolyte. Finally, the cellular response in terms of stimulation and electropermeabilization is determined.

4.2 Interdigitated device architecture

The study was performed with the same planar, two electrode devices that were used for the thickness study in chapter 3. Figure 4.1 shows the device architecture and dimensions. The interdigitated, gold electrodes have a width and spacing of 50 μ m and were coated with PEDOT:PSS with a thickness of 575 nm. This coating thickness was determined to be optimal in terms of charge injection and demonstrated a threshold of 30 V before slight changes in the stimulation performance were observed. The coating was extended 5 μ m to ensure full coverage of the underlying gold layer. Interdigitated electrode architecture was primarily chosen to establish a repetitive electric field and study the response of a large cell population where each individual cell situated between the fingers is exposed to an equivalent electric field.

Figure 4.1 | Device architecture. (A) Two electrode device with interdigitated finger configuration. The total area covered by the fingers is $3.6 \times 4.8 \text{ mm}$. (B) Tip of an electrode finger. (C) Cross section of an electrode finger and dimensions. The gold electrode width is 50 μ m. The PEDOT:PSS coating width and thickness are 60 μ m and 575 nm, respectively.

4.3 Current response and charge balance

The current response was determined in phosphate buffered saline (PBS) solution where the pair of interdigitated electrodes functioned as anode and cathode. Figure 4.2A shows the current responses for monophasic, 100 μ s pulses of various voltages (1-30 V) applied to uncoated and coated electrodes. The uncoated electrodes showed capacitive behaviour for 1 V

with an exponentially transient current of the form $I = I_0 \cdot e^{-t/\tau}$ with a resistance-capacitance (RC) time constant $\tau = 5 \ \mu$ s. At higher voltages, current reached a steady state, corresponding to the typical behavior of metallic electrodes, where a transition in charge injection mechanism occurs from the capacitive to Faradaic regime at increasing voltages. Figure 4.2B confirms this transition with a change in slope in the charge injection curve between 1 and 10 V. In this region the current is established by a combination of electrostatically coupled charges that accumulate at the electrode-electrolyte interface and electrochemical reactions with associated electron transfer across the interface. For voltages >10 V the charge injection depends linearly on the voltage implying resistive behavior that is characteristic for Faradaic dominated currents. PEDOT:PSS coated electrodes showed exponentially transient currents with a time constant $\tau = 430 \ \mu s$ and no change in the shape for voltages up to 20 V. The increased time constant reveals the relatively large capacitance of coated electrodes which has been characterized in chapter 3. For 30 V a steady state component appeared similar to the uncoated electrodes. This change in current shape is confirmed by the correlation coefficient of an exponential fit which is 0.99 up to 20 V, but decreases for higher voltages (Figure 4.3).

Figure 4.2 | Current response for monophasic voltage pulses. (A) Current response for various voltage amplitudes. (B) Charge injection as a function of voltage. The inset is focused on the 1-10 V range. The green/red color bar represents the transition from the capacitive to the faradaic regime for uncoated electrodes. (C) Current response for a 1 V pulse with a negative, discharging current after application of the pulse. The total charges involved throughout and after the pulse are indicated by the coloring under the curves. (D) Charge balance as a function of voltage.

Figure 4.3 | Correlation coefficient of the current response. The current responses are fitted an exponential function and the correlation coefficient of the fit as shown as a function of voltage.

The positive current during the application of the pulse was followed by a negative, transient current which represents the discharging of the electrode-electrolyte interface (Figure 4.2C). The two electrode types showed a large difference in discharge time with less than 1% of the

current remaining after 170 μ s for uncoated and 2.5 ms for coated electrodes. The ratio between the charge injected throughout the positive and negative phase of the current defines the charge balance. Figure 4.2D shows that the charge imbalance decreased rapidly as a function of voltage for uncoated electrodes, whereas coated electrodes showed >96 % charge balance for voltages <10 V.

High level of balanced charge implies that charging of the PEDOT:PSS coated electrodes in this voltage range is predominantly established by capacitive coupling and/or by reversible Faradaic processes and suggests the absence of irreversible electrochemical reactions. Importantly, the Faradaic component and charge balance depends on the coating thickness. Figure 4.4 shows that for a PEDOT:PSS coating of 125 nm thickness, a constant current appeared at 10 V, where only 63 % of the charge was balanced. The dependency of charge balance on the coating thickness suggests that saturation of the PEDOT:PSS bulk plays a role in the charging phenomena and underlines the need for a sufficient coating thickness to prevent Faradaic currents. Note that these observations also reveal a lower maximum repetition rate of stimulation pulses for coated electrodes since the application of consecutive pulses is limited by the time needed to discharge the electrodes.

Figure 4.4 | Current response and charge balance for 125 nm coating. (A) Current response for a 10 V pulse. (B) Charge balance as a function of voltage. The data uncoated and 575 nm coating are shown as well for comparison.

4.4 Stimulation induced generation of harmful species

To investigate the role of irreversible Faradaic processes and the associated species that are generated, colorimetric chemical indicators were used to visualize the change in pH. Similar indicators have been used previously to illustrate pH changes in electroporation applications (23). Figure 4.5A and B show the distribution of phenol red (transition from yellow to fuchsia for pH 6 to 8) in 0.1 M potassium chloride (KCl) when 150 pulses at 10 Hz were applied for 5, 8 and 10 V. For uncoated electrodes a clear color change was observed starting at 5 V which became more pronounced at higher voltages, indicating pH shifts above 8. Coated electrodes showed no color change in the solution indicating that pH remained constant.

Figure 4.5 | Colorimetric visualization of change in pH. (A) and (B) Coloring of phenol red for uncoated and coated electrodes induced by 150 pulses with a repetition frequency of 10 Hz and various voltages (5, 8 and 10 V). (C-F) Coloring of phenolphthalein (C and E) and congo red (D and F) for 500 μ m spaced electrodes for 10 pulses of 30 V with 5 Hz repetition rate. For all images the left and right electrodes are the anode and cathode, respectively. Images were taken 3 seconds after the application of the pulse.

In order to enhance visualization of pH changes at the anode and cathode, we examined devices with 500 μ m electrode spacing. Figure 4.5C-F show the coloring of phenolphthalein (colorless for pH < 8.2, fuchsia for pH >10) and congo red (red for pH >5.2, blue for pH <3) in 0.1 KCl for 10 pulses at 5 Hz for 30 V. Figure 4.6 shows the coloring for various voltages for uncoated electrodes. The color change of uncoated electrodes indicates that severe alkalization occurred at the cathode with pH values above 10 and acidification at the anode with pH values below 3. Coated electrodes showed no coloring at the cathode and only minor pH shifts at the anode obviating local acidic or alkaline environments.

Figure 4.6 | Voltage-dependent pH change for uncoated electrodes. (A) Coloring of phenolphthalein and (B) congo red in 0.1M KCl induced by 10 pulses with a repetition frequency of 5 Hz at various voltages.

Certain electrochemical reactions that influence pH involve the production of oxygen and hydrogen gas. Moreover, chlorine gas can be formed by oxidation of chloride ions present in the electrolyte. Indeed, gas bubbles were observed on the uncoated electrodes (Figure 4.7). The coated electrodes showed no bubbles confirming the absence of electrochemical reactions associated with the formation of gas. However, at high voltages we observed a minor swelling or inflation of the coating which appeared to be reversible as it returned to its initial state after the pulse. As shown in chapter 3, repetitive pulses will eventually damage the electrodes which depends on the voltage amplitude and number of pulses. No color shifts of the pH indicator were observed for uncoated electrodes in PBS or when a single pulse was applied. This confirms the cumulative effect of pH change, where the amount of generated species depends linearly on the total transferred charge. pH changing species are therefore also produced during a single pulse, but in smaller quantities.

Figure 4.7 | Formation of gas and reversible PEDOT:PSS delamination. (A) Formation of gas on uncoated electrodes after application of a single pulse for 10, 20 and 30 V. Bubbles are mainly formed on the cathode. (B) PEDOT:PSS coated electrodes after application of a single pulse for 10, 20 and 30 V. Note that the observed change is not formation of gas, but delamination and 'inflation' of the PEDOT:PSS film. (C) Reversibility of coating delamination for 10 pulses of 10 V with a repetition frequency of 5 Hz. Images shown are taken at 0, 20 and 40 seconds after application of the pulses.

In addition to pH changing species, the generation of reactive oxygen species (ROS) were measured with the fluorescent marker 2',7'-dichlorodihydrofluorscein (DCFH₂). Upon exposure to oxidizing species, the non-fluorescent DCFH₂ is converted to the highly fluorescent 2',7'-dichlorofluorscein (42, 43). As shown in Figure 4.8A, for uncoated electrodes the DCF fluorescence increased linearly at voltages above 10 V. This corresponds to the results of Figure 4.2 where it was found that at these voltages Faradaic currents dominate the charge injection and the current depends linearly on the applied voltage. Although charge was not balanced for 1 and 3 V pulses, no apparent change in ROS was observed for a single pulse. However, generation of ROS was confirmed at these voltages as well by applying multiple pulses revealing an increase in fluorescence with each pulse (Figure 4.9). Coated electrodes produced attenuated fluorescence indicating less generation of ROS. Almost no increase in ROS was observed at voltages below 10 V and a small, gradual increase at higher voltages, commensurate with the charge balance behavior.

Figure 4.8 | Stimulation induced generation of ROS. (A) DCF fluorescence response as a function of voltage for single pulses (electrode spacing 50 μ m). Values and error bars represent the average and standard deviation of 3 measurements, respectively. (B) Fluorescent images taken 60 seconds after a 20 V pulse (electrode spacing 500 μ m). (C) Intensity profile taken from the images in (B). The profile is taken perpendicular to the electrode fingers.

Figure 4.9 | Generation of ROS for multiple pulses. Fluorescence of DCF for 6 consecutive pulses of 3 V with 20 seconds interval.

Larger electrode finger spacing (500 μ m) and fluorescent images were used to minimize diffusion effects preventing clear visualization of the cathode and anode. As shown in Figure 4.8B and C, a significant difference in fluorescence was observed between coated and uncoated electrodes, but also at the cathode and anode of both types of electrodes. The intensity profile of the uncoated electrodes showed higher concentrations of ROS at the cathode. Coated electrodes showed no fluorescence responses at the cathode and relatively small increases at the anode, indicating marginal generation of ROS at only one of the electrodes. Note that small changes in pH were also observed at the anode, indicating coated electrodes favor electrochemical reactions at the positive electrode, which should be taken into account when setting pulse parameters for specific applications.

The PEDOT:PSS coating thus strongly attenuates electrochemical reactions at the electrode interface. The absence of severe changes in pH reveal a limited generation of hydrogen and hydroxyl. In combination with reduced production of ROS, this provides new insights in the distinctive electrochemical properties of this conducting polymer at higher voltages.

4.5 Functional response of cells to electrical stimulation

To determine the stimulation and electropermeablization performance of PEDOT:PSS coatings, we assessed the response of a glial cell line. This particular cell line (U87 glioblastoma) is sensitive to pulsed electric fields due to their endogenously expressed voltage-gated ion channels (44, 45). The electrical stimulation response was measured by monitoring changes in the intracellular calcium by loading the cells with the fluorescent indicator Fluo-4 and subjecting the cells to various voltages (46, 47).

Figure 4.10 | Fluo-4 response of U87 glioblastoma cells for single pulses. Fluo-4 fluorescent images before and after a 10 and 20 V pulse for (A) uncoated and (B) coated electrodes. Images were taken at the time of maximum fluorescence. Fluo-4 fluorescence time evolution for various voltages for (C) uncoated and (D) coated electrodes. (E) Fluo-4 dose-response. Data points are the mean of 3-5 experiments and error bars represent the standard deviation. The curve in (E) is the fit to a sigmoidal function.

Figure 4.10 shows the Fluo-4 response for coated and uncoated electrodes. For the uncoated electrodes, calcium signals started at 7 V and reached a maximum at 10 V, resulting in an EC₅₀ of 8.2 V and a Hill Slope of 0.97 determined by sigmoidal fitting of the dose-response. For coated electrodes, a higher stimulation threshold was found and a larger range between the unstimulated and maximal response. The Fluo-4 signal increased between 9 and 17 V leading to an EC₅₀ of 12.6 V and a Hill Slope of 0.31. The maximum possible Fluo-4 response in terms of calcium kinetics (amplitude and latency) was comparable for both type of electrodes, confirming both electrodes were capable of stimulating cells in a similar manner.

Figure 4.11 | PI response of U87 glioblastoma cells for single pulses. PI fluorescent images before and after a 10 and 20 V pulse for (A) uncoated and (B) coated electrodes. Images were taken 9 minutes after application of the pulse. PI fluorescence time evolution for various voltages for (C) uncoated and (D) coated electrodes. (E) PI dose-response. Data points are the mean of 3-5 experiments and error bars represent the standard deviation. The curve in (E) is the fit to a sigmoidal function.

Electropermeabilization was assessed by application of propidium iodide (PI), one of the most commonly used fluorescent indicator for assessing electropermeabilization of cells. PI is cell impermeant and non-fluorescent when outside the cell. Increased cell membrane permeability will allow PI to enter the cell and bind to nucleic acids which increases its fluorescence 20- to

30-fold (48). Figure 4.11 shows the PI response for coated and uncoated electrodes. For uncoated electrodes increase in PI uptake started at 8.5 V and for 20 V nearly all cells showed PI staining. On the contrary, marginal PI uptake was observed in cells stimulated with the coated electrodes for voltages up to 30 V. The electroporation voltages for the uncoated electrodes correspond to previously reported values for electroporation of cells with planar, interdigitated electrodes, whereas for coated electrodes no electroporation was observed with voltages greatly exceeding these thresholds (23, 49).

The difference in cell stimulation and electropermabilization performance implies that the cellular reponse does not depend exclusively on the injected charge and the associated electric field. Coated electrodes, compared to uncoated electrodes, showed higher or similar currents, yet higher stimulation thresholds and no electroporation were observed. Therefore, the nature of the charge injection established by electrochemical events appears to play an important role. Since the experiments were performed in HEPES buffered media to mitigate pH changes, it can be concluded that the generated ROS at the electrodes, and not the pH changing species, influence the stimulation and electroporation of cells. Indeed, it has been shown previously that cell membrane permeabilization involves and is stablized by ROS, which are known to cause phospholipid peroxidation in the plasma membrane (14, 16, 18). The findings presented here, demonstrate the absence of irreversible electrochemical reactions with PEDOT:PSS coated electrodes and confirm chemical changes of the cellular environment with metallic electrodes which indicates that the generated species are not merely a side effect, but rather represents a crucial mechanism in the cellular response.

4.6 Conclusion and discussion

Application of PEDOT:PSS coatings on metallic electrodes significantly changes the stimulation and electroporation performance. Coated electrodes reduced the Faradaic current, exhibited higher levels of charge balance and minimized electrochemical events that otherwise result in bubbles, pH changes and ROS. Moreover, PEDOT:PSS coatings slightly increased the Fluo-4 threshold and showed a strong reduction in uptake of PI, indicating nearly no electropermeabilization of cells.

For uncoated electrodes, a Faradaic component in the current appeared around 5 V, the same voltage where pH changes and considerable generation of ROS were initiated. At 7 and 10 V, an increase in Fluo4 and PI was observed, respectively. Above 10 V, the Faradaic component dominated the current, the charge balance was <45%, the generation of ROS showed a near linear dependency on the voltage and cell electropermeablization was increasingly more pronounced. PEDOT:PSS coated electrodes showed no Faradaic currents <20 V which was confirmed by high levels of charge balance indicating limited irreversible electrochemical processes. Consequently, marginal electrochemical events and no cell electrodes were able to deliver adequate electric fields to stimulate cells, excluding any possible attenuation effect of the coating.

The involvement of oxidative species in the electroporation phenomenon is gaining further evidence. In the published literature, however, the origin of the species that play a role seems controversial. Gabriel et al. and Breton et al. suggested that it is not the ROS from electrodes that causes oxidative jumps and lipid peroxidation, whereas Ruzgys et al. demonstrated the high

level of dependency of the electrode material on ROS generation and electroporation efficiency (16, 18, 20). Our results indicate that the extracellular generation of ROS caused by the electrode-electrolyte interface is strongly associated with the electroporation of cells. How this relates to intracellular ROS remains to be explored.

Investigation of which ROS species were generated and the molecular mechanisms of the interaction between the generated ROS and the cell was beyond the scope of this work. Besides lipid peroxidation, Rems et al. recently demonstrated the creation of pores in voltage-gated ion channels embedded in the cell membrane (50). Moreover, dissolved metal particles from electrodes have been shown to be harmful to cells in a concentration dependent manner (51). Besides lipid peroxidation, such mechanisms indicate the complexity of these chemical changes and the effect on cells. However, PEDOT:PSS coated electrodes enable the delivery of high voltage pulses with minimal electrochemical events at the electrode interface. This allows to experimentally explore electrical stimulation and electropermeabilization of cells and tissue with minimal chemical changes to the extracellular environment which could be used to investigate separate mechanisms that play a role.

The absence of electrochemical reactions for PEDOT:PSS coated electrodes supports the view where charge is injected through reversible processes. Avoiding irreversible reactions represents a crucial requirement for safe stimulation of nervous tissue. Our results indicate that this reversible charging mechanism also applies to the case of monophasic waveforms and voltages beyond the water window. Monophasic waveforms are more efficacious than conventional charge-balanced biphasic pulses (52, 53) and asymmetric waveforms can be used to modulate the spatial selectivity for neuronal stimulation (54). However, charge imbalance of monophasic pulses and the long activation phase of asymmetric pulses might result in undesired irreversible Faradaic reactions and are generally avoided in clinical applications. Application of PEDOT:PSS opens the possibility to employ alternative waveforms without adverse effects, which possibly improves desired stimulation effects and therapeutic outcomes. Furthermore, the absence of irreversible reactions is expected to mitigate metal dissolution, however, a more detailed study is needed to support this hypothesis.

The exact mechanism of PEDOT:PSS charging still needs further investigations where elementary steps should be studied individual (34). Some reaction products were observed at the anode which were not identified and could be further investigated to obtain a better understanding of the electrochemistry. Moreover, further studies are needed to determine the impact of our results for conventional, clinical electrode designs and microelectrodes that are used to improve the spatial resolution. For implantable applications, the in vivo performance has to be determined and the long-term stability of the coating needs to be guaranteed. We expect that future investigations will explore the protective properties of PEDOT:PSS for applications with alternative device architectures and different stimulation parameters which could benefit a wide range of applications, leading to better organic bioelectronic devices.

4.7 Materials and methods

Device fabrication: The fabrication of the devices is an adapted version of a previously published protocol (55). In short, after cleaning of the glass slides, a lift-off process was used to pattern 150 nm thick gold electrodes with a 10 nm chromium adhesion layer. For coated electrodes, a PEDOT:PSS dispersion of Clevios PH1000, glycerol, dodecyl benzene sulfonic acid and (3-glycidyloxypropyl)trimethoxysilane was spin coated on a photolithographically

patterned and etched parylene C layer that was peeled-off after 1 hour of PEDOT:PSS annealing followed by rinsing in deionized water. In order to achieve a PEDOT:PSS thickness of 575 nm, 4 layers were deposited consecutively.

Charge injection characterization: Measurements were performed in phosphate buffered saline (PBS). Constant voltage pulses were applied with an Avtech AV-1011-C pulse generator and currents were acquired with a National Instruments digital multimeter. Pulses have been applied in arbitrary order of voltage amplitude, multiple devices were used and for each pulse the device was washed and supplied with fresh electrolyte. Charge injection (CI) was calculated as $\int I \cdot dt$ and charge balance as $\frac{CI_{discharge}}{CI_{pulse}} \cdot 100\%$. The integration time to calculate the charge injection throughout the discharge was a variable parameter and was stepwise increased until CI_{discharge} reached a steady value.

pH measurements: Phenol red, phenolphthalein and congo red were used as pH indicators in 0.1 M potassium chloride electrolyte. Phenolphthalein needed to first be dissolved in ethanol, which was then added to the electrolyte. The color change upon pulses was imaged with an optical microscope. Images were taken 3 seconds after the application of the pulses.

ROS measurements: The fluorescent marker 2',7'-dichlorodihydrofluorscein (DCFH₂) was used as ROS indicator. 1 μ l of 1 mM DCFH₂ in dimethyl sulfoxide (DMSO) was added to 100 μ l PBS which served as the electrolyte. Fluorescence data was measured by illumination with a 490 nm pE-4000 CoolLed and recording with a CMOS Prime 95B Photometrics camera. Fluorescence intensity was measured before the pulse for 1 min to determine F₀. After the pulse, the electrolyte was mixed to homogenize the solution before measuring the fluorescence.

Fluo-4 and PI experiments: U87 cells (ECACC, Public Health England; 89081402) were maintained in culture in DMEM culture media with low glucose containing GlutaMAX supplement (Thermo Fisher, 21885108), supplemented with 10 % fetal bovine serum and 100 units/mL penicillin and streptomycin at 37 °C in 5 % CO₂. 10⁵ cells were seeded on the microelectrodes and incubated with 5 µM Fluo4-AM (AAT Bioquest, 20551) for 30 min at 37 °C, and washed in an isotonic saline solution (NaCl 145 mM, KCl 5 mM, KH2PO4 4 mM, CaCl2 1 mM, MgSO4 1 mM, Glucose 10 mM, pH 7.4). Experiments were performed in saline solution with 10 µL/mL added PI. Cells on devices were only pulsed once, for each voltage value a new cell culture was used. Fluorescent imaging was performed with a Zeiss LSM 800 inverted confocal microscope. U87 cells stained with Fluo4-AM and PI were stimulated with a single 100 µs pulse from 0 to 20 V for uncoated and from 0 to 30 V for coated electrodes. Images were recorded for 10 minutes, the pulse was applied after 60 seconds. Fluorescence imaging was analyzed using Image Analyst MKII (Image Analyst Software, Novato, CA). Kinetics and voltage dose-response were analyzed using the software Prism 6.01 (Graphpad Software, La Jolla, CA). Fitting of the voltage dose-response was performed with a sigmoidal function with variable slope of the form $\frac{\Delta F}{F_0} = min \cdot \frac{max - min}{1 + 10^{\log{(EC_{50} - V) \cdot HillSlope}}}$. Only cells between the finger electrodes on similar positions were taken into account for determing the Fluo4-AM and PI response.

4.8 References

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5. Electroporation Microchip with an Integrated Conducting Polymer Electrode Array for Highly Sensitive Impedance Monitoring

Real-time monitoring of impedance changes during electroporation-based treatments can be used to study the dynamics of the biological response and provide feedback regarding the treatment progression. Seamless integration of the sensing electrodes with the setup improves the non-invasiveness, whereas reduced electrode impedance improves the sensing capabilities. Here, we present a novel electroporation chip containing a pair of stimulation electrodes and an array of microelectrodes for in-situ impedance sensing. Coating the sensing electrodes with the conducting polymer PEDOT:PSS significantly enhances the sensitivity of the system to detect changes in the biological impedance during the growth of a confluent cell layer and treatment with electrical pulses. An equivalent circuit is used to model and verify the impedance contribution of, both, the electrodes and the cells. The results demonstrate that PEDOT:PSS coatings improve impedance monitoring with micro-scale electrodes, enabling high spatial resolution and increased sensitivity to study electroporation dynamics and improve treatment outcomes.

5.1 Impedance to assess cell barrier properties

Electroporation-based therapies and techniques rely on the permeabilization of cells and tissue upon exposure to pulsed electric fields. Nowadays applications include reversible electroporation for electrochemotherapy, gene electrotransfer and transdermal drug delivery, as well as irreversible electroporation for soft tissue ablation (1)(2)(3).

The success of electroporation techniques in terms of efficiency and cell viability depends on precise control of the delivered dose. Parameters such as applied voltage and treatment duration, as well as electrode configuration and type of cells and tissue determine the degree of increased membrane permeability (4)(5). In order to optimize the pulse parameters for the desired outcome, real-time monitoring of the membrane permeability can be utilized to determine treatment progression and provide feedback.

A promising, non-invasive method relies on the change in impedance of cells and tissue caused by a decrease in the cell membrane resistance and transmembrane transport of ions (6)(7). Impedance, or conductivity, measurements have shown to provide adequate monitoring of conductivity changes upon electroporation of liver and muscle, as well as in potato models (8)(9)(10). Moreover, electrochemical impedance spectroscopy (EIS) obtained with an array of electrodes in combination with modelling can provide information about change in tissue conductivity and the dimensions of the electroporated volume (11). In vitro, analysis of impedance measurements has shown to provide information regarding the structural and functional changes of epithelial cells (12) and cell coverage, adhesion and electroporation of mammalian cells (13). In addition to impedance monitoring, micro-scale electroporation devices can be used improved dosage control and cell viability (14)(15)(16). For instance, microfabricated electrodes with an interdigitated architecture have been used to achieve highly efficient transfection of nucleic acids in vitro (17) and in vivo (18). Miniaturized electrodes can combine electroporation and impedance sensing to effectively load adherent cells with macromolecules (19) and distinguish various types of cells (20). Moreover, microchips enable electroporation and real-time monitoring of dynamics of the impedance of individual cells (21)(22).

Measuring cellular or tissue impedance results in a combination of the impedance of the sensing electrode itself and the biological environment. Therefore, a lower electrode impedance increases the sensitivity which improves monitoring of changes in the biological sample (23). Reduction of electrode impedance can be realized by coating the electrode with the conducting polymer PEDOT:PSS (24)(25). This coating material increases the effective area for ionic-electronic interaction which results in a larger electrode capacitance, and hence, a lower electrode impedance (26)(27). PEDOT:PSS coated electrodes have shown adequate stability and enable the development of smaller electrodes which improves the spatial resolution (28).

Here, we report on the feasibility to employ PEDOT:PSS coated microelectrodes to assess changes in cell impedance during cell growth and electroporation treatment. We present a microfabricated devices that contains a pair of interdigitated stimulation electrodes in combination with an array of sensing electrodes. The performance of PEDOT:PSS coated electrodes is compared to uncoated electrodes to assess cell barrier properties by means of EIS. We show that the reduced impedance of the PEDOT:PSS coated sensing electrodes improves the monitoring of a growing, confluent cell culture which is verified by modeling with an equivalent circuit. Furthermore, we show impedance changes upon application of electric pulses of various voltages.

5.2 Materials and methods

Device and fabrication. The in vitro microchip containing a pair of interdigitated stimulation and 10 sensing electrodes is shown in **Fig. 1** and was fabricated by stacking of multiple layers of gold, parylene C and PEDOT:PSS. The stimulation electrodes with a width and spacing of 100 μ m and 250 μ m, respectively, covered a total surface area of $1.9 \times 1.9 \text{ mm}^2$ and consisted of gold only. To investigate the effect of the PEDOT:PSS coating for sensing capabilities, microchips were fabricated with and without PEDOT:PSS coated sensing electrodes. For all experiments, the diameter of the sensing electrodes was 100 μ m, except for **Fig. 2** where the impedance of different electrodes sizes are compared.

The microchips were fabricated on glass substrates and standard microfabrication processes were utilized for patterning of the materials. The glass substrates were cleaned with micro-90 soap, acetone and isopropanol, and the first metallic layer containing the sensing electrodes was patterned through a lift-off process with AZnLOF 2070 photoresist. Thermal evaporation was used to deposit 10 nm of chromium for adhesion followed by 150 mm of gold. After encapsulation of the sensing electrodes with a 1 µm parylene C layer, the second metallic layer containing the two interdigitated stimulation electrodes was patterned with the same process as the first metallic layer. The stimulation electrodes were encapsulated with a 2 µm parylene C layer and opened by dry etching with photolithographically patterned AZ10XT as an etch mask. For all depositions of parylene C, 3-(methacryloyloxy)propyl trimethoxysilane (A-174 silane) was used as an adhesion promotor. The opening and coating of the sensing electrodes was performed as follows. After spin-coating a layer of diluted micro-90 surfactant, a 2 µm sacrificial parylene C layer was deposited. The sensing electrodes were etched open with AZ10XT photoresist as an etch mask. After stripping of the photoresist, 4 layers of a mixture of Heraeus Clevios PH1000, glycerol, dodecyl benzene sulfonic acid, and (3glycidyloxypropyl) trimethoxysilane were spin-coated with a 110 °C baking of 1 min in between layers. This resulted in a PEDOT:PSS thickness of about 600 nm. After the removal of the sacrificial parylene C layer, the device was baked at 140 °C baking for 1 hour to complete annealing of the mixture. The final device was immersed in de-ionized water for 1 hour to remove excess low molecule weight compounds. A glass well was placed on top to confine the media used for characterisation and cell cultures.

Figure 5.1 | Electroporation microchip with integrated impedance sensing electrodes. (A) Multilayered design containing stimulation and sensing electrodes which are encapsulated by parylene C. (B) Cross section displaying two of the stimulation electrode fingers and one sensing electrode coated with PEDOT:PSS. (C) Optical image of the microchip containing 10 sensing electrodes.

Cell culture. Blood-brain barrier hCMEC/D3 cells were purchased from Sigma Aldrich. Cells were cultured in Endothelial Cell Culture Medium 2 (ECCM-2) supplemented with 0.02 ml ml⁻¹ fetal calf serum, 5ng ml⁻¹ epidermal growth factor, 10 ng ml⁻¹ basic fibroblast growth factor, 20 ng ml⁻¹ insulin-like growth factor, 0.5 ng ml⁻¹ vascular endothelial growth factor 165, 1 μ g ml⁻¹ ascorbic acid, 22.5 μ g ml⁻¹ heparin and 0.2 μ g ml⁻¹ hydrocortisone. Before seeding, the Electrodes were disinfected with 70 % ethanol and coated with collagen-I.

Impedance measurements. Electrochemical impedance spectroscopy (EIS) was performed in a two-electrode setup where for each measurement a pair of sensing electrodes functioned as the working and counter electrode. Each device contained 5 electrode pairs where the pairs consist of electrode 1 and 10, 2 and 9, 3 and 8, 4 and 7, and 5 and 6 (Figure 5.1C). EIS was measured with a PalmSens4 potentiostat in the frequency range of 1 Hz - 500 kHz at 4 points per decade and a voltage amplitude of 25 mV.

EIS monitoring during cell growth and modeling. To demonstrate the difference in sensitivity between PEDOT:PSS coated and uncoated electrodes, the impedance was monitored up to 144 hours (6 days) while a confluent layer of blood brain barrier cells grew on the device. For all time points, the EIS was measured between electrode 3 and 8 and 6 different devices were cultured simultaneously for both PEDOT:PSS coated and uncoated electrodes.

The EIS of the coated electrodes in PBS and the electrodes covered by a confluent cell layer were modelled with an equivalent circuit. Modeling was performed with the built-in equivalent circuit analysis tool of the PSTrace software. First, the R_s and C_{el} values of the electrodes in PBS were determined. For the EIS with confluent cells, the equivalent circuit was expanded by adding an R and C that represent the cell membrane and paracellular impedance and the values were fitted while keeping R_s and C_{el} fixed at the values as determined without cells.

Pulse experiments. The cellular response to electrical pulses in terms of permeability and electrical properties was assessed by measuring the change in propidium iodide fluorescence and EIS, respectively. Pulse experiments were performed with cell cultures that had grown for >72 hours to allow growth into a full confluent layer with all cultures exhibiting similar impedance spectra. We noticed a slight decrease in impedance in the first hour when devices with cells were taken out of the incubator, therefore, devices were taken out of the incubator 90 minutes before the start of the experiment to stabilize the impedance and ensure that any change observed in the impedance was due to the application of electric pulses. Electrical pulses were delivered to the cells by the pair of interdigitated, stimulation electrodes that were connected to an in-house built pulse generator. For each experiment, 8 monophasic pulses at 1 Hz with a pulse width of 100 μ s were applied. Pulses of 20, 30 and 40 V were applied to demonstrate the electric field dependency of cell electroporation and change in impedance. To verify delivery of the pulses, voltage and current were measured employing a Rohde&Schwarz RTM3004 oscilloscope with a voltage and current probe, respectively. Measurements were repeated in triplicates for all conditions.

PI staining and microscopy. The electroporation of cells was monitored by staining the cells with propidium iodide (PI) and monitoring the change in fluorescence. For PI signal experiments, 200 μ L of fresh supplemented ECCM-2 was placed in each well, and 10 μ l/ml of PI added. Fluorescence was measured with an inverted confocal microscope (ZEISS LSM 800) Imaging was performed with a ZEISS Axiocam MRm monochrome microscope camera and an illumination system for fluorescence microscopy (ZEISS HXP 120 C). Images were recorded using the ZEN software. Fluorescence data extraction was made using the software Image Analyst MKII (Image Analyst Software, Novato, CA). Only the cells in the region between the imaged pair of impedance sensors were taken into account, to correlate the imaging data with the impedance changes. First, the Regions of Interest (ROI) were drawn around individual cells.

Additionally, a ROI was drawn over a space devoid of cells, so that the background fluorescence fluctuations could be subtracted during later analysis. The ROIs signal evolution over time were exported as .txt files to be analyzed using custom-made Matlab scripts. Values of ROIs evolution were first normalized according to $\Delta F = \frac{F(t)-F_0}{F_0}$, where F(t) corresponds to fluorescence intensity at a given time t and F₀ the average fluorescence intensity calculated on the 5 seconds preceding the delivery of PEFs. Finally, the background fluctuation data were subtracted from all other ROIs. The mean value of fluorescence for every timestamp as well as the standard error to the mean was calculated.

EIS monitoring during pulses. The change in impedance upon the application of pulses was measured in frequency and time scan mode as follows. Before application of the pulses the EIS of the 5 pairs of electrodes (1 and 10, 2 and 9, 3 and 8, 4 and 7, and 5 and 6) was measured in the frequency range of 1 Hz - 500 kHz yielding the impedance of the full frequency spectrum. One pair of electrodes was selected to perform a time scan throughout which the impedance at 5 kHz was measured every second. At time t = 0 a time scan was started, at t = 5 min the 8 pulses were applied and at t = 25 min the time scan was stopped and the EIS of the full frequency range of all 5 pairs of electrodes was measured. For the 20 V pulses the time scan was continued for another 30 minutes.

5.3 EIS monitoring during cell growth

Figure 5.2 shows the magnitude of the EIS measured in PBS for coated and uncoated electrodes with a diameter of 15, 50 and 100 μ m. PEDOT:PSS coated electrodes exhibited a lower impedance compared to uncoated electrodes where, for the same electrodes size, the impedance was reduced by about two orders of magnitude for frequencies <1000 Hz. The reduced electrode impedance is explained by the larger effective surface area of coated electrodes which results in a larger electrode capacitance (29). As a result, the cut-off frequency, that separates the resistive and capacitive dominated parts of the impedance spectra, shifts to a lower frequency. For larger electrodes, the impedance reduced over the full frequency range due to an increased electrode capacitance and spreading resistance which scale with the electrode area and diameter, respectively (*30*). Note that the relatively small error bars confirm the reproducibility of the fabrication protocol.

Figure 5.2 | EIS for various electrode sizes. Data points and error bars represent the average and standard deviation of four different pairs of electrodes, respectively. Note that for most data points the error bar is smaller than the symbol. The dotted lines are a guide to the eye. The outlier for the 15 μ m, uncoated electrode was due to noise. The growth of a confluent layer of blood-brain barrier hCMEC/D3 cells was monitored with microchips containing 100 μ m diameter sensing electrodes. Figure 5.3A shows a bright field image of a confluent cell culture on top of the microchip. The evolution in time of the impedance spectrum and the magnitude of the impedance at 5 kHz during the cell growth for
PEDOT:PSS coated electrodes are shown in Figure 5.3B and C, respectively. Nearly no change was observed in the first 24 hours after seeding, whereas between 30 and 72 hours the impedance increased in the frequencies ranging from 50 Hz to 500 kHz. After 144 hours (6 days), no further increase of the impedance was observed indicating that after 72 hours a confluent layer had formed with stable barrier properties. The larger error bars for 48 and 72 hours in Figure 5.3C, indicate that the variation in impedance between various cell cultures was highest when the cell layer was growing, and the barrier properties of some layers were further established than others. For the remainder of this study, experiments were only performed on devices with cells that were in culture for >72 hours and displayed a comparable impedance spectrum.



Figure 5.3 | Impedance monitoring during blood-brain barrier cell growth. (A) Bright field image of a confluent cell culture 72 hours after seeding. (B) Magnitude of the impedance spectra for PEDOT:PSS coated electrodes at different time points after seeding. (C) Magnitude of the impedance at 5 kHz as a function of time for coated electrodes. (D) and (E), magnitude and phase of the impedance spectra with and without a confluent cell layer for PEDOT:PSS coated and uncoated electrodes. (F) Relative impedance change for various frequencies for coated and uncoated electrodes. All data points and error bars represent the average and standard deviation of 6 measurements. For (D), (E) and (F), the impedance with cells was measured 72 hours after seeding.

To demonstrate the difference in sensitivity, a confluent cell layer was also grown on devices with uncoated sensing electrodes. In Figure 5.3D and E the impedance spectra with and without a confluent cell layer are shown for PEDOT:PSS coated and uncoated electrodes. Further analysis of this data is shown in Figure 5.3F where the relative change in impedance is shown for various frequencies. For uncoated electrodes, the observed change in impedance was smaller and confined to a narrower frequency band. The largest change in impedance for uncoated electrodes was observed at 100 kHz where the impedance with cells was 3.5 times higher compared to without cells. For coated electrodes the impedance was 12 times higher at 1 kHz. Moreover, increased impedance was observed between 5 - 500 kHz for uncoated and between 50 Hz - 500 kHz for coated electrodes. These results reveal that the relatively high impedance of the uncoated electrodes obscured the effect of the confluent cell layer, whereas for coated electrodes, the sensitivity which improves the assessment of the barrier properties by means of EIS.

To extract quantitative, electrical parameters of the cellular barrier properties, the EIS was modeled with an equivalent circuit. Figure 5.4A shows the typical diagrams that can be used to

analyze the impedance spectrum of electrodes and confluent cells. For electrodes, the impedance is typically dominated by the electrode capacitance C_{el} and the spreading resistance R_s . The diagram for adherent cells accounts for the transcellular (cell lipid bilayer) and paracellular (junctions between cells) pathways with a parallel resistance R_{mem} and capacitor C_{mem} , and a resistance $R_{paracellular}$, respectively. The measured EIS without cells was modeled with a circuit containing C_{el} and R_s only, whereas for the EIS with cells the circuit in Figure 5.4B was used. Figure 5.4C shows the measured and modeled spectrum for PEDOT:PSS coated electrodes with and without confluent cells. Excellent agreement between measurement and model was found for $R_s = 5.9 \text{ k}\Omega$, $C_{el} = 61 \text{ nF}$, $R_{mem+paracellular} = 94 \text{ k}\Omega$ and $C_{mem} = 33 \text{ pF}$. The various components of the equivalent circuit can be noted at different frequencies in the spectrum. In the low and high frequency regions, the impedance was dominated by the electrode capacitance and the spreading resistance, respectively. Without cells, the resistive and capacitive part were separated by the cut-off frequency $f_c = 440 \text{ Hz}$. With cells, the capacitance of the confluent cells was observed at frequencies around 100 kHz, whereas the membrane and paracellular resistance dominated between 50 Hz and 50 kHz.



Figure 5.4 | Modeling with an equivalent circuit. (A) Typical equivalent circuits of electrodes and adherent cells. (B) Equivalent circuit used to model the EIS. (C) Magnitude of the impedance for the measured and modeled EIS.

Modelling of the EIS for uncoated electrodes revealed the best fit for $R_s = 5.8 \text{ k}\Omega$, $C_{el} = 0.25 \text{ nF}$, $R_{mem+paracellular} = 64 \text{ k}\Omega$ and $C_{mem} = 32 \text{ pF}$. The similar values for the spreading resistance and the adherent cells confirm the model. The difference between coated and uncoated electrodes was found in the electrode capacitance which is more than two orders of magnitude larger for coated electrodes. As shown in Figure 5.3D, the increased electrode capacitance is essential for adequate sensing of cell barrier properties with microelectrodes. Note that impedance measurements at frequencies >500 kHz would not provide additional information since the cell membrane capacitance is shorted here and the impedance equals R_s .

5.4 EIS monitoring during treatment with electric pulses

The effect of electric pulses on the impedance was examined by employing the interdigitated electrodes to apply 8 monophasic pulses with a pulse duration of 100 μ s at a repetition frequency of 1 Hz and various voltages. Figure 5.5A shows the impedance spectrum

before and 20 min after the pulses. The frequency range in which a decrease in the impedance was observed coincide with the cell dominated region.

In Figure 5.5B the change in impedance at 5 kHz is shown for various voltages. As shown in Figure 5.5C, the impedance decreased gradually in time and reached a stable impedance value \sim 20 min after the pulses. For higher voltages, a larger change in the impedance was observed. This observed decreased impedance as a function of voltage is in agreement with electric field dependent disruption of the paracellular junctions and cellular electroporation (*31*)(*17*).



Figure 5.5 | Impedance change upon application of 8 monophasic pulses, 100 μ s, 1 Hz of 20, 30 and 40 V (A) Impedance before and 20 minutes after the application of 40 V pulses. (B) Normalized impedance at 5 kHz before and after pulses of various voltages. (C) Time evolution of the 5 kHz impedance during the application of pulses.

5.5 Conclusion

A microchip was fabricated consisting of a pair of stimulation electrodes with an integrated electrode array for impedance monitoring during cell growth and treatment with electric pulses. An equivalent model, which distinguished the electrode from the confluent cells, was used to verify the impedance contribution of various components in the system. Coating the sensing electrodes with the conducting polymer PEDOT:PSS significantly reduced the electrode impedance which enhanced the sensitivity to assess changes in impedances caused by the growth of a confluent cell layer and application of electric pulses.

Such a microchip can be utilized to monitor cell growth and verify when a confluent cell layer with stable electrical barrier properties has formed. Throughout treatment with electric pulses, continuous impedance monitoring can provide feedback on treatment progression to optimize outcomes and study electroporation dynamics. PEDOT:PSS coated sensing electrodes allow smaller, micro-scale electrodes which could be exploited to improve the spatial resolution. Finally, the microchip can be fabricated on a flexible substrate for in vivo applications.

5.6 References

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In this thesis, the first steps were taken to investigate the application of organic materials for the treatment of glioblastoma by means of electroporation. More specifically, the performance of PEDOT:PSS coated electrodes for electroporation applications was investigated and compared with uncoated electrodes. The most important outcome of this thesis is that electrical pulses delivered with PEDOT:PSS coated electrodes do not permeabilize cells. Such a coating is therefore not suitable for electroporation-based therapies, however, other applications such as neuromodulation might benefit from this property. PEDOT:PSS coated electrodes exhibit superior sensing capabilities compared to uncoated electrodes which makes them ideal candidates for impedance monitoring during pulsed electric treatment. Therefore, the question 'Is PEDOT:PSS beneficial for electroporation-based applications?' has two answers, 'No' for stimulation electrodes and 'Yes' for sensing electrodes.

Main outcomes of this thesis are:

- 1. The stimulation performance of PEDOT:PSS coated electrodes depends on the coating thickness. The electrode impedance and charge injection can be optimized by tuning the thickness. Importantly, thicker coatings can endure more pulses and higher amplitudes without delamination and cracking of the film. Since stability is a critical electrode property, the coating thickness should be considered an important parameter in the design of bioelectronic devices.
- 2. **PEDOT:PSS coated electrodes do not permeabilize cells.** Coated electrodes primarily inject charge through reversible processes which strongly reduces electrochemical reactions that otherwise lead to changes in pH and the generation of reactive oxygen species. The absence of Faradaic currents and associated electrochemical reactions pleads for a capacitive electrode-electrolyte coupling. Moreover, the combined absence of by-products and cell electroporation strongly suggests that chemical species play an important role in cell membrane permeabilization.

Although not suitable for electroporation applications, the reversible charge injection mechanism of PEDOT:PSS coated electrodes is of great importance for the field of neuromodulation. Crucial requirement for long-term, safe stimulation of nervous tissue is to avoid irreversible electrochemical reactions and the generation of harmful species. The attenuation of cytotoxic species conferred by PEDOT:PSS could benefit cell integrity and viability, which is essential for optimal stimulation of nervous cells and tissue.

3. **PEDOT:PSS coated microelectrodes can adequately monitor impedance changes of biological samples.** The reduced electrode impedance of coated electrodes enhances the sensitivity to assess changes in the electrical barrier properties of confluent cell cultures. Such a system of microelectrodes can be used to study electroporation dynamics and provide feedback regarding treatment progression to optimize outcomes.

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Abstract :

Electropermeabilization has found successful biomedical applications such as electrochemotherapy, tissue ablation, gene therapy and transdermal drug delivery. Despite significant progress in the last decades, a better understanding of underlying mechanisms and advancement in technology could lead to improved outcomes. In this thesis, the performance of the conducting polymer PEDOT:PSS as an electrode coating material is explored in the context of electropermeabilization. Microfabricated PEDOT:PSS coated electrodes were investigated, both, to deliver stimulation pulses and to monitor changes in impedance as a consequence of cell electropermeabilization. The results can be summarized in three parts.

Firstly, increased PEDOT:PSS coating thickness improved the polymer-electrolyte interaction due to a higher electrode capacitance and showed higher stimulation thresholds before electrode failure such as delamination was observed. This emphasizes the importance of PEDOT:PSS coating thickness as a design parameter for optimized performance and increased stability of stimulation electrodes.

Secondly, electrical stimulation with PEDOT:PSS coated electrodes strongly reduced irreversible electrochemical reactions at the electrode interface that otherwise led to changes in pH and the generation of reactive oxygen species. In addition, coated electrodes showed nearly no electropermeabilization at voltages that greatly exceed the threshold for uncoated electrodes. These findings indicate that charging in PEDOT:PSS is dominated by reversible processes, also at voltage beyond the water window, which represents an important requirement for safe neuromodulation applications. Moreover, the absence of Faradaic reactions in combination with marginal electropermeabilization reveal that electrochemical events at the electrode surface play an important role in the cellular response. PEDOT:PSS separates the delivery of electric pulses from chemical changes which paves the way for future experiments that could lead to a better understanding of the underlying mechanisms.

Thirdly, to monitor treatment progression, an array of PEDOT:PSS coated sensing electrodes was integrated with uncoated stimulation electrodes. Reduced impedance of the sensing electrodes due to the PEDOT:PSS coating significantly improved the monitoring of the change in impedance of adherent cells upon electropermeabilization. Such microscale electrodes enable accurate measurements of changes in cellular impedance with high spatial resolution allowing better monitoring of treatment progression.

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Mots clefs : polymère conducteur, stimulation électronique, électrochimie, réponse cellulaire

Résumé :

L'électroperméabilisation, ou électroporation, a été utilisée avec succès dans des applications biomédicales telles que l'électrochimiothérapie, l'ablation tissulaire, la thérapie génique et la libération transdermique de médicaments. En dépit des progrès significatifs de ces dernières décennies, une meilleure compréhension des mécanismes sous-jacents ainsi que de nouveaux développements technologiques pourraient améliorer les résultats obtenus. Dans cette thèse, les performances du polymère conducteur PEDOT:PSS, utilisé comme revêtement pour les électrodes, sont étudiées dans le cadre de l'électroperméabilisation. L'utilisation des microélectrodes recouvertes de PEDOT:PSS a été investiguée pour, d'une part, délivrer des impulsions de stimulation, et d'autre part, surveiller les changements d'impédance résultants de l'électroperméabilisation de la cellule. Les résultats obtenus peuvent être classés en trois catégories.

Premièrement, l'augmentation de l'épaisseur du revêtement de PEDOT:PSS améliore l'interaction polymère/électrolyte grâce à une capacité d'électrode plus élevée et montre également des seuils de stimulation plus élevés avant la défaillance des électrodes, principalement due à une délamination du film de PEDOT:PSS. Ce résultat montre l'importance d'utiliser un revêtement de PEDOT:PSS pour optimiser les performances et augmenter la stabilité des électrodes de stimulation.

Deuxièmement, la stimulation électrique avec les électrodes recouvertes de PEDOT:PSS réduit considérablement les réactions électrochimiques irréversibles à l'interface avec l'électrode qui autrement produisent une modification du pH et des espèces réactives de l'oxygène. En outre, les électrodes recouvertes de PEDOT:PSS ne montrent quasiment pas d'électroperméabilisation pour des tensions qui excèdent largement le seuil pour des électrodes nues. Ces résultats montrent que charger le PEDOT:PSS est dominé par des processus réversibles. De plus, l'absence de réactions faradiques combinée avec une électroperméabilisation marginale indique que les processus électrochimiques à la surface de l'électrode jouent un rôle important dans la réponse cellulaire. Le PEDOT:PSS permet de séparer l'application d'impulsions électriques des changements chimiques, ce qui ouvre la voie à de futures expériences qui pourraient déboucher sur une meilleure compréhension des mécanismes sous-jacents.

Troisièmement, pour surveiller l'évolution d'un traitement par électroperméabilisation, une matrice de microélectrodes recouvertes de PEDOT:PSS a été intégrée avec des électrodes de stimulation interdigitées et nues. L'impédance réduite des électrodes recouvertes de PEDOT:PSS améliore grandement la surveillance des changements d'impédance des cellules lors de l'électroperméabilisation. Ces électrodes microscopiques conduisent à des mesures précises de changement d'impédance cellulaire avec une haute résolution spatiale, permettant ainsi une meilleure surveillance de la progression du traitement.