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Nanotechnology based implantable and interfaceable devices

iONE-FP7

Implantable Organic Nano-Electronics

PROJECT FINAL REPORT

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Name of the scientific representative of the project's co-ordinator, Title and Organisation:

Prof. Fabio Biscarini

(UNIVERSITY OF MODENA AND REGGIO EMILIA)

Tel:+39-0592055079

Fax: +39-059-2055410

E-mail: **fabio.biscarini@unimore.it**

Project website address: http://ione-fp7.eu

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1. An executive summary

The vision of I-ONE-FP7 was an active multifunctional device (AMID) to be implanted in an animal model of the spinal cord injury (SCI-contused mouse) and operated from the outside to deliver combination of electrical and chemical stimuli to the injury site. The aim was to recover the residual plasticity at the injury site and decrease the inflammatory events associated to acute and chronic SCI.

At month 36, the project iONE-FP7 demonstrated an AMID implanted in acute SCI murine models. AMID integrates four organic transistors, in EGOFET configuration and a microfluidics into a 4x1 mm2 biodegradable PLGA scaffold. First results of AMID stimulation on the animal response are encouraging, as an animal in terminal anaesthesia stimulated with AMID performs conditional movements of the lower libs that should be otherwise impaired by the injury. By the time the project was over, there was no time to assess whether these movements will be volunteer in an awaken animal. However, the final results of iONE-FP7 open the way to further investigation on the efficacy of the device in the chronic SCI. These studies are currently in progress within a continuing collaboration between some of the partners.

The project has been successful in most of the activities foreseen. Details are provided in the summary of context and objectives. As most relevant cases of success we mention:

- New fundamental knowledge on organic bioelectronics interacting with living matter was built;
- First electronic devices of greater complexity were demonstrated on biodegradable scaffolds;
- Technological steps, prototyping and integration were developed;
- Original instrumental solutions and tools were designed, as well as protocols elaborated, for the purpose of realizing experiments of increasing complexity;

- Methodological evolution from materials science, to in vitro assessment, to in vivo was proven to be effective also in minimizing the sacrifice of animals during the optimization of the device;
- Scientific production was substantial with 20 papers produced in three years,
- Dissemination activity included two showcases, more than 30 invited talks by iONE-FP7 members on the course of the project, newspaper, radio and TV interviews, a specific Linkedin forum with more than 125 attendees, and finally the organization of a Workshop on Implantable Organic Electronics with 40 attendees as satellite event of the Tenth International Conference of Organic Electronics (ICOE 2014);
- A Design History File database has been laid down;
- A joint patent is being written by partners, its potential exploitation being guaranteed by two partners;
- A partner has established a new start up company whose mission is bioelectronics.

Overall, the approach envisioned by iONE-FP7 has proven its potential in performing loco-regional therapies and treatments. This is important not only for SCI, but for a variety of other pathologies (e.g. Parkinson's), pain therapy, anaesthesia. The Consortium is actively formulating other EU-scale proposals with the aim to continue working on implantable organic bioelectronics.

2. A summary description of project context and objectives.

During the project "Implantable Organic NanoElectronics (iONE-FP7) we designed, developed and demonstrated a novel device called AMID (Active Multifunctional Implantable Device) for the treatment of Spinal Cord Injury in a murine model. AMID allows us to supply a combination of chemical, electrochemical and electric stimuli to the site of the injury.

Aims of AMID operations are:

- to control inflammation,
- to minimise foreign body reaction;
- to stimulate the residual plasticity and
- to allow the SCI injured animal to recover voluntary locomotion of the limbs.

Within the 36 month duration of the project, we built the fundamental knowledge on device/cell interactions and its control across the multiple length scales from nano- to millimetres; we developed original solutions for metrology, technology and tools to fabricate, assess and integrate materials into the device; we validated the device operations in vitro with neuronal cells obtained by differentiation of neuronal progenitor stem cells; we assessed the foreign body reaction elicited by AMID with activated cells of the immune system through quantitative analysis of biomarkers; we analysed the neuronal signals transduced onto the device with novel experimental setups developed *ad hoc* and with the aid of multiscale modelling; we designed AMID layout into progressively more sophisticated prototypes and finally arrived to the implantation into spinal cord contused mice.

We successfully achieved the transmission of electric signals to peripheral motor (sciatic) nerve upon pulsed electric stimulation supplied to the SCI injury site with an implanted AMID. This produces contraction and motion of the leg muscle in the paraplegic animal. Long-term efficacy, together with medical and functional assessment on chronic models of SCI, is under investigation.

In the latest prototypes of AMID for ultra-thin film organic field effect transistors (OFETs) are integrated together with a microfluidics channel onto a 20-micrometer thick film of FDA-approved biodegradable bio-resorbable polymer, i.e. poly-lactic-glycolic acid (PLGA). AMID unique features are:

- Integrated devices for bidirectional communication (sensing, transduction and stimulation of neuronal signals) with the central nervous system (CNS);

- Capability to perform a loco-regional therapy of SCI through supply of drugs, growth factors, neurotransmitters by microfluidics;
- 100% biocompatible;
- Made of more than 99.9% of bioresorbable material;
- Flexible and softer in time;
- Implanted according to a novel surgical protocol with no stitching or glue;
- Operated from the outside, also in a remote mode;
- Suited for interfacing to a front end electronics with wireless connection;

In the following we mention some of the achievements that have been accomplished in I-ONE-FP7 during this period explaining why they are important for the development of advanced SCI treatments.

1. **Methodology (WP1)**: we have elaborated clear specifications and application goals starting from the suggestions of the external advisory board (including an end user biomedical company, a neurosurgeon and a neurologist); the choice of materials to build AMID out of, has been selected from a large palette from literature and experience using an approach elaborated by us. We termed it decision matrix approach. The method has proven not only correct in ranking possible choices based on parameters identified by us and EAB, but also revealed to be readily adaptive and effective when the Consortium was reconfigured; with the choices of materials, the first generation AMID layout was designed.
2. **Technology (WP2)**: the choice to head towards an AMID fabricated on biodegradable substrate, keeping the biocompatible ones as a backup, has prompted us to solve the problem of fabrication and patterning of active materials whose processing windows are not strictly orthogonal to the ones of the scaffold, and the latter is not an ideal substrate for planar technology. As a result we have devised a rapid prototyping process based on laser scan writing of metal thin film of electrodes, and different strategies for photolithography. Some originally obvious choices of materials have been dropped in favour of PLGA. Chemically modified hyaluronic acid was also explored, without arriving to a demonstration of device operations. Metrological assessment of degradation time scales and mechanical properties have been devised using ad hoc multiscale patterns in conjunction with SPM metrology and coarse-grained modelling of the degradation process. Correlated swelling/erosion stages have been evidenced, with an overall softening of the device that leads to a fair matching with the Young' modulus of the relevant tissue. A systematic work on standardisation of samples has been carried out and a large flow of samples (more than 1600 throughout the course of the project) has allowed the consortium to work on a coherent fashion although in parallel on different experiments.
3. **Devices (WP3 and WP4)**: all individual active components, mainly OFETs, NOMFETs and OECTs have been demonstrated to work in physiological buffers as cellular media, exhibiting stability on a several days-few week time scale with marginal degradation during operations. Breakthrough has been the devising of the organic field effect transistors (OFET) in an Electrochemically-Gated architecture (EGOFET). Thanks to this configuration, gating is possible with much greater flexibility in the design with respect to bottom gate or dual gate ISFET-like devices. The architecture was proven successful as OFET transducer, biosensor of neurotransmitters and inflammatory cytokines (IL-6, IL4, and TNF-alpha, the latter currently in progress), as well as a to give rise to a frequency-modulated response of a NOMFET. First time NOMFET operations in physiological buffer were demonstrated, whereas the coupling to neuronal cell populations is scheduled in the forthcoming month (i.e. out the project period). Organic electrochemical transistors (OECT) and microfluidics were fabricated for the first time on biodegradable scaffold,

and first round integration started. OECT in a transistor mode of operations has proven extremely effective for the stimulation of the neuronal cell cultures, as well as for the transduction of bioelectric signals when interfaced to skin (ECG recording with flexible, impalpable electrodes).

4. **Integration with living cells (WP5)**: all materials and devices have been assessed for their interaction with neuronal stem cells, showing that not only adhesion and proliferation of NPCs but also their differentiation into neurons can be accomplished. In-depth proteomic profiling has allowed us to infer that cells actually have characteristics of neurons and neuronal networks formed give rise to communication (by Ca^{2+} imaging). The response of the living system to the potential implant (inflammation and foreign body reaction) has been evaluated by means of assays using cells of the microglia and T-cells, that are recruited by measuring a variety of biomarkers. Our results show that the idle devices activate these cells. Device operations appear to reduce the expression of such biomarkers.
5. **Neuronal signal transduction (WP6):** bidirectional communication between OFETs and neuronal networks from murine stem cells has been demonstrated. Devices exhibit sensitivity on the order of less than 100 microV of extracellular membrane potential. Devices have a bandwidth suited to transduce single firing events, and noise spectra reveal the occurrence of individual action potentials and syncronized transmission. Coupling the electrical measurements to calcium imaging allows us to correlate low frequency periodic signals (1 s time scales) to calcium waves triggered by the stimuli. Signal transmission along and away from an axon, the response of the device as well as the coupling between neurons and device has been studied using Hodgkin-Huxley model and finite element modelling of the Poisson equation. Ca imaging has allowed to have unambiguous evidence of the efficacy of electrical and electrochemical stimulation on neuronal activity, specifically on the capability to induce opening of voltage dependent ion channels involved in transmission. Finally, a new current-based detection scheme of the propagating Ca^{2+} wave has been devised and elucidated. It was demonstrated using glial cells and reveals itself extremely sensitive to transient changes of the electrode potential, induced by the propagating waves. This is first evidence of extracellular detection of the Ca^{2+} wave.
6. **Integration of discrete device components into the implantable device (WP7)**: the AMID layout was further revisited based on specifications for surgery and anatomy of the SCI site. The layout was transferred into a prototype and further optimized after first tests in WP8. The fabrication protocol has been standardized, and forms the basis of the first patent in preparation. The integration of microfluidics has required the design and construction of dedicated tools for the integration upon rapid prototyping. The device has been assessed for functionality, degradation in cellular culture buffers, and mechanical properties. The electrical and fluidic connections to the outside world have been optimized for the animal model. A few tens of devices were fabricated and delivered to UCAM for implantation.
7. **Demonstration of the implantable device in contusion SCI animal model (WP8)**: the AMID developed in WP7 has been implanted in acute SCI murine model, as well as on healthy animals as controls. The surgical procedure and the experimental design have required the setup of a protocol and the in vitro lab equipment has been adapted to the experiments in the stabularium. The experiments of stimulation of the SCI site, coupled to electrophysiology of the nerves have revealed communication between the SC and the sciatic nerve correlated to the stimulus supplied. The efficacy of the treatment in the chronic model, and the assessment of the inflammatory response due to the treatment are currently in progress.

8. **Safety, Regulation and Ethical Issues (WP9)**: *Safety and Regulation*: AMID has been assigned to Class III medical device – an active implantable device in direct contact with CNS, including medicinal product as an integral part, human cells, nanomaterials and electromagnetic effects. Before the clinical use of the medical device, there will be additional steps needed which will ensure safe and reliable therapy, e.g. phase III clinical trial should be conducted, albeit this is outside the scope of the present project. Therefore, relevant clauses for development of an electroactive biomedical device (EN 60601-2-10:2000) have been assessed for AMID. A Design History File (DHF) table has been created, containing a list of pointers to all pertinent documents prepared during the course of the project. *Ethical Issues*: The project has involved the use of mouse cells (NPCs and peripheral mononuclear cells) and in vitro assays, as well as in vivo pre-clinical tests. "3 Rs" policy (99/167/EC: Council Decision of 25/1/99) of Reduction (of the number of animals), Replacement (with non-animal methods) and Refinement (of methods that alleviate or minimize the potential pain, suffering or distress; while enhancing animal welfare) has been accounted in the workplan, and especially in the experiments in WP5-8 by carrying out most of the studies *in vitro.* The in vitro preparation and use of mouse NPCs and iPS cells do not involve ethical issues. The PPL (80/2457) and PIL are in place and already working. The PPL has been amended in line with the AMID implantation and with the option to perform MRI/PET in vivo imaging.
9. **Management, exploitation and dissemination (WP10)**: management has been always punctual and timeline fully respected. Communication between Consortium and Commission was always timely. The transfer of coordination and the change of partnership have been managed smoothly without disruption or delay of the activities. An extensive patent analysis in the sector of interest has been done in view of exploitation. An intense activity of dissemination was carried out by I-ONE-FP7 members through tenth of invited and contributed talks at universities, workshops and international conferences. I-ONE had a booth with several demonstrators at Euronanoforum 2013 in Dublin, gathering the interest of scientists and stakeholders. A satellite workshop on Implantable Organic Electronics has been held in Modena in June 2014, with iONE-FP7 partners speaking of the project results together with leading scientist from Europe, USA, Japan. It is worth highlighting the presence of Dr. Ph. Bergonzo, the coordinator of the sister project, NEUROCARE where carbon allotrope devices were the platform chosen for interfacing to the spinal cord.

In view of this, after 36 months from start we can state that the objectives envisioned have been successfully achieved. The short time has prevented us from carrying out the full assessment of the AMID on the chronic SCI model. This remains for the follow up. All deliverables have been accomplished, often thanks to novel solutions to problems that were originally unexpected. This has lead not only to publications and to IP generation, with a joint patent in preparation involving UNIMORE-CNR-SCRIBA-UNIBO-UCAM. Also inspired to the success of the project UCAM has established a start-up company dedicated to bioelectronics. UNIMORE and UALG are planning the creation of a joint startup company. Overall, the response from the partners has been extremely positive, and everyone involved in iONE-FP7 is willing to continue to work together in collaboration in future projects. UNIMORE and LiU have already got a bilateral Italy-Sweden grant on organic citokine biosensors for POC applications, and EU proposals in NMP (Biomaterials for Alzheimer's disease) and Infect ERA (personalised biosensors for influenza) have been submitted.

These achievements have prompted us to continue the collaboration outside of iONE-FP7, in order to arrive to the demonstration and assessment of the chronic model of SCI. This is

important also in view of suggesting further actions supported by EC (also in other thematic priorities of Horizon 2020) and from foundations funding research on paralysis.

3. Description of the main S&T results/foregrounds:

3.1. Technological interfaces, substrates and characterization

The activity of this WP can be divided in two sections. One section was devoted to the preparation of the suitable scaffold materials such as hyaluronic acid (HA) and Poly(D,L-lactide-co-glycolide) (PLGA) films. The other section was devoted to perform an integrated nanoscale characterization of the physical and chemical properties of biocompatible scaffolds as they were exposed to the biological buffers.

The activity on the scaffold materials was aimed to prepare a biocompatible and biodegradable scaffold to be incorporated in the implantable device. This activity involved several steps: (i) fabrication of films of controlled size and thickness, (ii) characterization of the films in terms of their macroscopic properties (transparency, thickness, surface energy), (iii) characterization in terms of surface nano-scale properties (roughness, porosity, elastic modulus), (iv) coupling with the organic semiconductor as active materials (Pentacene, Pedot:PSS).

The PLGA films were fabricated by casting a PLGA/dichloromethane solution in a silicone elastomeric pool onto a glass slide. The films should be flat, hole-free and transparent. The microscopic structure of the films (presence of cavities or pores) depends on the temperature during the solvent evaporation and the relative humidity. These pores could in fact hinder the fabrication of a good organic semiconductor thin film device. The films is preferably transparent to facilitate the manipulation during surgical implantation as well as to be compatible with the standard optical microscopy cellular assays. The wettability, that plays also an important role for the biocompatibility, has been assessed by measuring the contact angle of PLGA with physiological solutions.

The PLGA films were interfaced with the organic semiconductors (active materials) in the final implantable device. This step has required the assessment of the PLGA film compatibility with the fabrication techniques such as vacuum sublimation and spin coating. We have shown that the active materials used in iONE-FP7 are compatible with the PLGA scaffold. In addition, we prepared and studied several types of hydrophobized HA, mainly palmitoyl HA was used. Ultra-smooth films have been prepared (about 1 nm roughness). Those HA films are also biocompatible and biodegradable. We have studied the influence of the film on the cells. It was concluded that there is no negative impact on the cells.

To study the evolution of the scaffolds in the biological media we have developed a high resolution nanomechanical characterization method. This method was applied to study the early stages of swelling and degradation of PLGA and HA in phosphate buffer saline. To follow the changes of the PLGA over 14 days with nanoscale positioning accuracy, we have used micro- and nanoscale PLGA patterns fabricated by focused ion beam lithography. Force microscopy and spectroscopy methods have been used to follow the *in situ* evolution of those patterns. The evolution of the morphological properties, size and surface roughness shows two different periods. The first period, from day 0 to day 8, shows alternating changes in the volume. The second period, from day 9 until day 14 shows a slow decrease of the PLGA structures. On the other hand, the elastic response of the PLGA shows a continuous softening upon immersion in the buffer. The Young modulus shows a 200-fold decrease from an initial value of 2.4 GPa to 9 MPa. The softening rates have two high regions that match the days when the volume shows higher increases.

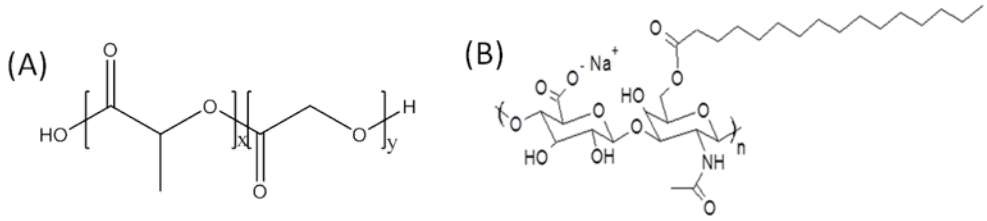

Figure 1. (A) Structure of Poly (lactic-*co*-glycolic acid) and (B) Hyaluronic Acid used in the experiments.

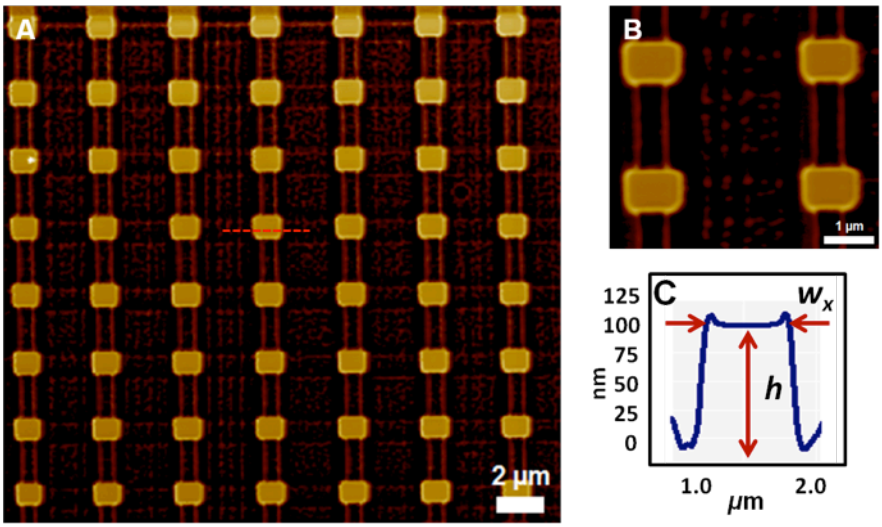

Figure 2. AFM topography images of PLGA nanopatterns on a silicon substrate. A. Aspect of PLGA patterns on silicon. B. Zoom of four PLGA patterns. C. Cross-section of a PLGA pattern.

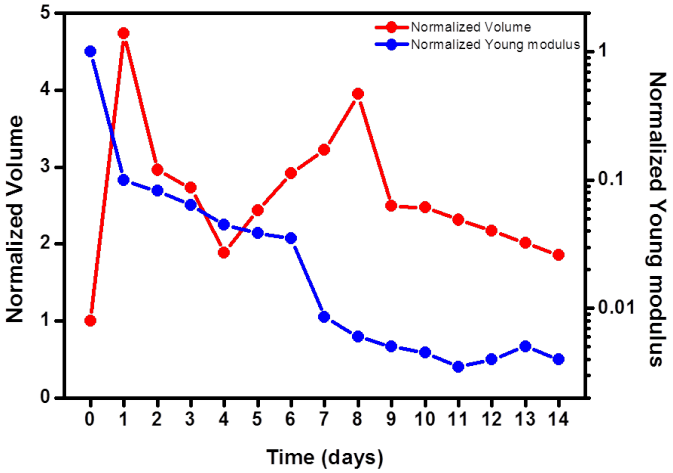

Figure 3. Young modulus and volume evolution upon immersion in PBS.

3.2. Design and fabrication of electro-active device components

The central technological innovation of iONE-FP7 is the active multifunctional implantable device (AMID). Its single electrically active components were developed in WP 3 lasting from M1 to M18 of the project. The WP3 focussed on three different functionalities to be implemented by a flexible, biocompatible organic electronics material platform as characterized in WP2: (i) amplified transduction of electrophysiological signals to be achieved by organic transistors; (ii) artificial synapse-like plasticity based on nanoparticle organic memory field effect transistors (NOMFET); (iii) sensing of inflammatory markers implemented as antibody functionalized organic field effect transistor. Each of these functionalities was developed independently in separate tasks. Critical constraints for fabrication technologies and operation conditions of components were identified in the beginning of the workpackage. In particular the later integration of components into a single AMID using a single bioscaffold as substrate necessitated the development of simple, solvent-free fabrication protocols. As a major technological breakthrough we introduced laser ablation to allow patterning of active layers on fragile bioscaffolds like poly(lactide-co-glycolide) (PLGA). Further, operation of devices under physiological conditions that comprise mechanical strain and immersion in saline solution for a period of days to weeks as typical for implantable neuro-prosthetics required a robust architecture of single components.
We identified the water gated transistor architecture as fundamental to this achievement. In contrast to encapsulation of active layers as is typical for silicon-based electronics, our approach exploits the water stability of organic semiconductors like pentacene or Pedot:Pss. Charge accumulation in the channel is controlled due to potential differences to the bath containing the biological system. The high capacitance of the resulting interfacial double layer enables then ultrasensitive detection of potential variations for recording or injection of capacitive currents for stimulation. In Fig.1 we visualize some of the main achievements in fabrication and operation protocols leading to the timely accomplishment of all deliverables and milestones of WP 3 and paving the way to successful integration in AMID tested under in-vitro and in-vivo conditions in later WPs.
As a highlight we emphasize the fabrication of electrophysiological transducers on bioresorbable scaffold which permits new approaches for minimally invasive loco-regional therapy of neurodegenerative diseases and which is currently being protected as patent.

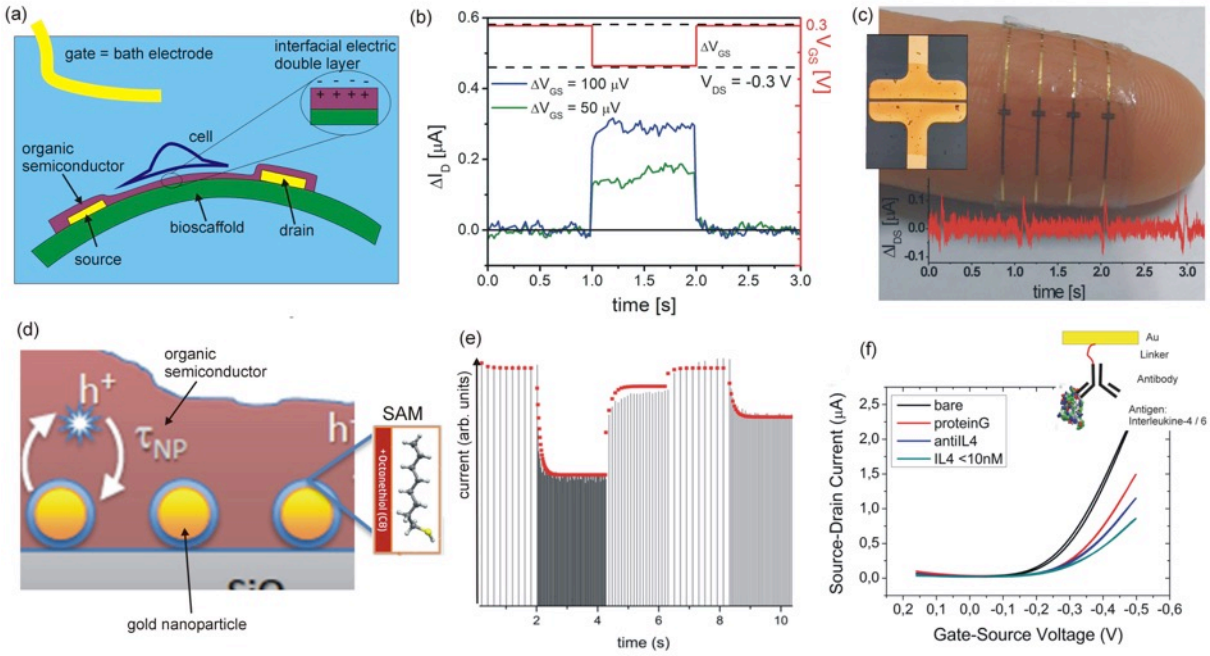

Figure 4: Single electrically active components of iONE-FP7 AMID technology: (a) scheme of organic transistor on bioscaffold acting as potentiometric transducer in solution. Fundamental to high sensitivity is the electric double layer building at the semiconductor/electrolyte interface as shown in

the inset. (b) recorded current-trace of transistor subjected to electrical stimuli affecting the potential at the water gate electrode V_{GS}. Amplification of potentiometric signals as small as 50 μV is demonstrated. (c) Exploitation of organic transistor fabricated on resorbable bioscaffold as electrophysiological transducer. Here detection of ECG signal by array of four transistors with conformal adhesion to skin is shown. The inset depicts a pair of source and drain electrodes and the patterned semiconducting channel here made of PEDOT:PSS. (d) Working principle of NOMFET representing an artificial synapse. Trapping of carriers in gold-nanoparticles leads to frequency dependent spike transmission as shown in (e). (f) Immuno sensor based on water gated organic transistor. Changes in the depicted transfer characteristics are due to increased interface capacitance and dipole as caused by subsequent adhesion of molecules to the active interface. The selective adhesion of inflammatory marker is guided by a tethered specific antibody as shown in the inset and allows detection of interleukine markers with concentration in the nM range.

3.3. Static and dynamic control of gradients

The specific workpackage, spanning M1 through M24, was focused on developing key technologies and processes for both static and dynamic cue gradients. The overarching aim was to grow the nascent technologies that the partners brought to the iONE-FP7 project into a toolbox of flow-based, non-flow-based, and topological methods to regulate cell development and inflammation response in the spinal cord injury site, all (potentially) to be integrated onto AMID devices. Specifically, this work package focused on (*i*) the initial liquid-flow-based microfluidic devices and geometries for the AMID device; (*ii*) non-flow-based ion pump (OEIP) chemical delivery adapted for iONE-FP7 applications; and (*iii*) nanopatterning of topologies for static cue gradients. The processes and methods thus included a range of fabrication techniques included microfluidic molding, photolithographic patterning, laser-assisted bioprinting, and laser ablative patterning. Characterization, conducted at all WP4 member institutions (LiU, Scriba, UniBo, CSIC), included methods such as atomic force microscopy, semiconductor current-voltage analysis, real-time liquid-flow microscopy, and computer modelling.

Initial microfluidic design (D4.1). Early on in the iONE-FP7 project, it became clear that the AMID device would be built up around a microfluidic system. This was the case, firstly, because the microfluidic channels and outlets are so much larger than the bioelectronic components (OECTs, OEIPs, and NOMFETs) to be incorporated onto the AMID. Secondly, it was clear that the microfluidic system itself could comprise the biodegradable substrate of the AMID itself. It was therefore of utmost importance to develop both the know-how and the actual methods for fabricating the microfluidic adapted for iONE geometries and requirements. D4.1 demonstrated this initial microfluidic system.

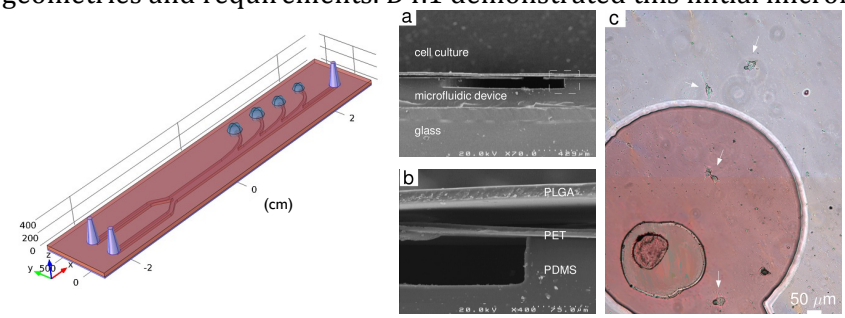

Figure 5: The figure shows the geometry of this first-stage microfluidic system: two inlet ports (at bottom left) feed to four delivery ports (fingers leading off toward left) and a single outlet. These initial devices were fabricated primarily using the standard microfluidic material PDMS (silicone). This was not the final biodegradable material chosen for iONE-FP7, but one used to demonstrate the fabrication. However, PLGA, the intended biodegradable material was used to demonstrate the positive biocompatibility of the microfluidic device. As seen in parts **a** and **b** of the figure, a layer of PLGA was placed above the oulet and provided a layer for stem cell adhesion. These cells can be seen below the white arrows in part **c** of the figure. This system provided an *in vitro* testbed for iONE-FP7 microfluidics, where physiological solution could be delivered to the cells under growth conditions. This microfluidic

design – and its accompanying fabrication methods – were used in later work packages and ultimately led to the integrated AMID prototype.

Electronically-controlled dynamic chemical gradients (D4.2). For the past several years, the LiU partner of the iONE-FP7 project has been developing a dynamic substance delivery system based on electrophoresis in organic bioelectronic materials. The result is an electronically-controlled delivery of precise amounts of small biochemicals, without requiring the flow of any liquid – only charged molecules (ions) are "pumped" toward the biological target region. The device, based on the familiar organic electronic material PEDOT:PSS, is thus called the organic electronic ion pump (OEIP). The two primary feature of interest within iONE-FP7 for such delivery is, first, the non-flow nature, and second, since delivery is not flow-based, a concentration gradient of the delivered substance is built up at the outlet, providing a means for dynamic gradient. The bulk of the effort in D4.2 was to adapt OEIP technology for iONE materials and geometrical requirements. Within WP4, this included efforts of patterning and processing OEIPs directly on biodegradable substrates (PLGA and HA) and biocompatible substrates (polyimide, PI). During the duration of WP4, the biocompatible substrate was chosen for demonstration (see D4.2 for details), however, more recent WP5 results with PEDOT:PSS on PLGA indicate that OEIPs on PLGA are feasible.

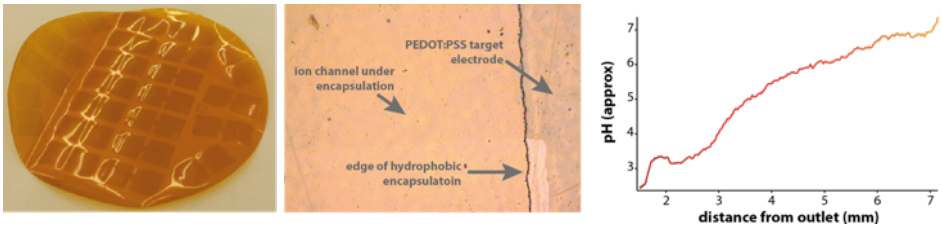

Figure 6: The figure shows successful fabrication (using high-precision photolithography) of multiple OEIPs on a 6 in PI substrate. The middle image is a microscope picture showing the well-defined ion transport channel and hydrophobic encapsulation required for OEIPs. Finally, the graph at right shows the results of H^+ delivery, resulting in a gradient of H^+ concentration, *i.e.*, a pH gradient, measured in mm from the outlet. H^+ was used as an easily-monitored delivery substance, and illustrates dynamics of iONE-adapted OEIPs. Indeed, the gradient shown in the figure was established by applying a driving voltage, and could be "turned on/off" with the flip of a switch. The OEIPs developed through WP4 could then be used (together with results from WP5 on PLGA) in final integrated AMID prototypes.

Static topochemical cues (D4.3, T4.4). In addition to active gradients of chemical cues, WP4 also focused on static/passive gradients to foster favourable conditions for cell growth, differentiation, and guidance. This effort incorporated three basic methods: (*i*) laser-assisted printing of biochemical cues on substrates, (*iii*) atomic force microscopy (AFM) of topochemical cues, and (*iii*) fabrication and modelling of passive release of substance-loaded materials.

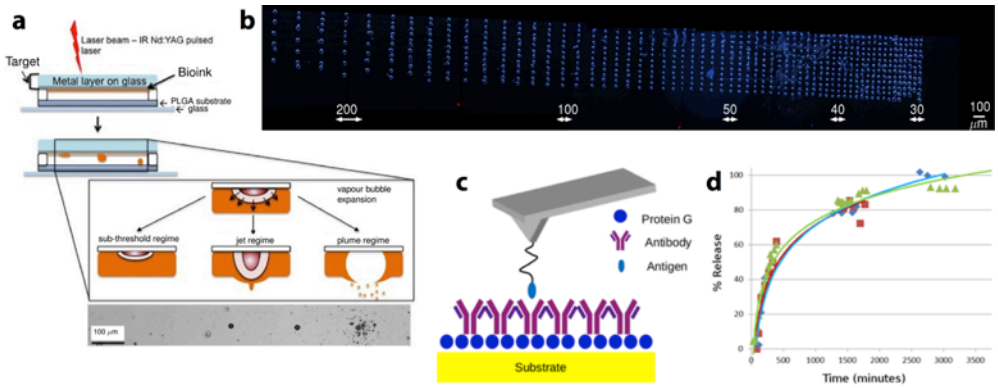

Figure 7: These efforts are summarized in the figure. Parts **a** and **b** show the laser-assisted bioprinting technique used to pattern a static gradient of the cell-adhesion protein laminin. These results are detailed in D4.3, and illustrate the versatility of the patterning technique to augment all future iONE-FP7 AMID devices with such gradients. Part **c** of the figure is a schematic of the AFM technique used to resolve topochemical cues at the nanoscale. The example shown here being the measurement of

patterned antigens by dragging the corresponding antibody along with the probe tip: antigens are resolved when/where the modified tip sticks to the surface. Part **d** of the figure shows a release-over-time curve for the anti-inflammatory drug minocycline loaded in a film of PLGA. This result shows the capability of loading the entire AMID substrate with inflammation-reducing compounds to prevent an immune response to the implant.

3.4. In vitro integration of stem and immune cells in devices

This **WP** was aimed at characterizing the biocompatibility of the electronic device and testing/optimizing its functionality. To do so we exploited customized *in-vitro* models.

The use of consistent/reproducible *in-vitro* cell culture systems allows for extensive exploitation of a wide range of conditions with a good prediction of the *in -vivo* outcomes, while reducing the unnecessary use of laboratory animals.
It is well established that the implantation of any kind of medical device triggers a non-specific immune response of the host tissue, called foreign body reaction (FBR), which constitutes a major impediment in the advancement of these technologies into clinically-applicable devices since it affects their long-term performance *in-vivo*. FBR causes, in fact, insulation, damage and overall rejection of the implant. We therefore established an *in-vitro* system to assess the FBR elicited by the different substrates identified and tested for the fabrication of the final device. Within this *in-vitro* assay, different cell types involved in a likely FBR within the central nervous system (CNS) were employed, among which microglial cells, astrocytes and bone marrow-derived macrophages. We characterized the immune/inflammatory response that results from the interaction between either of these cells and the materials. We compared the immunogenicity profile of our candidate organic, biodegradable material for device fabrication, poly-lactic-co-glycolic acid (PLGA), with that of poly-imide (PI), poly-ethylene naphtalate (PEN), poly-ethylene terephthalate (PET) and poly-dimethylsiloxane (PDMS). These are all organic (but not degradable) materials widely used in organic electronic and biomedical applications.
We observed that the direct contact only of immune cells with PLGA (and also the other materials) lead to the activation of macrophages and the generation of a pro-inflammatory reaction (**Fig. 8A-J**). Macrophages in fact easily adhered to PLGA (similarly to PI and PEN) (**Fig. 8A-G**) and produced pro-inflammatory mediators -such as interleukin (IL)-1β , tumour necrosis factor (TNF)-α and nitric oxide sinthase (NOS)-2 (**Fig. 8H-J**). We also analysed microglia and astrocytes, which showed a similar response upon contact with PLGA.
We then investigated the possibility of delivering drugs that are able to control this acute immune/inflammatory reaction elicited by the device. We focused on Minocycline, an approved antibiotic (already used in clinics) with anti-inflammatory properties, which has also been show to reduce inflammation *in-vivo* in animal disease models and being studied onto patients with chronic spinal cord injury (SCI) within phase 2 clinical trials.
We observed that the delivery of Minocycline trough micro channels built on PLGA substrates (called microfluidic channels, **Fig. 8K** and **L**) partially reduced the adhesion of macrophages (**Fig. 8M-P**) and completely block their shift towards a pro-inflammatory phenotype (**Fig. 8Q** and **R**). Thus, the delivery of Minocycline via device-integrated microfluidic channels might represent a valuable approach to avoid/reduce FBR *in-vivo*. The local release of Minocycline trough the device to reduce the FBR could also provide neuroprotection by reducing inflammation within the injured spinal cord. Furthermore, the PLGA composition can be tailored to control its degradation time. Once the pro-regenerative function of the device will not be needed any longer and the acute FBR reduced by Minocycline release, the device will slowly degrade (or at least more than 95% of it) reducing also the chronic consequences of FBR within the CNS, such as tissue scar formation, neuronal damage and chronic pain.

Successively the i-ONE FP7 consortium developed a PLGA-based organic transistor device for *in-vitro* experiments. This device is small (Area = 1cm^2), thin (thickness = 5μm) and transparent (fundamental for *in-vitro* tests) (**Fig. 9A**). It has gold electrodes and a thin layer of a (biocompatible and biodegradable) semiconductor material called PEDOT:PSS, which allows a better distribution of electrical charges during stimulation, thus improving its efficiency. We verified the functionality of the device using rodent neural stem/progenitor cells (NPCs)-derived neurons and rat cortical primary neurons (**Fig. 9B,** cell nuclei are in blue and cell bodies in white). A potential applied between the electrodes generated an extracellular current (**Fig. 9C**) that stimulated the cells by changing their membrane potential (that is the charge possessed by the cells in normal conditions). To identify the window of effective neuronal stimulation, we applied short pulses of stimulation (0.8 ms of duration) (**Fig. 9D**) at increasing current intensities. To monitor the response of the cells, we used a technique called calcium (Ca^{2+}) imaging that allows the *live* visualization of the intracellular increase of Ca^{2+} by means of changes in intracellular fluorescence intensity over time (**Fig. 9E** and **F**). Cells can, in fact, be loaded with a dye that emits fluorescent light

when it binds to Ca^{2+}. In our experiments, we stimulated a population of neurons for 60 seconds (with 5s of stimulation, after a delay time of 15s) to determine the range of currents able to stimulate the cells without damaging them. In this way we could determine the safe threshold of current to which cells respond to (**Fig. 2G**) without being damaged. We also established the ideal frequency of stimulation, which is another important parameter to be established for each given population of stimulated cells (**Fig. 9H**).

Since electrical stimulation of neurons can result in cell damage and toxicity it is important to evaluate that the observed response is effectively due to (*true*) membrane depolarization and consequent activation of specific ion channels, called voltage gated calcium channels (VGCCs), rather than (*false*) membrane damage (as consequence of electroporation) and consequent ion diffusion. The addition of VGCCs inhibitors, that are chemicals able to block the activation of VGCCs (**Fig. 10A**, red line), determined a reduction in the response of neurons upon stimulation. We also applied repetitive stimulations by using a protocol that may be likely applied *in-vivo* to boost neuronal activity and promote functional recovery. Multiple stimulations (up to 10 in 6 minutes) resulted in repeated and stable response of neurons (**Fig. 10B**). These results suggest that the identified protocol of electrical stimulation is physiologically activating neurons without acute toxic effects. Then we applied the same repetitive stimulation for one hour and we verified that this stimulation is safe and does not affect their viability in the long-term.
Finally, we looked also at possible effects of current stimulation onto non-neuronal cells, such as macrophages, since any electrical stimulation applied in proximity of the injury might also affect these cells, which play a crucial role in the inflammatory phase of the disease early after injury. Very interestingly, macrophages respond to the electrical stimulation, with electrically stimulated (ES) macrophages being less pro-inflammatory, as noted by the reduced expression of the RNA coding for the pro-inflammatory cytokine IL-1 β, when compared to non-ES macrophages (**Fig. 10C**).

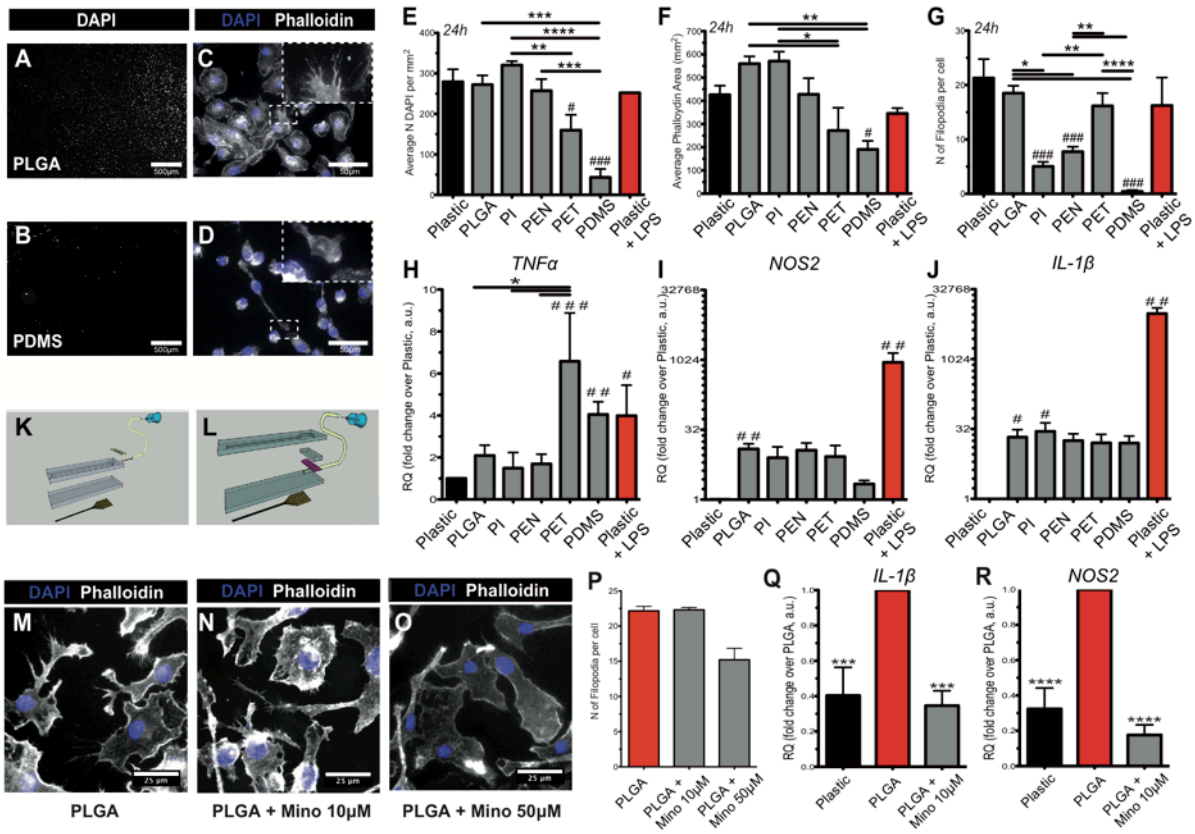

Fig 8. Minocycline administration reduce foreign body reaction caused by PLGA. (**A-D**) Representative images of macrophages adhering to PLGA (**A** and **C**) and PDMS (**B** and **D**). More macrophages (stained with DAPI, in white) are able to adhere onto PLGA (**A**) compared to PDMS (**B**). Macrophages also spread more and extend more processes (called filopodia, stained with Phalloidin in white) onto PLGA compared to PDMS (**C** and **D**). (**E**) Quantification of the numbers of macrophages adhering per mm^2 of materials, 24h after seeding. (**F**) Quantification of the area covered by macrophages on different materials. (**G**) Quantification of the number of processes (filopodia) generated by macrophages when seeded on different materials. In these graphs (**E-G**) macrophages adhering onto tissue culture plastic (black bar) are used as positive control because they generally

adhere perfectly to it. Plastic + Lypopolysaccharide (LPS) (red bar) is used, instead, as another control, where macrophage are pro-inflammatory activated by exposure to a bacterial stimulus (LPS). (**H-J**) The graphs show the production of pro-inflammatory mediators, namely TNFα, NOS2 and IL-1β, produced by macrophages upon contact (for at least 6h) with the different materials. In this case macrophages grown onto tissue culture plastic (black bar) are used as negative controls because the production of inflammatory mediators is generally low. Plastic + Lypopolysaccharide (LPS) (red bar) is used, instead, as positive control because macrophages are pro-inflammatory activated by LPS, thus producing these mediators. The production is measured as expression level of the genes encoding for these mediators. (**K** and **L**) The schematics represent the design of microchannels (called microfluidic channels) to allow delivery of drugs/chemicals via a PLGA device. (**M-O**) Representative images of macrophages adhering to PLGA in the presence of Minocycline. The drug partially reduces their adhesion (measured again 24h after seeding). (**P**) Quantification of the number of processes generated by macrophages onto PLGA with or without Minocycline. (**Q** and **R**) Quantification of pro-inflammatory meadiator production from macrophages upon contact with plastic (in black), PLGA (in red) and PLGA + Minocycline (in grey). Minocycline completely reduce the pro-inflammatory activation driven by contact with PLGA. In **C**, **D**, **M-O** cell nuclei are stained with DAPI (in blue), while cell body are stained with Phalloidin (in white). Scale bars represent 500μm in **A** and **B**, 50μm in **C** and **D**, 25μm in **M-O**. These symbols * or #, ** or ##, *** or ###, **** or ####, indicate the extent of the differences measured between the materials tested.

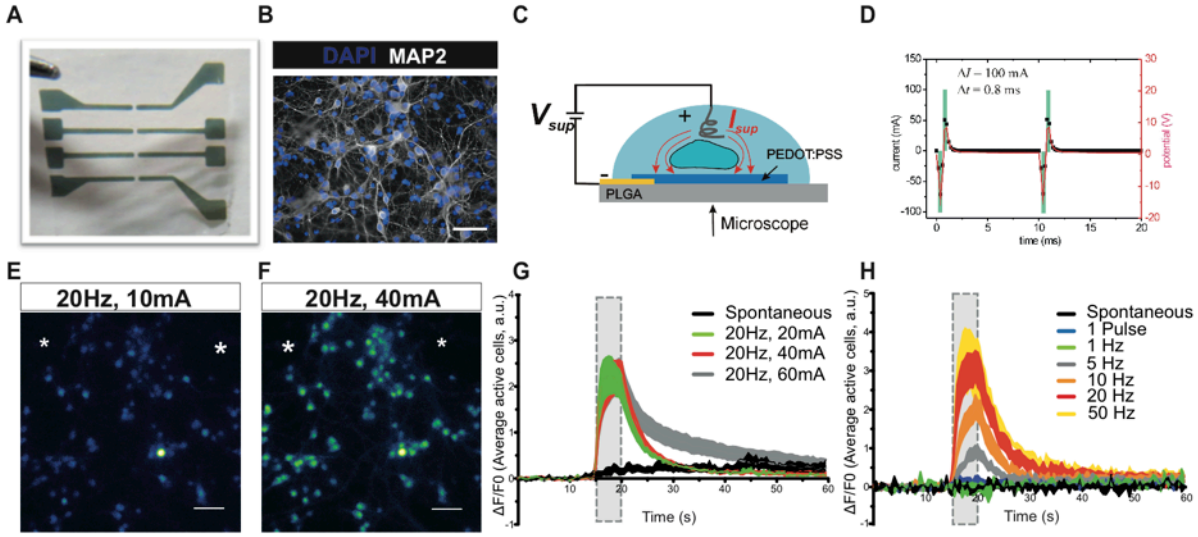

Fig 9. PLGA-based electronic devices can efficiently and reproducibly stimulate neurons. (**A**) Representative image of a PLGA-based organic electro-chemical transistor. The device is small and transparent, optimized for *in vitro* studies. It contains gold electrodes and a semi-conductor layer of PEDOT:PSS (**B**) Representative fluorescent image of neurons derived form rodent's brains, growing onto the device. Cell nuclei are stained with DAPI (in blue) and cell body are stained with MAP2 (in white). (**C**) Schematic representing the cross-section of the *in vitro* set-up. Neurons seeded onto the device are kept in solution while a potential difference is applied between the electrodes on the surface and the bath electrode (called gate). This generates a current perpendicular to the film as shown by the red arrows that changes the cell membrane potential. The transparency of the device allows us to image the cells from the bottom, as shown in the image. (**D**) Representative stimulation used in *in vitro* experiments. Short pulses (0.8ms) of biphasic current are applied at different intensities. (**E** and **F**) Representative fluorescent images of neurons loaded with a fluorescent dye to perform Ca^{2+} imaging. When neurons are stimulated at current intensity lower than a certain threshold, there is no response from neurons (**E**). When the threshold is reached neurons are activated and uptake Ca^{2+} from the medium and therefore we can observe an increase in their fluorescence (**F**). (**G**) The graph shows the changes of fluorescence over time (ΔF/F0) in a population of neurons when stimulated (5 seconds, grey dashed bar) at increasing current intensities, compared to the spontaneous (no stimulation) activity. (**H**) The graph shows the changes of fluorescence over time in a population of neurons when stimulated at increasing frequency. Scale bars represent 25μm in **B**, and 50μm in **E** and **F**.

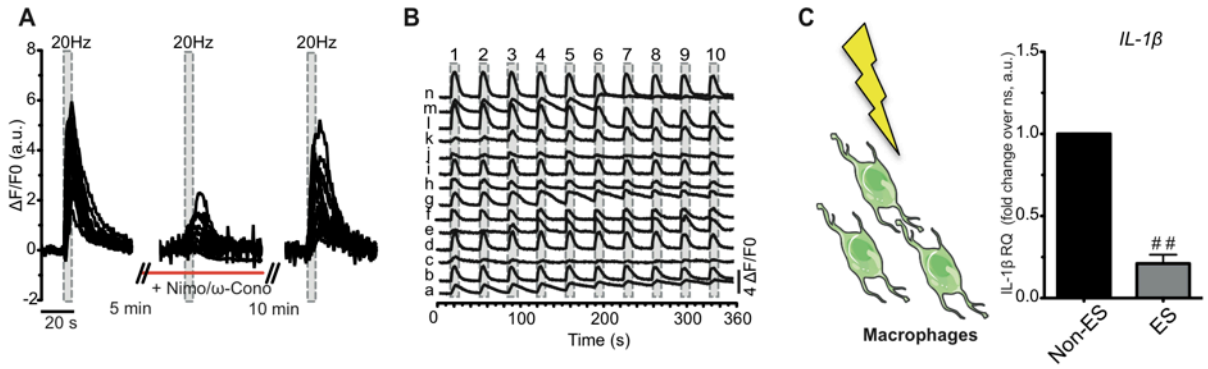

Fig 10. Repetitive electrical stimulation is safe in neurons and reduces pro-inflammatory response of macrophages. (**A**) The graphs shows traces of fluorescent changes over time (ΔF/F0) within single neurons during electrical stimulation (grey dashed bars), in normal conditions (on the left), in the presence of voltage gated calcium channels inhibitors (in the middle, red line), and after wash out of the inhibitors (on the right). The presence of inhibitors completely blocks neuronal response to electrical stimulation. After washing out of the inhibitors, the response of neurons to stimulation is re-established. This means that the response of neurons is due to the activation of voltage dependent ion channels rather then to damage of the cell membrane (called electroporation). (**B**) The graphs shows changes in fluorescence over time in single neurons upon repetitive (10 repetition in 6 min) stimulations (grey dashed bars). Most of the neurons respond consistently to all the stimulations, suggesting that this protocol is well tolerated by the cells. (**C**) A repetitive stimulation protocol (1h duration) optimized with neurons has been then applied to macrophages in order to understand the effect on non-neuronal cells. Interestingly electrically stimulated (ES)-macrophages showed a decreased production of pro-inflammatory mediators, such as IL-1β. Further analysis is running to better understand this effect.

3.5. Signal transduction in vitro

Cells communicate with each other by moving ions, such as potassium (K^+) and sodium (Na^+) across their membranes. These ionic movements cause minute electrical fluctuations. To understand and decode these messages, we have to fabricate electronic transducers that can detect and amplify these ultra-small ionic variations. These devices should have "friendly" surfaces to the cells (biocompatible), should not disturb the normal cell activity (non-invasive) and must translate the minute ionic fluctuations into electrical signals. In addition, they should also be able to stimulate the cells providing this way; a bi-directional communication interface with cells and tissues. These requirements pose numerous challenges to device engineering, ranging from an appropriate selection of materials, solving issues of stability and reliability to a proper design that optimizes the electrical coupling between biological environment and the electronics.

During the i-ONE FP7 project this process of device engineering and optimization was carried out in work-package named "Signal transduction in vitro". All the experiment was carried out using cell culture lines and steam cells. These experiments are named "in vitro". A large number of prototypes devices were fabricated and optimized to interact with different types of neuronal cells. The lifetime, the sensitivity of the device, and in general the overall performance gradually improved during the project. The experiments provide fundamental understanding about how to measure cells using electrical techniques and also to the development of new transducers with applications in life sciences. The most relevant in the context of this project is a multifunctional transducer designed to repair the spinal cord injury. However, other side-ways applications have emerged, for instance, drug screening platforms. These are devices that measure how cells react when exposed to new pharmaceuticals or toxic substances. Transducers that can generate naturalistic patterns of cell signals such as action potentials. These can be used to repair faulty cell signaling or stimulate neuronal circuits.

Detailed simulations closely accompanied the device optimization process. Modeling gave insight about how the bioelectrical signals connect to the electronic transducer, allows us to understand the shape amplitude and length of the measured signals. This information is then used to provide clear guidelines to optimize the transducer geometry and maximize the cell signal detection.

In the course of the project the simulations were used to provide; (i) proper description of electrode/dielectric/water/cells interface and cell responses to external stimuli and, (ii) obtain insight into drug deliver devices fabricated and measured during the project. These are based on a polymeric film (PLGA) loaded

with different amounts of a drug (minocycline). The simulations linked with real data made possible to quantify and model the diffusion coefficient of the minocycline inside the PLGA host film. This information was then incorporated into more advanced model used to predict the optimal PLGA formulation that can release the drug within the desired time. The major achievements of this work package are described in the following topics:

Demonstration of bio-electronic interfaces on flexible, transparent and bio-resorbable scaffolds

Innovative processing technologies were developed for the fabrication of devices operating in vivo for several days. These make use of, (i) laser printing of electrodes on polymer foils and (ii) Ink-jet printing of conductive polymers. The substrates used were PLGA and bio-cellulose. Bio-cellulose is biocompatible, and it is used as a skin substitute in burns. Bio-cellulose is not bioabsorbable, but it can be chemically modified to be resobable by living tissue. The degree of resorbability can be controlled to enable adequate device life-time. The fabrication methods are simple and versatile. The resulting prototype device combines optimized materials properties for implantation such as flexibility, transparency and bioresorption with excellent transducer capabilities for both recording and stimulating. This achievement is an important step forward for the fabrication of low-invasive electroceutical treatments

Transistor to measure cells signals produced by neuronal cells

Organic based transistors were optimized to be stable and to work under cell culture conditions for more than two weeks. These devices enable good cellular adhesion and efficient coupling of the ionic currents at the biological/device interface. These devices were used to stimulate and record collective signals from neurons (see Fig. 1). The knowledge gained was also exploited to fabricate skin adherent transistors used to measure high quality ECG recordings. These results demonstrate the stability, robustness, and relatively long-term functionality of the organic based transistors, fulfilling the challenging requirements for implantation.

Organic synapse-transistor (synapstor)

The consortium has successfully fabricated and characterized electronic devices having a synapse behavior. This means they can generate patterns of electrical signals with similar variations in speed and strength as the signals going from neuron to neuron.

These devices combines a memory and a transistor effect in a single device, therefore they are named organic synapse-transistor (synapstor) or alternatively, Nanoparticle Organic Memory Field Effect Transistor (NOMFET).

The synapstor-transistor has been engineered to operate in electrolyte environment at relatively low voltages (1 volt) and with a typical response time in the range 100-200 ms (see Fig. 2). These properties satisfy the minimum requirements to stimulate neuronal cells. The synapstor-transistor is part of the implantable transducer for the spinal cord. Its purpose is to generate partners of signals to stimulate the growth and the establishment of neuronal connections.

Electrical noise techniques to probe living cells

Cells do not always generated clear individual signals; normal metabolic activity generates a low-frequency electrical noise. Cells are noise generating machines. The cell bioelectrical noise should reflect cell activity and cell health state. When it is possible to distinguish these cell-induced fluctuations from the intrinsic background noise, we have a tool to monitor a cell population non-invasively in real time. This technique requires devices with intrinsic low-noise and efficient electrical coupling to the cells.

During the i-one PFP7 project and intensive effort was devoted to fabricating and optimize devices with an extremely low noise level. These devices were addressed by measuring current fluctuations instead of voltage. From an instrumental point of view, measuring electrical current is highly advantageous (comparing to voltage) because we can measure extremely weak current fluctuations. The handicap is that we cannot detect fast events or have high temporal resolution. However, most of interesting biological process occurs in a relatively slow time scale, usually less than one event per second.

The iONE-FP7 research team has developed devices with a noise floor of 100 fento-amps and capable of detecting pico-ampere fluctuations generated by living cells. The performance of these devices was demonstrated by recording the changes induced by the exposing neuronal cells to neurotransmitters and toxic drugs.

Simulation and modeling

The device optimization benefited from insight provided by physical models and the simulations. Two different approaches were followed; one empirical using equivalent circuit networks, other based on quantum chemical calculations and molecular dynamic simulations to mimic the device behavior and its interaction with living cells.

- **Equivalent circuit modeling**
  Equivalent circuits that described the cell device interface were devised. These circuits allow us to find how individual parts of the device contribute to the performance. For example, we determine the layer (polymer coating, or the electrolyte) that generates more electrical noise. This was achieved by systematically changing the dimensions, geometries and the materials used. For each modification the effect on performance was assessed. Following this methodology we have select the appropriate polymer coating, the substrate, the geometry and dimensions for maximum signal to noise performance.

- **Simulation of pentacene OFETs operation dynamics.**
  The response of pentacene under the effect of uniform electric fields was studied by carrying out extensive Molecular Dynamics simulations. Results showed that the applied electric field induces structural changes and defects in the pentacene crystal structure.

- **Description of electrode/dielectric/water/cells interface and cell responses to external stimuli.**
  In order to generate a library of signals based on simulated responses, UNIBO implemented a model (based on continuous methods) for the description of the electrode/dielectric/water/cells interface. The continuity equation for the electric currents was implemented in the computational tool (based on finite element method-FEM) to simulate the electrodynamic behaviour of the experimental interface used for the *in vitro* cellular investigation. Firstly, the gate of the transistor (the electrode) separated by the water layer through a small dielectric layer was modelled by solving the Poisson-Continuum Equation for a dielectric layer and a conductive layer with FEM. The successive step was to combine the extracellular/electrodic model with the already validated nervous cell one. The electrical behaviour of responsive cells was described using the Hodgkin and Huxley (HH) model that was then opportunely modified in order to introduce the external stimulation component. The effects of external stimulus and the cellular coupling with the extracellular electrolytic solution were taken into account through the introduction of a further term in the HH model. The developed model guided the experimental design of the transistor allowing: i) optimization of the recording geometry to maximize the cell signal detection; ii) elucidation of the transistor stimulus extracellular propagation and resulting cellular action in order to assess the optimal cell investigation/stimulation protocol. Furthermore this method will be essential in the comprehension of the experimentally detected bioelectrical signals.

- **Drug release kinetics in complex matrices.**
  Among the goals of iONE there is the achievement of controlled drug release through a passive transport mechanism. The release kinetics of drug from loaded polymeric films (PLGA) has been investigated using both experiments and theoretical calculations. The stochastic-kinetic program STEPS for the simulation of reaction-diffusion systems in 3D geometries has been used in order to predict the results. UV-VIS absorption spectroscopy and scanning electrochemical microscopy (SECM) allowed the detection of the release timing and kinetics of minocycline. Films loaded with different amounts of minocycline were measured and were found to have a similar kinetic behaviour. The degradation of PLGA itself was also taken into account. Simulation of passive transport mechanism has been carried out using Gillespie's algorithm (SSA) implemented in STEPS software. Phenomenological outcomes of the simulations have been linked to the analytical description of the system. First of all, a homogeneous model has been studied, in order to obtain the diffusion coefficient of minocycline inside the PLGA film. Then, an heterogeneous model based on experimental data and on previous simulations can be used to predict the optimal PLGA formulation yielding the desired release timing.

- **PLGA swelling behavior.**
  A mathematical model to gain understanding of the coupled diffusion-swelling process in PLGA has been proposed. The model predictions were validated with experimental data collected by CSIC partner. The numerical scheme is able to describe three regimes that has been experimentally observed, that is i) the initial swelling due to instantaneous and monodimensional diffusion of water into the PLGA; ii) the relaxation, where a slow response is triggered by internal stresses experienced by the polymer due to the presence of the diffusant. It involves the uncoiling / rearrangement of large segments of the polymer chains.

Macroscopically, the volume changes toward an equilibrium value by shrinking; iii) the final swelling, where the stresses are removed further uptake is possible. The polymer is solvated and the diffusion coefficient is no longer concentration dependent. The process is slow and the volume changes following a three-dimensional pace.

Measuring calcium oscillations

Inkjet-printed conducting polymer electrodes on bio-cellulose and in glass substrates are used to monitor propagating calcium oscillations generated by glioma cells *in vitro*. By measuring current instead of the conventional voltage, a high signal to noise ratio (40) is achieved (see Fig. 13). This allows the observation of detailed features of the calcium oscillation such as, replica waves, the traveling speed (50 μm/s) as well as the kinetics of the built-up of the cells synchronization process.

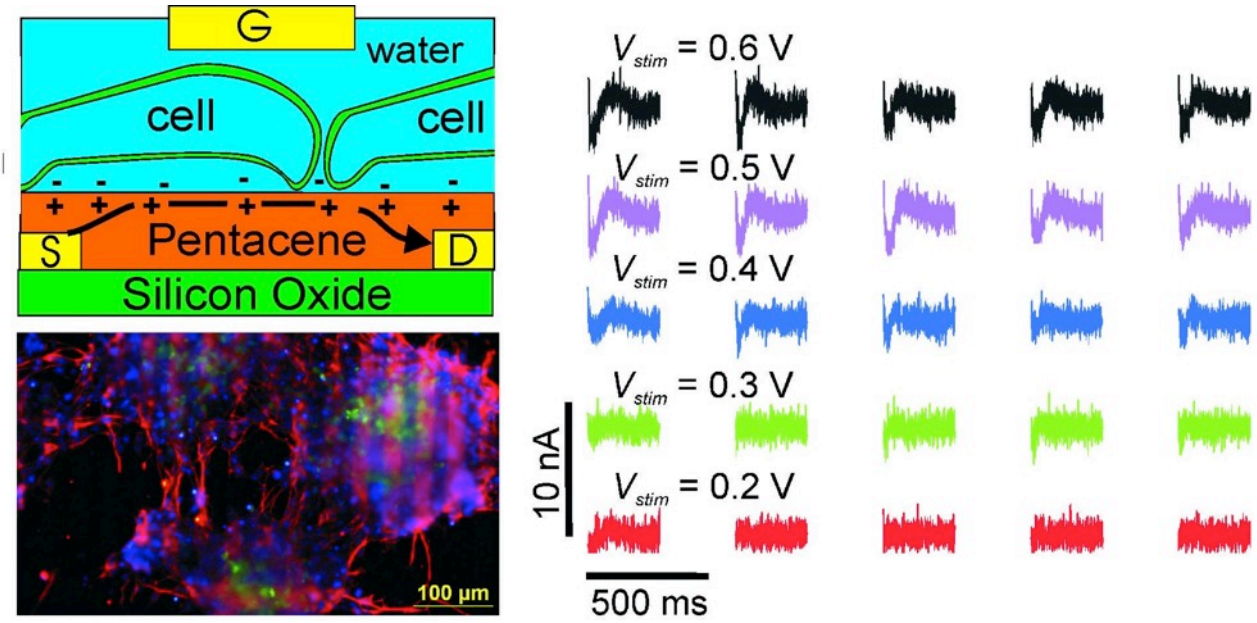

Figure 11. Liquid gated architecture for stimulation and recording of neuronal networks derived from stem cells. The micrograph shows immuno-fluorescence labelling of the neuronal network on top of the active transistor. The current time traces were measured after electrical stimulation of the network.

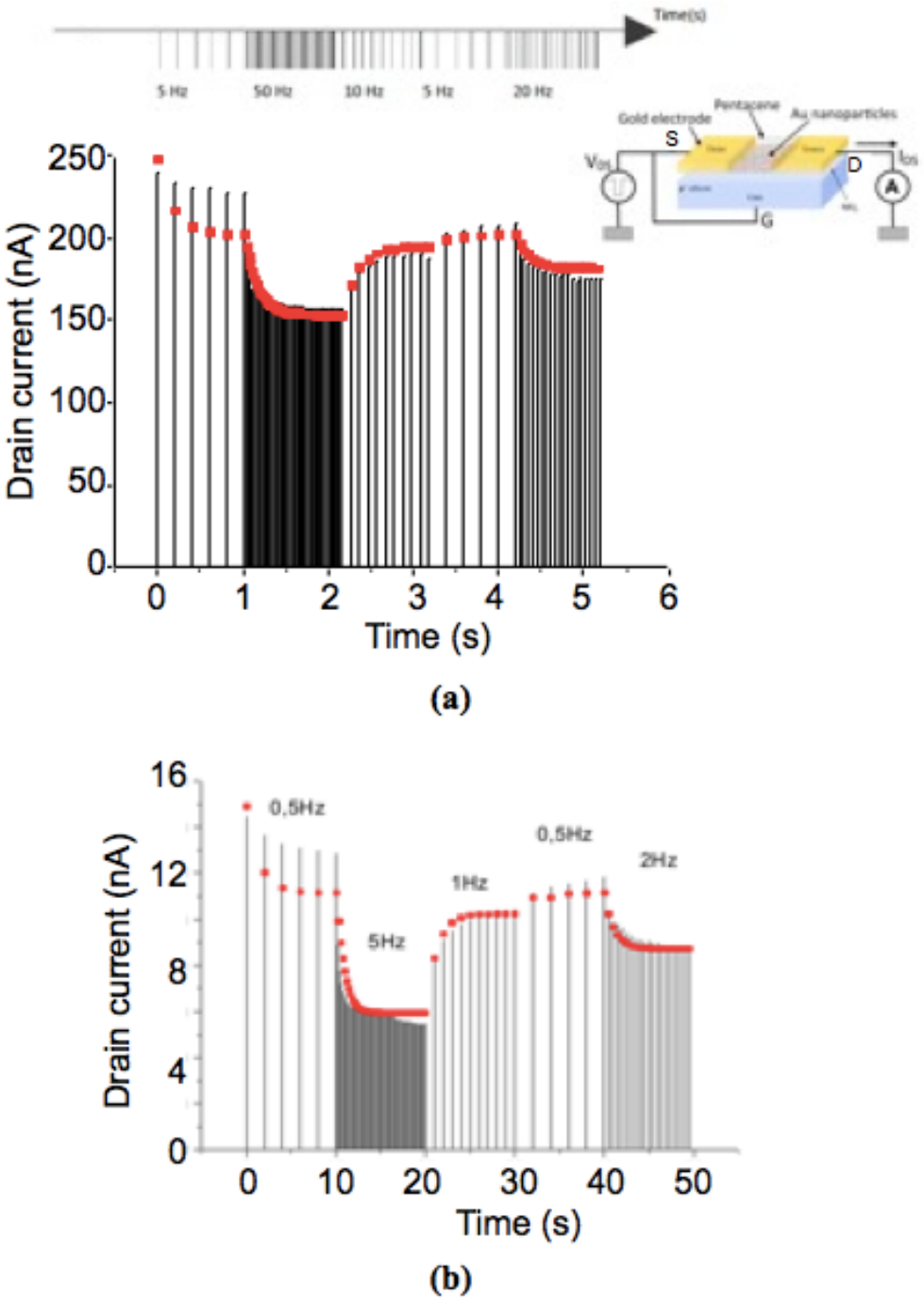

Figure 12. (a) Typical STP response of an optimized NOMFET (subjected to a sequence of spikes at various frequencies with a pulse amplitude of -1V. The red dots are fits with an analytical model from which we extract the characteristic response time constant of the NOMFET (here : 187 ms). (b) STP for a standard NOMFET (L= 1 µm, NPs of 10 nm) without the OTS treatment and for a pulse amplitude of - 10V and spike frequency sequence of 0.5/5/1/0.5/2 Hz (pulse width = 100 ms). The fit gives a STP time constant of 1.5 s.

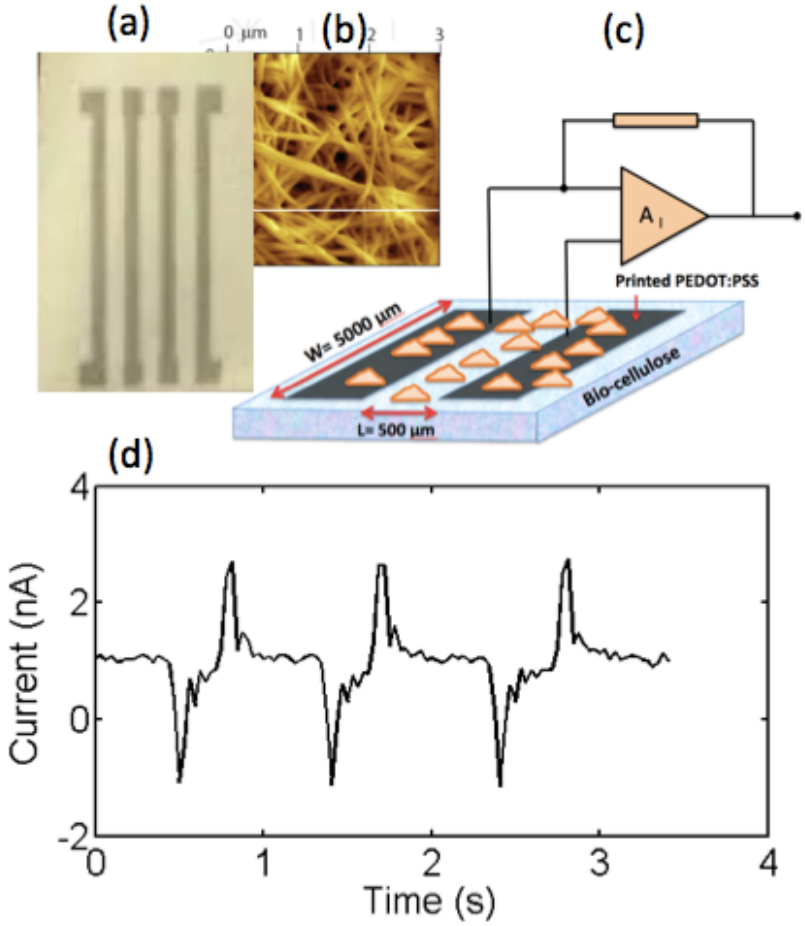

Figure 13. Devices fabricated using ink-jet printed conducting polymer on bio-cellulose substrates. (a) Optical photograph of the printed electrodes, (b) AFM micrograph of the bio-cellulose fibre structure, (c) the device structure and the recording instrumentation and (d) typical signals recorded with the sensing device from a population of glioma cells. These cells generate a quasi-periodic calcium wave.

3.6. Integration of discrete device components into the implantable device

The architecture of the AMID device has been developed during the project and is endowed with the functionalities to stimulate the spinal cord, record electrical activities by means of organic transistors, deliver drugs to the site of the lesion by means of integrated microfluidics. By means of this device clinicians can convey anti-inflammatory drugs and active molecules that are functional to the therapeutic process in a very targeted way. Unlike systemic therapies, it is proved that the loco-regional therapy, particularly with regard to recovery post implant, is more effective. Scriba has developed the process for fabricating devices with biodegradable material. This is innovative compared with existing products, as devices for perfusion of drugs or infusion with catheters are made of biocompatible but non-biodegradable polymers. The specificity of spinal cord injury justified the opportunity to build the devices completely biodegradable in a few months. The polymer selected is PLGA, used also in neural conduits for the regeneration of peripheral nervous tissue. The manufacturing process is based on technologies already widely used in the production of medical devices, just as the thermoforming and replica molding. Scriba developed the process for the manufacturing of the masters with laser technology, which are then replicated in the biodegradable polymer with the generation of a secondary master in polypropylene. These processes are compatible with the good manufacturing practices.

Scriba then realized the integration of this device on the microfluidic device containing electrodes for stimulation, developed by other partners (CNR and UNIMORE).

Potential areas of application of the device AMID are multiple and extend he application from the specific pathology of spinal cord injury because the stimulation electrodes, together with a microfluidic device of drug delivery is an integrated device, potentially with great impact from the therapeutic point of view. Moreover the microfluidic device in biodegradable material alone can be a useful tool in sensor applications where the appearance of biodegradability becomes particularly important

The whole know-how achieved by the consortium on (i) PLGA processing, (ii) the development and fabrication of bio-resorbable transistors, and (iii) the electrical stimulation of neural stem/progenitor cells has been exploited in the WP7, in order to build a robust integrated prototype, as a platform both for the in-vitro and in-vivo studies.
AMID devices were fabricated for in-vitro and in-vivo experiments with neural stem/progenitor cells and primary cortical neurons. In-vitro, cell culture was maintained more than 5 days on the active area of the device, while performing stimulation and recording of the extracellular activity.
Being almost completely biodegradable, the electrical characteristic of the AMID device are expected to change irreversibly in a period of time larger with respect to the extent of in-vitro study. Effort has been put in developing a fabrication process that keep a good interface between the biodegradable scaffold and gold surface of the electrodes, also in contact with complex medium.
Electrical characterization of small currents generated at the device's active interface demonstrates that the AMID device provides controlled extracellular stimulation to cells grown on top of the active area with ionic currents in the range of a few mA. The stimulation performance was maintained during the in-vitro experiment also in repetitive stimulation cycles. Stable performance was also observed on recording by means of the integrated organic transistors. At a sampling frequency of 1 kHz it is possible to detect extracellular potential variations in the range of 140 μV after five days of cell culturing.
Initial steps of validation have been pursued with implants of the AMID devices on mice subject to trauma on the spinal cord, recording the effect of controlled periodic stimulation after the surgery and during recovery on the hindlimb, or nearby the spinal cord. Results of these tests have great importance to optimize design, improve easy handling during surgery and improve stability of the implant to tearing and grasping of the mouse after recovery.

3.7. Demonstration of the implantable device in contusion SCI animal model

Within this WP we aimed at investigating the effects of device implantation and stimulation *in-vivo* in laboratory animals subjected to spinal cord injury (SCI).

As a first step the consortium developed an Active Multifunctional Implantable Device (AMID) (**Fig. 14A**), with size suitable for *in-vivo* implantation in a mouse model of contusion thoracic SCI.
The AMID is made of PLGA (**Fig. 14A**, in red), with interdigitated gold electrodes for electrical stimulation and recording of signals from the tissue (**Fig. 14A**, in yellow) and microfluidic channels (**Fig. 1A**, in grey) to deliver drugs/chemicals (such as Minocycline) at the site of implantation.
We initially sought to determine and verify its functionality after implantation at the level of the thoracic spinal cord segment of healthy mice. To this aim we applied an increasing range of current intensities to the thoracic/lumbar segment of the spinal cord. Similarly to the *in-vitro* experiments, we initially wanted to determine the threshold of neuronal activation monitored by recording of neural activity from the sciatic nerve. In fact, neurons residing at this level of the spinal cord project their axons to the hind limb muscles trough the sciatic nerve. Therefore, efficient stimulation of lumbar spinal cord neurons should result in neuronal activity (and recorded signal) within the sciatic nerve. We succeeded in generating a response, called electroneurographic (ENG) signal (**Fig. 14B**), in the ipsilateral sciatic nerve, thus demonstrating that the AMID is able to stimulate neurons *in-vivo* in the spinal cord (**Fig. 14C** and **D**), as previously demonstrated on neurons *in-vitro*.

To identify the current intensities needed to stimulate spinal cord neurons after experimental SCI, we induced thoracic contusion SCI in laboratory mice. In an aseptic surgical procedure, under controlled anaesthesia we can, using an impactor system (**Fig. 15A** and **B**), injure the spinal cord with a small rod (connected to a software-controlled piston) dropping onto the exposed spinal cord (T12 vertebral level) (**Fig. 15C-E**). This type of injury (**Fig. 15F**) is very reproducible and mimics in many pathological and behavioural aspects the human pathology (e.g. neuronal damage and degeneration, inflammation, bladder dysfunctions and paralysis, etc.). To minimize animals suffering and distress, mice were provided with analgesics and antibiotics following the surgery similarly to what is done for SCI patients in the hospital. Therefore, at different time points (1, 3 and 7 days) after the injury we implanted the device onto the spinal cord and stimulated at either above or below the injury site. The recorded ENG signals in the sciatic nerve suggest that the current intensity needed change depending on the site of the stimulation.

In particular at 1 and 3 days after injury the current needed above and below the injury were consistent, with higher values above the lesion. At 3 days post-contusion, the current needed to obtain ENG, stimulating above the lesion, was higher than 1 day. At 7 days post-contusion the current able to induce a neuronal response at 3 days was barely able to produce a mild response. Interestingly, the current needed below the injury site to obtain a neuronal response remained stable between 1 and 3 days post-contusion, while increasing dramatically at 7 days post-contusion.

We then stimulated below the injury site at these different time points for an hour (using the very same stimulation paradigm established *in-vitro*), using the very same current intensities derived from previous experiments. We hypothesized that the analysis of the stimulated tissue would reveal the possible activation of mechanisms related to tissue remodelling and neuronal plasticity. After the stimulation, the cord of the mouse was removed and cut into three segments (rostral, lesion epicentre and caudal), which were snap frozen for RNA analyses The corresponding tissue was collected also from the control (non-stimulated) mouse for each time point. We therefore extracted the RNA from the tissues and analysed for the expression of mRNAs relative to *Fos* and *Jun,* which are genes known to rapidly respond to Ca^{2+} variations within the cell (therefore called "early-genes"). It is known in fact that early genes are activated quite fast via the activation of L-type voltage gated channels during neuronal activity. Electrical stimulation boosts their activity, therefore promoting increased activation of early genes. These early genes are then associated with a number of downstream activation mechanisms, among which the up-regulation of regenerative associated genes (RAGs, e.g. *Bdnf* and *TrkB*). These are a family of genes that have been associated with regenerative processes within the injured nervous system. It has been shown that electrical stimulation enhances the expression of *Bdnf* and *TrkB (Al-Majed et al., 2000; Xu et al., 2014)*. Initially, as shown in Fig. 16B, we analysed how the relative expression of the genes analysed changes after injury (at the different time points) when compared to non-injured tissue. No significant differences in the expression of *Fos*, *Jun* or *TrkB* have been observed during this time point. On the opposite, we could observe a clear increase in the expression of the mRNAs encoding for the pro-inflammatory cytokine tumor necrosis factor-α (*Tnf-α*) both at D1 and at D21, while the pro-inflammatory cytokine interleukin-6 (*Il-6*) seemed to be reduced at all the time points analysed (with the exception of D7, which however showed a big variation). Then we compared the expression of the different mRNAs in electrically stimulated (ES) *vs* non-stimulated (nES) mice. As shown in Fig.16C-G, the expression of *Fos* increases in ES mice in all the time points analysed with the exception of D21 (Fig.16C), while *Jun* increases only at D3 and D7 (Fig.16D). On the opposite, the expression of *TrkB* does not seem to increase in ES *vs* nES mice (Fig.16E). As for *Tnf-α,* we could observe a great increase upon stimulation in the healthy mouse (D0) and a trend in its increase in injured animals over time, which, however, terminate at D21, when there is no difference between ES and nES mice (Fig.16F). Finally, current stimulation induces an increase in the expression of *Il-6* at all the time points analyzed (Fig.16G). In the last weeks, also, a new version of the implantable AMID has been generated in collaboration with CNR-UNIMORE. The main difference is relative to the size of the ziff connector and the flat cable. These reduced dimensions will allow a further improvement in the protocol of implantation. As a matter of fact, the flat cable will be retained under the skin of the mouse to finally exit through a 3D printed head connector secured on the skull of the mouse. This will guarantee a higher stability of the implant and less discomfort for the mouse. We will be able, also, to

perform chronic implantation studies combined to longitudinal electrophysiology on awake-freely moving mice.

Spinal cord stimulation at higher current intensities can be used, for example, to generate locomotion patterns (after injury) by providing an input stimulus that is generally generated by the upper brain but is lost after injury. Other researchers have already showed this recently. Alternatively, we believe that a lower current stimulation (sufficient to activate neurons without generating locomotion) might be useful to maintain a certain level of activity below the injury site, therefore reducing neuron degeneration and possibly boosting tissue remodelling (plasticity) and circuit reconnection. It is possible, in fact, that Ca^{2+} uptake within the stimulated neurons could promote regeneration/plasticity *in vivo*, as suggested by our and others *in-vitro* data. Moreover the use of lower currents would generate less heating at the site of stimulation and will also possibly result in less damage to non-neuronal cells populating the spinal cord.
In order to verify this hypothesis we are now looking at the effects of daily stimulations into injured mice. After lesion mice are implanted with the AMID and treated daily (20 Hz, 1h) with the stimulation paradigm established in the previous experiments. Analysis of behavioural outcome, tissue remodelling and electrophysiological data will provide us with a more precise idea about the role of electro-chemical stimulations in promoting spinal cord plasticity.
Although all the results collected so far are very promising, these experiments will open additional questions and new issues. In particular, protocols for optimal stimulation (both electrical and chemical) would need to be optimized prior to translation into clinical applications for humans.

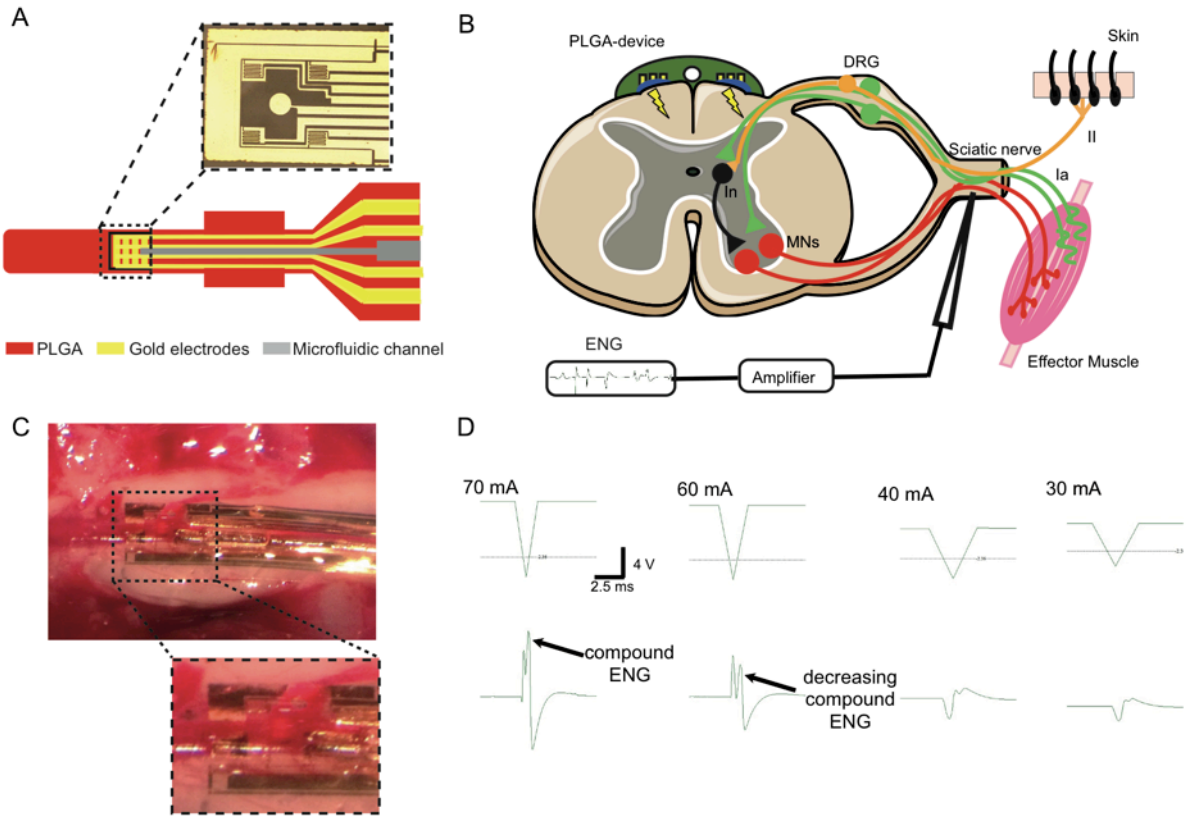

Fig 14. *In vivo* demonstration of device stimulation capacity. (**A**) Schematic showing the design of the AMID: PLGA substrate (in red), gold electrodes (in yellow) and microfluidic channel (in grey). The insert shows a high magnification view of the patterned gold electrodes on the AMID. (**B**) Schematic representation of the acute *in-vivo* electrophysiology experiments. The AMID is placed on the dorsal aspect of the spinal cord (of mice and rats) and electrical stimulation is applied while recording signals from the sciatic nerve (**C**) Representative image showing the implanted AMID on the spinal cord of laboratory animals. (**D**) Representative stimulation (top) and ENG recording (bottom) traces. The black

arrows in **D** highlight the compound ENG signals recorded from the sciatic nerve upon stimulation of the spinal cord with the AMID.

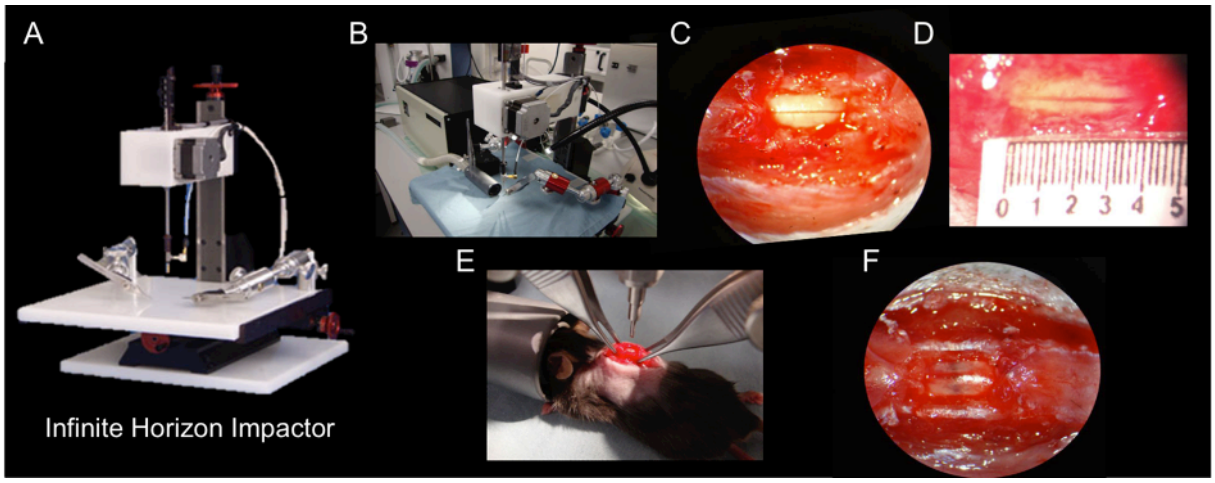

Fig 15. Experimental contusion spinal cord injury is performed on laboratory mice. (**A**) The picture shows the Infinite Horizon Impactor system used to deliver a controlled and reproducible contusion to the spinal cord of laboratory rodents. (**B**) The picture shows the surgical set-up with the impactor and the anesthetic mask to maintain the mouse sedated during surgery. (**C** and **D**) The pictures show the dorsal aspect of the spinal cord, exposed after laminectomy at the T12 vertebral level. At maximum the length of the laminectomy is 4mm. (**E**) The picture shows a mouse fixed on the impactor stage after laminectomy. A fine tip (attached to a piston) is then placed in proximity of the cord and contusion performed by the software controlled piston. (**F**) The picture shows the spinal cord after injury. Bruises are clearly visible immediately after contusion.

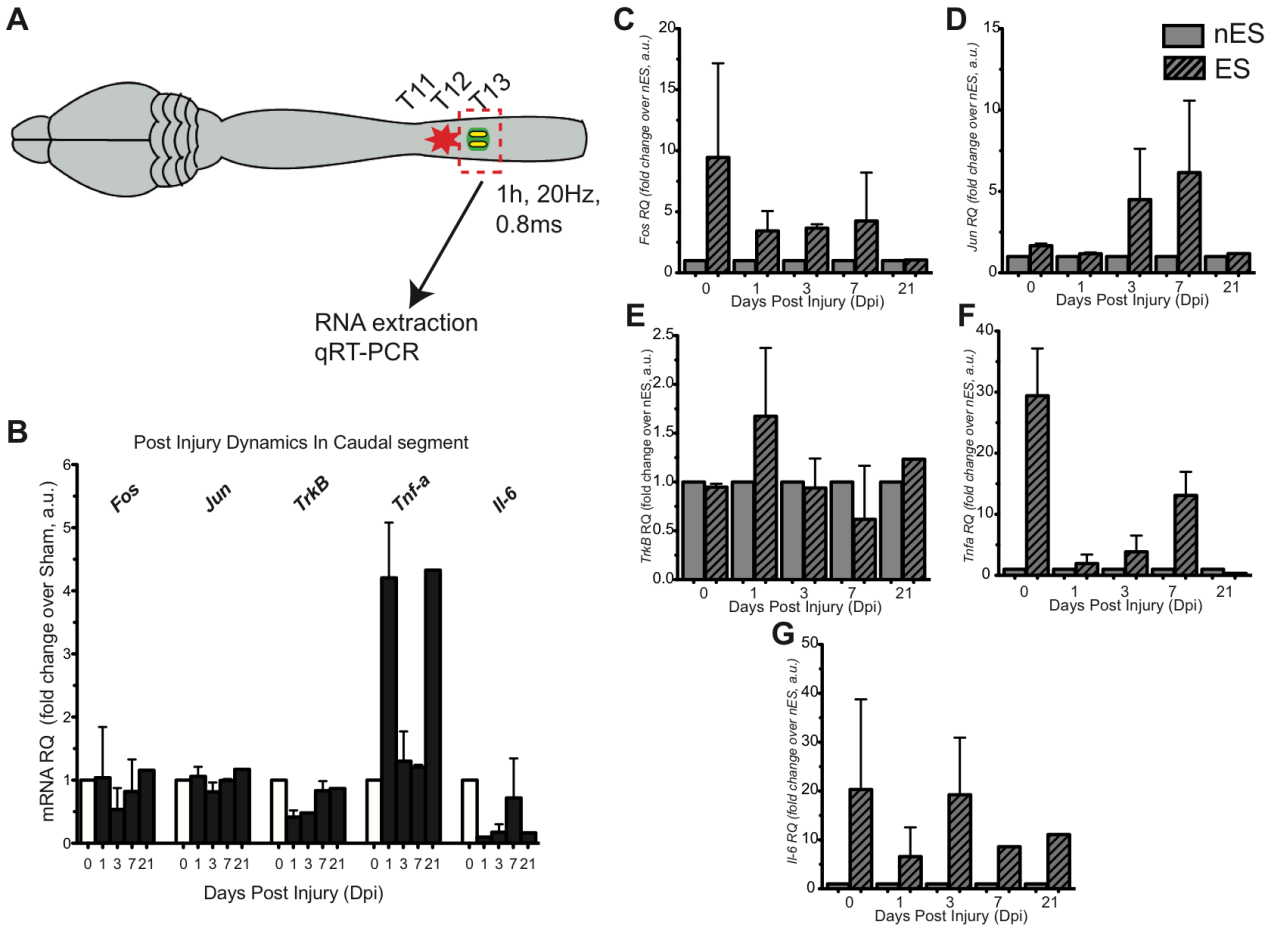

Fig. 16 Acute effect of ES paradigm onto the spinal segment below the injury site. 20 mice have undergone surgery, 16 of them received a 70 Kdyne contusion injury and 4 received only a laminectomy (sham operated). At 0, 1, 3, 7 and 21 DPI 2 mice were used for electrophysiological experiments. 1 of these mice was also subjected to ES paradigm (1h current stimulation) at the spinal segment below the injury site (A). At the end of the 1h stimulation RNA was extracted from 3 segments: T11 (Rostral), T12 (injury epicenter) and T13 (Caudal) and qRT-PCR performed. (B) The graph shows the dynamic changes of the mRNA analyzed (*Fos, Jun, TrkB, Tnf-a* and *Il-6*) in the caudal segment of non-ES mice. RQ is expressed over sham-operated mice. (C-G) The graph shows the expression levels of the mRNA when comparing ES *vs* non-ES segments at different time points. Data are expressed as mean (N=2) fold change over controls.

3.8. Safety, regulation and ethical issues

Objective of this work package was to provide a framework to safely and ethically design a medical device according to the EC/FDA regulations and ISO 13485:2003.

Safety aspects, regulations of medical device and ethical issues were considered from the very beginning of the project – so it has been ensured that basic questions of safety and ethics were solved from the beginning and through all the period of the project.

Deliverable D9.1. Design history file and registration report according the EC directives has been prepared in month 6 by former WP leader Polyganics. This deliverable gives an overview about the requirements and a guideline to follow while developing a medical device. In this deliverable there it was concluded that product resulting from this project is classified as medical device class III – an active implantable medical device with direct contact with central nervous system – it means the group of

medical devices with the highest potential risk for the patient. On the other hand when evaluating medical devices the ratio risk: benefits has to be always taken into account and this medical device means really great benefits for improving the quality of patient's life.

Countries have different regulations for classification and registration of medical devices – but ISO 13485 defines several phases of the design and development process, which should be documented. Most of registration or certification procedures accept documentation based on so called Design History File, which is actual file or list of all relevant documents, which had been prepared during the development.

That is why also during this project DHF list has been prepared. The list includes names of the documents, dates of creation, authors, location of their storage and contacts for the case – the document will be needed for creation of certification/registration documentation in the future.

It is also important to instruct continuously all project team members, which rules of the medical devices development process should be followed. Therefore, within this WP training sessions on such topics as medical devices certification procedures in EU and US have been held. During regular meetings presentations methods and principles of certification and marketing of a product were explained. All participants were provided with information how to ensure safety and efficacy of the product according to standards and legislative requirements, which must be followed. All team members were provided with references to those standards, regulations and directives, but also a several guidelines (MEDDEV) and public articles and discussions related to putting the medical device class III on the market.

During the period of this project some proposals on legislative changes have arisen. On September 26th, 2012 Regulation on medical devices, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 has been proposed. Main elements of the proposals include: stronger EU supervision of independent notified bodies, the position of notified bodies against manufacturers should be strengthened, for high risk medical devices; authorities could scrutinize the preliminary assessment conducted by notified bodies, stricter requirements for clinical evidence, to ensure patient and consumer safety, Unique Device Identification system and other changes. This change means stricter requirements and registration procedures mainly for products, which: include medicinal product as an integral part, include human cells, include nanomaterials, include electromagnetic effects and are active implantable devices – so this change will definitely affect this project product – that is why it was necessary to monitor the activities of European authorities and the process of approving of the new regulation.

Deliverable D9.2. Report on Ethical issues according to 99/167/EC: Council Decision of 25/1/99. has been prepared in month 6 by University of Cambridge, which is participant responsible for animal testing performed during this project. The project has involved the use of mouse cells (NPCs and peripheral mononuclear cells) and in vitro assays, as well as in vivo pre-clinical tests. Using these types of cell assays pose the question if testing can be ethically approved. The EU has drafted regulations that give guidelines with respect to this question, the so called "3 Rs" policy (99/167/EC: Council Decision of 25/1/99), of Reduction (of the number of animals), Replacement (with non-animal methods) and Refinement (of methods that alleviate or minimize the potential pain, suffering or distress; while enhancing animal welfare). In the deliverable the situation in UK has been described: each person who undertakes work under the Act must hold a personal license (PIL) which lists the techniques and species they may use and the establishment(s) at which they may work. At the same time, every project must be authorized in a project license (PLL), which is granted by the Secretary of State to the person who has overall responsibility for the program of work specified in the license. The place where regulated procedures are carried out is controlled by granting a certificate of designation (PCD) to a senior authority figure at the establishment, unless the nature of the work requires it to be carried out in other places, such as farms or fields. Also the main concepts of reduction, replacement and refinement are described and considered in the case of this concrete project. Concrete procedures of tests performed in animals are described in detail with focus on principles of 3Rs.

It has been concluded that UCAM fulfills all the rules and have all valid licenses allowing them to performed planned tests:
Following to the successful amendment applied to the PPL 80/2457 including all the requirements necessary to conduct the experiments described in iONE-FP7, Dr. Pluchino's lab has been fully allowed to proceed with the experiments described in the project. However most of the studies has been conducted *in vitro*.

4. The potential impact

Exploitation activities
It is widely accepted the focused medical target and selected technologies are not yet ready for full exploitation, however possible roadmap have been outlined. A joint patent between UNIMORE, CNR, SCRIBA and UNIBO on device design and manufacturing principles is under preparation.

Dissemination activities
Project partners present often at the top scientific conferences and publish their results in scientific articles in high impacted journals. The project generated over 20 scientific articles and it was presented in over 50 scientific events and conferences.

Several dissemination materials were prepared at the beginning of the project (leaflet, Roll-up, mock-up of iONE-FP7 device). Roll up with essential information about the project was produced and is available for any project partner purposes.

The project objectives and recent results were presented during ENF 2013 (EuroNanoForum 2013). The project exhibition stand did not offer only information on the project itself, but also gave an opportunity to see the first demonstrations of its outputs (e.g. mock up of the active implantable device for human therapy, transistor on biocompatible and on biodegradable substrate, microfluidic channels, scientific papers and posters). More than 40 conference participants that showed their huge interest in the presented concept and prepared samples, visited the stand. The stand was also visited by delegation of members of the NMP Programme Committee and by several World Class scientists (e.g. Prof. Dr. Klaus Müllen from MPI).

iONE-FP7 co-organized a one-day workshop on Implantable Organic Electronics on Monday 9th June 2014, which started the week full of events associated to the 10th International Conference on Organic Electronics (ICOE). Speakers like F. Biscarini (UNIMORE), G. Malliaras (EMSE), M. Berggren (LiU) , T. Someya (Tokio), R. Antognazza (PoliMI), T. Cramer (CNR), P. Bergonzo (Saclay), D. Simon (LiU), P. Greco (Scriba Nanotecnologie), S. Lacour (EPFL), R. Garcia (CSIC) and F. Zerbetto (UNIBO) presented their recent work in this field. The workshop has been organized in a beautiful lecture room of Accademia Nazionale di Scienze, Lettere e Arti in Modena and attracted over 30 participants out of 170 ICOE participants. The summary of this workshop was made available at the iONE-FP7 project website.

The video about iONE-FP7 will be recorded during the final meeting and will be distributed before the end of the project.

Last but not least, a Linkedin group on Implantable Organic Electronics was created, which unifies principal investigators, but mainly younger scientists. The group has more than 120 members, over 40 main contributions and it started to attract people from outside of the consortium as well.

https://www.linkedin.com/groups/Implantable-Organic-Electronics-4865869/about

5. The address of the project public website

http://www.ione-fp7.eu

Main contacts:

Project coordinator
Prof. Fabio BISCARINI
CNR-ISMN Bologna (Italy)
f.biscarini(at)bo.ismn.cnr.it
+39 051 6398522

Project manager
Rudolf FRYCEK, PhD.
AMIRES Sàrl (Switzerland)
frycek(at)amires.eu
+41 796 784 295