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New trends and challenges in the development of microfabricated probes for recording and stimulating of excitable cells

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I. Methods for the Recording of Electrical Signals from Cells in vitro and in vivo

1. Methods for the Recording of Electrical Activity from Cells In Vitro

1.1 Introduction

Excitable cells such as nerve cells communicate via signals transferred under the form of electrical potentials, the so-called action potentials. The communication is transmitted from one cell to another via numerous interconnections called synapses. This communication is critical for the life of higher organisms. Electrical activity of these cells can be studied using primary cell cultures, immortalized cell lines and acute slice preparations that are mostly brought in contact with a surface for adhesion or growth promotion. The study of single or groups of cells in these preparations is called 'in vitro' research. The study of the conduction of this electrical activity 'in vitro' and its impairment is of great importance in the development of new therapies for various neurological disorders such as Alzheimer's and Parkinson's disease, and epilepsia. Methods for studying action potentials can be conducted outside (extracellular recordings) or inside the cell (intracellular recordings).

The recording of the intracellular membrane potential requires either impaling the cell membrane with a sharp glass micro-electrode or establishing electrical access to the cell with a glass patch pipette. Extracellular recordings use either fixed or movable glass or insulated metal/metaloxide electrodes, positioned on the outside of the cell. In the following, a brief historical overview of the development of these techniques will be given, and new techniques based on micro-fabrication that gained a growing attention recently will be discussed.

The recording of the intracellular membrane potential provides the most precise description of the electrical behavior of a cell and, therefore, it requires specialized techniques. The use of sharp glass micro-electrodes for intracellular recordings is a challenging method and mostly limited to recordings in large cells from invertebrates. By impaling the cell with the sharp tip of the glass pipette, holding a Ag/AgCl electrode connected to a voltage follower, changes in the intracellular membrane potential can be measured. In addition, the pipette is usually filled with a high concentrated salt solution (KCl) to decrease the electrical resistance. The first studies on action potentials were performed on neurons of invertebrates using these intracellular glass micro-electrodes (Hodgkin (1939)).

1.2 Extracellular Recording

Although intracellular recordings provide the measurement of the intracellular potential of a cell, they are in any case invasive to the cell and its membrane. These recordings are, therefore, always limited in time. This rules out some investigations of important communication processes, such as late phase long-term potentiation (LTP) recordings. Potential changes of the membrane of a cell can also be measured from the outside of the membrane without making any physical contact to the cell. Glass micro-electrodes or thin, insulated metal electrodes can be used for extracellular recording of the membrane potential. Ionic movements across cell membranes are detected by placing a recording electrode close to the cell. The extracellular signal recorded upon the firing of an action potential is characterized by a brief, alternating voltage between the recording electrode and a ground electrode. Extracellular recordings with a glass electrode are thus advantageous in the investigation of long-term processes, such as LTP. Because the electrode is in close proximity but not in direct contact with the cell, the recordings are usually stable and not liable to mechanical instabilities. Although activity can be detected at the level of a single cell, recordings usually reflect the averaged response of a population of cells.

Despite the non-invasiveness of this method, the throughput of this type of experiments is rather low. The researcher has to manually bring the electrodes close to the cell membrane to be able to perform the recordings. With the progress in micro-fabrication techniques, planar micro-electrodes were developed that were able to record extracellularly from cultured cells grown on top of the electrode area. Planar micro-electrodes have been used as substrates for the culture support and non-invasive recording of cells, and electrical activity of single cells and networks of cells have been monitored successfully. In 1972, Thomas *et al.* described the first attempt to record electrical activity from cultured cells using a micro-electrode array (MEA) (Thomas et al. (1972)). They used gold-plated nickel electrodes on a glass substrate passivated with patterned photoresist. Embryonic chick heart cells were cultured in a glass chamber. Electrical activity was recorded extracellularly from the contracting heart cells simultaneously from many electrodes. Gross *et al.* used a similar system to record extracellular electrical responses from explanted neural tissue from the snail *Helix pomatia* (Gross et al. (1977)). Pine *et al.* were the first to report electrical recordings of dissociated neurons (superior cervical ganglia of neonatal rats) (Pine (1980)). Moreover, they combined the traditional method of intracellular recording using a glass micro-pipette with extracellular recording using a metal micro-electrode. Combining both techniques enables validation and calibration of the extracellular micro-electrode recording with the vast amount of information from intracellular recordings. These successes led to many groups using planar micro-electrodes for cultured cells (Droge et al. (1986); Eggers et al. (1990); Gross (1979); Gross et al. (1977); Martinoia et al. (1993); Novak & Wheeler (1986); Pine (1980); Thomas et al. (1972)).

The simultaneous stimulation and recording of cells is a logical next step and several researchers have already succeeded in stimulation and recording of embryonic chick myocytes cultured on planar micro-electrode arrays (Connolly et al. (1990); Israel et al. (1984)). In 1992, Jimbo and Kawana further expanded the possibilities with these systems by stimulation of neurites that were guided by micro-channels (Jimbo & Kawana (1992)). The same group later reported the simultaneous recording of electrical activity and intracellular $[Ca^{2+}]$ using fluorescent dyes, showing the combination of optical and electrical techniques (Jimbo et al. (1993)).

1.3 Active Multitransistor Arrays

Although micro-electrode arrays are of growing interest in electrophysiological and pharmacological research, there are still shortcomings in these devices. The most abundant disadvantages are the low signal quality and the small amount of electrodes on the chip, which are both technological aspects. Most micro-electrode arrays are passive arrays that only amplify the signal once it is led through wires connecting the electrodes. The capacitive load that is introduced in this way attenuates the signal significantly. The small amount of electrodes is based on the used micro-fabrication technology used in these systems. Technological improvements over the years, however, made it possible to address these shortcomings. In 1991, Fromherz *et al.* reported recordings of extracellular field potentials from Retzius cells from the leech *Hirundo medicinalis* measured by an integrated transistor (Fromherz et al. (1991)). Here, the neuron was directly coupled to the gate of a field effect transistor that consisted of silicon dioxide. The validation of the measured potentials was performed by injecting a microelectrode, which both stimulated the cell and monitored the intracellular voltage. Fromherz *et al.* used this system to further investigate the physics behind the coupling of the neuron and the transistor using an array of transistors below the neuron, as well as the capacitive stimulation of the neuron through the oxide layer (Fromherz et al. (1993); Fromherz & Stett (1995)). Recently, the same group showed the possibility of capacitive stimulation of specific ion channels using field effect transistors and recombinant HEK293 cells (Kupper et al. (2002)). In general, dense arrays of transistors, called multi-transistor arrays, have been used in increasing frequency for the recording of electrical activity from different cell types (Ingebrandt et al. (2001); Kind et al. (2002); Lorenzelli et al. (2003); Martinoia & Massobrio (2004); Martinoia et al. (2001); Meyburg et al. (2006)).

1.3.1 Cell-Chip Coupling

While these sensors show high signal-to-noise ratios, integration of read-out functionality and the possibility for downscaling, which makes these systems superior to passive MEA systems, this technology is still in an experimental phase and, therefore, very expensive. Furthermore, another crucial design and fabrication problem is the need for a biocompatible system. Most materials that are typically used in integrated circuitries are not optimized for use in liquids and with cultured cells. Both MEAs and arrays of FETs have been mostly used for recording from acute slices and large cells from invertebrates. Although some examples of extracellular recordings of mammalian cells have been demonstrated, single-cell addressability of small, mammalian cells remains challenging.

1.3.2 Recent Advances in Multitransistor Arrays

The electrical coupling between the cell membrane and the chip is mainly based on the contact between the lipid bilayer and the surface of the chip and the most important factor responsible for signal strength attenuation. Parameters that influence the cell-chip coupling are the distance between cell membrane and electrode and the electrical resistance that exists in this gap. The distance between the cell membrane and the surface was characterized extensively by Braun *et al.*, using fluorescent dye molecules to stain the membrane on silicon chips with microscopic oxide terraces (Braun & Fromherz (2004)). Using HEK293 cells on chips coated with fibronectin, the measured distance was $\sim$70 nm, independent of the electrical resistivity of the bath (Gleixner & Fromherz (2006)). Later, the same group used fluorescence interference contrast microscopy to calculate the distance between the cell membrane and chip surface. The separation between membrane and surface is caused by proteins in the membrane (glycoca-

lyx) and the surface coating on the chip. This gap could be narrowed down to 20 nm, when snail neurons were used on a laminin fragment that was anchored to the surface (Schoen & Fromherz (2007)). The electrical coupling between the chip surface and the cell depends on the electrical resistance of this thin layer between the oxide and the lipid bilayer. Fromherz *et al.* used a technique with alternating voltages applied to the chip to map this electrical resistance. The resistance and the capacitances of the surface (metal oxide) and membrane determine the voltage across the attached membrane. In normal culture medium, the sheet resistance was determined to be $\sim$10 MΩ. When the gap was 20 nm, the estimated resistance was $\sim$1.5 GΩ. The conclusion of these experiments was that the space between the cell membrane and the chip surface which was filled with cell medium, created a conductive sheet that prevented an effective interaction by direct electrical polarization. The resistance that exists in this gap is often referred to as the seal resistance (R_{seal}). One of the most important challenges in MEA recording is increasing the value of this seal resistance.
To enhance the signal-to-noise ratio when recording with MEAs, attempts have been made to hold or guide the cells. For example, if the cell can be positioned precisely on top of the sensor, the distance between the cell and the sensor surface can be decreased. Lind *et al.* proved this after performing a finite element analysis model of the extracellular action potential, whereby cells surrounded by extracellular fluid were compared with cells in grooves and cubic pits. The signal could be improved by as much as 700% when the extracellular space was confined by the external structures (Lind et al. (1991)). These modeling results were later confirmed by recordings of neurons from the snail *Lymnaea stagnalis*, cultured in a 10 μm wide, 1 μm deep groove (Breckenridge et al. (1995)).
In the Bioelectronic Systems Group of the Interuniversity Micro-Electronics Center (IMEC) in Leuven, Belgium, a multidisciplinary research team works towards the fabrication of micro-structured electrode arrays with three-dimensional electrodes. The concept lays in the fact that if the electrodes are small enough, the cell membrane will engulf the electrodes, creating a strong interaction between the membrane and the electrode surface. This would eventually lead to a stronger electrical coupling because of a higher electrical resistance in the gap between cell and chip. Preliminary data suggest strong engulfment of the electrode by the cell membrane, as could be observed by immunohistochemical actin filament staining and focused ion beam scanning electron microscopy (Figure 1)(Braeken et al. (2008); Huys et al. (2008); Van Meerbergen et al. (2008)). Moreover, the electrodes are spaced very close to each other, which allows for single cell recording and stimulation. However, this feature is highly dependent on the technology level that is used.

2. Methods for Recording of Electrical Activity from Cells In Vivo

2.1 Introduction

Understanding of the neural codes and the development of brain-computer interfaces for the normal and injured nervous system would require simultaneous selective recording and stimulation at multiple locations along the sensory-motor circuits. At present, there are several technological platforms that are capable of scaling to such recording and stimulation modalities.
The probes designed for deep brain recording need to penetrate the soft meninges and the underlying brain matter. Therefore, most of the designs implement either sharp tips or specialized add-ons for insertion. On the other hand, probes for surface recording, such as the surface arrays and the cuff electrodes, are flexible and are designed to adapt to the surface of the brain sulci or the nerves, respectively.

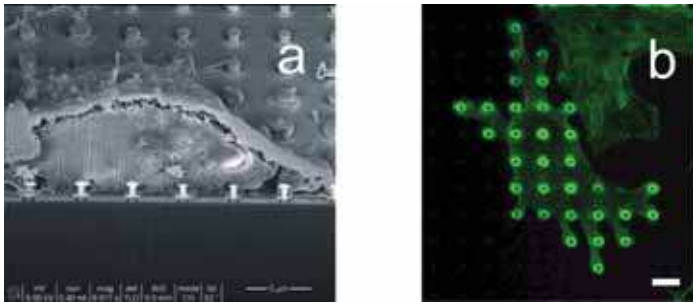

Fig. 1. Micro-structured electrode arrays developed in IMEC, Belgium. a) Focused ion beam scanning micrograph of a neuroblastoma cell on a nail bed. b) Actin filament staining of a single cardiomyocyte on a nail bed. Scale bar is 3 μm.

2.2 Silicone-based Probes

The first silicon-based electrode arrays for 'in vivo' recording were developed by Wise, Starr, and Angell in 1970 (Wise & Angell (1975); Wise et al. (1970)). They introduced the use of *integrated circuit* (IC) technology to develop micro-electrodes.

2.2.1 The Michigan probe

BeMent et al. (1986) reported for the first time the development of a micro-fabricated micro-electrode array from silicon having many recording contacts. These probes have evolved into the devices commonly known as the Michigan probes. Michigan probes are supported and distributed by the Center for Neural Communication Technology (CNCT) since 1994. There are multiple designs already disseminated through CNCT. Some of them are commercially available from the company NeuroNexus Technology.

The Michigan probes are based on a silicon substrate, the thickness and shape of which are precisely defined using boron etch-stop micro-machining. The substrate supports an array of conductors that are insulated by thin-film dielectrics. Openings through the upper dielectrics are inlaid with metal to form the electrode sites for contact with the tissue, and the bond pads for connection to the external world. The Michigan probe has also been modified for 3D configuration. Arrays of planar, comb-like multi-shank structures have been assembled into 3D arrays. Such three-dimensional structures can be constructed from the two-dimensional components using micro-assembly techniques. The procedure is based on inserting multiple two-dimensional probes into a silicon micro-machined platform that is intended to lay on the cortical surface. The Michigan probe process is compatible with the inclusion of on-chip CMOS (complementary metal-oxide semiconductor) circuitry for signal conditioning and multiplexing. Such active arrays were also validated in neural recording experiments. Bai & Wise (2001) reported the fabrication of "active" electrodes with monolithically integrated CMOS circuitry. High density probes for massively parallel recording, with on-chip preamplifiers to remove movement-related artifacts and reduce the weight of the headgear for small animals, were used to record simultaneously from the soma and dendrites of the same neurons (Csicsvari et al. (2003)).

2.2.2 The Utah Electrode Array

The group of Dr. Richard Normann at the University of Utah, USA, developed a microelectrode array referred to as the Utah Electrode Array. The Utah Array has a matrix of densely-packed penetrating shafts, which are between 1 and 1.5 mm long and project from a very thin (200 μm) glass/silicon composite substrate and are separated from each other by 400 μm. The device is formed from a monocrystalline block of silicon using a diamond dicing saw and chemical sharpening (Nordhausen et al. (1996)). It provides a multichannel interface with the cortex. The resulting silicon shafts are electrically isolated from one another with a glass frit and from the surrounding tissue with deposited polyimide or silicon nitride. The tip-most 50 to 100 μm of each shaft is coated with platinum to form the recording contact. Interconnection to the electrode sites is accomplished by bonding either individual, insulated 25 μm-thick wires or a polyimide ribbon cable having many individual leads to bond pads on the top of the array. The Utah array was originally designed with the goal to serve as an interface for a human cortical visual prosthesis (Branner & Normann (2000)). Performed experiments demonstrated numerous issues in favor of such an approach. Nevertheless, the device turned to be a successful research tool in animal experimentation. For example, it was used for acute and chronic recordings in the cat cortex (Maynard et al. (1997); Rousche & Normann (1998)). A modified design was also tested in cat peripheral nerve (Branner & Normann (2000); Branner et al. (2001)). The design of the Utah array was used for human motor cortical prosthesis spun-off in the company Cyberkinetics. There is an ongoing clinical trial authorized by FDA in five severely disabled patients to determine the usability of the technology (Hochberg et al. (2006)).

2.3 European designs

In Europe, there are co-ordinated efforts to build integrated probes for recording, stimulation and local drug delivery. Among the leading centers are IMTEK in Germany, Twente University in the Netherlands, IMEC in Belgium and EPFL in Switzerland. The devices are based on silicon micro-technology and are compatible with a CMOS process.

Several types of multi-electrode probes have been recently designed and fabricated at IMEC. Musa et al. (2008) reported the fabrication of single-shank passive probes for cortical recording. The probe implements a planar array of electrode contacts of varying sizes (4, 10, 25 and 50 μm). In some configurations, an additional larger reference electrode is placed close to the electrode array. Another probe design contains crescent-shaped electrodes. Two of the configurations are shown in Figure 2.

The devices produced in collaboration by IMTEK and IMEC[1] are based on the principle of modular assembly. The probes consist of needle-like structures made of silicon realized using deep reactive ion etching (Ruther et al. (2008)). The first generation devices come as single-shaft probes available in two lengths: 4 mm and 8 mm, each with cross-sections of 120 x 100 μm. In both cases, the probes have a row of nine equidistantly spaced, planar electrodes. The second generation of devices comprises comb-like rows of four probes, each of them with the same dimensions and number of electrodes as in the first generation. This two-dimensional array can be provided with a guide wire or with a thumbtack structure for insertion purposes. Another version of the device contains two such rows assembled in a back-to-back fashion.

Norlin et al. (2002) demonstrated the manufacture of a probe with 32 recording sites[2]. The silicon probes consist of 8 shafts with a minimal cross-section of 20 μm x 20 μm. The shafts

[1] part of the Neuroprobes research consortium
[2] part of the VSAMUEL research consortium

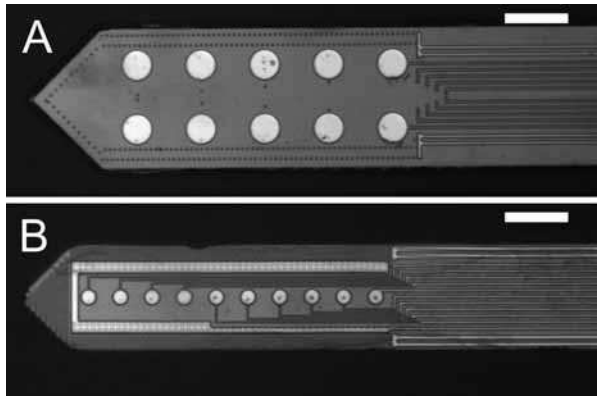

Fig. 2. First generation IMEC recording and stimulation probes

A – NP50 configuration. The probe contains 10 disk electrode sites arranged in a square lattice with diameters of 50 μm. The spacing between contacts is 100 μm. The tip angle is 90°. B – NP25 configuration. The probe contains a linear array of 10 disk electrode sites with diameters of 25 μm. The spacing between contacts is 50 μm. The tip angle is 90°. The shafts are long 2 mm; the cross-section is 200 x 200 μm. The active interface is realized from **Pt**. The probes are insulated with *Parylene C*. The fabrication approach is fully scalable. The fabrication process can be easily adapted to produce longer probes. Scale bars are 100 μm.

taper to very sharp tips (4°). Each of the shafts carries four **Ir** micro-electrodes (10 μm x 10 μm) as recording sites on their side.
Rutten et al. (1995) reported the fabrication of a 3D needle array with 128 recording sites on one electrode placed on the tip of a needle intended to serve as interface to peripheral nerves. The different lengths of the needles allowed selective stimulation of different volumes in peripheral nerves.

2.4 Non silicon-based hard substrates

Silicon-on-Insulator SOI electrodes can be also produced using silicon-on-insulator (SOI) technology (Cheung (2007)). SOI wafers use an insulating oxide layer to separate a thin silicon device layer (1 to 100 μm) from the thick silicon of the backside (about 500 μm thick). The SOI wafer gives excellent control over the final probe thickness. The buried oxide acts as an etch stop during a backside deep RIE of the silicon wafer. The same group presented SOI-based probes with integrated microfluidic channels, which permitted localized injections of chemical substances of very small volumes.

Ceramic-based The insulator ceramic (alumina, Al_2O_3) has been used as a substrate to reduce crosstalk between adjacent connecting lines (Burmeister & Gerhardt (2001); Burmeister et al. (2000)). Ceramic is a mechanically strong material which allows for development of micro-electrodes that can access much deeper brain structures (up to 5 – 6 cm versus 2 – 4 mm for silicon). Precise placement of the micro-electrode in tissue without flexing or breaking can be achieved. Individual devices have to be cut from the wafer either by a diamond saw or by a laser. Numerous four- and five-site platinum micro-electrodes

on ceramic substrates have been developed. Some designs are used for electrochemical measurements of neurotransmitters (Barbosa et al. (2008); Pomerleau et al. (2003)).

One of the very attractive features of the planar photo-engraved probes is the ability to customize design for specific experiments. The substrate can have any two-dimensional shape with single or multiple shanks, electrode sites can be of any surface area and can be placed anywhere along the shank(s) at any spacing, tips can be made very sharp or blunt, and features such as holes or channels can also be included.

2.5 Flexible substrates

The fabrication processes of flexible probes so far employed polyimide, parylene (DuPont) and benzocyclobutene as substrate materials. Polyimide films have been also used as top insulators for cortical micro-electrodes. Micro-electrodes less than 20 μm thick have been constructed with the use of parylene (Rousche et al. (2001)).

Polyimide probes have also been seeded with bioactive molecules such as neural growth factor (NGF) near the recording sites (Rousche et al. (2001)) with the idea to encourage neurite growth toward the active interface and to improve the stability in time (Metz et al. (2001)).

Benzocyclobutene can be used as an alternative to polyimide in the fabrication of neural interfaces. For example, Lee, He & Wang (2004) reported the fabrication of benzocyclobutene coated neural implants with embedded microfluidic channels (Lee, He & Wang (2004); Lee, Clement, Massia & Kim (2004)).

An important development direction in Europe is the development of flexible electrodes for cortical (Myllymaa et al. (2009); Rubehn et al. (2009)) and peripheral nerve recording (Navarro et al. (2001); Stieglitz & Meyer (1999)). Developed electrodes have been based on polyimide as carrier material.

Polymer-based implants using polyimide as both the structural and insulation material have been micro-machined with multilayer metallization for both acute and chronic nerve recording. Hybrid polyimide cuff electrodes embedded in silicone guidance channels have been fabricated for electrical stimulation of peripheral nerves (Stieglitz et al. (2005)). Polyimide sieve electrodes have been used in the regeneration and functional re-innervation of sensory and motor nerve fibers (Rodríguez et al. (2000)).

Rubehn et al. (2009) reported the fabrication of a micro-machined 252-channel ECoG (electrocorticogram)-electrode array made of a thin polyimide foil substrate enclosing sputtered platinum electrode sites and conductor paths. The array was designed to chronically interface the visual cortex of the macaque.

3. Commercialized Micro-electrode Arrays

Only recently, micro-electrode arrays are being increasingly used. However, valuable research was performed much earlier. The reason for earlier commercialization were the limitations of the computing technology available at that time. Because of their recent accessibility and affordability, interest in MEA systems has been renewed. Indeed, multi-electrode recordings accelerate the collection of sample sizes needed for valuable statistical analyses in drug screening assays. Today, MEAs suitable for routine electrophysiological recordings to monitor the activity of neuronal and cardiac populations *in vitro* are commercially available. Well-known manufacturers of these systems include Multichannel Systems, AlphaMed, Ayanda Biosystems and BioCell-Interface.

Most of the clinical neuronal probes used at present are fabricated by Medtronic. The Michigan probe was commercialized in the company NeuroNexus Technology.

4. Biomedical Applications of Micro-fabricated Arrays

4.1 Planar Micro-Electrode Array Systems

Micro-electrode arrays are fastly gaining interest as research instruments for the investigation of various disorders and diseases or the study of fundamental communication processes. Because they do not require highly trained personnel, various tissue preparations can be applied to the electrode surfaces, including acute slices of brain, retina and heart and primary dissociated cell cultures of different regions of the heart and central nervous system.

The biomedical applications are related to these preparations and can be classified into two categories: neuronal and cardiac. Neuronal electrophysiological research with micro-electrode arrays is conducted in various domains of neuroscience, for example long term potentiation from acute slice preparations (Dimoka, Courellis, Gholmieh, Marmarelis & Berger (2008); Dimoka, Courellis, Marmarelis & Berger (2008)) or organotypic slice cultures (Cater et al. (2007); Haustein et al. (2008)), electroretinograms (Wilms & Eckhorn (2005)) and microERGs (Rosolen et al. (2008; 2002)) and recordings from cortical, hippocampal or striatal primary cell cultures for various studies including network plasticity (Chiappalone et al. (2008); Wagenaar et al. (2006)) and memory processing and network activity (Baruchi & Ben-Jacob (2007); Pasquale et al. (2008)). Micro-electrode arrays are also widely used to study electrophysiological properties of the heart, such as gap junction functionality and impulse conduction (Reisner et al. (2008)), arrhythmias (Ocorr et al. (2007)) and experimental stem-cell derived cardiac research (Gepstein (2008); Mauritz et al. (2008)).

4.2 Neuroprosthetic and Neuromodulatory Applications

4.2.1 Development of Neuroprosthetic applications

The development of neural prostheses was influenced to a great extent by the successful clinical application of the cardiac pacemakers (review in Prodanov et al. (2003)).

In the 1970s, after two decades of continuous technological development, the pacemakers were adopted on a great scale in clinical practice. Similar was the case of the *respiratory pacemakers*, which were developed in parallel for patients suffered from cervical spinal cord injury. Stimulation of the phrenic nerves causes constriction of the diaphragm and inspiration. The first attempts to pace the diaphragm with implanted electrodes were carried out in 1948 – 1950 by Sarnoff et al. (1950). One of the most important prerequisites for the clinical acceptance of this technique was the introduction of the long-term electrical stimulation by the radio-frequency inductive method around the end of the 1950s (Glenn et al. (1964)). The first commercial phrenic nerve pacers were introduced in the early 1980s. *Restoration of hearing* was successfully introduced in the late 1950s based on the previous observations of Gersuni & Volokhov (1937). The proof of principle was demonstrated in the intraoperative experiments of Djourno & Eyries (1957) who stimulated the inner ear by an implanted electrode coupled inductively to an outside coil that was in turn connected to a microphone. The actual usefulness of the first experimental device was very limited since the patient could recognize only few words from the transmitted signal (*papa*, *maman*, and *allo*). The indications and contraindications for this implantation were elaborated in a broad debate between the clinicians and the pioneers of the cochlear prostheses. The general approval of the cochlear prostheses was given by FDA in 1984 after 20 years of design and trials. Over the past 20 years of clinical experience, more than 20 000 people worldwide have received cochlear implants. Cochlear implantation has a profound impact on hearing and speech perception in postlingually deafened adults. Most individuals demonstrate significantly enhanced speech reading capabilities during daily life. To restore the lost functions of the paralyzed leg muscles, experiments were

performed for the first time in 1961 by Liberson et al. (1961). The system was developed to compensate for the "drop foot" problem in hemiplegic stroke patients. The "drop foot" stimulation systems activate the nerve fibers in the peroneal nerve with the net effect of flexion in the tarsal joint.

From the presented cases, it is apparent that the successfully applied neural prostheses so-far have been developed for systems, which have either uniform topographic mapping, such as the phrenic or peroneal nerves, and/or inherent ability to learn the stimulation pattern - for example the auditory prostheses.

In contrast, in other sensory and motor systems so-derived principles apply to a limited extent and the performance of the neural prostheses is lower. For example, the usefulness of the *motor neural prostheses* is still insufficient for general clinical use. Motor tasks require orchestrated activation of many muscles, which in turn requires selective stimulation of only defined parts of the nerves or muscle groups. Existing leg and hand neuroprostheses are still far from providing such level of functional selectivity without extensive surgery. Steps towards improving the expected selectivity of stimulation were made by investigation of the topographic mapping of some peripheral nerves and spinal roots in rats (Prodanov (2006); Prodanov & Feirabend (2007; 2008); Prodanov et al. (2007)). However, those results still need to be translated to men. Other examples are some of the *hand prostheses and orthoses*. Most of the proposed implantable systems require extensive surgery in order to interface the hand nerves at several locations to improve selectivity. The surface stimulation systems need to combine several stimulation channels to provide an acceptable level of selectivity. The neuroprostheses have demonstrated improvement of the grasping function in clinical trials including stroke or spinal cord injury subjects. However, the grasp strategies that can be provided with the existing neuroprostheses for grasping are very limited and can only be used for a restricted set of grasping and holding tasks (review in Prodanov et al. (2003)).

Visual prostheses have been developed for the last 30 years (review in Prodanov et al. (2003)). Major research lines were focused on the development of cortical prostheses (Brindley & Lewin, 1968; Dobelle & Mladejovsky, 1974; Normann et al., 2001); retinal prostheses (review in Zrenner (2002)) and optic nerve prostheses (Veraart et al. (1998)). The results in the field demonstrate that generating perception of light patterns in blind people is feasible. However, true object recognition still can not be achieved. The *surface cortical microstimulation* (Brindley & Lewin (1968); Dobelle & Mladejovsky (1974)) could not provide useful images because of its limited spatial resolution and the fading of the induced phosphenes (sensations of light). Subsequent human trials with *penetrating cortical implants* (i.e. Utah arrays; see section 2.2.2) were more promising (Dobelle (2000); Normann et al. (1996); Schmidt et al. (1996)), but diminished neuronal excitation and the stability of spatial resolution were still unsolved problems even using high-resolution intracortical electrode arrays (Normann et al. (2001)). The group of Veraart at Université Catholique Louvain (UCL), Brussels, demonstrated that by stimulation of the optic nerve to have the patient recognize single spots of light (Veraart et al. (1998)).

In the end of the 1980s, several North American, Australian and European teams (Eckmiller (1997)) started developing retinal prostheses. Notably, these are the groups of M. Humayun (John Hopkins University) (Schmidt et al. (1996)) and that of J. Rizzo (Harvard University) (Rizzo et al. (2003)) in association with the Massachusetts Institute of Technology, which develop epiretinal implants. The epiretinal implant has no light-sensitive elements. In the epiretinal configuration a tiny camera-like sensor is positioned either outside the eye or within an intraocular plastic lens that replaces the natural lens of the eye. An alternative type of retinal prosthesis is the *subretinal implant* developed by Chow & Chow (1997) in Chicago and

Zrenner et al. (1997) in Tübingen. The Tübingen subretinal device is implanted between the pigment epithelial layer and the outer layer of the retina. The device consists of thousands of light-sensitive microphotodiodes equipped with micro-electrodes assembled on a very thin plate. The light falling on the retina generates currents in the photodiodes, which then activate the micro-electrodes and stimulate the retinal sensory neurons. Epiretinal and subretinal implants depend on the uniform topographic mapping of the retina. If the provided stimulation can trigger learning phenomena in the visual system, we could anticipate another successful clinical application.

4.2.2 Development of Neuromodulatory applications

Deep brain stimulation (DBS) and vagus nerve stimulation (VNS) can be regarded as examples for fast-developing neuromodulatory applications. DBS will be used further also to illustrate some of the challenges in the development of neural interfaces with the brain.

VNS uses an implanted battery-powered signal generator, which stimulates the left vagus nerve in the neck via a pair of spiral cuff-electrodes connected through a lead wire also implanted under the skin. In the case of VNS, the first experimental demonstrations of an anticonvulsant effect of VNS were made in 1980s (reviews in George et al. (2000) and Groves & Brown (2005)). So far the FDA approved the use of VNS as an adjunctive therapy for epilepsy in 1997 and for treatment resistent depression in 2005. Ongoing experimental investigations include various anxiety disorders, Alzheimer's disease, migraines (Groves & Brown (2005)), and fibromyalgia. Current implantable systems (notably the NCP system of Cyberonics Ltd) provide non-selective stimulation, which activates all A_α nerve fibers. Since the vagus nerve projects to three major brain stem nuclei [3], which in turn relay to other brain stem nuclei, such as the *reticular formation*, the *parabrachial nucleus* and the *locus coeruleus*, the effects induced by the electric stimulation of the vagus A_α nerve fibers are multiple and most probably interact with each other. Therefore, the beneficial effects of VNS most probably develop by plastic changes in all affected subsystems, i.e. a learning phenomenon.

Deep brain stimulation is a surgical treatment involving the implantation of electrodes in the brain, which are driven through a battery-powered programmable stimulator. Current versions of the therapy use high-frequency stimulation trains (i.e. in the range 80 – 130 Hz), which can modulate certain parts of the the motor circuits in the basal ganglia.

In 1991, two groups independently reported beneficial effects of thalamic stimulation for tremor suppression (Benabid et al. (1991); Blond & Siegfried (1991)). DBS is considered already as a standard and accepted treatment for Parkinson's disease (Deep Brain Stimulation in Parkinson's Disease Group, 2001), essential tremor, dystonia, and cerebellar outflow tremor (recent overview in Baind et al. (2009)). In the USA, the FDA approved DBS as a treatment for essential tremor in 1997, for Parkinson's disease in 2002 and for dystonia in 2003. There are undergoing clinical trials for epilepsy, depression, obsessive-compulsive disorder, and minimally conscious states (review in Montgomery & Gale (2008)). DBS offers important advantages over the irreversible effects of ablative procedures, including the reversibility of the surgical outcome and the ability to adjust stimulation parameters post-operatively to optimize therapeutic benefit for the patient while minimizing adverse effects (Johnson et al. (2008)).

The mechanisms of action of DBS are still subject to debate arising from conflicting sets of experimental observations. Early hypotheses proposed that stimulation mimicked the outcome of ablative surgeries by inhibiting neuronal activity at the site of stimulation, i.e. "functional

[3] *n. dorsalis n. vagi* (efferent), *n. tractus solitarii* (afferent), *n. ambiguus* (afferent)

ablation". This comprises the direct inhibition hypothesis. Several possibilities have been proposed to explain this view including (i) depolarization blockade, (ii) synaptic inhibition, (iii) neurotransmitter depression, and (iv) stimulation of presynaptic terminals with neurotransmitter release (see McIntyre et al. (2004)). Recent studies have challenged this hypothesis (reviews in Johnson et al. (2008); Montgomery & Gale (2008)), suggesting that, although somatic activity near the DBS electrode may exhibit substantial inhibition or complex modulation patterns, the output from the stimulated nucleus follows the DBS pulse train by direct axonal excitation. The intrinsic activity is thus overridden by more regular high-frequency activity that is induced by the stimulation. A number of alternative hypotheses about the mechanisms of DBS are offered in literature (Montgomery & Gale (2008)). These include (i) indirect inhibition of the stimulated nucleus possibly through thalamo-cortical loops; (ii) increased regularity of *globus pallidus internus* firing by decrease of the information content of the network output due to the regularity of the stimulation; and (iii) resonance effects through stimulation via reentrant loops. None of proposed hypothesis is entirely supported by the existing experimental evidence. However, in view of the recent experimental evidence, the direct inhibition hypothesis seems least probable.
If the same considerations apply also for neuromodulation, two similar principles of development can be stated. The successful neuromodulatory systems will be appplied in areas with uniform or discrete topology (for example the vagus nerve) and the overall effect of the applied stimulation should affect generic/systemic control mechanisms.

II. Biocompatibility of Micro-fabricated Devices

5. Introduction

When combining non-biological entity with living, biological matter, interaction between both is inevitable. When interfacing biological elements, whether they are peptides, proteins, cells or tissues, with non-biological elements, a new interface or situation is created. This interface situation is an interaction between two completely different milieus and, therefore, it is a crucial element for an optimal functioning of both. This interaction can either influence the role of both the biological and non-biological element in a manner that can change the original state of that element, and therefore, it can not be neglected. In this part, we will introduce the interfacing problems that originate from the contact between biological samples and tissues and non-biological materials present in implants and other bioelectronic devices. Biocompatibility issues and challenges will be presented for both in vivo and in vitro conditions, and future challenges and directions will be discussed.
Biocompatibility is extensively debated in biomaterials science and bioelectronic interfacing, but its definition is questionable and very broad. Because many different situations in biomedical engineering exist where biocompatibility is an issue, the uncertainty about the mechanisms and conditions is a serious impediment to the development of new techniques in biomedical and nanobiological research.
Biocompatibility refers to the ability of a material to perform with an appropriate host response in a speficic situation. This definition states that a material as such can not just exist inside a tissue or close to a biological organism, but has to fulfill three major requirements: (i) the response it evokes has to be appropriate for the application, (ii) the nature of the response to a certain material and (iii) its appropriateness may vary from one situation to another (Williams (1987; 2008a;b)). However, this definition is very general and so self-evident that it does not lead to an advancing knowlegde of biocompatibility. It is more likely that one concept cannot

apply to all material-biological element reactions in the widely spread applications such as brain implants, tissue engineering, prosthesis, biosensors or micro-electrode arrays. The nature of material itself plays a large role in the evoked response in the biological element. Some major material variables are material composition, micro- (or nano)-structure, morphology, hydrophobicity or hydrophilicity, porosity, surface chemical and topographical composition, surface electronic properties, corrosition parameters and metal ion toxicity. These parameters can all influence the functioning of the biological element (Williams (2008a)).

6. Biocompatibility of In Vitro Devices

6.1 Cytotoxicity

Although the degree of biocompatibility is much more complex at the level of implantable materials and devices, in vitro biocompatibility cannot be neglected, especially because of the growing amount of new materials and technologies. In the following, biocompatibility will be described in the specific situation of micro-electrode arrays, a fast growing field in bioelectronics. Micro-electrode arrays can consist of many different materials, for which some general cytotoxicity is known, but for others, very little information is available.

Cytotoxicity is strongly dependent on the type of cell or cell culture that is used. A material which is toxic for one type of cell is not necessarily toxic for another, or the lethal concentration (LC50) can be vastly different. Therefore, a cytotoxicity test should always be designed for the final situation were the system will be used. It is clear that immortalized cancer cell lines are more robust to cytotoxic agents than fresh, primary cell preparations (e.g., Olschlager et al. (2009)). It is, therefore, important that the biocompatibility test is carefully designed. For obvious reasons, cell cultures of excitable cells are interesting for cultivation on micro-electrode arrays. These cell cultures mostly include preparations of the heart (embryonic artrial, ventricular or whole heart cultures) and the central nervous system (embryonic cortical, hippocampal, spinal cord cultures) and retinal neuronal cultures. Viability assays for these cell cultures include visual microscopic inspection, trypan blue staining, cell death (apoptosis and necrosis) assays using fluorescent microscopy, bioluminescence imaging and cytofluorometry. At present, there is a variety of standardized ready-to-use assays available to investigate cell proliferation, adhesion and survival.

copper toxicity Materials used in modern micro-fabrication are typically chosen based on their durability, ease of processing, conductivity and price. However, this mostly appears to be at odds with their biocompatible properties. Although most commercialized micro-electrode arrays are fabricated with biocompatible materials including borosilicate glass, platinum, gold, titanium (nitride), and several metal oxides, more advanced technologies require unexplored materials. One of the most important materials used in advanced complementary metal oxide semiconductor (CMOS) technology is copper. Copper is a cheap material with excellent conducting properties which is relatively easy to process in micro-fabrication tools.

Because copper can migrate fast through other materials, it can cause problems in bioelectronic devices. Copper (Cu) is an essential trace element found in small amounts in a variety of cells and tissues with the highest concentrations in the liver. Cu ions can exist in both an oxidized, cupric (Cu^{2+}), or reduced, cuprous (Cu^{+}), state. Copper functions as a co-factor and is required for structural and catalytic properties of a variety of important enzymes, including cytochrome c oxidase, tyrosinase, and Cu-Zn superoxidase dismutase. Copper is known to be a highly cytotoxic material. Several reports show the

role of reactive oxygen species (ROS) in cell death induced by heavy metals (Houghton & Nicholas (2009)). Both cupric and cuprous ions can participate in oxidation and reduction reactions. In the presence of superoxide or reducing agents such as ascorbic acid, Cu^{2+} can be reduced to Cu^{+}, which is capable of catalyzing the formation of hydroxyl radicals from hydrogen peroxide. This is called the Haber-Weiss reaction, whereby copper catalyzes the formation of ROS by peroxidation of membranous lipids. The hydroxyl radical is the most powerful oxidizing radical likely to arise in biological systems, and is capable of reacting with practically every biological molecule (Buettner & Oberley (1979)). It can initiate oxidative damage by abstracting the hydrogen from an amino-bearing carbon to form a carbon-centered protein radical and from an unsaturated fatty acid to form a lipid radical (Powell et al. (1999)). Copper is cabable of inducing DNA strand breaks and oxidation of bases (Kawanishi et al. (2002)).

6.2 Interface Layers for Cell-based Biosensors

Another important aspect of in vitro biocompatibility is the growth of cell cultures on top of electrode surfaces. To perform successful experiments with cells on micro-electrode arrays, cells must adhere, grow and maintain on the surface of electrodes. Although some cells, such as immortalized fibroblast cell lines, can adhere easily to most surfaces, most cells need an interface layer to be present to adhere. The following describes straightforward methods for the adhesion and growth of various cell cultures.

An interface layer must mimic the normal environment of the biological element, creating optimal conditions for optimal functioning of the hybrid device. Although there are various strategies to construct interface layers, not all of them are suitable for cell-based biosensor technology. Self-assembled monolayers (SAMs) offer a reproducible manner of interfacing cells with sensor materials. Extracellular matrix peptides, polymers or proteins are also often used to attract and adhere cells. The enhancement of cell attachment and spreading through surface functionalization is a crucial parameter in the optimalization of the functioning of cell-based micro-electrode arrays.

6.2.1 Self-Assmbled Monolayers

Characterized by high temperatures and the formation of a monolayer, chemisorption is often used in the formation of SAMs. They provide a convenient, flexible and simple system that tailors the interfacial properties of metals, metal oxides and semiconductors. Self-assembled monolayers are organic assemblies formed by the adsorption of molecular constituents deposited from solution or vapor onto a solid surface. They organize spontaneously into crystalline structures. However, the experimental conditions for their development need to be strictly controlled to ensure clean, complete monolayer formation. The molecules that form SAMs have a chemical functionality, or 'headgroup', with a specific affinity for a substrate. In many cases, the headgroup also has a high affinity for the surface and displaces other adsorbed organic materials (Love et al. (2005)). The headgroup-substrate pair is typically used to define the individual SAM system. The most common examples are thiols (R-SH, where R denotes the rest of the molecule) on metals (e.g., gold, platinum) or silane-based molecules on metal/semiconductor oxides (e.g., silicon dioxide, tantalum pentoxide). Self-assembled monolayers are structurally well-ordered, and are therefore an ideal substrate for the binding of various extracellular matrix proteins. The proteins adsorbed on top of these layers can be structured and immobilized with a large density to promote attachment, spreading and migration of cells.

Self-assembled monolayers can be also engineered to prevent non-specific adsorption of proteins (Frederix et al. (2004)). The majority of applications that have been reported make use of polyethylene glycol or derivates, which excludes protein adsorption through mechanisms that depend on the conformational properties of highly solvated polymer layers. Another approach of SAMs for the adhesion of cells is the use of monolayers that present peptide fragments from extracellular proteins such as fibronectin. These peptides are ligands for some of the integrin family cell-surface receptors, which are an important class of receptors found on all cellular surfaces and that mediate attachment of cells to the extracellular matrix (Critchley (2000)). Many different peptides fragments have been used over the years to promote cell adhesion to electrode surfaces or chip surfaces (Huang et al. (2009); Tsai et al. (2009); Van Meerbergen et al. (2008)).

6.2.2 Extracellular Matrix Proteins and Polymers

Extracellular matrix proteins that are often used for cell adhesion are laminin, fibronectin, and collagen. These proteins directly bind to integrin receptors on the outside of the cell membrane (Critchley (2000)). In this way, they communicate in a direct way with the cytoskeleton of the cell, responsible for cell adhesion and spreading on surfaces. Although not inherently biological, many polymers have been used as well to promote cell adhesion, including poly-L/D-lysine, poly-L-ornithine and polyethyleneimine. The principle of cell adhesion using these artificial ligands is based upon the strong electrostatic binding of the cell membrane to the surface (Hategan et al. (2004)). The main advantage and the direct reason of their success is the availability and price of these synthetic molecules.

7. Biocompatibility of Implantable Devices

7.1 Regulatory Aspects

The selection and evaluation of materials and devices intended for use in humans requires a structured program of assessment to establish appropriate levels of biocompatibility and safety. Current regulations, whether in accordance with the US FDA (ISO 10993-1/EN 30993 standard, since 1995), the International Organization for Standardization (ISO), or EU regulation bodies (The EU Council Directive - 93/42/EEC), as part of the regulatory clearance process require conduction of adequate safety testing of the finished devices through pre-clinical and clinical phases (Bollen & Svendsen (1997)). An extensive account on the biocompatibility can be found in the standard ISO 10993-1/EN 30993. An implant can be considered biocompatible if it gives negative results on the following tests:

cytotoxicity The aim of in vitro cytotoxicity tests is to detect the potential ability of a device to induce sublethal or lethal effects on mammal cells (mostly on fibroblast cultures). Three main types of cell-culture assays have been developed: the elution test, the direct-contact test, and the agar diffusion test.

sensitisation The sensitization test recognizes a possible sensitization reaction (allergic contact dermatitis) induced by a device, and is required by the ISO 10993-1 standard for all device categories.

genotoxicity Genetic toxicity tests are used to investigate materials for possible mutagenic effects–that is, damage to the genes or chromosomes of the test organism (e.g. bacteria or mammal cells).

implantation Implantation tests are designed to assess any localized effects of a device designed to be used inside the human body. Implantation testing methods essentially attempt to imitate the intended conditions of use.

carcinogenicity The objective of long-term carcinogenicity studies is to observe test animals over a major portion of their life span to detect any development of neoplastic lesions (tumor induction) during or after exposure to various doses of a test substance.

skin irritation The ISO 10993-10 standard describes skin-irritation tests for both single and cumulative exposure to a device. Skin-irritation tests of medical devices are performed either with two extracts obtained with polar and nonpolar solvents or with the device itself.

intracutaneous reactivity The intracutaneous reactivity test is designed to assess the localized reaction of tissue to the presence of a given substance.

acute systemic toxicity is the adverse effect occurring within a short time after administration of a single dose of to the presence of given substances. ISO 10993-1 requires that the test for acute systemic toxicity be considered for all device categories that indicate blood contact. For this test, extracts of medical devices are usually administered intravenously or intraperitoneally in rabbits or mice.

subchronic and chronic toxicity tests are carried out after initial information on toxicity has been obtained by acute testing, and provides data on possible health hazards likely to arise from repeated exposures over a limited time.

As can be seen from Figure 4, undesirable interactions affecting biocompatibility can occur in most of the levels of the issue tree. For example, implants may be subject to continuous attacks by hydrolytic enzymes or free radicals produced by macrophages and/or cell lysis (Salthouse (1976)). Stability of implanted material is important not only for a stable function but also because degradation products may be harmful to the host organism. An overview on the biological reactions to implanted materials can be found in Ratner et al. (1996).
While the ISO standard addresses the general bio-compatibilty requirements of a medical device, it does not address specifically the interactions on the active tissue-device interface.

7.2 Interactions on the Active Interface

7.2.1 Chemical Properties of the Active Interface

Appropriate implant materials should be as chemically inert as possible. If chemical reactions are to be expected, they should be minimal and all resulting products should be inert. Candidate materials for use in neuroprotheses pass very rigorous testing since they must remain inert not only passively but also when subjected to electrical stimulation and when placed in contact with the biological tissue.
According to the literature the following criteria should be considered when choosing material for an implanted electrode: (i) the intensity of the tissue response, (ii) eventual occurrence of allergic response, (iii) electrode-tissue impedance, (iv) radiographic visibility and (v) MRI safety (Geddes & Roeder (2003)).
For electrodes that make Ohmic contact with tissues, **Au**, **Pt**, **Pt-Ir** alloys, **W**, and **Ta** are recommended as materials for the active interface (Geddes & Roeder (2003); Heiduschka & Thanos (1998)). The use of some metals should be avoided because of vigorous tissue reactivity. These pure metals are notably **Fe**, **Cu**, **Ag**, **Co**, **Zn**, **Mg**, **Mn**, and **Al** (Geddes & Roeder (2003)).
It can be necessary to distinguish between stimulating and recording electrodes. Good materials for *recording electrodes* are: **Pt**, **Ir**, and **Rh** and **Au**. Materials of choice for *stimulating*

Fig. 3. Loss of signal

The **immediate effects** are caused by the mechanical interaction of the device with the brain tissue during implantation. These are notably *vascular damage*, *hemorrhage* and *brain edema*.
During the **progressive phase**, the acute inflammation, cell death and nerve fiber degeneration predominate. The inflammation process is driven by the complement complex activation, the extravasation of neutrophils and mononuclear cells and the secretion of cytokines. The **late effects** involve gliosis or chronic inflammation and the tissue remodeling. Some processes, notably the micromotion and mechanical strain, act **continuously** during all stages.

electrodes are **Pt**, **Pt-Ir** alloys, **W**, and **Rh**. For capacitive stimulating electrodes, tantalum pentoxide (Ta_2O_5) has the highest dielectric constant, followed by iridium oxide (IrO_2). Aluminum oxide (Al_2O_3) is a candidate with a lower dielectric constant.
Glassy carbon or carbon fibers are also used as electrode materials, and they are biocompatible and stable, though they have a higher roughness than metals. Among the currently studied conducting polymers, polypyrrole (*PPy*) and poly-3,4-ethylenedioxythiopentane (*PEDOT*) appear the best candidates for materials. They provide interesting opportunities to incorporate other substances, for example peptides or growth factors, during the process of polymerisation in order to improve biocompatibility in vivo. Interesting candidates for materials are the nano-structured materials and notably the carbon nanotubes.

7.2.2 Biotic-abiotic Reactions

As recognized by many groups in the field, one of the central issues in favor of a closed-loop implantable system is the uncertain performance of the recording function in chronic conditions (Berger et al. (2007)). This can be attributed to causes related to the device, to the tissue or to the active interface (Figure 3). While it is generally believed that the brain tissue response to chronically implanted silicon micro-electrode arrays contributes to recording instability and failure, the underlying mechanisms are unclear. From the side of the tissue the loss of signal can be caused by several biological processes, which are part of the response to implantation. The loss of sources could occur due to neuronal cell death or spatial shift. *Neuronal cell death* can occur early after implantation when some neurons close to the insertion track die due to the trauma. At a later stage, some neurons can die due to the continuing process of neuroinflammation. It has been shown that activated macrophages migrate to the device-tissue interface and suggested that the presence of such devices are a persistent source of inflammatory stimuli (i.e. classical "foreign body" reaction). Since macrophages can be a source of neurotoxic cytokines, they could potentially induce cell death in the surrounding neurons. The effect of the activated macrophages on the quality of electrophysiological recording is still largely unexplored. In addition to the persistence of inflammatory cells, studies have observed significant reductions in nerve fiber density and neuronal cell bodies in the tissue