Development and use of bioanalytical instrumentation and signal analysis methods for rapid sampling microdialysis monitoring of neuro-intensive care patients

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I declare that all work presented in this thesis is my own original work unless otherwise acknowledged.

Delphine Feuerstein
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לעילוי נשמת
Abstract

This thesis focuses on the development and use of analysis tools to monitor brain injury patients. For this purpose, an online amperometric analyzer of cerebral microdialysis samples for glucose and lactate has been developed and optimized within the Boutelle group. The initial aim of this thesis was to significantly improve the signal-to-noise ratio and limit of detection of the assay to allow reliable quantification of the analytical data.

The first approach was to re-design the electronic instrumentation of the assay. Printed-circuit boards were fabricated and proved very low noise, stable and much smaller than the previous potentiostats.

The second approach was to develop generic data processing algorithms to remove three complex types of noise that commonly contaminate analytical signals: spikes, non-stationary ripples and baseline drift. The general strategy consisted in identifying the types of noise, characterising them, and subsequently subtracting them from the otherwise unprocessed data set. Spikes were effectively removed with 96.8% success and ripples were removed with minimal distortion of the signal resulting in an increased signal-to-noise ratio by up to 250%.

This allowed reliable quantification of traces from ten patients monitored with the online microdialysis assay. Ninety-six spontaneous metabolic events in response to spreading depolarizations were resolved. These were characterized by a fall in glucose by -32.0 µM and a rise in lactate by +23.1 µM (median values) for over a 20-minute time-period. With frequently repeating events, this led to a progressive depletion of brain glucose.

Finally, to improve the temporal coupling between the metabolic data and the electro-cortical signals, a flow-cell was engineered to integrate a potassium selective electrode into the microdialysate flow stream. With good stability over hours of continuous use and a 90% response time of 65 seconds, this flow cell was used for preliminary in vivo experiments the Max Planck Institute in Cologne.
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5 Dynamic metabolic response to multiple spreading depolarisations in the injured human brain

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</tr>
<tr>
<td>AC</td>
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</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>Ado</td>
<td>Adenosine</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial CerebroSpinal Fluid</td>
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<td>Silver/silver chloride</td>
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<td>AMPA</td>
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<td>Arterial Spin Labelling</td>
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<td>Adenosine TriPhosphate</td>
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<td>Blood Pressure</td>
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<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>Cerebral Metabolic Rate of Glucose</td>
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<td>h</td>
<td>Hour</td>
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<td>Internal Filling Solution</td>
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<td>IQR</td>
<td>InterQuartile Range</td>
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<td>International Union of Pure and Applied Chemistry</td>
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<td>potassium Tetrakis (4-ChlorPhenyl) Borate</td>
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<td>minutes</td>
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<td>Magnetic Resonance Imaging</td>
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<td>N- methyl-D-aspartate</td>
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<td>oxygenated haemoglobin</td>
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<td>PaCO$_2$</td>
<td>arterial partial pressure of carbon dioxide</td>
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<td>PCB</td>
<td>Printed-Circuit Board</td>
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<td>PEBBLE</td>
<td>Photonic Explorers for Bioanalysis with Biologically Localised Embedding</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>PG</td>
<td>ProstaGlandin</td>
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<tr>
<td>PID</td>
<td>Peri Infarct Depolarisation</td>
</tr>
<tr>
<td>REC</td>
<td>Research Ethics Committee</td>
</tr>
<tr>
<td>ROI</td>
<td>Region Of Interest</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
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<td>rsMD</td>
<td>Rapid Sampling MicroDialysis</td>
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<tr>
<td>$s$</td>
<td>second</td>
</tr>
<tr>
<td>SAH</td>
<td>SubArachnoid Haemorrhage</td>
</tr>
<tr>
<td>SD</td>
<td>Spreading Depolarisation</td>
</tr>
<tr>
<td>SDev</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SG</td>
<td>Savitsky-Golay</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-Noise Ratio</td>
</tr>
<tr>
<td>SPC</td>
<td>Slow Potential Change</td>
</tr>
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<td>SSE</td>
<td>Sum Squared Error</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TBI</td>
<td>Traumatic Brain Injury</td>
</tr>
<tr>
<td>THF</td>
<td>TetraHydroFuran</td>
</tr>
<tr>
<td>TTX</td>
<td>TetrodoToXin</td>
</tr>
<tr>
<td>UPS</td>
<td>Uninterruptible Power Supply</td>
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Chapter 1

Introduction

The brain is central to all bodily systems and controls every aspect of bodily function. It is the most complex and fascinating biological structure known. Damage to the brain can have devastating consequences. If not fatal, brain injury can lead to major permanent disabilities in intelligence, mobility and behaviour. In many cases, most damage takes place in the hours and days following the injury; this is called secondary brain injury.

The aim of this thesis is to develop new methods and tools to monitor the events associated with secondary brain injury, with the goal of understanding and ultimately preventing further neuronal deterioration.

1.1 Organisation and activity of the brain

1.1.1 Anatomical organisation of the brain

The two main components of the brain are the grey matter and the white matter. Grey matter structures (cortex, deep nuclei) process information. White matter is mainly formed of myelinated axons that transmit information to and from other parts of the brain and other organs.

The brain is protected mechanically by the thick bones of the skull and by cerebrospinal fluid (CSF) that acts as a "cushion" for the cortex. The brain is also protected chemically by the blood-brain barrier: endothelial cells in the walls of the blood vessels restrict the passage of microscopic objects (such as bacteria) and the diffusion of large or hydrophillic molecules into the CSF, while allowing the diffusion of small membrane permeable molecules (oxygen, carbon dioxide, hormones). Hydrophobic molecules needed by the brain (such as glucose) cross the blood-brain barrier via special carriers.

Despite these protections, the delicate nature of the human brain makes it susceptible to many types of damage that can irreversibly affect grey matter and cause major deficits.

1.1.2 Functional organisation of grey matter

Grey matter is composed of two main classes of cells, the nerve cells (neurones) and glial cells (glia), together with a rich network of arterioles, capillaries and venules. They are functionally and spatially inter-related, as illustrated by Figure 1.1:
Neurones have a large cell body, a long tubular projection (axon) and short branching processes (dendrites), as shown on Figure 1.1. They are excitable cells that can generate an electrical signal in response to stimuli, carry it along their processes and transmit it to other neurones by release of neurotransmitters (see section 1.1.2.1 for further details).

Astrocytes are the most numerous of glial cells. These cells have extraordinarily profuse processes that are interposed between neurones and blood vessels (see Figure 1.1). Fine processes cover most synapses and large diameter end-feet enwrap more than 99% of the vasculature [1]. Further details are given in section 1.1.2.2.

Astrocytes and neurones are spatially arranged around a dense network of cerebral arterioles and capillaries (see Figure 1.1). These intracerebral blood vessels supply them with essential nutrients to maintain normal brain activity (see section 1.3 for a detailed discussion).
1.1. Organisation and activity of the brain

1.1.2.1 Neurones

In response to a stimulus, neurones can generate an electrical signal (in the form of action potentials). This signal propagates from one end of the neurone (dendrites), down its axon and to the axon terminal. There, the arrival of action potentials causes an increase in intracellular calcium ($\text{Ca}^{2+}$). This leads to the vesicular release of neurotransmitters into the synaptic cleft, a space of circa 20 nm that separates two neurones. The neurotransmitter diffuses into the synaptic cleft and binds to specific receptors on the post-synaptic membrane. This results in the depolarisation of the postsynaptic neurone and the generation of an electrical signal in the form of another action potential [2]. Synaptic neurotransmission is represented in Figure 1.2 below:

![Synaptic neurotransmission](image)

**Figure 1.2: Synaptic neurotransmission.** When an action potential reaches the synaptic terminal of the presynaptic neurone, voltage-gated calcium channels open (1). This lets calcium in the cell (2) and vesicles containing neurotransmitter fuse with the pre-synaptic cell membrane (3). They expel their content (4) into the synaptic cleft. They finally bind to a post-synaptic receptor. Taken from [3].

There are many examples of neurotransmitters, such as glutamate (the most common excitatory neurotransmitter in the brain [4]), acetylcholine, dopamine and gamma-aminobutyric acid (GABA). Measuring neurotransmitters can be an excellent index of brain activity since it can give information directly on synaptic function. Techniques to do so are described in section 1.5.3.2.

Depolarisation of the post-synaptic neurone and generation of action potentials is the result of a shift in the electrochemical gradient across the post-synaptic membrane due to the opening of ion channels. As the neurotransmitter binds to designated receptors on the post-synaptic cell, it opens voltage-gated sodium ($\text{Na}^+$) channels. This results in a large influx of $\text{Na}^+$ into the cell, thus depolarising the membrane. At about +40 mV, the population of voltage-gated $\text{Na}^+$ channels gradually closes and voltage-gated potassium ($\text{K}^+$) channels open. As $\text{K}^+$ ions leave the cell, the
1.1. Organisation and activity of the brain

membrane repolarises. With this delayed increase in K\(^+\) efflux and a decrease in Na\(^+\) influx, the membrane recovers its resting potential of -65 mV. Figure 1.3 is an electrical representation of the current fluxes that occur through a neurone membrane at rest.

All these processes need energy (see Figure 1.4). In particular, depolarisation, repolarisation and maintenance of the resting membrane potential of the post-synaptic cell rely on ion pumps (Na\(^+\)/K\(^+\) ATPase) that actively transport two K\(^+\) from the extracellular space into the cytosol in exchange of three Na\(^+\). This is at the cost of one adenosine triphosphate (ATP) molecule. The role of the Na\(^+\)/K\(^+\) ATPase is illustrated in Figure 1.3.

![Figure 1.3: Current fluxes through a neurone membrane. The arrows indicate the movement of ions: the direction of the flux (arrow head) and the relative magnitude of the fluxes (sizes of the arrows). The membrane lipid bilayer acts as an electrical capacitance, \(C_m\), separating charges. At rest, the inside of the membrane is more negatively charged than the outside. Passive fluxes of Na\(^+\) (\(I_{Na}\)) and K\(^+\) (\(I_{K}\)) are due to 1) an electromotive force (represented by a battery, \(E\)) and 1) their passages through ion channels down their concentration gradients (represented by a conductance, \(g\)). These passive fluxes lead Na\(^+\) into the cell and K\(^+\) out of the cell (direction of the arrows). Chloride ions are passively distributed across the membrane (no flux). The Na\(^+\)/K\(^+\) ATPase actively transports 3 Na\(^+\) outside the cell and 2 K\(^+\) inside the cell (fluxes \(I'\)) against their concentration gradients. Note that \(I'_{Na}\) is 50% greater than \(I'_{K}\) (relative sizes of the arrows) and therefore \(I_{Na}\) is also 50% greater than \(I_{K}\) under steady-state. Model based on [2].](image)

1.1.2.2 Astrocytes

Astrocytes have two major roles that support neuronal function:

1. they take up and recycle neurotransmitters released during synaptic transmission,
2. they rapidly clear the excess accumulation of potassium in the extracellular space following depolarisation.

Both processes notably require pumping neurochemicals against their concentration gradients and therefore consume energy (see Figure 1.4).

Astrocytes are involved in other major functions in the brain, notably in the control of cerebral blood flow (see section 1.3.2 for further details), and in providing energy to neurones from the blood. Recently, it has been suggested that they could play a role in modulating synaptic neurotransmission [5]. This is notably suggested by their spatial organisation: astrocyte-delimited domains appear to define functional compartments, with all synapses lying within a given volumetrically defined
1.2 Brain metabolism

compartment under the influence of one single astrocyte [6].

1.1.2.3 Extracellular fluid (ECF)

Neurones and astrocytes are embedded in an extracellular medium formed of narrow gaps between cellular processes. These spaces form a complex three-dimensional mosaic filled with extracellular fluid (ECF), and they account for 12–25% of the brain’s volume [7].

Currents flow through this space, between the cells, due to the movement of ions. The extracellular space then acts as a volume conductor for a variety of electrical signals generated by both neurons and astrocytes [8] and brain electrical activity is often measured in the extracellular space (see section 1.5.3.1 for further details).

Additionally, the composition of the extracellular space reflects the chemical environment of the cells and it is therefore rich with information about the condition and health of the cells in the vicinity. The thesis will focus on monitoring neurochemicals in the extracellular space.

1.2 Brain metabolism

As mentioned previously, brain activity requires energy at all stages. In fact, the energy requirements of the brain are almost ten times more than predicted on a mass basis [9]. Most of the brain energy expenditure is to sustain grey matter signalling processes [10].

1.2.1 Energy requirements in the grey matter

The grey matter energy expenditure has been investigated by Attwell and Laughlin [11]. They based their “energy budget” on measured properties of individual ion channels and synapses to quantify the energy used:

- on maintaining membrane resting potential,
- on propagating action potentials,
- on pre-synaptic terminals — Ca$^{2+}$ entry and vesicular release
- by astrocytes — neurotransmitter uptake and recycling
- on post-synaptic responses — ion fluxes generated by postsynaptic current

Their findings are summarised in Figure 1.4 below.

According to their study, the primary energy requiring mechanism is spent on reversing the ion fluxes underlying electrical potentials by activation of the Na$^+$/K$^+$ ATPase pumps (Figure 1.4). The main difference between rodents and humans is the 3- to 10-fold lower density of neurones in humans with an unchanged density of synapses. This implies a 3- to 10-fold higher number of synapses per neurone. The direct consequence is the increased fraction of energy usage devoted to post-synaptic processes in humans (75%) compared to rodents (34%) (Figure 1.4).
1.2. Brain metabolism

Figure 1.4: Energy budget in rodents and primates. Diagram of an excitatory synapse with the percentage of energy use for processes associated with signalling in the grey matter of rodents (first number) and primates (second number). These were calculated using a model described in [11]. Almost all signalling is spent on reversing the Na\(^+\) and K\(^+\) fluxes across the cell membranes (represented by the ion pumps). Glial cells re-uptake glutamate (Glu) and thereby activate another type of ion pumps. Astrocytes then convert glutamate to glutamine (Gln). Mitochondrion icons illustrate the sites of ATP production. Taken from [12].

All this energy comes from the breakdown of ATP, the energy carrier. ATP itself comes from glucose, the energy substrate utilised to generate ATP.

1.2.2 Glucose metabolism in the brain

Glucose is present in the systemic circulation in a high, hormonally regulated, concentration, and rapidly transported across the blood-brain barrier via a carrier system [13]. After crossing the blood-brain barrier, it is taken up in both neurones and glial cells. There, glucose is metabolised into two pyruvate via glycoslysis, producing two moles of ATP/mole of glucose. Pyruvate then enters the mitochondrion and is further catabolised to carbon dioxide and water via the tricarboxylic acid cycle (or citric acid cycle) and the electron transport chain. This process yields 36 molecules of ATP per molecule of glucose. The net reaction is given in Equation 1.1 below:

\[
C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2
\]  

(1.1)

In the absence of oxygen, only glycolysis is possible, producing pyruvate. If this is turned into lactate, NAD\(^+\) (nicotinamide adenine dinucleotide) is regenerated, allowing glycolysis to continue (provided there is some glucose available in the first place). The net result is only two molecules of ATP per glucose molecule.

The brain also has some limited stores of glucose, in the form of glycogen. This is a high molecular-weight glucose polymer localised in astrocytes. Traditionally, glycogen was thought to serve as an
emergency energy store to be used only when glucose supply fails. Later studies showed that, even in the presence of normal levels of blood glucose, glycogen has a rapid turnover and may play a role in normal brain metabolism [14; 15].

A model developed by Magistretti et al. has challenged the traditional view that glucose is the sole energy substrate of neurones. Their model suggests the existence of a "lactate shuttle" [16]. The uptake of neurotransmitters into astrocytes triggers glucose uptake in the astrocytes and glycolysis. This results in the release of lactate from astrocytes that can be used by neurones as a metabolic substrate. There, lactate would be converted to pyruvate and enter the Kreb’s cycle for the production of ATP [17; 18; 19]. However, irrefutable evidence of this theory in vivo is still lacking [20].

A remarkable feature of the brain is that the magnitude of its energy utilisation is quantitatively related to the degree of its functional activation [21]. This is called the neuro-metabolic coupling: the increase demand in energy is met locally by the increase supply of energy substrate (glucose) by the blood to generate enough ATP. The increase supply of glucose is in turn achieved by a local increase in cerebral blood flow (CBF): this is known as the neurovascular coupling.

### 1.3 Neurovascular coupling

"The brain possesses an intrinsic mechanism by which its vascular supply can be varied locally in correspondence with local variations of functional activity."

This is the conclusion from Roy and Sherrington [22] who postulated, more than a hundred years ago, a tight coupling between neuronal activity and local cerebral blood flow (CBF). This coupling is both temporal and spatial. For example, models of somatosensory activation showed that the increase in neocortical CBF occurred within one second and was restricted to specific laminae [23] and specific whisker barrel [24]. Neurovascular coupling, also known as functional hyperaemia, is so tightly coupled to neuronal activation that haemodynamic signals are used as a surrogate measure for brain activity in many common imaging methods, such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) (see sections 1.5.4.1 and 1.5.4.2 for further details).

#### 1.3.1 The cerebral vasculature

The vascular supply of the brain is provided by four large arteries, the carotid and vertebral arteries, which merge to form the circle of Willis at the base of the brain. The arteries arising from the circle of Willis travel along the brain surface, giving rise to pial arteries (see Figure 1.5). The pial arteries branch out into smaller vessels, which dive into the brain parenchyma; they are called penetrating arteries and arterioles. They in turn give rise to capillaries (see Figure 1.5). Remarkably, brain capillary density is heterogeneous and correlates linearly with regional energy utilisation and blood flow over a fivefold range [25]. This has suggested a central role for capillaries in the local distribution of cerebral blood flow and blood volume [26; 27]. The structure of pial arteries, penetrating arterioles and micro-vessels are shown below in Figure 1.5.
1.3. Neurovascular coupling

Figure 1.5: The vascular bed of the brain. Large cerebral arteries run along the surface of the brain (pial arteries) and branch into smaller arteries and arterioles. Pial arteries are separated from the brain by the Virchow-Robin space. As the arterioles penetrate deeper into the brain, this space disappears and intracerebral arterioles and capillaries come into direct contact with the astrocytic end-feet or intrinsic neurones. Insert show cross sections of a pial artery, an intracerebral arteriole and a capillary, highlighting the cells controlling CBF. Taken from [28]

Pial arteries are densely innervated by perivascular nerves (see Figure 1.5) that originate from autonomic and sensory ganglia and contain many vasodilators and vasoconstrictors. However, deeper into the brain, intra-cerebral arterioles and capillaries are infrequently contacted by neural processes. Instead, astrocytic end-feet almost completely surround intraparenchymal blood vessels (see Figures 1.1 and 1.5).

CBF is directly controlled by the cells that form the walls of the intracerebral blood vessels. Smooth muscle cells (for arterioles) and pericytes (for capillaries) are contractile elements that control the diameter of the vessel. Their degree of contraction is regulated by vasoactive species that are released by endothelial cells, astrocytes and neurones (see section 1.3.2). Consequently, as the weaving pattern of neuronal activity takes place, a similar weaving pattern of focal hyperaemic regions appears. The arterioles and capillaries locally dilate or constrict to distribute the fixed cerebral blood volume to the active areas. This tight spatial and temporal coupling is achieved by the neurovascular unit.
1.3. Neurovascular coupling

1.3.2 The neurovascular unit

The current view is that neurovascular coupling is the result of the co-ordinated action of neurones, astrocytes and cerebral blood vessels. These cells constitute a functional unit known as the neurovascular unit. It is represented in Figure 1.6:

As shown on Figure 1.6, neurovascular coupling is quite complicated and is relayed by a number of chemical agents, that are released or synthesised by the participants in the neurovascular unit. Importantly, some substances have a vasoactive effect, i.e. they can directly act on smooth muscle cells and thereby determine the degree of constriction or dilation of blood vessels. These are briefly reviewed below and detailed discussion can be found in [12; 28; 29; 30; 31; 32; 33; 34].

**Neurotransmitters**

Some cerebral blood vessels at the surface and within the brain are directly surrounded by nerve fibres (recently reviewed by Hamel [35]). These nerves can release neurotransmitters and neuropeptides that can directly act on the smooth muscle cells. These include dopamine (DA),...
acetylcholine (ACh), serotonin (5HT), noradrenaline (NA) and gamma-aminobutyric acid (GABA) (see Figure 1.6 above). This direct control by neurotransmitters can account for a widespread and global change in CBF responses to activation but are not sufficient to account for the spatially restricted control of blood flow following neuronal activation [28]. Indeed, neuronal terminals are only in direct contact with vascular smooth muscle cells infrequently and diffusion of neurotransmitters over long distances is not compatible with a tight coupling in time and space.

Ions released during neurotransmission
Potassium (K\(^+\)) is the main ion released in the extracellular space during neuronal activation. Evidence suggests that activation of potassium channels on smooth muscle cells can lead to relaxation of the cerebral vessels [36]. One mechanism would be diffusion of potassium from the site of neurotransmission through the extracellular space to the blood vessels (see Figure 1.6). Alternatively, the uptake of potassium by astrocytic processes near synapses could be counterbalanced by the release of potassium at astrocytic foot processes surrounding blood vessels. This mechanism is known as K\(^+\) siphoning [37; 37] (see Figure 1.6). Recently, calcium sensitive potassium channels have been suggested as a preferential pathway to mediate the action of K\(^+\) on CBF [38].

By-products of metabolism
Some by-products of energy utilisation can have a direct impact on CBF. Adenosine, resulting from the breakdown of ATP, can activate dilatation [39]. An increase in hydrogen ions, notably due to the formation of lactate, could also mediate dilatation (see Figure 1.6). However, detailed studies of the time course of blood flow responses compared to the time course of H\(^+\) release have led to the conclusion that pH changes are unlikely to initiate the CBF response. Instead, it is likely that H\(^+\) ions could contribute to maintain the vasodilatation [40].

A cascade of enzymatic reactions and release of vasoactive species, mediated by intracellular calcium signalling in neurones and astrocytes
According to the current model, neurotransmission triggers a cascade of events in both neurones and astrocytes that would ultimately lead to the enzymatic synthesis of potent vasoactive substances.

In neurones, the binding of glutamate to post-synaptic receptors leads to an increase in intracellular calcium (Ca\(^{2+}\)) (see Figure 1.6). This leads to activation of two enzymes: nitric oxide synthase (NOS) and cyclo-oxygenase 2 (Cox2). These two enzymes release nitric oxide (NO) and prostaglandins (PG), which both cause vasodilatation [41; 42; 43].

In astrocytes, glutamate can bind to metabotropic glutamate receptors (mGluR). This causes an increase in intracellular calcium in astrocytes [44; 45], which in turn activates astrocytic enzymes that release epoxyeicosatrienoic acids (EETs) and PGs that cause vasodilatation [45; 46; 47] (see Figure 1.6). Remarkably, astrocytes can propagate the calcium signal over long distances [47]. The entry of inositol trisphosphate (IP3) through gap junction and the activation of purinergic receptors (P2Y) by ATP have been suggested to mediate the propagation of calcium waves between astrocytes [48]. This means that Ca\(^{2+}\) waves can spread over hundreds of micrometers in a time frame of a few seconds. This suggests that astrocytes are a key player in the spatial control of CBF over a large area.
and in the fine regional distribution of the vascular response by adjusting the constriction or dilation of the arteriolar-capillary network [29].

**Brain metabolism modulates the CBF response**

All the above mechanisms cause a local hyperaemia in response to functional activation. Given the fixed amount of cerebral blood volume, increase in CBF in some areas must be accompanied by a decrease in others. It is likely that the mechanisms of this constriction are also mediated by astrocytic calcium [26]. The increased astrocytic calcium would activate the synthesis of hydroxyeicosatetraenoic acids (HETEs) that have been shown to induce vaso-constriction [49]. A very recent study has shown that the prevailing level of oxygen dictates whether CBF would increase or decrease during activation [50]: low levels of oxygen would indeed result in glycolysis and the production of lactate, which would trigger the PG pathway and vasodilation of the arterioles. On the other hand, in the case of high levels of oxygen, vasoconstriction would occur via activation of the HETE pathway.

This shows how functional activity is tightly coupled to CBF, which is itself tightly related to metabolism. In the normal brain, this is all beautifully orchestrated but if one of the links in this chain is broken, this can have dramatic consequences. This is what happens during brain injury.

## 1.4 Brain injury

There are many causes to brain injury. In most cases, in addition to the damage caused at the moment of injury, brain injury causes *secondary injury*, a variety of processes which include alterations in cerebral blood flow and in the pressure within the skull. These secondary events typically take place in the hours and days following the injury, thus allowing a time window for therapeutic intervention. However, despite possible treatment, a majority of brain injuries are either fatal or result in major disabilities. To date, the known predictors of outcome for brain injury patients (such as hypotension, hypoxia, age, severity of the primary damage, etc.) can explain only 30% of the variance in outcome of brain injury patients. New therapeutic targets have therefore to be sought and this thesis proposes to monitor markers that can detect the development of secondary damage as it happens so as to, at a later stage, stop patients from deteriorating.

### 1.4.1 Focal brain injury

Brain injury includes haernorrhagic and ischaemic stroke, intracranial aneurysm rupture, and impact. The latter is generally referred to as a traumatic brain injury (TBI). Head trauma caused, for example, by vehicle or industrial accidents, is a leading cause of death in youth and middle age. Depending on the impact acceleration forces applied to the brain, TBI is usually sub-classified into two main groups [51]:

- *diffuse brain damage* due to acceleration/deceleration injury types resulting in concussions, diffuse axonal injury or brain swelling,
- *focal brain damage* due to contact injury types resulting in contusion and/or intracranial haemorrhage or haematoma.
1.4. Brain injury

This thesis concentrates on focal brain damage, which includes cases of TBIs but also subarachnoid hemorrhage (SAH) caused by spontaneous rupture of an intracranial aneurysm and spontaneous intracerebral hematoma (ICH) (bleeding in the cerebral parenchyma).

1.4.2 Primary and secondary brain injury

Immediately or within a few minutes after the injury, some brain cells die; this is known as the primary damage or primary insult. It is typically irreversible and refractory or inaccessible to most treatment. Within the subsequent hours or days after the acute event, pathological processes will develop and lead to further neuronal lesions and deteriorations with delayed clinical presentation [52]. This is known as the secondary damage and is treatable. Secondary damage can have a dramatic impact on the patient outcome [53], as seen in the example shown in Figure 1.7 below:

![Figure 1.7: Primary and secondary damages as seen by magnetic resonance imaging (MRI).](image)

Clinicians encounter secondary injury very frequently in patients with subarachnoid hemorrhage (SAH), who develop symptoms of ischaemia after an interval of days, and in patients with traumatic brain injury (TBI), who initially seem to recover, only to deteriorate at a later time.

1.4.3 Ischaemic core and penumbra

Focal ischaemic damage is characterised by complex spatial gradients of ionic, haemodynamic and metabolic disruptions. Within the centre or core of the ischaemic territory, CBF is dramatically reduced and the cells are deprived of vital energy substrates (oxygen and glucose). This causes a rapid depletion of ATP and cell death progresses within minutes [52]. The mechanisms of cell death have been reviewed notably in [51; 53; 55]. Briefly, the loss of energy fuels results in ionic imbalance, excessive excitatory amino acid release and inhibition of re-uptake (for example aspartate...
1.5. Strategies for monitoring the injured brain and prevent secondary injury

and glutamate) [56]. Binding of glutamate to N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors subsequently promotes excessive calcium influx. This accumulation of intracellular calcium triggers a cascade of enzymes which in turn increase the intracellular concentration of free fatty acids and free radicals that ultimately degrade membranes and proteins that are essential for cell survival. The failure of oxygen supply also directly affects the mitochondria (blocking the electron-transport chain) and this leads to the generation of oxygen radicals and release of death-inducing factors. Furthermore, excessive influx of sodium ions in both neurons and glial cells results in the co-transport of chloride ions and water, thus leading to cell swelling and oedema. Finally, the inflammatory cascades in response to ischaemia could further amplify tissue damage. All these factors, oxidative stress, excitotoxicity, energy failure and ionic imbalances are inextricably linked and contribute to ischemic cell death.

However, in the tissue surrounding the core within the flow-compromised territory, insults are milder, due to residual perfusion from collateral blood vessels. This ring of metabolically compromised but yet viable cells was coined the ischaemic penumbra, a term first put forward by Professor Strong and adopted in print for the brain in 1977 [57]. Traditionally, penumbral tissue has been defined based on cerebral perfusion threshold. As CBF decreases below 20 mL/min/100 g of tissue (in the primate brain), electrophysiological function is compromised. With lower perfusion, below 10 mL/min/100 g of tissue, cell membrane potential collapses and viability is lost in minutes without prompt re-perfusion. Between these two thresholds, cells are still salvageable and this defines penumbra [58; 59]. Recently, the definition of penumbra based exclusively on CBF thresholds has been challenged. Some studies have showed that peri-lesion tissue could be defined as tissue under metabolic stress with dysfunctional mitochondria without the necessary condition of a compromised perfusion [60; 61; 62; 63]. This issue is further explored in Chapter 5.

Penumbral tissue is the target of secondary injury with cells slowly dying as the penumbra collapses and is recruited into the core as the lesion expands over time (see Figure 1.7). The goal of this thesis is to monitor this penumbral tissue with suitable markers that can give predictive information about the sickening of peri-infarct tissue and therefore allow medical therapeutic intervention.

1.5 Strategies for monitoring the injured brain and prevent secondary injury

Current strategies to prevent secondary injury are mostly targeted at the neurovasculature. To restore perfusion in ischaemic stroke, thrombolytic therapy has been used: these are agents that break up the blood clot that occludes the arteries supplying the brain [64]. Alternatively, statins can improve vascular reactivity by enhancing NO synthesis from the endothelial cells [65]. However, the use of these treatments must be weighed against the risk of intracerebral haemorrhage and brain oedema.

Other approaches rely on monitoring some aspect of the brain, with the intention of keeping it within some “healthy” range.
1.5. Strategies for monitoring the injured brain and prevent secondary injury

1.5.1 Strategies based on monitoring physical parameters

Most commonly, brain injury patients are treated so as to maintain adequate cerebral blood flow by tight control of the cerebral perfusion pressure (CPP). CPP is the difference between the mean arterial blood pressure (MABP) and the intracranial pressure (ICP). To control CPP, adrenaline and inotropes can be used to increase cardiac output and thus blood pressure (BP). However, such treatment can enormously affect other vital organs, in particular the heart.

An alternative strategy is to control ICP. Under certain conditions, ICP can be reduced by carrying out decompressive craniectomy. The effect of this procedure on clinical outcome is currently under investigation [66]. More commonly, the intra-cranial pressure is continuously monitored to achieve a target CPP above 70 mmHg. This target value is controversial and there is a tendency to revise it to a lower value or to a value that could vary according to individuals [67]. The aim of a tight control of ICP is to maintain the patient in a "safe" zone where CBF is quasi-normal, around \( \approx 60 \) mL/min/100g of tissue. In a normal healthy brain, CBF is maintained in the normal range thanks to a process known as autoregulation. The endothelial cells are capable of adjusting the diameters of the arteries, and thereby the cerebral vascular resistance, to maintain a constant cerebral perfusion within the brain despite changes in blood pressure (or CPP) (Figure 1.8 a). However, in the injured brain, autoregulation is abolished and the CPP has to be controlled within a tight range to achieve normal perfusion (Figure 1.8 b).

![Figure 1.8: Abolishment of autoregulation in the injured brain.](image)

As Figure 1.8 b illustrates, the region of normal CBF is very narrow in brain injury patients. Consequently, this treatment based solely on physical parameters has only been partially successful in preventing secondary lesions. Other strategies for treatment have therefore to be sought.
1.5. Strategies for monitoring the injured brain and prevent secondary injury

1.5.2 Strategies based on monitoring cerebral blood flow

Restoration of adequate cerebral perfusion by directly monitoring and controlling CBF within a normal perfusion is an attractive strategy. This relies on sophisticated techniques to monitor CBF reliably. They are briefly reviewed below.

1.5.2.1 Magnetic Resonance Imaging (MRI)

To date, MRI is the method of choice to image brain injury. The technique exploits the intrinsic nuclear spin properties of protons in H_2O molecules to produce an image of the whole brain in terms of its water density. Using a powerful magnetic field and radio frequency pulses, the local environment of the protons can be obtained, thus yielding very detailed anatomical map of the tissue [68]. Importantly, MRI is a powerful diagnostic tool for brain injury as it can be used to precisely identify regions that are irreversibly damaged (the ischemic core). The penumbral zone can also be delineated: usually it is detected as the mismatch between the infarcted core detected by diffusion-weighted imaging and the hypoperfused region detected by perfusion-weighted imaging [69; 70].

MRI can also be used to visualise blood flow dynamically using a contrast agent. The two main perfusion MR techniques are:

1. bolus tracking (or dynamic susceptibility contrast imaging): it involves intravenous injection of a non diffusible contrast agent, typically a gadolinium-containing agent [54].
2. arterial spin labelling (ASL): it uses magnetically labelled water protons as an endogenous diffusible tracer [71].

Both methods are in a quite early stage of development and are at the moment only used to give information on relative perfusion [72].

The disadvantage of MRI is that the patient has to be inside the scanner for imaging. This can therefore only be used to give information at discontinuous time points during treatment and cannot be an on-going monitoring method. An alternative method is based on Doppler techniques, which give continuous measurements of CBF.

1.5.2.2 Doppler techniques

These measure blood flow based on the principle of the optical Doppler effect: waves emitted at a known frequency towards a moving target (such as the red blood cells) are backscattered at a shifted frequency. The difference in frequency between the incident and the reflected waves is known as the Doppler shift, which can be related to the velocity of the moving target. There are within this technique two main methods: 1) single point measurements using a laser Doppler probe, and 2) an imaging method, laser speckle.

Laser Doppler flowmetry (LDF)

Laser Doppler probes are commercially available. They usually consist of a 0.5–1 mm diameter fiber optic laser probe that is placed in contact with or within brain tissue and conducts scattered light back to a photodetector within the flow-meter sensor. The signal is processed to give a continuous voltage fluctuation versus time, which is linearly proportional to the red blood cell flux. LDF is considered an
1.5. Strategies for monitoring the injured brain and prevent secondary injury

excellent technique for instantaneous, continuous and real-time measurements of regional CBF and has been widely used to monitor neuro-intensive care patients [73; 74; 75]. The main limitations of LDF are that it is not a quantitative measurement and it records from an isolated point in the brain with a small tissue volume (about 1–2 mm³ [76]) and a limited sampling depth (about 500 µm to 1 mm) [77]. It is also prone to artefacts produced by patient movement or probe displacement, which has limited its clinical applicability.

Laser speckle imaging

A speckle refers to the grainy appearance of a diffuse object when illuminated with a coherent laser light [78]. This arises from the fact that the light backscattered from the object will form an interference pattern on the detector due to slightly different optical path lengths, known as the speckle pattern. When the scattering object is a population of particles in motion, these interferences will be de-correlated and the speckle pattern will be less pronounced. The speckle pattern is captured by a CCD camera and its temporal and spatial intensity variations are mathematically manipulated to produce a map of the velocity of the objects illuminated [78; 79]. When illuminating brain surface, this speckle pattern therefore corresponds to relative brain perfusion. This technique has been used to yield real-time dynamic (typically 100–500 ms temporal resolution) two-dimensional images (usually 10 to 25 µm spatial resolution) of CBF over a field of view ranging from 1 millimeter to several centimeters [80; 81]. An application of laser speckle to image the rat brain is discussed in Chapter 6. However, the use of laser speckle is restricted to experimental studies or intra-operative measurements because it requires an open cranial window for measurements.

Doppler techniques in general can only provide relative measurements of brain perfusion. This makes it difficult to assess the "sickness" of the tissue, in terms of perfusion threshold (between healthy and penumbral tissue and between penumbral tissue and ischaemic core) (see section 1.4.3 for further details on these thresholds). The only techniques that give access to absolute blood flow values are clearance techniques.

1.5.2.3 Clearance techniques

These techniques are based on the clearance of a marker from a source in the blood. The marker is inhaled or injected into the blood stream and its concentration in the brain is estimated. The higher the CBF, the faster the marker clears. Calculations are based on a model initially developed by Kety and Schmidt [82]. In their initial study, they used nitrous oxide as a tracer [83]. Today, this method is still used with some modifications. Other tracers that can be directly detected in the brain have been often used. Hydrogen clearance (with electrochemical detection) [84; 85] and fluorescent dyes (such as umbelliferone) [86] have been used experimentally.

Clinically, thermal clearance probes can be used. They rely on the tissue's ability to transport heat. A mathematical model can de-correlate the convective effects caused by tissue blood flow from the contributions from the intrinsic conductive properties [87]. New designs are based on implantable thermistors: a distal thermistor is heated to approximately 2°C above the tissue baseline temperature and establishes a constant spherical temperature field approximately 4 mm in diameter. The proximal sensor, which is located outside the thermal field, monitors tissue temperature and compensates
1.5. Strategies for monitoring the injured brain and prevent secondary injury

for baseline fluctuations. The power dissipated by the heated thermistor to maintain a constant temperature field provides a direct measure of the tissue's ability to transport heat [88]. The use of thermal clearance probes clinically is quite recent and problems such as movement artefacts, pyrexia and the effects of heat on local tissue are currently investigated.

1.5.2.4 Limitations of approaches based on cerebral blood flow

There is currently no monitoring method that can reliably measure absolute blood flow continuously (thermal clearance still being under validation). It is therefore very difficult to control CBF dynamically and quantitatively as the injury evolves. Furthermore, even if perfusion is known, it does not indicate whether CBF is sufficient to meet the needs of the cells. It does not give information on whether the cells have a sufficient supply of energy substrates to function normally.

1.5.3 Strategies based on monitoring brain activity

Techniques that can measure brain activity have instead been extensively used clinically to detect some pathophysiological processes and predict patients prognosis. They are mainly based on electrophysiological recordings, either from the surface of the cortex, known as electrocorticography (ECoG), or from the scalp, known as electro-encephalography (EEG). Based on the frequency and the pattern of the electrical recordings, abnormal activity associated with pathological events can be identified. Other markers of brain activity include neurotransmitters and ions involved in neuronal depolarisation.

1.5.3.1 Electrophysiological recordings

a. Origin of the electrophysiological signals

When the neurones generate action potentials, a sharp electrical spike (from resting potential of about $-65$ mV to $+40$ mV) is fired. One action potential lasts about 1 millisecond but action potentials can be conducted down the axon at a rate of 1 to 100 m/s. By placing a 1 µm-tip microelectrode in the extracellular space, just adjacent to the firing neurones (within a few micrometer), these action potentials can be recorded. They will appear as a train of spikes with an amplitude of a few millivolts (the amplitude of the signal decreases with the distance from the firing neurone). These are known as single- or multiple-unit activity, depending on whether the spikes are recorded from one single neurone or multiple cells respectively.

When an electrode is placed further away in the extracellular space, it will measure the current flows resulting from action potentials and neuro-transmission. When an action potential is generated, or when the neuro-transmitter binds to the post-synaptic receptors, the inflow of Na$^+$ into the active sites of a neuron appears as a current sink (inward currents); the current flows down the core of the dendrites or axon, and because of the continuity of current, inactive membrane sites act as a source (outward currents) for the active regions [89]. Due to the resistive properties of the extracellular space, this ionic flow causes a potential change that can be measured: this is the extracellular field potential. The extracellular field potential is composed of high frequency components (300–400 Hz), the multiple-unit activity, and the low-frequency components (below 300 Hz), the local field potential [90].
1.5. Strategies for monitoring the injured brain and prevent secondary injury

These types of extracellular recordings of the neuronal activity have been widely used experimentally and they are possible in the human brain when deep electrodes are implanted, such as in patients treated for epilepsy or Parkinson’s disease [91; 92; 93]. In the case of brain injury patients, techniques that are minimally invasive are preferred.

b. ECoG and EEG

Both methods measure the integrated extracellular field potential of mostly cortical pyramidal cells after attenuation and distortion by several layers of cerebral cortex, cerebrospinal fluid, pia mater, and arachnoid mater for ECoG and additionally dura, skull and scalp for EEG. As a consequence, the amplitude of ECoG recordings is usually in the order of about 10 mV whereas the amplitude of EEG potentials is in the order of 10 to 100 µV [94].

The main limitation of ECoG electrodes is that they are invasive and restricted to patients who require craniotomy (surgical removal of a part of the skull) to expose brain surface and implant the electrodes just below the dura mater. Subdural electrodes are usually arranged in a strip or grid pattern of various dimensions, having anywhere from 4 to 64 electrode contacts, usually in platinum or stainless steel, each typically 5 mm in diameter and spaced by 10 mm. Grid electrodes give better spatial resolution whereas strip electrodes can be removed without further surgery.

Contrary to ECoG, EEG is non invasive with electrodes placed directly on the scalp. They are usually caps or nets into which high-density arrays of electrodes (up to 256) are embedded. Since it is non invasive, EEG has a wide range of clinical applications, including the continuous monitoring of brain injury patients [62]. One disadvantage of EEG is the effect of the additional smearing of the signal caused by the skull and scalp. The direct consequence is that high frequency components that can be clearly seen in ECoG recording cannot be detected by EEG. In particular, EEG has a worse spatial localisation of the electrical activity. This means that it is difficult to identify electrical activity that originate from penumbral tissue as opposed to healthy tissue further away from the focal lesion.

In this thesis, we have used ECoG detection of spontaneous electrical events that develop in penumbral tissue. This will be described in Chapter 5.

1.5.3.2 Measurement of neurotransmitters

Other obvious markers of neuronal activity are neurotransmitters. There are three main techniques to measure neurotransmitters.

a. Microdialysis

Microdialysis is described in more detail in section 1.6.1. Briefly, it involves sampling chemical species from the extracellular space through an implanted probe [95]. Using this technique, glutamate [96], GABA [97] and dopamine [98] have been measured. The accumulation of glutamate in the extracellular space and its neuro-toxicity have been demonstrated using microdialysis [96]. However, measurement of glutamate with microdialysis has been controversial. This is because neither the increase nor the basal level of glutamate is blocked when local neurotransmission is stopped using the drug tetrodotoxin (TTX) [99; 100]. This suggests that microdialysate glutamate does not purely stem from synaptic activity but possibly from astrocytic spillover into the extracellular space [97].
1.5. Strategies for monitoring the injured brain and prevent secondary injury

b. In vivo biosensors for neurotransmitters

The glutamate signal seen in the brain that is sensitive to TTX comes from a 7 µm carbon fibre biosensor that can be placed sufficiently close to glutamate releasing sites [101]. There are however several issues with implanted biosensors that have restricted their use to experimental model and these are further discussed in section 1.5.4.2.

c. In vivo voltammetry

This technique involves applying voltage wave-forms to a microelectrode at a very high scan rate and measuring the resulting current passed. This has been used to measure dopamine in animals, allowing a sub-second temporal resolution and the study of behaviour [102]. One difficulty with this method is that the signals are very small and it requires subtraction of a large background current to extract the different contributions to the signal and reveal the characteristic oxidation and reduction peaks [103]. In vivo voltammetry has yet to be applied to the human brain. Difficulties notably include the fragility of the implanted carbon fibres.

1.5.3.3 Measurement of ions associated with neuronal depolarisation

The other chemical markers of cell membrane potential and therefore neuronal activity are ions, in particular potassium. Detection approaches are based on the selective binding of the ion by a ligand, known as the ionophore or ion carrier. Among the ionophore-based sensors, the most widely forms of transduction used are potentiometry and optical methods such as absorbance or fluorescence.

a. Ion-selective electrodes (ISEs)

The potentiometric detection of ions is based on ion-selective electrodes (ISEs). They are routinely incorporated into clinical analyzers for measurements of potassium, sodium, pH, etc. in serum and whole blood samples. Direct implantation of these electrodes into brain parenchyma is also possible [104]. It has been restricted to animal experiments for reasons of toxicity of the ionophore and polymer membranes. One solution is then to measure the ions from microdialysis samples and the development of such an ISE is the object of Chapter 6.

b. Optical methods

Detection of ions by optical methods include a whole range of dyes that can be loaded into cells and that selectively change their fluorescence or bioluminescence with the concentration of ions, such as calcium [105], sodium [106] and recently magnesium [107]. Dyes have also been encapsulated into nanosized sensors (20–100 nm) for intracellular measurements: these are known as PEBBLEs (Photonic Explorers for Bioanalysis with Biologically Localised Embedding) and have been used for calcium, potassium, magnesium, pH and other metabolites [108; 109; 110]. Problems with these measurements include ion interferences, non-specific binding of dye, autofluorescence, photodamage and photobleaching of the fluorescent indicators that all lead to erroneous measurement. Their use has been restricted to experimental models because of the possible risks of cytotoxicity of the dyes, the requirement of heavy instrumentation and of exposed cortex for detection.
1.5. Strategies for monitoring the injured brain and prevent secondary injury

1.5.3.4 Limitations of approaches based on brain activity solely

Measurement of neurotransmitters and ions is not feasible in the human as yet. However, electrophysiological recordings have widely been used as an aid to guide therapeutic treatments. One issue is that abnormal activity or large increases in neurotransmitters usually imply that local areas of cells have died. Although this information is very important, its predictive value is limited. It is good to have information about cells dying, but it is even better to have information that can predict it. It is our view that local availability of energy is so sensitive to both supply and local use that it can serve as a useful predictive marker of brain deterioration.

1.5.4 Strategies based on monitoring brain metabolism

The two main substrates of energy metabolism are glucose and oxygen. Various methods can be used to monitor their levels in the brain.

1.5.4.1 Measurement of markers of oxygen metabolism

Functional MRI and BOLD

Based on the same physical principles as MRI, functional MRI (fMRI) uses exogenously administered highly paramagnetic substances as contrast agents to produce transient changes in the MRI image as the agent passes through the brain. It is then possible to map both anatomy and changes in brain activity produced by functional activation [111]. This technique requires the administration of a contrast agent and has now been superseded by blood-oxygenation-level-dependent signal (BOLD).

BOLD is basically fMRI but using an endogenous agent to map brain activity: deoxyhemoglobin [112]. When the blood hemoglobin loses its oxygen, it becomes paramagnetic, which means it interferes with the magnetic field during MRI, creating a dip in the MR signals emitted by water protons [113]. Following activation, blood flow increases, bringing more oxygen to the activated region. This causes the relative percentage of deoxyhemoglobin to decrease, and therefore the BOLD signal to increase in the activated region. Since BOLD is completely non invasive, has a high spatiotemporal resolution, it has been extensively used in a wide range of cognitive studies [7; 68; 114].

However, the interpretation of the BOLD signal has been very controversial. First, the BOLD signal is minute (peaking at 1 to 3% of the total signal). This means that BOLD studies usually represent the average of many subjects with many trials per condition to reach an adequate signal to noise ratio [115]. Secondly, the origin of the BOLD signal is complex. It stems from the balance between oxygen consumption and oxygen supply locally, but is also depends on regional cerebral blood flow and cerebral blood volume [7; 116]. The interplay between these three parameters is complicated and has led to many controversies about findings using BOLD. Examples include the ongoing discussion surrounding the initial dip just preceding the positive BOLD response [90]. One major limitation for using BOLD in brain injury patients is that the BOLD signal is the result of neurometabolic coupling. However, in brain injury patients, this coupling is altered and many assumptions used to interpret the BOLD signal are not tenable. The other complication is that the patient has to be in a scanner for imaging, which is always a risk.
Near-infrared spectroscopy (NIRS)

Transcranial NIRS is a non-invasive measurement of brain oxygenation at the patient bedside: changes in the absorption patterns of near infrared light at specific wavelengths can be related to the concentrations of oxyhaemoglobin (oxHb) and deoxyhaemoglobin (deoxHb) in cerebral tissue [117]. Some monitoring NIRS system have been used in brain injury patients [117; 118]. They usually consist in two optodes (fibre optical bundles) and rely on spectral analysis and sophisticated mathematical algorithms to calculate the concentrations of oxHb and deoxHb based on the absorption and scattering of the infrared photons by the biological tissues. However, these algorithms are usually not robust enough, in particular in brain injury patients, and they have been mainly used as a relative measurement of blood oxygenation [119].

The main difficulty with NIRS in the intensive care unit is that it is highly susceptible to interference from ambient light and movement artefacts. The requirement of heavy and tight light shielding bandages has made it quite unpopular with the nursing staff and relatives.

Implanted electrodes for measurement of oxygen tension

Implantable oxygen electrodes are commercially available (Licox probes) and have been used to monitor brain injury patients. They are based on the Clark-type polarographic method whereby a permselective membrane separating an internal filling solution and the exterior solution is used to detect oxygen amperometrically at a platinum electrode. A potential is applied to the electrode to reduce oxygen under diffusion-controlled condition and the current that flows is proportional to the concentration of oxygen present [120]. In some clinical studies, the licox probes have helped detect periods of low oxygen tension in penumbral tissue, which has been associated with poor patient outcome [121; 122].

Limitations of approaches based on oxygen metabolism

Clearly, measurement of oxygen levels does have a role in detecting gross pathological changes, such as failure of local blood supply. However, oxygen may not always be a good marker for energy metabolism, in particular for energy utilisation. An important study by Fox and Raichle has indeed showed that, during stimulation, the increases in blood flow (by 50%) and glucose uptake (by 51%) were matched, whereas the oxygen consumption increased by only 5% [123; 124].

1.5.4.2 Measurement of markers of glucose metabolism

A better marker of brain energy metabolism is glucose. Its concentration in the extracellular space reflects the balance between supply by the blood and utilisation by active neurones. Lactate can also be used as a surrogate marker of oxygen.

Positron emission tomography (PET)

PET is a molecular imaging technology based on the detection of positron-emitting tracers by an external camera. An analogue of glucose is labeled with a radioisotope, $^{18}$F-2-fluoro-2-deoxy-D-glucose (FDG). Once injected in the subjects, it enters the brain using the same carrier system as glucose. It is then phosphorilated by hexokinase, the first reaction involved in glycolysis. The end product, FDG-6-phosphate, can neither be used as a substrate for subsequent reactions within
the glycolytic pathway nor exit the cells [13]. The tracer thus accumulates in metabolically active structures [10; 21; 125]. As the radionuclide decays, it emits positrons which annihilate with electrons to produce gamma rays. A PET scanner detects these gamma rays throughout the brain and various algorithms and models generate a three-dimensional map of the cerebral metabolic rate of glucose (CMRglu) [126].

PET has been widely used and has recently suggested that penumbral tissue could be metabolically impaired despite the absence of markers of ischaemia marker [63; 127].

However, similarly to MRI techniques, PET cannot be used as a continuous monitoring technique and needs patients to be in a scanner. Furthermore, it has a poor spatial resolution (compared to MRI) and acquisition times are usually long (30–40 minutes), limiting its use to detect dynamic events. It also requires a whole infrastructure to synthesise and manipulate the radioactive agents, which has made this technique limited to very few centres.

Implanted biosensors for metabolism
An alternative method is the use of electrochemical sensors directly implanted in brain tissue. The detection of glucose (and lactate) invariably relies on the use of enzymes that selectively react with the biological substrate. The challenge is the transduction of the enzymatic biochemical reaction into a measurable signal. This is generally achieved by measuring hydrogen peroxide (H$_2$O$_2$), a natural product of the enzymatic reaction [128; 129; 130]. The difficulty is then to eliminate endogenous interferents (notably ascorbate present at a concentration of 100 to 600 µM) that can be oxidised at a similar potential. Several strategies have been successful for in vivo measurement of glucose, lactate and glutamate notably. They include:

• the use of layers of polymer films to exclude interferents on the basis of size and charge (Nafion, cellulose acetate [101; 131], or phenyl [132]),
• the pre-oxidation of ascorbate [101; 131; 132],
• the subtraction of a blank electrode signal [133],
• and electrochemical mediation with a co-immobilised secondary enzyme [134; 135] or with a redox polymer [101].

Implanted biosensors have proved very useful in acute measurements in animal experiments [85; 136].

However, there are major issues with implanted biosensors. Firstly, they cannot be calibrated in situ. Consequently, any loss of sensitivity due to biological fouling of the electrode surface or loss of enzyme activity cannot be estimated and drifts are often observed after a few hours of implantation. In addition, they are invariably sensitive to physiologically-induced alterations of the interstitial environment, such as the partial pressure of oxygen, temperature and pH. These variables can be controlled experimentally but they are very likely to change spontaneously in brain injury patients and the signal is then difficult to interpret. Critically, their use in the human brain has been prevented, mainly because of the toxicity of products, such as H$_2$O$_2$, or mediators if leakage to the surrounding tissue. Furthermore, the requirement of sterility for use in the human brain is very difficult to achieve without destroying the enzymes used for detection. Hence, their use in the human brain has so far been impossible.
Microdialysis (MD)

An alternative approach is to couple detectors and biosensors to microdialysis sampling (see section 1.6.1 for more details about the technique). Although microdialysis suffers from a worse temporal (typically 600 seconds) and spatial (typically 0.1 cm$^3$) resolution than an implanted sensor, it can be coupled to various analytical techniques and therefore virtually allows the detection of any neurochemicals. Since the detection is ex vivo, there are no problems with biocompatibility and toxicity. Additionally, the detectors can be regularly calibrated to give a reliable continuous measurement. Importantly, MD probes are the only FDA approved technique for sampling the extracellular fluid in the human brain. Many clinical studies have used microdialysis to measure the concentrations of markers of glucose metabolism, in particular glucose, pyruvate and lactate.

The most common index is in fact the lactate/pyruvate ratio. It is believed to reflect the redox state of the cells and therefore mitochondrial (dys)function. An increase of the MD lactate/pyruvate ratio has been associated with severe hypoxic/ischaemic episodes in many studies in traumatic brain injury patients and subarachnoid hemorrhage patients [62; 137; 138; 140; 141]. However, we believe that the use of the lactate/pyruvate ratio as a marker for energy metabolism may be an over-interpretation. A study by Nortje et al. [142] showed that increased oxygen tension did not cause a significant (expected) reduction of the lactate/pyruvate ratio. Samuelsson et al. [143] also suggested that pyruvate levels may rather discriminate whether or not there is sufficient astrocytic capacity for glutamate-glutamine cycling in the brain. Consequently, elevated lactate/pyruvate ratios cannot always be interpreted as failing energy metabolism. From an analytical point of view, one important issue with the lactate/pyruvate ratio is that this ratio is highly sensitive to errors in measurements of pyruvate levels. Resting pyruvate levels are typically more than ten times lower than lactate levels and a pyruvate decrease simultaneously with a lactate increase exacerbates the sensitivity to errors.

Instead, extracellular glucose has been shown to be a reliable marker of physiological activity because of the tight coupling between blood supply and brain utilisation [144; 145]. Studies in traumatic brain injury patients showed that dialysate lactate increase and glucose depletion were associated with episodes of severe global hypoxia/ischaemia [146]. However, Cesarini et al. [147] showed that low glucose levels were correlated with a favourable outcome, and a year later, Vespa et al. showed that prolonged periods of low glucose levels were clearly associated with bad patient outcome [61]. These findings have left many clinicians sceptical about the clinical value of microdialysis. We suggest that one main limitation of these MD studies is the poor time resolution of the microdialysis analysis. This has been indirectly reported in a study by Kett-White et al. [148]. In their study, the MD markers of glucose metabolism missed or appeared later than hypoxic/ischaemic events detected by a continuous multi-modal sensor. This was because the duration of the hypoxic events was too short to be detected with a low time resolution microdialysis analysis.

In this context, we propose to couple microdialysis to high-time resolution sensors to track dynamic changes in brain energy metabolism in brain injury patients. The analyser is known as rapid-sampling microdialysis (rsMD) and more details about this assay and our approach are discussed in section 1.6 and Chapter 2.
1.5. Strategies for monitoring the injured brain and prevent secondary injury

1.5.5 Summary

The advantages and disadvantages of the monitoring techniques discussed in this section are summarised in Table 1.1. An ideal technique would need to have a high time resolution, a high spatial resolution, while providing quantitative information with no risk for the patient. As Table 1.1 shows, there is no such technique available as yet.
1.5. Strategies for monitoring the injured brain and prevent secondary injury

Table 1.1: Summary of the different methods to monitor the injured brain. Cells in green indicate advantages of the technique for monitoring secondary brain injury. Cells in red indicate that the disadvantages of the method. ICP: intra-cranial pressure. rsMD: rapid-sampling microdialysis.

<table>
<thead>
<tr>
<th>Measured parameter</th>
<th>Measurement method</th>
<th>Clinical use</th>
<th>Patient risk</th>
<th>Quantitative</th>
<th>Spatial resolution</th>
<th>Temporal resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP</td>
<td>Pressure transducer</td>
<td>ICU</td>
<td>invasive</td>
<td>yes</td>
<td>single site</td>
<td>continuous</td>
</tr>
<tr>
<td></td>
<td>MRI</td>
<td>scanner</td>
<td>tracer</td>
<td>no</td>
<td>3D, ≈1mm</td>
<td>poor</td>
</tr>
<tr>
<td>CBF</td>
<td>Laser Doppler Flowmetry</td>
<td>ICU</td>
<td>invasive</td>
<td>no</td>
<td>single site</td>
<td>continuous</td>
</tr>
<tr>
<td></td>
<td>Laser Speckle Imaging</td>
<td>no</td>
<td>exposed brain</td>
<td>no</td>
<td>2D, 10 to 25 µm</td>
<td>100-500 ms</td>
</tr>
<tr>
<td></td>
<td>Thermal clearance</td>
<td>ICU</td>
<td>invasive</td>
<td>possibly</td>
<td>single site</td>
<td>continuous</td>
</tr>
<tr>
<td>Electrical activity</td>
<td>ECoG</td>
<td>ICU</td>
<td>invasive</td>
<td>yes</td>
<td>≈1 cm</td>
<td>continuous</td>
</tr>
<tr>
<td></td>
<td>EEG</td>
<td>ICU</td>
<td>none</td>
<td>yes</td>
<td>poor</td>
<td>continuous</td>
</tr>
<tr>
<td>Synaptic activity</td>
<td>Sensors for neurotransmitters</td>
<td>no</td>
<td>invasive</td>
<td>yes</td>
<td>single site</td>
<td>milliseconds</td>
</tr>
<tr>
<td>Ion fluxes</td>
<td>Ion selective electrodes</td>
<td>no</td>
<td>invasive</td>
<td>yes</td>
<td>single site</td>
<td>seconds</td>
</tr>
<tr>
<td>Metabolic coupling</td>
<td>fMRI/BOLD</td>
<td>scanner</td>
<td>none</td>
<td>no</td>
<td>3D, ≈1mm</td>
<td>poor</td>
</tr>
<tr>
<td>Oxygen saturation</td>
<td>NIRS</td>
<td>ICU</td>
<td>none</td>
<td>disputable</td>
<td>poor</td>
<td>possibly continuous</td>
</tr>
<tr>
<td>Oxygen tension</td>
<td>O₂ electrodes</td>
<td>ICU</td>
<td>invasive</td>
<td>yes</td>
<td>single-point</td>
<td>continuous</td>
</tr>
<tr>
<td>Glucose utilisation</td>
<td>PET</td>
<td>scanner</td>
<td>radioactive tracer</td>
<td>yes</td>
<td>6 to 10 mm</td>
<td>poor</td>
</tr>
<tr>
<td>Glucose metabolism</td>
<td>Implanted biosensor</td>
<td>no</td>
<td>invasive</td>
<td>yes</td>
<td>single site</td>
<td>continuous but drift</td>
</tr>
<tr>
<td></td>
<td>Microdialysis</td>
<td>ICU</td>
<td>invasive</td>
<td>semi-quantitative</td>
<td>single-site</td>
<td>30 to 60 minutes</td>
</tr>
<tr>
<td></td>
<td>rsMD</td>
<td>ICU</td>
<td>invasive</td>
<td>semi-quantitative</td>
<td>single-site</td>
<td>30 to 60 seconds</td>
</tr>
</tbody>
</table>
1.6. Our approach

All the techniques with a red cell in Table 1.1 can be ruled out, as not suitable for human monitoring or for measuring dynamic events as the injury evolves. This leaves us with a few techniques that can be used to monitor secondary brain injury: ICP, thermal clearance, ECoG, tissue oxygen electrodes and rsMD.

As discussed in section 1.5.1, monitoring ICP has only had a limited impact to prevent secondary damage so far. Thermal clearance would be ideal to measure CBF and we have done it in some of the most recent patients monitored during this PhD (data not shown) but this technique still needs to be validated in a clinical setting. Tissue oxygen electrodes can be used (as in Patient 9 from Chapter 5), but brain oxygen may not be so tightly coupled to brain activity. This leaves us with ECoG and rsMD. This is the approach we have used to monitor brain injury patients.

1.6 Our approach: rsMD monitoring of markers of glucose metabolism coupled with brain activity

Rapid sampling microdialysis (rsMD) allows measurements of glucose and lactate from microdialysis samples with a high time resolution (30 to 60 seconds). Since markers of glucose metabolism are difficult to interpret on their own, we have coupled these measurements with ECoG recordings to assess brain activity. ECoG has already been presented in section 1.5.3.1 and more details will also be given in Chapter 5. We here give more details about the rapid-sampling microdialysis assay.

1.6.1 Principle of Microdialysis

Brain MD probes generally consist of a fine double lumen probe, sheathed at its tip in a semi-permeable hollow fiber membrane that ranges between 200 to 500 µm for its external diameter and 1 to 30 mm in length. The interior of the MD membrane is steadily perfused typically at 0.1 to 3.0 µL/min. As the perfusate flows pass the membrane, a concentration gradient is established across the MD membrane between the extracellular fluid and the dialysis lumen. This gradient results in the diffusion of analytes of interest from the extracellular space across the membrane into the dialysate (see Figure 1.9).

The MD membrane has a molecular-weight cutoff (typically from 15 kDa for neurotransmitters or energy metabolites to 100 kDa for peptides and proteins) that allows only molecules small enough to pass across the membrane. This excludes many of the components from the ECF and provides a sterile microdialysate (with no cellular debris, etc.) for chemical analysis.
1.6. Our approach

Figure 1.9: Microdialysis sampling. The tip of the microdialysis catheter is inserted into brain tissue. Molecules in the tissue diffuse through the tortuous extracellular space to reach the MD probe membrane. Only molecules below the cutoff go through and equilibrate with the perfusion fluid. The resulting dialysate concentration reflects the balance between the supply (by the blood and/or by the cells) and the utilisation by the cells (or clearance by the blood) of the analyte. Taken from [95].

The perfusate is a saline solution that ionically and osmotically resembles the brain extracellular fluid both to maintain fluid balance as well as ion balance across the dialysis membrane (details about this solution are given in section 2.1.3). Brain tissue is indeed highly sensitive to ionic concentration alterations, since such changes can alter neurotransmitter release and energy metabolism [149]. It is also important to limit liquid motion across the membrane. This can be due to hydrostatic pressure effects that can result in ultrafiltration. Alternatively, osmotic effects by diffusion of water through the membrane can be a problem. The risk is greater for large molecular weight cut-off probes and osmotic balancing agents such as dextrans or albumin are commonly added to microdilaysis perfusion fluid to prevent this effect [150].

1.6.2 Issues with Microdialysis

The major issues with MD sampling are related to trauma. This is mainly due to the large size and rigidity of the probes [151]. The damage caused by implantation of the MD probe not only puts increased stress on an already compromised area in a brain injury patient, but can also bias the sampling of the MD probe. The relationship between dialysate and true extracellular concentrations, called the microdialysis recovery, is indeed quite complicated. One way to exclude changes in MD levels that are due to fluctuations in the recovery of the MD probe (and not to real tissue changes) is to measure two species whose concentrations change in opposite directions under physiological conditions, such as glucose and lactate for example. Issues related to trauma and recovery are further discussed in sections 5.4.7.4 and 5.4.7.6.
1.6. Our approach

1.6.3 Analysis of the microdialysate

During the early stages of microdialysis sampling, the primary analytical detection methods used for analyte quantification were liquid chromatography coupled with various types of detectors. Liquid chromatographic separations methods are well suited to microdialysis samples because of the high salt content of the perfusion fluids. Salts are generally not retained by the LC stationary phase and are therefore eluted in the chromatographic void volume. For neuropeptides and proteins whose concentrations are usually in the ng/mL to pg/mL levels, more sensitive techniques, such as radioimmunoassays or liquid chromatography coupled to tandem mass spectroscopy (LC-MS2) can be used [152]. These analytical techniques for MD sampling have been reviewed in [150; 153]. These methods require sophisticated and expensive instrumentation and are invariably offline.

More recently, MD samples have been analysed with offline bedside analysers commercially available from CMA. They are based on enzyme detection and absorbance kinetics of a colored substance [154; 155] and are therefore very selective. They can perform multi-analyte analyses of glucose, lactate, pyruvate, urea, glycerol and glutamate. However there are still two main issues with these types of bedside offline analysers. Firstly, the time resolution is, at best, 15 to 30 minutes. This allows studies of long-term metabolic changes but is not sufficient to observe any event that may occur more dynamically. Secondly, it requires collection of sample vials that are connected to the end of the MD catheter and then placed onto the analyser. The sample problem seems trivial but in a clinical setting, this is much more problematic: samples are collected by very busy nursing/clinical staff, who are not analytical scientists and whose primary focus is patient care. Fifteen minute sampling gives 480 samples over a typical 5-day recording session. Finally, if the samples have to be stored for further offline analysis, a single error in sequence destroys the time line. The sample-collecting burden can quickly become intolerable at faster sampling rates or with multiple probes.

1.6.4 Online microdialysis

Online sampling of the microdialysis flow stream removes all these problems, allowing direct analysis of the dialysis stream, with no sample collection, and with improved time resolution. Another advantage of on-line monitoring is that the results can be continuously displayed for use by the clinical staff in guiding patient care.

The first online microdialysis detectors coupled enzymatic detection (dehydrogenase) with NADH detection by fluorescence: while oxidising the substrate, the dehydrogenase reduces NAD$^+$ present in excess in the assay [156; 157]. This has been used for detection of lactate and glutamate. However, it requires dilution of the microdialysate sample, and therefore suffers from a low sensitivity. Since highly sensitive fluorometers are cumbersome, they cannot be used at the patient bedside and their use is limited to experimental studies.

Alternatively, microdialysis has been coupled to amperometric biosensors. A landmark analyser developed by Boutelle et al. allows online detection of glucose and lactate [158]. To date, this is the only reliable on-line translational system that can be used both experimentally [159; 160; 161] and clinically [74; 162; 163; 164; 165] to monitor glucose and lactate at a 15-30 seconds time resolution. It is known as rapid sampling microdialysis (rsMD).
1.6. Our approach

1.6.5 rsMD: a second generation biosensor

The rsMD analyser is an enzyme based electrochemical sensor. It offers high specificity, a sufficient time resolution to resolve transient dynamic events, and stability and reproducibility for continuous use up to five days.

1.6.5.1 Bio-recognition

Two oxidase enzymes, glucose oxidase (GOx) and lactate oxidase (LOx), are used to selectively react with glucose and lactate respectively, according to the equilibria below:

\[ \beta-D-glucose + O_2 \xrightleftharpoons[GOx+Fd]{\text{GOx}} \text{gluconolactone} + H_2O_2 \]

and

\[ \text{Lactate} + O_2 \xrightleftharpoons[LOx]{\text{LOx}} \text{Pyruvate} + H_2O_2 \]

GOx and LOx oxidise their substrates and return to their active oxidised state by transferring electrons to molecular oxygen (O\(_2\)), resulting in the production of hydrogen peroxide (H\(_2\)O\(_2\)).

As oxidases undergo a reduction during the above reaction, it is theoretically possible to assay the substrate concentration by directly measuring the redox state of the enzymes. However, this is limited to a restricted number of small enzymes, mainly peroxidases and laccases [166]. In the case of GOx [167; 168] and LOx [169], the redox center is embedded more than 2 nm from the surface, which prevents electron tunneling to the surface of the electrode.

Alternatively, H\(_2\)O\(_2\) can be directly detected by oxidation on platinum at a potential of about 0.6 V vs silver/silver chloride (Ag/AgCl). However, because of the high oxidation potential, this is very susceptible to interference from other oxidisable components in the dialysate, such as ascorbate. Several approaches can be used to limit this interference, and some have been discussed in section 1.5.4.2. On the other hand, it is not practical to reduce H\(_2\)O\(_2\) because of its slow electrochemistry and its reduction potential around the same as that of O\(_2\). It has been achieved though, by modifying the electrode surface to increase the effective electrocatalytic surface area [170; 171]. A simpler solution is to reduce H\(_2\)O\(_2\) by a secondary enzyme with a high affinity for H\(_2\)O\(_2\) itself.

1.6.5.2 Transduction

Hydrogen peroxide can be selectively reduced to form water by horseradish peroxidase (HRP). The regeneration of HRP (gain of 2 electrons) can be achieved using a number of molecules, including dyes (such as Amplex Red) and electroactive molecules, such as ferrocene (Fc) and its derivatives [134; 135]. Ferrocene can shuttle electrons from the redox centre of the enzyme to the surface of an electrode: it reduces HRP in two separate steps to regenerate the active enzyme and is thereby oxidised according to the following equilibrium:

\[ H_2O_2 + 2Fc^{2+} \xrightleftharpoons[HRP]{\text{HRP}} H_2O + 2Fc^{3+} \]
1.7. Outline of this thesis

The oxidised species Fc\(^{3+}\) then diffuses to the electrode surface where it is detected by reduction at a low potential of 0.0 mV vs Ag/AgCl. This has been shown to be effectively immune to oxidisable interferents in brain dialysate for glucose and lactate [172]. Ferrocene is a good mediator since it has a very fast outer sphere electron transfer kinetics and its reduced form of ferrocene monocarboxylic acid, Fc\(^{2+}\), is stable enough to be used in a flow injection analysis (FIA) stream [172; 173]. As the dialysate stream is injected into the continuous accelerated flow of ferrocene buffer, these are the reactions that take place:

As ferrocene is reduced at the electrode surface, a current of a few µA is generated. Practical details about the rsMD assay are given in Chapter 2.

1.7 Outline of this thesis

This thesis starts in Chapter 2 by outlining common materials and methods used for the online microdialysis assay, for the in vitro work described in Chapter 6, and for data analysis of all the data presented in this thesis.

Chapter 3 describes the design and characterisation of miniaturised electronic instrumentation for the rsMD assay.

Chapter 4 presents generic signal processing algorithms that were developed to remove three classes of noise: spikes, non-stationary ripples and slow baseline drift. These were applied for de-noising all clinical and experimental data presented in Chapter 5 and 6.

Chapter 5 uses the signal processing techniques to quantify online microdialysis data from brain injury patients monitored before and during my PhD in collaboration with Professor Anthony Strong of King’s College hospital in London. The metabolic response to spontaneously occurring spreading depolarisations recorded by electrocorticography was investigated.

Chapter 6 describes the engineering of a flow-cell for measurements of potassium ions in the microdialysate stream. This was based on a potassium ion-selective membrane already optimised within the Boutelle group [174]. A new electrode body and a flow-cell using standard HPLC fittings were adapted and optimised for this purpose. They were characterised and applied in pilot experiments in vivo in collaboration with Professor Rudolf Graf of the Max Planck Institute for Neurological research in Cologne.

Chapter 7 concludes the thesis and proposes future work and directions.
Chapter 2

Common Materials and Methods

This chapter describes the common experimental methods (mainly the rapid sampling microdialysis (rsMD) assay for glucose and lactate) and common statistical tools used for analysing the data presented in the rest of the thesis.

2.1 Clinical microdialysis probe and adaptation for online analysis

2.1.1 Microdialysis probe

For clinical monitoring, we used a sterile single-use clinical microdialysis probe with 10-mm membrane and 20,000 Daltons cut-off (CMA 70, 250 µm tip diameter, 60 mm shaft, CMA Microdialysis, Sweden). Towards the completion of surgery, the MD probe was inserted obliquely into the cortex, to full membrane depth through a minimal pial incision. The position of the probe was checked on post-operative CT (gold thread at the MD probe tip). Figure 2.1 is a picture of the clinical microdialysis catheter:

![Clinical microdialysis catheter](image)

**Figure 2.1: Clinical microdialysis catheter.** The microdialysis probe is inserted into the brain tissue. It is perfused with sterile artificial cerebro-spinal fluid (aCSF) through its inlet tube. After equilibrium with the tissue through the dialysis membrane, the dialysate travels via the outlet tubing to the rsMD assay. Taken and modified from [95].

The MD probe was continuously perfused with sterile artificial cerebro-spinal fluid (aCSF) (CMA perfusion fluid CNS, Sweden) at 2 µL/min using a CMA 100 microinjection syringe pump (CMA 100, CMA Microdialysis, Sweden). The composition of the aCSF is identical to that described in section
2.2. Rapid-sampling microdialysis (rsMD)

2.1.3 below. The perfusion flow rate was chosen as a good trade-off between adequate recovery, overfilling of the sample loop and fast dialysate transit along the rsMD connection tubing.

2.1.2 Connection to the rsMD assay

The outlet tubing of the MD probe was adapted to connect to a dual online assay system. Typically, a one-meter length of low volume (1.2 µL per 10 cm length) connection tubing (FEP Tubing 4001005, Microbiotech, Sweden) was used between the patient and the rsMD assay. A tubing adapter (4001036, Microbiotech, Sweden) connected the FEP tubing to an injection needle (RN NDL 6/PK (22s/2°/3)L, Hamilton, Switzerland) placed into the injection port of the rsMD valve. The one-meter length of FEP tubing is necessary to isolate the patient from the assay equipment (electrical impedance and mains leakage tested by a duly authorised third party) and also facilitate patient movement and nurse care.

Both inlet and outlet tubing are primed before use with aCSF to ensure there is no blockage or back-pressure throughout the system. After use, the outlet tubing is thoroughly cleaned with water and methanol. The inlet tubing is discarded since non-sterile anymore.

2.1.3 Artificial cerebro-spinal fluid (aCSF)

The aCSF used to perfuse the MD probe was composed of:

- 147 mM sodium chloride (NaCl),
- 2.7 mM potassium chloride (KCl),
- 1.2 mM calcium chloride (CaCl₂),
- 0.85 mM magnesium chloride (MgCl₂).

For clinical monitoring, we used a ready-made sterile aCSF but for in vitro and in vivo experiments discussed in Chapter 6, we dissolved the above in de-ionised water and added 500 µL (for 1L stock-solution) of Kathon CG (5-chloro-2-methyl-4-isothiazoline-3-one) as a preservative.

The ionic composition of the aCSF used to perfuse the MD probe has a non-negligible impact on the composition of the tissue extra-cellular fluid [149]. The above aCSF is relatively close to the composition of the ECF, as determined by zero-net flux in the hippocampus of freely-moving rats [149].

2.2 Rapid-sampling microdialysis (rsMD)

This assay has been developed in the Boutelle group to measure glucose and lactate concentrations with a time resolution of up to 15 seconds. This is the only available online microdialysis assay that can be used both for animal models and clinical studies in brain injury patients [160; 161; 162; 164; 165; 175; 176]. During my PhD, I have been maintaining and operating the assay to monitor 13 patients in the Neurology Intensive Care Unit of King’s College Hospital for up to 5 days after surgical intervention.
2.2.1 Overview of the rsMD assay

A schematic representation of the rsMD assay is drawn below in Figure 2.2.

![Diagram of the rsMD assay](image)

**Figure 2.2: The online rapid sampling microdialysis analyzer.** The microdialysis probe is inserted into the brain tissue. The dialysate then enters a dual channel switching valve that alternatively injects the sample every 30 seconds into two separate enzyme reactors, one for glucose, the other for lactate. A flow injection analysis (FIA) buffer is used for the transduction of the biochemical reaction into an electrical current.

On leaving the MD probe, the dialysate is directly introduced into the sampling loop of a custom-built valve, typically with a volume of 200 nL. Separately, a HPLC pump continuously pumps a flow injection analysis (FIA) buffer (200 µL/min) from a reservoir to the valve. Before entering the valve, this buffer stream is split into two and enters into two separate ports of the valve. This produces two analysis streams, one which is designated to glucose and the other for lactate. The valve is automated to switch between the two channels at typical intervals of 15 to 30 seconds. As the content of the sampling loop is injected, the dialysate stream is accelerated through separate enzyme reactors containing the enzymes oxidase (GOx and LOx respectively) and HRP which have been adsorbed...
2.2. Rapid-sampling microdialysis (rsMD)

onto cellulose membranes. Any ferricinium ions produced by the enzyme reactors are detected as a reduction current at the radial flow glass carbon electrodes held at a potential of 0.0 mV vs Ag/AgCl. This yields two traces of current FIA peaks with time, one for glucose and the other for lactate, as shown below in Figure 2.3:

![Glucose and Lactate Peaks](image)

*Figure 2.3: rsMD signals: current peaks versus time.* Typical five minute recording with the rsMD assay. Current peaks for glucose (in red) and current peaks for lactate (in green) alternate every 30 seconds. The peak amplitudes are proportional to the concentrations of glucose and lactate respectively in the dialysate. In this example, 500 µM of glucose and lactate are measured.

At 0.0mV vs Ag/AgCl, the reduction current generated at the electrode is mass-transport limited, i.e. it is directly related to the flux of Fc\(^{3+}\) to the electrode surface according to:

\[ I = nF A J \]  

(2.1)

\(n\) is the number of electrons transferred per molecules, \(F\) is the Faraday constant (96,485 C/mol), \(A\) is the electrode area (in m\(^2\)), and \(J\) is the flux of material (in mol/m\(^2\)/s).

The flux of Fc is directly proportional to its concentration (constant flow rate of the FIA stream). This is related to the concentration of H\(_2\)O\(_2\) by the HRP kinetics, and hence to the concentration of glucose or lactate by the oxidase enzyme kinetics. The enzyme kinetics are pseudo-linear over the physiological range of glucose and lactate in the brain [172]. Therefore, the amplitude of each current peak from the rsMD assay is directly proportional to the concentration of glucose and lactate respectively.
2.2. Components of the rsMD assay

The detection unit is contained in a grounded Faraday box, pictured in Figure 2.4 below:

![Figure 2.4: rsMD box. Key elements of the rsMD assay: (1) injection valve, (2) FIA stream, (3) enzyme reactors, (4) electrode, (5) connectors to potentiostat.](image)

At the centre of the box, the valve (1) injects the dialysate sample into the glucose and lactate detectors. The flow injection analysis (FIA) ferrocene buffer is perfused via HPLC pump and tubing (2). The two enzyme reactors (3) are placed on both sides of the valve and the current is detected at downstream electrodes (4) connected and controlled by the instrumentation (5). These will be described successively.

2.2.2.1 Dual channel switching injection valve

The dialysate liquid is injected onto the system through an electrically actuated custom-built valve (Valco Instruments, Switzerland). It is based on a 6 port dual internal loop of 200 nL with low dispersion ports and sequential loop filling. A fill port (VISF-1, Valco Instruments, Switzerland) makes a direct sealed connection with a 22 gauge needle from the MD probe outlet FEP tubing. It is mounted on a 3 inch stand off to ensure easy access to it.

Since the signal is mass-dependent, it is critical that the exact same volume of dialysate is injected into the FIA buffer. This volume depends on the precision of the loop and/or the syringe pump. As with patients, it is very likely that the back-pressure in the connecting tubing can vary because of patient movement, nursing care, and others, we rely on the precision of the sampling loop and operate the system in a complete loop filling mode: according to HPLC good practice, the sample loop should be overfilled 5 times with the sample solution to eliminate any possible dilution effects due to Taylor dispersion [177; 178; 179]. Clinically, we use a 2 µL/min perfusion for a 200 nL loop injected every 30 seconds, so the loop is filled 2 to 3 times. In the animal work presented in Chapter 6, the perfusion flow rate (1.6 µL/min) was chosen to overfill the 200 nL loop twice while achieving a 15 seconds time-resolution.
2.2.2 Rapid-sampling microdialysis (rsMD)

2.2.2.2 FIA Buffer, pump and fluidics

FIA involves taking a small sample of the low flow, low pressure dialysate stream and injecting it into a high flow, higher pressure analysis stream. One liter of FIA buffer solution was prepared monthly or after each patient monitoring as follows:

- 1.5 mM ferrocene monocarboxilic acid (Fluka 46264, UK). This is the mediator used for the transduction of the enzymatic detection
- 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma ED, UK) is a chelating agent used to minimise the interference of di- and trivalent metal ions.
- 150 mM sodium chloride (Sigma S7653, UK) to maintain the concentration of chloride past the reference electrode at the same concentration of chloride as that in the aCSF (see section 2.2.2.4)
- 0.1 M sodium citrate (Sigma S4641, UK) to buffer the solution at pH=7.0, a condition required for optimum activity of the enzymes
- 1000 mL ultra pure water (18.1 MΩ cm² conductivity)

This solution is then continuously stirred and 500 µL of a biocide, Kathon (5-chloro-2-methyl-4-isothiazoline-3-one, Rohn and Hass, UK), is added to protect from bacterial scavenging. It is then filtered using a vacuum pump through 47-mm anodisc membranes (Whatman international) of 0.10 µm and 0.02 µm pore size. When not used, it is sealed and stored at 4°C.

The FIA buffer is pumped at 200 µL/min through the assay using a computer controlled high precision HPLC pump (Rheos 2000, Flux instrument, Switzerland). This is the optimum flow rate for maximum sensitivity and stability of the assay [180]. The HPLC pump is controlled by Janeiro II software running under Parallels 3.0 with Windows XP patch 2 on an Apple computer. A back-pressure regulator is inserted between the HPLC pump and the valve to ensure optimum conditions for the HPLC pump.

The fluidics, from the pump to the detecting electrode, consisted in standard HPLC 1/16 inch outer diameter PEEK® (PolyEtherEtherKetone) tubing and Sealtight® fittings from Upchurch Scientific.

The tubing used for connection between the pump and the valve are of 0.030 inch internal diameter while those between the valve, the enzyme reactors and the electrodes are of 0.005 inch inner diameter to minimise dead volume in the detection unit. A tee-flowpath adapter (Micro Valves for GC and LC, 660100, Valco Instruments, Switzerland) splits the FIA buffer stream upstream of the valve to yield two separate analysis stream, one for glucose and one for lactate.

Before and after use, the whole assay apparatus is flushed with water to wash away crystallised salts and methanol to eliminate any bacteria trapped in the tubing.

2.2.2.3 Enzyme reactors

Several generations of enzyme reactors were developed within the Boutelle group and we here used the latest enzyme beds that give increased sensitivity, stability for up to five days of continuous use [180]. They are based on two distinct layers of oxidase (GOx or LOx) and peroxidase (HRP) enzymes adsorbed onto cellulose membranes and enclosed in filter bodies, according to the procedure below:

1. glucose oxidase (1 mg), lactate oxidase (2 mg) and horseradish peroxidase (0.5 mg) (Genzyme Diagnostics, UK) are separately dissolved in 1 mL of filtered ferrocene buffer.
2. the dissolved enzymes are separately pumped, using a 1 mL plastic syringe, through nanoporous mixed cellulose ester membranes (0.025 µm pores, 25 mm discs (VSWP02500, Millipore UK membrane)) placed on a commercially available filter holder (SX0002500, Millipore, UK). They are pumped on
2.2.2 Rapid-sampling microdialysis (rsMD)

average five times to ensure maximum loading of the membrane with enzymes.

3. punched out discs are prepared and stored in ferrocene buffer at 4°C. To avoid any contamination
between the different enzymes all tools utilised (hole punch, tweezers, syringes, etc.) are dedicated
to a given enzyme and treated with a methanol solution after use.

4. a GOx (LOx respectively) disc and a HRP disc are then housed in high pressure inline filters (A-431,
Anchem, UK) with extra-frits filter end fittings (A-428X, Anachem, UK). It is important to make sure the
discs are in the centre of the reactors to allow maximum surface contact with the dialysate stream and
avoid unnecessary extra back-pressure.

5. the micro reactors are pressure-adjusted so that the FIA stream is equally distributed into the two
reactors when passively split upstream of the valve.

When the system is not in use, the enzyme reactors are filled with FIA ferrocene buffer and stored at
4°C.

It is paramount to use two distinct layers of adsorbed enzymes, the first one for the oxidase and the
second one for the peroxidase. They must be in this order in the direction of the flow. Randomly co-
localised enzymes can indeed lead to futile mediation: the ferricinium ions produced by the reduction
of HRP can mediate GOx directly, hence giving no signal at the expense of two glucose molecules
[172].

2.2.2.4 Radial flow glassy carbon electrodes

Two radial flow, thin layer electrochemical cells (Unijet kit, MF-2061, Bioanalytical Systems, Inc.,
USA) are used for the glucose and lactate streams respectively. The flow enters the cell through a
jet in a steel disc auxiliary electrode and hits the centre of a 3 mm diameter glassy carbon working
electrode. The glassy carbon electrode is set within PEEK™ together with an exposed Ag/AgCl
reference electrode with a 15 µm Teflon gasket (MF-1055, Bioanalytical Systems, Inc., USA).

The Ag/AgCl reference electrode is an example of a "thermodynamic electrode", meaning that its
potential (that must be kept constant) relies on a fixed concentration of chloride present in the mobile
phase. The electrode manufacturer recommends a 10 mM concentration of chloride in the flow-
through solution; a higher solution of chloride favours the dissolution of AgCl into the flow-through
solution. However, recent studies within the group showed that a higher concentration of chloride
(150mM) in the FIA buffer, so as to match the concentration in aCSF and dialysate, increased the
sensitivity and the stability of the assay [181]. To compensate for the change in electrode potential,
the potential of the working electrode is changed from -100 mV (for 10 mM Cl⁻) to 0.0 mV (for
150 mM Cl⁻) vs conventional Ag/AgCl (3M)) [181]. As a trade-off, it is necessary to regenerate the
AgCl layer on the reference electrode every week (instead of every month) by using a strong oxidant
reagent (Unijet Chloridising Solution CF-220, Bioanalytical System Inc.): a small drop of solution is
applied on the reference electrode (importantly not on the working electrode) for 5 minutes and then
washed off with water. The reference electrode then recovers its uniform dull bronze color. Failing
this chloridisation of the reference electrode, the signal becomes very noisy.
2.2. Rapid-sampling microdialysis (rsMD)

2.2.2.5 Instrumentation

The potential of the working electrode is controlled by custom-made potentiostats (Biostat III, Electrochemical and Medical Systems, UK). Chapter 3 describes the design of new potentiostats to miniaturise the rsMD assay.

The data are digitised at 200 Hz using a Powerlab 16/SP analogue/digital converter and Chart-5.5.6 software (ADInstruments, Oxfordshire, United Kingdom) running on an Apple MacBook Pro (A1212, Apple, CA, USA).

2.2.3 Online assay in the neurointensive care unit

The injection valve, enzyme beds, radial flow electrodes and the potentiostat headstages are all contained in a Faraday box. This box is placed on an extendable arm so that it can be situated near the head of the patient while the rest of the instrumentation (HPLC pump, potentiostats, PowerLab and computer) is placed behind the patient bed on a clinically certified trolley (Series 7000, CTL Medical, Essex, UK) to allow free access to the patient by the nursing staff. An uninterruptible power supply unit (APC, London, United Kingdom) feeds in all the equipment. This is to avoid interruption of the assay while moving the trolley from the lab to the intensive care unit (ICU). Below in Figure 2.5 is a picture of the whole rsMD analyzer in the ICU:

![Figure 2.5: All heavy instrumentation, analog/digital converter and laptop are placed on a trolley tucked behind the patient bed. They all feed into an uninterruptible power supply (UPS). Calibration solutions and other tools to adjust the enzyme reactors are kept in a (green) drawer beneath it. The Faraday box with the injection valve, enzyme reactors and flow cell electrodes is placed on an extendable arm close to the head of the patient. Nursing staff can enter comments such as “drug bolus”, “turn patient to left”, etc. using the comment marker. This is directly entered into the Chart file recording. A video camera also focuses on the head of the patient.](image)

The rsMD data are logged into a Chart file along with the EEG recording and other routine clinical data, such as blood pressure and intra-cranial pressure when available. The clinical findings will be
2.3. Calibration solutions

discussed in Chapter 5. As shown in Figure 2.5, we also use a video camera and comment marker for the nursing staff. This allows us to rule out events due to artefacts caused by patient movement or clinical care. No faces or names can be identified.

On some occasions, we also used an off-line bedside analyzer (ISCUS Clinical Microdialysis Analyzer, CMA Microdialysis, Sweden) for pyruvate, glycerol and amino acids (not shown on Figure 2.5). This can be used along with the rsMD assay since the valve is overfilled with dialysate. Figure 2.5 also illustrates that patients are surrounded with other monitoring equipment in the ICU. These can interfere with our signal and cause all kinds of noise: a first task during my PhD was to "clean" the signal and remove the artefactual noise to allow an automatic and reliable detection of the FIA peaks. The data processing strategies are discussed in Chapter 4.

2.3  Calibration solutions

Both the rsMD assay and the potassium electrode discussed in Chapter 6 need to be regularly calibrated to convert the electrical signal (current and potential respectively) into dialysate concentrations. They were systematically calibrated immediately before and immediately after use. The rsMD assay was also calibrated twice a day during patient monitoring and if the sensitivity of the enzyme reactors was below 0.2 µA/mM of glucose (or lactate), new reactors were made, set up in the Faraday box and calibrated.

2.3.1  Mixed lactate/glucose calibration solutions for rsMD assay

Calibration solutions were made with D-(+)-glucose (G 4750, 500 g, 99%, Sigma) and L-(+)- Lactic acid (L 7022, 10 g, 98%, Sigma). Typically, we proceeded as follows:

1. make a mixed solution of 2 mM glucose and 2 mM lactate in 50 mL de-ionised water (18.1 MΩ cm conductivity),
2. keep the solution in the fridge, at 4°C, to allow mutarotation of glucose during 24 hours,
3. filter the solution through a 0.02 µm membrane,
4. perform sequential serial dilutions to obtain 4–5 standard solutions ranging from 50 µM to 2 mM.

This covers the maximum physiological range for glucose and lactate in brain tissue. Aliquots of these standards were then stored at -18°C. Each calibrant was manually injected 3 to 4 times into the valve with a glass Hamilton syringe (1710RNR 100ul syringe, Hamilton). The rsMD response was linear in this range.

2.3.2  Potassium calibration solutions

The same salts as those used to make aCSF were used and the following method was used:

1. make 500 mL of aCSF-like background electrolyte with 147 mM NaCl, 1.2 mM CaCl₂, 0.85 mM MgCl₂;
2. dissolve KCl in 250 mL of the above solution to make a master solution of 100 mM KCl in a 250 mL aCSF-like background electrolyte;
3. perform sequential serial dilutions in this background electrolyte to obtain solutions ranging from 1.8 to 100 mM KCl (in an aCSF-like background);
4. store small vials of these calibrating solutions in the fridge at 4°C
2.4. Reference electrodes

These solutions were then used as free solutions to calibrate the potassium ion selective electrodes, or perfused with a syringe pump to calibrate the potassium electrode in the flow-cell.

2.4 Reference electrodes

The reference electrode for the rsMD assay has been described in section 2.2.2.4. This section applies to Chapter 3 and 6.

2.4.1 Commercial glass reference electrode

We used a commercial glass silver/silver chloride reference electrode (RE-5B Ag/AgCl Reference Electrode with Flexible Connector, 6 mm OD, glass body, Vycor frit, MF-2052, Bioanalytical Systems, Inc.). It is encapsulated in a glass-body with a porous ceramic frit at the solution interface. The filling solution is a 3 M NaCl gel that has been saturated with AgCl. The tip is kept wetted at all times and stored in 3 M NaCl when not in use.

2.4.2 Internal reference electrode for ion-selective electrodes

This internal reference electrode was made for the potassium selective electrode in Chapter 6. It consisted in a silver/silver chloride (Ag/AgCl) wire in an aCSF gel.

2.4.2.1 Ag/AgCl wire

This was made from an 8 cm-long teflon coated silver wire of 0.125 mm diameter (Ag 549411 99.99%, OD 0.125 mm, 5m, Gi 616/627, Advent, UK) as follows:

1. strip off the teflon insulation over 1–2 mm wire at both ends of the wire using a scalpel (blades from Swann Morton Ltd)
2. solder one end to a metal connector (24 way ultra low profile DIL socket, 0.3 inches, W30524/3SFTRC, Winslow). Test for the connection between the metal connector and the other end of the wire with a voltmeter.
3. chloridise the other end of the wire: a small drop of potassium dichromate dihydrate solution (Unijet Chloridising Solution CF-220, Bioanalytical System Inc., USA) is held in the tip of a plastic pipette. The silver chloride is formed when the wire tip turns brownish. This procedure has to be carried out with gloves and labcoat and all glassware or tissue in contact with the solution should be destroyed.

The chloridised wire was inserted into the ion selective electrode when filling the electrode with the internal reference solution as described in section 2.4.2.2. The chloridised end was positioned as close as possible to the ion selective membrane (without risking piercing it) and the metal connector was protruding out of the ISE body for safe connection to the amplifier.

2.4.2.2 aCSF gel

To avoid evaporation and/or modification of the ion concentrations in the internal filling solution of the electrode, and to allow manipulations of the electrodes upside-down when transported or fitted into the flow-cell, an aCSF gel was formed as follows:

1. add 3 wt-% (i.e. 6 g for 20 mL) of agar powder (Agar-Agar A7002, Sigma Aldrich) to 20 mL aCSF
2.4. Reference electrodes

2. stir the mixture with a magnetic stirring flea on a hot plate set at 80°C to dissolve the agar granules
3. heat the mixture agar-aCSF in the oven at about 100°C for 30–40 minutes. Regularly stir the mixture to remove bubbles.
4. meanwhile, insert the chloridised wire into the ISE, the chloridised end towards the membrane, and the metal connector protruding out of the tubing by 1 or 2 cm.
5. when a clear yellowish viscous gel is formed, quickly remove the beaker from the oven (using gloves) and fill the electrodes with the agar-aCSF gel using a syringe and a very thin flexible Microfil needle (MF28G-5, 97 mm, WPI Europe) that will fit in the tube in the presence of the wire. This has to be quite fast, while the agar-aCSF mixture is hot and viscous as it will quickly turn solid when it cools down
6. seal the open end of the tube to avoid the agar drying out and shrivelling inside the tube. This would break the connection between the membrane and the chloridised wire.
7. leave the gel to cool down for a couple of hours till it is solidified.

2.4.3 Radel Reference electrodes

For measurements in the flow-cell of Chapter 6, two reference electrodes were designed, based on a Radel tubing body (Radel R (polyphenylsulfone) tubing 0.030 In x 1/16 in x 5FT, UpChurch Scientific 1230).

2.4.3.1 Thermodynamic disc reference electrode

The first Radel reference electrode was fabricated as follows:
   1. make an Ag/AgCl wire as described in section 2.4.2.1
   2. cut a ≈ 5-cm length of Radel tubing and fit the Ag/AgCl wire in, with the chloridised end protruding slightly out of the tubing
   3. mix epoxy resin and hardener (mixture 1:1) to form a resistive glue
   4. use a pipette tip to apply a small amount of the mixture inside the Radel tube around the chloridised wire. Make sure not to spill some epoxy on the outside of the tube otherwise it will not fit into the ferrule.
   5. leave to dry for 2 hours
   6. cut the protruding chloridised wire (with a blade).

2.4.3.2 Saturated agar reference electrode

To limit the risk of a response to chloride ions in the analysis solution, we made a second Radel reference electrode with a saturated gel of 3M NaCl, so that the mass transport of chloride ions from the sample solution to the chloridised wire was considerably slower. This was made as follows:
   1. make a short 3-cm long Ag/AgCl wire as described in section 2.4.2.1
   2. cut a ≈ 5-cm length of Radel tubing and fit the Ag/AgCl wire in, with the chloridised tip fully inside the tubing, typically 2 or 3 cm recessed from the end of the tubing in contact with the solution
   3. prepare a 3M NaCl agar gel (3 wt% agar powder) following the procedure described above in section 2.4.2.2
   4. fill the Radel tube with the gel

These two Radel reference electrodes have been characterised in Chapter 6.
2.5 Common data analysis methods

2.5.1 Transit time

Many of our data were in-flow measurements involving connecting tubing between the sample or tissue and the analytical detector. A time delay during which the analyte is conveyed from the tissue to the detector must be taken into account when analysing the data. The transit time $t_{\text{transit}}$ can be estimated by:

$$t_{\text{transit}} = \frac{\text{Transit volume}}{\text{Flow rate}}$$  \hspace{1cm} (2.2)

For the rsMD assay separated from the patient by a one-meter long FEP-tubing (1.2 µL/10-cm length) and injection needle, the transit time should be of 7 minutes according to this calculation. In practice, we measured a time lag that was slightly longer, of about 9 minutes. This is likely to be due to Taylor dispersion inside the connection tubing [178; 179].

2.5.2 Statistical analysis

The statistical analysis was performed using either Igor Pro 6.0.1 (Wavemetrics, OR, USA) or Matlab 7.5 (The MathWorks Inc., MA, USA) run on a Power Macintosh dual G5 computer (Apple, CA, USA).

2.5.2.1 Normality test

The data collected during clinical monitoring was tested for normality before choosing an adequate statistical test. I used the D’Agostino-Pearson K2 normality test [182]. The calculations for this test are:

1. compute the skewness \(^1\) and the kurtosis \(^2\) of the distribution
2. compare these values to the values expected for a Gaussian distribution.
3. compute a p value from the sum of these discrepancies.

A small p value (<0.05) is evidence that the data do not follow a Gaussian distribution. The D’Agostino-Pearson K2 normality test is a versatile and powerful normality test and it was performed using the \textit{DagosPtest} function available for Matlab from [183].

2.5.2.2 Normally distributed data

Most of our measurements (time response, potential or current response, concentrations, etc.) could be described by a normal Gaussian distribution and are reported in this thesis using the formalism described below.

\(^1\) a measure of the asymmetry of the probability distribution  
\(^2\) an estimate of how the variance is due to infrequent extreme deviations as opposed to frequent modestly-sized deviations
2.5. Common data analysis methods

Central value and spread
The central value, $\mu_X$, of a Gaussian distribution with $n$ observations, $x_i$ and $1 \leq i \leq n$, can be estimated by the mean value, $m_X$, defined by:

$$m_X = \frac{\sum_{i=1}^{n} x_i}{n}$$  \hspace{0.5cm} (2.3)

The scatter of the data (made of $n$ observations $x_i$) around the mean value is given by the standard deviation, $\sigma_X$, and can be estimated by $s_X$:

$$s_X = \sqrt{\frac{\sum_{i=1}^{n} (x_i - m_X)^2}{n-1}}$$  \hspace{0.5cm} (2.4)

To measure the accuracy of the mean, the standard error of the mean (SEM) is used:

$$SEM_X = \frac{s_X}{\sqrt{n}}$$  \hspace{0.5cm} (2.5)

Most of the data reported in this thesis are presented as mean ± SEM.

The relative standard deviation (RSD) is used in Chapter 4 as a non-dimensional measure (in%) of the scatter of the signal and is estimated by:

$$RSD = \frac{s_X}{m_X} \times 100$$  \hspace{0.5cm} (2.6)

In the case of Chapter 4, X was a series of rsMD peak height values for a given analyte concentration.

Signal-to-noise ratio and limit of detection
The level of noise in our measurements is defined by the standard deviation of the blank signal, $s_b$, the blank signal referring to the recorded signal when no analyte is present. The signal-to-noise ratio (SNR) is defined as the ratio of the analyte signal over the level of noise. For the rsMD assay, this corresponds to the FIA peak height over the standard deviation of the current recorded between consecutive peaks.

The limits of detection (LOD) of the rsMD assay and of the potassium flow-cell of Chapter 6 are defined according to the IUPAC recommendations by:

$$LOD = k_D s_b$$  \hspace{0.5cm} (2.7)

with $s_b$ is the standard deviation of the blank signal, and $k_D$ is a numerical factor.

Using the upper limit approach of Bond et al. [184], we took the numerical factor, $k_D$, as equal to 2.326 for the 95% confidence limit. With this definition, the limit of detection corresponds to the smallest concentration change that can be resolved with a 95% certainty.
2.5. Common data analysis methods

Curve fitting

For the rsMD assay and the potassium electrode from Chapter 6, the calibration data were fitted by least-square linear regression ($y=ax+b$) on the n measured current peak heights or potential values ($y_i$) for standard concentrations (or activities) $x_i$. The final coefficients, a and b, of the linear regression were the ones that minimised the value of the Chi-square. The Chi-square is defined as:

$$\chi^2 = \sum_{i=1}^{n} \frac{(\bar{y}_i - y_i)^2}{\sigma_i^2}$$  \hspace{1cm} (2.8)

with $\bar{y}_i$ the fitted value for a given point $x_i$, $y_i$ the measured data value for the point $x_i$ and $\sigma_i$ the standard deviation for $y_i$.

The goodness-of-fit was estimated by the r-squared coefficient, which is the square of the Pearson product moment correlation coefficient, $r$, defined by:

$$r^2 = 1 - \frac{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2}{\sum_{i=1}^{n} (y_i - m_Y)^2}$$ \hspace{1cm} (2.9)

This coefficient of determination, $r^2$ can be understood as the fraction of the variance ($\sigma_Y^2$) in $\bar{y}_i$ that is accounted for by a linear fit of $x_i$ to $y_i$. It is between 0 and 1 and $r^2 = 1$ indicates that the fitted model explains all variability in $y_i$, while $r^2 = 0$ indicates no ‘linear’ relationship.

More generally, the Pearson product moment correlation coefficient can be used to estimate the correlation between two sets of data, Y and Z. It is then given by the following expression:

$$r = \frac{\sum_{i=1}^{n} (y_i - m_Y)(z_i - m_Z)}{(n-1)\sigma_Y^2\sigma_Z^2}$$ \hspace{1cm} (2.10)

The correlation is 1 in the case of an increasing linear relationship, -1 in the case of a decreasing linear relationship, and some value in between in all other cases, indicating the degree of linear dependence between the variables. The closer the coefficient is to either -1 or 1, the stronger the correlation between the variables. This was used in Chapter 5, section 5.3.3.3.

Student’s t test

A one sample Student’s t-test was used to test whether a population mean ($m_X$) differed significantly from zero (null hypothesis). The test statistics, t is given by:

$$t = \frac{m_X}{s_X/\sqrt{n}}$$  \hspace{1cm} (2.11)

The t value is then compared to tables of critical values given the degree of freedom (df=n-1) and the significance value $\alpha$ (we chose $\alpha=0.05$). If the calculated t value is greater than the critical t value, then the null hypothesis is rejected and the population mean can be considered significantly different from zero. A one sample student’s two-sided t-test was used for the mean slope of the regression lines in Chapter 5, section 5.3.6.
2.5. Common data analysis methods

It was also used to estimate the reliability of the least-square regression parameters, using 95% confidence intervals. These are calculated by:

\[ CI = t(v, \alpha)SEM \]  

(2.12)

with \( t(v, \alpha) \) the two-sided Student t critical value for \( v \) degree of freedom (\( v = n-2 \)) at the \( \alpha \) significance level (\( \alpha=0.05 \) for a 95% confidence level).

Using these confidence intervals, the regression slopes were compared to theoretical values in Chapter 6.

2.5.2.3 Not-normally distributed data

The clinical data reported in chapter 5 did not follow a normal distribution, as tested by the D’Agostino-Pearson K2 normality test (see section 2.5.2.1). We therefore report these data as follows.

Central value and spread

For not randomly distributed data, the central value is reported as a **median value**: the data is ranked and the median value is the value of the \( \frac{1}{2}(n+1) \)th (if \( n \) is odd) or \( \frac{1}{2}(n) \)th (if \( n \) is even) data point.

The dispersion of the data is defined by its **interquartile range (IQR)**. It is equal to the difference between the third and first quartiles, given by:

- first quartile (or 25th percentile): the cut off data point below which 25% of the observations \( (x_i) \) may be found,
- third quartile (or 75th percentile): the cut off data point above which 75% of the observations \( (x_i) \) may be found.

Not normally-distributed data will be reported as median (interquartile range). Graphically, they are represented by a box and whisker plot as schematically represented below:

![Box and whisker plot](image)

**Figure 2.6:** Box and whisker plot.

Data points that lie more than 1.5\( \times \) IQR lower than the first quartile or more than 1.5\( \times \) IQR higher than the third quartile are defined as outlying values.

Binomial test

A binomial test was used to test whether the distribution between positive and negative rsMD responses (see chapter 5) was due to chance or not. The test is performed as follows:

1. state the null hypothesis: the proportion of positive rsMD responses \( (p^*) \) is not different from that of negative rsMD responses \( (q^*) \), therefore: \( p^* = q^* = 0.5 \).
2. calculate the proportions from the sample, ie \( p = \frac{\text{number of rsMD responses that are positive}}{\text{total number of rsMD responses}} \) (and \( q = 1 - p \)).
2.5. Common data analysis methods

3. calculate the test statistic, $z$ given by $z = \frac{p - p^*}{\sqrt{\frac{p^*q^*}{n}}}$.

The decision about rejecting the null hypothesis is then given by comparing the $z$ statistics with tables of significance. We performed a binomial test with a 95% significance level.

**Fisher’s test**

This test is used to determine if there is non-random relationship between two dichotomous variables. It calculates the exact probability for a $2 \times 2$ contingency table of frequency data cross-classified according to two categorical variables, A and B, each of which has two levels or subcategories ($A_1$, $A_2$, $B_1$, $B_2$). A small p-value ($p<0.05$) will indicate that the association between the two categorical variables is not purely due to chance.

**Wilcoxon signed-rank test**

This is a non-parametric test that compares two independent sets of data, $X_a$ and $X_b$, by looking at the differences between the data points in the two sets. The resulting $p$ value answers the $H_0$ question: if the two sets of data were identical, what are the chances that random sampling would lead to a sum of ranked differences as far apart from zero? The smaller the $p$ value, the less likely it is that $H_0$ was rejected by chance. We used a 95% significance level two-tailed test based on the Matlab code from [185]. The Wilcoxon test statistic, $W$ is calculated as follows:

1. take the difference $X_a(i)-X_b(i)$ for each pair $i$ of data points,
2. omit the cases where $X_a(i)-X_b(i) = 0$,
3. rank the remaining differences without regard to their signs. Award average ranks for any ties.
4. sum the ranks corresponding to the positive differences and call this $W^+$,
5. sum the ranks corresponding to the negative differences and call this $W^-$,
6. the required test statistic is the smaller of these two values: $W = \min(W^+, W^-)$.

$W$ is then compared to a table of all possible distributions of ranks to calculate $p$, the statistical probability of attaining $W$ from a population of scores that is symmetrically distributed around the central point. As the number of data points, $n$, increases, the distribution of all possible ranks $W$ tends towards the normal distribution. Consequently, for $n \leq 15$, exact probabilities are calculated, and for $n > 15$, the normal approximation is used.

**Mann Whitney U test**

The Mann Whitney U test is another non-parametric test that can be used for unpaired data sets (contrary to the Wilcoxon signed rank test). It tests for chances of the two data sets, $X_a$ and $X_b$, to be from a single population (null hypothesis). This hypothesis is rejected for small values of $p$. We used a 95% significance level two-tailed test in Chapter 5.

1. rank all the data points from lowest to highest without regard to which sample they are in,
2. sum the ranks for the observations which came from $X_a$ (same calculations for $X_b$ respectively). This is $R_a$ (or $R_b$ respectively).
3. the required test statistic, $U$, is then given by: $U_a = R_a - \frac{n_a(n_a+1)}{2}$ (similar expression for $X_b$ using $n_b$ and $R_b$)
4. the required test statistic is the smaller of these two values: $U = \min(U_a, U_b)$.

The same procedure as that used for the Wilcoxon test follows: the $U$ value is compared to significance tables, with exact calculation for $n_a$ and $n_b$ below 15, and a normal approximation above.
Chapter 3

Miniaturisation of the instrumentation of the online microdialysis assay

Previous research efforts have focused on optimising the enzyme reactors of the rsMD assay for maximum sensitivity and stability, allowing them to monitor patients in the intensive care unit for up to five days. In this chapter, the design of new electronic instrumentation to control the electrochemical cells is presented. The aim is to:

• minimise the electrical noise affecting the analytical signal,

• enhance the stability of the signal for long-term monitoring,

• miniaturise the instrumentation

• reduce the costs of the instrumentation

To fulfill the above specifications, I used through-hole printed-circuit board (PCB) technology and several strategies:

• placing the instrumentation close to the electrochemical cell,

• using isolated instrumentation to allow positioning close to the patient while meeting safety requirements

• using screened cables and screened connectors to reduce deterioration of the signal from the electrochemical cell to the amplifier

• choosing high quality components

3.1 Potentiostat

The potentiostat fulfils two roles that are implemented by two electronic sub-circuits [186]:

1. control the voltage between the working electrode and the reference electrode to a constant value set by the user. This is usually implemented by injecting current into the electrochemical cell through the auxiliary electrode. This is achieved by the voltage follower circuit.

2. measure the current flow between the working and auxiliary electrodes. This is implemented by a current-to-voltage converter that forces the cell current to flow through a current measurement resistor and the voltage drop across the resistor is a measure of the cell current.
3.1. Potentiostat

3.1.1 Design of the printed circuit board

The PCB was designed using OrCAD 16.0 (Cadence, Berkshire, UK). It is composed of two main modules:

1. OrCAD Capture: for the design of the schematic, i.e. the electrical diagram
2. OrCAD Layout: generates a physical representation of a circuit, including the footprints of the parts (or electrical components) and the tracks between the parts with their actual dimensions

I learnt how to design a printed circuit board with OrCAD, thanks to online tutorials [187; 188].

3.1.1.1 Schematics

Figure 3.1 below shows the schematic I designed for the potentiostat:

![Schematics of the potentiostat.](image)

Figure 3.1: Schematics of the potentiostat. The two electronic sub-circuit are annotated (voltage follower in green, current-to-voltage converter in orange). The two operational amplifiers are indicated. Screened connectors were used to connect to the electrodes and to the analogue/digital converter (see section 3.1.1.2). The source of the voltage to apply to the reference electrode can be external or internal depending on the potential input selector. Four different gains can be chosen using the gain selector.

To simplify the electronic circuit, the working electrode was maintained at ground and the constant voltage was applied to the reference electrode.

Since reduction of ferrocinium ions in the rsMD assay can be done at 0.0 V versus the reference electrode, the potential input of the reference electrode can be forced to ground internally, thereby removing noise from an external voltage source (see section 3.1.2.1). It is also possible to apply a desired external voltage for other applications, such as to drive biosensors.

A given electrical circuit will inevitably suffer from a certain level of voltage noise (typically < 1 mV). The gain of the current-to-voltage converter defines the voltage equivalent to a given current and hence, determines the signal-to-noise ratio (SNR) for the potentiostat. I have included in the design of
3.1. Potentiostat

The potentiostat the possibility to adjust this gain to four different values to suit particular applications. Typically, the gains were:

- 1.0 and 0.1 µA/V specifically selected for the rsMD assay (lactate and glucose respectively),
- 10 nA/V for a microelectrode and/or biosensor,
- 0.1 mA/V for a macro-electrode.

Once the schematic is created, the logical representation of the circuit is output as a *netlist* file (.mn). It contains the list of the electrical connections (nets) and the list of the components (with their pins and designators).

### 3.1.1.2 Electrical components

The most important components in the circuit are the two operational amplifiers and we chose high quality components (particularly for the current-to-voltage conversion) to minimise noise interferences:

- AD 820 ANZ (Analog Device, Surrey, UK) for the voltage follower because of its high input impedance \((10^{13} \Omega)\), and low input voltage noise (2 µV peak-to-peak). It also has low input bias current (<25 pA), low input offset current (<20 pA) and meets the requirements for application into medical instrumentation.

- AD 549 JH (Analog Device, Surrey, UK) for the current-to-voltage conversion mainly because of its ultralow input bias current (<250 fA) and very high input impedance \((10^{15} \Omega)\). It also has low input offset current (<250 fA) and voltage (<1.0 mV), low offset drift (<20 µV/°C), and a low input voltage noise (4 µV peak-to-peak 0.1Hz to 10 Hz).

Noise interferences were minimised by including:

- *de-coupling capacitors* connected to the operational amplifier power input pins. These shunt the energy from AC signals, voltage spikes or other sources of transient noise from the power supply,

- *feedback capacitors* in parallel with the gain resistors of the current-to-voltage converter to limit the bandwidth of the operational amplifier, preventing ringing artifacts and gain peaking. These have very high specification with a high insulation resistance to stop current leakage. We chose polystyrene capacitors of 10 pF.

- *screened connectors* to carry the signal from the electrochemical cell to the potentiostat, and from the potentiostat to the digital-to-analog converter system. *Screened cables* (RG174, Tyco Electronics, UK), high insulation resistance (5 GΩ) gold-plated brass PCB sockets (SMB, Tyco Electronics, UK) and gold-plated brass electrode connectors for the radial flow cell (Bioanalytical Systems, Inc., USA) were used.

The full list of the components chosen for the PCB can be found in Table A.1 in Appendix A. The circuit was first tested on a breadboard before laying out the printed-circuit board.
3.1. Potentiostat

3.1.1.3 Layout of the PCB

To minimise noise contamination from the power lines, these were assigned to a different layer than
the signal tracks. As much as possible, they were also forced to run orthogonally to the signal tracks.
Additionally, the ground tracks all ran out from a central point, the star earth point (middle left on
Figure 3.2). This splits into power earth and signal earth tracks. This keeps current and noise from
one component from affecting other components.

In general, I tried to keep the tracks as wide and as short as possible. This lowers the DC resistance,
capacitance and inductance. It is also easier for the manufacturer to etch and easier to inspect and
rework.

Finally, a guard surrounds the output signal (running from the working electrode to the feedback gain
resistors, see Figure 3.2): it is at the same potential as the working electrode (ground in this design)
to limit any parasitic current leakage.

The layout for the PCB is given in Figure 3.2 below:

![Figure 3.2: Layout of the potentiostat. The board is outlined in yellow. The components (outline and pins in white) and tracks (red and blue) are laid out on the board. The two electronic sub-circuits are (artificially) delineated: voltage follower (green dotted lines), current-to-voltage converter (orange dotted lines). Four mounting holes are positioned on each corner of the board and not part of the electrical circuit. The star earth point refers to the point on the circuit from where all the local ground lines split. The guard is an additional track (bottom layer) that encloses the signal track from the working electrode down to all the gain resistors. It is at the same potential as the signal track, i.e. ground in this case.](image-url)
3.1. Potentiostat

The detailed procedure to lay out a PCB is as follows:

1. import the netlist from the schematics of the circuit
2. link footprints to components. The footprints are the physical representation of the components, including the dimensions of the part (particularly the dimensions and spacing of the pins and the outline of the component) and padstacks (areas of the board directly connected to the pin of the component). For generic components, most footprints are available in libraries but some were manually designed based on the datasheet of the components.
3. define rules for the layout:
   • the size of the board: 61 mm × 59 mm,
   • the number of layers: a two-sided board, one side for the signal tracks (bottom side) and one side for the power tracks (top side),
   • the widths of the tracks: 0.45 mm-wide signal tracks and 0.65 mm-wide power and ground tracks, clearance (spacing) between tracks and components: the default settings were used to avoid "hairline" short-circuits and other etching problems during manufacturing with too tight a clearance between tracks and pads.
4. place components on the board so as to minimise the crossing of connections. Additionally, the de-coupling capacitors were placed physically and electrically close to the operational amplifiers.
5. route the board (i.e. layout the tracks) according to the above rules with maximising 45° angles and ensuring that the tracks go right through the exact center of pads. This was done automatically and some tracks were manually edited according to considerations to minimise the noise discussed below.
6. check that the board abides by the design rules (design rule check tool implemented in Orcad Layout),
7. add mounting holes to attach the boards in the rsMD Faraday box,
8. complete the silk screen layer (layer for documentation) with outlines and designators of the components, identification of pin 1 (for component orientations) and free text (feedback gains, identification of the electrode connector, external/internal voltage positions, etc.),
9. output the layout as an extended Gerber format (RS274X) for fabrication of the PCB.

3.1.1.4 Manufacture and assembly of PCB

The PCBs were sent to an external manufacturer (Newbury Electronics Ltd., Berkshire, United Kingdom) who fabricate PCBs by etching. I specified a plated through-hole PCB, 1.6 mm-thick, made in FR4 with 2 oz copper weigh and immersion silver. These were electrically tested for the connectivity and impedance of the tracks and pads. The PCBs were then populated with the components mentioned above and the resulting PCBs are shown in Figure 3.3.

1There are rules for minimum track widths depending on the current, voltage and temperature ratings of the PCB. The widths of my tracks were far above these minima. Ground and power tracks should usually be as big as possible.
3.1. Potentiostat

The fabricated board is shown before (a) and after (b) populating it with the components.

The complete potentiostat PCBs were much smaller than the commercial potentiostats used for the rsMD assay (Biostat III, Electrochemical and Medical Systems, UK): 36 cm$^2$ as opposed to a box of 50 cm × 40 cm (≈1,200 cm$^2$). They were also much cheaper, ≈ £200 (including all the components, connecting cables and PCB fabrication) instead of ≈ £2,000 for a commercial potentiostat.

3.1.2 Testing of the potentiostat

Each part of the circuit (voltage follower and current-to-voltage converter respectively) were first separately tested to check the electronic design. Following these tests, the PCBs were used to drive real-world electrochemical cells for cyclic voltammetry and amperometric detection.

3.1.2.1 Tests of the electronic design

Voltage follower circuit

To test the performance of this part of the circuit, I joined the reference and auxiliary electrode leads to create a voltage follower. I then connected a voltage source (Powerlab, ADInstruments, Oxfordshire, United Kingdom) to the external voltage input connector of the PCB and measured the potential between the reference/auxiliary junction and the ground on the PCB. The voltage source was set to deliver a square waveform between +1.0 mV and -4.0 mV every 2 seconds (Figure 3.4 a).

When set to the external potential input position, the voltage applied to the reference electrode by the potentiostat (Figure 3.4 b1) exactly followed the voltage from the external source (Figure 3.4 a) . No offset and no time-lag were observed: when sampling at 40 kHz, the two traces (Figure 3.4 a and b1) exactly superimposed. Furthermore, the response of the potentiostat was very quiet. The noise (estimated as the standard deviation of the signal during 2 seconds) was 0.0305 mV for the voltage follower response (compared to 0.0106 mV from the voltage source) when no additional filtering was applied.
3.1. Potentiostat

When the potentiostat was set to internal potential, the potential of the reference electrode was 0.0 ± 0.0018 mV (Figure 3.4 b2) even when an external waveform was applied (Figure 3.4 a). The noise was therefore reduced by a factor of ten in this mode as compared to the external voltage mode.

**Current-to-voltage converter circuit**

To test the second part of the circuit, a DC precision current source (Keithley 6220, Ohio, United States) was connected between the reference/auxiliary junction and the working electrode lead. A chosen current was injected into the circuit and the corresponding voltage response was measured at the current-to-voltage output. The responses as a function of the input current are given for two gain resistors in Figure 3.5.

---

**Figure 3.4: Test of the voltage follower circuit.** (a) voltage waveform delivered by the voltage source. (b) potential of the reference electrode controlled by the voltage-follower circuit when set to the external voltage source mode (b1) or to the internal voltage mode (b2).
3.1. Potentiostat

The output signal was very quiet, with a noise (given as the standard deviation) below 0.0066 mV of the response for the 1MΩ resistor (Figure 3.5 a) and below 0.0330 mV for the 100 MΩ resistor (Figure 3.5 b). The accuracy (95% confidence limits) of the current-to-voltage conversion is also remarkable. Similar results were obtained for the other two gain resistors (data shown in Figure A.1 Appendix A).

3.1.2.2 Electrochemical cells

Cyclic Voltammograms (CVs)

Using EChem software (ADInstruments, Oxfordshire, United Kingdom), cyclic voltammograms were performed on a glassy carbon macro-electrode (3 mm diameter) and a gold micro-electrode (75 µm diameter) in a solution of ruthenium (III) hexamine (RuHex) in a background of 1 M potassium chloride. The reference electrode was a commercial silver/silver chloride electrode (see section 2.4.1). Exemplar cyclic voltammograms for the two cells are shown in Figure 3.6 below.

The principle aim of these experiments was to test the electronics, which worked very well at both current ranges (Figure 3.6 a and b). The noise of the potentiostat was estimated using the standard deviation of the signal over the first 20 points of the CVs: it was equivalent to 0.0511 mV for the 100 kΩ gain resistor (Figure 3.6 a) and 0.2661 mV for the 10 MΩ gain resistor (Figure 3.6 b) with no filtering applied.
Figure 3.6: Cyclic voltammograms with new PCB potentiostats. No filters were applied to the above data. (a): Gain resistor of 100 kΩ. 3-mm diameter glassy carbon electrode in 1 mM RuHex(III) and 1 M KCl and gentle nitrogen bubbling. Electrode polished with alumina prior to CV. Exemplar illustration for the calculation of the cathodic and anodic currents ($i_{p,c}$ and $i_{p,a}$) and potentials ($E_{p,c}$ and $E_{p,a}$) at 20 mV/s scan rate. (b): Gain resistor of 10 MΩ. 75 µm gold electrode in 10 mM RuHex(III) and 1 M KCl and gentle nitrogen bubbling. Electrode polished with alumina and cleaned in acid: 20 CVs in 0.5 mM sulfuric acid (potential ramp from 1.5 to -0.3 V).
3.1. Potentiostat

Additionally, these CVs presented the characteristics expected from a reversible redox couple.

For the macro-electrode (Figure 3.6 a), we observed:

- no change in the peak potentials, $E_{p,c}$ and $E_{p,a}$, with the scan rate,
- a peak width and a peak separation of 65 mV. This is slightly higher than the expected 59 mV but the difference is due to the quality of the electrode surface and not the electronics.
- a Nernst equilibrium potential $E_0 = \frac{E_{p,a} + E_{p,c}}{2} = -167$ mV,
- the peak currents, $i_p$, followed the theoretically predicted model [186]:
  \[
  i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} \nu^{1/2} c
  \]  
  (3.1)

where $n=1$ is the number of electrons transferred, $A=0.07065$ cm$^2$ is the surface area of the electrode, $D=7.9 \times 10^{-6}$ cm$^2$/s is the diffusion coefficient of ruthenium [189], $\nu$ is the scan rate in V/s and $c=1 \times 10^{-6}$ M/cm$^3$ is the concentration in ruthenium hexamine.

As an example, we measured $i_p=7.31$ and 11.33 µA at scan rates of 20 and 50 mV/s respectively, compared to the calculated values of 7.49 and 11.84 µA respectively.

For the micro-electrode (Figure 3.6 b), we observed:

- no current peaks at low scan rate,
- a Nernst equilibrium potential of −168 mV,
- the Tomes potential, $E_{Tomes} = |E_{1/4} - E_{3/4}| = 58.6$ mV, which is more far from the theoretical value of $\frac{RT}{nF} \ln(9) = 55.7$ mV at 21°C [190; 191].
- a limiting current, $|i_l|$, of 0.164 µA. In comparison, the theoretical value for a disk micro-electrode is given by [186]:
  \[
  |i_l| = 4nFcDr
  \]  
  (3.2)

where $F=96 485.3415$ C/mol is the Faraday constant, $c=10$ mM is the concentration in ruthenium hexamine, $D=7.9 \times 10^{-10}$ m$^2$/s is the diffusion coefficient of ruthenium [189] and $r=37.5$ µm is the radius of the electrode.

The numerical application gives 0.114 µA, which is clearly less than the measured current. One possible reason is that the bubbling of nitrogen in the solution increased the mass transfer to the electrode surface. Another reason would be that the electrode surface was not strictly an inlaid disk but more likely protruding slightly. In this case, a hemispheric model would be more appropriate. The relation between the limiting currents for a disc electrode compared to a hemispheric electrode is given by [186]:

\[
\frac{i_{hemi}}{i_{disk}} = \frac{2\pi}{4} \approx 1.57
\]  
(3.3)

Using the above equation, the predicted current is 0.179 µA, which is closer to the experimental value, 0.164 µA.

These results confirm that the PCB can drive an electrochemical cell with real-world issues, such as interfacial impedance and resistive drops among others.
3.2 Instrumentation amplifier for potentiometric measurements

**Amperometric detection**

Two PCBs were set up in the Faraday box of the rsMD assay (one for glucose and the other for lactate measurement), using the metal frame of the box as shields for the components. They were then used for *in vivo* measurement in the rat brain (see section 6.4 in Chapter 6 for more details). Exemplar glucose peaks recorded with the new PCBs are shown in Figure 3.7 below:

![Glucose peaks recorded with new PCBs](image)

*Figure 3.7: Application of the PCB to the rsMD assay.* These are raw data for glucose measurements in the rat brain with the new PCB potentiostats. No external potential was applied to the potentiostat and a 0.1 µA/V gain was used.

As shown on Figure 3.7, there is a baseline noise equivalent to 12.5 mV, which is much larger than the voltage noise attributable to the PCB (0.266 mV according to the CVs above in Figure 3.6). This is due to flow instability in the rsMD assay. Removing this noise is the object of Chapter 4. Nevertheless, the quality of the signal was very stable over time, with no deterioration during hours of continuous use of the assay. In addition, all the data presented in this Chapter were not filtered, but the full range of filters provided within the analogue/digital amplifier (ADInstruments, Oxfordshire, United Kingdom) can now be activated in a noisy environment if desired.

### 3.2 Instrumentation amplifier for potentiometric measurements

I also directed a student to design an instrument amplifier for potentiometric measurement of ion selective electrode (see Chapter 6). She used the ultra low input bias current instrumentation amplifier INA116PA (Texas Instruments, United States) and the PCB was designed following the exact same rules as those of the potentiostats above (such as de-coupling capacitors, two-sided board, same track widths, earth star points, input guards, screened cables and connectors). The resulting design is shown in Figure 3.8.
3.3 Isolated power supply

The PCBs were powered with a highly specified isolated DC-DC converters (THB 3-0523, Traco Power, Switzerland) to supply ±15 V to the operational amplifiers from 5 V DC. These are small, have very low output ripples and noise (<100 mV peak-to-peak), a 4000 VACrms isolation and they meet medical safety standards. The schematics and layout of the PCBs were straight-forward and are given in Figure 3.9 below:

This PCB was successfully tested for its time response, gain accuracy, stability and signal-to-noise ratio with potassium ion selective electrodes (data not shown).
3.4 Conclusion

The DC-DC converters were fed in with 5 V DC from a medically approved mains adapter (DPS52-M, Astec) with low ripple noise (<50 mV/peak-to-peak). When connected to the PCB (Figure 3.9 c), the output voltage was $\pm 15$ V with a typical ripple of maximum amplitude of 2 mV, and a standard deviation of 0.445 mV.

3.4 Conclusion

Potentiostat PCBs were successfully designed and manufactured to meet specifications:

- **low noise** with the additional benefit of having access to sophisticated hardware filters,
- **stable** over time in real-world conditions,
- **small** size,
- **cheap**.

Additionally, they have a wide range of applications and can be used for biosensors and a future generation of the rsMD assay. They are isolated from the mains, although an isolation amplifier will be required to create an isolation barrier between the data acquisition system and the PCBs. These would provide full isolation of the electronics and ultimately the rsMD box could be placed within centimeters of the patient. Work on this is currently in progress within the group.

Finally, the voltage noise controlling an electrochemical cell was of 0.051 mV (for a gain of 0.1 mA/V) and 0.266 mV (for a gain of 1 µA/V). That the noise in the rsMD system is greater than this, is due to other sources of noise. Removal of these is the topic of the next chapter.
Chapter 4

Development of signal processing tools for analytical signals

Experimental data are usually complicated by noise that may be due to interfering physical or chemical processes, instrumental noise, environmental noise, or any number of causes which result in spurious fluctuations of the signal generated by the detector. The rsMD assay is only one example of such an analytical signal that represents high-value data and that is used in an environment (the intensive care unit) where there are multiple sources of noise contamination. The main goal is therefore to devise techniques that provide a maximum signal-to-noise ratio (SNR). Efforts generally focus on maximising the signal by improving the sensitivity, precision, selectivity and limit of detection of the system. As an example, the rsMD assay has been optimised within the group to give a stable and sensitive signal for up to five days of continuous use. However, in real world conditions, analytical systems are frequently operated very close to their limits of detection, where the SNR is small. In this case, an otherwise small uncertainty in the background noise can induce a large error in the evaluation of the signal of interest. Examples of three types of noise typically encountered are given in Figure 4.1 below:

![Figure 4.1: Three types of noise.](image)

These are examples from clinical recordings with the rsMD assay in the intensive care unit. The signal-to-noise ratio (SNR) is defined in sections 2.5.2.2 and 4.2. (a): two analyte peaks with very low noise (b): two types of noise added to the signal: spikes (purple) and ripples (green). The SNR drops from 110 to 20. (c): two analyte peaks are masked and dramatically distorted by a slowly drifting baseline (gray). The SNR is very small.
Three classes of noise corrupt the signal of interest and can be identified and classified according to their characteristic time scales relative to that of the signal of interest, $t_{signal}$:

1. **spikes** are short signals with time scales ($t_{spike}$) very much shorter than the signal of interest, $t_{spike} \ll t_{signal}$. They can be larger or smaller in magnitude than the signal of interest. We will here distinguish "stereotypical spikes" that have a reproducible pattern, with a readily identifiable and constant time scale from other sorts of spikes that can include simple and complex spikes (i.e. clusters of simple spikes) of varying time scale. Stereotypical spikes can be inherent to the experimental process, such as sample injection to an analysis stream, while other spikes can be caused by various sources, such as noise on the power line, imperfection of electronic circuits, and environmental conditions.

2. **ripples** are quasi-periodic signals that frequently have a similar time scale $t_{ripple}$ to the signal of interest. Ripples can have any magnitude, distribution and shape, and can be non-stationary. They can arise for example from piston pump flow fluctuations, thermostatic valves, or cardiac and respiratory induced fluctuations in clinically-related measurements.

3. **baseline drifts** are signals that vary at a time scale that is large compared to the signal of interest: $t_{drift} \gg t_{signal}$. In particular, it is assumed that the gradient of the drift is much less than the gradient of the signal of interest.

The characteristic time scale of the signal of interest $t_{signal}$ is data-dependent. It can in general be defined as the rate limiting factor in the process, either 90% response time of the detector, or the typical time scale of the measured phenomenon, or the full width at half maximum (FWHM) for chromatographic peaks.

To improve the SNR when solutions to maximise the signal have already been implemented, methods to minimise the noise are necessary.

One approach to this is a hardware approach where a second "sentinel" sensor, that responds to interfering signals but not the analytical signal, is placed alongside the primary sensor. Modern operational amplifiers can then efficiently reject common-mode noise at all frequencies within a wide operational bandwidth. This has been used successfully for implanted electrochemical biosensors [101; 192]. Such an approach is very useful, but depends on the availability of a suitable sentinel sensor that can be combined with the analytical system.

Alternative approaches to increase the SNR of analytical signals have been based on digital signal processing methods. This chapter describes three generic methods I have developed to remove these three classes of noise. Their application to the rsMD assay is discussed as an example. They are relevant to a wide range of analytical time-domain signals where similar problems with noise are found.

### 4.1 Most widely used digital filters

The most widely used smoothing filters among analytical chemists include least-squares polynomial fitting popularised by Savitzky and Golay in 1964 [193], and a wide range of Fourier-based filters [194]. These have been recently reviewed by Komsta [195].
4.1. Most widely used digital filters

4.1.1 Least-squares polynomial filters

The idea of the filter is to perform a simple local least squares polynomial regression and provide its fitted value to the output. The fitted polynomial is "smoother" than the raw data. An example is given in Figure 4.2 below, with a simple signal of 9 data points.

![Figure 4.2: Least-squares polynomial filtering. The raw signal is shown in grey with 9 data points (black dots). It is fitted by a least-squares quadratic polynomial (in pink) and the filtered or smoothed data points are the blue crosses.](image)

4.1.1.1 Theory

A polynomial of order \( n \) is repetitively fitted to sections (or window) of \( 2m + 1 \) consecutive data points, the \( y_i \), according to equation 4.1:

\[
f_n(i) = \sum_{k=0}^{n} b_k i^k
\]  

(4.1)

where \( f_n(i) \) is the polynomial evaluated at point \( i \), \( i \) ranges from \( i=-m \) to \( i=+m \), \( b_k \) are the polynomial coefficients.

The polynomial coefficients, \( b_k \), are calculated so as to minimise the least-squares distance between the polynomial and the data points, which is equivalent to:

\[
\frac{\partial}{\partial b_k} \left[ \sum_{i=-m}^{i=m} (f_n(i) - y_i)^2 \right] = 0
\]  

(4.2)

Note that a filter with a polynomial order of 1 is equivalent to a moving average window filter.

In 1964, Savitzky and Golay showed that fitting by least-squares to polynomials could be carried out rather easily by a digital convolution of the data with a set of prescribed numbers of weights \( w_j \) [193],...
4.1. Most widely used digital filters

i.e. the filtered signal can be expressed as:

\[ f_n(i) = \sum_{j=-m}^{m} w_j y_j \]  

(4.3)

The \( w_j \) may be derived from a table [196] or analytically using the Gram polynomials (known to reproduce exactly the results of least-squares polynomial regression) [197]. The main advantage of the convolution is its linearity, which means that the filter coefficients, \( w_j \), can be pre-calculated and stored for a given polynomial order and window length. As compared to traditional smoothing methods, such as a moving average, the Savitzky-Golay (SG) filter performs much better and preserves the features of the distribution of points contained in the chosen window, including relative maxima, minima and width [193; 194]. For all these reasons, the Savitzky-Golay (SG) filter has become an almost universal method for increasing signal-to-noise ratio: it smoothes the data with minimal peak distortion and peak shift and it is computationally simple (convolution) and can do real-time signal processing (with a small delay).

4.1.1.2 Parameters of the filter

The smoothing performance of the filter function is linked to the width of the filter (length of the window) and the degree of the fitted polynomial.

Filter width

For a given degree of the polynomial, large-window filters effectively suppress noise at the expense of distorting smaller scale signal features, and generally features that are poorly approximated by a polynomial over a particular spatial scale [198]. Smaller-window filters preserve smaller scale features well with the disadvantage of being less effective at suppressing noise [197]. Willson and Edwards studied the effect of a quadratic SG polynomial on Gaussian and Lorentzian peaks when increasing the window length of the convolution as compared to the full width at half maximum (FWHM) of the peaks [199]. With increasing width, the polynomial fits distort the signal, shortening and broadening the peaks, as shown in Figure 4.3. In their study, they concluded that a window length equal to \( 0.7 \times \text{FWHM} \) of the narrowest Gaussian peak gave the best trade-off between noise reduction and signal distortion [199]. However, in a similar study carried out at the same time by Enke and Nieman [200], it was demonstrated that the best signal-to-noise enhancement from a quadratic smoothing occurs for a filter width that is twice as wide as the FWHM of the peak being smoothed. Hence, the optimum width of a single-pass smoothing filter will depend on the criteria set by the user. A further problem complicates the selection of the optimal size of the moving window: different window widths may be superior at various portions of the data.
4.1. Most widely used digital filters

**Figure 4.3: Effect of filter width on a Gaussian peak.** The Gaussian peak before filtering is shown in blue with its full width at half maximum (FWHM). The effect of increasing the width of a quadratic SG filter is shown by a succession of black curves: each curve corresponds to a given window width indicated by the ratio $\frac{\text{FWHM}}{\text{width}}$. Taken and modified from [199].

Recently, Browne *et al.* [201] suggested an unbiased method to choose the optimal window length on different sections of the signal. They used the residuals between the SG fitted data and the original data and proposed using the correlation (Pearson’s correlation coefficient) between the residuals over the window width as a criterion for optimal smoothing. They assumed that the noise is an uncorrelated process, so that if the residuals are significantly correlated over a given window width, then it means that some components of the signal are incorporated into the residuals and the window size is too large. In practice, the data is filtered using a gradually increasing width, until the point at which the residual correlations become significant. The optimal filter is of length less than the filter that produces significant residual correlations. This adaptive window SG filter resulted in very good noise reduction in flat regions whilst introducing relatively little bias near the higher dynamic regions. However, the improvement in SNR was not overwhelmingly better than a simple fixed window SG filter and the computation time was much longer.

**Polynomial degree**

Similarly to the window width, the degree of the polynomial will determine the level of noise reduction and of signal distortion. The minimum distortion will occur when the polynomial accurately describes the analytical data and will deviate as the polynomial departs from the true curve. Generally, a smaller polynomial degree maximises noise reduction and a larger polynomial degree maximises fidelity to the signal of interest [199]. Bromba and Zieger [198] showed that a SG smoothing filter of degree $2M$ exactly conserves every existing moment up to $m = 2M + 1$. Methods to adapt the polynomial degree to the nature of the signal have therefore been devised to achieve the best SNR enhancement. They are also based on statistical tests on the residuals between the fitted data and the actual data: the F-distribution of the residuals has been proposed to test the fitting quality of the polynomial function to the experimental data as the degree of the polynomial is increased [202]. Jakubowska *et al.* [203] showed that adaptive polynomial SG filters improved the shapes of voltammograms as compared to fixed degree SG filters.
4.1. Most widely used digital filters

4.1.2 Fourier filters

Historically, a solution to noise has been very heavy damping or filtering. Such an approach relies on a characteristic time scale for the signal of interest, $t_{\text{signal}}$, being so long that a simple low-pass filter effectively removed all types of noise. However, with the move of analytical instrumentation to "real-time" or rapidly responding approaches such as capillary electrophoresis, lab-on-a-chip, micro-bore columns, and flow injection analysis, this is no longer tenable.

4.1.2.1 Theory

The Fourier denoising is based on the Fast Fourier Transform [194], which converts the data from the signal domain to the frequency domain. The signal sequence, $y_1,...,y_N$, is transformed into the sequence of $Y_1,...,Y_N$ complex numbers with the following formula:

$$Y_n = \sum_{k=0}^{N-1} y_k e^{\frac{2\pi i k}{N}} = a_0 + \sum_{k=0}^{N-1} \left[ a_k \cos\left(\frac{2kn\pi}{N}\right) + ib_k \sin\left(\frac{2kn\pi}{N}\right) \right]$$  \hspace{1cm} n = 0,...,N - 1 \hspace{1cm} (4.4)

The frequency components, $Y_n$, are then multiplied with the filter coefficients that will eliminate the high frequency components (attributed largely to noise) while preserving the lower frequency components mostly contained in the signal. Then, the inverse Fourier Transform is performed on the result to construct a smooth(er) time-domain signal.

4.1.2.2 Parameter of the filter

A major issue with Fourier smoothing is that of determining the optimal spectral cutoff where noise is removed as much as possible without compromising signal integrity. When the signal and noise spectral distributions are completely separated, the cutoff is obvious, but when they overlap, which is often the case, it is less determinate.

There have been various methods suggested for determining the best frequency cutoff. These include:

- taking the frequency at which the Fourier amplitudes of the signal drop below 0.1% of maximum [204],
- taking the frequency at which the signal is five times greater than last few points of the spectrum [205],
- taking the frequency at which standard deviation of the spectrum significantly increases [206]
- taking the frequency which will preserve the narrowest peak, based on the equivalent width criterion (equivalent width defined as the ratio between the area and the height of the peak) [207]

Most of these methods are ad hoc methods and require prior knowledge of signal or noise. Alternatively, methods based on information-theory criteria can be applied to any sort of signal and noise combinations. Akaike [208] proposed combining a maximum likelihood cost function with an increasing (linear) function that penalises model complexity. Larivee et al. [209] later proposed a criterion based on a local maximisation of Shannon’s information entropy.
4.2. Common Materials

4.1.3 Limitations of these filters

The above methods have been successful in a wide number of cases but they usually fail in the case of spikes and ripples. In the first case, spikes appear as discontinuities that cannot be fitted by a polynomial and that cause ripples when using Fourier filters. In the second case, ripples can be non-stationary and can have the same time scale as that of the signal: hence, an SG window length cannot remove the ripples effectively without distorting the signal and there is no clear cut-off frequency that can separate noise and signal in the Fourier domain.

4.1.4 New strategy for data processing

Unlike these common filters that mainly aim at crushing the noise within the signal, my strategies are based on recognising the noise and then removing it from the otherwise unprocessed signal. The SG filter and the Fast Fourier transform have been used, but as intermediary steps to recognise and characterise the noise, rather than as the output result of the digital signal processing. The three types of noise that are frequently found in experiments and that have been briefly described above, are addressed: spikes, ripples and baseline drift (for completeness). The general approach consists in:

1. use prior knowledge about the noise to fit the noise types. In particular, spikes have a short characteristic time scale, $t_{\text{spike}}$ and ripples are quasi-periodic,

2. subtract the fitted noise from the otherwise unprocessed signal.

Using this two-step strategy, we can achieve both maximal noise reduction and minimal signal distortions.

4.2 Common Materials

4.2.1 Programming Language

All algorithms were coded using Matlab 7.2. One of the reasons for this choice is the possibility within the Chart software to directly export the data as a matrix format compatible with Matlab (*.mat).

A few other tools available in Matlab were extremely useful to develop the codes:

- it supports vector and matrix operations, thus limiting iterative point-to-point calculations (‘for’ loops). This is particularly significant when dealing with continuous 5 day-long data, which represent up to 86.5 million data points when sampling at 200 Hz;
- the signal processing toolbox has many filters and signal analysis tools already implemented, such as spectral analysis, a wide range of digital and analog frequency filters and other smoothing filters including a Savitzky-Golay filter (see section 4.3). These were invaluable for the despiking and derippling algorithms;
- a wide range of numeric computation functions are available, notably some non-linear optimization, at the core of detrending (see section 4.5);
- the Profiler function records the time spent executing each line of code. This is ideal for optimisation of the algorithm in order to minimise computation time, a pre-requisite for online data processing.
4.2.2 Test data set

The algorithms were first developed on a "test data set" recorded with rsMD. This test data set was recorded in the lab in the same conditions as those used when monitoring patients: two enzyme reactors were prepared and loaded onto the assay, the HPLC pump flow rate was set to 200µL/min and a 1 mM mixed glucose/lactate standard was continuously injected into the valve during a period of 3 hours. Before injecting 1mM standard solution, a period of blank signal was recorded: 30 minutes of ripples only, and another 30 minutes of ripples and spikes.

A 60-second snapshot from this test data is used to illustrate the algorithms. Examples of applications of the algorithms are from real clinical data recorded with the rsMD assay and general application to analytical signals is discussed.

In this chapter, we refer to the signal-to-noise-ratio (SNR), defined as the ratio of the peak height over the standard deviation of the baseline noise.

4.3 De-spiking

Spikes are fast signals with characteristic time scales very much shorter than the signal of interest, $t_{\text{spike}} \ll t_{\text{signal}}$. Spikes often mask details of the true data, which can lead to misidentification of the signal of interest and subsequently significant error in the quantification of the signal. To avoid these false readings, it is essential to remove the spikes reliably without introducing distortion into the rest of the curve.

4.3.1 Current strategies to remove spikes

Most of the methods used for noise reduction are not effective for spike removal. Methods such as Savitzky-Golay (SG) smoothing [197] or adaptive degree polynomial filter [202] fail in the presence of spikes, as a result of trying to fit a polynomial function across discontinuities. Similar problems occur when using Fourier filters, such as Butterworth or Chebyshev filters [210], because the filters produce ripples at sharp edges. In fact, these methods result in the propagation of the spike to the preceding and following points and change the signal shape undesirably. I have here developed an alternative method that does not use the filtered data as the denoised signal, but as an intermediate step for the localization of the spikes within the unprocessed raw data set.

De-spiking is a two-step procedure that achieves both maximal removal of spikes and minimal distortions of the signal of interest:

1. detection of the position and width of a spike or spike train (described in Section 4.3.2.1),
2. removal of the spikes in the original unprocessed signal by excision, linear interpolation and smoothing of the identified spike region (described in Section 4.3.2.2).

The main difficulty is step 1: the reliable identification of spikes. Manual detection of spikes is an easy operation, but automatic detection of short pulses is more complicated, especially when the data are complicated by other forms of random noise. Several different types of spike detection methods are described in the literature, usually based on outlier detection algorithms, as the spike may be treated
as an outlier from the correct experimental signal. These methods are generally based on examining higher moments, in practice skewness and kurtosis [211]. Non-linear smoothing algorithms have also been applied to Raman spectra [212; 213]. I here propose two other methods (methods 1 and 2 in section 4.3.2.1) that are easy to implement and fast to compute. They both exploit the main property of spikes: their short time scale, $t_{\text{spike}}$, as compared to the analyte signal.

### 4.3.2 De-spiking Algorithm

#### 4.3.2.1 Step 1: detection of the spikes

Two methods have been developed, depending on the type of spikes:

1. **Method 1**: for stereotypical spikes: they have a constant and readily identifiable time scale, $t_{\text{spike}}$ (Figure 4.4 a)
2. **Method 2** for non stereotypical spikes with a complex varying time scale (Figure 4.4 b)

![Figure 4.4: Stereotypical (a) or non-stereotypical spikes (b). These are rsMD signals during clinical monitoring.](image)

**Method 1: Stereotypical spikes**

The first method is applicable to stereotypical spikes whose time scale $t_{\text{spike}}$ can be directly estimated from the data set. It is based on locating the spikes by the subtraction of a quadratic SG filtered curve from the original data set. Using the known $t_{\text{spike}}$, one can significantly attenuate the spikes using a window width $W_{\text{spike}} \geq t_{\text{spike}}$, typically $W_{\text{spike}}=1.25 \times t_{\text{spike}}$. Taking Figure 4.4 (a) as an example, for an injection spike of $t_{\text{spike}} = 1$ second, I used a window $W_{\text{spike}}$ of 251 data points, corresponding to 1.25 second at 200 Hz sampling. However, using such a window also leads to significant distortions of smaller scale signal features, such as the very top of the analyte peak, hence reducing the corresponding peak height and causing error on estimation of the concentration of glucose (or lactate). Instead of using the SG filtered trace as the final de-noised signal, I subtract the filtered trace (Figure 4.5 b) from the original trace (Figure 4.5 a) to obtain an intermediate signal in which only regions of fast dynamics, in particular spikes and smoothing errors remain (Figure 4.5...
4.3. De-spiking

c). Spikes are then easily identifiable in this intermediary signal by thresholding.

![Graphs showing original, filtered, and spikes signals]

**Figure 4.5: De-spiking algorithm- Step1: Detection of Spikes.** (a) Original signal: one-minute long section from the test data set with one analyte peak and two spikes at 30 seconds interval. (b) Filtered signal with a Savitzky-Golay filter of window width equal to 251 data points and a second order polynomial. (c) Signal obtained when subtracting the filtered signal (b) from the original signal (a). Note that the current amplitude is different in (c).

**Method 2: Non-stereotypical spikes**

In the case when $t_{\text{spike}}$ cannot be determined or varies, method 1 is not always practical. Instead, a more computationally intensive method based on the derivative of the signal can be used to detect spikes: as spikes are rapidly varying signals relative to the signal of interest, their slopes are generally larger than those of the signal of interest. So when taking the derivative of the signal, large components will be ascribed to spikes in the original data. The derivative signal is therefore equivalent to Figure 4.5 c and spikes can be readily identified by thresholding.

**Methods 1 and 2: Thresholding and identification of the locations and widths of the spikes**

A threshold can be manually selected to detect the spikes in Figure 4.5 c based on the amplitude of the spikes. Alternatively, the threshold can be automatically selected using the method initially developed by Otsu for gray-level images [214]. Using a histogram of the difference signal, it calculates the threshold that best separates two classes of data points: spikes and other forms of random noise in our case. The optimum threshold is defined as the one that maximises the between-class variance, $\sigma_{12}$. In other words, the optimum threshold is the one that separates two tight classes with a minimum overlap between the two. This is a simple and fast to compute algorithm: the threshold is iteratively increased from an initial low value so that the variance of one class is increased while the variance of the other is decreased. It proceeds as follows:

1. Compute the histogram of the signal (with n bins).
2. Define class 1 as the data points with a magnitude less than the threshold, $k$, and class 2 the data points above this threshold. Initially $k=$bin 2.
3. Calculate the class probabilities $\omega_1$ and $\omega_2$, as follows:

$$\omega_1 = \frac{\sum_{i=1}^{k} p_i}{\sum_{i=1}^{n} p_i}$$

$$\omega_2 = \frac{\sum_{i=k+1}^{n} p_i}{\sum_{i=k+1}^{n} p_i} = 1 - \omega_1$$

with $p_i$ the number of points in bin $i$ over the total number of points.

4. Calculate the mean of each class, $\mu_1$ and $\mu_2$ defined by:

$$\mu_1 = \frac{\sum_{i=1}^{k} i p_i}{\omega_1}$$

$$\mu_2 = \frac{\sum_{i=k+1}^{n} i p_i}{\omega_2}$$

5. Calculate the between-class variance $\sigma_{12}$ defined as:

$$\sigma_{12} = \omega_1 \omega_2 (\mu_1 - \mu_2)^2$$

6. Repeat steps 2 to 5 for each possible threshold (bin $2 \leq k \leq \text{bin } n$)

7. The optimal threshold is the one that maximises the between-class variance $\sigma_{12}$.

The histogram and Otsu’s threshold for the case of Figure 4.5 c is given in Figure 4.6 below:

![Figure 4.6: Determination of the threshold by Otsu’s method.](image)

The signal in Figure 4.5 c is ordered by the absolute amplitude of the signal: a hundred amplitude levels (bins) are defined in this example. The number of data points within each absolute amplitude level is counted and a histogram is built. The optimum threshold is found by the method developed by Otsu [214]: here the optimum threshold is 0.085 (red vertical line).

This threshold determined by Otsu’s algorithm is then used to identify the positions of the spikes in the spikes signal (Figure 4.5 c for stereotypical spikes and 4.7 for non-stereotypical spikes). The position of the spikes is stored in an array of indices $(\text{spike}_1, \text{spike}_1, ...)$ on Figure 4.7), each defined as the central point of the segment of data points whose absolute amplitudes are above the threshold.

In the case of stereotypical spikes, the width of the spikes has been defined by $W_{\text{spike}}$. In the case of non-stereotypical spikes, the width of the spikes, $W_{\text{spike}}$, is defined using the method illustrated in
4.3. De-spiking

Figure 4.7.
1. estimate the number of data points, \( y \), whose absolute amplitudes are above the threshold
2. choose the biggest number, \( Y = \max(y) \)
3. define the width of the spikes: \( W_{\text{spike}} = a \times Y \) where \( a \) is a factor set by the user. By experience, \( a=20 \) was an appropriate choice for the data we analysed.

\[
W_{\text{spike}} = aY
\]

Figure 4.7: Identify the location and width of the spikes. The threshold is used to detect the spikes and their positions (indices in orange: spike1, spike2, spike2). A maximum width \( W_{\text{spike}} \) for non-stereotypical spikes is then defined based on the maximum (\( Y \)) number of data points (\( y \)) below the threshold.

4.3.2.2 Step 2: Removal of the spikes

After detection of the spike positions and widths, the data segments (of width \( W_{\text{spike}} \)) at the spike positions are first excised from the original signal. Then, the excised segment is replaced by a linear interpolation (Figure 4.8 b). The resulting signal is then smoothed using a quadratic SG filter with a window length \( W=3 \times W_{\text{spike}} \) (not shown on Figure 4.8). The output is then used to replace only the spikes in the original unprocessed signal. This illustrated in Figure 4.8 below.
4.3. De-spiking

Figure 4.8: Output of de-spiking. (a) Original signal from the test data set and represented in Figure 4.5 a. (b) Every identified spike is removed according to procedure above. (c) The de-spiked signal: the sections containing spikes were replaced (pink).

The de-spiked signal is identical to the original raw signal, except at the sections containing the spikes. A general overview of the de-spiking algorithm with a numerical example is represented in Figure 4.9.
4.3. De-spiking

Step 1: spike detection

Apply a quadratic SG filter with a window length 
\( W_{\text{spike}} = 1.5 f_{\text{spike}} \).

Calculate the difference signal by subtracting the output from the original signals

Compute the normalized histogram of the above
Apply Otsu’s thresholding method
Create \( x \) = array of all the indices of the points above the threshold

\[
\begin{align*}
x &= 3 \quad 4 \quad \ldots \quad 12 \quad 13 \quad 25 \quad 26 \quad \ldots \quad 33 \quad 34 \quad 56 \quad 56 \quad \ldots \quad 71 \quad 72 \quad 86 \quad 87 \quad \ldots \quad 94 \quad 95
\end{align*}
\]

Determine \( W_{\text{spike}} \):
1. Compute \( dx = \text{difference of } x \)
2. Create \( y = \text{indices of the elements in } dx > 1 \)
3. Compute \( dy = \text{difference of } y \) and its absolute maximum \( Y \)
4. \( W_{\text{spike}} = a Y \) (for our sampling rate, \( a = 20 \))

Define \textit{spike locations} as the middle of each set of consecutive indices of \( x \)
Define a \textit{spike region}: \( W_{\text{spikes}} \) data points centered on each \textit{spike location}

\[
\begin{align*}
x &= 3 \quad 4 \quad \ldots \quad 12 \quad 13 \quad 25 \quad 26 \quad \ldots \quad 33 \quad 34 \quad 55 \quad 56 \quad \ldots \quad 71 \quad 72 \quad 86 \quad 87 \quad \ldots \quad 94 \quad 95
\end{align*}
\]

spike1=8  spike2=29  spike3=63  spike4=90

Step 2: spike removal

Excise the \textit{spike regions}
Replace these with a linear interpolator.

Smooth the resulting signal using a quadratic SG filter with a window length of \( 3W_{\text{spikes}} \)

Replace only the \textit{spike region} with the output of this filter in the original unprocessed signal

Figure 4.9: De-spiking algorithm represented as a flow-chart with numerical examples.
4.3. De-spiking

4.3.2.3 Matlab code

The integrity of the Matlab code and some examples for this algorithm can be found on the DVD accompanying this thesis and in the Supplementary Materials of the paper published in Analytical Chemistry [215]. I here give the three key commands:

- \( y = \text{sgolayfilt}(x, \text{order}, \text{window}) \): the Matlab implementation of a Savitzky-Golay filter. The user defines the order of the interpolation polynomial and the window length.
- \([n, \text{amp\_range}] = \text{hist}(x, \text{nbins})\): bins the elements in an input vector \( x \) into \( \text{nbins} \) equally spaced containers and returns the number of elements in each container as a row vector \( n \) as well as the bin locations \( \text{amp\_range} \). This function was used to obtain Figure 4.6.
- \( y = \text{find}(x > \text{th}) \): returns \( y \), the indices of the elements of the array \( x \) that satisfy the logical expression \( x > \text{th} \) (\( \text{th} \) being the Otsu threshold in our case).

4.3.3 Results and Discussion

4.3.3.1 Application to clinical rsMD traces

The de-spiking method was very efficient at removing spikes. After de-spiking over 700 hours of data (i.e. over 85,000 spikes), 96.8% of the spikes were removed.

Figure 4.10 below is a 30-minute-long clinical trace from the first patient I monitored in the intensive care unit showing the output of de-spiking for stereotypical spikes. Here all spikes (100%) are removed effectively:

\[ a. \text{Raw signal} \quad b. \text{De-spiked signal} \]

![Figure 4.10: De-spiking stereotypical spikes. (a) Raw Signal showing 15 minutes of rsMD analysis peaks and spikes at injection of the dialysate sample. (b) Same signal after de-spiking. The window length for the Savitzky-Golaly filter was set to \( W_{\text{spike}} = 251 \) data points.](image)

Not only is this method very effective at removing spikes, it also causes minimal distortion of the original signal. This is because de-spiking alters the spike region only, while it conserves the raw signal everywhere else. In particular, in the worst-case scenario, when a spike occurs at exactly the same time as an analyte peak, the algorithm performs well. Such an example is given below in Figure 4.11:
4.3. De-spiking

Figure 4.11: De-spiking stereotypical spikes: minimal distortion. This is the case of patient 4 from Chapter 5. The window length for the Savitzky-Golay filter was set to \(W_{\text{spike}}=201\) data points.

De-spiking non-stereotypical spikes was also very effective and Figure 4.12 a below is a typical example with a variety of spikes that completely distort the signal:

Figure 4.12: De-spiking non-stereotypical spikes. The spikes in the raw signal have different magnitudes, some are positive and others negative, some are simple and others part of a train of multiple spikes. They mask the data and can foul an automatic peak detection algorithm (black dots in a). Once the signal is cleared of its spikes, the peaks can be reliably detected (black dots in b) for further quantification. This was used for Patient 7 in Chapter 5.

In the case of Figure 4.12, 93% of the spikes were effectively removed. From an analytical perspective, the de-spiking procedure enables reliable automatic estimation of the peak heights using an automatic local maximum detection algorithm, such as that embedded in the data acquisition software (Chart 5.5.6, ADInstruments). In the original data, most of the analyte peaks detected by the algorithm are in fact spikes (Figure 4.12 a). In the de-spiked data (Figure 4.12 b), all of the analyte peaks are identified correctly. This proved invaluable for further clinical interpretation of the data collected clinically with rsMD and clinical results are presented in Chapter 5. More examples and applications of de-spiking can be found on the DVD accompanying this thesis and online in the Supporting Information of the paper published in Analytical Chemistry [215].
4.3. De-spiking

4.3.3.2 Guidelines for a general use of de-spiking

The de-spiking method does not make any assumptions on the nature of the signal and can therefore be applied to any types of data provided that the conditions defining a spike \( t_{\text{spike}} < t_{\text{signal}} \) are met. Beside the rsMD clinical traces, I have also used de-spiking on aortic pressure data. They were "polluted" by some unidentified machinery in the catheter lab and stereotypical de-spiking was applied to de-noise this signal, as shown in Figure 4.13 below:

![De-spiking aortic pressure data](image)

**Figure 4.13:** De-spiking aortic pressure data. The window length was set to \( W_{\text{spike}}=61 \) data points.

On average (with rsMD data or aortic pressure data), the stereotypical de-spiking method is slightly more efficient than the non-stereotypical method (98% vs 90% of the spikes removed for all clinical traces tested) and faster to compute than the non-stereotypical method. This is because the polynomial fitting used by the SG filter is a linear operation: coefficients of the SG filter can be pre-stored in the programme and then applied to the signal with simple linear combinations of the polynomial coefficients [194].

However, the stereotypical method requires setting the window length \( W_{\text{spike}} \) using *a priori* knowledge of \( t_{\text{spike}} \), which is not always available, as in the case of patient 7 on Figure 4.12. From experience, the minimal window length \( W_{\text{spike}} \) should be \( W_{\text{spike}}=1.25 \times t_{\text{spike}} \). If a smaller window length is used, some spikes will be missed in the subtraction step. When using a longer window length, a somewhat larger region of the data set is affected by the spike removal step (step 2, section 4.3.2.2), which could be a problem when the spike occurs at the same time as the analyte peak as in Figure 4.11. In general, for data where the signal of interest is composed of peaks, the window length \( W_{\text{spike}} \) should be less than \( 0.7 \times \text{FWHM} \) (full width at half maximum) of the narrowest peak of interest. This causes minimal distortion of the peaks by an SG filter according to [199]. In cases where \( t_{\text{spike}} \) is not easily determined, then the non-stereotypical method is preferred.

Finally, the last parameter to choose is the polynomial order of the SG filter. When used in the spike detection step (step 1, section 4.3.2.1), it is not critical as signal integrity is not necessary in the spike identification step. However, for step 2 (section 4.3.2.2) and the removal of the spike with the second SG filter of width \( W = 3 \times W_{\text{spike}} \), it is preferable to select the order of the polynomial that will best approximate the signal of interest. Adaptive degree polynomial methods can be used for this purpose [202]. For chromatographic data, a quadratic filter is usually suitable, although the choice is also not very critical since it affects only a very limited segment of the final signal.
4.4. De-rippling

4.3.3.3 Comparison to other methods

Another spike removal technique is the moving median filter. In this method, the central value of an interval sliding along the curve is substituted by the median of the data in the interval. Originally described by Tukey [216], it has been applied to remove stereotypical spikes. However, it leads to considerable distortions of the curve shape, giving flat-topped truncated peaks [217], which has restricted its use in practice. I nevertheless tested it in the de-spiking method for stereotypical spikes. To avoid distortion of the peaks, I used it as a replacement for the SG filter used for the spike detection step (section 4.3.2.1); the window of the moving median filter was set to a length of half \( W_{\text{spike}} \) to effectively detect the spikes. Then, I subtracted this filtered data from the original data set and removed the identified spikes as described previously (step 2, section 4.3.2.2). It gave very similar results to the de-spiking method, with overlap of the output signals. Globally, 98% of the spikes were effectively removed with minimal distortion. However, it was computationally less efficient, usually 10 times slower than SG filters, which agrees with Stone’s observations [217].

A more recent algorithm designed for spike removal [218], based on a gross error statistical test, has been successfully used on voltammetric data, giving satisfactory spike removal while not distorting the curve. However, like the median filter, it requires sorting the data into either ascending or descending order, which makes it computationally slower than the de-spiking method.

In comparison to other de-spiking methods I have tried, the main advantage of my method (beside removing the spikes efficiently) is its computational speed, which makes it appropriate for real-time online processing as data are collected. The Matlab code is currently being re-written in C++ to implement de-spiking within the data collection software.

4.4 De-rippling

Ripples are defined as quasi-periodic fluctuations. They are neither random nor sinus-like and can be non-stationary, changing in morphology and distribution with time. As a further complication, they can have a characteristic time scale \( t_{\text{ripple}} \) very similar to that of the signal of interest. In the case of a periodic signal, as for example chromatographic peaks, the similar time scale between signal and ripple can lead to aliasing of the ripples on the peaks of interest. This affects the baseline, and over time, all parts of the peak. This in turn leads to slow variations in measures of the peak, such as height and peak area. To limit this problem, it is important to remove the ripples without compromising the signal of interest.

4.4.1 Current strategies to remove ripples

The most commonly used technique to remove periodic noise is based on Fourier filters [219]. This method works reasonably well when the typical time scale of the signal of interest is long, such as the broad analyte peaks in the case of traditional liquid chromatography [220]. However, with the introduction of faster chromatographic methods, the separation between noise and signal in the spectral domain is not so clear. In this case, it is very difficult to determine the optimal spectral cutoff that will remove noise without compromising signal integrity [210]. In any case, Fourier filters will
4.4. De-rippling

inevitably fail when noise and signal have the same time scales so that they overlap in the frequency domain. In such cases any Fourier filter will lead to dramatic distortion of the signal of interest, especially flattening and broadening of the analyte peaks, and therefore cannot be used in practice. An example of the effect of a low-pass Fourier filter is given in Figure 4.14 below:

![Figure 4.14: Low-pass filter distortion. (a) Original signal. (b) Output of a low-pass Fourier filter (cut-off of 0.1Hz) on the signal in (a). The Fourier filter effectively attenuate the ripples but also significantly distorts the peak, making it smaller and broader. There is no improvement in the signal-to-noise ratio (SNR).](image)

The Fourier filter effectively attenuates the noise, but it also attenuates and distorts the signal, which can lead to errors of quantification. Another main drawback of Fourier filters and other linear filters in general is their limited success with non-stationary signals.

An alternative method using the quasi-periodicity of the ripples is proposed here. It is again a two-step procedure:

1. estimate the ripples with a **template** that is a good representation of the noise (described in section 4.4.2.1).
2. **subtract** this template from the original unprocessed signal (described in section 4.4.2.2).

This is the de-rippling approach.

### 4.4.2  De-rippling algorithm

#### 4.4.2.1  Step 1: Creation of a ripple template

The template created from the ripple is computed as follows:

1. collect *n* ripples as a training set,
2. determine the temporal periodicity of the ripples, *t*_ripple by Fourier analysis (Figure 4.15),
4.4. De-rippling

Figure 4.15: Training set and its frequency spectrum. The training set is composed of the blank signal ripples (top). The power spectral density\(^1\) is calculated for the training set and the fundamental frequency (frequency of the first non-zero component of the spectrum) gives the periodicity of the ripples: here \(t_{\text{ripple}}=1/0.15=6.67\) seconds.

3. form an ensemble of \(n\) ripples of length \(1.2 \times t_{\text{ripple}}\) (Figure 4.16). If necessary, align them all using cross-correlation (see section 4.4.2.2),

4. subtract the mean from each ripple in the ensemble,

5. create the template as the average over the \(n\) ripples (Figure 4.16). The template is characterised by its magnitude, \(M_{\text{template}}\), and the variance across the \(n\) ripples, \(\sigma_{\text{template}}\).

Figure 4.16: Ensemble of \(n\) ripples and template. The training set is truncated into \(n\) ripples of \(1.2 \times t_{\text{ripple}}\) length that are all aligned together by correlation (see section 4.4.2.2). For each ripple, its mean is subtracted to form a zero-mean ensemble. The template is the average across the ensemble.

\(^1\)The power spectral density is actually the Fourier transform of the autocorrelation function of a stationary process. For a periodic signal, the non-zero components of the Fourier spectrum appear at discrete frequencies that are multiple of the fundamental (first) one.
4.4.2.2 Step 2: Subtraction of the template from the raw signal

The subtraction can be understood as an iterative process that moves along the signal by steps of \( t_{\text{ripple}} \), and works on sections of \( 1.2 \times t_{\text{ripple}} \) length (see Figure 4.17). The length of the section is defined as slightly longer than the length of the ripples so as to provide a sufficient number of data points overlapping at the "junction" between two consecutive subtraction steps.

For each section, before subtraction of the template, careful alignment of the template onto the signal is required to avoid distortion of the signal of interest. First, a series of tests is performed:

1. Is there a ripple in this section? If not, do not do anything. If yes, proceed to alignment (see below) and test 2.
2. Is there a change of the signal of interest in this section (for example, a chromatographic peak) occurring at the same time as a ripple? If not, subtract the aligned-template. If yes, we assume that the ripples are locally repetitive and subtract the previous aligned ripple. This procedure should ensure both that there is no contribution of the signal of interest when fitting the underlying ripple and that it gives the best chance of effectively zeroing the noise on the signal of interest.

The alignment of the template consists in:

1. time-alignment by cross-correlation (see next section for more details)
2. "level"-alignment by addition of the mean of the signal section to the template

After alignment, the template is subtracted, for each \( 1.2 \times t_{\text{ripple}} \)-long section from the raw signal according to the rules defined above (tests). An example is given in Figure 4.17 below:
4.4. De-rippling

Figure 4.17: De-rippling. As the algorithm moves along the raw signal (blue) by $1.2 \times t_{\text{ripple}}$-long sections, the de-rippled signal is progressively constructed (pink in b). For each section, tests and alignment (a) of the template (purple) are performed before subtraction (b). After subtraction, the algorithm moves along the signal by $t_{\text{ripple}}$ seconds so that there is an overlapping zone (orange rectangles) between consecutive sections. For section 1, the template is aligned onto the ripple preceding the peak (a left) and then subtracted from the raw signal (b. left). For section 2, the peak is detected so the template that was aligned on section 1 is only translated by $1.2 \times t_{\text{ripple}}$ (a middle) and then subtracted (b middle). The same thing applies for section 3 (middle traces). For the next sections, no peak is detected in the signal so the template is aligned on each ripple and subtracted from the raw signal, and the example for section 6 is given (traces on the right). For purpose of illustration, the de-rippled signal is shifted upwards by 0.6 µA (in reality, its baseline is close to 0.0 µA).

Time-alignment by cross-correlation

Initially, I used a few basic approaches to time-align the template onto the signal section. A time-lag was calculated based on one single feature of the template as compared to the same feature in the section. For example, I used the time of maximum, time of minimum, time of inflection point, etc. These initial methods worked on some clinical traces but failed on others, resulting in amplification of the ripples and distortions of the analyte peaks [221]. Alternatively, I also used two different features for time alignment (maximum point and inflection point for instance) and chose the best time-alignment. This was more robust but doubled the computation times which became unacceptable for 5-day-long data.

Using cross-correlation proved much more robust and applicable to a variety of "shapes" of the ripples. For two time-domain signals, $f(t)$ and $g(t)$, the cross-correlation function $\text{corr}_{f,g}(t)$ is defined by the following relation:

$$\text{corr}_{f,g}(t) = \int_{-\infty}^{+\infty} f(t + \tau)g(\tau) \, d\tau$$

(4.10)
4.4. De-rippling

The cross-correlation function can be understood as a comparison of the two time-domain signals at all possible relative positions \((t + \tau)\) in time. It works as follows:

- vary the relative position in time \(t\) of \(f\) (in our case the ripple template) compared to \(g\) (the signal section). For finite time signals \(f\) and \(g\) of maximum length \(T_L\), \(-T_L \leq t \leq T_L\).
- estimate how \(f\) and \(g\) match (in an inner-product sense) in that position, which gives a cross-correlation value at time \(t\).

This is illustrated in Figure 4.18 below representing the cross-correlation function (in green) of the signal section (in blue) and the template (in purple) when moved in 3 different time positions relative to the signal section:

![Cross-correlation function diagram](image)

**Figure 4.18: Cross-correlation function.** The central trace is the cross-correlation function (green). In reality, this is the cross-covariance (see section 4.4.2.3) so its amplitude is between \(-1\) and \(1\) and \(t-\tau\) varies between \(-1.2 \times t_{\text{ripple}}\) and \(+1.2 \times t_{\text{ripple}}\). Three cases of relative positions for the template compared to the signal section are illustrated: template moved backwards by 3 seconds (left, in black), template moved backwards by 2 seconds (top, in red) and template moved forwards by 3 seconds (right, in yellow). The alignment by \(-2\) seconds (top) corresponds to the best alignment.

As it reflects how well \(f\) and \(g\) match (in an inner-product sense), the cross-correlation shows a maximum peak at the time when \(f\) and \(g\) are the "closest" (see Figure 4.18 top). This is this time that I take for the time-lag to align the template on the signal section.
4.4. De-rippling

Update of the template to account for non-stationarity

Importantly, after each subtraction step, the template shape is updated to account for any changes in the patterns of the ripples (non-stationarity). Figure 4.19 is an example of a template that changed shape during de-rippling of a clinical trace.

![Figure 4.19: Update of the template.](image)

The decision to update the template with new ripples is determined by the variance of the template. This is calculated during the subtraction step. If this variance is much smaller than the amplitude of the ripples (typically 10 times smaller then the amplitude of the ripples after subtraction of the template), then the template is still adequate for subtraction. If the variance is above this critical value, then the template needs to adapt to the new morphology of the ripples. In this case, new ripples are added to the zero-mean ensemble as the algorithm moves along the trace and the "oldest" ripples are removed to keep the size of the ensemble constant.

A flowchart showing the decision tree for the whole de-rippling algorithm is shown in Figure 4.20.
4.4. De-rippling

**Step 1: creation of a ripple template**

1. Collect $n$ ripples as training set
2. Compute spectrum of the training set.
3. Determine the temporal periodicity $t_{\text{ripple}}$

- Truncate the training set into $n$ individual ripples, each of length $1.2 \times t_{\text{ripple}}$
- Form an ensemble of zero-mean ripples by:
  1. Aligning $n$ ripples in time using cross-correlation
  2. Subtracting the mean from each ripple

Define the template as the average over the $n$ ripples.
- $M_{\text{template}} = $ magnitude of the template
- $\sigma_{\text{template}} = $ variance across the $n$ ripples

**Step 2: subtract the template from the unprocessed signal**

1. Select a section of length $1.2 \times t_{\text{ripple}}$ in the original data.
2. Compute its magnitude $M_{\text{ripple}}$ and its mean $m_{\text{ripple}}$.

- **NO ripple in the section**
  - $M_{\text{ripple}} > \frac{M_{\text{template}}}{5}$?
    - Move on along the time axis by $t_{\text{ripple}}$

- **YES a ripple in the section**
  - Time align the template to the section by maximizing their cross-correlation.
  - Add $m_{\text{ripple}}$ to the template
  - Subtract the above from the section, estimate difference, $M_{\text{diff}}$

- **NO change in the signal**
  - $M_{\text{diff}} > \frac{M_{\text{template}}}{3}$?
    - Subtract the level- and time-aligned template from the section

- **YES a change in the signal**
  - Translate and subtract the template that was level- and time-aligned on the preceding section

- **NO**
  - $\sigma_{\text{template}} > \frac{M_{\text{diff}}}{10}$?

- **YES**
  - Update the template:
    1. add the section to ensemble
    2. subtract first ripple from ensemble

---

*Figure 4.20: De-rippling algorithm represented as a flow-chart.*
4.4. De-rippling

4.4.2.3 Matlab code

The complete Matlab code and some examples for this algorithm can be found on the DVD accompanying this thesis and in the Supplementary Materials of the paper published in Analytical Chemistry [215]. I here give the two key commands:

- \([P, \omega] = \text{periodogram}(x)\) computes the power spectral density (PSD) estimate, \(P\), of the time-domain vector \(x\). The PSD is the square of the magnitude of the windowed Fast Fourier Transform of \(x\) according to:

\[
P(e^{i\omega}) = \left| \frac{1}{\sqrt{N}} \sum_{k=1}^{N} x_k e^{-ik\omega} \right|^2
\]  

(4.11)

where \(\omega\) is the frequency in radians and \(e^{ik\omega}\) are the Fourier bases.

The periodogram is scaled so that the variance equals the mean of the periodogram, which is useful for picking out periodic components in the presence of noise. This command is used to determine the periodicity of the ripples (see Figure 4.15).

- \(\text{corr} = \text{xcov}(x,y)\) estimates the cross-covariance sequence between the two discrete vectors \(x\) and \(y\). The cross-covariance is simply the mean-removed cross correlation:

\[
corr_{xy}(j) = \sum_{i=1}^{N-j-1} (x_i - \mu_x)(y_{i+j} - \mu_y)
\]  

(4.12)

where \(\mu_x\) is the mean of vector \(x\).

This is simply the discrete equivalent of the cross-correlation function given in Equation 4.10. The output vector \(\text{corr}\) has elements comprised between \(-1.2 t_{\text{ripple}}\) and \(1.2 t_{\text{ripple}}\) with the zeroth lag of the covariance vector in the middle of the sequence (see Figure 4.18). As a consequence, the actual lag-time needed to align \(x\) and \(y\) is given by the time at maximum correlation \(+1.2 t_{\text{ripple}}\). This function is used for several key stages in the algorithm: to align the template onto the signal section (step 2, section 4.4.2.2), to align the ripples that form the initial ensemble (step 1, section 4.4.2.1) and during the update of the template (step 2, section 4.4.2.2).

The de-rippling algorithm is computationally more demanding than de-spiking and I have tried to minimise its computation time to allow fast processing of 5-day-long data. This is indeed desirable to allow fast "online" processing of the data ultimately.

Matlab offers a tool (Profile) to track the execution time of a programme by calculation of the execution time of the code line-by-line. Using this tool, I halved the time computing the de-rippling code: from 74 seconds to 38 seconds for a 500,000 data-point long signal.

4.4.3 Results and Discussion

4.4.3.1 Application to clinical rsMD traces

The de-rippling method has been applied to 10 clinical traces (see Chapter 5) and achieved significant enhancement of the signal-to-noise ratio. An example is given in Figure 4.21.
As shown in Figure 4.21 above, the baseline of the de-rippled trace is effectively zeroed (highlighted in pink in Figure 4.21 b) and the analyte peak shape is restored with no ripple artifact on its tail (highlighted in green in Figure 4.21 b). This was the case for all the clinical traces tested and the restored peaks could be fully fitted by a double-Gaussian peak function as described by Li [222]. As compared to the Fourier filter (see Figure 4.14 b) used to remove the ripples, the signal is not distorted (broader and smaller peak) and the SNR is improved (by 250% in Figure 4.21).

Improvement of the SNR is even more critical when it is particularly small in the raw signal, such as exemplified in Figure 4.22 below where the small analyte peaks are masked by the ripples:

In the case above (Figure 4.22), the concentration of glucose fell down below 150 µM, resulting in very small peaks that are swamped under the ripples: only an expert eye can recognise them. Quantification of the peak height is extremely difficult and subject to error with such a low SNR.
de-rippling, the peaks are clearly identifiable, the SNR is effectively improved by 350%, and the base and top of each peak can be defined to quantify the analyte concentration reliably. Further examples and applications of de-rippling can be found on the accompanying DVD and on the Analytical Chemistry website [215].

The improvement on the SNR has a direct impact on the analytical assay. It significantly reduces the error when estimating the magnitude of the peaks. When performing a calibration with \( n = 5 \) peaks per concentration, the relative standard deviation (RSD) within the peaks (calculated according to Chapter 2) is reduced by up to 136%. This is shown below in Figure 4.23:

As a result, there is less than 2% variation instead of 5.4% when the analyte levels reach 250 \( \mu \text{M} \). This means that the limit of detection of the assay improved from 70 \( \mu \text{M} \) to 25 \( \mu \text{M} \). This substantially improves the limit of detection of the assay, allowing detection of low concentration metabolites with a minimum error. This proved very valuable when analysing clinical data: as discussed in Chapter 5, the reliable detection of small concentrations of glucose could indicate a threshold of viability of the tissue.

### 4.4.3.2 Guidelines for a general use of de-rippling

Similarly to the de-spiking method, the de-rippling algorithm does not make any assumptions on the signal and could be applied to any types of data providing that a training data set of \( n \) ripples is available to compute a representative template. For this purpose, it is preferable to have a section of data with no signal of interest before the actual recording starts, but the algorithm can be trained using already recorded data as well. The number of ripples needed to have a good representation of the noise will depend on the initial SNR and the regularity of the ripples. From our experience, \( n \) of the order of 20 suffices even for poor SNR (less than 3). If the data set has spikes, the de-spiking
method should be applied first to avoid the presence of spikes within the training set, which would otherwise prevent the detection of the periodicity of the ripples.

Continual updating of the template as the subtraction occurs ensures that any non-stationarities in the ripples are accommodated. However, if, after 20 updates of the ensemble, the variance is consistently more than half the size of the ripples, then the ripples are not stable enough for this method and it will ultimately fail. There is also one assumption in the subtraction step: that the ripples superimposed on a signal change (a chromatographic peak for example) have a local consistency and a similar pattern to the ripple just preceding this change.

### 4.4.3.3 Comparison to other methods

As previously mentioned, the de-rippling method works well even for non-stationary signals where traditional linear filters usually fail. Some non-linear methods have been developed to overcome the problems associated with non-stationarity.

The most common of these methods is based on wavelets thresholding. The wavelet schemes involve projecting the time-domain signal into a wavelet domain where the basis functions are wavelets (Daubechies or Haar for instance) [194]. Contrary to sine and cosine (the Fourier basis functions), the wavelet functions are dually localised in both time and frequency domains, so that wavelet filters can be used in the time domain. Smoothing here relies on the fact that the energy of a signal will often be concentrated in a few coefficients in the wavelet domain while the energy of noise is spread among all coefficients [223]. Wavelet noise filters are constructed by calculating the wavelet transfer for a signal and then applying an algorithm that determines which wavelet coefficients should be modified (usually by being set to zero). Wavelet denoising is very efficient in cases where the wavelet basis functions best fit noise and signal of interest. As the bases functions are fixed, they do not necessarily match the wide variety of real-world analytical signals. Unlike the wavelet approach, the de-rippling method developed here is derived from the recorded noise itself. There is no assumption about the shape and distribution of the signal, which makes this method much more versatile and applicable to a much wider range of signals.

Another approach that is also completely data driven is empirical mode decomposition (EMD). In this scheme, the modes are derived from the signal itself, based on the sequential extraction of energy associated with various intrinsic time scales of the signal starting from finer temporal scales to coarser ones [224]. The problem is that the time scale of the ripples can be the same as that of the signal of interest, so that the ripples will be in the same mode as the signal itself and the EMD approach will thereby fail in separating ripples from signal. The de-rippling method avoids this pitfall.

The main advantage of de-rippling is that, once a sufficient training data set has been recorded (which can be prior to the actual measurement), it can be performed in real time while the wavelet filtering and empirical mode decomposition are post-acquisition approaches that transform the entire data set as a complete block. Once the code has been optimised (see section 4.4.2.3), the de-rippling algorithm is computationally fast: it takes approximately 50 seconds to process a trace with $10^6$ data points that included about $10^3$ ripples. It is therefore also suitable for online signal processing.
4.5 De-trending

4.5.1 Current strategies to remove baseline drift

The third type of noise identified at the beginning of this chapter is a slow baseline drift, which we define as noise that has a characteristic time scale that is longer than that of the signal of interest, commonly referred to as low-frequency baseline drift. This has been a major issue in traditional chromatography and a large number of digital methods have been developed for baseline correction. These include Fourier-based filters, whereby high-pass filters can remove the background drift assuming that the frequency components of this drift are in a lower range than those of the signal of interest. It was first used in spectroscopy by Atakan et al. [225]. The problem is the selection of the filter parameters: if the frequency cutoff is too low, some residual baseline drift will remain in the baseline-corrected spectra; if the spectral cutoff is too high, some of the signal peak frequency components will be removed along with the baseline and the signal peaks will be reduced.

Another very common approach is based on differentiation. The slopes of the background are in fact generally lower than those of signal peaks, so taking the first derivative will discriminate against slowly varying background components irrespective of their absolute values [226]. However, such methods lead to peak distortions with 90° phase shifts and attenuation of the signal [227].

To limit signal distortion, other methods have been devised, based on a SG filter subtraction [228] or median filter subtraction [227]. With both of these methods, the peaks of interest are smoothed out using a large window for an SG filter or a moving median filter. The output of this filter is therefore the baseline and its drift, without the peaks of interest. When this filtered data is subtracted from the original signal, it results in peaks on a flat baseline. The limitation is to determine the right window length that will preserve the broader peaks while removing the baseline from the sharper peaks, essentially the same problem as in high-pass filtering.

Many other techniques have been developed; using wavelet filtering, Gaussian filtering, artificial neuronal networks, etc. A good review can be found in [226].

Here, I have used a method that is similar to the homomorphic procedure described in 2001 by Michel et al. [229]. It is again a two step process and consists of:

- fitting the noise with a mathematical function (the model) (described in section 4.5.2.1),
- subtracting this model from the original data (described in section 4.5.2.2).

A huge variety of models can be used, depending on the nature of the drift, including polynomials, exponentials or power laws. The choice of the model is completely data-driven and in our case, the raw signal looked like that in Figure 4.24.

The analyte peaks are barely identifiable on top of the drifting baseline (Figure 4.24) and automatic quantification of their magnitudes is bound to fail. Instead, several PhD students previously within the group have tried to manually analyse these high-value clinical data (Patient 4 in Chapter 5): after spending three weeks on a three hour-long piece of the data, their results disagreed, due to inconsistent and unreliable estimation of the foot of the peaks. The aim of de-trending was therefore to retrieve the peaks to allow quantification of their magnitudes.
4.5. De-trending

4.5.2 De-trending algorithm

4.5.2.1 Step 1: fitting with a mathematical function

Based on the "shape" of the baseline drift in Figure 4.24, we chose an exponential function to fit the drift, as given by equation 4.13:

\[ I(t) = B - Ae^{-\frac{t}{T}} \]  \hspace{1cm} (4.13)

where \( I \) is the drift signal and \( A, B \) and \( T \) are constants.

The fitting was achieved using a non-linear optimisation algorithm (discussed in more details in the next section). To prevent non-convergence and extra computation time due to a very noisy signal, the baseline drift was first smoothed with a quadratic SG filter (window width of 21 data points) before calculation of the model.

4.5.2.2 Step 2: subtraction of the mathematical function

Once the coefficients (\( A, B \) and \( T \)) were estimated, the resulting analytical expression for the drift was subtracted from the baseline section and its following (baseline + peak) section, as illustrated in Figure 4.25:
4.5. De-trending

![Figure 4.25: Fitting of the drift and subtraction](image)

The exponential fit is shown in black on top of the signal (blue). It is given by \( I(t) = -0.037 - 0.047e^{-\frac{t}{5.72\times10^4}} \) for this baseline section. The model is then moved along by 15 seconds to the (baseline + peak) section.

The model coefficients were re-computed for each section of baseline drift preceding a section with analyte peaks. This allows for non-stationarities in the baseline drift.

### 4.5.2.3 Matlab code

The full Matlab code for de-trending can be found on the DVD accompanying this thesis and online in the Supplementary Information published in Analytical Chemistry [215]. The key step is the estimation of the exponential function best fitting the baseline, which was implemented using the Matlab command `fminsearch`. This performs what is called an unconstrained nonlinear optimisation based on the Nelder-Mead simplex method [230]. The mathematical theory underlying this method is beyond the scope of this thesis, and I will just give a brief explanation.

Basically, `fminsearch` finds the minimum of a multivariable scalar function. In our case, the function to minimise is the sum of square errors (SSE) between the actual signal and the exponential fit. This is a parameter commonly used for optimisation criteria. There are three variables in the problem: the parameters A, B and T. The problem was unconstrained, meaning that no conditions (such as A<B, for example) were imposed on the values of the coefficients. The calculation of the best fit is achieved using the Nelder-Mead simplex method, also called downhill simplex method. It is commonly used for a wide range of optimisation problems: in industrial process control for instance, it is used to find the parameter values that improve some performance measure [231]. A simplex in n-dimensional space is characterised by n+1 distinct vectors that define its vertices. In our case, n=3 (3 parameters to find), so the associated simplex is a triangular-based pyramid. The algorithm starts on the basis of initial conditions defined by the user. In our case, the starting set points were as follows, with \( I(t) \) being the time domain signal to model (signal from time \( t=0 \) and \( t=t_{final} \)).
4.5. De-trending

\[ B_0 = I(t_{final}) \]  
\[ T_0 = B_0 - I(0) \]  
\[ A_0 = -\log((B_0 - I(1)) \times T_0) \]

The minimal pyramid that contains these initial parameters is then computed.

At each step of the search, a new point in or near the current simplex is generated. The function value (here the SSE value) at the new point is compared with the function's values at the vertices of the simplex and, usually, one of the vertices is replaced by the new point, giving a new simplex.

This step is repeated until the diameter of the simplex is less than the specified tolerance. The termination tolerance used for these data was the Matlab default criterion: a function value (SSE value) smaller than \(10^{-4}\).

4.5.3 Results and Discussion

De-trending was effectively applied to clinical data recorded with rsMD (Patient 4 in Chapter 5). The de-trended signal for the data in Figure 4.24 is shown in Figure 4.26 below:

![Figure 4.26: De-trended signal](image)

This is the result of de-trending applied to the signal shown in Figure 4.24. This is the trace recorded for Patient 4 in Chapter 5.

The peaks are now readily identifiable on the de-trended signal with a stable baseline around 0.0 µA. Their magnitudes can be estimated unambiguously and automatically thus considerably reducing errors of quantification. It took less than three hours to analyse the 36 hour recording from this patient after de-trending.
Note that, in this particular case, de-spiking was combined with de-trending. The first step of the de-spiking procedure (see section 4.3.2.1), i.e. the spike identification step, was used to identify the baseline sections and the baseline+peak sections. The spikes were then excised as described in section 4.3.2.2. This prevented non-convergence of the Nelder-Mead method because of spikes. The de-trending procedure was then fully computed. Finally the linear interpolation of the spikes (see section 4.3.2.1) was completed.

This ad hoc method can be generalised to other sets of data, using different modelling functions. De-trending is particularly applicable to chromatography derived methods, including flow injection analysis, capillary electrophoresis, separation techniques using gradients of phases, where sections of data with no injection of the sample and only baseline drift alternate with sections containing the analyte peaks.

It can also be combined with other methods presented here to remove other types of noise. In our case, the de-trending procedure has allowed the quantification of high value clinical traces that could not possibly be analysed without removal of the drift. However, other methods for baseline correction may be preferable. This will depend on factors such as the nature of the measurement and noise, the magnitude of the signal-to-noise ratio, the computational and programming resources available, and the time scale for the required calculations.

4.6 Conclusion

Three different signal processing methods have been developed to remove three particular types of noise that are encountered in many analytical techniques. Key to the success of the methods presented here is the correct identification of these noise types based on their timescale relative to the analytical signal:

- **spikes** are much faster,
- **ripples** can have a similar characteristic time scale,
- **drifting baseline** is slower.

Unlike traditional filters, my methods are based on identifying specific patterns of the noise types and then subtracting the fitted noise from the original unprocessed signal. As a consequence, these methods remove the noise effectively with minimal distortion of the signal of interest, resulting in a significant improvement of the signal-to-noise ratio. Some parameters, including the length of the SG filter for the de-spiking procedure, the periodicity of the ripples, and the mathematical function describing the drift, have to be derived using a priori knowledge of the noise. In many cases, they can be easily determined from the recorded noise itself. They can be combined successively or simultaneously for denoising, permitting reliable subsequent automatic quantification of the data. I have used them all on clinical rsMD traces and the results in 10 patients are presented in the next Chapter. Given that de-spiking and de-rippling are non-intensive computationally and quite fast, these could ultimately be implemented on-line, as the data is collected. This could then be directly used by the clinical staff at the patient bedside. Finally, these signal processing tools are fully data-driven with no assumptions about the signal of interest. They are therefore applicable to a wide variety of time domain analytical signals.
Chapter 5

Dynamic metabolic response to multiple spreading depolarisations in the injured human brain

The signal processing algorithms developed in Chapter 4 have been applied to clinical rsMD traces collected between 2002 and 2008 in patients with acute brain injury. After noise removal, the dynamic changes in MD glucose and lactate in response to spontaneous waves of tissue depolarisation, known as spreading depolarisations (SDs), could be resolved. The main findings and their clinical implications for the management of brain injury patients are discussed in this chapter. A manuscript describing this work has just been submitted for publication.

5.1 Background on spreading depolarisations (SDs)

5.1.1 Discovery and definition of SDs

SDs were first observed in 1944 by Leao [232] while he was investigating the characteristics of the electrocorticogram (ECoG) of induced epileptic seizures in the rabbit brain. After stimulating the frontal pole electrically, he unexpectedly saw transient silencing of the ongoing normal electrical activity. This suppression of electrical activity slowly propagated over the cortex, from one recording electrode pair resting on the cortical surface to the next posterior electrodes, at a typical speed of 2–5 mm/min [232]. His original illustration is shown in Figure 5.1.

Leao coined this phenomenon spreading depression of cortical activity. He later demonstrated that the SD wave fronts were accompanied by a negative slow voltage variation from the cortical surface [233]. This change in cortical direct current (DC) potential is now recognised as the characteristic electrical signature of a mass neuronal and astrocytic depolarisation [234].
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A decade after its discovery, SD was still described as a "capricious and curious phenomenon of the cerebral cortex" [235]. Many studies followed and SD became well-documented not only at the surface of the brain [160; 161; 236; 237] but also in other internal structures of the brain, such as the hippocampus [238; 239], the cerebellum [240] and the brainstem [241]. SDs have since then been observed in a variety of species, from rodents [242; 243; 244; 245] through catfish [104] and turtle [240] to primates [246].

SD can generally be defined as a self-propagating front of mass depolarisation of neurones and astrocytes. Neuronal silence immediately follows, lasting for a few minutes [247]. It is characterised by a reversible negative extracellular slow voltage peak of the order of 10–30 mV and a 1–2 minute duration that spreads across tissue at velocities of a few millimetres per minute. The initial surface-negative wave is followed by a smaller but more prolonged positive phase [248]. The gold-standard to measure SDs experimentally is by extracellular recording of the DC potential with a microelectrode implanted in tissue or on the surface. The characteristic transient change in DC potential is sometimes referred to as "slow potential change" (SPC). Recently, SDs have also been imaged using fluorescence of a voltage sensitive dye [249].

5.1.2 Chemical characteristics of SDs

This massive tissue depolarisation is invariably accompanied by a "massive redistribution of ions between intracellular and extracellular compartments" [234]. Vyskocil et al. [242] were the first
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to measure an unparalleled increase in extracellular potassium using ion-selective microelectrodes in cortical tissue in the rat. This was confirmed by later studies, with peak values of potassium concentrations of 35 mM in the catfish cerebellum [104], and 55 mM in rat cortex [250]. Nedergaard et al. [237] reported a monophasic (1 to 60 mM) and fast transient K⁺ change in non-ischaemic tissue compared to a biphasic response to spontaneous depolarisations in ischaemic rat cortex (3 to 10 mM, then 10 to 60 mM during the depolarisation). We have also measured increases in extracellular potassium using microdialysis in a pilot study in the rat cortex, as will be discussed in Chapter 6. The potassium increase is accompanied by smaller, yet still pronounced, increases in extracellular hydrogen ions and drops in extracellular chloride, sodium and calcium ions [250; 251; 252; 253]. The time course and magnitude of the extracellular ion concentration changes is summarised in Figure 5.2 below:

![Figure 5.2: Ionic redistribution during an SD.](image)

This implies that the ionic channels have been opened such that K⁺ floods out of the depolarised cells. In exchange, Na⁺ and Ca²⁺ flood in down their respective concentration gradients, until there is a much lower potential gradient across the cell membrane due to relative equilibration of the ions [234]. Cations are not exchanged one for one between the intra- and extracellular solutions, for the reduction in [Na⁺]_{ECF} is greater than the increase in [K⁺]_{ECF}; the depolarisation effectively reverses the electrogenic effect of the normal Na⁺-K⁺-ATPase, which normally extrudes 3 Na⁺ and acquires 2 K⁺ from the extracellular space. The concomitant drop in [Cl⁻]_{ECF} indicates that some of the Na⁺
5.1. Background on spreading depolarisations (SDs)

entering the cells is accompanied by $\text{Cl}^-$ [254]. Nicholson suggested that the deficit in extracellular anions is made up by anions leaving the cytosol [252], such as glutamate that has been shown to be released during SDs. An exact picture of all the ion and chemical fluxes involved in SDs is not complete as yet.

With regard to extracellular pH, it first becomes alkaline and then acidic, with a local acidosis that outlasts the DC shift. It has been suggested that this sustained acidosis is the result of the production of lactic acid as a by-product of increased metabolic activity required for the activity of the membrane ion pumps and restoration of ion homeostasis [255].

Morphological studies also concluded that the movement of ions is accompanied by movement of water into the cytosol, cell swelling and subsequently an increase in the electrical impedance of the tissue [234].

5.1.3 Mechanism of SDs

Sixty-five years after their first discovery, the exact mechanisms that trigger SDs and sustain their propagations in brain tissue are still puzzling researchers. SDs can be elicited experimentally by high-frequency electrical pulses, direct current, mechanical stimuli (such as pressure on or puncture of the cortex), and a variety of chemicals, in particular potassium ions and glutamate [234]. The resistance of some tissues to the elicitation of SDs, such as the brainstem, long suggested that a brain tissue susceptible to SDs should comply with some requirements, such as a high density of neurons, a small extracellular space, a low density of myelin, and a relatively homogeneous brain tissue without boundaries that would hinder propagation of SDs. Recently however, Richter et al. [241] successfully elicited SDs in the brain stem (which has none of the above requirements for elicitation of SDs) by superfusing highly concentrated solutions of KCl. It had the effect of sufficiently increasing the neuronal excitability so as to easily induce SDs. Potassium indeed plays a central role for SDs and Grafstein pushed this observation further and suggested that potassium ions are the key to the initiation and evolution of the SD process [256]. With extracellular microelectrode recording, she observed that, at the initiation of the DC negativity, there was a brief but intense burst of single unit activity lasting some 2–3 seconds. This neuronal firing, she suggested, is accompanied by release of potassium that accumulates in the restricted interstitial spaces of brain tissue and this excessive extracellular potassium further depolarises the cells that released it in a vicious circle that leads to inactivation of neuronal excitability (and silencing of the ECoG activity). Some of the accumulated $\text{K}^+$ would diffuse through the interstitial spaces to neighbouring cells, which then also depolarise and go through the same cycle, thus producing a slowly propagating wave of depolarisation of the tissue. Potassium clearly plays an important role and it is widely accepted that any disturbance of $\text{K}^+$ homeostasis would predispose the brain region to SD [234; 241] but diffusion alone of potassium is too slow to explain the observed propagation speed.

Another major proposal was van Harreveld's glutamate hypothesis [257] that assumes that the release of the excitatory amino acid triggers neuronal sodium conductance changes. Evidence suggests that glutamate is released during SD [257], that high extracellular excitatory amino acids directly depolarise adjacent neurons, and that both elicitation and propagation of SD are exclusively sensitive to competitive and noncompetitive antagonists of the N-methyl-D-aspartate
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(NMDA) glutamate receptor [258]. However other studies have refuted glutamate as the key agent for SD induction [259]. Instead, acetylcholine and sodium channels have been suggested as necessary to trigger SDs [260] and a role for gap junctions and calcium waves in astrocytes for SDs propagation has also been proposed [254; 261].

In brief, there is no conclusive evidence for or against any of these hypotheses and SD initiation and propagation are likely to be a complex combination of some, or all, of the suggested processes. A detailed discussion is beyond the scope of this thesis and further information can be found in important reviews [234; 247; 254; 262].

5.1.4 SDs and metabolism

The current general consensus is that cell membrane repolarisation in the recovery phase of SD is highly energy dependent. To restore ionic homeostasis, the activity of Na\(^+\)/K\(^+\) ATPase pumps increases, thus requiring a huge amount of energy. As discussed in section 1.3, this is invariably accompanied by an increase in CBF. A major study on the CBF response to SD by autoradiography in the rat cortex [236] showed a biphasic response: a large hyperaemia (CBF increased by \(\approx 100\%\)) during the first two minutes after the SD front was followed by a prolonged oligaemia during 60 minutes. This was confirmed by the observation of a transient vasodilatation followed by a sustained vasconstriction of pial arterioles in the rat [263]. More recently, laser speckle imaging during SDs showed waves of hyperaemia propagating at a speed of 2–3 mm/min across the cortex, and closely coupled to DC potential shifts [80; 264]. These CBF waves are so tightly coupled to the depolarisation wave, that they have been used as a surrogate measure for SD waves in the cortex [160].

The response of tissue oxygen following SD waves was also studied. Using a microelectrode to measure tissue oxygen tension, Tsacopoulos et al. [265] showed that SDs also coincided with transient hypoxia of the tissue despite an increase in CBF, suggesting that the oxygen consumption transiently exceeded vascular delivery of O\(_2\). This was confirmed by Takano et al. [244] who also showed, combining micro-focal oxygen tension measurements with two-photon microscopy, that hypoxic pockets of tissue were located between capillaries during an SD event: the tissue adjacent to arteries and capillaries had consumed O\(_2\) at the expense of more distant tissue. From the observed inverse relationship between duration of the SD and the O\(_2\) supply, this group also suggested that hypoxia, combined with potassium, would drive the forward movement of SDs [244]. A recent study in the Lauritzen group further showed a prolonged tissue hypoxia, over 2 hours post-SD, with maintenance of the cerebral metabolic rate of oxygen during this period [245]. These data support the resort to non-oxidative metabolism, as confirmed by measurements of decreased glucose [266] and increased lactate [267] levels during and after the passage of SD waves. This was confirmed in our group using rsMD in a cat model [160] and will be discussed in more detail in the body of this chapter.

5.1.5 SDs in the injured brain

SD-like events, the so-called peri-infarct depolarisations (PIDs), can occur spontaneously in experimental models of stroke [237; 246] and contusional head injury [239]. It has been hypothesised that following insult, excitotoxicity and neuronal cell death results in a profound increase in the extracellular
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concentration of potassium in the ischemic core. This is sufficient to initiate diffusion of \( K^+ \) into the adjacent normally perfused cortex and trigger SD waves propagating from the rim of the ischaemic core through the surrounding penumbral tissue and away into the intact tissue during the early stages of focal ischemia. In these stroke models, the transient CBF response to SD is typically modified with increasing distance to the ischemic core: near the core, it is an almost monophasic decrease of CBF, whereas in the more distant periphery, CBF increases monophasically. In the zones in between, i.e., in penumbral tissue, biphasic patterns are observed [268]. This has the effect of locally reducing the tissue glucose content, caused by the increased demands and reduced supply of glucose [161]. This might further reduce the threshold for elicitation of SDs and in subsequent minutes and hours, further SD waves can be generated from the boundary of the ischaemic core provided that the chemical gradient is steep enough to support sufficiently intense diffusion of active substances away from the focus towards the intact cortex. Interestingly, these concentration gradients can be such that the SD wave is trapped in penumbral tissue and propagates circumferentially around the ischemic core with a periodicity corresponding to the length of the loop. This was first observed by Shibata et al. [269] and recently imaged with laser speckle [270; 271]. We also observed these cycling waves in the in vivo experiment described in section 6.4 of Chapter 6 and a video of these propagating hyperaemic waves can be found on the DVD accompanying this thesis.

These PIDs usually last longer than those elicited in healthy brain, and as they can repeat in a tissue where perfusion is already compromised (penumbra), they tend to exacerbate oligaemia [268; 272], hypoxia [244; 273] and glycopenia [161]. The net effect is the deprivation of high-energy phosphate for the cells to repolarise and this eventually leads to cell death. Mies et al. [266] showed a striking linear correlation between the number of PIDs and the ultimate size of the infarct lesion, each depolarisation actually increasing the infarct volume by 13%. Similarly, magnetic resonance imaging (MRI) and ATP-contents measurements showed a progressive expansion of the ischaemic volume at the expense of the penumbra volume as a consequence rather than a cause of a series of SDs [274; 275]. These findings are summarised in Figure 5.3. They show that SDs promote the expansion of damage by progressive infarction of the penumbra [275; 276], presumably due to a progressive depletion of glucose and accumulation of lactate in the penumbra [161].
5.1. Background on spreading depolarisations (SDs)

Changes in microdialysis concentrations from pre-clip levels /µM a. 

Figure 5.3: Effects of repetitive SDs on metabolism and infarct size in a model of focal ischaemia. In these 2 separate experiments, focal ischaemia was induced by occlusion of the middle cerebral artery (MCAO). (a) Spontaneously occurring SDs (PID marker) in the cat result in progressive decrease of glucose extracellular concentration and increase in lactate extracellular concentration. Taken from [161]. (b) The infarct size gradually expands after the passage of each SD wave (marked by a vertical arrow). Taken from [275].

5.1.6 SDs in the human brain

SDs were initially thought to be an epiphenomenon due to experimental conditions in animal studies and thus not to occur spontaneously in the human brain. In 1941, Lashley [277] was the first to report the occurrence of a phenomenon that could be similar to a wave of SDs in the human brain during his own migraine aura: using his knowledge of the retinotopic organisation of the visual cortex, he worked out that his scintillation-scotoma\(^1\) indicated a wave of intense excitation in the primary visual cortex that moved at the speed of 3 mm/min, followed by a longer period of inhibition.

\(^1\)area or island of loss or impairment of visual acuity surrounded by a field of normal or relatively well-preserved vision.
5.1. Background on spreading depolarisations (SDs)

Later studies showed that human cortical tissues do support the development of SDs with SDs observed in human cortical tissue \textit{in vitro} [278] and in hippocampus and striatum \textit{in vivo} during stereotactic brain surgery in patients with focal epilepsy [238]. More recently, Mayevsky [279] observed spontaneous SDs in the injured human brain, but this was a sporadic event that occurred in 1 patient out of 14. It was only in 2002 that the group of Strong and Boutelle [280] demonstrated the spontaneous spread of an SD wave in the perilesional area of the injured human cortex. This observation was confirmed and shown to occur in 55% of traumatic brain injury patients [281], 72% for aneurysmal subarachnoid haemorrhage [282] and essentially 100% for malignant hemisphere stroke [283]. When occurring adjacent to focal lesions in the injured human brain, depolarisations typically occur in temporal clusters of repetitive events spread out over up to 10 days post-injury [281; 282; 284].

Clinically, the exact role of SDs in the development of secondary neurological deterioration in the human brain is still unclear and the relation between SDs and patient outcome is currently under investigation by the COSBID group (Cooperative Study of Brain Injury Depolarisations: www.cosbid.org). Preliminary studies concluded that delayed ischaemic neurological deficit were time-locked to a series of recurrent SDs in subarachnoid haemorrhage patients [282] and it has been suggested that secondary deteriorations of stroke patients could also be associated with SDs [285]. However, physical evidence of deterioration of metabolism and recruitment of penumbral tissue into infarct core due to SD events is still lacking. The reason for this is mainly methodological. Although current sophisticated scanners, including MRI and PET, can measure metabolism in the human brain, they cannot in practice be used continuously for days, without compromising patient care. The current method of choice for the continuous assessment of metabolism is cerebral microdialysis [138; 139; 286; 287; 288; 289; 290]. Traditional microdialysis with its limited time-resolution in the order of about 30 to 60 minutes cannot resolve the effect of SDs dynamically since locally the passage of an SD wave may last only 2 to 5 minutes. We have therefore used rapid sampling microdialysis of peri-lesion tissue to determine the dynamic neurometabolic signature of recurrent spontaneous depolarisations in the injured human brain. A first clinical study of SDs with rsMD showed that the total number of SD events identified by their electrical signature was strongly correlated with the progressive reduction in dialysate glucose as shown in Figure 5.4 below:
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Figure 5.4: Changes in dialysate glucose levels vs the number of ECoG-identified SD events. The value for change in dialysate glucose is the mean value calculated over the second hour of monitoring, less the mean value recorded between 60 and 120 mins after the last depolarisation-like ECoG event, or at the conclusion of monitoring if no events occurred. Taken from [164].

This study provided evidence that rsMD could give information about the metabolic response to different variants of depolarisation events as seen on ECoG recordings, but did not provide a dynamic metabolic signature to individual SDs, and could not establish a causation link between SDs and the glucose drop. This was mainly because of the lack of an automatic error-free means to analyze the rsMD traces. Using the signal processing techniques presented in Chapter 4, I have carried out a retrospective analysis of the rsMD data from ten patients who were monitored in the ICU of King's College hospital between 2002 and 2008.

5.2 Methods

5.2.1 Patient recruitment and care

All human research procedures were approved by the local Research Ethics Committee (REC) of King's College Hospital, London, or by Cambridgeshire 4 REC. Patients requiring emergency craniotomy for traumatic brain injury (n=5), aneurysmal subarachnoid haemorrhage (n=2) or intracranial haematoma (n=3) were identified and research assent obtained from relatives or the authorised surrogate. Patients under 16 years of age, and/or with a Glasgow Coma Score (GCS)² below 4 at admission and/or with bilateral fixed and dilated pupils were excluded from this study. Only acute brain injury patients (with the ictus occurring less than 5 days before admission) were included.

²The GCS score is a measure of the conscious state of a patient, composed of a motor, verbal and eye response to a stimulus. The symptoms are severe (GCS \( \leq 8 \)), moderate (9 \( \leq \) GCS \( \leq 12 \)), or mild (13 \( \leq \) GCS \( \leq 15 \)).
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Towards the completion of surgery, a sterile clinical microdialysis probe (see section 2.1) was inserted along with a linear, six-platinum-contact electrocorticography (ECoG) recording strip (Wyler, 5 mm diameter contacts; Ad-Tech Medical, Racine, WI, USA). After surgery, patients were transferred to intensive or high dependency care unit. There, patients were monitored continuously for up to five days. The duration of monitoring depended on the condition of the patient and the quality of the recording.

Arterial blood pressure (BP) and intracranial pressure (ICP) were continuously recorded and copied by analogue link and analogue-to-digital converter into the research dataset. Blood gases, glucose and electrolytes were documented hourly or 4-hourly. Where required, patients were ventilated, typically to a target partial pressure of arterial carbon dioxide (PaCO₂) of 30 to 34 torr (4 to 4.5 kPa). Sedation was principally with fentanyl and midazolam, replaced with propofol during weaning. Target cerebral perfusion pressure was 60 mmHg, flexibly applied. Acute intravenous fluid therapy was typically Ringer's lactate or Hartmann's solution, 2 L/24 hours, supplemented with colloid for any additional volume requirements. Once tolerated, enteral nutrition replaced this, comprising in general 20 – 25 kcal/kg/day or 60 mL/hr, containing approximately 50% carbohydrate, 30% lipid and 20% protein, together with electrolytes and trace elements. Glycemic control was targeted to the range 70 to 140 mg/dL (3.9 to 8 mM) using actrapid insulin as a continuous intravenous infusion, typically at 1–2 unit/hour.

The outcome of the patients was assessed by telephone interview 6 months after discharge (when possible), using the extended Glasgow Outcome Score (eGOS) [291; 292]. Details about the patients included in this study are summarised in Table 5.1 below.

Note that patient 9 was diabetic: his initial glycaemia was estimated between 325 and 490 mg/dL (18 and 27 mM) at 21:00 on Day 0. His glycaemia was subsequently controlled with intravenous insulin infusion and the plasma glucose concentrations were maintained between 105 and 230 mg/dL (6 and 12.8 mM) during the rest of the monitoring period.
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<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Gender</th>
<th>Injury Description</th>
<th>Monitoring date</th>
<th>Duration of monitoring</th>
<th>Outcome (eGOS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>F</td>
<td>left parietal ICH</td>
<td>D0 to D2</td>
<td>72h</td>
<td>1 on D11</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>M</td>
<td>left frontal ICH, small MCA AVM</td>
<td>D1 to D2</td>
<td>31h</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>M</td>
<td>Dense left hemiplegia and facial weakness: spontaneous large right fronto-parietal ICH with MLS and mass effect secondary to a small frontal AVM.</td>
<td>D2 to D4</td>
<td>46h</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>57</td>
<td>F</td>
<td>Severe headache due to SAH</td>
<td>D4 to D7</td>
<td>55h</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>F</td>
<td>Right SAH with SDH</td>
<td>D2 to D7</td>
<td>55h</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>M</td>
<td>Collapsed outside a pub: TBI with large fronto-temporal SDH and MLS</td>
<td>D0 to D2</td>
<td>64h</td>
<td>1 on D4</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>M</td>
<td>TBI with contusions and left haematoma</td>
<td>D1 to D3</td>
<td>66</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
<td>M</td>
<td>Fell off skateboard: TBI with small bifrontal contusions</td>
<td>D1 to D2</td>
<td>24h</td>
<td>1 on D2</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>M</td>
<td>Right SDH with diffuse cortical SAH caused by TBI</td>
<td>D0 to D5</td>
<td>97h</td>
<td>1 on D6</td>
</tr>
<tr>
<td>10</td>
<td>41</td>
<td>M</td>
<td>TBI with left hemispheric acute SDH and frontal contusions</td>
<td>D1 to D5</td>
<td>120h</td>
<td>4</td>
</tr>
</tbody>
</table>

5.2.2 Implantation and location of the probes

The microdialysis catheter was inserted obliquely into the cortex, to full membrane depth through a minimal pial incision. The reason for this obliqueness is to maximise the cortex to white matter ratio surrounding the dialyzing membrane. Critically, the implantation was performed under direct vision to ensure that the MD probe was sited in penumbral cortical tissue, approximately within 1 cm of the focal contusion [290]. The ECoG strip was placed on the surface of the cortex accessible through the craniotomy, as closely as possible to the MD catheter, radiating away from the lesioned area and it was usually possible to site both probes on the same gyrus. Positions of the probes relative to each other, and relative to the ischaemic core are critical. These were documented by a sketch (Figure 5.5 a), a photograph (Figure 5.5 b) and, when possible, were assessed by CT scan after the operation (Figure 5.5 c).
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Figure 5.5: Probes location in the brain. The probes were placed under direct vision towards the end of the craniotomy. The locations of the probes were documented by a sketch (a), picture at craniotomy (b) and post-operative CT scan (c). This is the case of patient 9. The ECoG strip was lying horizontally on the right fronto-parietal cortex. The MD probe was situated between contacts 3 and 4 (closer to 3) and 1–2 cm inferior to ECoG strip. This patient had an intra-cranial pressure transducer (ICP) located in the cortical parenhyma near the ECoG strip. His brain tissue oxygen tension was also monitored with a Licox sensor. The letters A, B, C and D refer to the ECoG data channels. See section 5.2.3.

The MD probe and the ECoG strip were exited via a burr hole (hole in the skull exposing the dura mater) and tunneled out in-line 2 to 3 cm from the skin incision boundary. They were stabilised in position through suturing to the scalp during the whole monitoring period.

5.2.3 ECoG recording and SD detection

The origin of the ECoG signals was presented in section 1.5.3.1. Here, the electrocortical activity was recorded using a bipolar montage: each data channel (A to D on Figure 5.5 c) measured the difference between two adjacent electrodes. Having four channels of electrical activity along a certain length gave a temporal dimension to the measurement, and therefore allowed the detection of spreading electrical events. The advantage of using a bipolar montage between closely spaced electrode is the cancellation of far-field activity common to both electrodes, thereby obtaining sharp localisation of the response.

To reduce the impact of interference from ambient electrical noise in the ICU environment, appropriate reference and ground electrodes are necessary. The reference electrode was fixed to the mastoid (using conductive paste and an adhesive gel to attach to the skin). An Ag/AgCl ground plate electrode was placed on the back of the patient’s shoulder. All connections were checked every day.

The ECoG strip was connected to a high-gain differential input alternating current (AC) amplifier (Octal Bio Amp, ADInstruments, New South Wales, Australia), digitised at 200 Hz and continuously logged into the same data files as the rsMD data (Chart 5.5.6 software).

Spreading depolarisations were identified by their ECoG signature as defined by Fabricius et al. for the COSBID group [281]. Ideally, direct current (DC) recordings of the depolarisations are desirable to confirm that any observed ECoG suppression events indicate depolarisation. However, although such recordings are possible in an experimental laboratory, long-term intracranial DC recordings easily become unstable in a clinical intensive care unit. In addition, non-polarisable electrodes required
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for DC recordings would likely be neurotoxic. Instead, using appropriate filters (see Figure 5.6), SD waves are defined as the sequential onset in adjacent channels of a propagating, polyphasic slow potential change (SPC) (Figure 5.6 b) followed by a rapidly developing reduction in the power of the ECoG amplitude by at least 50% (Figure 5.6 c).

Very recently, a new montage, combining bipolar and monopolar ECoG recordings has been developed. It can be used with a signal processing tool that inverts the transfer function of the amplifier and thereby reconstructs the DC signal from the AC coupled amplifier [293]. This is to be used routinely within the COSBID group in the future.

5.2.4 rsMD data analysis

The rsMD assay has already been described in Chapter 2. The rsMD data for glucose and lactate were analyzed without knowledge of the ECoG analysis results. The traces were processed in Matlab according to the following procedure:

1. noise removal using one or a combination of the signal processing techniques developed in Chapter 4 [215]. Except for the data from patient 4 where de-trending was used, all other data were processed with de-spiking first, followed by de-rippling.
2. automatic peak detection using a standard local maximum algorithm ,
3. conversion to dialysate concentrations of glucose and lactate using calibrations performed twice-daily at the bedside throughout the monitoring period (see double scale for rsMD trace on Figure 5.6 d),
4. filtering (three-point moving average) to yield dialysate concentration time series,
5. time-alignment of the dialysate concentration time series with the ECoG trace corresponding to the closest electrode on the strip by subtraction of the 9 minutes time delay due to the one-metre length low volume connection tubing between the patient and the rsMD assay.

An example of an SD wave propagating at 4 mm/min in patient 4 is given in Figure 5.6 along with its rsMD response.

Epochs of 5 minutes prior- and 20 minutes post-ECoG-identified SDs were extracted from the whole glucose and lactate dialysate concentration data for further analysis. The onset of inflections of glucose and lactate dialysate concentrations were defined as time zero (example indicated as the dotted vertical line on Figure 5.6 d).
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Figure 5.6: Electro-metabolic signature of a spreading depolarisation in the acutely injured human brain. 25-minute recording of ECoG activity ((a), (b) and (c)) and rsMD signal (d). (a): raw ECoG signal as recorded from the AC coupled amplifier with full bandwidth 0.02 Hz to 200 Hz. (b): slow potential changes (SPC) recovered by a time integral of the ECoG signal followed by a subtraction of the general slope of the integral (300 s time constant decay). (c): AC signal after high pass filtering the signals (0.5–70 Hz). The arrows indicate a spread of the SD wave from channel D to A, corresponding to contacts 6 to 2 on the subdural strip. (d): rsMD response, time-aligned with the closest contact on the strip. Vertical dotted line for the onset of the rsMD response. Shaded rectangles for pre-SD basal levels (see section 5.2.4).
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Epochs were then characterised by:

- basal levels, defined as the mean of the recorded rsMD values during 5 minutes prior to time zero (as shown by the shaded rectangles on Figure 5.6)
- changes from basal levels in dialysate value at 10 minutes post-SD
- changes from basal levels in dialysate value at 20 minutes post-SD
- area under the curve (AUC) of dialysate change from basal levels for 20 minutes after SD onset.

The choice of 20 minutes post-SD was determined by the fact that it typically was the minimum interval time between consecutive SDs. When SDs repeated more frequently (7 cases), then the AUC was defined from time zero until the start of the following SD. Although this potentially underestimates the true AUC for these events, this conservative approach has been taken to avoid biasing the data.

Basal levels were defined as the mean over five minutes only to ensure that the “end” of a previous epoch was not included.

Epochs that showed parallel changes in glucose and lactate that had the same time course, percentage amplitude and polarity for both glucose and lactate were excluded from analysis on the basis that they might reflect fluctuation in MD probe recovery or detection of interfering species (such as ascorbate, or an electroactive drug or hydrogen peroxide, etc.) and not true physiological events. Events that were likely due to nurse activities or movements of the patients observed on anonymised video camera recordings were regarded as artefacts and also excluded.

5.3 Results

5.3.1 Number of rsMD epochs for each patient

Of 231 SD events detected by ECoG, 78 had no rsMD data recorded simultaneously, either because of an early removal of the MD probe, a dysfunction of the syringe pump perfusing aCSF or a too large back-pressure in the outlet tubing. This gave a possible 153 SD events to associate with the rsMD data. Table 5.2 below summarises the number of SD events and rsMD epochs for each patient.

62.7% of the ECoG events passed our inclusion criteria for glucose, and 36.6% for lactate. A few events were masked by noise despite post-processing with de-spiking and de-rippling and were therefore not regarded as reliable data (column “too noisy” in Table 5.2). Three events could not be resolved (number in brackets in Table 5.2) despite a “clean” signal, suggesting that less than 3.1% of the SDs led to changes in glucose and lactate smaller than our 95% confidence limit (see Figure 5.7) or no changes at all. To avoid bias, these are included in the analysis below. There is a discrepancy between the number of glucose epochs and the number of lactate epochs. This is because, for patients monitored at the beginning of the study, the data were either missing or unrecoverable for technical reasons. Clearly, we cannot apply the parallel shift exclusion criterion to these data.

For patient 10, no SD were detected. Six 25-minute control epochs were randomly chosen among the 120 hours of monitoring for inter-patient comparison. We also selected control epochs from the other nine patients when the ECoG recordings showed normal background activity (no SD events or epileptiform activity). These control epochs were characterised by their 20-minute area under the
5.3. Results

curve as defined above.

Table 5.2: Number of SD events and rsMD epochs. The numbers in brackets indicate epochs where no change in both glucose and lactate could be resolved. Patient 10 showed no SD events and is therefore not included in this table. See text below.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>ECoG SD</th>
<th>Artifact on video</th>
<th>Too noisy</th>
<th>Parallel transients</th>
<th>Glucose epochs</th>
<th>Lactate epochs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>28 (2)</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>0</td>
<td>10</td>
<td>2</td>
<td>15 (1)</td>
<td>15 (1)</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>153</strong></td>
<td><strong>11</strong></td>
<td><strong>22</strong></td>
<td><strong>24</strong></td>
<td><strong>96 (3)</strong></td>
<td><strong>56 (1)</strong></td>
</tr>
</tbody>
</table>

5.3.2 Baseline dialysate concentrations

The initial baseline microdialysis concentrations (an average of values recorded between 60 and 120 minutes after dialysis started) ranged between 0.3 to 2 mM for glucose and 0.4 to 2 mM for lactate. These are comparable to other microdialysis values reported in the human injured brain when accounting for the different recoveries of different probes and perfusion flow rates [61; 139; 146; 164; 294; 295; 296; 297]. This is a wide range but this is to be expected given the variability in the types of injuries, severity of the injuries, positions of the MD probe relative to the injury, among others.

5.3.3 Metabolic signature of SDs

We first sought to characterise the typical metabolic response to a typical SD event.

5.3.3.1 Dialysate changes in response to SD events

To evaluate the impact of SD events on the microdialysis concentrations of glucose and lactate, 25-minute epochs were compared. Seventeen events are shown in Figure 5.7 from three patients. All epochs are time-aligned with the onset of the rsMD response and basal-level-subtracted.

The general pattern of changes is similar in all cases: each SD is followed by a drop in glucose and a rise in lactate concentrations. However, there clearly is variability in the responses, as seen on Figure 5.7.
Figure 5.7: Changes in dialysate glucose and lactate in 3 patients. Time $t = 0$ indicates the onset of the rsMD changes, and dialysate change $= 0$ µM corresponds to the dialysate concentration at time $t = 0$. These are indicated by vertical and horizontal dotted lines respectively. The letter and figure on the plots identify the pair of traces to their corresponding ECoG event: a cluster of SD events (repeating within less than 40 minutes) is designated by a letter, and individual event within a cluster by a figure (for example A1, A2, A3, A4 are four SDs repeating in cluster A in patient 3). Note the different scales on the y-axis for each patient.
5.3. Results

In patient 4, the basal dialysate concentrations ranged from 640 to 930 µM for glucose, and between 1 and 1.5 mM for lactate. The first two SD waves (A1 and B1) were isolated SDs occurring on Day 12 between 00:30 and 2:30. They led to small changes in dialysate glucose and lactate. The other 4 SDs (C1 to C4) were observed as a temporal cluster of SDs repeating every 35–40 minutes on Day 12 between 14:00 and 16:00 and they resulted in larger changes in dialysate concentrations, all >100 µM. Globally, for these six SDs in patient 4, the glucose drops ranged from −10 to −96 µM and lactate rises from +130 to +300 µM at 10 minutes following the SDs. The time course of the changes also varied across events.

In patient 5, the changes were overall smaller than in patient 4. A first cluster of three SDs repeating every 40 minutes occurred on Day 2 from 23:45 (A1, A2, A3). The basal levels decreased from 1.1 (A1) to 0.9 (A3) mM for glucose and increased from 470 (A1) to 520 (A3) µM for lactate. An isolated event (B1) happened at 10:38 the following morning with a high basal values of 1.5 mM for glucose and 540 µM lactate. Finally a cluster of three SDs (C1, C2, C3) between 13:14 and 14:29 on day 3 resulted in a fall in glucose and increase in lactate. During this cluster, basal glucose dropped from 940 (C1) to 890 (C3) µM while lactate basal values were stable around 540 µM.

In patient 3, the changes were much smaller and uniform, ranging from −17 to −33 µM at 10 minutes following the SDs. The 4 SD waves shown here occurred on day 2 at regular intervals of 35 minutes between 8:03 and 09:43. The basal dialysate concentrations ranged from 440 and 540 µM.

Remarkably, 48% of the glucose changes and 39% of the lactate changes were small, i.e. below a 70 µM change. These were missed prior to noise removal (see Chapter 4). However, once the data were de-spiked and de-rippled, the baseline stability corresponded to a ± 8 µM smallest resolvable change (95% confidence limit) in the ICU (grey band on patient 3 plot in Figure 5.7). As a direct consequence, the number of rsMD responses to SD events that could be resolved increased from 43 to 96 after de-noising.

5.3.3.2 Area under the curve analysis

The variability in the rsMD responses in terms of magnitude and time course implies that a time average across the epochs is not appropriate. Instead, in order to integrate both the amplitude and the time course of the rsMD changes, an area under the curve (AUC) analysis was performed: for each 20 minutes period following an SD event, the area under the curve of dialysis concentration changes was calculated. The distribution of these AUCs is shown for all glucose and lactate events of Table 5.2 in Figure 5.8 below.
These distributions clearly show that the AUCs for glucose are negative, indicating a deficit of dialysate glucose during the 20 minutes following SD events, while the AUCs for lactate are positive, indicating an excess of dialysate lactate during the 20 minutes post-SD. This is confirmed by the very high significance of the binomial test (p<0.0001), rejecting an equal distribution of positive and negative AUC values, both for glucose and for lactate (see section 2.5.2.3 in Chapter 2 for further details about this test).

The distribution histograms also graphically indicate that the population of AUCs cannot be described by a normal Gaussian distribution, although the total number of events (n=96 for glucose and n=56 for lactate) could be sufficient to invoke the central limit theorem. The Gaussian distribution was rejected by the low p-values test obtained with the D’Agostino-Pearson’s K2 tests for normality (p<0.001 for the glucose AUCs and p=0.0391 for the lactate AUCs) [182; 183]. Instead the distributions are left-skewed for glucose (skewness γ₁=-0.9829) and right-skewed for lactate (skewness γ₁=0.334) according to a skew-normal data-fitting [298; 299]. As a consequence, a non-parametric statistical analysis was carried out on the AUCs for each patient. This is summarised in the box and whisker plot of Figure 5.9.

The distribution histograms also suggest two sub-groups of SD responses, distinguishable by an AUC of 500 µM.min. The responses in the sub-group smaller than this (37% of the cases for glucose and 43% for lactate) are tightly grouped. In the sub-group larger than this, the responses are widely scattered with extremes of -7300 µM.min for glucose and +6518 µM.min for lactate.
5.3. Results

Figure 5.9: Per-patient analysis of the 20-minute areas under the curves. Lactate AUCs are plotted in green on the top and glucose AUCs in red on the bottom of the graph. The zero-AUC values are marked by dotted horizontal lines on both part of the graph. For patient 10, six epochs of 20-minute changes in rsMD glucose and lactate were analysed for their AUCs. Details about the box and whisker plot can be found in section 2.5.2.3. A two-tailed Wilcoxon signed-rank test was performed for each patient and the results are indicated on the figure with their n number and significance levels: ns: p>0.5; *: p<0.05; **: p<0.01; ***: p<0.005.

As Figure 5.9 shows, when considering each patient separately and using the two-tailed Wilcoxon signed rank test, the null hypothesis of a zero-AUC change (i.e., a zero-change of dialysate concentrations during the 20 minutes following an SD) was rejected very significantly, provided there were more than three SD events occurring in a patient. In contrast, in patient 10 who had no SDs, the six 20-minute AUCs were not significantly different from zero. We can confirm that this is not due to a dysfunction of the rsMD assay for this particular patient since we observed physiological metabolic transients. Indeed, on day 2, this patient had an episode of high cerebral perfusion pressure (above 70 mmHg) for about 10 minutes. During this period, the glucose concentration dropped by 200 µM while lactate increased by 1.5 mM.

Secondly, pooling the results from all 10 patients, we confirmed that the 20-minute AUCs in response to SDs were significantly different from zero and from control 20-minute AUCs (n=41 for glucose and n=29 for lactate). Figure 5.10 shows the resulting box and whisker plot.
5.3. Results

Figure 5.10: SD vs control responses compared by their 20-minute AUCs. 20-minute AUCs in response to SDs were pooled across all 9 patients. Similarly control epochs were grouped across all 10 patients. A two-tailed Wilcoxon signed-rank test compared the two groups of AUCs to zero-AUCs. A Mann Whitney U test compared SD to control AUCs. AUCs and the results are indicated on the figure with their n number and significance levels: ns: p>0.5; *: p<0.05; **: p<0.01; ***: p<0.005.

Overall, the 96 glucose AUCs and 56 lactate AUCs were very significantly different from zero (p<0.0001 for both glucose and lactate). In contrast, the control AUCs were not significantly different from zero (p=0.984 and p=0.308 respectively) despite a relatively high n. We also compared control and SD epochs using a two-tailed Mann Whitney U test (see section 2.5.2.3, Chapter 2 for details on this test) that concluded that the SD changes were significantly different from control changes based on the 20-minute AUC (p<0.0001 in both cases). All together, these results strongly confirm that the dialysis changes in glucose and lactate concentrations are due to SD events.

5.3.3.3 Dynamic and sustained changes after the passage of the SD wave

The median and inter-quartile ranges for dialysate concentrations at 10 minutes and 20 minutes post-SD event are summarised for all the 96 events across the nine patients in Table 5.3.

The relatively wide interquartile range, even within patients, indicates that a variety of rsMD responses was observed. Overall, the changes for glucose and lactate were respectively −43.4 µM (−92.2 to −17.8 µM) and +41.7 µM (+8.2 to +93.9 µM) at 10 minutes following SDs. The changes in dialysate glucose at 10 minutes showed a significant correlation with the duration of the suppression of ECoG AC signal (Pearson product-moment correlation: r=−0.25, p=0.05). The longer the suppression, the greater the fall in dialysate glucose seen. In most cases no signs of recovery to the basal values were observed at 20 minutes after the SD events: overall, the concentration changes at 20 minutes after SDs were of −32.0 µM (−92.3 to −18.4 µM) for glucose and +23.1 µM (+5.5 to +93.6 µM) for lactate. These values were significantly different from zero (two-tailed Wilcoxon signed rank test) provided that there were more than three events for the patient (p values in Table 5.3 below).
### Table 5.3: Dialysate changes at 10 and 20 minutes post-SD.
Values are given as median (interquartile range). \( p \) values for the two-sided Wilcoxon signed rank test of the 20 minutes changes.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>10 minute change /µM</th>
<th>20 minute change /µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Lactate</td>
</tr>
<tr>
<td>1</td>
<td>-72.4 (-122.2 to -26.3)</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>-21.3 to -42.4</td>
<td>56.1 to 66.5</td>
</tr>
<tr>
<td>3</td>
<td>-30.1 (-43.4 to -17.6)</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>-46.2 (-61.9 to -13.8)</td>
<td>+137.6 (+54.9 to +226.4)</td>
</tr>
<tr>
<td>5</td>
<td>-66.3 (-113.9 to -31.7)</td>
<td>+22.1 (+11.9 to +47.4)</td>
</tr>
<tr>
<td>6</td>
<td>-15.6 (-20.3 to -9.8)</td>
<td>+34.6 (-35.9 to 54.1)</td>
</tr>
<tr>
<td>7</td>
<td>-220.1 (-272.6 to -199.6)</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>-57.0 (-108.1 to -47.6)</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>-101.5 (-193.2 to -238.0)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td>-43.4 (-92.2 to -17.8)</td>
<td>+41.7 (+8.2 to +93.9)</td>
</tr>
</tbody>
</table>

To summarise, despite an obvious variability in the metabolic responses to SDs, the changes in glucose and lactate were characterised by a significant and sustained drop in glucose and increase in lactate over the 20 minutes following an SD.
5.3. Results

5.3.4 CSDs and PIDs, two different types of SDs?

Given the range of dynamic responses, we tried to sub-categorised SDs. Based on the metabolic responses and the 20-minute AUC distributions, we could already discriminate small and large changes with a cut-off at 500 µM.min for the AUC.

In the literature, SDs have been sub-categorised into two classes according to their ECoG signature [281]:

- **cortical spreading depressions (CSD)**: spreading biphasic slow potential change accompanied by a transient depression of the fast AC activity
- **peri-infarct depolarisations (PID)**: spreading biphasic slow potential change without depression of the AC activity because this background activity is already silenced

Using these electrical characteristics to differentiate CSDs and PIDs, we also observed differences in terms of the time interval between consecutive events. Both types of SDs repeat in clusters of regularly repeating events. However, the time interval between consecutive PIDs of a cluster was generally shorter and more uniform (between 17.5 min and 20 min) than between consecutive CSDs in a cluster (24 to 40 minutes).

This raises the question: do CSDs and PIDS have different metabolic responses? Using an AUC analysis, I compared the rsMD responses to PIDs with those to CSDs and this is summarised in the box and whisker plot in Figure 5.11 below:

![Figure 5.11: CSD vs PID vs control responses.](image)

The 20-minute AUCs in response to SDs were sub-divided into CSD responses and PID responses and pooled across all 9 patients. Control-AUCs are also shown. A two-tailed Wilcoxon signed-rank test compared the three groups of AUCs to zero-AUCs. A Mann Whitney U test compared CSD- to PID-AUCs, CSD- to control-AUCs and PID- to control-AUCs. The results are indicated on the figure with their n number and significance levels: ns: p>0.5; *: p<0.05; **: p<0.01; ***: p<0.005.
5.3. Results

This AUC analysis suggests that, on the basis of the metabolic response, CSDs and PIDs are not different from each other (p=0.409 for glucose, p=0.1515 for lactate respectively). However they are both clearly different from a zero or control metabolic change.

We then asked the question whether PIDs were more likely to occur at certain levels of MD glucose. We built the following contingency table, using a band of [250:1000] µM for the basal levels of glucose, \([G_b]\), just before the SD event:

<table>
<thead>
<tr>
<th>([G_b])</th>
<th>CSD occurrences</th>
<th>PID occurrences</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>250&lt;([G_b])&lt;1000</td>
<td>60</td>
<td>11</td>
<td>71</td>
</tr>
<tr>
<td>([G_b])&lt;250 and ([G_b])&gt;1000</td>
<td>14</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>22</td>
<td>96</td>
</tr>
</tbody>
</table>

A two-tailed exact Fisher test was performed and the result was very significant with a p value of 0.0058. This means that, when an SD occurs while the basal glucose levels are inside the band [250:1000] µM, it is very likely to be a CSD. For the last sections of this chapter, we will continue to collectively refer to both CSDs and PIDs as SDs, and will specify PIDs only when necessary.

5.3.5 Cumulative effects of recurrent SDs

We have investigated the cumulative effect of SDs on the glucose concentrations as measured by microdialysis. These are presented for four patients where we represented each glucose epoch by the basal levels of glucose and the levels at 20 minutes (or the basal value before the next SD if an SD repeated before 20 minutes). Figure 5.12 below represents the 8 rsMD epochs we resolved in patient 6:

![Figure 5.12: Stepwise fall of glucose in patient 6.](image) Glucose basal levels and 20-minute post-SD levels versus time. Time indicated as time of the day /HH:MM. The ECoG events are identified by the dots at the top of the plot. Blue dots indicate PIDs.
5.3. Results

Figure 5.12 shows a final cluster of SD events in Patient 6 with a very short time-interval between consecutive SDs of only 15 to 20 minutes. After each SD event, the glucose concentrations fell by 10 to 92 µM. Over the duration of this cluster, the pre-SD glucose values progressively fell from 334 µM to 134 µM. At the end of the cluster, the dialysis glucose concentrations fell below 150 µM and remained there for the following two hours. Note that the SD presented on Figure 5.12 were PIDs: the ECoG background activity was depressed for the whole monitoring period from 01:00 on Day 2. After this series of PIDs (Figure 5.12), the tissue under the ECoG strip was completely silent electrically, with no background activity or depolarisation events observed for the remainder of the monitoring period. Patient 6 died on day 4.

Most patients showed more complex patterns of glucose response with repetitive SDs. For example, Figure 5.13 below represents the 14 epochs resolved in Patient 4.

The first six SDs in the early morning of Day 5 (Figure 5.13 a) had a net effect of driving down microdialysis glucose from 324 µM to 228 µM over a period of 4 hours and 10 minutes. However, the glucose levels never went below 200 µM, as they did in Patient 6 and the background AC activity of the ECoG was normal. Interestingly, the first SD of each cluster pushed the glucose level down by at least 20 µM whereas the second SDs led to changes just above the detection limit of the assay. From 11:00 on Day 5 (Figure 5.13 b), a cluster of 11 SDs in total were recorded on the ECoG (the rsMD signal was too noisy for the first three and they are therefore not shown here). The time intervals between each SD (32 minutes) were strikingly constant. The changes in glucose concentrations were either negative (of the order of −30 µM) or slightly positive (+15 µM). Remarkably, the basal glucose tended to go up, starting from 640 µM at 11:10 and rising to 927 µM at 13:46. A few other SDs followed but globally, the rsMD glucose and lactate and the ECoG activity remained fairly stable throughout and patient 4 recovered well: at six months following her surgery, she complained of occasional dysphasia but was completely independent and self-caring.
Figure 5.13: Two types of clusters in patient 4. Time indicated as time of the day /HH:MM. The square brackets at the top of the plots indicate clusters of regularly repeating SDs. The concentration scales are different on the two plots. (a): SD events progressively led to a moderate fall in glucose concentrations. (b): SD events hardly drove glucose down. The concentrations increased between clusters.
Patient 3 also showed these two types of clusters of SDs, as represented in Figure 5.14 below:

![Graph showing two types of clusters in patient 3](image)

**Figure 5.14: Two types of clusters in patient 3.** The plots are given in chronological order. Time indicated as time of the day /HH:MM. The square brackets at the top of the plots indicate clusters of regularly repeating SDs. The scales in concentrations are different between the two plots. **(a):** Each individual SD drives glucose down. The glucose concentrations recover between consecutive clusters of SDs. **(b):** Glucose progressively falls with repeating SDs with no recovery between events.

During the first period of monitoring (Figure 5.14 a), either clusters or isolated SDs occurred. They all individually pushed glucose levels down but moderately (mostly down by \(-25\) µM). Overall, the basal levels drifted upwards. Later on Day 3 though (Figure 5.14 b), clusters of SDs repeating every 20 to 50 minutes over a period of six hours drove glucose down just below 400 µM. More SDs occurred later
on Day 3, separated by at least one hour. They did not cause a further drop in glucose. Dialysate concentrations never fell below 200 µM during the whole monitoring period and he recovered well despite epileptiform activity since discharge.

Finally, Patient 9 also showed an interesting pattern, as plotted for three and a half days of continuous monitoring in Figure 5.15 below:

Figure 5.15: Stepwise depletion of brain glucose in patient 9. Three days of data are shown here. Time indicated as time of the day /HH:MM. The square brackets at the top of the plots indicate clusters of regularly repeating SDs. The blue dots indicate PID events.

In his case, there was a wider range of time intervals between consecutive SDs (see dots in Figure 5.15 above). During clusters of frequently occurring SDs glucose gradually decreased: from 2.0 mM to 591 µM on Day 1 and from 1.2 mM to 140 µM between 12:00 on Day 2 and 08:00 on Day 3 (see Figure 5.15). When the gaps between SDs were long enough (at least eight hours), the basal levels could fully recover their initial values. The final cluster of SDs on Day 3 pushed the glucose level down to 106 µM and the patient never recovered and deceased on Day 6.

Globally, the patterns of rsMD glucose changes with frequent SDs are complex and vary across patients. Nevertheless, there is a general trend, whereby when SD events repeated within less than 50 minutes, the glucose concentration did not recover fully to the initial pre-SD level before the onset of the following SD event, thus leading to a progressive stepwise fall in glucose concentration. When the interval between SDs was over 50 minutes, the basal glucose levels were similar or greater before the onset of subsequent SDs.

Furthermore, although an anecdotal observation, when MD glucose levels were forced below 200 µM by SDs, the patient’s prognosis was poor.
5.3. Results

5.3.6 Influence of plasma levels on dialysate concentrations

All the above have focused on the effect of SDs on the rsMD concentrations without considering any other factors. Clearly, given the complexity of the injured human brain, many variables play a role in the response. In particular, the supply of glucose and lactate from the blood is an important factor that can influence dialysate glucose and lactate. Figure 5.16 below summarises the relations we observed between dialysate concentrations and plasma levels.

Figure 5.16 indicates that there is no relation between rsMD lactate and either plasma lactate (Figure 5.16 b) or plasma glucose (Figure 5.16 c).

However a case can be made for rsMD glucose compared to plasma glucose levels. It is first noticeable that, despite active control with continuous intravenous feeding and infusions of insulin in the ICU, brain injury patients display a wide range of plasma glucose (Figure 5.16 a).

Patient 9 was diabetic and both his plasma and brain glucose were very high at the beginning and gradually controlled within a normoglycemic range. Since in diabetic patients, the activity of the blood-brain barrier glucose transporters is different from that in non-diabetic patients, the relation between his brain glucose and his plasma glucose is likely to be very different from that of the other patients. We have therefore excluded this patient from further analysis.

Patient 8 displayed a very high single data value above 15.0 mM plasma glucose, and given the great uncertainty of the plasma measurements, it is likely to have been an error and this data point was excluded for calculating the least-square linear regression.

Individually, the linear regressions show a poor goodness-of-fit between rsMD glucose and plasma glucose. However, on average, the levels of plasma glucose could influence the microdialysis glucose concentrations with a sensitivity of $40.8 \pm 10.7 \mu M/mM$ plasma glucose change (mean $\pm$ SEM, $n=6$). This is significantly different from zero as indicated by a two-tailed Student’s t-test ($p=0.0124$, $t = 3.8145$). This means that large changes in blood glucose are a confounding variable. This effect is further discussed in section 5.4.7.3.
5.3. Results

Figure 5.16: Comparison of microdialysis concentrations with plasma levels. rsMD concentrations were determined by the mean value over 5 minutes just before the blood sample was taken. The error bars indicate the standard error of the mean. Least-squares linear regression were performed for each patients between: rsMD glucose and plasma glucose (a), rsMD lactate and plasma lactate (b), and rsMD lactate and plasma glucose (c). The resulting equations are given in the tables beside the plots.
5.4 Discussion

There are four principal findings from the data presented in this Chapter. First, SD waves in the penumbra lead to a fall in extracellular glucose concentrations and a rise in extracellular lactate concentrations. Second, the effect of SDs on glucose and lactate could be resolved dynamically using rsMD in the ICU and showed a wide range of patterns of changes. Third, the metabolic response to the passage of the SD wave is sustained, with no sign of recovery at 20 minutes after SD. Finally, frequently occurring spontaneous SDs in the human penumbra lead to a stepwise progressive depletion of the extracellular glucose pool.

5.4.1 Fall in glucose and increase in lactate following SD waves

We have seen a characteristic metabolic response to SD waves in the human brain: a fall in dialysate glucose and an increase in dialysate lactate concentrations. Such changes in extracellular glucose and lactate levels have been observed in experimental models of stroke with induced or spontaneous SD waves. Mies et al. [266] found a decline in glucose in brain slices following the passage of a single SD wave, and a microdialysis study showed a marked increase in lactate during the repolarisation phase in the rat striatum [267]. Selman et al. [300] observed a marked decrease in extracellular glucose concentration and increase in lactate concentration at the SD wavefront and after the passage of the SD in rats using a freeze trapping technique. More recently, the metabolic response to induced SD was quantified in cats using the same rsMD assay, with results [160] very similar to our values now described for the human brain. I also measured similar changes in a pilot study in the rat brain (see Chapter 6). This suggests that rsMD is a reliable translational method to resolve the metabolic response to SD dynamically and that this response is similar across species (though differences in magnitudes and time courses have been observed).

Clinically, Parkin et al. [164] previously demonstrated a clear correlation between the total number of SD events and the reduction of dialysate glucose (see Figure 5.4 in the introduction to this chapter). However, although the association between low glucose concentrations and SD events was observed, the causation could not be demonstrated, for example from a typical time course of metabolic changes linked to single depolarisation events. Here, we show the first evidence that SD waves in the injured human brain do indeed lead to a decline in extracellular glucose concentration and increase in extracellular lactate concentration.

5.4.2 Dynamic changes in the metabolic response

We observed a wide variety of glucose and lactate responses to SD in terms of amplitude and time course of the changes as exemplified in Figure 5.7 and Table 5.3. The variability of these responses is much greater than the random noise from the rsMD assay, given that the reported changes were all above our limit of detection. Therefore, the observed variety in the responses is physiological and likely to reflect different responses of the injured tissues to SD waves.

Recent findings by the COSBID group indicate that SDs in SAH patients can be associated with different patterns of haemodynamic response: either physiological or inverse [75]. The former
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corresponds to a hyperaemic response that delivers enough oxygen and energy metabolites to repolarise the tissue and hence sustain recurrent SDs. This could correspond to small changes in the rsMD data both in amplitude and in duration. The inverse response is seen as a transient (but sometimes sustained) oligaemia (low blood flow) that spreads across the tissue in a similar pattern as the SD waves (and has been termed "cortical spreading ischaemia" [75]). This inverse coupling results in tissue hypoxia and could account for the larger prolonged responses observed in the rsMD data. A future study combining CBF measurements with rsMD in the human brain would be very valuable.

Another factor that could account for the variability of the rsMD responses is the duration of depolarisations. In support of this, we found a positive correlation between the duration of the depression of the background ECoG activity and the amplitude of the glucose drop at 10 minutes post-SD. These findings suggest that the amplitude and duration of the glucose and lactate changes could serve as measures of the insult severity associated with SD waves in cortical peri-lesion boundary zones in human brain injury.

Finally, different types of SDs have been identified according to their patterns of propagation across the cerebral cortex (radial and circumferential propagation) when visualised by laser speckle imaging [270; 271]. The cause for these different patterns is not clear and probably depends on the concentration gradients between core, penumbra and the border with the healthy tissue. Preliminary animal experiments suggest that the haemodynamic and metabolic responses might be different for these two types of SDs and some evidence is shown in Chapter 6: waves propagating radially away from the lesion usually lead to a more severe metabolic stress, with subsequent oligaemia and large changes in glucose and lactate measured with rsMD compared to waves propagating circumferentially around the lesion [180]). Due to methodological issues, imaging of the SD waves is difficult in the human brain, but a circumferential pattern would suggest a very regular periodicity between subsequent SDs (likely reason for the clusters of SDs) as opposed to the radial pattern. More investigation is needed on that matter, but this could be another source of variability in the responses we report here.

5.4.3 Sustained changes after the passage of the SD wave

Despite variability in the dynamic responses, the metabolic changes were on average still present 20 minutes after SD, with no sign of recovery observed. Increased utilisation of glucose in cortical tissue during the depolarisation phase of the SD wave has been demonstrated [301]. It has been attributed to the activation of the ATP-dependent ionic pumps to restore ionic homeostasis following the depolarisation. However, this alone cannot explain the sustained decrease in glucose at 20 minutes since at this time, cells are no longer depolarised, but are hyperpolarised. Alternatively, glucose may be used at this stage to replenish the astrocytic glycogen. Hertz et al. [302] suggested that glycogenolysis offers a higher anaerobic yield of ATP than fresh glucose and is thus likely to dominate ATP supply when there is acute energy demand. A significant reduction of glycogen levels after the passage of an SD wave has indeed been observed [300] and glycogen synthesis is therefore likely to be required between 10 and 20 minutes after SD.
5.4. Discussion

The concomitant sustained increase in lactate concentrations suggests a possible lack of oxygen locally and/or the activation of non-oxidative metabolism to sustain the additional metabolic workload following SDs in penumbral tissue. Hypoxia has been observed to coincide with the passage of SD waves [244; 245; 265]. It has also been suggested that the early increased aerobic metabolism in response to massive depolarisation [303] could switch to glycolysis at a later stage [304]. Hyperglycolysis was also observed in other microdialysis studies in brain injury patients [146; 305] and ultimately leads to increased lactate concentrations, as observed here.

5.4.4 Cumulative stepwise fall of glucose with recurrent SDs & implications for management of brain injury patients

We show that the sustained drop in glucose concentrations after depolarisations, combined with their capacity to recur in clusters at intervals of less than 50 minutes, leads to a progressive depletion of the cerebral extracellular glucose pool in the injured brain. A similar stepwise decrease in glucose concentration was observed and linked to spontaneous peri-infarct SDs following experimental middle cerebral artery occlusion [161]. Similarly, Takeda et al. [306] described a depletion of tissue glucose and production of lactate with recurrent SDs in rats.

5.4.4.1 A vicious circle

The frequency of SDs is closely linked to the availability of glucose from the blood. It was earlier shown that intravenous insulin increases SD frequency in rats [307], and, conversely, hyperglycaemia reduced the frequency of peri-infarct SDs occurring after middle cerebral artery occlusion [308]. In cats, the frequency of peri-infarct SDs increases as plasma glucose falls, although remaining within the (clinically defined) normal range [161; 309]. This combination of findings led to the suggestion of a vicious circle [161; 164] in which recurrent SDs drive brain glucose down, in turn increasing the frequency of SDs as proposed earlier [308], and in turn further lowering tissue glucose concentrations. The interaction of frequency of recurrence of depolarisations with delayed restoration of tissue glucose that we have demonstrated here suggests that this concept of a vicious circle, strengthened by mild hypoglycaemia, can indeed be applied to patients. To this threatening combination of factors (perhaps present to some degree in injured tissue even without depolarisations) must now be added the impact of microvascular spasm promoted by depolarisations in ischemic brain, as first characterised experimentally as "cortical spreading ischemia" [243] and recently demonstrated in patients with SAH [75].

5.4.4.2 SDs and patient outcome.

Several clinical studies have demonstrated a correlation between low brain glucose concentrations and poor patient outcomes. An initial study reported "undetectable" (lower than 50 µM) dialysate glucose in most patients who died after severe brain injury [295]. More recently, a strong relationship between periods of low brain glucose concentrations (lower than 200 µM for two hours) and outcome was demonstrated in TBI patients [61] and in aneurysmal SAH patients [310]. In all these studies, clinical factors (for example, seizures, hypoglycaemia, activated macrophages, neutrophils or elevated glutamate concentrations) either did not have a time course that corresponded well to the...
observed increased glucose utilisation [63] or simply accounted for only a minority of the observed prolonged periods of low glucose values [61]. We suggest that clusters of frequent SDs are an important mechanism leading to the low brain glucose concentrations measured in these studies, augmenting the likely effect of mitochondrial failure. The resulting accumulated failure of glucose supply to meet the energetic demand for the repolarisation of the cells could compromise the viability of the tissue and hence lead to further expansion of the lesion and poor neurological outcome. However, given the small number of patients (n=9) and the diversity of their primary pathologies (TBI, SAH and ICH), we cannot presently conclude that repetitive SDs are the dominant cause for low brain glucose. A future prospective study would be required to address this question.

5.4.5 Defining a metabolic penumbra

Evidence of persisting disturbance of metabolism, particularly tissue glycopenia, strongly supports recent arguments for a new definition of the penumbra as a "metabolic" penumbra rather than an "ischaemic" penumbra [311]. Classically, the penumbra in the context of focal cerebral ischemia is defined by marked reductions in cerebral perfusion (see section 1.4.3) and availability of oxygen to tissue with a resulting decrease in electrophysiological function [312], an increase in oxygen extraction fraction [313], and increased probability of delayed depolarisation and infarction [314]. The concept is probably also applicable in SAH patients [315; 316; 317], although the spatial distribution of penumbral cortex must be expected to be different, and perhaps more patchy than after, for example, occlusion of the middle cerebral artery. However, evidence for a well-defined ischemic penumbra in ICH and TBI patients is more equivocal. Thus a recent study showed that ischemia was not present in perihematoma tissue after ICH [63] and little or no increase in the oxygen extraction fraction was observed in the peri-traumatic region [62]. Increases in perihematoma glucose metabolism [63] and very low rates of oxygen use despite increased lactate/pyruvate ratio [62] and low extracellular glucose [61] were observed. In addition, evidence suggesting mitochondrial dysfunction is accumulating [60; 62]. Our findings do support this concept of metabolic penumbra.

5.4.6 Implication for glycaemic control of neuro-intensive patients

There has been an ongoing discussion regarding the optimal plasma glucose target in management of patients with acute brain injury. A number of clinical studies found that hyperglycemia is an unfavorable prognostic indicator in traumatic brain injury [318; 319] and stroke patients [320; 321]. It has been suggested that an abundant supply of glucose during ischaemic conditions allows continuation of anaerobic metabolism with accumulation of lactate and hydrogen ions, resulting in intracellular acidosis that triggers a cascade of calcium entry into the cells and eventual destruction of neurones [322]. Consequently, a large study on patients with severe brain injury concluded that morbidity and mortality were dramatically reduced when patients received intensive insulin therapy to tightly maintain their plasma glucose at a level between 4.5 and 6.0 mM as opposed to those whose plasma glucose varied between 10 and 11 mM [323]. Recently, the utility of tight glycemic control has been challenged. Vespa et al. [297] demonstrated that intensive insulin therapy resulted in a net reduction in microdialysis glucose (by 70% of baseline concentration) without improving functional outcome from traumatic brain injury. More ominously, in a study of ICU patients in which glycemic control was tight (4.4–6.7 mM) versus intermediate (6.8–10 mM) range, tight systemic glucose levels
were associated with a higher prevalence of low cerebral MD glucose (68 versus 36%) and brain energy crisis (25 versus 17%) than intermediate levels. Adjusting for intra-cranial pressure and cerebral perfusion pressure, it was concluded that systemic glucose concentration and insulin dose independently predicted brain energy crisis, which in turn correlated with increased mortality [324]. Recent reviews have argued in favour of a more limited use of intensive glycaemic control and a higher glycaemic target for patients with acute brain injury [325; 326], and thus particularly in the context of frequent, spontaneous SD events [327; 328]. Since these data indicate an additional cause of low brain glucose in acute brain injury, the availability of “adequate” plasma glucose, especially to the ischemic brain, must necessarily become critical in such circumstances. Considering the risks associated with tight glycemic control, Schlenk et al. [329] recently recommended a moderate control of plasma glucose, with levels up to 7.8 mM for subarachnoid haemorrhage patients. The data presented in this chapter shows that repetitive SDs can drive glucose down to a viability threshold based on MD glucose ($\approx 200 \, \mu$M). Given that there is some sensitivity of blood glucose on MD glucose, we would suggest a higher glycaemic target that would help patients with frequently occurring SDs not to reach this threshold.

5.4.7 Limitations of this study

There are several limitations to this study, which are primarily due to the disadvantages of the microdialysis sampling technique.

5.4.7.1 Localisation of the MD probe

The results are highly influenced by the localisation of the probe since the MD probe samples only local tissue. Since it is a one-site measurement, it cannot estimate global cerebral metabolism or regional differences in metabolism. Furthermore, localisation within the peri-contusional tissue is crucial: it is only when placed in the highest-risk territory that MD changes are expected to be at all clinically useful, as recommended in 2004 by a general consensus on microdialysis [330].

Another issue is the depth of the probe: it was observed that the MD signals may be quantitatively smaller in human white matter compared to grey matter [294; 331; 332]. This would result in the dilution of changes in the cortex by less abnormal white matter dialysate, in which case true losses in glucose related to depolarisations may in fact be greater than recorded here. For all these reasons, implantation under direct vision, intra-operatively, and obliquely to full membrane depth into the cortex, all aim to control the exact location of the MD membrane. The probe was also secured in position through suturing to the scalp during the whole monitoring period, but movement of the patient or nurse activity could have displaced the probe slightly and this cannot be excluded. When possible, post-operative CT scans confirmed the correct position of the MD probe, but these were not systematic. A future prospective study will require systematic assessment of the exact location of the MD catheter tip as to grey/white matter and proximity to focal lesions and oedema in each case to allow correct interpretation of the MD data.
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5.4.7.2 Depletion of glucose

The MD probe can act as a "sink" for the sampled analytes, and thus could potentially deplete the tissue locally and disturbs normal function. To evaluate this potential hazard, we estimated the amount of glucose drained by the MD probe. I first approximated that the tissue sampled by the MD probe as a ring of 2.5 mm diameter centered on the MD probe. This corresponds to 194.4 μL of tissue, or $1.94 \times 10^{-3}$ grams of tissue sampled (assuming that the tissue has the same density as water). If we consider that 250 μM of glucose diffuse to the probe, a perfusion of 2 μL/min would be equivalent to the removal of $5 \times 10^{-4}$ μmol of glucose per minute. When scaling for a 100 grams of tissue, this corresponds to 0.26 μmol/min/(100g of tissue) of glucose removed. This is non-negligible and corresponds to 69.7% of the metabolic rate of glucose at rest in human when comparing to the values from Fox and Raichle: $\text{CMR}_{\text{glu}} = 0.37 \pm 0.0053$ μmol/min/(100g of tissue) [124].

However, we can also estimate the amount of glucose supplied by the blood: the concentration of glucose in the blood is circa 6 mM and blood flow is normally of approximately 60 mL/min/(100g of tissue), thus supplying 360 μmol/min/(100g of tissue) of glucose. The depletion in glucose caused by the MD probe represents only 0.07% of the supplied glucose. In penumbral tissue, perfusion is lower, with a value of minimum 20 mL/min/(100g of tissue). This would in theory supply only 120 μmol/min/(100g of tissue) of glucose. This is nevertheless sufficient to provide significantly more glucose than that removed from the MD probe (0.26 μmol/min/(100g of tissue)).

From these broad estimates, we can conclude that, although a substantial quantity of glucose is removed by the MD probe, it is not sufficient to significantly deprive the cells of glucose and bias our results.

5.4.7.3 Effect of plasma glucose

We estimated that changes in plasma glucose would affect the brain glucose with a sensitivity of $\approx 40$ μM per mM changes in plasma glucose. This agrees with a previous study by Diaz-Parejo et al. [333] who measured MD glucose in penumbra during normoglycemia and hypoglycemia: their measurement indicated a sensitivity of 230 μM per mM changes in plasma glucose. Accounting for the differences in MD in vivo recovery between the two studies, our sensitivity would be equivalent to 220 μM per mM plasma glucose changes at their perfusion flow rate (0.3 μL/min in their study) (see section 5.4.7.6 about the influence of perfusion flow rate on the MD recovery).

On that basis of a sensitivity to plasma glucose, we re-evaluated the cumulative depletion of glucose shown in Figures 5.12–5.15. For patient 4 for example (Figure 5.13) the blood glucose varied between 7.5 and 8.2 mM during Day 5. Given the 40 μM per mM changes in plasma glucose, this would account for a change in MD glucose of 28.5 μM. This is clearly far less than the changes we see (range from 228 to 927 μM). For the diabetic patient (Figure 5.15), the effect of plasma glucose is more difficult to assess. Nevertheless, during Days 2 and 3 his plasma glucose was under insulin control between 11.5 and 12.0 mM and during this time, his MD glucose fell dramatically, by more than 1 mM.
5.4. Discussion

We can therefore conclude that plasma glucose changes alone are not sufficient to explain the changes observed during clusters of SDs. Nevertheless, a future study will have to address the extent of the effect of plasma glucose as compared to the effects of SDs on MD glucose.

5.4.7.4 Invasiveness and tissue damage

Knowledge about the traumatic effect of MD probes on the tissue comes from experimental studies, mainly in rodents. Within the first two hours following implantations, studies found evidence for oedema [334], ionic imbalances, disturbances in microcirculation and increased local glucose metabolism [335]. On a cellular level, histological examination of the tissue in the vicinity of the MD with electron microscopy have found both regions of apparently normal neurons and regions of damaged or degenerating neurons in the immediate vicinity of the dialysis probe [334; 336]. It was also demonstrated that there was a gradient of damage moving away from the probe: an ≈ 50% decrease in neuronal and synaptic density within 400 µm of a probe was observed [336], whereas 1.4 mm away from the probe, tissue disruption, including swollen axons, was still observed but neuronal and synaptic densities were not different from those of the control tissue at this distance. Other studies reported no signs of loss of dopaminergic fibers in the vicinity of the MD probe [337]. These findings all suggest that great care must be taken when implanting MD probes, and a very slow insertion of the probe is recommended to minimise trauma caused by acute implantation. West et al. [338] recommended inserting the probe at a controlled slow speed of 3 µm/s over a 25–30 minute period, in which case they recorded functional dopaminergic terminals in the vicinity of the acutely implanted microdialysis.

Most experimental studies suggest that the tissue response is back to normal within 24 hours after implantation; local cerebral blood flow and glucose utilisation in the region around the probe are indistinguishable from control values [335; 339] and neurotransmitter levels measured with microdialysis are most closely linked to neuronal activity at 24 h [340]. It is therefore good microdialysis practice to allow a 12 to 24 hour interval between implantation and data collection. However, a very recent study showed that even at 24 hour post-implantation, signs of ischaemic disruption, though reduced compared to the first four hours, were still evident [341]. In most human microdialysis studies though, transient neurochemical perturbations have been observed following implantation with a normalisation of dialysate metabolite levels within one to two hours [286; 294].

Finally, some studies also found that the blood-brain barrier was impaired 3 and 24 hours after MD-probe implantation in the rat, presumably because blood vessels rupture as the probe is pushed through the tissue [342], while earlier studies showed that the blood-brain barrier becomes intact again within a few hours of probe insertion [96]. In the human brain, the effect of MD probe implantation on the blood brain barrier integrity is largely unknown.

In our case, decision of implantation and removal of the MD probe were guided by the patient condition and on two or three occasions, the surgeons decided not to implant a MD catheter. When implanted, no adverse effects, such as bleeding, oedema or infection were observed in the immediate vicinity of the MD probe. Fixation of the MD catheter was also an important precaution to avoid damage from movement of the probe in the brain parenchyma. It is our view that insertion of a MD probe is far less traumatic than the initial brain injury of these patients in the first place. In addition, in
5.4. Discussion

this study, the probe was perfused at least a couple of hours before starting rsMD monitoring, allowing normalisation of the tissue surrounding the probe after implantation and surgery. As evidence, steady levels of glucose and lactate were obtained within the first 15–30 minutes of monitoring.

Finally, it has been suggested that MD implantation could cause SDs [335] and therefore that adverse tissue response to SDs would be amplified due to the mere presence of the MD probe. However, it was shown in an experimental model with induced SDs that the microdialysis probe does not affect the propagation of the SD waves and their associated haemodynamic responses as measured by laser speckle [160]. We made the same observations in in vivo experiments described in Chapter 6.

5.4.7.5  Long term measurements

Within a few days following implantation of the MD catheter in the rat, glial processes have been observed to start expanding towards the track of the MD probe which is engulfed in a glial layer by three days post-implantation [334]. This results in blunting neuronal chemical responses and makes the measurement of neurotransmitters very difficult after a few days. Cell swelling due to progressive injury could also impair diffusion of the analytes towards the probe and thus impair MD sampling.

However, clinical studies generally observed fluctuations in the dialysate levels of glucose, pyruvate and lactate also several days (up to 11 days) following implantation [138; 287]. Studies in epilepsy patients also showed that potassium-evoked increase in microdialysate adenosine and GABA did not diminish over a time period of 7–16 days following probe implantation [343]. More recently, the use of urea as an endogenous reference compound to assess the in vivo performance of the MD probe provided evidence that the dialysate concentrations of a passive species were remarkably stable over 120 hours of continuous microdialysis, thus suggesting that no diffusional barrier was impairing the diffusion of this compound over this time [344].

In the present study, we can also rule out the presence or extension of a diffusional barrier with time. Indeed, if it were the case, we would have observed a simultaneous decrease of microdialysate glucose and lactate. However, lactate concentrations generally increased with time, and, although the general trend for glucose was a progressive decrease, we observed glucose levels increasing to high concentrations provided sufficient time between SD events.

5.4.7.6  Microdialysis recovery of extracellular compounds

An inherent problem with MD is that the dialysate concentration does not equal the true interstitial level, but rather a fraction thereof. Different methods have been devised to calibrate MD in vivo, the most commonly used being the no-net-flux method [345]. The analyte of interest is perfused at various concentrations through the probe (C\textsubscript{in}) and the concentration of this analyte is then measured at the outlet of the probe (C\textsubscript{out}). The perfusion concentrations are chosen to bracket the actual concentration of the analyte in the tissue. The plot of C\textsubscript{in}−C\textsubscript{out} vs C\textsubscript{in} yields a straight line and the point of no-net-flux across the membrane is the x-intercept where C\textsubscript{in}=C\textsubscript{out}, according to Equation 5.1.
\[ C_{in} - C_{out} = E(C_{in} - \frac{R}{E}C_{tissue}) \] (5.1)

where \( E \) is the extraction efficiency, i.e. the ability of the tissue to collect material from the dialysate fluid in the MD probe, \( R \) is the relative recovery, i.e. the ability of the MD probe to collect from the extra-cellular space, \( C_{out} \) is the measured dialysate concentration, \( C_{in} \) is the concentration in the perfusate, and \( C_{tissue} \) is the analyte tissue concentration far away from the probe.

\( R \) and \( E (<1) \) characterise the ability of an analyte to diffuse to and from the MD probe respectively. They therefore depend on several physicochemical parameters of the analyte and of the MD probe but also on tissue processes that can influence the mass transport of the analyte \textit{in vivo}. If \( R=E \), the point of no-net-flux corresponds to the true extracellular concentration of the analyte. This has traditionally been accepted as a reasonable assumption. However, evidence from fast scan cyclic voltammetry in conjunction with microdialysis sampling of dopamine suggests that this is not always the case [346; 347; 348]. This can be understood when considering that \( E \) and \( R \) are the results of a combination of mass transport resistances, as initially formulated by Bungay \textit{et al.} [349; 350]:

\[ E = 1 - e^{-\frac{1}{Q(\rho_d + \rho_m + \rho_t)}} \] (5.2)

where \( Q \) is the perfusion flow rate through the MD probe, \( \rho_d, \rho_m, \) and \( \rho_t \) are a series of mass transport resistances for the dialysate flow channel, dialysis membrane, and extracellular tissue.

The dependence on the perfusion flow rate, \( Q \), stems from the fact that \( Q \) determines the residence time of the perfusate within the dialysis membrane. Decreasing \( Q \) allows more time for the analyte to diffuse through the MD membrane and equilibrate with the internal perfusate concentration.

The combined mass transport resistance contributions from \( \rho_d \) and \( \rho_m \) can be experimentally determined \textit{in vitro} by collecting dialysates at different flow rates. A plot of the natural logarithm of \((1-E)\) vs. \( 1/Q \) should yield a straight line, which can be regressed to determine the additive values for \( \rho_d \) and \( \rho_m \) [150].

The tissue contribution to mass transport of the analyte to the MD probe, \( \rho_t \), is much more difficult to estimate experimentally and has been the subject of many studies over the last ten years. Parameters such as the tortuosity of the tissue (which will effectively decrease the diffusion coefficient of the analyte [150]), the blood flow in the capillaries and arterioles [351], the extra-cellular volume fraction, the kinetic of release and uptake from and into the surrounding cells [352; 353] and the traumatised layer in the vicinity of the probe all influence the mass transport of the compound to the MD probe. In particular, the effect of active processes, such as release and uptake of neurochemicals, can affect the mass transport to the probe and from the probe differently, thus leading to \( E \neq R \). This has been confirmed by elegant \textit{in vivo} electrochemical measurements in the proximity of MD probe by Michael \textit{et al.} [346; 354] along with theoretical models by different groups [347; 348; 350; 355]. Experiments and theoretical models concluded that inhibiting uptake causes the recovery of dopamine to increase [346; 347; 348]. This can be understood by considering concentration gradients in the tissue surrounding the probe; as the analyte is recovered by the probe, it creates a concentration gradient at the membrane/tissue interface. When uptake is inhibited, this concentration gradient is steeper and therefore the recovery is enhanced [150; 353]. For the same reason, the extraction fraction from the probe to the surrounding tissue decreases with uptake inhibition [349]. Similarly the rates of release of a compound will affect the recovery and extraction fraction of the MD probe.
In particular, the effect on release and uptake is very complicated in the close proximity of the probe. Some observations suggest that damage preferentially affects the sites of release of dopamine [355], while other models assume a passive layer of tissue surrounding the probe [347], which could apply for gliosis of chronically implanted MD probes or for analytes such as glucose. A key parameter to consider is the distance from which an analyte is sampled by the MD probe. This depends on the distance over which the analyte can diffuse in tissue. This diffusion length is usually estimated using Equation 5.3 [356]:

\[ x \approx \frac{0.693}{\sqrt{D/k}} \]  

where \( D \) is the diffusion coefficient of the species in the extracellular space and \( k \) the pseudo-first-order rate constant of uptake of the species.

For dopamine, this distance has been estimated to be \( x_{DA} \approx 4.8 \, \mu m \) [356]. This means that dopamine cannot diffuse through a layer of damaged tissue of 400 \( \mu m \) around the MD probe [336] and that therefore, the dopamine sampled by the MD probe is that within the damaged layer in the vicinity of the MD probe, where sites of release of dopamine are preferentially affected. In this case, the dopamine levels in dialysate reflect only the *apparent* concentration in the vicinity of the probe and not the true extracellular concentration further away, beyond the damaged layer. This explains the controversial underestimation of extracellular concentrations of dopamine by microdialysis sampling.

However, in the cases of glucose and lactate, the diffusion lengths, \( x_{glucose} \) and \( x_{lactate} \), are much greater. We estimated the pseudo-first-order rate constant for glucose based on calculations of the metabolic rate of glucose and measurements of the concentrations of extracellular glucose:

\[ k_{glucose} = \frac{CMRGlu}{[Glucose]_{ECF}} \]

Using CMRGlu = 0.37 \( \mu mol/min/(100 g \, \text{of tissue}) \) [124] and \([Glucose]_{ECF} = 400 \, \mu M \) [136; 144; 357], a pseudo-first-order rate constant for glucose uptake is approximately \( k = 1.5 \times 10^{-4} s^{-1} \). Using this value in equation 5.3 and an apparent diffusion coefficient for glucose in the extracellular space of 0.4 \( \mu m^2/s \) [358], this gives a diffusion length for glucose of \( x_{glucose} = 1.12 \, \text{mm} \) in the extracellular space. We did the same calculations for lactate and found a diffusion length \( x_{lactate} = 376.55 \, \mu m \) (using \( k_{lactate} = 0.0021 \, s^{-1} \) derived experimentally after stimulation in the rat cerebellum [359] and \( D_{lactate} = 0.62 \, \mu m^2/s \) calculated by nuclear magnetic resonance spectroscopy [358]). Both values for \( x_{glucose} \) and \( x_{lactate} \) are considerably larger than the estimated diffusion length of dopamine (4.8 \( \mu m \) [356]). This would mean that, contrary to dopamine, glucose and lactate can diffuse through a layer of "passive" damaged tissue and be detected by the MD probe. The MD glucose and lactate concentrations thus reflect the true extracellular concentrations in glucose and lactate. This was indirectly confirmed by the agreement between estimates of the extracellular concentrations in glucose of \( \approx 400 \, \mu M \) with MD sampling [144; 357] and with an implanted biosensor [136].

However, we cannot exclude that the probe recovery will not change over time, as has been reported for glucose under conditions of extreme energy demand when using time-resolved no-net-flux experiments [360]. This is especially true in brain injury patients: the cerebral perfusion is usually unsteady, the extracellular space volume can decrease due to excitotoxic oedema and inflammation can occur over time.
5.5. Conclusions

5.4.7.7 Timing of the metabolic responses

A particular issue here is the accurate determination of the time of the onset of the rsMD event as compared to the time of the passage of the SD wavefront on the ECoG strip. In this study, this time was estimated to be within five minutes after the occurrence of the depolarisation on the closest electrode on the ECoG strip, and corrected to account for the direction of propagation of the wave and position of the MD catheter relative to the subdural contact. However, we cannot precisely determine the exact time when the SD wavefront reached the MD probe. We aim at using online microdialysis measurements of potassium, as a marker of depolarisation, to correct for this inaccuracy. The development of a flow-cell for online microdialysis measurement of potassium is discussed in Chapter 6.

5.5 Conclusions

Rapid sampling of cerebral microdialysate for glucose and lactate, together with the use of noise-reduction post-processing, demonstrates dynamic and sustained reductions of tissue glucose following individual SD events. At 20 minutes post-SD, glucose has fallen by −32.0 µM (range: −92.3 to −18.4 µM, n=96) and lactate has increased by +23.1 µM (range: +5.5 to +93.6 µM, n=49). Furthermore, these changes are additive and clusters of frequent SDs lead to the progressive depletion of glucose in penumbral tissue. When MD glucose concentrations fell below concentration of 200 µM, this was associated with poor patient outcome on an individual case basis. Finally, we suggest that SDs are an important cause for the unexplained prolonged periods of low glucose observed in other independent studies. In these studies, low glucose was associated with poor patient outcome. A case can therefore be made for greater use of rapid-sampling microdialysis as an aid to clinical management of such patients in order to prevent brain glucose levels to fall below a viability threshold.

A prospective study would be required to determine whether SDs are the dominant mechanism that causes a drop in brain glucose concentration, independent of other factors, such as plasma glucose. Such a study could also address additional questions, such as 1) whether there is a difference in the responses to SDs when they occur at early versus late days following injury, 2) whether the magnitudes of the glucose drops are correlated with the frequency of SD events, and 3) whether different sub-types of SDs can be identified by the severity of their metabolic responses. Other markers of ischaemia, such as cerebral blood flow, tissue oxygen content and pyruvate concentration could also be relevant for a multi-modal study.
Chapter 6

Development and application of a flow-cell for online measurements of potassium in the microdialysate stream

One particular issue we are facing with the clinical data is the timing between the ECoG detection of the SD waves and the onset of the chemical response seen by the microdialysis probe (see Chapter 5). To resolve this temporal uncertainty or “fuzziness”, it would be very valuable to have a direct marker for the passage of SD waves that can be seen on the dialysate stream. One obvious candidate is potassium, that has been shown to increase at the wavefront of spreading depolarisations [104; 237; 250].

This chapter presents our approach to engineer a flow cell that can integrate a potassium ion selective electrode into the dialysate flow stream and be coupled to the rsMD assay. The flow-cell should fulfill a few specifications:

- a good sensitivity and selectivity for reliable detection of potassium in the dialysate,
- a good time response to resolve potassium changes during the passage of SD waves,
- a sufficient stability and signal-to-noise ratio for long-term measurements in noisy environments (intensive care unit).

In vitro optimization of a potassium ion selective electrode and a compatible flow-cell are presented and preliminary in vivo results are reported.

6.1 Background on ion selective electrodes

Methods to measure ion concentrations in vivo have already been discussed in section 1.5.3.3. Here, we have used an electrochemical method based on the technique of ion selective electrode (ISE). ISEs present a few advantages over other techniques (mainly based on fluorescent dyes), such as:

- they do not remove ions from the sample solution since the measurement is based on equilibration of ions on both sides of the membrane,
- they have a wide dynamic range: 1.0 to $10^{-6}$ M,
- they can be easily calibrated with standard ionic solutions,
- they are small and portable,
- they are relatively inexpensive, particularly with regards to the instrumentation.
ISE is a potentiometric technique, whereby a potential difference is generated between two sides of a membrane which is selective for the specific analyte ion to detect, the primary ion. In this chapter, the primary ion is potassium, and more generally, we will refer to the primary ion as $I$.

### 6.1.1 Different types of ISE

#### 6.1.1.1 First electrodes: glass membrane ISEs

Glass membrane electrodes were the first ISEs [361] based on pioneering work by Cremer in 1906 [362]. This type of ISE has a good selectivity and an excellent chemical durability, with incomparable stability and lifetime. The most common of the glass membrane electrodes is the pH electrode. However, these glass electrodes are rigid and the mounting of glass membranes is not easily adaptable to a flow-cell that would integrate into the dialysis stream. This first type of ISEs has been superseded by liquid solvent polymeric electrodes.

#### 6.1.1.2 Liquid solvent polymeric electrodes

Liquid membranes are based on a permselective membrane containing an ionophore — a liquid soluble molecule that can "carry" a particular ion — and mobile ion-exchange sites supported by a polymer matrix. The solvent polymeric membrane is physically a water-immiscible liquid of high viscosity that is commonly placed between two aqueous phases, the sample and the internal filling solution (IFS). The inside of the membrane equilibrates with the IFS while the outside of the membrane equilibrates with the sample solution. As the primary ion is selectively transferred from the sample solution to the hydrophobic membrane phase, a potential difference is generated between the two sides of the membrane. This potential is, ideally, a linear function of the logarithm of the activity ratio of $I$ in the two solutions contacting the membrane [363; 364; 365; 366].

Compared to glass electrodes, polymeric ISEs are less mechanically and chemically resistant but they offer much more flexibility. They can be cast into virtually any shapes and hence can be more compact. They are also quite simple to make and relatively low cost. Most importantly their properties (such as sensitivity, selectivity, response time) can be tailored to particular applications by optimising the composition of the membranes.

In particular, very high selectivity is possible using an appropriate ionophore which selectively binds to the primary ion. Here, we have used a neutral ionophore which is well-characterised for its selectivity to potassium: valiomycin (see section 6.1.2 for further details about the ionophore). To prevent extraction of the counter-ions into the membrane while maintaining electroneutrality in the membrane, charged mobile lipophilic salts are added to the polymeric membranes. These salts are referred to as ion-exchanger sites in the ISE literature. The general working principles of the extraction process for a cation ISE with a neutral ionophore is schematically represented in Figure 6.1 below:
6.1. Background on ion selective electrodes

![Diagram of solvent polymeric membrane and internal filling electrolyte](image)

**Figure 6.1: Working principles of the solvent polymeric ion selective membrane.** Sample solution composed of the salt, IA, with the primary cation $I^+$ and its counter-ion $A^-$. $I^+$ selectively binds with the neutral ligand, L, to form positively charged complexes, $[IL^+]$. Lipophilic anionic exchangers, $R^-$, protect against anionic interferences from $A^-$ in the sample solution.

Both the ionophore and the anionic additives are critical for the performance of the ISE membrane. Typically, this type of ISEs has a working range of 1 to $10^{-6}$ M [366].

Traditionally, solvent polymeric membranes have been casted onto a glass substrate and they have been used for intra- and extracellular measurements [363; 365]. However, there are several limitations associated with glass, mainly the requirement of silanisation of the glass to reliably bind the valinomycin membrane [364]. This is usually a difficult process that can lead to an inhomogeneous interface with the membrane and unstable potential responses. Additionally, glass is rigid and fragile, which is not appropriate for our application. Instead, we developed our ISEs using a polymer substrate.

6.1.1.3 All solid-state ion selective electrodes

The hindrance of liquid membranes is their internal filling electrolyte that prevents miniaturization of the devices. The challenge is the transduction of the ion detection at the membrane to a measurable signal.

The first all solid state ISEs were based on field effect transistors (FETs). In such electrodes, the drain current of the FET is measured. ISFETS have been developed for a few ions, including potassium [365]. However, the production of ISFETS is based on silicon semiconductor techniques, which requires the setup of special and elaborate facilities [367]. Furthermore, the electrochemical interface between the membrane and the gate insulator is not fully well-defined and this has typically led to long-term instabilities of the ISFETs responses.

More recently, different groups have tried to develop solid contacts to PVC membranes using an ion-to-electron transduction. This has been reviewed in [368] and the main techniques include:

- **coated wire electrodes**: the metal wire is simply dipped into a solution of the membrane mixture. However, the lack of a defined interface between the membrane and the wire leads to erratic drifts [369].
- **metal loaded epoxy substrates**: they adhere better to the membrane but they generally suffer from oxygen interference [367].
- **hydrogels**: a gel layer is formed to make contact with the membrane. The problem is their fragility since PVC is permeable to water [368]
- **conducting polymers**: they are currently the most promising approach [369; 370]. They can be functionalised by covalent bonding, or immobilization of functional doping ions and hence be tailored to specific applications. As an example, potassium electrodes were successfully
6.1. Background on ion selective electrodes

fabricated with polypyrrole doped with tetrphenylborate [368]. More research is needed to fully characterise these ISEs.

- **single walled carbon nanotubes**: the transduction is based on the high charge transfer of the nanotubes that sense the phase-boundary potential change of the ion selective membrane [371; 372]. These ISEs are currently under development.

6.1.2 The ionophore: valinomycin

The use of ionophores for electrochemical measurements started with the discovery, in 1964, that some antibiotics (valinomycin for example) induced ion transport in mitochondria [373]. A few years later, Stefanac and Simon [374] showed that the phenomenon was mainly due to the selective formation of complexes between these compounds and certain cations. They introduced the first neutral-carrier based ISE and demonstrated that these antibiotics induce *in vitro* selectivities similar to those observed *in vivo*. The first valinomycin-liquid membrane for selective detection of potassium was born [375]. Since then, a large number of natural and synthetic, charged and uncharged ionophores have been developed for anions and cations [366]. Figure 6.2 shows the structure of valinomycin before and after complexing potassium ions.

![Valinomycin](image)

**Figure 6.2**: Valinomycin in its free uncomplexed form (a) and when complexed with potassium ions (b). These are X-ray stereoviews taken from [366]

Valinomycin is a macrocyclic ionophore (Figure 6.2 a): it has a ring structure so that potassium is fully enclosed in its internal polar cavity (Figure 6.2 b). This cavity is of the same size as potassium ions. The outer surface of the complex is hydrophobic [365]. This enables valinomycin to translocate in the polymeric membrane in its free and complexed forms in the process of transporting the ion from a region of high to low concentration [376]. This is at the origin of the potential response of the valinomycin based potassium-selective electrode.

In general, for an ionophore to provide good selectivity and ISE response, it must fulfil a few requirements:

1. the ligand should strongly *complex* the primary ion and only weakly all the others. The coordination number and the cavity size (compared to the ion radius) of the ionophore determine the preference for a certain cation [364].

2. the complexation process should be *kinetically fast* so that thermodynamic equilibrium can be
6.1. Background on ion selective electrodes

established quickly, thus avoiding long response times [377]. Ionophores are thus required to have some degree of "pre-organisation", ie the structure of the free ligand resembles that of the complex, so that the overall free energy barrier of conformations of the two forms is small enough for complexation to occur quickly.

3. the ionophore must be lipophilic. so as not to leach from the membrane into the aqueous phase over time [377].

6.1.3 ISE response: the classical models

Ion-selective electrodes are typically studied under zero-current conditions in a galvanic cell such as the following:

![Principle of the ion-selective electrode measurement](image)

Figure 6.3: Principle of the ion-selective electrode measurement. An ISE is made of a selective membrane with the ionophore, an inner filling solution and an inner reference electrode. The potential is measured at zero-current using a high-impedance voltage measurement and an external reference electrode.

The electromotive force (EMF) across this cell is the sum of all individual potential contributions:

\[
EMF = E_{const} + E_J + E_M
\]  

(6.1)

where \( E_{const} \) includes sample-independent contributions, \( E_M \) is the membrane potential, \( E_J \) is the liquid junction potential at the sample/bridge electrolyte interface.

The liquid junction potential, \( E_J \), can be either be kept reasonably small and constant under well defined conditions or be estimated according to the Henderson formalism (beyond the scope of this thesis) [366].

The response of the ISE membrane, \( E_M \) is a complex time-dependent phenomenon. It depends on the composition, thermodynamic, and kinetic properties of the membrane, the sample solution and the membrane/sample interface. Several models have been developed to account for all of the above.
and details can be found elsewhere in reviews [366; 368; 378]. We will here describe the phase-boundary model and its semi-empirical extension, the Nickolskii-Eisenman equation [379; 380].

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6.1.3.1 The phase-boundary potential model

Formally, the membrane potential can be described as in equation 6.2 [364; 378]:

\[ E_M = E_{PBsample} + E_{PBint} + E_D \]  

(6.2)

where \( E_D \) is the membrane diffusion potential due to the diffusion of ions within the membrane phase, and \( E_{PBsample} \) and \( E_{PBint} \) are the boundary potentials related to the exchange processes of ion I at the membrane/sample phase-boundary and the membrane/internal filling solution phase-boundary respectively.

The phase-boundary potential model assumes that:

1. \( E_{PBsample} \) governs the membrane response. In other words, \( E_D = 0 \) (migration effects in the membrane are ignored) and \( E_{PBint} = \text{constant} \) (\( E_{PBint} \) is sample-independent). These assumptions are valid in most cases of practical relevance [366].

2. the electric potentials and the concentrations of ions in the phases in contact are independent of the distance (ie bulk activity is equal to surface boundary activity) and of time [368]. This means that the Guggenheim’s concept of the electrochemical potential, \( \mu_I \), can be applied [381]:

\[ \tilde{\mu}_I = \mu_I + z_I F \phi = \mu_I^0 + RT \ln(a_I) + z_I F \phi \]  

(6.3)

with \( \mu_I \) the chemical potential of ion I in the phase (\( \mu_I^0 \) under standard conditions), \( z_I \) the ion valency, \( a_I \) the activity of the uncomplexed ion I, \( \phi \) the electrical potential in the phase, and \( R, T, F \) the universal gas constant (\( R=8.314=J/K/mol \)), the absolute temperature (in Kelvins, K) and the Faraday constant (\( F=96,485.34 \text{ C/mol} \)).

3. the organic phase-boundary in contact with the sample is in electrochemical equilibrium with the aqueous sample solution (true if the phase transfer reaction is much faster than the relevant diffusion processes in both the aqueous and organic phase). This means that for each phase, the electrochemical potentials are equal: \( \mu_I(\text{org}) = \mu_I(\text{aq}) \).

Hence, the membrane phase-boundary potential can be written as:

\[ E_M = E_{PBsample} + \text{constant} = \bar{E}_I^0 + \frac{RT}{z_I F} \ln \frac{a_I(\text{aq})}{a_I(\text{org})} \]  

(6.4)

with \( \bar{E}_I^0 \) including the constant \( E_{PBint} \) and the standard potential \( E_I^0 \) for ion I.

Under the condition that \( a_I(\text{org}) \) is independent of sample activity changes, it can be incorporated in the constant terms and the membrane potential reduces to the Nernst equation for the primary ion I:

\[ E_M = \bar{E}_I^0 + \frac{RT}{z_I F} \ln a_I(\text{aq}) \]  

(6.5)

Therefore, the potential response of an ISE is proportional to the logarithm of the activity of the primary ion in the sample solution. The proportionality constant (or slope) is equal to \( 58.17/z_I \text{ mV/decade at } 20\degree \text{C} \).
This model has been verified experimentally. The linear portion of an ISE calibration curve that follows the Nernstian response towards the primary ion has been defined as the working range of an ISE [382]. This definition is part of the nomenclature of ISE from the International Union of Pure and Applied Chemistry (IUPAC) in 1976. A typical ISE calibration is shown below in Figure 6.4.

![Graph showing the linear Nernstian part of the calibration curve of an ISE defining its working range (limited by blue dotted vertical lines). The detection limits of the ISE are defined by the cross-section of the two extrapolated linear calibration curves. Modified from [382].](image)

The Nernstian working range of a cation-selective electrode is limited by:

- the upper limit of detection: due to co-extraction of interfering anions with increasing activity of the primary cation. This is also referred to as the Donnan exclusion failure [383].
- the lower detection limit: due to interference by competing cations in the sample solution or modification of the activity of ions at the interface of the organic phase with primary ions leaching from the membrane to the sample solution [384; 385]. Traditionally, this detection limit is of the order of the micromolar [364; 365; 386] but recent work by Bakker et al. has pushed it down to the nanomolar range using trans-membrane ion fluxes (see section 6.1.6 for further details).

### 6.1.3.2 The Nikolskii-Eisenman model

Equation 6.5 does not take into account the presence of ions other than the primary ion, I, in the sample solution. A semi-empirical model has been proposed to account for mixed sample solutions (with ions I and J). This is the Nikolskii-Eisenman equation [379; 380]:

---

_Figure 6.4: Working range of an ISE according to the IUPAC recommendations._ The linear Nernstian part of the calibration curve of an ISE defines its working range (limited by the blue dotted vertical lines). The detection limits of the ISE are defined by the cross-section of the two extrapolated linear calibration curves. Modified from [382].

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6.1. Background on ion selective electrodes

\[ E = E^0 + \frac{RT}{z_J F} \ln \left[ a_I(aq) + \sum_J K^\text{pot}_{I,J} (a_J(aq))^{z_I z_J} \right] \]  
\[(6.6)\]

where the \( K^\text{pot}_{I,J} \) are the Nikolskii selectivity coefficients of the membrane for interfering ion \( J \) relative to primary ion \( I \).

The smallest the \( K^\text{pot}_{I,J} \), the most selective the electrode. The selectivity of an ISE depends mainly on the ionophore but also on the other constituents of the membrane, in particular additional lipophilic anionic sites.

The \( K^\text{pot}_{I,J} \) can be understood as the equilibrium constant of the extraction of the primary ion \( I \) (in the aqueous phase) into the membrane by complexation with the ligand \( L \) (stoechiometry \( n \)) in the presence of interfering ions \( J \) (for simplification, \( I \) and \( J \) are cations but anions can be treated similarly):

\[ IL^n_+(org) + J^{z_J+}(aq) \rightleftharpoons I^{z_I+}(aq) + JL^{z_J+}_n(org) \]  
\[(6.7)\]

For primary and interfering ions of the same charge, and neutral carrier that predominantly form 1:1 complexes (such as valinomycin for potassium), the selectivity coefficients simplify to \( K^\text{pot}_{I,J} = \beta_{IL} / \beta_{JL} \) where \( \beta_{IL} \) and \( \beta_{JL} \) are the equilibrium constant for the complexation of the ion \( I \) and \( J \) respectively with the ionophore \( L \). Therefore, in an ideal case, the ion selectivity of neutral carrier membranes is completely governed by the complex formation properties of the carrier [364].

The Nikolskii-Eisenman model has been extended in the 1990s to heterovalent ions — case where \( z_I \neq z_J \) — by Bakker et al. using an extended definition of the selectivity coefficient and a theoretical model for mixed ion solutions of different charges [378; 387; 388; 389].

Experimentally, the Nikolskii coefficients (\( K^\text{pot}_{I,J} \)) are usually determined using [383]:

- the separate solution method: electrode responses to two solutions, each containing either a salt of the primary or interfering ion only are compared
- the fixed interference method: calibration curves for the primary ion are determined in a constant background of interfering ion.

However, large discrepancies in the experimental values of the \( K^\text{pot}_{I,J} \) have been reported for similar ISE membranes, suggesting that the selectivity is not an intrinsic physical property of a given membrane but depends on the composition of the sample, the history of the electrode, and the experimental conditions [390; 391; 392]. It was acknowledged that these huge variations were due to improper extrapolations of the Nikoskii coefficients using Equation 6.6. The Nikolskii-Eisenman equation is indeed only valid for an electrode that exhibits a Nernstian response towards both the primary ion and the interfering ion [393]. However, it is often the case that ISEs have a non-Nernstian slope, particularly for highly discriminated interfering ions. In these cases, the experimental \( K^\text{pot}_{I,J} \) can be overestimated by many orders of magnitude. Solutions to this issue have been suggested and recently adopted by the IUPAC [394]:

- the matched potential method (MPM) [391]: the potential change upon increasing the primary analyte activity in a starting solution is measured. Interfering ions are then added to an identical starting solution until the same potential change is observed. The ratio of the changes in the
6.1. Background on ion selective electrodes

activity of the analyte and interfering ion is the selectivity factor. The MPM is applicable in cases of non-Nernstian responses. The issue is that the selectivity coefficients determined by this method are very dependent on both the initial concentration of primary ion and its increment [392].

• methods that restore a Nernstian response by counteracting ion fluxes through the membrane (see section 6.1.6 for further details) [393; 395; 396; 397].

• a new conditioning procedure that yields Nernstian response slopes even for highly discriminated ions [398; 399]. Before any contact with the preferred primary ion, the membrane is first exposed to the discriminated ion. This allows the discriminated ions to completely saturate the membrane and the respective electrode gives a Nernstian response.

For a valinomycin-polymeric membrane with the same formulation as that used in this chapter, the following selectivity coefficients have been reported (using the new conditioning approach):

- \( \log(K^{pot}_{K^+,Na^+}) = -4.5 \)
- \( \log(K^{pot}_{K^+,Mg^{2+}}) = -7.5 \)
- \( \log(K^{pot}_{K^+,Ca^{2+}}) = -6.9 \)

These selectivity values are sufficient for our particular application, with good selectivity towards the interfering ions mostly present in the extracellular fluid, Na\(^+\).

6.1.4 Time response of ISEs

The dynamic response of neutral-carrier ISEs has been modelled by Morf et al. [400; 401; 402; 403]. It is given by equation 6.8 for a step change in activity of the primary ion I from \( a_i^0 \) to \( a_I \):

\[
E(t) = E_\infty + S \log \left[ 1 - \left(1 - \frac{a_i^0}{a_I} \right) \frac{1}{\sqrt{t/\tau} + 1} \right]
\]

(6.8)

where \( E(t) \) is the potential value found after time \( t \), \( E_\infty \) the total potential change reached at final steady state, \( S \) is the experimentally determined Nernstian slope for the electrode and \( \tau \) is a time constant.

The time constant, \( \tau \), in equation 6.8 is characteristic of the ISE. The smaller its value, the faster the electrode. It depends on the diffusion of the primary ion I within the membrane phase. It is given by equation 6.9

\[
\tau = \frac{D_{org} K^2 \delta_{aq}^2}{D_{aq}^2}
\]

(6.9)

where \( D_{org} \) and \( D_{aq} \) are the diffusion coefficients of ion I in the membrane and in the aqueous sample phase respectively, K is the partition parameter of the ion I between the sample solution and the membrane, and \( \delta_{aq} \) is the Nernst boundary layer at the interface membrane/sample solution.

The time response of an ISE depends on \( \tau \) but also on the magnitude and direction of the activity change. This is illustrated by Figure 6.5 which represents the solution to equation 6.8 with different step changes:
6.1. Background on ion selective electrodes

Figure 6.5: Dynamic response of neutral-carrier based cation-selective electrodes. The time constant \( \tau \), and the magnitude and direction of activity change all affect the response time of an ISE. Taken and modified from [400].

This model has direct practical implications. The response time of an ISE can be improved using:

- non-polar membrane components (reduces K) [400]. For example, PVC-based ISEs were shown to be twice as fast as a silicon rubber matrices [401].
- ion-exchanger sites into the membrane (reduces K) [401; 403]
- a thin membrane (reduces \( \tau \)) [400], as confirmed previously within our group [174]
- a stirred flow-through cell (reduces \( \delta_{aq} \)) [400]: this is one of the main challenges for the design of an adequate flow-cell, object of this chapter.

When fully optimised (1 \( \mu \)m thin membrane, wall-jet arrangement, etc.), values for \( \tau \) down to a few milliseconds were reported [401; 403; 404].

Experimentally, the response time of ISEs has rarely been characterised by their \( \tau \) values. Instead, they are generally characterised by their \( t_{90\%} \), defined as the time taken for the potential to change from \( E_0 \) (at activity \( a_0^i \)) to \( E_\infty + 0.9 \times (E_\infty - E_0) \) (same conventions as in equation 6.8) [383; 405]. Reported values for \( t_{90\%} \) for valinomycin potassium ISEs when increasing potassium activities are of the order of the second [405]. We will use \( t_{90\%} \) to characterise the response time of our flow-cell in the rest of this Chapter.

6.1.5 Stability and lifetime of ISEs

Besides mechanical defects, electrical leakage pathways, severe surface contaminations and membrane poisoning, both the drift and the lifetime of ISE mainly depend on leaching of membrane components into the sample solution [363; 365].
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Models have been developed to estimate the kinetics of the loss of ionophores and ionic additives and concluded that the main parameter to control was the lipophilicity of the respective species [406]. For the membrane we use in this work, the lipophilicity of valinomycin ($\log_{10}(p_{TLC}) = 7.8–8.6$ [406]) is such that the lifetime of our ISE membrane should be over three months of continuous operation according to previous reports [407].

6.1.6 Trans-membrane ion fluxes

Recently, Bakker et al. [408; 409] have developed a new formalism to describe the ISE response. It is based on an extension of the phase-boundary potential and can also describe the behaviour of the ISE at the detection limit. In this model, the dynamic interactions between the internal filling solution (IFS) and the sample solution are accounted. These are the result of diffusion of the complexed and free ionophore within the membrane. The ISE membrane potential is thus given by Equation 6.10 as the sum of two phase-boundary potentials, one at the membrane/sample interface (designated by ' in Equation 6.10) and the other at the membrane/IFS interface (designated by " in Equation 6.10):

$$E_M = E^0 + \frac{RT}{F} \ln \frac{c'_I[IL]}{[IL]} + \frac{RT}{F} \ln \frac{c''_I[IL]}{[IL]}$$

(6.10)

where the [ ] denote the concentrations in the membrane phase and c denote the concentrations of species in the aqueous phases. The activity coefficients have been assumed to be constant for simplicity.

In the normal working range of ISEs ($10^{-6}$ M $\leq c_I \leq 1$ M), the concentration at the membrane/sample interface, $c'_I$, can be assumed to equal the bulk concentration, $c_I$ and the terms in blue in equation 6.10 can be considered constant or sample-independent. Therefore, equation 6.10 reduces to the phase-boundary potential model (equation 6.4).

At the detection limit however ($c_I < 10^{-6}$ M), trans-membrane ion fluxes appear and only Equation 6.10 can account for these. The mechanism for these ion fluxes are described below for a cation-selective electrode with primary ion $I^+$. 
6.1.6.1 Mechanisms of the ion fluxes

Primary ion only in solution

Ion fluxes for the primary ion, $I^+$, are schematically represented in Figure 6.6 below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Liquid membrane</th>
<th>IFS</th>
<th>Sample</th>
<th>Liquid membrane</th>
<th>IFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Normal working range: $10^{-5} &lt; c_I &lt; 10^{-1}$ M</td>
<td>$c_I$(IFS)$=10^{-3}$ M</td>
<td></td>
<td>1) Normal working range: $10^{-5} &lt; c_I &lt; 10^{-1}$ M</td>
<td>$c_I$(IFS)$=10^{-3}$ M</td>
<td></td>
</tr>
<tr>
<td>2) Detection limit: $c_I &lt; 10^{-9}$ M</td>
<td>$c_I$(IFS)$=10^{-3}$ M</td>
<td></td>
<td>2) Detection limit: $c_I &lt; 10^{-9}$ M</td>
<td>$c_I$(IFS)$=10^{-3}$ M</td>
<td></td>
</tr>
<tr>
<td>Case 2.1: $c_I$(IFS)$=10^{-3}$ M</td>
<td></td>
<td></td>
<td>Case 2.2: $c_I$(IFS)$=10^{-10}$ M</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.6: Primary ion fluxes (a), concentration gradients (b) across a solvent polymeric membrane and ISE response (c) under three conditions: at normal working range (Case 1), at the detection limit (Case 2) when the sample bulk concentration is much lower than the IFS bulk concentration (Case 2.1) and when the IFS bulk concentration is much lower than the sample bulk concentration (Case 2.2). Only the equilibrium with the primary ion is considered at both interfaces: membrane/sample (') and membrane/IFS ('). There is conservation of the total mass of ionophore in the membrane. $\delta_{\text{aq}}$: Nernst diffusion layer in the sample solution. $\delta_{\text{org}}$: membrane thickness. See text below for discussion.

Generally, the IFS is quite concentrated in primary ion ($c_I$(IFS)$\approx 10^{-3}$ M). At normal working range (Figure 6.6 a Case 1 and b Case 1), there are concentration gradients in $I^+$ across the membrane, but they are very small and close to zero. The ISE response to the bulk concentration $c_I$ is Nernstian and follows the phase-boundary equation 6.4 (linear response with a slope of 59 mV/decade at 25°C as in Figure 6.6 c Case 1).

However, at the detection limit, the sample solution is very dilute in primary ion ($c_I < 10^{-9}$ M). There is therefore a high gradient in [I] across the membrane with $c_I$(IFS)$\gg c_I$ (Figure 6.6 a and b Case 2.1). To diffuse down its concentration gradient across the membrane, $I$ interacts with the ionophore $L$ in the membrane. This causes first a polarization of ionophores $L$ towards the inside of the membrane, where they complex $I$ from the IFS. These [IL$^+$] then diffuse down their concentration gradients towards the sample side. Here [IL$^+$] releases $I$ at the sample interface. This 1) results in an increase of the sample boundary concentration in $I$ ($c_I'>c_I$) and 2) maintains the concentration gradients in [L] ([L]$>[L]^+$).
6.1. Background on ion selective electrodes

As a consequence of these dynamic fluxes, the ISE response is sub-Nernstian: it responds to the boundary concentration of primary ion, \(c'_i\), regardless of how small the bulk sample concentration becomes (Figure 6.6 c Case 2.1).

The leaching out of primary ions from the membrane into the sample has been confirmed by atomic absorption spectrometry [384] and scanning electrochemical microscopy [385]. This is the reason why the detection limit of ISE has been traditionally limited to the micromolar range [395].

A solution to this problem, suggested by Bakker et al. [397; 409], is to reduce the concentration in I in the IFS. This is schematically illustrated in Figure 6.6 Case 2.2. This sets up similar ion fluxes but in the reverse direction, and critically, of much smaller magnitudes since both \(c_i\) and \(c_i(IFS)\) are low and of the same order of magnitude. These ion fluxes can nevertheless result in a super-Nernstian response of the ISE. The electrode indeed responds to a phase-boundary primary ion concentration lower than the sample bulk concentration (\(c'_i< c_i\)).

**Primary ion and interfering ion in solution**

In practice, both sample and IFS contain interfering ions, \(J^+\). At the detection limit, \(c_i< c_J\) in the sample and the interfering ion \(J\) can displace the primary ion in the membrane. The ion fluxes and gradient concentrations in these conditions are given in Figure 6.7 below:

**Detection limit:** \(c_i<10^{-9} \text{ M}, c_J=10^{-3} \text{ M}\)

<table>
<thead>
<tr>
<th>Case</th>
<th>Primary ion concentration</th>
<th>Interfering ion concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(c_i(IFS)=10^{-3} \text{ M})</td>
<td>(c_J(IFS)=10^{-4} \text{ M})</td>
</tr>
<tr>
<td>2</td>
<td>(c_i(IFS)=10^{-10} \text{ M})</td>
<td>(c_J(IFS)=10^{-2} \text{ M})</td>
</tr>
</tbody>
</table>

**Figure 6.7:** Interfering ion fluxes (a) and concentration gradients (b) across a solvent polymeric membrane. * indicates the membrane/sample interface and " the membrane/IFS interface. \(\delta_{aq}\): Nernst diffusion layer of the aqueous phase. \(\delta_{org}\): membrane thickness. When there is an excess of interfering ions \(J\) as compared to \(I\) in the aqueous phase, the interfering ion \(J\) is co-exchanged for \(I\) with the ligand \(L\): this is true on the sample side in **Case a** and on the IFS side in **Case b**.

With traditional IFS compositions (Figure 6.6 Case 2.1), ion fluxes for the primary ions occur, as discussed previously for Figure 6.6 Case 2.1. The ISE response is therefore sub-Nernstian for I. However, in the case of a mixed sample solution, there also is \(c_J> c_i(IFS)\) and therefore the ion fluxes...
6.1. Background on ion selective electrodes

for J counter-balance the fluxes in I. In other words, J diffuses inwards, from the sample to the IFS. This has the same consequences as those discussed for Figure 6.6 Case 2.2 but for J. The difference is that the fluxes of J within the membrane are less than for I because of the preferential binding of L with I. Nevertheless, they are sufficient to induce a super-Nernstian response of the ISE towards J. As a result, the ISE is now responding to changes in the activity of J and not I. This is the reason why unexpectedly large values of selectivity coefficients have been reported in the literature when using the separate solution method [399].

The solution is to use an IFS with high concentrations of J and low concentrations in I: this reverse the fluxes through the membrane (Figure 6.7 Case 2). The ISE response towards the primary ion I is restored, even in the presence of large amounts of interfering ions J in the sample and the selectivity is improved.

6.1.6.2 Methods to reduce the trans-membrane ion fluxes

We can now understand the different methods that have been proposed to limit the fluxes of ions within ISE membranes. The aim was primarily to improve the lower detection limit of ISEs [370; 410] but in fact, these strategies should also improve reproducibility. These methods include:

1. the use of an optimised IFS with low activity of the primary ion and high activity of the interfering ion [397; 408; 411]. This prevents the primary ion to leach out from the internal filling solution to the sample side. This way, the detection limits of ISEs have been reduced down to $5 \times 10^{-9} \text{ M}$ for potassium ISEs [408] and down to $5 \times 10^{-12} \text{ M}$ for lead ISEs [411]. In this chapter, we used a mixed IFS with interfering ion concentrations that match that of our sample (brain CSF). This minimises the ion fluxes.

2. the reduction of the concentration of ionophore: this will limit the flux of $I^+$ down its concentration gradients through the membrane [409] and increase the working range of the ISE towards lower concentrations.

3. the reduction of diffusion within the membrane by either increasing the thickness of the membrane, or lowering its plasticiser content, or covalently binding the ionophores to the membrane. These methods have been reviewed in [370; 412]. A limit here will be the reduction in speed of the response.

4. the reduction of the thickness of the Nernst diffusion layer using sample stirring [385; 411], rotating electrode configurations [413; 414] or wall-jet systems [415]. This reduces the difference between phase-boundary and sample bulk concentrations in $I^+$. However, the limit is noise introduced by the stirring procedure.

5. the application of currents across the membrane to counteract the spontaneous zero-current ion fluxes [416; 417; 418]. However, this requires an elaborate setup to apply the appropriate current pulse. Furthermore, the determination of the optimal current that needs to be applied to counteract the zero-current ion fluxes is difficult in practice. This is due to the interdependence of the current and concentration driven ion fluxes [419].

On the other hand, the ion fluxes that result in a different phase-boundary concentration in I as compared to the bulk sample concentration have in fact been used for the so-called "backside calibrations" [420]. Both IFS and sample are stirred and the composition of the IFS is altered so
6.2 Development and characterisation of a potassium ion selective electrode

as to compensate for the ion fluxes across the membrane [418; 420]. When this "no-flux" point is reached, the concentration in I in the sample can be determined. However, this backside calibration requires a sophisticated setup and is not easily reproducible [393].

6.2 Development and characterisation of a potassium ion selective electrode compatible with a flow-cell.

The first step was to design a potassium ISE compatible with a flow-cell. The ion-selective membrane itself had previously been developed and optimised within the Boutelle group [174]. We have used this membrane with a new electrode body and characterise the new electrode in terms of sensitivity, temporal response, and stability. The selectivity of the membrane had previously been assessed as sufficient for our application [174].

6.2.1 Ion-selective membrane

6.2.1.1 Composition of the membrane

The ion-selective membrane is composed of the neutral ionophore, immobilised into a polymer film composed of a matrix, solvent and plasticiser. Anionic sites are added to the membrane to improve the performance of the ISE. This is the membrane we have used for our ISEs:

<table>
<thead>
<tr>
<th>Component</th>
<th>Details</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ionophore</td>
<td>valinomycin (Fluka 60403, selectophore)</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>anionic additive</td>
<td>potassium tetrakis(4-chlorophenyl)borate (KT-CIPB) (Fluka 60591, selectophore, &gt;98.0% )</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>matrix</td>
<td>high molecular weight PolyVinylChloride (PVC) (Fluka 81392, selectophore)</td>
<td>66.0 mg</td>
</tr>
<tr>
<td>plasticiser</td>
<td>bis (2-ethylhexyl) sebacate (DOS) (Fluka 84818, selectophore, &gt;97.0% )</td>
<td>150.0 mg</td>
</tr>
<tr>
<td>solvent</td>
<td>tetrahydrofuran (THF) (Fluka 87362, Bio-Chemika, for luminescence, &gt;99.5% )</td>
<td>3-4 mL</td>
</tr>
</tbody>
</table>

The properties of the ionophore have already been discussed in section 6.1.2. The other components of the membrane will also influence the general performance of the ISE.

The matrix: Polyvinylchloride (PVC)

The main role of the matrix is to provide the necessary physical properties, such as mechanical and chemical stability (that will determine the lifetime of the ISE), while allowing the mobility of the ionophore within the polymer [364; 366]. PVC is the most common polymeric matrix for ISE membrane because of the high flexibility of its polymer chain. It allows a sufficiently high mobility of the incorporated ionophore and therefore results in reversible reaction processes [363]. It is also quite simple to use. Typically, polymeric membranes contain ≈ 33 wt-% PVC. The polymer matrix
6.2. Development and characterisation of a potassium ion selective electrode

can influence the overall polarity of the membrane, its resistance (that increases with PVC content), the diffusion coefficient of the ionophores and other components in the membrane phase [363; 366]. Since PVC has a glass transition temperature above ambient temperature (≈ 80°C), it has to be used with a plasticiser.

The plasticiser and solvent: bis(2-ethylhexyl)sebacate (DOS) and tetrahydrofuran (THF)

In order to give a homogeneous organic phase, the plasticiser and solvent must be physically compatible with the polymer, i.e. exhibit excellent plasticiser properties: they have to be a swelling agent for the polymer, which is held in place by solvation forces [363]. Solvent polymeric membranes are usually based on a matrix containing about 66% of plasticiser/solvent: such a high amount of plasticiser provides optimum physical properties and ensure relatively high mobilities of their constituents [364]. Properties of the plasticiser and solvent such as polarity viscosity and dielectric constant will influence the resistance, response time and working range of the ISE.

Additional lipophilic anionic sites: potassium tetrakis (4-chlorophenyl) borate (KTCIPB).

A lipophilic anionic salt is incorporated into the membrane to add permanent mobile cation-exchange sites, thus reducing the interferences by lipophilic anions in the sample [421]. It also reduces the membrane’s electrical resistance [422], and thus the response time [423]. The lipophilicity of the ionic additive must be sufficient to guarantee a certain sensor lifetime (or it will leach out). Importantly, the molar ratio of ionic sites to ionophore will strongly influence the selectivity of the ISE [423; 424; 425; 426]. The optimum ratio between the lipophilic sites and the ionophore concentrations is dependent on the ion charges, the ion-ligand complex stoechiometries and the complex stability constant (tables of the optimum ratios can be found in [427]). Previous work on this membrane showed that a lipophilic anionic site/ ionophore ratio of 10 wt-% was appropriate for our application [428; 429; 430].

6.2.1.2 Preparation of the membrane

Before preparation of the membrane, some precautions must be taken:

1. wear gloves and a lab-coat and work under a fume cabinet during preparation of the membrane. This is to protect from the toxic chemicals. Once dry, the membrane can be manipulated without particular personal protection.
2. use rubber “suba-seal” stoppers (Leyrto/17/red, VWR International Ltd, United Kingdom) for all solutions containing THF (including the stock-bottle). This is to avoid evaporation of THF and contamination with humidity in the air. The suba-seals work best with round-bottom flasks (201-1334, VWR International Ltd, United Kingdom)
3. for all volume transfers, use a 1 mL glass syringe (plastic syringes will be dissolved by THF) with a long needle that can pierce through the suba-seals. These immediately re-seal when removing the needle.
4. thoroughly clean and dry all glassware (round-bottom flasks, beaker, glass syringe and needle): rinse thoroughly with de-ionised water, oven-dry, then blow with nitrogen gas to remove any remaining traces of water. This is critical to avoid contamination of the membrane (see Figure 6.8 b).
6.2. Development and characterisation of a potassium ion selective electrode

To minimise errors when weighing the valinomycin and tetrakis borate salt, two separate concentrated solutions of THF/tetrakis borate and THF/valinomycin respectively have to be pre-made. They were:

- a 2 mg/mL ionophore/THF mixture — weigh 5.0 mg of valinomycin and add 2.5 mL of THF with a 1mL glass syringe.
- a 0.1 mg/mL tetrakis borate/THF mixture — weigh 2.0 mg of KTClPB and add 20 mL of THF with the same 1 mL glass syringe.

Both solutions were made in round-bottom flasks, sealed with suba-seals and swirled gently for homogenization. They could be stored in the fridge (sealed) and used for preparing several membranes.

The actual membrane was prepared in a 5 mL glass beaker as follows:

1. weigh 66 mg of PVC (directly in the glass beaker)
2. add 164 µL of DOS (equivalent of 150 mg) with a micropipette (200 µL, Gilson)
3. add in 1 mL of ionophore/THF mixture (glass syringe through subaseal)
4. flush the glass syringe with THF (not the stock solution though) to avoid cross-contamination with ionophore residues
5. add 2 mL of borate/THF mixture in the glass beaker
6. flush the syringe to avoid contamination of the stock-solution of THF with KTClPB
7. add 1 mL of THF in the glass beaker
8. stir the mixture with a stirring flea in the fume cupboard at ambient condition and at moderate speed for 2 hours, capping the glass beaker with parafilm to prevent evaporation of the THF
9. when completely dissolved, clear and homogeneous, remove the cap and leave it to evaporate till it sets as a transparent plastic.

After evaporation, the membrane was inspected: it had to be a clear, transparent and homogeneous film. If water was present at any stage of the preparation, the membrane would appear cloudy and would degrade the selectivity and response time of the ISE. Examples of clear and cloudy membranes are illustrated below in Figure 6.8:

![Figure 6.8: Potassium-selective membranes.](image)

Cloudy membrane (Figure 6.8 b) were discarded and only the clear membrane (Figure 6.8 a) were used to construct the ISEs.
6.2. Development and characterisation of a potassium ion selective electrode

6.2.1.3 Construction of the potassium ion selective electrode

The ISE consists in:

- an electrode body,
- the ion-selective membrane on one end,
- an internal filling solution,
- an internal reference electrode

**Electrode body**

The ISE had initially been developed within the group using a plastic conical pipette tip (200 µL polypropylene pipette, Gilson) for the electrode body [174]. However, it was very difficult to incorporate this pipette electrode into a flow cell with easy and reproducible controlled flow conditions. This was mainly due to the difficulty of making a seal with conical shape. Instead, we have used a new electrode body that can be readily incorporated in a flow-cell. It is based on Radel polymer cylindrical tubing of similar dimensions and shape to that of the tubing in our online microdialysis assay (Radel R (polyphenylsulfone) tubing 0.030 in x 1/16 in x 5FT, UpChurch Scientific 1230). The Radel polymer tubing was chosen for the following reasons:

- it is chemically resistant and therefore will not dissolve when in contact with THF,
- it is strong and resistant enough for in-flow applications,
- its translucent light amber colour allows direct visual inspection of the quality of the ISE,
- it is suitable for medical applications (as ultimately it withstands sterilization),
- it can easily be used with the standard HPLC fittings we use in our online system (for 1/16" outer diameter tubing) and can be incorporated into commercially available flow-cells for controlled flow conditions.

**Casting the electrode membrane**

Batches of 10 to 20 electrodes were fabricated using the following procedure:

1. re-dissolve the membrane prepared above by adding 1 mL THF. This allows a more reliable quality of the casted membrane [428].
2. continuously and gently stir this semi-viscous polymer with a glass transfer pipette for about 5 to 10 minutes while THF evaporates in the fume cabinet.
3. when the viscosity is correct, dip a 6-cm long Radel tubing into the superficial layer of the mixture for circa 1 second. The distal end of the tubing coated with a thin film (2 to 3 mm at this stage) of polymer by capillary action.
4. leave the electrodes upright with the membrane at the bottom for the film to set and dry at room temperature for 24 hours.

It is difficult to assess when the viscosity of the polymer is adequate. By experience, it is so within 5–10 minutes of stirring and evaporation of THF in the fume cabinet. *A posteriori*, if the membrane is too liquid, it will form a thick film and will not properly bind to the tubing. If instead the membrane is too viscous, capillary action will not be sufficient to coat the tubing at all.

Any excess of ISE membrane can be stored in the fridge for future use.

Once dry, the electrode tips were examined under a microscope and exemplar electrodes are given in Figure 6.9.
6.2. Development and characterisation of a potassium ion selective electrode

Electrodes on Figure 6.9 a2 and b2 were discarded. Only electrodes that fulfil the following criteria were kept for further tests:

- the coated film is colourless with no foreign particles or debris
- a uniform thickness of 1–2 mm membrane covers the tip of Radel tubing — a thicker membrane will slow down the response time of the ISE
- no air bubbles are trapped in the film — this can increase the resistance of the ISE
- there is a tight bond between the inner surface of the Radel tube and the membrane
- the film is restricted to the tip of the electrode: if it protrudes, it will be difficult to fit in the fittings and the membrane may be more fragile when in the flow; if it is recessed, then a dead volume is created, thus reducing the temporal resolution of the response when in the flow-cell.

Figure 6.9 proves that the Radel tubing can be used for an ISE, since the membrane can bind to the polymer tubing. We also observed that the film often formed a "U shape" (Figure 6.9 b1). This is because the film slightly shrinks when drying out. It is therefore important to first coat the tube with a 2–3 mm thick film to finally get a 1–2 mm thin film. This "U shape" film did not seem to affect the performance of the ISEs (see section 6.2.2.1).

**Internal reference electrode**

The internal reference electrode is a \(~8\) cm-long silver/silver chloride wire (see section 2.4.2.1 for further details) inserted into an aCSF gel (see details of the preparation in section 2.4.2.2). This internal reference solution was made of artificial cerebro-spinal (aCSF) to best match the ionic environment in the brain and minimise adverse effects due to trans-membrane ion fluxes (see section 6.1.6).
6.2. Development and characterisation of a potassium ion selective electrode

The chloridised end of the wire was placed as close as possible to the membrane (without risking piercing it) and the metal connector protruded out of the ISE body for safe connection to the amplifier.

A picture of the final ISEs is given in Figure 6.10 below:

![Figure 6.10: Pipette and Radel tube ion-selective electrodes. (a) Pipette tip ion selective electrodes. (b1) Radel tube ion selective electrodes that are compatible with standard 1/16” HPLC ferrules and nuts (b2). Both types of electrodes were constructed using the method above.](image)

We initially used a Blu-Tack bung to seal the ISEs (as on Figure 6.10) but it proved unreliable when strain was applied to the wire. Additionally, some plasticisers from the Blu-Tack could leach out and contaminate the ISE membrane. Instead, thermoglue (Power Adhesives 180/1/RS) is now used. It proved more robust when strain was applied onto the wire and presented no risk of contamination for the ISE membrane.

Once sealed, the electrodes can be stored in the fridge (4°C) for later use.

6.2.2 Characterisation of the Radel ISEs

To evaluate whether the Radel tubing was suitable as an ISE body, we tested the Radel electrodes for their sensitivity, response time and long-term stability.

6.2.2.1 Sensitivity

Methods

Calibration solutions Solutions of varying concentrations of potassium chloride (KCl) were prepared in a constant background electrolyte similar to aCSF (147mM NaCl, 1.2 mM CaCl₂ and 0.85 mM MgCl₂). Two sets of calibration solutions were used:

1. solutions between 1 and 100 mM — this wide range was used for functionality tests
2. solutions between 1 and 10 (or 20) mM — this range brackets the expected range of
6.2. Development and characterisation of a potassium ion selective electrode

concentration changes in MD potassium during SDs [174]. Intermediary concentrations were regularly spaced on a logarithmic scale: 1.8, 2.7, 4.3, 6.5 and 10 mM KCl. All calibration solutions were made according to the serial dilution method described in chapter 2.

**Measurement of the electromotive force.** The reference electrode was a commercial glass silver/silver chloride reference electrode (see section 2.4.1 for more details).

The ISE and the reference electrode were immersed in KCl solutions contained in small beakers in order of increasing concentration in potassium.

The potential response was measured with a high impedance electrometer (pH amplifier 239, ADInstrument) connected to a Powerlab and Macbook laptop as described in chapter 2.

All measurements were performed in a Faraday cage to minimise electromagnetic noise interference with the signal.

The set-up is shown below in Figure 6.11.

**Figure 6.11: Set-up for the calibration experiments in free solutions.** Each ISE is calibrated against a commercial reference electrode. Both electrodes are placed in a grounded Faraday cage for measurements. The potential is measured with a high-impedance pH amplifier and the signal is digitised via a Powerlab. The signal is visualised and analyzed with Chart 5.5.6 software.

For each calibration solution, the ISE and reference electrode were immersed in solution till a steady-state potential response (within 1 mV) was recorded. The mean of the potential values at steady-state was defined as the potential response for the given concentration of potassium. The phase-boundary potential model was then used to trace the calibration curve $E$ versus $\log(a_K)$. The slope, intercept and their 95% confidence limits were calculated for each calibration curve (see section 2.5.2.2 for
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further details on these calculations).

**Calculation of the activity of potassium ions in an aCSF background electrolyte**  With the aCSF background electrolyte, the solutions were not diluted enough to be considered ideal solutions. The activity coefficient were therefore considered to account for the chemical interactions between charged species in solution, according to the following equation:

\[ a_I = \gamma_I c_I \]  \hspace{1cm} (6.11)

where \( a_I \) is the activity of ion I, \( c_I \) its molar concentration and \( \gamma_I \) its activity coefficient \((0<\gamma_I<1)\).

\( \gamma_I \) can be measured experimentally or determined theoretically using the Debye-Hückel limiting law or the extended Debye-Hückel equation with Davies modification depending on the ionic strength of the solution \(^1\) [431]. In our case, the ionic strength was between 154 mM to 253 mM (0 to 100 mM of KCl in fixed background electrolyte), so the extended Debye-Hückel model was applicable and the activity coefficients, \( \gamma_I \), were calculated according to equation 6.12:

\[ \log \gamma_I = -\frac{A z_I^2 \sqrt{I}}{1 + B d_I \sqrt{I}} + C z_I^2 I \]  \hspace{1cm} (6.12)

where A and B are temperature and solvent dependent parameters equal to 0.51 and 0.33 respectively for water at 25°C, C is a solute and solvent specific parameter characterizing the solvation of the ions and equals 0.1 for water at 25°C, \( d_I \) is the radius of the ion I, and \( I \) is the ionic strength of the solution, defined in equation 6.13 as:

\[ I = 0.5 \sum c_I z_I^2 \]  \hspace{1cm} (6.13)

The activity coefficients for the primary ion, potassium, are calculated according to equation 6.12 for all calibration solutions and for a radius \( d_K \) of 138 Å. The results of the calculations are summarised below in table 6.2. Details about the calculations and activity values for other concentrations of potassium are given in Appendix B.3

\(^1\) The Debye-Hückel limiting law considers ions as point charges and does not include the ion-solvent effects: it is therefore a good approximation only for ionic strength below \( 10^{-2} \)M. The extended Debye-Hückel equation with Davies modification however, considers ions as sphere charges with radius \( d \) and the parameter \( C \) includes the ion-solvent interactions.
6.2. Development and characterisation of a potassium ion selective electrode

Table 6.2: Activities of potassium ions in calibrating solutions with an aCSF-like background electrolyte.

<table>
<thead>
<tr>
<th>Concentration $c_K$ /mM</th>
<th>Ionic strength /mM</th>
<th>Activity coefficient $\gamma_K$</th>
<th>Activity $a_K$ /mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>154.15</td>
<td>0.6534</td>
<td>0.6534</td>
</tr>
<tr>
<td>2.000</td>
<td>155.15</td>
<td>0.6526</td>
<td>1.3052</td>
</tr>
<tr>
<td>3.000</td>
<td>156.15</td>
<td>0.6518</td>
<td>1.9553</td>
</tr>
<tr>
<td>4.000</td>
<td>157.15</td>
<td>0.6509</td>
<td>2.6038</td>
</tr>
<tr>
<td>5.000</td>
<td>158.15</td>
<td>0.6501</td>
<td>3.2506</td>
</tr>
<tr>
<td>10.00</td>
<td>163.15</td>
<td>0.6461</td>
<td>6.4613</td>
</tr>
<tr>
<td>50.00</td>
<td>203.15</td>
<td>0.6172</td>
<td>30.8615</td>
</tr>
<tr>
<td>75.00</td>
<td>228.15</td>
<td>0.6015</td>
<td>45.1102</td>
</tr>
<tr>
<td>100.0</td>
<td>253.15</td>
<td>0.5871</td>
<td>58.7102</td>
</tr>
</tbody>
</table>

The activity coefficients are different from one and decrease with increasing potassium concentration. Clearly, the solution is not ideal, and goes further from ideality with increasing potassium concentration.

Results and discussion

An example of raw data when calibrating a Radel electrode (Radel 3) is shown below in Figure 6.12:

![Figure 6.12: Voltage response for the calibration of a Radel ISE.](image)

Figure 6.12: Voltage response for the calibration of a Radel ISE. Each step in the potential response corresponds to a change in concentration of potassium (as indicated by the graded blue bar at the top of the plot). Dipping artefacts (one is indicated by the circle) are observed as infinite potential values when changing the calibrating solution.

Except for dipping artefacts, the Radel ISEs quickly reached a steady-state potential when changing the concentration of potassium. A mean potential value is calculated for each potassium
6.2. Development and characterisation of a potassium ion selective electrode

concentration step. Using the calculated activities (Table 6.2), a calibration curve is computed. Six Radel ISEs calibration curves are shown in Figure 6.13 below:

![Calibration curves for six Radel ISEs](image)

**Figure 6.13: Calibration curves for six Radel ISEs.** Radel electrodes 1–4 came from the same batch of ISEs. The average linear regression is given by the pink line. Radel 5 and 6 came from a later batch and were calibrated only over one order of magnitude range of potassium concentrations. Linear regressions for both of them are shown.

Electrodes from the same batch showed uniform calibration curves (Figure 6.13 Radel 1 to 4). Calibration curves for ISEs made two months later by re-dissolving the same ion selective membrane had a similar slope than the previous batch but a somewhat different intercept (Figure 6.13 Radel 5 and 6). The slopes, intercepts and their 95% confidence limits are summarised in Table 6.3 below:

**Table 6.3: Slopes and intercepts of the calibration curves of six Radel ISEs.** All values are given for a least-square linear regression ± 95% confidence limit calculated with Igor Pro 6.01. n is the number of data points for each response curve. Lab temperature ≈ 20°C.

<table>
<thead>
<tr>
<th>Radel electrode</th>
<th>Slope / mV/decade</th>
<th>Intercept / mV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radel 1</td>
<td>58.0 ± 2.2</td>
<td>232.3 ± 5.2</td>
<td>9</td>
</tr>
<tr>
<td>Radel 2</td>
<td>54.7 ± 5.9</td>
<td>228.4 ± 11.1</td>
<td>7</td>
</tr>
<tr>
<td>Radel 3</td>
<td>56.4 ± 3.0</td>
<td>226.8 ± 6.6</td>
<td>7</td>
</tr>
<tr>
<td>Radel 4</td>
<td>59.1 ± 9.3</td>
<td>232.9 ± 21.6</td>
<td>4</td>
</tr>
<tr>
<td>Radel 5</td>
<td>57.7 ± 2.8</td>
<td>217.8 ± 6.4</td>
<td>6</td>
</tr>
<tr>
<td>Radel 6</td>
<td>58.8 ± 3.7</td>
<td>228.1 ± 9.1</td>
<td>5</td>
</tr>
</tbody>
</table>

On average, the sensitivity slope for these six Radel ISEs was 57.4 ± 1.7 mV/decade (mean ± 95% confidence limit). The Radel ISEs performed as well as the pipette ISEs that had an average slope of 58.7 ± 2.3 mV/decade (n=6) (calibration curves given in Appendix B.4).

In addition, the theoretical Nernstian slope of 58.17 mV/decade (at 20°C) is included in the 95% confidence intervals of the slope values for the average as well as for each individual electrode. This confirms that the new electrode responded appropriately to potassium ions and that issues related
6.2. Development and characterisation of a potassium ion selective electrode

to the physical attachment of the membrane to the Radel tubing ("U shape" observed on the ISE tips as in Figure 6.9 b1) were not a limitation.

6.2.2.2 Temporal response

We used the $t_{90\%}$ value to characterise the ISE response time, according to the IUPAC recommendations for ISEs [383]. It was defined previously in the introduction of this chapter and Figure 6.14 below shows how to determine $t_{90\%}$ from the recorded data in Chart:

![Figure 6.14: Determination of $t_{90\%}$](image)

The ISE responses (raw data shown in Figure 6.12) were quite fast and difficult to quantify because of the "dipping artefact" that masked the electrode change (Figure 6.12). To solve this issue, we used the injection method described in [405; 432]. The electrode was kept immersed in one solution with a low K$^+$ concentration and a measured volume of concentrated solution of KCl was added. With stepwise additions, a range of KCl concentrations can be covered. The volumes and concentrations of the aliquots used are given in Appendix B.5. This method minimised the artefact when changing the activity of potassium and we managed to quantify the response time, $t_{90\%}$. The results for three Radel ISEs are given in Table 6.4.
Table 6.4: \( t_{90\%} \) for four Radel ISEs. The time response \( t_{90\%} \) is determined as described before for each concentration step. The electrode time response is the average ± SEM of all the step change \( t_{90\%} \) for this electrode.

<table>
<thead>
<tr>
<th>([K^+]) step /mM</th>
<th>ISE 2</th>
<th>ISE 3</th>
<th>ISE 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 → 5</td>
<td>2.45</td>
<td>2.40</td>
<td>1.73</td>
</tr>
<tr>
<td>5 → 10</td>
<td>1.92</td>
<td>1.76</td>
<td>1.79</td>
</tr>
<tr>
<td>10 → 50</td>
<td>2.01</td>
<td>1.58</td>
<td>1.37</td>
</tr>
<tr>
<td>Electrode ( t_{90%} )/s</td>
<td>2.12±0.16</td>
<td>1.91±0.25</td>
<td>1.63±0.13</td>
</tr>
</tbody>
</table>

On the whole, most ISEs had a short \( t_{90\%} \) of \( \approx ± 2 \) seconds or less. This value is quite consistent between electrodes and is in accordance with that previously determined within the group for pipette ISEs [174].

I could not fit the recorded response with the theoretical model by Morf et al. [400; 403; 404] because of noise that masked the rise in E as we added KCl in the solution (opening and closing of the Faraday cage). However, the traces had the expected shape (Figure 6.14 compared to the analytical solution in Figure 6.5 in the introduction of the chapter). We also observed that the response time was faster with thinner membranes (data not shown), as predicted by the model.

This \( t_{90\%} \) of 2 seconds is largely sufficient for our application, since SD waves propagate past the microdialysis probe within a few minutes.

6.2.2.3 Stability, robustness and longevity

The Radel ISEs showed a baseline stability (prior to any filtering or post-processing) with a drift below 0.1 mV/hour and a standard deviation of 0.11 mV when left overnight immersed in aCSF. This amounts to a smallest resolvable change of 29 µM at the 95% confidence limit within this time (see Chapter 2 for the details of this calculation). Part of this drift was due to changes in temperature in the lab overnight. This was measured and showed slow variations between 18.5 to 20.5°C, which can alter the ISE potential response by up to 0.4 mV. In a temperature-controlled environment, such as in the ICU, this drift would be expected to be less.

The electrodes were quite robust over time and could be stored for two months without apparent deterioration of their performances. They also sustained transport between hospitals with no change in sensitivity or stability.
6.3 Development and optimisation of a flow-cell for potassium ISEs

To measure potassium concentration in the dialysate, an in-flow device has to be designed to meet the following requirements:

- **reproducible and easy to set up** flow conditions for application in the clinical environment,
- a sufficient **sensitivity** to detect dialysate changes in potassium concentrations during a spreading depolarization. Due to the recovery of the microdialysis probe, changes will be lower than those measured with intra-parenchymal electrodes (≈ 70 mM) [433].
- a **temporal resolution** sufficient to resolve the transient changes in dialysate potassium as the SD wave moves past the MD probe,
- a good **stability** over days of continuous monitoring,
- a maximal **signal-to-noise ratio** so that measurements can be performed in the electrically hostile ICU environment.

6.3.1 The flow cell: a Y-connector

The main reason for using the Radel tubing as a new electrode body was its compatibility with a commercially available 3-port-flow-cell (Y’s Micro Volume Connector, 0.15mm bore, MY1XCPK, Valco Instruments). It is shown in Figure 6.15 below:

![Diagram of the flow-cell and direction of the flow-path within it](image)

**Figure 6.15**: Picture and schematic of the Y-flow-cell for the Radel ISE. (a) Picture of the three-port Y flow-cell with standard nuts and ferrule fittings (from [434]). (b) Diagram of the flow-cell and direction of the flow-path within it (arrows in blue): the dialysate stream enters in from one arm and branches past the potassium ISE and out to a downstream reference electrode. A tiny bore (in red) is connecting the three arms of the Y-connector. Nuts and ferrules are threaded in the ring for a perfect seal. (c) Schematic of the side-view of the flow cell. Ring and insert are perfectly aligned through the bore connection. In this diagram the two ports of the flow-cell are at 180° for illustrative purposes only. Taken from [434].

The flow-cell is compatible with HPLC fittings (Figure 6.15 a) and consists in two parts:

1. the ring with the threads for 1/16" ferrule and nut fittings to allow a precise seal,
2. the insert where a precisely machined bore (0.05 inches long and 0.006 inches in diameter) connects the three arms of the flow-cell with minimum dead-volume (of 0.0232 μL).

With this flow-cell, the dialysate flow enters one of the ports (bottom right on Figure 6.15 b) and bifurcates into the ISE and reference arms (see Figure 6.15 b).

The whole flow cell is relatively small, 8 mm radius, and can therefore fit in the Faraday box of the online microdialysis assay. Finally it is also relatively cheap (≈ £60) as compared to the custom-made flow-cell that was developed previously for the pipette ISE (≈ £600) [174].
6.3. Development and optimisation of a flow-cell for potassium ISEs

6.3.2 Characterisation of the Y-connector

6.3.2.1 Experimental set-up of the flow-cell

Before setting up the flow-cell experiments, the functionality of the ISEs was always checked using a 2- or 3-point calibration in free solutions as detailed in the previous section 6.2.2.1.

Importantly, before measurements in the flow-cell, all air bubbles possibly trapped in the flow-cell were removed using the following method:
1. block the outlet port with a blind fitting (High pressure PEEK plug, 1/16” fingertight, ZP1FPK, Valco Instruments)
2. pump a potassium solution at high flow-rate (usually 50 µL/min) so that it fills up the flow-cell and flows out from the ISE port
3. flip the flow-cell horizontally placing the ISE port upwards to move all the bubbles up outside of the flowcell
4. thread the ISE and stop the flow
5. repeat steps 2 and 3 for the outflow port

The same calibration solutions previously used to test the ISE response in free solution were used to test the ISE response in the flow-cell (see section 6.2 for more details). The solutions were loaded into syringes and pumped using a syringe pump (KDS 101, KD Scientific). The syringes were connected to the flow-cell via a low-volume FEP tubing (see chapter 2) and a tubing sleeve that directly fitted into the linet arm of the flow-cell (Tubing sleeve 1/16” OD x 630-670um (0.027”) yellow FEP (EA), TS-010, Kinesis).

After use, the flow-cell was systematically cleaned by flushing it with water at high perfusion flow rate (while blocking ports sequentially). This is to remove salts that could crystallise and obstruct one of the connecting bores.

6.3.2.2 Experimental results

A remote Ag/AgCl reference electrode was placed in a beaker containing a KCl solution. The flow-cell was set-up in the grounded Faraday cage and perfused with a 5 mM potassium solution at 5 µL/min. Figure 6.16 below is a picture of the experimental set-up:
A steady state potential of 16.2 mV was recorded in response to 5 mM KCl perfusion. The perfusion solution was then changed to 10 mM potassium. After fifteen, then thirty minutes, nothing happened: the potential very slightly drifted upwards by 2 mV whereas we expected the potential to reach 31.0 mV (potential response of the ISE in a free solution of 10 mM KCl). This slow rise continued overnight: after more than 9 hours, the potential had reached 26.2 mV, i.e. about 70% of the expected final potential. The raw data is shown below in Figure 6.17:

Figure 6.17: Sluggish response with the potassium flow cell. This is a compressed view of the voltage response (E) versus time. At time t=0, we changed the perfusion solution from 5 mM KCl to 10 mM KCl. 9 hours and 40 minutes later, the potential just reached 70% of its final value. These data were smoothed with a 51-point Savitzky-Golay filter.
The flow-cell was thus responding to changes in potassium, but with a $t_{90\%}$ response longer than 9 hours and 40 minutes! The experiment was repeated one more time with similar results. This is obviously not a viable response time for detecting dynamic potassium changes. In comparison, the $t_{90\%}$ of the ISE itself was negligible ($\approx 2$ seconds). Hence, we concluded that this long response time was due to a very slow mass transport to the ISE membrane when in the Y-connector.

The rate-limiting step in the path of the solution towards the ISE surface can only be within the connecting bore: there, the sample solution can only diffuse to reach the surface of the ISE. The connecting bore is shown in Figure 6.18 below:

![Diagram](image)

**Figure 6.18: Diffusion path in the Y-connector.** (a) The insert of the flow-cell and the connecting bore highlighted in red. (b) close-up on the connecting bore of 1.27 mm length and 0.015 mm diameter.

A first strategy to improve the response time is to reduce the diffusion length that slows down mass transport to the ISE surface. To estimate the maximum length of the diffusion path that would give a reasonable $t_{90\%}$, a model was built to study the diffusion through the connecting bore (Figure 6.18).

### 6.3.2.3 Mathematical modelling of the diffusion path within the Y-connector

The connecting bore can be considered as a cylindrical tube, in which transient diffusion is governed by Fick’s second law of diffusion given in Equation 6.14. It is assumed that the bore has an axisymmetric geometry in two dimensions (2D) and a spherical geometry in three dimension (3D):

$$\frac{\partial C}{\partial t} = D \frac{\partial}{\partial r} \left(r^k \frac{\partial C}{\partial r}\right)$$

(6.14)

where $C$ is the concentration, $t$ is time, $r$ is the radial co-ordinate, $D$ is the diffusion coefficient, and $k = 0$ in 1D, $k = 1$ in 2D and $k = 2$ in 3D. The diffusion coefficient of potassium chloride in aqueous solution has been estimated by Harned *et al.* as $D=1.9 \times 10^{-9}$ m$^2$/s [435].

Using an analytical approach and then a numerical finite element model, we tried to answer mathematically the two following questions:

1. Is the model a good approximation of our experimental findings and is the calculated response time similar to the experimental response time?
2. How small should the diffusion path (i.e., the bore) be for the response time to be in the order of a few seconds or one minute?
6.3. Development and optimisation of a flow-cell for potassium ISEs

Analytical approach

All analytical results discussed here were calculated by a mathematician from the department [436].

One main assumption is necessary to obtain analytically solvable equations: the conditions are such that Equation 6.14 can be reduced to a single variable differential equation. The new variable is \( \eta = \beta r^{-\alpha} \) with \( \alpha = -1/2 \) and \( \beta = \frac{1}{\sqrt{4D}} \). C is only a function of \( \eta \).

The boundary conditions of the problem are:
1. \( C = C_0 \) at \( r=0 \), i.e. there is a source of concentration at the entry of the bore
2. \( C \to 0 \) as \( \eta \to \infty \), i.e. there is no potassium after an "infinite" length down the bore

Using the assumption above and the boundary conditions, the concentration profile \( C(r,t) \) is defined by Equation 6.15:

\[
C = \tilde{A} \int_{2\sqrt{Dt}}^{\infty} u^{-k} e^{-u^2} du \tag{6.15}
\]

Intermediary calculations can be found in Appendix B.6.

Analytical solution in one dimension

In one dimension, the solution is given by Equation 6.16:

\[
C = C_0 \text{erfc}(\frac{r}{2\sqrt{Dt}}) \tag{6.16}
\]

with \( C = C_0 \) at \( r=0 \) and \( \text{erfc} \) is the complementary error function defined by \( \text{erfc}(x) = \frac{1}{\sqrt{\pi}} \int_{x}^{\infty} e^{-t^2} dt \).

The concentration profile as a function of \( t \) at \( r=a=1.27 \) mm (i.e. where the ISE is positioned) is given in Figure 6.19 a. The potential response corresponding to this concentration change is shown in Figure 6.19 b.

![Figure 6.19: Analytical solution for diffusion in a one dimensional tube.](image)

\( C_0 = 1 \) mM ([K⁺] at the entry of the tube). (a) Concentration profile (C versus log(t)) after diffusion in a one dimensional tube of length 1.27 mm. \( C_0(1-\epsilon) \) represents 90% of \( C_0 \) and is reached at \( t_{90\%} \). (b) Voltage response to a step change from 2.7 to 3.7 mM assuming the concentration changes with time according to (a). The potential response was assumed to have a perfect slope of 58.17 mV/decade. The 90% potential response is reached at \( t_{90\%} \).
6.3. Development and optimisation of a flow-cell for potassium ISEs

According to the concentration profile (Figure 6.19 a), at a distance a=1.27 mm from the entry of the tube where the potassium concentration is 1mM, it takes circa 20,000–30,000 seconds to reach 0.9 mM (90% of $C_0$). This is equivalent to a $t_{90\%}$ for the potential response of about 27,000 seconds (Figure 6.19 b). The $\tau_{90\%}$ can be calculated using an asymptotic expansion at $\infty$ of the erfc function. Full details of the calculations are given in Appendix B.6. It leads to the following expression for $t$:

$$t_{90\%} \approx \frac{a^2}{D\pi \epsilon^2}$$  (6.17)

Using $D=1.9 \times 10^{-9}$ m$^2$/s [435], $C_0 = 1$ mM, a=1.27 mm and $\epsilon =0.1$, we obtain $\tau_{90\%} = 27021.2$ seconds. In other words, it takes approximately 7.5 hours for the concentration at the surface of the $K^+$ ISE to reach 90% of the concentration at the entry of the bore connector. Diffusion within the connecting bore is therefore the rate-limiting step in the potential response of the ISE in the flow-cell. The diffusion time calculated by the analytical model agrees well with the experimental $t_{90\%}$.

**Analytical solution in two dimension**  
To solve the problem in two dimensions, an extra condition must be assumed: at the entry of the tube, the flux of potassium ions is supposed to be constant and equal to $F$. In this case, the solution is given by:

$$C = \frac{F}{4\pi DH} E_1 \left( \frac{r^2}{4Dt} \right)$$  (6.18)

with $E_1 = \int_{x}^{\infty} e^{-t} dt$ and $h$ is the third dimension, assumed to be small.

details of the calculations leading to Equation 6.18 are given in Appendix B.6.

An asymptotical approximation of $\tau_{90\%}$ is more difficult and was not calculated.

**Analytical solution in three dimensions**  
In three dimensions, there is no obvious appropriate physical boundary conditions that could be used to simplify the problem and obtain an analytical solution [436].

Hence, the analytical model cannot be used to estimate the maximum length of connecting bore that is possible for a small response time. We used a numerical model to address this question.

**Numerical results**  
We modelled transient diffusion through the bore of the Y-connector using COMSOL Muliphysics 3.4, a finite element analysis and solver software package. Contrary to the analytical approach, the partial differential equation are solved exactly. However, the exact solutions are determined on a discrete number of points only (the nodes) within the defined geometrical domain (here, the domain is the bore) and given appropriate boundary conditions and initial conditions. The solutions on the node are then interpolated for each element defining the space according to a mesh and interpolating function (shape function), as described here.
The model was built using the following parameters, summarised in Figure 6.20 a:

1. **equation**: transient diffusion based on Fick's second law, given in Equation 6.14
2. **geometry**: the space is defined in cylindrical polar coordinates \((r, z)\) with a 2D-rotational symmetry about the \(z\) axis. Using this symmetry, only half of the bore is modelled (1.27 mm long and 0.075 mm wide) to half the computations
3. **domain**: it is assumed that diffusion of potassium is isotropic with a diffusion coefficient \(D=1.9 \times 10^{-9}\) m\(^2\)/s according to [435].
4. **mesh**: a simple mesh with uniform triangular element was used, since there were no sharp gradients or discontinuities in the bore. A simple mesh saves computation power and time.
5. **shape function** used to interpolate the solutions for each element was a Lagrange quadratic function (polynomial function of degree two).
6. **boundary conditions** (see Figure 6.20 a):
   - \(C=C_0\) at the entry of the bore,
   - symmetry axis for the boundary on the left
   - insulation boundaries for the others. This means there is no flux through them: \(\vec{n}.\vec{N}=0\) where \(\vec{n}\) is the vector normal to the boundary and \(\vec{N}=-D\vec{\nabla}c\) is the diffusive flux, i.e. the concentration gradient of \(K^+\)
7. **initial conditions**:
   - \(t<0\): \(C(t<0)=0\) in the domain: the bore is empty
   - \(t=0\), \(C(z=0,t=0)=C_0=1\) mM: the concentration of potassium at the entry of the bore is of 1 mM

The solutions of the model are represented as colour-coded surface maps showing the concentration of \(K^+\) across the domain for given time points, as represented in Figure 6.20 b.

**Figure 6.20**: Numerical model: parameters and surface plots. (a) The geometry, mesh, boundary conditions (BC) and initial conditions (IC) used to model transient diffusion in the Y-connector bore. (b) Maps of concentration across the Y-connector bore: concentrations from 0 to 1 mM are coded in colours across the volume of the bore at different time points \((t=5, t=30, t=100, t=500\) and \(t=1,000\) s).

The first result from the model is that the concentrations are uniform across the radial co-ordinate, \(r\),
6.3. Development and optimisation of a flow-cell for potassium ISEs

at a given cross-section along the bore.

Furthermore, it can be inferred from Figure 6.20 b that, after 1,000 seconds of diffusion time, the concentration of potassium is not uniform all along the tube. The time to reach 90% of the initial concentration at the surface of the ISE electrode (top of the tube in Figure 6.20) can be inferred to be comprised between \( t = 500 \) and \( t = 1,000 \) seconds.

A more accurate estimation of \( \tau_{90\%} \) was obtained with a cross-sectional concentration-time plot at the ISE position (\( z=1.27\text{mm} \)). This is plotted in Figure 6.21 below:

![Figure 6.21: Concentration change at the surface of the ISE predicted by the COMSOL model. \( z=1.27\text{mm} \). The predicted \( \tau_{90\%} \) is 900 seconds.](image)

According to both the analytical model (Figure 6.19 b) and the numerical model (Figure 6.21 above), there is no increase in the concentration at the surface of the ISE for the first 50-60 seconds, then a steep rise till \( t \approx 650 \) seconds and finally the ascent slows down for \( t > 800 \) seconds. The numerical model estimates that \( \tau_{90\%} \) at the surface of the electrode is approximately 900 seconds, that is 15 minutes. In comparison to the experimental and analytical model, this is much shorter. It is likely that assumptions in this simple model underestimate the true \( \tau_{90\%} \). Even if underestimated, a 15 minutes response time is very long.

The advantage of the numerical model is that is that we can estimate how small the diffusion path needs to be for a \( t_{90\%} \) of the order of 30 to 60 seconds. Figure 6.22 below shows the concentration - time plots at different cross-sections along the bore:
6.3. Development and optimisation of a flow-cell for potassium ISEs

Figure 6.22: Concentration versus time at different cross-sections along the Y-connector bore. Four concentration - time curves with t varying from 0 to 500 seconds. z = 1.27 mm (position of the ISE) (blue), z = 635 µm (purple), z = 320 µm (magenta) and z = 100 µm (red). The end point of the concentration-time curves (at t = 500 s) is also shown on the colour-code map.

We can estimate that, for a diffusion length of only 100 µm, \( \tau_{90\%} \) is about 150 seconds. We found that to obtain \( \tau_{90\%} = 60 \) seconds (or 30 seconds respectively), the diffusion length should be no more than 50 (20 respectively) µm! Since the model underestimates the \( \tau_{90\%} \), it is in fact likely that the distances are over-estimated. In any case, a precision of a few micrometers will never be achieved with standard machining equipment.

6.3.2.4 Summary

The three studies (experimental, analytical and numerical) to characterise the Y-connector all reached the same conclusion: diffusion in the 23.2 nL bore is the rate-limiting step in the response of the K\(^+\) electrode in the flow-cell. Mathematical models confirmed that the \( t_{90\%} \) in the bore is of a few hours. This is therefore not surprising then that, in biological systems, diffusion distances are reduced to the nanometer (for example, the synaptic cleft is no more than 20 nm wide). In our case though, we cannot match the precision required to sufficiently reduce the diffusion length. Another strategy is needed to improve the response time: diffusion has to be eliminated and replaced by convection.
6.3. Development and optimisation of a flow-cell for potassium ISEs

6.3.3 Optimisation of the flow-cell by convection assisted mass transport

The flow-cell arrangement has to be re-designed to achieve convection assisted mass transport of the dialysate flow stream to the K$^+$ ISE surface, as shown in Figure 6.23.

![Figure 6.23: Convection for the flow cell.](image)

The dialysate flow needs to flow past the ISE arm to achieve convection mass transport towards the ISE membrane. To maintain an electrical path to the reference electrode, it now needs to be integrated into the flow-cell. We made our own reference electrodes based on Radel tubing.

6.3.3.1 Effect of the Radel reference electrode on the potential response

Two types of Ag/AgCl Radel reference electrodes were fabricated according to the method given in details in section 2.4.3:

1. a thermodynamic disc electrode
2. a saturated agar electrode

The results of the calibrations of the Radel ISE against the two types of Radel reference electrodes in free solutions are summarised in Figure 6.24.

The theoretical Nernstian slope of 58.17 mV/decade is included in the 95% confidence band, which confirms that we can use these Radel electrodes as reference electrodes.
6.3. Development and optimisation of a flow-cell for potassium ISEs

Figure 6.24: Results of the calibration of the ISE versus the Radel Reference electrode. Several Radel ISEs were calibrated versus the two types of Radel reference electrodes (legend in the table) in free solutions. The table below summarises the slopes and intercepts of each calibration curve with ± 95% confidence limit.

<table>
<thead>
<tr>
<th>Reference electrode</th>
<th>Slope / mV/decade</th>
<th>Intercept /mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermodynamic disc</td>
<td>57.4±11.9</td>
<td>181.2±45.5</td>
</tr>
<tr>
<td>Thermodynamic disc</td>
<td>54.0±5.6</td>
<td>152.9±13.4</td>
</tr>
<tr>
<td>Thermodynamic disc</td>
<td>62.2±7.0</td>
<td>168.7±17.0</td>
</tr>
<tr>
<td>Thermodynamic disc</td>
<td>59.7±1.1</td>
<td>163.9±2.6</td>
</tr>
<tr>
<td>Thermodynamic disc</td>
<td>61.8±2.2</td>
<td>169.4±5.2</td>
</tr>
<tr>
<td>Agar 3M NaCl</td>
<td>49.7±4.7</td>
<td>159.4±12.0</td>
</tr>
<tr>
<td>Agar 3M NaCl</td>
<td>51.9±12.0</td>
<td>164.1±31.0</td>
</tr>
</tbody>
</table>

However, in two instances with the thermodynamic disc reference electrodes (cases 3 and 5), the slopes were above the Nernstian value, suggesting a possible contribution of chloride ions to the reference potential. Assuming that the thermodynamic reference electrode response is strictly Nernstian towards the chloride ions in the calibrating solutions, this would indeed account for a $\Delta E = -10.22$ mV over the range of concentrations we used. This is substantial and can lead to false reading in practice. However, the calibration curves above in Figure 6.24 suggest a smaller effect. To quantify this effect, we measured the response of the Radel reference electrodes versus a commercial glass reference electrode for our range of KCl solutions: we measured negative slopes of -0.56 and -0.08 mV/decade for the thermodynamic disc and saturated agar reference electrodes respectively. This suggests that the saturated agar reference electrode is less prone to changes in chloride ions and this is the one we used.

It is also worth mentioning that, in reality, the potential contributions of the chloride ions to the EMF response will be further reduced in the Y-connector. In this new configuration, mass-transport to the reference electrode will be achieved by diffusion only. This is, as demonstrated above, a very slow process, so that ultimately, the response to chloride ions will be spread over hours, whereas the response to potassium activity changes should be of the order of seconds or minutes. Such a different time scale should limit false readings due to chloride ions.

6.3.3.2 Convection through "a controlled leak"

The main challenge is to create "enough" convection assisted mass transport so that $t_{90\%}$ is small enough while at the same time the flow conditions are well-controlled.
6.3. Development and optimisation of a flow-cell for potassium ISEs

The first strategy was to force the dialysate flow stream to "leak" through the ISE arm. A number of approaches have been tried:

1. loosening the fitting, either by loosening the nut or by removing the ferrule of the K⁺ ISE fitting (Figure 6.25 a),
2. designing an imperfect fitting with a groove within it (Figure 6.25 b).

They are illustrated in Figure 6.25 below:

a. Loose nut / no ferrule b. Modified fitting

![Diagram of flow-cell](image)

**Figure 6.25: Convection through a controlled leak.** The reference electrode (REF) is tightly fitted in the flow cell. The dialysate stream is forced to flow through the K⁺ ISE arm because of a loose nut or the absence of ferrule (a), or because of a grooved fitting (b).

The first approach was not very reproducible whereas the "grooved fitting" approach was quite reliable.

The first approach was tested with a HPLC pump and a manual sample injector valve (Rheodyne Model 8125 Low Dispersion Injector, Valco Instruments). However, this proved unreliable at low flow rates (< 10 µL/min) and prone to complicated earthing effects that led to long term baseline instability.

The second approach was tested with a syringe pump and a liquid switch for concentration changes (Liquid switch, 400-1056, Royem Scientific Ltd). The set-up of the flow-cell was similar to the method described in section 6.3.2.1 (removal of air bubbles, cleaning).

In the interest of brevity, the results, in terms of response time, are summarised in Table 6.5 for the two approaches:
6.3. Development and optimisation of a flow-cell for potassium ISEs

Table 6.5: Time responses of the potassium flow-cell with a controlled leak. Approach 1: loose fitting. Approach 2: modified fitting. Rise $t_{90\%}$: response time for a step change of increasing K$^+$ concentration. Fall $t_{90\%}$: response time for a step change of decreasing K$^+$ concentrations. Values given as mean $\pm$ SEM. n is the number of step changes for each flow rate.

<table>
<thead>
<tr>
<th>Flow rate / µL/min</th>
<th>Rise $t_{90%}$ /s</th>
<th>Fall $t_{90%}$ /s</th>
<th>n</th>
<th>Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.3±0.2</td>
<td>89.0±2.7</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>16.1±5.3</td>
<td>90.1±32.7</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>65.7±8.4</td>
<td>155.5±18.1</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>108.6±6.4</td>
<td>186.4±24.4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>194.7±2.7</td>
<td>309.1±21.5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>436.4±56.1</td>
<td>985.0±254.0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

The response time is now of the order of a few minutes for microdialysis flow rates, which is a remarkable improvement from the previous 9.5 hours in the diffusion mode. Nevertheless this is still quite slow compared to the response time of the ISE itself: of the order of 3 and 5 minutes for 2 and 1 µL/min respectively versus $\approx$ 2 seconds for the ISE. It is also slower than we would expect: a volume of 23.2 nL (volume of the flow-cell bore) should be filled twice within circa 3 seconds at 1 µL/min. This discrepancy is likely to be due to extra dead volume introduced in the flow-path. A slight recess of the ISE from the flow cell insert can introduce extra diffusion volume of the order of the µL and therefore significantly increase the response time.

As shown on Table 6.5, the response time is asymmetric, much slower with decreasing activities (fall $t_{90\%}$) than when increasing activities (rise $t_{90\%}$) for the same magnitude of change. This is typical of ISEs: theoretical models of the dynamic response of ISEs indeed predict a much slower response (at least 10 times) when decreasing the activity of the primary ion (see Figure 6.5 in the introduction of this Chapter and [400; 405]).

One of the main limitations of the "controlled leak" method is that we are throwing away the dialysate after potassium detection. To solve this issue, I engineered a modified Y-connector with controlled convection and a means of collecting the dialysate after detection with the ISE.

6.3.3.3 Convection through a modified flow cell

Machining of a vent and attachment of a connection system to the Y-flow-cell

A vent was added to the Y-connector. The exact position and size of this vent onto the ISE arm of the Y-connector were critical.

The vent had to be located just downstream of the ISE surface to prevent diverting the flow before reaching the ISE surface. On the other hand, it had to divert the flow "early" enough to induce convection.

It also had to be small enough to fit exactly within the flow path and not impinge on the insert of the Y-connector. If it did, it would create extra dead-volume and damage the seal of the Y-connector. The minimum size is limited by precision with small size drills (small drills tend to wander more easily).
6.3. Development and optimisation of a flow-cell for potassium ISEs

The decisions were guided by the engineering diagram of the Y-connector on Figure 6.26 a (courtesy of Valco Instruments).

For carrying the flow out of the Y-connector, a Nanoport assembly was used (Nanoport Assembly Headless 10-32 Coned 1/16" N-333, Valco Instruments): this is a connection system with a PEEK threaded nut and ferrule that is commonly used in microfluidics to seal to glass or PDMS chips. Such an assembly is shown in Figure 6.26 b.

![Engineering diagram of the Y-connector](a). Engineering diagram of the Y-connector. Dimensions are in inches. The position and approximate size of the vent are represented by the disc in magenta. (b) Schematic of the Nanoport assembly: a PEEK conical threaded nut and a flat-bottomed coned Nanoport are sealed to the surface of a chip with an adhesive ring, while a gasket ensures perfect seal. This is designed for 1/16 inches outer diameter tubing. Dimensions are in millimeters.

Given the above specifications, the vent and Nanoport connection was performed as follows:

1. drill a hole of 0.3 mm diameter (smallest hole achievable), 2.38 mm deep, at the position indicated in Figure 6.26 a,
2. drill a concentric hole of 1/16 inches down to 1 mm depth. This is to fit a 1/16" HPLC tubing down the vent, very close to the ISE membrane.
3. attach the Nanoport according to instructions and use a 1/16" tubing to ensure alignment between the Nanoport and the vent,
4. cure the whole assembly in a dry oven at 175°C for one hour.

The end-result is shown in Figure 6.27 below:

![Flow-cell with a vent and a nanoport assembly](a). Vent in the Y-connector. (b): Assembly with the Nanoport. The flow is now going past the ISE membrane and up through the vent and Nanoport, as indicated by the white arrows.
6.3. Development and optimisation of a flow-cell for potassium ISEs

Correct flow circulation through the flow-cell and Nanoport was checked. No significant back-pressure or volume loss were measured. A detector can now be placed downstream of the K\(^+\) flow-cell.

**Performance of the new potassium flow-cell**

The final test was to measure a potassium response with this new flow-cell.

A calibration of the new flow-cell for increasing K\(^+\) concentrations is given in Figure 6.28 at a perfusion of 10 µL/min:

![Figure 6.28: Calibration in the new flow-cell. KCl concentrations from 1.8 mM to 10 mM (step changes indicated in the blue bar at the top of the plot). The potential response was de-spiked with a 101 point window (see Chapter 4 for further details).](image)

The potential response was faster than with the "controlled leak" approaches: \(t_{90\%} = 32.2 \pm 1.8\) seconds (n=4) at 10 µL/min and \(t_{90\%} = 64.8 \pm 1.5\) seconds (n=4) at 2 µL/min. This \(t_{90\%}\) increased to 122.1 \(\pm\) 9.25 seconds when decreasing the activity of KCl (10 µL/min).

This \(t_{90\%}\), though much faster than previously, was still far from the \(t_{90\%}\) of the ISE itself. Taylor dispersion within the Y-connector would add an additional 4 seconds (estimated from [178]), which is far less than the flow-cell response time. There is no other model that can describe our "non-straight" flow path within the flow-cell and we can only infer that this long \(t_{90\%}\) may be due to void volume, changes in tubing/connection diameters, expansion of the bore to the surface of the ISE, or other phenomena that complicate mass transport of the dialysate to the ISE membrane.

As shown on Figure 6.28, the response of the new flow-cell was also low noise: the standard deviation over the 3-minute plateau for each potassium concentration was under 0.25 mV. When repeating a similar calibration at 2 µL/min, the same low-noise response was measured. Calibration curves for these two flow rates are shown in Figure 6.29 below:
6.3. Development and optimisation of a flow-cell for potassium ISEs

Figure 6.29: Sensitivity of the new flow-cell. Calibration curves are plotted for different conditions: two flow rates in the flow cell (2 and 10 µL/min) and two calibrations in free solutions, one before and one after the flow-cell calibrations. The slopes and intercepts of each calibration curve are given in the table on the right with 95% confidence limit (see Chapter 2 for details on this calculation).

Overall the slopes for all calibrations were smaller than the expected Nernstian slope of 58.17 mV at 20°C. This is true both in the flow-cell and in free solutions, suggesting that this is inherent to the electrode, which was probably old and used for months in many other experiments.

In addition, the four slopes from Figure 6.29 are not different, with the 95% confidence bands overlapping, whether in the flow-cell or in free solutions. The flow-cell calibration data points overlap, hence suggesting that the flow-cell potentiometric response is not flow-rate-dependent.

The intercepts are not significantly different from each other either (see 95% confidence limit on Figure 6.29). This is because they intercept at $\log(a_{K^+}) = 0$, i.e. for $a_{K^+} = 1$ M. However, at physiological concentrations, there is a clear voltage offset between the flow-cell calibration and the calibrations in free solutions. This means that we cannot use a free solution calibration when doing measurements in the flow-cell. This could be an issue in practice, when monitoring patients: a whole calibration in the flow-cell can take up to 30 minutes and we want to minimise interfering with the system during monitoring. Nevertheless, there are two possible solutions to this issue:

1. either use a one point measurement in the flow-cell and use the slope of the calibration in free solutions
2. or do a complete calibration at a high flow rate to reduce the duration of interruption of the monitoring system
6.4. In vivo application

6.3.3.4 Integration to the rsMD assay

The new flow-cell could be placed in the Faraday box of the rsMD assay. We could use it upstream of the rsMD assay, which greatly improved our signal-to-noise ratio (no injection spikes, see Chapter 4 for further details). In terms of stability of the flow-cell in the Faraday box of the rsMD assay, we observed:

1. long-term drifts of the potential: when continuously flowing a solution of potassium through the flow-cell overnight (so with no other ambient noise), we observed a drift of +136 mV over about eight hours,
2. sudden erratic abrupt shifts of the potential without apparent reasons.

After several attempts to ground various parts of the system, we came to the solution of using a little self-contained Faraday box for the potassium flow-cell with a grounded plane. We also earthed the solution and the valve, which resulted in a stable potential when we left the flow cell continuously perfused with aCSF, while the rsMD assay was running in the background: over 7 hours, the mean potential was -11.0 mV with a standard deviation of 0.12 mV (prior to despiking), which is equivalent to a smallest resolvable change in potassium of 71 µM during this time (95% confidence limit, see Chapter 2 for further details for this calculation).

6.3.3.5 Conclusions

We have successfully engineered a new portable, easy to set-up and reliable flow-cell for online measurement of dialysate potassium ions. It can be integrated into the current rsMD assay and is characterised by:

- a response time $t_{90\%}$ of $\approx 1$ minute at a dialysate flow rate of 2 µL/min,
- the sensitivity and selectivity of a standard ISE,
- a robust and stable response over hours

6.4 In vivo application

I had the opportunity to collaborate with the Max Planck Institute for Neurophysiological research in Cologne, Germany. We started a series of pilot experiments looking at the influence of oxygen and glycaemia on the metabolic responses to spreading depolarisations. These experiments are still under way and I will here present the result of one experiment where I used the Nanoport-potassium flow-cell.

6.4.1 Method

Wistar rats were anaesthetised with isoflurane and artificially ventilated. The femoral artery was catheterised for monitoring blood pressure and blood gases.

The skull was then thinned to provide a large field of view of the left hemisphere for laser speckle imaging of cerebral blood flow (see section 1.5.2.2 about the laser speckle technique). Details about the set-up and analysis of the speckle images are given in [160; 437].
6.4. In vivo application

Three burr holes were made in the cranial window:

- one for the induction of SD waves by epidural application of 3 M potassium onto a cotton ball positioned 7 mm posterior and 2 mm lateral from Bregma. (No duroctomy)
- one for surface recording of the DC and AC potentials associated with SD waves. A silver/silver chloride wire in a glass capillary was inserted superficially (< 0.5 mm) without craniotomy, 4 mm posterior to Bregma. It was referenced against a sintered silver/silver chloride electrode in the cervical subcutaneous tissues. The DC component was extracted by a low pass filter with a cut-off frequency of 0.1 Hz and the AC activity by a high pass filter cutoff of 0.1 Hz and low-pass filter cutoff of 50 Hz.
- one for the microdialysis probe (MAB 6.14.2, 15kDa, OD 0.6 mm, Microbiotech, Sweden) implanted through a small duroctomy 2 mm lateral and 2 mm posterior from Bregma. It was inserted very slowly with a micromanipulator, obliquely to the brain surface, to place the full length of 2 mm membrane into the cortex. To avoid compression of the tissue surrounding the MD probe, it was retracted by about 30 µm. The MD probe was perfused at 1.6 µL/min with aCSF buffered at pH=7.3 (see chapter 2 for the details about the composition of aCSF).

Figure 6.30 is a picture of the thinned skull cranial window with the different probes:

![Thinned skull cranial window with MD probe, DC electrode and SD induction site](image)

**Figure 6.30:** Thinned skull cranial window with MD probe, DC electrode and SD induction site. Thin skull preparation for laser speckle imaging. This is a picture in control conditions prior to SD induction. MD probe inserted obliquely 2 mm lateral and 2 mm posterior from Bregma. DC surface electrode 4 mm posterior to Bregma. Site of SD induction 7 mm posterior and 2 mm lateral from Bregma (cotton ball not in place yet). Two region of interests (ROIs) for the laser speckle analysis are shown. They are of 0.2 mm in diameter, 1 mm anterior to the MD probe (dark blue) and 0.5 mm posterior to the MD probe (light blue). The results for these two ROIs are plotted in Figure 6.34.

The potassium flow-cell was placed in a separate Faraday box above the rsMD assay: the FEP connection tubings between the MD probe outlet and the flow cell, and between the flow-cell and the rsMD valve corresponded to a 5 min 15 seconds delay between the tissue and the flow-cell response and 8 min delay for the rsMD assay. Control baseline measurements of laser speckle, DC potential, and microdialysis were recorded for thirty minutes. This was sufficient to allow glucose and lactate to stabilise to control values that were between:

- 200 and 800 µM for dialysate glucose
- 300 and 600 µM for dialysate lactate
6.4. In vivo application

If the control dialysate glucose concentrations were below or lactate above these ranges, then the animal was not included in the study.

Blood gases were also measured to ensure that the systemic conditions of the animal were correct, according to the following normality criteria:

- arterial blood pressure between 70 and 120 mm Hg.
- $pO_2 > 80$ mmHg (normoxia).
- $pCO_2$ between 35 and 40 mmHg
- pH between 7.35 and 7.45
- plasma glucose above 4.5 mM

Following the control period, we induced SDs by application of 3M KCl onto a cotton ball for 30 minutes. In the experiment discussed here, this led to a series of 5 SDs, one immediately following the KCl application and four later SDs at 6 to 8 minutes interval. DC, MD and speckle were recorded during the SD and we allowed a 60 minutes period of recovery. We also measured blood gases during the application of the KCl cotton ball and during the recovery period and ensured that the systemic values were in the inclusion ranges. We will here focus on the potassium data.

6.4.2 Results and Discussion

6.4.2.1 Calibration of the potassium flow-cell

During preparation of the animal the ISE was fully calibrated in free solutions. Then a two-point calibration in the flow-cell was performed: aCSF was perfused, followed by 10 mM KCl (in aCSF-like background) and back to aCSF. The potential readings were stable for these two concentrations (183 mV for 2.7 mM and 214 mV for 10 mM KCl). The potassium ISE calibrated with the same sensitivity in the flow-cell as in the free solution. We did not correct for activity changes given that the range of concentration changes was small enough to have a less than 1% effect on the ionic strength (and therefore on the activity coefficients). We therefore used the following calibration equation ($E$ in mV and $[K^+]$ in mM):

$$E = 160.6 + 53.2\log[K^+]$$

(6.19)

6.4.2.2 Voltage offset on insertion of the MD probe

During the 30 minute control period for MD levels, the ISE potential stabilised at 211.4 mV, which corresponds to 9.0 mM potassium concentration in the dialysate. This is quite a high value for a dialysate extracellular concentration. Admittedly the brain is locally traumatised by the insertion of the MD probe, but most of the preparation (thinning of the skull in stead of craniectomy for instance) is aimed at minimizing brain damage and the basal concentration of potassium should be closer to normal values, i.e. about 2.7–3 mM. Furthermore the dialysate glucose and lactate concentrations were in the normal range. We therefore tried to check whether this potential was solely due to potassium and not to an earthing offset. We therefore disconnected the outlet tubing from the MD probe for 2 minutes and then connected it back. Figure 6.31 is the potential we measured during this procedure:
6.4. In vivo application

Figure 6.31: Potential offset when connected to the animal. Potential response when the flow-cell is connected (blue section) and disconnected (magenta section) from the MD probe implanted in tissue. The potential is about 215 mV when connected and 184 mV when disconnected. This value corresponds to the potential measured for 2.7 mM potassium when calibrating the flow-cell. Data were smoothed with a second order Savitzky-Golay filter and a 101-point window.

When the needle of the outlet tubing is disconnected from the MD probe for such a short period of time, the flow-cell is still full of dialysate. Therefore the ISE potential is solely governed by the potassium concentration in the dialysate. In this case, the potential value is equal to 184 mV. This is the same potential as our 2.7 mM calibration point. This means that the potential measured when the MD probe is in the tissue is the sum of the potential response to 2.7 mM potassium in the dialysate and an offset potential of 31 mV. This offset is likely to be due to an earthing path to other equipments via the animal. To account for this voltage offset and calculate absolute dialysate concentration of potassium, we added 31 mV to the calibration points. For the analysis that follows, we therefore used the following equation for converting potentials to potassium concentrations:

\[
E = 185.3 + 53.2 \log(K^+) \tag{6.20}
\]

6.4.2.3 Baseline stability

During the recovery period, we recorded a stable baseline potential value of 208.8 mV ± 0.19 mV (mean ± SDev) for 32 minutes. According to the statistical analysis described in Chapter 2, this means we have a 0.475 mV uncertainty on the baseline value (95% confidence limit). This is equivalent to a 55 µM smallest resolvable change in potassium in vivo (95% confidence limit), which should be sufficient to resolve transient increases associated with SDs.
6.4. In vivo application

6.4.2.4 Global ischaemia: a potassium peak

First, to validate our in vivo potassium measurement, we tried to measure big changes, such as those associated with global ischaemia. Figure 6.32 shows the electrical and chemical changes observed at the onset of global ischaemia.

The dotted line in Figure 6.32 indicates the time when the brain perfusion was approaching zero (as indicated by laser speckle) and the blood pressure of the animal was below 30 mmHg. We here observed a "classic" electrical, ionic and metabolic response to global ischaemia. As expected, the loss of brain perfusion instantly resulted in a final irreversible anoxic depolarisation of glial and neural cells and complete silencing of the electrical activity. Indeed, the cells are deprived of all energy substrate, so the Na⁺/K⁺ ATPase pump is not functional any more and the cell membrane ion permeability collapses with no possibility to maintain a resting membrane potential. As the cells depolarise, extracellular potassium increases. Glucose utilisation far exceeds supply so that the extracellular level drops to nearly nothing. Lactate, produced by anaerobic metabolism with the little glucose available accumulates in the tissue.
Figure 6.32: Electrical and chemical signature of global brain ischaemia. This is the surface electrode and microdialysis recording during euthanasia of the animal. An anoxic depolarisation (DC) with suppression of the spike activity (AC) is shortly followed by an increase in dialysate potassium and lactate and a decrease in glucose. The dotted line indicates the onset of the anoxic depolarisation.
6.4. In vivo application

Figure 6.32 also shows a tight coupling between the onset of the DC shift (about 20 mV) and the onset of the potassium increase. As the DC potential slowly continues to rise, so does potassium. The time course of both the DC and potassium changes are very similar to previous findings in the rat cortex during anoxic depolarisation induced by paralysing the respiration of the animals [242]. There is also a tight coupling between the onset of the potassium increase and the onset of the glucose and lactate responses. This confirms that our potassium recording can resolve our initial problem of timing between the electrical activity of the tissue and the metabolic response as seen on the rsMD.

However, the amplitude of the change in potassium is relatively small: the ISE potential increased by 12 mV, which corresponds to a peak concentration of only 4.5 mM dialysate potassium. This is extremely small compared to the 100 mM potassium concentrations recorded by Vyskocil et al. with parenchymal ion-selective electrodes [242]. Although dialysate concentration changes are smaller than true extracellular concentration changes, this cannot solely account for such a large discrepancy. Possible explanations include:

1. a poor in vivo recovery of the MD probe — it is unlikely since I measured glucose and lactate changes during SD events (data not shown) that were similar to previous results in the cat with the same MD probe [160].
2. the MD probe partly samples white matter: the MD membrane length is 2 mm whereas the rat cortex is only 1 mm thick, so it is likely that some of the membrane is in white matter, which would result in smaller changes in the dialysate — we cannot rule out this possibility but we observed very big changes in glucose (down to 15 µM vs a baseline of 200 µM) and lactate (up to 700 µM vs a baseline of 250 µM) during the global ischaemia event, which argue against sampling white matter.
3. a change of the in vivo recovery of the MD probe during global ischaemia since the properties of the tissues are dramatically altered— this can be ruled out because if that were the case, glucose and lactate would not change in opposite directions (as per Figure 6.32).
4. a smaller recovery for potassium than for glucose and lactate: the mechanisms of release and uptake of potassium in the tissue means that the net diffusion length of potassium to the MD probe is less than that of glucose and lactate. This could explain smaller changes in dialysate potassium than in dialysate glucose or lactate. However, it is likely that the active processes for potassium uptake are abolished during anoxic depolarisation, thus weakening this argument for this particular case.
5. a change in the gain of the ISE instrumentation: we measured an electrical offset when connected to the animal and it is not excluded that other complicated electrical effects could affect the ISE instrumentation. We cannot rule out this hypothesis with the current data.

More experiments will be needed to confirm one or the other hypothesis. In particular, a calibration in situ would confirm/rule out point 5. An in vivo no-net-flux experiment to compare the recovery of potassium with that of glucose and lactate (the potassium concentrations will have to be limited to 5 mM to avoid adverse affects such as spreading depolarisations) would address point 4. Nevertheless, we now have an upper limit for the in vivo potassium changes: if we can see any changes with an SD wave, these will be within a maximum of 2 mM change.
6.4. In vivo application

6.4.2.5 Potassium transients during spreading depolarisations

The topical application of highly concentrated potassium was quickly followed by a first SD wave (within the next minute). The electrical, ionic and haemodynamic responses are given in Figure 6.33:

A reduction in the ECoG signal amplitude, characteristic of Leao's spreading depression, is observed. A DC shift (depolarisation) is accompanied by a time-locked potassium increase, which confirms that our potassium sensor can act as an accurate time marker of the electrical activity of the tissue. The potassium response is slower, which possibly reflects the delayed opening of voltage-gated channels.

Figure 6.33: DC, potassium and CBF responses to the first SD wave. The onset of the DC shift is marked by a black vertical dotted line. The horizontal dotted lines on each trace indicate the control levels prior to the SD wave. CBF levels were taken as the inverse of the laser speckle contrast and are given in arbitrary units. The potassium data was time aligned with the speckle and electrical recordings by accounting for the time delay due to the connection tubing from the MD probe to the flow-cell. The K⁺ data were de-spiked (see Chapter 4) and smoothed with a Savitsky-Golay filter of 101 points.
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potassium channels following neuronal depolarisation. Both the DC and potassium changes are transient, with the DC shift shorter than the potassium change, as previously reported [104; 242]. The DC shift is also followed by a hyperpolarisation of the tissue with a DC level below normal resting potential as observed by Leao [248]. The period of hyperpolarisation seems to lead eventually to a reduced potassium level. This is in agreement with [242] and is to be expected due to the reduced potassium efflux from cells at a hyperpolarised potential in the face of continued activity of Na\(^+\)/K\(^+\) ATPase activity.

The CBF response (shown here in arbitrary unit) is the classic biphasic haemodynamic response to an SD wave in "healthy" brain: a transient hyperaemia followed by a sustained oligaemia, as observed earlier [236]. The response seems slightly delayed as compared to the electrical and potassium change onsets, confirming that the haemodynamic response is a consequence of the SD wave.

Remarkably, the DC shift has the same amplitude as the anoxic depolarisation (+15 mV above resting potential). The only difference between the two is that the electrical changes are transient for the SD wave.

The first SD was followed by a cluster of four SD waves, as shown by the electrical and CBF traces given below in Figure 6.34:

![Figure 6.34: A series of 5 SD waves.](image)

The dotted lines indicate, for each SD, the onset of the DC shift. CBF levels were taken as the inverse of the laser speckle contrast and are given in arbitrary units (a.u.). The laser speckle data were analysed for two regions of interest (ROIs) of 0.2 mm in diameter, as shown in Figure 6.30: relative to the induction site of the SD waves, the dark blue ROI was situated before the MD probe on the path of the SD wave ("pre-MD probe") whereas the light blue ROI was located after the MD probe on the path of the SD wave ("post-MD probe").
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From an electrical perspective, these are classic SDs in a “healthy” brain: transient global depolarisation of the cell membranes as indicated by the DC shifts, transient silencing of the spiking activity (AC amplitude reduction) (Figure 6.34). They are all accompanied by a transient hyperaemia observed on the laser speckle images. The time delay between the DC shift and the onset of the two ROIs for the CBF responses corresponds to an (expected) SD wave propagation speed of $2.43 \pm 0.2$ mm/min (mean ± SEM).

The CBF responses to SD waves were virtually identical in the 2 ROIs analyzed here (Figure 6.34), with a time delay that corresponds to the speed of the wave. This confirms that the presence of the MD probe does not interfere with the propagation of the the propagation of the SD waves and their associated haemodynamic responses (also verified in previous experiments [160]).

As suggested by Figure 6.34, the first SD wave was different from the following cluster of four SDs (SD2–5). The differences are recapitulated in Table 6.6 below. A video recording of the experiments by laser speckle is available on the DVD accompanying this thesis.

<table>
<thead>
<tr>
<th></th>
<th>SD 1</th>
<th>SD 2–5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC shift amplitude</td>
<td>15 mV</td>
<td>3–5 mV</td>
</tr>
<tr>
<td>AC silencing duration</td>
<td>5 minutes</td>
<td>2–3 minutes</td>
</tr>
<tr>
<td>CBF change</td>
<td>biphasic</td>
<td>hyperaemia</td>
</tr>
<tr>
<td>Propagation pattern</td>
<td>radial</td>
<td>circumferential</td>
</tr>
<tr>
<td>Time interval between consecutive SDs</td>
<td>15 minutes</td>
<td>7–8 minutes</td>
</tr>
<tr>
<td>rsMD glucose and lactate changes</td>
<td>50 µM</td>
<td>18 µM</td>
</tr>
</tbody>
</table>

All SD events were in fact accompanied by small transient changes in the potassium ISE potential response. These are shown for all 5 SDs in Figure 6.35.

Though small, the changes in potassium could be resolved for each SD wave: they were all time-locked with the DC shifts (indicated by the vertical dotted line on Figure 6.35), again showing that the ISE response was a reliable marker of the SD wave at the MD probe site.

1A video of the SDs can be found on the DVD accompanying this thesis. The propagation patterns are clearly visible. Such types of propagation have been commonly observed using laser speckle imaging in stroke models across different species [270; 271].
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Figure 6.35: ISE potential response to 5 SDs. The potential responses were time-aligned with the passage of the SD wave on the MD probe using the laser speckle images (and accounting for the connecting tube induced time delay). The unsteady decreasing potential two minutes preceding SD 2 was an artefact in the dialysate flow stream, as confirmed by the simultaneous loss of the glucose and lactate peaks. These data were de-spiked (see Chapter 4) and smoothed with a Savitsky-Golay filter of 101 points.

There is the possibility that the voltage changes measured by the ISE instrumentation could be, as in Figure 6.31, due to DC voltages in the tissue that accompany SD events. However, this can be ruled out as the potassium changes are time-shifted by 5 minutes and 15 seconds compared to the "real-time" of the DC changes. Hence, we can safely conclude that these are purely chemical events, due to changes in the potassium concentration in the tissue.

Finally, these ISE transients also show a slight difference between the first SD wave (in blue on Figure 6.35) and the subsequent four SD waves that form a very uniform group with their overlapping potassium responses (SD 2–5 in Figure 6.35).

The ISE potential transients were converted to concentrations according to the calibration curve and offset potential described previously. We defined a pre-SD basal level of dialysate potassium as the mean concentration over one minute prior to the SD event and estimated the changes in concentration following the SD event. The results are summarised in Figure 6.36 below:
6.4. In vivo application

![Graph showing changes in dialysate potassium concentration over time from SD event.](image)

**Figure 6.36: Summary of the potassium responses to SD events 2–5.** The average potassium concentration changes for the cluster of 4 SDs is plotted. The table summarises the basal potassium levels prior to each SD event, and the maximum changes. For SD 2 we could not calculate the basal concentration as the mean over one minute given the artefact, but we defined the basal concentration as the concentration at time t=0 when the wave moves across the MD probe.

<table>
<thead>
<tr>
<th>SD event</th>
<th>Basal dialysate [K⁺] /µM</th>
<th>Max [K⁺] change /µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD 1</td>
<td>3025.4</td>
<td>307.7</td>
</tr>
<tr>
<td>SD 2</td>
<td>2720.8</td>
<td>328.9</td>
</tr>
<tr>
<td>SD 3</td>
<td>2807.2</td>
<td>207.4</td>
</tr>
<tr>
<td>SD 4</td>
<td>2834.7</td>
<td>271.1</td>
</tr>
<tr>
<td>SD 5</td>
<td>2873.4</td>
<td>232.5</td>
</tr>
</tbody>
</table>

On average, for the group of 4 SDs, the maximum change in potassium concentration was $220 \pm 26$ µM (mean ± SEM) at 2 min and 11 seconds ± 16 seconds (mean ± SEM) following the SD wave. These are small changes but, given our detection limit of 55 µM, they can be reliably resolved.

Previous results in the literature showed a potassium peak to 40–60 mM within 20 seconds following the SD wavefront [242]. Here, the response is much smaller in magnitude and much longer in time (Figure 6.36). Reasons for such discrepancy in magnitude have already been discussed in section 6.4.2.4. An additional line of argument is a finding by Vyskocil *et al.* [242] who reported that SD-induced potassium changes decreased with depth and were virtually inexistent 2 mm deep. Hence, if part of our MD membrane was inserted too deep, the measured potassium changes would be diluted. Another important explanation is due to the $t_{90\%}$ of our flow-cell. It is of circa one minute. It means that brief transients that have a time scale shorter than this time cannot be fully resolved. Instead they will be detected as smaller potential changes that spread over a longer period.

Interestingly, while the first SD wave was followed by a prolonged decrease in the basal potassium level, the following four SD waves each led to a progressive rise in the basal concentrations of potassium prior to the SD waves. A parallel can be drawn to our clinical findings for glucose: for frequently repeating SDs, the basal levels of glucose preceding each SD became lower and lower.

Finally, combining the potassium flow-cell, the rsMD assay for glucose and lactate with laser speckle for CBF measurement, we could identify an ionic and metabolic signature of SDs. This is shown below for SD 5 in Figure 6.37:
The passage of an SD wave is associated with a transient increase in extracellular potassium concentrations and a transient hyperaemia (Figure 6.37). However, despite this hyperaemic response, it is accompanied by an increase in lactate and decrease in glucose (Figure 6.37). This has been previously observed in the cat [160]. It suggests that, even in the non-injured brain, an SD wave leads to a sheer metabolic stress of the tissue, with glucose consumption exceeding supply.
(since the net extracellular level drops) and ionic homeostasis being not restored completely even 5 minutes after the SD wave (potassium still high).

With SDs repeating every 7–8 minute, a good time resolution is critical to fully resolve the chemical response to SD waves: with a data point every 15 seconds for glucose and lactate, this is just sufficient and the continuous measurement of potassium is very valuable, acting as a chemical marker for the onset and termination of the SD wave.

6.5 Conclusions

A potassium flow-cell has been engineered for reliable measurements of dialysate potassium concentrations. Its time resolution, sensitivity and robustness allowed the detection of SD waves in animal experiments. These first results suggest that MD potassium is tightly coupled to the electrical depolarisation. It can therefore be used as a marker of SD waves to resolve the issue of timing between the ECoG activity and the MD response in a clinical setting.

The in vivo experiments also pointed out to some difficulties with the current flow-cell in vivo. In particular, earthing effects were identified. In general, to resolve this issue, some strategies can be developed:

• measure the voltage offset during the experiment or clinical monitoring (see Figure 6.31 in this experiment),
• perform a calibration of the flow-cell in situ,
• use isolated electronics (see Chapter 3)

More experimental and clinical data need to be collected to confirm the reliability of the flow-cell in vivo, notably to confirm the magnitude and duration of the potassium changes with SDs.
Chapter 7

Conclusion and future work

7.1 Conclusions

The mechanisms of secondary neuronal damage following brain injury are poorly understood, the main reason being the lack of reliable methods to monitor these patients. Microdialysis is currently the only tool that gives access to neurochemicals in the human brain. Although a very powerful technique, there are major challenges in monitoring microdialysis samples with a system that is clinically useful at the patient bedside. Boutelle et al. developed such a system in 2002 [162]. This system allowed online measurement of glucose and lactate from microdialysis samples every 30 to 60 seconds. It has recently been optimised for sensitivity and stability to allow continuous clinical monitoring for up to five days [180]. However, despite these significant improvements, many clinical data could not be reliably interpreted and quantified. This was because of a poor signal-to-noise ratio (SNR), mainly due to the multiple sources of noise in the intensive care unit. The initial aim of this thesis was therefore to improve this SNR, using first a hardware approach and subsequently a software method.

In Chapter 3, printed-circuit boards were designed with high quality components and screened cables and connectors. They proved to have very low noise and to be stable, even when no filtering was applied. Thanks to their very small size, they could be placed in the Faraday box of the analyser. This has two implications: 1) the instrumentation is very close to the detectors, hence limiting signal deterioration, and 2) the rsMD system is more compact and self-contained, facilitating access to the patient.

However, electronic noise was not the only type of noise and significant baseline fluctuations in the rsMD signal persisted. These were mainly due to flow instability within the system but also to ambient sources of noise from the neuro-intensive care unit. In Chapter 4, digital signal processing algorithms were developed to remove three classes of noise that are typically encountered in real-world analytical signals: spikes, non-stationary ripples and slow baseline drift. Three generic methods were developed. Traditional filters were used as an intermediary step to detect and remove spikes in the signal with 96.8% success. Adaptive ensemble average subtraction was developed to remove non-stationary ripples that have similar time scales as the signal of interest. This method increased the signal-to-noise ratio by up to 250% and led to minimal distortion of the signal, unlike conventional Fourier filters. Finally the removal of baseline drift was achieved by subtraction of a mathematical model for the baseline. The three algorithms are computationally fast and applicable to a wide range of analytical techniques provided prior knowledge of the noise types.
7.1. Conclusions

Using these data processing tools, a retrospective analysis of clinical rsMD data was carried out in Chapter 5. In ten patients, a total of 96 spontaneous metabolic events were associated with spreading depolarisations (SD) detected by electrocorticography. For the first time, a dynamic signature typical of these events was identified in the human brain: a fall in glucose by $-32.0 \, \mu$M (range: $-92.3$ to $-18.4 \, \mu$M, $n=96$) and increase in lactate by $+23.1 \, \mu$M (range: $+5.5$ to $+93.6 \, \mu$M, $n=49$). The changes were sustained for 20 minutes after the SD events and were highly significant, using an area under the curve analysis ($p<0.0001$). This was very similar to previous investigations of the metabolic response to SDs in animal models using rsMD [160; 161]. Furthermore, as the SDs frequently repeated, they led to a progressive depletion of brain glucose, sometimes below a viability threshold that we estimated to be around 200 \, \mu$M dialysate glucose. It was indeed previously shown by other groups that prolonged periods of low brain glucose were associated with poor patient outcome [61; 438]. Our study was not powered to reach this conclusion, but on an individual case basis, this trend was verified. We also showed that SDs are one important mechanism to account for otherwise unexplained instances of depletion of brain microdialysate glucose. This could have direct implications for the management of brain injury patients. In particular, it makes a case for a higher glycemic target range in patients with acute brain injury than in those with purely extracerebral pathology.

Finally, to improve the temporal coupling between the metabolic data and brain electrical signals, a flow-cell for online potassium measurement in the dialysate flow stream was developed in Chapter 6. The flow-cell uses standard HPLC components to be readily integrated into the online amperometric assay. It was optimised to give a 90\% temporal resolution of 65 seconds at a dialysis flow rate of 2 \, \mu$L/min. This was a large improvement from the initial 9 hour response time, but remained far longer than the response time of the underlying potassium selective electrode. \textit{In vivo} experiments, in a model of induced SDs in the intact rat brain, showed that the online potassium response was very stable with smallest resolvable changes of the order of 55 \, \mu$M. Potassium changes measured \textit{in vivo} were tightly coupled to electrical depolarisations and could be used as a chemical marker for the onset of neuronal depolarisation. Additionally, potassium changes associated with spreading depolarisation could be resolved: five SDs in one animal were detected by a transient increase in dialysate potassium, of $220 \pm 26 \, \mu$M (mean $\pm$ SEM) at 2 minutes and 11 seconds $\pm$ 16 seconds following the SD wave. However, these changes were very small compared to typical values reported in the literature [242; 252] and reasons for this still need investigation. If we manage to use potassium measurements as a reliable marker for the detection of SD waves, this would ultimately mean that we could measure SDs even in patients where no ECoG strip can be implanted. This would extend the range of patients monitored to many stroke cases where craniotomy is not required. These patients are usually monitored for intra-cranial pressure with triple lumen bolts, wherein microdialysis probes can be inserted.
7.2 Future work and new directions

7.2.1 Microdialysis sampling

One main issue with microdialysis probe is the damage caused upon implantation [336], which is primarily due to the rigidity and large size of the probes [439]. Microfabricated probes would be less traumaticising and more flexible, thus minimising this problem. Typically, a key length scale is 70 µm, the inter-capillary distance. However, smaller probes would need improved sensitivity of the detectors since the sampling area is smaller.

Microdialysis recovery is another important issue that cannot be neglected during long-term monitoring, especially in brain injury patients where the extracellular volume, blood flow, and other complex pathological events can interfere with the sampling. Three solutions exist:

1. sampling at a very low flow rate — to maintain the high time resolution of the rsMD assay at this low flow rate, the sensitivity of the detectors would need to be improved to assay smaller volumes.
2. no-net-flux methods — in the clinical context, this is difficult due to sterility issues. Furthermore, the time taken for analysis removes the possibility of high time resolution.
3. use a passive measure of recovery, such as urea — this would need the development of sensors for urea with a high temporal resolution.

7.2.1.1 Improved real-time detectors

Continuous sensors in the flow stream

Although the analyser is online with a typical temporal resolution of 30 seconds, it is not continuous, and a better time resolution may be desirable in some cases, such as:

- very frequent SD events, such as those presented in Chapter 6 that repeated every 7–8 minutes.
- very brief events, such as seizures, which are well-recognised secondary insults following primary brain injury and have the potential to exacerbate tissue damage [440]

One approach would be the use of biosensors based on enzyme modified electrodes that could be placed directly in the dialysate flow stream. This would simplify the system (no valve, no pump). The new printed circuit boards are designed to be fully functional for typical current ranges from biosensors. However, the difficulty is to make this a robust and stable system in a clinical environment.

Increase the range of analytes

Hyperkalemia and hyponatremia are of increasing clinical interest, as they would tend to lower the threshold for the occurrence of seizures. The development of a flow-cell for ion selective electrodes in Chapter 6 proved the feasibility of detecting potassium online in the microdialysis stream. Similarly, a sodium selective electrode could be implemented in a similar flow-cell system and such an electrode has already been developed within the group. The two ion selective electrodes could be concatenated in a single flow-cell, placed upstream of the rsMD valve and as close as possible to the microdialysis probe. These two ions would give two signals changing in opposite directions, which would help rule
out recovery artefacts. Another ion that seems to play a role in seizures is magnesium and a similar approach could be used to develop online measurement of this ion. Issues regarding earthing effects, \textit{in situ} calibration and long-term stability in an intensive care unit still need to be addressed.

Additionally, measuring pyruvate would be useful as it would help compare our data with other clinical microdialysis studies. It can be measured off-line initially, but time resolution matching that of glucose and lactate would be desirable. This could be achieved in the short term by developing enzyme reactors for pyruvate oxidase and using a third stream of ferrocene. However, it would complicate the existing system and pyruvate oxidase is a more challenging enzyme. The long term goal would be microelectrode arrays placed directly in the flow stream.

A more interesting analyte to measure would be ATP. Evidence suggests that there is ATP in the extracellular space. ATP would provide a direct link into the availability of energy in the tissue. Preliminary work in the group has consisted in setting up a HPLC assay. Ultimately, what is really needed is an ATP biosensor.

Minimise the distance between the rsMD analyser and the patient

A one-meter length of connecting tubing is necessary to meet electrical safety requirements for the patient (leakage currents). However, this means a net delay of 9 minutes between the response in the tissue and the rsMD response. It is also accompanied by Taylor dispersion that leads to peak broadening and smeared transient changes. Three strategies can be proposed to reduce the length of connecting tubing:

1. introduce an isolation barrier for the electronic components. Preliminary work has been carried out with the choice of an isolated power supply and the testing of an isolation amplifier. However, the isolation process can introduce significant noise.
2. design wireless electronics, which would have the advantage that the patient could be mobile with respect to the bedside analyser. The issue with wireless technology is its adverse effect on the SNR with the introduction of digitisation noise.
3. develop flow-segmentation techniques: these consist in forming discrete droplets of the microdialysate using an immiscible carrier flow stream in microfluidic channels. The difficulty is to develop sensors that have a suitable longevity and that can survive exposure to a non-aqueous medium.

7.2.2 Online event detection

The algorithms developed in Chapter 4 are computationally fast and could be run online whilst data are collected. A student has been working on an implementation of the codes into C++ that can be interfaced with our data acquisition software. This would greatly simplify data analysis and could be readily interpreted by the nursing staff at the patient bedside. A set of thresholds/alarms could be used to alert clinical staff to potentially important MD events for the direct benefit of the patient.

Currently, all electrocorticography channels are post-analysed manually by a highly-trained neuro-physiologist. This is very time-consuming and with the increasing volume of data collected by the COSBID study group, it will soon become impossible to carry out this analysis by hand. Automatic detection of SD events with pattern recognition algorithms could be an alternative.
7.2. Future work and new directions

7.2.3 Further clinical studies

All the above suggestions would ultimately improve the reliability of the data collected clinically with the rsMD assay and more studies could be designed.

SDs and brain glucose

Following our findings in Chapter 5, a prospective study should be carried out to establish whether spreading depolarisations are the primary mechanism that depletes brain glucose, independently of other variables. To do so, more patients are to be recruited to perform a multi-variable analysis. The possibility to use microdialysis with triple-lumen bolts in traumatic brain injury patients who do not require a craniotomy will drastically extend the range of patients monitored so far. Such a study would also need better quality data, not only rsMD data but also a better documentation of other parameters, including:

- systematic scans with the exact positions of the MD probe relative to the core of the injury and to the ECoG strip
- two-hourly standardised measurements of plasma glucose. This frequency could be increased in case of frequently occurring SDs, isolated aberrant glycemic levels or very low MD glucose.

Other sub-questions could be addressed, such as:

- whether there is a difference in the responses to SDs when they occur at early versus late days following injury,
- whether there is any correlation between the magnitudes of the glucose drops and the frequency or duration of the SDs.

Other events associated with metabolic changes

Intermediate patterns of ECoG activity, known as periodic epileptiform discharges, have been identified as spontaneously occurring in acute brain injury patients. They are not typical seizure patterns, but may be considered ictal [441]. Currently, there is controversy whether to treat them with anti-epileptic drugs. Detection and characterisation of their metabolic effects on tissue would be one way to determine their severity and impact on tissue viability. In particular, the acute effects on glucose and lactate, and the long-term effect on baseline brain glucose would be good markers.
References


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


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REFERENCES


REFERENCES


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REFERENCES


Appendix A: Supplementary information about the printed-circuit boards

A.1 Components of the Potentiostat PCB

They are listed in Table A.1 below:

<table>
<thead>
<tr>
<th>Component identification</th>
<th>Role in the circuit</th>
<th>Unit price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operational amplifier AD549JH</td>
<td>current-to-voltage converter</td>
<td>£20.23</td>
</tr>
<tr>
<td>Operational amplifier AD820ANZ</td>
<td>voltage follower</td>
<td>£3.15</td>
</tr>
<tr>
<td>Capacitor ceramic 0.1 µF</td>
<td>de-coupling capacitor for AD549</td>
<td>£0.12</td>
</tr>
<tr>
<td>Capacitor ceramic 0.01 µF</td>
<td>de-coupling capacitor for AD820</td>
<td>£0.16</td>
</tr>
<tr>
<td>DIL switch 4 spst</td>
<td>gain selector</td>
<td>£2.05</td>
</tr>
<tr>
<td>DIL changeover 1 spdt</td>
<td>potential input selector</td>
<td>£1.55</td>
</tr>
<tr>
<td>Resistor Radial 10 kΩ</td>
<td>Gain 100 µA/V</td>
<td>£4.69</td>
</tr>
<tr>
<td>Resistor Radial 1 MΩ</td>
<td>Gain 1 µA/V</td>
<td>£4.69</td>
</tr>
<tr>
<td>Resistor Radial 10 MΩ</td>
<td>Gain 100 nA/V</td>
<td>£1.30</td>
</tr>
<tr>
<td>Resistor Radial 100 MΩ</td>
<td>Gain 10 nA/V</td>
<td>£1.30</td>
</tr>
<tr>
<td>Polystyrene 10 pF capacitor</td>
<td>feedback capacitors</td>
<td>£1.10</td>
</tr>
<tr>
<td>AuPtSMB male straight PCB socket</td>
<td>screened signal connector</td>
<td>£1.40</td>
</tr>
<tr>
<td>0.040&quot; male cell connector, gold-plated brass (Bioanalytical systems)</td>
<td>working electrode connector</td>
<td>£43</td>
</tr>
<tr>
<td>0.060&quot; female cell connector, gold-plated brass (Bioanalytical systems)</td>
<td>reference electrode connector</td>
<td>£43</td>
</tr>
<tr>
<td>0.040&quot; female cell connector, gold-plated brass (Bioanalytical systems)</td>
<td>auxiliary electrode connector</td>
<td>£43</td>
</tr>
<tr>
<td>Header square pin 0.1&quot; 3 way + KK housing female, 3 way + KK Crimp terminal anti-fishhooking</td>
<td>Connector between PCBs</td>
<td>£0.60</td>
</tr>
<tr>
<td>DC/DC converter THB 3-0523 Traco</td>
<td>Isolated power (+15/-15V)supply for operational amplifiers</td>
<td>£55</td>
</tr>
<tr>
<td>Mains adapter DIPS52-M</td>
<td>Output 5V DC for the DC/DC converter</td>
<td>£48</td>
</tr>
<tr>
<td>Panel mounted power socket 2.5 mm centre plug</td>
<td>Connection of the power PCB to the mains converter</td>
<td>£3.5</td>
</tr>
</tbody>
</table>
A.2 Results of the tests of the current-to-voltage converter

The results for two gain resistors are shown in Chapter 3. Here, I give the results for the other two gain resistors:

<table>
<thead>
<tr>
<th>Gain Resistor</th>
<th>Current / mA</th>
<th>Output voltage / V</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 kΩ</td>
<td>9.960 ±0.0046 V/µA</td>
<td></td>
</tr>
<tr>
<td>10 MΩ</td>
<td>9.9853±0.00257 mV/nA</td>
<td></td>
</tr>
</tbody>
</table>

Figure A.1: Test of the current-to-voltage converter. The output voltage from the potentiostat is plotted against the current applied to the working electrode: each point on the curve is given as the mean ± standard deviation. Note the different scales in current depending on the gain resistor. The slopes (in blue) are given with 95% confidence.

The current-to-voltage conversion is very accurate and very low noise for the gain resistor shown in Figure A.1 above and in Chapter 3.
Appendix B: Supplementary information about the potassium electrode and flow-cell

This appendix gives full details about:

1. the calculation of the activity of potassium ions in various solutions
2. the calibrations of the initial potassium ISEs made with pipette tips
3. the concentrations and volumes used for the calibration of the ISEs with the injection method to determine their response time
4. the intermediary calculations for the analytical solutions of diffusion through the bore of the Y-connector
5. shape functions commonly used in finite element numerical modelling

B.3 Calculations of the activities of potassium ions in various solutions

The activities of potassium ions in different concentrations in an aCSF-like background electrolyte were calculated using the following Matlab programme:

```
x=[0.0018 0.0027 0.0043 0.0065 0.01 0.015 0.02];
I=0.5.*(147e-3*1+x.*1+1.2e-3*4+0.85e-3*4+x.*1+147e-3*1+1.2e-3*2*1+0.85e-3*2*1);
H=-(0.51*1.*sqrt(I))./(1+138e-10.*0.33.*sqrt(I))+0.1.*I ;
G=10.*H
a=G.*x
```

where x represent the concentration values of $K^+$ in mol/dm$^3$, I are the corresponding ionic strength (with the cation contribution first, and then the anion contribution) in mol/dm$^3$, H are the log of the activity coefficients, G are the activity coefficients and a the activities in mol/dm$^3$.

The results for a small range of $K^+$ concentrations with regularly spaced values on a logarithmic scale are summarised in Table B.1 below:
B.4. Calibration curves for pipette ion selective electrodes

A batch of six pipette ISEs served as standard electrodes to check the quality of the potassium selective membrane and compare with the new ISE design. These pipette ISEs were not fabricated once the Radel ISEs were validated as functional ISEs. Here, we give the calibration curves and parameters of the linear regressions for these six pipette ISEs. The calibration curves are plotted below in Figure B.2:

![Calibration curves for six pipette ISEs](image)

**Figure B.2: Calibration curves for six pipette ISEs** The calibration curves are very uniform for these six ISEs from the same batch. A linear regression for the average response is shown in pink.

The slopes, intercepts and their 95% confidence limits are summarised in Table B.2.

### Table B.1: Activities of potassium ions in calibrating solutions with an aCSF-like background electrolyte.

<table>
<thead>
<tr>
<th>Concentration $c_K$ /mM</th>
<th>Ionic strength /mM</th>
<th>Activity coefficient $\gamma_K$</th>
<th>Activity $a_K$ /mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>154.95</td>
<td>0.6527</td>
<td>1.17</td>
</tr>
<tr>
<td>2.7</td>
<td>155.85</td>
<td>0.6520</td>
<td>1.76</td>
</tr>
<tr>
<td>4.3</td>
<td>157.45</td>
<td>0.6507</td>
<td>2.79</td>
</tr>
<tr>
<td>6.5</td>
<td>159.65</td>
<td>0.6489</td>
<td>4.21</td>
</tr>
<tr>
<td>10</td>
<td>163.15</td>
<td>0.6461</td>
<td>6.46</td>
</tr>
<tr>
<td>15</td>
<td>168.15</td>
<td>0.6422</td>
<td>9.63</td>
</tr>
<tr>
<td>20</td>
<td>173.15</td>
<td>0.6384</td>
<td>12.8</td>
</tr>
</tbody>
</table>
B.5 Injection method for quantification of the temporal resolution of the ISEs

Table B.2: Slopes and intercepts of the calibration curves of the pipette ISEs

<table>
<thead>
<tr>
<th>Radel electrode</th>
<th>Slope ±95% confidence limit /mV/decade</th>
<th>Intercept ±95% confidence limit /mV</th>
<th>Number of data points, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette 1</td>
<td>57.4 ± 2.92</td>
<td>232.0 ± 7.4</td>
<td>7</td>
</tr>
<tr>
<td>Pipette 2</td>
<td>57.3 ± 1.8</td>
<td>232.8 ± 4.7</td>
<td>7</td>
</tr>
<tr>
<td>Pipette 3</td>
<td>58.7 ± 2.4</td>
<td>234.7 ± 6.14</td>
<td>7</td>
</tr>
<tr>
<td>Pipette 4</td>
<td>58.1 ± 5.3</td>
<td>232.6 ± 13.4</td>
<td>7</td>
</tr>
<tr>
<td>Pipette 5</td>
<td>57.7 ± 2.6</td>
<td>234.0 ± 6.7</td>
<td>7</td>
</tr>
<tr>
<td>Radel 6</td>
<td>63.0 ± 6.21</td>
<td>244.5 ± 15.0</td>
<td>7</td>
</tr>
</tbody>
</table>

B.5 Injection method for quantification of the temporal resolution of the ISEs

Figure B.3 below is a copy of the Excel table we used to calculate the volume of known concentration of potassium we needed to add to the initial volume and concentration to reach a given concentration, given the pre-made calibration solutions available:

**Figure B.3: Calculations of the volumes and concentrations needed for calibrations by addition.** The initial volume is each time incremented with the new volume added to the solution. The initial concentration is the target concentration at the preceding step. The titre concentration is the known concentration that is going to be add and is usually quite a high concentration from one of our calibration solution. The titre volume is the volume that is calculated to be added to reach the target concentration given the initial conditions and the titre concentration.
B.6. Analytical approach to diffusion within the bore of the Y connector

The problem is that of transient diffusion through a cylinder, governed by Fick’s second law for a cylindric tube, with axisymmetry in 2D and spherical symmetry in 3D:

\[
\frac{\partial C}{\partial t} = D \frac{\partial}{\partial r} \left( r^k \frac{\partial C}{\partial r} \right)
\]

where \( r \) is the radial coordinate, \( c \) the concentration, \( D \) the diffusion coefficient, \( t \) time, \( k=0, 1 \) or 2 in 1, 2 or 3D respectively. The solution below was developed by Jennifer Siggers from the Department of Bioengineering [436].

This problem can be re-written, using another coordinate, \( \eta = \beta r t^\alpha \), so that it can be solved analytically: indeed we get:

\[
t^{-2\alpha} \frac{\partial C}{\partial t} + \frac{\alpha \eta}{t^{1+2\alpha}} \frac{\partial C}{\partial \eta} = \frac{\beta^2 D}{\eta^k} \frac{\partial}{\partial \eta} \left( \eta^k \frac{\partial C}{\partial \eta} \right)
\]

To simplify the problem, we can choose \( \alpha = -1/2 \) and \( \beta = \frac{1}{\sqrt{4D}} \), which imposes boundary conditions on the system. If we then assume that \( C \) is only a function of \( \eta \), then equation 2 becomes:

\[
0 = \frac{1}{\eta^k e^{\eta^2}} \frac{d}{d\eta} \left( \eta^k e^{\eta^2} f \right)
\]

with \( f = \frac{dC}{d\eta} \)

Equation 3 can be integrated twice for \( \eta \), which leads to the following expression for \( C \):

\[
C = B - A \int_{\eta}^{\infty} \eta^{-k} e^{-\eta^2} d\eta
\]

The boundary conditions can be chosen as:

1. there is a source at \( r = 0 \) so that \( C = C_0 \)
2. \( C \rightarrow 0 \) as \( \eta \rightarrow \infty \)

This leads to \( B = 0 \) and \( A \) is negative (\( \tilde{A} = -A \)). When using \( \eta = \frac{r}{\sqrt{2Dt}} \), then

\[
C = \tilde{A} \int_{\frac{r}{\sqrt{2Dt}}}^{\infty} \eta^{-k} e^{-\eta^2} d\eta
\]

B.6.1 Solution in one dimension

For \( C = C_0 \) at \( r = 0 \), then

\[
C = C_0 erf \left( \frac{r}{2\sqrt{Dt}} \right) = \frac{2C_0}{\sqrt{\pi}} \int_{\frac{r}{2\sqrt{Dt}}}^{\infty} e^{-t^2} dt
\]

The concentration profile at the position of the \( K^+ \) ISE \( (r = a = 1.27 \text{mm}) \) in the Y-connector is given in Figure 6.19 in Chapter 6.

The asymptotical behaviour of \( C \) can be determined for large \( t \) to estimate the time when the concentration \( C \) reaches 90% of \( C_0 \) respectively.
B.6. Analytical approach to diffusion within the bore of the Y connector

Asymptotical behaviour at large $t$

For large $t$, using the asymptotic expansion of $\text{erfc}$, we can determine when $C$ reaches $C_0(1 - \nu)$ with $\nu \ll 1$ for position $r = a$:

$$C_{|r=a} = C_0(1 - \frac{2}{\sqrt{\pi}} \frac{a}{2\sqrt{D}t} + \text{smaller terms})$$

(7)

Therefore, when neglecting smaller terms, $\nu = \frac{a}{\sqrt{D}t\pi}$, so $t = \frac{a^2}{\pi D\nu}$. Numerical applications are given in Chapter 6.

B.6.2 Solution in two dimensions

We start from Equation ???. We need another assumption to solve the problem in 2D. We can no longer set a boundary condition at $r = 0$ on $C$ because we already imposed $C \to \infty$ as $r \to 0$. However, we can impose a boundary condition on the flux through a circle at $r = r_0$:

$$\text{Flux} = -2\pi r_0 hD \frac{\partial C}{\partial r} |_{r=r_0} = -2\pi h D \tilde{A} e^{-\frac{r_0^2}{4Dt}}$$

(8)

And if $r_0 \to 0$, then this flux $\to 2\pi hD\tilde{A}$, so we can have a prescribed flux boundary condition at $r = 0$, $F$. We then get the solution:

$$C = \frac{F}{2\pi Dh} \int_{\frac{r}{\sqrt{4Dt}}}^{\infty} \frac{1}{\eta} e^{-\eta^2} d\eta$$

(9)

This can be re-written using $E_1 = \int_{x}^{\infty} e^{-x^2} dx$ as:

$$C = \frac{F}{4\pi Dh} E_1(\frac{r^2}{4Dt})$$

(10)

The asymptotical behaviour for small $t$ is given by a Taylor expansion of $E_1$ to find $t$ when $C_{|r=a} = \frac{F}{4\pi Dh} e^t$:

$$e = E_1(\frac{a^2}{4Dt}) \approx \left( \frac{4Dt e^{-2/4Dt}}{a^2} + \text{smaller terms} \right)$$

(11)

Re-arranging Equation 11 gives:

$$\frac{a^2}{4Dt} = \ln(\frac{1}{e}) - \ln(\frac{a^2}{4Dt})$$

(12)

The first term is much bigger for $e \ll 1$, so an expression for $t$ is given by:

$$t \approx \frac{a^2}{4Dln^2 e}$$

(13)

Numerical applications are given in Chapter 6.