

Biomaterials Science and Engineering Series

BIOSENSORS IN THE BODY

Continuous *In Vivo* Monitoring



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An Introduction to *in vivo* Biosensing: Progress and Problems

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1.1 SETTING THE SCENE

This book provides a realistic and up-to-date guide to the continuous monitoring of chemical species *in vivo*. It summarises the clinical challenges and the commercial motivation behind efforts to implement such monitoring: it treats the key technologies—notably biosensing—that appear to offer solutions to those challenges: it reviews the obstacles to the success of these technologies, and finally it identifies the key areas in which progress must be made before the benefits of such monitoring can become widespread.

This introductory chapter provides a review of the progress and problems associated with *in vivo* biosensing. In subsequent chapters, leading researchers provide commentaries on particular aspects of *in vivo* monitoring. These contributions will provide more detail for those readers already familiar with one or more facets of *in vivo* sensing: clinical monitoring, clinical chemistry, point-of-care and critical care medicine, immunology, cell biology, enzymology, microelectronics fabrication. Although many readers may well have expertise in some of these fields, I believe that they will benefit from the overview that this first chapter represents. Without dwelling on the intricacies of the vast range of biosensor technologies that may be employed, I shall describe the basic concepts that must be considered in any attempt to develop and use an *in vivo* diagnostic method. Owing in part to the recent trendiness of biosensors, a large number of books and reviews have appeared on the subject (Hall, 1990; Buerk, 1993; Fraser, 1994a–c). Most of these texts contain details of the construction and operation of electrochemical and optical biosensors. In this chapter, only those aspects which influence *in vivo* behaviour of such devices will be examined. In many cases, particular devices are adapted for particular clinical settings, with the need to function in particular body

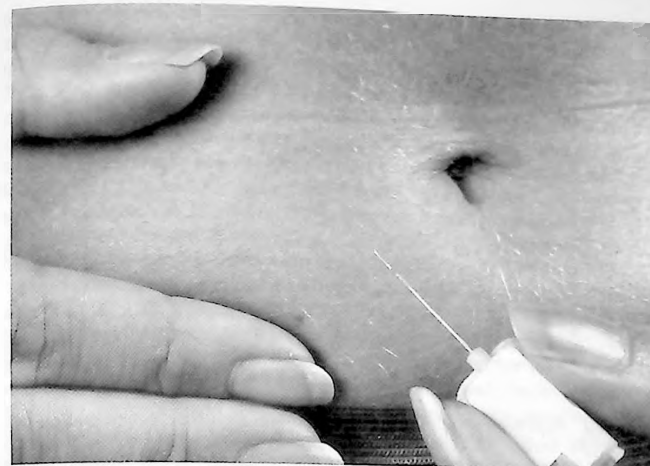


Figure 1.1 A needle-type glucose sensor, ready for subcutaneous implantation in the abdominal region. Reproduced with permission from Disetronic Medical Systems

compartments and fluids, and so it is difficult to introduce the devices without reference to where and how they are used. Equally, it is difficult to describe the clinical settings and results without some description of the devices used. Consequently, this chapter will tend to jump back and forth between consideration of devices and their application. Work on *in vivo* sensing has been heavily biased towards (commercially-attractive) monitoring of glucose, and this book will necessarily reflect that bias (Figure 1.1).

1.2 THE NEED FOR CONTINUOUS *IN VIVO* MONITORING: BIOLOGICAL, CLINICAL AND COMMERCIAL GOALS

In fact, is there a need for continuous *in vivo* monitoring? Clinical and analytical chemists already possess a huge arsenal of diagnostic methods used in health care. These diagnostic tests are based on chemical reactions (particularly those catalysed by enzymes) and/or physical molecule–molecule interactions (particularly antigen–antibody binding). A huge variety of *in vitro* diagnostic tests are available, for analytes ranging from ions to small metabolites, from drug moieties to peptides and proteins. The tests often have detection limits in the picomolar range, and some tests give results in minutes.

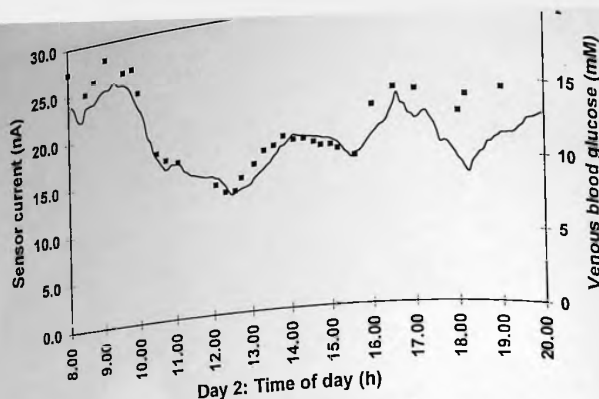


Figure 1.2 Real-time monitoring of subcutaneous glucose concentration by a needle-type sensor (continuous trace, rolling average current at 5 minute intervals). Data are current readings (nA, left axis) and not calibrated glucose concentrations. Reference venous blood samples were measured for glucose concentrations. Reference venous blood samples were measured for glucose concentrations. Data are current readings (nA, left axis) and not calibrated glucose concentrations. Reference venous blood samples were measured for glucose concentrations. The two sets of data are (in mM, right axis) with a YSI benchtop meter (■). The two sets of data are superimposed merely as a guide to the eye. Reproduced with permission from Disetronic Medical Systems

Such tests have contributed massively to diagnosis and therapy of many illnesses and diseases, and have improved the quality of life of countless patients. Self-monitoring of blood glucose (SMBG) by diabetics represents a market in excess of 100 million dollars worldwide. A huge, global industry is based around diagnostics manufacture in general. So what more do we want?

The important proviso is that these diagnostic tests are generally performed by an analytical device *in vitro*—i.e. in some sort of receptacle. The level of the analyte is estimated from samples that are expelled from or extracted from the body (urine, blood, faeces, whole cells or tissues etc.). The sample is often transported, manipulated and stored before it reaches the analytical apparatus, although point-of-care diagnostics (including *in vitro* biosensors) are reducing these delays (Kost, 1993). Analysis is discontinuous: *in vitro* monitoring can only provide a limited number of data points: these data must be extrapolated or interpolated to judge what is almost always a changing situation, so although absolute levels at a particular moment are known, trends and rapid fluctuations are harder to detect. A continuous stream of data is clearly preferable (Figure 1.2).

Measurement of the levels of distinct analytes directly in the body would be ideal. *In vivo* continuous monitoring promises to make as much difference to patient care as *in vitro*, discontinuous measurement has already done: almost

every area of medical care could benefit (Siggard-Andersen *et al.*, 1995). Indeed, the term “monitoring” has become associated with continuity, as opposed to “analysis” which is associated with discrete measurements (Kost, 1993). Such monitoring *in vivo* would not necessarily involve the identification and measurement of new analytes but rather would provide a new dimension to analysis of the most popular current analytes: glucose, ethanol, lactate, amino acids, oxygen, pH, carbon dioxide, and anions and cations like Na^+ , K^+ , Mg^{2+} , Ca^{2+} , and Cl^- would still remain the most frequently requested tests (Christiansen & Jakobsen, 1995): tests for larger, proteinaceous analytes are almost non-existent in continuous *in vivo* monitoring because such analytes fluctuate slowly enough for *in vitro* analysis to suffice.

The continuous monitoring of analytes can cover a vast range of situations, from diagnosis to treatment, from hospital-based use to ambulatory care, from mass-markets touching millions of people to highly specialised research applications (Connolly, 1995). Although the major commercial interest is *in vivo* monitoring of humans, veterinary medicine and basic research on laboratory animals could also benefit. Continuous *in vivo* monitoring often avoids the need to sacrifice an animal, and could allow more humane use of laboratory animals.

1.2.1 A PLETHORA OF OPPORTUNITIES FOR *IN VIVO* SENSING

The analytes that have been investigated by continuous *in vivo* monitoring may be grouped in a number of ways: (i) By chemical nature—one could distinguish gases such as oxygen, carbon dioxide or NO (nitric oxide); ions such as H^+ , Ca^{2+} , Cl^- , Na^+ ; or small organic molecules such as glucose, glutamate and dopamine. (ii) By location in a certain body compartment or tissue—one could distinguish blood-borne analytes such as glucose, oxygen, hydrogen ions, carbon dioxide, ionised calcium, sodium ions etc.; compounds common in neural tissue (again glucose, but also compounds with neuroactive or neurotransmitter roles such as ascorbate, glutamate, dopamine and derivatives (Broderick, 1993; Cooper *et al.*, 1995), or compounds in the extracellular fluid of subcutaneous tissue; excreted body fluids such as sweat and urine are also candidates for estimating the concentrations of compounds in the blood (Mitsubayashi *et al.*, 1994), although there is often little correlation with concentrations in blood, or the equilibration between blood and fluid is prohibitively long (Guilbault *et al.*, 1995). (iii) By clinical context—methods suited to intensive care situations, i.e. on-line monitoring of blood gases such as oxygen, carbon dioxide, and blood pH during surgery (Meyerhoff, 1993; Gumbrecht *et al.*, 1994); long-term, ambulatory applications like monitoring glycaemia in diabetics; use of sensors implanted in neural tissue in laboratory animals for research purposes.

To give an idea of the breadth of application of *in vivo* monitoring, there now follows a non-exhaustive list of clinical and research applications where

in vivo monitoring and related methods used advantageously to replace *ex vivo* or frequent *in vitro* measurements.

1.2.1.1 Opportunities in Critical Care

- (1) Monitoring of blood gases, electrolytes and key metabolites (Meyerhoff, 1993) in critical care situations such as major surgery, problematic childbirth, accidental trauma, blood acidosis, or septic shock induced by severe bacterial infections (Landolt *et al.*, 1994; Telting-Diaz *et al.*, 1994; Baker & Gough, 1995).
- (2) Lactate monitoring with a biosensor performed during open-heart surgery (Kyroläinen *et al.*, 1993). Equally, heart muscle pH can be monitored during open-heart surgery with miniaturised implantable ISFET sensors (Covington *et al.*, 1993).
- (3) Subcutaneous pO_2 monitoring in critically-ill patients by fluorescence oximetry, as a guide to the state of blood perfusion of the tissues (Powell *et al.*, 1995).

1.2.1.2 Manipulation and Study of Metabolism

- (1) Ambulatory, mass-market, long-term measurement of glycaemia in insulin-dependent diabetics (Fischer, 1995): the study of the efficacy of insulin therapy and the effect of changes in activity and diet on glycaemia.
- (2) Measurement of circulating levels of administered medicaments *in vivo*, in order to define their pharmacodynamics (Müller *et al.*, 1995) or to tune therapy.
- (3) Measurement of common metabolites in order to measure the effect of other pharmacologically active compounds (Broderick, 1993).
- (4) The measurement of lactate to determine metabolic state in shock: the measurement of ethanol to determine intoxication (Hu *et al.*, 1993; de Boer *et al.*, 1993, 1994a,b; Baker & Gough, 1995).
- (5) Monitoring of the uptake and metabolism of pyruvate and glucose in individual sheep embryos developed *in vitro* (Gardner *et al.*, 1993).

1.2.1.3 Measurement of Physiological Performance

- (1) The analysis of metabolites in sweat as a measure of athletic condition; most studies have relied on *in vitro* biosensor measurements of a few sweat samples collected manually after intense exercise (Mitsubayashi *et al.*, 1994). This *in vitro* method has been referred to as "non-invasive" sensing (Guilbault *et al.*, 1995) which should not be confused with *in vivo* measurements made by spectroscopic methods (Chapter 3).
- (2) Transcutaneous pCO_2 -monitoring can be used to reveal the anaerobic threshold in athletic performance but does not yet seem reliable enough

to replace blood lactate measurement (Breuer *et al.*, 1993; Larsen *et al.*, 1993; Urhausen, 1993).

- (3) Implanted pacemakers measure the changes in blood pH, pO_2 and pCO_2 which occur upon exercise, thus adapting cardiac stimulation to the extent of bodily activity (Connelly & Group, 1993; Carlsten *et al.*, 1994; Ovsyshcher *et al.*, 1995).

1.2.1.4 Studies of Neural Tissue

- (1) Measurement of the release and metabolism of neurotransmitters and neural analytes (Boutelle *et al.*, 1986; Crespi *et al.*, 1993, 1994; O'Neill 1993, 1994, 1995; Lambas-Senas *et al.*, 1995) aiding basic research, diagnosis of mental illness and modulation of therapy.
- (2) Drugs of abuse: the effect of cocaine on neurotransmitter release in rats has been studied by *in vivo* electrochemistry (Broderick, 1993), as has the involvement of excitatory amino acid pathways in the development of morphine withdrawal symptoms (Hong *et al.*, 1993).
- (3) Monitoring the concentration of choline (the precursor of acetylcholine) in rat brain with a microelectrode (Garguilo & Michael, 1993, 1994).
- (4) The study of the effect of stress, electroconvulsive shock and drug administration on lactate levels in rat brain (Korf *et al.*, 1993).
- (5) Microdialysis (with off-line measurements) to monitor directly extracellular lactate levels in the human hippocampus during seizures (Dahl-Jorgensen *et al.*, 1994).
- (6) Non-invasive, infrared spectroscopic monitoring of the gerbil brain to demonstrate changes in protein and lipid composition as a result of stroke/cerebral ischaemia (Carney *et al.*, 1993).

1.2.1.5 Miscellaneous Studies

- (1) Monitoring gastric pH using optical indicators embedded in plastic optical fibres (Baldini *et al.*, 1994).
- (2) The efficacy of cancer treatment: in some poorly-vascularised tumours, chronic hypoxia results in a low extracellular pH. This has been measured with an needle-type pH-sensor and is a useful prognostic indicator of future tumour response to thermoradiographic therapy (Engin *et al.*, 1994).
- (3) The characterisation of cardiac function and disease with implanted sensor arrays for pH and K^+ (Cosofret *et al.*, 1994a-c, 1995).
- (4) Replacement of some *in vivo* estimates of toxicity (e.g. the Draize test for ocular toxicity of cosmetics in rabbit) by *in vitro* or *in vivo* biosensors (Catroux *et al.*, 1993).
- (5) As a diagnostic tool in gynaecology, an ISFET pH sensor has been used to measure intravaginal pH (Oelssner *et al.*, 1993).

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- (6) During transport of organs destined for transplantation, the state of perfusion has been studied with implantable oxygen microsensors (Barrow & Cefai, 1993); measurements of ionised calcium and magnesium during organ transplantation (Kost, 1993).
- (7) Sweat analysis with a lactate sensor as diagnosis for Frey's syndrome—gustatory sweating (Laccouraye *et al.*, 1993).
- (8) Measurement at carbon-fibre microelectrodes of chemical secretions (possibly insulin) by isolated pancreatic β -cells *in vitro* (Kennedy *et al.*, 1993).
- (9) Measurement with miniaturised amperometric sensors of the oxygen in the tracheal system of butterfly pupae (Hetz *et al.*, 1993).

So, a wide spectrum of opportunities exists. To illustrate how one particular challenge is being tackled, the next section examines *in vivo* monitoring of glucose levels in diabetics, potentially the most popular (and profitable) area. Many of the examples in this chapter used to illustrate more general points will in fact be taken from one particular *in vivo* system—the subcutaneously implanted, needle-type, glucose biosensor, based on the enzyme glucose oxidase. This is due to the huge health-care and commercial potential of such a device, and the subsequent attention it has received in the learned-journal and patent literature.

1.2.2 A CASE IN POINT: CONTROLLING GLYCAEMIA IN DIABETICS

The most obvious need for continuous *in vivo* monitoring is in diabetes mellitus therapy, and as such is the main factor fuelling research into biosensors. Diabetes mellitus is a relatively common, life-threatening and debilitating disease of glucose metabolism which looks to become more prevalent with the tendency of the populations of the world's emerging economies to consume a more energy-rich diet. It exists in two forms, Type I (also referred to as juvenile-onset or IDDM (insulin-dependent diabetes mellitus)) affecting about 0.1% of the world population, and Type II (adult-onset or NIDDM (non-insulin-dependent diabetes mellitus)) affecting up to 1.5%. Type I is characterised principally by the pancreas' loss of ability to produce and release insulin in response to rising blood glucose levels. This absence is due to death of the β -cells in the islets of Langerhans, which normally act as the body's glucose sensor, and regulate insulin secretion. Why the β -cells die is not yet known: it has been suggested that the body destroys them in an autoimmune reaction. Type II diabetes involves a number of factors such as reduced secretion of insulin, erratic release of glucose by the liver, poor control of lipid metabolism, lack of sensitivity of the peripheral tissues to insulin and resulting failure to take up glucose into the cells. Apart from the immediate need to avoid the coma and possible death associated with hypoglycaemia, harmful long-term effects exist due to the slow chemical reaction of glucose with the

structural biomolecules of the body (Harding, 1985). Neuropathy (leading to impaired motor function), retinopathy (leading to blindness), nephropathy (microalbuminuria and albuminuria, leading to kidney failure) and micro- and macrovascular complications (leading to amputation of limbs and cardiac disease; Dahl-Jorgensen *et al.*, 1994) are the principal consequences of this long-term glycosylation.

However, the debilitating consequences of this disease can be dramatically reduced by attempting to control glycaemia (blood glucose level) as tightly as possible. That was the main conclusion of the Diabetes Control and Complications Trial study (The DCCT Research Group, 1993; Réach, 1993a; Santiago, 1993) carried out on 1441 Type I diabetics in centres across the US from 1983 to 1993, for an average of 6.5 years. Intensive therapy (at least four self-tests of glucose per day, accompanied by continuous, pumped insulin infusion or by an equivalent number of insulin injections) was clearly shown to reduce cardiovascular problems, neuropathy, retinopathy, and nephropathy (microalbuminuria and albuminuria). The only major disadvantage was an increased incidence of hypoglycaemic incidents caused by increased insulin injection, although generally these were not life-endangering. At present, good glycaemia control can only be approximated by frequent insulin self-administration by injection or infusion, and this depends on having precise knowledge of blood glucose concentration. How accurate such monitoring needs to be, and the real accuracy of devices currently commercially available for SMBG, is still subject to debate (Clarke *et al.*, 1987; van't Sant *et al.*, 1994). Currently, a wide range of accurate, portable and relatively user-friendly "glucometers" (some of which are based on biosensor technology) are available for self-measurement of the glucose concentration in a drop of fresh capillary blood. These devices produce data which are life-saving data but infrequent; the pain of lancing a finger (or toe) a few times a day to obtain a blood drop, coupled with the discomfort due to the constantly damaged state of those tissues, discourages diabetics from more frequent testing. Even though some authors suggest less painful, alternative methods for obtaining a blood droplet, (for example by firing a pulse of laser light; Fonseca *et al.*, 1994), this remains a problem.

Continuous estimation of blood glucose by a sensor would provide the mass of information that could lead to more effective control of insulin administration, be it in a closed-loop system (where the means for introducing insulin is controlled by the sensor) or open-loop system (where the patient decides on the quantity to introduce). The "artificial pancreas" or "artificial β -cell" are often-used terms (Bernardi, 1993; Pfeiffer, 1993; Shichiri *et al.*, 1994); the dream of researchers, health-care companies and diabetics alike is to ally an external insulin infusion pump (where a subcutaneous-implanted catheter delivers insulin) or a fully-implanted pump (Hepp, 1994) to a reliable glucose sensor (Poitout *et al.*, 1993).

Large, bedside models do exist, for example the "Biostator" (Pfeiffer *et al.*,

1974) commercialised by Miles-Ames (Elkhart, IN, USA), and these can successfully maintain a fixed blood glucose concentration (or "glucose clamp"). Such machines have not found very widespread use in improvement of patient well-being (they are not portable, and rely on frequent blood sampling via an intravenous catheter) but are valuable tools in diabetology research. Numerous algorithms for matching insulin release to glucose concentration have been published (Brunetti *et al.*, 1993; Candas & Radziuk, 1994). The portable artificial pancreas was first announced in 1982 (Shichiri *et al.*, 1982) but almost 15 years later, despite frequent announcements of semi-commercial systems (e.g. Pfeiffer, 1994), there is no sign of a real commercial device for the mass-market, everyday, continuous, ambulatory use by diabetics. The reasons for this failure are numerous and will be dealt with in Section 1.4. In terms of sensing techniques, a wide variety of devices have been proposed. The different sorts of *in vivo* monitors are presented briefly in the next section.

1.3 MEETING THE NEED: THE TECHNOLOGY BEHIND BIOSENSORS AND BIOPROBES

The need for continuous *in vivo* monitoring exists. But which technologies and techniques can meet this need? This book intends to show that biosensing devices are prime candidates. By virtue of their conception and construction, biosensors inherently possess many of the characteristics needed: small size giving portability, rapid response times giving real-time data and high specificity for the chosen analyte, giving detailed information on one species in a milieu containing possibly many hundreds or thousands of different substances.

1.3.1 DEFINING BIOSENSORS

The term biosensor has been interpreted so widely that no single definition is universally accepted. My definition is as follows:

A biosensor is a deliberate and intimate combination of a biomolecule and a man-made transducer.

The biomolecule provokes a recognition event, either by binding the analyte or causing the analyte to react, whilst the artificial transducer detects the resulting change in a chemical or physical parameter, causing an electrical signal to be created or modified. The definition of a biosensor as an intimate and deliberate combination of the two components, the bioelement and the sensor element, gives a clue as to the inherent power of biosensing devices. Intimate because the best biosensors are those where the division between the two halves of the device is blurred: the tighter the coupling, the better the

specificity of signal transduction from the biological element into an electronic circuit. Deliberate because modern science and technology has made possible the purification of thousands of different molecules of biological origin, which can then be deposited in or on transducers. The biomolecules used in biosensors are almost always enzymes or antibodies, and so the analytes are almost always enzyme substrates or antigens: most analytes of medical interest are essential metabolites. This means that in some convenient microorganism, plant or animal source there are necessarily enzymes that act on these metabolites. The enzymes can be extracted, purified, and then used as the biocomponent in biosensing devices. Organic, host-guest chemistry has not yet been able to match the specific catalysis or specific binding of which enzymes and antibodies are respectively capable, though the creation of "biomimetic sensors" (Kriz *et al.*, 1995) is attracting increasing interest.

Equally, manufacturing technology and materials development has enabled the fabrication of a vast range of electric/electronic transducers. Implicitly, almost all physicochemical parameters—electric (current, voltage, impedance) and radiative (intensity, wavelength, directionality) are identified with some sort of sensor. Combining biological molecules and human-made transducer devices makes it possible to sense almost any analyte via a change in one of several physicochemical properties.

1.3.2 DIFFERENTIATING BIOPROBES FROM BIOSENSORS

This book will also consider devices which are not strictly biosensors, but can yield very similar information to that provided by "true" biosensors. A good example would be the comparison between implantable enzyme-electrodes (see Section 1.3.5 and Chapter 2) and the non-invasive spectrometers working in the near- or mid-infrared (see Section 1.3.4 and Chapter 3). These two classes of devices work on very different principles—the former are implanted in the body and produce a signal via enzymatic and electrochemical reactions, whilst the latter emit electromagnetic radiation into the body tissue and measure the intensity that is transmitted, scattered or reflected. The former are biosensors, the latter are not, but the results are very similar: continuous estimates of blood or tissue glucose concentration. Whatever the method of measurement, the devices should provide the patient, doctor or research scientist with a real-time read-out of the intravascular or tissue level of a non-labelled analyte—what matters is that the device works!

Going even further away from biosensors, one meets devices which neither incorporate a biomolecule as part of their operating mechanism nor give information on a specific molecule. They are referred to as "bioprobables" because although they measure in a biological system, they probe a purely physical parameter like temperature, pressure, nuclear relaxation time or the emission of nuclear radiation. In general, this book will not deal with such bioprobables, but will concentrate on those devices that yield information on the

level of a specific, non-labelled chemical species in the body, by detecting either the chemical reaction involving that species, the specific effect of that species on labelled compounds, or the characteristic spectral features exhibited by that species. A borderline case is magnetic resonance spectroscopy and imaging, which can give specific quantitative information on the amount and the spatial distribution of phosphorus-containing molecules such as creatinine phosphate. Other physical parameters such as hydrodynamic flow can be investigated: an invasive integrated sensor for blood-flow has been proposed (Kersjes *et al.*, 1993). The level of oxygen *in vivo* can be estimated by electron spin resonance spectroscopy of oxygen-sensitive, spin-labelled nitroxides in injected blood-borne microspheres (Liu *et al.*, 1994). Another remarkable example is the measurement of an electrical property, the *in vivo* potential difference across the cell mucous membranes of the nasal passage, for prediction of the efficacy of gene transfer treatment of cystic fibrosis (Labow *et al.*, 1995). Radiation-sensitive sensors have been implanted into tumours in mice to measure in real-time the dose received during radioimmunotherapy with ^{90}Y -labelled antibodies (Gladstone & Chin, 1995). So it is clear that the barrier between biosensors and bioprobes is somewhat arbitrary, and the fields of application certainly overlap.

1.3.3 IN VIVO, EX VIVO AND IN VITRO SENSING

The separation of sensing schemes into *in vivo*, *ex vivo* and *in vitro* analysis is also somewhat arbitrary. Taking the example of an electrochemical sensor, "true" *in vivo* monitoring means implantation within a tissue, i.e. where the sensing part of the device has crossed an epithelial layer, or within a closed body cavity. This would mean either a monolithic device (like a pacemaker) or a needle-type sensor implanted under the skin or through the wall of a blood vessel. However, very similar results might be obtained by *ex vivo* monitoring. This would be performed by cannulating a blood vessel to establish an extracorporeal blood loop. The blood flows past a sensor and is then returned to the body (Gumbrecht *et al.*, 1994). This has been adopted by some companies who seem to have conceded that direct *in vivo* measurement of blood gases and pH is not yet sufficiently reliable (Meyerhoff, 1993; Zimmerman & Dellinger, 1993; Shapiro, 1995). The *ex vivo* term can equally be applied to the extraction of extracellular fluid across or through epithelium, with measurement of the analyte being performed in a device that sits on the skin or near the site of extraction but not inside the body. An example would be a combined suction device/glucose sensor (Kayashima *et al.*, 1995): (i) under negative pressure, the suction device sucks out extracellular fluid across the skin (although the relatively dry, impermeant layer of dead cells of the epidermis has first to be removed, e.g. by repeated sticky tape-stripping); (ii) the sample enters a classical glucose sensor incorporated within the suction device; (iii) the fluid enters a detachable reservoir.

Another example is the microdialysis system: a probe made from a hollow fibre of semipermeable material is implanted into the subcutaneous fatty tissue (Bolinder *et al.*, 1993; Amine *et al.*, 1995). Physiological buffer contained in an external reservoir is continuously or discontinuously pumped into the probe: chemical species diffuse across the semipermeable membrane into the buffer carrier stream, which then returns to the exterior via the catheter's central lumen. The amount of analyte (e.g. glucose or glutamate; Zilkha *et al.*, 1994) is analysed in a sensor placed outside the body (Laurell 1992, 1993) but which can be compact enough to be attached to the body or clothing, allowing portability. The overall system gives similar performance to the needle-like, percutaneous electrochemical sensor.

1.3.4 THE SPECTROSCOPIC SENSOR

A completely different approach is that of the spectroscopic sensor (Lübbers 1993, 1995; Arnold, 1996), which may be non-invasive or invasive (see Chapters 3 and 8). The optical properties of tissue are monitored in order to determine one or more specific chemical components. Most purified, distinct chemical compounds have unique, "finger-print" spectra when studied by most spectroscopic techniques. Some form of electromagnetic radiation (usually infrared or near-infrared for *in vivo* studies) is directed into well-vascularised tissue (such as the earlobe, lip or finger). The near-infrared (NIR) corresponds to wavelengths of 700–2500 nm (or 14 300 to 4000 cm^{-1} when referring to wavenumbers—number of wavelengths per centimetre). The radiation will then interact with the tissue, being variously reflected, scattered, and adsorbed: a detector placed appropriately can then record the intensity across a spectrum of wavelengths. The specific spectral peaks of the analyte are then sought amongst the overall spectrum. This technique has been most developed for measuring the different forms of haemoglobin in the blood, which is present in high concentrations and has strong spectral features depending on whether a gaseous ligand such as oxygen, carbon dioxide or carbon monoxide is bound to the haem groups in haemoglobin. Each form has a distinct spectrum, so the sensor primarily detects the relative fractions of all subspecies of haemoglobin. As the stoichiometries and kinetics of the interaction of haemoglobin with blood gases and protons are reasonably well described, the local pO_2 can be estimated from the relative distributions of haemoglobins. This technique is known as oximetry and has revolutionised monitoring in anaesthesia and neonatal care (O'Brien *et al.* 1993; Rolfe, 1995) and can even be used to monitor cerebral oxygenation of an intrapartum foetus (Faris *et al.*, 1994).

Pulse oximetry is a variant with enhanced accuracy through measurement of the spectral changes during the pulsatile flow provoked by the pumping of the heart; this is treated in more detail by Peter Rolfe in Chapter 8, who also introduces a range of invasive optical sensors: these tend to be based on fibre

optic light guides, which have the advantage of being narrow (diameters of a few hundred micrometres), flexible (allowing their insertion via catheter) and amenable to the immobilisation of biological molecules and chemical sensing molecules (for example, dyes that change their fluorescent properties upon a change in local pH or pO_2 (Seitz, 1991; Zhang *et al.*, 1995)). Miniaturisation allows several sensing spots to be deposited on the same fibre tip (Li & Walt, 1995).

Another promising (semi-invasive) technique aimed at long-term sensing is the use of *in vivo* oxygen-sensitive fluorescent probes, incorporated in subcutaneously implanted pads of silicone. Light from a laser source is guided along an external optical fibre and shines through the skin and into the implanted pad, with the returning, oxygen-dependent amount of fluorescent light being measured. The light source can be a relatively small laser diode, and measurement of the fluorescent lifetime or phase delay between excitation and emission minimises errors associated with measuring absolute fluorescence intensities (Bambot *et al.*, 1994, 1995a,b). Biocompatibility of the implant may be less of a problem because oxygen diffuses quickly through most substances and thus isolation of the implant by fibrous encapsulation is less likely to impair the function. This work is currently sponsored by the company SpectRx Inc. (Norcross GA, USA).

A valuable complement to oximetry is the sensing of transcutaneous oxygen with a skin surface electrode. If the subject is haemodynamically stable, the transcutaneously-measured values correlate well with blood values, although the technique is best suited to neonates with thin skin (Larsen *et al.*, 1993; Poets & Southall, 1994). The electrode is heated to 42°C to increase local blood flow and to permeabilize the epidermis (Harsanyi *et al.*, 1994): long-term use can lead to skin damage.

1.3.5 A CASE IN POINT: SENSORS FOR CONTINUOUS *IN VIVO* MONITORING OF GLUCOSE

Thus a range of diagnostic devices exist, and each device has advantages and disadvantages for a given clinical use. Which device is best suited to a particular clinical problem? For glucose monitoring *in vivo*, the situation can be summarised as follows (much more detail is provided in Chapters 2, 3 and 4).

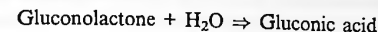
The simplest tactic has been to adapt *in vitro* glucose sensors for *in vivo* use by miniaturisation: this usually means a subcutaneously-implantable needle. A large number of devices have been proposed by various university groups and companies (Bindra *et al.*, 1991; Shaw *et al.*, 1991; Johnson *et al.*, 1992; Atanasov & Wilkins, 1993a; Ikeda *et al.*, 1993; Koudelka *et al.*, 1991; Moussy *et al.*, 1993; Abdel-Hamid *et al.*, 1994; Fischer *et al.*, 1994; Ward *et al.*, 1994; Csoregi *et al.*, 1995).

Although some researchers attempt to measure directly the oxidation of glucose molecules at an "electrocatalytic electrode" (Lemke & Lustermann,

1991; Lager *et al.*, 1994a,b; Xu *et al.*, 1994), this approach is extremely unlikely to be reliable *in vivo*, because of the wide range of other chemicals that can also be oxidised, i.e. such sensors are not specific for glucose. Most glucose biosensors contain the flavoprotein enzyme glucose oxidase (GOD), which provides the necessary specificity by catalysing the following reaction:



which is then followed by a non-enzymatic hydrolysis:



Thus, if a layer of GOD is associated with a suitable transducer, the concentration of glucose can be estimated by measuring the following:

- (1) The consumption of oxygen, via measurement of remaining oxygen by electrochemical reduction at an electrode.
- (2) The production of hydrogen peroxide (H_2O_2) via its subsequent electrochemical oxidation or reduction at an electrode (Zhang & Wilson, 1993; Csoregi *et al.*, 1994a).
- (3) Substitution of the O_2/H_2O_2 pair by "foreign", redox-active molecules called "mediators" (Zakeeruddin *et al.*, 1992). The oxidised form of the mediator is reduced by GOD and then reoxidised at an electrode: the reoxidation current is proportional to glucose consumption.
- (4) The acidification due to gluconic acid production, using transducers such as pH ISFETs or conventional, miniaturised, glass pH electrodes.

Needle sensors are similar to insulin infusion catheters in that they allow easy, anaesthetic-free, subcutaneous self-implantation without the intervention of medical staff. Disadvantages include the need for miniaturisation to minimise tissue damage, making fabrication processes more expensive; this miniaturisation also makes the device more fragile and prone to damage during implantation. To avoid infection of the puncture site, needle-like devices should be changed every few days, and if different implantation sites are used, the results may be different, so an *in vivo* calibration may be needed each time (see Section 1.4.4).

Other, related devices use an *in vivo* sampler with an *ex vivo* biosensor measurement. For example, electrochemical glucose sensors can be coupled to needle-like, *in vivo* microdialysis probes (Moscone & Mascini, 1992, 1993; Bolinder *et al.*, 1993; Keck & Kerner, 1993; Meyerhoff *et al.*, 1993, 1994). This has the advantage that the *ex vivo* sensor can be larger than a needle-type sensor, allowing more flexibility in design and fabrication. Much experience in microdialysis *in vivo* has been acquired in a range of tissues, in human and animal models (Laurell, 1992, 1993; Bolinder *et al.*, 1993). The company

CMA Research AB (Stockholm, Sweden) produces a range of microdialysis probes and accessories, as does Bioanalytical Systems BAS (West Lafayette IN, USA; Yang *et al.*, 1995). The possibility of automatic (re)calibration of the sensor by use of an internal standard solution also exists, though this does not remove the need for a calibration of the efficiency of the microdialysis probe, which is subject to the same problems of biocompatibility as a similarly shaped needle-type sensor (see Section 1.4.3 and Chapter 5). Local inflammation may alter the amount of analyte in the extracellular fluid around the probe: calibration based on a concurrent blood measurement is still necessary. The efficiency of extraction of analyte from the tissue is also in doubt. Elaborate methods involving recirculation of dialysate, or changing the flow rate systematically with measurement of the recovery of analyte (Lönnroth & Strinberg, 1995) can yield this sort of information in the laboratory but this is not feasible for a mass-market, home-use device. Compared to needle sensors, microdialysis systems are more bulky, as they need buffer reservoirs and disposal containers, and need larger batteries to drive the pump. A very precise pump is needed because a small variation in the flow rate of dialysis buffer leads to significant change in the recovery of metabolites. As flow increases, recovery decreases, and vice versa: such artefacts would be interpreted as changes of blood glucose.

A similar technique, where *in vivo* sampling is followed by *ex vivo* analysis, is "reverse iontophoresis". An anode and cathode are placed on the skin (Kim *et al.*, 1993; Rao *et al.*, 1993; Tamada *et al.*, 1995) and upon application of a potential, positively-charged and negatively-charged ions in the extracellular fluid of the underlying tissue migrate towards the cathode and anode respectively. The flow is accompanied by movement of water (typically at a rate of a few microlitres per minute) which in turn carries other substances (i.e. glucose) into the iontophoresis device. The fluid can then enter a collection device, which can be contiguous with an *ex vivo* biosensor. Potential problems here may be the limited volumes of fluid collectable by this method (hence a time delay) and the low concentrations of glucose, typically ten- or a hundred-fold more dilute than in blood or interstitial fluid (hence a need for extremely accurate glucose biosensors). The long-term reaction of skin subjected to μ A currents has not been well documented. This technology is currently being developed for glucose by Cygnus Therapeutic Systems (Redwood City CA, USA). Superficially similar are devices which rely on extraction of interstitial fluid by suction across stripped skin, coupled to a glucose sensor (Kayashima *et al.*, 1995).

Fully-implantable, electroenzymatic glucose sensors have been also proposed. These are fully-integrated units comprising a biosensing electrode, a potentiostat, a power supply, a signal transmitter and antenna/receiver, and are destined for long-term subcutaneous implantation. In terms of invasive sensing, this is clearly the ideal solution but it will only become practical if long-term performance is guaranteed. Updike and co-workers have accumulated data out

to 3 months in dogs (Gilligan *et al.*, 1994; Rhodes *et al.*, 1994; Shults *et al.*, 1994; Updike *et al.*, 1994) and these results are described by these authors in Chapter 4. Another group has also recently reported some promising, short-term results (Wilkins & Atanasov, 1995). For total implantation, surgical intervention under local or general anaesthetic would be necessary. The device would have to be reliable and long-lived enough to avoid frequent surgical removal or replacement.

Non-invasive spectrometers for *in vivo* determination of glucose have also been frequently proposed. The most popular spectral regions are the infrared and near-infrared, between 750 and 2500 nm (13 000 to 4000 cm^{-1}). Raman spectroscopy in the 200–3500 cm^{-1} range has also been suggested (Erckens *et al.*, 1993). Glucose has particular spectral features: an O–H stretching resonance, vibrational modes of its C–O, C–O–H and C–O–C bonds; C–O stretches and O–H deformations, together the overtones of these resonances due to interaction with the surrounding water (Christison & MacKenzie, 1993). These features have permitted accurate *in vitro* quantification of physiological concentrations of glucose or other analytes such as galactose, ethanol, lactate (for example in fermentation media (Picque *et al.*, 1993)). The challenge has been to measure *in vivo*, when many other chemical species are present, and when the background spectrum is fluctuating. In Chapter 3, Dr Heise describes such efforts in more detail. At the time of writing, orders were being taken in Germany and the UK for a non-invasive, *in vivo* glucose monitor (The Diasensor™ 1000, marketed by Diasense (UK) Ltd (St Albans) and produced by Biocontrol Technology, Pittsburgh PA, USA), though approval for sale in the US was refused by the Food and Drug Administration, due to concern over poor accuracy. Other, similar devices appear to be in the final stages of testing (Simpson *et al.*, 1994). The long-term accuracy and reliability of such devices is not yet known.

Furthermore, glucose can interact with light to cause a rotation of the plane of polarisation, due to the fact that biological glucose is exclusively the D-rotamer (March *et al.*, 1982; Rabinovitch *et al.*, 1982). According to other authors (Barnikol & Weiler, 1995), glucose may be responsible for over 90% of the rotative effect of blood plasma. The change in rotation of a beam of plane-polarised light passed across the cornea, through the aqueous humor of the eye has been documented (March *et al.*, 1982; Rabinovitch *et al.*, 1982). Also, subcutaneous microdialysis can be used to obtain glucose-containing ultrafiltrate samples, which are then examined *ex vivo* for their rotative power (Barnikol & Weiler, 1995). Invasive, optical sensors are also feasible: oxygen- or pH-sensitive optical sensors can be coupled to GOD to give a glucose sensor. The semi-invasive technique with oxygen-sensitive fluorescent probes incorporated in subcutaneously implanted pads of silicone (Bambot *et al.*, 1994, 1995a,b) is also being extended to detection of glucose.

A novel technique that has been evaluated for non-invasive blood glucose measurement *in vitro* is laser photoacoustic spectroscopy (LPAS). The IR

frequencies for bond resonance in glucose are excited by a laser. Part of the optical energy adsorbed is converted into thermal energy via non-radiative transitions. The localised thermal expansion produces a sound shock wave, the intensity of which is related to glucose concentration (Christison & MacKenzie, 1993; Takamoto *et al.*, 1994).

1.4 OBSTACLES TO CONTINUOUS IN VIVO MONITORING

Why have the few biosensor success stories been almost exclusively limited to one class of device and one type of application (determination of blood glucose *in vitro* with a GOD-based electrode)? And this despite the fact that behind the very conception of the biosensor (attributed to Leland C. Clark) was the intention to use the device for continuous monitoring during surgery (Clark & Lyons, 1962)? That goal has barely been achieved more than 30 years later. Clark's work did, however, trigger a minor revolution in glucose analysis during the 1970s and 1980s. Development of Clark's basic ideas by the Yellow Springs Instrument Company gave rise to a successful, widely-sold, semi-portable, clinical instrument for *in vitro* analysis of blood glucose. The subsequent major success in the mass diabetes care market was that of the Exactech self-test glucometer from MediSense (Cambridge MA, USA), now part of Abbot Diagnostics, which relies on mediation of GOD by ferrocene derivatives. An increasing variety of similar devices are now on the market, from companies such as Johnson & Johnson Lifescan, Boehringer Mannheim and Bayer. However, the success story stops there. *In vivo* (glucose) sensing has so far proved too tough a nut to crack on the commercial level. The field has suffered from much early hype, although some authors are promoting a more prudent attitude (Pickup, 1993; Gough & Armour, 1995). The next sections consider why *in vivo* sensing has not broken through. The obstacles are numerous and can be divided into several classes.

1.4.1 PROBLEMS OWING TO THE WAY THAT BIOSENSORS WORK

In much the same way as the inherent properties of biosensors make them ideal for use in continuous *in vivo* monitoring, the obstacles to achievement of that goal are also related to these properties: Such problems are also present *in vitro* to some extent but are often magnified *in vivo*.

1.4.1.1 Perturbation of the Analyte Concentration by Continuous Biosensor Operation

This is a problem when the biosensor is based on an enzyme which catalyses the reaction of the analyte. When the rate of consumption of analyte is less

than or comparable to the rate of arrival of analyte into the device, local analyte levels will decrease and will no longer be representative of tissue levels. For example, operation of an oxygen sensor based on the Clark electrode (reduction of ambient dissolved oxygen having diffused across a gas-permeable membrane) will necessarily consume oxygen. If oxygen supply to the sensor site is poor, the analyte concentration will fall and stable readings of low concentrations will not be possible. Oxygen limitation around an implanted sensor, which may also result from high local cellular activity due to inflammation, wound-healing and all processes associated with the foreign body response, or the presence of a fibrous capsule limiting oxygen diffusion (see Chapters 4 and 5), is also a potential problem for all biosensors based on oxidases (Zhang & Wilson, 1993) i.e. those that need oxygen as a cosubstrate in the analyte (substrate) reaction. If, for enzymatic catalysis, cosubstrate becomes limiting, the sensor signal will reflect cosubstrate levels rather than substrate levels, however abundant this latter might be.

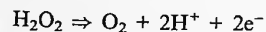
Several approaches for overcoming oxygen limitation have been employed. The most favoured tactic has been to decrease the sensor requirement for oxygen by restricting the amount of analyte (e.g. glucose) that enters the sensor: oxidase-based sensors are often covered with an outer membrane (usually a polymer such as NafionTM, polyurethane or acrylate) which slows glucose diffusion whilst allowing oxygen to diffuse relatively unhindered (van Strooe-Biezen *et al.*, 1993; Moussy & Harrison, 1994; Moussy *et al.*, 1994a,b). This is achieved by carefully balancing the hydrophilic/hydrophobic character of the polymeric membrane (Burczak *et al.*, 1994), either by appropriate choice of the constituents monomers or by blending together a number of distinct polymers. Analytes tend to be small hydrophilic molecules which diffuse only through hydrated, hydrophilic regions, whilst oxygen diffuses well through hydrophilic and hydrophobic regions. Limiting the analyte has four main advantages: (i) the sensor can continue to operate with low local oxygen concentrations; (ii) the upper limit of glucose concentrations to which the sensor responds linearly (otherwise determined by the inherent enzyme kinetics) is increased; (iii) the outer membrane material can be chosen to increase the biocompatibility of the device (see Section 1.4.3 and Chapter 5); (iv) the outer membrane may also contribute to the rejection of interferent, low-molecular weight, electrochemically active molecules (such as paracetamol, uric acid and ascorbic acid, see Section 1.4.1.3) that give rise to superfluous signals at the sensor electrode. The main drawback is that response times are lengthened and sensitivity is reduced, although a happy medium can usually be found (Zhang *et al.*, 1994). Another approach has been to introduce an "internal" supply of oxygen. This can be generated at an electrode, by application of a high, positive potential, evolving oxygen by breakdown of water. This may be achieved by intermittently changing the potential of the working electrode from the H₂O₂-detecting potential (usually +650 mV) to over 1 volt for oxygen evolution. This can also be done at a separate, dedicated electrode.

1.4.1.2 Production of Other Chemical Species During Sensor Operation

The enzymatic reaction used to recognise substrate almost always involves the production of other species. As mentioned above, most GOD-based, *in vivo* biosensors produce and then detect H_2O_2 , but H_2O_2 itself can react with GOD to cause irreversible loss of enzyme activity (Tse & Gough, 1987). Furthermore, although a proportion of the H_2O_2 is consumed by oxidation at the electrode, most diffuses out of the sensor into the surrounding tissue. Despite the widespread presence of catalase and various peroxidases in intracellular and extracellular fluids, and the general and well-recognised need to avoid carrying over exogenous, leachable, toxic and irritant components from sensor production (see Section 1.4.3), the possible effect on tissue of this endogenous "leachable" has not been well documented. H_2O_2 is itself a strong oxidising agent but more seriously, reaction of H_2O_2 with trace levels of Fe^{2+} ions via a Fenton-type mechanism can generate highly reactive hydroxyl radicals, against which the body has no enzymatic defence (Ali *et al.*, 1994). Iron can indeed be present in electrochemical sensors, as during some sensor production schemes the silver/silver chloride (Ag/AgCl) pseudoreference electrode is produced by chemical oxidation of a silver layer by immersion in an Fe^{3+} solution. Despite rinsing, traces of iron may be left in and on the sensor. One solution for reduced H_2O_2 escape is to include a superficial layer of catalase enzyme (Urban *et al.*, 1994a,b; see also Section 7.6.3).

Yet another example from the GOD-based biosensor: the enzyme-catalysed oxidation produces one proton per glucose consumed, which may lead to acidification inside the enzyme layer, changing the enzyme activity. pH 5 is the optimum for catalysis by GOD free in solution, though upon immobilisation this is usually seen to shift to nearer pH 7 (Wilson & Turner, 1992). In most cases, this is not a problem because the limiting factor in sensor response is not enzyme activity but the diffusion of glucose across an outer membrane into the device, which is less likely to be affected by pH changes. However, for some membrane materials, the change in pH may lead to protonation of ionizable groups, giving a change in the net charge of the polymer which alters the diffusion characteristics for glucose in such a case, sensor output will indeed change despite constant glucose concentration in the surrounding milieu. Changes in pH will also diminish the long-term stability of the enzyme.

For an amperometric sensor, the net proton/hydroxyl ion consumption of the electrochemical reactions at the working and counter electrodes may also influence sensor performance and lifetime, and even the body's response to such a device. For an H_2O_2 -oxidising electrode, the reaction is:



In order that the working electrode remains poised at the same potential relative to the reference electrode, the potentiostat allows the counter electrode

to drift to whatever potential is necessary to achieve the same current density as the working electrode. Although the most probable reactions are reductions of H_2O_2 ($\text{H}_2\text{O}_2 + 2\text{e}^- + 2\text{H}^+ \Rightarrow 2\text{H}_2\text{O}$) and oxygen ($\text{O}_2 + 2\text{e}^- + 4\text{H}^+ \Rightarrow 2\text{H}_2\text{O}$), other, undefined electrochemical reductions of small metabolites of biological fluids can occur. The effect of species generated at the counter electrode on working and reference electrode function (and on the surrounding tissue) has hardly been studied.

Mediated amperometric sensors avoid some of the problems and can also operate at lower applied potentials, although they have their own particular disadvantages. On one hand, the mediator molecule must be free to diffuse, shuttling electrons between the active site of the enzyme and the electrode surface. On the other hand, for *in vivo* use, the mediator should be prevented from diffusion out of the sensor into the body. Mediators used currently in commercially-available, disposable sensor strips intended for *in vitro* use have been shown to be toxic (Kulys *et al.*, 1992) or mutagenic (Gasiorowski *et al.*, 1995). However, immobilisation of the mediator usually compromises sensor performance, by slowing mediation and increasing the possibility of oxygen reacting in place of mediator, rendering the sensor once again sensitive to fluctuating local oxygen levels. To allow use of mediated sensors, several groups have covalently immobilised the mediator in some way, by covalently bonding the redox-active groups to a support. The best results have been achieved by using fast redox-couples linked to particularly flexible polymer backbones (Csoregi *et al.*, 1994a,b, 1995). These workers claim that during prolonged exposure, leaching of redox-active centres does not occur (or is below measurable levels). Whether this is the case *in vivo* is less clear: in general, the aggressive *in vivo* environment provides new pathways for faster biomaterial degradation than *in vitro*. In my opinion, until much more thorough studies have been carried out, *in vivo* use of any mediated system is unwise.

Just as worrying is the difficulty in maintaining a stable reference electrode *in vivo*. For electrochemical sensors, be they amperometric or potentiometric, a simple, stable reference electrode is a prerequisite for stable operation for days or weeks. The current produced at the measuring electrodes depends directly on applied voltage, so if the reference drifts, the effective working potential drifts too. The most frequently used reference electrode is the Ag/AgCl electrode, because of its ease of fabrication (chemical or electrochemical oxidation of the surface of a silver wire, paste or thin film) and its resistance to contamination. In fact, the Ag/AgCl electrode is a "pseudoreference" because the electrode will respond in a Nernstian manner to chloride ions (59 mV shift in potential for a ten-fold change in chloride activity), though in most cases researchers assume that the chloride concentration at the implantation site does not change enough to change the reference potential by more than a few mV.

The Ag/AgCl electrode has been shown to be moderately well accepted by the body. Nevertheless, in some implantation studies, the AgCl layer seems to

dissolve, leading to possible local toxicity for the tissues, and fluctuation of the reference (and hence working) potential of the sensor. However, in most electrochemical sensors, the reference Ag/AgCl electrode is also covered by the same polymeric, diffusion-limiting membrane as the GOD-covered working electrode, and this has been shown to afford greater stability (Moussy & Harrison, 1994). One great advantage of optical sensors is the lack of necessity of an external reference.

1.4.1.3 The Leakiness of the Bioelement/Sensor Junction, Giving Rise to Non-specific Interference

Biosensors represent the marriage between a biological molecule and a transducer: this union is not always as solid as one might hope! The ultimate biosensor would be a device where the biocomponent/sensor interface is blurred or has disappeared: the scheme of operation would be much like that of a ligand-gated membrane receptor containing an ion-channel: a highly specific binding or reaction of analyte with the surface of the transducer should trigger a conformational molecular change leading directly to the modification of an electrical parameter. In reality, the junction between the two components is leaky and can thus be a source of interference; rarely is the biomolecule the only element to produce a change in the physicochemical variable being measured by the transducer.

Taking once more the GOD-electrode as an example of a "leaky junction": at the potential necessary to detect H_2O_2 at most electrode materials, a number of other molecules present in the surrounding fluid (endogenous species such as urate and cysteine, the semi-endogenous ascorbate (vitamin C); some medicaments such as the popular headache cure (called paracetamol in the UK and acetaminophen in the USA), can also undergo electrochemical oxidation, and thus produce sensor signal unrelated to glucose concentration. In a further possible complication of the *in vivo* environment, ascorbic acid has been shown to react homogeneously with H_2O_2 (Lowry *et al.*, 1994), thus diminishing the sensor signal.

Various strategies are used to avoid such interference:

- (1) Mediated sensors, permitting a lower operating potential and hence less interference.
- (2) Electrode materials such as platinum black (Johnson *et al.*, 1994b) or particulate iridium which can catalyse H_2O_2 oxidation at lower potentials than smooth metallic electrodes (but which can also catalyse oxidation of interferents at lower potential!).
- (3) Electrode materials that catalyse H_2O_2 reduction rather than oxidation.
- (4) Covering the electrode with membranes that reject interfering molecules, either by a charge-repulsion mechanism, e.g. negatively-charged Nafion, cellulose acetate or other hydrogels rejecting positively charged urate and

ascorbate (Turner *et al.*, 1991; Higson *et al.*, 1993; Vaidja & Wilkins, 1994; Zhang *et al.*, 1994) or size discrimination—polyphenol or polydiaminobenzene are more permeable to H_2O_2 than to interferents of higher molecular weight (Sasso *et al.*, 1990). Sometimes such membranes are used to trap/immobilise the biocomponent on an electrode: the disadvantage of such an approach is that analyte may be prevented from diffusing into the enzyme layer, and sensor sensitivities and response times increase.

- (5) A "dummy electrode" which is identical to the working electrode but lacks active biocomponent. Non-specific interference is "recorded" at this electrode and subtracted from the current produced at the real working electrode. Nevertheless, adding an extra electrode adds to the size of device.

Similar sources of non-specific interference can be envisaged for most transducers: stray background light in fibre optic sensors, non-specific surface adsorption of proteins on mass-optical density-sensitive optical wave guides, surface-plasmon crystals or piezoelectric crystals, though similar schemes can be envisaged for correcting for interference. In summary, biosensors are more specific for a given analyte than transducers lacking a bioelement but there is still room for improvement.

1.4.1.4 Problems Specific to Non-invasive Sensing

Although an analyte such as glucose is present in relatively high concentrations relative to other individual blood components, when all those other components are taken together, the glucose signal is no longer distinct—it gets swamped out. The measured spectrum is a superposition of the individual fingerprint spectra of all the tissue components added together—too many "smudged" fingerprints are not useful. In some spectral regions, water has a particularly strong signal, obscuring analytes. Temperature changes can lead to large fluctuations in background intensity. As a result of these fluctuations, elaborate statistical deconvolution techniques must be employed to identify spectral features due to glucose (see Chapter 3). These often have to take into account a large number of reference spectra previously recorded in the individual with simultaneous, *in vitro* measurement of glucose blood. However, fluctuation in the relative proportions of the blood components seems too great to ensure sufficiently accurate measurements, especially in diabetics whose body chemistries are already perturbed and unstable. At least this reinforces one of the central themes and saving graces of biosensors as opposed to physisensors—their specificity, i.e. the ability to pull a glucose signal out of a sea of potential interferences.

Finally, the instrumentation needed to produce and analyse the infrared radiation is not yet small enough for convenient, mobile use, although with

the current rate of progress in solid-state physics and microelectronics, this could soon change. Despite the apparent clumsiness of non-invasive spectroscopic methods, one huge potential advantage is that, with ideal deconvolution, one spectrum could yield data simultaneously for many of the major components of the blood components—protein, urea, cholesterol, fatty acids etc. This is something that biosensors are not likely to be able to do for a while.

1.4.2 PROBLEMS OWING TO THE CHOICE OF BODY SITE/COMPARTMENT

For *in vivo* monitoring of a particular chemical, several body compartments might be considered: systemic arterial blood; venous blood; the microvascular system; in the subcutaneous tissues; in the peritoneal cavity; in the brain etc. The final choice depends on a number of parameters.

In critical care situations, systemic blood values are necessary. These are thought of as the "gold standards"; they allow easier comparison with other patients, other analytical apparatus and other clinical studies; overall they give the best indicators of patient status. For *in vivo* sensing to contribute all its inherent advantages, in-blood measurement would be most desirable. Nevertheless, intravenous operation invasive devices is subject to serious blood-compatibility problems, principally blood clotting on and in the device. Even widely-used, well-protected devices such as cardiac pacemakers do have a proportion of device failures due to thrombus formation which may, over months, trigger radical changes in the surrounding tissue (see Chapter 5): collagenous capsules have even been observed to differentiate into bone-like tissue (Stokes *et al.*, 1995). Long-term, intravascular biosensing has not convincingly been demonstrated. Short-term sensing with successive implantations of disposal sensors risks infection and damage to the blood vessels.

Persistent problems with blood compatibility have lead to an appraisal of other body sites/fluids for *in vivo* biosensing which contain measurable levels of analyte, preferably correlated in time and concentration with blood levels. The most obvious choice is extracellular fluid. This can be viewed as an ultrafiltrate of blood plasma: clotting problems are avoided whilst the level of many analytes is similar to that found in blood. Most of the current attempts to biosense glucose probe this fluid in some way; by insertion of a needle, by suction across skin (Kayashima *et al.*, 1995), or by reverse iontophoresis (Kim *et al.*, 1993; Rao *et al.*, 1993; Tamada *et al.*, 1995). The most popular implantation sites are in the fatty tissue around the waist, in the forearm, or on the wrist. Definitive studies comparing the sensing results at various body sites for a single individual or across groups are not available. Furthermore, doubt remains as to the relationship of subcutaneous glucose to blood glucose concentrations (Rosdahl *et al.*, 1993; Schmidt, F.J. *et al.*, 1993; Réach & Poitout, 1994; Sternberg *et al.*, 1994a). Various groups have experimentally

determined different figures for subcutaneous glucose, ranging from 50% to 100% of blood glucose, with 75–90% the most common range. How this varies in a population, or whether this is constant for a given individual from one day to the next, has not been well documented. However, this debate may be of little importance, if a device has to be calibrated *in vivo* after implantation.

More problematic is the use of continuous monitoring in neural tissue: "minimally-invasive" implantation is hardly possible. Intracranial implantation of microelectrodes is an option during surgery but long-term monitoring will not be possible until highly miniaturised, fully autonomous implantable units are available. Even then, apart from some specialised instances such as the need to give prior warning of violent seizures, the widespread application of such techniques is unlikely. Nevertheless, as shown in Chapter 6 by Dr Robert O'Neill, biosensing in neural tissue is a valuable research tool in humans and laboratory animals to aid diagnosis and description of the mechanisms of mental illness, and to study the efficacy and side-effects of neuroactive drugs.

1.4.3 BIO(IN)COMPATIBILITY OF INVASIVE DEVICES

This section will introduce the issues which are treated in greater detail by Ziegler & Schlosser in Chapter 5. An invasive sensor is necessarily in contact with the chemicals that it senses. It can never be isolated from its milieu like a temperature sensor or pressure sensor: complete encapsulation preventing physical contact is impossible, for example. *In vivo* this becomes a serious problem.

In my opinion, the greatest obstacle to *in vivo* biosensing is device biocompatibility—or rather, lack of it. Why should this be so? Over the last few decades, many hundreds of materials and objects—artificial hip and knee joints, heart valve replacements, cardiac assist devices, cardiac pacemakers, intraocular lenses, dental and facial reconstructs (Williams, 1989) have been fairly successfully implanted in the body, with residency times of hours to years, depending on function. The vast worldwide activity in biomaterials is testimony to the ability of the body to tolerate long-term implants of non-living materials under certain circumstances, so why should biosensors be any different? For biosensors, mere toleration of the device is not sufficient. Biocompatibility works both ways: even if the sensor is well tolerated by the body, the body is not always well tolerated by the device (Bobbioni-Harsch *et al.*, 1993; Pickup *et al.*, 1993). In the vast majority of cases, the biosensor progressively loses all function, be it after a few hours, days, weeks or exceptionally months (Armour *et al.*, 1990; Gilligan *et al.*, 1994).

A catalogue of reasons for this failure can be built up. The most striking example is that of the blood-incompatibility of intravenous sensors (Marconi, 1993). After a few seconds of blood contact, a layer of blood proteins adsorbs

on the sensor surface. This layer may then change over time (seconds, minutes) in terms of protein composition and structure. It is thought that some of the proteins in the layer change their shape from their "native" configuration (i.e. that of the protein when circulating in blood) to a denatured configuration. This exposes new surfaces that can interact with components of the blood-clotting cascade, which sooner or later is triggered. The end result is thrombus formation—a thick layer of fibrin protein fibres, with entrapped blood cells and platelets. Despite progress in developing materials that are less thrombogenic (the term "non-thrombogenic" is too optimistic), where the time-scale for clotting is shifted from seconds or minutes to tens of minutes, clotting is inevitable. For some sensors, this clotting is fatal—the sensor "window" is blocked and analyte cannot diffuse into the sensor. In other cases, such as gas or pH sensors, even if the clot is permeable and some function is maintained, *in vivo* recalibration may be necessary. However, preventing the adsorption of protein (or species of molecular weight greater than a few thousand (Elbicki & Weber, 1989)) is far from being the only problem in biocompatibility of implantable devices. Small molecules (such as the sulphur-containing amino acid cysteine, or a metabolite such as glutathione) may also adsorb to form a blocking layer: furthermore, those which are electrochemically active may well react at the electrode to form insoluble products, thus "poisoning" the sensor. Hydrogen peroxide oxidation at a noble metal electrode is an electrocatalytic process involving surface-adsorbed peroxide and derivatives, so it is also possible that certain small metabolites or ions prevent or slow this catalysis.

Despite progress in the miniaturisation of electronic sensors, all implantable devices are relatively large (from hundreds of μm to tens of cm) compared to cells or small blood capillaries (1–100 μm). For example, needle-type glucose sensors are small enough to avoid use of local anaesthetic for self-implantation but their insertion of a biosensor into subcutaneous tissue creates widespread damage to capillaries, and with millions of cell–cell contacts broken. Even though nano- and microelectrodes (those made from carbon-fibres, or finely-drawn glass tubes) have diameters on the micrometre scale, the associated wire connections and encapsulation push up the size of the whole device. Elaborate mechanical means have been proposed for automatic implantation of needle sensors (Mastrototaro, 1994; Lord *et al.*, 1995), although my own experience is that implantation by hand is well accepted by patients. Sensor size, texture and shape (Figure 1.3) are also likely to have consequences for biostability of a device (see Chapter 7).

Using a novel application of laser Doppler analysis (Anderson *et al.*, 1994), the implantation of microdialysis catheters has been shown to cause acute local changes in tissue perfusion around the implantation site out to a radius of a few cm, and these changes persist up until to one hour post-implantation. Nevertheless, in all such cases, doubt remains as to what the sensor signal during the first hour or two really means: most groups admit that a "run-in" is

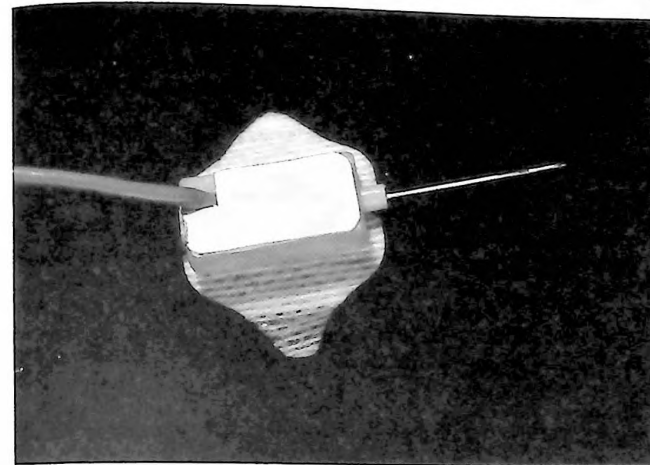


Figure 1.3 Work is proceeding on resolving packaging and handling issues, to allow needle-type sensors to be integrated into a semi-disposable format. Reproduced with permission from Disetronic Medical Systems

inevitable. Although this is clearly not ideal, it would be acceptable if the sensor continued to work perfectly after this period.

Immediately upon contact with extracellular fluid (and inevitably some blood from damaged vessels), proteins and other molecules will adsorb on and diffuse into the sensor, blocking the membrane and poisoning the electrode. Changes in native protein conformation may provoke the activation of the complement system or elements of the immune response. Acute inflammation accompanies the trauma of sensor implantation and is inevitably part of the foreign-body response to the sensor. Cellular and humoral responses are elicited. The tissue damage accompanying sensor implantation is followed over the next four days by the recruitment of white blood cells, i.e. neutrophils, monocytes (days 1–2) and phagocytic macrophages (days 2–3) (Park & Lakes, 1994). In a so-called "respiratory burst", macrophages secrete a battery of chemical weapons (Fernandez-Urrusuno *et al.*, 1995) such as hydrogen peroxides, superoxide radicals and lytic enzymes which can have dramatic effects on the chemical structure of implants (Labow *et al.*, 1995). The environment around the implant becomes rapidly acellular, filled with cell debris and degraded material from the implant. The pH and ionic composition of the fluid may change. This attack on the implant and the surrounding tissue is then generally followed by a period of intense reconstruction (days

3-9); fibroblasts migrate to the implant-tissue interface and begin to deposit collagen fibres (days 4-12). A fibrous capsule is progressively formed, in most cases acellular and not permeable to the analytes of interest. Few capillaries are present and thus the region becomes hypoxic or anoxic (notably having severe consequences for sensors detecting blood gases, or those which are based on oxidases), though in some cases thin, viable capsules have been seen (Menconi *et al.*, 1995). The sensor becomes isolated, deprived of analyte and the *in vivo* signal fails.

In the worst cases, chronic inflammation occurs around the sensor implant site. This can be caused by several factors. The loss of chemicals from the implant, either those originally present (leachables, e.g. unreacted monomer from a polymeric membrane, or the biomolecule used to detect analyte) or those formed through biodegradation (see Chapter 5), can have toxic and mutagenic effects. The enzyme and antibodies used in biosensors are never of human origin and as a result are often highly immunogenic. Leakage of the sensor's biocomponent(s) is further discussed in Section 1.4.6. The release of particulate micrometre-scale matter from the sensor provokes the activation of macrophages which secrete eicosanoid and cytokine compounds (strong stimulators of the inflammation response) together with active oxygen species from a respiratory burst (Fernandez-Urrusuno *et al.*, 1995). Carcinogenesis can be associated with particulate debris in rats, though it is thought to be less likely in humans. Nevertheless, in a novel *in vitro* cell culture "metabolic co-operation" test, it was suggested that silicone and polyetherurethane polymers were tumour-promoting (Tsuchiya *et al.*, 1995).

The two main humoral lines of defence are the immunoglobulin and complement systems. Recent controversy over the use and safety of silicone breast implants (Kossovsky & Freiman, 1995) has fuelled a debate as to whether all or certain implanted materials provoke a specific response by the immune system. Recent evidence suggests that common biosensor membrane materials such as cellulose acetate do evoke specific antibody secretion (Schlosser *et al.*, 1994; Ziegler *et al.*, 1994; and see Chapter 5 by these authors). These would bind to the implant surface and mark it for attack by macrophages or cytotoxic T-cells, which engulf or attach to the foreign body and secrete a large number of surface-active peptides and potent hydrolases. Even though the implant may not be directly affected by these agents, the release contributes to chronic inflammation. A further humoral mechanism is the complement system of blood: this has been blamed for certain incompatibilities seen during venous dialysis over cellulose-based membranes. The complement system is comprised of a series of proteins in the blood, and indeed shares similarities with the "cascade" clotting of blood. The natural role is to kill blood-borne bacteria or parasites by puncturing their outer membrane. For implants, foreign material binds one of complement proteins, altering its conformation. The altered protein triggers a change in conformation of the next link in the system, and so on, magnifying the effect. One

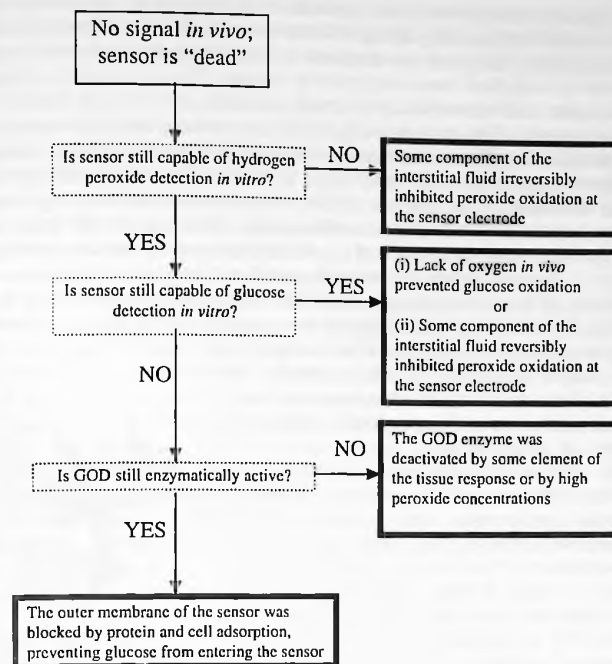


Figure 1.4 Post-implantation *in vitro* behaviour can often reveal the source of biostability, although often several deactivating mechanisms operate simultaneously. Reproduced with permission from Advanstar Communications

effect of complement activation is promotion of monocyte adhesion to the foreign surface (McNally & Anderson, 1994).

In broad terms, it is possible to tell which sensor-deactivating mechanisms are operating by testing aspects of *post vivo* sensor response (Figure 1.4). In practice, the answer is often most or all! Histological analysis of the implant site to study capsule growth and structure is possible in some case by explanting the sensor with the surrounding tissue: this is excluded in humans and is becoming less acceptable in laboratory animals. Withdrawal of a percutaneous sensor before study of the implant site is unreliable, as it is likely to damage the morphology of the wound and complicate histological analysis of the exposed surface of the explanted sensor.

These phenomena have led to a search for surfaces which seek to improve biocompatibility by manipulating or diminishing the device's interactions with body proteins—this search is not specific to biosensors but is performed for all manner of body-fluid contacting medical devices. This can mean modifying the existing surface material of a device, or adding a new outer layer of a special material. The simplest methods involved creating particular chemical functionalities on the existing surface, for example hydroxyl, carboxyl, amine, sulphonate or phosphate groups. This is done either by reaction with derivatising chemicals, or by exposure of the surface to reactive ions (e.g. a gas plasma) or ionising radiation. In some cases, adsorption of one particular protein can indeed be diminished or slowed by covering the device with a particular chemical group: for example, a surface that is anionic by virtue of an excess of ionised carboxyl groups may reduce adsorption of an anionic protein. Nevertheless, the biological milieu contains so many different proteins that there will always be some able to adsorb, and indeed surface treatment may increase adsorption of certain proteins while decreasing adsorption of others. Some materials are thought to be "inert": diamond-like carbon has been proposed as a biocompatible composite barrier for glucose sensors (Higson & Vadgama, 1993).

It is naive to think that biocompatibility can be achieved with such simple schemes. Other chemical approaches are based on more complex physico-chemical considerations. One of these is to graft poly(oxyethylene) (PEO) chains onto a surface. These chains tend to line up parallel to each other and perpendicular to the surface to present a water-rich phase that resists penetration of many protein molecules (Claesson, 1993). The outer surface is hydrophilic and uncharged by virtue of the end-hydroxyl groups. The way in which PEO is hydrated is thought to be responsible for these effects. PEO-containing membranes have been applied to implantable glucose sensors (Quinn *et al.*, 1995). Another strategy that has met with some success is the creation of a blood-contacting surface that bears phosphorylcholine (PC) groups, i.e. a surface thought to resemble the phosphatidylcholine-rich, outer leaflet of the lipid bilayer membrane of red blood cells (RBCs), these being haemocompatible by definition. It should, however, be pointed out that the RBC membrane contains many proteins (particularly glycosylated proteins) that project far into the extracellular space, so the RBC surface is probably not the lipid bilayer.

The diminution of protein adsorption on PC-containing surfaces is well-proven, though the underlying mechanism is not clear. The PC group is zwitterionic at physiological pH, i.e. it contains an anionic (phosphate) and cationic (choline) group in close proximity, and is also highly and strongly hydrated, so this may explain some of its ability. PC-containing polymers (such as polyacrylates and polyurethanes) are also used in biomedical devices such as dialysis membranes (Ishihara *et al.*, 1995) and elastomers as joint replacements (Williams *et al.*, 1995). *In vivo* biosensors have been derivatised

or synthesised to include PC-containing groups/monomers although few results are available (Ishihara *et al.*, 1994). The poly(oxyethylene) and PC approaches have recently been combined in one polymer (Ishihara *et al.*, 1996).

Another approach for increasing blood compatibility has been to use molecules with biological activity such as the glucosaminoglycan heparin, the well-known anticoagulant, which may be temporarily immobilised on the surface of a device by physisorption, or permanently fixed by covalent chemical bonding to polymers (Marconi *et al.*, 1993). An intelligent approach is to use biological functionalities (for example fibronectin or RGD-peptides that promote cell attachment; Anderheiden *et al.*, 1992) to modulate the host response to an implant; this tactic should be adopted for implantable sensors.

A recent, novel approach to avoid bioincompatibility is the so-called "open flow microperfusion" as applied to a subcutaneously-implanted glucose biosensor (Kyröläinen *et al.*, 1995; Rigby *et al.*, 1995). The aim is to have a slow, constant flow of externally-stored buffer across the outer surface of the sensor. This is not microdialysis because the buffer does not leave the body and return to an external sensor. Apparently, this microflow prevents (or at least delays) adsorption of proteins whilst permitting glucose to diffuse across the mobile buffer layer and into the sensor. A claimed advantage is that once the sensor is calibrated *in vitro* with microflow, the same sensitivity is observed *in vivo*, thus eliminating the need for *in vivo* calibration (see below). This would be a major step forward but at the time of writing, the long term performance of such a system had not been reported.

1.4.4 LACK OF STRATEGIES FOR IN VIVO CALIBRATION

As mentioned above, changes at the sensor-body interface mean that *in vivo* sensitivity (the current per concentration unit of analyte) falls rapidly below the pre-implantation, *in vitro* sensitivity. At the same time, the *in vivo* "background current", i.e. that produced by the sensor when hypothetically zero analyte is present (i.e. the intercept on the current axis of a current/analyte concentration plot) *in vivo* is likely to be higher than *in vitro* due to current produced by electrochemical interferents. If the *in vitro* "factory parameters" for slope and intercept are used, the sensor would therefore grossly underestimate the analyte concentration (Figure 1.5).

This means that to establish a useful relationship between *in vivo* current and the level of analyte actually present around the sensor, an *in vivo* calibration cannot be avoided. The simplest approach is a one-point *in vivo* calibration. A venous or capillary blood sample is taken from the patient and analysed with a conventional *in vitro* test. Then the current output (minus the assumed background) at the moment of the blood-sampling (or with a time-lag) is divided by the blood analyte concentration to yield the momentary *in*

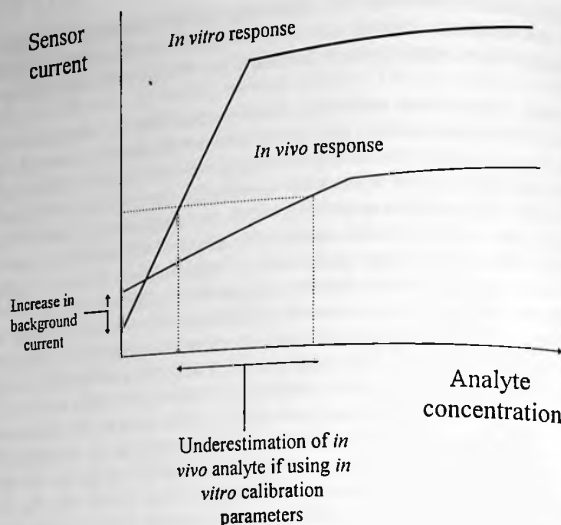


Figure 1.5 Schematic diagram showing the likely change of sensor response when comparing *in vitro* and *in vivo* analyte sensing

vivo sensitivity. The background is often assumed to be the same as *in vitro*, or a higher but arbitrarily fixed value. In order to better estimate the *in vivo* background, a two-point calibration can be used; two blood samples are taken, ideally one point when blood analyte is low and one when it is high. The sensor response is assumed to be linear over this range, and these two points are linked to provide a straight line with a slope and an intercept.

Nevertheless, if these procedures were used in a commercial device, they would probably need to be performed/overseen by a medical professional, thus increasing cost and inconvenience and making the sensor less attractive. This will only be a valid approach if *in vivo* sensitivity stabilises for a length of time, thus decreasing the frequency of subsequent *in vivo* calibrations. Section 2.3.6 addresses these issues. The general problem has also been reviewed in more detail (Sternberg *et al.*, 1994b).

1.4.5 THE TECHNICAL DIFFICULTY OF MASS PRODUCTION OF SENSING DEVICES

The fabrication of sensing devices is treated in more detail in Chapter 7: the main issues can be summarised as follows:

Most biosensor devices used in preliminary *in vivo* studies have essentially been made by hand: a series of fine wires or optical fibres wound or glued together, surrounded by insulating housings, with the tips being dipped in solutions of the biocomponent and any additional polymeric materials. Much has been borrowed from the pipette-pulling technique to make traditional miniaturised glass electrodes, used for observing changes in cell membrane potential via the patch/clamp technique. Recent interest in the attractive electrochemical properties of microelectrodes and nanoelectrodes (high signal-to-noise, ability to observe very fast electron transfer) has prompted the use of micrometre platinum wire and carbon fibres (Seddon *et al.*, 1995), but these techniques are still essentially manual.

More complex sensors are made using the techniques that led to the microprocessor revolution, i.e. based on the deposition and patterning of thin films of metals, insulators and semiconductors. Nevertheless, the presence of biomolecules and other polymeric materials used in sensors but not in standard microelectronics means that sensor fabrication can be divided into generic process steps (where the conducting and insulating sensor structures are created and patterned) and sensor-specific steps (for example, where the biocomponent is deposited and patterned). These latter steps tend to be delicate, costly and time-consuming, and are potential barriers to mass fabrication. Biomolecules are usually not compatible with the high temperatures, UV light, organic solvents, and extreme pH and redox conditions often used in thin-film technology. Most sensors have an active window through which analyte diffuses, and thus the layer which defines this layer also serves to electrically insulate underlying structures. Not all the insulating materials used in thin-film technology are biocompatible, so they must be chosen with care (Bowman, 1986 #267). Nevertheless, some elegant sensor fabrication schemes based on thin-film technology have been developed (Morff *et al.*, 1990; Mastrototaro *et al.*, 1991; Johnson *et al.*, 1992, 1994a,b; Steinkuhl *et al.*, 1994). Similar thin-film sensors for glucose, pH, potassium and calcium ions have been extensively tested for biostability and biocompatibility *in vivo* (Lindner *et al.*, 1993, 1994, 1995; Cosofret *et al.*, 1994a–c, 1995; Buck *et al.*, 1995). Biocompatible microstructures are also of interest as cell culture substrates, as neural implants and prostheses (Schmidt *et al.*, 1993), and as base materials for bioartificial organs (Meyer & Biehl, 1995).

Fabrication methods which have proved successful for producing *in vitro* diagnostics/biosensors may not be applicable to *in vivo* devices. Screen-printing (serigraphy) is one example. This "thick-film" technique allows millions of disposal biosensing strips to be produced, being well-suited to planar devices on the centimetre/millimetre scale, with layers of bio- and electrode components tens or hundreds of micrometres thick (Goldberg *et al.*, 1994; Galan-Vidal *et al.*, 1995). A wide range of flexible and rigid, plastic and ceramic substrates may be used. The ink materials are essentially particulate pastes containing electrode materials (carbon powder, silver/silver chloride,

gold, palladium and platinum pastes), solvent and polymer fillers. Biomolecules can also be incorporated in such pastes, with careful choice of the other components. The pastes are printed by pressure through a mask onto the support, to define contact pads, connecting tracks, electrode windows and so on, and are then fired, cured or dried. The present tendency is to use pastes which cure at low (<60°C) temperatures, in order to preserve the activity of any constituent biomolecules and to permit use of flexible plastic supports.

Insulating layers can also be printed to define electrode windows and to cover connecting tracks. However, the precision of screen-printing is lower than for thin-film techniques: the smallest objects currently printable are generally in the 90–100 µm range, with a resolution of 25–50 µm. Nevertheless, when allied with laser machining to expose conducting materials by punching holes in an overlying insulating layer (Seddon *et al.*, 1994), smaller (10–15 µm) features such as electrode windows can be produced. Laser machining has also been used to make the sampling lumen for catheter-tip, intravenous pO₂ sensors (Spencer *et al.*, 1995). Fully implantable devices with fewer size restrictions can use centimetre-scale, screen printed electrodes. This would have the advantage of increased surface area and thus increased current. However, production of thick-film electrodes for *in vitro* glucose testing do appear to suffer from variability in sensor performance. Despite the experience acquired by several companies in this domain, such sensor strips still need batch calibration before use.

As noted above, the presence of the biomolecule complicates the biosensor fabrication process. Apart from the need to avoid certain conditions during manufacture, it is also difficult to immobilise biocomponents, densely packed and with high spatial resolution. Biomolecules are often amongst the most costly components per unit weight in biosensor manufacture. To minimise cost, and also to reduce the risk of leakage mentioned below, it is advisable to deposit the biocomponent only where it is needed; for example, glucose oxidase should only be deposited on the working electrode of a glucose sensor. In most *in vitro* biosensors with commercial pretensions, the biocomponent is deposited by screen-printing or ink-jet printing. However, for miniature sensors for *in vivo* use, limiting the deposition of the biomolecule to electrodes or sensing windows on the micrometre scale becomes difficult. Despite the availability of micropipettes that can deposit submicrolitre volumes with good spatial and volume precision, such individual-sensor techniques are too slow for mass production, where whole-batch or whole-wafer steps are preferable. Biomolecule-containing inks must contain a certain amount of water, buffer or other solvent to be printable: when the ink dries, the biomolecule is exposed to high salt concentrations. Variation in the rate at which drying occurs will also introduce varying losses in biocomponent activity, and thus possible variation in sensor performance. If long-term sensing is to be achieved, a sufficient reserve of biocomponent must be contained in a sensor. Biomolecules are not always highly soluble in solution,

and so multiple drops must be applied and dried sequentially in order to achieve the desired loading. Nevertheless, some UV-polymerisable enzyme pastes compatible with standard photolithography have been proposed (Rohm *et al.*, 1995). Some form of chemical crosslinking between protein molecules is usually needed to retain them durably on the transducer (Wong, 1992).

Probably the best technique used to date is electrodeposition (Johnson *et al.*, 1994a). Most biomolecules contain groups that are either positively or negatively charged when ionised in solution. The relative proportions of these determine the pH at which the molecule has no net charge (pK_a) and thus by choice of a buffer pH on one side or the other of the pK_a, the net global charge is known. Next, the sensor electrodes are immersed in solution and polarised with the opposite charge. There is some debate as to the exact mechanism of deposition: real electrophoresis where attraction of opposing charges leads to adsorption on the electrode, or precipitation of the biomolecule on the electrode caused by the local pH change resulting from the sometimes extreme electrode potential used (Im *et al.*, 1995). Nevertheless, the end result is a thick cake of biomolecule deposited only where the electric field was present, thus solving the problem of how to spatially control deposition. The deposited mass is then chemically crosslinked (usually with glutaraldehyde) to achieve maximum, irreversible immobilisation (Wong, 1992). Proteins can also be co-deposited from solutions containing metal salts such as those of ruthenium (Sakslund *et al.*, 1995), or from solutions containing monomers such as pyrrole, phenylenediamine or phenol, which can be oxidised at the electrode to form adsorbed linear and branched polymer chains, thus simultaneously trapping the protein (Malitesta *et al.*, 1990).

Non-invasive, spectroscopic systems, which require high precision and stability to allow reliable measurement of small optical changes, are also difficult to produce in mass, cheaply and in a portable format that allows ambulatory monitoring. In some cases, "portable" seems only to describe the attachment of the light source to the body, and ignores the need for such things as a notebook computer, a N₂-pumped layer and other paraphernalia (Brennan *et al.*, 1993). The weight of batteries needed to power some optical devices appears also to be prohibitive. The first non-invasive glucose monitor proposed commercially (The Diasensor™ 1000, marketed by Diasense, UK Ltd, St Albans, UK, and produced by Biocontrol Technology, Pittsburgh PA, USA) is the size of a small television set and is not portable—unless portable is taken to mean unplugging, carrying and plugging-in somewhere else. Nevertheless, given the current pace of progress in solid-state light sources and detectors, these technical problems will probably be solved (Spanner & Niessner, 1996).

Additionally, any device destined for implantation must be sterilised by destroying the biomolecules of any bacteria and viruses present. This also destroys some of those of the biosensor. The techniques currently used include gamma- and electron-beam radiation, sometimes combined with a disinfecting

surface wash of H_2O_2 (von Woedtk *et al.*, 1994). Luckily, a sufficient excess of biomolecule in the sensor, coupled with clean production methods and minimum doses of radiation allows devices to survive sterilisation with sufficient remaining activity. For long-term *in vitro* biosensing, stability of the biocomponent is an issue (Katakis & Dominguez, 1995). At the moment, this is less important for *in vivo* work because poor biocompatibility causes device failure long before the activity of the biomolecule becomes limiting. However, if long-term biocompatible implantable sensors are ever achieved, this may become an issue. The group of Updike (see Chapter 4) has calculated that maximum lifetime of their glucose oxidase containing sensor would be around two years under ideal circumstances (Rhodes *et al.*, 1994). Some researchers have proposed "refillable" sensor designs which allow replacement of deactivated GOD (Atanasov & Wilkins, 1993b; Xie *et al.*, 1994).

1.4.6 CONTAINMENT OF THE SENSOR'S BIOMOLECULE

The biomolecules contained in biosensors tend to be extracted from microbial or plant sources (in the case of enzymes), from mouse hybridoma cells (in the case of monoclonal antibodies) or mammalian blood plasma (in the case of polyclonal antibodies). If such a "non-self" biomolecule is not sufficiently immobilised or retained in an implanted sensor, it will escape into the body, where it may exert a toxic effect. Even though many sensors are covered with membranes that are supposedly semi-permeable, i.e. they allow diffusion of small molecules like glucose while holding back large ones like proteins, the biocomponent will inevitably, if slowly, leak out if not completely immobilised; if a membrane is permeable enough to analyse to obtain a response on the timescale of seconds or minutes, this means that the biosensor's large biocomponent, although possessing a diffusion coefficient hundreds or thousands of times smaller than the analyte, will diffuse out on a timescale of hundreds or thousands of seconds or minutes. GOD has an acute toxicity by virtue of its consumption of oxygen and generation of hydrogen peroxide although the amount contained within a typical sensor would be too small to cause systemic damage, and would be quickly cleared from the body. A more serious consequence would be acute inflammation and immune response directed against the biocomponent. If a semi-disposable, needle-type sensor were to be used, sensitisation might be provoked by the repeated introduction and leakage of the sensor's biocomponent. Nevertheless, recent experiments have shown the absence of anti-GOD antibodies in rats with implanted GOD-based sensors (Schlosser *et al.*, 1994; Ziegler *et al.*, 1994) but more data are needed.

1.4.7 ECONOMIC AND REGULATORY FACTORS

Mass fabrication of disposable (bio)sensors has been achieved in three main areas:

- (1) *In vitro* self-monitoring of glucose.
- (2) Rapid point-of-care testing for blood glucose, urea, gases and electrolytes (i-STAT Corporation, Princeton NJ, USA; Cozette *et al.*, 1991; Woo *et al.*, 1993).
- (3) *Ex vivo* blood gas monitoring.

In the previous section, it was suggested that mass fabrication of sensors suitable for *in vivo* use—for example disposable, needle-type sensors costing \$10–20 is feasible, but costly and time-consuming. Tens or hundreds of millions of dollars of capital will be needed. Few companies are large enough and healthy enough to make this sort of high-risk investment, despite the potential returns. This consideration excludes many small and medium sized companies from sensor development. Nevertheless, my rough estimate is that 15–20 companies worldwide (see Table 1.1) are working on implantable sensors, related microdialysis devices, or non-invasive spectrometers. Whether any of this activity will provide a world-beating product remains to be seen.

Because few *in vivo* sensing devices have actually been submitted for sale to the medical sector (let alone the general public), regulatory issues have rarely been discussed. In the mid-1990s, the tendency in Europe is towards ever tighter control of medical devices of all classes, in an effort to protect consumers (Schmalz, 1995). At the same time in the USA, the regulatory body for such instruments, the Food and Drug Agency (FDA), is attempting to accelerate device approval (or rejection) by a series of administrative reforms, without necessarily relaxing standards. One possible source of confusion is the classification of such devices as active medical devices, where the residency time in the body determines the stringency of testing requirement prior to device approval. The longer the device stays implanted, the stricter the criteria for approval. For example, a device which is implanted for longer than 24 h must pass a bank of tests to show that the device and its constituents are not potentially mutagenic or carcinogenic (Eloy, 1994).

There is also a fine line between device and drug in some cases of implantable sensors. Devices do not achieve their action principally by pharmacological means. But how should one treat an intravenous glucose sensor covered with an antithrombotic enzyme (Ryu *et al.*, 1994) or an analogue of a blood clotting inhibiting factor? The sensing mechanism does not have much pharmacological significance but its components may do, in order to prolong and protect sensor function. Usually, the non-sensing elements of the device (heparin, for example) will have been used in previously approved pharmaceutical preparations, and do not represent major obstacles to approval of the whole device. Matters become more complicated when pharmacological agents are used together with sensor to ensure their optimal functioning. The situation is then greatly complicated as, in general, drugs undergo far more extensive regulatory scrutiny than do medical devices.

Table 1.1 Commercially-oriented in vivo sensing projects and their status

Company	Type of device	Probable status	References
Amplicific SRL (Milan, Italy)	Subcutaneous microdialysis with <i>ex vivo</i> glucose sensor	Prototype	Bernardi, 1993
AVL (Linz, Austria)	Subcutaneous microdialysis with <i>ex vivo</i> glucose sensor	Pre-production	Skrabal <i>et al.</i> , 1995
Biomedical Sensors/Pfizer (High Wycombe, UK)	Intravenous, catheterised blood gas monitor	Production	Company brochures
Cygnus Therapeutic Systems (Redwood City CA, USA)—recently acquired by Becton Dickinson	Reverse iontophoresis unit on wrist coupled to glucose sensor	Pre-production	Tamada <i>et al.</i> , 1995
Diasense/Biocontrol Technology (Pittsburgh PA, USA)	Non-invasive, infrared spectrophotometer for glucose determination	Orders being taken in UK and Germany	Barnes <i>et al.</i> , 1995; company brochures from Diasense (UK) Ltd (St Albans)
Disetronic Medical Systems AG (Burgdorf, Switzerland)	Needle-type glucose sensor	Prototype	Fraser <i>et al.</i> , 1996
Futrex Inc., (Gaithersburg, Maryland, USA)	Non-invasive, infrared spectrophotometer for glucose determination	Prototype/pre-production	Rosenthal <i>et al.</i> , 1991, 1995
E. Heller & Company (San Francisco, USA)	Needle-type glucose sensor	Prototype	Coregi <i>et al.</i> , 1995; Quinn <i>et al.</i> , 1995
Eli Lilly & Co. (Indianapolis IN, USA)	Needle-type glucose sensor	Prototype	Morff <i>et al.</i> , 1990; Mastroiardo <i>et al.</i> , 1991; Johnson <i>et al.</i> , 1992
Markwell Medical Institute, Inc. (Madison WI, USA)	Fully-implantable, subcutaneous glucose sensor	Prototype	Rhodes <i>et al.</i> , 1992
MimiMed Inc. (Sylmar CA, USA)	Needle-type glucose sensor	Prototype	Cheney & Van Antwerp, 1995; Lord <i>et al.</i> , 1995
Nat. Applied Science (Portland OR, USA)	Needle-type glucose sensor	Prototype	Poitout <i>et al.</i> , 1993
N.V. Nederlandsche Apparenten Fabriek (De Groenlo, Netherlands)	Fully implantable, subcutaneous glucose sensor	Unknown	Hogen Esch, 1994
SpectRx Inc. (Norcross GA, USA)	Fluorescence spectrometry of subcutaneously implanted silicone pad: determination of oxygen/glucose	Prototype	Bambot <i>et al.</i> , 1995a,b
Ultrasonic Sensing & Monitoring Systems, Inc. (Kansas City MO, USA)	Vascular stent with glucose sensor incorporated on inner surface	Prototype	Winston & Neet, 1995
Unitec Ulm (University of Ulm, Germany)	Subcutaneous microdialysis with <i>ex vivo</i> glucose sensor	Prototype	Pfeiffer, 1994
Vuman Ltd/Sensor Solutions Ltd (Manchester, UK)	Needle-type glucose sensor with open flow microperfusion	Prototype	Rigby <i>et al.</i> , 1995

1.4.8 SAFETY CONSIDERATIONS

The goal of many researchers working on *in vivo* biosensors is the closed-loop system. Despite numerous patents and papers claiming a portable artificial pancreas, only devices intended for bedside use and clinical study have been marketed. By virtue of frequent intravenous sampling and *ex vivo* analysis followed by intravenous insulin release, such devices can indeed "clamp" blood glucose concentration about a mean value with some acceptable fluctuation (Heinemann & Ampudia-Blasco, 1994). The situation for non-intravenous body sites such as subcutaneous tissue is more complicated because of the increased time lags involved: the lag between a change in glucose concentration in blood and that in subcutaneous tissue, and the lag between insulin release into subcutaneous tissue and its effect on systemic blood glucose. The end result is, however, still likely to mean far better control of glycaemia than at present achieved by many diabetics (even those on intensive insulin therapy) and therefore a worthwhile goal.

All such plans for closed-loop systems pre-suppose that the sensor itself functions perfectly but if a sensor were to fail, i.e. with a signal falling to background despite a high ambient glucose concentration, the constant high glycaemia would rapidly lead to complications. If a sensor malfunction were to produce a large signal unrelated to the real glycaemia, insulin would be introduced in possibly fatal quantities. If perfect functioning cannot be assured, and continuous monitoring use has to be backed up by regular fingersticks, such a system rapidly loses its added-value and becomes non-viable from a marketing point of view. If the luxury of continuous *in vivo* measurement is accompanied by dubious reliability, such devices will have little or no advantage over reliable *in vitro* methods. Clearly, until (if ever) 100% sensor reliability is achieved, only open-loop systems are feasible, and insulin dosage will remain the decision of diabetic people and their medical advisers.

1.4.9 COMPETING TECHNIQUES

In exceptional circumstances, diabetes can be treated by whole pancreas transplantation. This is already performed for diabetics with kidney failure (Larsen *et al.*, 1994; Lacy, 1995) but needs subsequent life-long immunosuppression. More importantly, numerous reports of work on the "bioartificial pancreas" are being published, where β -cells from humans or pigs are encapsulated in semi-permeable polymers to (i) allow insulin release and (ii) protect their rejection by the immune system (Réach, 1993b, 1995; Lelli *et al.*, 1994). The same tactic has been used for transplantation of cells to the nervous system (Gentile *et al.*, 1995). Nevertheless, biocompatibility and long-term survival of active insulin-secreting cells is still problematical and even here, a reliable *in vivo* glucose sensor could be useful tools for checking the correct function of a bioartificial pancreas.

1.5 COMMERCIAL IN VIVO (BIO)SENSING PROJECTS

In Table 1.1, a (certainly incomplete) list of companies known to be working on (or to have recently worked on) *in vivo* sensing projects is presented. Entries are listed alphabetically. I cannot guarantee that the information is complete or up-to-date. Companies range from large, research-driven pharmaceutical or diagnostics companies (e.g. Eli Lilly, Becton Dickinson) to medium-sized companies with activities related to insulin delivery (e.g. Disetronic Medical Systems, MiniMed) to small, venture-capital-based enterprises founded entirely on research from particular university research groups (e.g. Futrex, Cygnus, E-Heller & Co.) to university-run start-up companies (e.g. Unitec Ulm, Sensor Solutions).

1.6 CLOSING REMARKS

In vivo (bio)sensing is at a crucial moment in its evolution. One question is whether reliable *in vivo* biosensing can be introduced on a mass-market scale before progress in other branches of medical science makes diseases such as diabetes curable or preventable. In this latter case, although the underlying causes of diabetes are probably numerous and complex, it may eventually be possible to diagnose genetic predispositions to diabetes and then prevent destruction of the native β -cells, by somatic gene therapy, for example.

Large companies are reluctant to invest in *in vivo* biosensing because of the hard truth: reliable, long-term, *in vivo* biosensing has never been achieved, mainly due to poor biocompatibility of devices, and poor understanding of this poor biocompatibility. This illustrates the paradox of *in vivo* biosensing: to study, understand and ultimately master the phenomena governing loss of sensor function *in vivo*, large numbers (at least thousands) of sensors with identical *in vitro* performance must be available. However, because the investment needed to produce thousands of identical sensors is great, such sums will not be made available unless the biosensor is shown to work reliably *in vivo* which is only possible once biocompatibility issues are overcome, and so on, and so on!

Can the public sector respond by coordinated, multinational efforts? In the last few years, Europe-wide programmes (Coughlan & Alcock, 1991; Alcock & Turner, 1993; Machado *et al.*, 1994; Réach *et al.*, 1994; Fischer *et al.*, 1995) have sought to coordinate and fund *in vivo* chemical sensing in universities, and indeed a glossy brochure aimed at industry has resulted (Alcock & Turner, 1995). However, with some exceptions (Rigby *et al.*, 1995), few new insights have resulted from such programmes. The fundamental problem is that for a given sensor in a given subject, the working lifetime cannot be predicted or controlled. The most important effort in the *in vivo* biosensing field should be to characterise why some sensors "work" and some do not and

this should be aided by an industrial production of large numbers of sensors with identical *in vitro* performance.

More basic research on the foreign-body response needs to be done. The only hope for long-term, invasive sensing seems to lie in manipulation of the inevitable host response to an implanted object. For example, a "dormant" sensor could be implanted and then activated some time later, after stabilisation of the foreign-body response. Deliberately biodegradable/resorbable polymers (e.g. polylactides and polyglycolides) may be useful outer coatings for sensors, especially as the resorption rates can be tuned by choice of monomer and morphology. Equally, sensor membranes containing polymers functionalised with biological motifs, such as sequences from adhesion proteins or growth promoters, may be worth investigation. Another possibility would be to accompany device implantation with pharmacological intervention, to direct the vascularisation of a foreign-body capsule. In the particular case of a microdialysis system linked to a sensor, addition of pharmacologically-active substances to the perfusion buffer for modulation of the host response is a distinct possibility.

Although biosensing is traditionally viewed as an interdisciplinary field, some techniques remain under-exploited. For example, *in vitro* cell culture, which can reproduce many of the elements of the *in vivo* environment, has been under-used in the biosensor area, with a few notable exceptions (Zhang *et al.*, 1991; Ziegler *et al.*, 1994). More work is also needed on intra- and interindividual variations in diabetes metabolism: for example, how does the correlation between subcutaneous glucose and blood glucose vary from day to day, or depend on general physiological/psychological state, or the presence of medications or state of local tissue perfusion? Furthermore, for subcutaneous, needle-type sensors, there are no definitive studies comparing forearm or abdominal implantation sites.

In conclusion, I believe that there is a time window of 10–15 years during which *in vivo* sensors must achieve mass-market, commercial success: if they do not, then other therapies will out-mode them. The competence to achieve success exists but is scattered worldwide, and across academia and industry. Rather than thinking about the dilution of nebulous, future profits, the main "players" in the invasive and non-invasive *in vivo* sensing games should think of forming multi-partner ventures to share the cost of the decisive, market-making research and development.

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2

Electrochemical Sensors for in vivo Glucose Sensing

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2.1 INTRODUCTION

The need for implantable glucose sensors allowing continuous monitoring of physiological glucose levels has been recognised for more than a quarter of a century. It is generally agreed that for patients with Type I diabetes mellitus,

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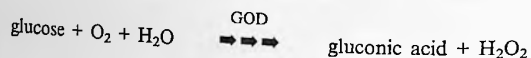
only continuous monitoring of glucose levels will permit something approaching normoglycaemia (Tchobroutsky, 1991), thus preventing both short-term and long-term complications (for example, hypoglycaemic episodes in the former case and retinopathy, neuropathy and nephropathy in the latter). However, as shown in the DCCT trial (DCCT, 1991), improved normoglycaemia control is often accompanied by a greater risk of severe hypoglycaemia, due to the so-called hypoglycaemia unawareness syndrome. Continuous glucose monitoring must also seek to decrease this risk.

The control of glucose levels by an artificial device was first demonstrated by Albisser *et al.* (1974): similar performance with specifically an implantable sensor was shown by Shichiri *et al.* (1982, 1983, 1984). These landmarks have been followed by an impressive number of papers developing various concepts for implantable and extracorporeal glucose sensors. The ultimate goal is the development of a closed-loop insulin delivery system, where insulin would be delivered by a pump controlled by a glucose sensor. However, there are many other situations where an implantable glucose sensor could be very useful for monitoring insulin-dependent diabetics or any patient with unstable body chemistry. Examples would be a overnight alarm for hypoglycaemia, or a monitor during surgery, intensive care, childbirth and initiation and modulation of insulin therapy. All these applications are of a limited duration and, considering the present state-of-the-art of the implantable glucose sensors, constitute the most realistic targets for the first stage of continuous, intracorporeal glucose monitoring. Recent consensus shows a short-term hypoglycaemia alarm to be the first clinical priority.

Many different strategies for potentially-implantable glucose sensors have been proposed. However only a few have so far been submitted to serious *in vivo* evaluation. These are principally the electrochemical devices, and more specifically amperometric enzyme electrodes using glucose oxidase (GOD) immobilised on an amperometric transducer. Enzyme electrodes coupled with microdialysis have been also investigated. Microdialysis is an *in vivo* sampling technique allowing a continuous sampling of metabolites from blood or interstitial fluid by infusing a tissue via a semipermeable, double channel catheter. By coupling the dialysate from a microdialysis probe to a glucose sensor, extracorporeal glucose monitoring can be performed. The principles of glucose sensors are described briefly below. The emphasis is on those sensor aspects that are directly linked to *in vivo* applications.

2.2 ENZYME ELECTRODES FOR GLUCOSE

Glucose oxidase catalyses the following reaction



and this can be monitored using either potentiometric or amperometric techniques. Despite considerable research effort on potentiometric systems based on glucose ENFETs (enzyme field effect transistors, see Section 7.6.2), no *in vivo* feasibility has been demonstrated, due to the difficult problem of the variation in the buffer capacity of the enzymatic membrane, as well as that of the biological fluid.

The amperometric approach dominates glucose sensing and was first proposed by Updike & Hicks (1967). Using a two- or three-electrode electrochemical cell, either oxygen consumption or peroxide formation can be monitored. In both cases, an external potential is applied and the resulting current is measured. This, for a well designed sensor, is directly proportional to the glucose concentration. An obvious problem with this scheme is the requirement of oxygen as a co-substrate. To circumvent this, several so-called "mediators" have been investigated: these act as a substitute for oxygen. Ferrocene and its derivatives are the most commonly used (Claremont *et al.*, 1986). These diffusional mediators decrease the influence of the local oxygen concentration. Moreover, by allowing lower working potentials (100–200 mV relative to the Ag/AgCl electrode compared to 600–700 mV for direct peroxide detection), the problem of electroactive interferences is also minimised. However, leaching of diffusable mediator results in a loss of sensitivity and more importantly, when used *in vivo*, the high risk of introducing the mediator into the body. These problems have led to the abandonment of this approach for *in vivo* devices. Recently, "electrical wiring" of the enzyme has been employed, by which the enzyme is associated with the electrode via a long polymer having a dense array of covalently-attached mediator groups (Csöregi *et al.*, 1994).

2.3 CRITERIA FOR BIOSENSOR PERFORMANCE

Besides the obvious requirement concerning safety to the patient, which is of primary concern for an *in vivo* use, other functional requirements of implantable sensors can be divided into general requirements common to all sensors and specific requirements for *in vivo* use. General parameters include specificity, linearity in the required concentration range, adequate response time, detection limit and resolution, acceptable signal/noise ratio, reasonable storage and operational lifetime. Specific factors include miniaturisation, sterility, independence of hydrodynamic and oxygen variations, accuracy in the normal glucose concentration levels and stringent demands on biocompatibility. A number of these issues are addressed below.

2.3.1 THE TRANSDUCER

To decrease the overall sensor size, a two electrode set-up, with a Pt, Pt-Ir or Pt-black working electrode, has been most commonly used. The second

electrode, which in this case serves as both the reference and counter electrode, is usually an Ag/AgCl wire but can even be an external electrode, i.e. attached to the skin surface. The fabrication process uses thin metallic wires, i.e. each sensor is handmade.

Notably, two sensors described in the literature are based on microfabricated, thin-film, three-electrode transducers on either a polyimide (Mastroiuto *et al.*, 1991) or a silicon (Gernet *et al.*, 1989) substrate. The former consists of Pt-black working and counter electrodes while the latter uses simple Pt thin films. In both cases, a thin film Ag/AgCl reference electrode is used. Another approach has been based on microcontainment technology, where the Pt thin film electrode is in a cavity (500 μm diameter), which is then filled with an enzyme membrane (Steinkuhl *et al.*, 1994): this sensor was later used in a microdialysis system (Steinkuhl *et al.*, 1996). Another transducer design was used in the sensor based on oxygen detection reported in (Armour *et al.*, 1990). This was composed of two Pt wire electrodes and an Ag/AgCl reference. These were integrated into a flexible, silicone rubber catheter. Silicone, being highly permeable to oxygen but impermeable to glucose, allows the relative ratios of glucose to oxygen diffusion to be modulated by adjusting the respective areas of the enzyme layer/silicone. This sensor was used in a differential mode against a reference O_2 sensor, which was similar to the glucose sensor but lacked the enzymatic membrane.

2.3.2 THE ENZYME MEMBRANE

In the vast majority of sensors, the GOD (usually in large excess), either alone (Kerner *et al.*, 1993; Pickup *et al.*, 1993) or mixed with bovine serum albumin (BSA) (Gough *et al.*, 1985; Bindra *et al.*, 1991; Moussy *et al.*, 1994; Gernet *et al.*, 1989), is immobilised by glutaraldehyde cross-linking. By adjusting concentrations of various components, gel-like membranes having good mechanical stability and thickness of several μm are formed. In some cases, catalase is co-immobilised with GOD in order to decrease inactivation by H_2O_2 (Tse & Gough, 1987). GOD immobilisation on Sepharose has also been described (von Woedtke *et al.*, 1994a,b).

Less conventional enzyme immobilisation can be performed by galvanostatic electrodeposition of a layer of GOD and BSA of about 5 μm thickness, which is later crosslinked with glutaraldehyde (Johnson, 1991). This method allows spatial control of the enzyme deposition, which is an important advantage for miniature sensor fabrication.

For the "enzyme-wired" electrodes, the conducting redox hydrogel was formed by poly((vinylimidazole)Os(bipyridine) $_2\text{Cl}$) $^{+2+}$ redox polymer, complexed with GOD and crosslinked with poly(ethyleneglycol) diglycidyl ether (Csöregi *et al.*, 1994).

2.3.3 THE INFLUENCE OF OXYGEN

The problems which may arise from low and non-uniform oxygen tensions in the subcutaneous tissue have motivated the development of sensors using mediators (Claremont *et al.*, 1986) or sensors with vascular implantation sites (Armour *et al.*, 1990). In the subcutaneous tissue, oxygen pressures vary in the range 20 to 30 mmHg (Fischer *et al.*, 1989; Zhang & Wilson, 1993) corresponding roughly to 1×10^{-5} M, whilst the glucose concentration can rise to 2×10^{-3} M. Therefore the risks of a non-linear glucose response and/or response saturation are very high. The most common approach for avoiding or at least decreasing the oxygen influence on the sensor signal is the use of an outer, diffusion-limiting membrane with properties that are designed to reduce diffusion of glucose with respect to oxygen (Shichiri *et al.*, 1983, 1984). Beside lowering the oxygen dependence, this membrane has several other effects on the sensor behaviour such as the minimisation of influence of enzyme- and electrode kinetics on the sensor response, a lower temperature coefficient and greater linear range (increase of the apparent K_m in the diffusion-limited regime). It serves also as protection for the sensor, and since it is the outer membrane, its properties with respect to biocompatibility and stability in biological fluids will largely determine the functional characteristics of the sensor. Polyurethane (PU) membranes are the most frequently used, followed by Nafion (Moussy *et al.*, 1994), perforated polyethylene (von Woedtke *et al.*, 1994a,b) and photopolymerisable derivatised poly(ethylene oxide) (Csöregi *et al.*, 1994).

2.3.4 BIOCOMPATIBILITY

This has been defined as the ability of a material to perform with an appropriate host response in a specific application (Braybrook, 1993). It is a dynamic and two-way process that involves the time-dependent effect of the biosensor on the host and *vice versa*. Understanding the pathophysiological mechanisms is becoming critical at the present stage of the sensor development, and these are treated in more detail in section 1.4.3 and Chapter 5. Biocompatibility will ultimately determine the feasibility of clinical use, not only in terms of the presence or absence of adverse reactions (toxicity, acute and chronic inflammatory and immune reactions) but also because the host-sensor reaction will determine sensor function (drift, response time and operational lifetime).

One of the rare studies of the toxicity of biosensors has been performed on glutaraldehyde-crosslinked membranes (Zhang *et al.*, 1991). *In vitro* cell culture tests have shown that freshly-prepared, ethylene oxide sterilised sensors showed moderate toxicity, mainly due to the presence of non-reacted glutaraldehyde. If a polyurethane polymer membrane was present, residual solvents also leached. However, the toxic effect was removed by soaking the

sensor for about one week in phosphate buffer. Immunogenicity of sensors implanted in rats has been tested by detecting specific IgG-antibodies produced against various components of the sensor such as cellulose acetate, polyethylene, polyurethane and enzyme membrane (Ziegler *et al.*, 1994). The highest immune response was observed with cellulose acetate and regenerated cellulose, which, however, are usually used as inner, anti-interference layers and thus are not in direct contact with the tissue. The polyurethane was shown to be of low immunogenicity, and no cytotoxicity was observed *in vitro* using monolayer cell cultures. The biocompatibility studies of subcutaneously-implanted, alcohol-sterilised Nafion membranes have shown the rapid onset of fibrosis, resulting in the formation of a fibrous capsule (Turner *et al.*, 1991). However, the thickness of the capsule remained relatively thin ($<100\text{ }\mu\text{m}$ after more than three months) and well vascularised with a near steady-state achieved after 43 days. A different situation was observed for intravenous implants where fibrosis progressed steadily up to at least 100 days. However, no chronic inflammation was observed. It was concluded that the biocompatibility of Nafion was comparable to that of silicone rubber.

2.3.5 THE SITE OF SENSOR IMPLANTATION

This is a critical consideration. Implantation in the vascular space would be the most straightforward choice, since most clinical information deals with blood glucose levels. So far, only one sensor has been successfully implanted in the vascular space (Armour *et al.*, 1990). The major concern over thrombosis and infection has forced another choice of implantation site, namely the subcutaneous tissue. This site has been validated using reference methods such as microdialysis (Jansson *et al.*, 1988; Bolinder *et al.*, 1989) or a wick technique (Fischer *et al.*, 1987; Brückel *et al.*, 1989) by showing that the slow changes in blood glucose concentrations were reflected with an acceptable time lag (5–10 min) in the subcutaneous tissue. A more complicated situation (longer time lag or a complete lack of blood/tissue equilibration) can exist for faster variations of glycaemia. Despite this doubt, and the controversy surrounding the precise glucose concentration in extracellular fluid, the subcutaneous tissue is presently the favoured site of implantation. Other reasons to favour the subcutaneous site are that it is already used for implantation of needles for insulin perfusion, and that percutaneous access is more convenient if a periodic exchange of sensors is envisaged.

2.3.6 CALIBRATION AND IN VIVO SENSITIVITY

The initial results obtained with the subcutaneously implanted sensors were expressed in nA without any transformation of the sensor current output into a glucose concentration. Attempts to use the *in vitro* sensitivity to calculate the subcutaneous glucose concentration gave confusing estimates, which varied

between 20 and 95% of the blood glucose levels. This showed clearly the necessity of *in vivo* calibration for subcutaneous monitoring.

Using two steady-state conditions for low and high blood glucose levels (basal/insulin-induced hypoglycaemia, and glucose infusion/bolus hyperglycaemia respectively), a two-point *in vivo* calibration method has been used (Poitout *et al.*, 1993). The *in vivo* sensitivity and the background current (i.e. the current that would be obtained in the absence of glucose) were determined from (respectively) the slope and the intercept of a straight line representing current vs. blood glycaemia. The *in vivo* sensitivity was then used to calculate an apparent subcutaneous glucose concentration and an excellent correlation between blood and subcutaneous glucose levels was found. These results were confirmed in the same animal model (the rat) using the same technique with constant hypo- and hyperglycaemic plateaux (Koudelka *et al.*, 1991). The two-point calibration method was also used in non-diabetic patients, where the basal and plasma glucose levels after an oral glucose load were paired with the concomitantly measured sensor currents (Pickup *et al.*, 1993). It was also used for diabetic patients, where the glucose levels were adjusted by feedback-controlled dextrose and insulin infusions (Kerner *et al.*, 1993). The possibility of using a one-point calibration was investigated (Bobbioni-Harsch *et al.*, 1993; Csöregi *et al.*, 1994): this is only valid if the background current is assumed or observed to be small compared to the sensitivity. More complicated calibration methods based on both *in vivo* and *in vitro* sensor parameters have been used (Johnson *et al.*, 1992; Gilligan *et al.*, 1994). The *in vitro* calibration adjusted for oxygen sensitivity (against a reference oxygen sensor) was employed in the case of oxygen-based sensors implanted in the dog vena cava (Armour *et al.*, 1990).

In most cases, *in vivo* studies using different animal models or humans show that the *in vivo* sensitivities were almost always lower compared to the *in vitro* ones, and that the background currents were higher. An exception is the intravascularly implanted sensor where the original pre-implantation sensitivity was used throughout the test (Armour *et al.*, 1990). Pre-implantation sensitivity for subcutaneously implanted sensors has been used in the initial stages of one experiment (Updike *et al.*, 1994). The reasons for this impaired *in vivo* sensitivity are not definitely known, although several explanations have been proposed. The most likely is the formation of a protein or cellular coating at and around the sensor, hindering glucose diffusion. The frequently observed re-establishment of the initial, *in vitro* sensitivity following the sensor explantation and rinsing may confirm this hypothesis (Rebrin *et al.*, 1992; Gilligan *et al.*, 1994; Thomé-Duret *et al.*, 1996). Scanning electron micrographs of explanted sensors showed that while functioning sensors had minimal visible coating of cellular or other material, the non-functioning ones had thick deposits and damaged sensor membranes (Pickup *et al.*, 1993). However, in this paper, no information was given on whether the pre-implantation sensitivity was re-established *in vitro* after explantation. The

extent and the time evolution of this unavoidable sensor-host interaction, resulting in the outer membrane fouling and/or a fibrous capsule formation, will depend strongly on the biocompatibility of the sensor.

Another hypothesis suggests the inhibition by as yet unidentified low molecular weight substances present in subcutaneous extracellular fluid and plasma, which diffuse through or adsorb onto the polyurethane membrane (Kerner *et al.*, 1993). The so-called inhibition factor "Q" leads to a drift in sensor signal and finally to sensor deactivation. Also in this case, the sensitivity of the explanted sensors was rapidly restored in a buffer solution. The participation of the peroxide in the biodegradation of polymer materials, resulting in stress-cracking and leaching of small molecular weight substances was discussed by Updike *et al.* (1994). During an inflammatory reaction, the exudate has been shown to have lower glucose concentrations (Rebrin *et al.*, 1992) than the interstitial fluid: this might also contribute to the impaired *in vivo* sensitivity. However, in most of the previously cited work, no visible inflammatory reaction or bleeding was observed. The different components of biocompatibility—physicochemical interactions at the interface, material degradation, and local and systemic host response will ultimately determine the longevity and performance of implanted sensors. This stresses the importance of these issues in sensor development (Kyrolainen *et al.*, 1995). A successful transition from animal models to clinical investigation implies the solution of these problems.

2.3.7 INTERFERENCES

The problem of electrochemical interference is particularly acute at the high anodic potentials required for hydrogen peroxide oxidation. To date, the effects of the few most likely interferents have been studied: endogenous species such as ascorbate and urate, and exogenous species such as paracetamol (acetaminophen). The outer membranes used on sensors provide minimal protection only. Inner, low molecular weight cut-off (<200 MW) membranes of cellulose acetate (Brückel *et al.*, 1989; Bindra *et al.*, 1991) or electropolymerised poly(o-phenylenediamine) (Moussy *et al.*, 1994) have been investigated. Both are effective in screening out the endogenous interferences at the physiological levels. The paracetamol problem is more complicated (Moatti-Sirat *et al.*, 1992b). To date, the most efficient paracetamol screening was obtained using a membrane composed of several alternating layers of cellulose acetate and Nafion (Moatti-Sirat *et al.*, 1994; Zhang *et al.*, 1994). Elimination of interferences by peroxidase-catalysed oxidation was performed by Csöregi *et al.* (1994). The limited life-time of this layer led to a different approach in which electro-oxidation of the interferents was avoided by using a low (95 mV vs. SCE) operating potential (Csöregi *et al.*, 1995).

2.3.8 STERILISATION

Although most of the initial animal experiments were performed with non-sterile devices, device sterility is obviously a prerequisite for all human intracorporal measurements. More recently, chemical or irradiation sterilisation procedures have been employed. For the former, glutaraldehyde (Kerner *et al.*, 1993; Pickup *et al.*, 1993) or ethylene oxide (Poitout *et al.*, 1993) were used. The question of the remaining toxic residues after chemical sterilisation needs to be clarified. The irradiation sterilisations consisted either of a 2.5 MRad e-beam (Johnson *et al.*, 1992) or gamma dose (Koudelka *et al.*, 1991; Bobbioni-Harsch *et al.*, 1993). A lower, 0.65 Mrad dose of gamma-irradiation combined with several days' exposure to a peroxide solution has been proposed (von Woedtke *et al.*, 1994b). Complete telemetric and sensor assembly sterilisation has also been performed using a dilute thimerosal saline solution for 24 hours (Gilligan *et al.*, 1994).

2.4 *IN VIVO* EVALUATION OF IMPLANTABLE SENSORS

Ten years ago (Shichiri *et al.*, 1986), the successful use of a subcutaneously-implanted glucose sensor coupled to an insulin or glucagon infusion pump was described. By exchanging the sensor after four days, normalised glycaemic profiles of diabetic subjects were obtained for one whole week. One is tempted to ask how far the state of the art has progressed since then. With three notable exceptions (Armour *et al.*, 1990; Gilligan *et al.*, 1994; Updike *et al.*, 1994), all other *in vivo* sensor evaluations were performed for periods ranging only from a few hours to several days. At this point, one might pose a fundamental question about the feasibility of *in vivo* glucose monitoring. The answer is far from being straightforward and a differentiation between short-term (up to several days, a week) and long-term (several months, a year) applications should be made. The use of a sensor *per se* (e.g. in a hypoglycaemia alarm without controlling insulin delivery) is of real clinical and economical importance. Thus, even though it may be considered as a step back in the scheme of *in vivo* glucose monitoring, the development of a night-time hypoglycaemic alarm is entirely justified (Pickup, 1993). In addition, as already mentioned in the introduction, other short-term uses of implantable glucose sensors would be also of clinical interest.

In all studies but one (Armour *et al.*, 1990), sensors were implanted subcutaneously. Most of the tests describe short-term glucose monitoring, and were conducted on animals such as rats (Moatti-Sirat *et al.*, 1992a; Bobbioni-Harsch *et al.*, 1993; Csöregi *et al.*, 1994), dogs (Poitout *et al.*, 1992; Rebrin *et al.*, 1992; Moussy *et al.*, 1994), rabbits (Johnson *et al.*, 1992) and sheep (Brückel *et al.*, 1989). It is impossible to compare the results obtained because

not only were different sensor designs and different animal models used but also different protocols were applied: glucose infusions, intraperitoneal glucose loads and glucagon-insulin administration, different blood withdrawal sites for the reference measurements and different implantation sites, etc.

One of the rare observations common to most of these experiments was the so-called run-in period, i.e. the time after implantation necessary to obtain a stable current output. However, the duration of this period, during which the current decreases and then stabilises, varied from one hour (Bobbioni-Harsch *et al.*, 1993; Moussy *et al.*, 1994), to 2–4 hours (Johnson *et al.*, 1992; Kerner *et al.*, 1993) to overnight (Moatti-Sirat *et al.*, 1992a). A second common observation of all these short-term animal experiments was the post-implantation decrease of the sensor sensitivity, although the extent varied. In some cases, the *in vivo* sensitivity first decreased but then remained relatively stable for implantations of several days (Moatti-Sirat *et al.*, 1992a; Bobbioni-Harsch *et al.*, 1993). Following an *in vivo* calibration, the *in vivo* sensitivity was used to calculate *a posteriori* the subcutaneous glucose levels, which were compared with the reference blood or plasma glucose concentrations. The graphical representation of the correlation plots was performed either by simple regression lines or by the more clinically oriented Clarke error grid analysis (Clarke *et al.*, 1987). In the former, the accuracy of data is expressed by a correlation coefficient, while in the latter, a percentage difference related to the clinical requirements for accuracy is used. Thus, more clinically meaningful evaluation of the accuracy between the sensor values and the reference glucose values is obtained (Moatti-Sirat *et al.*, 1992a).

The results of the short-term animal studies confirmed previous results (for the pre-1988 situation, see Pfeiffer & Kerner, 1988), demonstrating that a subcutaneously implanted sensor can follow correctly the variations of glycaemia for several days. Automated feedback control of the subcutaneous glucose concentration in diabetic dogs before and during an OGTT has been demonstrated (Rebrin *et al.*, 1989). In this paper, the relatively high failure rate of the subcutaneously implanted sensors was correlated with the problem of tissue reaction. The results of the animal experiments have also been confirmed in non-diabetic patients (Johnson *et al.*, 1992; Pickup *et al.*, 1993; Poitout *et al.*, 1993). Tests lasting up to 72 hours showed good correlations between the subcutaneous glucose concentrations and blood glucose values during standard glucose tolerance tests (OGTT) or normal meals (Johnson *et al.*, 1992).

As already mentioned, *in vivo* calibration was performed *a posteriori*, i.e. upon completion of the experiment. Although acceptable for initial sensor evaluations, this is completely unacceptable for clinical applications. A wearable control and monitoring unit with built-in software allowing real-time *in vivo* calibration has been described (Poitout *et al.*, 1993). A two point calibration was used to determine the *in vivo* sensitivity—at basal level, and at

a value 60 min after an OGTT (which corresponds to a slightly increased glucose plateau in non-diabetic patients). Performed 14 hours after implantation, the sensors could reliably follow the plasma glucose levels following the OGTT.

The longer-term evaluations have been performed in dogs, using either a catheter-shaped, intravascularly-implanted sensor (Armour *et al.*, 1990) or a modified Direct 30/30™ sensor implanted in the subcutaneous tissue (Gilligan *et al.*, 1994; Updike *et al.*, 1994). The former uses an enzyme electrode of two-dimensional construction (flexible silicone rubber catheter with an enzyme membrane at the tip) based on oxygen detection, while the latter is based on peroxide detection, using a three-electrode transducer and commercially available, polyurethane-based anti-interference and enzyme membranes. Both use implanted telemetry systems, either near the electrode (Armour *et al.*, 1990) or integrated with the electrode (Shults *et al.*, 1994). The telemetry systems are not small; dimensions of $5 \times 5.5 \times 1.1$ cm for the former and $7 \times 3.2 \times 1.2$ cm for the latter, with weights of 27 g and 70 g respectively.

The catheter-type oxygen-based glucose and a reference oxygen sensor used in the differential measurement mode was evaluated during several weeks' implantation in the *vena cava* of non-diabetic dogs (Armour *et al.*, 1990). In total, six sensors were implanted so that they free-floated in the blood stream: five had telemetry and one had percutaneous leads. After a healing period of 1–2 weeks, the sensor performance was assessed using various glucose infusion protocols. Results obtained on different days after implantation show that sensors could indeed track the glucose variations using their pre-implantation calibration. Different failures related to electronics and wire-breaking complicated the assessment of sensor lifetime. The longest implantation was 108 days, and this particular sensor was still functional upon explantation, with minimal changes in post- vs. pre-implantation *in vitro* sensitivity. Except for one dog, in which the sensor adhered to the vessel and was encapsulated with a thin layer of tissue, no adherent clots or necrotic regions were found in post-implantation examinations.

The second longer-term evaluation concerns the sensors integrated within a telemetry system which were implanted in the subcutaneous tissue of dogs. The run-in period (instability of the sensor signal upon implantation) up to 18 days was associated with inflammation followed by the development and maturation (vascularisation) of the foreign body capsule. A stable sensor output was then obtained, and glucose monitoring in freely-moving dogs was performed with three sensors from 20 to 94 days using intravenous glucose infusions (Updike *et al.*, 1994). It was observed that the sensor lifetime was limited by the biodegradation of the polyurethane membrane. The addition of a bioprotective membrane (material not disclosed) seemed to improve the sensor lifetime (Gilligan *et al.*, 1994). In this case, 10 implanted sensors were evaluated, for durations ranging from 20 to 114

days. An important observation concerned the decrease of vascularisation of the fibrous capsule after about 3 months, resulting in oxygen limitations (>15 mM glucose). This confirms the importance of stable vascularisation. The feasibility of long-term glucose monitoring in the subcutaneous tissue would thus depend on the formation of a defined and well-vascularised tissue capsule, characterised by stable glucose and oxygen access to the implanted sensor. What the duration of this unstable period is, and whether it is acceptable to patients, has yet to be determined. This will depend on the sensor's subsequent functional lifetime. For example, if the sensor functions for at least several months or one year, a run-in period of a few days or weeks respectively might well be acceptable.

2.5 OTHER IMPLANTABLE ELECTROCHEMICAL SENSORS

The sensor developed by Wilkins *et al.* (Atanasov & Wilkins, 1993) differs from the classical enzyme electrodes described so far, although it is also an enzyme electrode. It consists of two parts—an amperometric peroxide detector of a wire-type and an enzyme microbioreactor—enclosed in a 1 cm-diameter plastic tube. The microbioreactor is filled with fine carbon powder, onto which GOD has been immobilised. Nafion-pre-treated polycarbonate is used as a diffusion limiting membrane. The sensor housing provides an inlet and an outlet, so that the reactor can be refilled when necessary with a fresh carbon/enzyme suspension. This approach addresses the problem of loss of enzyme activity over time. The device is connected to a miniaturised potentiostat and signal transmitter, and has been tested in plasma and whole blood *in vitro*. Very preliminary, short-term, subcutaneous implantation in a sheep has also been performed.

A completely different type of sensor is the electrocatalytic device. This is based on direct, anodic oxidation of glucose at Pt-based electrodes. The electrochemical cell is usually completed by a Pt counter and an Ag/AgCl reference electrode. Due to the complexity of the resulting electrochemical signal, complex measurement methods have to be used to extract a meaningful glucose signal: charge-compensated cyclic voltammetry (Sarangapani *et al.*, 1988), impedance (Preidel *et al.*, 1990) or differential pulse voltammetry (Lemke & Luster mann, 1991). The measurements are complicated by the adsorption of the reaction products and various other substances, causing a rapid fouling of the electrode surface, with concomitant loss of sensitivity. Thus, despite the option of using a protective membrane, frequent electrochemical reactivations of the electrode have to be performed. The short-term, *in vivo* evaluations of the electrocatalytic sensors were performed in dogs using catheter-type sensors implanted either intravascularly or subcutaneously (Lemke & Luster mann, 1991). An electrocatalytic sensor within a flow-through cell for extracorporeal glucose monitoring was evaluated in the blood

circulation of a sheep (Preidel *et al.*, 1990). In later work, an improved design of the flow-through cell was used for longer-term experiments (Preidel *et al.*, 1993). Using intravenous glucose tolerance tests (IVGTTs), the sensor behaviour was evaluated over several weeks, with good stability of the calibration up to 33 days.

Recently, a new strategy to overcome the loss of *in vivo* sensitivity of subcutaneously implanted sensors was proposed (Rigby *et al.*, 1995). It uses slow flow (60 μ l/h) of an isotonic solution over the tip of a classical glucose sensor, to form a liquid film at the sensor/tissue interface. This film should help to reduce or eliminate the biofouling and thus to improve the sensor stability. Several such sensors were implanted subcutaneously in rats and evaluated up to four hours. Using the *in vitro* calibration values, good correlations with blood glucose levels were obtained.

2.6 MICRODIALYSIS

Microdialysis combined with an *ex vivo* glucose sensor provides an interesting alternative to the implantable glucose sensor. Microdialysis as an *in vivo* sampling technique was developed more than 20 years ago for measurement of neurotransmitters. Its role in the validation of the subcutaneous tissue implantation site was mentioned above (Jansson *et al.*, 1988; Bolinder *et al.*, 1989). The system consists of a hollow-fibre microdialysis probe, a microcell with a glucose sensor, and a precision pump. The hollow fibre (outer diameter 200–500 μ m), constantly perfused with a physiological solution, is implanted subcutaneously and connected to the extracorporeal pump. Due to the concentration gradient at the hollow-fibre/tissue interface, glucose and other small molecular weight substances (molecular-weight cut-off of the fibre is typically 20–50 kDa) diffuse into the fibre, and the dialysate then flows to the detector cell. The most important parameters that determine the system performance are the hollow-fibre length and perfusion rate. These determine the relative recovery, i.e. the ratio between glucose present in the tissue and that leaving in the dialysate. Similarly to the implantable glucose sensors, local electrochemical interferences can still be present. Several groups are currently working on continuous glucose monitoring using the microdialysis approach (Aalders *et al.*, 1991; Meyerhoff *et al.*, 1992; Moscone & Mascini, 1992; Pfeiffer *et al.*, 1993; Hashiguchi *et al.*, 1994; Steinkuhl *et al.*, 1996). All except the last-cited have been tested *in vivo* in animals, or non-diabetic and diabetic patients.

A microdialysis probe (16 mm length, 500 μ m o.d.) was implanted in 13 non-diabetic and seven diabetic patients and evaluated up to 21 hours post-implantation (Meyerhoff *et al.*, 1992). Perfusion rates of 7 μ l/min were used, and the test consisted of an OGTT for the healthy subjects and a meal for the diabetics. The sensor (a two-electrode, hydrogen peroxide detector) was

calibrated using a one-point *in vivo* calibration, using the *in vitro* background current correction. The relative recovery of the microdialysis varied for each individual, although after individual calibration, the changes of blood glucose concentrations were correctly monitored via the subcutaneous dialysate. Time-lags of up to 18 minutes were observed. The interindividual variability of the recovery was explained by differing tissue reactions and by the differences in the tissue resistance to glucose diffusion. Comparable microdialysis probes were investigated in eight diabetic patients under steady-state and dynamic variations of blood glucose levels (Keck & Kerner, 1993). Perfusion rates of up to 9 $\mu\text{L}/\text{min}$ gave good glucose recovery values, while higher rates resulted in much lower recoveries. Dynamic experiments showed that for increasing glucose concentrations, there was an interindividually variable time-lag of 10 to 40 minutes, while only short time lags were observed upon decreasing blood glucose concentration.

Results of continuous glucose monitoring up to 72 hours in healthy and diabetic volunteers have been described (Pfeiffer *et al.*, 1993). Following an oral intake of carbohydrate, a time-lag of about 20 minutes was observed. Taking this together with the dead volume of the tubing into account, good correlations with the i.v. and the subcutaneous glucose were obtained. Another study by the same group was designed to estimate the real glucose concentration in the subcutaneous tissue of healthy volunteers (Sternberg *et al.*, 1995). It was based on recirculation of the perfusing solution in the microdialysis probe until complete equilibration was achieved between the glucose outside and inside the probe. Using a recirculation rate of 2.5 $\mu\text{L}/\text{min}$ and a number of cycles ranging between 33 and 52 (cycle duration = 3.7 min), the subcutaneous glucose concentration measured was 72 ± 6 and $78 \pm 6\%$ of the blood glucose measured in the fasting state and during a hyperglycaemic clamp, respectively.

Short-term experiments on non-diabetic subjects following an OGTT showed good correlation of the blood and dialysate glucose values (Moscone *et al.*, 1992). A sharp decrease in sensitivity was observed after two hours of implantation: this was explained by the release, during the inflammatory reaction, of an interfering compound which reacted with the enzyme or electrode. A complete system has been investigated—the perfusion solution reservoir, a waste bag and micropump were integrated into one unit and the electronics into a second one (Hashiguchi *et al.*, 1994). After evaluation of the system in animals for seven days, it was tested on five healthy and eight diabetic patients. Perfusion rates of 2 $\mu\text{L}/\text{min}$ were used and the system was calibrated *in vivo* prior to the experiment. The calibration factor could be used for up to four days of implantation during which close agreement between blood glucose values and glucose concentrations in the dialysate was obtained. Due to the decrease of the recovery after four days of implantation, the recalibration either had to be performed daily (i.e. on days 5, 6, 7, 8) or the hollow-fibre probe had to be changed. The decrease of the recovery was also

observed in saline solutions containing 10–50 g/L albumin. The biofouling of the hollow-fibre by various proteins was visible under the scanning electron microscope. By recalibrating the system, glucose monitoring could be performed for up to seven days.

The approach adopted by Aalders *et al.* (1991) was different, since it used a glucose oxidase-containing saline solution (0.15 mg/ml) for perfusion at 20 $\mu\text{L}/\text{min}$. The probe was subcutaneously implanted in non-diabetic and diabetic patients, and tested intermittently up to nine days. Good correlation between the dialysate and the blood glucose values was obtained, with time-lags of 4.4 and 8.2 minutes for rising and decreasing glucose concentrations for the non-diabetics, and a maximum delay of 22 minutes for the diabetic patients. In order to decrease the risk of the enzyme leakage into the body, a more sophisticated, double-membrane, hollow-fibre microdialysis probe was developed (Schmidt *et al.*, 1992). It was tested in 12 non-diabetic and 12 diabetic patients using steady-state glucose clamps, allowing two-point calibration of the sensor to be performed. The *in vivo/in vitro* coefficients for both non-diabetic and diabetic patients correlated well, with low inter-individual variations. The conclusion of this work was that when *in vitro* calibration factors are used, the sensor estimated subcutaneous tissue glucose to be $43 \pm 9\%$ of the blood glucose value.

2.7 REVERSE IONTOPHORESIS

Iontophoresis is a well established technique, which uses an externally applied potential difference (electric field) to promote molecular transport across the skin. Interest in this technique is increasingly motivated by the possibility of performing controlled drug delivery via the transdermal route. The mechanisms and the kinetics of fluxes across the skin (electromigration, electro-osmotic and osmotic fluxes) are quite complex (Burnette, 1989). In brief, while an enhanced flux of charged molecules is due to electromigration, the concomitantly observed enhancement of the flux of neutral molecules is attributed largely to electro-osmotic flow. The skin is believed to be negatively charged at $\text{pH} > 4$ and is therefore permselective to cations: this provokes a pre-dominant electro-osmotic and osmotic flow of solvent from anode to cathode. The flow can be used to enhance transdermal flux of glucose in the subcutaneous extracellular fluid, thus allowing minimally-invasive glucose monitoring. The transdermally-extracted samples seem to be less aggressive to the sensor than blood or subcutaneous tissue itself, and thus less problems from biofouling might be expected. However there are several points that need to be clarified before considering long-term use of transdermal glucose monitoring by reverse iontophoresis. Among them are the long-term effects of the current on the skin permeability and biology; site-to-site variability of glucose recovery; response time (at least 15 minutes are needed to extract a

volume containing a measurable amount of glucose) and, as is the case for most implantable devices, the frequency of sensor recalibration.

The feasibility of reverse iontophoresis for glucose monitoring has been demonstrated *in vitro* using mouse skin (Rao *et al.*, 1993). Iontophoresis was performed for two hours at a current density of 0.36 mA/cm^2 , and the amount of glucose was measured using either a classical enzyme electrode or a modified copper electrode. Using the former, a good correlation was obtained between subdermal (1–18 mM glucose range) and extracted (cathodic compartment) glucose concentration. Using two 0.4 ml extraction chambers attached to the skin of diabetic patients, the feasibility of the transdermal glucose monitoring has been demonstrated (Tamada *et al.*, 1995). Extracted glucose concentrations closely followed the changes in blood glucose levels induced by an OGTT, followed by the administration of insulin (albeit with a time-lag of 20 minutes). In this case, an HPLC was used for glucose assay. A current of 0.32 mA/cm^2 was applied during 15 minutes, with a reversal of the polarity at half-cycle. The extracted glucose values were correlated with blood glucose levels measured every 7.5 minutes. Using Clarke error grid analysis, good correlation between blood and transdermally extracted glucose concentrations was obtained for all five subjects and 12 experiments. Minor skin irritations were observed at the test site immediately after removal of the extraction chambers.

Experiments performed on non-diabetic subjects, using 0.25 mA/cm^2 and extraction chambers of 0.55 ml, have confirmed the feasibility of reverse iontophoretic glucose monitoring (Rao *et al.*, 1996). Chromatography with pulsed amperometric detection was used for glucose analysis. Investigating the influence of the extraction time on the correlation of the subdermal and extracted glucose concentrations showed inconsistent results for short extraction times (approx. 15 min). Measurements of the passive glucose diffusion (i.e. without applied current) showed high initial extractions of glucose. It was postulated that this glucose comes from the stratum corneum where it was produced by deglycosylation of glucosylceramides and that it is not correlated with the systemic concentration. With sampling times of 1 h, good correlations were obtained, although in absolute terms these differed between individuals. For longer extraction times, slight skin irritation was also observed.

2.8 CONCLUDING REMARKS

The development of short- and long-term glucose sensors remains a key target of *in vivo* monitoring. Miniaturisation of the amperometric enzyme electrodes already used successfully in laboratory analysers and in disposable self-test strips has appeared as the most straightforward technological choice.

Although not yet entirely solved, the technological aspect of producing reliable sensors (see Chapter 7) does not constitute the most critical issue hindering the introduction of sensors into clinical practice. Different sensor configurations have been shown to possess the required *in vitro* characteristics, including reasonable functional and storage lifetimes. In the case of implantable sensors, the main challenge concerns the improvement of the sensor's biostability. No consensus has been reached on how to achieve this goal, for either tissue or intravenous implantation. This issue concerns the microdialysis probes which are also subject to biofouling, leading to a change of recovery with implantation time. Although the feasibility of reverse iontophoresis has been demonstrated, detailed investigation of its long-term effect on skin permeability must be carried out before deciding on the clinical usefulness of the method. As can be seen for all the approaches described above, there are fundamental questions—by no means trivial—that require urgent answers.

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