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J.D. Newman

Advanced Manufacturing Processes for the Production of Biosensors

Supervisor: Prof. A.P.F. Turner

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Abstract

Biosensors were first described over thirty five years ago, but commercialisation did not occur until the early 1970s. Interest was subsequently sparked by the market for large numbers of disposable devices required for self-testing of blood glucose levels by diabetic patients. The introduction of automated production technology, for the manufacture of the millions of devices required, became a necessity. Screen printing has been the most successful of these technologies to date, but there are many other techniques which could be applicable to biosensor production.

This thesis reviews the main technologies used in sensor construction and describes the manufacture of some devices using a range of these processes. The construction of transducers and subsequent chemically- and biologically-sensitive layers, as well as membrane structures are considered. Most of the work presented here concerns the manufacture of electrochemical devices, but many of the techniques described would be equally applicable to certain aspects of the production of other classes of biosensors.

I am a sundial, and I make a botch
Of what is done far better by a watch.

Hilaire Belloc
(On a sundial)

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Primary Papers

I Biosensors: Principles and Practice

II Ink-Jet Printing for the Fabrication of Amperometric Glucose Biosensors

III TTF-Modified Biosensors for Hydrogen Peroxide

IV Catalytic Materials, Membranes and Fabrication Technologies Suitable for the Construction of Amperometric Biosensors

V Development of a Mass-Producible Glucose Biosensor and Flow-Injection Analysis System Suitable for On-Line Monitoring During Fermentations

Secondary Papers

VI Monitoring of the Glucose Concentration During Microbial Fermentation Using a Novel Mass-Producible Biosensor Suitable for On-Line Use

VII Lactate Sensors: Sensing Sites and Fabrication

VIII Silicon-on-Insulator Integrated Optical Biosensors for Environmental Monitoring

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Advanced Manufacturing Processes for the Production of Biosensors

This thesis is based on the following papers, referred to in the text by their roman numerals.

PRIMARY PAPERS

I. **Biosensors: Principles and Practice.**
J.D. Newman and A.P.F. Turner
Essays in Biochemistry, **27** (1992), 147 - 159.

II. **Ink-Jet Printing for the Fabrication of Amperometric Glucose Biosensors.**
J.D. Newman, A.P.F. Turner and G. Marrazza
Analytica Chimica Acta, **262** (1992), 13 - 17.

III. **TTF-Modified Biosensors for Hydrogen Peroxide.**
L. Bifulco, C. Cammaroto, J.D. Newman and A.P.F. Turner
Analytical Letters, **27** (8) (1994), 1443 - 1452.

IV. **Catalytic Materials, Membranes and Fabrication Technologies Suitable for the Construction of Amperometric Biosensors.**
J.D. Newman, S.F. White, I.E. Tothill and A.P.F. Turner
Analytical Chemistry, **67** (1995), 4594 - 4599.

V. **Development of a Mass-Producible Glucose Biosensor and Flow-Injection Analysis System Suitable for On-Line Monitoring During Fermentations.**
S.F. White, I.E. Tothill, J.D. Newman and A.P.F. Turner
Analytica Chimica Acta, **321** (1996), 165 - 172.

SECONDARY PAPERS

VI. Monitoring of the Glucose Concentration During Microbial Fermentation Using a Novel Mass-Producible Biosensor Suitable for On-Line Use.

I.E. Tothill, J.D. Newman, S.F. White and A.P.F. Turner

Enzyme and Microobial Technology, **2 0** (1997), 590 - 596.

VII. Lactate Sensors: Sensing Sites and Fabrication.

G. Marrazza, M. Mascini, J.D. Newman, G. Palleschi and A.P.F. Turner

In: In vivo chemical sensors: Recent developments (Eds. S.J. Alcock and A.P.F. Turner). Cranfield Press, UK (1993), 47 - 51.

VIII. Silicon-On-Insulator Integrated Optical Biosensors for Environmental Monitoring.

J.D. Newman, A.P.F. Turner, A.G. Rickman, A.P.R. Harpin, M.P. Shaw and J.S. McKenzie.

Proceedings of IEEE conferenence "Biosensors for environmental monitoring", IEEE, London (1995), 3/1 - 3/6.

1. Introduction

A biosensor has been described as an "analytical device in which a biological component is integrated within, or in intimate contact with a physico-chemical transducer" (Turner *et al.*, 1987). The aim is usually to produce a real-time, digital, analytical signal, which is proportional to an analyte, or a set of analytes.

Biosensors are particular types of chemical sensor and are more complex extensions of physical sensors. Indeed, many of the transducer technologies developed and commercialised for physical sensors form the basis of chemical and biological sensors. Biosensors are highly varied in their method of construction, but always contain at least three components: an active surface; a transducer; and some form of electronics and software. In addition, the device may contain a membrane layer and it may be incorporated into a more complex instrument such as a flow-injection analyser (FIA) or large, automated, laboratory-based machine, such as a clinical analyser.

Biosensors are relatively new measurement devices. The technologies for producing them evolved largely from advances in chemical sensors and in biochemistry. In the latter case, it was primarily developments in protein immobilisation which formed the backbone of the enabling technology for biosensors. Table 1 depicts an historical outline of some of the defining events in biosensor development and commercialisation

The most notable event on the illustrated timeline is undoubtedly the description of the first biosensor in 1962 by Clark and Lyons, which was based on Clark's earlier invention, the oxygen electrode. The key concept was the entrapment of biological components close to the electrode surface by the use of a semipermeable membrane. This allowed the free diffusion of analyte within the sensor without the loss of the crucial components, such as the enzyme. This procedure formed the basis of the first commercially-available product, the glucose biosensor produced by Yellow Springs Instruments (YSI, Ohio, USA) in the early 1970s. Devices produced by YSI and some other manufacturers today are still based on this simple construction technique.

Date	Development	Reference
1916	First report on the immobilisation of proteins: adsorption of invertase to activated charcoal.	[1]
1922	First glass pH electrode.	[2]
1956	Invention of the oxygen electrode.	[3]
1962	First description of a biosensor: an amperometric enzyme electrode for glucose.	[4]
1964	Coated piezoelectric quartz crystals as sensors for water, hydrocarbons, polar molecules and hydrogen sulphide.	[5]
1969	First potentiometric biosensor: acrylamide-immobilised urease on an ammonia electrode to detect urea.	[6]
1970	Invention of the ion-selective field-effect-transistor (ISFET).	[7]
1972-74	First commercial biosensor: glucose analyser based on glucose oxidase enzyme electrode (Yellow Springs Instruments).	
1975	First microbe-based biosensor: cellulose-immobilised *Acetobacter xylinium* on an oxygen electrode for ethanol.	[8]
	Invention of the pO_2/pCO_2 optrode.	[9]
	First immunosensor: ovalbumin on a platinum wire electrode for ovalbumin antibody.	[10]
1980	First fibre optic pH sensor for *in vivo* blood gases.	[11]
1982	First fibre optic-based biosensor for glucose.	[12]
1983	First surface-plasmon resonance immunosensor	[13]
1984	First mediated amperometric biosensor: ferrocene used with glucose oxidase for the detection of glucose.	[14]
1987	Launch of the ExacTech™ (MediSense) blood glucose biosensor for self-use by diabetic patients.	
1990	Launch of BIACore (Pharmacia, Sweden) surface-plasmon resonance-based biosensor system	

Table 1: Some Defining Events in Biosensor Development

References from Table 1

[1] Nelson and Griffin (1916). [2] Hughes (1922). [3] Clark (1956). [4] Clark and Lyons (1962). [5] King (1964). [6] Guilbault and Montalvo (1969). [7] Bergveld (1970). [8] Davies (1975). [9] Lubbers and Opitz (1975). [10] Janata (1975). [11] Peterson *et al.* (1980). [12] Shultz *et al.* (1982). [13] Liedberg *et. al.* (1983). [14] Cass *et al.* (1984).

Biosensors have been the focus of a number of books to which the reader is directed for further general information (Turner *et al.*, 1987; Cass, 1990; Hall, 1990; Turner, 1991, 1992; Turner and Yevdokimov, 1995; Kress-Rogers, 1997). A general introduction to biosensors is provided in the main body of this thesis by Paper I, which discusses biosensor components, selected principles of operation and gives some examples of devices. This introduction will therefore concentrate on aspects of biosensors directly relevant to the theme of this thesis, namely fabrication technologies and their application to the production of practical biosensors. The marketplace is discussed, and additional background is provided about the biosensor technologies used in the featured selection of publications.

2. Biosensor Markets

In discussing the practical aspects of biosensors, it is necessary to consider the marketplace for such products, since this defines the problems facing their more widescale introduction and points to where their introduction is most likely in the future.

2.1 Medical Diagnostics

Without doubt, the driving force behind the development of biosensors has been provided by the often very demanding requirements of the clinical laboratory (Griffiths *et al.*, 1992). This is a high expenditure industry, which is constantly searching for improvements in analysis time and reduction in expense. The area is broadly split into centralised, decentralised and consumer testing, each of which has its particular needs and constraints.

2.1.1 Centralised Testing

Centralised laboratories are usually highly automated, being equipped with a small number of large, complex multi-analysers. In this area, it can be argued that the main impact of biosensors could be to improve on existing approaches and to expand the range of analytes accessible to a particular analyser. In many ways, this is the simpler option for the introduction of biosensors, since the analysers are able to control the local environment, manipulate samples and perform automated calibration checks. Reagent-free operation is, therefore, not a major consideration. On the other hand, high standards of accuracy, precision and reliability must be met and most importantly, the analysis must be fast for high throughput operations such as these. Biosensors for many analytes have been introduced by autoanalyser manufacturers and this is a trend that is expected to continue. However, large numbers of biosensors are not usually required and these are often produced by conventional, manual approaches.

2.1.2 Decentralised Testing

Decentralised testing is defined here as that carried out in the physician's office, accident / emergency ward, operating theatre, intensive care ward, health centre, pharmacy, ambulance or sports medicine clinic. It is much more fragmented than centralised testing and the biosensors applicable to this market are highly variable. Such an environment often places more demands on the versatility of the device. There will, typically, be much less sample preparation (automatic or manual) than in centralised laboratories and control of the ambient conditions may be less rigorous. In such a situation, the electronics and software associated with the sensor may be required to calibrate the device for fluctuations in, for example, the local temperature.

2.1.3 Consumer Testing

Consumer tests are those purchased and used by the general public. Such tests are now widely available, but in a limited application area. The monitoring of blood glucose by diabetics is the market in which the first really successful biosensor products became established. The most successful of these, the ExacTech™ device (formerly MediSense, Cambridge MA, US, and now Abbott Diagnostics, Illinois, US) succeeded for many reasons. It is compact, easy-to-use and gives accurate and rapid results. It is also well packaged and uses disposable sensors, which is reassuring to the user, since it overcomes the problems of contamination from blood samples. This combination of features results in a strong consumer appeal and has continued to lead to an erosion of the market share that is held by more conventional devices based on photometric methods. All of the other major players in this area, such as Boehringer (Mannheim, Germany), Miles (Slough, UK) and Lifescan (Milpitas, California, US), have now introduced similar biosensor-based systems.

The spread of consumer testing has, however, been limited. There are only a relatively small number of home tests which do not arouse controversy amongst doctors. These include consumer tests for pregnancy or ovulation, alcohol, cholesterol, uric acid and minor allergic conditions. Even alcohol testing is contentious, since it has legal implications through drink-driving. Another

problem involves the interpretation of the results of tests, such as those for cholesterol. Other applications, involving tests for serious disease states such as cancer or HIV infection are less likely to be widely accepted. Another aspect of these examples is that it may not be necessary to use biosensor technology in many of these cases. Where a simple yes or no answer is required, it is often dipstick technology which is more attractive. This is certainly the case in pregnancy testing, where the lack of a requirement for an instrument has led to the enormous success of chromatographic dipstick devices such as ClearBlue™ (Unipath, Bedford, UK).

Politics also plays a major role here, since governments worldwide are fighting healthcare costs. The widespread introduction of new technologies is likely to be restricted unless significant cost benefits can be demonstrated. Despite the problems outlined above, there has been considerable research and development in this area for analytes other than glucose (Gilmartin and Hart, 1994, 1995; Hart *et al*, 1996).

2.1.4 *In Vivo* Biosensors

In vivo diagnostics concerns analyses which are carried out inside the body. Accordingly, this area is extremely specialised and complex. The technical difficulties caused by allergenicity and biocompatibility are enormous and the consequences of malfunction are severe. In addition, there are considerable legal and ethical barriers to introducing devices of this kind.

The area is, nevertheless, the subject of considerable research activity (Alcock and Turner, 1993). The use of integrated circuit (IC) technology is one of several ways which have been proposed to manufacture reliable, miniature devices suitable for such demanding applications (Buck *et al.*, 1995). However, success remains limited.

2.2 Environmental Diagnostics

Widespread public awareness of environmental issues and increasingly stringent legislation have stimulated activity in the environmental area recently. This has become apparent through, for example, the increase in funding opportunites for new analytical methods for environmental monitoring in the various European funding initiatives (Alcock and Turner, 1997).

Biosensors could be useful for monitoring air, land or water. In particular, there is considerable interest in small, portable instruments for use "in the field". The measurement of pollutants, particularly those which affect the water supply is a growth area. Biosensors could be of use to the water industry, farmers, general industry and the various policing bodies. Targets for analysis include chemical residues (Stephens *et al.* 1997), pesticides (Palchetti *et al.*, 1997), herbicides (Skladal and Kalab, 1995), toxins (Dennison *et al.* 1996) and microorganisms (Hobson *et al.*, 1996).

Environmental applications are diverse and well served by a wide range of simple, manual test kits. This may appear to discourage expensive biosensor development programmes aimed at a mass market. However, there are numerous similar groups of analytes which could be tackled with generic sensor designs, but with modified surfaces. Another possibility involves the use of arrays of sensors which are widely applicable, but can be *trained* for a particular application. This is a variation on the electronic nose theme (Persaud and Travers, 1997).

2.3 Food Applications

Food production is a major processing industry which is fragmented into a myriad of different companies, products and services worldwide. Although the industry is very large, profit margins are comparatively low. Food products can also vary substantially within and between national boundaries. Traditionally, the industry

has been slow to adopt modern methods of analysis and with profit margins being low, there has been reluctance to invest in expensive analytical equipment.

Increasing concern over health implications of food has been catalysed by a number of high-profile issues. These include the *salmonella* contamination of eggs controversy of the late 1980s, the *E. coli* contamination of meat, such as that which occurred in Lanarkshire, UK in 1997 and the recent problems encountered by the possibility of transferring bovine spongiform encephalopathy (BSE) from infected cattle to humans.

Any means which reassure the public will clearly be politically attractive. Biosensors are one way of checking food quality and safety, but they will have to compete with other technologies if they are to be successful. In food monitoring applications, in particular, costs must be minimised and speed and simplicity are essential (Stephens *et al.*, 1997).

2.4 Other Applications

There are many other potential uses for biosensors. They could be used in many process industries, but their introduction has, so far, been somewhat limited by the diverse nature of these applications and the often demanding environments encountered (Brooks *et al.*, 1991).

The various international defence organisations have been quick to see the possible advantages of biosensors over other forms of rapid test procedures. The portability and speed of analysis, coupled to high sensitivity and selectivity is a clear benefit in field-type applications. Sensors for such measurements need to be simple to use, but the cost-per-test is less of an issue than, for example, food applications. Nevertheless, because of their inherent simplicity, disposable, single-shot sensors are often attractive in such environments. There are exceptions to this, since devices such as Biacore's range of instruments are reusable. However, it is extremely advantageous, when reversibility of antibodies is an issue, to have disposable devices.

3. Commercialisation of Biosensors

For several years, biosensors have been hailed as the solution to many analytical problems. Indeed, such devices offer some attractive features and benefits (Table 2). There is general agreement that biosensors have the capacity to offer easy-to-use, cost-effective and rapid analyses in a wide variety of operating environments (Griffiths *et al*, 1992). The use of a biological recognition system typically confers a high degree of selectivity on the sensor, which is an advantage over many chemical sensors, for example. This advantage is significant and hence, the area has attracted considerable attention, both academically and commercially. The biological component also results in one of the major drawbacks of devices such as this. Due to the often labile nature of biochemicals, biosensors are poor at withstanding extremes of environmental conditions. They are particularly sensitive to high temperatures and changes in pH beyond a comparatively narrow range.

Feature	Benefit
Targeted Specificity	Versatility, accuracy
Electronic Processing	Ease of Use, Versatility
Selectivity in Complex Samples	Decreased User Time, Ease of Use
Rapid Measurement	Increased Workload, Decreased Time
Continuous Measurement	Increased Efficiency
Small Size	Versatility, Economical

Table 2: Features and Benefits of Biosensors

The high number of academic publications and comparatively low number of commercial products has been commented upon frequently at meetings and in the literature (Newman and Turner, 1994; Alvarez-Icaza and Bilitewski, 1995). Problems with the biological component are undoubtedly a factor in this, but it is

also true that many biosensors are designed for use in the laboratory and are produced by hand. These technologies often do not lend themselves directly to manufacture, a factor underestimated by many scientists.

There are many practical aspects to consider when designing a biosensor. The choice of biocomponent and transducer are certainly important, but it is necessary to spend time considering how it will be used and how it will be produced. Some of the factors are outlined in Table 3.

Factor	Possibilities
Mode of application	In-line, at-line, remote, invasive, non-invasive
Sampling	Manual, automated, direct
Sample preparation	None (direct), filtration, centrifugation, dilution, reagent addition
Mode of operation	Single-shot, intrermittent, continuous
Production	Hand-built, automated
Cost	Totally re-usable, re-usable instrument / disposable sensor, totally disposable.

Table 3 Some Factors to Consider When Designing a Biosensor

Many of these factors have a profound influence on the type of biosensor that is suitable and the demands that will be placed upon it. For example, in-line sensors must be insertable into the measurement medium or process, without

compromising the process itself. They will, additionally, have a need to meet high demands for long-term stability and reliability, since it may not be simple or convenient to change them. These constraints would not be the same for a single-use device. Here, there are other problems. Cost would often be much more of an issue, but accuracy without calibration would frequently be important. It is in applications such as this that automated manufacturing processes are particularly advantageous.

4. Automated Production Techniques

There are a great many production techniques which can and have been used for biosensor production. Generally, these fall into two types of process, depending on what is being produced. The first types are used for production of the base transducer and the second for handling the chemicals and biochemicals deposited onto this structure. There is some overlap, since some procedures can be used to produce the transducer and the bioactive layer. The types of process are generally 'borrowed' from other industries and adapted for biosensor use. They are usually rapid and require minimal operator skill or involvement. The equipment can be complicated and expensive, but running costs are often low.

The work presented in the papers in the main body of this thesis features many of the automated techniques featured here. The following provides an overview of the techniques and highlights some of the principle features of each technology.

4.1 Screen-printing

Screen-printing, or as it is sometimes known, silk-screening, is a thick film manufacturing process that has been used for many years for reproducing patterns on T-shirts, for artistic purposes (Andy Warhol specialised in the technique) and, more recently, for the production of miniature, robust and cheap electronic circuits. It is really from the latter application that screen-printing evolved into a viable method for the production of chemical sensors and biosensors (Goldberg *et al.*, 1994).

The process is particularly suited to sensor applications, as long as a planar sensor is acceptable. It is possible to print on non-planar surfaces, but it is much more difficult and many of the advantages of the process are often lost. Screen-printers are not particularly expensive and the total set-up cost makes it economically viable to produce even small numbers of devices.

The process involves pressing a paste (usually referred to as an ink) through a screen onto an underlying substrate. The screen consists of a frame, over which is stretched a mesh, which was traditionally made from silk, but now is more likely to be made from nylon or stainless steel. The mesh is coated with an emulsion which contains openings to allow the ink to pass and, hence, form an image underneath. The ink is forced through the openings in the screen by a flexible blade, known as a squeegee, which is usually made from a material such as neoprene. On a typical automated screen-printer, there are many parameters which can be adjusted to optimise print quality. These include squeegee speed and pressure, the type of squeegee used and whether a flood bar is used (to draw ink over the surface prior to printing). Optimisation of the process is usually carried out using a mixture of experience with trial and error, although recent attempts at modelling the process appear to have simplified this with the introduction of software.

The resolution of the screen-printing process is dependent on several factors, but the most important are the mesh size, the emulsion thickness and the physical properties, such as the viscosity, of the ink. As a general guideline, an approximate resolution of 100μm is possible with typical inks printed through a 325-mesh screen. The ultimate resolution may be much less than this, but practically it becomes very difficult to print features with a resolution of below 50μm.

Inks generally consist of several components. These consist of an active material (often in powdered form), an organic binder and a solvent. The compositions of these inks can become considerably more complex than this in order to achieve optimum print quality for a particular application. From a sensor point of view, it is particularly important that the properties of an ink remain consistent over extended time periods. This is not straightforward to achieve and can involve such complications as a continuous ink feed and a local atmosphere which is saturated with the solvent employed, for example.

Once printed, inks can either be fired at comparatively high temperatures, or they can be cured at relatively low, or even room temperatures. The inks and the

substrates used for these processes are often very different, as is the end product. The high temperature process involves the removal of much of the binding material and considerable chemical and physical modification of the material of interest. At low temperatures, however, the ink is simply cured by evaporation of the solvent, leaving the material of interest held within a binder matrix.

There are many types of ink available for screen-printing. By printing layers one at a time, multilayered structures can be produced which contain all of the features required in the biosensor. Some commercially-available inks, which are frequently used in biosensor applications, are shown in Table 4.

Conductors	Semiconductors	Resistors
Gold	Tin oxide	Alumina
Platinum		Silica
Carbon		Many polymers
Metalised-carbon		
Silver		
Silver/Silver Chloride		
Palladium		

Table 4 Examples of Commercially-Available Screen-Printing Inks

The list above is far from comprehensive. Much of the challenge to the sensor developer involves ink formulation (Wang *et al.*, 1995). This is particularly true of biosensors, since the biological component must be incorporated into a screen-printable paste (Nagata *et al.*, 1995). This clearly rules out high temperature processing and often the use of various solvents, but over the last ten years or so, many biological reagents have been screen-printed. Most screen-printed biosensors have been electrochemical in nature, but there is no reason why the process cannot be used in optical or piezoelectric applications, although production of the transducer may not, then, be as straightforward as it is for

electrochemical devices.

Screen-printing is often thought of as a mass-production process, possibly due to the success of the technique commercially. MediSense (at Abingdon, UK), for example, produced hundreds of millions of sensors per year. However, the process is equally suitable for a research student wishing to produce merely hundreds of devices. Even at this level, the process has some significant advantages over hand fabrication. Once the screens have been produced, it is a very rapid way of producing sensors. The reproducibility is superior to most hand-fabrication processes. There is, however, a loss of flexibility of design.

Screen-printing has been used extensively in the papers featured in this thesis, but in particular, it is used in papers II, IV, V and VI, which give details of some of the print parameters. Further discussion of the process can be found in many sources (Turner and Bergveld, 1993; Turner *et al.*, 1995).

4.2 Photolithography

In photolithography, a photomask is used to shield a patterned area on an underlying layer. The mask usually takes the form of a glass or quartz plate onto which a metal pattern is deposited. This layer is opaque to UV light and enables the selective exposure of the underlying area on exposure to this light source. This is a process which is widely used in the microelectronics industry and hence is highly developed for silicon applications. Due to this, the process will be described here for silicon processing, although it can be adapted for other types of base material.

The mask can either be placed in contact with a photoresist surface (a contact mask), or near it (a proximity mask). Contact masks offer higher resolution, but degrade faster through wear. They can also be used only for printing 1:1 images. Magnification or reduction are possible with proximity masks, but the image produced is usually less well defined. A three dimensional structure can be built up by a succession of deposition, exposure and etching steps.

The first step in photolithography, with silicon as a substrate, is to grow an oxide layer on the silicon surface. This is usually carried out by heating the silicon in humidified oxygen at a temperature of 900-1150^{o}C. As the first step of the photolithography process itself, a thin layer of an organic polymer, which is sensitive to UV radiation, is deposited on the surface. A uniform layer is achieved by spin-coating this material. The reproducibility of the final device is affected by the accuracy of deposition of this layer, which should vary by less than 1% ideally (+/- 15 nm for a 1.5 micron film).

The resist layer is then exposed through a mask to UV light of wavelengths between 150-500 nm. Development transforms the latent image into an image in the resist. During this process, selective dissolvong of the resist takes place. There are two general types of photoresist: positive tone and negative tone. Upon exposure, the photochemical reaction strengthens the negative resist and weakens the positive resist.

The practical resolution of photolithographic strucures is due to a number of parameters, including alignment and flatness of the wafer, but the major restriction is due to diffraction of light at the edge of opaque features in the mask. Diffraction causes an edge to become blurred or diffused. The wavelength of the light is, hence, important in determining the resolution, which is improved by the use of shorter wavelengths. For contact printing, with a wavelength of 400 nm and a 1 micron thick resist, the maximum resolution is slightly less than 1 micron. Diffraction is increased in proximity printing and resolution is limited to about 3 microns under similar conditions.

Developments in the integrated circuit (IC) industry are continuous. The latest advances include the use of X-rays, electron beams and ion beams. It is predicted that it will be possible to improve resolution by at least ten-fold in the next few years. From a biosensor standpoint, this will enable smaller and more complex designs to be produced.

Processing costs can be low if total production volume is high. In order to minimise the overall cost, the size of the devices produced should be as small as possible, since silicon wafers are an expensive component of the overall cost.

Production equipment costs are high and hence it is common for devices to be produced by a specialist silicon processing company, which will act as a contractor.

The technique, despite its attractions, is still not widely used in biosensor construction, but examples of its use exist (Vopel *et al*, 1991). The success of i-stat (California, US), whose devices are produced in this way, and who have their own foundry, points to a promising future for this technology.

4.3 Vacuum Evaporation

Evaporative techniques are based on the boiling off or sublimating of a heated material onto a substrate through a screen. This is generally carried out under a high vacuum. Heating typically takes place by passing a high current through a refractory metal containment structure, which holds the material to be evaporated. Other heating techniques include electron beam and radio frequency induction evaporation.

As a mass manufacturing process, vacuum evaporation is somewhat limited by the time taken for each process step and the problems of scale-up. Nevertheless, the process is relatively simple and the equipment required is fairly inexpensive. It is not possible, at present, to produce structures as small as is possible with photolithography, but evaporation is an improvement over thick-film deposition in this respect.

Many materials can be deposited in this way and complex structures can be built up. However, great care is often necessary, since it is very simple for small amounts of dust or dirt to be deposited on the surface of these structures. Since the deposited layers are usually comparatively thin, this can result in pin-holes in the surface, which expose the underlying layer. This can be catastrophic in some types of device. Clean room facilities may therefore be necessary for reliable production.

4.4 Sputtering

In a sputtering process, the target (a disc of the material to be deposited) is bombarded at a high negative potential with positive argon ions from the plasma. The target material is removed (sputtered) by momentum transfer and ejected surface atoms are deposited onto the substrate, which is placed on the anode. For conductors, a direct current (DC) set-up can be used; for insulators a radio frequency (RF) power supply is required.

4.5 Chemical Vapour Deposition (CVD)

CVD is based on diffusive-convective mass transfer. The constituents of a vapour phase, often diluted with an inert carrier gas, react at a hot surface to deposit a solid film. The sample surface chemistry, its temperature and its thermodynamics determine the compounds deposited. CVD is used extensively in the semiconductor industry and has played an important role in transistor miniaturisation by making it possible to deposit very thin films of silicon. It is also the principle building technique in surface micromachining.

CVD was one of the techniques used to produce the optical tracks in Paper VIII of this thesis.

4.6 Ink-Jet Printing

With the exception of screen-printing, the fabrication techniques discussed so far are used, in sensor applications, for the production of the underlying transducer structure only. Ink-jet printing, on the other hand, is usually used to deposit the chemical or biological components of a biosensor onto the surface of the transducer. It is a very rapid method of depositing small droplets of liquid onto a surface.

Ink-jet printers take many forms, but there are two types which are the most widely used. The traditional office-based ink-jet printer heats an ink, causing a little of it to vapourise, which ejects a droplet. This heating stage is a problem where thermally-labile materials, such as those of biological origin, are concerned. The industrial ink-jet, on the other hand, ejects droplets under pressure, with droplet formation controlled by a pressure pulse from a piezoelectric crystal. The latter system can be either drop-on-demand (Kimura *et al.*, 1988), or continuous (Newman *et al.*, 1992).

One of the attractions of the industrial ink-jet process is the high deposition speed possible with devices such as this. Commercial applications include printing onto cartons and packages on production lines, where a conveyor belt may be travelling at considerable speeds. Other attractions are that it is a non-contact process and with the selection of appropriate nozzles and conditions, very small droplets (less than 1 nl) can be handled with ease.

There are some drawbacks to ink-jet printing, however. It is not always simple to achieve good print quality due to a number of factors:

a) Bubble formation in the print-head causes problems due to their effect on the piezoelectrically-induced shock wave. This is a particular problem in aqueous applications, where dissolved gases form bubbles in the print head. It is also a problem where the solvent has a low boiling point. Acetone, for example is difficult to print as it vapourises in the print head leaving voids.

b) Splashing can result when the droplet hits the underlying substrate. This is difficult to cure completely, but the problem decreases with droplet size.

c) The viscosity of the printed solution can change during printing due to solvent evaporation. This changes the required printer settings, which means that frequent adjustments are necessary to maintain print quality.

d) Particulates must be avoided, since the nozzles are easily blocked. It is common to employ a filter upstream of -the print head. However, precipitation of salts from the printed solution can occur downstream of the filter and the filter itself can become blocked in some instances.

e) Most biological reagents appear to be relatively unaffected by the printing process. There are, however, some materials which appear particularly susceptible to the shearing forces involved. Lactate oxidase, for example, becomes rapidly inactivated and some protein solutions suffer from excessive foaming.

The ink-jet process is discussed in more detail in Paper II of this thesis.

4.7 Dip Coating

Dip coating is the simplest way to apply a membrane to a substrate. A large number of laboratory-based biosensors feature dip-coated layers. To form such a layer, the sensor may be simply dipped into an appropriate solution of a material and a solvent. A thin layer is then left by evaporation of the solvent. Although scale-up would appear straightforward, the technique is mainly useful during research, since it can be difficult to control and is not easily adaptable to the coating of selected regions of a complex sensor structure.

4.8 Spin Coating

Spin coating has been optimised for the deposition of thin layers of photoresist on silicon wafers. The process works by dropping the solution to be deposited onto the substrate surface, which is spinning at high speed. The balance between the centrifugal force and the surface tension result in a controlled thin-film of material to be deposited onto the surface.

Spin-coating is used for chemical sensor and biosensor production. However, it is not ideally-suited to the production of thicker membranes, such as those often encountered during sensor fabrication. Also, it is best for flat, circular substrates (such as silicon wafers). Biosensors are rarely shaped like this and hence the techniques has some drawbacks.

4.9 Cavro Printing

A Cavro printer is an automated syringe pump connected to a nozzle. The substrate, onto which the dosed liquid is dispensed, is typically placed on a microprocessor controlled X-Y table. The printed material is deposited in dot-matrix or linear form, usually as a touch-off process. Droplet sizes are typically of the order of 0.1-10 μl, depending on the nozzle and syringe employed. Print speeds are slow compared with the ink-jet process, but the system is simpler to set up and operate, and cheaper. Since the conductivity of the printed fluid is not important, the Cavro technique is also more flexible than ink-jet and it is possible to print most liquids, without modification, with this approach. Cavro is also less susceptible to blockage than ink-jet and is also much simpler to unblock and clean.

Recent developments include a hybrid process, whereby a syringe pump is connected to a print head, which contains a piezoelectric crystal, similar to that used in the industrial ink-jet process. The droplet produced by the syringe pump can, thus, be ejected in a more controlled manner. This results in a smaller droplet capability and improved deposition accuracy.

Cavro deposition is frequently used in applications such as high throughput screening for drug discovery in the pharmaceutical industry. However, It has not yet featured prominently in biosensor applications. It is particularly useful in small to medium scale applications where good control over the deposition is required and where deposition speed is not crucial. In a development programme where small batches of devices may be produced, each with slight variations of

composition, Cavro offers advantages due to its flexibility and the ease with which it is possible to change from one deposited material to another.

One example of the use of Cavro deposition in the fabrication of membranes on planar substrates, such as screen-printed sensor devices. The membrane material is dissolved in an appropriate solvent and deposited onto the surface as a solution. Curing (in this case, simple evaporation of the solvent) results in a membrane structure which covers the appropriate region of the sensor. In this way, many common membranes can be fabricated onto sensor surfaces. The use of Cavro printers would overcome many of the problems encountered during the manufacture of membranes on biosensors using screen and ink-jet printing (Turner *et al.*, 1995).

5. Conclusions

Biosensors have, undoubtedly, progressed a great deal since the first description of such a device by Clark in 1962. The field took off in the 1980s, resulting in the first truly commercially-successful biosensors. There have been many significant achievements in biosensor research. Electron transfer in amperometric biosensors has been advanced considerably due to the introduction of mediated technology. More recently, a combination of enzyme engineering and surface modification has led to further improvements. Optical sensors have evolved from crude devices based on coated optical fibres to those in use today, which are based on such principles as surface plasmon resonance (SPR), interferometry and others.

Understanding of surface interactions has improved considerably, not least due to the development of scanning probe microscopy techniques. The effect of surface interactions is vital in the field of biosensors, particularly in the case of immunosensors, which can be prone to problems from non-specific binding and the orientation of the immuno-reagent for example.

Finally, consideration of manufacturing processes has become more widespread. As devices have been introduced to the marketplace and commercial success has become a reality, it has become clear that how biosensors are fabricated, is a key issue to their ultimate success. It is essential for commercial production that biosensors can be produced at least on a medium batch scale, and with appropriate quality assurance. Product engineering must be included as part of development if further commercialisation is to be realised.

Fabrication technology is developing at a rapid rate in many industries. The main developments are occuring in the microelectronics and telecommunications fields, where huge sums are being invested. The adoption and adaption of these technologies will present major opportunities in biosensor development and commercialisation.

6. Advanced Manufacturing Processes for the Production of Biosensors

The papers featured in this thesis relate to a programme of work intended to explore and exploit automated production processes, which may be suitable for biosensor manufacture. Some of the techniques have already become widely adopted in both academic and commercial applications and here, further exploitation of their capabilities was the aim. Screen-printing, for example, has been used for biosensor production for at least fifteen years and has evolved from the laboratory to full-scale production, most notably for the manufacture of disposable blood glucose biosensors, such as the MediSense range of devices. The other processes have not yet achieved the commercial success of screen-printing in biosensor applications, but their uses are becoming more widespread. The manufacture of rapid strip-type technologies, such as those use for pregnancy testing, for example, rely heavily on ink-jet and air-brush printing techniques.

The overall aim of the work was, undoubtedly, to identify processes which were of industrial significance for biosensor production, but there was a further in-house use for such techniques in research and development applications. Large scale production was therefore important, but was not the sole aim of the work. This has been reflected by the subsequent use of the processes during many student projects, where only small-scale production was required, but where accuracy and precision were prerequisites.

Paper I provides an introductory overview of biosensors. The breadth of their potential application and their attractions are discussed and examples of the components which make up biosensors are described. Brief theory of operation is given for each of the fundamental technologies, with references supplied for further reading in each case. Examples of some of the key biosensor technologies are provided, with descriptions of their fundamental operating principles. Finally, some conclusions and future directions are discussed.

The following paper (Paper II) describes the first application of continuous ink-jet technology for the production of biosensors. An industrial ink-jet printer was modified for use with biological reagents and was used to deposit enzyme solutions onto amperometric transducers to produce biosensors. The paper was predated by a description of the use of a related device for depositing an enzyme layer on an ion-selective field-effect transistor (ISFET) potentiometric transducer (Kimura *et al.*, 1988).

The main difference between the two ink-jet printers is the possible deposition speed. The industrial ink-jet is significantly faster, primarily since it prints in a different way to the drop-on-demand device. In the industrial ink-jet, a continuous stream of droplets is emitted by the nozzle and these are selectively charged and printed. This enables very rapid control of the deposition of the droplets, since it is not necessary to control both the X and Y axes of an X-Y table, as is required in drop-on demand printing, which does not provide the facility to direct the droplets. There are, however, some advantages to the drop-on-demand process. The technique is less susceptible to small fluctuations in the make-up of the printed solution, which cause variations in the droplet deflection. This is one of the main problems in the use of the continuous ink-jet printer, since evaporation of the solvent during recirculation can change the properties of the printed solution over extended print runs.

Paper III describes some preliminary work aimed at expanding the range of mediated amperometric biosensors which could be produced by automated means. The use of mediators has been demonstrated widely, as described in the paper, for reducing the operating potential and overcoming the reliance of such devices on the local oxygen partial pressure. In addition, the measurement of hydrogen peroxide itself is of significant commercial interest due to its widespread use in industrial applications. Further applications of this work are discussed in Paper VII.

Paper IV is the first of three related papers (with Papers V and VI) which chart the development of a prototype fermentation analyser which was designed for on-line use. The first paper explores suitable working electrode materials and assesses fabrication technologies which could be used for the production of biosensors

suitable for incorporation into a flow-injection analyser and sampler, which could be used for the on-line analysis of glucose concentrations during fermentations. Glucose was selected as the initial analyte, following a questionnaire circulated amongst fermentation experts, which revealed glucose as the primary analyte to be monitored. The design of the sensor, however, was such that other analytes, such as ethanol and lactate could be measured without a fundamental design change.

In designing such a biosensor, several parameters were of key importance. These were also assessed partially from the results of the questionnaire, which revealed that users did not require highly extended biosensor lifetimes, but that they would not wish to have to change a sensor during a typical fermentation. These were of very varied timescales, but the majority of fermentations were not expected to last more than seven days. This was, therefore, chosen as the target lifetime of a biosensor. It was also apparent that the measurement ranges of different users were substantially different. In order to accommodate as many of these as possible, flow-injection analysis was chosen, since it enables simple sample handling and dilution. There are many other advantages of the flow-injection approach which are detailed in the three papers, but flexibility was the key issue.

Two approaches were considered: catalytic surfaces, which selectively enhanced the detection of hydrogen peroxide, and mediated techniques. Mediators, whilst highly effective in "one-shot", disposable devices, could add to stability problems, since leaching of the mediator could cause problems in such applications. The biosensor design that was chosen involved the use of a rhodinised carbon working electrode which was suitable for screen-printing. Rhodinised carbon has significant advantages over the more commonly used platinised carbon. It exhibits high current density and significantly lower interference, although it is more expensive. Since the aim was to produce semi-disposable biosensors, the cost of the rhodinised carbon was of concern, placing increased emphasis on ensuring a low-cost manufacturing process that used small quantities of materials. Screen printing enabled rapid production, whilst minimising utilisation of such costly reagents.

Simple construction was a target throughout this work. The minimisation of the number of steps required during the printing process was considered, which resulted in the step of including the enzyme with the catalytic material, for printing in one step. This required the use of a water-soluble binder for use during the printing of such a layer. This was provided by hydroxyethyl cellulose (HEC), which proved highly amenable to the screen-printing process. A membrane was now, therefore, required to prevent the dissolution of the working electrode during use in aqueous application. Had this been the only reason for including a membrane, the advantage of the use of HEC in a one-step process would have been lost, since a further step was still required. However, a membrane was also required to exclude high molecular weight compounds which could cause interference and also to extend the linear range of the biosensor. As discussed in Paper IV, a non-linear signal is expected from the kinetics of glucose oxidase, and the use of a diffusion barrier extends the linear range of such devices.

The design of the biosensor card for incorporation into the flow-injection analyser remained substantially as originally envisaged, but the design and its production evolved during the course of the project. Paper V describes the optimised design which took account of both scientific reasoning for the layers printed and the commercial aspects of production.

Screen-printing was chosen for the production of all layers, since this was required for producing the base structures anyway. Thus, purchase of other equipment was avoided and a simpler production environment would be possible. Other problems with ink-jet printing included the requirement for modifying cellulose acetate inks to facilitate printing and that, in this application, ink-jet printing did not conveniently print sufficient material. The adopted approach was demonstrated to be suitable for producing biosensors for application in an FIA system for on-line use.

Paper VI presents results obtained with the analysis system described in Papers IV and V. It was decided to test the biosensors in a cross-section of fermentations, which were of commercial relevance. Good correlations were generally obtained between the FIA / biosensor and the test kit method. It was, however, clear that care was necessary in complex samples, especially at low glucose

concentrations.

The use of sensors for monitoring lactate levels in alternatives to blood in human patients is discussed in Paper VII. Several types of biosensor were used and results were compared with those obtained from blood samples. Improvements in reproducibility were obtained by the use of screen-printing to produce sensors, but lifetimes were poor. This work pre-dated that presented in Papers IV, V and VI, but in hindsight, it is clear that the biosensors described in these three papers would have overcome the difficulties encountered at the time.

The work presented in the papers so far has focused on electrochemical biosensor applications. The fabrication techniques are, however, equally suitable for some optical biosensor fabrication requirements. Paper VIII combines a description of a novel method for the production of interferometric optical structures with a means of incorporating biological recognition elements. Interferometric devices were produced using silicon-manufacturing technology, which enables low-cost production in high volume environments. Furthermore, small, highly reproducible devices can be produced with relative ease. Applications of this technology could be very broad. A generic transducer structure could be produced, which would be suitable, with appropriate modification for a wide range of immunosensor applications.

This thesis has described work involving biosensors. It should not be forgotten, however, that biosensors may not always be the most suitable device for a particular application. Test-strip technologies, for example, are becoming ever more sophisticated and ELISA tests are unlikely to be replaced in the foreseeable future. The work presented here, whilst intended for biosensor applications may find use in many other bioanalytical applications and, indeed, other areas where controlled reagent handling is important.

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Paper I

Biosensors: Principles and Practice
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11

Biosensors: principles and practice

Jeffrey D. Newman and Anthony P.F. Turner

Biotechnology Centre, Cranfield Institute of Technology, Cranfield, Bedford MK43 0AL, U.K.

INTRODUCTION

Biosensors have been proffered as solutions to multifarious analytical problems in clinical chemistry, food analysis, biotechnology, environmental monitoring and defence. The enormous breadth of proposed applications is matched only by the variety of permutations of transducer and biological components that have been described in the literature. Selected examples include home monitoring of glucose by diabetics, rapid detection of microbial contamination in food and the measurement of ethanol for fermentation process control. The relative activity in different areas of biosensor technology (amperometric, potentiometric, optical and others) is illustrated in Figure 1.

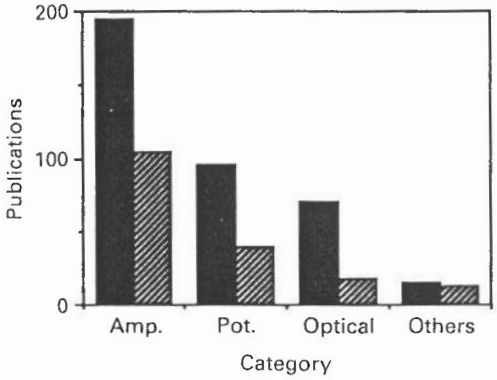


Figure 1. Biosensor publications in 1990
Solid boxes indicate papers; hatched boxes indicate patents.

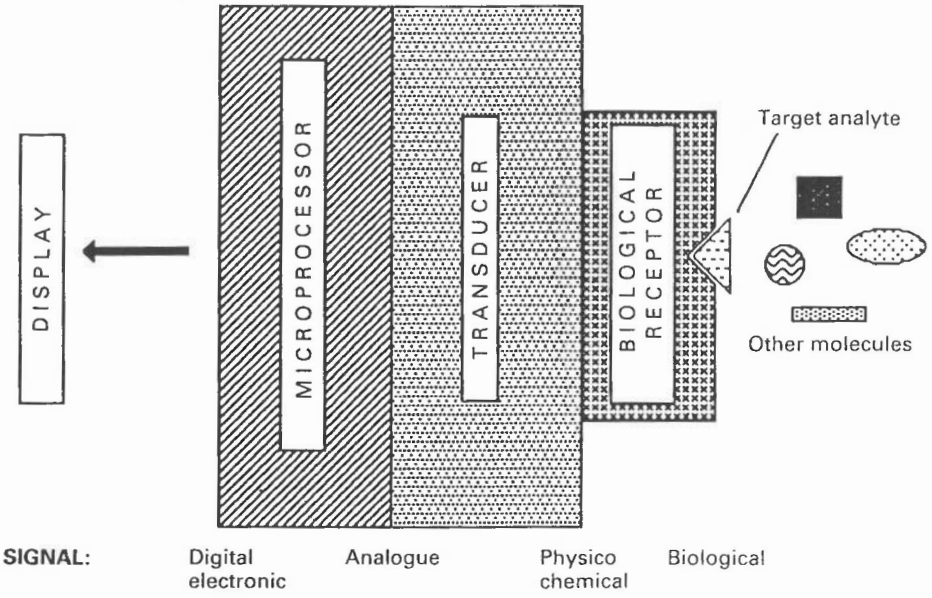


Figure 2. Generalized scheme of biosensor operation

Early success[1] with electrochemical devices together with their relative simplicity and flexibility accounts for the dominance of research activity in this area. Optical technology, however, has advanced rapidly in recent years as a result of activity in the telecommunications industry and offers interesting opportunities, particularly in combination with biological affinity reactions. The considerable investment of time and money worldwide in the field of biosensors over the last decade is beginning to crystallize in the form of novel commercial devices for analysis.

This essay briefly reviews the essential elements of biosensor technology and illustrates a selection of the most important developments that have led, or are leading, to practical devices. Biosensors have been the subject of several books recently[2–5], in which a thorough documentation of the subject area can be obtained. In this text, the components that make up biosensors will be described and some selected examples will be given that illustrate important principles and advances.

BIOLOGICAL COMPONENTS

One of the chief attractions of biosensors is the remarkable specificity that biological molecules confer on them. Biological components can be used to make either catalytic or affinity sensors. In both cases, it is binding reactions that provide the specificity, but with catalytic sensors it is a change in concentration of a component due to the catalysed reaction that is detected, whereas with an affinity sensor it is the binding event itself that is monitored.

Enzymes are by far the most commonly used components in catalytic biosensors, but microbial, plant or animal cells, or subcellular organelles, have been used where the use of enzymes has proved impractical or impossible. Many of the known enzymes are now available commercially, and advances in enzyme technology should enhance

biosensor development by increasing enzyme availability. Recently, there has been a rapid increase of interest in the operation of enzymes in non-aqueous media. When used in the organic phase, enzymes have been shown, not only to retain activity, but to offer certain features and benefits. Improved thermal stability and altered substrate specificity, together with the possibility of detecting previously inaccessible analytes, is of interest for biosensor applications. This novel area has been reviewed recently[16].

Most affinity sensors reported to date are based on immunological principles, but other receptor molecules can be used. Lectins, plant proteins that bind specific carbohydrates, can be used in biosensors and cell receptors have been used in gas sensing. Also, bioreceptors, synthetic molecules with affinity properties, can be applied in biosensors. Devices need not be restricted to the use of a single biological component. Various combinations, such as more than one enzyme or an antibody and an enzyme can be co-immobilized to provide new or improved analytical capabilities.

The component parts of a biosensor must be held in close proximity to one another to facilitate efficient operation. The localization of biological components at or close to the transducer surface permits continuous or repeated use, maximizes the response by concentrating the biocomponent and often increases stability, particularly in enzymes. The methods used, collectively known as immobilization techniques, can be separated into: entrapment with a membrane, gel or microcapsule, physical or chemical adsorption, crosslinking between molecules and covalent bonding to insoluble supports. The area has been covered comprehensively in a recent article[17].

TRANSDUCERS

In a biosensor, a transducer is used to convert the biological event, be it a catalytic response or a binding reaction, into a form where it can be read directly or further processed by a microprocessor. The mode of operation of a generalized biosensor is outlined in Figure 2. This shows that a biological event is detected as a physical or chemical change by the transducer which converts this into an analogue signal that can be digitized by a microprocessor and displayed.

Since the transducer is in intimate contact with the biological component and the sample medium, it must be compatible with the sample and amenable to an appropriate immobilization procedure. A wide range of transducers may be used in biosensors and these can be broadly divided into electrochemical, optical, piezoelectric and calorimetric devices. Examples of these are outlined in Table 1, and are discussed more fully in the following sections.

Table 1. Examples of transducer types

Transducer	Examples
Electrochemical	Clark oxygen electrode, mediated electrodes, ion-selective electrodes, chemical field-effect transistors, light-addressable potentiometric sensors
Optical	Photodiodes, waveguide systems, integrated optical sensors
Piezoelectric	Quartz crystals, surface acoustic wave devices
Calorimetric	Thermistor, thermopile

Electrochemical devices

Many biological systems consume and/or produce electroactive species which can be monitored by electrochemical techniques such as amperometry, potentiometry or by conductimetric methods.

Amperometry has been used for the measurement of a wide variety of species in many applications, not least in biological media. Under suitable conditions, it is possible to measure concentrations in the nanomolar range directly, with a dynamic range of up to four orders of magnitude or more. The technique classically utilizes a three-electrode system in which a potential is applied between a working electrode and a reference electrode by the use of a counter or auxiliary electrode. In this way, effectively zero current is passed through the reference electrode so that the reference potential is not perturbed. The resulting current measured is due to the oxidation or reduction of the species of interest at the working electrode. The current is directly proportional to the concentration of the analyte at the working electrode surface and is therefore dependent on the rate of mass transfer of this component to the surface and to the charge transfer kinetics.

For many practical devices, it is more convenient to combine the counter and reference electrodes. Since a current is passed, this causes a drift in the reference potential, but for short periods of operation this may not be a problem. Amperometric devices may be subdivided into cathodic (reducing) or anodic (oxidizing) sensors. Both types have been extensively used in biosensors. The classical working electrode material is mercury. For biosensors however, solid electrodes based on platinum, carbon and gold have been preferred. Finding a suitable method for surface preparation and the occurrence of fouling during use, especially in biological samples, are particular problems with solid electrodes. The Clark electrode[1], incorporating an oxygen-permeable polymer membrane that excludes interferents, overcomes many of these difficulties and is still commonly used, particularly in research applications.

A related constant-potential technique is coulometry, which involves the measurement of the total charge transfer rather than the current. This technique follows Faraday's law[6]:

$$N = Q/nF$$

where N = number of moles of reactant consumed, Q = total charge passed (coulombs), n = number of electrons involved in the reaction and F = Faraday constant (96 487 coulombs/mole).

It is therefore an absolute method, independent of the electrode and reaction kinetics. The technique is usually carried out with small sample volumes to reduce the time required to consume all of the electroactive species.

A potentiometric sensor operates under conditions of near-zero current flow and measures the difference in potential between the sensing element and a reference electrode. The response of these devices is usually proportional to the logarithm of the activity of an ion in solution, characterized by the Nernst equation[6]:

$$E_{ISE} = E_0 + 2.303\ (RT/zF)\ \log a_i$$

where E_0 = a constant, independent of a_i, R = gas constant (8.314 J/K), T = temperature (K), z = charge number of ion i and F = Faraday constant.

This corresponds to a linear relationship between the measured potential, E_{ISE} and the logarithm of the activity of the ion i (a_i) with a slope of 59.16 mV for each 10-fold change of activity of a monovalent ion at 25 °C.

Ion-selective electrodes are the most important of this class of transducer, with many applications in biosensors. Specificity is conferred by selective membranes which may be formed from metal salts or polymer membranes containing ion-exchangers or neutral carriers. The construction and operation of these ion-selective electrodes are discussed in detail elsewhere[8].

Miniaturized potentiometric devices can be fabricated by deploying field effect transistors as transducers. This approach was discovered in 1970 by Bergveld[9] who, instead of connecting an ion-selective electrode to an impedance-converting field effect transistor, used the transistor directly as an ion-selective electrode. Deposition of a wide range of permselective membranes on the silicon nitride gate layer of the field effect transistor enables a wide variety of ions to be detected. The small dimensions and ready availability of microelectronics fabrication technology makes the field effect transistor an attractive transducer for biosensor applications. However, problems in depositing and maintaining biological molecules on devices of this type have limited their success. A possible area where developments are possible is in the production of multi-sensors, in which a single device may contain many sensing elements.

Light-addressable potentiometric sensors are based on a silicon wafer possessing a chemically modified surface. A measurement is made by illuminating the back of the wafer with a light-emitting diode which causes a photocurrent to flow. Modulation of the light results in an alternating current which is pH-dependent. A chemical reaction changing the pH at the sensor surface alters the current. If an external potential is applied to maintain a constant current, the reaction rate can be monitored by the change in this potential.

Suggested further reading: Bard & Faulkner (1980)[6], Wang (1988)[7].

Optical devices

Emission and absorption of electromagnetic radiation are useful techniques that have been used in chemical analysis for over 100 years. Emission spectroscopy, atomic absorption, and Raman scattering are some examples of techniques that have been used. Modern advances in miniaturization make possible the incorporation of optical analysis into small, cheap and simple-to-operate sensors.

The change in the light absorbance characteristics of a reagent layer on interaction with an analyte is the simplest form of optical detection. Optical fibres are commonly used to carry light to and from a reaction area. Suitable materials for the construction of optical fibres, which transmit light by multiple internal reflections, include glasses and plastics. Indicators immobilized on optical sensors can be used to react reversibly with an analyte to form a product with different optical properties to the original reagent. This principle has been used for many analytes and is typified by an optical pH sensor in which an indicator in its base form is protonated by the analyte, the hydrogen ion. These sensors have a working range of 1–2 pH units around the pK_a of the indicator. Considerable research has been focussed on indicators suitable for use in the physiological pH range, but little has been done outside this range. Most oxygen sensors based on optical principles utilize fluorescence quenching. The fluor-

escent material can be incorporated onto the sensor in the solid phase, or it can be in solution separated from the sample by an hydrophobic oxygen-permeable membrane. The measurement can also be based on a change of intensity or a shift in the spectrum.

A light beam emanating from a medium of higher refractive index (n_1) striking the interface with a medium of lower refractive index (n_2) undergoes total internal reflection if the angle of reflection is larger than the critical angle (ϕ_c):

$$\phi_c = \sin^{-1}(n_2/n_1)$$

When light is reflected in an optical device, there is a decay of energy away from the point of reflection into an external medium of a different refractive index. This electromagnetic phenomenon is known as the evanescent wave and can be used to detect a change in refractive index in close proximity to the optical interface. There are three principal techniques, commonly used in optical biosensors, that utilize this effect. Attenuated total reflection relies on the absorption of light of specific wavelengths by molecules at the waveguide surface. This absorption is proportional to the concentration of these molecules present. Total internal reflection fluorescence occurs when the evanescent wave is used to excite fluorescent molecules bound to the waveguide surface. It is argued that a washing step, as required in conventional immunoassays to separate bound from unbound antibody, is no longer required with this technique since fluorescent molecules in the bulk solution are not excited. When a glass substrate is coated with a thin metal layer, it is possible to excite an electromagnetic wave (a surface plasmon) which propagates along the metal surface, by using a light beam which undergoes total internal reflection at the glass surface. At a certain angle of incidence of the light beam, surface plasmon resonance occurs and is observed as a sharp minimum of reflectance intensity. This critical angle is very sensitive to variations in the refractive index of material lying a few hundred nanometres outside the metal film. The increase in reflected light can be measured, which corresponds to the system moving out of resonance as molecules are bound to the surface.

Integrated-optical sensors have been developed recently[12] which utilize grating couplers on planar waveguides. These can be used to respond either to a change in the refractive index of a liquid sample covering the waveguide, or to the adsorption and desorption of molecules from a gaseous or liquid sample. When the incident light beam strikes the miniature diffraction grating on the waveguide, dispersion of light into the sample medium occurs. The dispersion is sensitive to the refractive index of the sample and results in a shift in the incoupling angle or the incoupled power, which can be detected by a photodetector.

Suggested further reading: Dakin & Culshaw (1988, 1989)[10,11].

Piezoelectric devices

Exploitation of acoustic techniques in biological science has received scant attention until recently. Developments in piezoelectric transducers, especially expansion of the upper ultrasonic frequency limits, has led to an increased interest in this area over the last few years. Piezoelectric crystals are mass produced cheaply for the electronics industry, an attraction for biosensor applications. The major limitations of devices

such as these is, at present, the poor understanding of the acoustical characteristics of biological samples.

Piezoelectric materials are able to generate and transmit acoustic waves in a frequency-dependent manner. At a certain frequency, acoustic resonance is induced, which is a function of the crystal mass. Adsorption of molecules onto the crystal surface leads to a frequency change (ΔF) according to the Sauerbrey equation:

$$\Delta F/F = -\Delta m/A\rho t$$

where Δm = mass of adsorbed material (g), A = crystal face area (cm^2), ρ = density of adsorbed material (g cm^{-3}), and t = thickness of adsorbed layer (cm).

ΔF is highly sensitive to Δm, with values varying between 500 and 2500 Hz/μg with pg detection limits available from commercial quartz crystals. In solution, device performance is limited by response to the solution components such as electrolyte concentration, conductivity and also by non-specific adsorption. In such cases, the Sauerbrey equation is not obeyed strictly and the response is dependent on viscoelastic effects at the surface.

Surface acoustic waves can be formed by applying radiowave frequencies to one of two pairs of interdigitated electrodes (transmitter) on the surface of a piezoelectric material. An acoustic wave (Rayleigh surface wave) propagates along the surface of the crystal and is detected by a second pair of electrodes (receiver). The depth of penetration of the wave is of the order of a wavelength, and its transmission is dependent on material deposited and/or on the surface.

Suggested further reading: Clarke *et al.* (1989)[13]; Guilbault (1990)[14].

Calorimetric devices

Two transducers presently dominate research into thermal biosensors. Thermistors are temperature-sensitive, semiconductor-based sensors. Thermopiles are arrays of thermocouples designed to increase sensitivity. The former is a resistivity-based and the latter a voltage-based measurement. A third device, developed recently, utilizes a light-activated micro-fabricated resonating silicon bridge. The resonant frequency is extremely sensitive to temperature, allowing changes of millidegrees to be detected.

Measurement of temperature changes due to a biological reaction is almost universally applicable and appears straightforward. However, thermal interference is a problem which often requires elaborate and expensive equipment to overcome.

Suggested further reading: Danielsson (1989)[15].

BIOSENSOR EXAMPLES

The number of permutations of transducer and biological component is very large. In the following section, several examples of biosensors will be described that illustrate important concepts.

Oxidase enzyme electrodes based on oxygen electrodes

The first, and most widely used, enzyme in biosensor applications is glucose oxidase, which catalyses the reaction:

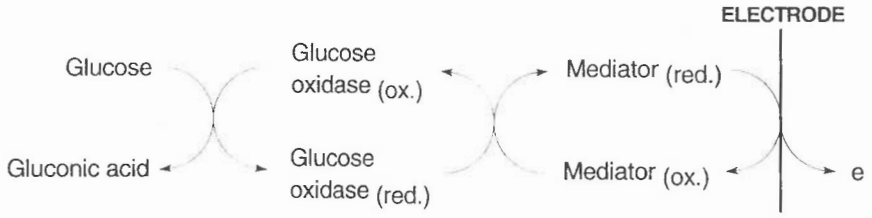
$$\text{glucose} + O_2 \rightarrow \text{gluconic acid} + H_2O_2$$

Glucose concentration can be monitored by measuring, for example, the fall in oxygen tension or the production of hydrogen peroxide. This can be achieved amperometrically by the use of a platinum working electrode poised at –0.6V (versus saturated calomel electrode) for the detection of oxygen or at +0.7V for the detection of hydrogen peroxide. The relatively extreme potentials required make this system prone to interference from other electroactive species. A further problem is the reliance of the reaction on the oxygen concentration in the sample.

Glucose biosensors based on the Clark oxygen electrode (as described earlier) contain an outer polycarbonate membrane which entraps glucose oxidase close to the electrode surface and excludes large molecules and cells from the external solution. An inner gas-permeable membrane restricts the species that can reach the electrode, decreasing interference. In hydrogen peroxide-detecting systems, an inner acetate membrane replaces the gas-permeable membrane, allowing the passage of hydrogen peroxide, oxygen, salts and water, but excluding glucose, ascorbic acid and some other potential interferents. Membranes, as used in this example, have proved successful in excluding most major interferents, but the approach is not ideally suited to mass production techniques.

Mediated electrochemical biosensors

In the previous example, oxygen was present as an electron acceptor. It is possible to substitute artificial electron acceptors (mediators) so that oxygen is no longer required, and hydrogen peroxide, ideally, is not produced. Using glucose oxidase as an example again, the following reaction scheme can be applied:



Electrochemistry occurs at the redox potential of the mediator, which can be chosen to minimize interference from other electroactive species. In addition, a successful mediator will be stable and have rapid kinetics. In particular, the reduced form must be stable in the presence of oxygen. Some of the most commonly used mediators include benzoquinone, potassium ferricyanide, tetrathiafulvalene, tetracyanoquinodimethane and the ferrocenes.

The most commercially successful biosensor to date, the ExacTech (MediSense, Cambridge, MA, U.S.A.) blood glucose meter is based on this principle[18], utilizing a ferrocene derivative as a mediator. A disposable enzyme electrode strip as used in this meter is shown in Figure 3.

Biosensors based on the fluoride ion-selective electrode

The enzyme peroxidase can be used to catalyse the cleavage of the covalent carbon–fluorine bond in certain organofluorine compounds such as 4-fluorophenol with

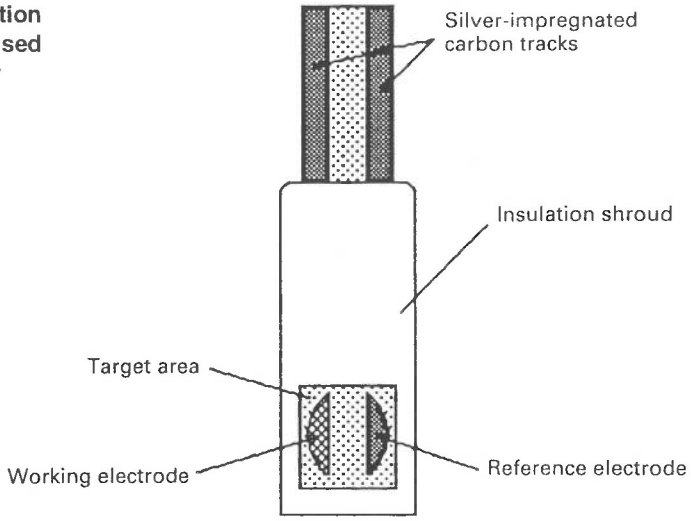


Figure 3. Schematic representation of the enzyme electrode strip used with the ExacTech glucose meter

the elimination of fluoride ions. In its simplest form, hydrogen peroxide can be monitored directly by immobilizing peroxidase close to a fluoride ion-selective electrode[19] and providing 4-fluorophenol as a substrate, but the system is highly versatile. It can be coupled to any enzyme catalysed reaction in which peroxide is released. For example, glucose can be monitored by co-immobilizing glucose oxidase and peroxidase. A further advantage lies in the remarkable specificity of the fluoride electrode, which has no significant interferents at or close to neutral pH.

Electrochemical immunosensors and immunoassays

Antibodies can be labelled with electroactive molecules so that both amperometric and potentiometric measurement can be employed. There are many such systems which have been proposed and developed.

Field-effect transistors are charge-measuring devices that appear attractive for the direct detection of immunochemicals which are bound to the sensitive gate area. However, to date, attempts at achieving this have been largely unsuccessful. The reasons for this have been discussed in detail recently[20].

Enzyme-labelled antibodies can be used to catalyse a reaction releasing an electroactive product. Furthermore, a secondary enzyme can be incorporated as an amplifier which can greatly increase the apparent activity of the label. This technique has been demonstrated for the determination of prostatic acid phosphatase using an alkaline phosphatase label which catalyses the dephosphorylation of $NADP^+$ to NAD^+. This is followed by the catalytic cycling of NAD^+ to NADH in a redox cycle generating ferrocyanide (Figure 4). The rate of ferrocyanide production can be directly related to NAD^+ and hence to alkaline phosphatase concentration, and can be monitored coulometrically.

A total DNA assay system based on light-addressable potentiometric technology features disposable assay sticks which are inserted into an instrument for measurement. The system is rapid and the sensitivity is claimed to be comparable to that of conventional radiolabelling techniques. The sample is incubated with two binding

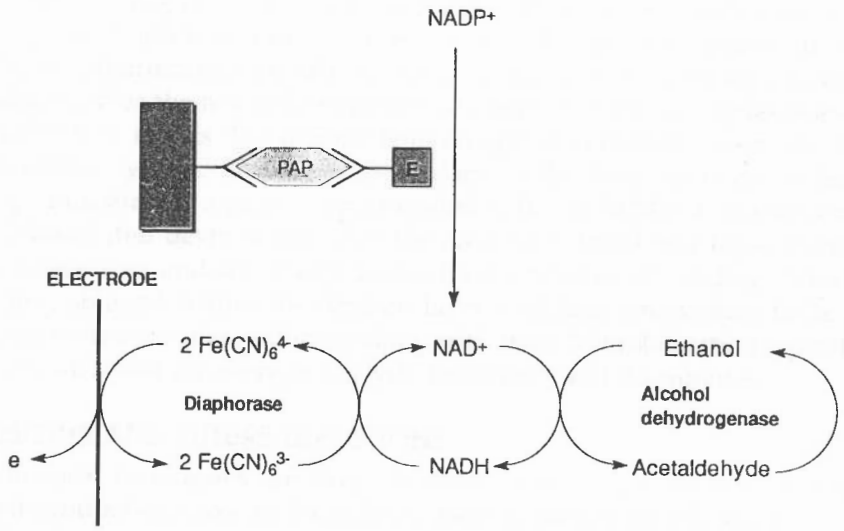


Figure 4. Schematic of electrochemical immunoassay

protein conjugates and the single-stranded binding protein is conjugated to a hapten, which makes a specific link to a capture membrane. An anti-DNA monoclonal antibody is conjugated to an enzyme, such as urease, and the labelled complex transferred to a filtration unit, where it is concentrated onto the measurement site. Unbound enzyme conjugate is washed from the membrane which is then brought into contact with the sensor. In the presence of urea the rate of change in pH can be monitored, which is proportional to the amount of DNA present (Figure 5).

Enzyme optodes

Optical oxygen and pH sensors, based on fluorescence quenching, can be used to monitor many enzyme-catalysed reactions. For example, glucose oxidase has been immobilized in a sensing layer at the end of a fibre optic light guide. The catalytic oxidation of glucose to gluconic acid lowers the local pH which can then be monitored by the quenching of a fluorescent pH-sensitive dye.

Optical immunosensors

A biological binding event can be conveniently converted into a quantitative result by use of optical techniques. Solid phase immunoassay with optical detection allows measurement of both high and low molecular mass analytes in either competitive or sandwich formats. The two optical technologies currently receiving the most attention are fluorescent evanescent wave and surface plasmon resonance.

The fluorescence capillary-fill device consists of two parallel plates, with a 0.1 mm separation. The assay capture system is covalently coupled to the baseplate, which also acts as an optical waveguide. Other reagents are deposited on the top plate, so that they dissolve upon addition of sample to the device. On completion of the immunoassay, bound fluorophore can be discriminated from free fluorophore by the evanescent optics of the baseplate waveguide. Two examples of assays that have

been developed using this device are for *Rubella* antibody in serum and for human chorionic gonadotrophin, a hormone used as an indicator in pregnancy testing.

The BIAcore (Pharmacia, Uppsala, Sweden) system is a surface plasmon resonance prism which enables the researcher to monitor the formation of complexes and reveal steric and allosteric effects. The unit contains an optical detection system, autosampler and microfluidic system. The sensing interface of the BIAcore is an exchangeable sensor chip consisting of a glass support coated with a gold film and a biocompatible layer of carboxylated dextran bound to the gold film. This latter layer increases the effective surface area and sensitivity and reduces non-specific binding. A biospecific ligand is immobilized within the dextran layer, enabling interactions to be studied directly, in complex samples, without prior purification. Immobilization typically takes about 30 minutes and an average analysis between 5 and 10 minutes.

CONCLUSIONS AND FUTURE DIRECTIONS

Electrochemical techniques are likely to continue to dominate both research and commercial products in enzyme-based biosensors in the foreseeable future, and many other applications for the techniques will continue to be found. Optical techniques are extremely promising, particularly in affinity applications. The predicted advances in optical technology and materials resulting from the large amount of research in telecommunications and optical computing should increase opportunities in optical biosensors. At present, other transducer-based systems offer interesting possibilities

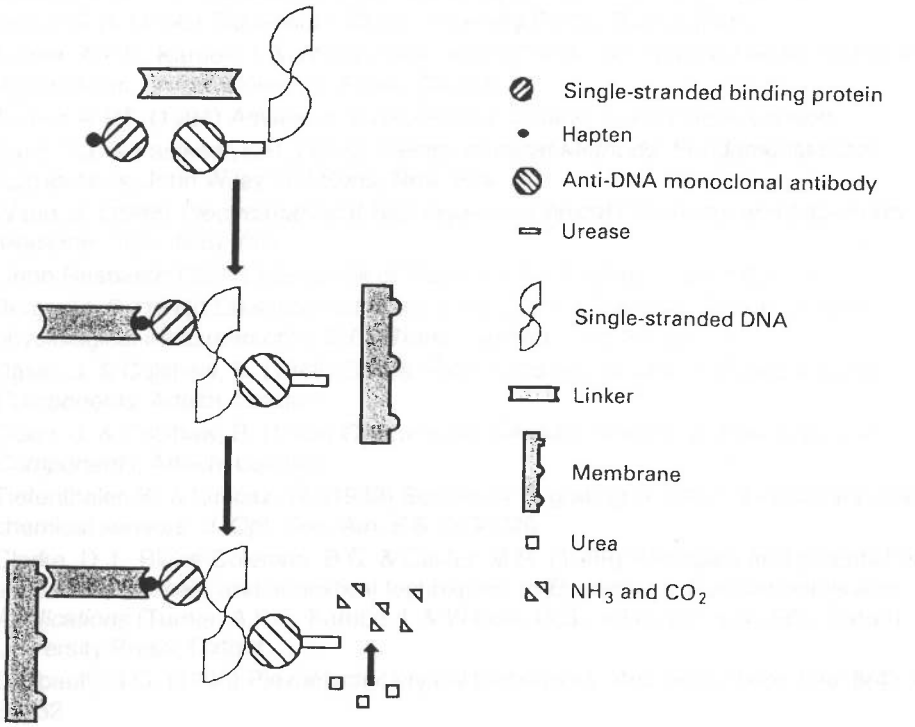


Figure 5. Schematic of light-addressable potentiometric sensor assay

for certain applications, but appear unlikely to challenge electrochemical or optical methods in the majority of situations.

Medical diagnostic requirements have been a major driving force in biosensor development: the instant analysis of clinical samples is appealing to both patient and physician. A lot of early work in this field was directed at the determination of blood glucose in insulin-dependent diabetic patients. Recently, attention has been directed towards *in vivo* sensors, which represent a particular challenge, since workers face not only the traditional problems of biosensor development, but the need for biocompatibility. In recent years, the number of biosensor applications outside of the clinical sector has been increasing. New legislation and increasing awareness of environmental issues has fuelled research into biosensors for pollutants such as pesticides, herbicides, microbial contamination and gases such as carbon monoxide. Likewise, hygiene regulations and the need for tighter quality control in an increasingly competitive market are convincing the food industry of the benefits of biosensors. Military applications are focussed on specialized requirements such as biological and chemical defence, but the development of generic technologies could have spin-offs in other applications.

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Paper II

Ink-Jet Printing for the Fabrication of Amperometric Glucose Biosensors

Newman, J.D., Turner, A.P.F and Marrazza, G.

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Ink-jet printing for the fabrication of amperometric glucose biosensors

J.D. Newman and A.P.F. Turner *

Biotechnology Centre, Cranfield Institute of Technology, Cranfield, Bedford MK43 0AL (UK)

G. Marrazza

Sezióne di Chimica Analytica, Dipartimento di Sanità Pubblica, Epidemiologia e Chimica Analytica Ambientale, Università degli Studi di Firenze, Via G. Cappioni 9, 50121 Florence (Italy)

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Abstract

Ink-jet printing has been demonstrated as a manufacturing technique that facilitates the rapid, reproducible and economical production of amperometric glucose biosensors. Glucose was chosen as the analyte for demonstrating the process. For eight electrodes produced, the relative standard deviation of the response was $< 5\%$. The technique is extremely versatile, and will enable a wide variety of reagents to be placed on virtually any sensor design. This technique will be of particular benefit for the mass manufacture of intricate devices, where existing production techniques, such as screen-printing, may not be suitable.

Keywords: Amperometry; Biosensors; Enzymatic methods; Ink-jet printing; Enzyme electrodes; Printed enzyme electrodes

Over the last few years, there has been a great deal of interest in the production of a wide range of amperometric enzyme electrodes [1,2]. The use of mediators to circumvent the reliance of many of these electrodes on the local oxygen partial pressure has become widespread [3] and is amenable to mass production using established techniques such as screen-printing. The commercial launch of the ExacTech glucose meter, based on ferrocene-mediated enzyme electrodes [4], has highlighted this approach to the production of amperometric devices for the analysis of glucose and other analytes. More recently, tetrathiafulvalene (TTF) has been shown to be a good mediator, promoting electron transfer from glucose oxidase [5] and lactate oxidase [6] to graphite electrodes.

Whereas single electrodes may be fabricated in the laboratory, the complications introduced by intricate designs tends to hinder the large-scale production of inexpensive and reproducible devices. Further, the immobilization of all of the components of a biosensor is critical for continuous use and, for microfabricated devices, the accurate spatial distribution of one or more enzymes or other components is also required. The deposition of biological reagents such as enzymes can be achieved with an ink-jet nozzle [7]. It is also possible, by selection of an appropriate solvent, to deposit a mediator solution. The technique allows fluid to be placed on almost any surface, irrespective of shape or delicacy, facilitating great flexibility in the design of the finished device. In addition, the fluid is deposited as small

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droplets (typically about 1 nl) which can be placed accurately and at high speed.

A TTF-mediated, amperometric glucose biosensor was chosen for initial studies, as biosensors for this substrate have been widely reported, enabling the ink-jet printing process to be compared with a variety of other fabrication techniques.

The reactions involved at the electrode are the following:

$$\text{glucose} + 2\text{TTF}^+ \rightarrow \text{gluconic acid} + 2\text{TTF} + 2\text{H}^+ \quad (1)$$

$$2\text{TTF} \rightarrow 2\text{TTF}^+ + 2\text{e} \ (200 \text{ mV vs. SCE}) \quad (2)$$

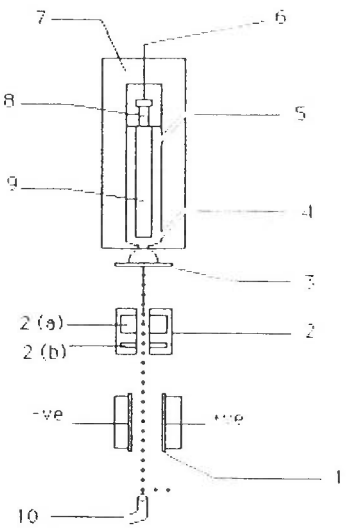


Fig. 1. Schematic diagram of ink-jet print-head: 1 = high-voltage deflector plate; 2 = charge electrode featuring (a) electrode and (b) detector; 3 = nozzle; 4 = ink bleed; 5 = ink feed; 6 = modulation voltage supply; 7 = gun body; 8 = piezoelectric crystal; 9 = drive rod; 10 = return tube.

EXPERIMENTAL

Materials

Chemicals were of analytical-reagent grade where not specified otherwise. Glutaraldehyde (25% aqueous solution), bovine serum albumin (BSA) and β-D-glucose were obtained from Sigma (Poole). Glucose oxidase (GOD) (type VII from *Aspergillus niger*, 10^5 U g^{-1}) was obtained from ABM Chemicals (Stockport). Tetrathiafulvalene (97%), potassium dihydrogenphosphate and dipotassium hydrogenphosphate were supplied by the Aldrich (Gillingham), tetrabutylammonium perchlorate (> 98%) by Fluka (Buchs) and ethanol (99.8%) by BDH (Poole).

Screen-printed electrodes

Screen-printed carbon electrodes were fabricated using a Model 245 screen-printer (DEK, Weymouth). The electrodes were printed on PVC substrates purchased from Sericol (London) using silver (Electrodag 477 SS RFU), carbon (Electrodag 423 SS) and UV-cured insulating (product number 451) inks obtained from Acheson Colloids (Plymouth). The screens were obtained from DEK, with 200 counts in^{-1} screens used for the carbon and silver inks and 390 counts in^{-1} screens used for the insulating ink. The electrodes were constructed by printing arrays of eight silver conducting tracks (22 × 1 mm) and, once dry, overlaying carbon working electrodes (8 × 3 mm) at one end of the tracks. Finally, an insulating layer was printed over the electrodes so that only the graphite layer at one end and a contact at the other were exposed. The silver and graphite inks were cured overnight at room temperature (it is possible to accelerate this process by heating the inks), and the insulating ink was cured by exposure to a UV source (power output 7.0 mW cm^{-2}, 302 nm) for 2 min.

Ink-jet nozzle

The print-head of the ink-jet printer (Biodot, Diddington) is shown in Fig. 1. The fluid to be printed is forced under pressure (typically about 3 bar) through a small hole in the nozzle (75 μm is commonly used). As the fluid passes through the gun body and nozzle, the drive rod vibrating under the influence of an oscillating piezoelectric crystal breaks the fluid into individual droplets at the rate of about 64 000 s^{-1} (depending on the modulation voltage). The stream is aligned so that the droplets enter the return tube to be

recycled. Printing is achieved by charging the droplets with the charge electrode and then deflecting them between the parallel high-voltage deflector plates. The printed pattern is built up in dot-matrix form controlled by a microprocessor.

Preparation of glucose sensors

A 75-μm nozzle was fitted into the ink-jet print-heat, providing a droplet size of 1.4 nl. The mediator, TTF, was dissolved in ethanol (0.05%, w/v). Tetrabutylammonium perchlorate (1%, w/v) was added to increase the conductivity of the mediator solution (a conductivity-based level detector, actuating a transfer pump in the printer, was not able to detect the presence of the mediator solution alone owing to its low conductivity). A rectangular print pattern was selected with the microprocessor in which a matrix of 16×21 droplets was deposited with each pass of the print-head. GOD (0.05%, w/v) was dissolved in phosphate buffer (0.1 M, pH 7) and deposited using the same print pattern. A layer of BSA (0.05%, w/v, in phosphate buffer) followed by a layer of glutaraldehyde (5%, w/v, in phosphate buffer) were deposited on some sensors to aid retention of the enzyme. The amounts of mediator and enzyme deposited on the electrode surface were varied by passing the electrode under the print-head several times, so only a single concentration of each solution was required. All electrodes were stored dry and refrigerated at 4°C.

The linear range of the response can be increased by covering the surface of an enzyme electrode with a semi-permeable membrane, which restricts the mass transport of the analyte into the catalytic layer adjacent to the base electrode [8]. This technique has been widely used, especially for the production of glucose sensors, where the electrode response can be extended into the higher clinical levels possibly encountered with diabetic patients, but is not of importance in demonstrating the ink-jet process as a means of depositing enzymes and mediators in a rapid, economical and reproducible manner. Therefore, uncoated electrodes were used throughout this work.

Electrochemical measurement

A three-electrode system was used, consisting of a saturated calomel reference electrode (SCE) (Russell, Auchtermuchty), a platinum foil auxiliary electrode and the printed working electrodes, as described earlier. The electrodes were connected to an Autolab electrochemical analyser (EcoChemie, Utrecht) and immersed in 15 ml of 0.1 M phosphate buffer (pH 7). A potential of 200 mV vs. SCE was applied for the amperometric determination of glucose.

RESULTS AND DISCUSSION

Preliminary measurements were made in order to check that the ink-jet printed enzyme was functional and able to couple with the TTF giving a significant signal. A glucose sensor was prepared as described earlier, with one pass of the print-head for both TTF and GOD deposition, followed by glutaraldehyde/BSA immobilization. A calibration graph for one of these devices is shown in Fig. 2 (plot a). The linear range of the glucose sensors prepared with the enzyme immobilized in this way was much extended when compared with those where the enzyme was simply adsorbed on the graphite electrode surface (plot b). The functional range of these sensors was much as expected for a device with no semi-

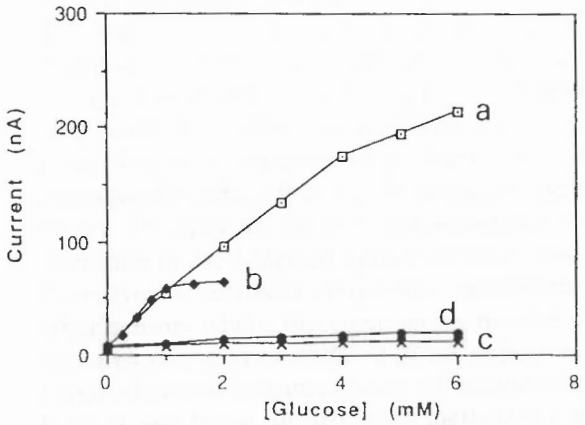


Fig. 2. Response of ink-jet printed sensors: (a) enzyme (glutaraldehyde/BSA immobilized) and mediator; (b) enzyme (adsorbed) and mediator; (c) mediator only; (d) enzyme only.

permeable membrane coating [9]. Comparison of plots a and b shows that a greater retention of enzyme at the electrode surface occurs with glutaraldehyde immobilization. Calibration graphs obtained from electrodes prepared with TTF only (plot c) and GOD only (plot d) gave negligible deviations from the background current.

In order to study the effect of varying the enzyme and mediator concentrations, the electrodes were passed under the print-head several times. Figure 3 shows the effect of increasing the enzyme concentration, by printing one pass (plot a), six passes (plot b) and twelve passes (plot c), at constant mediator concentration (ten passes), on the electrode response. As can be seen, the slope and operating range of the sensors increased with increasing enzyme concentration.

For a disposable single-use sensor, reproducibility is vital. The screen-printing process provides a means of producing electrodes with well defined areas and the ink-jet process allows the accurate deposition of a mediator and enzyme. The reproducibility of a batch of eight electrodes produced with twelve passes of enzyme and ten passes of mediator is shown in Table 1. This compares very favourably with other techniques [10].

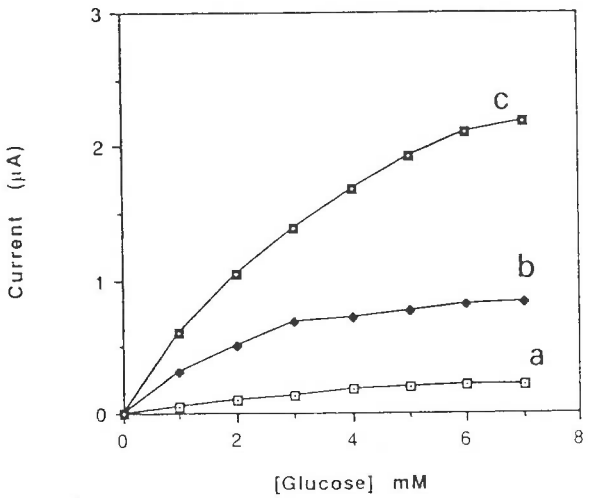


Fig. 3. Effect of enzyme concentration on electrode response: (a) one pass of print-head; (b) six passes; (c) 12 passes.

TABLE 1

Reproducibility of eight ink-jet printed glucose sensors

[Glucose] (mM)	Mean current (μA)	Standard deviation (μA)
0.0	0.007	0.001
1.0	0.618	0.016
2.0	1.064	0.045
3.0	1.383	0.036
4.0	1.664	0.040
5.0	1.893	0.040
6.0	2.114	0.092
7.0	2.177	0.102

Mediated enzyme electrodes have been reported to have response times of the order of 1 min [6,11]. The ink-jet printed glucose sensors also had response times in this range.

Conclusions

It has been demonstrated that it is possible to deposit GOD and TTF solutions on electrodes using an ink-jet printer in order to make amperometric biosensors for glucose.

Planar screen-printed carbon electrodes were chosen as transducers, but it would be possible, with an ink-jet printer, to deposit material on a wide variety of objects, irrespective of their shape or, because it is a non-contact process, delicacy. This may allow the mass production of biosensors where a planar substrate is not the ideal choice.

One of the most significant aspects of ink-jet printing is the economy of the process. Because the enzyme and mediator solutions are only placed where they are required, there is very little wastage. For example, for each pass of the print-head, with the pattern used in this work, only about 470 nl of solution were deposited. This corresponds to about 0.28 μg of enzyme for twelve passes. This may not be the most important consideration in the design of a glucose sensor, where the enzyme is relatively inexpensive, but for many other sensors where the components may be extremely valuable it could be of prime importance. Electrochemical polymerization of enzymes has been shown to be an attractive method for the immobilization of enzymes [12] in which the amount and spatial distribution of the immobi-

lized enzyme can be accurately controlled. Ink-jet printing offers these advantages without the complexity of the electrochemical process.

Further advantages of this technique include the ease with which multi-sensors could be manufactured. It would be possible to use a row of ink-jet nozzles in order to deposit several different enzymes in a desired sequence so that multi-sensors or multi-layer sensors could be manufactured. Also, as the droplets are small, the technique could be very useful for the manufacture of microelectrodes.

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Paper III

TTF-Modified Biosensors for Hydrogen Peroxide
Bifulco, L., Cammaroto, C., Newman, J.D. and Turner, A.P.F.
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ANALYTICAL LETTERS, 27(8), 1443-1452 (1994)

TTF-MODIFIED BIOSENSORS FOR HYDROGEN PEROXIDE

Key Words: Enzyme electrode, hydrogen peroxide, mediator, TTF.

Laura Bifulco[a], Cosimo Cammaroto[a], Jeffrey D. Newman[b], Anthony P.F. Turner[b]

[a]*Hydrocontrol, Centro di Ricerca e Formazione per il Controllo dei Sistemi Idrici, Poggio dei Pini, 09012 Capoterra (CA), Italy*

[b]*Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedfordshire MK43 0AL, UK*

ABSTRACT

Amperometric biosensors for hydrogen peroxide were produced, based on TTF-modified graphite disc electrodes, incorporating immobilised peroxidase. At the optimum operating potential (-200 mV vs. calomel electrode), a linear response to hydrogen peroxide was obtained in the range 0-2 mM (Km value = 1.98 mM). Low sensitivity to pH fluctuations and good stability at room temperature were exhibited.

INTRODUCTION

Amperometric approaches to biosensing have continued to dominate in biocatalytic devices. The combination of sensitivity, low cost, simplicity and versatility still offer considerable benefits when compared to alternative transduction methods[1]. Electrochemical mediators have gained in popularity over the past decade due to the advantages offered by, for example, the reduction in the operating potential and the ability to overcome the reliance of some sensors on the local oxygen partial pressure[2].

Ferrocene ethanolamine and hexacyanoferrate have so far proved the most successful mediators in commercial devices such as the ExacTech (Medisense) and the Glucocard (Kyoto Daiichi) respectively. TTF, however, offers a viable alternative[3] and has attracted increasing attention[4,5].

Hydrogen peroxide sensors are of interest due to the fact that H_2O_2 is the product of the reactions catalysed by a large number of highly selective oxidase enzymes. A great deal of work has been published in this area using, for example, tissue-based[6], whole-cell[7] and enzyme-linked sensors[8]. Furthermore, cheap and rapid determination of hydrogen peroxide in food products (where it is frequently used as a sterilising agent), pharmaceuticals[9], rain and fogwater is becoming increasingly important.

Horseradish peroxidase (E.C. 1.11.1.7) catalyses the oxidation of a "hydrogen donor" by converting hydrogen peroxide to water. The enzyme exhibits a broad specificity to the "hydrogen donor", which in this case is the electrochemical mediator TTF. A two electron transfer reaction takes place:

$$TTF + H_2O_2 \xrightarrow{HRP} TTF^{++} + 2\,H_2O$$

Electrochemical reduction of the oxidised TTF can then be carried out:

$$TTF^{++} + 2\,e^- \longrightarrow TTF$$

Thus, TTF is able to act as an electron transfer mediator, the oxidised form of which can be reduced at the electrode surface. The resulting change in current can be directly related to the hydrogen peroxide concentration. The aim of this work was to demonstrate the suitability of TTF as a mediator in biosensors for hydrogen peroxide.

EXPERIMENTAL

Instrumentation

Electrochemical experiments were carried out using a PSTAT10 AUTOLAB electrochemical analyser, controlled by a DSL personal computer using the

software GPES3. The analyser and software were supplied by ECOCHEMIE (Utrecht, Netherlands). A three electrode system employed a biosensor, as described below, a saturated calomel reference electrode (Russell pH, Auchtermuchty, Fife, UK) and a platinum wire counter electrode.

A water jacketted cell, thermostatically controlled by a Grant model SE15 water bath (Cambridge, UK), was used in all experiments.

Reagents and solutions

Horseradish peroxidase (HRP) (EC 1.11.1.7), glutaraldehyde (25%), hydrogen peroxide (100 volumes, 30%) and bovine serum albumin (BSA) were obtained from Sigma (Poole, Dorset, UK). Tetrathiafulvalene (TTF) was obtained from Aldrich (Gillingham, Dorset, UK). Epoxy Resin (Araldite) and silver-loaded epoxy resin were supplied by RS Components (Corby, Northants, UK). All other chemicals were of reagent grade. A Tris-maleate-NaOH buffer was used for all experiments.

Biosensor preparation

Electrodes were prepared from 5mm diameter carbon foil disks (Graphoil, Union Carbide, Sheffield, UK), washed with acetone and soaked in boiling water for 1 hour. An electrical connection was made via a nickel-chromium wire which

was attached to the internal face of the disk by silver loaded epoxy resin. This assembly was then sealed into a glass tube with epoxy resin. The electrode surface was finally cleaned with acetone prior to use and checked for electrochemical interference by cyclic voltammetry.

The electrodes were then modified by deposition of a solution of TTF in acetone and were allowed to dry in air for 1 hour. This was followed by the application of 20 µL of enzyme/BSA solution onto the surface of the modified electrodes. This solution contained 25 mg/ml HRP and 50 mg/ml BSA in TRIS-maleate buffer. After 2 minutes, 10 µL of glutaraldehyde solution (25 mg/ml in distilled water) was added and the electrodes were left to dry in air for two hours to facilitate enzyme cross-linking. The biosensors were then washed in buffer for approximately 15 minutes and stored at 4°C overnight before use. All optimization and calibration experiments were carried out using biosensors prepared on the previous day, in this way.

RESULTS AND DISCUSSION

As expected[5], a reversible two electron transfer reaction was indicated by cyclic voltammetric analysis of electrodes modified with TTF. On the basis of this, biosensors were prepared as described above, using 5 µl of 50 mg/ml TTF in acetone. These were used for the optimisation of the applied potential, which was studied in the range +300mV to -400mV (fig.1). As can be seen, a good plateau

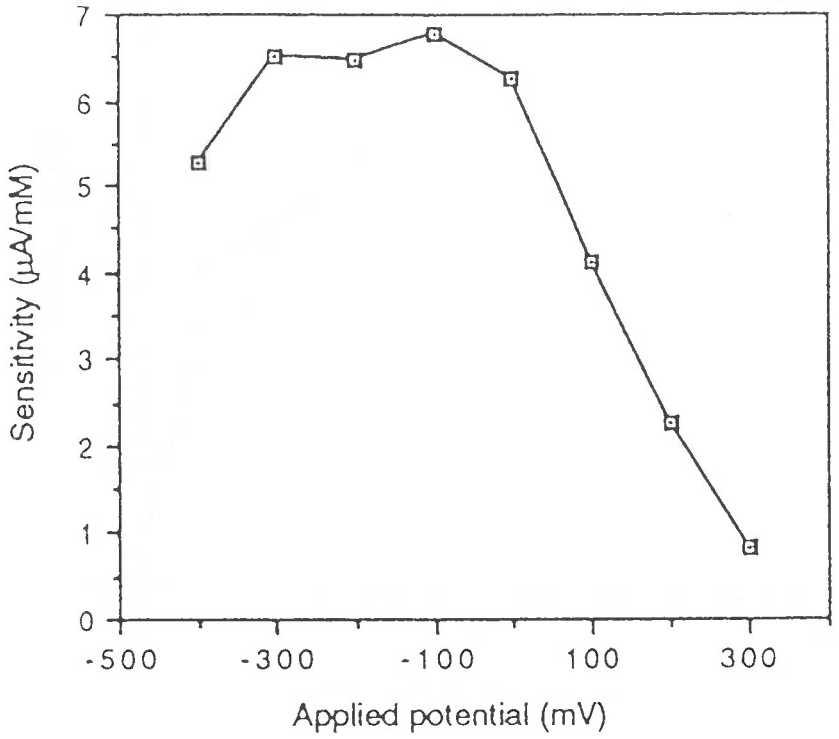


FIG.1 Optimisation of applied potential (pH = 7.0, Temperature = 25°C)

in sensitivity can be observed between -100mV and -300mV. In addition, the residual current, response time and noise sequentially increased as the applied potential was made more positive. From these results, it was decided to use an applied potential of -200mV for further studies.

Optimisation of the amount of TTF used was carried out by depositing between 0.5 and 50 μl of a 10 mg/ml solution on the electrode surface and preparing biosensors as above. A plateau of sensitivity was indicated (fig. 2) above approximately 20 μl. To ensure that the TTF was not rate limiting, however, 50 μl were added in all further experiments.

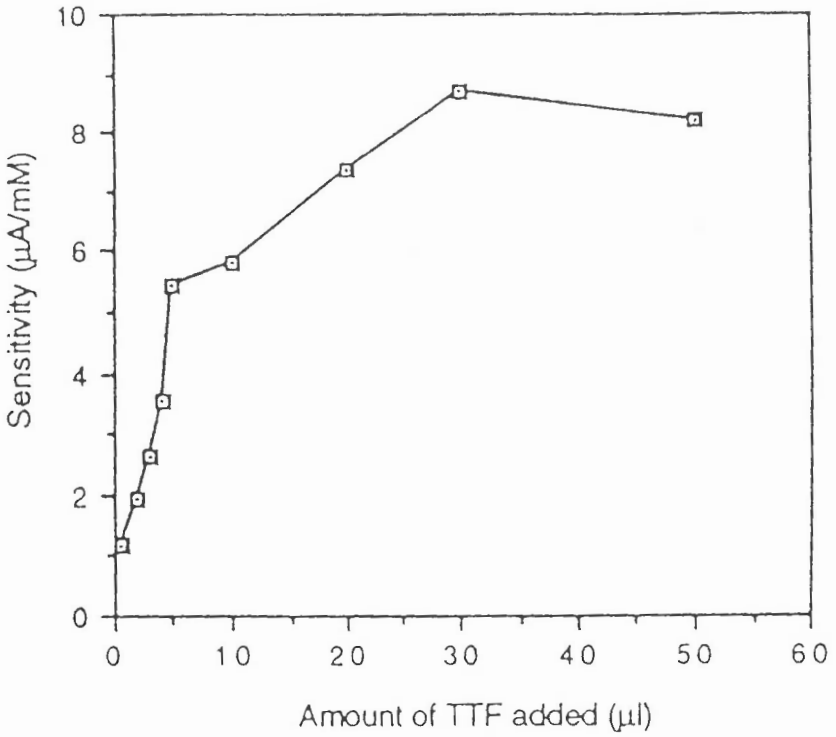


FIG.2 Effect of TTF concentration on biosensor response (pH = 7.0, Temperature = 25°C)

The temperature dependence of the biosensor sensitivity was tested between 15 and 35°C (fig.3), in which range, an increase in the steady state current of approximately 3.0% / °C was observed. Over the pH range 5 to 9, the biosensor was essentially independent of pH, which is in marked contrast to non-mediated methods of electrochemical hydrogen peroxide measurement. The optimised biosensor displayed a linear response up to approximately 2mM hydrogen peroxide. The Michaelis constant, K_M, was calculated by Hanes plot, and was found to have a value of 1.98mM.

Multiple use of these biosensors was limited by the sharp drop in sensitivity during repeated use. It is believed that this is caused by leaching of TTF from the

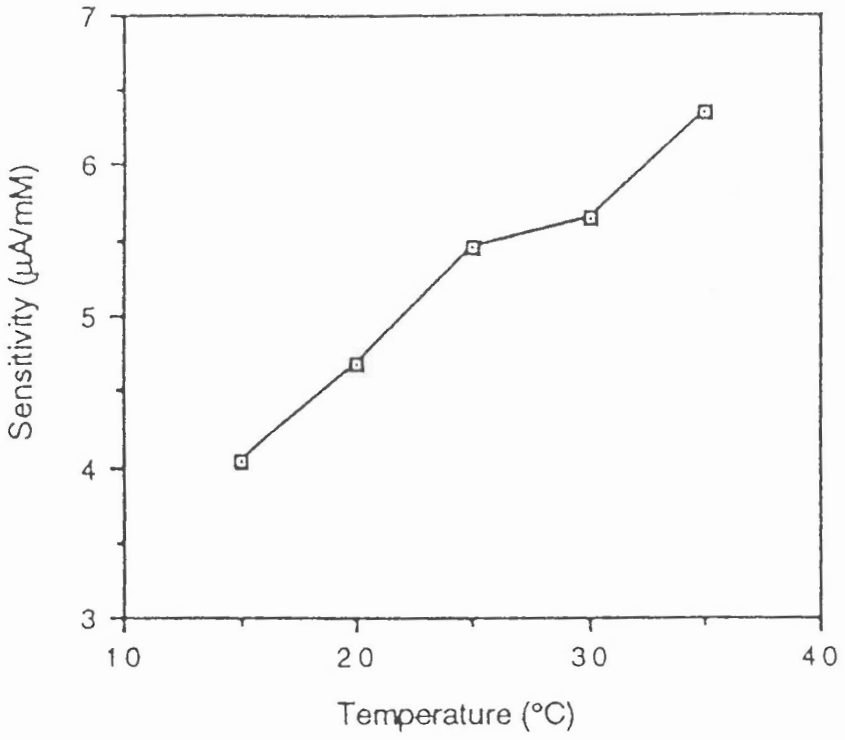


FIG.3 Effect of temperature on biosensor response (pH = 7.0)

TABLE 1. Precision data of TTF-peroxidase biosensors

[Hydrogen Peroxide] (mM)	0.22	1.1	1.98
Coefficient of Variation (n=23)	14.3%	10.7%	8.7%

electrode surface. A further drawback arises from the relatively poor between-device reproducibility which was obtained (Table 1). The use of automated manufacturing techniques, such as screen-printing or ink-jet printing should enable inexpensive and reproducible sensor fabrication, which would overcome these drawbacks.

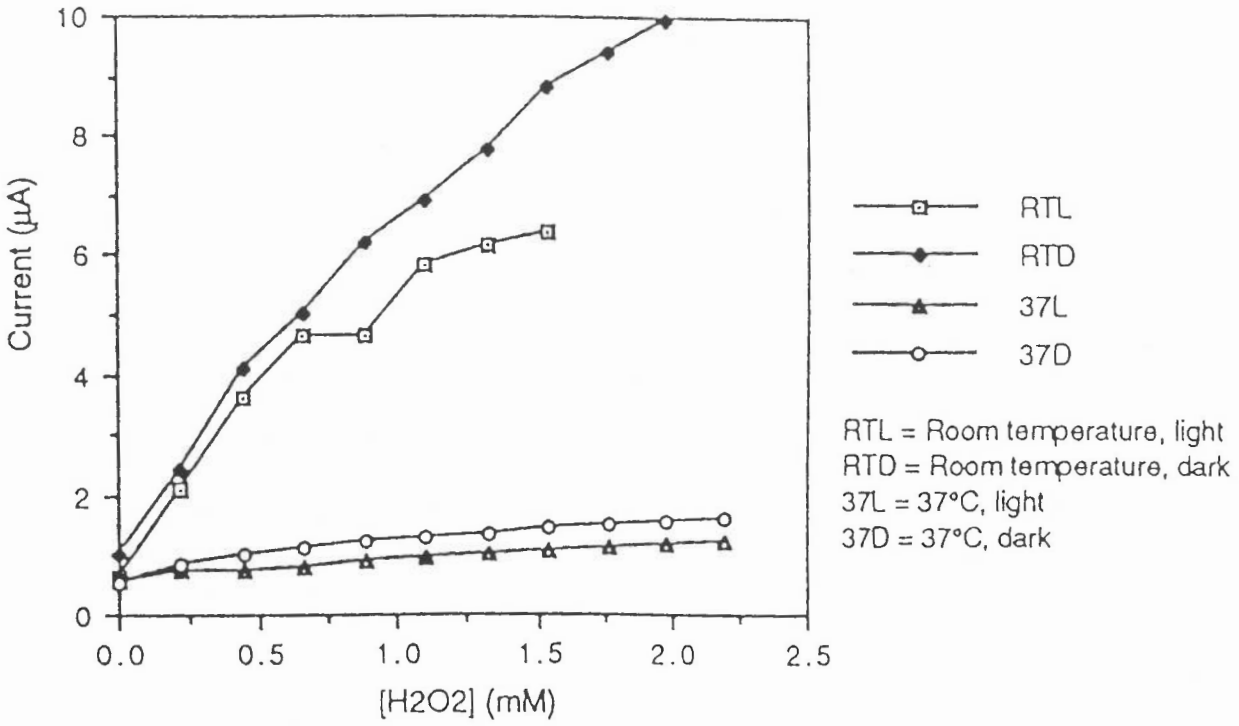


FIG.4 Storage stability of biosensors (30 days)
(Sensors tested at pH 7.0 at 25°C)

Storage stability was found to be good at normal room temperature, but dropped off sharply at elevated temperatures (fig. 4). Storage in the dark appeared to improve stability slightly, presumably due to the light-sensitivity of TTF.

CONCLUSIONS

TTF-mediated amperometric biosensors for hydrogen peroxide determination can be constructed simply and at low cost. The described device requires no addition of further reagents and exhibits an extended operational pH range. Furthermore, the configuration of this biosensor lends itself to mass production technologies, such as screen-printing, ink-jet printing and air-brush deposition.

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Paper IV

Catalytic Materials, Membranes and Fabrication Technologies Suitable for the Construction of Amperometric Biosensors

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Anal. Chem. 1995, *67*, 4594–4599

Catalytic Materials, Membranes, and Fabrication Technologies Suitable for the Construction of Amperometric Biosensors

Jeffrey D. Newman,* Stephen F. White, Ibtisam E. Tothill, and Anthony P. F. Turner

Cranfield Biotechnology Centre, Cranfield University, Cranfield, Beds MK43 OAL, U.K.

A selection of recently available catalytic carbon powders were assessed and compared with the more conventionally used platinized material. Their suitability for incorporation in amperometric biosensors is discussed. In conjunction with this study, methods of applying membranes to the surfaces of these devices were investigated. Advanced fabrication technologies, potentially suitable for scale-up of sensor production, such as screen printing and ink-jet printing, were used for manufacture of the complete sensor structure. Hydrogen peroxide-sensing electrodes and glucose biosensors were produced as model systems, demonstrating the advantages of these approaches. The commercially available rhodinized carbon MCA4 produced a high current density at low potentials over a plateau region (300–400 mV vs SCE). In addition, direct oxidation of glucose (seen with platinized carbon) was not observed at the chosen potential of +350 mV. Further interference studies using fermentation media highlighted its suitability as an electrode material for use in complex samples. Ink-jet printing proved to be a successful method for the deposition of Nafion membranes of defined and reproducible geometry.

A number of approaches to the construction of amperometric biosensors have been adopted since the conception of the first device by Clark in 1962.[1] The first biosensors were based on the Clark oxygen electrode, detecting the depletion of oxygen resulting from the action of an oxidase enzyme.[2] Alternatively, hydrogen peroxide, generated as a result of the enzymatic reaction, can be detected and related to the analyte concentration.[3] Further refinements involved the use of mediators to circumvent the reliance of the sensor on the local oxygen concentration, to reduce the operating potential and hence enhance selectivity.[4] In addition, the use of metals such as platinum and rhodium, coated onto carbon electrodes as a fine dispersion, has been widely adopted.[5,6] This results in a highly catalytic surface as long as the particle size of the deposited metal is comparable to that of the electrical double layer.[7] In this instance, the difference in the electronic work functions of platinum and carbon result in an increase in the electron density on the platinum.

Several methods for the incorporation of the metal into the carbon matrix have been reported. These include electrochemical deposition using, for example, cyclic voltammetry,[8] sputtering[9] and straightforward mixing of the metal in a carbon paste.[10] The conventionally preferred working electrode material, platinized carbon, is able to reduce greatly the oxidation potential of hydrogen peroxide. Unfortunately, this material is also highly electrocatalytic for a large number of other substances, such as glucose and other reducing sugars. In complex samples, the presence of such interfering compounds will clearly compromise specificity. To overcome this drawback, selectivity can be enhanced by the use of membranes, typically constructed from polymers such as cellulose acetate, polyurethane, Nafion, and a variety of others.[11] Membranes are also a convenient way of extending the linear range of a biosensor. They form a diffusion barrier, and substrate/product concentration profiles within this layer are, therefore, a function of both diffusion and reaction. One parameter limiting the linear range of such an electrode is the K_m of the enzyme, which exhibits saturation kinetics, and a nonlinear signal is anticipated. If the diffusional effects are dominant, the limitation on the linear range, due to a low K_m value, becomes negligible. In such situations, "apparent" kinetic parameters are often used.[12]

A drawback in the use of membrane-modified electrodes arises from the time-consuming and inconsistent manual fabrication methods typically employed. Automated methods, such as screen printing[13] and ink-jet printing,[14] can be used to accelerate and regulate production. This is an area which is often overlooked when considering the commercialization of such devices.[15]

This paper reports on the suitability of some recently available catalytic carbon powders and examines methods of depositing reproducible membrane structures on screen-printed electrodes constructed of such materials. In addition, this manufacturing process enables biological components such as enzymes to be printed, since it involves a room temperature curing process

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EXPERIMENTAL SECTION

Electrode Construction and Test Procedure. Screen-printed electrodes were used throughout these investigations and were constructed in planar arrays of eight electrodes. These were fabricated using a DEK 247 screen-printer (DEK Printing Machines, Weymouth, UK) in a multistage printing process whereby a silver conducting track (Electrodag 477SS RFU, Acheson Colloids, Plymouth, UK) was placed on a PVC substrate (Genotherm, Sericol, Surrey, UK). A graphite pad (Electrodag 423 SS, Acheson Colloids) was printed at one end of the silver track, and an insulation shroud (Matt Vinyl White M.V. 27, Apollo Colours, London, UK) was deposited over these layers, such that only a contact pad at one end and a working electrode (8 mm × 2 mm) at the other end were exposed. In each case, the printed layer was cured for 1 h at 40 °C.

A range of commercially available metalized carbon powders (Table 1) were obtained from ETEC and MCA Services (Royston, UK) and investigated. The MCA powders are proprietary materials consisting of highly dispersed precious metals and containing promoters. For studies of the response of the catalytic materials to hydrogen peroxide, the powders were mixed with a 2.5% (w/v) hydroxyethyl cellulose (HEC; Fluka, Gillingham, UK) solution dissolved in 0.1 M, pH 7.0 phosphate buffer. The metalized carbon powder was incorporated into the HEC solution in the ratio 1:2 (w/w). The ink was mixed using a rotary stirrer until a uniform consistency was achieved (~1 h at 25 °C). Finally, the resultant ink was screen-printed onto the graphite pad. Despite this layer being slowly soluble in the test solution, it remained bound to the working electrode surface for sufficient time to enable the analysis to be carried out.

Experiments were carried out in a batch mode using a three-electrode amperometric system consisting of a saturated calomel reference electrode (SCE; Russell, Auchtermuchty, Fife, UK), a 2 mm diameter platinum wire counter electrode, and the working electrode as described above. All experiments were performed under stirred conditions at 25 °C using 0.1 M sodium phosphate buffer containing 0.1 M potassium chloride at pH 7.0. Aliquots of hydrogen peroxide were added to the buffer solution, and the dynamic current response was monitored and recorded using an Autolab electrochemical analyzer (EcoChemie, Utrecht, Netherlands), which was controlled by the software package GPES3.

Enzyme Electrode Construction. Having determined the preferred composition of the electrode base material, the next stage was to incorporate the enzyme into the structure. Glucose oxidase (Glucox PS, ABM, Stockport, UK) was chosen as a model system. Two sensor configurations were investigated. First, the enzyme was mixed with the catalytic powder to form a printable ink (electrode types A). Second, the enzyme was printed over the catalytic powder in a separate layer (electrode types B).

The following ink formulations were used: type A, 1 g of 2.5% (w/v) HEC in phosphate buffer (pH 7.0) solution, 100 mg of glucose oxidase, and 400 mg of catalytic graphite powder; type B, the catalytic graphite layer deposited as described earlier and containing no glucose oxidase. The enzyme was subsequently incorporated into an ink consisting of 1 g of 3% HEC, 100 mg of glucose oxidase, and 400 mg of T10 graphite (Acheson Colloids).

After printing, all electrodes were left to dry for 2 h at room temperature. Because the organic binder is soluble in aqueous solution, an outer protective membrane was required. We investigated two such materials, Nafion (Aldrich, Gillingham, UK), a persulfonic charge exclusion material, and cellulose acetate (Aldrich), a size exclusion barrier. Three techniques were used to apply these membranes: ink-jet printing, dip-coating, and spin-coating. Ink-jet printing requires a charged solution for deposition and is therefore suitable for applying the Nafion layer. Cellulose acetate solutions, which are nonconducting, require the presence of a charged species, which can be provided by the inclusion of an organic salt such as tetrabutylammonium toluenesulfonate (TBATS). Dip-coating and spin-coating are not subject to this constraint and were suitable for the application of both membrane materials.

Nafion was supplied as a 5.0% (w/v) solution, dissolved in a mixture of lower aliphatic alcohols and water. It was necessary to dilute this to reduce the viscosity for printing and coating. Water was added to make a final concentration of 1% (w/v) for ink-jet printing and 0.5% (w/v) for spin-coating and dip-coating. Cellulose acetate was prepared as a 2.0% solution in acetone.

Ink-Jet Printing. A Biodot printer (Biodot, Diddington, UK) was used for all ink-jet experiments. Such a printer operates by forcing fluid under pressure through a small nozzle (75 μm). As the fluid passes through the print head, a drive rod actuated by an oscillating piezoelectric crystal produces a shock wave which breaks the jet into a series of regular droplets. This occurs at a frequency of ~64 000 Hz, dependent on the modulation voltage. Printing is achieved by charging individual droplets via a charge electrode which is controlled by a microprocessor. The printed pattern is constructed in a dot matrix format.

An array of 16 × 21 droplets of membrane solution (total printed volume, 0.47 μL) was printed onto the electrode surface such that it completely covered the active area and overlapped the insulation shroud by ~2 mm. The amount of solution was varied by repeatedly passing the electrode array under the print head.

Spin-Coating. The electrodes (individually) were mounted on a purpose-built spigot and rotated at 2000 rpm. An aliquot (10 μL) was pipetted onto the surface of the working electrode, which was left to rotate for 15 min, allowing the solvent to evaporate, leaving a thin membrane deposit. All spin-coated electrodes were prepared using this approach.

Dip-Coating. The enzyme electrode was immersed in the membrane solution and immediately removed. Following immersion, the electrodes were suspended vertically and left to dry for 30 min (allowing the solvent to evaporate).

Glucose Biosensor Test Procedure. Glucose stock solutions (0.1 M) were all prepared in 0.1 M, pH 7.0 phosphate buffer containing 0.1 M KCl. These solutions were allowed to mutarotate overnight prior to use. All glucose experiments were carried out under the conditions described earlier for hydrogen peroxide determination. pH optimization was carried out using five electrodes which were tested at 25 °C in the appropriate buffer under stirred conditions, with the working electrode potential poised at +350 mV (vs SCE). The following buffers were used:

pH 5–5.5	0.1 M sodium acetate
pH 6–7.5	0.1 M sodium phosphate
pH 8–8.9	0.1 M Tris-HCl

When the electrode had reached a steady state, a 5 mM concentration of glucose (prepared in the appropriate buffer) was added to the buffer, and the electrode response was monitored. Three measurements were made at each pH.

Table 1. Catalytic Graphite Analysis: Electrochemical Response to Hydrogen Peroxide in the Range 0–2.5 mM at 25 °C and pH 7.0

material	hydrogen peroxide response (μA/mM) and correlation coefficient (r, in parentheses) at the indicated applied potentials				
	100 mV	200 mV	300 mV	400 mV	500 mV
ETEC (10% Pt)	−23.4 (−0.9975)	13.9 (0.9995)	29.1 (0.9998)	29.3 (0.9997)	
MCA1 (5% Ni, 5% Cr)	−0.1 (−0.9165)	0.1 (0.9165)	0.8 (0.9820)	2.7 (0.9750)	5.1 (0.9979)
MCA2 (5% Pt)	−22.3 (−0.9992)	8.2 (0.9997)	33.1 (0.9937)	41.7 (0.9934)	
MCA3 (0.1% Pt)	−2.0 (−0.9943)	4.6 (0.9991)	14.8 (0.9987)	22.9 (0.9989)	
MCA4 (5% Rh)	−10.9 (−0.9961)	15.0 (0.9973)	27.3 (0.9925)	26.8 (0.9982)	
MCA5 (0.1% Rh)	no response	no response	no response	no response	0.8 (0.9586)
MCA6 (5% Ru)	−13.1 (−0.9971)	−11.2 (−0.9985)	−9.1 (−0.9991)	−0.3 (−0.9846)	52.8 (0.9976)
MCA7 (0.1% Ru)	no response	no response	no response	no response	7.6 (0.9952)
MCA8 (10% Pd)	−14.3 (−0.9994)	7.9 (0.9984)	23.5 (0.9998)	31.0 (0.9998)	
MCA9 (5% Pt, 5% Pd)	−30.4 (−0.9993)	4.4 (0.9898)	28.9 (0.9982)	44.5 (0.9985)	
MCA10 (5% Pd, 0.5% Ru)	−22.8 (−0.9990)	2.0 (0.9999)	27.4 (0.9998)	36.6 (0.9995)	
MCA11 (5% Ni, 5% Cr, 5% Pd)	−1.8 (−0.9948)	0.9 (0.9853)	3.7 (0.9935)	7.4 (0.9988)	
MCA12 (10% Ag)	no response	no response	no response	no response	no response

Interference Studies. To validate the potential use of the selected catalytic material as a suitable base for practical biosensors, interference studies were carried out by studying the direct oxidation of glucose and the electrode response in fermentation media. For these experiments, the non-enzyme electrodes were used. All experiments were carried out under the conditions described above, with the potential interferent solutions being added in place of hydrogen peroxide. The first six media investigated were sterile samples in which no cells had been grown. Following this, tests were carried out in cell-free extracts;,- obtained during two bacterial fermentations and one yeast fermentation. The media were prepared according to the following (amounts given in grams per liter):

medium 1 (medium for yeast)	
KH_2PO_4	12
$MgSO_4 \cdot 7H_2O$	5
$FeCl_3 \cdot 6H_2O$	0.2
yeast extract	20
hycase	20

medium 2 (medium for bacteria)	
$CaCl_2 \cdot 4H_2O$	0.1
$MgCl_2 \cdot 6H_2O$	2
K_2SO_4	5
NaCl	2
KCl	10
yeast extract	20
hycase	20
K_2HPO_4	2

medium 3 (LB medium)	
bactotryptone	10
yeast extract	5
NaCl	10

medium 4 (brain–heart infusion medium)	
calf brain	1.25
beef heart infusion solids	5
proteose peptone	10
NaCl	5
dextrose	2
Na_2HPO_4	2.5

medium 5 (reinforced clostridial medium)	
yeast extract	3
lablemco powder	10
peptone	10
soluble starch	1
dextrose	5
cysteine hydrochloride	0.5
NaCl	5
sodium acetate	3
agar	0.5

medium 6 (MRS broth)	
bacteriological peptone	10
beef extract	8
yeast extract	4
Na_2HPO_4	2
sodium acetate	5
triammonium citrate	2
$MgSO_4 \cdot 4H_2O$	0.2
$MnSO_4 \cdot 4H_2O$	0.05
Tween 80	1

Fermentation Broths. Fermentations were carried out in 250 mL shake flasks, containing 50 mL of inoculated media. The organisms grown were *Escherichia coli*, *Lactobacillus plantarum* LPCO-10, and *Saccharomyces cerevisiae*.

E. coli was grown with shaking at 37 °C in nutrient broth consisting of 10 g/L beef extract, 10 g/L peptone no. 1, and 5 g/L NaCl. Samples were taken at regular intervals over a period of 7.5 h.

L. plantarum was grown in a stationary mode at 30 °C in medium 6 (outlined above). Once more, samples were taken over a 7.5 h period.

S. cerevisiae was grown in a stationary mode at 30 °C in a medium consisting of 7 g/L yeast extract, 10 g/L mycological peptone, and 20 g/L glucose. Aliquots were removed at intervals over a 24 h period.

RESULTS AND DISCUSSION

Selection of Electrocatalytic Material. The criteria used to select the catalytic material were based on (i) high current density, thus maximizing the signal; (ii) low operating potential, with a response plateau, such that small fluctuations in the applied potential have a negligible effect on precision; (iii) low interference from other electroactive compounds, a problem highlighted earlier for metal/carbon electrodes; and (iv) linear response in the working range.

Electrochemical Response to H_2O_2. The response to hydrogen peroxide over a range of concentrations between 0 and 2.5 mM was determined amperometrically for each of the electrode types over a series of applied potentials between 100 and 500 mV. Results of these measurements are summarized in Table 1. From this, it can be seen that several catalytic carbons show significant electrocatalytic activity. This is characterized by a high positive slope value at low potentials. From these data, it is apparent that the following materials exhibited the highest current densities in this range: ETEC Pt–C, MCA2, MCA3, MCA4, MCA8, MCA9 and MCA10.

The electrodes which exhibited the best plateau responses were ETEC Pt–C and MCA4, which both displayed this feature between 300 and 400 mV. Electrodes which produced a significant signal were all linear over the tested hydrogen peroxide concentration range.

Direct electrooxidation of glucose has been previously described.[16] Generally, the anodic oxidation was carried out using

(16) Giner, J.; Malachesky, P. Proceedings of the Artificial Heart Conference, Washington, June 1969, U.S. Dept. of Health, Education and Welfare; 1969; p 839.

Table 2. Electrode Material Response to Hydrogen Peroxide and Glucose

material	hydrogen peroxide	glucose
ETEC	+++	++
MCA1	+	+++
MCA2	++	–
MCA3	+	+
MCA4	+++	–
MCA5	+	+
MCA6	+++	++
MCA7	++	+
MCA8	++	+
MCA9	++	–
MCA10	++	+
MCA11	++	–
MCA12	+	–

[a] –, no noticeable response; +, slight response; ++, significant response; +++, strong response.

either pure (Au, Pt) or adatom (Pb, Tl, Bi)-modified noble metal electrodes. Similar behavior has been reported at platinized carbon electrodes. The effects of direct glucose oxidation may cause problems due to electrode fouling and may complicate the biosensor kinetics, leading to a poorly defined relationship between the glucose concentration and the recorded signal. It is also likely that if glucose is electrochemically active at these electrode surfaces, then other organic compounds, such as alcohols and carbohydrates, will also exhibit this characteristic.[6] Hence, the electrodes were tested for their sensitivity to direct glucose oxidation using cyclic voltammetry. The potential was cycled between 0 and 700 mV at a scan rate of 50 mV/s in the buffer solution described above, in buffer containing 1.0 mM hydrogen peroxide, and finally in buffer containing 25.0 mM glucose. The magnitude of the response in each case, which represents a qualitative interpretation of the cyclic voltammograms, is shown in Table 2.

It is apparent from these results that MCA4 offers the best response to hydrogen peroxide with no noticeable interference from the direct oxidation of glucose. The cyclic voltammograms of the MCA4 material in buffer, buffer + hydrogen peroxide, and buffer + glucose are shown in Figure 1. As can be seen, the scans for the buffer and the buffer + glucose are virtually identical. The scan when hydrogen peroxide is present, however, clearly shows a large electrocatalytic oxidation of hydrogen peroxide. As a result of these experiments, it was concluded that MCA4 is the best of the materials tested. It offers a significant advantage, when compared with the commonly used platinized carbon material, in that it does not suffer interference from direct oxidation of reducing sugars, and hence it was used for all subsequent experiments.

Results of Interference Studies. A three-electrode amperometric cell, as described earlier, was poised with an applied potential of +350 mV. Initially, the electrodes were immersed in 20 mL of phosphate buffer (pH 7.0) under stirred conditions at 25 °C. When the electrode response had reached a steady current (~60 s), buffer was removed and replaced with the same volume of the appropriate media or fermentation sample over a dilution range between 1:20 and 1:1. Following the addition of the media sample, hydrogen peroxide was added to ensure that the operational characteristics of the electrode were unimpaired. For all of the samples tested, no significant interference was observed.

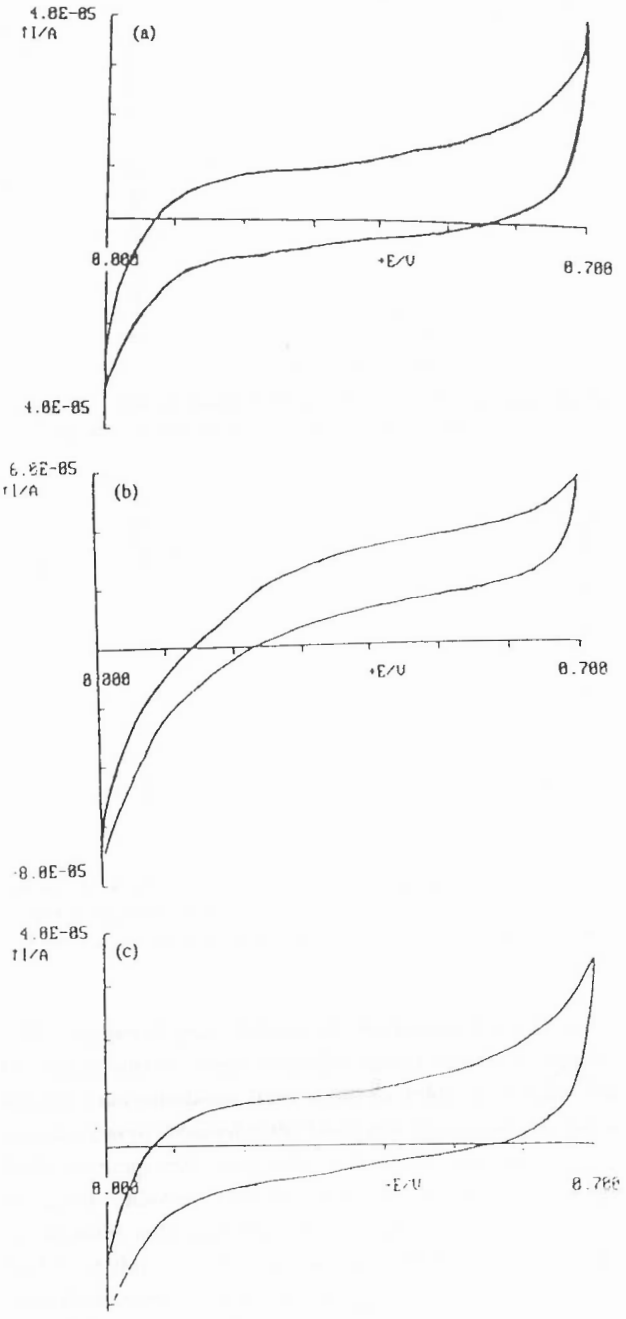


Figure 1. Cyclic voltammograms of MCA4-based electrodes. (A) Response to 0.1 M, pH 7.0 phosphate buffer. (B) Response to 1.0 M hydrogen peroxide in phosphate buffer (as in A). (C) Response to 25.0 mM glucose in phosphate buffer (as in A).

This suggests that this electrode material may be well suited to operation in complex samples, where potential electrochemical interferences may be present, such as in fermenters.

Enzyme Electrodes: Initial Membrane Test Results. Table 3 illustrates the mean responses from five of each type of electrode to a 5 mM addition of glucose. Electrode types A and B are as defined earlier. All of the electrodes fabricated using dip-coating as the method of membrane application produced very small currents and exhibited poor reproducibility.

It can be seen that type A electrodes (enzyme and catalytic powder in the same layer) produced significantly higher currents and were found to be more stable than those of type B. Furthermore, producing electrodes via this approach eliminates the need for a separate step during manufacture. Nafion-coated

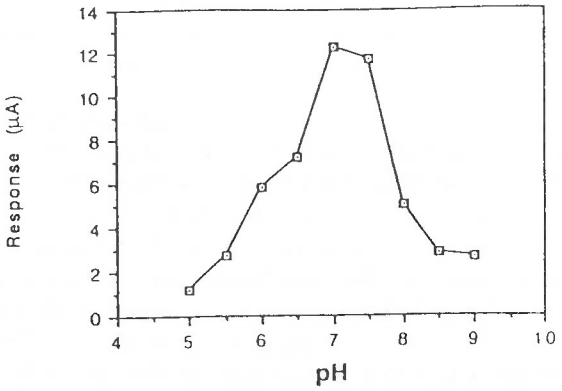


Figure 2. pH profile of glucose biosensor response at a glucose concentration of 5 mM at 25 °C under stirred conditions.

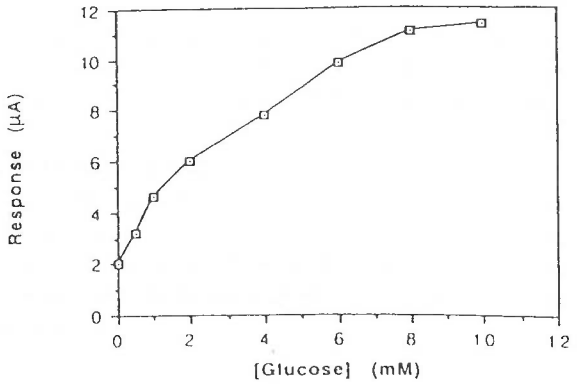


Figure 3. Typical calibration plot for a screen-printed glucose biosensor with an ink-jet-printed Nafion membrane.

Table 3. Results of Membrane Fabrication Studies

	response to 5 mM glucose (μA)		
electrode and membrane type	mean	SD	comments
type A/ink-jet Nafion (8 passes)	15.8	1.2	low noise
type B/ink-jet Nafion (8 passes)	4.8	0.4	noisy
type A/spin-coated cellulose acetate	10.7	1.0	noisy
type B/spin-coated cellulose acetate	1.8	0.6	very noisy
type A/spin-coated Nafion	17.7	1.2	noisy
type B/spin-coated Nafion	3.5	1.0	very noisy

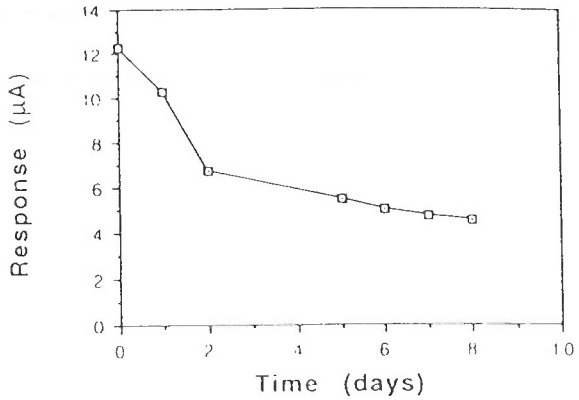


Figure 4. Biosensor stability over an 8 day period. Sensor stored dry at 4 °C between measurements. Plot depicts biosensor response to a glucose concentration of 5 mM at 25 °C under stirred conditions

electrodes produced higher currents with less noise than those coated with cellulose acetate. Ink-jet printing proved to be the most controllable method of applying the membranes. Spin-coated, Nafion-covered electrodes produced marginally higher currents, but the response was significantly noisier. From these results, it was decided that all future electrodes would be constructed in the type A format, with initial work being carried out using an ink-jet-printed Nafion membrane.

Investigation of the pH Profile. The graph shown in Figure 2 depicts the electrode response over a pH range between 5.0 and 9.0. Obviously, the optimum applied potential for hydrogen peroxide detection and the activity of the enzyme will be influenced by the pH. However, for simplicity of associated instrumentation of an analytical device based on this technology, it is useful to operate at a fixed potential. Glucose oxidase operates over a wide pH range (2.7–8.5) and the optimum is usually reported to lie between 4.8 and 6.0 for the free enzyme utilizing dioxygen as the electron acceptor.[17] Numerous publications describing glucose biosensors have described a shift in the pH optimum exhibited due to the use of artificial electron acceptors.[18] Immobilization is another factor which may alter the operating characteristics of the enzyme.[19] Hence, the observation that the optimum value is 7.0 is a function of the electrode and its mode of operation at a constant voltage and is not necessarily an indication that the behavior of the enzyme has been altered.

Calibration Curves. A series of enzyme electrodes (five of each), prepared using different amounts of Nafion (between 2.35 and 22.09 μL, calculated by multiplying the mean droplet volume by the number of printed droplets) which was ink-jet-printed onto the sensor surface, were tested for their response to a range of glucose concentrations from 0 to 15 mM. Generally, linear responses were obtained at the lower glucose concentrations, with the linear range extending as the amount of Nafion deposited was increased. Above 22.09 μL, the current response declined significantly, indicating that the membrane thickness restricted the flux of glucose to the immobilized enzyme. A representative calibration graph is shown in Figure 3.

Stability. Using the same operating conditions as those described above (phosphate buffer, pH 7), an electrode was tested for stability over 8 days. Three electrodes were tested against a 5 mM glucose concentration daily over this time. When not in use, the electrodes were stored in buffer at 4 °C. Figure 4, which depicts the response of a typical enzyme electrode, indicates that an initial sharp decline in activity is followed by a more gradual decrease and that after 8 days, the sensor was still producing a significant response.

Nafion is a perfluorosulfonic ion-exchange membrane material which differs from conventional ion-exchange materials in that it is not a cross-linked polyelectrolyte, but a thermoplastic polymer with pendant sulfonic acid groups partially or completely neutralized to form salts. This also provides a negatively charged surface. It is believed that the membrane, by virtue of the electrostatic interaction between enzyme and membrane, is helping to retain the enzyme electrostatically as well as physically. Furthermore, this immobilization procedure may lead to a greater rigidity, enhancing physical resilience of the enzyme. Another effect may

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(18) Wilson, R.; Turner, A. P. F. *Biosens. Bioelectron.* **1992**, *7*, 165.
(19) Gorton, L.; Csoregi, E.; Dominguez, E.; Emneus, J.; Jonsson-Pettersson, G.; Marko-Varga, G.; Persson, B. *Anal. Chim. Acta* **1991**, *250*, 203.

be that the use of a membrane of this type may stabilise the local microenvironment.

CONCLUSIONS

Of the catalytic powders investigated, MCA4 proved to have a response comparable to the best of the other materials, coupled with reduced interference effects. Experiments to determine the best way of depositing the enzyme and catalytic graphite demonstrated that it is preferable that both components are screen-printed in the same layer as an water-based ink using hydroxyethyl cellulose as the polymeric binder.

Nafion proved to be amenable to deposition via several different methods. Compared to cellulose acetate, Nafion-coated sensors produced higher currents and were more stable. Of the methods investigated, ink-jet printing proved to be more controllable and reproducible. Furthermore, sensors fabricated by this method exhibited the highest signal-to-noise ratios.

Using these materials and fabrication methods, biosensors can be produced via an automated process, with its inherent advantages of mass production capability and reproducibility.

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Paper V

Development of a Mass-Producible Glucose Biosensor and Flow-Injection Analysis System Suitable for On-Line Monitoring During Fermentations

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Development of a mass-producible glucose biosensor and flow-injection analysis system suitable for on-line monitoring during fermentations

Stephen F. White *, Ibtisam E. Tothill, Jeffrey D. Newman, Anthony P.F. Turner

Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedforshire MK43 OAL, UK

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Abstract

Screen printed amperometric glucose biosensors were constructed and optimised for use in a flow injection analysis (FIA) system. The sensors were fabricated using a catalytic metallised carbon based ink, allowing a working potential of +350 mV (Ag/AgCl). Overall the sensors displayed a high degree of reliability and robustness. A linear response was obtained over the range 0.1 to 25 mM glucose with a slope of 1.35 ± 0.20 μA/mM. The within electrode coefficient of variation at each concentration tested ($n = 10$) was less than 2% (or 0.1 mM for concentrations below 5 mM). In addition, it was demonstrated that a single sensor can be used in the FIA system over a seven day period indicating a high level of operational stability. These sensors fulfil the criteria necessary for a mass produced device, which would be suitable for the on-line monitoring of glucose consumption during microbial fermentation.

Keywords: Flow injection; Biosensors; Fermentation

1. Introduction

A critical feature of any production procedure, including industrial fermentations, is the application of an effective method of process monitoring. An optimised bioprocess should lead to more efficient downstream purification, a reduction in materials costs and improvements in quality control. A number of fermentation parameters are routinely monitored including pH, redox potential, degree of agitation, pO_2 and pCO_2. Unfortunately, there are few instruments currently available for the continuous monitoring of parameters closely reflecting the cell status, e.g., biomass, metabolite concentration, substrate utilization and waste product accumulation.

Generally, methods of monitoring can be classified into three basic categories: off-line, on-line and in situ. Off-line methods involve extracting samples from the bioreactor for analysis by wet chemical or automated laboratory instrument systems in remote or local sites. One notable approach, which has been commercially available for more than a decade, is to use enzyme electrodes [1]. These biosensors are recognised for their ability to detect a desired analyte with a high degree of accuracy and selectivity [2]. In situ systems involve the siting of instruments in direct contact with the fermentation media, providing

* Corresponding author.

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a continuous measurement. Again, enzyme electrode biosensor-based systems have been developed and successfully used to monitor fluctuations in the concentration of a desired analyte during a number of fermentations [3,4]. Despite the advantages offered (real time monitoring, continuous operation and no requirement for a sampling system) only a relatively few "dip in" enzyme electrode sensors have been reported. Finally, on-line systems involve continuous or discontinuous sampling of a process stream, under sterile conditions, to provide a rapid (frequency controlled) measurement of a desired analyte.

An important contribution to the advancement of on-line systems has been the development of flow injection analysis (FIA) [5]. This liquid handling technique has proved to be flexible in adapting to a large number of chemical and biochemical reaction procedures [6]. Although there are a number of reports on the use of enzyme electrode biosensors with FIA, there are relatively few descriptions of their combination and application to monitoring fermentations. Recent reports [7–10] have demonstrated that this combination both improves the performance of analytical methods and suggest that this arrangement is a practical approach to solving process analytical problems.

In order to enhance the feasibility of using biosensors with FIA, we report here the development of a mass-producible glucose sensor, fabricated using a thick film screen-printing technique. This fabrication method fulfils a number of the criteria which are required for successful mass production of bioanalytical devices [11]. The use of this manufacturing approach is not new; indeed, the MediSense (Cambridge, MA) ExacTech blood glucose analyser, the Worlds' most successful commercial biosensor to date in terms of sales, is constructed using this method [12]. These sensors are designed for disposable "one-shot" applications, but it is also possible to produce multiple use devices using the same technology. The biosensors described in this report differ from previously reported screen printed devices in that they are suitable for use over at least seven days. This is achieved using a novel design of the biosensor card onto which a three electrode configuration is printed. A previous study [13] has shown that the promoted rhodinised-carbon powder, designated MCA 4 (MCA Ltd. Cambridge, UK), can be used successfully as a base material for the construction of the working electrode of amperometric sensors. Electrodes formed from this material display high current densities in the presence of H_2O_2, whilst maintaining a significant degree of selectivity.

In order that the biosensor card could operate in a confined flow stream, consideration was given to the choice of reference electrode. Conventional saturated calomel (SCE) and Ag/AgCl reference electrodes are typically large, constructed of glass and require periodic maintenance. For most laboratory applications this is not a problem. It would, however, be inconvenient in an FIA system where very small flow channels are employed. A Ag/AgCl reference electrode was therefore screen-printed onto the biosensor card and chloride ion supplied in the running buffer.

The Ag/AgCl reference and a carbon counter electrode were used in conjunction with a working electrode, in which the enzyme was retained in a water soluble polymer, which was mixed with the catalytic carbon powder. This layer was retained by a thin, water insoluble, polymeric membrane, which was required for several reasons:

(1) The integrated enzyme/catalytic layer is held within a water soluble matrix. Without a protective layer this material would be washed away in the flow stream.

(2) A membrane offers a degree of protection from potential interferents.

(3) Membranes offer a convenient way of extending the linear range of a biosensor by controlling the diffusion zone above the sensor surface.

Furthermore this allows the enzyme to be maintained in an aqueous matrix permitting free access to both the analyte (glucose) and the catalytic material. Unlike most conventional enzyme immobilisation procedures used in biosensor fabrication, in particular covalent binding, this approach overcomes losses of activity due to steric hindrance and binding at the active site. The response of the electrodes is further enhanced by the unique T-shaped design which maximises the exposed area of all three electrodes, but keeps the contacts well separated from the flow stream.

The planar sensors are designed to be accommodated easily in a purpose-built flow cell, in which the

calibration and exchange of sensors can be carried out simply and rapidly. By using the FIA system, small variations in the operating characteristics between individual sensors and drift in the response can be compensated for by a simple calibration step. A dedicated software package was designed to control all flow regimes. In this prototype instrument, the samples were manually mixed with a suitable buffer, although this step will be automated in the next phase of instrument design. The result of this will be that a wide range of glucose concentrations will be measurable using a single biosensor card.

2. Experimental

2.1. Reagents

All reagents used were of analytical grade and obtained from Aldrich. Experiments were carried using 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1 M potassium chloride. Glucose stock solutions were prepared in phosphate buffer and allowed to mutarotate for at least 12 h before use. Glucose oxidase from *Aspergillus niger* (Glucox PS, 76,000 U/g) was obtained from Rhone Poulenc.

2.2. Biosensor fabrication

The biosensors were constructed as a screen-printed, three electrode amperometric system, whereby hydrogen peroxide (enzymatically generated by glucose oxidase in the presence of glucose) was oxidised at +350 mV (Ag/AgCl). The subsequent recorded current was related to the concentration of glucose present in the sample.

Fig. 1 illustrates the compact design of the screen printed sensors which were used throughout these investigations. All the biosensors were constructed in a multi-stage printing process whereby three parallel silver conducting tracks (Acheson Colloids) were deposited onto a 400 μm thick PVC substrate (Genother) and dried for 2 h at 40°C (the temperature and time period used for all subsequent drying stages). A graphite pad (Electrodag 423SS, Acheson Colloids) was printed at one end of both the working electrode and counter electrode. This graphite pad formed the basis of the counter electrode and provided a stable contact point for the subsequent working electrode ink. Following drying, silver/silver chloride ink (MCA Services) was printed over the area designated for the reference electrode. After a further drying step an insulation shroud (matt vinyl white M.V., Apollo Colours) was deposited over all the layers, such that only the contact points and the electrode faces were left exposed. The ink formulation for the working electrode was prepared using the following:

1.0 g of a 3% (w/v) solution of hydroxyethyl cellulose (a water soluble organic binder) in 0.1 M phosphate buffer pH 6.8
100 mg of glucose oxidase
400 mg of MCA 4 (promoted catalytic carbon powder)

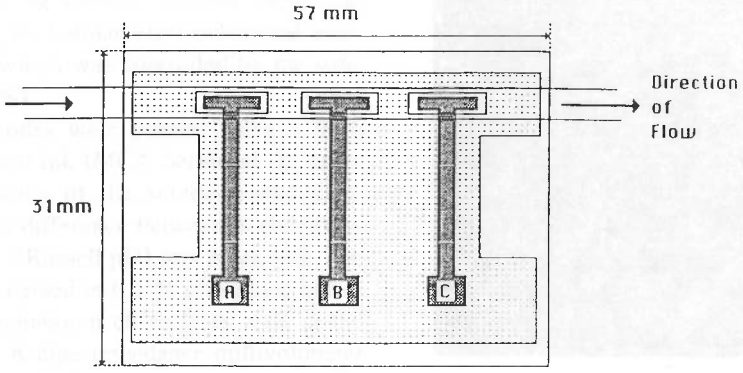


Fig. 1. Screen printed biosensor design. A = Reference electrode; B = working electrode; C = counter electrode (dimensions: 10 × 2 mm).

This quantity of ink was sufficient to print at least 75 biosensors. Thorough mixing was carried out using a rotary stirrer until a uniform consistency was achieved. Deposition of this ink over the working electrode face was carried out and the sensors left to dry for 2 h at 25°C. Finally, an outer membrane was deposited (using screen printing) over the working electrode face.

Cellulose acetate was the preferred choice of membrane material. This material has been demonstrated to be particularly effective in improving the selectivity (by size exclusion) of H_2O_2-based enzyme electrodes [14]. The pore size is dependent on the molecular weight of the polymer molecules and the method and conditions of membrane casting. For this approach, the pore size was greatly affected by the volatility of the solvent used to dissolve the cellulose acetate. The membrane printing solution was prepared using 2.5% (w/v) cellulose acetate (Sigma) dissolved in 1:1 (v/v) of cyclohexanone–acetone.

If used as the sole solvent, the comparatively low volatility of cyclohexanone led to a very slow drying of the membrane, concomitant with small membrane pore sizes. In contrast, the rapid evaporation of acetone led to large membrane pore sizes and produced an ink which dried too quickly for practical printing purposes. Mixtures of cyclohexanone and acetone produced solutions which were amenable to screen-printing and allowed fine control of the final pore sizes. A series of biosensors was prepared using different thicknesses of membrane, achieved using multiple printing steps. Initially the sensors were tested under stirred conditions at 25°C. Aliquots of the prepared glucose solutions were added to the buffer solution and the current response monitored and recorded using an Autolab electrochemical analyser (EcoChemie) which was controlled by the software package GPES3.

Reference electrodes were printed using a 90% Ag/10% AgCl based ink (MCA Services). In order to verify the accuracy of the screen-printed electrodes, the potential difference between 8 electrodes and a standard SCE (Russell pH) was measured. The electrodes were immersed in 0.1 M phosphate buffer containing 0.1 M potassium chloride as used in the FIA buffer stream. A high impedance millivoltmeter was used and the solution temperature controlled using a water jacketed beaker connected to a thermostatted water bath.

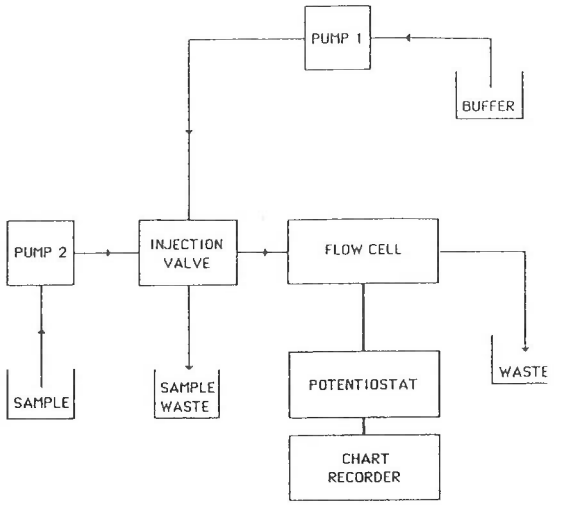


Fig. 2. Diagram of the FIA system used throughout all the experiments.

2.3. FIA system

The general layout of the flow system is illustrated in Fig. 2 and was used for all the FIA experiments. The biosensor was retained in a purpose built flow cell (Fig. 3), constructed using Plexiglass. Alignment of the electrodes with respect to the flow stream was achieved via two locating pins. Two holes were drilled into the biosensor cards (accurate location of these was realised via a purpose built

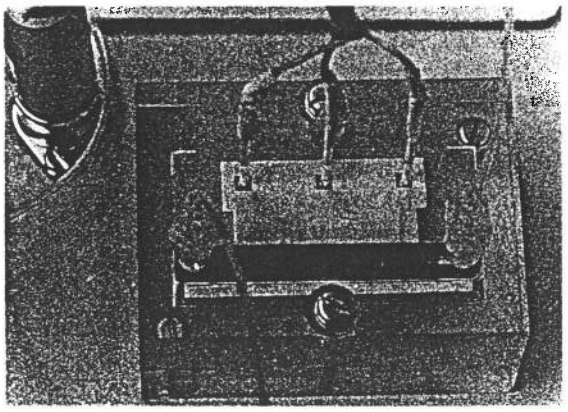


Fig. 3. Flow cell incorporating a biosensor.

drilling jig). All three electrodes were connected to a potentiostat, which applied a potential of +350 mV (Ag/AgCl). Measurements were recorded on a single channel chart recorder. Sample and buffer streams were propelled, using diluter syringe pumps (Model 401C, Gilson) with a capacity of 5 ml, through the flow cell and out to waste. Sequential, timed injections of the sample solution into the buffer stream were carried out using a Gilson 817 injection valve actuator. PTFE tubing (0.5 mm i.d., Omnifit) was used to connect all of the FIA components. Control of the system, including opening and closing of the injection ports in conjunction with the pump operation was maintained using a software driven key pad control device (Gilson). After use, the flow system was flushed with distilled water to remove all traces of salts.

2.4. Biosensor optimisation

Initial experiments involved determining the optimum flow rate for the sensor, with the aim of achieving the maximum peak current for the minimum baseline reversion time. This was accomplished using a standard 5 mM glucose solution, in phosphate buffer (as described previously). Using the control key pad, the rate of buffer delivery was pre-set to the required flow rate. The range of flow rates investigated was from 0.1 to 0.9 ml min^{-1}. Maximum peak currents and the time required for the peak signal to revert to a stable baseline were recorded at each flow rate. Using this flow regime, a number of parameters were investigated:

1. Optimum number of cellulose acetate membrane depositions required
2. Operating range of the sensor
3. Stability
4. Precision

All of the calibration and stability experiments were carried out in a temperature controlled room. Calibrations were carried out with a range of glucose standard solutions. Peak responses were recorded on the chart recorder. Furthermore, the maximum current responses were recorded via the digital display unit located within the potentiostat unit. This allowed two parameters to be used to determine the glucose concentration. The maximum current or peak integral could be related to the glucose concentration of the sample solutions. It was clear that the simplest approach was to use the peak current (height of response).

The long term operational stability of the sensors was assessed by running the FIA system continuously over a seven day period at 30°C in a constant temperature room. During this time the sensors were constantly flushed with buffer solution and periodic (every 30 min) injections of a 10.0 mM standard glucose solution were made.

Storage stability was assessed over a nine month period during which time a batch of biosensors were stored at 4°C in the dark. A card was withdrawn from this batch at intermittent intervals and tested for its response to a 1 mM stepped glucose increment in stirred solution. A previously unused card was tested each time.

The temperature response of the biosensors was tested using the FIA system in a constant temperature room. Each time the temperature was altered, the system was given 1 h to reach a steady temperature. The solutions used were split into batches and kept in a series of water baths at the required temperatures in the equilibration process to ensure that the temperature at the sensor was the same as that of the room. Ten measurements were made at each temperature using a glucose concentration of 10 mM. Due to the delay imposed by the equilibration step, it was important to take account of the fact that a loss of activity would be likely between readings. To compensate for this, values were adjusted by using a 25°C calibration between measurements.

3. Results and discussion

The most important feature of the printed reference electrode was that a consistent reference potential was exhibited between electrodes under identical conditions, thus allowing close control of the potential applied to the working electrode. The difference between the screen-printed and conventional reference electrodes was 49.0 mV (standard deviation, S.D. = 0.8 mV, $n = 8$) at 25°C. This precision indicates that performs in a similar way to a conventional reference electrode. The working electrode potential selected was based on a plateau response observed between +300 mV and +400 mV [13]

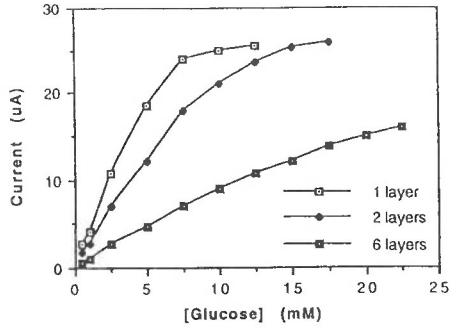


Fig. 4. Response from sensors prepared with a range of cellulose acetate membranes.

Table 1
Effect of flow rate on the maximum peak current response and the time taken for the signal to regain a stable baseline

Flow rate (ml min^{-1})	Current response (μA)	Baseline reversion time (s)
0.1	12.4	400
0.2	9.0	270
0.3	8.1	255
0.4	7.2	240
0.5	7.0	225
0.6	6.4	200
0.7	6.1	180
0.8	5.9	165
0.9	5.2	135

using this system. Therefore, any small fluctuations in the reference potential (which we have demonstrated to be minimal) will have an insignificant effect on the electrode signal.

The results from experiments to determine the optimum number of printed cellulose acetate membranes are shown in Fig. 4. From this, it can be seen that the linear range is extended and the magnitude of the response is decreased as the thickness of the membrane layer is increased. Initial tests were carried out in a stirred solution, not in the FIA system. In the latter system the linear range can further be adjusted by altering the profile of sample dispersion in the delivery tubing. As a result of these experiments, it was decided to utilise the larger response of the thinner membranes. Combining the higher sensitivity of these sensors with the flexibility of the FIA system led to larger signals over an extended range of glucose concentrations. Therefore, sensors with two layers of cellulose acetate were used for all subsequent work.

The effect of flow rate on the biosensor response, including the time taken for the signal, from a 5 mM glucose sample, to return to a stable baseline is illustrated in Table 1. During the timescale of these experiments, negligible baseline drift was exhibited (< 50 nA) and noise levels were insignificant ($<$ 0.5% of the lowest response).

From this, it can be seen that a flow rate of 0.2 ml/min allowed a measurement frequency of 12 samples h^{-1}, in conjunction with a large signal response. Consequently, this flow rate was chosen for all future experiments.

In order to assess the reproducibility of the fabrication technique, two separate batches of 50 sensor cards were produced and five biosensors were randomly selected from each of these. The sensors were subsequently calibrated over a range of glucose concentrations between 0.5 and 50 mM. The mean value of the slope of the linear portion (0 to 25 mM) of the calibration graphs for the 10 sensors was 1.35 μA/mM and the standard deviation was 0.23 μA/mM. A typical calibration profile is shown in Fig. 5. Ten measurements were made at each concentration with each biosensor so that the precision could be assessed. The within electrode coefficient of variation was less than 2% (or 0.1 mM for concentrations below 5 mM). These results meet the high precision necessary for on-line monitoring and are comparable with other reports of on-line glucose monitoring using biosensors with FIA [15].

Three biosensors were tested to determine their operational stability. These were all able to operate

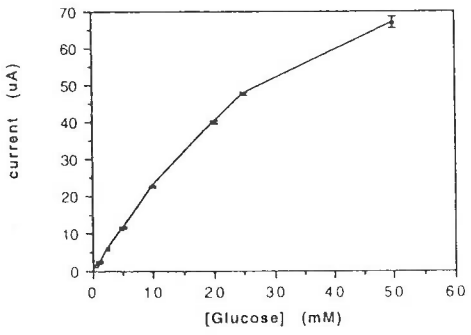


Fig. 5. Calibration profile of a typical biosensor

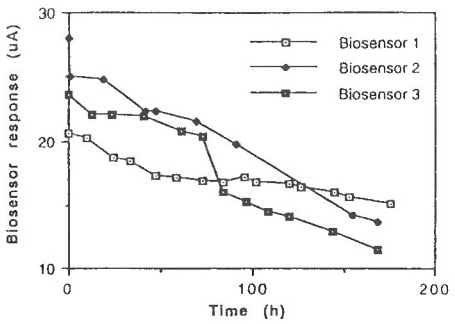


Fig. 6. Operational stability of three sensors tested continuously over a seven day period.

continuously over a seven day period, during which time over 300 measurements were made of the standard 10 mM glucose solution. The decline in activity over this period is illustrated in Fig. 6, which shows losses of 27%, 44% and 42% for each sensor. At first glance, this may appear to be a large drop in activity, but it must be borne in mind that the initial activity was very high and that this decline had occurred over a relatively long time. Combined with negligible drift and noise, the sensors displayed a high level of operational stability. Indeed, at the end of the seven day period, all the biosensors could still measure glucose concentrations in the range 0.5 to 25 mM, as determined at the start of the test. On the basis of these results, it was concluded that sufficient operational stability was achieved to allow successful on-line monitoring for practical applications, such as during fermentations.

The results from investigations into the effects of long term storage are shown in Table 2. From this table it can be seen that despite a variation between the sensors, a significant degree of stability is retained. This variation between sensor response is not

Table 2
Long-term storage stability

Day	Response (μA)
1	2.5
8	4.1
15	4.3
22	4.3
40	3.0
111	2.7
263	2.6

Table 3
Effect of temperature on the response of the FIA/biosensor system

Temperature (°C)	Response (μA)	C.V. [a] (%)
15	12.3	0.97
20	15.4	1.21
25	17.3	0.68
30	16.8	1.89
35	15.9	1.65

[a] Coefficient of variation ($n = 10$).

important when they are used in conjunction with the FIA system, since the calibration step will account for these changes. If this were a problem (in other applications) these fluctuations could be significantly reduced by carrying out production under more closely controlled conditions of temperature and humidity.

The temperature response of the FIA/biosensor system is depicted in Table 3, which shows peak activity at 25°C. The reason for the decline in activity between 25°C and 35°C, where the response reverted to that seen at the lower temperatures is unclear. It was expected that the response would show a plateau, but at a higher temperature. It is envisaged that subsequent instruments will incorporate temperature compensation. The next phase of development will include a detailed examination of the sensor in the new instrument, which will incorporate a built-in temperature measurement system, similar to the envisaged in the final device. Due to the small sample size (typically 20 μl), the sample temperature is relatively unimportant, since it will rapidly attain the temperature of the instrument.

4. Conclusion

We have demonstrated the practical combination of a mass-producible (screen-printed) amperometric glucose biosensor with FIA for on-line monitoring. This approach allows glucose concentrations between 0.5 and 1000 mM to be measured using three different dilution ratios. It is envisaged that a single biosensor could be used for fermentations of up to seven days duration. For longer periods of operation, such as fed-batch and continuous culture, the design

of the flow-cell, coupled to the low cost of the biosensor card, allows exchange of spent sensors to be carried out simply and rapidly, without disrupting the fermentation or compromising sterility. Future work will involve the investigation of real fermentation samples. In addition, despite the wide range of fermentations and hence the potential for a large number of possible interferents (electrochemical and surface fouling), the combination of a selectively catalytic carbon working electrode and a size exclusion membrane should minimise these effects.

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Paper VI

Monitoring of the Glucose Concentration During Microbial Fermentation Using a Novel Mass-Producible Biosensor Suitable for On-Line Use

Tothill, I.E., Newman, J.D., White, S.F. and Turner, A.P.F.
Enzyme and Microbial Technology, **20**, (1997), 590-596

ELSEVIER

Monitoring of the glucose concentration during microbial fermentation using a novel mass-producible biosensor suitable for on-line use

Ibtisam E. Tothill, Jeffrey D. Newman, Stephen F. White, and Anthony P. F. Turner

Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedfordshire, United Kingdom

A flow-injection analysis system was combined with a mass producible, disposable biosensor and was used to monitor glucose concentrations during several microbial fermentations. The biosensor was manufactured using thick film, screen printing technology. Unlike previous devices of this type, the biosensor was designed to make multiple measurements over extended periods rather than to operate as a "one-shot" sensor. One yeast, one lactic acid, and three E. coli *bioreactor cultures were tested using either defined or complex media. Results from the sensor were compared with a standard spectrophotometric test kit. In samples containing glucose concentrations within the range of the biosensor and the test kit, good correlations were obtained between the two methods. In addition to glucose, microbial growth and pH were recorded.* © *1997 by Elsevier Science Inc.*

Keywords: Biosensors; glucose; fermentation; flow-injection analysis (FIA); process monitoring

Introduction

The effective control of biotechnological processes requires the measurement of as many significant parameters as possible as frequently as possible; therefore, the measurement of glucose which is often not only the main carbon source but also the growth-limiting substrate, is of particular interest. Operationally, excess glucose concentration can be responsible for such problems as overproduction of lactic acid which can have detrimental effects such as decreasing pH. Furthermore, the concentration of glucose can be used to switch cell metabolism between glycolytic and oxidative states and can affect uptake and utilization of alternative carbon sources such as amino acids. Analytical systems for monitoring glucose concentration in biotechnological processes such as fermentation should fulfill at least three requirements: they should not compromise sterility, they should allow readings over a wide range of concentration, and they should give rapid results.[1]

Complications result from the high complexity of reaction mixtures which are composed of many different metabolites whose influence on the enzymatic analysis is, so far, largely unknown. This makes the adaptation of glucose sensing technology, which is well characterized (and commercialized) in other applications, particularly challenging; nevertheless, a number of approaches to the problems of monitoring glucose in biotechnological processes have been studied with some success.

Amperometry formed the basis of the first true biosensors[2] and has remained the quintessential underlying principle since the conception of the first device over thirty years ago.[3] Most commonly, the enzyme glucose oxidase (EC 1.1.3.4) is used to convert glucose to gluconic acid:

$$\text{Glucose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{Gluconic Acid} + H_2O_2 \qquad (1)$$

Thus glucose can be monitored amperometrically by measuring either the fall in O_2 concentration, the production of H_2O_2, or by facilitating electron transfer from the enzyme to the electrode by using, for example, mediators.[4,5] Traditionally, platinum electrodes poised at −600 mV and +700 mV (vs. SCE), respectively, have been used to monitor the following reactions:

Address reprint requests to Dr. I. E. Tothill, Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedfordshire MK43 0AL, United Kingdom
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655 Avenue of the Americas, New York, NY 10010

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$$O_2 + 4\ H^+ + 4\ e^- \rightarrow 2\ H_2O \quad (2)$$

$$H_2O_2 \rightarrow O_2 + 2\ e^- + 2\ H^+ \quad (3)$$

Hydrogen peroxide detection has become the more popular approach[6]; however, biosensors based on this approach can be prone to interference due to the high potentials required. A further problem can arise from fluctuations in the local oxygen tension which affects the enzyme-catalyzed reaction above. Interference can be minimized by using materials, particularly platinum group metals dispersed on carbon, which selectively reduce the potential required to oxidize hydrogen peroxide.[7] The conventional approach was to use platinized carbon; however, it has been shown that rhodinized carbon has superior properties.[8] We have shown recently that a new catalytic powder based on finely dispersed, promoted rhodium on carbon, designated MCA4 (MCA, Royston, U.K.), is highly effective for this purpose. This powder exhibits very low sensitivity to interferences commonly found in fermentation media.[9] This material was successfully incorporated into an ink suitable for manufacturing glucose biosensors, capable of carrying out multiple measurements over a seven-day period.[10] The biosensor was designed to be operated in a flow-injection analysis system.

In terms of bioreactor process monitoring, there are two possible strategies available using biosensors:

1. The sensor is used outside the reactor, i.e., external enzyme sensor
2. The sensor is placed *in situ* in the reactor

For *in situ* sensors, placing of the sensor in the bioreactor could be advantageous in situations when sample extraction and transport could cause difficulties; however, certain problems can arise. The sensor must be sterilizable; the mode of operation does not permit treatment of the sample or manipulation of the sample environment (with respect to pH, buffer capacity, or oxygen concentration); recalibration during use is difficult; and it is necessary to operate at the process temperature and pressure. A number of approaches have been used in order to overcome this drawback including disinfection by chloroform,[11] injection of a suspension of the enzyme into the autoclavable body of the enzyme electrode after autoclaving,[12] and introduction of the enzyme electrode which is disinfected into an autoclavable housing with an organism-impermeable membrane separating the enzyme from the broth.[13]

A number of options are available if the biosensor is to be used outside the fermenter. One possibility could be to perform an analysis off-line (i.e., in a remote analytical laboratory) where a device such as a Yellow Springs analyzer could be used. It is also possible to measure glucose in off-line fermentation samples using the ExacTech (Medisense, Abingdon, U.K.) blood glucose sensor,[14] however, such approaches are rarely suitable for any form of real-time process control since methods of this type require time-consuming sample collection, preparation, and analysis.

On-line analysis, by contrast, enables effective process control and is, in many ways, a simple option. The problem of sensor sterilization is completely avoided and any sample preparation required such as dilution to within the linear range of the sensor, pH adjustment by addition of an appropriate buffer, and measurement of temperature can be accomplished easily. An efficient sampling technique with the ability to obtain a representative sample of the culture is very important for this form of process monitoring. A technique which can easily be interfaced with such a sampling system is flow-injection analysis (FIA). This is an analytical technique which has found broad acceptance and has been the subject of many papers and reviews recently.[15–17]

This paper describes the performance of a dedicated sampler, FIA system, and a flow cell containing a previously described glucose biosensor[10] which are suitable for use in on-line fermentation monitoring.

Materials and methods

All reagents were analytical grade (Sigma Chemical Co. Poole, Dorset, U.K.) unless otherwise stated. Glucose standards were dissolved in 0.1 M sodium phosphate buffer pH 7.0, containing 0.1 M KCl (to maintain the reference electrolyte concentration) and were allowed to mutarotate overnight at 25°C. All solutions were degassed by ultrasonic treatment and the application of a vacuum prior to use to minimize the effect of air bubbles in the flow-injection tubing and the flow cell.

Thick-film amperometric biosensors based on glucose oxidase at a catalytic carbon-working electrode[9] combined with a carbon counter and silver/silver chloride reference electrode, screen-printed on a planar substrate as described in a previous publication,[10] were used throughout. The biosensor was located within a purpose-built flow cell (*Figure 1*). This design enables the biosensor to be positioned accurately and facilitates rapid and simple exchange of the spent sensor. During the experiments, samples were collected manually via the filtration unit and analyzed on the FIA system. Glucose concentrations were determined using the peak height response recorded on a chart recorder. Throughout all the cultures, the sensors were periodically calibrated to ensure the accuracy of the results.

All bioreactor culture experiments were performed in a 100-l fermenter (Bioengineering AG, Basle, Switzerland). In every case, the fermenter was inoculated during the previous evening and a small amount of glucose (0.5 g l^{-1}) was added. The remaining glucose was added on the following morning. This reduced the lag phase sufficiently to maximize the number of samples which could be acquired during the period of a working day.

In order to obtain a cell-free sample from the fermenter, it was necessary to include a sampling device (Persep, Techsep, France) directly linked to the FIA system. The filtration module was located in a fermenter port such that the revolving membrane (1,500 rpm during sampling and 500 rpm when no sampling occurs) was immersed in the culture broth. A diagram illustrating the layout of the system used is shown in *Figure 2*.

Three cultures were prepared using *Escherichia coli* K12 (National Collection of Type Culture, U.K.) with defined, complex, and industrial media. A beer fermentation was performed using the yeast, *Saccharomyces cerevisiae* (Vina, Liverpool, U.K.). Finally, a lactic acid fermentation was performed using the bacteria *Lactobacillus plantarum* (National Collection of Food Bacteria, U.K.). Glucose was measured by FIA and compared with the results obtained using a Boehringer D-glucose test kit (D-glucose UV-test 716251, Food Analysis, Boehringer Mannheim, Mannheim, Germany) using the manufacturer's instructions.

The media and conditions used for the *E. coli* fermentations were as follows:

1. Defined medium-KH_2PO_4, 1.7 g l^{-1}; $K_2HPO_4 \cdot 3H_2O$, 2.85 g

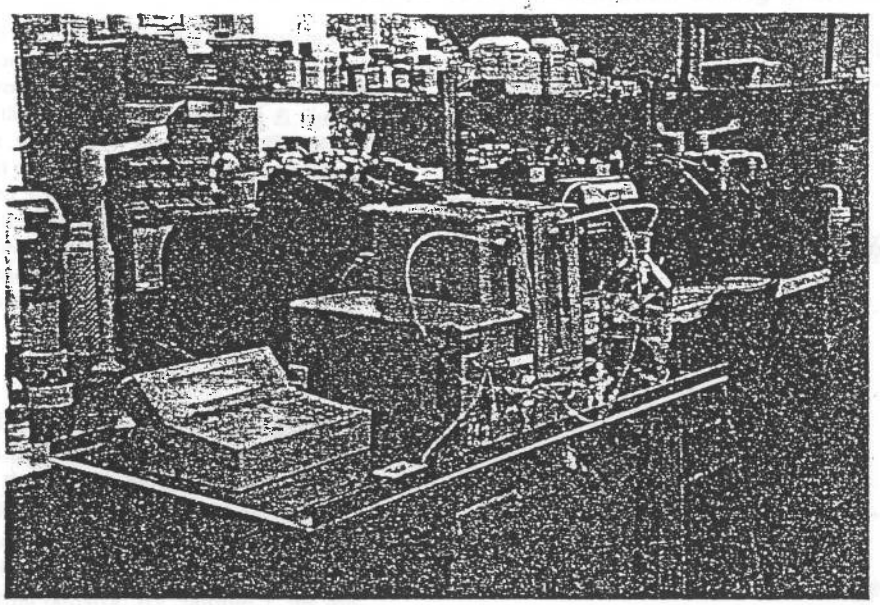


Figure 1 Flow-injection analysis (FIA) system. The flow cell containing the biosensor can be seen in front of the injection valve. Also shown to the left of the FIA unit is the microprocessor control unit

l^{-1}; Na_2SO_4, 0.1 g l^{-1}; ($NaHCO_3$, 0.42 g l^{-1}; $CaCl_2 \cdot 2H_2O$, 0.002 g l^{-1}; $FeCl_3 \cdot 6H_2O$, 0.002 g l^{-1}; $MgCl_2 \cdot 6H_2O$, 0.16 g l^{-1}; and glucose, 2.7 g l^{-1}. Fermentation conditions were as follows: temperature, 37°C; pH 7.2; stirring speed, 300 rpm; and air, 30 l min^{-1}. Samples were taken at 30-min intervals and stored at −20°C until required. Glucose was added after sterilization.

2. Complex medium-tryptone, 0.4%; yeast extract, 0.4%; glucose, 0.4%; castenholty minerals, 10.0%. Castenholty minerals consist of nitriloacetic acid, 1.0 g l^{-1}; $CaSO_4 \cdot 2H_2O$, 0.6 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 1.0 g l^{-1}; NaCl, 0.08 g l^{-1}; KNO_3, 1.03 g l^{-1}; $NaNO_3$, 6.89 g l^{-1}; Na_2HPO_4, 1.11 g l^{-1}; $FeCl_3$, 0.0028 g l^{-1}; trace element solution (10 ml l^{-1}) which consists of H_2SO_4, 0.5 g l^{-1}; $MnSO_4 \cdot H_2O$, 2.2 g l^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.5 g l^{-1}; H_3BO_3, 0.5 g l^{-1}; $CuSO_4$, 0.016 g l^{-1}; $NaMoO_4 \cdot 2H_2O$, 0.025 g l^{-1}; and $CoCl_2 \cdot 6H_2O$, 0.046 g l^{-1}. Fermentation conditions were as follows: temperature, 37°C; pH 5.96; stirring speed, 300 rpm; air, 30 l min^{-1}; polypropylene glycol (10 ml added to the fermenter). Samples were taken at 30-min intervals and stored at −20°C until required. Glucose was added after sterilization.

3. Industrial medium-KH_2PO_4, 4.0 g l^{-1}; H_2SO_4, 0.125 g l^{-1}; $(NH_4)_2SO_4$, 0.5 g l^{-1}; $CaCO_3$, 4.0 g l^{-1}; KCl, 1.0 g l^{-1}; molasses (containing 50-60% carbohydrate), 8.0 g l^{-1}; and corn steep liquor, 3.5 g l^{-1}. Fermentation conditions were as follows: temperature, 37°C; pH 5.43; stirring speed, 300 rpm; air, 30 l min^{-1}; and polypropylene glycol (10 ml added to the fermenter). Samples were taken at 45-min intervals and stored at −20°C until required. Molasses was added after sterilization.

The medium used for the *S. cerevisiae* fermentation was as follows: A commercial home-brew beer kit (Vina, Liverpool, U.K.) was used to prepare 60 l medium for the yeast fermentation. Two kg of glucose were added according to the recommendations of the kit supplier. The beer kit composition (amounts unspecified) were: barley syrup, barley malt extract, hops, hop extract, and yeast. Fermentation conditions were as follows: temperature, 25°C; pH 4.94; stirring speed, 100 rpm; air, 10 l min^{-1}; and polypropylene glycol (10 ml added to the fermenter). Samples were taken at 60-min intervals and stored at −20°C until required.

The medium used for the lactic acid fermentation (*Lactobacillus plantarum* LPCO–10) was as follows: Skimmed milk powder, 60 g l^{-1}; yeast extract, 5.0 g l^{-1}; bactopeptone, 5.0 g l^{-1}; and glucose, 8.0 g l^{-1}. Fermentation conditions were as follows: temperature, 37°C; pH 6.31; stirring speed, 200 rpm; anaerobic conditions; and polypropylene glycol (10 ml added to the fermenter). Samples were taken at 60-min intervals and stored at −20°C until required.

Samples were also taken from all the fermentations for both pH and optical density determinations (at 600 nm). For all the fermentations, samples were extracted using the filtration module and treated prior to analysis according to the recommendations of the Boehringer test kit. The samples were then divided into two for analysis by the kit and the FIA system. The FIA experimental conditions were as follows: temperature, 25°C; running buffers,

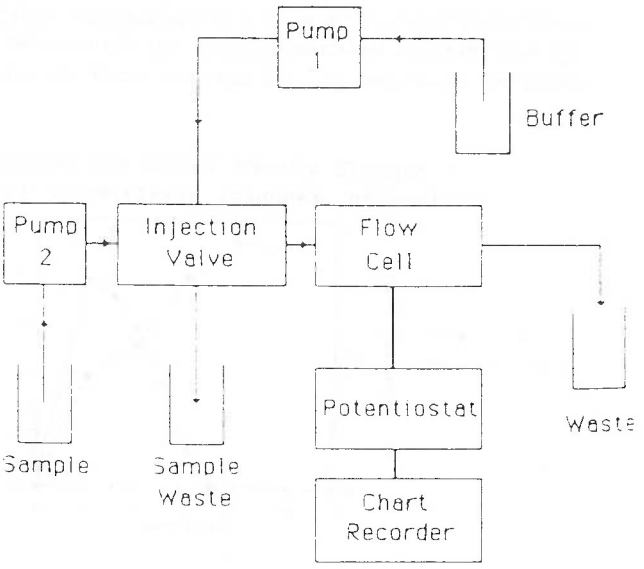


Figure 2 Schematic of FIA system

0.1 M sodium phosphate pH 7.0 containing 0.1 M potassium chloride; flow rate, 0.2 ml min^{-1}; volume of buffer between sample injection, 1 ml; time between injections, 60 s; and operating potential, +350 mV. For all the samples taken from the *E. coli* fermentations, a 1:1 dilution with 0.2 M phosphate buffer pH 7.0 containing 0.2 M potassium chloride was made. The yeast fermentation samples were diluted 1:10 with 0.11 M sodium phosphate buffer pH 7.0 containing 0.11 M potassium chloride. For the *L. plantarum* fermentation, the samples were prepared with either a 1:10 dilution with 0.11 M phosphate buffer pH 7.0 containing 0.11 M KCl or by a 1:3 dilution with 0.125 M buffer and KCl.

Results

Figure 3 shows the relationship between OD (microbial growth) and glucose utilization during the *E. coli*-defined medium culture. During the growth period, the pH declined from 7.2 to 6.3. When the microbial density had reached a steady state, the pH remained constant at 6.35. A decrease in the level of glucose during the fermentation is matched by increase in the microbial density. By Sample 7, the glucose concentration had fallen to approximately 0.08 mM and remained at this level for the remainder of the fermentation. Furthermore by Sample 7, the microbial growth curve had reached a plateau. The results indicate that the glucose concentration was a limiting factor for microbial growth. In order to assess the accuracy of the biosensor, samples were also examined using the Boehringer test kit. *Figure 4* shows the correlation between these two sets of data. The correlation coefficient was 0.993 (n = 6), indicating a high degree of agreement between the two methods.

When *E. coli* was cultured in the research medium, the initial pH was 8.04. At the onset of microbial growth, the pH declined to a minimum of 5.92 followed by an increase up to 7.5. As with the first fermentation, the microbial growth was limited by the glucose concentration in the medium. From a starting concentration of 14 mM, the glucose level had fallen to approximately 0.42 mM by Sample 7 (*Figure 5*). *Figure 6* illustrates the correlation between data obtained using the biosensor and the Boehringer test kit. Again, the two sets of results were in good agreement. This was illustrated by a correlation coefficient of 0.998 (n = 7).

The final *E. coli* fermentation was performed using an industrial medium incorporating molasses (containing 50–60% carbohydrate) as the carbon source. The forms of carbohydrate present in the molasses were undefined and no further glucose was added to the fermentation broth. *Figure 7* shows the changes in glucose concentration and microbial growth (OD). Throughout the fermentation, the pH remained confined to a narrow range of values from 5.2 to 5.9. Microbial density, however, more than doubled between Samples 1–5. After this time, the density remained constant. It can also be seen that the initial glucose concentration was found to be very low in comparison to the total available carbohydrate in the molasses. The glucose was rapidly metabolized by the bacteria and indeed by Sample 5, the concentration was negligible. *Figure 8* illustrates the relationship between glucose values determined using the FIA system and the Boehringer test kit. The correlation coefficient

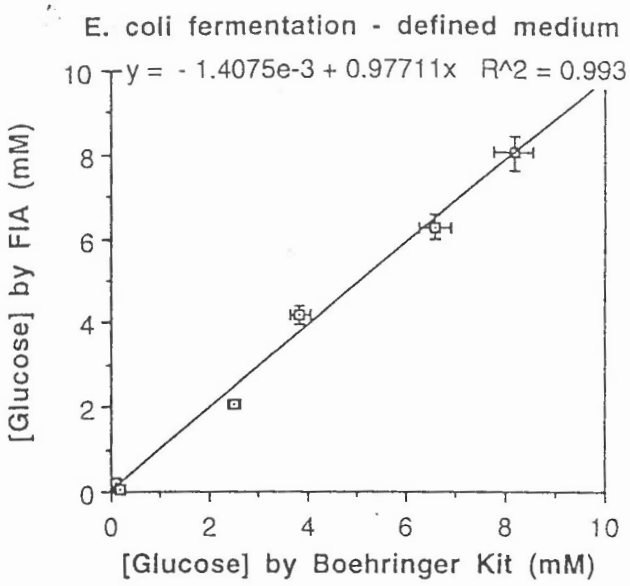


Figure 4 Correlation values obtained between measurements made using the FIA/biosensor system and a commercially available (Boehringer) glucose test kit for the *E. coli* fermentation with defined medium

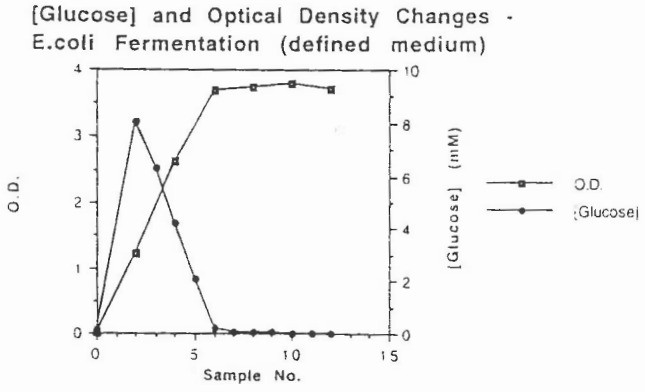


Figure 3 Relationship between OD (microbial growth) and glucose utilization (measured by FIA/biosensor) during the first *E. coli* fermentation using defined medium

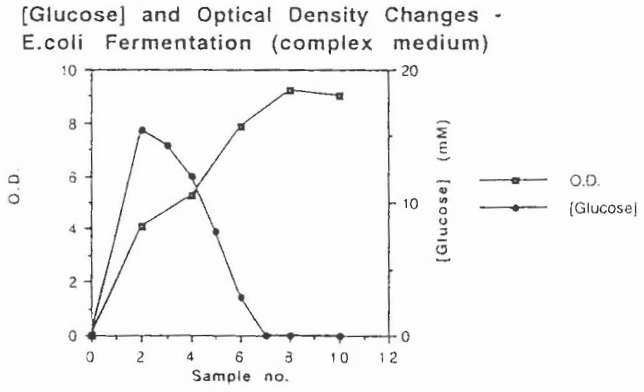


Figure 5 Glucose utilization (measured by FIA/biosensor) and microbial growth of the *E. coli* fermentation performed using complex medium

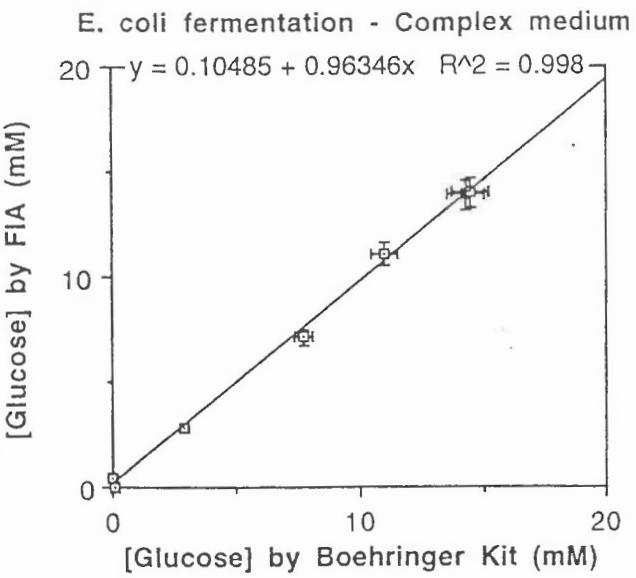


Figure 6 Correlation values obtained from measurements using the FIA/biosensor system and the test kit for the *E. coli* fermentation using complex medium

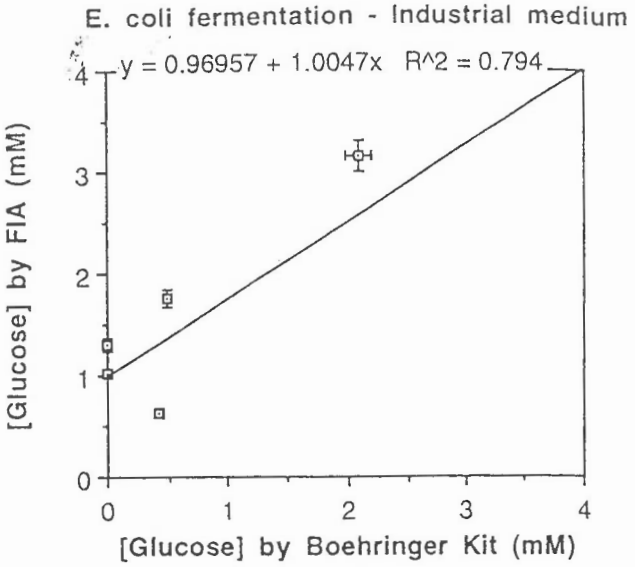


Figure 8 Relationship between glucose concentrations determined using the FIA/biosensor system and the test kit for the *E. coli* fermentation using an industrial medium

was 0.794 (n = 5), a value significantly lower than that achieved with previous fermentations. There are several possible reasons for this low coefficient value. The levels of glucose were quite low and a dilution ratio of 1:1 was used for the analysis. Higher dilution ratios are beneficial in complex (and viscous) media. Potential interferences may, therefore, have been quite high compared to the level of glucose in this case.

The glucose level and OD during the yeast fermentation are shown in *Figure 9*. Over the period monitored, the pH declined steadily from 4.92 to 3.45. In contrast, microbial growth accelerated during the initial stages (between Samples 1 and 2). Following this high growth rate before addition of the final glucose concentration, the density increased gradually. The fermentation was not taken to completion since enough samples were collected during a 9-h period to enable a satisfactory comparison between sensor and test kit values to be made. *Figure 10* illustrates the relationship between the sensor and test kit values. Again, an excellent correlation coefficient of 0.997 (n = 8) was obtained. This indicates a high degree of compatibility between the two sets of data.

The final microbial fermentation was performed using *Lactobacillus plantarum*. *Figure 11* shows the changes in glucose concentration and OD during this fermentation. Following addition of glucose, the pH declined from 3.85 to 3.63. Microbial growth was rapid prior to the addition of the bulk glucose into the fermenter. After the addition of glucose, the growth appeared to be limited. This indicated that the fermentation had reached near completion with only the initial low glucose addition. Nevertheless, the objective of these experiments was to verify the accuracy and reliability of the biosensor under real fermentation conditions; therefore, samples were still taken and analyzed. The comparison between test kit values and those obtained using the sensor

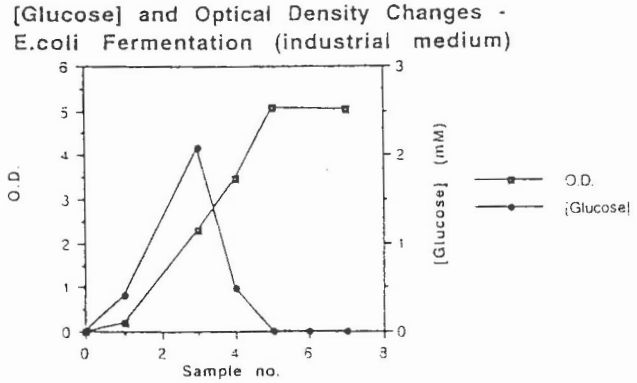


Figure 7 Changes in glucose concentration (measured by FIA/biosensor) and microbial growth (OD) throughout the *E. coli* fermentation using an industrial medium

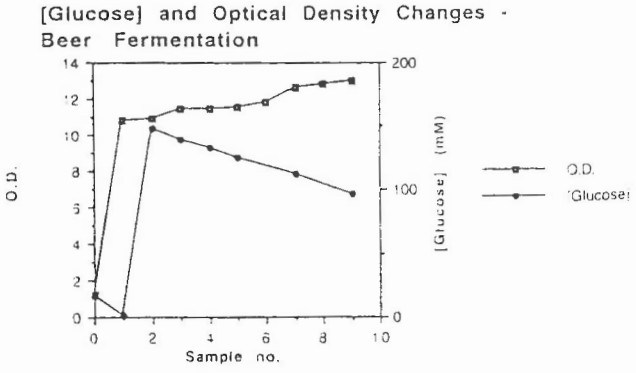


Figure 9 Changes in glucose concentration (measured by FIA/biosensor) and microbial growth (OD) during the yeast fermentation

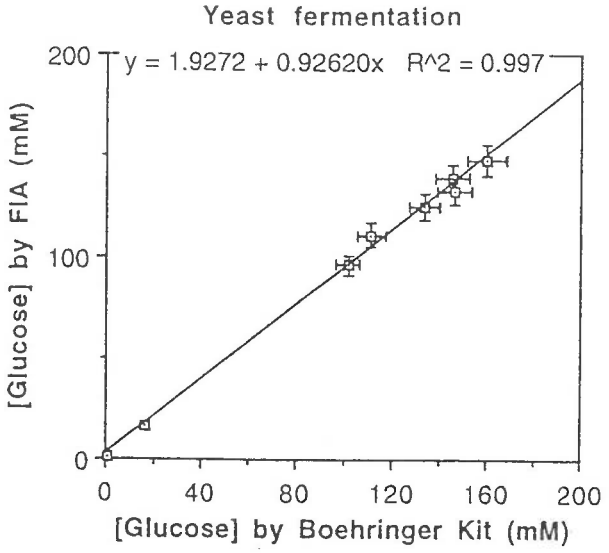


Figure 10 Relationship between FIA/biosensor and test kit values for the glucose concentrations during the yeast fermentation

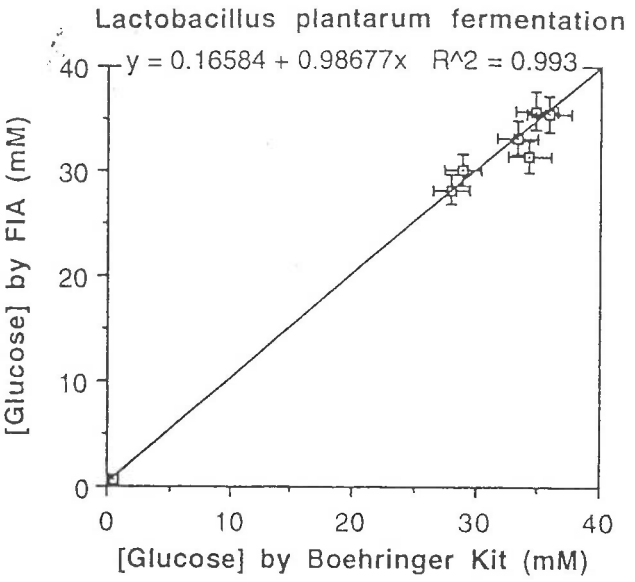


Figure 12 Correlation between glucose concentrations determined using the FIA/biosensor system and values obtained using the test kit for the *Lactobacillus plantarum* fermentation

are shown in *Figure 12*. The correlation coefficient was 0.993 (n = 8), again indicating an excellent agreement.

Conclusions

The objective of this work was to demonstrate the viability of a novel on-line monitoring system for glucose determination based on a mass-producible biosensor housed in a purpose-built FIA system. Previous reports[9,10] have demonstrated the advantageous features of this device. In particular, the precision, reproducibility of the manufacturing process for the sensor (including the low cost), ease of use, and potential for automated monitoring were highlighted. In the current work, the accuracy of this system (when used to monitor glucose consumption in fermentations) was established. Different microorganisms with various fermentation mediums were used in order to subject the biosensor system to distinct conditions. With the exception of one medium (*E. coli*, industrial medium), the sensor system was in excellent agreement with values obtained using a standard off-line assay kit.

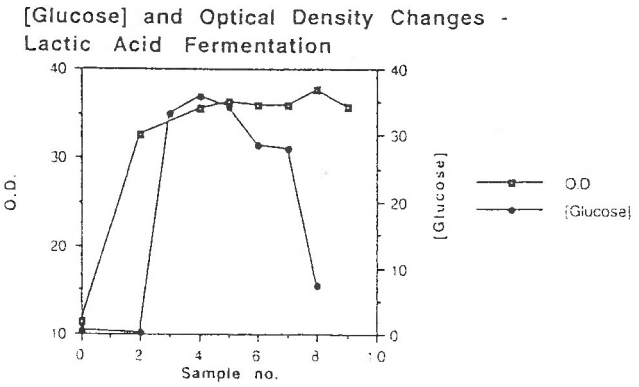


Figure 11 Changes in glucose concentration (measured by FIA/biosensor) and microbial growth (OD) during the *Lactobacillus plantarum* fermentation

Using the FIA system, recalibration of the sensor can be performed rapidly and automatically, thereby enabling the system to be left unattended throughout the fermentation. As was previously reported,[10] the biosensor can be left for at least seven days during which time, up to 1,500 measurements can be made between exchange of the device. The flow cell and biosensor were designed to facilitate a rapid and simple exchange of the sensor card without disrupting the fermentation or compromising sterility. This process takes less than 1 min to perform and the total downtime (including equilibration of the biosensor) is less than 10 min. For the experiments reported in this publication, results were collected on a chart recorder and analyzed after the fermentation was completed. Future developments will include a data logging system using dedicated software to improve the interpretation of the measurements and the accuracy.

The pH of the fermentation media did not affect the operation of the sensor. Dilution of the samples with the running buffer helped to maintain the optimum conditions required for the sensor. Future developments will include an automatic variable dilution step which allows a wide range of glucose concentrations to be monitored and automatic temperature compensation.

A recent report[3] highlighted the current range of commercially available biosensor-based systems. It also emphasized the paucity of such instruments for the on-line analysis of glucose during fermentation; hence, direct comparison of this approach with such devices is not straightforward. Perhaps the nearest devices for such a comparison are those offered by the Yellow Springs Instrument (YSI) Company and Ismatec SA; however, the YSI glucose monitor is intended for off-line measurements with all the inherent dis-

advantages of this approach. In contrast, the Ismatec unit is intended for on-line use. The system relies on the use of enzyme-packed columns followed by electrochemical detection. The cost of the enzyme columns could be a disadvantage. Our approach, which integrates the enzyme and electrode array in a biosensor format, allows the use of mass-fabrication technologies to produce a cost-effective, reliable device.

The combination of economically viable and disposable biosensors with FIA, fully integrated with a sampler unit has been shown to be a powerful tool, thereby, enabling rapid acquisition of data. The approach will enable improved monitoring of fermentations and eventually could be incorporated into a control regime.

Acknowledgments

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Paper VII

Lactate Sensors: Sensing Sites and Fabrication
Marazza, G., Mascini, M., Newman, J.D., Palleschi, G. and Turner, A.P.F.
In: In vivo Chemical Sensors: Recent Developments
(Eds S.J. Alcock and A.P.F. Turner), Cranfield Press, UK, 1997, 590-596

6

Lactate Sensors: Sensing Sites and Fabrication

G. Marrazza,[1] M. Mascini,[1]
J.D. Newman,[2] G. Palleschi[3] and A.P.F. Turner[2]
[1] Dipartimento di Sanita Pubblica, Epidemiologia e Chimica Analitica Ambientale, Universita Degli Studi di Firenze, Italy
[2] Biotechnology Centre, Cranfield Institute of Technology, UK
[3] Dipartimento di Scienze e Technologie Chimiche, II Universita di Roma 'Tor Vergata', Italy

Elevated lactate levels are indicative of insufficient tissue oxygenation. The lactate concentration in blood is used to assess the degree of metabolic acidosis following surgery or other shock conditions and continuous *in vivo* monitoring of lactate may prove valuable during intensive care, cardiac surgery or in monitoring foetal distress. Conditions associated with L-lactic acidosis include diabetes, heart disease, leukaemia, glycogen storage disease, ethanol ingestion, spontaneous lactic acidosis and meningitis. Lactate measurements are also increasingly used in sports medicine to assess physical fitness.

The demand for non-invasive lactate analysis is increasing, particularly for people with blood collection problems. The measurement of lactate in saliva using an enzyme electrode was examined as an alternative to taking blood samples. The concentration of lactate in saliva ranges from 0.1 to 0.2 mM. Salivary lactate was measured in eight fasting subjects; following a meal the lactate concentration increased in all subjects. A subject showed a consistent increase in salivary lactate after physical exercise (Figure 6.1).

Lactate concentration in blood ranges from 0.5 to 2 mM. Lactate measurements using an enzyme electrode in four human sera correlated well with conventional spectrophotometric methods. Recovery studies were carried out adding lactate to a pooled serum and results were excellent.

An enzyme electrode in conjunction with a bedside artificial pancreas was used to continuously monitor blood lactate concentrations *ex vivo* after a 'glucose clamp' experiment in a diabetic patient. Results showed that lactate increased after insulin infusion and after the patient did a simple physical

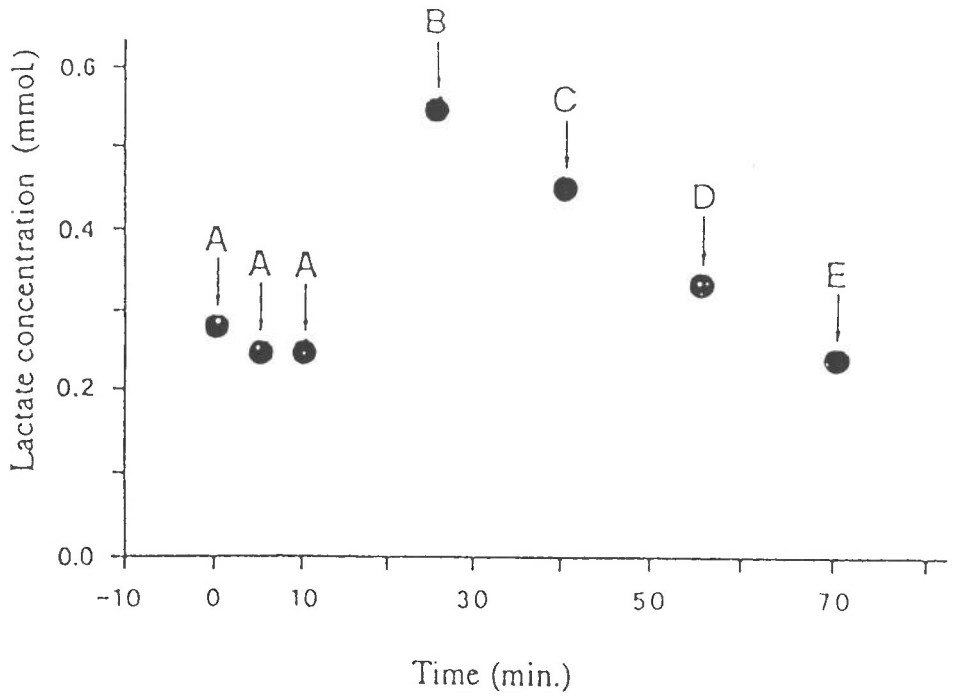


Figure 6.1 Lactate acidaemic curve. A, Saliva lactate measured while the subject was in the resting state; B, C, D and E, saliva lactate measured immediately, 15, 30, 45 and 60 min, respectively, after physical exercise.

exercise. Results correlated well with those obtained on intermittent blood samples drawn every 15 minutes (Figure 6.2).

The anaerobic threshold is the point at which a change from exclusively aerobic to a partially anaerobic energy supply, by formation and accumulation of lactate, occurs in graded exercise tests. Continuous measurements of lactate were carried out by *ex vivo* monitoring in amateur and high level athletes. The experiments yielded precise, real-time measurement curves during the exercise periods with a well-defined transition from the aerobic to anaerobic mode.

Several fabrication methods were used to construct enzyme electrodes for lactate ranging from complex, hand-crafted design to techniques suitable for the mass production of reproducible, inexpensive devices.

Lactate was measured in blood using an enzyme electrode based on a Clark-type oxygen probe consisting of a Ag/AgCl reference electrode and a platinum wire covered with a gas permeable (teflon or polypropylene) membrane. The sensor tip was covered with lactate 2-monooxygenase EC

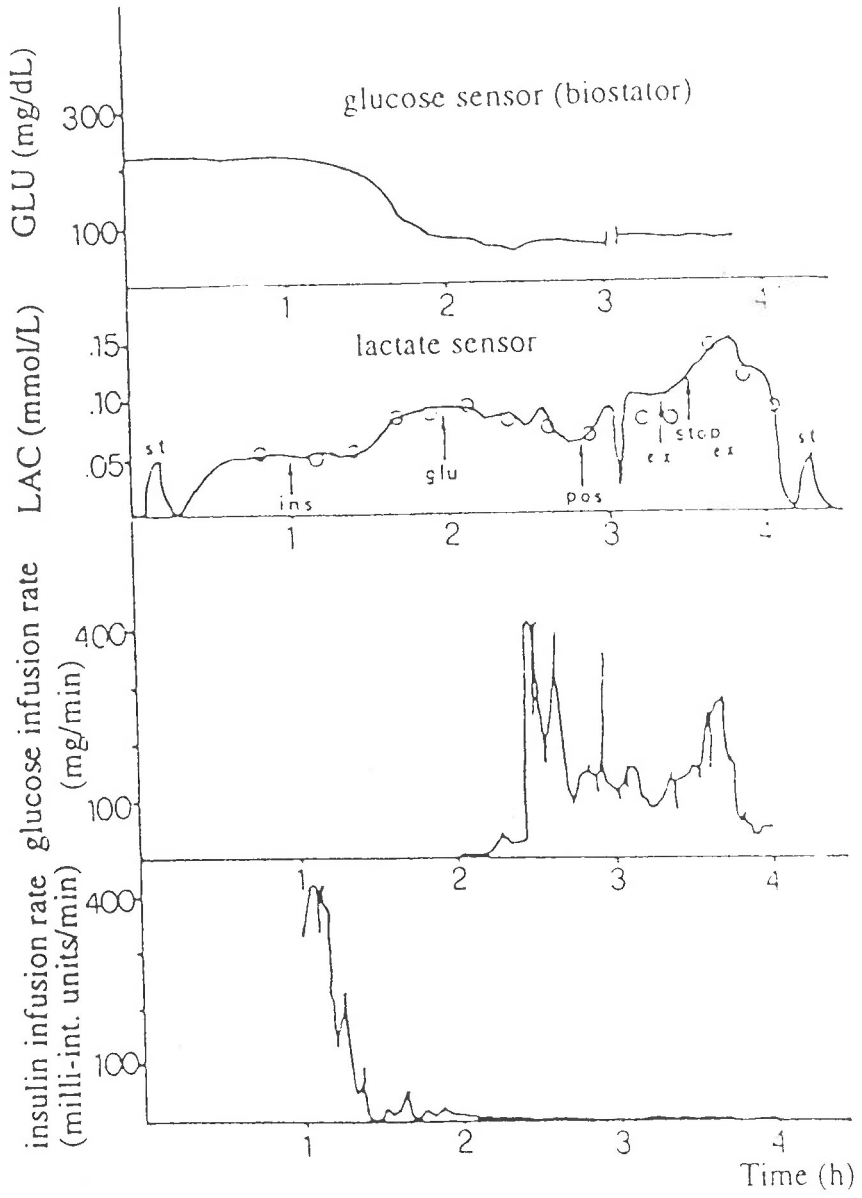


Figure 6.2 Glucose clamp experiment in a diabetic patient. Ex, exercise

1.13.12.4 (from *Mycobacterium smegmatis*) immobilised on a nylon net. This enzyme consumes oxygen, but does not produce hydrogen peroxide.

For *ex vivo* measurements lactate oxidase from *Pediococcus* species was used. In this case either oxygen consumption or hydrogen peroxide production was measured. The hydrogen peroxide sensor consisted of a platinum wire coated with a cellulose acetate membrane (molecular weight cut off 100) and an Ag/AgCl reference electrode. An outer dialysis membrane was used to protect the immobilised enzyme in both the oxygen and hydrogen peroxide electrode configurations (Figure 6.3). When this probe is used as oxygen sensor the cellulose acetate membrane is replaced with a gas membrane.

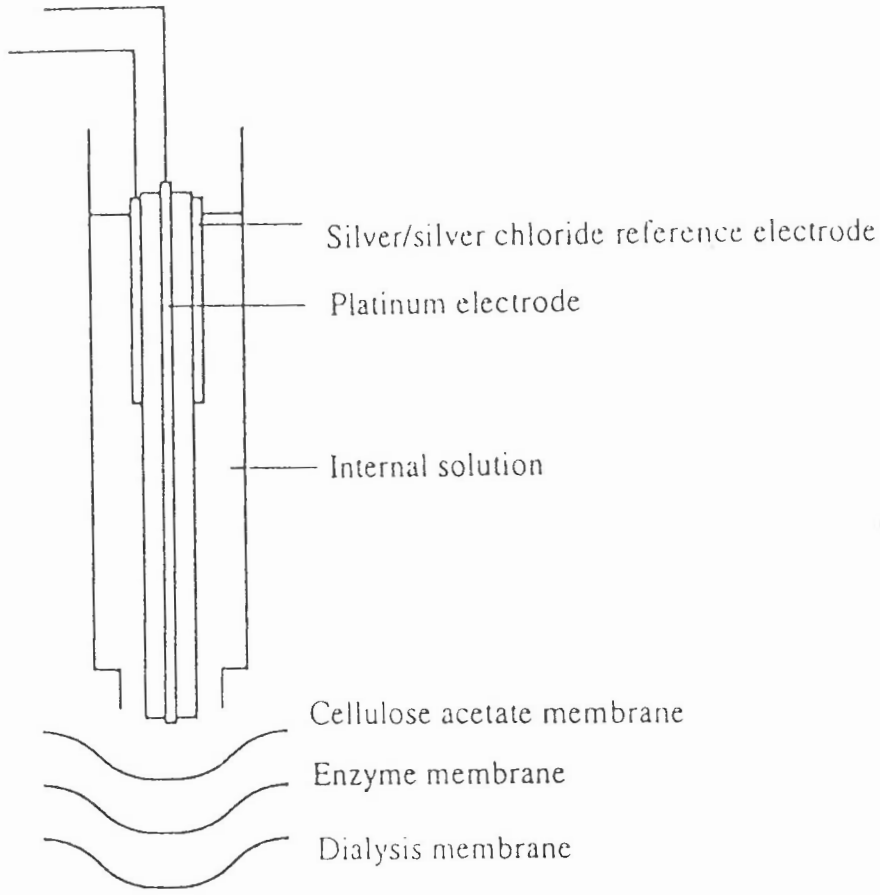


Fig 6.3 Enzyme probe based on a hydrogen peroxide electrode.

The use of mediators to replace the natural electron acceptor for lactate oxidising enzymes in enzyme electrodes should remove the need for oxygen in the sample, reduce the dependence of the signal on oxygen concentration, widen the range of enzymes that can be used, reduce the operating potential (and hence interference), reduce any production or involvement of hydrogen peroxide (which is implicated in enzyme inactivation) and reduce the effect of variations in pH. This will be achieved, however, at the expense of introducing a further potentially unstable and toxic component. A number of enzymes were tested with ferrocene-modified carbon electrodes and lactate oxidase was found to give the most satisfactory results. Tetrathiafulvalene (TTF) was also used as a mediator to modify carbon foil electrodes prior to absorption of lactate oxidase. Calibration curves were obtained with both 1,1′-dimethyl-ferrocene- and TTF-modified electrodes in the range 10^{-4} to 10^{-3} M lactate. The linearity was influenced by the method of enzyme immobilisation and the presence or absence of a membrane.

In order to improve the reproducibility of electrodes, automated fabrication technologies were investigated. Carbon ink electrodes, with and without

ferrocene as a mediator, were screen printed onto a PVC base. Following absorption of lactate oxidase, lactate could be monitored in buffer either by the detection of hydrogen peroxide (electrodes without mediator) or by mediated electrochemistry. Electrodes could be used for single assays, multiple assays or continuous monitoring, but stability in the latter cases was poor. It should be possible to improve the retention of enzyme and mediator, adjust the linear range and facilitate measurement in biological samples by screen printing a membrane over the electrode. An ink jet printer was also used to produce mediated and unmediated enzyme electrodes. Lactate could be monitored successfully in buffer using either hydrogen peroxide detection or ferrocene monocarboxylic acid as a mediator, but problems were experienced with other mediated sensors. Further work with mediators is proposed.

Paper VIII

Silicon-on-Insulator Integrated Optical Biosensors for Environmental Monitoring

Newman, J.D., Turner, A.P.F, Rickman, A.G., Harpin, A.P.R., Shaw, M.P. and McKenzie, J.S..

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SILICON-ON-INSULATOR INTEGRATED OPTICAL BIOSENSORS FOR ENVIRONMENTAL MONITORING

Jeffrey D. Newman[a], Anthony P.F. Turner[a], Andrew G. Rickman[b], Arnold P.R. Harpin[b], Matthew P. Shaw[b], James S. McKenzie[b]

Introduction

The use of optical transduction in environmental sensors has been well documented in the scientific literature[1]. Techniques such as surface plasmon resonance (SPR)[2], reflectometric interference spectrometry (RIFS)[3] and interferometry[4] have all been demonstrated in research laboratories. The performance of these devices is suitable for use in a wide variety of applications, including the detection of pesticides, hydrocarbons and microorganisms. The inherent capability of being able to detect directly specific binding interactions, without the need for labels offers significant advantages over the other widely adopted biosensor approach of electrochemical detection. Depending on the size of molecule, detection limits of the sub-nanomolar range are possible[4]. Devices based on these technologies have been incorporated into commercially available instruments. For example an instrument which utilises surface plasmon resonance based detection in conjunction with a miniaturised fluid handling regime has been available for several years[5]. However, much of the potential of this type of technology remains untapped due to technical limitations and the high production costs associated with existing level of miniaturisation of optical technologies.

The integrated optical technologies employed for evanescent field optical chemical/biological sensors reported to date have been defined by the requirement for low optical confinement waveguides. These waveguides provide the basis of selective evanescent sensing elements, but they are incompatible with the practical demands of high accuracy integrated optical measurement circuits. Hence, integrated optics has been restricted either to forming part of a bulk optical sensing system[6] or to limited sensitivity and dynamic range compared with bulk optical measurements. A viable integrated optics sensor technology must combine, at a realistically low cost, both sensing optical waveguides and active waveguide circuits. Bookham Technology have developed a manufacturing process to meet these needs known as Active Silicon Integrated Optical Circuits (ASOC™) which brings to chip manufacture a highly efficient production method which is based on the same processing techniques as those used in the production of silicon electronic chips[7]. The major benefits of ASOC™ are that the key components such as couplers, splitters, phase modulators, laser diodes, photodiodes and self-aligning optical fibre / waveguide interfaces can all be integrated on a single chip[8].

A further requirement of sensor production is the ability to deposit chemically or biologically sensitive layers onto the waveguide surfaces. Many sensors have been demonstrated in the laboratory which have relied on manual fabrication technology and have subsequently failed to reach the marketplace. A number of reports have emphasised the importance of automated production methods[9,10]. This is not simply due to the ability of these techniques to be scaled up to mass production, but because of the inherent reproducibility of such processes.

a. Cranfield Biotechnology Centre, Cranfield University, Cranfield, Bedfordshire, MK43 OAL.
b. Bookham Technology Ltd., Central Microstructure Facility, Rutherford Appleton Laboratory, Chilton, Oxfordshire, OX11 OQX.

Ink-jet printers are most familiar in the form of the office-based printers. The industrial ink-jet is a similar technology, which is conventionally used to print bingo cards, which have a different set of numbers on each card and information such as sell-by dates on food packaging. The office-based printer operates by heating the ink and using the expansion to eject a droplet from a nozzle. This renders the process unsuitable for use with labile materials such as biological solutions.

The industrial ink-jet, however, operates by circulating the solution through a small hole (typically 35-75 μm) in a nozzle under a pressure of approximately 3 bar. Shock waves are sent through the fluid via a drive rod which breaks the jet into individual droplets at a rate of about 64 kHz (dependent on the modulation voltage). The jet is aligned such that it enters a return tube for recirculation. Printing is achieved by charging individual droplets, under the control of a microprocessor, using a charging electrode. The charged drops are then deflected on passing between two parallel high voltage deflector plates (Figure 1). A pattern is hence built up in dot-matrix form. Due to microprocessor control, it is extremely easy to change the print pattern and the volume of reagent dispensed with an ink-jet printer. It is also a non-contact process, which means that it is suitable for deposition of material onto non-planar and delicate surfaces, should the need arise. The solution must, however, be electrically conducting (so that the droplets can be charged) and particulate free. The solution is only deposited where it is required and due to the small size (in the order of 1 nl) of each droplet, the process is economical with reagents, although there is a relatively large volume (approximately 5 ml) required for circulation.

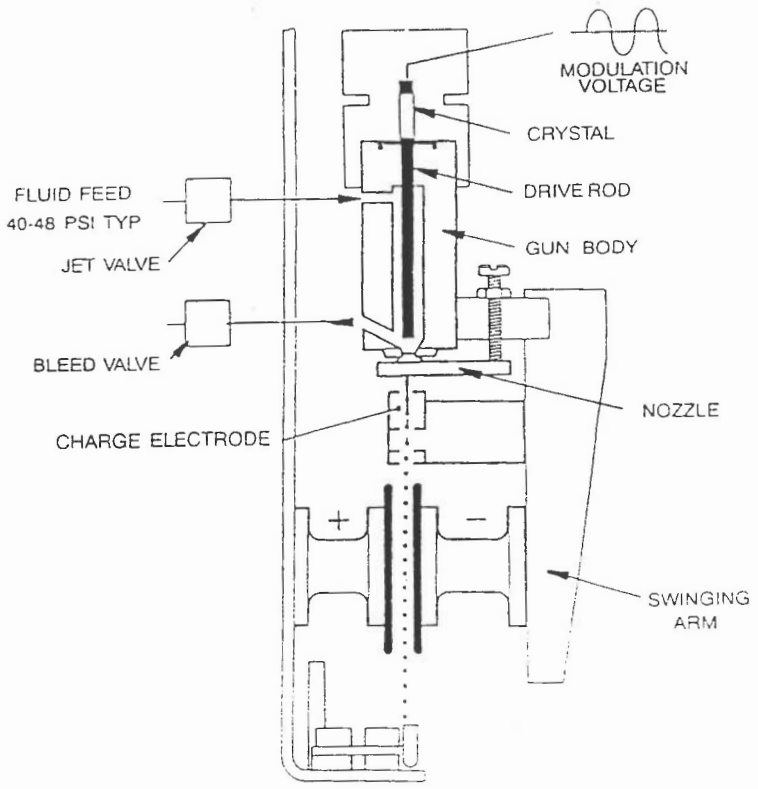


Figure 1. Ink-jet Print Head Assembly

Integrated Optical Biosensors

Integrated optical chemi/biosensors are based on the interaction of the evanescent field of a guided optical wave with a surface receptor layer. Figure 2 illustrates the waveguide structure. It consists of a lower cladding layer, a waveguide core and surface receptor layer. The surface layer is chosen to alter its optical properties (real refractive index and/or absorption) when in contact with the target analyte. The change in the optical properties of the receptor layers results in a change in the propagation coefficient in the waveguides (ie optical path length). The absorption can be measured by the variation in transmitted power and the propagation coefficient can be very accurately measured by placing the sensor waveguide in one arm of an optical interferometer. ASOC™ technology can be used to produce interferometers on a chip.

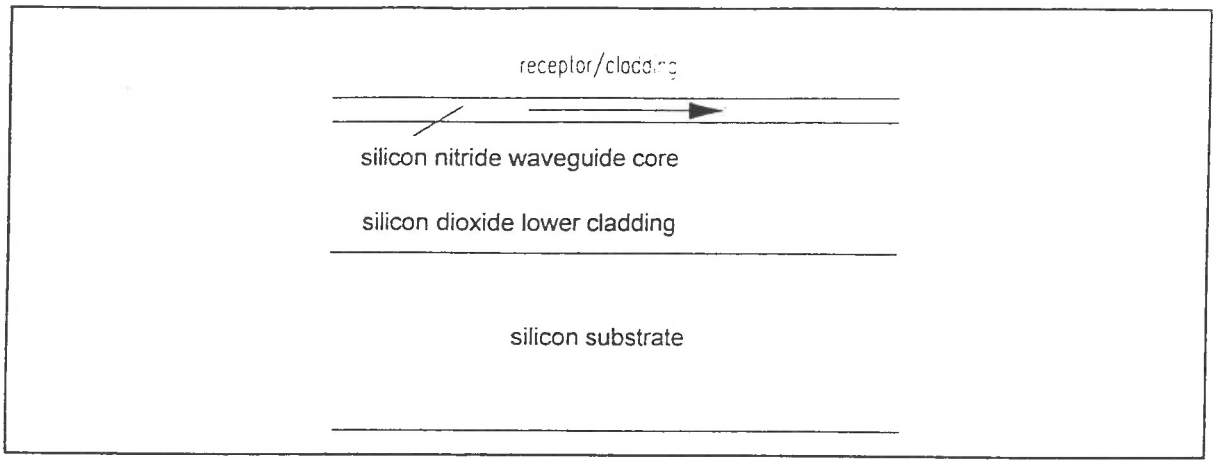


Figure 2. Optical Evanescent Field Chemical and Biological Sensing

An important condition placed on the sensing waveguide structure by the ASOC™ manufacturing process is that it must be fabricated from materials commonly used in the production of silicon chips. Hence, silicon dioxide and silicon nitride, which have low optical loss in the visible and near infrared, are obvious choices. It was also determined from computer modelling that the optimum waveguide structure for evanescent field sensing would have a core thickness of approximately 100 to 200 nm, a refractive index of 2.0 and have a lower cladding with a refractive index of 1.5. Silicon nitride is thus suitable for the outer layer and silicon dioxide was suitable for the inner layer. Figure 3 illustrates this structure. It was found with this arrangement that a refractive index change of 1 ppm in the receptor layer resulted in a change in the waveguide propagation constant of 0.994 ppm for the TE_o mode. A further advantage of this structure is that most chemical and biological reagents have a refractive index between 1.35 and 1.6, and hence the refractive index of silicon nitride (2.0) is likely to be much larger than any conceivable receptor film, therefore, the structure should be applicable to most situations.

Waveguide Butt Coupling and Incorporation of an Interferometer

A low cost method of integrating the silicon nitride waveguide to the silicon waveguide was devised using butt coupling. This was expected to result in three optical loss mechanisms; reflection, scattering and electric field overlap loss. The reflected loss can be minimised by choice of the film thicknesses to about 0.1 dB. The total loss for this structure is at present about 6.0 dB, which is considered acceptable for practical device applications.

Having determined the possibility of integrating a silicon nitride sensor waveguide with a silicon waveguide, the structure was considered in the context of a whole sensor device. The silicon waveguides have active electro-optic capability which is unavailable in the nitride waveguides. Therefore an active interferometer can be produced (Figure 3). The use of an electro-optic phase modulator in the reference arm allows the resolution of an interference fringe to better than 1 ppm (using null point detection by active phase dither).

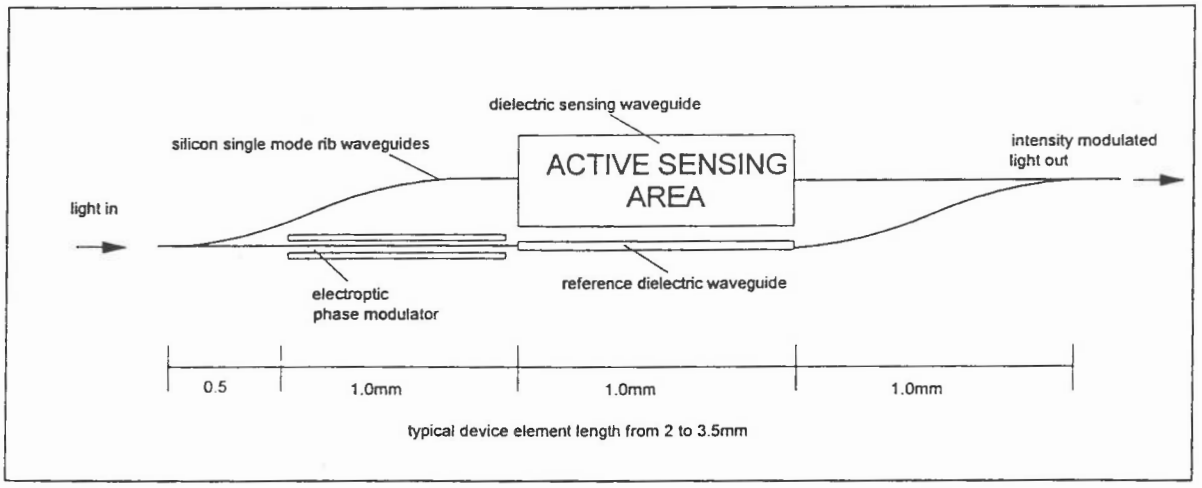


Figure 3. Schematic of Active Interferometer

Over a sensitive waveguide of say 1000 wavelengths (approximately 1 mm in this context), the effective refractive index change of the sensor waveguide could be measured to 1 ppb. Thus, the physical aspects of the system appear suitable for the detection of low levels of biological or chemical moieties.

Non-Specific Binding

One of the major problems with sensors of this type is that of non-specific responses. This can be partly reduced by careful selection of the chemical or biological receptors and the method by which they are immobilised on the waveguide surface. In addition, there are a range of optical parameters which can be used to help differentiate the signal from the interference. These include:

a) Using different wavelengths of light, such that strong specific absorptions can be exploited.

b) The combination of a range of optical channels with different receptor reagents or different waveguide dimensions.

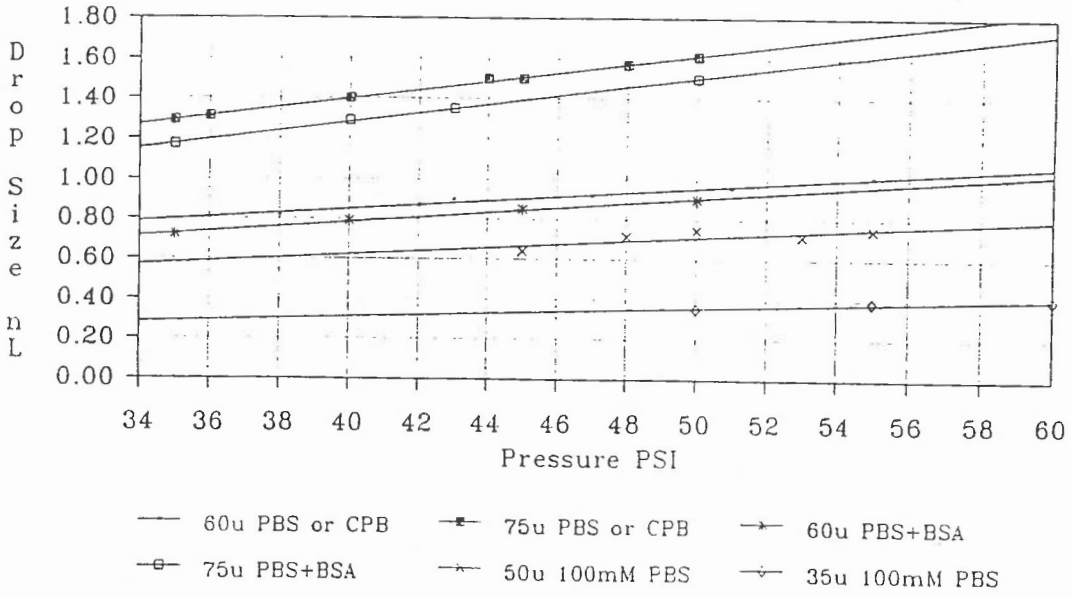
c) The use of an interferometer with sensing waveguides in both arms, but with one layer chemically deactivated to the target analyte.

d) Using the relationship between refractive index change and absorption to provide a further sensor dimension.

It is a major advantage of the ASOC™ manufacturing process that it is relatively straightforward to incorporate such features onto the sensor due to its active capability.

Ink-jet Printing

In order to best utilise the capabilities of integrated optical sensor platforms, a controllable method of depositing the receptor layer is required. Ink-jet printing allows droplets of the order of 1 nl to be printed. However, the droplet size is determined by the selection of the appropriate nozzle and is sensitive to the pressure applied and to a lesser extent to the viscosity and density of the printed solution. Figure 4 illustrates the droplet sizes obtained with different solutions over a range of pressures using 35, 50, 60 and 75 µm nozzles. These values were calculated by collecting the printed solution over 60 seconds, measuring its volume and using the known droplet formation rate to calculate the volume of each drop.



PBS = Phosphate Buffered Saline 50mMole
CPB = Citrate/Phosphate Buffer 100mMole
PBS+BSA = PBS + Bovine Serum Albumen mix

Figure 4. Ink-jet Printer Drop Sizes

Conclusions

It is clear from this work that the fabrication of a family of silicon based evanescent optical field sensors can be produced using well established silicon microelectronics processes. Such devices offer advantages of low cost and high sensitivity compared with conventional technologies. A further cost advantage arises from the generic nature of the technology. This is largely due to the active features of silicon circuits. New sensors will only require the development of appropriate receptor layers.

Ink-jet printing provides the means to produce highly controlled droplets of very low volume. These can be printed at high speed and are controlled by a microprocessor. It is therefore possible to produce small receptor film regions. It is, however, desirable to further reduce the droplet size to match the miniaturisation capabilities of the optical structure.

These technologies offer exciting possibilities, particularly in the area of environmental analysis, where cost is often the rate limiting step during the introduction of a new measurement method. The approach of interferometry has been well documented in the scientific literature as being sensitive enough to meet many modern requirements. With further development, this approach should enable optical environmental sensors to become a commercial reality.

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