

Enzyme Biosensors

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Summary

The biosensor field has grown enormously since the first demonstration of the biosensor concept by Leland C. Clark Jr. in 1962. Today's biosensor market is dominated by glucose biosensors, mass-produced enzyme-electrodes for the rapid self-diagnosis of blood glucose levels by diabetes sufferers. Here we take a historical look at the inception, growth, and development of the enzyme biosensor field from a strong commercial viewpoint. The current status of the technology is evaluated and future trends in this dynamic and fast-moving field are also anticipated.

Key Words: Biosensor; enzyme; electrodes; screen-printed; electrochemistry; amperometric; glucose; glucose oxidase; mediator; electrocatalyst; immobilization; membrane; bioreceptor; transducer; biomimic.

1. Introduction

For our purposes, a biosensor can be defined as a compact analytical device incorporating a biological or biologically derived sensing element either integrated within or intimately associated with a physicochemical transducer. The usual aim of such a device is to produce either a discrete or a continuous digital electronic signal that is proportional to a single analyte or a related group of analytes (*1*).

This definition allows us to identify clearly Professor Leland C. Clark Jr. as the father of the biosensor concept. In 1956, Clark published his definitive paper on the oxygen electrode, a schematic of which is shown in **Fig. 1 (2)**. Based on this experience and addressing his desire to expand the range of analytes that could be measured in the body, he made a landmark address in 1962 at a New York Academy of Sciences symposium. In this he described "how to make electrochemical sensors (pH, polarographic, potentiometric, or conductometric) more intelligent" by adding "enzyme transducers as membrane enclosed sandwiches." The concept was illustrated by an experiment in which

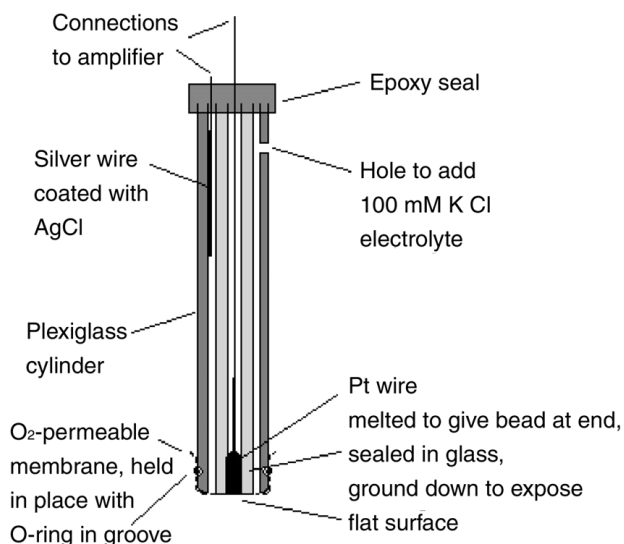


Fig. 1. Schematic of Clark oxygen electrode.

glucose oxidase was entrapped at a Clark oxygen electrode using a dialysis membrane. The decrease in measured oxygen concentration was proportional to glucose concentration. In the published paper (3), Clark and Lyons coined the term “enzyme electrode,” which many reviewers have mistakenly attributed to Updike and Hicks (4), who expanded on the experimental detail necessary to build functional enzyme electrodes for glucose.

The sensor, essentially invented by Clark, was the basis of numerous variations on the basic design, and many other (oxidase) enzymes were immobilized by various workers as a result. Indeed, Clark’s basic design was so successful that many research biosensors and at least one commercial biosensor are still produced using the original concept of oxygen measurement. However, nowadays the preferred alternative is to measure hydrogen peroxide. Most notable of the commercially available biosensors today is probably the range of biosensors sold by the Yellow Springs Instrument Company (YSI; Yellow Springs, Ohio, USA). Their glucose biosensor was successfully launched commercially in 1975 and was based on the amperometric detection of hydrogen peroxide. This was the first of many biosensor-based laboratory analyzers to be built by companies around the world.

Guilbault and Montalvo (5) were the first to detail a potentiometric enzyme electrode. They described a urea sensor based on urease immobilized at an ammonium-selective liquid membrane electrode. The use of thermal transducers for biosensors was proposed in 1974 and the new devices were christened “ther-

mal enzyme probes" (6) and "enzyme thermistors" (7), respectively. Lubbers and Opitz (8) coined the term "optode" in 1975 to describe a fiberoptic sensor with an immobilized indicator to measure carbon dioxide or oxygen. They extended the concept to make an optical biosensor for alcohol by immobilizing alcohol oxidase on the end of a fiberoptic oxygen sensor (9). Commercial optodes are now showing excellent performance for in vivo measurement of pH, pCO₂, and pO₂, but enzyme optodes are not yet widely available.

The biosensor took a further fresh evolutionary route in 1975, when Diviès (10) suggested that bacteria could be harnessed as the biological element in "microbial electrodes" for the measurement of alcohol. This paper marked the beginning of a major research effort in Japan and elsewhere into biotechnological and environmental applications of biosensors.

In 1976, Clemens et al. (11) incorporated an electrochemical glucose biosensor in a bedside artificial pancreas; this was later marketed by Miles (Elkhart, IN) as the Biostator. Although the Biostator is no longer commercially available, a new semicontinuous catheter-based blood glucose analyzer has recently been introduced by VIA Medical (San Diego, CA). In the same year, La Roche (Basel, Switzerland) introduced the Lactate Analyser LA 640, in which the soluble mediator, hexacyanoferrate, was used to shuttle electrons from lactate dehydrogenase to an electrode. Although this was not a commercial success at the time, it turned out in retrospect to be an important forerunner of a new generation of mediated biosensors and of lactate analyzers for sports and clinical applications. A major advance in the in vivo application of glucose biosensors was reported by Shichiri et al. (12), who described the first needle-type enzyme electrode for subcutaneous implantation in 1982. Companies are still pursuing this possibility, but no device for general use is available as yet.

The idea of building direct "immunosensors" by fixing antibodies to a piezoelectric or potentiometric transducer had been explored since the early 1970s, but it was a paper by Liedberg et al. (13) that was to pave the way for commercial success. They described the use of surface plasmon resonance to monitor affinity reactions in real time. The BIAcore (Pharmacia, Uppsala, Sweden) launched in 1990 is based on this technology.

It was during the 1980s, however, that large-scale commercial success was first achieved. YSI had built up a steady and thriving business, but it was not in the same league as the success that had been predicted and, indeed, widely expected. The basic problem lay largely with the cost of producing the biosensors of the time, which made them uncompetitive with the other technologies widely used in the massive rapid testing sector. It was within this sector that the hopes were pinned, since it was (and still is) a huge market.

In 1984, a much-cited paper on the use of ferrocene and its derivatives as an immobilized mediator for use with oxidoreductases was published (14). These

were crucial components in the construction of inexpensive enzyme electrodes, and formed the basis for the screen-printed enzyme electrodes launched by MediSense (Cambridge, MA) in 1987 with a pen-sized meter for home blood-glucose monitoring. The electronics have since been redesigned into popular credit-card and computer-mouse style formats, and MediSense's sales showed exponential growth, reaching \$175 million per year by 1996, when they were purchased by Abbott (Abbott Park, IL, USA). Boehringer Mannheim (now Roche Diagnostics [Basle, Switzerland]), Bayer (Fernwald, Germany), and LifeScan (Milpitas, CA, USA) now have competing mediated biosensors and the combined sales of the four companies dominate the world market for biosensors and are rapidly displacing conventional reflectance photometry technology for home diagnostics.

Academic journals now contain descriptions of a wide variety of devices exploiting enzymes, nucleic acids, cell receptors, antibodies, and intact cells, in combination with electrochemical, optical, piezoelectric, and thermometric transducers (15). Within each permutation lie myriad alternative transduction strategies and each approach can be applied to numerous analytical problems in health care (16), food and drink (17), the process industries (18), environmental monitoring (19), and defense and security.

A summary of some of the key events in the evolution of biosensors is shown in **Table 1**. As can be seen, the 1980s was a very inventive decade, with commercialization being the theme of the 1990s. There appears to be no sign of the latter theme changing in the early 21st century.

This demonstrates that, in some areas, biosensors have become a mature technology. It must be remembered, however, that this commercial success and maturity are limited to a small number of applications and that they came as a result of a great deal of research and development, and they have really taken place only where market size or share justified significant financial investment.

2. Looking into the Technology

2.1. Back to Basics

There are numerous components to any biosensor configuration. Over the years a great many combinations have been proposed and demonstrated, though far fewer have been commercial successes. A generalized schematic of a biosensor is shown in **Fig. 2**. The basic principle is to convert a biologically induced recognition event into a usable signal. In order to achieve this, a transducer is used to convert the (bio)chemical signal into an electronic one, which can be processed in some way, usually with a microprocessor.

2.2. Assembling the Picture

One of the chief attractions of biosensors is the remarkable specificity that their biological component confers on them. Enzymes are the most commonly

Table 1
Defining Events in the History of Biosensor Development

| Date | Event |
|---------|---|
| 1916 | First report on the immobilization of proteins: adsorption of invertase on activated charcoal |
| 1922 | First glass pH electrode |
| 1956 | Invention of the oxygen electrode |
| 1962 | First description of a biosensor: an amperometric enzyme electrode for glucose |
| 1969 | First potentiometric biosensor: urease immobilized on an ammonia electrode to detect urea |
| 1970 | Invention of the ion-selective field-effect transistor (ISFET) |
| 1972–75 | First commercial biosensor: Yellow Springs Instruments glucose biosensor |
| 1975 | First microbe-based biosensor First immunosensor: ovalbumin on a platinum wire Invention of the pO_2/pCO_2 optode |
| 1976 | First bedside artificial pancreas (Miles) |
| 1980 | First fiber optic pH sensor for in vivo blood gases |
| 1982 | First fiber optic-based biosensor for glucose |
| 1983 | First surface plasmon resonance (SPR) immunosensor |
| 1984 | First mediated amperometric biosensor: ferrocene used with glucose oxidase for the detection of glucose |
| 1987 | Launch of the MediSense ExacTech blood glucose biosensor |
| 1990 | Launch of the Pharmacia BIACore SPR-based biosensor system |
| 1992 | i-STAT launches hand-held blood analyzer |
| 1996 | Glucocard launched |
| 1996 | Abbott acquires MediSense for \$867 million |
| 1998 | Launch of LifeScan FastTake blood glucose biosensor |
| 1998 | Merger of Roche and Boehringer Mannheim to form Roche Diagnostics |
| 2001 | LifeScan purchases Inverness Medical's glucose testing business for \$1.3 billion |
| 2003 | i-STAT acquired by Abbott for \$392 million |
| 2004 | Abbott acquires Therasense for \$1.2 billion |

used reagents, but many other biologicals and biomimics have also been featured. These include antibodies; whole cells, including microbial, plant, and animal cells; subcellular organelles; tissue slices; lectins; and numerous synthetic molecules with affinity or catalytic properties similar to biologicals, extending to those obtained through parallel synthesis and imprinted polymers.

Since biological components offer such exquisite selectivity (and often sensitivity), why are synthetic molecules so attractive? The answer is frequently that biological reagents are often poorly stable outside of their normal environment.

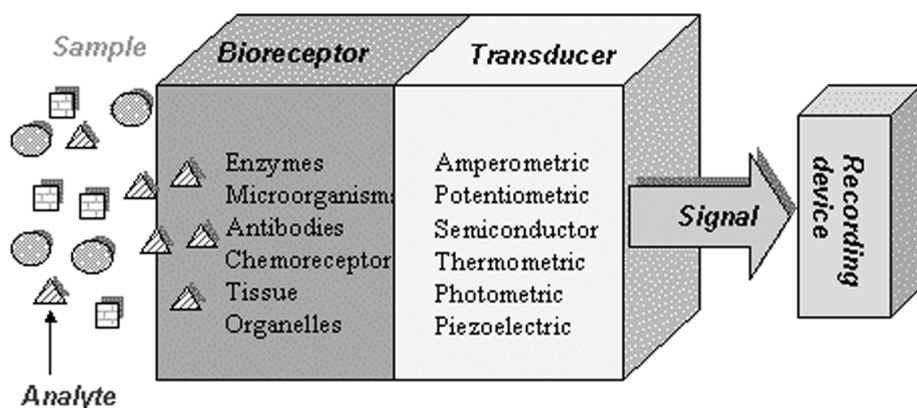


Fig. 2. Generalized schematic of a biosensor.

Table 2
Examples of Transducers Used in Biosensor Construction

| Transducer | Examples |
|-----------------|---|
| Electrochemical | Clark electrode; mediated electrodes; ion-selective electrodes (ISEs); field-effect transistor (FET)-based devices; light addressable potentiometric sensors (LAPS) |
| Optical | Photodiodes; waveguide systems; integrated optical devices |
| Piezoelectric | Quartz crystals; surface acoustic wave (SAW) devices |
| Calorimetric | Thermistor; thermopile |
| Magnetic | Bead-based devices |

Thermal stability is usually poor, resulting in short lifetimes and limited ranges of application. In addition, pH sensitivity is often troublesome, as is the need for cofactors and other reagents in some instances. The aim of the biomimicry approach is to utilize the best features of the biological reagent (sensitivity, specificity, etc.) in a more stable matrix.

There are also numerous transduction mechanisms available. Some of the more popular examples are given in **Table 2**. For further explanation of the transduction principles involved, the interested reader could refer to any of the excellent texts referred to in the **References** at the end of this report. Several shorter descriptions have also been written, including an article by Newman and Turner (20).

2.3. Integration Is Key

Biosensor technologists strive for the simplest possible solution to measurement in complex matrices. While notable success has been achieved with indi-

vidual sensors, pragmatic solutions to many problems involve the construction of a sensor system in which the carefully optimized performance of the sensor is supported by associated electronics, fluidics, and separation technology.

In process monitoring, for example, the process must remain inviolate while the sensor frequently requires protection from the process and its products. One solution is an integrated system (21) consisting of a rotary aseptic sampling system with flow-injection analysis incorporating a reusable, screen-printed electrode. In this instance, the enzyme electrode utilized glucose oxidase immobilized in a hydrophilic gel and detected hydrogen peroxide at a catalytic electrode made of rhodinized carbon. While the enzyme electrode alone exhibited enhanced stability and interference characteristics, a complete solution of the monitoring problem demanded the optimization of the whole system.

There are increasing demands for a systems-oriented approach in other sectors: environmental monitoring places demands on sensor technology that, in many cases, are unlikely to be met by isolated sensors; in clinical monitoring microdialysis offers a useful way forward for measurement *in vivo*.

The sensor/sampling system biointerface is a key target for further investigation, involving tools such as evanescent wave techniques, and atomic force and electrochemical microscopy to aid further understanding of interactions between biological molecules or their mimics and surfaces. Work on *in vivo* sensing systems for both glucose and lactate has confirmed the effectiveness of phospholipid copolymers in improving hemocompatibility. Immunosensors offer a further general example where microseparations, using, for example, immunochromatographic methods, can be coupled with electrochemical or optical detectors to yield simple dipstick-style devices combining the speed and convenience of sensors with the specificity and sensitivity of immunoassays. The advent of micromachining makes these and other techniques amenable to such a high degree of miniaturization that the distinction between sensor and analytical instrument becomes hazy.

2.4. Sensor Arrays—Looking at the Bigger Picture

The success of single-analyte sensors has been followed by the formulation of arrays of sensors to offer menus relevant to particular locations or situations. The most obvious example is in critical care, where commercially available handheld instruments provide clinicians with information on the concentration of six key analytes in blood samples and benchtop instruments on the ward can measure 16 analytes. These instruments commonly feature biosensors for glucose, lactate, urea, and creatinine.

The dual demands for detection of an increasing range of analytes and minituarization are driving biosensors toward micro- or even nanoarrays.

Thinking in this area is being stimulated by the demands of the pharmaceutical industry where high-volume, high-throughput drug screening, involving low volumes of analyte, is essential for survival. In the longer term, arrays of 1 million sensors/cm² are a realistic target. As can be seen in the later sections, photolithography, microcontact printing, and/or self-assembly techniques offer routes to high-density arrays, but laser desorption is particularly promising and offers the ability to “write” proteins to surfaces with very high resolution.

2.5. Supporting Technologies

The basic components of the biosensor, as outlined above, are only the start of the story. In order to obtain functional devices, which can be manufactured within the necessary performance and cost constraints, numerous other components and technologies are needed.

2.5.1. Membranes and Immobilization

The very first biosensors relied on membranes for their functionality. The Clark oxygen electrode contains a gas-permeable membrane, which allows oxygen to pass while excluding undesirable, interfering species. Many other membranes have been used and these have a wide variety of purposes. They may be used to retain the biological component while allowing the analyte to pass. Another useful function is their ability to extend the (linear) range of a biosensor by acting as a mass transport barrier (22).

Membranes have attracted a great deal of attention in many application areas, including process monitoring (21), food (23), environmental (24), and medical applications (25). However, they have been most prominent, at the forefront of development efforts, in the quest for improved *in vivo* devices (26). A more detailed description of membrane application in biosensors is provided by Cunningham (27).

The many approaches to immobilization of reagents on biosensors are outside the scope of this report, but the interested reader can obtain details from numerous texts (1,27,28).

2.5.2. Fabrication Techniques

It has long been realized that advanced fabrication techniques are a key to the successful development of commercially viable biosensors in many applications (29). Fortunately, many technologies have been developed for other applications, such as the microelectronics industry, and are therefore available with much greater reliability and at a much lower cost than would otherwise be the case, although they obviously require certain modifications and considerable development.

2.5.3. Screen-Printing

Screen-printing is a thick-film process that has been used for many years in artistic applications and, more recently, for the production of miniature, robust, and cheap electronic circuits. The main developments, from a biosensor viewpoint, have involved ink formulation. There is now a wide array of inks suitable for producing biosensors. Most of the applications to date have involved electrochemical devices, but the technique is applicable to the production of any planar device.

Since the technique has been developed for mass production, it is possible to produce very large numbers of reproducible, inexpensive devices at high speed. The process has been one of the major reasons for the commercial success of many biosensors and is the process by which MediSense (now Abbott) produces more than 1 billion biosensor strips annually.

2.5.4. Liquid-Handling Techniques

The ability to handle small volumes of liquids with high precision is one of the key areas in the development of some of the next generation of biosensors. As devices become smaller and more sophisticated, it becomes increasingly difficult to handle the analytical reagents involved in production. Some of the latest advances in transducer design, for example, make the production of 1 million measurement points on a 1-cm² chip a possibility. Currently, the most difficult aspect of the production of these devices is incorporating the biological reagents onto the surface of such arrays.

Ink-jet techniques (30) are suitable for depositing droplets of less than 1 nL in volume. This can be achieved at very high speeds (kHz), but the resolution of the droplets is comparatively poor. In addition, although the volume appears very small, the droplet size of 50–100 μm is relatively large compared to the size of the transducer structures that can be produced.

Other liquid-handling techniques include automated syringe-type processes, the best known of which is often referred to as Cavity deposition and usually involves “touching off” a droplet onto a surface. Another method involves picking up reagents on a “pin” that possesses a concave head and depositing it onto the surface of the device, a technique adapted from pharmaceutical applications.

2.5.5. Photolithography

Lithographic techniques are able to produce very small structures with well-defined geometries. Recent advances have enabled submicron structures to be fabricated using oblique evaporation and short-wavelength light and electron beams for fabrication of suitable masks. The lithographic process is very popular, largely due to its applications within microprocessor production.

2.6. Improving Performance

Improvements in the performance of analytical devices are a continuing theme in all areas of their application. Legislators, particularly in environmental applications, change consent levels, often based on what it is possible to detect. Clinicians demand simpler, longer-lasting, less-expensive devices with improved accuracy. The development community itself continuously pushes the boundaries of what is possible.

2.6.1. Sensitivity

Clinicians, food technologists, and environmentalists all have an interest in generally increased sensitivity and limits of detection for a range of analytes. While today's demands for precision may be modest in these respects, few would contest the longer-term benefits of reliable detection of trace amounts of various indicators, additives, or contaminants. With the advent of atomic-force microscopy we can consider single-molecule detection in the research laboratory, but great strides have also been made with conventional sensors. Enzyme electrodes have been designed that preconcentrate the analyte of interest (31).

Advances are not limited to the liquid phase. A gas-phase microbiosensor for phenol, in which polyphenol oxidase was immobilized in a glycerol gel on an interdigitated microelectrode array (32), has been reported, in which phenol vapor partitioned directly into the gel where it was oxidized to quinone. Signal amplification was enhanced by redox recycling of the quinone/catechol couple, resulting in a sensor able to measure 30 ppb phenol. Detection limits of parts per trillion volatile organic carbons are feasible with this approach.

Ultra-low detection limits are achievable with many affinity sensors and electrochemical detection may be readily integrated with chromatographic techniques to yield user-friendly devices (33), an approach that overcomes the need for multiple sample manipulation steps, which was a major drawback of many early sensors of this type. In an alternative approach, double-stranded DNA may be used as a receptor element. "Sandwich"-type biosensors based on liquid-crystalline dispersions formed from DNA-polycation complexes may find application in the determination of a range of compounds and physical factors that affect the ability of a given polycation molecule to maintain intermolecular crosslinks between neighboring DNA molecules (34). In the case of liquid-crystalline dispersions formed from DNA-protamine complexes, the lowest detectable concentration of the hydrolytic enzyme trypsin was 10^{-14} M. Elimination of the cross-links caused an increase in the distance between the DNA molecules that resulted in the appearance of an intense band in the circular dichroism spectrum and a "fingerprint" (cholesteric) texture. Work is in progress to develop mass-producible films and inexpensive instrumentation.

2.6.2. Stability

Arguably the most obvious disadvantage in exploiting the exquisite specificity and sensitivity of complex biological molecules is their inherent instability. Many strategies may be employed to restrain or modify the structure of biological receptors to enhance their longevity. One way, which has been recently demonstrated as a means of stabilization, is to use sol gels as an immobilization matrix. The effectiveness of such materials has been clearly demonstrated in, for example, an optode for glucose detection, using simultaneous fluorescence quenching of two indicators [(2,2'-bipyridyl) ruthenium(II) chloride hexahydrate and 1-hydroxypyrene-3,6,8-trisulfonic acid]. In such a format the excellent optical properties of the gel and enhanced stability of glucose oxidase are highly advantageous (35).

Some desirable activities, however, remain beyond the reach of current technology. Methane monooxygenase is one such case where, despite reports of enhanced stability in the literature, the demands of hydrocarbon detection require stability far beyond that exhibited by the enzyme. In these cases it is valuable to resort to biomimicry to retain the essence of the biocatalytic activity but to house this within a smaller and more robust structure. For example, a simple and rapid method for quantifying a range of toxic organohalides based on their electrocatalytic reaction with a metalloporphyrin catalyst has been demonstrated. This approach can be used to measure lindane and carbon tetrachloride (representative of haloalkane compounds) perchloroethylene (a typical haloalkene) 2,4D and pentachlorophenol (aromatics) and the insecticide DDT (36).

2.6.3. Selectivity

Improvement in the selectivity of biosensors may be sought at two levels: the interface between the transducer and the biological receptor may be made more exclusive, thus reducing interference, and new receptors can be developed with improved or new affinities. The use of mediators as a strategy to improve performance in amperometric biosensors has proved extremely popular and work in this area has continued to explore these possibilities. For example, it has been reported (37) that it is possible to use pyrroloquinoline quinone as a "natural" mediator, in combination with glucose oxidase, in an enzyme electrode for the measurement of sugar in drinks.

Alternatively, electrocatalytic detection of the products of enzymatic reactions may be enhanced by the use of chemically modified electrodes such as rhodinized (22) or hexacyanoferrate-modified (38) carbon. The latter method results in a Prussian blue coating on the electrode, which may then be used for amperometric detection of hydrogen peroxide at both oxidative and reductive potentials in enzyme electrodes for lactate and glucose.

Arguably, a more elegant solution is to seek connection of the redox center of an enzyme to an electrode via a molecular wire. Much has been published about so-called “wired” enzymes, but these papers have generally been concerned with immobilized mediators on various polymer backbones. An alternative approach is to use molecular wires, in their pure sense, for long-distance electron transfer effected via a single molecule with delocalized electrons. Novel heteroarene oligomers, consisting of two pyridinium groups, linked by thiophene units of variable length (thienoviologens) are promising candidates for such conducting molecular wires and may be used in conjunction with self-assembly techniques to produce an insulated electrode that transfers electrons specifically along predetermined molecular paths (39). This design should produce enzyme electrodes free from electrochemical interference.

Advances in computational techniques now allow the modeling of both electron transfer reactions and receptor binding interactions with increasing accuracy. This not only enhances understanding of the receptor/transducer interface, but also allows consideration of the design of new receptors based on biological molecules. To obtain improved binding ligands for use in an optical sensor for glycohaemoglobin (HbA1c), a novel synthetic peptide library composed of 1 million L-amino acid hexapeptides was constructed from 10 amino acids using combinatorial chemistry (40). The hexapeptide library was screened against HbA1c, HbA1b, HbAF, and HbA0 and selected ligands sequenced. Individual ligands or arrays of ligands in conjunction with pattern recognition techniques are being used to design a sensor with improved selectivity.

3. Technology Foresight

It is often difficult to predict the future in any high-technology area, as breakthroughs can happen at any time, are often not the expected, and can occur in fields that are apparently unrelated. There are, however, numerous technologies that have emerged recently, which will have an impact on the future direction of biosensors. There are also many others that will continue to evolve technologically and into new application areas. We will now examine some of these.

3.1. *An Insider's Guide to the Technology*

Academic research into biosensors continues unabated, and this has been matched by commercial development. The important issues are largely the same as they have always been. End users want devices that last forever, require no calibration, involve no sample extraction or preparation, and, above all, give results that are 100% reliable. We need hardly add that these demands are somewhat excessive. So how far along these lines has the field advanced? In order to answer this, we need to have a close look at the different application areas, as advances have varied.

Table 3
Commercially Available Glucose Biosensor Characteristics

| | LifeScan One-Touch Ultra | Roche Diagnostics Accu-Chek Advantage | Bayer Diagnostics Glucometer Elite XL | TheraSense FreeStyle | Medisense Precision Q.I.D. |
|------------------------------------|--------------------------------|--|--|-------------------------|----------------------------------|
| Alternate site testing | Yes | No | No | Yes | No |
| Sample size (μL) | 1 | 3–4 | 2 | 0.3 | 3.5 |
| Test time (s) | 5 | 40 | 30 | 15 | 20 |
| Capillary action strip | Yes | Yes | Yes | Yes | No |
| Temp. range ($^{\circ}\text{C}$) | 5–44 | 8–39 | 10–39 | 10–35 | 18–30 |
| Test memory | 150 | 100 | 120 | 250 | 10–125 |
| Data downloading | Yes | Yes | Yes | Yes | Yes |

3.1.1. Glucose Biosensors

Many of the issues associated with glucose biosensors have very little to do with the device itself. In virtually every case, the basic design of the commercially successful biosensors has not changed significantly for some time. Mediated amperometric designs similar to the original MediSense ExacTech device, which was first launched 15 yr ago, are still the norm.

The active area of the biosensor is universally smaller than it was in the past, enabling smaller samples to be analyzed. As can be seen in **Table 3**, samples need be no larger than a few microliters. Modeling of the response characteristics has also made quicker analyses possible. A result can now be obtained in just 10–15 s.

The way in which a sample is obtained is also an issue undergoing considerable research and discussion. As sample sizes have become smaller, questions have been asked about how representative the sample becomes. This is particularly true in the case of the most minimally invasive of the sampling regimes, which often measure glucose in interstitial fluid.

3.1.2. Sampling Regimes

Most of the current blood-glucose biosensors measure the glucose in a small sample of capillary blood, which is obtained using a lancet (**Fig. 3**). Even though the required samples need be no greater than a few microliters, there has been considerable effort aimed at enabling measurements to be made in other media, thus removing the need to draw blood. The process of drawing blood is inconvenient and uncomfortable, particularly for young diabetics and parents of diabetic children, who find this particularly stressful.

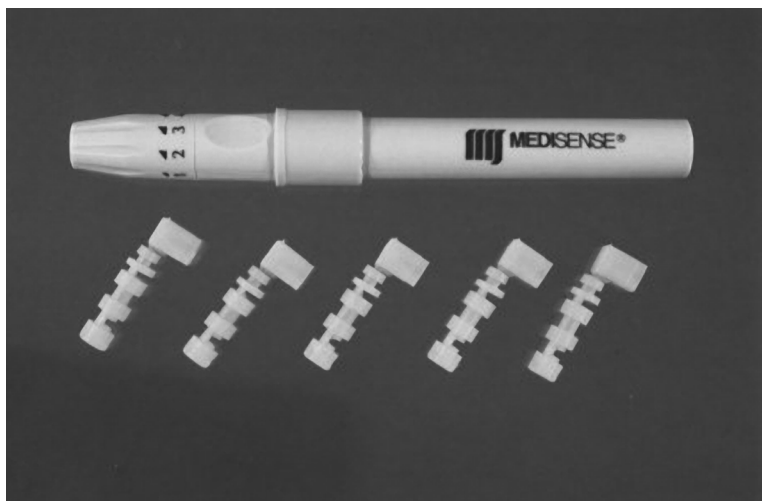


Fig. 3. Lancet for obtaining a sample of capillary blood.

The Bayer Microlet Vaculance® is suitable for non-finger use. The endcap is removed to insert a lancet and the lancet cap removed. After replacing the endcap, the puncture depth is adjusted by rotating and aligning the transparent endcap to one of four settings. The Vaculance is pressed against the puncture site and the lancet fired by completely depressing the plunger. An airtight seal is then formed by slowly releasing the plunger, which creates a vacuum. This causes the skin to bulge into the endcap, dilating the puncture and increasing the flow of blood (**Fig. 4**). The transparent endcap enables the operator to see when sufficient blood has been collected, at which point the vacuum is released by partially depressing the plunger. If this step is omitted, any residual vacuum creates aerosols.

Blood is then applied to a glucose test strip in the usual way. The used lancet is removed, recapped, and discarded. The use of the Vaculance is limited to glucose-measuring systems that aspirate (by capillary draw) small sample volumes of approx 3 μL . Bayer designed it for use with the Glucometer Esprit™ and Elite™.

Several methods have been proposed for obtaining small samples of interstitial fluid (ISF) in a painless manner. The most promising (and those with the most commercial backing) involve laser ablation, ultrasound, or reverse iontophoresis. Laser ablation uses short laser pulses to open up small pores in the epidermis, allowing a small sample of interstitial fluid to be released (**Fig. 5**). The process is painless and causes minimal damage to the skin. Ultrasound can also be used to open pores in the skin. The original application of this technology was envisaged to be opening pores to allow drug administration, but it has



Fig. 4. Bayer Microlet Vaculance®.

been demonstrated for diagnostic applications by Sontra Medical (Cambridge, MA), which has used the technology to open pores to extract a sample. Reverse iontophoresis relies on glucose being carried in an ion flow induced by electrolysis (**Fig. 6**). The flow of (mainly sodium and potassium) ions carries glucose to the surface of the skin, where it can be measured.

With all the above techniques, relatively conventional measurement technologies can be applied. Other principles are under development, however, that rely on completely different approaches, as discussed in the following sections.

There is, however, some controversy about the validity of measurements made in interstitial fluid. ISF is located in the outermost layers of the skin, above the nerve endings. Hence, withdrawal of ISF from the interstitial space

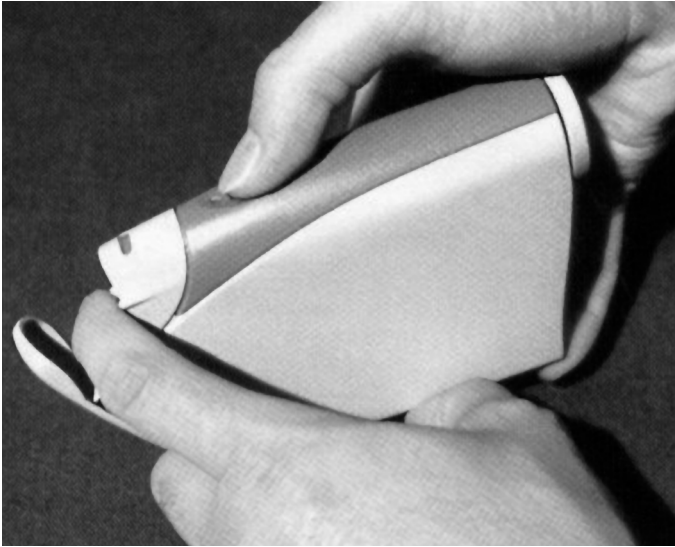


Fig. 5. Cell Robotics Lassette® laser ablation blood sampling device.

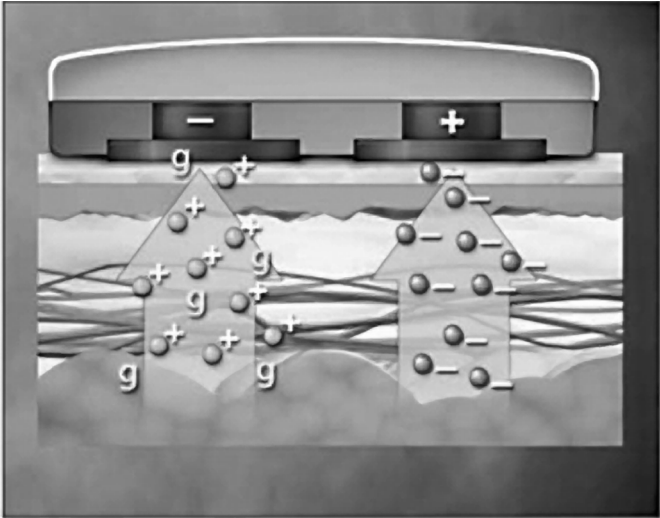


Fig. 6. Principle of reverse iontophoresis.

above the nerve endings draws no blood and, according to the manufacturers, causes little or no pain. Some research studies conclude that ISF glucose measurements are accurate for diabetes glucose testing, but others say this has not been completely proven, and more research is necessary.

David Klonoff, MD, clinical professor of medicine at the University of California, San Francisco, School of Medicine, is cautious about the use of ISF, stating: "It has not been studied sufficiently for definite answers to be known. Most people agree that interstitial fluid and blood contain essentially identical glucose concentrations, as long as the blood glucose levels are steady and not rising or falling. If the blood levels are rapidly changing, then interstitial fluid glucose levels may trail or possibly precede serum glucose levels." If this lag time is found to be significant, diabetic patients are at an increased risk of becoming hyper- or hypoglycemic without adequate warning. Companies that use ISF measurements claim that clinical studies have shown that this lag time either does not exist, or that it is rarely more than about 5 min or so, and is insignificant.

The first alternative site system to receive FDA approval was Amira's AtLast™ Blood Glucose System. Significantly, it measures blood, rather than ISF glucose. The measurement sites are on the forearm or thigh, which have a lower density of nerve endings than the fingertips. The success of this technology prompted the acquisition of the company by Roche Diagnostics in November 2001. Similar devices have followed from several other major manufacturers.

The fact that ISF-measuring products have been slow to get approval is indicative of the problems that interstitial fluid presents. Nevertheless, the area is being heavily pursued and the recent launch of the Cygnus Glucowatch® Cygnus (Redwood City, CA, USA) is a significant success.

It has also been suggested for some time that it may be possible to measure glucose in saliva, urine, or teardrops. There is some evidence that these are possible alternatives and that correlations with blood glucose levels can be established. However, the issue of a lag time between glucose concentrations in these media and those of blood is even more of a problem in each of these cases.

3.1.3. *Noninvasive Glucose Sensors*

It has long been suggested that it would be possible to measure blood glucose in a similar way to which blood oxygen can be measured using near-infrared light spectroscopy. This approach was most notably pursued by Futrex (Gaithersburg, MD, USA), for example.

Unfortunately, the glucose spectrum in this light region is diffuse and complex and falls in a region plagued by interference, making analysis difficult. Nevertheless, the approach has attracted a great deal of interest, primarily because of the opportunities presented. The method would allow continuous measurement if necessary and would be completely painless.

The measurement site has attracted a considerable amount of attention. Some of the proposed sites have included the finger, arm, and earlobe. Unfortunately, the problems encountered have been the same for virtually all these sites, as the measurement matrix in each of these is extremely heterogeneous.

One possible way of making an optical measurement, which is less problematic, is to look at the back of the eye, using a reflectance technique. The matrix in this case is much simpler and homogeneous, and the rear of the eye has a blood supply close to its inner surface (www.ieee.org/organizations/pubs/newsletters/leos/apr98/opticalsensor).

3.2. *Biomimetics*

Despite their numerous advantages, biological materials suffer from several major drawbacks. In some cases, they may be difficult to obtain and hence would be prohibitively expensive. Sometimes there may even be no available biomaterial for a particular task. Those that exist may have limited pH or temperature ranges, which may be inappropriate for the envisioned application. However, it is undoubtedly the poor stability of biological components that has led researchers to look at alternative materials for use in biosensor-type devices. The principle is based on biological inspiration and even biological building blocks (amino acids are a popular example) and chemical synthesis to provide improved materials.

In many ways this approach is not new. One of the earliest ionophores, for use in ion-selective electrodes, is valinomycin (for potassium). This material is biological in nature (it is an antibiotic) and has been in use for more than 30 yr. As far back as the 1960s, chemists have been seeking to imitate materials such as valinomycin with synthetic substitutes, which have improved function, especially stability. This early work focused on relatively simple structures, such as crown ethers, and modeling of the structure featured heavily in these efforts.

More recently, there have been a number of advances in some key technologies, which have greatly increased the efficiency of designing and fabricating potentially useful compounds for diagnostic applications. These advances have largely evolved from the increased complexity of computer-modeling algorithms and some improved manufacturing processes.

Such has been the recent interest in this area that the definition of the biosensor has been changed to incorporate biomimetics and the World Congress on Biosensors, in 2002, included a mini-Congress on biomimetics.

3.2.1. *Imprinted Polymers*

Work on molecular imprinted polymers (MIPs) began more than 25 yr ago, when Wulff and his coworkers from Dusseldorf University carried out experiments on the preparation of synthetic polymers with receptor properties for sugar derivatives. The process involved the formation of a complex between functional monomer and guest molecules (template) in an appropriate solution and the “freezing” of this complex by polymerization in the presence of a high concentration of cross-linker (**Fig. 7**). The templates were then removed by

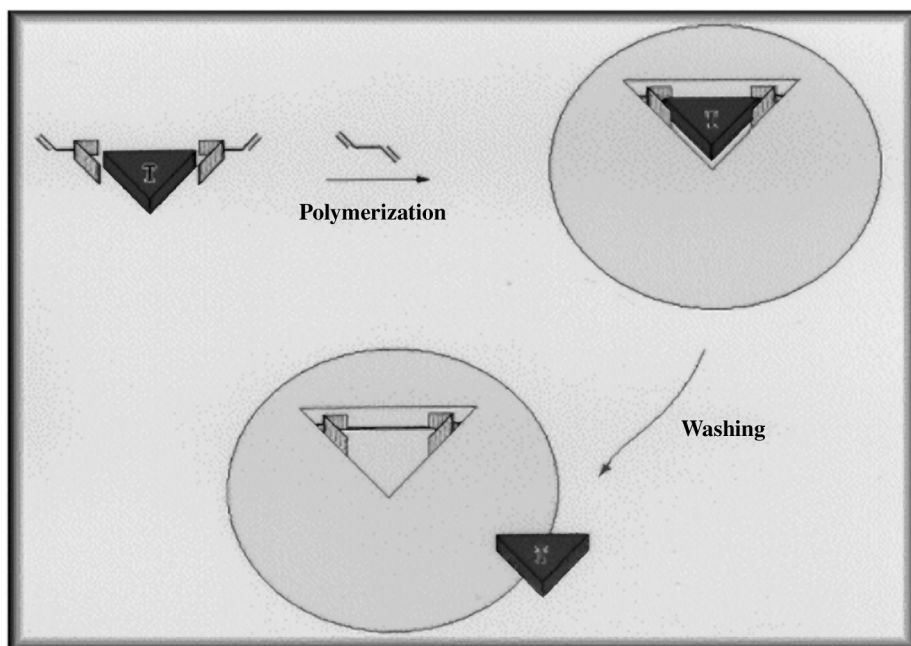


Fig. 7. Formation of imprinted polymers.

simple washing, resulting in the polymer binding sites (imprints), which are structurally specific or complementary to those of template molecules.

Over the years this method, called imprinting or template polymerization, has attracted broad interest from scientists engaged in the development of chromatographic sorbents, sensors, catalysts, enzymes, and receptor mimics. From a sensor point of view, it is interesting to obtain information about the nature of the molecular recognition phenomenon. Primarily, however, interest lies in their considerable advantages in comparison with natural receptors and enzymes, due to their superior stability, low cost, and ease of preparation.

Using such an approach, one can envisage (1) the development of assays and sensors for drugs, toxins, and environmental pollutants using artificial MIP-based receptors and (2) the development of systems for the high-throughput screening of chemical libraries on ligands possessing biological activity. In order to achieve this, work needs to be carried out on the structural analysis of imprinted polymers and computer modeling of the MIP–template interactions.

As was mentioned earlier, it is arguable whether or not devices constructed using these techniques are, indeed, biosensors. It is beyond doubt, though, that they have attracted interest within the biosensor community and, under the banner of biomimetics, they are often included as biosensors.

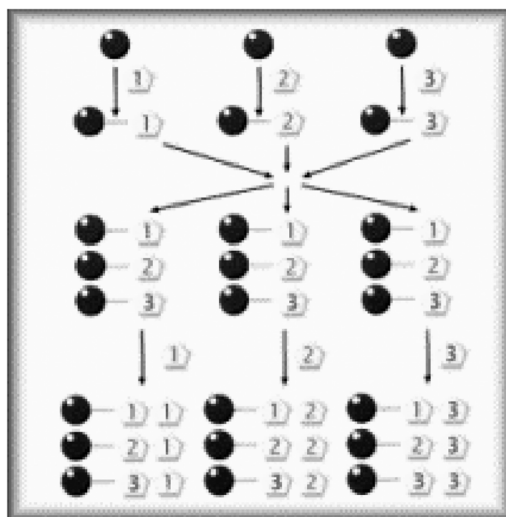


Fig. 8. Schematic of split-and-mix combinatorial synthesis.

3.2.2. Combinatorial Chemistry and Solid-Phase Organic Synthesis

Combinatorial chemistry and solid-phase organic synthesis techniques can be used to prepare libraries for the screening of novel affinity ligands for sensor applications. Current approaches involve the use of peptide libraries using natural and unnatural amino acids as building blocks. Various types of resins, such as Merrifield, Wang, Trityl, and PEGA, are used to prepare libraries using split-and-mix synthesis (**Fig. 8**). The libraries are screened and tethered onto the resin beads, and the positive results can be visualized under a microscope. When libraries are screened in solution, techniques such as surface plasmon resonance (SPR) can be used to measure the binding. Potentially useful ligands can then be structurally analyzed and subsequently resynthesized on a large scale for evaluation and kinetics study.

The use of combinatorial methods for the production of receptors for biosensors is still in its infancy. However, the technique is very widely used in drug discovery by the major pharmaceutical companies. The level of commitment to this technology for pharmaceutical applications means that advances in understanding and equipment will have natural spin-off benefits for analytical applications. However, the investments in terms of time, equipment, and personnel to obtain a ligand with the desired characteristics are not cheap and this may make the technology applicable only to biosensors with considerable market value.

Most importantly, biomimetics of all types have the potential to overcome some of the shortfalls associated with biological components, primarily poor

stability and high cost of production. The successful introduction of such materials would enable biosensors to be used in many currently difficult environments.

An alternative way of creating affinity ligands is to design them rationally using a molecular modeling approach. Furthermore, this approach can be used in parallel with combinatorial chemistry or molecularly imprinted polymers to guide the selection of building blocks and to visualize and evaluate the interactions between ligands discovered from combinatorial libraries and the target analytes.

Traditional modeling techniques could involve obtaining protein structural data from a databank and importing it into a modeling program. The next step would involve refining and correcting the protein structure to a reasonable model, followed by visualizing and defining the binding pocket of the protein. From here a designing tool could be used to generate lead ligands for organic synthesis and testing.

3.3. Molecular and Cell Biology, Proteomics, and Genomics

The above approaches are largely synthetic and it could even be argued that biology has very little part in the construction of such “biomolecules.” A truly biotechnological approach involves the use of molecular biology to engineer natural receptor molecules and to produce completely new receptors.

Successes in this area have been surprisingly scarce, but it is possible to produce antibodies and antibody fragments for unusual analytes, including mercury, which was considered too small for this approach until recently.

A large amount of information regarding the human genome and its expression will become available over the next few years. This will create new opportunities for biosensors, among other diagnostic devices. These will involve not only gene sequences but also patterns of gene expression and may even report information back from tissues *in situ*. The information, when coupled to computational interpretation, will permit new precision with regard to disease diagnosis and prognosis and we are clearly in for interesting times as we gain an understanding of the molecular basis of life itself.

3.4. Fabrication Issues

The ability to produce devices in large numbers and at a low cost is certainly a major requirement for many biosensors, particularly those aimed at self-testing markets, where one-shot use has many advantages (not least commercially, in terms of continued after-sales). It is also true that the ability to produce better-defined and smaller structures will open up new possibilities. This section looks at the advances already under way and the obstacles that need to be overcome, and then examines some of the opportunities available.

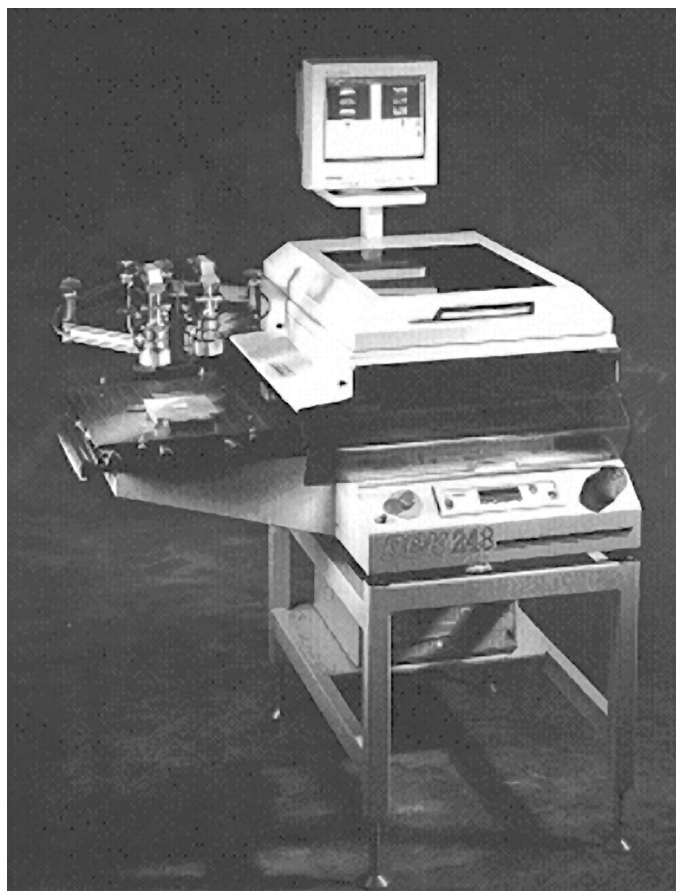


Fig. 9. DEK 248 Screen-Printer.

3.4.1. Screen-Printing Advances

As was discussed earlier, screen-printing has had a huge impact on the commercialization of biosensors. Many of the most successful electrochemically based devices to date have used the technique. A typical screen-printer (for relatively modest production requirements) is shown in **Fig. 9**.

However, the full commercial impact of the technology has been somewhat limited by the range of inks available for the construction of inexpensive devices. The main reason for this is the need for high-temperature curing with many current inks. Whereas carbon inks are widely available for low-temperature applications, those for many metals are not. Temperatures of up to 850°C are often required to cure inks employing gold and many other useful electrode

materials and, hence, costly ceramic substrates are often required. It is not impossible to produce relatively inexpensive devices using ceramic bases, but polymeric materials are cheaper and easier to process.

Among others, Gwent Electronic Materials (Pontypool, Gwent, UK) is developing a new range of low-fire materials. Using a new technology, the firing temperature of metallic-based pastes has been reduced from the normal temperatures of 400–450°C to the range of 150–350°C. This type of system means that it is now possible to fire onto polymeric rather than ceramic materials. Additionally, Gwent has found that it is possible to produce a precious-metal coating that is able to connect directly to base-metal systems at a temperature below which oxidation would normally be a problem. These modifications to the metallic film give the ability to render the surface easily solderable. It is expected that these developments will open up new opportunities for inexpensive, precious-metal-based chemical sensors and biosensors.

As a result of the intensive research and expertise acquired in this area, Palintest (Tyne and Wear, UK) has developed screen-printed sensors for the detection of lead, cadmium, and copper. These devices contain no biological components, but they are an interesting spin-off from what was originally a technology developed for biosensors.

3.4.2. Miniaturization

Miniaturization of fabricated structures is reaching the stage where it is possible to imagine devices in the future that have greater capabilities than at present, yet are far smaller. Extrapolation of the recent advances could even predict bioelectrochemical or bio-optical devices that surpass the capabilities of the human brain. Such devices have, of course, featured heavily in science fiction programs, but how close are they to reality?

The limits of what can be constructed, and what can be constructed within realistic timescales and budgets, are currently vastly different. It is possible, for example, to use various technologies to construct monolayers of reagents. Langmuir-Blodgett technology has been around for some time for achieving this. It is also possible to deposit single atoms onto a surface using atomic-force microscopy-based methods. However, in order to produce reasonable numbers of devices, we are limited by methods that are both feasible and economically viable for mass production.

Connecting tracks is one area where extreme miniaturization is important. This applies equally to electrochemical as well as optical biosensors, both of which currently offer the best scopes for miniaturization (compared to the other possible transduction mechanisms). The same problem affects both types of transduction, albeit in different ways. The drawback involves the wavelength of light.

In the case of optical transduction, this means that any confining pathway must have a smallest dimension of the same order of magnitude as the wavelength of the light being transmitted through it. This restricts the size of any structure to being of the order of a micron or so. This limitation also applies to electrochemical devices, although indirectly. In order to manufacture a mask to produce structures using vacuum evaporation, the wavelength of light, once more, comes into play, since the photolithographic technique used to produce such masks is limited by the light used. Once again, structures of many microns are the result.

Recent advances in fabrication have improved matters. It is possible to produce submicron tracks using oblique evaporation, for example. In this case, the tracks are produced using a shadowing effect behind a suitably engineered structure. The obvious method, however, is to use shorter wavelengths of light or electron beams. The microprocessor industry has pioneered advances in this area and at the time of writing, chips with track dimensions of 0.125 μm are in production. These advances are available for biosensor applications when required. However, the cost of adapting the technology, at present, would be considerable. As with all new technologies, one must consider the advantages, which must outweigh the costs. At the moment, it is doubtful whether it is worth pursuing this further, as there are many other problems, which are more important, to be tackled—as we will see later in this report.

3.4.3. Liquid Handling

In order to produce arrays of sensors, reagents need to be handled and deposited onto the correct part of the array. For large arrays, this means that many different liquids need to be deposited at high speed. This is not simple to do in a reliable way. There are many ways of tackling this problem and many companies have become involved in producing deposition equipment. It should be stated that the driving force behind this comes from the high-throughput screening programs within drug discovery businesses. The issues are broadly the same and, indeed, biosensors are one solution to the problems faced within this industry.

The Packard Instrument Company (Meriden, CT), for example, now offers a complete turnkey solution to microarray manufacture and analysis that enables the production and interrogation of DNA chips. The BioChip Arrayer combines PiezoTip™ technology with a robotic arm and stage, with real-time pressure sensing. The PiezoTip, which is licensed from, and was co-developed with, Microdrop GmbH, allows deposition of droplets with a volume of approximately 325 pL. The high resolution X-Y-Z stage allows positioning of the droplets to within a 200- μm spacing (center-to-center), depending on the dried drop size. Another company, Biodot (Huntingdon, UK), produces many different droplet deposition machines based on continuous ink-jet and other related

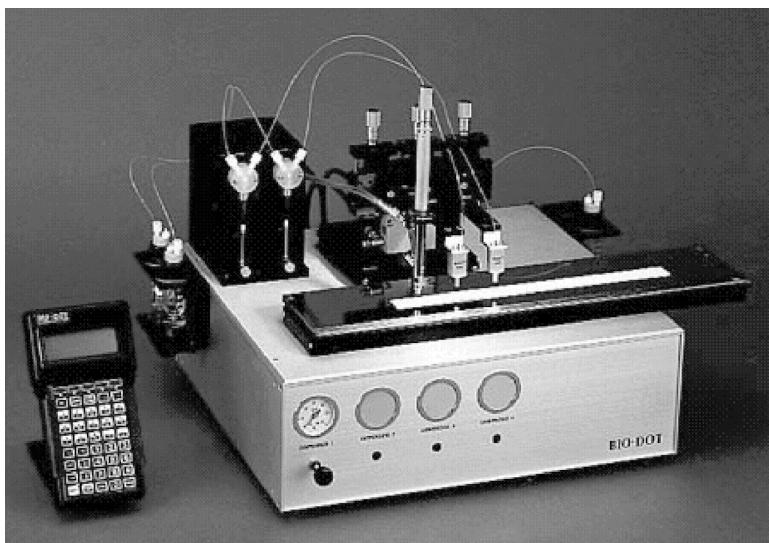


Fig. 10. Biodot Multi-Head Deposition Instrument.

technologies as well as Cavity deposition and “pin” technologies, some of which are illustrated in **Fig. 10**.

A satisfactory solution to the problem of liquid handling will revolutionize the multisensor array field, since production costs and time will be lowered and production volumes raised.

For microsensor arrays, it would also be highly desirable to reduce the volume size still further. It may sound as though 325 pL is a very small volume, but the dimensions of such a droplet are still relatively large, since the sphere will be more than 80 μm in diameter and will spread a little as it is deposited (compared to the submicron structures that can be produced using cutting-edge lithographic processes). The problems of handling such small droplets are significant and complex, but it is predicted that improvements should be possible to the required levels.

3.5. Selective Membranes

Membranes have been a common feature of biosensors since their inception. Clark's original devices were based on an oxygen-permeable membrane, for example. They are incorporated into biosensors for numerous reasons, including exclusion of interferents (size, charge), immobilization of biological components, and extension of (linear) range of sensor.

Advances in conventional membrane design have been ongoing for some years and will probably continue for the foreseeable future. An exciting prospect

is the ability to be able to control the membrane porosity or even charge distribution at will. Some of the more promising work in this area suggests that such membranes are a very real possibility and, since they are activated by nothing more complicated than the application of a voltage across the membrane, there is no reason why they could not be manufactured using relatively conventional technology, yet offer the potential of biosensors that can be made to make different responses, depending on environmental conditions.

3.6. Arrays, Computational Intelligence, Noses, and Tongues

The aim of using computational intelligence in conjunction with biosensors (or any other sensors) is to apply computational methods for solving analytical problems, which are complex and problematic using other approaches. It is possible to apply a wide variety of techniques including chemometrics, neural networks, mathematical modeling, expert systems, and data mining to an equally wide range of problems in the fields of data analysis, knowledge discovery, and bioinformatics.

In the field of instrumental data analysis, chemometric techniques such as principal components analysis (PCA), multilinear regression (MLR), time-series analysis, and artificial neural networks (ANNs) are used to extract useful information from analytical responses. These techniques are regularly applied, for example, to various spectra, electrochemical voltammograms, and sensor array responses. For maximum impact, these activities need to be augmented by the development of methods for the effective visualization of multidimensional data produced by such techniques.

An example of a technology that utilizes these methods is the DiagNose instrument (**Fig. 11**) developed by Cranfield University (Bedfordshire, UK). This consists of an electronic “nose,” which detects disease states through their characteristic odor. The technology will dramatically reduce the diagnosis time for urinary tract infections (UTIs), which are the initial disease targets. Current tests take between 24 and 48 h and are sent away to a laboratory. DiagNose returns results in a matter of hours and could be installed in most surgeries. The new test can also be done for a fraction of the present cost of UTI diagnosis. The test works by sniffing out characteristic odors of infecting microorganisms from a patient’s urine when it is mixed with a specially engineered growth medium. As the microorganisms multiply, they produce odors that give their presence away. Each microorganism produces a different odor, allowing DiagNose to determine the underlying infection so that correct treatment can be given. Furthermore, by detecting the effect of antibiotics added to the medium on the level of bacterial growth, the technology has the potential to provide information on antibiotic resistance and hence determine the most effective therapy.



Fig. 11. DiagNose instrument.

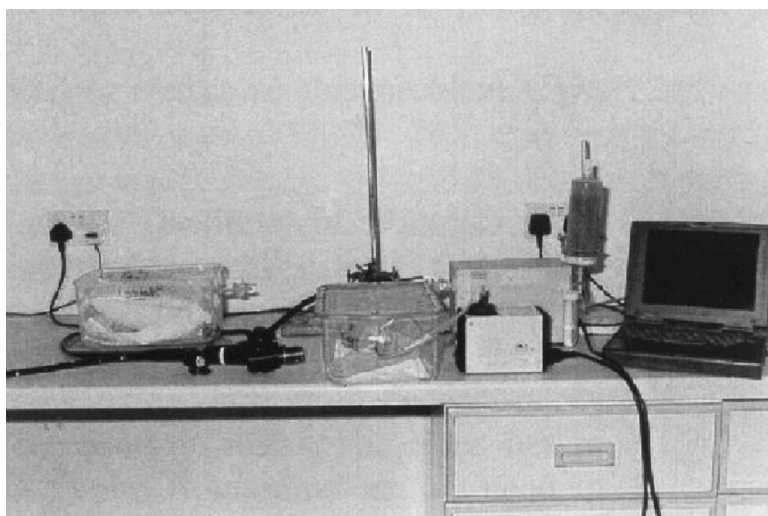


Fig. 12. Sniffing endoscope.

Laboratory trials for the new test have proved very successful, and 80% of UTIs can already be detected. Clinical trials starting shortly will give a full evaluation of DiagNose and diagnostic companies are currently being sought to bring the technology to the market. The Sniffing Endoscope (**Fig. 12**) is a related technology.

Mathematical modeling and analysis of large databases of analytical responses can harness the information present in analytical data to add power to analytical techniques. This can yield a superior understanding of the mechanism of measurement, thereby accelerating the development of new analytical systems, particularly where transducer arrays or complex sample matrices are involved. In addition, new analytical areas open where, previously, complex separation and detection systems were once required. Techniques employed in this area include analysis of variance (ANOVA) and Bayesian belief networks.

All of the above techniques promise advanced sensing possibilities based on “fingerprinting-type” approaches. There is, however, another advantage in using multiple-sensor arrays. It is possible with such technologies to incorporate self-checking capabilities and to have some built-in redundancy. These approaches help to overcome the problems associated with the lability of biological components and concomitant decay in the sensor signal, which is often apparent with such devices.

3.7. Instrumentation Simplification

One of the drawbacks of biosensors compared with other diagnostic techniques is, very often, the need for relatively expensive instrumentation. This is particularly true when they are compared with the various dipstick technologies, which rely on color changes or the appearance of bands, indicating the presence or absence of a particular material. It is often true that such devices are either not quantitative, or only semiquantitative, but the cost differential between them and biosensors can be prohibitive.

There are several technologies that have attempted to overcome this problem. Two examples are shown below.

Holographic biosensors have been developed at the Institute of Biotechnology (University of Cambridge, UK) and are the subject of several patents and patent applications. Academic research to date has centered on the marriage of holography with new polymeric thin films. Data on their performance have been obtained through monitoring the optical properties of the holograms using custom-made laboratory reflection spectrometers. The future of holographic sensors in real applications lies in their use as directly visible image holograms. These “smart holograms” do not need an electronic instrument, as they are a virtual instrument. Holographic sensors are test strips that provide a changing optical image (color, alphanumerics, messages) as the test result. A reflection hologram provides an image when it is illuminated by white light. The image is stored in a thin polymer film using patented photosensitizing technology. The polymer film is also chemically sensitized to react with a

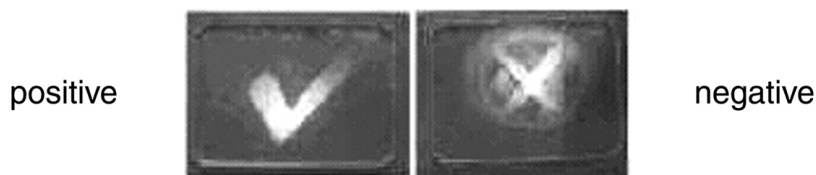


Fig. 13. Holographic sensor display.

substance in, for example, a sample of bodily fluid. During the test, the target substance reacts with the polymer, leading to an alteration in the image displayed by the hologram as shown in **Fig. 13**. In order to take commercial advantage of the opportunities offered by this technique, a spin-off company, Holometrica Limited (Cambridge, UK), has been formed to develop and manufacture sensitive holographic devices for the consumer health care and environment-monitoring markets. The first product is to be aimed at replacing the current alcohol breath test.

In a sensor technology that was developed by researchers at the Center for Fluorescence Spectroscopy at the University of Maryland School of Medicine (Baltimore, MD), only the human eye is needed to detect the sensor response. It consists of a fluorescent probe and an oriented fluorescent film. The probe responds to the analyte while the film does not. Incident light fluoresces the transparent film and is polarized. The same light that fluoresces the film then does the same to the sample. In this case, the fluorescent light is not polarized. At this point the filter removes the incident light and selects only the wavelengths needed for analysis. This light, with the appropriate wavelengths, is sent through a dual polarizer, which polarizes half the light vertically and the other half horizontally. An adjustable polarizer is rotated manually so that both sides of the dual polarizer are of equal brightness. By taking into account the angle of rotation and relating it to a calibration curve, the concentration of the analyte can be measured. Early applications of the technology include glucose, pH, oxygen, and calcium measurement, but the technique is generic.

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