Biosensors and flow injection analysis

Chien-Yuan Chen and Isao Karube

National Taiwan University, Tapei, Taiwan and University of Tokyo, Tokyo, Japan

Combining flow injection analysis with a biosensor is a novel biosensing process which has allowed speedy and accurate analysis. Diagnostic analysis is the most important application for biosensing flow injection analysis, but other applications include bioprocess monitoring, analysis of food and agricultural products, as well as environmental analysis. In addition, the analysis of compounds, such as explosives and abused drugs, and monitoring of *Salmonella*, the microorganism that causes food poisoning, have been reported.

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Introduction

It is difficult to give an exact definition of a biosensor. Widely speaking, a biosensor can be considered as an analytical device that responds to biological substances selectively and reversibly. According to this definition, any system that can be used to analyse a biological material can be classified as a biosensor. This definition, however, would include almost all physical and chemical sensors and is therefore obviously too wide. Thus, a more generally acceptable definition would be analytical devices that use a biological material as the sensing element.

Three elements are required for the construction of a biosensor (shown in Fig. 1): a recognition element composed of biological materials that react selectively with the specified substrate; a conversion element for converting the related information from the bio-catalytic reaction

into electrical signals; and an element for recording these electrical signals.

The sensing elements used in biosensors can be generally classified into four groups: proteins, organelles, cells and tissues. Biosensors themselves can be divided into several groups according to the biological materials used or the reaction type involved, and a list is shown in Fig. 2. On the other hand, conversion elements used in biosensors employ most methods used in the fields of physical and chemical analysis. Spectrophotometry, amperometry, potentiometry, thermometry, fluorimetry and resonance luminescence are widely adopted for this purpose.

The use of biosensors started in 1962 when Clark and Lyons [1] combined an oxygen probe with glucose oxidase to determine glucose levels. The fact that biosensors still attract a lot of interest three decades after they were

Fig.1. General biosensor construction and function. Chemical substances, heat, light, sound and electrical signals are produced during bio-catalyzed reactions in the bio-functional membrane. These are converted into electrical signal which are then detected by electronic devices.

Abbreviations

FIA—flow injection analysis; G-6-P—glucose-6-phosphate; TNT—trinitrotoluene.

Fig. 2. Classification of biosensors according to the sensing elements used. Proteins, organelles, cells and tissues are major biomaterials used for constructing biosensors.

first developed demonstrates their importance in many fields of analysis.

Although the technique of flow injection analysis (FIA) is not new, the combination of FIA with a detection device is novel in many analytical systems. There are still many studies concerned with the theory or operation of FIA. Some have tried to devise good methods for calibrating data and reducing interference [2]. Others have tried to establish mathematical models [3,4], and still others have carried out error analysis of signals and then tried to establish a more reasonable operation process [5]. All of these have improved FIA to some extent. A biosensing FIA system is constructed by combining a biosensor with an FIA system. Most of the biological materials used as recognition elements in biosensors have the disadvantage of losing their activity when sterilized by heat or chemical treatment. However, a biosensor does not need to be sterilized if the sample is not returned to its source. Another problem is the decrease in response intensity if the biological material being used is kept in contact with its substrate for a long period of time. This can be overcome by using an FIA system to minimize the contact period between a biosensor and its substrate and to rinse the biological material with the carrying solution. In addition, it is possible to obtain almost-real-time data even though the sensor is not directly connected with the system under investigation, for example a fermenter. Other merits of a biosensing FIA system include the possibility of on-line calibration of the sensor and the manipulation of the sample before contact with the sensor.

Glucose determination for clinical analysis

Glucose determination is the most widely studied application of biosensors. This is partly because the high stability and satisfactory specificity of the glucose-related enzymes, including glucose oxidase and glucose dehydrogenase, make it quite easy to get good results. The major

reason, however, is the applicability of glucose sensors to many fields, especially clinical analysis.

Glucose sensors have been studied for some decades and over this time have been much improved. Amperometric glucose enzyme sensors can be divided into three generations according to the electrodes and mediators used. The first generation sensors (Fig. 3) detect hydrogen peroxide formation or oxygen consumption in the reaction. The second generation sensors use a mediator to transfer electrons between the enzyme and the electrode and the third have an electrode made from a special material which allows direct transfer of electrons to it, from the enzyme. Conductive organic complex salts, for example Fulvadene-tetracyanoquinodimethane, have been used as electrode materials for this purpose. A recent study compared these three generations of sensor combined with FIA in determining glucose concentration in undiluted whole blood [6•]. The second generation sensors seem to be better than the others, although the problem of mediator leakage from the electrode must be taken into consideration because most of the mediators used are small molecules that are difficult to immobilize together with enzyme.

Glucose sensors might also be improved by altering the materials and methods used for enzyme immobilization. Nylon has been used as the insoluble carrier for glucose oxidase immobilization and the effects of various spacers have been investigated in order to improve the efficiency of enzyme immobilization [7]. A nylon membrane with enzymes immobilized using spacers was combined with an FIA system and used to determine the concentration of glucose in blood serum [8]. Immobilization of enzymes in electropolymerized polypyrrole film on the surface of a platinum electrode was reported to provide a convenient sensor for flow injection glucose analysis [9•]. The technique of electropolymerization has also been applied to prevent interference and electrode fouling [10]. If, however, whole blood instead of serum is used as the sample, the influence of the blood cells should be taken into account. This problem can be solved by improv-

Fig. 3. A first generation enzymatic glucose sensor showing sensing and conversion elements. Hydrogen peroxide is generated from glucose and oxygen through the action of glucose oxidase immobilized to a membrane. This hydrogen peroxide reacts at the anode to produce two hydrogen ions, one oxygen atom and two electrons. The electrons are measured by an ammeter (A).

ing the operation of the FIA system, for example, by equipping it with on-line dialysis and segmented sample injection [11•]. A similar study used segmented sample injection, membrane-deposited reagent and membranes of low permeability. This system was used to determine L-lactate and creatinine in addition to glucose in whole blood samples [12]. Other studies have tried to eliminate interference [13] or to evaluate the amperometric determination of glucose in blood serum or whole blood [14].

As a result of these studies, application of biosensing FIA systems in the determination of glucose concentration for clinical purposes seems very attractive. The enzyme system can be immobilized, packed as a column and connected to the FIA system at a point before the sample passes through the detector, or immobilized directly onto the working surface of an electrode. In both cases, the reusability of the immobilized enzyme system effectively reduces operation costs by decreasing the consumption of enzyme. Other merits are the fast response time and the ability to alter the sample solution to meet the conditions optimal for the enzyme system so that longer operation periods are possible.

Other clinical applications

Analysis systems composed of a biosensor and an FIA system have many other applications in clinical analysis. The measurement of serum cholesterol, believed to be an important factor in coronary heart disease, has become a routine diagnostic analysis. An FIA system for determining serum cholesterol was designed using immobilized cholesterol esterase and cholesterol oxidase [15]. An anion exchange resin was used to remove materials causing interference prior to analysis in order to increase accuracy and precision. The accuracy of the results conformed to the guidelines of the national cholesterol education program of Canada.

An increase in the activity of serum creatine kinase is a valuable indicator for some health disorders including myocardial infarction, cerebral diseases, Duchenne's muscular dystrophy, Reye's syndrome and carbon mono-

xide poisoning. This enzyme catalyzes a reaction that transfers the phosphate group of creatine phosphate to ADP. The ATP produced in this reaction can be used in the conversion of glucose into glucose-6-phosphate (G-6-P), catalyzed by hexokinase. The G-6-P is further converted to gluconolactone-6-P by G-6-P dehydrogenase and the coenzyme NADP. The reduced form of NADP (NADPH) produced in this reaction can then be determined spectrophotometrically or spectrofluorimetrically. In accordance with this reaction sequence, an immobilized enzyme column containing hexokinase and G-6-P dehydrogenase, in conjunction with an FIA system, was used to determine the activity of creatine kinase in serum samples [16•]. The activity of two other enzymes that are valuable indicators of health, glutamate pyruvate aminotransferase [17] and lactate dehydrogenase [18], can also be determined using procedures that incorporate enzymatic reactions in series.

In most cases, the quantification of FIA operation is usually calculated by a univariate calibration based on the height or the area of the peak. For very complex biological samples with many interfering materials, however, a multivariate calibration system is more convenient. A photodiode array detector was applied to produce such a multivariate signal and this system was used to determine teniposide, an antitumor agent, in blood serum [19].

The components of urine can also be determined by a biosensing FIA system. Oxalate, a marker of calculosis, has been determined by a kinetic-enzymatic procedure [20]. The enzymes used in this analysis include oxalate decarboxylase and formate dehydrogenase; the former catalyzes the decarboxylation reaction of oxalate to produce formic acid, which is then converted to carboxylic acid by the latter. NADH is formed in this reaction and monitored spectrophotometrically at 340 nm.

Although NADH itself is detectable by photometric or fluorimetric methods, the application of NADH oxidase to convert NADH into NAD^+ and produce hydrogen peroxide in the same reaction has been reported as a more sensitive way of determining NADH [21]. Higher sensitivity was obtained by mixing the hydrogen peroxide with luminol and potassium ferricyanide and measuring the emitted chemiluminescence with a photon counter. Us-

ing this method, the detection limit of NADH is 10 pmol. It is possible to link this system to any NAD-dependent reaction and it has been used to determine magnesium in human serum by linking it to hexokinase and G-6-P dehydrogenase catalyzed reactions.

Techniques for determining several substrates simultaneously have also been investigated by using an arrangement of several immobilized enzyme columns in an FIA system. Glucose, ethanol and lactate in serum samples were measured simultaneously by a parallel configuration of three columns containing immobilized glucose oxidase, alcohol oxidase and lactate oxidase [22•]. A 16-way switching valve was designed and adopted as the sample injection port for this system. An ascorbate oxidase column was used to pretreat the sample and a uricase column was connected before the immobilized enzyme columns to eliminate interference from ascorbic acid and uric acid. A schematic diagram of the multichannel FIA system is shown in Fig. 4.

Other clinically interesting analyses have been performed such as the determination of the concentration of bile acids in serum, which has been measured by a fluorimetric enzymatic FIA system [23] and a combination of FIA and liquid chromatographic processes [24]. Amino acids in serum have also been determined: L-alanine was measured using a packed-bed enzyme reactor comprising alanine dehydrogenase immobilized on poly(vinyl alcohol) beads [25]. Trace amounts of thiocyanate in saliva samples were measured by a kinetics spectrophotometric assay of a flow injection system [26].

Determination of drug components

Bio-field-effect transistors combined with an FIA system have been used to determine penicillin G, penicillin V and cephalosporin C concentrations during a fermentation process [27] and gave results that matched well with off-line liquid chromatographic data. This was achieved by immobilizing penicillin G amidase, penicillinase and

cephalosporinase onto the gate of a pH-sensitive field-effect transistor. These bio-field-effect transistors for penicillin G, penicillin V and cephalosporin C are usable for up to 3 months, 4 months and 1–2 days, respectively.

Other drugs including tetracycline, chlortetracycline, oxytetracycline, doxycycline and methacycline have been determined by FIA with a spectrophotometric detector [28] or a flow-through sensor [29]. The operation rate of the latter was emphasized as one hundred samples per hour. Berberine and benzethonium in drugs have been determined spectrophotometrically by batchwise and flow injection methods after a solvent extraction process [30].

Applications in fermentation processes, the food industry and agricultural products

Fermentation monitoring, as discussed further by Raju and Cooney (this issue, pp 40–44), is an essential part of bioprocess control. A schematic diagram for connecting fermenters with an FIA system is illustrated in Fig. 5. In this diagram, a recycling sampling system composed of a pump and a filter is shown. This design is suitable for a small jar-fermenter only. For a big fermenter for large-scale production (e.g. 300 M³ ton capacity) a sampling rate of a few milliliters every minute makes no difference to the final volume. Therefore, there is no reason to take the risk of contamination by recycling the sample solution. The dialyzer may be unnecessary if the flow system is designed to flush the surface of the sensor effectively or if the surface of the sensor is covered by materials that effectively obviate the adhesion of components of the medium.

Glucose is still the major compound of interest in the field of fermentation. A chemically modified graphite electrode with immobilized glucose dehydrogenase was used as the detector in an FIA system to determine glucose concentration in wine production [31•]. This system was reportedly used for 3 days of continuous operation with

Fig. 4. Schematic diagram of a multichannel flow injection analysis system. I, lactate reactor; II, glucose reactor; III, ethanol reactor; IV, urate-eliminating reactors; V, ascorbate-eliminating reactor. Buffer A, phosphate (0.1 M, pH 8.0); buffers B and C, phosphate (0.1 M, pH 7.0).

Fig. 5. Incorporation of a fermenter with a flow injection analysis system.

no interference from alcohol observed. Another FIA system using a double injection process to measure glucose in a fermentation broth has also been reported [32]. The sample solution and enzyme solution were injected individually into a carrier stream. The hydrogen peroxide produced after passing a reaction coil was measured amperometrically.

Amino acids are another important class of compounds in fermentation. L-lysine is an important essential amino acid. Commercially, it is mainly produced by fermentation and used as an additive in fodder. The concentration of L-lysine in fermentation broths and fodder concentrate was determined with high specificity by a combination of an immobilized L-lysine monooxygenase column, a dissolved oxygen probe and an FIA system [33]. L-lysine was also measured using the same FIA and immobilized enzyme column, but with a different detection system: the hydrogen peroxide produced reacted with peroxidase, phenol and 4-amino antipyrine to produce a red quinoneimine dye which was detected spectrophotometrically at 500 nm [34]. L-glutamic acid, an important amino acid used to prepare monosodium L-glutamate, which is widely used in the food industry was measured, was by an amplification system based on substrate recycling [35••]. L-glutamate was converted to 2-oxoglutarate by L-glutamate oxidase. The glutarate produced was subsequently converted back to L-glutamate by glutamate

pyruvate aminotransferase. Using this method, sensitivity is increased 20–30-fold and concentrations of L-glutamate as low as 1 picomolar could be detected. L-alanine in serum and beverages can be determined by FIA with a fluorimetric detector [25]. Some other amino acids in cheese have been determined chemiluminescently by FIA [36].

Aspartame is an important artificial sweetener composed of aspartic acid and phenylalanine. The concentration of this dipeptide can be determined by FIA using two enzyme columns containing peptidase and aspartate aminotransferase, respectively, and an enzyme sensor with immobilized L-glutamate oxidase [37]. Aspartame is hydrolyzed into aspartic acid and phenylalanine by the peptidase. Aspartic acid is then converted into glutamic acid by aspartate aminotransferase and the product, L-glutamic acid, can then be determined by the glutamate sensor.

L-ascorbic acid is also an analyte that has attracted much attention. L-ascorbate oxidase converts ascorbate into dehydroascorbate. The hydrogen peroxide produced at the same time is detectable using a hydrogen-peroxide-sensing electrode. Ascorbate itself, however, is electroactive at the potential used to detect hydrogen peroxide. A system for ascorbate determination using FIA, an immobilized L-ascorbate oxidase column and a blank blank column with no enzyme was designed. On passing through the enzyme column some of the L-ascorbic acid was con-

verted into the dehydro form and the decrease in response (measured against the blank column) was used to calculate the concentration of ascorbate [38]. Another system using cucumber juice as the carrier solution has also been used to determine ascorbate [39]. In still another system, measurement of the total ascorbic acid concentration has been reported using kinetic fluorimetric FIA and two serial injection valves [40].

Many carbohydrates have also been determined by FIA coupled to a biosensing system. In addition to fermentation broths (described above, [31•]), systems for measuring the concentration of glucose in milk [41] and alcoholic beverages [22•] have also been described. In contrast with glucose oxidase, which oxidizes the C-1 hydroxyl group of β -D-glucose, pyranose oxidase oxidizes the C-2 hydroxyl group of the pyranose ring in hexoses and pentoses. This enzyme has been used in the determination of monosaccharides including glucose, xylose and galactose produced during the hydrolysis of cellulosic materials [42•]. This system can give a relative measurement of the total pyranose concentration in a sample of crude broth or an absolute value of an individual substrate in a sample without competing analytes. In another study, the determination of starch by FIA and a multi-enzyme system was reported [43•]. Amyloglucosidase, mutarotase and glucose oxidase were co-immobilized on a nylon membrane and bound to a platinum electrode. The concentration of starch in solution, when pretreated with α -amylase at room temperature for one hour, was determined by measuring the hydrogen peroxide produced. In one recent report, an immobilized mannitol dehydrogenase column combined with FIA was used to detect D-mannitol (e.g. in celery and chewing gum) [44] and the NADH produced in this enzymatic reaction was measured fluorimetrically.

Applications in environmental protection

Environmental protection has become the focus of much attention in recent years. FIA systems equipped with biosensors have been used to measure indicators of environmental pollution, such as pesticides and herbicides. Paraoxon, an organophosphorus insecticide with a wide range of activity that is very toxic to mammals, was determined by both continuous flow and stopped flow systems in combination with an immobilized cholinesterase column. As an organophosphorus compound, paraoxon can form a stable complex with cholinesterase and inhibit its activity. Paraoxon determinations were made at a rate of 60 per hour [45•] on the basis of its ability to inhibit enzymatic hydrolysis of α -naphthyl acetate and the subsequent reaction of the product, α -naphthol, with p-nitro-benzenediazonium fluoroborate.

The concentration of phosphate in water is considered to be another important indicator of pollution. An amperometric FIA system with an immobilized enzyme reactor has been designed for determining phosphate in water samples [46•]. In the enzyme reactor, an amplification system for substrate recycling using purine nucleoside

phosphorylase and alkaline phosphatase is co-immobilized along with xanthine oxidase. This amplification system increases sensitivity up to 12-fold.

Applications to microbiological and enzymatic reactions

The activities of many enzymes have been determined using biosensing FIA systems, often by incorporating additional enzymes to convert the product formed by analyte catalysis into a detectable material. Two examples of this, as mentioned above, are glutamate pyruvate transaminase (an indicative enzyme of liver function) [17] and lactate dehydrogenase. The activity of lactate dehydrogenase can be determined using an immobilized pyruvate oxidase membrane on an oxygen probe in an FIA system [47]. Another system that can determine the activities of lactate dehydrogenase and glutamate pyruvate transaminase simultaneously was developed by the same group and is based on measuring the pyruvate produced with pyruvate oxidase immobilized on an oxygen probe [48]. Guanase activity has been determined by measuring the hydrogen peroxide produced in the analyte-catalyzed reaction in conjunction with xanthine oxidase and uricase [49]. The FIA system used consisted of series-injection, stopped-flow and merging-zone methods. Another multi-enzyme FIA system, for determining the activity of creatine kinase in serum samples by photometric and fluorimetric detection has also been reported [50].

Microorganisms, bioreactions of microorganisms and cell components can also be monitored using biosensing FIA systems. The presence of *Salmonella* in food preparations causes severe food poisoning. To check for *Salmonella* in food products by the traditional method is time consuming, costly and often difficult because it is only present at very low concentrations. An FIA immunoassay system has been developed [51•] for the purpose of detecting microorganisms belonging to this genus. A sandwich system composed of antibody, microorganism and glucose oxidase labeled antibody was constructed by immobilizing the antibody onto the inner wall of a Tygon tube through which the sample solution and enzyme labeled antibody solution were pumped in succession. Finally, a glucose solution was pumped through the tube and hydrogen peroxide determined electrochemically.

Another system, combining FIA with a membrane introduction tandem mass spectrometric system, was used to monitor liquid and gas components during the production of 2,3-butanediol by *Bacillus polymyxa* or *Klebsiella oxytoca* in these microorganisms [52•]. Although this study did not use a biosensor for measuring the analytes, we have included it because it was used to monitor a fermentation process.

An FIA system incorporating a fibre-optic biosensor to determine ATP and NADH has been designed [53•]. Three enzymes, firefly luciferase, bacterial oxidoreductase and bacterial luciferase are co-immobilized onto a polyamide membrane. The first enzyme is used to measure ATP and the other two enzymes to measure NADH.

By changing the reagents, ATP or NADH can be determined with the same sensing element. The activity of enzymes that produce ATP or NADH in their catalyzing reactions, for example kinase and dehydrogenase, can be determined by this system.

Other applications

A flow-injection biosensing system has been constructed using an immobilized alkaline phosphatase column, a planar pH-sensitive field effect transistor and a flow injection system. This system was used to determine the concentration of zinc(II) ions by activation of the immobilized cofactor-free apoenzyme in the column [54•]. A chelating agent, 2,6-pyridine dicarboxylate, was injected between successive samples to regenerate the column.

Two immuno-FIA systems have been developed for determining methamphetamine [55•], a drug that is often abused, and trinitrotoluene (TNT) [56], an organic explosive. The latter involves mixing TNT with anti-TNT and determining the decrease of fluorescence of anti-TNT after mixing. The former incorporates immobilized albumin conjugated to methamphetamine on the surface of an AT-cut quartz crystal. The decrease of the resonant frequency of the crystal after contacting a solution of methamphetamine and monoclonal antibody against methamphetamine is measured, with the frequency decrease correlating with the concentration of anti-methamphetamine in the flowing solution. At a fixed concentration of anti-methamphetamine, the frequency decrease was found to be attenuated by the concentration of methamphetamine that was present in the solution. Concentrations of methamphetamine as low as 0.02 ppm can be detected in about 5 min.

Conclusion

The papers cited in this review demonstrate the wide applicability of a combined biosensing FIA system to many fields including clinical diagnosis, the food industry, agricultural products and environmental control. The range and number of these systems is expected to increase as immobilization, flow injection and detection techniques improve. To analyze a biomaterial using traditional methods may be time consuming and even then the data may be of little value because the result is obtained hours or perhaps days after the sample was obtained. The acquisition of real-time, or almost-real-time, data is the most important advantage of biosensing FIA systems. Increasing use of this kind of system may be expected in the future.

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C-Y Chen, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1, Komaba, Meguro-Ku, Tokyo, Japan and Department of Agricultural Chemistry, National Taiwan University, 1, Sec. 4, Roosevelt Road, Taipei, Taiwan.

I Karube, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1, Komaba, Meguro-Ku, Tokyo, Japan.