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A Biosensor Array Based on Polyaniline

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This paper describes the fabrication of polyaniline-based microsensors and microsensor arrays for the estimation of glucose, urea, and triglycerides. Microelectronics technology has been used to produce gold interdigitated microelectrodes on oxidized silicon wafers. Polymer deposition and enzyme immobilization has been done electrochemically. Electrochemical potential control has been used to direct enzyme immobilization to the chosen microelectrodes and prevent it at other microelectrodes in contact with the enzyme solution. This has enabled the immobilization of three different enzymes on three closely spaced microelectrodes, resulting in a sensor array which can analyze a sample containing a mixture of glucose, urea, and triolein in a single measurement using a few microliters of the sample. This strategy is quite general and can be extended to other enzyme−substrate systems to eventually produce an "electronic tongue".

Recently, there has been considerable interest in the development of probes for the detection of biologically significant molecules using conducting polymers.[1−6] Early reports were concerned with the use of polypyrrole for the amperometric detection of glucose.[1−3] The idea essentially was to provide an electronically conducting polymer matrix for the immobilization of the enzyme glucose oxidase. Since then, there has been considerable work along these lines. We have recently described a new generic concept[4] which is based on the fact that the conductivity of this class of polymers is very sensitive to the chemical potential of the microenvironment within the polymer matrix.[7−9] In this concept, the conducting polymer acts as the immobilization matrix as well as the physicochemical transducer to convert a chemical signal (change of chemical potential of the microenvironment) into an electrical signal (change in electronic conductivity of the polymer). In the present work, we describe proof-of-concept studies for the detection of glucose, urea, and triglycerides simultaneously from a single sample of analyte using a biosensor array.

There are two principal challenges in the fabrication of such a biosensor array. The first is miniaturization of the single-substrate sensor, and the second is preventing cross-talk between neighboring sensors on an array. Microelectronics technology has been used to fabricate interdigitated microelectrode elements and miniaturize the devices. Chemical cross-talk between neighboring devices can occur if the immobilization of the enzyme that imparts specificity to the sensor is not localized to the designated microelectrode pair. This is particularly difficult if the microelectrode pairs are closely spaced. We have made use of the electrostatic interaction between the charged surface of a conducting polymer and the charges on biomolecules to target the adsorption of a specific enzyme to a specific microelectrode pair.

EXPERIMENTAL SECTION

Microelectrode Layout and Fabrication. Two different electrode layouts were used, as shown in Figures 1 and 2. Figure 1 shows the layout for a single-substrate microsensor. One interdigitated microelectrode pair (IMP) was used for the sensor device and the other for a reference device. Figure 2 shows the layout for a sensor array with three IMPs for three different sensors and one IMP for a reference device. The masks for these layouts were designed on a Sun-Sparc work station using EESOF Academy Tools. A hard copy was obtained on Flashscan Photoplotter.

The starting substrates were (100) p-type Si of 5 Ω-cm resistivity. They were cleaned using the RCA procedure and oxidized in dry oxygen. The oxidation temperature was 1000 °C, and the oxidation time was set to obtain an oxide thickness of 100 nm. A 10 min postoxidation anneal in dry nitrogen was performed at the oxidation temperature.

Chrome−gold was deposited with the substrate heated to 250 °C in a chamber with pressure better than 10^{-6} Torr. The thickness of the chrome layer was about 20 nm and that of gold 80 nm. However, this thickness of the gold film gives rise to relatively high resistance, which leads to considerable joule heating and delamination of the gold film if high current densities are used during electropolymerization. Hence, it was decided to increase the thickness of the gold film to 1 μm by electroplating gold onto the gold evaporated on the wafers. A thicker gold film was also required for gold wire bonding to the contact pads of the device. The patterning of the gold film was done using photolithography. A positive photoresist was used to define the patterns shown in Figures 1a and 2a. The gold layer was etched in KI (50 g), iodine (12.5 g), and 150 mL of deionized water for 30 s. The photoresist was stripped, and the chrome layer was etched in $K_3Fe(CN)_6$ (25 g), NaOH (12.5 g), and 150 mL of deionized water for 30 s, with the gold pattern acting as the mask.

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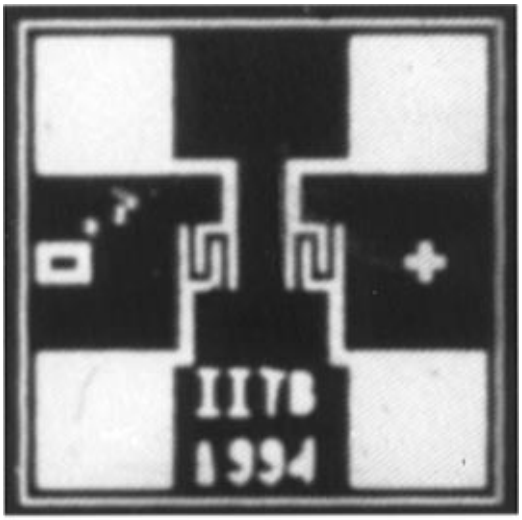


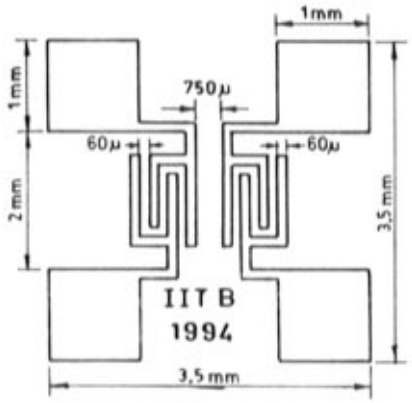
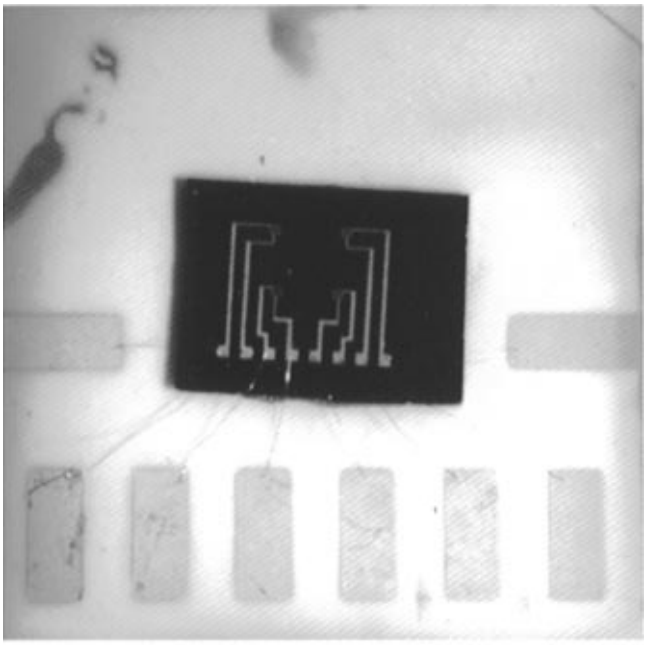


Figure 1. (a, bottom) Layout for a single substrate microsensor. One IMP was used for the sensor film, and the other was used for a reference film. (b, top) Enlarged view of a single-substrate sensor.



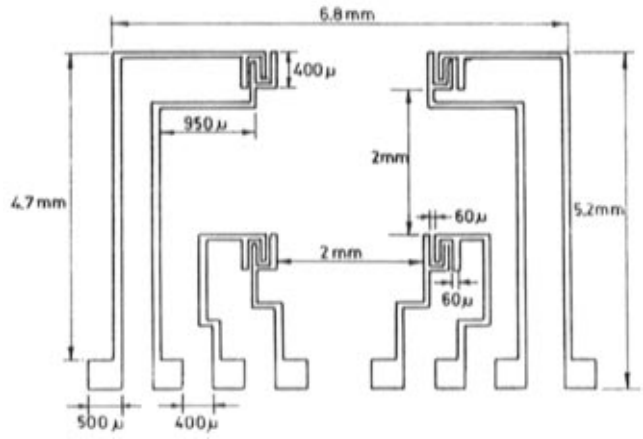


Figure 2. (a, bottom) Layout for a sensor array. Three IMPs were used for depositing sensor films, and the fourth was used for a reference film. (b, top) Enlarged view of a sensor array mounted on a ceramic base.

After copious rinsing in deionized water, the wafers were scribed to separate the devices. The dies were mounted on ceramic bases which had gold contact pads evaporated on them. The contact pads on the diced wafers were gold wire bonded to the contact pads on the ceramic base from which connections to the instrumentation were made. Figures 1b and 2b show the photographs of the gold microelectrodes for the single-substrate sensor and the sensor array.

Deposition of the Polymer and Immobilization of the Enzyme. The diced wafers with gold microelectrodes were cleaned by boiling in acetone to remove traces of the photoresist deposited during lithography. Polyaniline was deposited on the microelectrodes electrochemically. To restrict the contact of the electrolyte to the IMP region, the arrangement shown in Figure 3 was used. The electrolyte was contained in a disposable syringe barrel, and electrolytic contact to the IMP was made through a tiny drop of electrolyte extruded onto the IMP. The syringe also housed a platinum wire quasi-reference electrode and the counter electrode, which were introduced through the piston of the syringe. The syringe needle was positioned just above the device so that the electrolyte drop rested on the IMP without being detached from the needle.

Prior to polymer deposition, the gold microelectrodes were cleaned electrochemically by potentiodynamic cycling in 0.5 M H_2SO_4 between the potentials −0.2 and +0.5 V vs SCE. A polyaniline film was deposited on the IMP by electrochemical polymerization from a solution containing 0.1 M aniline in 0.5 M H_2SO_4. The electrode potential was cycled between −0.2 and +0.8 V vs SCE. The gap between an interdigitated pair was bridged by polyaniline film in about 5 min under these conditions.

Enzyme immobilization in the polymer film was not as straightforward as one would expect, since the pH at which most enzymes are stable is not the optimum for high rates of polymerization and bridging of the IMP. Only in the case of glucose oxidase, which is relatively stable at pH 4, was entrapment during electropolymerization possible. Thus, glucose oxidase, EC 1.1.3.4 (*Aspergillus niger*), was immobilized in the conducting polymer matrix by electropolymerization from a pH 4 phthalate buffer containing 0.1 M aniline and 250 units/mL of GOx. The potential was cycled between the limits −0.2 and +1.2 V. The polymerization was carried out for 2 h. This resulted in a two-layer structure as described in ref 4. Lipase (triglycerol acylhydrolase), EC 3.1.1.3 (*Pseudomonas* sp.), was immobilized from a pH 5.2 acetate buffer by adsorption on a bridged IMP. Adsorption was carried out for 2 h. Urease, EC 3.5.1.5 (Jackbean meal), was similarly immobilized by adsorption from a pH 5.2 acetate buffer. In the microelectrode pattern shown in Figure 1b, the adjacent IMPs are sufficiently close that it was not possible to restrict the

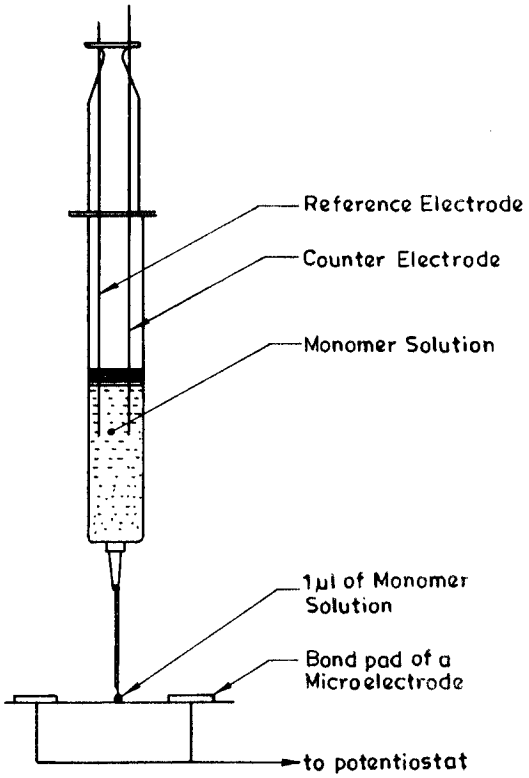


Figure 3. Electrochemical setup used for the deposition of a conducting polymer film on the IMPs.

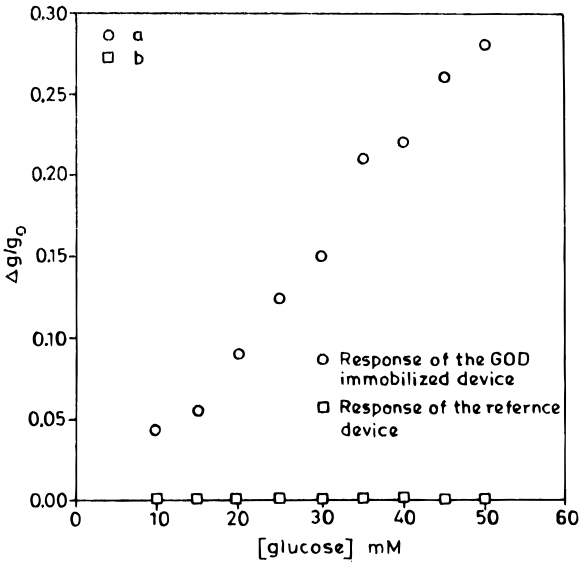


Figure 4. Response of a glucose microsensor. (a) Response of the sensor film. (b) Response of the reference film.

drop of enzyme solution to only one IMP. In these cases, the IMPs on which immobilization was not desired were maintained at −0.2 V.

Chemicals and Instrumentation. Chemicals used during microelectrode fabrication were MOS grade. The monomer aniline was AR grade, distilled and stored under dry nitrogen. The sulfuric acid was also AR grade, purified further by distillation under dry air. Salts for the preparation of buffers were AR grade and were used without further purification. Glucose oxidase and urease were obtained from Sisco Research Labs, and lipase was from Amano Chemicals. Glucose, urea, and triolein were obtained from SRL. All solutions were made in water purified from a MilliQ water purification system.

Table 1. Comparison of the Performance of Macro- and Microsensors

	sensitivity (mM^{-1})			limiting concn (mM)		
	glucose	urea	lipid	glucose	urea	lipid
macrosensor	0.010	0.005	0.014	30	80	80
microsensor	0.027	0.015	0.076	50	80	90

Electrochemical potential control was provided using an EG&G PARC Model 273 potentiostat, and the polymerization process was monitored by recording voltammetric traces on a Linseis LY 16100 *XYt* recorder. The resistance of the polymer film was measured using small signal ac excitation. This reduces the possibility of polarization at the polymer/solution interface, and because phase sensitive detection is used, the effect of noise on the measurement is reduced. A current of 100 nA rms (at 1.33 kHz) was forced through the sensor, and the in-phase voltage across the sensor was measured using an EG&G PARC Model 5208 two-phase lock-in analyzer. The ratio of the in-phase voltage to the current gave the resistance. The conductance was obtained by taking the inverse of the resistance, assuming that it is a reasonable estimate of the conductance of the polymer film. The sensor "response" is represented by $\Delta g/g_0$, where g_0 is the conductance of the sensor in the buffer without the substrate and $\Delta g = g - g_0$, where g is the small signal conductance of the sensor in the presence of the substrate. Representing the response as a ratio, $\Delta g/g_0$, normalizes the sensor response and minimizes variations from sensor to sensor.

RESULTS AND DISCUSSION

Electrodeposition of polyaniline was carried out by potentiodynamic cycling between −0.2 and 0.8 V vs SCE. After a polymer bridge was established across the IMP, potential cycling was stopped, the surface was rinsed thoroughly with water, and enzyme immobilization was carried out as described earlier. Figure 4 shows the response of a sensor on which glucose oxidase had been immobilized. The sensor response is represented as the change in conductance of the polymer film in the presence of the substrate glucose as compared to the conductance measured in the buffer in the absence of the substrate. The change in conductance is normalized with respect to the value measured in the buffer. This dimensionless sensor response function eliminates the variation due to slight differences in the polymer film conductance from sensor to sensor. The response of the glucose microsensor is linear up to a glucose concentration of about 50 mM, above which it saturates. The macrosensor, on the other hand, showed linearity only up to a concentration of 30 mM.[4] The sensitivity of the microsensor is 0.027/mM, as compared to 0.010/mM for the macrosensor (see Table 1).

The differences in sensitivity and linearity of response between micro- and macrosensors can be understood as follows. For sensor applications, the change of conductance of the active device is the parameter of interest. The conductance of the active device depends on several factors: (i) the contact resistances between the metal electrodes and the polymer film, (ii) the geometric factors of the film, i.e., the length, width, and thickness of the film between the electrode pair, and (iii) the film conductivity, which depends on several factors, such as analyte pH, temperature, polymer film potential, substrate concentration, enzyme

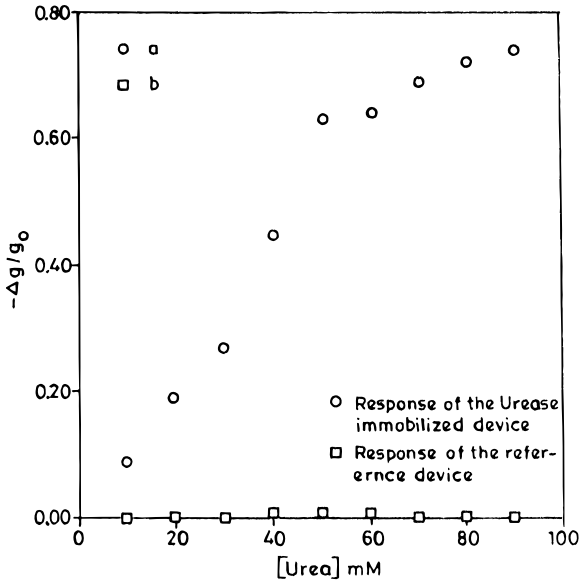


Figure 5. Response of a urea microsensor. (a) Response of the sensor film. (b) Response of the reference film.

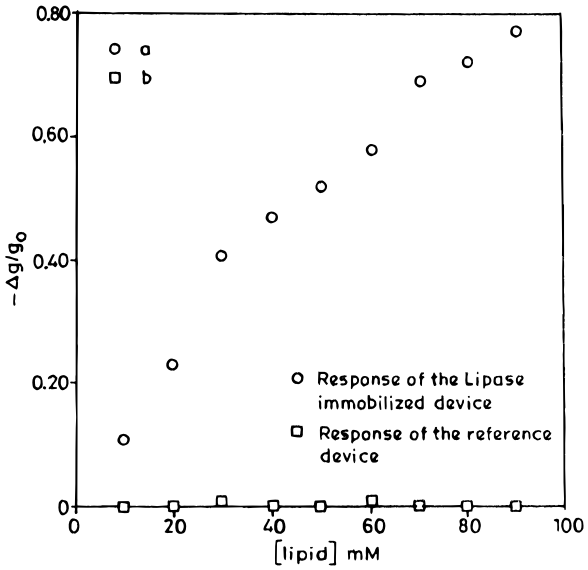


Figure 6. Response of a lipid microsensor. (a) Response of the sensor film. (b) Response of the reference film.

loading, the diffusion coefficients of reactants and products in the polymer film, and the diffusion layer thickness. The measured conductance also depends on the conductance of the parasitic path between the electrodes via the analyte. Devices made on microelectrode pairs are more sensitive because of more favorable geometric factors of the active device, effectively higher and more uniform enzyme loading, and a smaller contribution from the parasitic path. The increased range of linearity can be attributed to the shorter distances that reactants and products need to diffuse in the polymer for the microsensors as compared to macrosensors.

The enzyme-catalyzed reaction in the polymer matrix can be represented as

$$\text{glucose} + O_2 + 2H^+ \rightarrow \text{gluconolactone} + H_2O_2$$

The production of gluconolactone in the micropores of the polymer matrix lowers the pH in the microenvironment. This lowers the conductivity of the polymer. Even though the polymer film is immersed in a buffer of pH 7, the diffusion of protons from the microenvironment of the polymer matrix is sufficiently slow to give a stable response.

Urease was immobilized by adsorption on the polymer film at open circuit. The open circuit potential of the polymer film is +0.326 V vs SCE. At this potential, the polymer has a net positive charge and is swollen due to the influx of hydrated counterions and solvent molecules, which help to shield the charge between adjacent polymer chains.[10] Under these conditions, some of the counterions are probably replaced by the negatively charged urease. Therefore, the immobilization of urease can be inhibited by maintaining the polymer at a potential where the net charge on the polymer is either negative or zero. With polyaniline, this can be achieved by maintaining the polymer at −0.2 V vs SCE. Figure 5 shows the response of such a urea microsensor. The response of the sensor film is represented by "a", and the response of the reference film on the adjacent IMP is represented by "b". The immobilization of urease on the reference film was suppressed by potentiostating it at −0.2 V, while immobilization was carried out on the sensor film. The small and constant response of the reference film shows that the above procedure is effective in targeting the adsorption process to the desired IMP. Thus, the immobilization of the enzyme on any of several closely spaced IMPs is possible by using this technique. This will be useful in the fabrication of miniaturized sensor arrays. The sensitivity of the urea microsensor was 0.015/mM, as compared to 0.005/mM for a macrosensor.[12]

The enzyme-catalyzed reaction at the urease-loaded polymer film can be represented as

$$NH_2CONH_2 + H_2O \rightarrow 2NH_3 + CO_2$$

Considering that 2 mol of ammonia is generated for every 1 mol of CO_2 and that the pK_a for NH_3 is 9.25, the net effect of the enzyme-catalyzed reaction is to raise the pH of the microenvironment in the polymer matrix and consequently lower the conductivity of the polymer film.

Lipase was also immobilized by adsorption with the polymer film held at open circuit. Immobilization of the enzyme on the adjacent IMP (reference film) was similarly inhibited by maintaining it at −0.2 V vs SCE. The response of the lipid sensor is shown in Figure 6. The response of the sensor film is represented by "a", and response of the reference film is represented by "b". The reference film shows only a small and constant response, confirming that lipase immobilization has been inhibited effectively on this film. The enzyme-catalyzed reaction at the lipase-loaded polymer film can be represented as

$$\text{triolein} + H_2O \rightarrow \text{oleic acid} + \text{glycerol}$$

Oleic acid, being insoluble in water, either forms micelles or remains solubilized by Triton X, while glycerol goes into solution. Independent control experiments have shown that the addition

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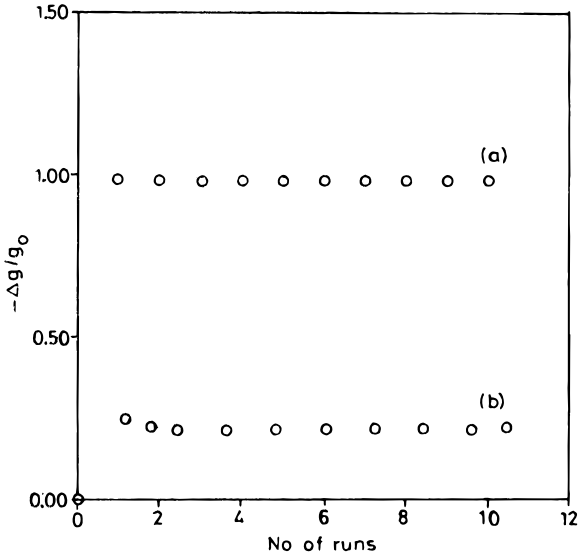

Figure 7. Repeatability of the response of the lipid and urea microsensors to 10 independent measurements. (a) Response of the lipid microsensor to 60 mM lipid solutions. (b) Response of the urea microsensor to 60 mM urea solutions.

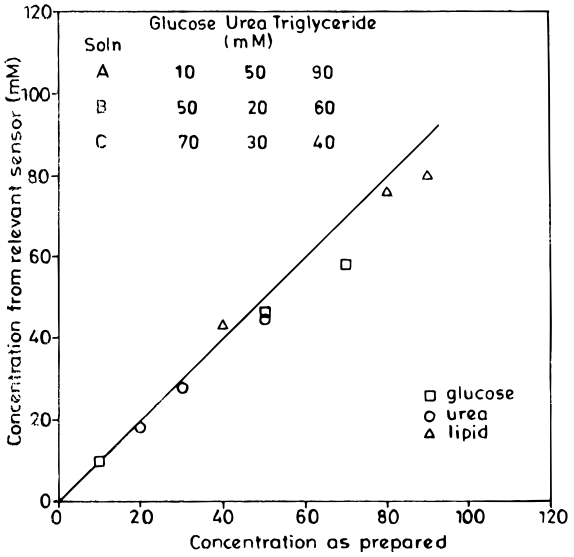

Figure 8. Analysis of three different mixtures of glucose, urea, and lipid using a sensor array.

of glycerol and Triton X solution to the buffer in concentrations similar to those used here results in an increase in the pH of the solution. This probably accounts for the increase in pH of the microenvironment in the polymer matrix and consequently in the lowering of its electronic conductivity. This is in agreement with the observations recorded in Figure 6.

Stability of the sensor activity is important in view of the fact that the immobilization of the enzyme involves relatively weak electrostatic interactions and physical entrapment. Therefore, the sensor response as a function of repeated use has been examined. The results of these studies are shown in Figure 7 for the urea and triglycerides sensors. The sensor response is fairly constant, even after 10 independent measurements, which include washing with a buffer solution between every two measurements. This demonstrates that the immobilization technique is effective and the extent of leaching is not significant in both these cases.

The results described so far clearly demonstrate that (i) the immobilization of glucose oxidase, urease, and lipase can be directed to the desired IMP, (ii) adsorption of the enzyme can be inhibited by holding the polymer film at a potential where it is either neutral or negatively charged, and (iii) the immobilization is sufficiently strong to permit multiple usage. Based on these results, we have fabricated a sensor array consisting of glucose, urea, and lipid sensors as well as a reference sensor. The electrode layout used was that shown in Figure 2. At first, all the four IMPs were coated with polyaniline. Glucose oxidase, urease, and lipase were separately immobilized from their respective solutions on one IMP each, while the fourth IMP was used as a reference sensor. The polymerization and immobilization procedures were as described earlier. The sensor response to each substrate was determined independently, and the resulting calibration plots were used to obtain concentrations of these substrates when present in a mixture. Each IMP was independently addressed, and the conductance of each patch of polymer film could be determined when all the four IMPs were exposed to a common pool of analyte solution. Figure 8 shows the results from the analyses of three mixtures of glucose, urea, and lipid. The compositions of the three mixtures are shown in the inset. The concentrations calculated from the sensor response are plotted against the concentrations as prepared. The experimental data show excellent agreement with a line of unity slope, which is to be expected if the array behaved ideally.

CONCLUSION

It has been shown that an analyte containing a mixture of three substrates can be analyzed by a single measurement using a single sample of a few microliters. The problem of cross-talk arising from uncontrolled adsorption of the enzyme on all the polymer-coated IMPs in contact with the enzyme solution has been solved by appropriate control of the electrochemical potential of the polymer film. Design and fabrication of independently addressable microelectrodes has been achieved using microelectronics technology. This strategy is quite general and can be extended to other enzyme−substrate systems, eventually leading to the development of an “electronic tongue”. Recently Meyer et al.[11] have described an array of 400 sensors, each independently addressable. They have used it to obtain a two-dimensional concentration profile of glucose by immobilizing glucose oxidase on all the sensor elements. The strategy described by us can be combined with the large array of Meyer et al. to fabricate an “electronic tongue”.

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