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Electrochemical Sensor for Measurement of Urea and Creatinine in Serum Based on ac Impedance Measurement of Enzyme-Catalyzed Polymer Transformation

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Enzyme-catalyzed polymer transformation with electrochemical ac impedance detection has been employed for the measurement of urea and creatinine in serum samples. A polymer, based on poly(methylvinyl ether)/maleic anhydride modified by esterification with *n*-octanol, which is stable at pH 7.4 and which is transformed rapidly in response to alkaline pH changes, was linked to enzymatic reactions between urease and urea or creatinine deiminase and creatinine to produce a disposable sensor system. The polymer was screen-printed onto interdigitated screen-printed carbon electrodes and the electrodes overlaid with absorbent pads containing the relevant enzyme. Application of serum samples, "spiked" with either urea or creatinine, resulted in rapid polymer transformation, and resultant changes in the capacitance of the polymer-coated electrodes were analyte-concentration dependent. Additional information on the mechanisms of polymer transformation was obtained from dynamic quartz crystal microbalance measurements.

Electrochemical-impedance measurements have been used mainly to investigate nonbiological systems such as protective coatings covering a metal substrate.[1−3] However, this technique is beginning to be used for novel applications such as the modeling of biological systems from the impedance response using networks of electrical components.[4] Impedance methods are also beginning to be used for bioanalytical applications and have been reviewed by Bergveld.[5] For example, Bataillard et al.[6] and Souteyrand et al.[7] described how a Si/SiO_2 surface can be modified with antibody and used to measure antigen-binding events. Song et al.[8] used electrochemical-impedance spectroscopy as an analytical method for the characterization of cytochrome *c* adsorbed to alkanethiolate self-assembled monolayers on gold-film electrodes. Recently, Cornell et al.[9] have combined electrodes coated with lipid layers containing ion channels with impedance measurements to produce an ion-channel switch biosensor. Such a switch has a high gain: a single channel facilitates the flux of up to 1 million ions/s.

In a previous paper, we presented a novel impedance sensor based on measuring a change in capacitance of an enzyme-catalyzed breakdown of polymer-coated screen-printed gold-ink electrodes.[10] A commercially available polymer, Eudragit S-100 (Dumas (U.K.) Ltd, Kent, U.K.), was used to demonstrate this principle. A specific change in pH was caused by the action of urease on urea, producing ammonium and hydroxide ions, which resulted in polymer breakdown and a 4 orders of magnitude change in capacitance in the presence of excess urea. In comparison, parallel measurements of the change in potential due to the pH change induced by the urease reaction resulted in an overall change in potential of approximately 120 mV. This demonstrates the potential sensitivity of impedance analysis compared with simple pH measurement. The method was demonstrated for the measurement of urea in buffer and for an immunoassay of human IgG. The main disadvantage in using Eudragit S-100 for clinical analysis was its low pH-breakdown threshold (pH >7.0). Thus, addition of samples of either whole blood, serum, or plasma at physiological pH 7.4 would break down this polymer in a nonspecific manner.

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In this paper we present significant advances, in the type of polymer used and the electrode design, toward a simple, integrated, disposable, sensor design for clinical use. The polymer used is a modification of poly(methyl vinyl ether)/maleic anhydride. This type of polymer has been described by Heller et al.[11] as a method of controlled drug release through polymer dissolution due to an increase in pH. It was shown that esterification using various alcohols could alter the dissolution pH. An impedimetric assay for urea and creatinine in serum is demonstrated using a polymer which is stable at pH 7.4, but which breaks down at a more alkaline pH.

Clinical Relevance of Urea and Creatinine. Elevated levels of urea and creatinine are pathognomic of renal insufficiency[12] and it has been shown that inadequacy of dialysis can be directly linked to mortality.[13,14] Increasing use is being made of plasma creatinine values, alone, to assess changes in renal function, and it has been shown that plasma creatinine is more sensitive than creatinine clearance in detection of changes in glomerular function.[12] In addition, there is diagnostic value in the plasma urea/creatinine ratio.

EXPERIMENTAL SECTION

Reagents. Carbon ink (no. C20723D12) was obtained from Gwent Electronic Materials Ltd. (Pontypool, Gwent, U.K.). Poly(methyl vinyl ether)/maleic anhydride copolymer (Gantrez AN-139) was a gift from ISP Europe (Guildford, Surrey, U.K.). *n*-Octanol, sulfuric acid (95−98%), di(propylene glycol) methyl ether, tri(propylene glycol) monoethyl ether, and dibutyl phthalate were obtained from the Aldrich Chemical Co. (Gillingham, Dorset, U.K.). 1-Ethoxy-2-propanol was obtained from Acros Chimica (Hyde, Cheshire, U.K.). Urease (EC 3.5.1.5, code URE3) was obtained from Biozyme Laboratories Ltd. (Blaenavon, Gwent, Wales, U.K.). Creatinine deiminase (EC 3.5.4.21) was obtained from Toyobo (Japan). Nyco control serum was obtained from Nycomed Pharma AS (Birmingham, U.K.). Urea was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Sodium chloride and Tween 20 were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). Wicking material 939 was obtained from Ahlstrom Filtration Inc. (Mt. Holly Springs, PA). Buffer components were prepared using AnalaR grade reagents from BDH Ltd. Water produced by reverse osmosis and delivered through a Milli Q (Millipore) ion-exchange system was used to prepare all solutions.

Polymer Modification. 1-Octanol (150 mL) and 30 g of Gantrez AN-139 copolymer were refluxed in a 200-mL round-bottomed flask with constant rapid stirring for 48 h. This was followed by the addition of 540 μL of concentrated sulfuric acid, and the mixture refluxed for a further 2 h and 25 min. This reflux time must be as exact as possible and is the most crucial factor in obtaining a polymer with the desired transformation pH. The polymer solution was then poured into 200 mL of methanol, with stirring, and allowed to cool to room temperature. Water was added gradually, with constant stirring, to precipitate the polymer. Only the minimum amount of water was used. The supernatant was discarded and the remaining polymer redissolved in 400 mL of acetone. Again, water was added with constant stirring to precipitate the polymer. The supernatant was discarded and the polymer freeze-dried. This process was repeated four times.

Ac Impedance Measurements. Ac impedance measurements were performed using a 1253 Gain Phase Analyzer with an SI 1286 Electrochemical Interface (Solartron, Farnborough, Hampshire, U.K.). The instruments were connected to a PC via a IEEE interface card (National Instruments U.K., Berkshire, U.K.). Instrument operation and data acquisition were controlled using software written "in-house". All impedance measurements were performed in the two-electrode mode at a frequency of 20 kHz unless stated otherwise, with an AC amplitude of 40 mV peak to peak. DC off-set potential was 0 V.

Quartz Crystal Impedance Measurements. Polished 10 MHz AT-cut quartz crystals with gold electrodes on both sides were obtained from Elchema, USA. To study the mechanism of polymer degradation by quartz crystal impedance spectroscopy, the process had to be slowed sufficiently. Therefore, fairly thick (about 1 μm) films of Eudragit S-100 or the esterified maleic anhydride copolymer (EMAC) were spin-coated onto a quartz crystal at a spinning speed of 4000 rpm from a 7.5% solution of polymer in acetone. Only the polymer-coated side of the crystal was exposed to the electrolyte solution. Quartz crystal impedance measurements were carried out at a series of frequencies close to the resonance frequency of the quartz crystal using a Hewlett-Packard 4192A LF Impedance Analyzer. Initial spectra were taken in a buffer solution pH 6.1 containing 140 mM NaCl and 10 mM phosphate. Eudragit S-100 breakdown was initiated by increasing the pH to 7.0. EMAC transformation was initiated by increasing the pH first to 9.0 and later to 11.0. It should be noted that spectra might be slightly deformed because the system was changing during the course of the measurement.

Electrode Screen Printing. Carbon-interdigitated electrodes were screen printed manually, 10 at a time, using a stainless screen (Type 230 mesh, 13 μm emulsion thickness from DEK Precision Screen Division, Weymouth, Dorset, U.K.) onto a thin (0.23 mm) PVC substrate. The carbon ink was first mixed thoroughly for at least 20 min and then spread evenly over the screen pattern. A rubber squeegee was then drawn over the screen, with pressure and speed applied as evenly as manually possible, imprinting the PVC substrate with the electrode pattern. The ink was then dried at 60 °C overnight. Figure 1 illustrates the electrode pattern and dimensions.

Polymer Screen Printing. A screen-printing polymer solution was prepared containing 47.2% (w/w) di(propylene glycol) methyl ether, 11.3% tri(propylene glycol) monoethyl ether, 5.3% 1-ethoxy-2-propanol, 1.6% dibutyl phthalate, and 34.6% EMAC. The stainless screen for printing polymer consisted of two rows of five "open" squares with dimensions and spacing exactly over the positions of the "heads" of the electrodes. A line of polymer solution was poured just above one line of open squares. A rubber squeegee was drawn, with pressure and speed applied as evenly as manually possible, across the open squares, pulling and pressing the polymer solution over the squares and thus imprinting the polymer over the entire working area of the interdigitated electrodes.

Enzyme-Electrode Preparation. Urease was dissolved in water containing 0.1% (v/v) Tween 20 to give a final concentration

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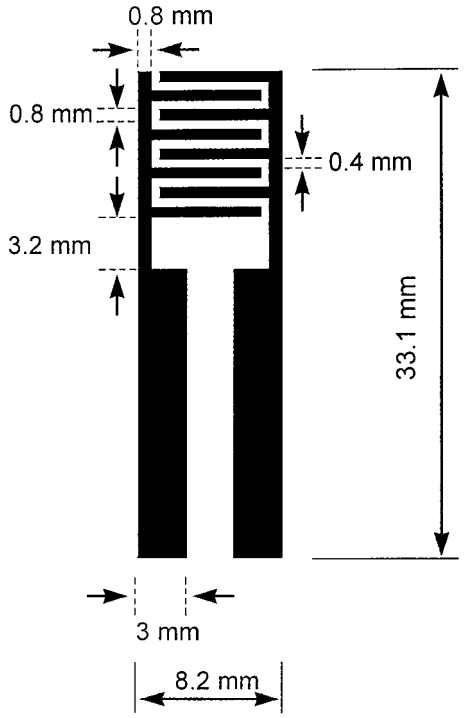

Figure 1. Design of the screen-printed carbon-interdigitated electrodes used, showing all dimensions.

of 10 mg mL^{-1} (4790 units mL^{-1}). To a piece of Ahlstrom wicking material (50 mm × 10 mm × 0.5 mm), 400 μL of the urease solution was added. The strip was allowed to dry overnight at room temperature. Pads were then quartered and stored at −20 °C until required. Similarly, pads saturated with a solution of creatinine deiminase, 54 mg mL^{-1} (617 units mL^{-1}), containing 0.1% (v/v) Tween 20, were also prepared. The carbon tracks leading from the interdigitated section of the electrode were insulated from solution by applying masking tape over the tracks. A piece of double-sided tape (10 × 6 mm) was placed on top of the masking tape at the edge of the interdigitated electrode and an enzyme-loaded pad (12 mm × 10 mm) placed over the polymer-coated electrode just in contact with the double-sided tape (Figure 2).

Urea and Creatinine Assay in Serum. Nyco control serum was reconstituted with 10 mL of water. The endogenous-urea concentration in this serum was 3.3 mM. The pH was checked and adjusted to pH 7.4 with concentrated HCl. Standards were prepared by spiking the serum with urea or creatinine. Urea-standard solution (110 μL) was pipetted onto the urease pad (Figure 2). Simultaneously, impedance measurements were started and the response measured over a period of 10 min. The electrode and pad assembly was then discarded and replaced by another. The results were compared with a reference laboratory method[15] in the Hospital Diagnostic Laboratory of the Department of Clinical Biochemistry. Additionally, serum samples were spiked with "unknown" urea concentrations and measured. The urea concentrations of the unknown samples were determined by comparison of impedance responses obtained with a calibration curve constructed using Nyco control serum-standard solutions. Assays for creatinine in serum using creatinine deiminase in place of urease were also performed essentially as described above.

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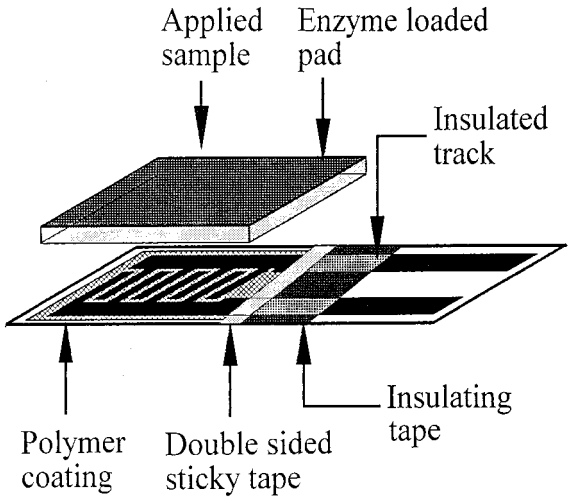

Figure 2. Arrangement of the electrode system for the impedimetric determination of urea and creatinine in serum.

RESULTS AND DISCUSSION

Electrode Reproducibility. The reproducibility of the initial capacitance of bare-screen-printed carbon-interdigitated electrodes was determined by repeated measurement in 10 mM sodium phosphate buffer containing 140 mM sodium chloride, pH 7.4. Twenty electrodes were examined.

The capacitance of each electrode was measured every 10 seconds for twenty consecutive measurements. The imaginary component of the impedance ($-Z''/\Omega$) was used to calculate a value for the capacitance (C/F) using the equation below:

$$C = -\frac{1}{2\pi f Z''}$$

Here, f is the ac frequency (Hz). The average initial capacitance was 257 ± 31 nF (CV = 12%). In all cases, the capacitance was observed to decrease slightly, on average 32 nF, over the measurement period of 10 min. This probably represented the gradual penetration and wetting of the carbon ink by the buffer solution. The interelectrode coefficient of variation for the 20 consecutive measurements varied between 9 and 13%. It would be expected that automated screen printing would further improve the electrode reproducibility by fabricating larger batches of electrodes and thus reducing variations resulting from the manual process employed in this study.

Polymer Transformation. Reaction of Gantrez AN-139 with long-chain alcohols leads to esterification of the anhydride groups. The degree of esterification is dependent upon the reaction time and determines the pH at which the polymer degrades. The greater the degree of esterification, the lower the fraction of free carboxyl groups present. It is the ionization of these groups that causes the polymer to break down. The final result is a polymer that requires an increasingly alkaline pH to initiate transformation. The degradation mechanisms of Eudragit S-100 and of the modified Gantrez AN-139 (EMAC) have been investigated using impedance spectroscopy and other electrochemical techniques.[16] The results showed that Eudragit S-100 dissolved forming pores

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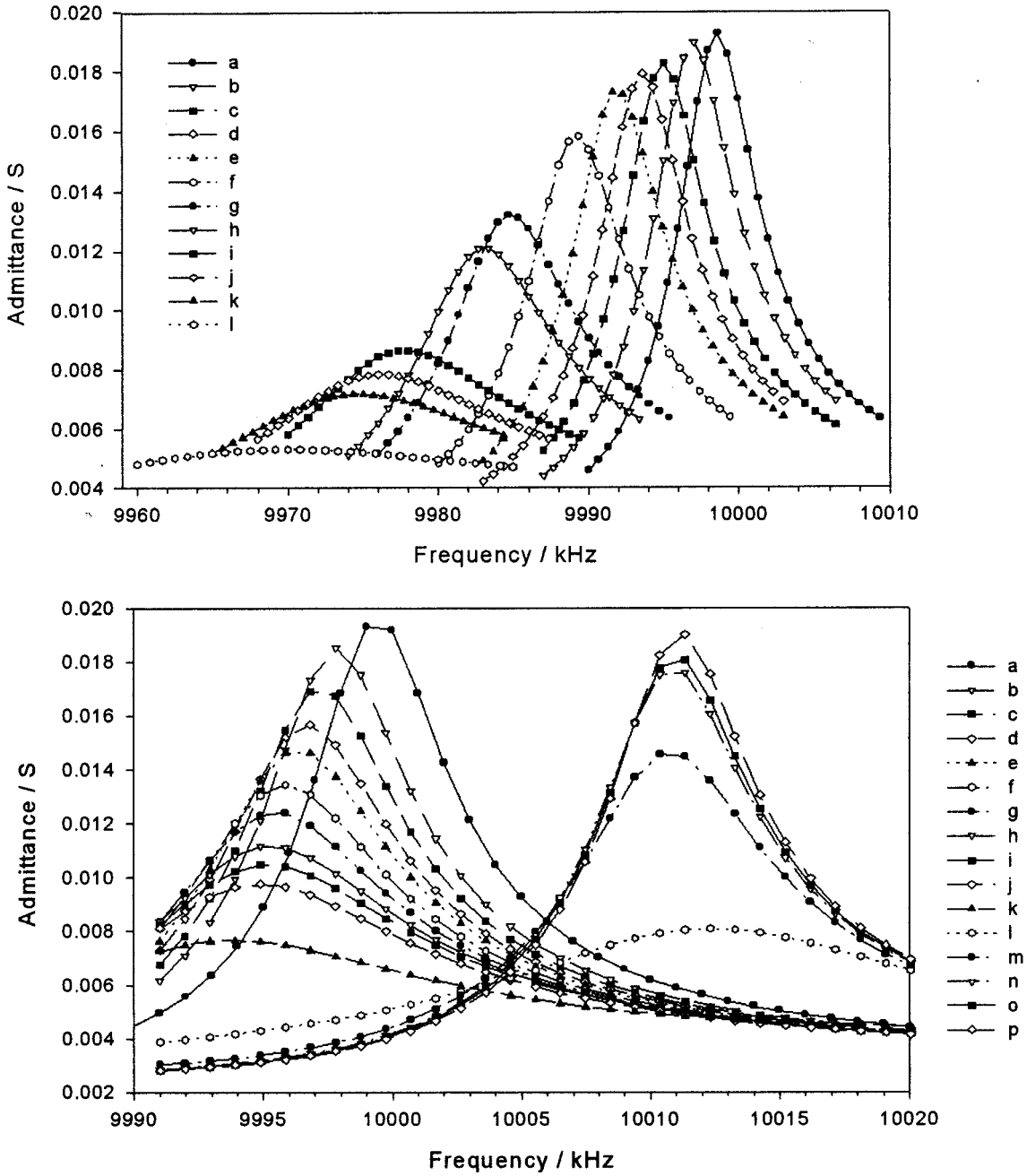

Figure 3. (A) Admittance spectra of an EMAC coated quartz crystal taken dynamically during the transformation of the polymer film. (a) At pH 6.1 prior to transformation, (b) 8 min at pH 9, (c) 16 min, (d) 26 min, (e) 39 min, (f) 62 min, (g) 103 min, (h) 158 min, (i) 3 min at pH 11, (j) 17 min, (k) 52 min, (l) overnight. (B) Admittance spectra of an Eudragit S-100 coated quartz crystal taken dynamically during the transformation of the polymer film. (a) at pH 6.1 prior to transformation, (b) 1 min at pH 7, (c) 4 min at pH 7, (d) 10 min, (e) 15 min, (f) 17.5 min, (g) 18.5 min, (h) 20 min, (i) 21.2 min, (j) 22.5 min, (k) 23.8 min, (l) 25.2 min, (m) 26.5 min, (n) 30 min, (o) 32 min, (p) 45 min and bare electrode.

through which electrolyte penetrated to the gold and spread along the electrode surface.

In contrast to the Eudragit S-100, EMAC films did not dissolve, probably due to higher molecular weight and long alkyl side chains of the resulting polymer. Instead, ionization of free unconjugated carboxyl groups resulted in an influx of water and cations into the polymer matrix, thus causing the polymer to swell, leading to a resultant decrease in the impedance. Since the polymer contains free carboxyl groups, its behavior can be compared to a cation-exchange membrane bearing weak-acid groups. The influx of ions therefore increases the conductivity, which ultimately approaches the conductivity of the bulk solution. This type of polymer transformation is very uniform and hence more reproducible than that of the Eudragit S-100.

To obtain additional information about the transformation mechanisms, quartz crystal impedance spectra were acquired dynamically during the course of degradation of both polymers (Figure 3A and B). The spectra of an uncoated crystal (Figure 3B, spectrum p) and of crystals coated with Eudragit S-100 (Figure 3B, spectrum a) and EMAC (Figure 3A, spectrum a) immersed into a pH 6.1 buffer solution show a similar peak admittance and peak width, i.e., both polymer films are rigid prior to degradation. During the course of transformation (Figure 3A, spectra b−l), the resonance frequency of the EMAC coated crystal decreased by

29 kHz, i.e., the mass coupled to the quartz crystal increased due to uptake of water and sodium ions into the film. The decrease in peak admittance observed during the transformation of the film is known to be caused by energy dissipation.[17] This can be explained by a transformation of the rigid film into a viscoelastic film as a result of the swelling. Apparently, no significant dissolution of the EMAC film occurred even after overnight treatment at pH 11 (Figure 3A, spectrum l). For Eudragit S-100, an initial decrease in frequency by only 4 kHz, with a drastic decrease in peak admittance, was observed (Figure 3B, spectra a−k). In addition to swelling, the decrease in the resonance frequency may be caused by an increase in surface roughness due to pore formation and the trapping of electrolyte in these pores. Comparison with results presented in the literature regarding the influence of surface roughness and viscoelasticity on quartz-crystal measurements led to the conclusion that the extreme energy dissipation in this case cannot exclusively be explained with swelling or increased porosity.[17−19] It is proposed that the energy loss was caused by a slow detachment of the film from the gold surface because of the spreading of the electrolyte beneath the polymer film. The increase of the resonance frequency at a later stage of degradation (Figure 3B, spectrum l) indicates the liftoff of the film. Eventually, an increase in the peak admittance occurred (Figure 3B, spectra m to p) until the admittance spectrum corresponded to that of an uncoated electrode (Figure 3B, spectrum p). This is consistent with a removal of polymer residues from the electrode surface. The results presented here confirm the degradation mechanisms proposed for the two different pH-sensitive polymers.[16]

The pH-of-transformation behavior of the EMAC polymer produced was investigated by screen printing the polymer onto carbon-interdigitated electrodes. The polymer-coated electrodes were then placed into buffer solutions with the pH ranging from 7.4 to 7.9. Desired characteristics for application to serum analysis were stability at pH 7.4 with rapid transformation at pH >7.4. To compensate for variations in the initial capacitances of individual electrodes, the capacitance for each electrode was normalized by taking ratios of the capacitance change (C) over the initial capacitance (C_o). As shown in Figure 4, *n*-octanol demonstrated the desired pH stability and transformation behavior. This polymer was relatively stable between pH 7.4 and 7.6, and above this value there was a sharply defined pH threshold for polymer transformation. An increase of only 0.1 pH unit resulted in an increase in the capacitance, which was indicative that polymer transformation was occurring.

Figure 5 shows a Bode plot, obtained in 50 mM sodium phosphate buffer, pH 7.4, of an electrode screen printed with one coat of the polymer (circles) prior to transformation. The slope of the impedance (approximately −1) and the corresponding phase angle (approximately 90°) indicate that the impedance measured is capacitive in nature over the frequency range 20 kHz to 4.5 Hz. The capacitance measured is the geometric capacitance of the polymer. Complete transformation of the polymer in 1 M NaOH (Figure 5, squares) shows an almost identical impedance spectrum to a bare electrode (triangles). This means that the conductivity of the swollen polymer must be very similar to the bulk solution conductivity; thus, the impedance measured is due to the properties of the underlying electrode. The equivalent circuit of the bare-electrode spectrum may be described by a capacitor and a resistor in series. The resistor represents the resistance of the bulk solution, and the capacitor represents the double-layer capacitance.

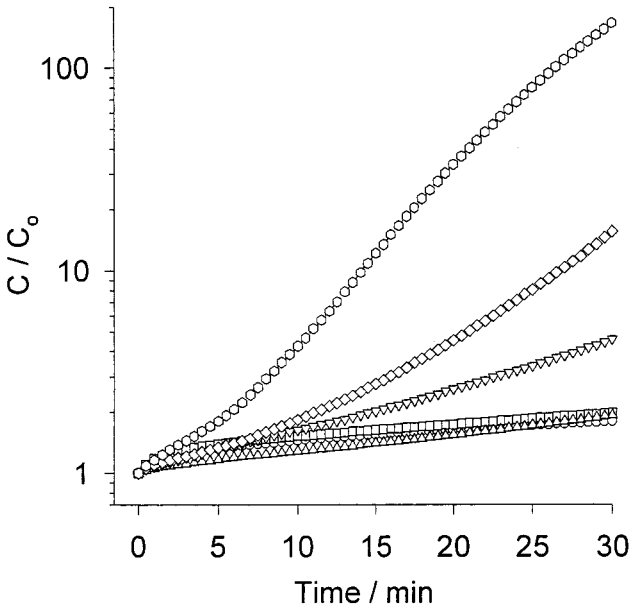

Figure 4. Stability of EMAC polymer in 50 mM sodium phosphate buffer at various pH values: pH 7.4 (△); pH 7.5 (○); pH 7.6 (□); pH 7.7 (▽); pH 7.8 (◇); pH 7.9 (○). Polymer transformation occurs at pH > 7.6.

Urea Measurement. An impedimetric assay for urea in serum was demonstrated using this polymer screen printed onto carbon-interdigitated electrodes. The average initial capacitance of polymer-coated electrodes was 0.80 ± 0.08 nF (CV = 10%, n = 5) as compared with 257 nF for a bare electrode, demonstrating that the polymer layer has decreased the capacitance of a bare electrode by 99.7%. Figure 6A shows the time course for polymer transformation followed over 10 min. The transformation was characterized by an increase in the capacitance of the polymer coating. As shown in Figure 6A, equilibrium capacitance values were reached for all urea concentrations within 10 min. A urea concentration of 30 mM resulted in a final capacitance which approached the capacitance of a bare electrode, i.e., the polymer had fully broken down. Lower concentrations of urea led to lower values for the equilibrium capacitance. These values represent a stable polymer coating in intermediate stages of transformation. With high concentrations of urea, there was sufficient substrate present to generate enough hydroxide ions to completely transform the polymer. The stable intermediate capacitances indicated that with lower urea concentrations (<20 mM) less hydroxide ions were being generated. Since polymer transformation depends on deprotonation of carboxyl groups, equilibrium must be reached when all the hydroxide ions produced in the enzymatic reaction have reacted, thus halting the transformation process and resulting in a stable capacitance value. Both the absolute rate of polymer breakdown as well as the final capacitance depends on the urea concentration and can be used as the sensor signal.

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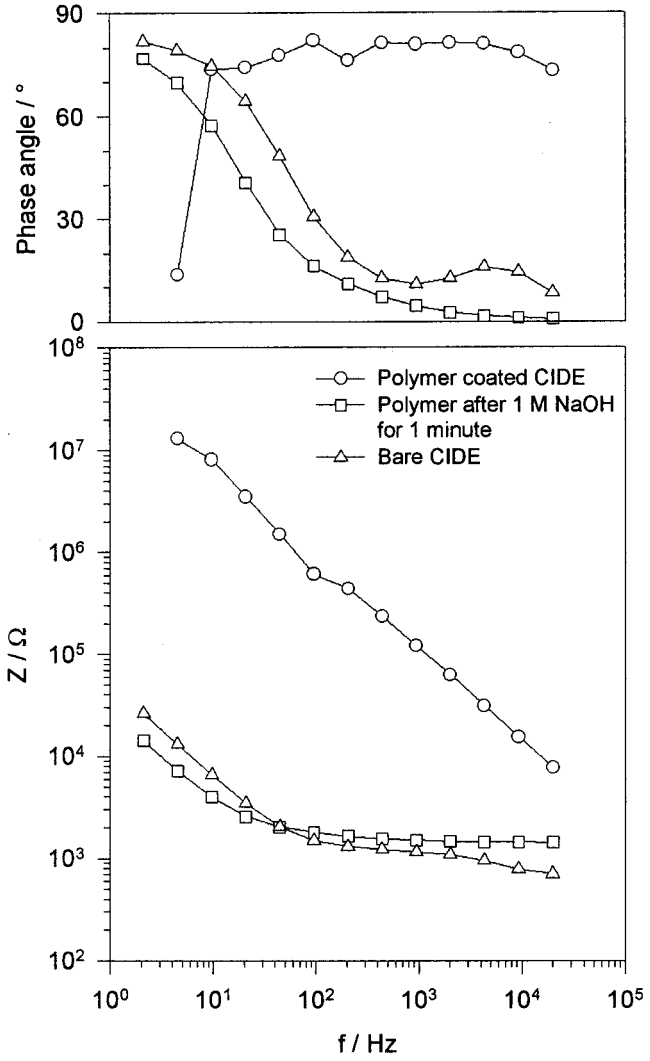

Figure 5. Bode plots of a bare CIDE (△), EMAC coated CIDE (○), broken down EMAC coated CIDE after 1 min in 1 M NaOH (□).

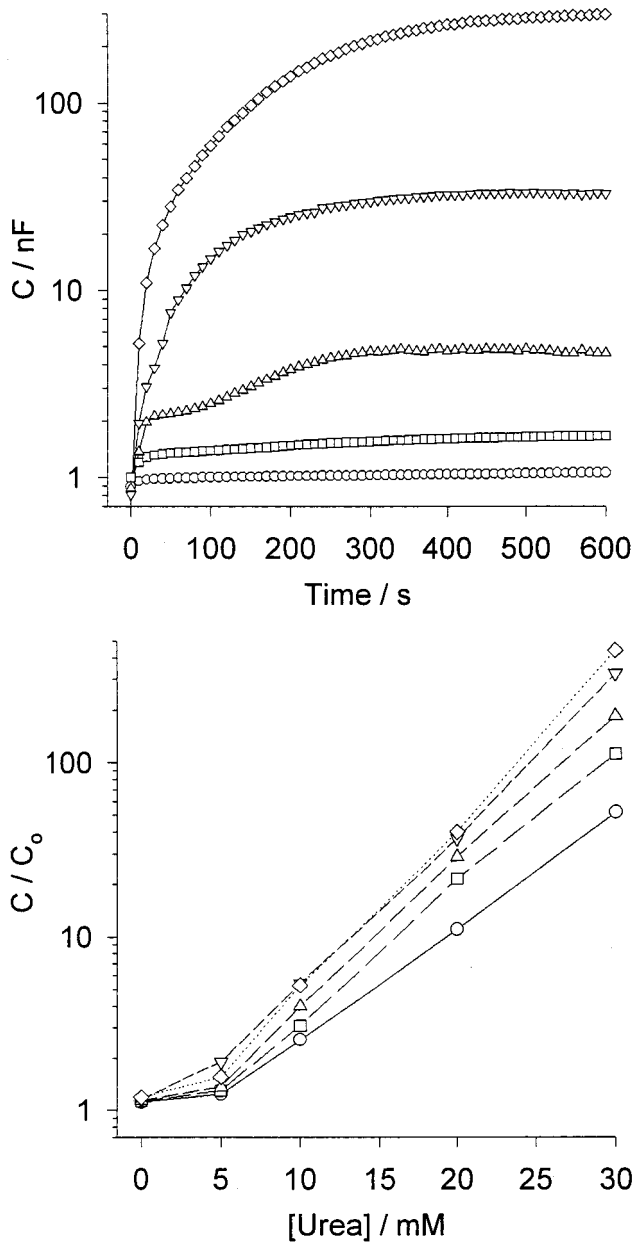

Figure 6. (A) Transformation of polymer as a function of time for five EMAC coated CIDEs at various standard urea concentrations in serum: 0 mM (○); 5 mM (□); 10 mM (△); 20 mM (▽); 30 mM (◇). (B) Calibration curve for urea measurement in serum derived from Figure 6A at five selected transformation times: 1 min (○); 2 min (□); 3 min (△); 5 min (▽); 10 min (◇).

Variations in the initial capacitance of the coated electrodes were compensated for by plotting the ratio of the measured capacitance over the initial capacitance, i.e. C/C_o, as shown in Figure 6B for a urea calibration curve. The data are displayed as a series of curves at various measurement times obtained from the results shown in Figure 6A. At short times, the data represent a mainly rate-based response, while after 10 min an equilibrium response was obtained. After 1 min, at least 5 mM of urea could be measured. Results at longer measurement times resulted in increasing resolution of urea concentrations below 5 mM. Using such curves, the urea concentration of unknown samples, prepared by standard addition of urea to normal serum, was determined. In a representative series of experiments, serum samples were spiked to contain urea levels of 7, 15, and 25 mM as determined by a reference laboratory method[15] in the Hospital Laboratory of the Department of Clinical Biochemistry. After five separate determinations, the results obtained were as follows: 9.2 ± 2.5, 17.6 ± 1.1, and 22.7 ± 2.7 mM. The precision and accuracy of this method does require improvement although in considering that crude manual methods of screen printing were employed, the results are quite satisfactory. As already mentioned, an automated screen-printing machine would not only provide greatly improved reproducibility in the printing of the electrode ink, but also in the printing of the polymer layer.

The main problem with the polymer transformation measurement method was the sensitivity of the system. While thin polymer coats were advantageous for measuring low concentrations of urea, this also proved to be a disadvantage when attempting to measure high urea concentrations under the same conditions, i.e. greater than 20 mM where the measurement system was, in fact, too sensitive since the polymer was completely transformed in a very short time. Typically, measurement of 20 mM urea in serum after 10 s showed a capacitance change of the order of $75 \pm 12\%$ compared with the initial value. This made it difficult to initiate the instrumental-impedance measurement after addition of sample

before transformation had occurred. Possibly, for urea concentrations greater than 20 mM, thicker polymer coats could be used. This would also extend the range of urea concentrations which could be measured.

The measurement of creatinine in serum using this method was also investigated. Ahlstrom pads loaded with creatinine deiminase were used; however, the method lacked sensitivity since only 2 mM creatinine could be detected after 20 min. Although the use of Ahlstrom pads for enzyme "immobilization" had the distinct advantage that as much enzyme activity could be applied onto the absorbent pad as was required, problems for creatinine measurement still remained since the creatinine deiminase enzyme could only be obtained commercially at much lower activity than urease. Creatinine deiminase supplied by Toyobo (Japan) only had an activity of 11.5 units mg^{-1} solid, compared with a urease activity of 479 units mg^{-1}. The lack of sensitivity is a problem for measurement of creatinine over the clinical range. The normal ranges for creatinine in serum are 62−115 (male) and 53−97 μM (female). In patients with kidney dysfunction or muscle disorder, however, creatinine levels can increase to at least 1000 μM. Therefore, although the system works at high creatinine levels, which would be found in cases of acute or chronic renal failure, more work is required to obtain the necessary sensitivity.

We have developed an innovative technique employing a degradable polymer layer on an electrode and the capacitance measurement of its transformation due to a specific enzymatic reaction. Thus we have introduced a new sensing concept which offers the possibility of greater sensitivity of measurement. Screen printing the electrode ink and the polymer layer is a convenient method for the mass production of cheap, single-use, disposable electrodes. The sample introduced to the sensor ideally should be whole-blood, and in a recent development we have incorporated Cytosep membranes (Ahlstrom Filtration Inc.) over the top of the sensor electrodes. These membranes will remove the cellular component and allow measurement in plasma using a whole-blood sample. The results of this study will be published in due course.

The measurement concept presented herein is generic since this type of sensing can be applied to any reactions which cause transformations in specifically designed coatings on electrode surfaces thereby leading to capacitance changes.[20]

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