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Electrochemical Sensors Based on Impedance Measurement of Enzyme-Catalyzed Polymer Dissolution: Theory and Applications

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A novel sensor approach based on ac impedance measurement of capacitance changes produced during enzyme-catalyzed dissolution of polymer coatings on electrodes, leading to a 4 orders of magnitude change in capacitance, is described. Electrodes were coated with an enteric polymer material, Eudragit S 100, which is based on methyl methacrylate, and dissolution was exemplified by utilizing the catalytic action of the enzyme urease. The resulting alkaline pH change caused dissolution of the polymer film with a consequent large increase in capacitance. A mechanism for polymer breakdown is proposed which has been validated experimentally using both ac impedance measurements and electron microscopy. The large changes in capacitance that are apparent using this technique allow much greater sensitivity of measurement than, for example, potentiometric electrodes. The potential broad clinical analytical application of this technique is demonstrated in this report by application to urea measurement and to enzyme immunoassay. Urea measurement between 2 and 100 mM has been achieved with a change in response over this concentration range by over 4 orders of magnitude. We have taken account of both the effect of protein adsorption on the surface of the polymer-coated and bare electrodes and the effect of buffer capacity when carrying out these measurements in buffered solutions containing 8% (w/v) protein and have demonstrated that the method should allow simple, interference-free measurement of urea in serum and whole blood. In addition, both competitive and noncompetitive enzyme immunoassays for human IgG based on the use of urease–antibody conjugates are reported. Human IgG, or goat anti-human IgG (Fab specific), were immobilized covalently onto cellulosic membranes via a diamine spacer group and the membranes placed over enteric polymer-coated electrodes. Specific measurement of IgG in both formats was achieved over the concentration range 0.0001–100 μg mL^{-1}. The performances of the impedance-based enzyme immunoassays were compared directly with identical assays employing spectrophotometric detection.

The concept of sensors based on an electrochemical transducer sensitized with a biological moiety is both simple and elegant and offers the prospect of reagentless clinical analysis with minimum sample preparation. The major advantage of this approach for medical use is ease of operation, thus allowing deployment of sensors in decentralized laboratories and facilitating a more rapid return of clinical information, the net benefit being an earlier institution of appropriate therapy.[1] In efforts to decrease overall analysis time and to produce methods suitable for decentralized laboratory measurement, attempts have been made to produce electrochemical sensors for clinically important analytes. To date, these have mainly been based on amperometric or potentiometric measurement using enzyme electrodes which for certain analytes have drawbacks for biosensor exploitation. The purpose of this report is to introduce a new electrochemical approach to circumvent these problems and to produce a specific, sensitive technique suitable for interference-free clinical measurement of analytes such as urea and creatinine and for application to immunoassay. In the method described in this report, we have investigated the feasibility of constructing sensors based on enteric polymer coatings which dissolve in the presence of analyte leading to highly sensitive impedance changes at underlying electrodes. Relatively little effort has been directed at the exploitation of electrode impedance measurements which have, potentially, distinct advantages for analysis including a dynamic range extending over 4 orders of magnitude and the lack of an absolute requirement for a reference electrode.

Principle of the Proposed Method. The impedance of an electrode is determined by applying a sinusoidal potential of small peak-to-peak amplitude to the electrode and measuring the resultant sinusoidal current. The frequency range used for measurement of electrode impedance is typically between 10^5 and 10^{-3} Hz. There is generally a phase difference (θ) between the potential and current so that the ratio of potential to current is essentially a vector quantity (Z) which has magnitude ($|Z|$) and direction (θ). The impedance of an electrode can be changed in many ways. For example, the adsorption of protein to an electrode will cause the electrode impedance to change.[2] However, in order to be useful as a sensor, the change of impedance must be highly specific to the substance being measured and give high sensitivity. The capacitance of the electrical double layer at an electrode can

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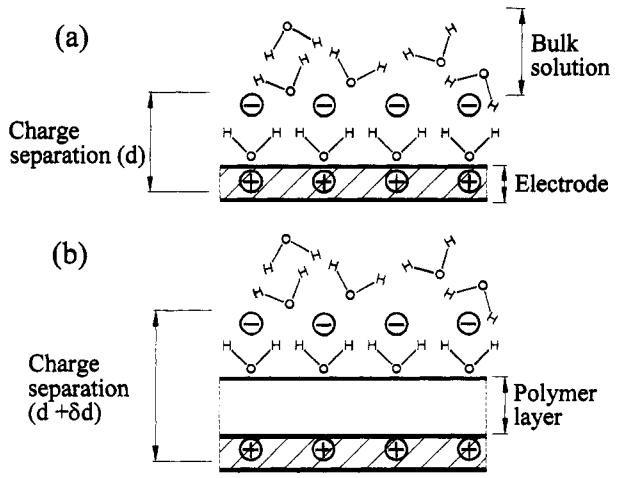

Figure 1. Schematic diagram of (a) an uncoated electrode in contact with electrolyte and (b) a polymer-coated electrode. The polymer layer increases the distance between the electrode and electrolyte by δd, thus decreasing the capacitance.

be calculated from the equation

$$C_{dl} = \epsilon_o \epsilon_r A/d$$

ϵ_o is equal to the permittivity of free space, ϵ_r is the dielectric constant of the material that separates the electrode from the mobile charges, A is the surface area, and d is the distance of closest approach of the mobile charges to the electrode surface. For a planar electrode in direct contact with an aqueous solution (Figure 1a), the double-layer capacitance value is ~20 μF cm^{-2}. If the surface of an electrode is coated with an electrically insulating layer of known dielectric constant, a dielectric, then the distance d is increased, ions are forced further from the surface of the electrode, and the capacitance value decreases (Figure 1b). The value of ϵ_r is generally also reduced, which further reduces the capacitance. This effect is small, however, in comparison to the large change produced by the increased charge separation. If a planar electrode is covered with 1 μm of an insulating polymer, the capacitance due to the double layer would be expected to change by ~4 orders of magnitude. Upon removal or partial degradation of the polymer film, a return to the original capacitance value would be observed. By coupling such large changes in capacitance values to a sensor format by using enzymes to catalyze the formation of polymer-degrading products, it is envisaged that metabolite assays and immunoassays with extended dynamic ranges and improved sensitivities in comparison to other sensor formats may be produced. We have chosen to demonstrate the application of this principle to metabolite assay and immunoassay by examining the effect of immobilized urease, in the presence of urea, on enteric polymer-coated electrodes.

Enteric Polymers. By definition, enteric coatings are polymers used in the coating of dosage forms for orally administered drugs, with the purpose of delivering the drug to specific regions of the gastrointestinal tract. These pH-sensitive coatings are moisture resistant and are known to be stable as polymer layers in contact with aqueous solutions provided that the solution acidity is high, e.g., in the acid environment of the stomach, but dissolve at higher pH, in most cases as a result of the loss of a proton from a carboxyl group, e.g., in the alkaline environment of the small intestine.[3] The pH sensitivity of enteric polymers depends on the hydrophobicity of the backbone polymer, the coating thickness, and the degree of derivatization within the acidic functional group.[4] The degree of derivatization is of vital importance to enteric polymer design, as it is the presence of ionizable groups that determines the exact dissolution pH. A sufficient portion of these acidic groups ~10%, must be ionized for water solubility to be achieved. This degree of ionization corresponds to the point at which pH rises to within one pH unit of the pK_a value. A development in enteric polymer technology are the methyl methacrylate copolymers, known by the trade name Eudragit (Röhm Pharma, Germany). The particular material used in this study, Eudragit S 100, begins to dissolve at pH 7 and is resistant to water absorption below the dissolution pH. Eudragit S 100 becomes highly soluble at pH values above 7.[5-7] In contrast, other Eudragit polymers, such as Eudragit RL, which incorporate quaternary ammonium groups, are designed for use as delayed release coatings and, instead of dissolving, swell and become permeable in aqueous solution (independent of the solution pH).[5,7]

Enteric polymers can be deposited on an electrode by solvent evaporation, and by generating OH^- ions adjacent to a polymer-coated electrode as a result of immobilization of an enzyme in intimate contact with the electrode, it should therefore be possible to sense low levels of an analyte, since 50% of the locally generated OH^- ions will react with the polymer, whereas if the OH^- ions were generated homogeneously in solution, a much smaller proportion would diffuse to, and react at, the polymer-coated electrode.

Measurement of Urea. Urea is one of the most requested analytes in the central hospital diagnostic laboratory in both routine and emergency situations. Indirect methods based on the determination of NH_3 released by the action of urease (EC 3.5.1.5) are now established as the methods of choice.[8]

Electrochemical sensors for urea have concentrated mainly on the use of urease in combination with ion-selective electrodes to produce potentiometric sensors for ammonium ions and ammonia gas.[9-11] Ammonium ion-sensitive electrodes may suffer from interference from Na^+ and K^+, while ammonia gas electrodes are prone to error due to the background levels of endogenous ammonia nitrogen. There are few reports of practical amperometric sensors for urea,[12-14] but only one method, based on the

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use of oxygen detection via horseradish peroxidase, is used commercially.

Enzyme Immunoassay. Theoretically, urease has advantages over both horseradish peroxidase (HRP) and alkaline phosphatase (AP) in enzyme immunoassay as it has considerably higher activity on a molar basis,[15] therefore allowing a greater turnover of substrate to measurable product, and thus potentially creating assays with improved limits of detection and sensitivity. This should be particularly true when used in combination with the electrode impedance measurement system described in this report.

A number of urease-labeled immunoassays have been developed which utilize pH-sensitive chromogens as indicators.[16-18] Typically, urease has been used in semiquantitative, yes/no, immunoassays, due to the sharp and unequivocal color change produced. Potentiometric immunoassays based upon urease conjugates have also been produced.[19,20] Meyerhoff and Rechnitz[19] reported the use of urease conjugates with potentiometric detection, using an ammonium ion-selective electrode, in a model immunoassay for BSA and a fully optimized competitive immunoassay for cAMP. Such sensors suffered from the drawbacks usually associated with potentiometric measurement, i.e., interference from sample components and poor sensitivity.

Relatively recently, urease-based sensors based on the measurement of conductance have been produced.[21-23] An immunoassay based on this principle has been developed by Thompson et al.,[24] using a steel rod electrode, which could be lowered into standard polystyrene microtiter wells in which a two-site immunoassay, using a urease-labeled second antibody, for human chorionic gonadotrophin (hCG) had been performed.

Immunosensors not based on enzyme labels have also been developed which measure the change in the dielectric properties of an electrode as a direct result of a specific binding interaction between antibody and antigen.[25-28] It was shown by Gardies and Martelet,[27] using silicon/silicon dioxide electrodes to which anti-α-fetoprotein had been covalently coupled, that after exposure to serum containing α-fetoprotein, concentration-dependent changes in electrode capacitance could be measured. However, the direct binding of protein on an electrode surface produced impedance changes of typically less than 15%,[29] a change considered too small to be of practical use in a commercial device. In addition, nonspecific binding is a major problem when such small changes in signal are considered.

In this paper we report preliminary studies of both competitive and noncompetitive enzyme immunoassay formats for human IgG based on the use of urease conjugates and impedance measurement of enteric polymer dissolution.

EXPERIMENTAL SECTION

Reagents. Jack bean urease (Type VI), β-NADH (disodium salt), α-ketoglutarate (disodium salt), L-glutamate dehydrogenase (EC 1.4.1.3, Type III from bovine liver), human immunoglobulin G (h-IgG, technical grade), goat anti-h-IgG (Fab specific), bovine serum albumin (BSA, Fraction V), glutaraldehyde (Grade II, 25%), Tween 20, putrescine (tetramethylenediamine, 98%) and 1,1′-carbonyldiimidazole were obtained from the Sigma Chemical Co. (Dorset, UK). Goat anti-h-IgG–urease conjugate (heavy- and light-chain specific) was obtained from Biogenesis (Bournemouth, UK). Urea was obtained from BDH Chemicals Ltd. (Dorset, UK). Dibutyl phthalate and bromocresol purple (indicator grade, sodium salt) were obtained from Aldrich (Dorset, UK). Regenerated cellulose membranes (0.2-μm pore size) were obtained from Sartorius AG (Göttingen, Germany). Eudragit S 100 polymer was obtained from Dumas (UK) Ltd. (Kent, UK). Acetone was obtained from Fisons Scientific Equipment (Loughborough, UK). Buffer solutions were prepared using AnalaR grade reagents from BDH Ltd. All buffers and solutions were prepared using distilled water passed through a Milli-Q purification system (Millipore).

Apparatus. Impedance measurements were performed using a Schlumberger Solartron 1253 gain-phase analyzer and Schlumberger Solartron 1286 electrochemical interface (Schlumberger Technologies, Hampshire, UK). The Schlumberger Solartron equipment was interfaced to an IBM-compatible personal computer via an IEEE card obtained from National Instruments UK (Berkshire, UK). Instrument operation and data acquisition was controlled using "in-house" software. All impedance measurements were performed in the two-electrode mode. The counter electrode was a 4-mm-diameter glassy carbon disk sealed in a 10-cm-long PTFE tube. This was placed directly opposite and parallel to the gold ink working electrode in the template with a separation of ~3 mm. All impedance measurements were performed at zero dc potential, with respect to the counter electrode, with an ac peak-to-peak amplitude of 30 mV using a 5-s integration time. Electrodes for impedance measurements were manufactured by Gwent Electronic Materials Ltd. (Wales, UK) using a gold organometallic ink screen printed onto a ceramic substrate. The electrodes were fitted into a 10-well Perspex template containing silicone O rings, which exposed a 6-mm-diameter (0.28 cm^2) working area at the bottom of a well to applied solutions. The total capacity of each well was 1 mL. Electrodes were spray-coated with Eudragit S 100 polymer solution using an air brush system (BioDot Ltd., Cambridgeshire, UK). Spectrophotometric measurement of urease activity was carried out using a Titertek Multiscan MCC/340 microtiter plate reader (IQ Systems, Cambridge, UK).

Spray Coating of Electrodes with Eudragit S 100. A 1.1-g sample of Eudragit S 100 polymer was dissolved in 13.7 g of acetone containing 0.25 g of dibutyl phthalate. A total of three layers (three spray passes) were sprayed over the working area of the gold ink electrodes, with each layer being allowed to dry for ~20 min before the next layer was applied. To ensure effective drying of the polymer film the electrodes were left for at least 24 h at room temperature before use.

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Polymer Dissolution Mechanism. Initial impedance measurements were carried out in 2 mM phosphate buffer (pH 5.2) containing 1 M NaCl. The dissolution of the polymer was then initiated by removing the measuring solution and adding 100 μL of 0.1 M phosphate buffer (pH 7.8) over the electrodes in the template described previously. In order to halt polymer dissolution at different stages during the breakdown, the experiment was interrupted by removing the pH 7.8 phosphate buffer, rinsing the template well with deionized water and adding the same electrolyte as for the initial measurements. Electron micrographs of the polymer film-coated gold ink electrodes were taken at the same stages of dissolution.

Immobilization of Urease on Membranes. A 450-mg aliquot of 1,1′-carbonyldiimidazole (CDI) was dissolved in 10 mL of acetone (total 4.5% w.v), and 100 6-mm-diameter disks of regenerated cellulose membrane were placed into the solution. The contents were gently mixed by rotation for 16 h at room temperature. The disks were then removed and washed several times with acetone. The CDI-activated membranes were stored in acetone at room temperature until required. Urease was dissolved in 0.1 M sodium carbonate buffer (pH 9.6) to a concentration of 10 mg mL^{-1}. CDI-activated membranes were removed from the acetone, blotted dry on tissue paper, and placed into the enzyme solution. The membranes were incubated with agitation for 16 h at 4 °C. The membranes were then removed, rinsed, and stored in 140 mM sodium chloride solution (pH 6.5) containing 0.2 mM EDTA at 4 °C until required. The activity of the urease-loaded membranes was measured using a coupled enzyme reaction employing glutamate dehydrogenase.[8] The rate of NADH consumption was followed by measuring the change in absorbance at 340 nm. A standard curve with soluble urease was used to estimate the amount of urease activity immobilized onto the membrane disks.

Urea Assay Based on Impedance Measurement. A 6-mm-diameter urease-loaded membrane disk was placed over the working area of an Eudragit S 100-coated gold ink electrode. The membrane was gently pressed down and the electrode loaded into the template. A silicone O ring was placed on top of the membrane and used to ensure a leak-proof seal between the electrode and the template. A solution consisting of 140 mM NaCl and 0.2 mM EDTA, adjusted to pH 6.5 using 0.1 N HCl, was used as the electrolyte from impedance measurements and for the preparation of urea standard solutions. The template well was filled with 200 μL of urea standard solution (1−100 mM), the counter electrode was placed in position, and impedance measurements were carried out at a fixed frequency of 20 kHz using an external measuring resistor of 10 kΩ. In addition, to investigate the effect of buffer capacity and protein in a simulated serum matrix, urea solutions in 10 mM phosphate buffer (pH 6.5) containing 8% (w/v) BSA, 0.2 mM EDTA, and 0.14 M NaCl were prepared and used as described above.

Immobilization of h-IgG. Fifty regenerated cellulose membranes (6-mm diameter) were activated with CDI as described previously. After activation, the membranes were placed into 10 mL of 0.5 M sodium carbonate buffer (pH 9.6) containing 0.3 M putrescine and mixed by rotation for 16 h at room temperature. The membranes were then removed and washed several times with distilled water before addition to 10 mL of a 25% (w/v) solution of glutaraldehyde. Thereafter, the membranes were mixed by rotation for 30 min. The disks were then washed several times with distilled water before placing the membranes in 3 mL of a 3 mg mL^{-1} solution of h-IgG in 0.5 M sodium borate buffer (pH 10). The membranes were then mixed by rotation for 4 h at room temperature, then placed in 5 mL of 0.1 M Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1% (w/v) glycine to neutralize any unreacted glutaraldehyde. After mixing for 30 min, the membranes were removed and washed several times with 0.1 M Tris-HCl buffer (pH 7.5) prior to storage at 4 °C in this buffer until required. Prior to use in a competitive immunoassay format, potential protein binding sites on the membranes were blocked using 5 mL of 0.1 M Tris-HCl buffer (pH 7.5) containing 1% (w/v) BSA.

Impedimetric Competitive Immunoassay. A range of h-IgG standards were prepared in 0.1 M Tris-HCl buffer (pH 7.5) containing 1% BSA, and 100 μL of each standard competed against 100 μL of a 1:250 dilution of anti-IgG−urease conjugate for IgG-binding sites on regenerated cellulose membranes for 1 h. Thereafter the membranes were removed and washed several times with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1% (v/v) Tween 20. They were then rinsed with deionized water and the urease activity of the membranes, after competitive binding of anti-IgG−urease conjugate, was assessed by impedance measurement. Individual membranes were placed over the working surface of polymer-coated gold ink electrodes. The electrodes were inserted into the template, and each template well filled with 90 μL of 0.2 mM EDTA containing 140 mM NaCl (pH 6.5). The initial impedance of each electrode at 20 kHz was measured, using an external measuring resistor of 10 kΩ and then 10 μL of 1 M urea in EDTA/NaCl solution added. The impedance was then monitored over a 1 h period. Final capacitance values were calculated and the ratio of final to initial capacitance (C_f/C_o) taken. In addition, for comparative purposes, the urease activity of each membrane was measured spectrophotometrically at 540 nm using bromocresol purple color reagent[16] according to the following procedure. After the immunological reaction had been carried out and the membranes washed, each membrane was immersed in 200 μL of substrate solution. This was prepared by dissolving 8 mg of bromocresol purple in 1.5 mL of sodium hydroxide and diluting with 100 mL of water to which 100 mg of area and 7 mg of EDTA were added. The pH of this solution was adjusted to 4.8 with dilute sodium hydroxide. Optical density measurements after a fixed time were made in microtiter plate wells after removing a 50-μL aliquot of substrate solution.

Immobilization of Anti-h-IgG. Regenerated cellulose membranes were activated with CDI, putrescine, and glutaraldehyde exactly as described previously. A 10-μL aliquot of a 2.3 mg mL^{-1} solution of goat anti-h-IgG in phosphate-buffered saline (pH 7.4) was then spotted onto the activated membranes. The spotted membranes were then stored in Tris-HCl buffer at 4 °C until required. Prior to the use in a two-site noncompetitive immunoassay format, potential protein binding sites on the membranes were blocked in 5 mL of a 0.1 M Tris-HCl buffer (pH 7.5) solution containing 1% (w/v) BSA.

Impedimetric Sandwich Immunoassay. Individual anti-h-IgG-coated membranes were incubated in 100 μL of a range of h-IgG standards in 0.1 M Tris-HCl/1% BSA buffer (pH 7.5) for 1 h at room temperature. The membranes were washed several times with Tris-HCl/0.1% Tween 20 and then individually incubated with 100 μL of anti-h-IgG−urease conjugate solution diluted 1:500 with 0.1 M Tris-HCl/1% BSA buffer for 1 h at room

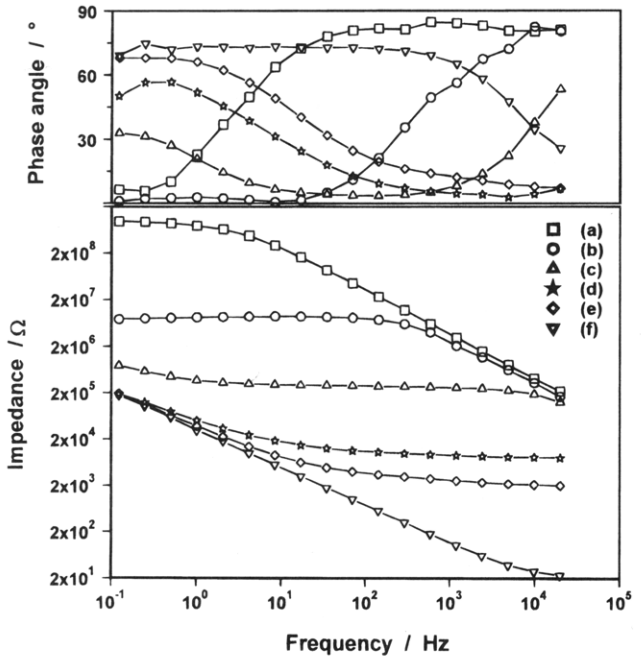

Figure 2. Bode plots of (a) a polymer-coated electrode prior to breakdown, (b–e) partially degraded polymer films, and (f) a bare electrode.

temperature. This was followed by washing several times with Tris-HCl/0.1% Tween 20. The amount of urease labeled bound to the membranes was assessed by both impedance and spectrophotometric measurement exactly as described previously.

RESULTS AND DISCUSSION

Mechanism of Polymer Breakdown. We have carried out simple qualitative experiments to demonstrate that Eudragit S 100 dissolves completely at pH values greater than 7. This involved titrating a suspension of the polymer (1 g in 50 mL of distilled, deionized water, pH 4.5) with 0.1 M NaOH and observing the formation of a homogeneous solution above pH 7. The mechanism of dissolution of the enteric polymer coated on gold ink electrodes was then investigated using impedance spectroscopy. Basically, three different states of the Eudragit S 100-coated gold ink electrodes were examined, the initial film, bare gold electrode, and intermediate stages of partial dissolution of the polymer.

The impedance spectrum of the initial film showed capacitive behavior over a large frequency region (spectrum a in Figure 2); i.e., the phase angle was close to 90°. This indicated that the Eudragit S 100 films provided good insulating properties. The geometric capacitance calculated from a fit of the spectrum was 177 pF cm^{-2} and corresponded to a dielectric constant of $\epsilon_r = 7$. From the spectrum of a bare electrode (Figure 2, spectrum f), a double-layer capacitance for the gold/electrolyte interface of ~25 μF cm^{-2} was determined, as would be predicted from theoretical considerations.

Impedance spectra of partially dissolved films are represented in spectra b–e in Figure 2. Interestingly, polymer dissolution from the electrode surface could be observed visually during the course of these experiments. For the first stage of breakdown (spectrum b), the geometric capacitance of the polymer was 188 pF cm^{-2}, i.e., nearly the same as for the initial state (spectrum a). The low-frequency region of the spectrum showed a resistive behavior, which can be ascribed to the formation of pores in the polymer

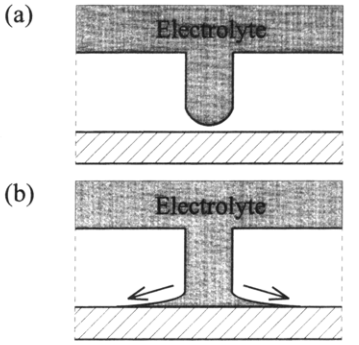

Figure 3. Schematic diagram showing the proposed mechanism of pore formation in the polymer film in alkaline solution: (a) Electrolyte begins to penetrate the polymer; (b) electrolyte penetrates the polymer to the gold electrode and spreads across the surface beneath the polymer.

layer. The resistance reflected the conductivity of the electrolyte in the pores and allowed an estimate of the relative porosity of the film. For this purpose, the resistance calculated from the impedance data was divided by the resistance of an electrolyte layer that would occupy the same space as the initial polymer (0.034 Ω cm^2). For spectrum b, a porosity of 1.7×10^{-8} was obtained. In the frequency range used for the measurements, the limiting low-frequency behavior of spectrum b was purely resistive. Therefore, in this instance the electrolyte did not penetrate the polymer and contact the gold surface (Figure 3a). At later stages of breakdown, as shown in Figure 2, spectra c–e, where the electrolyte had penetrated the polymer film to contact the electrode surface, the low-frequency impedance was capacitive and represented the wetted area of the gold electrode. For these partially dissolved films, the ratio of the double-layer capacitance of a polymer-covered electrode to a bare electrode gave the fraction of the gold surface wetted with electrolyte.[30]

At the second stage of breakdown (Figure 2, spectrum c), the geometric capacitance of the polymer was still the same (188 pF cm^{-2}). However, the porosity of the film had increased to 4.9×10^{-7} and the double-layer capacitance, by comparison with that of a bare electrode, showed that ~8.5% of the gold surface was wetted with electrolyte. The relatively high fraction of wetted electrode surface in combination with the small porosity of the film can only be explained by spreading of the electrolyte on the gold surface beneath the Eudragit S 100 polymer layer (Figure 3b).

These results were confirmed by the impedance spectra of films which had been broken down further. For spectrum d in Figure 2, a geometric capacitance of 425 pF cm^{-2} and a porosity of 1.5×10^{-5} were calculated, while ~90% of the surface area was wetted. At the next stage of breakdown, spectrum e, the geometric capacitance could no longer be measured. From the electrolyte resistance in the pores, a relative porosity of 5.6×10^{-5} was estimated. Since the double-layer capacitance obtained from spectrum e was identical to the capacitance of a bare electrode, essentially 100% of the gold surface was covered with electrolyte.

From the results described, a probable mechanism for polymer dissolution on the electrode surface can be derived. When the polymer film is exposed to a pH higher than 7.0, initially partial pores are formed. This was confirmed by electron micrographs (Figure 4), which showed the formation and growth of holes in

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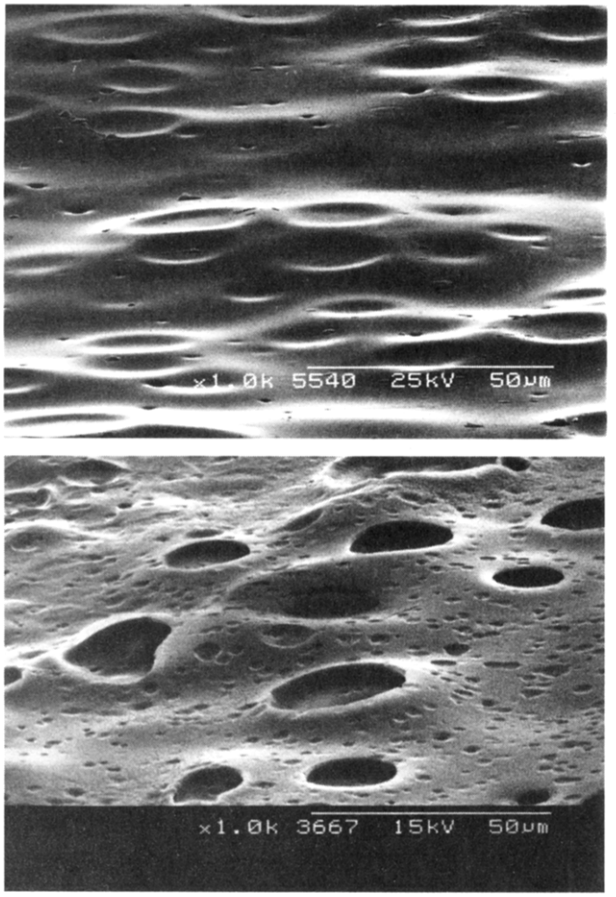

Figure 4. Electron micrographs of (a, top) a polymer-coated electrode prior to breakdown and (b, bottom) a partially broken down polymer film on the electrode showing pore formation.

the film that, in cross section, was ~36 μm thick. Further dissolution resulted in the penetration of the electrolyte to the gold electrode where it spread across the surface (Figure 3b). Further investigations are in progress which will provide a more detailed explanation of the dissolution mechanism.

Urea Measurement. The polymer-coated electrodes with urease membranes in place initially exhibited capacitive behavior at a frequency of 20 kHz. This enabled the initial capacitance of the electrode to be calculated (C_o). The initial C_o values of electrodes prepared in an identical fashion showed some degree of variation (0.4 ± 0.1 nF cm^{-2}, n = 11).

Using the coupled enzyme assay method, the activity of the membrane-bound urease was determined to be 0.135 unit/disk based on a specific activity of the urease preparation of 13.5 units mg^{-1}. Incubation of an uncoated membrane disk with the assay reagents gave no significant changes in absorbance.

The impedance of the electrodes was measured as a function of incubation time with urea standard solutions over the range 1–100 mM. The imaginary impedance component (Z'') at 20 kHz was used to calculate the electrode capacitance (C_f) at all times. The time-dependent values of C_f/C_o as a function of urea concentration are shown in Figure 5. By using the ratio C_f/C_o we should be able to account, to a large extent, for the initial variability in capacitance. For example, after 10 min the coefficient of variation of the C_f/C_o ratio for five replicate measurements of 10 mM urea was 3.6%. Using the data generated after a 10-min incubation, it was apparent from C_f/C_o vs log [urea] calibration curves that, over the range of urea concentrations from 2 to 100

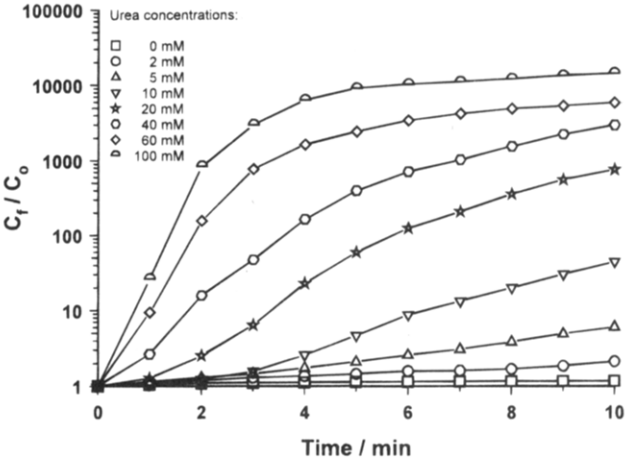

Figure 5. Effect of urea concentration in 140 mM NaCl and 0.2 mM EDTA (pH 6.5) on the time-dependent change in C_f/C_o ratio using a urease-active cellulose membrane laid over an Eudragit S 100 polymer-coated electrode. A calibration curve of ln C_f/C_o vs ln [urea] generated using the 10-min incubation data gave a linear response over the range 2–100 mM ($y = 2.21x - 0.88$, $r = 0.99$).

mM, this ratio changed by over 4 orders of magnitude. However, due to the complex mechanism of polymer removal, the capacitance ratio increased nonlinearly with urea concentration. The mean value (n = 10) of C_f/C_o after a 10-min incubation in the absence of urea was 1.1 ± 0.1, indicating that the use of the capacitance ratio did significantly improve the signal variation. Thus these results and the data shown in Figure 5 demonstrate clearly that the full potential of the method could be achieved in these measurements. Obviously a prime future concern is the development of methodology that would provide highly reproducible values of C_o, and to this end we are currently investigating the use of spin-coating of the polymer onto the electrodes.

When considering changes in capacitance at electrode surfaces in biological samples for the ultimate development of sensors capable of measurement in undiluted serum or whole blood, it is obviously important to take account of both the effect of protein adsorption on the surface of the polymer-coated and bare electrodes and the effect of buffer capacity. Adsorption of proteins is known to cause impedance changes at electrodes. For example, Lacour et al.[29] have shown that the capacitance of a gold electrode will decrease by 15–20% when exposed to a solution of bovine serum albumin (BSA) for 30 min. However, at a polymer-coated electrode, protein adsorption will not appreciably change the effective thickness of the insulating dielectric layer and therefore will not give rise to a measurable change in capacitance. We have verified experimentally that this is the case by measuring the capacitance of Eudragit S 100-coated gold ink electrodes in a 10 mM phosphate buffer solution containing 10 mM urea, 0.2 mM EDTA, and 0.14 M NaCl in the presence and absence of 8% (w/v) BSA. This buffer and protein concentration was used to simulate the situation that would be encountered in serum samples since although the major buffering system in serum is related to the balance between bicarbonate and dissolved CO_2/carbonic acid, proteins also significantly contribute to the buffer capacity. The capacitance of a polymer-coated electrode after a 30-min exposure to BSA was 0.38 nF cm^{-2}. This was identical to the value measured in the absence of BSA after the same time. Therefore, protein binding to the intact polymer should not be a source of interference in this method. Removal of the polymer film using

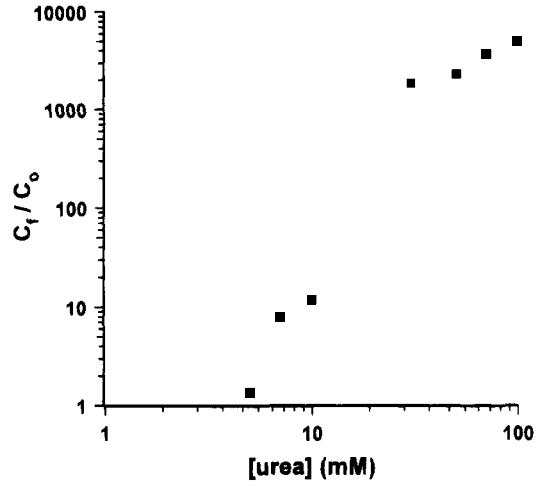

Figure 6. Calibration curve (10-min incubation) for urea assay in a simulated serum matrix [10 mM phosphate buffer (pH 6.5) containing 8% (w/v) BSA, 0.2 mM EDTA, and 0.14 M NaCl] using a urease-active cellulose membrane laid over an Eudragit S 100 polymer-coated electrode.

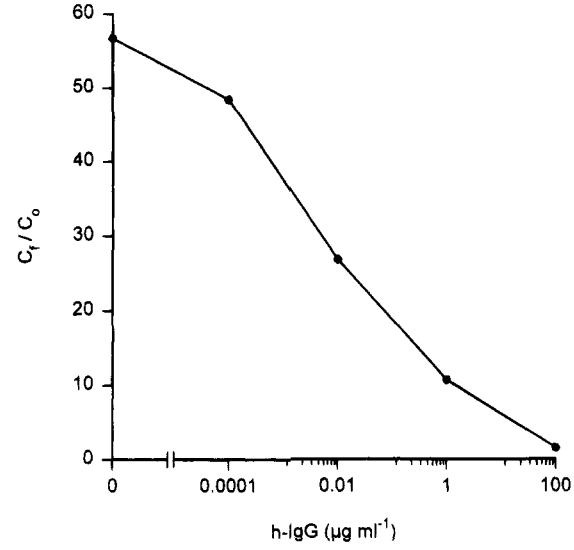

Figure 7. Calibration curve for impedimetric competitive immunoassay for h-IgG.

a urease membrane located over the electrode caused the capacitance to change to a final value of 5.7 μF cm^{-2} in both cases, although it was noticed that the rate of polymer breakdown was slowed by ~20% in the presence of protein. In parallel with these experiments, the change in potential due to the pH change upon the action of a urease membrane with the same 10 mM buffered urea solution was monitored. An overall change in potential of ~120 mV was observed (equivalent to a 2 order of magnitude change in H^+ concentration) compared with a simultaneous 4 order of magnitude change in capacitance. This demonstrated the potential sensitivity of impedance analysis compared with simple pH measurement.

To investigate further the effect of buffer capacity and protein during urea-catalyzed polymer dissolution, we generated calibration curves after a 10-min incubation with urea standard solutions prepared in 10 mM phosphate buffer (pH 6.5) containing 8% (w/v) BSA, 0.2 mM EDTA, and 0.14 M NaCl (Figure 6). It was apparent that although the presence of buffer and protein decreased the rate of polymer dissolution, such that the detection limit after 10 min was increased from 2 to 5 mM (cf. Figure 5), the system still displayed a 4 order of magnitude change in impedance. It is thought that the decrease in dissolution rate was probably caused to some extent by protein entering the pores during formation; however, since the polymer acts by dissolving rather than swelling this does not cause serious nonspecific interference.

The experiments carried out have shown clearly that the method described in this report can operate in protein-containing buffered solutions. While it is recognized that protein may bind to the bare metal after complete removal of the polymer film, by making measurements at a particular time when the polymer is being removed, this source of potential interference will be avoided. It should also be stressed that protein binding directly to the electrode surface may alter the capacitance by ~15%[29] while removal of the dielectric enteric polymer will cause a 4 order of magnitude change.

Work is currently in progress to increase the rate of polymer dissolution in the presence of protein and buffer by use of wicking materials[31] and also to produce prototype instrumentation based on single-frequency capacitance measurement for urea determination in whole blood or serum. To achieve this, the synthesis of enteric polymers that are stable at the pH of these matrices is being undertaken. Heller et al.[32] reported that for a series *n*-butyl and *n*-pentyl half-esters, depending on the degree of esterification, the dissolution pH can be controlled to occur anywhere in the region pH 5–7.5. Further, they described that esterification of *n*-hexyl and *n*-heptyl half-esters to ~60% will produce polymers with dissolution pH's between 7.5 and 8.

Impedimetric Immunoassay. A competitive immunoassay for h-IgG was carried out using h-IgG and a limiting amount of membrane-bound h-IgG competing for goat anti-h-IgG–urease conjugate in solution. Figure 7 shows a typical competitive assay response curve produced using capacitance measurement. As would be expected, similar behavior was observed when colorimetric detection using bromocresol purple was employed. Membranes that had no h-IgG immobilized to the surface but that were blocked using BSA showed no significant responses in either the capacitance or colorimetric measurement formats when exposed to the goat anti-h-IgG–urease conjugate. Thus, the degree of nonspecific binding to the cellulose membrane surface was minimal.

Colorimetric detection demonstrated poor sensitivity at low h-IgG concentrations (<0.01 μg mL^{-1}) due to the sharp and unequivocal color change of the pH indicator dye, bromocresol purple. Optical density values, at 540 nm, of the low standards reached a plateau after 15 min of incubation (data not shown). In contrast, no such "saturation" effect was observed using capacitance measurement. The curve shown in Figure 7 was obtained 1 h after the addition of 100 mM urea, and discernible concentration-dependent responses could also be obtained after 15 min.

The two-site assay format, with F_{ab}-specific anti-h-IgG immobilized at the surface of regenerated cellulose membranes, produced typical noncompetitive immunoassay standard curves using capacitance measurement (Figure 8). Colorimetric detec-

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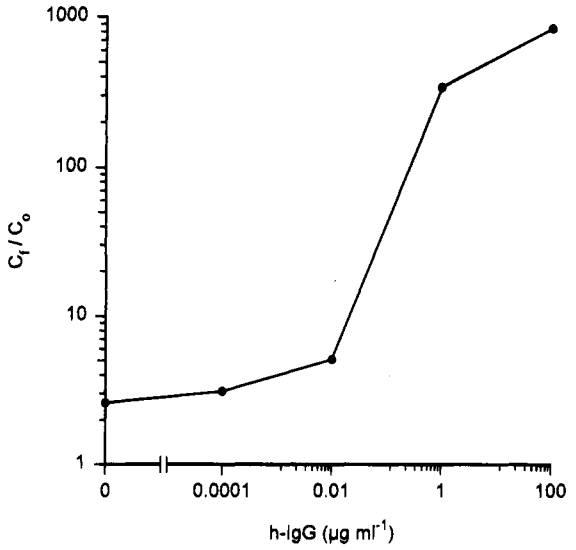

Figure 8. Calibration curve for impedimetric noncompetitive immunoassay for h-IgG.

tion using bromocresol purple once again proved to have limitations since the optical density measurements at 540 nm for h-IgG standards above 0.1 μg mL^{-1} reached a plateau value after a 30-min incubation (data not shown). Using capacitance measurement, the standard curve shown (Figure 8) was obtained after a 1-h incubation with 100 mM urea, although it was possible to measure concentration-dependent responses after a 20-min incubation with substrate (data not shown).

The capacitance ratios (C_f/C_o) shown for both the competitive and two-site assays were once again used to minimize the effect of the degree of interelectrode initial capacitance value irreproducibility. It was apparent that in both h-IgG immunoassay formats capacitance measurement produced an increased dynamic range compared with spectrophotometric detection. Further optimization of both immunoassay formats, in terms of electrochemical cell design and the possible use of wicking systems as the immunological capture phase,[31] should allow a significant decrease in substrate incubation time for capacitance measurement.

In summary, we have demonstrated the feasibility of a new concept for electrochemical sensors based on the measurement of the change in electrode impedance upon the degradation of polymer coatings as a result of specific interactions. We are currently extending this approach to the interference-free measurement in blood of other clinically important analytes such as creatinine. The major advantages of this technique for such analytes are the wide dynamic range available and the inherent sensitivity of the method. In addition, we are examining the use of alternative approaches to polymer breakdown such as Fenton chemistry based upon free radical attack on electrode coatings such as *cis*-polyisoprene.[33,34]

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