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1a. REPORT SECURITY CLASSIFICATION Unclassified			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY ELECTED			3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited.		
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE 17 1989			5. MONITORING ORGANIZATION REPORT NUMBER(S) ARO 23106.3-LS		
4. PERFORMING ORGANIZATION REPORT NUMBER(S) D 03			7a. NAME OF MONITORING ORGANIZATION U. S. Army Research Office		
6a. NAME OF PERFORMING ORGANIZATION Research Triangle Institute		6b. OFFICE SYMBOL (If applicable)	7b. ADDRESS (City, State, and ZIP Code) P. O. Box 12211 Research Triangle Park, NC 27709-2211		
6c. ADDRESS (City, State, and ZIP Code) Research Triangle Park, NC 27709		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAAL03-86-K-0036			
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U. S. Army Research Office		8b. OFFICE SYMBOL (If applicable)	10. SOURCE OF FUNDING NUMBERS		
8c. ADDRESS (City, State, and ZIP Code) P. O. Box 12211 Research Triangle Park, NC 27709-2211		PROGRAM ELEMENT NO	PROJECT NO	TASK NO	WORK UNIT ACCESSION NO
11. TITLE (Include Security Classification) Protein-Modified Electrochemically Active Biomaterial Surface					
12. PERSONAL AUTHOR(S) Mirtha Umana					
13a. TYPE OF REPORT Final		13b. TIME COVERED FROM 2/1/86 TO 1/31/89		14. DATE OF REPORT (Year, Month, Day) December 1988	
15. PAGE COUNT 9					
16. SUPPLEMENTARY NOTATION The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	Biomaterial, Enzymes, Glucose Oxidase, Electropolymerization, Pyrrole, Organic Polymers		
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
This research was initiated with a novel approach to electrode immobilization of an enzyme, glucose oxidase, by electropolymerization of pyrrole in the presence of the enzyme. The enzyme was entrapped by the polypyrrole film growing on the electrode surface. Since polypyrrole is an electronically conducting organic polymer, the effect of this immobilization was to enfold the enzyme in a conducting electrode. Continued on back side					
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL			22b. TELEPHONE (include Area Code)		22c. OFFICE SYMBOL

In addition, the products of the enzyme reaction with glucose were produced in the immediate proximity of this conducting material. The electropolymerized enzyme is active and serves as the basis for a glucose electrode system which remained active for several days. The conditions for electropolymerization and the characteristics of the immobilized enzyme system were reported in the open literature.

PROTEIN-MODIFIED ELECTROCHEMICALLY ACTIVE BIOMATERIAL SURFACE

FINAL REPORT

Mirtha Umaña

December, 1988

U.S. ARMY RESEARCH OFFICE
Contract Number: 03-86-K0036

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OBJECTIVES

The overall goals of this three-year research program were:

- 1) to immobilize proteins on an electrode surface by using immobilization media not previously explored, such as the conducting organic polymer;
- 2) to study the reactivity of enzyme and antibody biocatalysts in this novel microenvironment;
- 3) to obtain a fundamental understanding of protein behavior in relation to the new microenvironment, which may exhibit a mildly hydrophobic character similar to that of biological matrices;
- 4) to study the stability and chemical reactivity of the polymer/protein system attached to the surface of the electrode; and
- 5) to exploit the reaction chemistry of the immobilized proteins for the development of new, sensitive, and specific analytical biosensors. In order to achieve high specificity, enzymes and antibodies were employed.

Potential benefits that may result from this research are specific biosensors of interest to clinical analysis, biotechnology industries and the Department of Defense.

SUMMARY OF RESULTS

This research was initiated with a novel approach to electrode immobilization of an enzyme, glucose oxidase, by electropolymerization of pyrrole in the presence of the enzyme. The enzyme was entrapped by the polypyrrole film growing on the electrode surface. Since polypyrrole is an electronically conducting organic polymer, the effect of this immobilization was to enfold the enzyme in a conducting electrode. Figure 1 shows a schematic representation of this immobilization. In addition, the products of the enzyme reaction with glucose were produced in the immediate proximity of this conducting material. The electropolymerized enzyme is active and serves as the basis for a glucose electrode system which remained active for several days. The conditions for electropolymerization and the characteristics of the immobilized enzyme system were reported in the open literature [1].

Initial experiments included:

- (1) preparation and characterization of polypyrrole-modified electrodes under conditions compatible with enzyme immobilization,
- (2) demonstration that polypyrrole-modified electrodes are relatively stable and applicable for the indirect determination of electrochemically active products of glucose oxidase-catalyzed reactions that occur in a solution contacting the electrode, and
- (3) demonstration that glucose oxidase becomes immobilized in a thin polypyrrole film, electropolymerized onto surfaces in the presence of the enzyme.

The following efforts focussed on preparing glucose-oxidase/polypyrrole-modified electrodes (GOx/PP/electrodes) with better long-term stability and improved response to glucose in aqueous media. Initial poor stability was attributed to GOx leaching out of the polymer matrix over time. To minimize this effect, a procedure for cross-linking the polymer was investigated whereby the freshly prepared GOx/PP/electrodes were exposed to an aqueous solution of glutaraldehyde. Several conditions for crosslinking were tested including glutaraldehyde concentrations (1% to 25%), time of exposure at room temperature (1 to 24 hours), and atmospheric conditions (air and N₂). It was determined that 2.5% glutaraldehyde over 18 to 20 hours at room temperature under an inert atmosphere gave the best results as measured by the total absence of GOx leaching into the storage solutions [2].

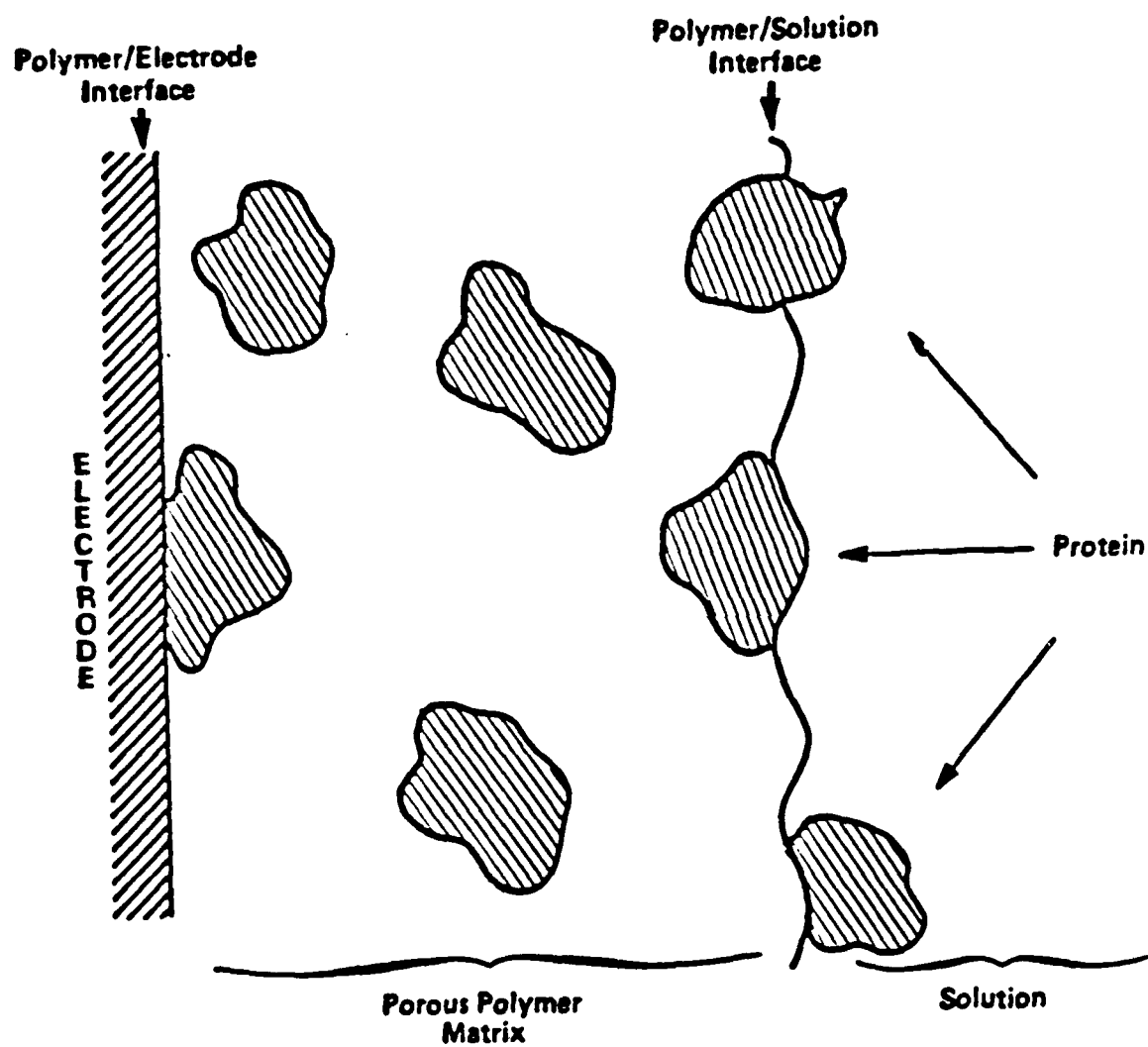
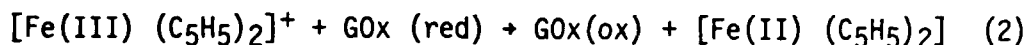


Figure 1. Schematic representation of biocatalyst trapped in polymer matrix by co-polymerization.

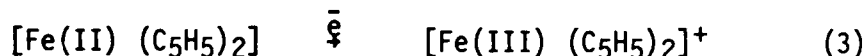
Once a stabilizing procedure was found, a cross-linking GOx/PP/electrode was used to measure glucose over a range of concentrations (5×10^{-4} to 5×10^{-2} M). The [glucose] vs. current plot was linear at low concentrations up to approximately 1×10^{-3} M. Using a procedural blank solution with a cross-linked GOx/PP/electrode, the limit of detection was estimated (at three times the background noise) as $30 \mu\text{A}$, which corresponds to 4×10^{-5} M [glucose] [3].

The following efforts were focussed on strategies for electrochemical immunoassays.

After the demonstration of the feasibility of determining glucose with a protein-modified electrode, we proceeded to utilize the same principle with the goal of developing an immunoassay using a protein-modified electrode. Experiments were performed demonstrating the use of GOx/PP/electrodes as the base for immunoassays. This was accomplished by using ferrocene as an electron transfer mediator. Ferrocene can interact with the glucose oxidase active site after its reaction with glucose:



The reaction product is then detected at the electrode:



When ferrocene is used as an electrochemical label attached to an antigen, reaction (2) is inhibited by the binding of antibody and antigen, and thus, reaction (3) could not be detected at the electrode surface. If the system is then exposed to a competitive binding in the presence of an unlabeled antigen (the analyte), the ferrocene-labeled antigen is released from the antibody, can react with GOx, and can be detected at the electrode. Thus, the system becomes a sensor for the antigen [4,5].

From our preliminary electrochemical immunoassay results, we concluded that:

1. The electrochemical response of the GOx/ferrocene carboxylic acid system is dependent on the enzyme and ferrocene carboxylic acid concentration.

2. The electrode shows a measurable response in the range of 10 to 400 μ M ferrocene carboxylic acid and 0.5 to 10 units/mL GOx.
3. In the range of 20 to 40 mM glucose concentration, the electrochemical response is independent of the glucose concentration.
4. Based on these preliminary results, the GOx/ferrocene system may be used as an electrochemical sensor where the ferrocene can act as a label for analytes of interest.

In Figure 2, the overall strategy for the electrochemical immunoassay is presented. This scheme is based on our technique to immobilize glucose oxidase (GOx) into a conducting organic polymer.

To study the scheme, we synthesized a ferrocene-antigen (FeII-Ag) conjugate using phenytoin as a model antigen. We also characterized the conjugate in terms of identity (NMR, IR, MS), purity (chromatography and elemental analysis), electrochemical behavior (cyclic voltammetry), and immunological response [6,7].

We studied the overall reaction of ferrocene and ferrocene-Ag conjugate with GOx in the presence of excess glucose and concluded that the catalytic cycle shown in Figure 2 is feasible and can be the basis for an electrochemical immunoassay [8].

To understand the fundamental processes involved in the enzyme catalytic behavior, we performed experiments to attempt a direct electron transfer from the electrode (in our case polypyrrole) to the center of the enzyme. Preliminary experiments indicate that redox behavior is observed when glucose oxidase is immobilized in polypyrrole, in the absence of any electron mediator (such as ferrocene or oxygen). This is the first reported successful attempt to achieve this direct electron transfer to the active site of a protein. However, from our results we also concluded the electron transfer process is very slow, as indicated by the scan rate response of the cyclic voltammogram. Thus, the use of polypyrrole as the electron transfer medium may not be the most advantageous for practical purposes.

An electrochemical immunoassay in solution was developed for phenytoin. The system was characterized in terms of its electrochemical behavior and its immunological response. Some kinetic parameters were calculated and the range of applicability was determined. In addition, initial experiments aimed at producing a completely immobilized immunoassay system were performed.

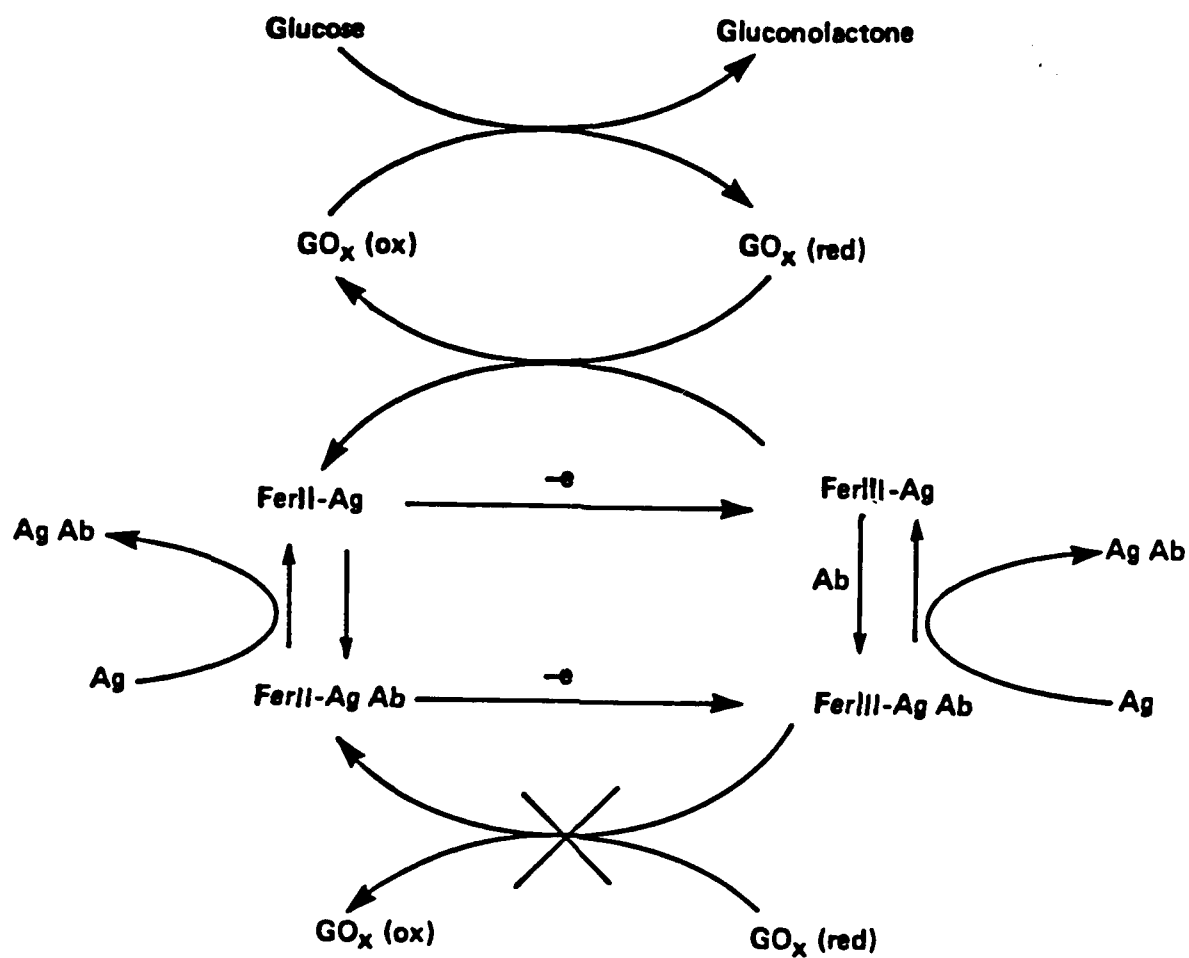


Figure 2. Schematic Representation of the Overall Reaction.

Finally, we initiated research aimed at demonstrating the feasibility of assembling a completely immobilized electrochemical immunoassay sensor. The sensor design would require no reagents and would provide a direct measurement of the analyte.

For our initial experiments, we prepared a low crosslinked polyacrylamide gel, an ionically conducting polymer. This gel is adsorbed on the surface of an electrode assembly containing a glassy carbon electrode, a platinum auxiliary electrode, and a silver pseudoreference electrode (See Figure 3). The reagents required for the immunoassay include ferrocene derivatives, glucose oxidase, glucose and antiserum. We successfully immobilized ferrocene derivatives, glucose oxidase, and glucose. We found that the solubility of the ferrocene derivatives is critical for the stability and reproducibility of the experiment. Ferrocene derivatives which are insoluble in water, such as ferrocene and ferrocene-phenytoin conjugate, require the addition of up to 30 percent acetonitrile to the gel. The addition of solvent does not appear to have a negative effect on the gel or the enzyme; instead, it gives a reproducible and stable electrochemical response. However, 30-50% acetonitrile has a negative effect on the antiserum and thus the use of lower amounts of the solvent, ideally 10%, is recommended.

The final procedure utilizes 10% acetonitrile-water medium. The reagents (glucose, glucose oxidase, ferrocene-DPH conjugate, and anti-DPH serum) were all immobilized in the gel and allowed to equilibrate for 30 minutes. The analyte, DPH, is then added to the gel and the current increase is recorded. This experiment is the proof-of-concept for a completely immobilized electrochemical immunoassay sensor [8].

Further work in this area should include a complete characterization of the sensor including determination of the limit of detection, linear range, accuracy, precision, interferences, shelf life, durability, ruggedness, and other potential analytes.

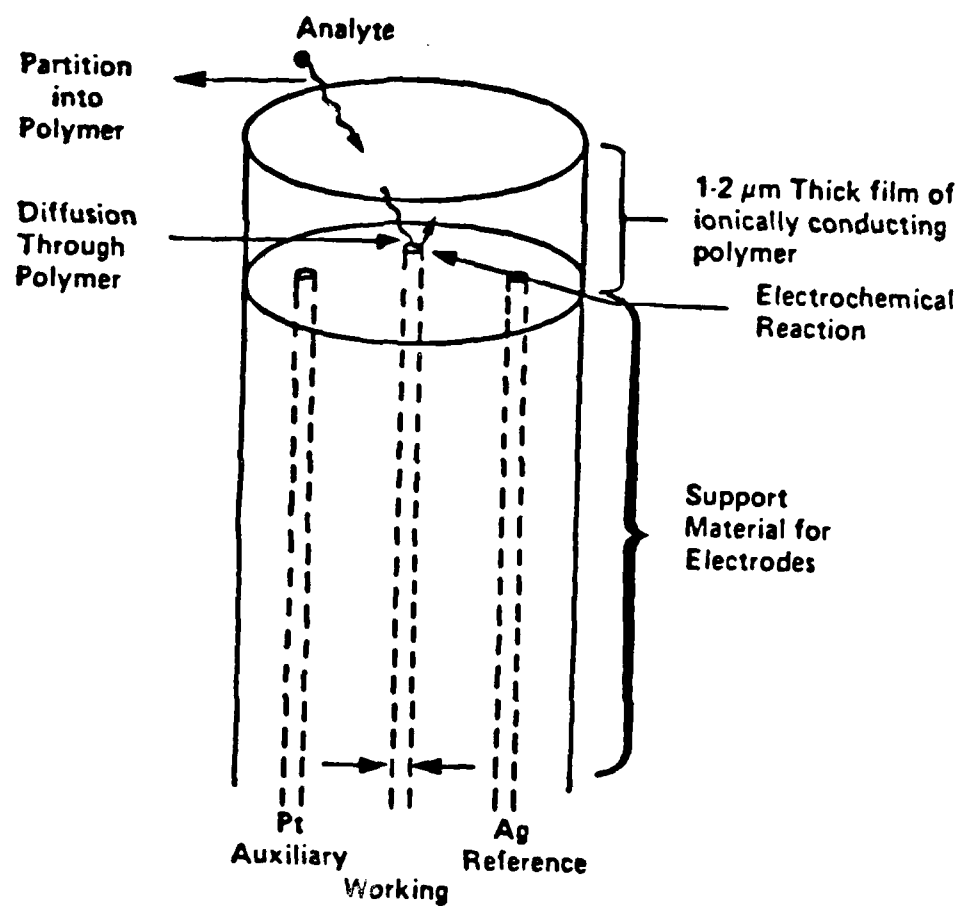


Figure 3. Schematic representation of microelectrode system.

LIST OF PUBLICATIONS AND PRESENTATIONS

- [1] "Protein-Modified Electrodes. I. Glucose Oxidase/Polypyrrole," M. Umaña and J. Waller, *Anal. Chem.*, 58, 2979, 1986.
- [2] "Protein-Modified Electrodes II. The Glucose Oxidase/Polypyrrole System." M. Umaña and J. Waller. Proceedings of U.S. Army CRDEC Scientific Conference on Chemical Defense Research, Aberdeen Proving Ground, MD. November 18-21, 1986.
- [3] "Biosensors Based on Protein-Modified Electrodes," M. Umaña and J. Waller. Army Research Office, Fourth Biodefense Workshop, Cashiers, NC, 1986.
- [4] "Protein-Modified Electrodes," M. Umaña, C. Whisnant, E. Cook, and J. Waller, International Bioanalytical Workshop, University of Kansas, Lawrence, Kansas, 1987.
- [5] "Protein-Modified Electrode Approach to Biosensors." M. Umaña, C. Whisnant, E. Cook, Symposium on Accuracy in Trace Analysis-Accomplishments, Goals, Challenges, National Bureau of Standards, Gaithersburg, Maryland, 1987.
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- [7] Electrochemical Immunoassay for Phenytoin," M. Umaña, C. Whisnant, and E. Cook, Army Research Office Workshop, Cashiers, NC, 1988.
- [8] "Reagentless Electrochemical Immunoassay Sensor", M. Umaña, C. Keller, manuscript in preparation.

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