volume of the binding site was calculated and displayed with the "set map", and this cavity was then rotated and viewed in 3-D, with the help of the Chem-Movie facility in Chem-X.

Comments on the Method. It is well-known that molecular mechanics calculations of this type are more accurate for neutral molecules than those involving polar interactions. The success of the current study probably can be attributed to the following. First, the binding site of visual pigments is known to be hydrophobic. The only major polar interaction in these analogues is near the common imino center. Thus, any possible errors associated with polar interactions at such centers could well be present in a similar amount, thus mutually cancelled when comparing differences in energy. Second, and probably more important, the ease of binding interaction for all the analogues, a point of concern here, is probably determined primarily by steric interactions rather than any polar interaction. A meaningful observation along this line is the close homology between the unprotonated blue cone pigment and the protonated green and red cone pigments.[40] The ability of the 11-cis chromophore to occupy these binding sites is apparently unaffected by the presence or absence of such polar interactions.

(40) Nathans, J.; Thomas, D.; Hogness, D. S. *Science* **1986**, *232*, 193-202.

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Registry No. *all-trans*-Retinal, 116-31-4; 7-*cis*-retinal, 24315-14-8; 9-*cis*-retinal, 514-85-2; 11-*cis*-retinal, 564-87-4; 13-*cis*-retinal, 472-86-6; 7,9-*dicis*-retinal, 56085-53-1; 7,11-*dicis*-retinal, 67737-35-3; 7,13-*dicis*-retinal, 56085-54-2; 9,11-*dicis*-retinal, 67711-05-1; 9,13-*dicis*-retinal, 23790-80-9; 11,13-*dicis*-retinal, 564-88-5; 7,9,11-*tricis*-retinal, 67737-38-6; 7,9,13-*tricis*-retinal, 56085-55-3; 7,11,13-*tricis*-retinal, 67737-39-7; 9,11,13-*tricis*-retinal, 67737-36-4; *all-cis*-retinal, 67737-37-5.

Supplementary Material Available: Tables listing calculated bond lengths (Table IV) and calculated dihedral angles (Table V) of retinal isomers (3 pages). Ordering information is given on any current masthead page.

Amplified Mass Immunosorbent Assay with a Quartz Crystal Microbalance

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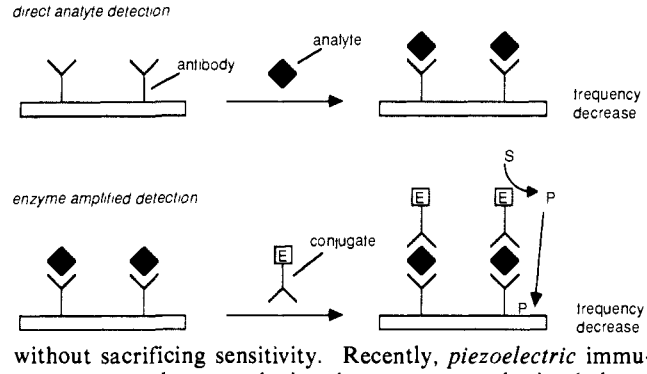
Abstract: Amplified mass immunosorbent assays (AMISA) using a quartz-crystal microbalance (QCM) are described for the detection of adenosine 5'-phosphosulfate (APS) reductase and human chorionic gonadotropin (hCG). APS reductase detection is accomplished by binding APS reductase to an anti-APS reductase antibody immobilized on the surface of the QCM, followed by addition of an anti-APS-alkaline phosphatase reductase conjugate. Subsequent exposure of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to the bound sandwich complex results in enzymatically amplified deposition of the oxidized dimer of BCIP on the QCM surface, resulting in a frequency change that corresponds to the level of APS analyte. The enzymatic amplification leads to significant enhancement of the detection limits; levels of ca. 5 ng/mL (10^{-14} M) of APS reductase can be detected whereas the direct binding of APS reductase at even more elevated concentrations cannot be measured. Assay methodology providing for a reusable biosensor is also described. In this example, hCG is detected with a horseradish peroxidase conjugate in which a sandwich complex is immobilized on a separate nylon membrane positioned in close proximity to a polyvinylferrocene (PV-Fc) film on the QCM. This enzyme catalyzes the oxidation of I^- to I_2/I_3^-, which subsequently reacts with the PV-Fc film with concomitant insertion of I_3^-, resulting in an observable decrease of the QCM frequency. Since the formation of PV-Fc$^+I_3^-$ can be reversed electrochemically to regenerate the original PV-Fc film, this configuration provides for reuse of the detection crystal.

Immunoassay methodology for the diagnostic determination of biological analytes (drugs, enzymes, metabolites, hormones, antigens, etc.) has proven valuable for clinical analyses, primarily because of the highly specific recognition between analytes and antibodies elicited for those analytes.[1-3] Although extensively used, the cost and time intensiveness of these procedures and the safety hazards of radioimmunoassay have stimulated investigations of new methods. Much attention has been given to "biosensors" in which an immunological reaction that occurs at the interface of a transducer results in output of an electrical signal. Critical features of biosensors are low cost, simplicity, and disposability, without sacrificing sensitivity. Recently, *piezoelectric* immunoassay procedures employing the quartz-crystal microbalance (QCM) and surface acoustic wave (SAW) devices have been described in attempts to address these criteria.[4] The fundamental

Scheme I



(1) *Enzyme Labelled Immunoassay of Drugs and Hormones*; Pal, S. B., Ed.; de Gruyter: New York, 1978.
(2) Engvall, E., Pesce, A. J., Eds.; *Scandinavian Journal of Immunology*; Blackwell: Oxford, 1978; Supplement No. 7, Vol. 8.
(3) Lunte, C. E.; Heineman, W. R. *Top. Curr. Chem.* **1988**, *143*, 1, and references therein.

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principle in these studies involved the change in resonant frequency of these devices that resulted from the mass increase associated with binding of an analyte to a complementary antianalyte immobilized on the surface of the piezoelectric material (Scheme I). For example, the resonant frequency of a shear mode quartz crystal of the QCM depends upon the mass according to the Sauerbrey relationship[5] (eq 1) where Δf is the measured frequency

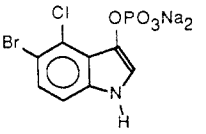
$$\Delta f = -\frac{2f_0^2\Delta m}{A(\rho_q\mu_q)^{1/2}} \quad (1)$$

shift, f_0 the parent frequency of the quartz crystal, Δm the mass change, A the piezoelectrically active area, ρ_q the density of quartz (2.648 g cm^{-3}), and μ_q the shear modulus (2.947×10^{11} dynes cm^{-2} for AT-cut quartz).

Analytical applications of piezoelectric detection relying on the direct binding of an analyte to antibody-modified QCM surfaces are attractive, owing to the versatility and simplicity of the method. However, the sensitivity of these approaches can be limited by several factors. Relatively small changes in areal density (i.e. $\Delta m/A$) are to be expected, owing to the relatively small numbers of analyte entities that can specifically bind to the limited number of complementary binding sites in a single antianalyte layer on the QCM surface. Reduced signal to noise due to frequency damping when the analyses are performed in liquid media can reduce the sensitivity of the QCM. Ex situ methods wherein the binding occurs in solution but the frequency difference is measured in the gas phase can be compromised by retention of solvent.

In order to overcome these limitations, we have investigated amplification schemes using methods analogous to sandwich enzyme-linked immunosorbent assay (ELISA). ELISA methods ultimately rely on the catalytic generation of detectable products by enzymes linked to antibodies that are specifically bound to the analyte. The amplified concentration of the products, which corresponds to the amount of analyte, is then more readily determined by rather conventional methods such as optical and electrochemical detection. In the case of piezoelectric transduction, a *mass* change at the QCM surface must be induced by the enzymatic reaction product (Scheme I). Therefore, this approach is referred to herein as amplified mass immunosorbent assay (AMISA).

We describe herein two approaches using the AMISA concept. The first employs histochemical staining agents[6] that are enzymatically converted to insoluble products that deposit on the QCM surface. The resulting mass increase can be detected by a frequency change, with the magnitude of change corresponding to the level of analyte bound to the antibody-modified QCM surface. Specifically, we describe the detection of adenosine 5′-phosphosulfate (APS) reductase with an antibody-modified QCM, an alkaline phosphatase–anti-APS conjugate, and 5-bromo-4-chloro-3-indolyl phosphate (I; BCIP) as a substrate.



I

The other approach is exemplified by a sandwich assay for human chorionic gonadotropin (hCG), in which the enzyme horseradish peroxidase (HRP) of a conjugate reagent catalyzes formation of I_2/I_3^-, which subsequently oxidizes a redox active polyvinylferrocene (PV–Fc) film on the QCM surface with concomitant insertion of the massive I_3^- ion.[7] The soluble nature of the I_2/I_3^- product coupled with the electrochemical reversibility of the film redox process allows the fabrication of a reusable biosensor. In both these approaches, detection is not limited by the areal density of the specifically bound analyte but rather the catalytic accumulation of mass on the QCM surface.

Experimental Section

Materials. Adenosine 5′-phosphosulfate reductase enzyme was isolated and purified to homogeneity from a pure culture of *Desulfovibrio desulfuricans* according to the method of Odom.[8] Antibody to APS reductase was prepared by first immunizing rabbits with purified enzyme protein in a standard immunization adjuvant. The antibody was then purified by affinity chromatography from immune rabbit serum with an APS reductase–sepharose gel as described by Tijssen.[8c] Affinity gel for the purification was prepared by coupling the purified APS reductase (20 mg) to cyanogen bromide activated sepharose (700 mg). The resulting APS reductase–sepharose affinity gel was added to pooled immune serum (100 mL), which had been previously diluted with an equal volume of buffer (0.5 M phosphate, 0.1 M sodium chloride). The mixture was equilibrated overnight at 4 °C with constant stirring. The resulting APS reductase–sepharose–antibody gel was washed four times with the buffer (0.5 M phosphate, 0.1 M sodium chloride) to remove residual serum constituents. Antibody was recovered by treatment of the gel at acidic pH (2.1). The pH of the recovered supernatant fluid was rapidly readjusted to pH 7.0 by addition of 0.5 M phosphate buffer. The recovered antibody was then concentrated by ammonium sulfate precipitation. Purified antibody (20 mg) was dissolved in 6 mL of solution containing 5 mM phosphate, 30 mM sodium chloride, and 30 mM sodium azide for storage.[1b] The conjugate reagent comprised of anti-APS reductase antibody and alkaline phosphatase was prepared according to the method of Imagawa, et al.[9]

The hormone hCG was obtained commercially from Calbiochem (LaJolla, CA) as a lypholized preparation (Cat. No. 869029). The material was hydrated in purified water and then diluted to the desired concentration in 50 mM Tris buffer (pH 7.4). Anti-hCG was purchased from Hybritech Inc. (San Diego, CA) as a monoclonal antibody directed toward the α subunit of hCG. For attachment to the QCM or membrane surfaces, the antibody preparation was diluted in PBS buffer to 100 μg/mL. The anti-hCG–HRP conjugate was purchased commercially as a Target hCG test kit from V-Tech, Inc. (Pomona, CA). The liquid conjugate reagent was used directly (50 μL/test). Polyvinylferrocene obtained from Polysciences was used as obtained. The 5-bromo-4-chloro-3-indolyl phosphate (BCIP) phosphatase substrate reagent, purchased from Sigma Chemical Co. (St. Louis, MO, Cat. No. 710-3) as a 2.3 mM solution in 2-amino-2-methyl-1-propanol buffer with magnesium ions (pH 10.2), was used as obtained. Nylon 0.65-μm Immunodyne membranes were obtained from PALL, Inc. Phosphate-buffered saline (PBS) solution containing 120 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer at pH 7.4 was prepared by dissolving the contents of a PBS buffer concentrate purchase from Sigma Chemical Co. (Cat. No. 1000-3). Tris test buffer contained 50 mM tris(hydroxymethyl)aminomethane at pH 7.5, 75 mM NaCl, 0.1% w/v bovine serium albumin

(4) (a) Ngeh-Ngwainbi, J.; Foley, P. H.; Kuan, S. S.; Guilbault, G. G. *J. Am. Chem. Soc.* **1986**, *108*, 5444. (b) Muramatsu, H.; Dicks, J. M.; Tamiya, E.; Karube, I. *Anal. Chem.* **1987**, *59*, 2760. (c) Shons, A.; Dorman, F.; Najarian, J. *J. Biomed. Mater. Res.* **1972**, *6*, 565. (d) U.S. Patent 4,236,893. (e) Roederer, J. E.; Bastiaans, G. J. *Anal. Chem.* **1983**, *55*, 2333. (f) U.S. Patent 4,242,096.

(5) Sauerbrey, G. *Z. Phyzik* **1959**, *155*, 206.

(6) (a) Kaplow, L. S. *Am. J. Clin. Pathol.* **1975**, *63*, 451. (b) Graham, R. C., Jr.; Karnovsky, M. J. *J. Histochem. Cytochem.* **1965**, *13*, 448. (c) Nakane, P. K. *J. Histochem. Cytochem.* **1968**, *16*, 557. (d) Nineham, A. W. *Chem. Rev.* **1955**, *55*, 355. (e) Sternberger, L. A.; Joseph, S. A. *J. Histochem. Cytochem.* **1979**, *27*, 1424. (f) Holt, S. J.; Wither, R. F. J. *Proc. R. Soc. London, B* **1957**, *B142*, 160, 520. (g) Levey, A. I.; Bolam, J. P.; Rye, D. B.; Hallanger, A. E.; Demuth, R. M.; Mesulam, M.-M.; Wainer, B. H. *J. Histochem. Cytochem.* **1986**, *34*, 1449. (h) Baskin, D. G.; Mar, H.; Gorray, K. C.; Fujimoto, W. Y. *J. Histochem. Cytochem.* **1982**, *30*, 710. (i) Mesulam, M.-M. *Tracing Neural Connections with Horseradish Peroxidase*; Wiley: New York, 1982; p 1. (j) Tsou, K.-C.; Cheng, C.-S.; Nachlas, M. M.; Seligman, A. M. *J. Am. Chem. Soc.* **1956**, *78*, 6139. (k) Feinstein, R. N.; Lindahl, R. *Anal. Biochem.* **1973**, *56*, 353. (l) Feidberg, R. S.; Datta, P. *Science (Washington, D.C.)* **1970**, *170*, 1414. (m) Horwitz, J. P.; Freisler, J. V. *J. Med. Chem.* **1970**, *13*, 1024. (n) Wolf, P. L.; Horwitz, J.; Mandeville, R.; Vazquez, J.; von der Muehll, E. *Am. J. Clin. Pathol.* **1969**, *30*, 663. (o) Horwitz, J. P.; Easwaran, C. V.; Wolf, P. L. *Biochim. Biophys. Acta* **1972**, *276*, 206. (p) Wolf, P. L.; Horwitz, J. P.; Freisler, J. V.; Vazquez, J.; von der Muehll, E. *Biochim. Biophys. Acta* **1968**, *159*, 212. (q) Horwitz, J. P.; Easwaran, C. V.; Wolf, P. L.; Kowalczyk, L. S. *Biochim. Biophys. Acta* **1969**, *185*, 143.

(7) (a) Ward, M. D. *J. Electroanal. Chem. Interfacial Electrochem.* **1987**, *236*, 139. (b) Ward, M. D. *J. Phys. Chem.* **1988**, *92*, 2049.

(8) (a) Odom, J. M. *Abstracts of the American Society for Microbiology*: 1987; Vol. K-77, p 215. (b) Odom, J. M., submitted for publication in *J. Appl. Environ. Microbiol.* (c) Tijssen, P. *Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theory of Immunoassays*; pp 110–114.

(9) Yoshitake, S.; Imagawa, M.; Ishikawa, E.; Niitsu, Y.; Urishizaki, I.; Nishiura, M.; Kanazawa, R.; Kurosaki, H.; Tachibana, S.; Nakazawa, N.; Ogawa, H. *J. Biochem.* **1982**, *92*, 1413.

(BSA), and 0.02% sodium azide. Tris diluent contained 50 mM tris(hydroxymethyl)aminomethane at pH 7.5. Citrate buffer contained 35.6 g of $NaHPO_4 \cdot 2H_2O$ and 21.0 g of citric acid in 300 mL of purified water. The solution was adjusted to pH 5.0 and diluted to a final volume of 360 mL.

Immunoassay Procedures. Assays for APS reductase were performed stepwise. Initially, quartz crystals were equilibrated with 2 mL of 100 $\mu g\ mL^{-1}$ anti-APS reductase antibody in PBS buffer solution for 4 h. The crystals were then washed with PBS buffer containing 0.1% BSA, followed by washing with PBS buffer three times to remove excess anti-APS reductase. The modified crystals were then exposed to varied concentrations of APS reductase in 1.5 mL of Tris buffer for 20 min. After washing with 50 mM Tris buffer to remove nonspecifically adsorbed APS reductase, the crystals were exposed to 1.5 mL of Tris buffer solution containing 30 μL of anti-APS reductase–alkaline phosphatase conjugate for 20 min. The crystals were then washed with Tris test buffer and once with 50 mM Tris solution and placed in the QCM cell. Then 0.5 mL of Tris test buffer was added. After the frequency stabilized, 0.5 mL of the BCIP reagent solution was added and the frequency monitored.

Assays for hCG were also performed stepwise, initially incubating the Immunodyne membranes with 2 mL of 100 $\mu g\ mL^{-1}$ anti-hCG antibody in PBS buffer solution for 24 h at 4 °C. The membranes were then sequentially washed with PBS buffer containing 0.1% BSA followed by three washings with PBS buffer. The membranes were then exposed to hCG in 0.1% BSA–PBS buffer solution for 30 min, washed with PBS buffer, exposed to 2 mL of PBS buffer containing 20 μL of stock anti-hCG–HRP conjugate for 20 min, and finally washed with 0.1% BSA–PBS buffer solution. The membranes were then placed in the QCM cell as described below, and a mixture of equal amounts of the citrate buffer and a 0.25% NaI solution were added to the cell. After the frequency stabilized, 10 μL of a 0.01% H_2O_2 solution was added and the frequency monitored.

Apparatus. The experimental apparatus comprised a 5-MHz AT-cut quartz crystal (Valpey–Fisher) and a homemade oscillator designed to drive the crystal at its resonant frequency. Gold electrodes (2000 Å thick) were deposited on chromium underlayers (200 Å) on both sides of the crystal by evaporative techniques. The patterns were arranged so that the gold leads from the outer edges of the crystal to the center circular pad on opposite sides did not overlap. The sensitivity of the QCM as given by eq 1 is 17.68 ng $Hz^{-1}\ cm^{-2}$. An asymmetric electrode format was used, with the side having the larger gold pad facing the solution (A = 0.35 cm^2 electroactive area working side, A_{piezo} = 0.18 cm^2 on the opposite side); in this case a 1-Hz shift was equivalent to 3.2 ng. The quartz crystal was mounted between two O-rings confined by standard glass fittings and a metal clamp. Electrochemical access to the gold electrode facing the solution was provided by a Princeton Applied Research Model 273 potentiostat with the QCM electrode at hard ground, as described previously.[7b] In experiments using a nylon membrane to support the sandwich structure, the membrane was inserted between the glass O-ring joint and the O-ring. This resulted in a gap of approximately 2 mm between the quartz crystal and the membrane. The frequency of the QCM was monitored with a Hewlett Packard 5384A frequency counter and recorded with a Digital Equipment Corp. PDP-11/73 computer. Spectrophotometric interrogations of BCIP dimer deposits on the QCM surface were accomplished with a Hewlett Packard 8451 spectrophotometer.

Results and Discussion

Immunoassay by Enzymatically Amplified Mass Deposition. The simplest operational mode for piezoelectric detection of biological analytes is measurement of the frequency change due to direct binding of the analyte to a QCM surface that has been modified with an antianalyte reagent possessing binding sites complementary to the analyte. Preparation of the antibody-modified QCM was accomplished by immobilization of rabbit anti-APS reductase antibody onto the QCM surface, either directly onto the gold electrode, or following surface modification by spin-coating polymer films (e.g. polystyrene, polyvinylferrocene) or reaction of the gold surfaces with benzyltrichlorosilane. The adsorption of antibody onto the gold, polymer, or silane surfaces was consistent with the hydrophobic qualities of these surfaces and proved to be adequate for these investigations. Given the known reactivity of disulfide moieties with gold surfaces,[10] we

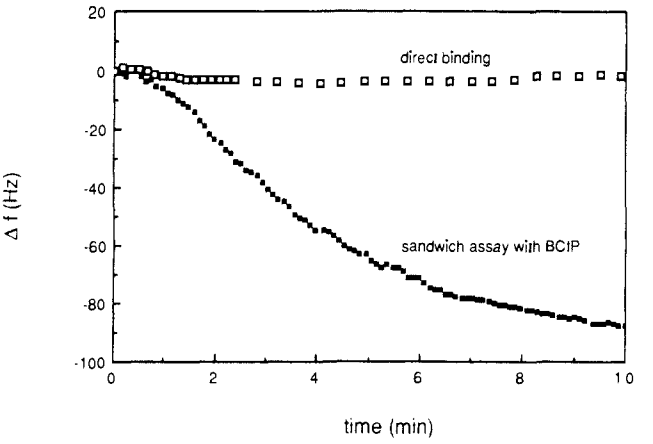
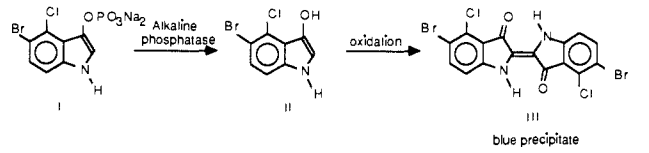


Figure 1. Response of the QCM (a) upon addition of APS reductase to a polystyrene film modified with anti-APS reductase (direct binding) and (b) upon addition of BCIP to the film in (a) that had been exposed to anti-APS reductase–alkaline phosphatase conjugate. The dosage of APS reductase was 100 ng mL^{-1}.

Scheme II



presently cannot discount antibody attachment to the gold surface via binding of the antibody disulfide groups, with retention of the F(ab) antigenic binding sites. The presence of antibody on these surfaces was evidenced by the immunological response (vide infra).

In principle, the change in mass associated with the binding of APS reductase to the anti-APS reductase antibody immobilized on the QCM surface should result in a corresponding decrease in the resonant frequency of the QCM. However, exposure of APS reductase to these antibody-modified surfaces did not result in significant frequency changes (Figure 1) in the concentration range investigated (5–200 ng/mL). This is consistent with previous reports that suggested poor sensitivity for piezoelectric detection of direct binding.[4e] The lack of sensitivity is not unexpected since the mass change at the QCM is limited by the number of APS reductase molecules that can bind to the limited number of antibody binding sites on the surface. Assuming proper orientation of all the antibody sites on the QCM surface, a molecular density of 1 g cm^{-3} for both the antibody and APS reductase, and a molecular weight of 190 000 for APS reductase, approximately 22 ng of APS reductase, would specifically bind to the piezoelectrically active area (0.2 cm^2). This would result in a −7 Hz shift in frequency, which is actually similar to that observed. However, under these conditions, small shifts such as this approach the limit of detectable changes in frequency and are of marginal analytical utility.

An amplification strategy was therefore adopted in which antibody–enzyme conjugate reagent comprised of anti-APS reductase antibody linked to an alkaline phosphatase enzyme was employed to catalytically amplify the mass change. Once antibody has been attached to the QCM surface, the immunoassay can be performed as outlined in Figure 2. The antibody-modified QCM is first sequentially equilibrated with APS reductase and washed with buffer solution. The QCM is then equilibrated with rabbit anti-APS reductase-alkaline phosphatase conjugate and washed with buffer solution to remove excess conjugate. During these steps the conjugate binds with APS reductase to form a conjugate-APS reductase complex bound to the antibody-modified QCM surface. In this manner, a molecular sandwich complex is formed, similar to that employed in sandwich ELISA methods.[1,2] Alternatively, the sample and conjugate reagents can be combined and equilibrated simultaneously with the antibody-modified QCM. In this way, the complexity of the testing procedure is simplified.

(10) (a) Nuzzo, R. G.; Fusco, F. A.; Allara, D. L. *J. Am. Chem. Soc.* **1987**, *109*, 2358. (b) Nuzzo, R. G.; Zegarski, B. G.; Dubois, L. H. *J. Am. Chem. Soc.* **1987**, *109*, 733. (c) Whitesides, G. *Langmuir* **1988**, *2*, 365.

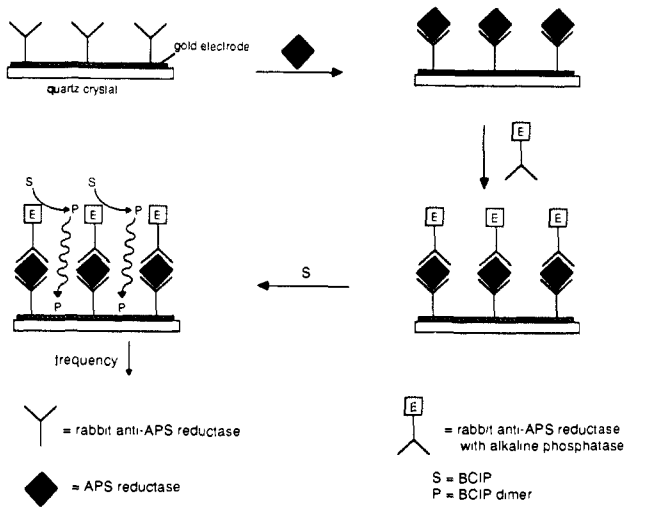


Figure 2. Schematic representation of the sandwich enzyme-linked immunosorbent assay procedure used with the QCM. The final step involving conversion of the BCIP substrate to the insoluble blue BCIP dimer, which deposits on the QCM, results in a decrease in the resonant frequency of the quartz crystal.

The actual detection step comprises hydrolysis of the phosphate moiety of the BCIP substrate (I) by the alkaline phosphatase conjugate, as shown in Scheme II. This results in the formation of the enol product (II), which is subsequently oxidized in air to the insoluble blue BCIP dimer (III).

The BCIP dimer product (III) is a member of the class of indigo dyes,[11] and its intense color and negligible solubility has been exploited to locate enzymatic activity on cell walls, membranes, and chromatographic gels since it precipitates in the vicinity of the enzyme. Likewise, we have found that the presence on the QCM surface of a sandwich complex possessing alkaline phosphatase will deposit product on the QCM to give an analytically useful response. Instead of optical or electrochemical detection of an enzymatically generated product, the deposition of III results in a decrease in the QCM resonant frequency corresponding to the increase in mass of the precipitate. The frequency change of the QCM is therefore measured in situ following addition of BCIP substrate solution to the sandwich complex bound to the QCM surface. In these sandwich assays the conjugate enzyme is only present when APS reductase is bound to the surface, and therefore the amount of precipitate directly reflects the quantity of APS.

Although direct binding of APS reductase did not produce an analytically useful frequency shift, APS reductase detection was readily accomplished by this sandwich AMISA procedure. Addition of 0.5 mL of BCIP substrate solution to 0.5 mL of Tris buffer solution covering the APS reductase conjugate sandwich on the QCM resulted in a smooth decrease in frequency (Figure 1) after a small induction period (<30 s). The induction period is attributed to the time required for the concentration of the BCIP dimer to exceed its solubility product, subsequent nucleation for crystal growth, and adhesion of the nuclei to the QCM surface. The frequency change continued for long periods of time, generally exceeding 1 h, after which the experiments were terminated. After long reaction times the surface of the QCM was noticeably blue due to the accumulation of the BCIP dimer (III). These dimer deposits were strongly bound to the surface, such that copious washing or agitation in aqueous media did not result in any visible loss of material. Although the reasons for this strong adhesion are not known, it seems reasonable to suggest that nature of the BCIP dimer adsorption process is identical with that occurring in histochemical studies, possibly involving hydrophobic interactions, hydrogen bonding, or $\pi-\pi$ interactions between III and protein residues of the sandwich array on the QCM surface.

(11) Gordon, P. F.; Gregory, P. *Organic Chemistry in Colour*; Springer-Verlag: New York, 1983; p 82.

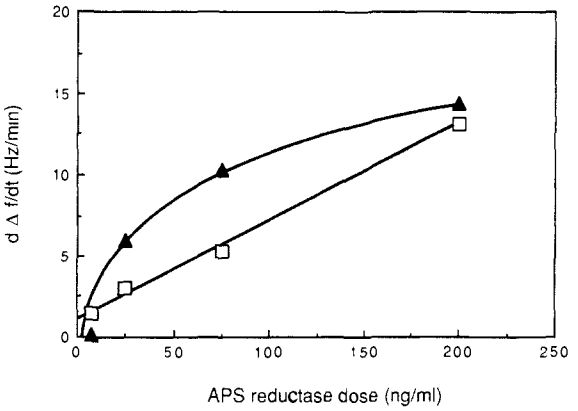


Figure 3. Rate of change in frequency of the QCM after addition of BCIP to Au–quartz-crystal surfaces (▲) and PV-Fc-coated Au–quartz crystals (□) that were modified with anti-APS reductase, exposed to different dosage levels of APS reductase, and treated with anti-APS reductase–alkaline phosphatase conjugate. The correlation of $d\Delta f/dt$ with APS reductase concentration indicates that the amount of bound APS reductase is modulated by its solution concentration.

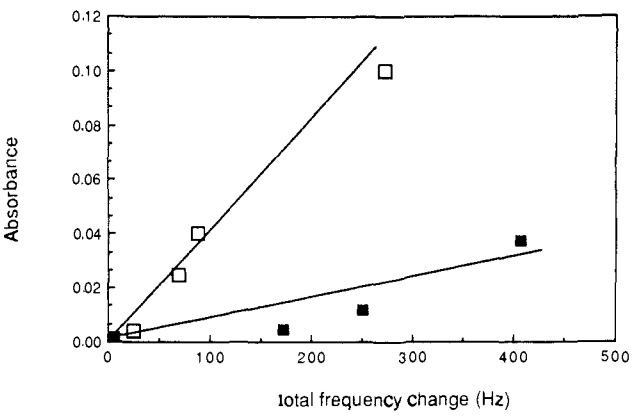


Figure 4. Comparison of the optical density of the deposited BCIP layers with the total frequency change observed in the QCM after immunoassay.

The rate of frequency change ($d\Delta f/dt$) and the total frequency change (Δf_T) were dependent upon the dosage of APS reductase exposed to the antibody-modified QCM (Figure 3). This indicated that the amount of bound APS reductase was modulated by its solution concentration in the range investigated (5–200 ng/mL), as dictated by its equilibrium binding constant with the antibody. The observation of a well-behaved dose response suggests that this procedure can be exploited as a quantitative immunoassay technique.

The response for crystals with antibody attached directly to the gold surfaces was slightly larger than that for polymer-coated crystals, suggesting greater coverage or immunological activity of the anti-APS reductase antibody on the bare gold surfaces. In both cases, APS reductase was readily detectable at the lower end of the concentration range investigated. Since the molecular weight of APS reductase is 190 000, this corresponds to detection limits of approximately 25 fM. The sensitivity indicated by these results is several orders of magnitude greater than that reported for direct detection of IgG[4c] and IgG phospholipase A antibody,[4d] although recently a detection limit of 1 ng/mL was claimed for human IgG.[4a]

The QCM frequency response during immunoassay was corroborated following each experiment by measurement of the optical density of the strong absorption band at 650 nm exhibited by the blue BCIP dimer. Spectrophotometric analysis of the quartz crystals was accomplished by aligning the crystals in the beam of the spectrophotometer so that the region of quartz nearest the gold electrodes was interrogated uniformly for different crystals. The strong adsorption of antibody onto the quartz surfaces of the crystals was also consistent with previously reported

affinity of proteins for silica surfaces.[12] Very good correlations between the optical density and Δf_T were observed for the polyvinylferrocene-coated films over the concentration range investigated (Figure 4). The correlation was not quite as good for the unmodified crystals, probably reflecting differences in antibody adsorption or precipitant adhesion to the gold and quartz surfaces.

The AMISA approach has several key advantages. Contrary to previously reported piezoelectric methods, the actual detection step is not sensitive to many factors influencing the immunological reactions. Rather, the production of mass resulting from addition of BCIP constitutes the measurement step. Interferences from the biological entities at this stage are thereby minimized. Rapid precipitation also serves to obviate product inhibition of the enzyme, thereby eliminating the need for mechanical product removal from the vicinity of the enzymatic reaction. Optical clarity is not required during the measurement step as is generally necessary for optical detection methods, and electrode poisoning or interferences by undesirable electrochemically active substances than can arise during amperometric detection are avoided. It was also generally observed that activity could be detected more easily with the QCM than by spectrophotometric analysis of the crystals. This is readily understood when the sensitivity of the two methods is compared. For example, a 50-Å-thick film of the BCIP dimer would afford an approximately 30-Hz shift, which is well within the detection limits of the QCM. However, the optical density of a film with this thickness would be difficult to detect visually as well as spectrometrically. Most notably, the *rate* of the enzymatic mass conversion can be continuously monitored, contrary to most immunoassays, which rely on reaction endpoints. This measurement capability affords early detection and minimizes the need for blank corrections.

Immunoassays with Polyvinylferrocene Films. A Reusable Biosensor. Recently, we reported that PV-Fc films were readily oxidized by aqueous I_2/I_3^- mixtures, with concomitant insertion of I_3^- ion to maintain electroneutrality in the film (eq 2).[7] In-

$$\text{PV-Fc}^0 \xrightarrow{I_2/I_3^-} \text{PV-Fc}^+I_3^- \quad (2)$$

$$\text{PV-Fc}^+I_3^- + 3e^- \rightarrow \text{PV-Fc}^0 + 3I^- \quad (3)$$

vestigations of this process using the QCM[7b] demonstrated that the insertion of the massive I_3^- ion resulted in large frequency shifts. Specificity of I_3^- insertion into the PV-Fc$^+$ films was significant even in the presence of large concentrations of other electrolytes. Additionally, electrochemical reduction of the PV-Fc$^+I_3^-$ film was found to result in rapid expulsion of I_3^- from the film in the form of I^- (eq 3).

The specificity and affinity of the process depicted by reaction 2 suggested the incorporation of I_3^- into a PV-Fc film immobilized on a piezoelectric quartz crystal could be configured to form a reusable immunoassay biosensor. For example, in a sandwich immunoassay a conjugate containing horseradish peroxidase could catalyze the oxidation of I^- to I_2/I_3^-, resulting in formation of PV-Fc$^+I_3^-$ with a concomitant change in the resonant frequency. Unlike the BCIP methodology described above, in this case, the enzymatic I_3^- reaction product is soluble. Therefore, I_2/I_3^- generated by an HRP conjugate of a sandwich complex immobilized on a surface separate from the piezoelectric crystal could be detected, provided the conjugate on the secondary support was located in proximity to the PV-Fc film on the QCM surface. Following assay detection, the PV-Fc film could be regenerated by exploiting the reversibility of the film redox process.

The feasibility of this approach was demonstrated by the detection of the hormone human chorionic Gonadotropin. In this assay the sandwich complex was immobilized on a nylon membrane facing opposite a polyvinylferrocene film spin coated on a quartz crystal (Figure 5). The nylon membrane provides a large surface area for adsorption of anti-hCG in order to enhance binding of hCG. The sandwich structure was prepared stepwise by (1) immobilization of anti-hCG on the nylon membrane, (2) exposure of the membrane to hCG and (3) exposure of the anti-hCG–hCG complex to an anti-hCG–HRP conjugate. Each step was followed by washing with buffer solution to eliminate nonspecific binding. The membrane was then placed opposite to the PV-Fc film on the QCM, separated by a Teflon-coated O-ring, thereby forming a reaction chamber with an approximate volume of 0.2 mL. The chamber was filled with citrate buffer containing NaI, and, after the frequency of the QCM stabilized, 10 μL of a 0.01% H_2O_2 solution was added to the chamber.

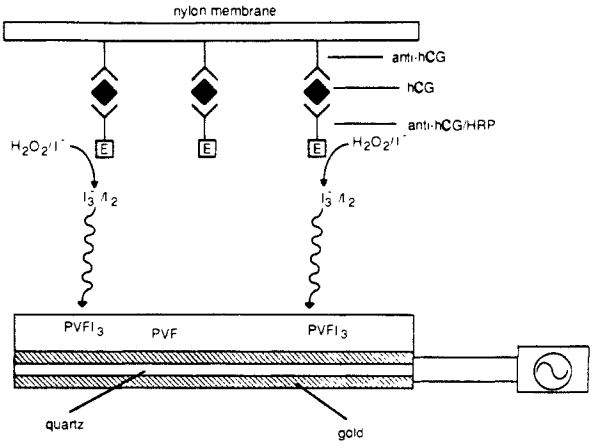


Figure 5. Schematic representation of the cell and the reactions involved in the analysis of hCG.

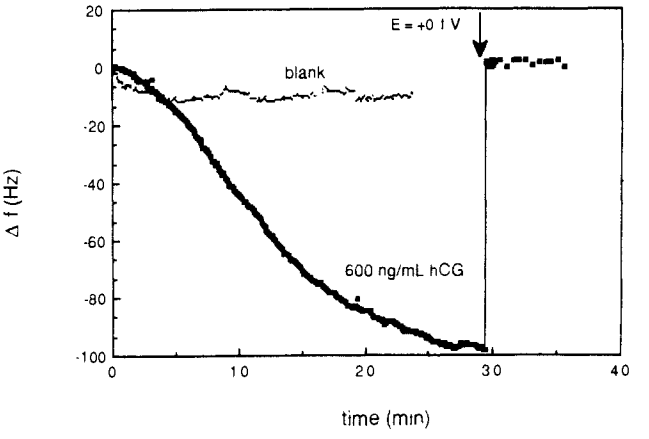


Figure 6. Comparison of the frequency response upon addition of a 10-μL 0.01% H_2O_2 solution for (a) a blank nylon membrane not exposed to hCG and (b) a membrane exposed to 600 ng mL^{-1} hCG. In both cases the membranes were exposed to a anti-hCG–HRP conjugate. Following the assay, the original QCM frequency was reestablished by expulsion of I^- via electrochemical reduction of the film at +0.1 V (vs Ag/AgCl).

Blank experiments performed in this manner, in which anti-hCG surfaces were not exposed to hCG but were treated with conjugate, gave no significant frequency response upon addition of the H_2O_2 reagent (Figure 6), indicating that nonspecific binding of the conjugate was not operative. Conversely, exposure of the anti-hCG membrane to hCG followed by binding of the conjugate resulted in a readily observable frequency decrease upon addition of H_2O_2, due to insertion of I_3^- into the PV-Fc film after its oxidation by I_2/I_3^- generated by the HRP of the conjugate. The PV-Fc film could be regenerated to its original neutral state by applying a potential of +0.1 V. This results in expulsion of I_3^- (as 3 equiv of I^-) with concomitant return of the QCM frequency to its original value (Figure 6). Accordingly, the same PV-Fc film can be used for repetitive hCG analysis by simply inserting another nylon membrane with a sandwich array into the QCM cell. This constitutes a sensor that is reusable with regard to the PV-Fc film and quartz crystal and disposable with regard to the biologically active component. Therefore, this methodology has a potential advantage over that in which antibody is attached directly to the QCM. It should be noted that this procedure has not yet been optimized, as the nylon support exhibited a small affinity for I_3^-

(12) Kozin, F.; Millstein, B.; Mandel, G.; Mandel, N. *J. Colloid Interface Sci.* **1982**, *88*, 326.

as evidenced by its change in color during the assay, thereby inhibiting the response at the QCM associated with reaction 2. Investigations with more inert supports are currently in progress.

Concluding Remarks

The results described above demonstrate that amplification routes via catalytic processes, such as enzymatic reactions, can significantly extend the detection limits of piezoelectric assay. Since antibodies can be elicited for a wide variety of analytes, the methodology can be generally applied to various substances (e.g. proteins, enzymes, hormones, etc.). The method requires, in addition to an antibody, only an appropriate anti-analyte–enzyme conjugate to convert the substrate to a product capable of inducing a mass change at the surface of the quartz crystal. Detection can be accomplished by simple deposition of insoluble assay products or by reactions of the assay products with films on the QCM that produce mass changes due to adsorption–complexation processes or changes in ion population. As demonstrated here, the latter is particularly advantageous if reusable sensors are desired.

One key advantage of enzymatically amplified sandwich methods is that detection limits are independent of the mass of the analyte. Therefore, detection of low molecular weight analytes such as hormones and drugs is possible under conditions where detection of direct binding will be difficult. The versatility regarding the ease of modification of the QCM surface makes piezoelectric detection widely applicable toward a variety of analytes and suggests that chemical design of new receptors on the surface of the QCM will play a significant role in expanding the scope of piezoelectric immunoassay.

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Registry No. BCIP, 38404-93-2; hCG, 9002-61-3; Pv-Fc, 34801-99-5; APS reductase, 9027-75-2; quartz, 14808-60-7.

Synthesis, Characterization, and Reactivity of Oxomanganese(IV) Porphyrin Complexes

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Abstract: The preparation, isolation, and characterization of two types of oxomanganese(IV) porphyrin complexes are described. The reaction of chloro(5,10,15,20-tetramesitylporphyrinato)manganese(III) [Mn^{III}TMP(Cl), **1**] with 1.2 equiv of tetramethylammonium hydroxide (TMA(OH)) and 1.2 equiv of *m*-chloroperoxybenzoic acid (*m*-CPBA) in CH_2Cl_2 produced a second complex formulated as Mn^{IV}TMP(O) (**2**). The reaction of **1** in CH_2Cl_2 containing excess tetra-*n*-butylammonium hydroxide (TBA(OH)) at +1.20 V generated a stable oxomanganese(IV) porphyrin complex, [Mn^{IV}TMP(O)(OH)] (**3**). When the reaction stoichiometry was altered, mixtures of complexes **2** and **3** could be prepared. The aerobic reaction of **1** in $CHCl_3$ containing 6 N NaOH and a phase-transfer catalyst resulted in the formation of a similar complex, Mn^{IV}TMP(O)(X) (**2a**). The addition of excess TBA(OH) to CH_2Cl_2 solutions of either **2** or **2a** resulted in the quantitative formation of **3**. The EPR spectra of **2**, **2a**, and **3** all displayed a strong broad resonance at $g \sim 4$ and a weak unresolved signal at $g \sim 2$ consistent with a high-spin ($S = {}^3/_2$) assignment of the Mn^{IV} ions. The Mn^{IV}=O stretching frequency in **2** was identified at 754 cm^{-1} by FT-IR spectroscopy. In the case of **3** the Mn^{IV}=O stretching frequency was at 712 cm^{-1}. The reaction of **2a** with *cis*-β-methylstyrene under anaerobic conditions produced a mixture of *cis*- and *trans*-epoxide in a ratio of 0.17. The reaction of **2** with *cis*-β-methylstyrene under aerobic conditions produced a different *cis*-epoxide/*trans*-epoxide ratio and product distribution than those of the identical reaction run under anaerobic conditions. In the presence of $H_2{}^{18}O$, the stereoisomeric epoxides showed a significantly different ^{18}O content. Further, ^{18}O was found to reside in the oxidation products when this reaction was carried out in the presence of $^{18}O_2$. Mechanisms for the epoxidation of olefins by **2** under anaerobic and aerobic conditions are discussed, which involve atom transfer from both oxomanganese(V) and oxomanganese(IV) species.

The oxygen activation and transfer reactions of cytochrome P-450 have attracted attention for over a decade. The remarkable reactivity of P-450 is believed to derive from an oxoiron(IV) porphyrin cation-radical species, which has been suggested as the ultimate oxidant in this enzymatic system.[1] Oxidized synthetic metalloporphyrin complexes have been extensively studied as simple active site models for monooxygenases and peroxidases and have provided a means to isolate and characterize reactive intermediates implicated in these biological systems.[2] Indeed, structurally characterized oxometalloporphyrins of iron[3] and chromium[4] are known to be capable of oxidizing organic substrates. Further, dioxoruthenium(VI) and oxoruthenium(IV) porphyrin complexes have recently been identified as active species in the catalytic aerobic epoxidation of olefins.[5]

(1) (a) Sligar, S. G.; Murray, R. I. *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Oritz de Montellano, P. R., Ed.; Plenum: New York, 1986; pp 443-479. (b) Guengerich, F. P.; MacDonald, T. L. *Acc. Chem. Res.* **1984**, *17*, 9-16. (c) White, R. E.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315-336. (d) Groves, J. T. *Adv. Inorg. Biochem.* **1979**, 119-145.

(2) (a) Wolberg, J.; Monassen, J. *J. Am. Chem. Soc.* **1970**, *92*, 2982-2991. (b) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1976**, *17*, 187-251. (c) McMurry, T. J.; Groves, J. T. *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Oritz de Montellano, P. R., Ed.; Plenum: New York, 1986; pp 1-28.

(3) (a) Groves, J. T.; Haushaulter, R. C.; Nakamura, M.; Nemo, T. E.; Evans, B. J. *J. Am. Chem. Soc.* **1981**, *103*, 2884-2886. (b) Penner-Hahn, J. E.; McMurry, T. J.; Renner, M.; Latos-Grazynsky, L.; Elbe, K. S.; Davis, I. M.; Balch, A. L.; Groves, J. T.; Dawson, J. R.; Hodgson, K. O. *J. Biol. Chem.* **1983**, *258*, 12761-12764. (c) Boso, B.; Lang, L.; McMurry, T. J.; Groves, J. T. *J. Chem. Phys.* **1983**, *79*, 1122-1126. (d) Penner-Hahn, J. E.; Elbe, K. S.; McMurry, T. J.; Renner, M. R.; Balch, A. L.; Groves, J. T.; Dawson, J. H.; Hodgson, K. O. *J. Am. Chem. Soc.* **1986**, *108*, 7819-7825. (e) Chin, D. H.; Balch, A. L.; La Mar, G. N. *J. Am. Chem. Soc.* **1980**, *102*, 1446-1448. (f) Chin, D. H.; La Mar, G. N.; Balch, A. L. *J. Am. Chem. Soc.* **1980**, *102*, 4344-4350. (g) Balch, A. L.; La Mar, G. N.; Latos-Grazynski, L.; Renner, M. W.; Thanabal, V. *J. Am. Chem. Soc.* **1985**, *107*, 3003-3007.

(4) (a) Groves, J. T.; Kruper, W. J., Jr. *J. Am. Chem. Soc.* **1979**, *101*, 7613-7615. (b) Groves, J. T.; Haushaulter, R. C. *J. Chem. Soc., Chem. Commun.* **1981**, 1163-1166. (c) Groves, J. T.; Kruper, W. J., Jr.; Haushaulter, R. C.; Butler, W. M. *Inorg. Chem.* **1982**, *21*, 1363-1368. (d) Penner-Hahn, J. E.; Benfatto, M.; Hedman, B.; Takahashi, T.; Sebastian, D.; Groves, J. T.; Hodgson, K. O. *Inorg. Chem.* **1986**, *25*, 2255-2259.

0002-7863/88/1510-8628$01.50/0