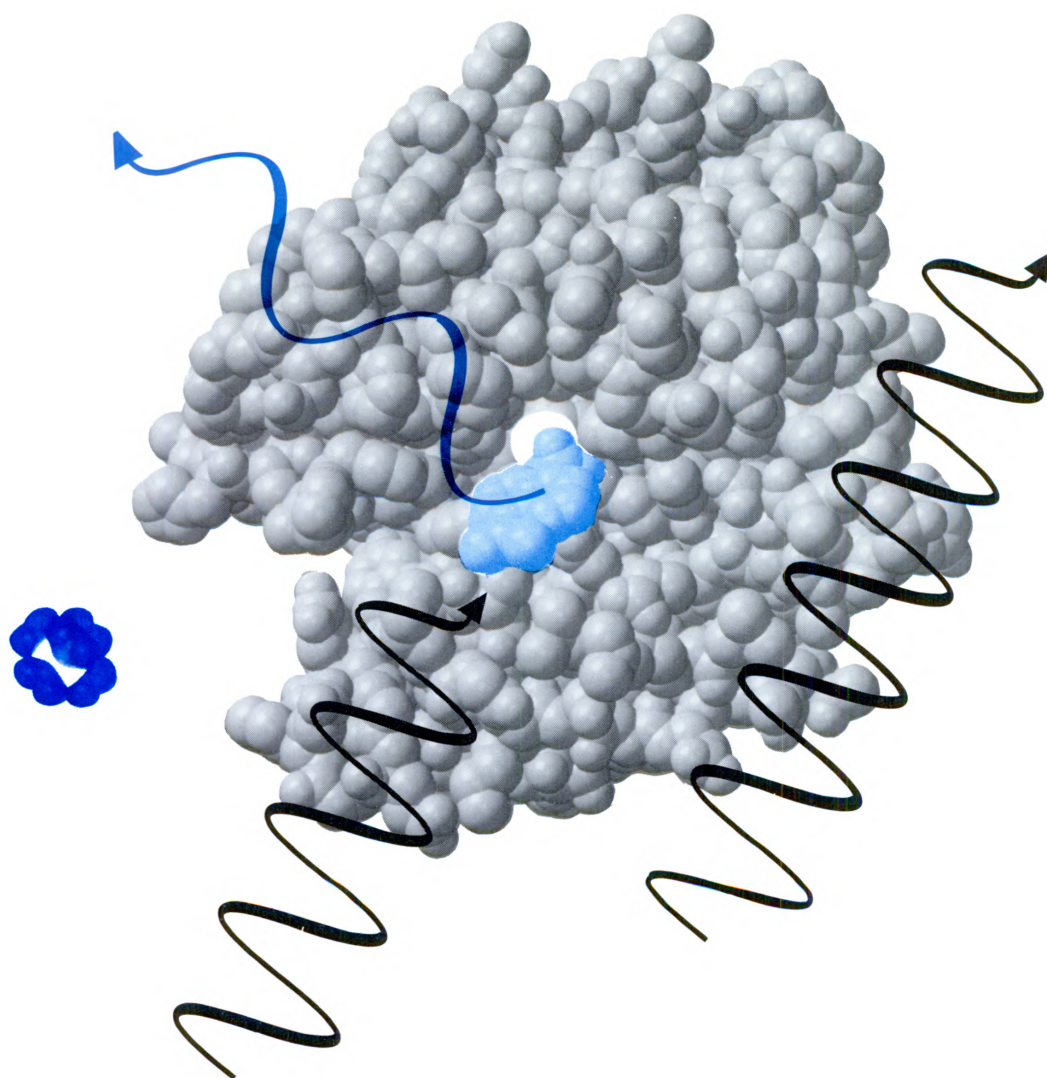


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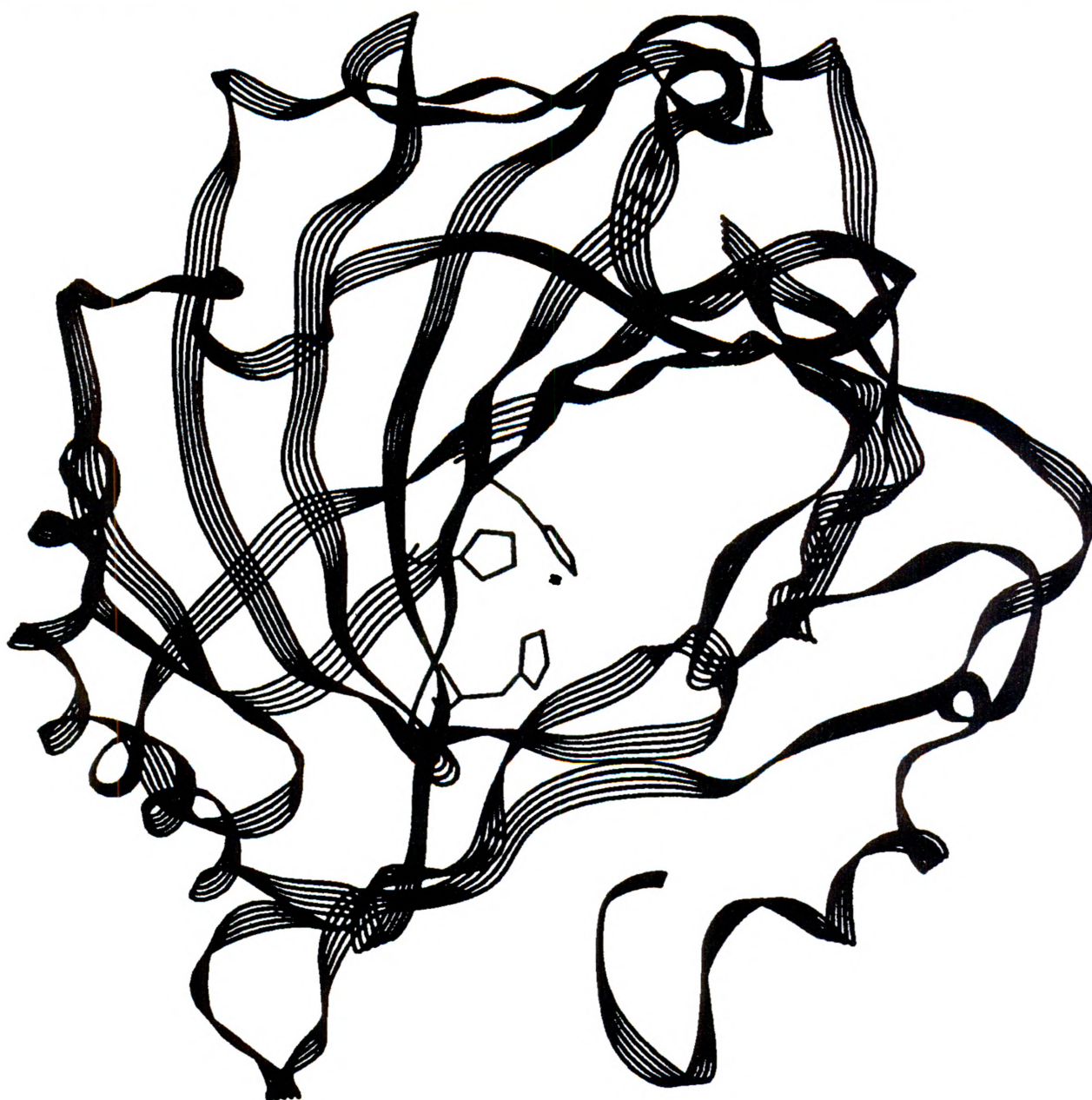
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Ribbon drawing of the backbone of carbonic anhydrase showing the three histidyl residues which form the binding site for Zn^{++} at the bottom of the cleft. This view is of the rear of the carbonic anhydrase molecule shown on the front cover, so that the binding cleft now appears on the right side instead of the left side.

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Cartoon depicting early version of carbonic anhydrase-based zinc biosensor (see Chapters 4 and 5). Carbonic anhydrase is shown with Zn^{++} (white sphere) bound at the bottom of their binding cleft. **Biomolecular recognition** of dissolved Zn^{++} (floating as the hydrated metal at 8 o'clock just outside the binding cleft) requires dehydration and diffusion at the bottom of the cleft. A fluorophore (an aryl sulfonamide, shown as a blue molecule pointing towards the observer) then complexes with the bound Zn^{++} , but not with the free Zn^{++} . When excited with ultraviolet light (the black sinusoidal arrows), the fluorophore complexed to the bound Zn^{++} emits blue light whereas the free fluorophore (the green molecule at 2 o'clock) emits green light. The **transduction signal** for bound Zn^{++} (and hence for free Zn^{++} is the ratio of intensities of blue and green light emitted by the bound and free fluorophores (see blue and green sinusoidal arrows), respectively.

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Biosensor Technologies: Perspectives and Promise

*Randall S. Alberte,
Guest Editor
Office of Naval Research*

The detection, assessment, monitoring or quantification of trace chemicals or biologics in the atmosphere, water, sediments/soils or in living systems such as the blood stream have depended, until recently, exclusively upon sampling and analytical separation and quantification methodologies. These approaches are extremely challenging for most analytes of interest and suffer from sampling problems, losses and contamination during sample handling and significant technological issues associated with separation, detection and verification in backgrounds of a large diversity of often similar analytes which are present at significantly higher concentrations. Advances in separation technologies including high pressure liquid chromatography (HPLC), gas chromatography (GC) and other solid- and gas-phase separation technologies, the availability of new materials (chromatographic resins, super critical fluids, etc.) and high resolution and highly sensitive detection technologies (mass spectrophotometric techniques, analyte-specific electrodes, etc.) have yielded significant improvements in detection even to levels of 10^{-19} M (attomolar) in environmental samples. Though it is certain that analytical capabilities will be enhanced in the future, the shortcomings of sampling and sample handling will limit their utility simply because they will not be useful in real-time applications, in remote untended applications and in situations where power requirements and environmental stability may prohibit their application.

Nature, not too surprisingly, has evolved extremely sensitive sensory systems for trace chemicals. For example, certain moths can detect as few as a couple of molecules of a specific pheromone from a source that is at a distance through diffusion of the analyte from the source through the atmosphere. Some of these systems are highly selective but lack the ability to detect related molecules, while other systems provide a broad sensitivity to a wide range of analytes with lower selectivity, utilizing signal processing and amplification provides the sensitivity needed for precise detection or quantification. This strong interplay between sensitivity and selectivity has served as a major driving forces for the evolution of a broad diversity of sensor systems in biology. This interplay is also driving new thinking about how we can exploit Nature's secrets to mimic or use biological capabilities to overcome the shortcomings of analytical techniques and to achieve a new plateau of capabilities. Anticipated capabilities target highly sensitive, inexpensive and real-time

detection and even quantification capabilities of biologics (e.g., bacterial cells or spores) or chemical species (analytes) in natural systems, in industrial and operational production streams and in operational or manufacturing systems for maintenance assessment.

Sensors are comprised of a detector which recognizes the analyte and a transduction systems which translates the recognition event into a signal which can be quantified. Common transduction systems are optical, electro-optical or electrochemical and this variety offers many opportunities to tailor biosensors for specific applications. Optical transduction systems offer significant signal amplification and examples of such systems are provided in Chapters 3 (Ligler et al.), 4 (Thompson and Walt) and 6 (Holmes). In the example described by Holmes, whole cell sensors generate light in the form of bioluminescence when the cell detects a specific analyte, and the light generated can be quantified directly or as current on a photomultiplier. In the system described by Thompson and Walt, fluorescence is actively excited by a laser through fiber optic and the light signal is analyzed to ascertain analyte binding. In many new applications not described here where antibodies are used in biosensor applications, the antibodies are bound to a thin film of gold which is held above an optical waveguide which "collects" the light which passes through the antibodies with bound analyte. Since the amount of light passing through antibody layer varies with the amount of analyte bound, the amount of light collected by optical fibers at the end of the waveguide varies proportionally with analyte binding yielding a sensitive means to detect and quantify a specific target compound.

A range of sensor types are described in the volume with the primary focus on biologically-based sensors. In Chapter 2, however, Johnson and Jannasch describe an in situ analytical sensors for oceanographic applications. This sensor is based upon a laboratory analyzer, called an autoanalyzer, for quantification of nutrient ions in seawater. The system is a miniaturized version which exploits new technologies to provide near real-time, remote quantification of nitrite (NO_3^-) in seawater. This capability, when more widely exploited, will revolutionize monitoring and quantification of chemical ions in seawater. We can, however, expect that biosensors will perform this same function, with greater efficiency, lower costs and with a minimum of maintenance that will extend operational lifetime from months to years and in almost all

oceanic environments. Such sensors will have an enormous impact on oceanographic research as well as on environmental quality assessment and pollution monitoring.

Biosensor applications for the Navy in the future will be diverse and highly specialized for specific applications. Of high Navy priority are inexpensive, highly sensitive sensors with real-time capabilities to detect remotely trace chemical or biological species in water and in air to allow for classification and detection of analytes that might compromise naval operations or signal friend or foe. Biosensors will be essential for the assessment and monitoring of a host of chemical/biological materials associated with shore and shipboard naval operations and support facilities and will be critical to environmentally sound Navy activities world-wide. Many of the current technologies lack the required reliability for wide-area evaluations, are not sufficiently rapid, are labor intensive, or are compromised by environmental features characteristic of many of the important operational regions for the Navy.

Biosensors offer non-conventional, remote real-time or near real-time sensor technologies that embody (1) high signal-to-noise, (2) high levels of discrimination, (3) multi-target capabilities, (4) potential expendability, (5) long-term unattended field deployment capabilities and (6) broad ranges in area coverage. Biosensor technologies have a bright future and will see wide-ranging uses in the future within the Navy and DoD, and will see broad application in the civilian sector targeted at needs ranging from environmental monitoring and assessment and food quality and safety to industrial process control and fisheries and coastal resource management.

Analytical Chemistry Under the Sea Surface: Monitoring Ocean Chemistry *In Situ*

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Introduction

Increasing attention is being focused on ocean chemistry and its role in controlling biological processes in the sea. One often hears the expression “the (open) ocean is a desert”¹. This is true, not for lack of water, but for lack of essential nutrient chemicals such as NO_3^- , $\text{H}_4\text{Si}(\text{OH})_4$, Fe^{3+} , and Co^{2+} that are required to support biological processes. The concentrations of most elements in seawater are less than 10^{-9} M (Figure 1). This paucity of many of the biologically essential chemicals has a significant impact on biochemical processes in the ocean. It regulates the structure of oceanic ecosystems and the absolute levels of biomass^{2,3}. These biological features, in turn, regulate optical properties of seawater such as light transmission and bioluminescence.

The interactions of ocean chemistry with biological and physical processes are complicated. The natural cycles of many nutrients are characterized by large, temporal changes in concentration. Daily variability of these chemicals in the surface waters can be driven by photosynthesis, respiration, photochemical reaction, tidal mixing and overturn of the water column forced by solar heating. Variations in chemical concentration can also occur on a seasonal cycle due to changes in light, temperature, vertical mixing rates and growth of phytoplankton and bacterioplankton (Figure 2). Interannual changes in chemical distributions over large areas of the ocean

basins occur due to climatic processes such as the El Niño-Southern Oscillation (Figure 2), the Monsoon in the Arabian sea and other large scale land/ocean/atmospheric interactions.

Concentrations of biologically essential chemicals in the ocean are difficult to predict because there are few instruments available that allow chemical concentrations to be remotely monitored in the ocean⁴. Chemical measurements can be made only when ships are present on station to collect water samples for analysis in shipboard or land-based laboratories. As a result, the biogeochemical cycles that control oceanic ecosystems and the associated properties of the water column are not well understood, and elude reliable prediction.

These problems require that a new generation of chemical instrumentation be developed that can operate unattended on remote moorings or on packages towed by ships. We briefly review the progress that has been made to develop such instrumentation for *in situ* observations of ocean chemistry.

Why Go *In Situ*?

The advantages of making chemical measurements *in situ*, rather than collecting samples and transporting them to the laboratory for analysis, are numerous. High resolution profiles allow oceanographers to resolve small scale processes which cannot be detected by conventional sampling methods^{5,6,7}. Further, continuous, long-term data sets will allow for

a better understanding of temporal variability in the abundance and distribution of chemicals of interest⁸.

In situ sensors and analyzers also minimize the probability of chemical changes in the sample due to reactions or contamination that can occur during transfer and storage of conventionally collected samples⁹. Contamination can be a particularly severe problem for the determination of trace elements in seawater¹⁰. Accurate measurements of the most contamination prone elements in seawater, such as iron and zinc, require that metal-free sampling equipment, including winches equipped with Kevlar line be brought to sea. Portable laboratories with filtered air are required to transfer samples from the sampling bottles to Teflon storage containers without contamination. These problems are avoided by performing analyses *in situ*.

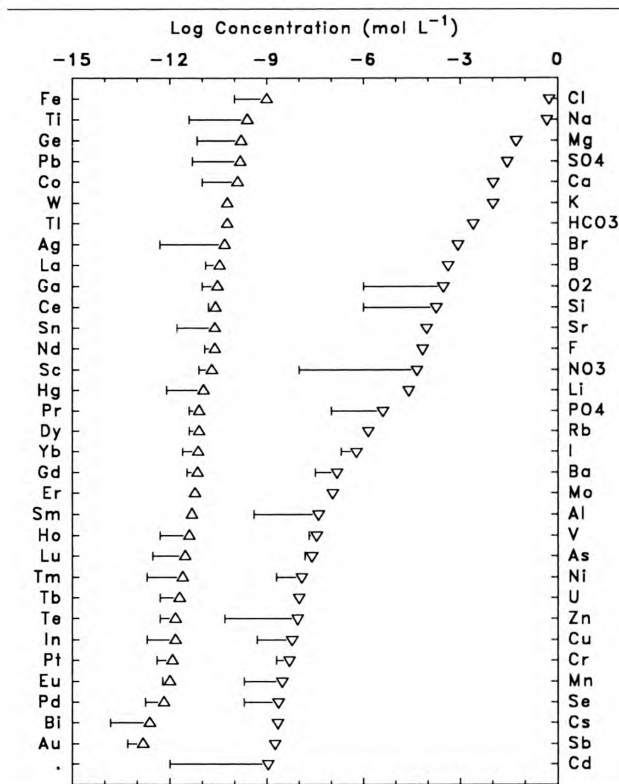
In situ chemical measurements are also required to monitor chemical concentrations at remote sites over intervals longer than a typical oceanographic cruise period (up to 4 weeks). Most oceanographic stations are occupied only for several days at a time, which results in a paucity of data on the temporal variability of chemicals in the ocean. This temporal variability of chemical properties on short- and long-time scales can be used to study processes such as primary production¹¹ and metal cycling¹². Similarly, long-term *in situ* analyzers would also be highly useful for studying the effects of time-varying processes such as El Nino and global warming on ocean chemistry and biological processes. Monthly or longer-spaced time-series, as collected by shipboard sampling, often miss crucial events occurring on more rapid time scales, including blooms, local weather events such as storms, ocean eddies, and episodic upwelling, making interpretation of the data more difficult and conclusions more tentative⁸.

Technical Approaches to *In Situ* Analysis

There are two general approaches that could be used for the determination of chemical concentrations *in situ*: chemical sensors and chemical analyzers. Chemical sensors are devices in which passive diffusion transports the chemical species to determined to the detector. Chemical analyzers move a sample by mass transport through the instrument, which then carries out the analysis. Both of these approaches to chemical analysis will face common challenges when used to detect dissolved chemicals in seawater. In particular, the combination of high salt concentrations (Na^+ , Mg^{2+} , Ca^{2+} , Cl^- , SO_4^{2-}) and the low concentrations of the nutrient chemicals require a high *selectivity* and *sensitivity*. For example, the concentrations of Mg^{2+} and Mn^{2+} in seawater are about 0.05 and 1×10^{-9} M, respectively. The valence states and radii of the ions are similar, which complicates the discrimination of the ions. An analytical method for Mn^{2+} must be very *selective* to discriminate

Figure 1.

Concentrations of dissolved elements in open ocean seawater.



against the 5×10^7 times higher Mg^{2+} levels and it must be very *sensitive* to detect the low nanomolar concentrations.

Chemical sensors and chemical analyzers also exhibit specific differences with relative advantages and disadvantages. These features are summarized below.

Chemical Sensors

Chemical sensors are typified by electrochemical sensors for molecular oxygen (O_2) and H^+ . These are the only chemical sensors that are routinely used *in situ*. Their main advantage is extreme mechanical simplicity. They generally lack any mechanism for self-calibration *in situ*, and therefore can only be calibrated at the beginning and end of a deployment period. The utility of the chemical sensor is therefore limited by the nature of the instrumental drift between these two calibrations. It is possible, however, to develop chemical sensors with sufficient stability for long-term observations. For example, the development of pulsed O_2 electrodes¹³ resulted in a marked increase in the long-term stability of these devices. As a result, they are being used to monitor changes in O_2 concentration on oceanographic moorings over periods of several months¹⁴.

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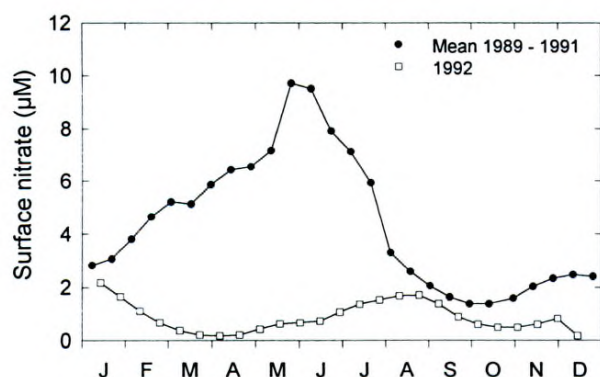
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Figure 2.

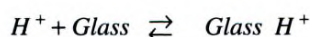
Annual cycle of nitrate measured in Monterey Bay, California. Solid circles show the average values determined over a three-year period from 1989 to 1991. The open squares show nitrate values measured during 1992, which was an El Nino year. Each point represents concentrations averaged over many locations within the Bay. Figure courtesy of F. Chavez, Monterey Bay Aquarium Research Institute.



Oxygen and pH electrodes operate on fundamentally different principles. Oxygen electrodes consume dissolved O_2 by converting it to water by the following reaction:



The electrical current produced by the reaction is proportional to the diffusive flux of O_2 into the electrode to replenish the O_2 that was consumed. This flux is determined, in part, by the O_2 concentration in solution. On the other hand, a pH electrode does not consume protons. Protons bind to the glass membrane of the electrode via a reversible chemical equilibrium as follows:



The number of protons at the surface is proportional to the bulk solution concentration, and this number changes reversibly as the bulk concentration changes. The proton concentration is determined by measuring the electrical potential created by the protons bound at the glass surface.

Like the pH sensor, most chemical sensors are based on reversible binding reactions. For example, an extremely sensitive sensor for trace levels of dissolved zinc has been developed in which Zn^{2+} binds with the apo-enzyme of carbonic anhydrase and a second reagent, dansylamide¹⁵ (see Chap. 4, Thompson and Walt). The product of this binding reaction is a highly fluorescent complex that can be used to report Zn^{2+} at low nanomolar concentrations.

Chemical sensors based on equilibria have a fundamental difficulty when chemicals at trace concentrations must be detected. To quantitatively bind a large fraction of a trace chemical such as Zn^{2+} , the equilibrium constants for the metal-ligand complex used to detect the metal must be quite large. The dissociation constant (K_D) is equal to the ratio of the rate constants for dissociation of the metal-ligand complex (k_{off}) and formation of the complex (k_{on}) as follows:

$$K_D = \frac{k_{off}}{k_{on}}$$

A consequence of small K_D values is that the dissociation rates of the complex must be very slow. The sensor signal may require days to reach a steady value following a large drop in metal concentration as the metal slowly dissociates from the ligand and diffuses away from the sensor.

This problem can be solved by modifying the sensor to operate in a mode similar to that of an O_2 sensor. That is, the metal-ligand complex can be removed from the sensor by pumping a solution containing the ligand through the detection area and sweeping out the complex. Metal ions will continue to diffuse into the sensor area and be detected, but the resi-

Figure 3.

Fiber optical sensor for the determination of carbon dioxide partial pressure in seawater using a flowing reagent stream¹⁷. Components are a) reagent delivery capillary; b) reagent exit capillary; c) fiber optic from source; d) fiber optic to detection system; e) white silicone rubber membrane; f) white silicone sealant; g) epoxy; h) O-ring; i) sensor housing; j) fiber optic cable. Figure courtesy of M. DeGrandpre, Woods Hole Oceanographic Institution.

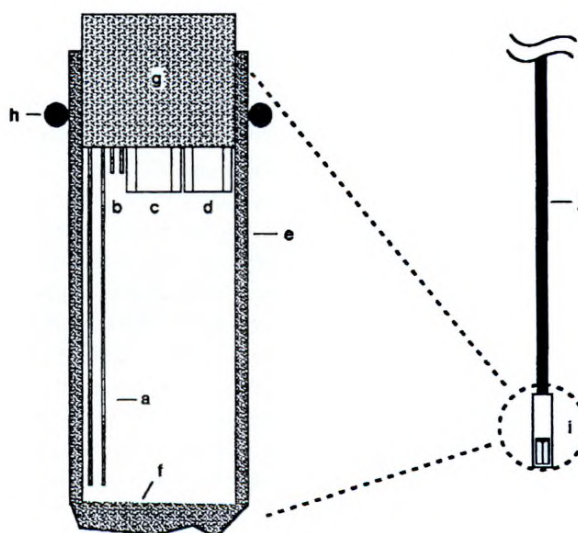
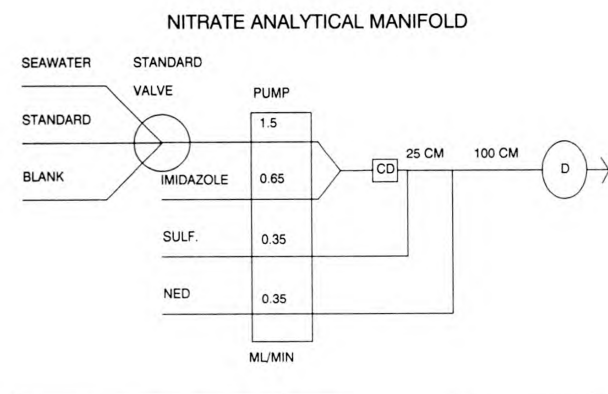


Figure 4.

Scanner block diagram modified from Johnson et al.²². The lines are Teflon capillaries. CD is a cadmium column for reduction of nitrate and D is a photometric detector that measures light absorption of the dye generated by reaction with the nitrate



dence time of the metal and the metal-ligand complex will be limited by the flow of the reagent solution. This concept, called a renewable reagent sensor, was pioneered by Inman et al.¹⁶, at the Naval Ocean Systems Center. The renewable reagent sensor concept has been applied to oceanographic processes by DeGrandpre¹⁷, who developed a prototype sensor for dissolved carbon dioxide (Figure 3).

Chemical Analyzers

Chemical sensor systems in which the reagents are continually renewed are a hybrid between a sensor and an analyzer. The analyte enters the sensor by diffusion, but it is removed by mass transport. The complexity of the system is greater than a simple sensor, but response rates can be increased considerably. The next level of complexity in chemical analysis involves building systems in which the sample is actively transported into the instrument. This confers several advantages, but at the cost of additional complexity. First, it is feasible to add a valve that allows the sample inlet to be switched to a standard solution with a known concentration of the analyte when the sample is actively moved through the analyzer. Calibration *in situ* is possible in such an analyzer.

It is also relatively easy to perform multi-step reactions in an analyzer by merging the flowing sample stream with reagents at various points along the flow path. This is a significant advantage because it is often difficult to find reagents that will bind many of the nutrient chemicals with sufficient selectivity to discriminate them from the high salt background in seawater. For example, in seawater many divalent cations cannot be discriminated at a sensor surface because the large concentrations of Mg^{2+} and Ca^{2+} overwhelm the sensor signal.

However, multistep reactions make it possible to develop much more selective detection schemes.

Segmented continuous flow analyzers (Autoanalyzers) have been widely used in oceanographic research for chemical analyses onboard ship. These systems use air bubbles to segment the liquid sample stream, which limits dispersion of the sample in the turbulent flow. Segmented analyzers are not well suited for chemical analysis underwater because air bubbles are used to segment the sample stream.

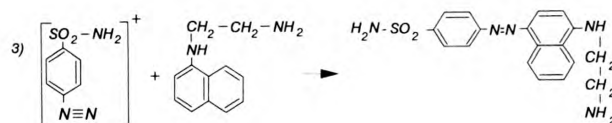
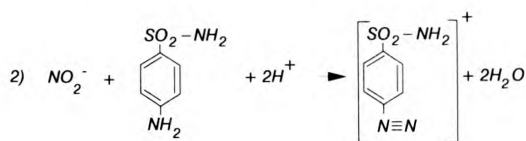
The development of Flow Injection Analysis (FIA) demonstrated that continuous flow analyzers could operate equally well without air bubbles to segment the sample stream if small diameter tubing (≈ 0.5 mm, i.d.) was used to carry the sample¹⁸. Flow is laminar in these systems and radial diffusion is sufficiently rapid to limit dispersion of the sample. A continuous flow analyzer, based upon the principles of FIA, was developed in 1984 for *in situ* measurements in hydrothermal systems¹⁹. This Submersible Chemical Analyzer (commonly called a Scanner) was capable of simultaneously measuring two chemicals by colorimetry, as well as monitoring dissolved O_2 in the sample stream and temperature at the sample inlet.

A schematic layout of a Scanner system configured for the analysis of dissolved NO_3^- is shown in Figure 4. The determination of NO_3^- requires three reaction steps, each of which is essentially irreversible (Figure 5). Nitrate is reduced to NO_2^- by buffering the sample to a neutral pH and pumping it through a cadmium column. The nitrite is then reacted with sulfanilamide in acid to form a diazonium ion. Finally, the diazonium ion is reacted with N-(1-naphthyl)-ethylenediamine to form a brightly colored product, an azo dye molecule which can be quantified spectrophotometrically.

The sample and reagents are propelled through the reaction manifold by a multi-channel pump and delivered to a colorimeter. The amount of light absorbed by the dye solution

Figure 5.

The 3-step reaction sequence used for the determination of dissolved nitrate.



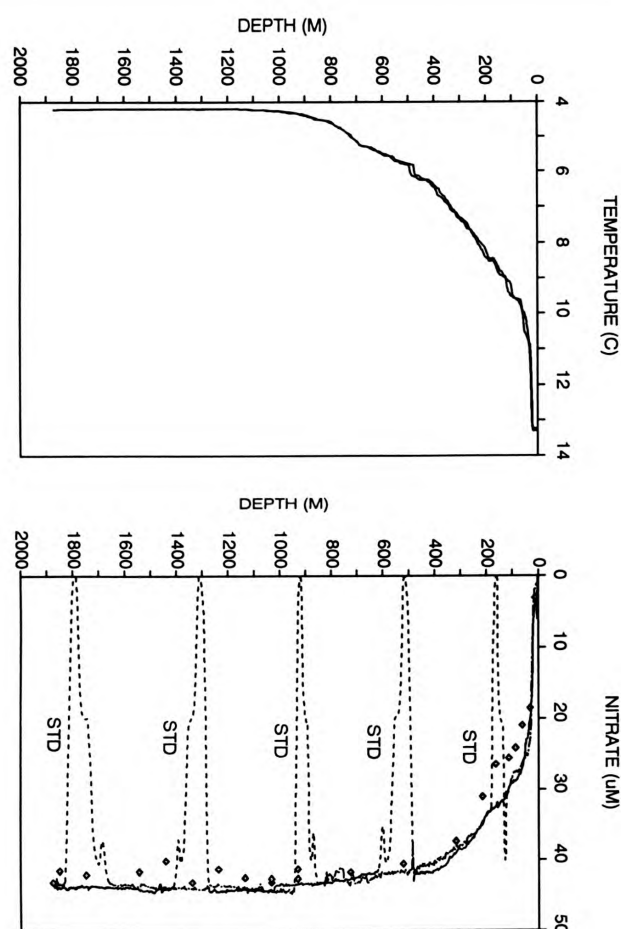
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Figure 6.

Vertical profile of nitrate determined *in situ*²². Measurements were made in the Santa Cruz Basin in the Southern California Borderland (33°39.1N, 119°32.8W). The solid line shows the concentration of nitrate. The dashed portions of the lines show time periods when the instrument calibrated itself *in situ* by analysing a blank solution, a solution containing 20 $\mu\text{M NO}_3^-$ and a solution containing 40 $\mu\text{M NO}_3^-$. Each complete calibration required 6 minutes. Nitrate concentrations determined in shore based laboratories on samples collected by conventional hydrocasts are shown as diamonds, for comparison.



is a function of the initial NO_3^- concentration. The system can be calibrated by switching the valve on the inlet to select either sample, a blank or a standard solution with a known NO_3^- concentration.

Submersible chemical analyzers are the only means available today that allow remote monitoring in the ocean of the concentrations of dissolved nutrients⁴. Work on *in situ* analyzers is ongoing in several laboratories, and it is now possible to

monitor concentrations of nutrient species for periods of time greater than one month. Recent advances have made it possible to produce systems with greatly reduced complexity and increased reliability for long-term deployments²⁰.

It is our belief that analyzer technology provides a near term solution to the need for *in situ* chemical analysis of ocean waters. Below, we summarize some of the work that has been done to develop chemical analyzers that will operate underwater.

Oceanographic Applications of Submersible Chemical Analyzers

Two classes of applications exist for Scanners in oceanography: high resolution instruments for the determination of chemical distributions in the presence of large spatial gradients and low resolution instruments for long-term observations on oceanographic moorings (Table 1). High resolution instruments are typically lowered through the water column from a stationary ship or towed behind a moving ship to determine the spatial distribution of an analyte. This application requires a Scanner that responds rapidly to the changes in concentration in regions of large chemical gradients. Nutrient concentrations may change by 100% or more in a few meters within the thermocline where large vertical temperature gradients prevent warm, nutrient depleted surface water from mixing with cold, nutrient rich deep waters, or within ocean fronts where two water masses with different properties meet. The 90% response time must approach a few seconds to produce useful data in such areas. The instrument response must also be relatively immune to large temperature and pressure changes. Calibration *in situ* is not mandatory if the Scanner is mounted with a device to collect a few discrete samples, which can provide calibration points for the detector signals.

The second application involves placing a chemical analyzer on a fixed mooring or drifting array. This application requires a device that is capable of operating for extended periods of time (up to 1 year for open ocean moorings) without operator intervention. The instrument must be self-calibrating to control for detector drift. However, the requirements for response rate are more relaxed than in the case of towed analyzers. Moored analyzers will need to have a longer endurance, resistance to biofouling and stable chemical reagents.

High Resolution Analyzers

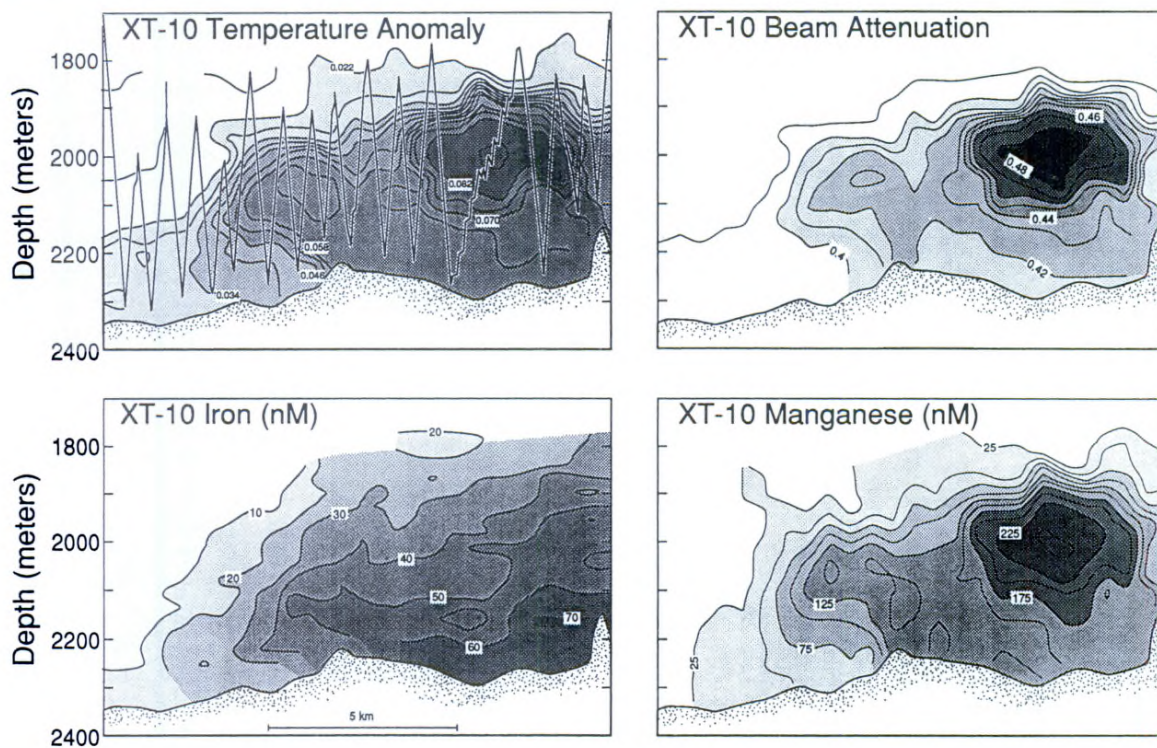
Scanners designed for high resolution measurements of chemical distributions have been used in a variety of oceanographic studies. These studies have ranged from measurements of chemical distributions around deep-sea hydrothermal vents^{6,7,21} to observations of the vertical distribution of NO_3^- (Figure 6)^{22,23}.

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Figure 7.

Contoured section of temperature anomaly (A), light attenuation anomaly (B), dissolved iron (C) and dissolved manganese (D) measured along a sawtoothed tow track over the crest of the South Cleft segment of the Juan de Fuca Ridge²¹. The left hand side is 44°52.8N, 130°13.0 W on the eastern flank and the right hand side is at 44°50.6N, 130°21.0W on the western flank. The tow track of the chemical analyzer package is plotted together with the contoured section of temperature anomaly. The temperature anomaly is reported in units of m°C (0.001°C).



Scanner systems have been particularly useful in studies of processes that control metal distributions in hydrothermal plumes^{7,21}. Figure 7 shows a vertical section of dissolved Mn^{2+} and iron (as $\text{Fe}^{2+} + \text{Fe}^{3+}$) measured *in situ* while a Scanner was towed in a sawtooth pattern across the Juan de Fuca Ridge off the coast of Washington State. Approximately 3700 sets of measurements of manganese and iron were made *in situ* and used to contour this section. The distribution of Mn^{2+} is highly correlated with the temperature anomaly in the water column, which is derived from hydrothermal heat. Iron, on the other hand, is only poorly correlated with temperature anomaly. This occurs because hydrothermal iron is soluble as the Fe^{2+} ion under the reducing conditions found within the mid-ocean ridge. The hydrothermal Fe^{2+} rapidly oxidizes to Fe^{3+} in seawater, and the Fe^{3+} precipitates as iron oxide particles (FeOOH) that produce the light attenuation anomaly. These iron oxide particles are not detected chemically with the reagents used *in situ*. Results from this type of work have been used to predict the existence of hydrothermal vents with

unique metal:heat signatures. These vents were subsequently found during dives with the manned submersible Alvin²¹.

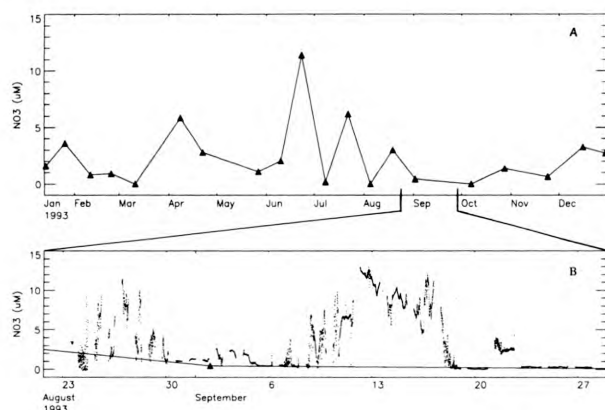
Time Series Analyzers

Chemical analyzers designed to operate for long periods on oceanographic moorings are needed to monitor ocean chemistry when ships cannot be present to collect samples⁴. Even the most ambitious programs cannot provide enough ship time to keep chemists on station for long time periods. The Scanners used for vertical profiling are generally not well suited to long-term applications because the pumps used to propel the reagents and sample require frequent maintenance. Significant progress in extending the endurance of Scanner systems has been made by replacing mechanical pumping systems with pumps that use osmotic pressure to propel sample and reagents²⁰.

The application of osmotic pumps represents a significant advance for several reasons. First, when immersed in liquid they will pump continuously without an electrical power

Figure 8.

The concentration of nitrate determined at a single station in Monterey Bay, California at biweekly to monthly intervals (A) and the concentration of nitrate determined continuously over a one-month period with an osmotically powered Scanner deployed on a mooring at 10 m depth in the Bay (B). The solid line and triangles in B are from the data set in A. Panel A courtesy of C. Sakamoto, Monterey Bay Aquarium Research Institute.



source. Secondly, they have only one moving part (a collapsible bag) and they are extremely reliable for long-term (ca. 1 year) operation. Thirdly, they can produce constant liquid flow rates in the microliter per hour range, which means the instruments will require only 10 to 20 ml of reagents for chemical analyses over year-long time periods. Finally, the simplicity and small size of osmotic pumps allows the entire instrument to be greatly reduced in size and cost.

Osmotic NO_3^- analyzers have been deployed in Monterey Bay for month-long periods. The results of this work illustrate the great variability in chemical concentrations that can occur over very short time periods (Figure 8b). This variability is severely undersampled when ships are used as sampling platforms to estimate natural chemical variability (Figure 8a). The continuous measurements show rapid concentration changes that were completely missed by the biweekly sampling program. Although these analyzers have so far only been used for the determination of NO_3^- , this approach can also be used to determine trace metal concentrations.

Future Needs

Future research that is directed towards the development of methods for *in situ* chemical analysis is required in two broad areas. The detection limits for dissolved trace metals need to be reduced by factors of 10 to 100. Though metals such as Mn^{2+} and Fe^{2+} can be detected in hydrother-

mal plumes, the concentrations in these plumes are elevated as much as 10,000 times above the concentrations in the surrounding seawater^{7,10}. Detection limits of current Scanner systems are not low enough to detect dissolved metals in areas where concentrations are near the ambient levels. New methods of metal detection, such as the enzyme based Zn^{2+} biosensor developed by Thompson et al.¹⁵ (see Chapter 4), need to be adapted to chemical analyzer systems to achieve the needed sensitivities required for measurements at ambient levels in the ocean¹⁷.

Considerable work is also needed to develop methods for the determination of a broader suite of chemicals. The list of elements that may regulate ocean ecosystem structure has grown from NO_3^- , PO_4^{3-} , and $\text{H}_4\text{Si}(\text{OH})_4$ to include many trace elements such as Mn^{2+} , Fe^{2+} , Fe^{3+} , Zn^{2+} , and Co^{2+} . *In situ* detection of CO_2 partial pressure is essential in order to assess the fate of fossil fuel carbon dioxide in the ocean. Significant advances in our understanding of marine ecosystems will come about when the technology is developed to monitor these elements *in situ*.

Many chemicals also have the potential to cause significant harm to marine ecosystems. For example, in the Baltic Sea, concentrations of the nutrient compounds NO_3^- and PO_4^{3-} appear to have doubled in the last years²⁴. As a result of similar nutrient increases, the concentrations of dissolved O_2 have decreased in many bays and coastal systems, with serious consequences to the ecosystems. In some enclosed harbors, concentrations of metals, particularly tributyl tin compounds, have increased dramatically. These changes can only be interpreted with well resolved time-series measurements of chemical concentration. There simply are not enough resources to accomplish this with conventional monitoring programs²⁵. New technologies for continuous chemical monitoring will be required to fully understand the impacts of these chemical changes and to distinguish anthropogenic influences from natural chemical variability.

Table 1.

Property	Profiling Devices	Moored Devices
Response Rate	1/s	2/h
Lifetime	24 h	1 yr
Temporal Stability	hours	months
Resistance to Biofouling	low	high
Selectivity	high	high
Sensitivity	high	high
Reagent and Standard Stability	low	high

Biographies

Kenneth S. Johnson is Professor of Chemical Oceanography at the Moss Landing Marine Laboratories (MLML) of the California State University and a Senior Scientist at the Monterey Bay Aquarium Research Institute (MBARI). He received his B.S. degrees in Chemistry and in Oceanography from the University of Washington in 1975 and a Ph.D. in Oceanography from Oregon State University in 1979. His research interests include the development of automated methods for trace chemical analysis that operate on board ship and *in situ*, and the application of these systems to studies of chemical cycling in the ocean. This work has been funded by the Office of Naval Research since 1982. These methods were recently used to map dissolved iron concentrations at sea during the ONR and NSF funded Open Ocean Iron Fertilization Experiment conducted south of the Galapagos Islands to test the Iron Hypothesis of the late John Martin.

Hans W. Jannasch received his Sc.B. in Chemistry from Brown University in 1978 and his Ph.D. in Chemical Oceanography from the University of Washington in 1990. Following a joint post-doc at MBARI and MLML to develop osmotically powered chemical analyzers, he was appointed as Assistant Scientist at MBARI. His current work focuses on developing *in situ* chemical detectors for studies of chemical variability in seawater.

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The Antibody-Based Biosensor: A Technology For Today

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Center for Bio/Molecular Science and Engineering
Naval Research Laboratory

The fiber optic biosensor was developed as an ultrasensitive detection system to make simple, rapid measurements of analytes in clinical and environmental samples. It integrates the spatial discrimination of evanescent wave measurements, the rapid signal transduction capability of fiber optics, the signal-to-noise discrimination capability of fluorescence measurements, and the sensitivity and specificity of biological recognition molecules. The sensor has evolved over the last five years from a system that weighed 100 pounds to one the size of a voltmeter. The geometry of the probe has been improved to the point that the sensitivity is enhanced 100-fold. And a wide variety of tests for hazardous materials in real-world samples has been developed using antibodies to provide the molecular recognition.

Sensor Hardware

The antibodies immobilized on the fiber surface bind the analyte of interest (and the complex of analyte and fluorescent reagent) with such high affinity that the current major limitation in sensitivity is the ability of the device to preserve and discriminate the returning fluorescent signal. Three advances in the sensor technology have had a major impact in addressing this problem and in producing a sensitive, easy-to-use device: refined probe geometry, diode lasers, and new fluorescent dyes.

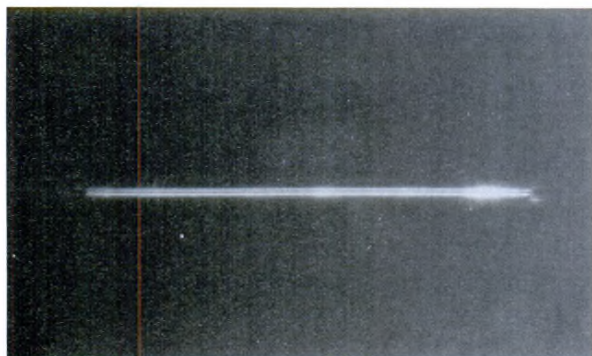
Figure 1 shows an optical fiber immersed in a solution of fluorescent dye. Only the fluorophores at the surface of the

fiber are excited. No light reaches the fluorophores which are more distant from the fiber surface. The optical field at the fiber surface is called the evanescent wave. Under the conditions described here, the excitation light extends about 150 nm into the surrounding sample. The use of the evanescent wave configuration to perform a homogeneous immunoassay was first described by Kronick and Little¹. The idea is to immobilize an antibody on the surface and measure fluorescence excited by radiation in the evanescent field as the antibody binds its antigen. In this way, no removal of unbound analyte or fluorescent reagent is required prior to generating a signal, and measurements can be made in real time.

Because we wanted to analyze potentially hazardous samples, we needed to physically isolate both the operator and the optics from the sample. To this end, we employed long optical fibers which can be easily manipulated and introduced into closed sample containers, enclosed flowing streams, or bio-hazard hoods. However, these long fibers were not very effective in preserving the returning fluorescent signal. Over most of the fiber, the glass core is clad with silicone. Over the last 10 centimeters, the plastic cladding is removed to fabricate the sensing region. Antibodies are attached, and the fiber is immersed in an aqueous sample. As the fluorescent light returns up the fiber it passes from the unclad region of the fiber into the clad region. At this point, much of the emitted light was lost into the cladding. Changing the geometry of the fiber, as shown in Figure 2, solved this problem²⁻⁴. Tapering the fiber enhanced both the distribution of excitation light throughout

Figure 1.

Excitation in the evanescent wave. Light extending beyond the surface of the unclad fiber only excites fluorophores near the surface.

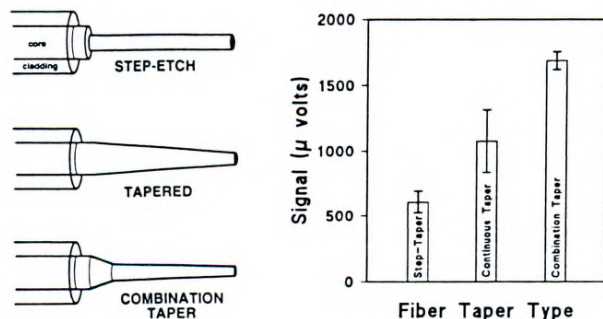


the length of the probe region and the preservation of the returning fluorescent signal.

To excite the fluorescence, various light sources have been utilized during the development of the fiber optic biosensor. The various lasers were combined with complementary optoelectronic components to yield the different fiber optic fluorimeters. The first laboratory breadboard device included an HeNe laser and weighed 100 pounds^{5,6}. It could be operated at 488 or 514 nm excitation light to measure the fluorescence from fluorescein and rhodamine dyes. The next version of the system employed a frequency doubled neodymium YAG laser operating at 532 nm, was compatible with the rhodamine dye Texas Red, and weighed 40 pounds. We have recently tested

Figure 2.

Geometry of the fiber probe. Tapering the fiber in various shapes (left panel) increases both the uniformity of the excitation power in the evanescent wave and the preservation of the fluorescent signal (right panel).



our ability to make fiber optic biosensor measurements using 650 nm and 785 nm diode lasers and the newly available dyes Cy5 and NN 384⁷. Figure 3 provides an idea of the size difference in these systems. In collaboration with a small optics company, we have just completed construction of a diode-laser based sensor the size of a voltmeter with the capability of simultaneously analyzing signal from 4 fiber probes. Surprisingly, no sensitivity was lost as we transitioned to the handheld systems. Because the samples and fibers have much less background fluorescence at the longer wavelengths, signal-to-noise actually improves. With the 650 nm system, we have obtained more than 10-fold improvement in sensitivity over that obtained using the 514 nm system.

Immunoassays

The wide variety of immunoassays developed for fluorescence measurements can be adapted for use with the fiber optic biosensor. While not discussed here, we are also developing DNA probe/PCR-based techniques and ligand-binding assays using non-antibody proteins for measurements at the surface of the optical fiber. Common to all these assays, however, is the requirement for a method to immobilize proteins at high density on the fiber surface without damaging the recognition function. The immobilized proteins must also be stable during long periods of storage prior to use. Methods for covalent protein immobilization were developed to satisfy the above requirements⁸. Fibers coated with antibodies using this procedure can be stored up to 2 years prior to use.

A sandwich immunoassay is conducted at the surface of the fiber as shown in Figure 4. The fiber is coated with a capture antibody and then exposed to a sample containing the analyte. This sample may also contain the fluorescent antibody, or alternatively, exposure to sample and fluorescent antibody can take place sequentially. A signal is generated as the fluorescent complex forms at the surface of the fiber. To date, we have developed assays for 4 different protein toxins, varying in molecular weight from 13,000 to 160,000, and 2 different bacterial proteins⁹⁻¹¹. Monoclonal antibodies, polyclonal antibodies, and combinations of the two have been used. Signal could be measured within the first minute after the addition of the fluorescent antibody. In all cases, the assays were conducted at 514 nm and produced limits of sensitivity of 1-10 ng/ml. Initial tests indicate that the handheld systems are capable of at least an order of magnitude higher sensitivity. Most importantly, accurate quantitation could be obtained in samples as viscous as serum and as opaque as whole blood (Figure 5).

To perform a sandwich assay, however, requires that the analyte to be detected is sufficiently large that it has two sites for antibody binding. Many pollutants, drugs, and explosives are small molecules which can only be bound by a single antibody. For analysis of such small molecules, we have

developed a competitive immunoassay at the fiber surface. To conduct this assay, we prepare not only an antibody-coated fiber, but also a dye-labelled version of the analyte to be detected. We then compare the signal generated by the binding of a known amount of the fluorescent analyte to the fiber with the signal generated when a mixture containing sample and the fluorescent analyte is added to the fiber. Unlabelled analyte competes with the fluorescent analyte for antibody binding sites on the fiber. The reduction in signal is proportional to the amount of analyte in the sample. The small standard errors in Figure 6 indicate the precision with which a small molecule such as the explosive trinitrotoluene (TNT) can be detected.

A rather unusual assay (11, patent pending) has been developed for detection of microorganisms. In this assay, biological components of the sample are stained using a mem-

brane, lipid, protein, or nucleic acid dye which excites at the appropriate wavelength. The capture antibody on the fiber binds only the bacteria or virus for which it is specific, immediately generating a fluorescent signal. For 3 different bacteria, we have been able to detect as few as 100 cells/ml in a 250 l sample. The assays have been performed both in serum and in samples collected from the environment using an air sampler.

Potential

A portable device capable of simultaneous interrogation of multiple fiber probes is being manufactured. Not only will the use of multiple fibers provide for simultaneous detection of multiple antigens, but it will also facilitate improved discrimination of the fluorescence signal from background interferences and provide controls for false positive and false negative responses. Manufacturing techniques for producing the tapered fiber probes inexpensively and in large quantities can be developed. While bound antigens can be removed and the probes reused, the immobilized antibody is still sensitive to proteolytic degradation in dirty samples. Procedures for minimizing fouling and protease degradation of the antibody-coated fibers must be developed for specific applications that require extended periods of use.

Once these problems are solved, the fiber optic biosensor will be used for environmental and clinical monitoring. A variety of monospecific recognition materials (i.e. antibodies, lectins, ionophores, synthetic binding elements) will be attached to the fiber optic probes. Upon antigen recognition, the optical signal will be translated into an immediate readout from a portable device or sent through a fiber optic network to a central alarm system. Optical fibers and networks are immune to radio frequency or electromagnetic pulse interference. Lightweight and reliable, fiber optic biosensors will be adapted to monitor for the presence of toxic agents, hazardous

Figure 3.

Sensor development. The light source for the 100 pound instrument is compared with both the 40 pound system and the diode laser being incorporated into the handheld system under development.

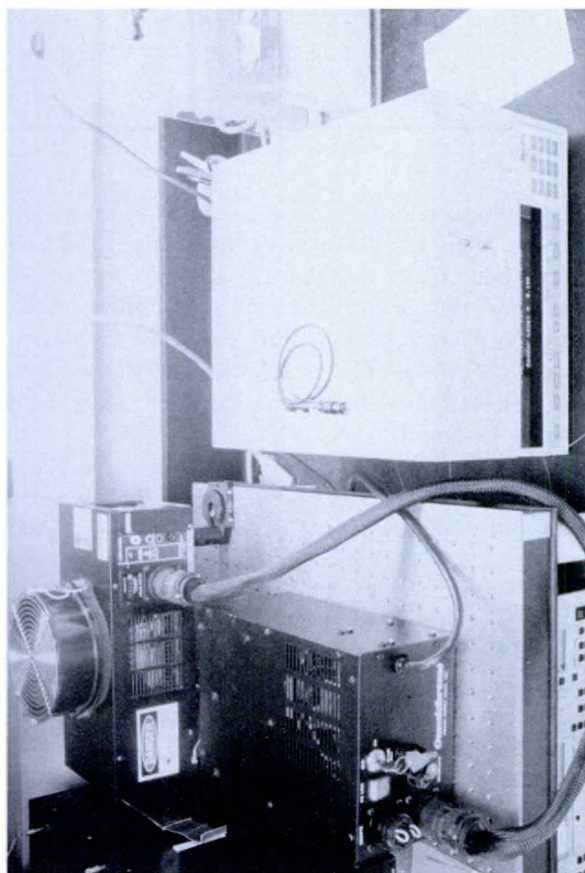
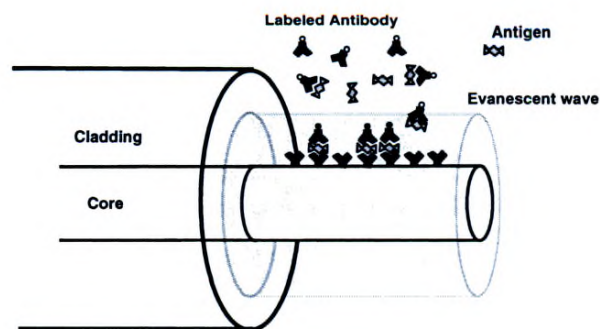


Figure 4.

Sandwich immunoassay.

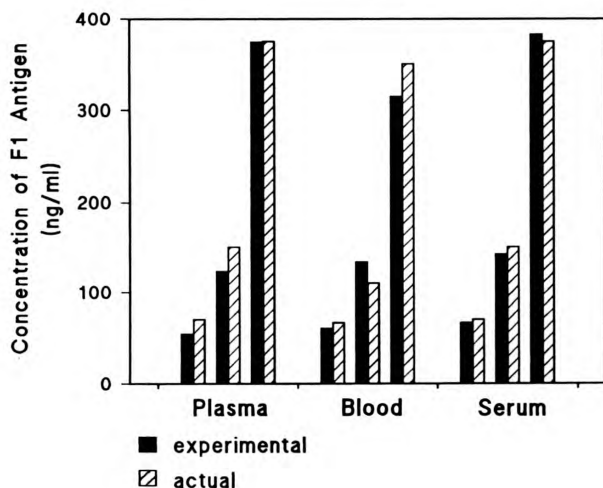


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Figure 5.

Detection of bacterial antigen in viscous and opaque samples. The F1 antigen of *Y. pestis* was introduced into samples of serum, plasma, and blood, and the samples tested blind. Actual values represent the signal as predicted from a standard curve obtained by measuring F1 in phosphate buffered



chemicals in storage or production facilities, hazards in closed environments such as engineering spaces or magazines, explosives and byproducts of explosive manufacture, pollutants, drugs or pathogenic organisms.

Acknowledgements

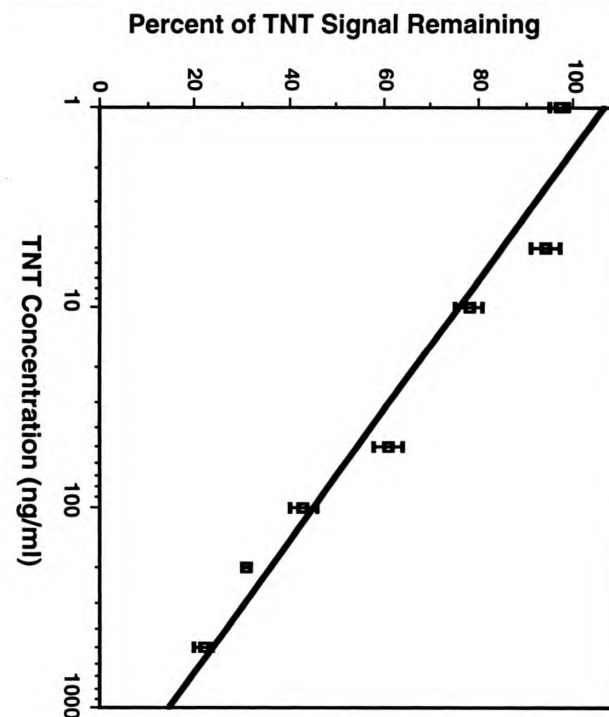
We would like to thank summer students Jennifer Doyle, Kristen Breslin, Kara Schmetzer, and Stephanie Stratton for their contributions to this work. We also thank our collaborators at US Army Medical Research Institute for Infectious Disease (USAMRIID), Drs. Ed Brown, John Ezzell, John Hewetson, and John Middlebrook, and LCDR James Burans at the Naval Medical Research Institute who provided reagents and advice. The work was supported by the Office of Naval Research, the Naval Medical Research and Development Command, and the US Army Medical Material Development Agency. The views expressed here are those of the authors and do not represent those of the U.S. Navy, or the Department of Defense.

Biographies

Frances S. Ligler is currently Program Manager for the Biosensors Programs in the Center for Bio/Molecular Science and Engineering at the Naval Research Laboratory. She earned a PH.D. from Oxford University, Oxford, England and com-

Figure 6.

Detection of a small molecule. A competitive immunoassay for TNT was conducted on 4 fibers and the results averaged ($\pm$ S.E.).



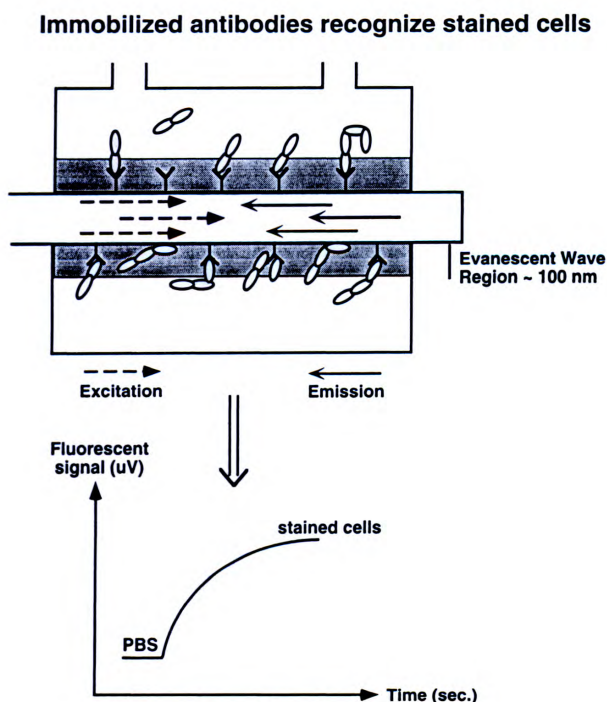
pleted Postdoctoral Fellowship at University of Texas Health Science Centers at San Antonio (Biochemistry) and Callas (Immunology). After five years in academic research, she spent five years in industry (DuPont) and then joined NRL in 1985. Dr. Ligler has been awarded patents and has published in many scientific journals. She is the winner of the National Drug Control Policy Technology Transfer Award and the American Chemical Society's Hillebrand Award.

Lisa C. Shriver-Lake came to the Center for Bio/Molecular Engineering at NRL in 1987. Before, she was with the Biochemistry Department of the University of Maryland. She received a MS in Chemistry from George Mason University, Fairfax, Virginia, in 1991. Ms. Shriver-Lake has been working in the area of optical biosensors for the last eight years. She has also been involved with controlled immobilization of proteins on solid supports. Ms. Shriver-Lake has numerous publications in referred journals. She also has a U.S. Patent on immobilizing proteins to solid supports.

Joel P. Golden received his BS degree in electrical engineering from the University of Maryland in 1984 and received his MS degree in electrical engineering from Johns Hopkins University in 1992. He has been at the Naval Research Laboratory since 1984. His work at the Center for Bio/Molecular

Figure 7.

Detection of whole bacteria. Schematic of assay in which all cellular components of the sample are stained and the capture antibody on the fiber binds only the cells of interest.



science and Engineering has focused on development of fiber optic biosensors for Navy applications. Other research interests have included resistance of holograms to radiation damage and numerical Fourier transform holographic image propagation. He is a member of IEEE and SPIE.

George P. Anderson received his BS and Ph.D. (1986) in biochemistry from The Ohio State University. His thesis research focused on chemical medication of the electron transport protein plastocyanin. As a post-doctoral fellow at The Ohio State University, College of Medicine, he studied activation mechanisms of the platelet Fc receptor. Since 1991, he has been at NRL working on the development of fiber optic biosensors. He received support in this work from a National Research Council-NRL Research Associateship.

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Profiles in Science



Dr. Carol Ann Fierke

Dr. Carol Ann Fierke is Professor of Biochemistry at the Duke University Medical Center and a member of the Duke University Cancer Center. For five years, she has been a principal investigator of the Office of Naval Research researching protein variants.

She earned a Ph.D from Brandeis University investigating structure-reactivity relationships in biological catalysts. Next, she worked in the laboratory of Professor Stephen Benkovic of Pennsylvania State University where she was introduced to molecular biology and recombinant DNA technology which are techniques essential for the success of her ongoing research.

In 1987, she collaborated with Professor David Christianson at the University of Pennsylvania to determine the high resolution structure of many enzyme variants using X-ray crystallography. This combined structure-function approach has led to the identification of structural features in carbonic anhydrase essential for: binding substrates, inhibitors and metals; catalysis of CO₂ hydration and ester hydroly-

sis; and protein stability. These studies provided a perfect backdrop for the preparation of carbonic anhydrase variants for use in a fluorescent carbonic anhydrase-based zinc biosensor concurrently being developed by Professor Richard Thompson at the University of Maryland. This three-way collaboration, funded by ONR, provides a model for the design and optimization of protein variants for practical use in a variety of applications, including field-deployable optical biosensors. Professor Fierke's laboratory is currently exploring methods of screening large libraries of variants to facilitate this process. Her group is also investigating metal binding and reactivity in a variety other enzymes, including RNA and DNA nucleases.

Professor Fierke is a member of the American Society for Biochemistry and Molecular Biology and the American Association for the Advancement of Science and has served on a National Science Foundation Advisory Panel and as the Chair of the Nominating Committee for the American Chemical Society.

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Emerging Strategies for Molecular Biosensors

Richard B. Thompson
University of Maryland School of Medicine

David R. Walt
Max Tishler Laboratory for Organic Chemistry
Tufts University

Background and Introduction

Our sense of smell is remarkable. We can detect the presence of many different molecules at the part per billion level. Even more remarkable, however, is our ability to make a split second judgment regarding the nature of the smell, i.e., pleasant, noxious, irritating. The olfactory system, therefore, is not only sensitive, but is interfaced to the brain which makes value judgements very rapidly. Finally, these judgements can be used to decide on a course of action, e.g., aversion, give a sound of approval "Mmm", close a valve, etc.

In a rudimentary way, biosensors attempt to mimic these biological sensing systems. In contrast to most traditional analytical instrumentation, sensors are continuous and are used in a monitoring, rather than discrete sampling, mode. Sensors employ one of four transduction mechanisms for operation. They may detect changes in electrochemical, mass, thermal or optical parameters. In general, sensors operate by incorporating a recognition element, such as an indicator, selective polymer, enzyme or antibody into the transducer. The presence of the analyte is sensed by the recognition element which signals a change in the transducer resulting in the sensor's output. Historically, and to a large extent presently, most sensors have been designed to exhibit the greatest degree of selectivity possible - that is, they are designed to react to one analyte with as little interference as possible from other chemical species. Although this approach may work in a well-controlled and well-understood environment, it is by

design not anticipatory. In other words, most sensors can only detect what they are designed to detect and the presence of an unusual or new chemical species will go undetected. This approach contrasts sharply with biological systems. For example, extremely low levels of an inorganic thiol or amine can be invariably detected by the olfactory system whether or not an individual has ever been challenged with these chemicals previously. This flexibility, broad selectivity and high sensitivity provides organisms with a tremendous survival advantage. It is the goal of today's biosensor scientist to develop sensing systems that have a broad range of flexibility while maintaining the sensitivities represented by the one sensor-one analyte paradigm.

The Navy has long been interested in sensors for its specialized interests and applications. More recently, attention has been focused on the environmental impact of Navy ships, aircraft, personnel, and operations, and the role that chemical sensors might play in assessing and minimizing that impact. For instance, shipboard wastewater treatment and discharge standards have changed, requiring much closer control and analysis of the wastewater before it is pumped overboard. Similarly, the Navy has a longstanding interest in understanding the marine environment, as this has become increasingly important tactically. Finally, in circumstances where environmental remediation may be required as a result of Navy activity, chemical sensors are crucial to evaluating the extent of the problem, and the success of the remediation. For all these reasons, since 1990 the Navy has pursued the devel-

opment of chemical sensors using biological synthesis and transduction principles as an integral part of its research and development program.

Sensor and Biosensor Operating Principles

The basic architecture of a biosensor is depicted in Figure 1. The sensors we are discussing are chemical sensors, and are intended to measure the presence or level of some chemical species and convey that information to the operator in some usable form. Chemical species are construed broadly to include everything from atoms and ions through small molecules such as amino acids and pesticides, up to macromolecules and biological polymers such as proteins. Note that still larger entities of analytical interest such as viruses and bacteria can be identified by chemical sensors sensitive to the molecules attached to the surfaces of these organisms.

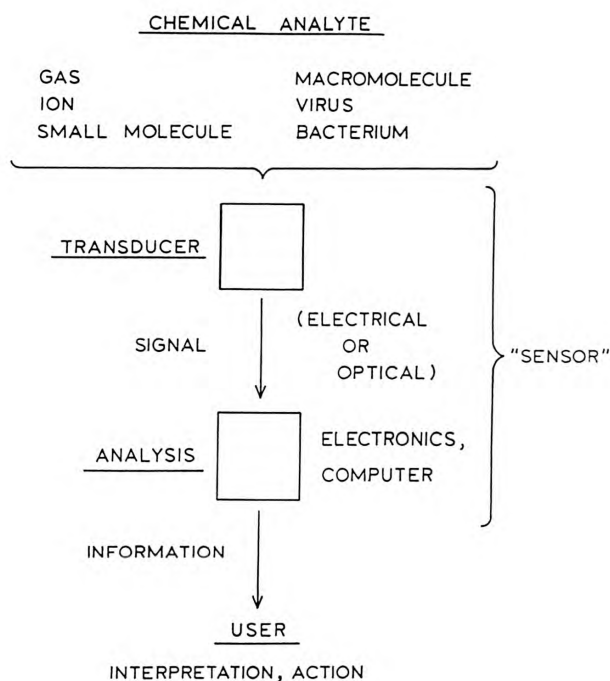
The heart of the sensor is its *transducer*, whose function is to transduce the presence or level of the analyte as a signal which may be interpreted. This definition originated with the work of Malmstadt, Enke and Crouch¹, who introduced the paradigm of data domains. The basis of the transducer in our case is a molecule of biological or biomimetic origin which has some selective interaction with the analyte, such that a signal is produced. It is typically incorporated into the transducer in the form of a coating or layer within a multi-lamellar coating. The signal can be optical, electrical, electrochemical, or some other form with favorable characteristics for conversion, communication, or subsequent analysis. For instance, a sensor based on electrochemical transduction might produce a small current in the presence of the analyte, which would be subsequently amplified and converted into some interpretable form by a picoammeter. The operator, a computer, or a neural network would then interpret the value in picoamps as corresponding to some level of the analyte.

A wide variety of transduction techniques exists that can be used to make measurements. Even within the electrochemical sensor field, techniques such as voltametric, conductimetric and potentiometric sensors all can be used based on different detection mechanisms. Similarly, with optical sensors, fluorescence, absorbance, and reflectance have all been employed as transduction mechanisms. Even within these different mechanisms, a variety of sub-mechanisms exist; for example, fluorescent sensors can be based on either emission intensity, ratios of intensity or excited-state lifetimes of the fluorescence species. The critical component of the sensor is the transducer coating – it is what provides the sensor with its selectivity and specificity. Two examples will illustrate the principle as shown in Figure 2. In Figure 2a, an electrochemical sensor is shown. The electrochemical sensor contains an ionophor that can bind selectively and carry a cation into a thin membrane film covering the electrode (the transducer). An

Figure 1.

Sensor paradigm. The presence or amount of some chemical species is transduced as a change in some signal such as a current or light flux, which is detected and analyzed to provide information to the operator.

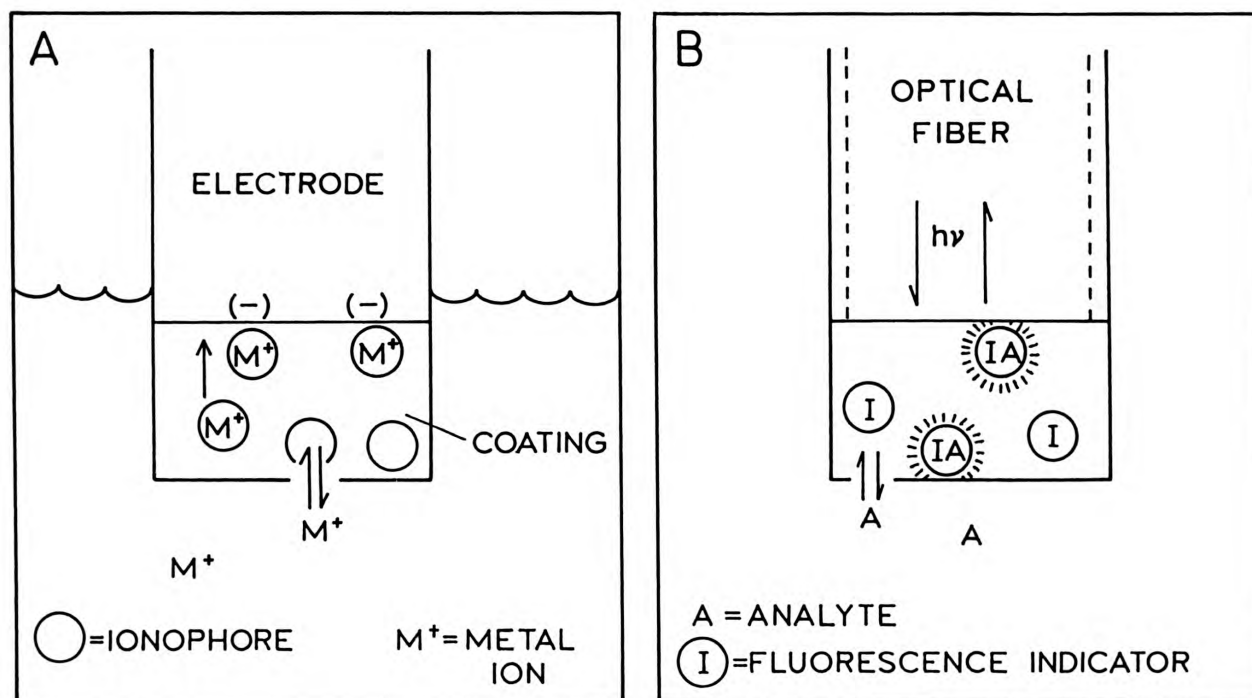
CHEMICAL SENSOR PARADIGM



example of an ionophor is valinomycin, a cyclic peptide isolated from fungi which selectively transports potassium across membranes. By carrying positive ions into the membrane, an electrical potential is generated at the membrane's surface. The electrode detects the potential which is proportional to the concentration of the ion of interest, i.e., the higher the ion concentration the greater the potential – the lower the concentration the lower the potential. Thus, the key to selectivity is the ability of the ionophor to bind selectively to different ionic species. In Figure 2b is shown an optical sensor. For purposes of discussion we will focus on a fluorescence optical sensor. The coating layer contains a fluorescent indicator that responds to the presence of analyte. In this case the analyte diffuses into the polymer layer (a coating) and reacts with a bound indicator that changes its fluorescence properties in proportion to the amount of analyte in solution. The indicator may be a fluorescent dye, a mixture of dyes, or a dye in combination with a biomolecule. Light of one color excites the

Figure 2.

Schematic electrochemical (A) and optical fiber (B) sensors. In the electrochemical sensor, an ionophore dissolved in the coating selectively binds a metal ion M^+ and carries it to the vicinity of the electrode surface, where it induces a potential change. In the optical sensor, some fluorescent indicator I binds the analyte A to form a complex with different fluorescence properties; the fluorescence of the complex is excited, and its fluorescence collected through the optical fiber.



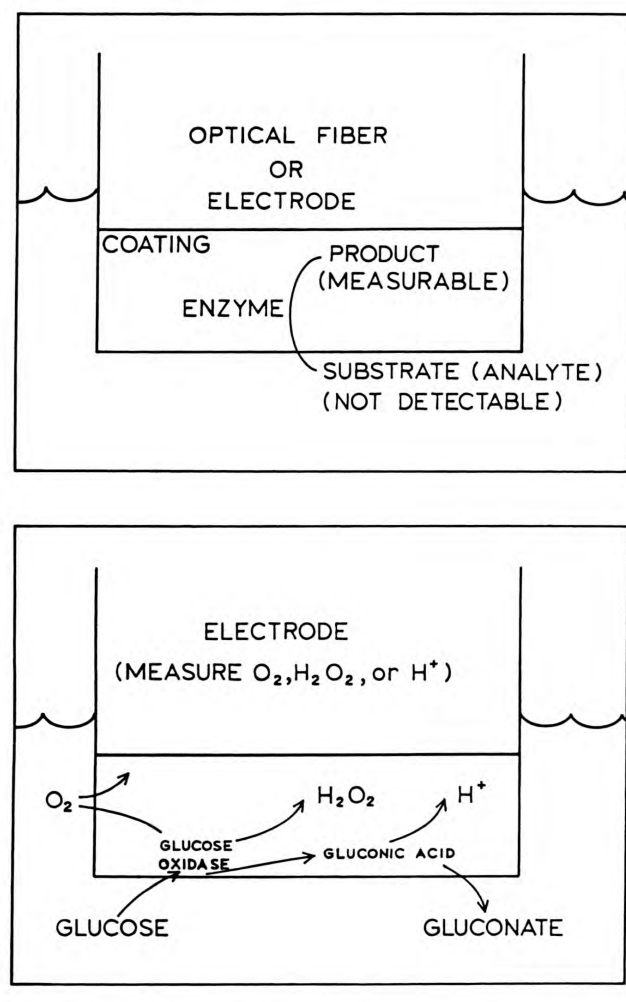
indicator and emits fluorescence of a different color. The emitted light returns through the same fiber and is sent to a detector system that correlates the intensity or lifetime of the fluorescence with the analyte concentration.

Biosensors contain a biologically-active element as an integral part of their sensing mechanism. These biological species may include enzymes, antibodies and cell receptors, but may also contain naturally-occurring receptors such as cyclodextrins and ionophors such as valinomycin. Figure 3 illustrates the operating principles of an enzyme-based biosensor with glucose as the target species. In this case, the enzyme acts to convert an analyte species into a detectable entity. It is extremely difficult to find chemistry that is selective for glucose, particularly when other sugars may be present. Consequently, one takes advantage of the specificity of the enzyme glucose oxidase, which utilizes glucose exclusively. Glucose oxidase catalyzes the reaction between glucose and oxygen to ultimately generate gluconic acid and hydrogen peroxide as shown in Figure 3. In a glucose biosensor the enzyme-catalyzed reaction can be followed by monitoring the consumption of O_2 or the production of H_2O_2 or protons (lower pH). For instance, the gluconic acid product dissociates into gluconate

and a hydrogen ion, which lowers the pH in the sensor coating relative to the concentration in solution. Thus, the amount of gluconic acid produced by glucose oxidation can be measured by coupling it to a H^+ sensor such as a pH electrode or a pH optical sensor. Alternatively the consumption of O_2 can be monitored with an electrochemical O_2 sensor, or hydrogen peroxide may be detected amperometrically. In this case, depletion of oxygen from the coating caused by the catalytic enzyme reaction is measured and is indicative of the glucose concentration. The obvious limitation of biosensors based on the activity of a selective catalyst (glucose oxidase) is that they depend on the measurement of a second species such as oxygen or H^+ . These parameters must either be controlled or measured independently in order to ascertain the concentration of the analyte of interest. Alternatively, one can measure the rate of evolution of H^+ or H_2O_2 , or the consumption of O_2 under conditions where the amount of glucose oxidase is not limiting; this approach is often used for greater precision. The important point, however, is the operating principle of the sensor which is based on coupling the catalytic reaction of an enzyme to the measurement of an easily measurable species. The catalytic reaction causes a change in the species of interest

Figure 3.

(Upper) Schematic of enzyme electrode/optode. The analyte is a substrate for the enzyme incorporated into the coating; the enzyme converts it to a product which may be measured optically or electrochemically. (Lower) Glucose oxidase enzyme electrode. Glucose oxidase converts glucose and oxygen to gluconic acid and hydrogen peroxide; the electrode quantitates glucose by the disappearance of oxygen or the appearance of peroxide, or H^+ , as the gluconic acid dissociates.



creating a "micro-environmental" change in the coating layer. An issue in applying such biosensors is the stability of the enzyme; recent work with exotic thermophilic bacteria suggests that some enzymes can be made very rugged indeed (see below).

The second most common type of biosensor is based on the binding specificity of an antibody or other macromolecule. Antibodies are exquisitely-selective binding proteins that specifically recognize the antigen to which they have been generated. This specificity has both positive and negative

consequences. On the positive side, the antibodies can be extremely selective recognizing only the antigen of interest with no tolerance in structural variation. However, the antigen-antibody complex often has such a high binding constant that the two are almost permanently bound (see below). Consequently, antibodies rarely can be used in a continuous monitoring mode where the system comes to equilibrium in a brief time; rather, they can be used in a discontinuous fashion by measuring the rate of signal change when exposed to an analyte-containing solution. Once saturated, however, the antibodies must be replaced if subsequent measurements are to be made. An example of such an immunoprobe is shown in Figure 4. In this case the antibody, depicted as the Y-shaped species, combines with the antigen shown as triangles. As long as vacancies exist in the binding sites of such antibodies the capacity to detect antigen exist^{778s}. Once binding has been completed and all the binding sites are occupied, a new probe must be used to make subsequent measurements. Alternatively the binding between antibody and antigen can be disrupted using chaotropic ions or other chemical reagents. Unfortunately such treatments can destroy some of the binding capability of the antibodies and limit the usefulness of such processes. The other challenge with antibody-based biosensors is the need to couple the binding event with signal generation. Unlike enzymes, which have catalytic activity and cause a change in the chemistry of the analyte, antibodies simply bind to the analyte. Often times antibodies are labelled with an electroactive or optically-changing species that becomes altered in its properties upon antigen binding. Alternatively, a competition reaction between a labelled antigen and unlabelled antigen is employed in which the binding of the labelled antigen species can be detected easily (via an electrochemical or optical label). The presence of such labels is inversely proportional to the concentration of the solution analyte of interest. Unfortunately, such sensors typically require exogenous reagents that add complexity. [A third approach is detailed by Ligler, et al in Chapter 3.]

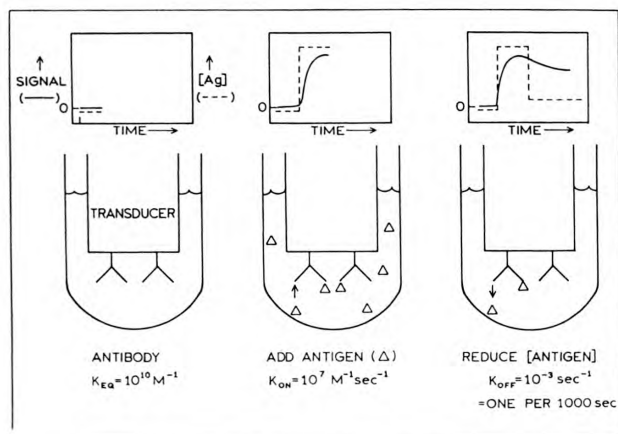
The third and final type of biosensor is the cell or tissue based biosensor. These biosensors employ whole cells or sections of living tissue to obtain the requisite specificity and sensitivity (see Holmes, Chapter 6). The basis of their operation is varied. Some operate because they contain an enzyme that catalyzes a reaction of interest and are coupled to the detection of an easily measurable species as discussed above for the enzyme-based biosensors. Other cell and tissue biosensors are based on receptor molecules located at the cell surface that generate changes in electrical activity or cause a chemical signal that can be measured using the transducer.

Biomolecular Transducers

A variety of sensors have been described that use biomolecules as the transducing element. In most of these cases,

Figure 4.

Effect of binding kinetics on immunosensor response. A tight-binding antibody ($K_D = 10^{-10} M$) is attached to the transducer. When antigen (triangles) is added, it binds with an association rate constant of $k_{on} = 10^8 M^{-1} sec^{-1}$, causing a rapid increase in signal. When the antigen concentration is reduced by immersing the transducer in an antigen-free solution, the signal goes down slowly in this case because the dissociation rate constant is small ($k_{off} = 10^{-2} sec^{-1}$).



the sensing biomolecule is a macromolecule, such as an enzyme, immunoglobulin (see Ligler, *et al* Chapter 3.), polynucleotide, or receptor. The choice of biomolecule as recognition element is driven not only by the characteristics of the biomolecule's interaction with the target, but also the means of transduction. Thus the level of the target molecule glucose can be transduced as an electrical signal as discussed above, or glucose can be quantitated optically by its ability to displace a fluorescent-labeled sugar from a saccharide-binding protein called a lectin². There are advantages to both approaches, and the utility of each depends on the circumstances of the problem.

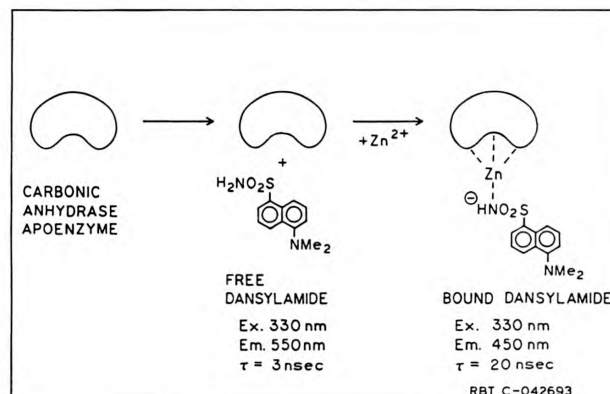
There are several reasons for employing biomolecules as the transducing element in a sensor. Probably most important is selectivity. For instance, a metallofluorescent indicator like hydroxyquinoline sulfonate can transduce the presence of a metal ion like zinc by a change in the intensity of its fluorescence. However, about twenty-five other metal ions also can affect the indicator's fluorescence, so that in complex mixtures it would be difficult to ascertain how much of each ion is responsible for the fluorescence yield. By comparison, certain biomolecules bind and may transform particular ligands with specificity. For instance, enzymes catalyze the reaction of a small molecule, such as the decarboxylation of an amino acid,

with such specificity that no measurable reaction occurs with other stereoisomers of the target amino acid. Similarly, metalloenzymes are usually quite selective for metals they bind in their active sites, and of the metals bound only a handful will serve in catalysis, typically at much lower rates. The reason for this selectivity is the extreme complexity of cells and organisms when viewed as mixtures of chemicals; if cellular processes (reaction sequences) such as carbohydrate metabolism, protein biosynthesis, DNA replication, intracellular signaling, or the immune response took place with anything less than absolute fidelity, catastrophe would result. This is evidenced in inherited diseases where such problems occur due to genetic flaws.

Another advantage is sensitivity. In many cases the non-covalent interactions that take place between targets and recognition biomolecules are very strong: strong enough to permit target detection at subpicomolar ($10^{-12} M$) concentrations. While other methods of chemical analysis such as mass spectrometry, atomic fluorescence, and high performance chromatography offer very high sensitivity in favorable cases, they usually require substantial pretreatment of all but the most highly purified samples. Often these same methods are ill-suited to rapid or continuous determination, or use outside the laboratory. A major reason for this sensitivity is that some biosensors have a built-in amplification factor. For instance, if the target molecule is an inhibitor which affects the efficiency of the enzyme as a catalyst, one can quantify the target by the rate of substrate conversion to product by the enzyme.

Figure 5.

Zinc biosensing scheme. Apo-carbonic anhydrase does not bind the inhibitor dansylamide in the absence of zinc, resulting in a weak, greenish, short lifetime fluorescence of dansylamide free in solution. When zinc is present in the active site, dansylamide binds tightly to give a strong, long lifetime, blue fluorescence.



The enzyme-catalyzed reaction can be left to run for a period of time to yield product concentrations large enough to measure using simple instruments; e.g., spectrophotometers. Similarly, where the target is a nucleic acid, it can be amplified by cloning or polymerase chain reaction such that in favorable circumstances single molecules (single gene copies) can be detected. This latter approach is powerful and has many applications such as forensics, but is difficult to automate and is not amenable to real-time readout.

There are other advantages to sensors with biomolecule transducers. With the advent of biotechnology such transducers can now be produced in quantity. For instance, monoclonal antibodies have now almost superseded antibodies isolated from blood because they are homogenous, reproducible indefinitely, and available in multigram quantities. Similarly, enzymes, nucleic acids, and molecules previously expressed only at minute levels, such as growth factors and receptors, are now routinely available in adequate quantities using recombinant technology. Also, our increasing understanding of structural biology at the molecular level suggests that it will be practicable to insert transducers in rugged structures and materials, to miniaturize them, and to configure transducers in arrays (see below) by manipulating the genetic material that codes for such structures. These molecular biological techniques, along with the classical techniques and knowledge base of biochemistry, provide a firm technology base of procedures, reagents, organism strains, and equipment for biosensor development.

Biomolecule transducers have disadvantages as well. Foremost among these is fragility. Most of the biomacromolecules used as transducers have relatively narrow tempera-

Figure 6.

Ratios of emission from free (560 nm) and bound (450 nm) dansylamide as a function of zinc concentration; note logarithmic scales.

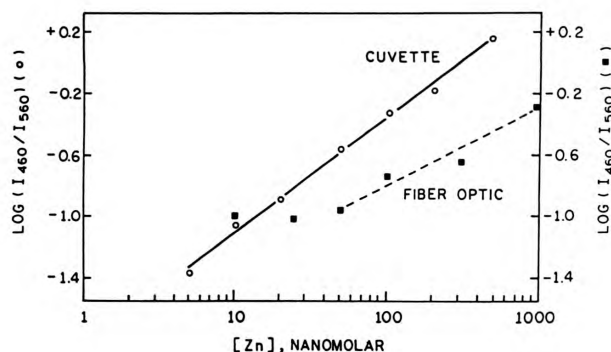
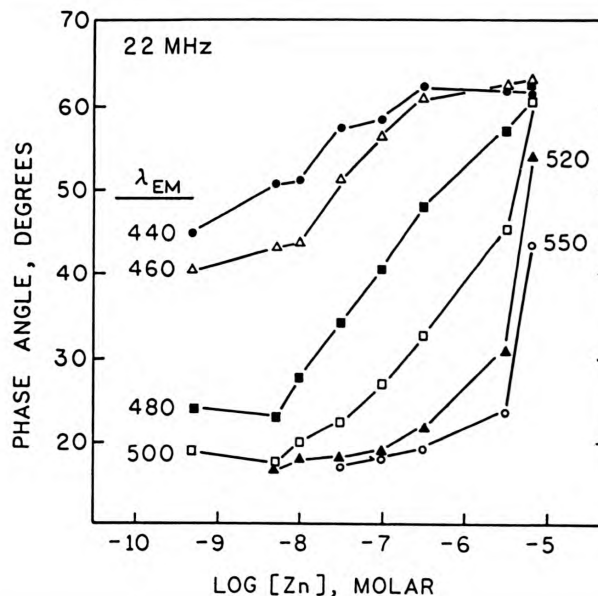


Figure 7.

Fluorescence phase angles measured at 22 MHz for different emission wavelengths of dansylamide as a function of zinc concentration at 2.5 μ M apo-carbonic anhydrase, 9 μ M dansylamide. Phase angles are proportional to the fraction of longer lifetime species present, and thus bound dansylamide.

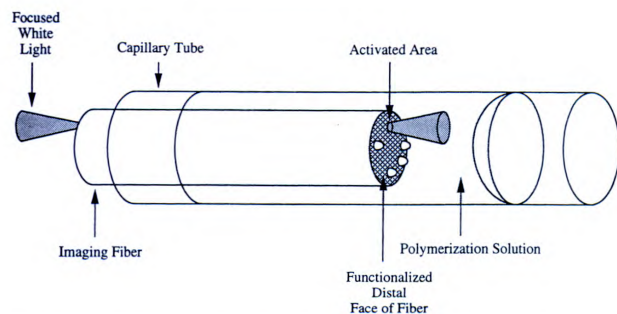


ture optima for remaining functional, as indeed most organisms do. For instance, an enzyme will exhibit differing catalytic kinetics at 25°C than at 37°C; at 0°C or 50°C the enzyme may be inactive or structurally disrupted, perhaps irreversibly. This narrow range of temperature contrasts with the much broader ranges specified for storage and use of prepackaged meals or electronic equipment. Moreover, steps must be taken to prevent contamination of biomolecules by microorganisms which feed on them. Biomolecules are seldom optimized for a sensing task, in the sense that they produce a signal easy to monitor. For instance, few biomolecules by themselves provide useful changes in fluorescence upon binding some ligand; the best examples are antibodies, which typically exhibit a negligible change in their fluorescence upon binding their cognate antigen. Happily, attaching fluorescent moieties to the antibody is straightforward, again making use of extensive experience in fluorescent labeling for purposes of analytical flow cytometry. Recently, Chalfie, et al.,³ demonstrated that such labeling could be performed at the genetic level, using the Green Fluorescent Protein from *Aequorea*.

The trade-off between tight binding and response time was introduced above with regard to antibody-based sensors. Some noncovalent binding interactions are rather strong, (K_D values less than 10^{-12} M), with many antibody binding con-

Figure 8.

Optical fiber array is fabricated by photopolymerizing different indicating chemistries on the distal tip.



stants in the range of 10^{-6} to 10^{-9} M. Relatively tight binding is a virtual necessity if analytes present in low concentration are to occupy a significant fraction of binding sites. However, if we consider the equilibrium constant as the ratio of association and dissociation constants $K_D = k_{\text{off}}/k_{\text{on}}$, a problem becomes apparent. Suppose our biomolecule binds the analyte relatively tightly ($K_D = 10^{-10}$ M) and the association rate is as fast as possible: diffusion controlled reaction for a small molecule in water at 25°C is about $10^8 \text{ M}^{-1} \text{ sec}^{-1}$. Consequently, the dissociation rate is about 10^{-2} sec^{-1} , or about once every two minutes.[See Figure 4]. The dissociation rate imposes a lower limit on the time constant (actually reset time) of the sensor. If we equilibrate the sensor with this biomolecule in a solution of the analyte, then reimmerge it in a second solution containing no analyte, the analyte molecules bound to the biomolecule in this example will only diffuse away in minutes. Thus many antibody-based sensors, while very sensitive, cannot respond to rapid changes in analyte level and are effectively “alarms” in most scenarios.

Electrochemical sensors mainly do not depend on equilibrium binding, and usually their time constants are of the order of seconds; processes limiting their speed include diffusion rates across membranes, mass transfer of analytes to the surface of an electrode, and low frequency response of bio-compatible conductors. These barriers can be surmounted in some cases by clever design and materials.

Nevertheless, while dissociation constants represent a barrier, it is not insurmountable. Examples in Nature suggest that some organisms have overcome this limitation. For instance, the blue crab *Callinectes* is able to detect concentrations of amino acids in water as low as picomolar, and follow a concentration gradient of such analyte to a food source.⁴ Similarly, flying insects follow concentration gradients of pheromones at part per trillion levels in the air to find their (nonflying) mates. In both cases, the sensory organs must have a low time constant to be useful for guidance of the animal as it flies or swims. Yet an amino acid receptor on the crab with

picomolar equilibrium constant would require minutes to reset, which is too slow to be of use to the crab. Clearly the crab is able to overcome this limitation. Bush and Rechnitz⁴ have proposed an array of sensors and neurons is responsible for the crab’s rapid response. Other approaches (see below) make use of transduction methods capable of quantitating very low fractional binding site occupancy, permitting the use of binding sites on the transducer biomolecule with lower affinity.⁵

Engineering Specificity and Sensitivity in a Zinc Biosensor: A Case Study

Recently, one of us (RT) developed an optical sensor for zinc (II) which addresses many of these issues. The transduction principle of the sensor is illustrated in Figure 5. Human carbonic anhydrase II is a metalloenzyme with a zinc ion coordinated in its active site which is required for activity. Enzymologists discovered decades ago that carbonic anhydrase was very selective for the metal ions it would bind in its active site, and that it is thus much more selective than typical ligands like EDTA. Enzymologists also found that the zinc could be removed and subsequently replaced. The problem then became how to transduce the binding of the zinc to the active site ligands. The work of Chen and Kernohan⁶ suggested a means to address the problem. Several workers had established previously that carbonic anhydrase could be inhibited by members of a family of compounds termed aryl sulfonamides. These compounds inhibit the enzyme by binding to its active site zinc in place of the hydroxide ion ordinarily found there. Many such aryl sulfonamide inhibitors are known because they have therapeutic value in treating glaucoma. The aryl sulfonamide introduced by Chen and Kernohan⁶, dansylamide, is weakly fluorescent in aqueous solution (Figure 5). When bound to the enzyme as an inhibitor, the quantum yield of the dansylamide goes up dramatically; its emission shifts 100 nm to the blue, and its lifetime increases seven-fold. Thus, if we remove the zinc from the enzyme and add dansylamide, one observes the weak greenish fluorescence typical of the free inhibitor. If we subsequently add zinc ion to the solution, it binds to the enzyme, permitting the dansylamide to bind to it in the active site, and we observe bluish emission. It is important to note that the ratio of fluorescence intensities in the blue and green is proportional to the fraction of the enzyme with zinc bound to the active site, and thus the concentration of zinc. This result can be seen in Figure 6, which depicts the ratio of blue (450 nm, corresponding to bound dansylamide, and therefore zinc) to green fluorescence (550 nm, corresponding to unbound dansylamide) measured for different concentrations of zinc ion.⁷

The ratiometric approach has several advantages over sensors based on changes in fluorescence intensity. In particu-

lar, the ratio of intensities is insensitive to many artifacts that affect accuracy and stability, and make calibration difficult. For instance, a change in the turbidity of a sample containing a fluorescent indicator might affect the apparent fluorescence intensity, but the ratio of fluorescence intensities at two different wavelengths is insensitive to turbidity, and thus will provide a true readout. The very successful calcium indicators developed by R. Tsien and his colleagues are also ratiometric, and are used very widely by cell biologists for this reason.

We found that the enzyme and dansylamide could be incorporated into a fiber optic sensor and provide a detection limit in the nanomolar range. To determine zinc in complex media like seawater or wastewater, this range is nearly as good as atomic absorption spectrophotometry. However, we found that even the best optical fiber exhibited a blue background photoluminescence with ultraviolet excitation and this photoluminescence mimics the emission from the bound dansylamide, thus compromising the detection limit. A second limitation is the difficulty in ordinary fluorometers to accurately measure concentrations over a very wide range. For instance, it has been shown that zinc concentrations in the ocean vary over three orders of magnitude at least, and other analytes vary over an even wider range (see Johnson and Jannasch, Chapter 2).

It appeared difficult to address the problems of photoluminescence interference and dynamic range easily with the ratiometric approach. Recently, however, Szmajda and Lakowicz⁸ showed that by using a somewhat different approach with a ratiometric pH indicator called Carboxy-SNAFL-2, they could quantitate pH accurately over a range of 5 units using a single indicator by measuring changes in the apparent fluorescence lifetime of the indicator at selected excitation and emission wavelengths. By observing the emission at wavelengths where mainly the acid form emits (such that a very low percentage of acid form can be seen in the presence of excess base form at high pH) and taking advantage of the nonlinear behavior of lifetime measurements in the frequency domain, they were able to measure pH accurately up to three units above the pK_a , corresponding to a fraction of the acid form of 0.1% or less. Another advantage of the lifetime-based approach is that, to a large degree, it discriminates against the type of photoluminescence background we encountered.

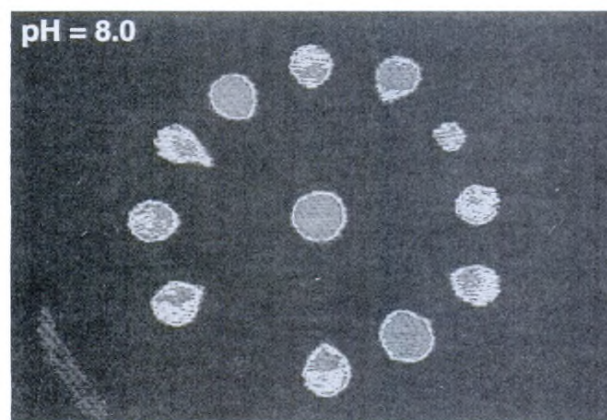
The carbonic anhydrase/dansylamide indicator system is nearly ideal for lifetime-based zinc sensing because of the large differences in wavelength of emission and lifetime of the free and enzyme-bound forms of the dansylamide. In a case where part of the dansylamide is bound to the enzyme, the time dependence of the emission will represent a weighted average of the fluorescence lifetimes of the free and bound forms. If we measure the phase shift of the dansylamide/apocarbonic anhydrase emission with respect to that of excitation sinusoidally amplitude-modulated at some frequency, it will increase

with zinc concentration, reflecting the greater fraction of the long lifetime bound form from Figure 7. The curves in Figure 7 are effectively calibration curves, in that they are reproducible under the same conditions and permit a one-to-one correspondence between phase angle and zinc concentration. As is evident from the figure, we can measure zinc concentration accurately over a wide range simply by varying the emission wavelength. The lifetimes themselves are properties of the molecules, and to a first approximation do not change. We have demonstrated that such lifetime measurements can be made through optical fibers with precision and accuracy comparable to what we achieve with a laboratory instrument. Thus, by using lifetime-based sensing we can address the issues of dynamic range and background photoluminescence.

Some issues remain with our zinc sensor. First, the high attenuation of optical fiber in the ultraviolet and the poor absorbance of dansylamide make truly remote sensing (beyond 50 M) very difficult. We would prefer to operate at longer wavelengths, preferably those that permit the use of a diode laser for excitation. Diode lasers are inexpensive, small, efficient, reliable, and rugged, and therefore, desirable for most practical applications. Also, the kinetics of the sensor are too slow for most applications. Partly this is due to our sensing tip design, which in future versions will incorporate a different coating. Ultimately, the apoenzyme and dansylamide may be incorporated into a thin (5 μm) layer of polymer covalently¹⁴ attached to the end of the fiber, permitting very fast kinetics. (see below). However, the known kinetics of zinc binding to apocarbonic anhydrase suggest that the molecules themselves will become rate limiting before the configuration of the

Figure 9.

CCD image fluorescence intensities emanating from a pH sensor array containing thirteen sensors on a 500 micron diameter imaging bundle.



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sensing tip, for the reasons discussed above (Figure 4). Thus, we have embarked on a collaboration with Professors Fierke and Christianson to modify apocarbonic anhydrase using combinatorial mutagenesis to obtain mutants with improved kinetics, (see Chapter 5). Moreover, we plan to examine these and other mutants for altered specificity in metal binding, in order to sense other ions of interest such as Pb, Co, Cu, Ni, Hg.

Emerging Architectures

We are now ready to discuss some of the exciting new prospects that are emerging in the area of biosensors. Because many of the supporting technologies are discussed elsewhere in this issue, we will focus specifically on some of the exciting new technologies being explored in biosensor preparation and implementation.

Micro- and Nano-Fabrication

In contrast to the bulky pH electrodes of a decade ago, the size of sensors has decreased significantly. This reduced size is a consequence of a variety of contributing factors. First, the ability to fabricate both micro- and nano-structures has grown out of the electronics industry from application of a variety of new lithographic techniques. Tremendous advances have been made in materials that allow them to maintain their strength and performance at exceedingly small dimensions. Another important contributing factor is the tremendous improvement in our ability to detect small signals, whether electrical or optical. These advances have enabled shrinkage of the sensors to nanometer dimensions due to the ability to sensitively detect exceedingly small signals. With optical sensors for example, the ability to do single photon counting has enabled sensors with nanometer diameters to be fabricated – a consequence of the tremendous advances in cooled detector technology that have arisen in recent years. Enzyme-based micro-biosensors have been fabricated for glucose⁹ while Wightman and co-workers can detect the presence of single-vesicular packets of neurotransmitter being released from the ends of neurons.¹⁰ These vesicles contain several thousand neurotransmitter molecules, illustrating the tremendous sensitivity of such micro-sensors. This sensitivity underscores an important point about size – the smaller the sensor, the more sensitive the measurement can be as a consequence of reduced background due to a reduced surface area or volume¹¹.

Sensor Arrays

The advent of lithographic techniques as applied to sensor fabrication has also enabled the preparation of sensor arrays. This technology has the potential for dramatically enhancing the utility of sensors. Other than thermal sensors, all three (electrochemical, optical and piezoelectric) other types of sensors have been fabricated into arrays. In some of these systems, the arrays are simply technical expedients in that they

are based on identical operating principles as discussed above but have all the sensors integrated into a sequentially or concurrently addressable array configuration. Such arrays will perhaps have a major impact for enzyme biosensors in which it will be possible to measure both the analyte of interest (e.g., glucose) and the measurable species (e.g. pH, O₂) with both sensors disposed on the same array substrate. Fabrication should be easier and the precision of the sensors should be superior to the conventional approach of having to make two independent sensors. A more striking advantage of such arrays, is the relaxation of absolute specificity for the sensor chemistry. In this approach an array of semi-selective sensors can be fabricated in which cross-reactivity between different sensor elements exists. Rather than rely on the specificity of a particular recognition chemistry, it is the pattern generated by the array that provides the specificity. By looking at a large number of sensor elements, various computational approaches can be employed to deconvolute the complex pattern and specify the precise concentrations of a variety of analytes. This approach more closely parallels the way the olfactory system operates.¹²

Imaging Optical Sensor Arrays: A Case Study

One of our laboratories (DW) has created optical arrays using fiber optic imaging bundles.^{13,14} These bundles are fused-fiber arrays containing thousands of individual optical fibers aligned coherently within a single sensing element. The coherent alignment of the fibers allows sensing chemistries disposed on the distal tip of the fiber to carry their signals individually thorough the array and maintain spatial resolution on the fiber's proximal tip. The emanating signal from the proximal end of the fiber is focused onto a CCD detector enabling intensity information to be correlated with spatial position. Sensors are fabricated using photopolymerization Figure 8. Light is focused through a pinhole onto the proximal end of a fiber. The fiber's distal tip, which has been silanized to facilitate adhesion of the polymer, is placed in a solution containing a monomer crosslinker and polymerizable indicator. Upon illumination, the light is transmitted through the fiber coherently such that only the area that has been illuminated on the proximal end is illuminated on the distal tip. The emerging light causes the solution to polymerize only in the region where light emanates from the fiber. In this manner, a polymer spot is created containing a selective indicator located in a precise position on the distal fiber tip. A second indicator can be placed in precisely the same manner by positioning the illuminating light on a different region of the fiber. For each indicator chemistry used, a different polymer spot must be created. We have fabricated such arrays to contain upwards of a dozen individual sensors on a single 350 μ fiber. The sensor chemistries developed to date for arrays include pH, CO₂, O₂, and hydrocarbon as well as a penicillin biosensor. An example

of a fluorescent image collected by a CCD camera is shown in Figure 9.

In an alternative approach the same array type sensors can be used to immobilize a variety of non-selective chemistries that can be recognized as patterns by neural network programs. Such arrays ultimately may prove to be superior to selective indicating chemistries as they provide the chemist with the ability to detect many components simultaneously without needing to develop separate highly-selective indicating chemistries for each analyte of interest.

Development Issues and Supporting Technology

Heretofore we have focused on the transduction aspects of sensors, but as is apparent in Figure 1, there are other characteristics of sensors which are also important to their development.¹⁵ These aspects, while mundane, often determine the utility of a sensor for a particular task, or its cost-effectiveness in a particular application. Several applications and scenarios of interest to the Navy such as operation at sea, on board ships or aircraft, or at extremes of temperature impose stringent limitations on sensors.

A complete list of sensor characteristics/parameters is difficult to compile, but some of the more important ones are summarized below, with emphasis on biosensors. Selectivity and sensitivity have been discussed at length already. The molecular basis of response time was also discussed but its importance varies a lot with scenario. For instance, a buoy-based sensor intended for monitoring long-term variations in oceanic CO₂ might only measure once a day, making a fast response irrelevant. In most scenarios of chemical oceanography, chemical process monitoring, or wastewater outflow monitoring, however, a time constant of less than a minute is clearly desirable. Weight, size, power consumption, and cooling are all interrelated, and usually determined by the transduction mechanism. For optical sensors incorporating gas lasers, these are all significant issues, especially if the sensor must be put aboard an aircraft or carried on someone's back. A large part of the impetus towards incorporating light emitting diodes and diode lasers into optical sensors is their very low weight, size, power consumption and need for cooling. The sensor's propensity to fouling and corrosion are important, particularly for measurements in the environment. Historically, our understanding of these processes and their prevention was wholly empirical, but recent results in corrosion and fouling research supported by ONR have led to better understanding and will ultimately lead to very robust systems. Resistance to shock, movement, pressure, humidity, and thermal changes remain important issues, particularly for biosensors whose transducing molecules probably evolved to operate in a narrow temperature range. Optical fibers and solid state optoelectronic components are at least as robust as their electronic cousins in

these respects, so that one might anticipate taking an optical sensor anywhere one finds sensors with electrical transduction. Sensitivity to electromagnetic interference remains a drawback for electrically-transduced sensors. In many scenarios, such as use aboard aircraft or ships, transmitting weak currents or voltages through lengths of cable with good fidelity is difficult if the cable moves through the Earth's magnetic field or is near generators or radars. In cases such as the conductivity/temperature/depth sensors used in oceanography, the signals are often digitized, amplified, and converted to frequency shift-keyed format before transmission to the surface. Automation and data reduction, transmission, storage, and retrieval can be difficult depending on conditions. The advent of satellite communications has been a boon, but the INMARSAT link used to collect data from buoys has an very slow data rate. As the cost and regulatory burden of having trained people perform chemical analyses has gone up, consequently so has the desirability for automation. Calibration, reproducibility, accuracy, and the availability of standards become particularly important given the legal importance of some chemical analyses, and the need to compare data sets collected years apart, on a global scale. Manufacturability has always been important, and fortunately, much of the sensor apart from the transducer can usually be assembled off the shelf. The introduction of microelectronic and integrated optic technologies promises to make transducer manufacturing more straightforward as well. Finally, cost continues to be an important factor in any sensor application. Sensors are typically very cost-effective compared to having a discrete sample collected, transported, and analyzed by a skilled person on an expensive laboratory instrument. However, the development of a sensor for a demanding application such as trace analysis of an analyte in a complex medium in some hostile environment is seldom quick and easy, particularly if there is some regulatory requirement to be met. Prolonged development is costly, and may make the sensor uneconomical even if it performs well.

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Biographies

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Molecular Biosensors: The Roles of Molecular and Structural Biology

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Introduction

Among the first row transition metals, zinc is second only to iron in terms of its abundance and importance in biological systems. In seawater, zinc and other metals (such as iron and manganese) are found at low concentrations and exhibit a variation with depth characteristic of nutrients, including surface depletion¹ (see Johnson and Jannasch, Chap. 2). Low concentrations of trace metals may in fact be limiting factors for plankton growth in the ocean². However, identification and quantification of trace constituents in a complex mixture, such as seawater, remains a formidable task requiring both high sensitivity and selectivity. Current methods of quantifying zinc are costly, time-consuming and subject to contamination. One way to circumvent these problems is to apply the selectivity and avidity of metalloproteins, such as carbonic anhydrase, in molecular biosensors³ (see Thompson and Walt, Chap. 4). Metalloproteins have been optimized through evolution to recognize and bind specific target metals contained in the complex mixture of cations present in biological systems. For example, different naturally-occurring metalloproteins are known to be specific for the binding of zinc, iron, manganese, nickel, cobalt, cadmium, and mercury. The properties of each such metalloenzyme can then be further optimized for use as molecular biosensors by combining the tools of molecular and structural biology.

Zinc is a versatile metal ion in biology because it may perform structural as well as functional roles⁴⁻⁶. Since zinc is spectroscopically colorless, the only biophysical techniques that can be used to study zinc metalloproteins are X-ray crystallography or multidimensional NMR spectroscopy, and only X-ray crystallography can be used to study proteins larger than 25,000 kDa. The functional role of zinc is governed by its environment in the metalloprotein structure, particularly its binding site which consists of 3-4 amino acid side chains which coordinate to the metal ion (such amino acids are referred to as "ligands"). In general, the properties of a protein-bound metal ion can be altered by engineering its ligands, and it is possible that the desired properties for a protein-based transition metal biosensor can be conferred upon a single, naturally-occurring metalloprotein by engineering its metal ligands.

The biophysical properties most relevant to the design and construction of a protein-based zinc biosensor are protein-zinc affinity and the kinetics of protein-zinc equilibration (i.e., zinc association and dissociation rate constants). Obviously, the easiest way to affect protein-zinc affinity is to alter the zinc ligands. If the three-dimensional structure of the metal binding site is known, and if the structure of the engineered metal binding site can be determined, then this approach is quite rational in its design and execution. In other words, such an approach would be termed "structure-assisted protein design".

X-ray crystallography plays a critical role in structure-assisted protein design since it is the only technique of modern structural biology that provides a direct visualization of the atoms of a wild-type or redesigned protein.

Although it might be relatively easy to determine the three-dimensional structure of a protein for use in protein design experiments, it is not necessarily easy to draw up a molecular “blueprint” for what a (re)designed protein should look like – the current state of knowledge is such that we are still learning how to make accurate blueprints. In order to learn how to make blueprints for the structure-assisted design of a protein-zinc binding site with predictable properties, we must literally dissect a naturally-occurring site to probe the structural features which contribute to zinc affinity and equilibration. Once we establish valid structure-function relationships, desired properties of zinc affinity and equilibration can be engineered into the same metalloprotein for exploitation in metal biosensor applications.

We note that the conventional notation to indicate amino acid substitutions in proteins consists of the three-letter abbreviation for the wild-type amino acid and sequence number, followed by a right arrow and the three-letter abbreviation for the substituted amino acid. For example, in a carbonic anhydrase variant where wild-type residue histidine-94 is replaced by cysteine, the amino acid substitution is indicated by the expression “His-94→Cys”. This notation is used throughout this Review, and three-letter abbreviations for other amino acids relevant to our discussion include alanine, Ala; asparagine, Asn; aspartate, Asp; glutamate, Glu; glutamine, Gln; and threonine, Thr.

Carbonic Anhydrase – A Prototypical Zinc Enzyme

Here, we summarize our progress in the engineering of the zinc binding site of a metalloprotein, one of the human carbonic anhydrase (CA) isozymes. Ultimately, the engineered CA molecule will be used in an optimized zinc biosensor³ (see Chap. 4). Wild-type CA contains one catalytically-obligatory zinc ion bound to a single polypeptide chain of 260 amino acids^{5,7,8}. The only known biological function of CA is the hydration of CO₂ to yield HCO₃⁻ plus a proton with kinetics that approach those of a diffusion-controlled reaction ($\approx 10^8 \text{ M}^{-1}\text{s}^{-1}$)⁷. The structure of CA from human blood has been determined by X-ray crystallographic methods⁹ and reveals a zinc ion located at the bottom of a 15 Å deep cavity where it is liganded by hydroxide ion and the imidazole side chains of three histidine residues: His-94, His-96, and His-119 (Figures 1 and 2). These zinc ligands also make secondary hydrogen bonds with the surrounding protein scaffolding and are nested in a hydrophobic shell of protein atoms. The proposed mechanism of catalysis involves direct nucleophilic attack by the zinc-bound hydroxide on CO₂ to

Figure 1.

Ribbon drawing of the backbone structure of carbonic anhydrase⁹, rendered with graphics software MOLSCRIPT^{39,40}. Note that the protein structure consists of short α -helices and longer β -strands, secondary structural elements discovered by a former Office of Naval Research grantee, Linus Pauling. The zinc binding site is located in the middle of a central β -sheet superstructure and consists of three protein ligands: His-94, His-96, and His-119.



form zinc-bound bicarbonate. Sulfonamides inhibit the catalytic activity of CA by coordinating to zinc and displacing zinc-bound hydroxide (Figure 3); these inhibitors bind to zinc through a negatively-charged nitrogen atom that essentially mimics the negatively-charged oxygen atom of the hydroxide ion which it displaces.

The metal binding site of carbonic anhydrase binds zinc very tightly ($K_D \approx 10^{-12}\text{M}$); only copper, cadmium and mercury bind with an affinity similar to that of zinc¹⁰. However, of these four possible metal ion variations, only the zinc-substituted enzyme is an active catalyst and binds sulfonamide inhibitors. The binding of zinc to CA can be observed fluorimetrically by coupling it to the binding of an aryl sulfonamide such as dansylamide (Figure 3), which causes a large increase and blue-shift in the dansylamide fluorescence. Hence, dansylamide fluorescence is proportional to the concentration of zinc-bound carbonic anhydrase, which in turn is proportional to the zinc concentration when the concentration of CA is larger than the K_D . These properties are relevant to the design of a zinc biosensor³ (see Chap. 4). However, to

achieve the goal of a biosensor capable of continuously monitoring the zinc concentration, this initial design must be further optimized. In particular, the slow dissociation rate constant of zinc from CA ($t_{1/2} \approx 2$ weeks at 4°C for the wild-type enzyme) significantly limits the practical utility of this biosensor. Slow equilibration with zinc is also dictated by the association rate constant which is much slower than diffusion-controlled ($\approx 10^4 \text{ M}^{-1}\text{s}^{-1}$). This problem can be overcome by engineering CA variants with altered zinc binding dynamics.

It is not surprising that the *de novo* design of zinc binding sites in other proteins relies on the structurally-characterized His₃ zinc binding site of CA as a paradigm, and much of this work has been supported by the Office of Naval Research. For example, His₃ zinc binding sites have been constructed in a metalloantibody^{11,12} and an α -helix bundle¹³, and some of these engineered proteins have been used as first-generation zinc biosensors¹⁴. Hence, the structural biology of the zinc binding site in CA and its variants is relevant not only to the development of CA-based biosensors, but also zinc biosensors based on other related systems.

Molecular Biology: Preparation and Screening of CA Variants

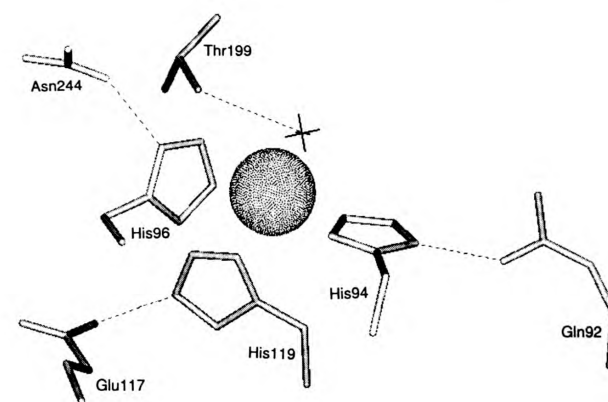
Site-directed Mutagenesis

The tools of molecular biology are essential for preparation of CA variants with altered functional properties which arise from amino acid substitutions. To this end, the gene for the second isozyme of human CA has been cloned and then subcloned into a self-replicating genetic element called a plasmid¹⁵, which allows for high level expression of human CA protein in bacteria (100 mg CA L^{-1}). The structural and functional properties of this recombinant protein are identical to CA isolated from human blood^{16,17}. Using this plasmid, it is possible to prepare CA variants with single or multiple amino acid substitutions at designated positions in the following way (designated "site-directed mutagenesis"). First, a short DNA oligonucleotide is chemically synthesized containing the altered portion of the CA nucleotide sequence. This oligonucleotide is hybridized to single-stranded plasmid DNA containing the wild-type CA sequence and then incorporated into the CA gene by serving as a primer for DNA synthesis catalyzed by DNA polymerase. Changing selected amino acids in CA allows analysis of structure-function properties and optimization of CA for use as a biosensor.

We are currently preparing a variety of single amino acid variants of CA to probe the functional importance of the following conserved structural features of zinc binding sites. First, catalytically-active zinc ions in proteins are usually liganded by three amino acid residues which are some combination of histidine, aspartate, glutamate, and cysteine side

Figure 2.

Close-up of the structure of the active site of wild-type CA taken from the crystal structure of Alexander et al.¹⁷, showing the zinc coordinated to His-94, His-96, His-119 and a solvent molecule (star) in a tetrahedral geometry. The residues that form hydrogen bonds with the zinc ligands are also shown. The imidazole NH groups of His-94, His-96 and His-119 donate a hydrogen bond to the side chain carbonyl oxygen of Gln-92, the backbone carbonyl oxygen of Asn-244, and one of the side chain oxygens of Glu-117, respectively. Finally, the zinc-bound solvent molecule (water or hydroxide) donates a hydrogen bond to the side chain hydroxyl of Thr-199.



chains (Figure 4), as well as a water molecule⁴⁻⁶; these ligands can be varied in order to alter the structural and functional properties of the protein-bound zinc ion. Studies of CA variants with altered zinc ligands additionally indicate that the imidazole ligands are determinants of zinc binding affinity and reactivity¹⁸⁻²³. Furthermore, amino acid substitutions at these zinc ligands can effectively modulate the kinetics of zinc equilibration. For example, the His-94→Glu substitution increases the dissociation rate constant significantly (about 10^3 -fold) while increasing the association rate constant 2-fold²².

A second conserved structural feature in the CA zinc binding site is the formation of second shell hydrogen bonds between the histidine zinc ligands and other amino acid residues in the following pairs (Figure 3): His-94/Gln-92, His-96/Asn-244 backbone, and His-119/Glu-117. This type of hydrogen bonding interaction is observed for all histidine ligands which bind metals, including iron, calcium, copper and zinc^{24,25}. In CA we have demonstrated that these hydrogen bonds each increase zinc affinity by 3- to 10-fold probably by decreasing the entropic cost of organizing the zinc binding site²². Furthermore, substitutions at Glu-117 (for example, Glu-117→Ala, Gln or Asp) increase both the association and dissociation rate constants for zinc dramatically (10^2 to 10^3 -fold). Additionally, X-ray crystallographic analysis of relevant

variants (for example, Gln-92→Ala and Glu-117→Ala) reveals that the structure of each zinc binding site is identical to that of the wild-type enzyme²³. The increased zinc equilibration of these variants is an important first step in the optimization of CA as a biosensor with practical utility.

Finally, the direct metal ligands in all metalloproteins are embedded within a larger shell of conserved hydrophobic groups²⁶. This may increase metal binding by restricting the flexibility of the site and thereby reducing the entropic cost of metal binding, or by enhancing the electrostatic interactions between the metal and amino acid ligands. We postulate that substitutions in this region of CA, which primarily consists of Phe-93, Phe-95, and Trp-97, will provide variants with altered zinc affinity and, perhaps, an increased rate constant for zinc association.

Combinatorial Mutagenesis

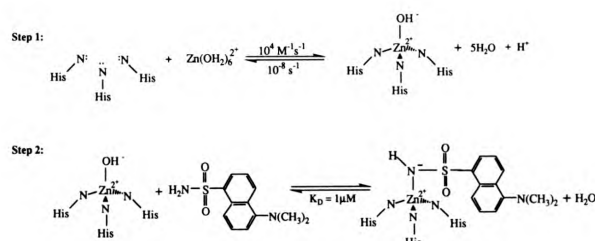
Site-directed mutagenesis is ideal for testing hypotheses about the role of specific conserved structural features essential for metal binding. However, in some cases the structural determinants of a given functional feature are unknown. For example, in CA the molecular mechanism limiting the association rate constant for zinc binding is not well understood; it may be limited by movement of the β -sheet¹⁹, reorganization of the histidine ligands, or desolvation of the zinc ion. In such cases, the method of choice is to screen a large number of enzyme variants in order to identify a few with the desired properties (such as rapid zinc binding, for example). Determination of the amino acid substitutions which lead to these altered functions should provide clues to the underlying mechanisms of the mutational effects and lead to improved rational engineering of CA.

Libraries of CA variants can be easily generated using a number of methods. Chemical mutagens will generate random mutations within a DNA segment; however, this method often results in a high proportion of wild-type sequences and a low diversity of substitutions²⁷. Therefore, we have chosen to mutagenize the CA gene utilizing site-directed mutagenesis with an oligonucleotide chemically synthesized using nucleotide precursors contaminated with a small percentage of each of the three incorrect nucleotides at each synthesis step²⁸. This technique allows substitutions to occur with equal frequency at each base; however, the size of the region that can be mutagenized is limited by the length of the synthetic oligonucleotide (≤ 100 nucleotides). A method which circumvents this difficulty uses amplification of DNA via the polymerase chain reaction with a low fidelity DNA polymerase²⁹. This technique produces a random distribution of base substitutions in DNA segments up to 1200 bases in length with a high mutagenesis efficiency, and is the best method currently available for mutagenizing large segments of DNA.

The success of any combinatorial mutagenesis approach is highly dependent on the ingenuity and efficiency of screen-

Figure 3.

Scheme illustrating the linkage between zinc and dansylamide binding to carbonic anhydrase. In step 1, zinc coordinates with the imidazole nitrogens of His-94, His-96 and His-119 of CA. Dansylamide does not bind to apo-CA. In step 2, the sulfonamide nitrogen of dansylamide coordinates to zinc, displacing the zinc-bound solvent molecule.



ing and/or selecting variants with the desired properties from the random library. We have successfully identified CA variants with increased dansylamide binding out of a random library by screening colonies lysed on nitrocellulose paper for increased fluorescence intensity upon the addition of dansylamide²⁸. Similarly, variants with altered zinc affinity could be identified by incubation with a combination of a zinc/chelator buffer and dansylamide, thereby coupling dansylamide fluorescence to zinc binding³ (Figure 3) (see Chapt. 4). These are examples of screening protocols.

However, a procedure to select CA variants with altered zinc affinity would be advantageous since a larger number of variants can be rapidly tested. Recently developed methods for the expression of foreign proteins on the surface of filamentous phage, combined with affinity chromatography of these phage, allow facile screening of large libraries of variants (about 10^9)^{30,31}. Since expression of the variant protein on the phage surface is spatially linked to the replication element of the phage (see Figure 5), this method allows for isolation of both the variant proteins with the desired properties and the genes encoding these proteins. A variety of proteins can be displayed in a functional form on the surface of filamentous phage as an N-terminal fusion with the minor coat protein encoded by gene III (pIII) (Figure 5). Phage displaying pIII-CA fusion proteins can then be fractionated according to their zinc avidity by binding to sulfonamide affinity resin in the presence of various concentrations of zinc chelators. Furthermore, this technique can be modified to select for CA variants with increased dissociation and association rate constants for zinc or variants displaying altered binding of sulfonamide inhibitors. The functional and structural properties of these CA variants can then be investigated in detail in order

to further probe the molecular determinants of zinc and sulfonamide affinity.

Structural Biology of Zinc Binding Sites

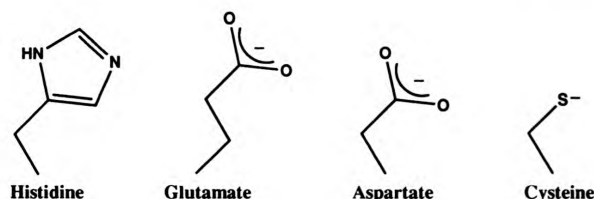
A thorough understanding of three factors contributing to stable metal binding sites must precede the (re)design of a protein-zinc binding site, particularly where the fine-tuning of affinity and equilibration time are involved. First, the chemical nature of the target metal must be considered. It could be “hard”, like the small, highly-charged magnesium ion; it could be “soft” like the large, highly polarizable mercury ion; or, it could be intermediate between these two extremes (i.e., “borderline”)³². An optimized metal binding site in a protein molecule will exhibit complementary hardness to its target metal ion. For example, the binding site of magnesium will usually contain several negatively-charged oxygen atoms of aspartate and glutamate, and the binding site of mercury will usually contain one or more polarizable cysteine sulfur atoms. Zinc is a borderline metal and as such is satisfactorily coordinated by harder ligands aspartate and glutamate, borderline histidine, and the softer ligand cysteine.

Second, the conformations of the amino acid side chains which comprise the metal coordination site must be favorable. Metal ligands should not adopt unfavorable side chain torsion angles^{33,34}, nor should they incur an unfavorable energetic cost in order to coordinate to a metal ion. For example, one would not obtain an avid zinc binding site with an engineered cysteine side chain if the cysteine-zinc interaction requires unfavorable conformational change on the part of the protein.

Finally, and perhaps most importantly, the separation and stereochemistry of ligand-zinc coordination must be optimal in order to have tight protein-metal binding. The geometric preferences of metal ion interactions with carboxylate^{35,36} (glutamate or aspartate), imidazole^{24,37} (histidine), and thiolate³⁸ (cysteine) ligands have been delineated in analyses of refined molecular structures contained in the Cambridge Structural Database and the Brookhaven Protein Data Bank. The results of these studies guide protein design experiments and provide criteria by which designed metal binding sites can be evaluated and optimized in modeling experiments prior to their actual construction. That is to say, structural information gained from the study of naturally-occurring protein-metal sites helps us to draw blueprints for new or redesigned zinc binding sites. Sometimes, however, comparison of these blueprints to the actual structure of the designed metal site points to shortcomings in current modeling approaches, for example, modeling these sites as static structures. Many proteins, including CA, demonstrate a large degree of structural plasticity in the protein scaffolding which accommodates a variety of amino acid substitutions.

Figure 4.

Amino acid side chains that commonly serve as metal ligands.



For instance, consider the thiol side chain of cysteine which typically coordinates to a metal ion as the ionized cysteine thiolate. The $C\alpha-C\beta-S \rightarrow \text{metal}$ torsion angle should preferentially adopt a value of 180° or $\pm 90^\circ$ in order to avoid unfavorable interactions between the bound metal ion and the cysteine methylene group at $C\beta$ ³⁸. Thus, as histidine $\rightarrow$ cysteine zinc ligand substitutions are considered in the active site of CA, the first step of the redesign strategy is to ensure that optimal cysteine $\rightarrow$ zinc separation and stereochemistry can be achieved as protein ligand groups are substituted or added to the active site zinc ion. Since the engineered side chain histidine is shorter than the wild-type side chain histidine (Figure 4), some degree of structural plasticity in the protein scaffolding will also be required to maintain zinc coordination.

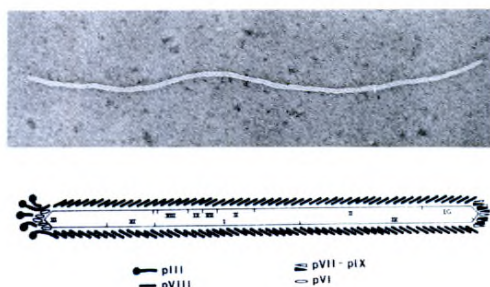
In recent work from our laboratories funded by the Office of Naval Research, cysteine residues have been substituted for each of the naturally-occurring histidine zinc ligands in CA. Additionally, a cysteine residue has been engineered which displaces the nonprotein zinc ligand, hydroxide ion. This “cysteine scan” of the CA zinc binding site provides a recent example of novel structural and functional behavior on the part of the metal site and its surrounding protein scaffolding. Correlation of structural results with accurate functional measurements allows for a detailed description of structure-function relationships in the protein-metal binding site, as summarized below. Moreover, an in-depth knowledge of structure-function relationships in a protein-metal binding site helps us to draw more accurate blueprints of subsequently-designed metal sites. This structure-assisted approach will allow us to take protein design another step forward toward the optimization of a CA variant for zinc biosensor applications.

The His-94 $\rightarrow$ Cys Variant of CA

Under the first crystallization conditions, this variant surprisingly crystallized in a new crystal form (referred to as a “space group”). The crystal structure was solved by molecular replacement and revealed an active site devoid of zinc¹⁹. Apparently, zinc binding was sufficiently compromised (by a factor of about 10^4) to allow facile equilibration between

Figure 5.

Electron micrograph of filamentous phage f1, schematic representation of the location of the coat proteins, and a genetic map. Each phage contains one molecule of single-stranded DNA encoding several proteins, including the coat proteins, packaged within a protein tube composed of mainly the major coat protein (pVIII). Both minor coat protein (pIII) and carbonic anhydrase-pIII fusion protein are displayed on the tip of the phage. The numbers in the diagram indicate the locations of the phage genes and IG indicates the intergenic sequence containing the complementary-strand origin. (Courtesy of R. E. Webster, Duke University)



zinc-bound and zinc-free forms. In subsequent work, crystals of this variant were grown in the presence of added zinc and under anaerobic conditions; these crystals formed in the wild-type space group and revealed a fully-bound metal ion in the active site^{22,23} (Figure 6).

Importantly, the structure of metal-bound His-94→Cys CA reveals substantial plasticity on the part of the β -strand which contains Cys-94. As previously mentioned, the replacement of a histidine zinc ligand with a shorter cysteine ligand has serious structural consequences. If metal coordination is to be retained in the cysteine variant, either the metal ion and/or the protein structure must move in order to maintain satisfactory ligand-metal coordination separation. In His-94→Cys CA, the metal ion moves about 0.6 Å toward the shorter cysteine side chain, while the cysteine side chain along with its polypeptide backbone move about 1 Å toward the metal ion (Figure 7). Thus, the metal binding site and its surrounding protein structure can be sufficiently plastic in order to facilitate favorable thiolate-zinc coordination, but the price of this structural plasticity is reflected in the 10⁴-fold loss of protein-metal affinity and 100-fold increase in the dissociation rate constant^{19,22,23}. Furthermore, the substitution of cysteine for histidine at position 94 affects the properties of the zinc-bound solvent molecule; the acidity decreases significantly (2 pH units) and the reactivity decreases by a factor of about 1000.

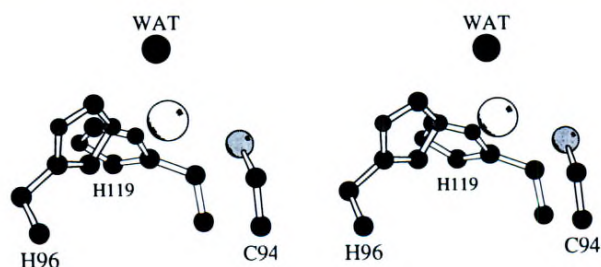
These data clearly indicate that it is possible to change the zinc affinity, equilibration time and, possibly, metal ion specificity, simply by changing the chemical nature of the zinc ligand. The plasticity of the protein scaffolding can often compensate for the structural differences in the side chain and allows the new ligand to coordinate with the metal. Although the zinc affinity of the His-94→Cys CA variant has decreased compared to wild-type, it is still sufficiently tight to measure the nanomolar concentrations of zinc normally observed in seawater. Furthermore, the properties of this variant might be further modulated by additional amino acid substitutions.

The His-96→Cys Variant of CA

However, substitution of a cysteine for a histidine ligand does not always produce a His₂Cys zinc binding site. In the His-96→Cys variant of CA the three-dimensional structure reveals a metal-bound form in which the zinc ion is coordinated by His-94, His-119, and two solvent molecules²³. Unfortunately, the Cys-96 side chain does not coordinate to zinc; instead, it packs against a moderately hydrophobic wall in the enzyme active site. In this variant there appears to be insufficient plasticity on the part of the β -strand at position 96 to allow for the shorter Cys-96 side chain to replace His-96 as a ligand to zinc. Moreover, zinc remains in its wild-type position. Thus, the energetic costs associated with the plastic

Figure 6.

Stereogram of the zinc-binding site of His-94→Cys carbonic anhydrase, in which a cysteine thiolate is substituted for a naturally-occurring histidine zinc ligand. Each amino acid side chain is designated by its one-letter code (H = histidine, C = cysteine) and sequence number. The structure of this variant has been determined by X-ray crystallographic methods, and the rms deviation in atomic coordinates is on the order of 0.2 Å^{22,23}. A water molecule occupies the fourth tetrahedral coordination site of zinc. This variant binds to zinc about 10⁴-fold less tightly than the wild-type enzyme due to a slight conformational change of the β -sheet superstructure which accommodates the amino acid substitution. Carbon and nitrogen atoms are represented by filled and cross-hatched small spheres, respectively; oxygen and sulfur atoms are represented by darkly and lightly cross-hatched larger spheres, respectively; and the central zinc ion is represented by the largest sphere.

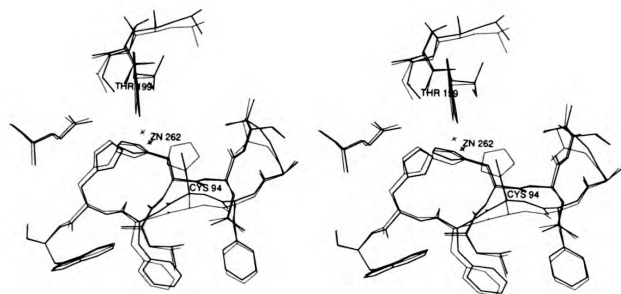


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Figure 7.

Stereogram of the least-squares superposition of His-94→Cys (thick bonds) and wild-type (thin bonds) carbonic anhydrase structures. A stick-figure representation is employed which highlights the structural changes accommodating the amino acid substitution. Residue 94, Thr-199, and zinc are indicated. Note that zinc moves toward Cys-94, and Cys-94 (along with its associated polypeptide backbone) moves towards zinc to reach a structural and energetic compromise with regard to optimal zinc binding, side chain conformation, and side chain packing interactions.



potent thiolate→zinc interaction. However, the dissociation constant of zinc is reduced only 2-fold relative to the His 94→Cys variant suggesting a delicate energetic balance for formation of the thiolate→zinc interaction in both variants²². The differences between the metal sites in the His-94→Cys and His-96→Cys variant enzymes could only be visualized by the method of high resolution X-ray crystallography. Clearly, the His-96→Cys CA variant is not suitable for ultimate incorporation into a zinc biosensor. However, given the complementary softness of the cysteine side chain with larger metal ions, this variant might be a useful starting point for the design of a biosensor specific for the biohazardous metals mercury and lead.

The His-119→Cys Variant of CA

In this variant, the engineered Cys-119 side chain coordinates to zinc with favorable stereochemistry but a lengthy separation of 2.9 Å. Second, the metal ion moves in an attempt to optimize thiolate→zinc interaction. Finally, as found for His-94→Cys CA, these plastic structural changes exact a cost on protein-metal affinity and are likely to be responsible for the observed 1.2×10^4 -fold loss of protein-zinc affinity relative to the wild-type enzyme²². In this case, the decrease in zinc affinity is accompanied by a 1000-fold increase in the dissociation rate constant for zinc. These data suggest that either of the His-119→Cys or the His-94→Cys CA variants are useful starting points for a second round of optimization for use in a

zinc biosensor. Combining these substitutions with substitutions at Glu-117 (which also increase zinc equilibration) should provide variants that equilibrate rapidly with zinc at nanomolar concentrations. Furthermore, these studies have provided valuable lessons in protein design which should confer improved predictability as we draw blueprints for subsequent metal site designs.

The Thr-199→Cys Variant of CA

Genetic-structural studies of this variant demonstrate that it is indeed possible to enhance protein-zinc affinity, although this approach does not involve the engineering of wild-type zinc ligands provided by the protein. Instead, an additional protein ligand to zinc is introduced which displaces the relatively weakly-bound nonprotein zinc ligand, hydroxide ion. This is achieved by amino acid substitutions at Thr-199, which normally accepts a hydrogen bond from zinc-bound hydroxide^{20,21}. Interestingly, it is not readily apparent in molecular modeling experiments that the near-isosteric substitution Thr-199→Cys would allow for optimal zinc coordination distance and geometry by Cys-199. However, residue 199 is contained within a plastic loop in the CA active site, which is observed to respond to amino acid substitutions in the active site hydrophobic pocket¹⁸. Thus, in this example the plasticity of the protein scaffolding facilitates the coordination of the engineered metal ligand to zinc, and in so doing zinc affinity is modestly improved²⁰. This work illustrates a very simple route toward enhancing protein-zinc affinity, namely the replacement of a non-protein zinc ligand with a protein zinc ligand; i.e., make the protein molecule tetradentate instead of tridentate (Figure 8). This approach yields a 20-fold more potent zinc binding site in the Thr-199→Glu variant of CA, where the engineered carboxylate group displaces zinc-bound hydroxide and coordinates to the metal ion. Unfortunately, this fourth protein ligand blocks dansylamide binding; hence this variant is not useful in the CA biosensor as currently designed.

In summary, this cysteine scan of the CA zinc binding site reveals that the polypeptide backbones of two zinc ligands, namely His-94→Cys and Thr-199→Cys, are sufficiently plastic to allow for optimal cysteine thiolate-zinc coordination. However, the plastic accommodation of the former mutation incurs a substantial loss of protein-metal affinity, whereas the accommodation of the latter mutation confers a slight gain in affinity. Zinc binding is also observed in His-119→Cys CA. A plastic structural response of the polypeptide backbone is not required for this particular substitution, although the zinc ion moves slightly toward the cysteine thiolate in order to optimize the coordination interaction.

Conclusions

Structure-function studies of carbonic anhydrase variants demonstrate that changes in the primary and tertiary structure of this protein can modulate its sulfonamide affinity as well as the zinc affinity, reactivity and equilibration rates. That these properties can be engineered by a structure-assisted protein design approach eminently qualifies CA for use not only in a zinc biosensor, but also in biosensors specifically engineered to detect other metal ions such as the environmental toxins mercury and lead. It is logical to employ a naturally-occurring metalloprotein as a metal ion sensor: protein-metal binding sites have naturally evolved to bind their target metals with high affinity and exquisite specificity, even as these target metals are contained in a complex mixture of cations present in seawater, blood, etc.

To illustrate the first steps of a structure-assisted design protein approach, we outline the structure-function correlations revealed in a "cysteine scan" of the CA active site. This work illuminates structural features that contribute to protein-

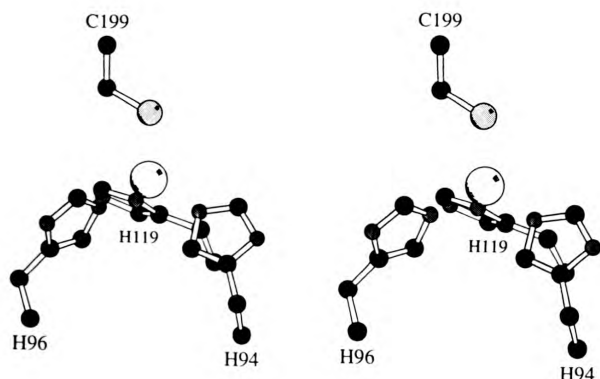
zinc affinity, and it also reveals the selective plasticity of the protein scaffolding which accompanies certain point mutations. For instance, why does a plastic change in the central β -sheet facilitate zinc coordination by a cysteine residue at position 94, but not at position 96? Such effects may be impossible to predict by molecular modeling, but X-ray crystallographic analysis of protein variants will at least reveal these subtle yet important structural changes. The underlying rationale of structure-assisted protein design is that structure-function correlations established by the study of first-generation designs will lead to improvements in subsequent designs.

How are the structure-function correlations determined in this work useful for the optimization of a zinc biosensor design based on a naturally-occurring metalloprotein? The cysteine scan of the CA zinc binding site reveals that it is possible to modestly improve zinc affinity, but only by adding another protein ligand to the metal binding site. However, the single-site amino acid variants in the zinc ligands and second shell hydrogen bonds drastically increase zinc equilibration rates. From extrapolation of our current data, we therefore predict that a CA variant containing two substitutions, His-94→Cys and Glu-117→Asp, would have a K_D in the tens of nM range and equilibrate with these concentrations of zinc with a half-time less than one second. Thus, by combining the techniques of structural and molecular biology we have already produced metalloprotein variants with zinc equilibration rates sufficiently rapid for use in a zinc biosensor. The properties of any variant can be further optimized for biosensor use by subsequent rounds of mutagenesis.

In future studies, the combination of random mutagenesis approaches with traditional structure-function studies will allow further dissection of the rules governing the affinity and selectivity of protein-metal binding sites, particularly for metals other than zinc. This approach will allow for the optimization of CA for use in a zinc biosensor. Furthermore, our approach will serve as a paradigm for the general design of protein biosensors specific for molecules even more complex than zinc, such as heavy metals or small organic compounds that pose considerable threat to the environment.

Figure 8.

Stereogram of the zinc-binding site of Thr-199→Cys carbonic anhydrase. Each amino acid side chain is indicated by its one-letter code (H = histidine, C = cysteine) and sequence number. The thiolate side chain of the engineered cysteine at position 199 displaces the zinc-bound solvent molecule present in wild-type carbonic anhydrase in order to complete a tetrahedral His3Cys zinc-binding site. This site binds to zinc four times more tightly than the wild-type protein, because the protein is able to "grasp" (i.e., bind) the metal ion with four "hands" (i.e., ligands) instead of three. Carbon and nitrogen atoms are represented by filled and cross-hatched small spheres, respectively; the sulfur atom is represented by a larger cross-hatched sphere; and the central zinc ion is represented by the largest sphere.



Biography

David W. Christianson received the A.B. degree from Harvard College in 1983, and the A.M. and Ph.D. degrees from Harvard University in 1985 and 1987. In 1988 Christianson was appointed Assistant Professor of Chemistry at the University of Pennsylvania, and was promoted to Associate Professor in 1993. He is also Adjunct Associate Professor at the Wistar Institute. Christianson received a Young Investigator Award from the Office of Naval Research in 1989, and he has also received a Searle Scholar Award (1989), an Alfred P. Sloan Research Fellowship (1992), and a Camille and Henry Dreyfus Teacher-Scholar Award (1993). As a protein crystal-

lographer, Christianson's research interests focus on structure-function relationships in metalloproteins and proteases, and mechanisms of enzyme inhibition.

Carol Fierke – see "Profiles in Science".

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Bioluminescent Biosensors

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Introduction

A number of different species of bacteria, many of them marine, are bioluminescent. They generally emit light in the yellow-green range due to an enzyme-catalyzed oxidation of a long chain aldehyde. Two *lux* genes (A and B) encode an enzyme, termed luciferase, that carries out this oxidation, emitting a quantum of light in the process. Three other *lux* genes (C, D and E) encode enzymes that are involved in the recycling of the oxidized aldehyde back to the reduced form. All five genes are clustered as an operon that is regulated by two other genes (*lux I* and *R*). When the operon is being fully expressed as many as 10^4 photons per sec per cell may be produced and the eerie glow of a culture of the bacteria is readily seen by the naked eye in a darkened room. In general, when the bacteria are found singly in the ocean, the regulatory genes have inactivated the structural genes and no light is emitted. However, when the bacteria occur at high concentrations, for example in the light organs of certain fish, the regulatory genes activate transcription of the structural genes resulting in light production.

Bioluminescent biosensors are based on the concept of using genetic engineering to remove the natural regulatory genes of the *lux* operon and replacing them with other genetic regulatory regions that respond to the presence of compounds of interest. These genetic constructions are then placed in *E. Coli* or some other suitable host. In this review the properties of two such constructions will be described, one that emits light in the presence of copper and the other in the presence of mercury. Because the constructions can be used to detect compounds they are called living cell "biosensors."

Under well-defined conditions of growth and with defined chemical solutions the biosensors emit light in proportion to the concentration of mercury or copper with an acceptable statistical level of accuracy and in a useful range of concentrations. In this regard they appear to meet or exceed the specifications of popular physicochemical analytical procedures such as atomic absorption spectroscopy (AA).

A crucial question, however, is whether the biosensors are useful for measuring their target analytes in more complex situations such as in environmental samples. Such samples might contain compounds that are inhibitory to the metabolism of the biosensor cells, or to the *lux* enzymes themselves, resulting in no light or diminished light even if the target analyte is present. Preliminary work in this area demonstrates that this problem exists in some, but not all, environmental samples, and research is in progress to evaluate the extent of the problem and to determine if it can be overcome. One area in which the biosensors could offer a significant advantage over conventional analytical techniques is in the evaluation of "bioavailability". Because the biosensors are living organisms and because the compounds that they detect must be available for cellular uptake one could argue that they represent the most direct way yet devised to assess bioavailability.

The *Lux* Gene System

All bioluminescent bacteria studied use essentially the same pathway and enzymes for the production of light. In this review only the biochemistry and genetics of the systems that are relevant to the understanding of the biosensors will be described. More comprehensive descriptions can be found in

a recent article in Naval Research Reviews (Nealson, 1994)⁹ and in several recent reviews.⁷⁻⁹

Figure 1 illustrates the *lux* system in the marine bacterium *Vibrio fischeri*. It consists of five structural genes A-E. Genes A and B encode different subunits of the enzyme luciferase. Luciferase catalyzes the oxidation of a reduced long chain aldehyde resulting in a quantum of light. The emitted light can be easily measured with a luminometer. Genes C, D and E encode fatty acid reductases that form a complex and are involved in the recycling of the oxidized aldehyde back to the reduced form. The flavin reductase enzyme that recycles the redox factor FMN is encoded by a structural gene that is not part of the *lux* operon but is found in all bacterial cells as part of their normal metabolic machinery.

The reaction requires reducing power (NADPH)⁺, energy (ATP) and O₂. It is important that these are available and are not rate limiting when the biosensors are being used to measure the concentration of their target analytes. The significance of this is that attention must be paid to the metabolic activity of the biosensor cells and, as we will see, this is not uniform during the growth of a bacterial culture.

The genetic region that controls the expression of the five structural genes consists of two regulatory genes, *R* and *I*. The mechanism by which they exert this regulation is reviewed by Nealson.¹⁰

Using standard techniques of genetic engineering the *R* and *I* genes were removed and replaced by genetic control regions regulated by mercury or copper respectively. The resulting constructions were then introduced into *E. coli* on plasmids.

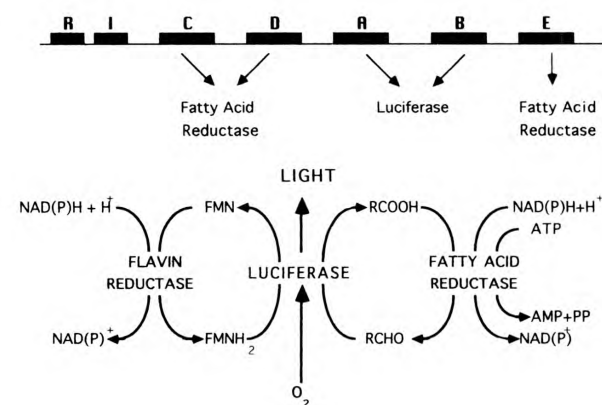
The Mercury Detoxification Gene System

A number of different species of bacteria are able to detoxify inorganic or organic mercury using a mercury reductase enzyme to reduce Hg⁺² to Hg⁰.^{13,14} Hg⁰ is volatile and its production facilitates the removal of Hg⁺² from the immediate environment of the bacteria into the air. The genes involved in mercury detoxification appear to be related in many bacteria and a simplified scheme of how they work is shown in Figure 2A.

There are three essential structural genes *T*, *P* and *A* under the control of one regulatory gene, *R*. Genes *T* and *P* encode proteins that transport Hg⁺² into the cell and gene *A* encodes the mercury reductase enzyme. Gene *R* encodes a protein that can bind to the promoter region just upstream from the structural genes. In the absence of Hg⁺² this protein binds to the promoter preventing transcription of the structural genes. In the presence of Hg⁺² it first binds Hg⁺² and the then resulting complex binds to the promoter region activating transcription of the structural genes. This results in the synthesis of T and P proteins which then actively transport Hg⁺² into the cell further

Figure 1.

Representations of the *lux* operon genes from the marine bacterium *Vibrio fischeri* and the biochemical reaction catalyzed by the five structural *lux* genes A-E.



enhancing the transcription of the structural genes. Meanwhile, the mercury reductase enzyme is busy reducing Hg⁺² to Hg⁰. When no more Hg⁺² remains, the *R* protein, minus bound Hg⁺², inactivates transcription of the structural genes and the operon shuts down.

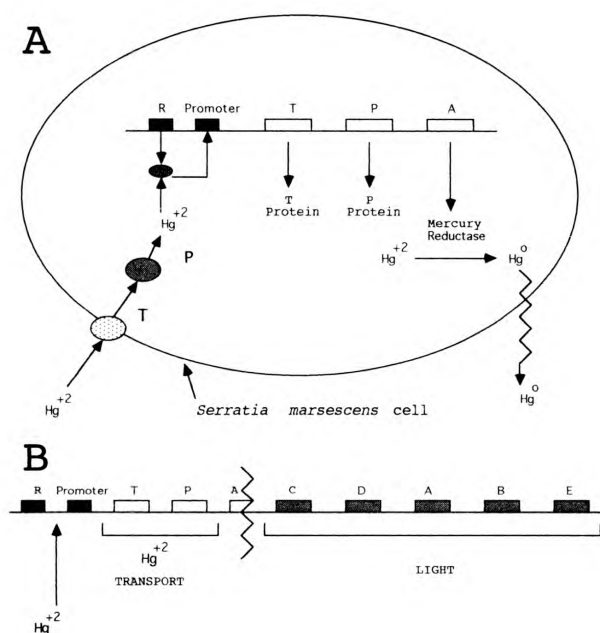
A mercury biosensor was constructed by using genetic engineering to splice the mercury control region, *R*, together with the genes *T* and *P* next to the structural genes of the *lux* operon, minus their natural control genes *R* and *I* (Figure 2B). The resulting plasmid termed pDU1003 was introduced into *E. coli* and tested for light production with and without added Hg⁺².^{2,5} Note that the *A* gene was cut in half in this construction and that, consequently, no active mercury reductase enzyme is produced. In contrast, the *T* and *P* genes are still active permitting Hg⁺² to be actively taken up by the cells. This results in a biosensor that is extremely sensitive to low levels of Hg⁺² but which is inactivated at higher concentrations because it cannot dispose of the Hg⁺² as Hg⁰. A similar construction has been made in which the *lux* genes have been placed downstream from the mercury reductase gene leaving it fully functional.¹² This biosensor does not detect as low a level of Hg⁺² as pDU1003 but is active at higher concentrations of Hg⁺². Thus, by using the two different genetic constructions the dynamic range of Hg⁺² detection can be extended.

As indicated earlier the light output of the biosensors is dependent upon culture conditions and the age of the culture. This phenomenon can be seen in Figure 3. In this experiment Luria broth, a rich bacterial culture medium, was inoculated with a 1% sample of actively growing Hg⁺² biosensor cells. At the same time the Luria broth was inoculated with 1μM Hg⁺² (HgCl₂) and the culture incubated at 25°C with vigorous

Figure 2.

(A) Representation of a mercury resistant *Serratia marcescens* cell illustrating the three structural mercury resistance genes *T*, *P* and *A* under the control of the regulatory gene *R*.

(B) The genetic construction of the mercury biosensor. The light emitting lux genes *A-E* were fused next to the mercury resistance genes *T* and *P* bringing the two genes under the control of the mercury resistance regulatory gene *R*. The mercury resistance gene *A*, encoding the reductase enzyme, has been cut in two during the genetic splicing and is not active.



shaking. When light output per live cell is measured as a function of time it can be seen that there are two peaks of activity at about 6 and 13 hours respectively. Further analysis shows that, under optimal conditions, and when the bacterial culture is at 6 hours, it takes only 15 minutes for the light genes to become activated and only 60 minutes for maximal light expression. The two peaks of light output in the growing bacterial culture are most likely due to the metabolic state of the cells. The earlier peak at 6 hours coincides with mid-log growth when the cells are most actively dividing and presumably reflects their intense metabolic activity. However, the reason for the second peak of activity during stationary phase, when the cells are no longer dividing, is not known. A variety of different biosensors, created using different promoters exhibit this phenomenon and thus the two peaks of activity are not an intrinsic property of the genetic control regions.

Whatever the explanation turns out to be for the two peaks of activity the important point, at this juncture, is to recognize that attention must be paid to this phenomenon and careful standardization must be achieved so that the light output of the biosensor denotes the concentration of the analyte being measured and not the metabolic state of the cells.

There are at least two possible solutions to this problem. The first is to accept the problem and with the use of proper controls standardize all the measurements to reflect the light output at peak activity which, at 25°C, is about 6 hours. This is not particularly difficult to achieve in a bacteriology laboratory but it adds a level of inconvenience for a non-bacteriologist.

The second solution to the problem is to maintain the cells at peak metabolic activity. This can be achieved in a continuous culture bioreactor in which materials are fed in, and cells and metabolic products are removed, at a rate that maintains the resident cells in a rapidly dividing state. The whole operation can be computer controlled and miniaturized.¹⁵

Properties of the Hg⁺ Biosensor

In Luria broth and under defined conditions of growth time (6 hours), oxygen supply, temperature, pH and time of application of the sample, the biosensor cells produce light in response to concentrations of Hg⁺² between 0.1 nM and 1μM. Maximal light is produced in response to 1μM Hg⁺²; above this concentration the amount of light diminishes sharply presumably due to the toxicity of the Hg⁺². In the absence of added Hg⁺² the biosensor cells produce only 4x10⁻⁵ of the amount of light observed in the fully induced state; i.e., the background, in the absence of Hg⁺², is only 0.004%.

The Hg⁺² biosensor was tested with a range of concentrations of other metal ions including CdSO₄, CoCl₂, NiSO₄, ZnCl₂ and CuSO₄. None produced light above background. Both Hg⁰ and organic mercury produced light, but since both of those could be converted metabolically to Hg⁺² it is difficult to tell what the biosensor is reporting.

At peak metabolic activity (6 hr.) and at 25°C it takes 15 minutes after the addition of 1μM Hg⁺² to observe light at a level that is statistically significant above background. With lower concentrations of Hg⁺² the time required is proportionally longer. This reflects (1) the time it takes for the Hg⁺² to enter the cell (recall that, initially, there are few, if any, molecules of the Hg⁺² transport proteins present per cell) (2) for the operon to be transcribed into mRNA (3) for the mRNA to be translated into proteins and (4) for the proteins to function as enzymes. Independent genetic studies indicate that it takes about 5 minutes at 37⁰ before mRNA can be detected after gene activation and we suspect that the more extensive time (15 minutes) before light can be observed in the biosensor system probably reflects a slightly slower metabolic activity of cells

at 25°C compared to 37°C. Unfortunately, light is not produced at 37°C because the luciferase enzyme is inactive at this temperature.

Of interest, at the fundamental level, is the observation that the background light, in the absence of exogenously added Hg^{+2} , is only 0.004%. A rule of thumb states that a typical regulated bacterial gene is expressed at about 0.1% of its induced state. We do not know whether the substantially reduced level of expression of light in the absence of Hg^{+2} reflects a peculiarity of the mercury regulatory system or whether other estimates of gene activity (using more complex methods to analyze gene activity) are less accurate than using light.

Because light is so easy to measure, genetic constructions utilizing light as a reporter system to determine promoter activity are proving very important in helping to determine the basis of gene regulation. It is easy to imagine how, by manipulating DNA sequences in the regulatory region and measuring the resulting changes in light output, an understanding of the molecular basis of gene regulation can be deduced. Another advantage of the light system is that it is non-destructive in contrast to other techniques used to measure gene activity (which normally require that the cell is broken to measure mRNA or enzyme levels). Because it is non-destructive the effects of different conditions or compounds on gene regulation can be evaluated successively on the same cells.

Properties of the Cu^{+2} Biosensor

A genetically engineered plasmid (pCULux) has been constructed in a similar fashion to that described for the Hg^{+2} biosensor except, in this instance, a genetic regulatory region responsive to copper was used to activate the *lux* genes.⁵ The copper responsive regulatory region used was originally derived from a copper-resistant *E. coli* isolated from a piggery where copper was being used as a food supplement to decrease bacterial infections.

The mechanism of copper resistance involves the activation of three structural genes in an operon that encode intracellular copper binding proteins. One of these copper binding proteins is thought to pass through the bacterial membrane carrying bound copper to the outside thus lowering the intracellular concentration of copper.⁶ The copper biosensor contains only the genetic regulatory region from the operon and thus the *E. coli* host that harbors the plasmid bearing the biosensor genes has only the normal level of resistance to copper. One could envisage constructing a second type of biosensor containing the copper detoxification genes as well as the copper regulatory region, thus extending the range of copper detection, but this has not been done yet.

When pCULux was introduced into a laboratory strain of *E. coli* it emitted light in response to added Cu^{+2} . Similar to

the mercury biosensor it was observed that there were two peaks of light, the first in mid-log at about 6 hours and the second in stationary phase.

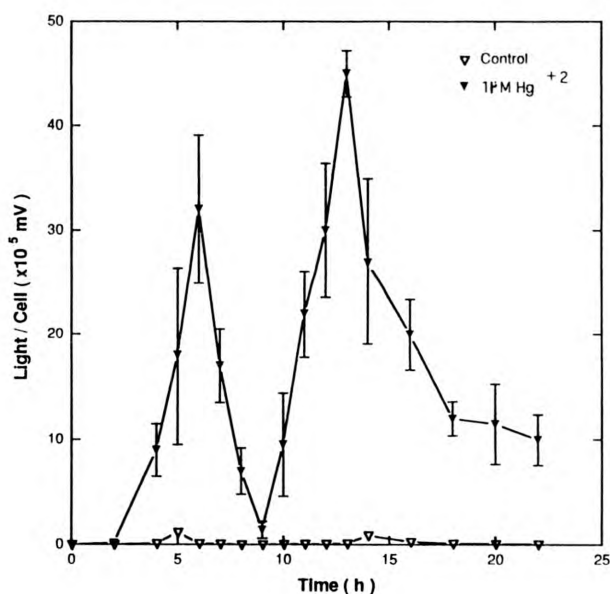
Under optimal conditions the copper biosensor produces light in proportion to Cu^{+2} concentration (CuSO_4) in the range 0.1 μM to 1 mM, with maximum light produced at 1 mM Cu^{+2} . In the absence of added Cu^{+2} the biosensor cells emit 0.2% of the amount of light compared to the fully induced state. Atomic absorption analysis indicates that the bacterial growth medium contains about 0.05 μM Cu^{+2} due to copper contamination of the reagent-grade chemicals used to formulate the growth medium and this copper probably is responsible for the background light. Removal of this copper by chelation inhibits bacterial growth because it is an essential element. A careful analysis of how low the endogenous copper level can go before impairing bacterial growth has not been done, but the requirement for copper as a growth factor will ultimately set the lower limit of sensitivity of the copper biosensor to exogenously added copper.

A variety of other metal ions were tested and it was found that Zn^{2+} and Ni^{2+} were able to induce light above background to the levels of 0.5% and 0.3%, respectively, of the maximal level induced by Cu^{+2} .

In the examples described the light emitted from the biosensor cells was measured in a luminometer. An alternative

Figure 3.

A plot of light per cell versus time in a growing bacterial culture with or (∇) or without (∇) addition of 1 μM Hg^{+2} . Six hours corresponds to about mid-log and 13 hours to stationary phase in the growth cycle of the biosensor bacteria.

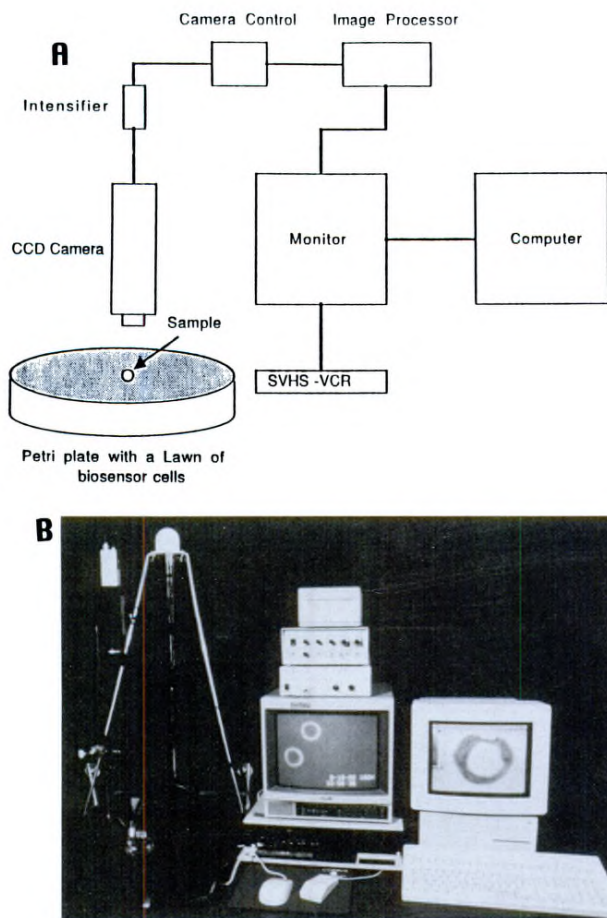


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Figure 4.

(A) Diagrammatic representation of the set-up to visualize light emitted from a petri plate of biosensor cells stimulated with an appropriate sample. The computer contains the NIH Image Analysis program. The VCR is for continuous recording of images. (B) An example of the hardware diagrammed in (A) above.



approach is to measure light using a CD camera and image analysis. A typical set-up to measure light in this way is illustrated in Figure 4.

A petri plate of nutrient agar is seeded with a lawn of biosensor cells and the liquid sample to be analyzed is placed as a drop in the center of the plate. As the target analyte diffuses out from the sample it lights up the bacteria in proportion to its local concentration. Image analysis can be used to quantitate the light. Examples of such images are shown in Figure 5.

Two advantages of this approach are that continuous data can easily be collected and quantitated and multiple samples can be recorded simultaneously. The approach could also

conceivably be used to measure diffusion rates of target analytes through different materials such as filters, ceramics, membranes etc.

Environmental Applications

A summary of the properties of the copper and mercury biosensors is given in Table I. The biosensors are sufficiently specific and sensitive to detect their target analytes in useful ranges of concentration. However, these analyses have been done in relatively defined chemical solutions, and the question arises as to whether they can report compounds in more complex samples, such as environmental samples.

There are potentially many difficulties for analyzing and interpreting light from biosensors in environmental samples. For example target metals might exist in more than one oxidation state or in more than one chemical compound or they could be bound to soil particles with a variety of dissociation constants. Interference of light measurement might come from the soil particles and metabolic inhibitors of the biosensor cells could come from toxic compounds in the sample. The presence of soil microorganisms might also interfere with the metabolic properties of the biosensor cells, although growth of such microorganisms is not anticipated to be a severe problem since incubation times are expected to be short (hours).

One potential advantage of using the biosensors for measuring compounds in environmental samples is that they may represent a relatively simple and unique way of measuring bioavailability. The bioavailability of a compound is an important property to know because it correlates with the extent to which the compound can be bioremediated and bioremediation is becoming an increasingly used technique to decontaminate soils and wastes. Bioavailability also correlates with the extent to which a compound is washed by natural processes into the ground water, or is it taken up by living material.

The traditional way to measure bioavailability is to wash the sample in an aqueous solution under standardized conditions of time, temperature etc and to analyze the amount of the compound that enters the aqueous wash using a technique such as AA. The residue, after the wash, is also subjected to AA to determine the bound, or non-bioavailable fraction of the compound.

In preliminary studies (unpublished) we have analyzed ten environmental samples including soils, sludges, ashes and composts, using both the copper biosensor and AA for comparison. The samples were subjected to the standard aqueous wash and the Cu^{+2} concentration was determined in the aqueous wash and the residue by both AA and analysis using the copper biosensor. In 8 out of the 10 samples the biosensor reported exactly the same level of Cu^{+2} in the aqueous wash as was determined by AA. In the remaining two samples the biosensors reported less Cu^{+2} in the aqueous wash compared to AA. Subsequent analysis revealed the presence of cobalt in

these samples at levels that were inhibitory to the biosensor cells. However, if the samples were first diluted then the toxic effect of the cobalt disappeared and the biosensors again reported the same levels of Cu^{+2} as determined by AA. In this case one could say that dilution is the solution. However, it is anticipated that environmental samples exist in which the ratio of the inhibitory compound(s) to the target analyte is too high to be diluted out and another answer to the problem must be found.

Of particular interest in the studies on the environmental samples was the fact that the biosensor cells reported the presence of bioavailable Cu^{+2} in the residue left after the aqueous wash. Clearly, this Cu^{+2} was "bioavailable" to the biosensor cells and the traditional chemical approach underestimates this fraction. It now remains to be determined whether "bioavailability" to the biosensor cells is a general indication of bioavailability or whether the biosensor cells are special due to the genes used in their construction. For example, the Hg^{+2} biosensor cells contain Hg^{+2} transport proteins and so are more effective at extracting Hg^{+2} from their environment than the average soil microorganism. Probably there will never be a generally accepted definition of "bioavailability" but the biosensors might offer unique opportunities to investigate the concept of bioavailability.

Future Directions

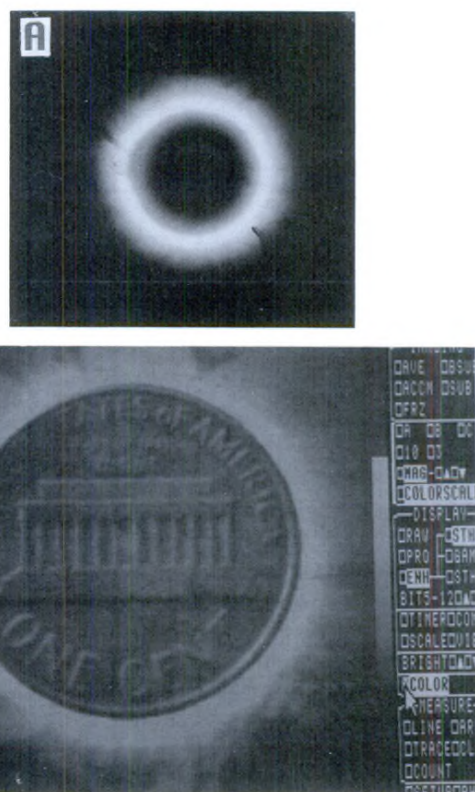
Many genes, in both prokaryotes and eukaryotes, are regulated at the genetic level by intracellular signals such as metabolites or by external signals such as O_2 levels, temperature, pH and various environmental compounds. In principle, genetic engineering or classical genetic manipulation could be used to place the *lux* genes under the control of any genetic control regions creating a variety of biosensors responding to numerous compounds of interest. In addition to copper and mercury, there exist biosensors for naphthalene and salicylate⁴ and also to aluminum, arsenate and arsenite (Michael Dubow and Simon Silver, personal communications). Clearly, in the future, many more biosensors will be created exploiting the enormous extent and diversity of genetic regulatory regions.

Whereas the biosensors created to date function effectively as detectors of their target analytes in relatively defined chemical solutions in the laboratory, considerable work remains to be done in order to assess their ability to detect compounds in complex environmental samples. The problem is that the biosensor cells, are living organisms and need to be maintained in an active metabolic state in order for the *lux* genes to be expressed and for the light reaction to occur. Toxic compounds could clearly interfere with either or both of these requirements. As described above preliminary experiments indicate that, in certain cases at least, it may be possible to dilute out the toxic compound(s) while retaining a sufficient concentration of the target analyte for effective reporting.

However, in cases where this cannot be achieved another solution must be found or else the use of biosensors in these instances must be curtailed. This problem raises the issue of using the proper controls. Specifically, in this case, one needs as a control a biosensor that expresses light constitutively, that is, continuously and without activation by a specific compound. When used side-by-side with the appropriate biosensor, this control should exhibit no change in light output unless a toxic compound is present. One variation of this idea, frequently discussed at tea time, is to genetically engineer a biosensor cell so that it contains two sets of *lux* genes. One set, expressing red light for example, could be attached to the specific control region of interest, while the other set, expressing green light, is constitutive. The wavelength of the light output could be monitored using appropriate filters. If an environmental sample contained the target analyte but no toxic compound, red light output would increase, while green light output would remain steady, a sort of living traffic light. In contrast, if the sample contained a toxic compound in addition

Figure 5.

Example of images visualized by the equipment shown in Figure 4. (A) filter disc containing 1mM Cu^{+2} . (B) a US one cent coin. Both images were made using only the light emitted by the copper biosensor cells. The image in B is false-colored.



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to the target analyte then the green light output would decrease, signalling a problem. It might still be possible, in simple cases, to detect the level of the target analyte by measuring the red light output and taking into account its diminution by the toxic compound as reported by the green light. However, it might also be very difficult to do the accounting properly! Time will tell.

A second problem is to understand exactly what the biosensor cells are reporting in an environmental sample. Obviously the target analyte must be taken up by the cell and so in a sense must be "bioavailable". But what does "bioavailability" mean? What is bioavailable to one organism might not be bioavailable to another. How long does one allow the biosensor to interact with the environmental sample? This is important because bioavailability is also controlled by chemical equilibria and by diffusion parameters. All of these questions need to be explored. At the very least the bioluminescent biosensors could become important research tools to explore issues related to the bioavailability of compounds in the environment.

Whereas a considerable amount of research needs to be done to establish the role of bioluminescent biosensors in the armamentarium of accepted sensing devices they are already proving of exceptional value in helping us to understand fundamental aspects of gene regulation. By splicing genetic regulatory regions next to the *lux* genes one can readily estimate promoter strength and time of gene induction and one can explore the interaction of the regulatory region with other aspects of gene regulation and cell metabolism.^{3,11} One can

Table 1

Properties of the Hg⁺² biosensors described in this report

	Cu ⁺² Biosensor	Hg ⁺² Biosensor
Concentration showing maximum light output	1mM	1μM
Range	0.1μM - 1mM	0.1nM - 1μM
Specificity to target metal	99.2%	100%
Optimal Temperature °C	25	25
Optimal pH	6.5	6.5

also alter the regulatory region by site-directed mutagenesis and monitor the effect of these mutations on light production, thus providing a superior way to unravel the molecular mechanisms underlying gene regulation.¹

Biography

Professor Holmes received his Bachelor of Science degree in Natural Sciences in 1969 from Trinity College, Dublin, Ireland and his Ph.D. in 1973 in Biochemistry from the Cali-

fornia Institute of Technology. After 2 years of postdoctoral work in the Chemistry Department at CalTech he took a position as Assistant Professor at the State University of New York in Albany. He subsequently worked at the Research and Development Laboratories of General Electric, Rennselaer Polytechnic Institute and Clarkson University. He is currently professor titular at the University of Chile, Santiago.

His research interests lie in the genetic engineering of microorganisms for industrial and environmental applications. He has been involved for the past thirteen years in studies of microorganisms used for metal recovery in biohydrometallurgical operations and, more recently, in the development of biosensors for environmental pollutants.

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Epilogue: Biosensors for the Future

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This issue of *Naval Research Reviews* has served to highlight selected aspects of the current biosensor research effort supported by the Office of Naval Research (ONR). In three of the articles (Chaps. 2,3 and 6), the authors have focused specifically on approaches to sensing and biosensing that they themselves have initiated and developed in their laboratories. Chapter 2 introduces the new generation of submersible chemical analyzers. While these devices in their present configurations are not biomimetic in any sense, they represent an evolved and adapted technology that offers a standard as well as a perspective to the biosensor presentations in the chapters that follow. The highly developed family of antibody-based fiber optic devices described in Chapter 3 has symbolic as well as analytical significance because it suggests that the Navy may utilize fiber optic biosensors for the first time. In Chapter 4, the authors discuss a range of emerging biomolecular transduction options as well as introduce their own work as case studies (the carbonic anhydrase-based zinc biosensor from the Thompson laboratory and the imaging optical sensor array from the Walt laboratory, both of which are fiber optic fluorescence devices). The state of the art of protein engineering using the tools of high resolution X-ray crystallography and molecular biology, is described in Chapter 5 as a means of redesigning the folded polypeptide framework of carbonic anhydrase to meet metal ion biosensor specifications. Finally, in Chapter 6, an entirely biological approach to sensing is discussed using genetically-engineered light-emitting microorganisms.

There are a number of exciting developments in the biosensor program at ONR which include, but are not limited to, those described in this volume. The issues addressed by these developments involve the desirability of cross-reactive biosensor arrays, the type of biomolecular receptor and the means of generating the large number of receptor variants to display on the array, the transduction mechanisms required for efficient imaging of the array and the means by which the imaged array patterns will be deconvoluted.

Cross-Reactive Receptor Arrays

Today's biosensors usually achieve selectivity by binding one analyte (ligand) more tightly than all others (the antibodies in Chapter 3, for example, and carbonic anhydrase in Chapters 4 and 5). When the binding becomes very tight (as alluded to in Chapter 2 and discussed in Chapter 4) the dynamical behavior of the biosensor is compromised. By moving from the one biosensor/one analyte format to a differentially cross-reactive array, where the receptors in each element of the array cross-react with different analytes in the mixture in a unique way, it becomes possible to identify and quantify the analytes by examining the *pattern* of binding across the array. Because the use of very tight-binding receptors can be avoided, such arrays should display useful response/reset times (at least down to nanomolar levels of analytes). Other advantages of arrays include the ability to deal with mixtures of very similar analytes at varying concentrations in time and space (a common occurrence in practice), the expansion of the dynamic range of

the device, easier identification of false positive signals and the possibility of self-calibration. Such devices will also be reagentless.

Choice of Biomolecular Receptors and Generation of Receptor Variants

Most biomolecular receptors used in today's biosensors (e.g., the antibodies in Chapter 3 and the carbonic anhydrase in Chapter 4) are off of Nature's shelf and quite large. While these globular structures can be re-engineered and perfected to satisfy biosensor specifications (Chapter 5), there is still considerable interest at ONR in searching for smaller frameworks that, under strong selective pressure in Nature, have evolved rugged features more resistant to the harsh insults to which biosensors will be subjected. Efforts are also underway to evaluate natural and designed polypeptide channel structures (in contrast to the globular architectures of antibodies and carbonic anhydrase, for example) as the basis for biosensors. When placed in a membrane, such channels permit ions to flow across the membrane. If the channel contains a suitable positioned binding site for an analyte then that analyte may block, or gate, the ion flux in a characteristic manner. In the much longer term, it may be possible to convert the folded polypeptide backbone of either globular receptors or channels to a "bullet-proof" non-natural backbone and, in the case of channels, to even contemplate transduction schemes which avoid binding steps altogether.

Given a suitable biomolecular receptor or channel framework, the emerging technology of encoded combinatorial synthesis can be used to generate the desired receptor variants for the array. The protocol involves (partial) randomization of the polypeptide sequence, display of the resulting sequence variants in a library, selection or screening in the library for desired variants (e.g., by analyte binding criteria) and, finally, scaled-up synthesis of the preferred receptor variants. If the randomization/display/selection cycle is repeated many times, one has molecular evolution in a test tube! A specific example of the library technology is discussed in Chapter 5 under the heading "Combinatorial Mutagenesis".

In the long term, it may be possible to use a single, small, robust receptor framework that is capable of recognizing all shapes and types of small and medium-sized analytes for biosensor applications (in much the same way that the immune system uses a molecular motif). It should be noted that analytes as large as bacterial or viruses can be sensed using specific antibodies (see Chapter 3).

Transduction Schemes for Biosensors

An ideal transduction scheme would generate a signal when an analyte occupies the receptor binding site and an entirely different signal when the binding site is empty. For example, in the case of fluorescence transduction, the lifetime of the fluorophore might be significantly different in the occupied versus empty state (see the case of carbonic anhydrase in Chapter 4). Furthermore, the signal indicating site occupancy should be independent of the analyte occupying the site, and the transducer molecule (or feature) must be an integral part of the sensor and not added as an external reagent. While these requirements represent an important unsolved problem in the basic science of biosensing, it is likely that combinatorial library technology will be a useful approach to its solution.

Signal Processing

The olfactory system defects and discriminates odorants far better than any physical or chemical device now available. Attention is therefore being given at ONR to the use of physiologically-based computational models of the olfactory bulb, as well as conventional neural networks, that focus on both the temporal and spatial attributes of the sensor signal arrays.

These, then are some of the issues driving basic science at ONR in the field of biosensors. While many problems remain (sampling strategies and biofouling, to name but two) the prospect of advanced biosensor arrays able to satisfy both naval and civilian requirements is very real and will open a broad range of new possibilities in sensing, detecting and monitoring chemical and biological species in Nature.

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