REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 24, 2002	Final Report February 1990-30 Sept.9

4. TITLE AND SUBTITLE
Fiber Optic Metal Ion Biosensor

5. FUNDING NUMBERS
N00014-91-J-1572

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Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution unlimited

13. ABSTRACT *(Maximum 200 words)*

This report describes the initial conception and reduction to practice of a fluorescence-based fiber optic metal ion biosensor. The sensor transducer is a variant of a biologically derived molecule, human apocarbonic anhydrase II, which binds metal ions such as Cu(II), Zn(II), Cd(II), Co(II), and Ni(II) with high affinity and specificity. Several approaches were developed to transduce the presence or level of the metal ion as a change in fluorescence intensity, wavelength, lifetime, or anisotropy (polarization). Sensors were constructed capable of determining Zn(II) and Cu(II) at part per trillion levels in the presence of Ca(II) and Mg(II) at part per thousand levels.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Fluorescence, Metal Ion, Biosensor, Copper, Zinc, Fiber Optic	12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclass	Unclass	Unclass	UL

FINAL REPORT

Grant No. N00014-91-J-1572

PRINCIPAL INVESTIGATOR: Richard B. Thompson

INSTITUTION: University of Maryland School of Medicine

GRANT TITLE: Fiber Optic Metal Ion Biosensor

AWARD PERIOD: 01 February 1990 to 30 September 1999

OBJECTIVE: The central objective of this project was to see if a fluorescence-based sensor employing a biological or biomimetic molecule as a transducer could demonstrate high sensitivity, selectivity, as well as rapid and reversible response to an analyte such as Cu(II) or Zn(II). A key goal was to see if the sensor figures of merit could be optimized by modification (mutagenesis)of the transducer molecule; among the figures of merit were the affinity, selectivity, stability, and response speed of the sensor. Results of that work are described in the ONR grant Final Reports of Drs. Carol Fierke and David Christianson.

APPROACH: The basic approach was to take advantage of the known propensity of human apocarbonic anhydrase II to bind certain metals in its active site. The approximate affinities of the wild type enzyme are Cu(II): 0.1 pM, Zn(II): 4 pM, Cd(II): 10 nM, Ni(II): 40 nM, and Co(II): 100 nM; importantly, neither Ca(II) nor Mg(II) appear to bind at all, a key consideration for measurements in sea water or serum, where these cations are present at millimolar levels. Because the binding is reversible, the fractional saturation of the binding site is a simple function of the free metal ion concentration and therefore is easily calculated from it.

Several means were developed to transduce the binding of particular metal ions as changes in fluorescence intensities, spectra, lifetimes, or anisotropies (polarizations). Chronologically the first approach was that of Thompson and Jones, which took advantage of the results of Chen and Kernohan, wherein a fluorescent aryl sulfonamide inhibitor for carbonic anhydrase, dansylamide, was found that bound as a fourth ligand to the active site Zn(II). Thompson and Jones found that dansylamide did not bind in the absence of zinc, and thus the fractional occupancy of the binding site and the associated free zinc concentration are easily determined from changes in the fluorescence intensity, spectrum, and lifetime. The dynamic range of zinc determination could be expanded by varying the wavelength at which the lifetime is measured.

This approach was improved upon by the development of other fluorescent aryl sulfonamides with better fluorescence properties, such as ABD-N, ABD-M, Dapoxyl sulfonamide, and BTCS. We constructed a fluorescent energy transfer-based transducer by attaching a fluorescent label to the apoprotein and using a colored aryl sulfonamide which could serve as an energy transfer acceptor for the label; in the presence of zinc the acceptor would bind and accept the labels's energy, reducing the lifetime and intensity, whereas in the absence of zinc no energy transfer occurs and the label remains unquenched. We showed that the response of such transducers can be optimized by placement of the label using site-directed mutagenesis.

We showed that similar sensitivity could be achieved in a "reagentless" format without the diffusible aryl sulfonamide small molecule, and this sensor responded to metal ions other than zinc. In particular, Cu(II), Ni(II), and Co(II) exhibit weak d-d absorption bands which are too weak (extinction coefficients one hundred to one thousand-fold weaker than typical organic dyes)to be analytically useful, but which serve admirably as energy transfer acceptors. Thus by placing the donor label in a suitable position and choosing its spectral properties to overlap well with the metal absorbance, very large responses were achievable, and free Cu(II) concentrations were detectable down in the sub-picomolar (parts per quadrillion) range. The protein transducer could be incorporated into a lifetime-based optical fiber sensor.

While lifetime-based sensing is well-suited to remote sensing using optical fiber, measurements of fluorescence lifetimes in the nanosecond range require relatively complex and costly instrumentation. Because of this, we demonstrated anisotropy-based determinations of metal ions using a similar approach, but which only requires simple steady state measurements. This approach was demonstrated using both reagentless and reagented configurations. The approach was also demonstrated with two-photon excitation using infrared excitation from a mode-locked titanium sapphire laser.

More recently, these approaches were adapted to measuring free copper in sea water at sub-picomolar levels in real time through a length of optical fiber.

Related work was carried out under ONR grants N00014-94-1-0353, N00014-98-1-0475, N00014-98-1-0685, and N00014-00-1-0921 (all to Richard Thompson), and grants awarded to Carol A. Fierke (N00014-93-1-1245 and N00014-95-1-0573) and David W. Christianson.

ACCOMPLISHMENTS: While the details of the program accomplishments

are described in greater detail in the listed references, a few "firsts" may be noted. While not the first fluorescene-based metal ion biosensor, our sensor remains the most selective and sensitive sensor of any kind for zinc, and probably for copper. We were the first to demonstrate a ratiometric metal ion biosensor, a lifetime-based metal ion sensor, an energy transfer-based metal ion sensor, and the determination of metal ions of any kind by fluorescence anisotropy. Our colleagues Carol Fierke and David Christianson were the first to show that the metal ion affinity, selectivity, and/or binding kinetics could be dramatically improved by subtle changes in the protein structure, arrived at either by design or combinatorially. By comparison, this remains difficult to achieve in organic fluorescent indicators for zinc and copper. Twenty years after the development of Fura-2, the most sensitive fluorescent indicators for zinc (Fura and TPEN derivatives from several laboratories) are still one hundred-fold less sensitive than ours, and the few (Newport Green and FuraZin-1)that are sufficiently selective to be used in the presence of millimolar calcium and magnesium are one million-fold less sensitive than ours. While not the first to demonstrate real time sensing of copper in sea water, we were the first to show that fluorescence anisotropy determinations could exhibit an expanded dynamic range, and the first to image the release of zinc in real time from the brain in response to an electrical stimulus.

CONCLUSIONS On the basis of this work we conclude that fluorescence-based biosensors employing variants of carbonic anhydrase as the transducer molecule are capable of determining free metal ions such as Cu(II) and Zn(II) at picomolar levels and Co(II), Ni(II), and Cd(II) at nanomolar levels in real time, through a length of optical fiber, in media as complex as sea water or cerebrospinal fluid.

SIGNIFICANCE This work shows that biological molecules are not only workable but preferred as sensitive and selective recognition elements in biosensors, and offer the best hope for sensing (e.g., continuous, real-time determination of an analyte) in complex media. While we have transduced the recognition as a change in fluorescence which can be measured through a length of optical fiber, transduction could also be electrochemical, or by other means. It also suggests that biosensors may be useful in a variety of applications currently not served by existing technology, particularly environmental monitoring and clinical sensing.

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