Chemical Communication in Water Using Fluorescent Chemosensors

ANTHONY W. CZARNIK*

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

Received November 29, 1993

Introduction

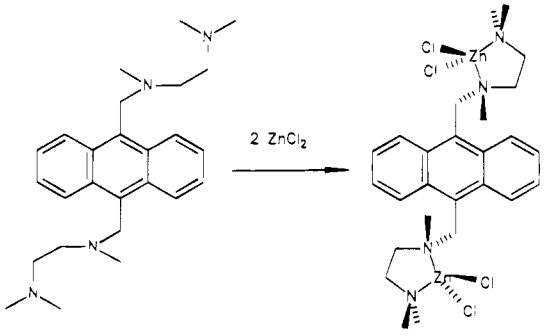
The modern era in fluorescence commenced with the work of George Stokes, who in the mid 19th century first reported that fluorescence emission occurs at a longer wavelength than excitation. This wavelength shift, termed the Stokes shift, provides the primary conceptual basis for the enormous sensitivity of fluorimetric analyses. Stokes also outlined the relationship between the concentration of a fluorophore and observed fluorescence intensity, describing the quenching of fluorescence at high concentration and by the presence of foreign substances. Neither of these effects occurs in absorption spectroscopies, which led Stokes in 1852 to propose that fluorescence be used for the detection of organic substances.[1]

Compounds incorporating a binding site, a fluorophore, and a mechanism for communication between the two are called *fluorescent chemosensors*. Because the selective binding of metal ions from water is considerably easier than that of anions or neutrals, the development of fluorescent chemosensors for metal ions occurred early and boasts a substantial literature. Goppelsröder reported in 1867 that morin forms a strongly fluorescent chelate with Al(III).[2] Fluorimetric methods for the determination of many other metal ions, with vastly differing degrees for ion discrimination, have been described in the anteceding century.[3] These sensing molecules were invariably, until only recently, heterocycles bearing chelating arrays of donor atoms, which we have classified as *intrinsic* chemosensors. The past 25 years have witnessed substantial progress in the *de novo* design of abiotic receptors, in an endeavor progressively termed host–guest chemistry, biomimetic chemistry, molecular recognition, and supramolecular chemistry. An ability to design receptors from scratch complements an ability to utilize (or generate) such receptors from biotic sources and has proven useful in the mimicking of enzyme catalysis and in transport phenomena. The application of supramolecular receptors to fluorescence sensing was first described by Sousa in 1977.[4] When naphthalene is outfitted with a crown ether binding site, the association of alkali earth metals is signaled by changes in the naphthalene fluorescence; because all of the donor atoms remain insulated from the fluorophore π-system, we have classified such compounds as *conjugate* chemosensors. The purpose of this Account is to summarize advances in the design of conjugate chemosensors, particularly as applied to their use in aqueous solution. It is only fair to note that our group's work receives disproportionate air time here; a more comprehensive review of contributions from other laboratories is available.[5]

Metal Ion Sensing

In 1987, we published the syntheses of 9,10-bis[[[2-(dimethylamino)ethyl]methylamino]methyl]anthracene (**1**) and its binuclear $ZnCl_2$ chelate **2**.[6] Our intent had been to demonstrate metal ion catalyzed amide hydrolysis within compound **2**'s acrylamide cycloadduct, which afforded instead one of our group's more noble failures. However, the beautiful fluorescence of most anthracenes regularly provided us with an opportunity, rare in organic chemistry, to enjoy the colors of our products. Thus, each NMR sample containing a new anthracene derivative was subjected to the hand-held UV lamp. It therefore came as something of a surprise to observe that anthracene **1** in DMSO did not exhibit the characteristic blue fluorescence of anthracene itself, and more of a surprise that anthracene **2** did (Scheme 1). In retrospect, the observed fluorescence signaling of a nonfluorescent species ($ZnCl_2$) should have captured our

Scheme 1



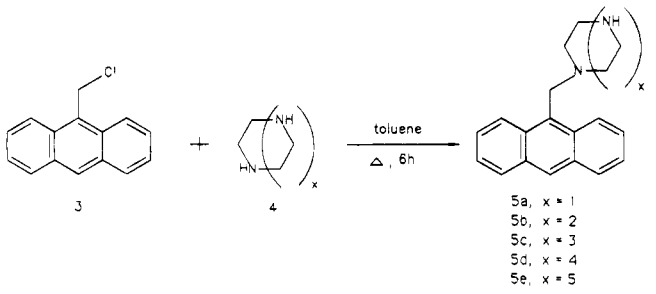
Anthony W. Czarnik was born in Appleton, WI, in 1957 and received his B.S. from the University of Wisconsin in 1977. He received his graduate training at the University of Illinois under the guidance of Prof. Nelson Leonard and from 1981 to 1983 was an NIH Postdoctoral Fellow at Columbia University with Prof. Ronald Breslow. In 1983, he joined The Ohio State University, where the work described in this Account was carried out. He received DuPont and Merck awards for new faculty and was named an American Cyanamid awardee in 1986, an Eli Lilly awardee in 1988, a Fellow of the Alfred P. Sloan Foundation in 1989, and a Teacher-Scholar Fellow of the Camille and Henry Dreyfus Foundation in 1990. In 1993, he joined Parke-Davis Pharmaceutical Research as Director of BioOrganic Chemistry. His current research interests are in molecular diversity as applied to drug discovery, small molecule antagonists of nucleic acid receptors, and fluorescent chemosensors.

* Present address: Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48105.

(1) Stokes, G. G. *Philos. Trans. R. Soc. London, A* **1852**, *142*, 463.
(2) Goppelsröder, F. *J. Prakt. Chem.* **1867**, *101*, 408.
(3) (a) West, T. S. *Complexometry with EDTA and Related Reagents*; BDH: 1969; esp pp 65–73. (b) Seitz, W. R. In *Fiber Optic Chemical Sensors and Biosensors*; Wolfbeis, O. S., Ed.; CRC Press: Boca Raton, 1991; Vol. II, pp 4–6.
(4) Sousa, L. R.; Larson, J. M. *J. Am. Chem. Soc.* **1977**, *99*, 307.

0001-4842/94/0127-0302$04.50/0

Scheme 2



imagination immediately; in fact, it required several years of fermentation before we decided to follow up on this result.

Our findings, reported in 1988,[7] implicated fluorescence quenching via photoinduced electron transfer from nitrogen to anthracene. Chelation-enhanced fluorescence (CHEF) on $ZnCl_2$ addition thus results when metal ion complexation of the amine lone pairs decreases amine oxidizability such that it cannot reduce the anthracene S_1 excited state. Amine quenching of anthracene fluorescence intermolecularly is well-known and predicted from the Weller equation;[8] the efficiency of intramolecular amine quenching of naphthalene's fluorescence, in particular that by benzylic amines, has been described previously by Thomas[9] and by Davidson;[10] we have made the logical extension of these findings to anthracene.[11] Furthermore, H. Bouas-Laurent[12] and A. P. de Silva[13] had previously and independently reported similar CHEF effects using anthrylmethyl monoazacrown ethers by an identical mechanism. The more generic question, how to transduce a binding event into a fluorescence event, has now received numerous other mechanistic solutions whose review has been provided recently by de Silva.[14] Intracellular calcium sensing is a particularly powerful technique requiring fluorescence signal transduction upon calcium complexation to a variety of selective chemosensors.[15] Our group's work has sought more to explore which aqueous binding mechanisms intersect with one useful signal transduction scheme, photoinduced electron transfer, utilizing anthracene.

Neither chemosensor **1** nor the original chemosensors of Bouas-Laurent or de Silva function in aqueous

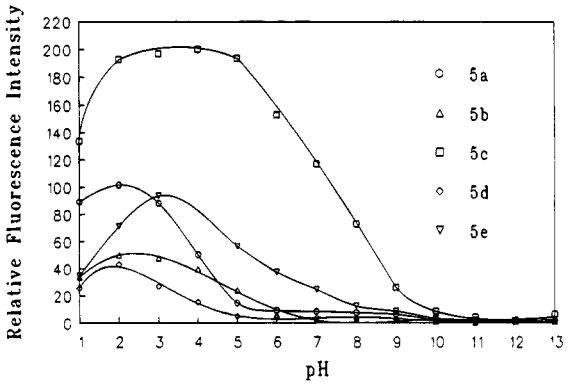


Figure 1. pH–fluorescence profiles for 10 μM solutions of anthryl azamacrocycles **5a** (○), **5b** (△), **5c** (□), **5d** (◇), and **5e** (▽). Excitation was at 335 ± 3 nm; emission was measured at the emission maximum centered near 416 nm. pH was maintained using the following solutions (all 0.1 M): trichloroacetate (pH 1); dichloroacetate (pH 2); chloroacetate (pH 3); acetate (pH 4 and 5); MES (pH 6); HEPES (pH 7 and 8); CHES (pH 9); CAPS (pH 10 and 11); tetrabutylammonium hydroxide (pH 12); NaOH (pH 13).

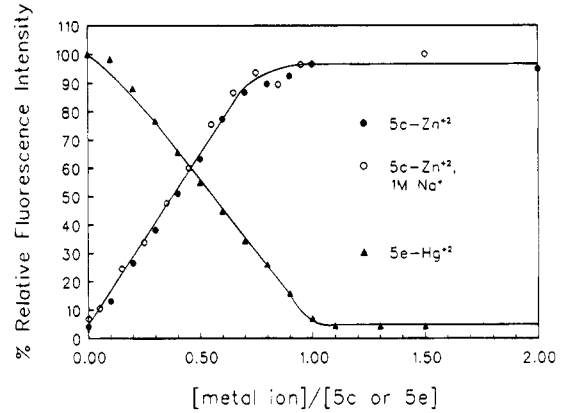


Figure 2. Titration of the fluorescence of anthryl azamacrocycles **5c** and **5e** (10 and 100 μM, respectively) by added metal ions: (●) titration of **5c** in pH 12 buffer with $Zn(ClO_4)_2$; (○) titration of **5c** in pH 12 buffer with $Zn(ClO_4)_2$ and 1 M $NaClO_4$; (▲) titration of **5e** in pH 6 buffer with $Hg(ClO_4)_2$. The CHEQ (chelation-enhanced quenching) of **5e** by Hg(II) ion was accomplished at pH 6 because, while the azamacrocycle is sufficiently deprotonated at this pH to bind Hg(II), it is also sufficiently protonated to display some background fluorescence (Figure 1). Excitation was at 335 ± 3 nm; emission was measured at the emission maximum centered near 416 nm.

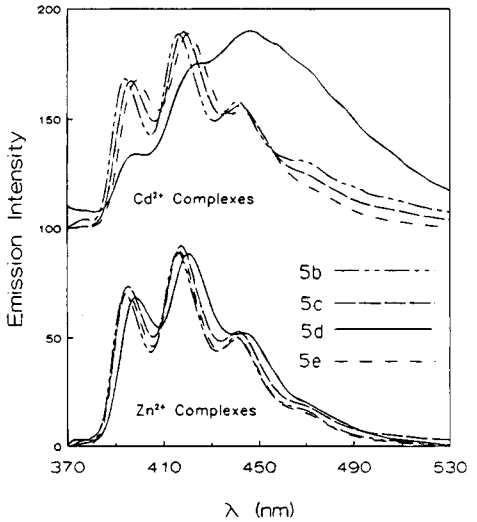


Figure 3. Normalized fluorescence spectra of 10 μM solutions of **5b–e** containing $Cd(ClO_4)_2$ (above) and $Zn(ClO_4)_2$ (below) in 0.1 M pH 10 CAPS buffer. Excitation was at 335 ± 3 nm.

(5) For first-hand accounts of contributions by other principal investigators in this area, including L. R. Sousa, B. Valeur, A. P. de Silva, H. Bouas-Laurent, A. Ueno, T. Bell, R. Y. Tsien, M. Kuhn, D. Masilamani, and G. Krafft, see: *Fluorescent Chemosensors for Ion and Molecule Recognition*; Czarnik, A. W., Ed.; American Chemical Society: Washington, DC, 1993.

(6) Nanjappan, P.; Czarnik, A. W. *J. Am. Chem. Soc.* **1987**, *109*, 1826.
(7) Huston, M.; Haider, K.; Czarnik, A. W. *J. Am. Chem. Soc.* **1988**, *110*, 4460.
(8) Rehm, D.; Weller, A. *Isr. J. Chem.* **1970**, *8*, 259.
(9) Chandross, E. A.; Thomas, H. T. *Chem. Phys. Lett.* **1971**, *9*, 393.
(10) Brimage, D. R. G.; Davidson, R. S. *J. Chem. Soc., Chem. Commun.* **1971**, 1385.
(11) Beeson, J. C.; Huston, M. E.; Pollard, D. A.; Venkatachalam, T. K.; Czarnik, A. W. *J. Fluoresc.* **1993**, *3*, 65.
(12) Konopelski, J. P.; Kotzyba-Hibert, F.; Lehn, J.-M.; Desvergne, J.-P.; Fages, F.; Castellan, A.; Bouas-Laurent, H. *J. Chem. Soc., Chem. Commun.* **1985**, 433.
(13) de Silva, A. P.; de Silva, S. A. *J. Chem. Soc., Chem. Commun.* **1986**, 1709.
(14) Bissell, R. A.; de Silva, A. P.; Gunaratne, H. Q. N.; Lynch, P. L. M.; Maguire, G. E. M.; McCoy, C. P.; Sandanayake, K. R. A. S. *Top. Curr. Chem.* **1993**, *168*, 223.
(15) See especially the reviews by Tsien and Kuhn in ref 5, as well as the following: Tsien, R. Y. *Chem. Eng. News* **1994**, *72* (July 18, 1994), 34.
(16) Akkaya, E. U.; Huston, M. E.; Czarnik, A. W. *J. Am. Chem. Soc.* **1990**, *112*, 3590.
(17) Huston, M. E.; Czarnik, A. W. *J. Am. Chem. Soc.* **1990**, *112*, 7054.

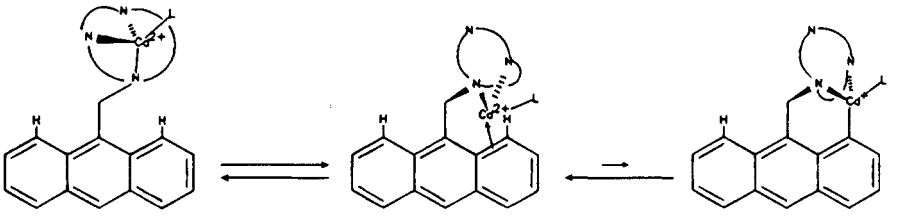
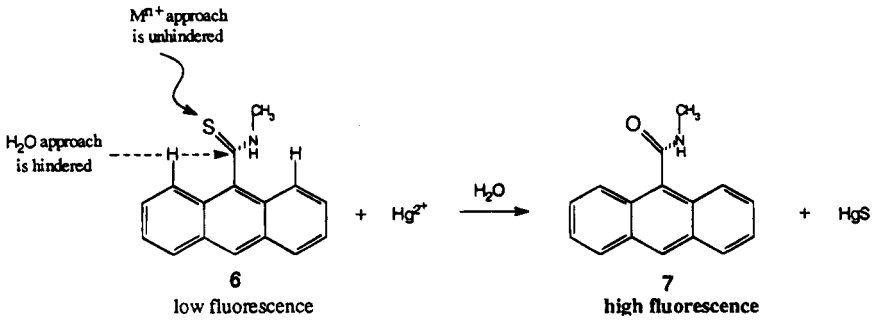


Figure 4. A proposal rationalizing the observed fluorescence pertubation and selective deuteration of the **5d**–Cd(II) complex.

Scheme 3



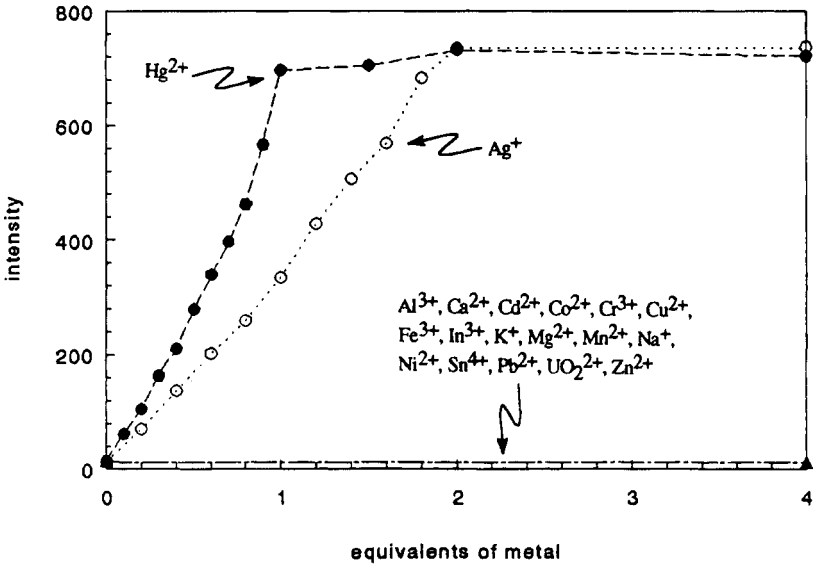


Figure 5. Fluorescence metal titrations of **6**. Solutions are 23 μM **6** in 0.01 M HEPES buffer, pH 7. Measurements shown were made after 70 h, principally to demonstrate the stability of **6** toward nonindicating metals. The Hg(II) reaction is 87% complete and the Ag(I) reaction 73% complete after 10 min under stoichiometric conditions at room temperature.

solution, owing to weak metal ion complexation; nonetheless, water is the solvent in which most useful analyses will be conducted. Because polyazamacrocycles afford tight complexation of selected transition metal ions in water, compounds **5a**–**e** were prepared[16] (Scheme 2). The pH–fluorescence profiles (Figure 1) are consistent with a PET mechanism for cation (in this instance, proton) signaling, all compounds displaying low fluorescence under basic conditions. Zn(II) complexation in water is now possible (Figure 2), without interference by the nonbinding sodium ion even in large excess. Zn(II) and Cd(II) ions afford fluorescence enhancement; these are nonquenching ions, and their chelation affects only amine oxidizability. It was not at all clear what the net effect of chelation by inherently quenching metal ions [e.g., Cu(II), Hg(II)] would be, as influences on fluorescence intensity oppose one another. We observed net intensity decreases with these ions, observable when viewed under nonbasic conditions.

Because the azamacrocycle ligands in compounds **5b**–**e** bind both Zn(II) and Cd(II) strongly, one expects no ion discrimination based on binding affinities in the eight 1:1 possible permutations. Indeed, the lack of sufficient binding discrimination has stubbornly resisted solution by ligand engineering. Selectivity in biotic receptors does not lie in binding discrimination alone, but with combined nonbinding recognition such as use as a substrate, transportability, etc. In the same way, it seems likely that one solution to the problem of ion discrimination will be the overlapping of a secondary selectivity mode over a binding selectivity mode. We observed that the **5d**–Cd(II) combination uniquely displays a new emission with λ_{max} 446 nm (Figure 3). Our best explanation for the observed

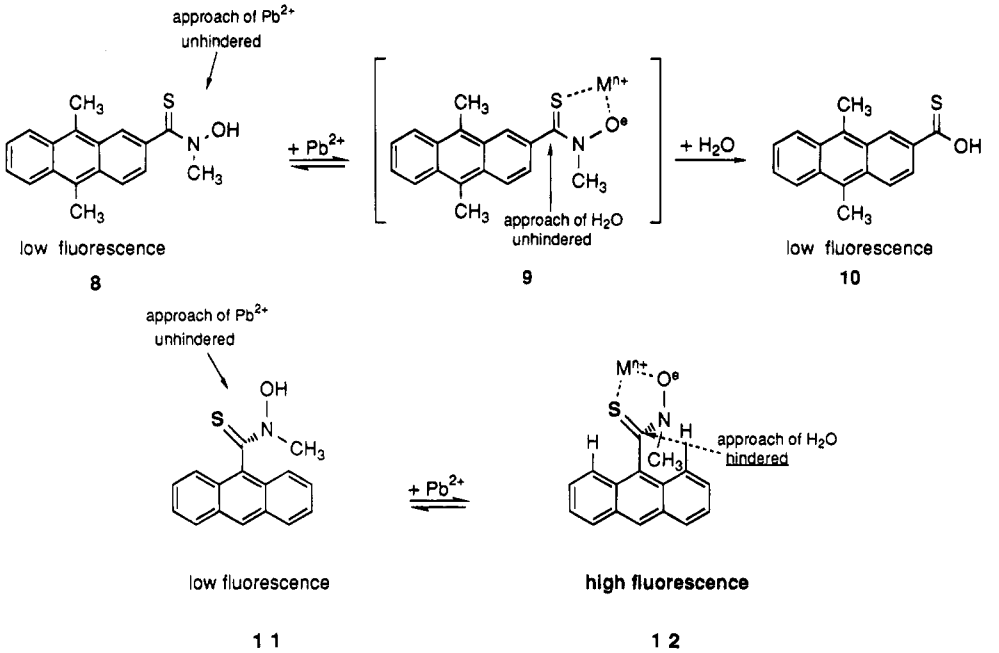
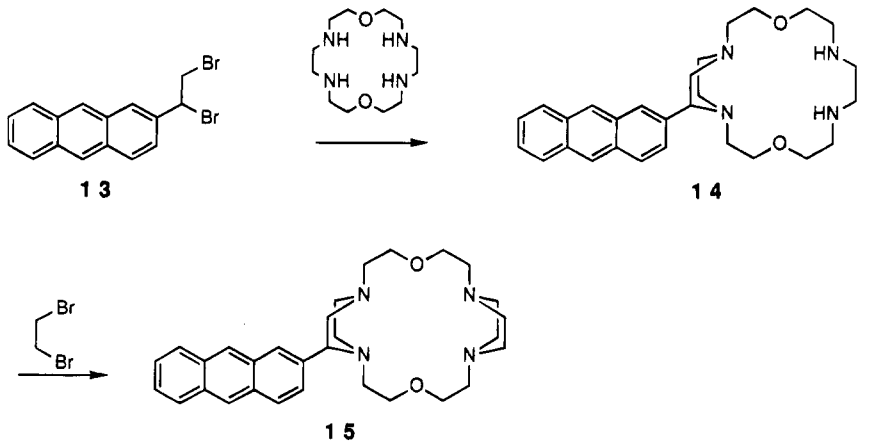


Figure 6. A rationalization for the observed difference in stabilities of compounds **8** and **11** toward Pb^{2+} in water.

Scheme 4



secondary selectivity is ground state Cd(II) π-complexation, as shown in Figure 4.[17]

Because titrations affording increasing signal have sensitivity advantages over those affording decreasing signal, it is useful to evaluate methods in which inherently quenching metals might be indicated via fluorescence enhancement. One such approach is outlined in Scheme 3 and Figure 5, in which Hg(II) and Ag(I) concentrations are indicated dosimetrically and with selectivity based on their selective reactivities with the thioamide.[18] Sensors, however, require real-time, nondestructive associations with analytes.

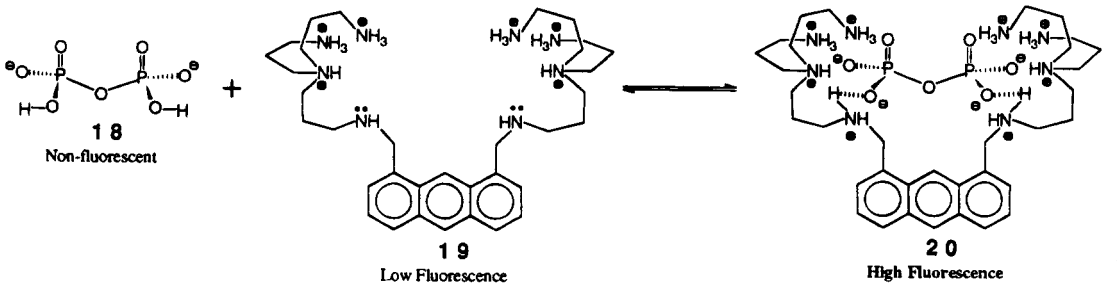
We therefore evaluated two potential CHEF chemosensors for Pb(II) indication, based on different rationales. The well-known affinity of Pb(II) for thiohydroxamic acids led us to synthesize chemosensors **8** and **11** (Figure 6).[19] Because the thiohydroxamate group is itself strongly quenching, we envisioned that coordination to the less strongly quenching Pb(II) might lead to net CHEF. In fact, full complexation of Pb(II) to **11** affords a 13-fold enhancement of fluorescence, in accord with the prediction; of course, the initial fluorescence intensity of **11** is very low. Anthrylhemicryptand **14** and anthrylcryptand **15** (Scheme 4) bear nitrogeneous ligands with selectivity for Pb(II) as reported by Hancock, and we hypothesized that metal ion encapsulation might lessen heavy atom quenching. Instead, methanolic complexations display fluorescence signal decreases rather than increases.[20]

Anion Sensing

While fluorimetric methods for metal ion determination have precedent going back over a century, methods for inorganic nonmetal ion sensing are very few. This state of affairs may be understandable given the substantial utility provided by metal ion chelation, but there are so many other analytes worth measuring

(18) Chae, M.-Y.; Czarnik, A. W. *J. Am. Chem. Soc.* **1992**, *114*, 9704.
(19) (a) Chae, M.-Y.; Czarnik, A. W. *J. Fluoresc.* **1992**, *2*, 225. (b) Chae, M.-Y.; Czarnik, A. W. Submitted.
(20) Chae, M.-Y.; Cherian, X. M.; Czarnik, A. W. *J. Org. Chem.* **1993**, *58*, 5797.
(21) Huston, M. E.; Akkaya, E. U.; Czarnik, A. W. *J. Am. Chem. Soc.* **1989**, *111*, 8735.
(22) Van Arman, S. A.; Czarnik, A. W. *Supramol. Chem.* **1993**, *1*, 99.
(23) Vance, D. H.; Czarnik, A. W. *J. Am. Chem. Soc.* **1994**, *116*, 9397.
(24) For example see: Tombler, E. R.; Deutsch, D. G. *BioTechniques* **1993**, *15*, 1060.
(25) Van Arman, S. A.; Czarnik, A. W. *J. Am. Chem. Soc.* **1990**, *112*, 5376.
(26) Hong, S.-Y.; Czarnik, A. W. *J. Am. Chem. Soc.* **1993**, *115*, 3330.
(27) Lorand, J. P.; Edwards, J. D. *J. Org. Chem.* **1959**, *24*, 769.
(28) Yoon, J.-Y.; Czarnik, A. W. *J. Am. Chem. Soc.* **1992**, *114*, 5874.
(29) Yoon, J.-Y.; Czarnik, A. W. *Bioorg. Med. Chem.* **1993**, *1*, 267.

Scheme 5



in real time that the current state is unacceptable. The few available methods all rely on an inherent quenching property of the ionic analyte (e.g., iodide), but this mechanism is not applicable in most instances. During the course of our work, we noted that the binding of an anion to an incompletely protonated azamacrocycle would change the effective pK_a's experienced by free amine groups, in most cases raising them. If that amine were benzylic, then its protonation would lead to CHEF. It proved fortuitous that benzylic amines are about 1–1.5 pK_a units less basic than structurally analogous alkylamines, because this key group was unlikely to protonate first in a polyamine binder.

We thus synthesized anthryl polyamines **16** and **17** (Figure 7) as potential fluorescent chemosensors for anions.[21] We were thus gratified to observe CHEF upon introduction of many anions, including phosphate, sulfate, and even acetate. Straightforward application to the monitoring of enzymatic ATP hydrolysis was successful, although not at a desirable pH.[22] It proved important in all these studies to carefully control for adventitious transition metal ions, which are present in varying concentrations in all commercial samples of anions; EDTA solved this problem for **16** and **17**, which are predictably weak binders of monoanions. (One solution to the metal ion conundrum is presented in Polymeric Anion Sensing, the following section.)

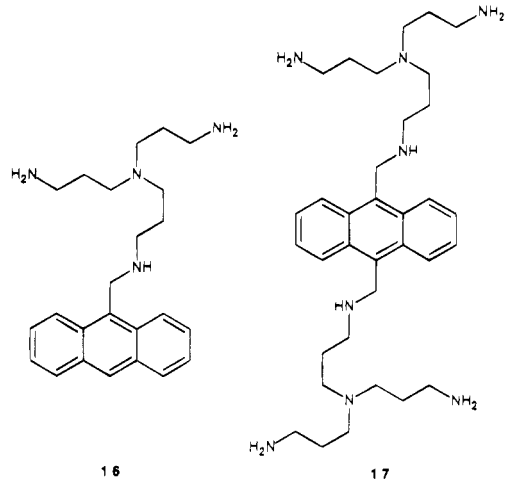


Figure 7. Structures of anthryl polyamines **16** and **17**.

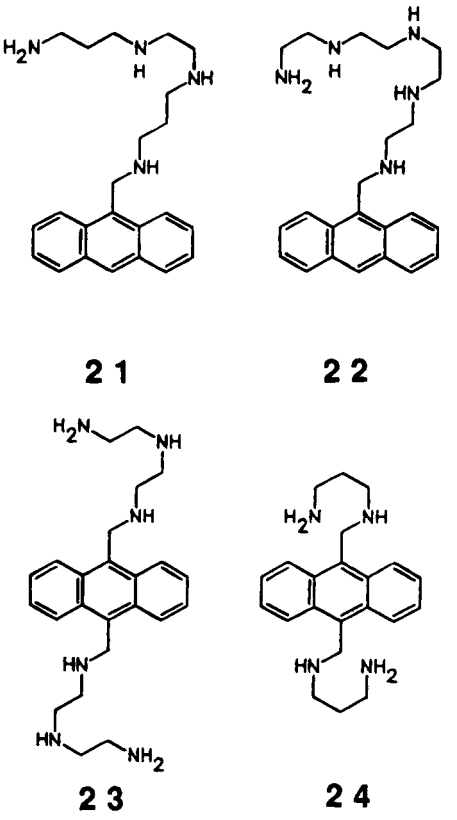


Figure 9. Structures of anthryl polyamines **21**–**24**.

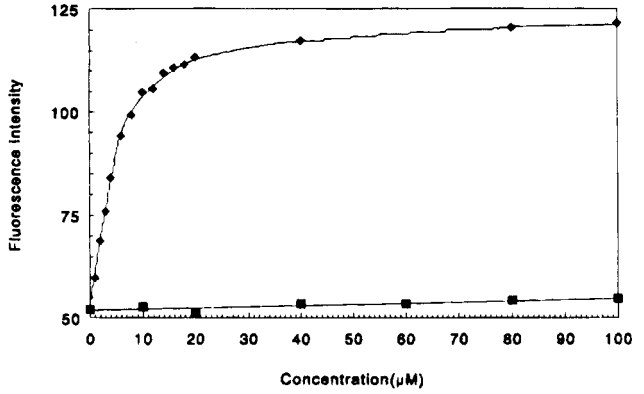


Figure 8. Fluorescence titrations of chemosensor **19** with pyrophosphate (◆) and phosphate (■): 1 mM cyclen, 50 mM HEPES, pH 7.

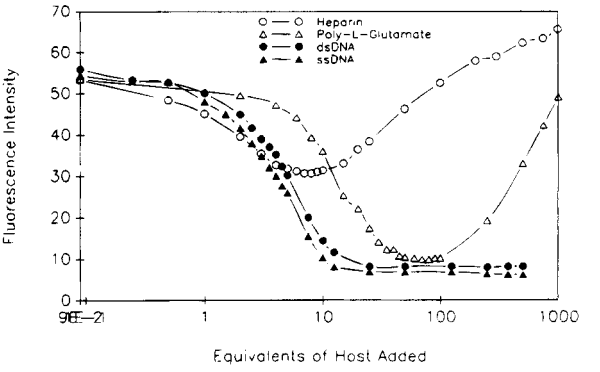


Figure 10. Effects on the fluorescence of anthryl polyamine **23** upon titration by four biological polyanions.

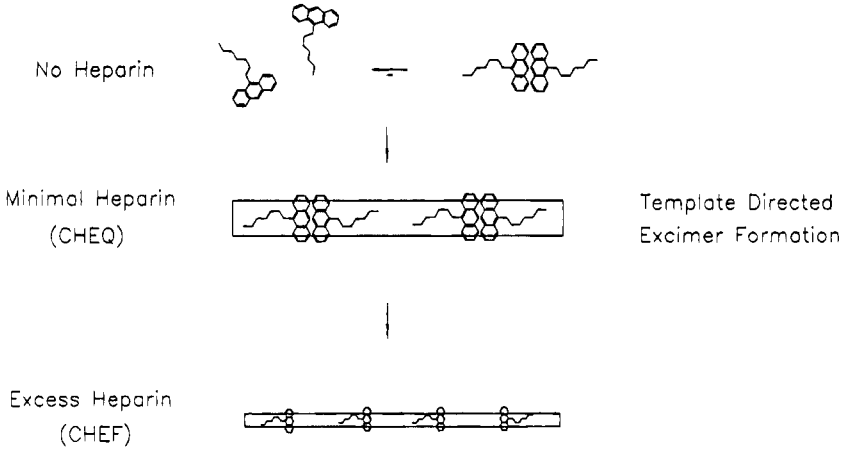
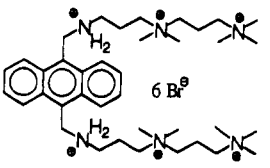


Figure 11. Proposed mechanism for template-directed excimer formation.



25

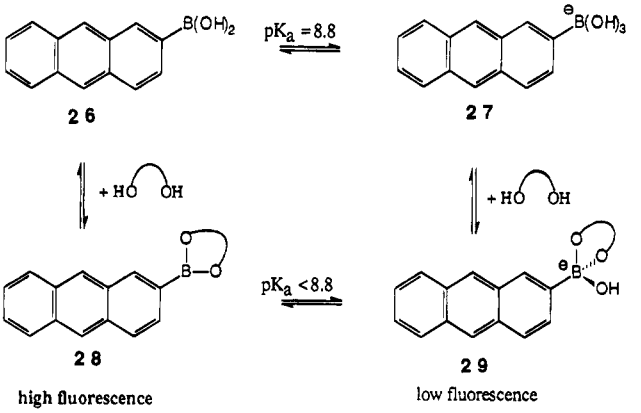
Figure 12. Structure of metal ion insensitive polyanion chemosensor **25**.

Selectivity is ultimately the result of strong binding by species of interest and lesser binding by species of noninterest. Of course, the phrase "complete selectivity" is a misnomer; an observed selectivity will always be determined as useful or not depending on the constraints of a given application. However, the ability to increase selectivity is always valuable. Selective anion sensing is likely to result mainly from selective anion binding, engineered by complementarity of host–guest charge and shape. We thus designed and prepared chemosensor **19**, in which the 4.8 Å distance between polyammonium sites permits cooperative pyrophosphate chelation but not phosphate chelation (Scheme 5).[23] As designed, chemosensor **19** displays a 2200-fold binding selectivity for pyrophosphate (K_d = 2.9 μM) over phosphate (K_d = 6.3 mM) (Figure 8). At [pyrophosphate] = 20 μM, the resulting hydrolysis to [phosphate] = 40 μM is thus expected to result in a fluorescence decrease of nearly 50%. Using this method, we reported a real-time assay for monitoring the enzyme inorganic pyrophosphatase. Unlike assays based on coupled enzyme reactions, the method utilizing chemosensor **19** is nondestructive and does not require a supply of reagent to work. Such methods are not common, but of great utility in the design of remote sensing schemes with fiber optic waveguides.

Polymeric Anion Sensing

The ethidium ion complexes to double-stranded DNA with a concomitant enhancement of its fluorescence; the resulting analytical applications have been many and varied.[24] However, there are other polyanions of interest (e.g., heparin) that do not afford π-stacking interactions, for which fluorescent sensors have consequently not evolved. Because of their high charge density, anthryl polyamines such as those already described are anticipated to associate with polyanions tightly; because the analyte is anionic, the anticipated result of complexation is CHEF.

Scheme 6



Thus, we prepared anthryl polyamines **21**–**24** (Figure 9) based on biogenic amines with known electrostatic affinity for DNA and for heparin.[25] Fluorescence changes did occur on binding to double-stranded DNA; more interesting were effects with single-stranded DNA, heparin, and polyglutamate in which intercalation does not play a role in binding. Unexpectedly, the fluorescence titrations of chemosensor **23** were observed to be biphasic as a function of heparin and polyglutamate concentrations (Figure 10). Spectroscopic investigation led us to propose template-direct excimer formation (Figure 11) on the surface of the

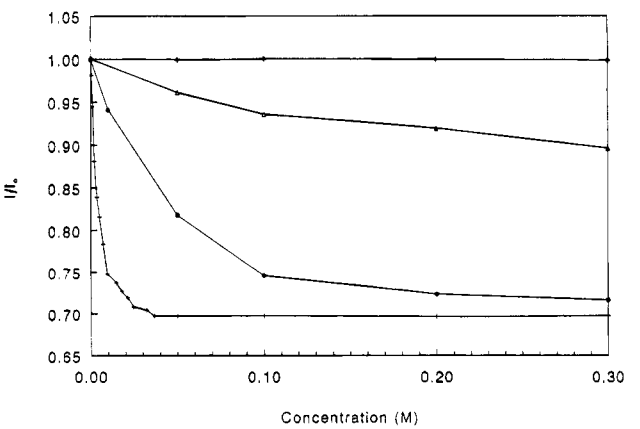
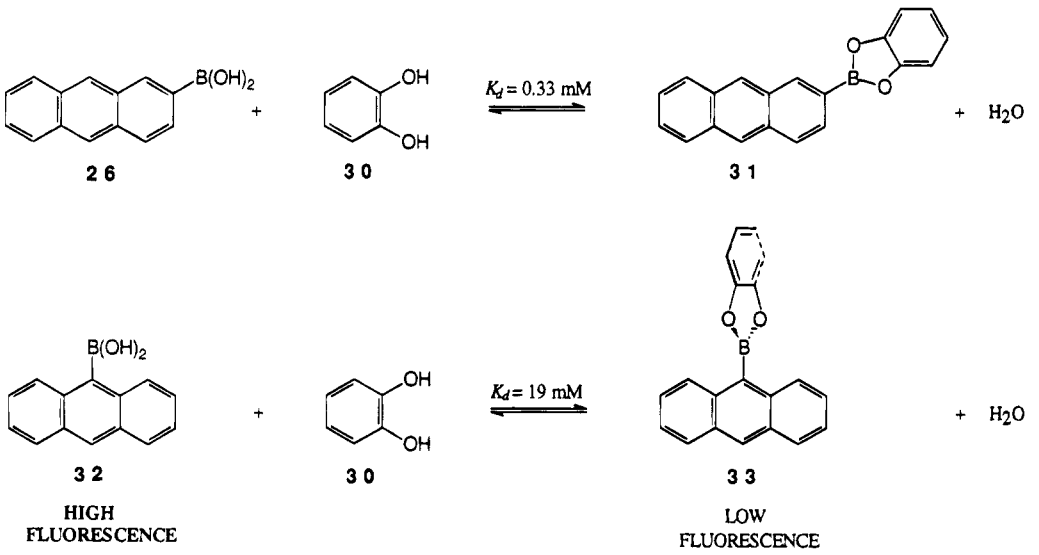


Figure 13. Fluorescence titrations of anthrylboronic acid **26** (0.75 μM) at pH 7.4 (20 mM phosphate buffer) as a function of polyol concentration: (+) fructose; (●) 1,1,1-tris(hydroxymethyl)-ethane; (△) glucose; (+) ethylene glycol (no effect on fluorescence). All solutions contain 1% (v/v) DMSO.

Scheme 7



polyanion as the mechanism for fluorescence decrease at low polyanion/chemosensor ratios. Indeed, the excimer emission spectrum is visible and its maximum intensity coincides with the titrated fluorescence minimum in Figure 10.

Transition metal ion impurities interfere with the utility of compounds **21**–**24**; in response, we prepared chemosensor **25** (Figure 12) in which anion binding remains but metal ion chelation is not possible.[26] At 0.75 μM and pH 7, the fluorescence emission spectrum of **25** is unaffected by 1000 equiv of Cd(II), Co(II), Mn(II), Zn(II), Cu(II), Ni(II), Mg(II), or Ca(II). Use of this metal ion insensitive polyanion chemosensor permitted the real-time assay of DNase I from calf thymus, and **25** was also used for a survey of the metal ion activating effects on Bal 31 nuclease from *Alteromonas espejiana*.

Neutral Analyte Sensing

Not all analytes of interest are charged. There have been very few reported efforts toward host–guest chemistry of sugars in water, in part because the absence of an electrostatic "handle" limits the options available for designing complementary binding sites competitive with bulk water. Our group does not discriminate between covalent and noncovalent methods for molecule recognition studies, which proves essential in the present case. One of the very few methods available to complex sugars from water is the reversible reaction with boronic acids, now known for over 35 years as a result of the work of Lorand and Edwards.[27] Even so, not every mechanism for binding provides overlap with mechanisms for fluorescence signaling. However, we became convinced that the change in pK_a experienced by phenylboronic acid upon diol complexation might result in fluorescence modulation in some fluorescent boronic acids. This is observed, as shown in Scheme 6.[28] Titrations of compound **26** with weakly and strongly binding diols are shown in Figure 13. The fructose K_d of 3.7 mM is a good start for the evolution of better carbohydrate sensors.

The catechol group complexes to phenylboronic acid in water, with substantially greater affinity than that of carbohydrates. As a potential analyte, catechol carries the additional advantage that it is rather easily oxidized and, like amines, might be capable of exhibiting PET quenching of anthracene. This proves to be the case. 2- and 9-anthrylboronic acids (Scheme 7) reversibly complex catechol in water (K_d = 0.33 and 19 mM, respectively), with resulting 20-fold decreases in fluorescence intensities.[29] Predictably, the biologically important catecholamine dopamine displays similar behavior, providing a starting point for the construction of real-time chemosensors for this neurotransmitter.

Several conclusions and predictions can be drawn from the experience in our labs. First and foremost, the PET mechanism for fluorescent chemosensor construction provides a highly versatile tool for the real-time signaling of analytes in water, many of which would not readily prove amenable to chemosensors based on UV or other spectroscopic means. In our opinion, this tool provides an important utility for host–guest chemistry, and thus a rationale for continued investigation into more fundamental receptor design strategies. However, the argument for utility places a burden of comparison upon any new technique. The binding selectivity that exists in biotic receptors (i.e., proteins, nucleic acids, and polysaccharides) has made their incorporation into biosensor devices logical and productive. This must not, however, serve to stifle the continued investigation of wholly abiotic molecules with useful selectivity indices. Molecular devices displaying enhanced stability, decreased cost, and facile manipulation of receptor structure are certain to result.

This work summarizes the creative contributions of Michael E. Huston, Karl W. Haider, Engin U. Akkaya, Scott A. Van Arman, Susan Stahly, Xavier M. Cherian, Mi-Young Chae, Juyoung Yoon, Mark Mortellaro, Sungyeap Hong, John C. Beeson, Patrick Kan, Douglas A. Pollard, T. K. Venkatachalam, and David H. Vance, with whom I have been honored to work. Our research would not have been possible without the support of the National Science Foundation, the Office of Naval Research, The Ohio State University, the Camille and Henry Dreyfus Foundation, and the A. P. Sloan Foundation.