SENSORS BASED ON IMMOBILIZED FLUOROGENIC REAGENTS

LINDA ANNE SAARI
University of New Hampshire, Durham

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SENSORS BASED ON
IMMOBILIZED FLUOROGENIC REAGENTS

BY

Linda A. Saari
B.S., University of Massachusetts, Amherst, 1974

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Chemistry

May, 1983
This dissertation has been examined and approved.

M. Rudolf Seitz
Dissertation director, W. Rudolf Seitz
Associate Professor of Chemistry

Christopher F. Bauer
Assistant Professor of Chemistry

James D. Morrison
Professor of Chemistry

Paul J. Ossenbruggen
Professor of Civil Engineering

Frank L. Pilar
Professor of Chemistry

4/4/83
Date
to Larry,

to my mother, Marian Laskey,

and

in memory of my father, Warren M. Laskey
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ABSTRACT

SENSORS BASED ON IMMOBILIZED FLUOROGENIC REAGENTS

by

Linda A. Saari

University of New Hampshire, May 1983

Sensors based on the fluorescence of immobilized fluorogenic reagents are prepared and their characteristics are defined. The fluorogenic reagent is chemically immobilized on a solid support and attached to the common end of a bifurcated fiber optic bundle. Fluorescence is excited through one arm of the fiber optic and observed through the other arm.

The fluorogenic reagents fluoresceinamine, morin, calcein, and quinalizarin are immobilized on powdered cellulose via cyanuric chloride. Reagents immobilized on cellulose via cyanuric chloride are stable up to pH 8. Above pH 8 the reagent is hydrolyzed from the surface. Fluoresceinamine is also immobilized on controlled pore glass.

Consistent with previous studies of the pH dependence of fluorescein, the fluorescence from the immobilized fluoresceinamine sensor increases with pH. Immobilized fluoresceinamine serves as a viable pH sensor, but fluoresceinamine immobilized on cellulose can not be used above pH 8 because of stability limitations.
Immobilized morin serves as a sensor for Al(III) and Be(II). When immobilized morin is placed in a solution containing Al(III) or Be(II), fluorescence is observed from the morin-metal complex. Response is linear from $10^{-6}$ to $10^{-4}$ M Al(III) or Be(II) at pH 4.8 and 5.2, respectively. The detection limit, which is established by variations in background signal, is $1 \times 10^{-6}$ M Al(III) or Be(II). The response time is 1-2 minutes. Conditional binding constants for the immobilized morin-metal complexes are on the order of $10^3$ to $10^4$ for the pH range 3 to 5. Under conditions of this study, the sensor extracts about 1% of the Al(III) or Be(II) in the sample when operating in the range of linear response and, thus, serves as a true equilibrium sensor which does not perturb the system. Fe(III) and Cu(II) interfere by quenching the fluorescence of the immobilized morin-metal complex.

The fluorescence from immobilized calcein is quenched by Cu(II), Co(II), and Ni(II) in the pH range 5 to 7. Strong calcein-metal complexes are formed as evidenced by conditional binding constants for immobilized calcein on the order of $10^9$ to $10^{12}$ (Cu(II), pH 5 and 7, respectively). Immobilized calcein extracts a high percentage of metal and does not function as a true sensor. Because immobilized calcein is effective in extracting metal, it can be used as a chelating resin or as an indicator for chelatometric titrations.
CHAPTER I

INTRODUCTION

The ideal device for many analytical measurements is a "sensor" with the following characteristics. It should be easily immersed into the system of interest, be selective for the analyte, respond continuously to the analyte, be easily calibrated, and, very importantly, not perturb the system. Because the immersion of a sensor does not perturb the system, the concentrations of ions participating in other equilibria can be determined. The use of sensors for continuous analysis is another important advantage since sensors give chemical information as a function of time.

Potentiometric electrodes have most of the above characteristics. In potentiometry the basis for the measurement is the development of a potential which is related to the concentration of the analyte. Potentiometric electrodes are widely used for analysis of monovalent ions such as H\(^+\), Na\(^+\), K\(^+\), F\(^-\), Cl\(^-\), and Br\(^-\); and divalent ions such as Ca\(^{2+}\), Cu\(^{2+}\), and Cd\(^{2+}\) (1,2). However, potentiometric electrodes have limitations such as poor selectivity in many cases and susceptibility to electrical interference. They are also subject to fouling and are not available for many ions. Therefore, it would be desirable to develop other types of sensors which respond to different analytes or avoid some of the difficulties associated with electrodes. Recent
research has investigated the possibility of developing optical sensors in which the basis for the measurement is a change in the optical properties of a sensing element on the end of a fiber optic. These changes can involve absorbance, fluorescence, reflectance, chemiluminescence, or phosphorescence.

To date there has been very little published dealing with optical sensors. A pH sensor based on dye absorption and fiber optics has been reported by Peterson (3,4). This sensor is based on phenol red (phenolsulfonphthalein). In the physiological pH range the dye behaves as a weak acid of pK 7.9 and exists in two tautomeric forms, each having a slightly different absorption spectrum (3). As the pH of a solution containing indicator is varied, the relative size of each tautomer's absorption peak varies in proportion to the changing relative concentration of the acid and base forms of the dye (3). Therefore, the absorbance of the dye at one of the absorption maxima serves as an indicator of pH. Phenol red is bound to microspheres of polyacrylamide and packed along with smaller microspheres of polystyrene (to promote light scattering) into an envelope of dialysis tubing at the end of a pair of plastic optical fibers as shown in Figure 1. This 0.4 mm diameter, flexible sensor measures pH over the range 7.0 to 7.4 to the nearest 0.01 pH unit. The sensor does not require an electrical connection which is an advantage for pH measurements in vivo (3).

In addition to the sensor reported by Peterson, sensors
Figure 1: Diagram of Peterson's fiber optic pH probe (4).
for oxygen (5) and chlorine dioxide (6) based on chemiluminescence (CL) have been reported. A pH sensor based on fluorescence has also been reported, based on research described in this thesis (7). CL sensors are based on two phase CL systems in which a reagent required for a CL reaction is immobilized. When the sample contacts the reagent phase the analyte diffuses across the interface under a concentration gradient (8). Once in the reagent phase, the analyte reacts to produce CL, and the CL intensity is measured (8). The oxygen measuring device (5) consists of a reagent chamber containing tetrakis 1,4-dimethylaminoethylene separated from the analyte by a Teflon membrane. When the membrane contacts an oxygen-containing sample, CL is observed as oxygen diffuses through the membrane to react with the aminoethylene. A steady state is achieved when the rate at which oxygen diffuses through the membrane is equal to the rate at which oxygen reacts. The oxygen sensor offers the advantages of selectivity and sensitivity, but suffers the disadvantages of reagent degradation and the need to exclude ambient light (5). The chlorine dioxide sensor (6) consists of a CL flow cell with a silicone-poly-carbonate copolymer membrane separating the luminol-peroxide reagent from chlorine dioxide. CL intensity varies with analyte concentration and reagent flow. Dissolved chlorine dioxide can be measured at sub ppm levels (6).

Although the number of publications on optical sensors is still quite small, there is considerable interest in
their development. Ongoing research is described in recent news articles in *Science* (9) and *Chemical and Engineering News* (10), and in the FOCUS section of *Analytical Chemistry* (11). These articles use the term "optrode," which comes from optical electrode to describe optical sensors. The major goal in the research is to develop optrodes for remote sensing. Fiber optic based sensors are attractive for remote sensing because no electrical connection is needed, offering safety and freedom from electrical interference. In addition, fiber optics have high data carrying capacity, are resistant to corrosive atmospheres, and are relatively low in cost (10).

The news articles describe research by Tomas Hirschfeld and coworkers at Lawrence Livermore Laboratory to develop apparatus for measuring the response of various optrodes. A diagram of the apparatus is shown in Figure 2. Three ways of using the fiber optic as an optrode are described and shown in Figure 3. One can use a cuvette on the fiber to measure the fluorescence of analyte (cuvette optrode), focus the signal from the fiber using a sapphire ball (sapphire ball optrode), or use a membrane to separate the analysis region from sample (membrane optrode) (10). Work is reported to be in progress on optrodes to measure temperature, pressure, pH, dissolved oxygen, chloride, iodide, iron, uranyl, plutonium, and sulfate ions (10,11). Operating characteristics for the optrodes are not mentioned, but many of these are based on an indirect reaction forming a
Figure 2: Diagram of Hirschfeld's apparatus for Remote Fiber Fluorimetry (RFF) (10)
Figure 3: Diagram of Hirschfeld's "optrodes" (10)
substance whose fluorescence can be measured. For example, an iodine optrode based on the quenching of rubrene fluorescence is mentioned (11).

The sensors that are the focus of this thesis are based on the fluorescence of immobilized fluorogenic reagents. A reagent whose fluorescence characteristics change with pH and/or metal ion activity is chemically immobilized on an insoluble solid substrate. The immobilized reagent is attached to the end of a bifurcated fiber optic bundle. The fluorescence of the immobilized reagent is excited through one arm of the fiber optic and measured through the other arm. This is illustrated in Figure 4. The fluorescence of the reagent can be measured as a function of pH or metal ion activity. The fluorescence intensity can then be used as a measure of pH or metal ion activity.

The goals of the research were to demonstrate the feasibility and capability of sensors based on immobilized fluorogenic reagents by the characterization of several specific ion sensors. The instrumentation developed to measure the fluorescence of the immobilized reagents is described in Chapter II. The characteristics of a pH sensor based on immobilized fluorescein amine are described in Chapter III. Sensors for Al(III) and Be(II) based on immobilized morin are described in Chapter IV. Properties of immobilized calcein as a possible sensor for Cu(II), Co(II), and Ni(II) are outlined in Chapter V, and work on other immobilized reagents not suitable for sensors is mentioned in Chapter VI.
Figure 4: Diagram of Bifurcated fiber optic with immobilized fluorogenic reagent (F) on the common end. Excitation (EX) of the fluorescence of the immobilized reagent is accomplished through one arm and emission (EM) is observed through the other arm as metal ion concentration (M) is varied.
CHAPTER II

FLUORESCENCE SENSORS:
INSTRUMENTATION AND TECHNIQUES

Introduction

The instrumentation for measuring the fluorescence of immobilized fluorogenic reagents was built around the basic idea depicted in Figure 4. The instrumentation developed was basically a fiber optic fluorometer consisting of a source, a bifurcated fiber optic bundle (which consists of two bundles of optical fibers that meet in a common end (12)), interference filters, and a photomultiplier tube. Because the characterization of the immobilized reagent requires measurement of fluorescence spectra, a method for measuring fluorescence intensity as a function of excitation wavelength was needed. In order to obtain excitation spectra, an SLM 8000 spectrofluorometer was modified so that the source and detector could be attached to the fiber optic arms and the scanning capability of the manual excitation monochromator could be utilized.

In this experimental chapter the fiber optic fluorometer and the SLM spectrofluorometer and its attachment are described. Procedures for measuring the characteristic response of the immobilized reagents on the optic surface and in solution are presented. Other instrumentation used in the project is also described.
Instrumental

Fiber Optic Fluorometer

The fiber optic fluorometer is diagrammed in Figure 5. It consists of a tungsten halogen lamp (250 W, 5000 lumens, Edmund Scientific), a source housing with cooling fan and the capacity to hold filters, a bifurcated fiber optic bundle threaded to fit the other components (4.5 mm bundle diameter, glass fibers, Fiber Optic Technology), a photomultiplier tube (RCA 1P21 operated at 700 V), a photomultiplier tube housing with a variable slit width and the capacity to hold filters, and a digital photometer/power supply (SPEX DPC-2). The signal from the photometer is recorded on a Heath SR-255B strip chart recorder. The sample is contained in a 15 mL beaker covered by a light-tight aluminum casing with an injection port. A shutter is located between the source and fiber optic to prevent possible decomposition of immobilized reagent when the source is on, but the system is not being used. Another shutter is located in front of the detector and excludes light when the common end of the fiber optic is exposed to ambient light. Filters used to select the excitation and emission wavelengths were dielectric interference filters (2" x 2" filters from Oriel, 1" x \( \frac{1}{2} \)" filters from Edmund Scientific) with 7-10 nm bandwidth at half maximum transmittance.

The SLM Spectrofluorometer and Attachments

The SLM spectrofluorometer which is diagrammed in
Figure 5: Diagram of Fiber Optic Fluorometer
Figure 6 is a modular instrument which contains an LH-450 xenon arc lamp, and OP-450 optical module, an MC 300 concave holographic manual monochromator, and a photomultiplier tube with a variable voltage supply for each of two channels, A and B. Three polarizers are contained in the instrument. A Glan-Thompson calcite prism is contained between the source and the sample compartment, and two Polaroid HNP'B polarizers are in front of the detectors. The sample compartment for solution measurements contains a cuvette holder with magnetic stirring and temperature control. The SLM electronics offer a background subtraction feature.

Attachments to the SLM spectrofluorometer for measurements on the optic surface are diagrammed in Figure 7. The cuvette holder and stirrer portion of the sample chamber are removed and the excitation and emission arms of the bifurcated fiber optic are attached to the source and detector lens housings in the sample chamber by means of light-tight aluminum fittings and O rings.

Other Instrumentation

An Orion Digital Ionalyzer/501 was used to measure pH. Fluorescence excitation and emission spectra of solutions were measured on a Perkin Elmer 204 spectrofluorometer. A Bausch and Lomb spectrometer was used to measure UV-VIS absorption. Infared spectra were measured on a Perkin Elmer 283B Infared spectrometer. Elemental analysis (C,H,N) was done on a Perkin Elmer 240B Elemental Analyzer.
Figure 6: Simplified diagram of the SLM spectrofluorometer and the interior of the OP-450 (from user's manual). FH = filter holder; BSC = beam splitter control; PW = polarizer housing; SC = shutter control; PMT = photomultiplier tube
Figure 7: Modification of the SLM sample chamber for use with the bifurcated fiber optic. The sample holder is removed from the compartment before the fiber optic is attached.
Techniques

Fluorescence Measurements on the Optic Surface

Fluorescence measurements of the immobilized fluorogenic reagents were made by attaching the solid support to the end of the fiber optic and immersing the optic into solution. A thin layer of the support was spread on a piece of cellophane tape (Scotch Brand Transparent Tape) by placing the tape over a vial containing the support. The vial was shaken until a uniform layer formed. The tape was held in place on the end of the optic by a piece of Tygon tubing. This is illustrated in Figure 8. A reproducible thin layer of support (about 1 mg) is obtainable. The tape is not completely satisfactory because it becomes cloudy when wet resulting in reduced transmittance and significant scattering.

Because of the limitations of the tape, other adhesives were evaluated. None of these was as satisfactory as tape. Two of the better adhesives tried were collodion (Mallinckrodt, USP) and Duco adhesive (Dupont). A small amount of the adhesive was spread on the end of the optic and was allowed to partially dry and become sticky. The powder was then applied as a thin layer. Both these adhesives remained clear with use, but neither was found to be satisfactory. There was difficulty in forming a thin, uniform layer. The collodion also loses its adhesive properties with time. The Duco adhesive can withstand continued use and is easily removed with acetone.
Figure 8: Method of attachment of immobilized reagent to the common end of the fiber optic
The response of the immobilized reagent as a function of pH or metal ion concentration was determined by monitoring the fluorescence intensity using the fiber optic fluorometer or, in some cases, the SLM spectrofluorometer. Micro-liter amounts of solutions of known concentration of the species of interest were added to 15 mL of the sample through the injection port using a syringe (50 or 100 μL, Hamilton). The sample was an acid for the pH sensor and a buffer for the metal ion sensors. The responses of the immobilized reagents to changes in metal ion concentration were also determined by immersing the sensor in buffered solutions of varying metal ion concentration. The solution was magnetically stirred as measurements were taken.

**Fluorescence Excitation Spectra of Immobilized Reagents**

The SLM spectrofluorometer was used to measure all excitation spectra of the immobilized reagents on the optic surface. In order to measure the spectra, the reagents were attached to the end of the fiber optic as described in the last section. In order to correct for the variations in source intensity with wavelength, measurements were taken in the ratio mode as shown in Figure 9. The fluorescence signal which was detected through Channel A was divided by the source intensity which was monitored through Channel B. A neutral density filter was positioned between the source and the detector in Channel B. The output was read as the ratio A/B. Output was corrected for dark current by the instrument electronics. The monochromator slit width was
Figure 9: Arrangement of the SLM for use in the ratio mode.
set at 8 nm for maximum throughput and the excitation and emission polarizers were set at 90°. The excitation wavelength was varied manually and recorded every 5 or 10 nm.

**Solution Fluorescence Spectra**

To determine the wavelengths for excitation and emission of the immobilized reagents, fluorescence spectra were measured for the nonimmobilized reagent in solution using the Perkin Elmer 204 spectrofluorometer.

The response of the nonimmobilized reagent in solution to pH or metal ion concentration changes was evaluated with the fiber optic fluorometer by immersing the end of the fiber optic into a solution of the reagent. Microliter amounts of species of interest were then added through the injection port. Solution measurements were also made with the SLM spectrofluorometer. Microliter amounts of the species of interest were added to a solution of the dissolved reagent. A cuvette was used and the solution was stirred magnetically.

Solution excitation spectra were measured for comparison with the spectra of the immobilized reagents using the SLM spectrofluorometer and a cuvette. Spectra were measured in the same manner as for the immobilized reagent.

**Preparation of Solutions**

All chemicals used to prepare solutions were reagent grade or better. Solutions were prepared with deionized water which was again deionized by passing through a mixed bed ion exchange resin (Ultrapure, Fisher), and then distil-
led in an all-glass still (Corning, Megapure).

Buffer solutions were made by titrating volumes of aqueous solutions of the acid or base with solid KOH or concentrated HCl until the desired pH was obtained as measured using a glass pH electrode. The solutions were then diluted to the volume desired and the pH was again measured. The pH meter was calibrated with two buffers covering the pH range of interest.
CHAPTER III

pH SENSOR BASED ON IMMobilIZED FLUORESCEINAMINE

Introduction

The first system that was investigated in detail was a pH sensor based on the fluorescence of immobilized fluoresceinamine. This system was chosen because it was easy to work with. Amino compounds are readily immobilized on solid supports and fluorescein is a fluorophor with excellent spectroscopic properties. It has a high absorptivity close to 490 nm which permits efficient excitation using a variety of sources (13). Fluorescein is an efficient fluorophor with a quantum yield close to unity. The green emission of fluorescein, which peaks near 520 nm is in the useful range of common photomultiplier tubes. In addition, since acid-base reactions are fast, no complications were expected due to slow kinetics. Therefore, the pH sensor provided a simple system both to test out the fiber optic fluorometer and to demonstrate the viability of sensors based on immobilized reagents.

The structure of fluorescein (Isomer I) is shown in Figure 10. It can be immobilized on controlled pore glass by the reaction shown in Figure 11 and can also be immobilized on powdered cellulose via cyanuric chloride as shown in Figure 12. The acid-base behavior of immobiliz-
Figure 10: Structure of fluoresceinamine and abbreviation used in the immobilization reactions.
Figure 11: Reaction for immobilization of fluoresceinamine on Glycophase-G controlled pore glass.

\[
\begin{align*}
\text{-Si(CH}_2\text{)}_3\text{OCCCOH} &\quad \rightarrow \quad \text{RCOHNF} \\
\text{RCOHN} &\quad \rightarrow \quad \text{RCOHNF}
\end{align*}
\]

R-C-OH represents controlled pore glass.
Figure 12: Reaction for immobilization of fluoresceinamine on powdered cellulose. \( R-C-OH \) represents cellulose.
ed fluoresceinamine should be similar to that of fluorescein because these immobilization procedures tie up the amino group.

The fluorescence characteristics of fluorescein are extremely dependent on pH (14,15,16). Fluorescein can exist in various forms depending on pH (See Figure 13). These have different fluorescence excitation and emission maxima along with different fluorescence efficiencies. The temperature coefficient of fluorescein fluorescence is low, so temperature control during fluorescence measurements should not be needed (13).

Experimental

Apparatus

The apparatus for measuring the fluorescence of the immobilized fluoresceinamine is described in Chapter 2. The interference filters used were 1" x \( \frac{1}{2} \)". The excitation filter has peak transmittance at 480 nm and a bandwidth of 7.1 nm at half maximum transmittance. The emission filter has peak transmittance at 520 nm and a bandwidth of 8.2 nm. The SLM spectrofluorometer was not used in this study of immobilized fluoresceinamine.

Reagents

Fluoresceinamine (Isomer I) and cyanuric chloride were purchased from Aldrich. Glycophase G Controlled pore glass was purchased from Pierce. Powdered cellulose (microcrystalline for TLC, through 60 mesh sieve, Baker) was used
Figure 13: Ionic and Molecular forms of fluorescein (16).

- **A**: Ionic form
- **B**: Molecular form
- **C**: Molecular form
- **D**: Molecular form
- **E**: Molecular form
as the cellulose support.

**Immobilization Procedure**

The fluoresceinamine was immobilized on controlled pore glass by a modification of a previous procedure (17). Potassium periodate was used in place of sodium periodate to oxidize the glass support. The oxidized glass was soaked in a solution saturated with fluoresceinamine in borate buffer at pH 8.5 for 10 days at room temperature before washing. The sodium borohydride reduction step was omitted (17). The derivatized glass was air dried and stored at room temperature.

Other batches of fluoresceinamine immobilized on glass were prepared and were more intensely colored signifying a higher coverage. The more intensely colored products were less fluorescent, however, and not adequate for use. This loss of fluorescence with higher coverage was thought to be a concentration quenching effect (13). To test whether this was a concentration quenching effect, some of the glass was soaked in 1 M KOH to remove some of the fluoresceinamine. After the soaking, the glass was washed with water. The glass was then fluorescent when observed under UV light, using a hand-held source (Blak-ray, UV Products).

The fluoresceinamine was immobilized on cellulose by a modification of a previous procedure (18). Preliminary experiments were performed by using this procedure on strips of filter paper (Whatman #1). Because of the thickness, the fluoresceinamine immobilized on filter paper responded very
slowly to changes in pH. Therefore, powdered cellulose was used in later experiments. The powdered cellulose was soaked in a solution of 1 M KOH for 15 minutes. After washing away the excess base, the cellulose was immersed in a solution of cyanuric chloride in acetone (0.5 g/20 mL) and 20 mL of water was added immediately. The cellulose was then washed with 100 mL each of water and acetone and soaked in a solution of fluoresceinamine in acetone (0.1 g/20 mL) for 1 hour. The product was washed first with acetone and then several times with water until no fluoresceinamine fluorescence was visible when the washings were exposed to UV light.

**Determination of Amount of Fluoresceinamine Bound**

The amount of fluoresceinamine bound per gram of solid was found by titrating the neutral form of the immobilized fluoresceinamine with base. The immobilized fluoresceinamine was soaked in a solution 0.10 M in HCl and then washed with water and dried. A weighed amount of the product was titrated with 0.00209 M KOH (standardized against KHP) under nitrogen in 5.00 mL of 0.1 M potassium nitrate. The titration was followed potentiometrically using a glass electrode. The endpoint was taken as pH 9. The amounts titrated were 0.0060 g for the cellulose and 0.0021 g for the glass. Blank titrations on equivalent amount of underivatized support were also performed. The blank values were 7% and 21% of the sample values for the cellulose and glass substrates, respectively. In this titration
the neutral form of fluorescein is converted to the dianion. The pK values for forming the mono- and dianion are 4.4 and 6.7, respectively (14,15).

Fluorescence Measurements

Preliminary experiments. In order to select the proper wavelengths for excitation and emission of the immobilized fluoresceinamine, solution spectra of dissolved fluorescein were run at high pH (12, 0.1 N KOH), near neutral pH (5, distilled water), and low pH (0.1 N HCl) with the Perkin Elmer 204 spectrofluorometer. When these experiments were performed, there was no method available for obtaining spectra of the immobilized fluoresceinamine on the optic surface. Therefore, the solution data was used in selecting wavelengths.

In early experiments using the fiber optic fluorometer to determine the response of the immobilized fluoresceinamine to changes in pH, various combinations of glass long pass and band pass filters were used to maximize the signal to background ratio. The combinations are listed in Table 1. The background was estimated by attaching glass or cellulose to the optic and measuring the intensity. Difficulties were found with the glass filters. When used as the excitation filter, the glass was subject to cracking due to thermal stress produced by the heat of the 500 W tungsten-halogen source. None of the filter combinations used were satisfactory because the wavelength ranges for excitation and emission overlapped allowing a large component of scattered
Table 1:
Glass Filters Used in Preliminary Experiments with Immobilized Fluoresceinamine and their Characteristics

Filters Used (Listed by code)

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Emission*</th>
</tr>
</thead>
</table>

*Various combinations were used with each of the two excitation filters.

Characteristics of Filters Used**

Long Pass (LP) Filters:

<table>
<thead>
<tr>
<th>Code</th>
<th>1%T (nm)</th>
<th>80%T (nm)</th>
<th>Maximum %T</th>
<th>Wavelength (nm)</th>
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</thead>
<tbody>
<tr>
<td>LP518A</td>
<td>500</td>
<td>533</td>
<td>87</td>
<td>590</td>
</tr>
<tr>
<td>LP518B</td>
<td>500</td>
<td>533</td>
<td>87</td>
<td>585</td>
</tr>
<tr>
<td>LP524</td>
<td>497</td>
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<td>597</td>
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<td>680</td>
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<td>680</td>
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<td>LP580A</td>
<td>565</td>
<td>599</td>
<td>88</td>
<td>680</td>
</tr>
<tr>
<td>LP580B</td>
<td>565</td>
<td>598</td>
<td>87</td>
<td>680</td>
</tr>
<tr>
<td>LP599</td>
<td>584</td>
<td>618</td>
<td>86</td>
<td>710</td>
</tr>
</tbody>
</table>

Band Pass (BP) Filters:

<table>
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<th>Maximum %T</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
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</tr>
<tr>
<td>BP507</td>
<td>194</td>
<td>70</td>
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<td>BP510</td>
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<td>42</td>
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</tr>
<tr>
<td>BP515</td>
<td>135</td>
<td>9</td>
<td>515</td>
</tr>
<tr>
<td>BP525</td>
<td>60</td>
<td>40</td>
<td>525</td>
</tr>
</tbody>
</table>

**Compiled by Julie Pflug
***Filter width at half maximum transmittance
radiation to reach the detector and causing a low signal to background ratio. Furthermore, the filters available did not allow the best combination of wavelengths to maximize the change in fluorescence intensity with pH. Dielectric interference filters were then obtained. The use of dielectric interference filters improved the signal to background ratio by cutting down on the scattered radiation, and improved the observed response vs pH since appropriate wavelengths of excitation and emission could be selected. These filters can withstand greater changes in temperature.

In these preliminary experiments instrumental problems such as light leaks in the system and reflection of light off the aluminum casing and stirrer were identified. The light leaks were corrected by using modeling clay and the reflection was eliminated by painting the inside of the chamber with flat black paint.

**Response to pH changes.** Fluorescence measurements were made on the immobilized fluoresceinamine by attaching the support to the end of the fiber optic as described in Chapter II. The optic was then immersed into a solution of 15 mL of 0.1 M acetic acid. Other acids used were citric and phosphoric. Measurements were taken as the pH was varied by adding microliter amounts of 4.0 M KOH. Blanks were run on solvents and the underivatized glass or cellulose to determine how much of the signal was due to scattering or impurities. The pH changes of the buffer system were also measured on a pH meter. The pH changes were
measured both with and without immobilized fluoresceinamine (0.0010 g). The presence of fluoresceinamine did not affect the pH.

The reversibility of the response of the glass-bound fluoresceinamine was tested by taking measurements of the fluorescence intensity of the glass-bound fluoresceinamine on the optic surface as it was immersed in 0.1 M acetic acid and the pH was varied by adding microliter amounts of first 4.0 M KOH and then 4.0 M HCl. The reversibility of the cellulose-bound fluoresceinamine was not determined since the fluoresceinamine is cleaved from the cellulose at high pH.

For comparison, fluorescence measurements were also made on fluorescein in solution with the fiber optic fluorometer. The optic was immersed in a solution prepared by combining 14.00 mL of 0.1 M acetic acid and 1.00 mL of fluorescein solution in ethanol (2.9 x 10^{-4} M), to make the solution 1.9 x 10^{-5} M in fluorescein. The pH was varied and measured as above.

**Results and Discussion**

**Response vs. pH**

The fluorescence from the immobilized fluoresceinamine sensor increases with pH as shown in Figure 14. The glass-bound and cellulose-bound fluoresceinamine behave similarly up to pH 8. Above pH 8 the glass-bound fluoresceinamine response decreases. In solution, fluorescein behaves
Figure 14: Relative fluorescence intensity as a function of pH for immobilized fluoresceinamine on glass (o) and cellulose (△). The values for fluoresceinamine on glass have been multiplied by a factor of ten so they fit on the same scale as the values for fluoresceinamine on cellulose.
similarity to the glass-bound fluoresceinamine as shown in Figure 15. The fluorescence increases significantly from pH 3 to 6 and then increases only slightly above pH 8. The fact that the pH response for immobilized fluoresceinamine and soluble fluorescein agree is evidence that, as expected, the amino group is tied up in the immobilization procedure. The variation of the fluorescence of dissolved fluoresceinamine with pH was found to differ significantly from the behavior of the immobilized fluoresceinamine and soluble fluorescein. This is shown in Figure 16.

The observed variation in fluorescence with pH is consistent with previous studies of the pH dependence of fluorescein (19). This pH dependence is complex because it is influenced not only by protonation and deprotonation, but also by the fact that neutral fluorescein occurs as three different tautomers.

The response of the pH sensor will depend on wavelength. At the excitation wavelength of 480 nm, the monobasic form of fluorescein is excited more efficiently than the neutral form. Thus, the observed response reflects spectral shifts as well as changes in the fluorescence efficiency with pH.

The reversibility of the response of the glass-bound fluoresceinamine is shown in Figure 17. The behavior is approximately reversible, but does not yield exactly the same results in the reverse direction. This could be due to background shifts or to a change in the ionic strength
Figure 15: Relative fluorescence intensity as a function of pH for fluorescein in solution.
Figure 16: Relative fluorescence intensity as a function of pH for fluoresceinamine in solution.
Figure 17: Relative fluorescence intensity as a function of pH for fluoresceinamine immobilized on glass as the pH is raised (○) and then lowered (●).
of the solution. The reversibility of the cellulose-bound fluoresceinamine was not determined because at high pH the fluoresceinamine is cleaved from the support by hydrolysis resulting in a reduction in fluorescence intensity. The hydrolysis is accompanied by a momentary increase in intensity followed by a decrease as shown in Figure 18.

**Operating Characteristics**

The pH measurements were typically made with 1.0 mg of derivatized support material on the fiber optic surface. This amount of support holds about $2 \times 10^{-7}$ mol of immobilized fluoresceinamine as determined by titration. Titration curves are shown in Figures 19 and 20.

When pH is changed by injection of base, it takes about 15-30 s for the fluorescence intensity to reach a new steady state. Typical data are shown in Figure 21. Since the acid-base reaction itself is fast, the response time reflects the rate of mass transfer of solution into the layer of immobilized fluorophor. This is not unreasonable since the layer is on the order of 0.1 mm thick.

The signal to noise ratio is much poorer for the immobilized fluoresceinamine than for dissolved fluorescein. The noise can be seen in the data of Figure 21. Two effects contribute to this. One problem is the presence of a significant background signal due to scattering for the immobilized fluoresceinamine. For cellulose-bound fluoresceinamine, the background is 40% of the signal at pH 3. The signal to background ratio improves as pH increases because
Figure 18: Typical fluorescence signal for fluoresceinamine immobilized on cellulose as hydrolysis from the support occurs. (B= base added)
Figure 19: A plot of pH as a function of volume base added for determination of the amount of fluorescein-amine immobilized on glass. Results are shown for sample (○) and blank (△).
Figure 20: Plot of pH as a function of volume base added for determination of the amount of fluorescein-amine immobilized on cellulose. Results are shown for sample (○) and blank (△).
Figure 21: Typical raw data showing changes in fluorescence intensity in response to added base. (Data are for fluoresceinamine on cellulose.)
the background is constant with pH. For the glass-bound fluoresceinamine the situation is poorer. The background is twice the signal at pH 3. The background due to scatter would be much lower if the excitation and emission wavelengths were further apart. For example, at an excitation wavelength of 420 nm and an emission wavelength of 488 nm, both selected by interference filters, the background is barely detectable above detector noise. Unfortunately, in the case of immobilized fluoresceinamine the excitation and emission wavelengths need to be closer together to maximize the change in fluorescence with pH.

The other problem is low fluorescence intensity from the immobilized fluoresceinamine. The intensity per mole of cellulose-bound fluoresceinamine is about two orders of magnitude lower than intensity per mole of dissolved fluorescein in the solution below the fiber optic. Although the comparison is crude, there is no doubt that we are getting much less light from the immobilized fluoresceinamine. Part of the decrease in intensity is due to attenuation of the source by adsorption and scattering. However, this effect by itself can not account for such a large decrease in intensity since the thin layer of immobilized cellulose has considerable transmittance in water. The fluorescence efficiency must be lower for immobilized fluoresceinamine than for dissolved fluorescein. The fact that hydrolysis of the immobilized fluoresceinamine causes a momentary increase in intensity is further evidence for this. (See
Figure 18.) The decreased fluorescence efficiency may reflect the different environment of immobilized fluoresceinamine relative to dissolved fluorescein, or it may be a consequence of the changes in structure accompanying the immobilization process. The reduction in intensity is greater for fluoresceinamine on glass. Intensity per mole of glass-bound fluoresceinamine is about 15% of the intensity for the cellulose-bound fluoresceinamine.

"Concentration quenching" is common among fluorophors such as fluorescein when several molecules are in close proximity (13). This may be another reason for the lower intensity of the immobilized fluoresceinamine relative to fluorescein in solution. The mechanism responsible for this is apparently not adequately understood, but it is thought to involve resonance energy transfer among the fluorophors because of the overlap between the fluorescence excitation and emission spectra. Evidence supporting the idea of concentration quenching of immobilized fluoresceinamine is that when there is a substantial amount of fluoresceinamine bound, the resulting product is nonfluorescent when observed under UV light. When this nonfluorescent product is soaked in strong base to remove some of the fluoresceinamine, the product becomes fluorescent.
CHAPTER IV

SENSORS FOR Al(III) AND Be(II)
BASED ON IMMOBILIZED MORIN

Introduction

The characteristics of sensors for Al(III) and Be(II) based on the fluorescence of immobilized morin-metal complexes are described in this chapter.

Morin (3,5,7,2',4'-pentahydroxyflavone) belongs to the large class of flavonoid compounds which are aromatic phenols of the general structure C₆-C₃-C₆ (20). Flavones are compounds of plant origin, which have been studied in relation to subjects such as the fermentation of tea, tanning of leather, manufacture of cocoa, and the flavor of foods (21).

Flavonoids forms complexes with metal ions including Al(III), Be(II), Fe(III), Mo(VI), U(VI), Th(IV), and Zr(IV). Many of these complexes are highly fluorescent and are yellow or orange (21).

Complexation ties up nonbonding electrons which causes them to be lower in energy. Therefore, it is fairly common to have molecules that have n-π * lowest energy singlets when uncomplexed, and π-π * lowest excited singlets when complexed with a metal ion. Complexation can cause a nonfluorescent ligand to become fluorescent by changing the nature of the lowest excited singlet (22).
Flavones (Figure 22, structure I) can form analytically useful chelates through (23):

1. the hydroxyl group and the carbonyl (shown in the flavylidium structure II),
2. the 5-hydroxyl group and carbonyl (shown in III),
3. two hydroxyl groups present ortho to each other in ring B of the flavone molecule as shown in structure IV, and
4. the formation of multi-ligand complexes incorporating metal, flavone, and some other ligands (antipyrine, perchlorate, sulfate, etc.

Flavonoids have been used as reagents for fluorometric analysis of metals which form fluorescent complexes as well as for spectrophotometric analysis of metals which do not form fluorescent complexes (20-22). The fluorescence and color of the metal complexes formed depend on the number and position of the hydroxy groups in the flavone molecule. The hydroxy groups at the 3, 5, and 2' positions show the greatest effect on the fluorescence of the complex. Therefore, morin and datiscetin (3,5,7,2'-tetrahydroxyflavone) are the most reactive and sensitive among the flavones studied (23).

Morin, whose structure is shown in Figure 23, occurs in the wood of *Artocarpus integrifolia* (24) and *Toxylon pomiferum* (24) and can be synthesized (25,26).

Morin is probably the most studied of the flavones and is widely used for fluorometric analysis of Al(III) (20,21,23,27). An alcoholic solution of morin reacts with Al(III) in neutral or slightly acid solution to give an intensely green fluorescent complex (21). With the 1:1 morin complex, the sensitivity for the fluorometric
Figure 22: Analytically useful chelates of flavone molecules (23).
Figure 23: Structure of morin (23).
determination of Al(III) is 0.001 µg/mL (23).

Morin is also one of the most sensitive fluorometric reagents for analysis of Be(II) (20,21,23,28,29). In alkaline solution Be(II) produces a yellow-green water soluble complex with morin which is stable toward alkali (21). The absorption maximum is 420 nm and the fluorescence emission maximum is 513 nm (23). The most sensitive chemical test for Be(II) is that based on the fluorescence of the complex developed when morin reacts with Be(II) in NaOH at a pH greater than 11.0 (29). The detection limit is 0.004 µg. This test is subject to many interferences and Be(II) must be separated first (29). Paper impregnated with morin and a morin crayon have been used for determining Be(II) (21).

Morin can be immobilized on cellulose using cyanuric chloride via the phenolic group instead of an amine group in the same way as fluoresceinamine. (See Figure 12.) The product should be a mixture with the morin bound at different sites. As long as the groups responsible for complexation are not completely tied up, the immobilized morin should form fluorescent complexes with Al(III) and Be(II). The material can then serve as the basis for a sensor for Al(III) and Be(II).

In the present work, immobilized morin was first studied as a sensor for Al(III). Be(II) was shown to be an interference and, consequently, immobilized morin was also studied as a sensor for Be(II). The characteristics
of the two sensors were found to be similar. An unusual feature of the sensor for Be(II) is the large spectral shift in the excitation spectrum of the immobilized complex relative to the complex in solution.

Theory

In this section the theory is developed to describe the relative response of the immobilized morin sensor as a function of metal ion concentration. It is assumed that the total number of immobilized morin molecules, \( C \), is much less than the number of metal ions in solution and that the equilibrium constant for complex formation is relatively small. Under these conditions the insertion of the sensor will not significantly affect the metal ion concentration in solution.

The equilibrium constant for metal binding to immobilized ligand can be represented (assuming a 1:1 complex):

\[
K = \frac{ML/L(a_m)}{4-1}
\]

where \( L \) is the number of immobilized ligands not associated with metal, \( ML \) is the number of immobilized morin ligands associated with ligand, \( a_m \) is the metal ion activity in solution, and \( K \) is the equilibrium constant.

Because the total number of immobilized ligand molecules, \( C \), is fixed:

\[
C = L + ML. \quad 4-2
\]

Since morin is essentially nonfluorescent by itself
and the morin-metal complex is fluorescent, the fluorescence signal will depend on the amount of metal bound to the ligand:

$$I = kKL$$  \hspace{1cm} 4-3$$

where $I$ is the fluorescence intensity and $k$ is a proportionality constant relating fluorescence intensity to the amount of metal bound to ligand. It is assumed that the conditions are such that intensity is proportional to the number of sites (i.e., no inner filter effects).

By substituting equation 4-2 into equation 4-1 and rearranging, it is possible to express $ML$ in terms of $C$, $a_m$, and $K$:

$$ML = a_mKC/(1 + a_mK).$$  \hspace{1cm} 4-4$$

By substituting equation 4-4 into equation 4-3, an expression for fluorescence intensity as a function of metal ion activity is obtained.

$$I = kKCa_m/(1 + a_mK).$$  \hspace{1cm} 4-5$$

To determine $K$ for the sensor a linear form of equation 4-5 can be used:

$$a_m/I = a_m/kC + 1/kKC.$$  \hspace{1cm} 4-6$$

A plot of $a_m/I$ versus $a_m$ will be linear with a slope of $1/kC$ and an intercept of $1/kKC$.

The experimental equilibrium constant, $K$, is a
conditional constant that depends on pH because metal displaces hydrogen ions when binding to morin as shown in Figure 24.

Experimental

Apparatus

The apparatus for measuring the fluorescence of the immobilized morin is described in Chapter II. The interference filter used for excitation was a 2" x 2" interference filter with maximum transmittance at 420 nm and a bandwidth of 7.1 nm at half maximum transmittance. For the Al(III) sensor, the emission filter had maximum transmittance at 488 nm and a bandwidth of 7 nm at half maximum transmittance; for the Be(II) sensor the filter had maximum transmittance at 520 nm and a bandwidth of 8.2 nm at half maximum transmittance. Both filters were 1" x ½".

Reagents

Morin was purchased from Aldrich.

Immobilization Procedure

Morin was immobilized on powdered cellulose using cyanuric chloride according to the procedure in Chapter III. The concentration of the cyanuric chloride solution was 0.14 M in acetone and the concentration of the morin soaking solution was 0.0076 M in acetone. The product was washed with acetone, dried by suction, and stored at room temperature. Because there was batch to batch
Figure 24: Formation of the morin-Al(III) complex.
variability, a single batch of immobilized morin was used for all characterization studies on both the Al(III) and Be(II) sensors.

Morin was also immobilized as its Al(III) complex, because this should prevent the functional groups involved in complexation from reacting with cyanuric chloride. The soaking solution consisted of acetone-water (3:2), 0.0021 M morin and 0.014 M Al(III) (from aluminum sulfate). For comparison a batch of uncomplexed morin was immobilized under the same conditions, but omitting the aluminum. The morin bound uncomplexed gave slightly higher intensity than the morin bound complexed when both were saturated with aluminum and, consequently, morin bound in the uncomplexed form was used for all experiments. The amount of morin bound per gram of cellulose was found to be slightly greater when morin was bound as the complex. Thus, it is not clear why this preparation failed to respond more sensitively to Al(III). This is an interesting question, but was not pursued.

**Determination of Amount of Morin Bound**

The amount of morin bound/gram of cellulose was found by stripping the morin from the cellulose in 1 M KOH and measuring the morin concentration by spectrophotometry. Weighed amounts (0.0311 g and 0.0356 g) of the immobilized morin were soaked in 1 M KOH solutions (10.00 mL) until the cellulose lost color (4 hours). The absorbance of 1:10 dilutions of the soaking solutions was measured at 405 nm.
along with morin standards in 1 M KOH. The standards were allowed to soak a comparable amount of time as the samples since morin absorbance may deteriorate with time. Absorbance spectra were measured for the standards and soaking solutions from 350 to 600 nm.

The amount of active aluminum binding sites contained/gram of cellulose was determined by two methods. The first method was based on the difference in Al(III) concentration of a solution after contact with the immobilized morin. Knowing the mass of the support and the Al(III) concentration both before and after soaking, one can calculate the number of active binding sites. Weighed amounts (0.0077 g and 0.0115 g) of the immobilized morin were soaked in solutions of aluminum sulfate (5.45 x 10^{-4} M, 0.400 mL) at pH 5.1 for 15 minutes. The decrease in aluminum concentration of the soaking solutions was then determined by taking 0.100 mL of each soaking solution, adding morin (0.00683 M, 0.175 mL), diluting to 25 mL with pH 5.1 acetate buffer, and measuring the fluorescence with the SLM spectrofluorometer. The excitation wavelength was set at 429 nm and the emission wavelength was selected using the 488 nm interference filter. The unknown concentration was determined by comparing intensity for the unknown to intensities for a series of aluminum standard solutions of varying concentration. Since this method involves the morin-Al(III) system at equilibrium, the equilibrium constant for binding at pH 5.1 (Table 3) was used in
calculating the number of active binding sites from the decrease in aluminum concentration.

The second method used to determine the number of active aluminum binding sites was based on saturation of the binding sites with Al(III) and then stripping off the Al(III) and measuring how much was bound. Weighed amounts (0.0215 g and 0.0197 g) of the immobilized morin were each soaked in a solution of aluminum sulfate \((7.56 \times 10^{-4} \text{ M})\) at pH 4.8 for 0.5 hours. This concentration of aluminum is sufficient to saturate all binding sites. The excess solution was washed away with four 10 mL portions of distilled deionized water on a sintered glass funnel. In deionized water the kinetics of complex dissociation are so slow that the washing step may be completed without significant dissociation of the complex. The cellulose was then soaked in 10 mL of 20% acetic acid for 1 hour. The cellulose was fluorescent under UV light after the aluminum soaking and washing, but nonfluorescent after the acid treatment, indicating that all the aluminum had been stripped off. The solution was decanted and the cellulose was rinsed with 5 mL of distilled water, resulting in 15 mL of solution. The pH was adjusted to 4.7 with KOH and the solutions were analyzed for aluminum by adding morin (0.1 mL, 0.00777 M) and measuring the fluorescence intensity using the fiber optic fluorometer. Standards of varying aluminum concentration, at the same pH, acetate concentration, and morin concentration were used to
determine the amount of aluminum.

The number of binding sites for Be(II) was not measured. It should be the same as for Al(III).

**Fluorescence Measurements**

**Fluorescence spectra.** To determine the wavelengths for excitation and emission, fluorescence spectra were measured on the Al(III)-morin and the Be(II)-morin complexes in solution using the Perkin Elmer 204 spectrofluorometer.

Excitation spectra of both immobilized and free morin and their Al(III) complexes were measured at a fixed emission wavelength of 488 nm at a pH of 4.8 using the SLM spectrofluorometer as described in Chapter II.

Excitation spectra of both immobilized and free morin and their Be(II) complexes were measured at a fixed emission wavelength of 520 nm at a pH of 5.3 using the SLM spectrofluorometer. There was a large difference between the solution and immobilized spectra. It was thought to be due to different morin-Be(II) complexes formed by dissolved morin relative to those formed by the immobilized morin. To determine if this was the reason for the large difference, excitation spectra were measured at various Be(II): morin ratios. Because the ratio of Be(II): morin did not significantly influence the excitation spectra of the complex, spectra of the complex in solution were measured at various pH values using the SLM spectrofluorometer. This was done in order to determine if the excitation spectrum for the immobilized morin-Be(II) complex reflects
a difference in the effective pH of the complex on the surface.

Response to Al(III) and Be(II). Fluorescence measurements on the immobilized morin sensor as Al(III) or Be(II) concentration was varied were made as described in Chapter II. Aluminum or beryllium concentration was varied both by adding microliter amounts of aqueous standard solutions of aluminum sulfate or beryllium sulfate to the same buffered solution and by immersing the sensor into buffered solutions of varying aluminum or beryllium concentration. Measurements were made on morin in solution for comparison to the immobilized morin. The concentration of morin used in the solution studies was $8.00 \times 10^{-5}$ M.

The reversibility of the sensor response was determined by adding Al(III) or Be(II) to the sensor in buffer solution until the response was constant, and then immersing the sensor into a buffer solution without Al(III) or Be(II). The sensor was then submerged into a fresh buffer solution and more Al(III) or Be(II) was added. Because Al(III) forms strong complexes with EDTA, the aluminum was also stripped off the immobilized morin by immersing the sensor into a solution of EDTA (0.0255 M at pH 7) and washing with distilled deionized water.

Effect of pH. The effect of pH on the response to Al(III) was studied using acetate buffers (0.1 M) of varying pH in the range 2.9 to 6.5.

The effect of pH on the response of the sensor to
Be(II) was studied using buffers of varying pH. Acetate buffers of pH 3.5 and 5.3 were used, along with phosphate buffers of pH 7.0 and 11.2. All buffers were 0.1 M. Measurements were made on dissolved morin for comparison.

**Effect of interferences.** Interferences were studied by adding various ions separately to the immobilized morin sensor when it was responding to aluminum or beryllium. Metal ions tested were Al(III) (Be(II)), Ca(II), Mg(II), Fe(III), Cu(II), and Co(II).

**Results and Discussion**

**Response to Al(III)**

The fluorescence of the sensor based on immobilized morin increases with aluminum concentration as shown in Figure 25. This is expected since aluminum forms a fluorescent complex with morin. The response is fairly linear from $10^{-6}$ to $10^{-4}$ M aluminum concentration. Above $10^{-4}$ M there is a change in slope and the response levels off. This levelling off is due to saturation of the morin with aluminum. The same type of response is observed for morin in solution as shown in Figure 26.

The effect of pH on the immobilized morin-Al(III) complex is similar to the effect on the morin-Al(III) complex in solution as shown in Figures 25 and 26. The complex is most intensely fluorescent at about pH 4.8. At lower pH values, the complex is less intensely fluorescent and above pH 5, the fluorescence of the complex is also
Figure 25: Relative fluorescence intensity of the immobilized morin sensor as a function of Al(III) concentration at pH 3.8 (△), pH 4.8 (□), and pH 5.9 (○).
Figure 26: Relative fluorescence intensity of dissolved morin as a function of Al(III) concentration at pH 3.5 (⋆), pH 4.8 (□), and pH 6.5 (△).
Response to Be(II)

The fluorescence of the sensor based on immobilized morin increases with beryllium concentration as shown in Figure 27. This result is similar to that obtained for the sensor in the presence of aluminum. The response is fairly linear from $10^{-6}$ M to $10^{-4}$ M beryllium. Above $10^{-4}$ M there is a change in slope and the response levels off due to saturation of the morin with beryllium. The complex formed is weaker than the morin-Al(III) complex as the break in the curve is not as sharp. The relative fluorescence intensity of the immobilized morin-Be(II) complex is higher than that for the immobilized morin-Al(III) complex at saturation.

A similar type of response is observed for the morin-Be(II) complex in solution, except for the large effect of pH on the fluorescence intensity as shown in Figure 28. The morin-Be(II) complex is most intensely fluorescent at high pH (12.0). In order to obtain greater sensitivity in using immobilized morin to determine Be(II), a high pH should be used. However, the immobilized morin is stripped off of the cellulose at high pH resulting in a lower intensity than would be expected from the solution results. At high pH we are most likely measuring the fluorescence of the morin-Be(II) complex which has been cleaved from the cellulose because of hydrolysis. The intensity is lower than expected for dissolved morin because of the increased
Figure 27: Relative fluorescence intensity of the immobilized morin sensor as a function of Be(II) concentration at pH 3.45 (○), pH 5.25 (△), pH 7.00 (□), and pH 11.23 (●).
Figure 28: Relative fluorescence intensity of dissolved morin as a function of Be(II) concentration at pH 3.45 (O), pH 5.25 (△), pH 7.00 (□), and pH 11.23 (●).
distance from the optic and limited transmittance of the cellulose support on the optic surface.

At pH 7.0, the emission intensities for both dissolved and immobilized complexes are low. One reason for the lower intensity is a slight shift in the excitation and emission wavelengths for the complex at pH 7.0. These shifts are shown in Table 2 for the complex in solution. Another explanation is that the species responsible for the fluorescence at pH 7.0 is a less efficient fluorophor. This is shown when intensities are compared for solutions with the same concentration in morin and Be(II) as shown in Table 2.

At low pH (2.74) there is again a low intensity for both dissolved and immobilized complexes and the same reasoning can be used to explain the result. Another reason for low intensity at low pH could be incomplete complexation because at low pH morin is in the protonated form.

Because the sensor is not stable at high pH and is only weakly fluorescent at pH 7.0 and 2.7, a pH of 5.0 is most suitable for Be(II) measurements as indicated in Figure 27.

**Determination of Binding Constants**

The fluorescence intensities and aluminum concentrations were plotted in the form of the theoretical linear equation modeling the system (see equation 4-6) yielding results which are shown in Figure 29. The linear model is adequate for the system at the 95% confidence level as
<table>
<thead>
<tr>
<th>pH</th>
<th>Wavelength (nm)</th>
<th>Fluorescence Intensity (Relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Excitation</td>
<td>Emission</td>
</tr>
<tr>
<td>2.74</td>
<td>435</td>
<td>490</td>
</tr>
<tr>
<td>4.77</td>
<td>420</td>
<td>520</td>
</tr>
<tr>
<td>7.00</td>
<td>440</td>
<td>510</td>
</tr>
<tr>
<td>12.50</td>
<td>435</td>
<td>515</td>
</tr>
<tr>
<td>13.54</td>
<td>435</td>
<td>520</td>
</tr>
</tbody>
</table>
Figure 29: A typical plot showing Al(III) concentration divided by relative fluorescence intensity as a function of Al(III) concentration in which the linear model (equation 4-6) is represented.
shown by the appropriate regression analysis. From the slope and intercept, values for the conditional binding constant, K, were determined for the immobilized morin in solutions of varying pH. The results are shown in Table 3. These values reflect changes in protonation of the immobilized morin and formation of hydroxide complexes of aluminum. At high pH the formation constant decreases because more of the aluminum is in the form of hydroxide complexes. At low pH, the conditional formation constant decreases because the ligand is protonated. The binding constant for Al(III) to morin in solution has been reported as $2.96 \times 10^6$, but the pH was not specified (21,23).

Using the slope and intercept of the linear plot at pH 4.8, the theoretical equation to describe the response of the sensor (equation 4-5) is plotted in Figure 30. Since the data is represented adequately by the theory for a 1:1 Al(III)-morin complex, this suggests that the complex is primarily in this form.

The fluorescence intensities and beryllium concentrations were also plotted in the form of the theoretical linear equation modeling the system (See equation 4-6.). The results for pH 5.25 are shown in Figure 31. The linear model is adequate for the system at the 95% confidence level as shown by the appropriate regression analysis. From the slope and intercept, values for the conditional equilibrium constant, K, were determined for the immobilized morin in solutions of pH 5.25 and 3.48. The results
Table 3:
Experimental Values of the Conditional Equilibrium Constant, K, for Aluminum Binding to Immobilized Morin

<table>
<thead>
<tr>
<th>pH</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>$4 \times 10^3$</td>
</tr>
<tr>
<td>4.1</td>
<td>$1.2 \times 10^4$</td>
</tr>
<tr>
<td>4.6</td>
<td>$1.4 \times 10^4$</td>
</tr>
<tr>
<td>4.8</td>
<td>$1.7 \times 10^4$</td>
</tr>
<tr>
<td>5.1</td>
<td>$3.3 \times 10^4$</td>
</tr>
<tr>
<td>5.5</td>
<td>$2.8 \times 10^4$</td>
</tr>
<tr>
<td>5.9</td>
<td>$3 \times 10^4$</td>
</tr>
<tr>
<td>6.5</td>
<td>$1.8 \times 10^3$</td>
</tr>
</tbody>
</table>
Figure 30: A plot of the theoretical response of the immobilized morin sensor to aluminum (curve) represented by equation 4-5 and experimental points (O) at pH 4.8.
Figure 31: A typical plot showing Be(II) concentration divided by relative fluorescence intensity as a function of Be(II) concentration in which the linear model equation 4-6 is represented. Results are at pH 5.2.
are $5.0 \times 10^4$ at pH 3.48 and $1.0 \times 10^4$ at pH 5.25. The theoretical curve for Be(II) at pH 5.25 is shown in Figure 32.

**Determination of Amount of Morin Bound**

The amount of morin bound/gram of cellulose is $4.2 \times 10^{-5}$ mole as determined by spectrophotometrically measuring the amount of morin stripped off the cellulose in strong base. The calibration curve is shown in Figure 33. The absorbance spectra are identical for the morin in base and the solutions containing the stripped morin. This indicates that treatment with base yields free morin rather than a morin-cyanuric chloride conjugate. The spectra are shown in Figure 34.

The number of moles of active complexing sites/gram is $1.1 \times 10^{-5}$ moles Al(III)/gram of immobilized morin. The same result is obtained by each of the two methods. This means that the number of moles of active complexing sites is 26% of the number of moles of morin bound/gram of cellulose. That the number of active sites is less than the total implies that the group responsible for complexation is partially tied up in the immobilization procedure. It is not known which of the morin phenolic sites the cyanuric chloride binds to, but the product is most likely a mixture with the cyanuric chloride bound to different sites.
Figure 32: A plot of the theoretical response of the immobilized morin sensor to Be(II) (curve) represented by equation 4-5 and experimental points (*) at pH 5.2.
Figure 33: A plot of absorbance as a function of morin concentration for determination of the amount of morin stripped from cellulose. Experimental points (*) are shown along with the least squares line.
Figure 34: Absorbance spectra (in 1 M KOH) for morin (A) and for morin stripped from cellulose (B). (Absorbance is in arbitrary units and the spectra are placed above each other for comparison.)
Operating Characteristics

Operating characteristics for the sensor in the presence of Al(III) and Be(II) are similar. The optimum pH for the sensor response to Al(III) is 4.8. The response to Al(III) at various pH values is shown in Figure 25. The optimum pH for the sensor response to Be(II) is 5.0. The response to Be(II) is shown in Figure 27. At low pH the kinetics of the response are very slow and the intensity is low for the sensor in the presence of both Al(III) and Be (II). At higher pH values the fluorescence intensity of the sensor in the presence of Al(III) is low due in part to the hydrolysis of Al(III). Fluorescence intensity versus time data at different pH values are shown in Figure 35 for Al(III); the same trend is observed for Be(II). The response time for the sensor to a change in Al(III) or Be(II) concentration is 1-2 minutes. When the sensor is saturated and placed in a buffer solution without Al(III) or Be(II) the fall time is about 3 minutes. The response time when the immobilized reagent is inserted into solution for the first time is 3 to 4 minutes.

Measurements were made with 1 mg of derivatized support on the optic surface. This corresponds to $4.2 \times 10^{-8}$ moles of morin on the optic surface as determined by absorbance measurements.

Using the following relationship which is valid at any aluminum level:
Figure 35: Response as a function of time for the immobilized morin sensor to Al(III) at pH 3.8 (△), pH 4.8 (□), and pH 6.5 (★).
number of Al(III) ions bound = intensity
                  total number sites      intensity at saturation

It is possible to show that only 1% of the Al(III) is extracted from solution when operating in the region of linear response to aluminum. Because of the similar concentrations used, the result is assumed to be similar for beryllium. This supports the assumption in the theory that insertion of the sensor does not significantly affect concentration.

The signal to background ratio is 2.5:1 at aluminum concentrations of $1 \times 10^{-3}$ M. Dark current represents 50% of the background with the remainder resulting from scattered and reflected light plus residual morin fluorescence. The signal to background ratio can be improved to 7:1 at $1 \times 10^{-3}$ M aluminum using the SLM spectrofluorometer as the source and detector in which case dark current represents 25% of the background. The signal to background ratio is similar to the sensor in the presence of beryllium: 2:1 at beryllium concentration of $1.5 \times 10^{-4}$ M. Dark current represents 50% of the background.

The detection limit for the sensor, defined as the concentration equivalent to two times the standard deviation of the background signal, is $1 \times 10^{-6}$ M for Al(III) (0.027 ppm) and $1 \times 10^{-6}$ M for Be(II) (0.009 ppm). Although the fluorescence intensity of the morin-Be(II) complex is higher than for the morin-Al(III) complex, the detection limit is not lower because it is still limited
by the variation in background.

**Excitation Spectra**

**Spectra of the morin-Al(III) complex.** The excitation spectra (not corrected) of the immobilized morin, its aluminum complex, and the morin-Al(III) complex in solution are shown in Figure 36. The spectra of the immobilized complex and the complex in solution are similar with the spectrum of the immobilized complex showing a slight broadening. The excitation spectrum of the immobilized morin shows a small peak similar in shape to the spectrum of the complex superimposed on a flat, continuous background. This indicates that the background includes a contribution from weak morin fluorescence plus a signal due to reflected and scattered radiation.

**Spectra of the morin-Be(II) complex.** Excitation spectra for the immobilized morin-Be(II) complex and the complex in solution are shown in Figure 37. The spectrum of the immobilized morin-Be(II) complex is shifted a large amount relative to the spectrum of the complex in solution. When measurements are made at various Be(II):morin ratios, both the immobilized and solution spectra remain similar as shown in Figure 38. The shift in the spectrum seems to be due to a difference in the effective pH of the immobilized complex on the surface, because a solution of the complex at pH 9.91 exhibits a similar shift in the excitation maximum. (See Figure 39.)
Figure 36: Excitation spectra (uncorrected) of the morin-Al(III) complex in solution (△), the immobilized morin-Al(III) complex (○), and the immobilized morin without added aluminum (□) at pH 4.8.
Figure 37: Excitation spectra (corrected for variations in source intensity with wavelength) of the morin-Be(II) complex in solution (△), the immobilized morin-Be(II) complex (○), and the immobilized morin without Be(II) (●) at pH 5.2.
Figure 38: A) Excitation spectra of the morin-Be(II) complex in solution at ratios of Be(II):morin of 0.070 (△) and 28.0 (□) at pH 5.2.
B) Excitation spectra of the immobilized morin-Be(II) complex at ratios of Be(II):morin of 10.4 (△) and 167 (□) at pH 5.2. (All spectra are not corrected.)
Figure 39: Excitation spectra (corrected for variations in source intensity with wavelength) of the morin-Be(II) complex in solution at pH 9.91 (O) and the immobilized morin-Be(II) complex at pH 5.2 (●).
Interferences

The species Be(II), Ca(II), and Mg(II) were evaluated as possible interferences with the Al(III) determination because they form fluorescent complexes with morin. The optimum pH for the formation of these complexes, however, is above 7 (21,23). The ions Fe(III), Cu(II), and Co(II) are all heavy metal ions and could quench fluorescence by complexation or by the heavy atom effect in solution. The effect of all these ions on the aluminum response is shown in Table 4. The major interferences are Be(II) which increases the signal, and Fe(III) and Cu(II) which quench fluorescence to an appreciable extent. Because of the differences in excitation maxima for the immobilized morin-Al(III) and morin-Be(II) complexes, analysis for aluminum in the presence of beryllium can be carried out by proper selection of excitation wavelength. The signal from Al(III) decreases in the presence of Cu(II), but the sensor still responds to further increases in the aluminum concentration. The quenching by Cu(II) may be due to Cu(II) ions in solution or to weak complexation, rather than due to strong complexation as in the case of Fe(III). Morin is used to determine Fe(III) spectrophotometrically by measuring the absorbance of the strongly-absorbing, non-fluorescent morin-Fe(III) complex (21,23). After being quenched by the addition of Fe(III) the sensor no longer responds to added aluminum. To use the sensor in the presence of Fe(III) it would be necessary to reduce Fe(III)
Table 4:
Effect of Interferences on the Al(III) Response of the Immobilized Morin Sensor

<table>
<thead>
<tr>
<th>Interference</th>
<th>Concentration (M)</th>
<th>Signal</th>
<th>Signal w/Int*</th>
<th>%Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(II)</td>
<td>3.32 x 10^{-3}</td>
<td>0.088</td>
<td>0.090</td>
<td>+2 **</td>
</tr>
<tr>
<td>Mg(II)</td>
<td>2.83 x 10^{-3}</td>
<td>0.108</td>
<td>0.118</td>
<td>+9 **</td>
</tr>
<tr>
<td>Be(II)</td>
<td>3.19 x 10^{-4}</td>
<td>0.084</td>
<td>0.103</td>
<td>+23 **</td>
</tr>
<tr>
<td></td>
<td>4.73 x 10^{-4}</td>
<td>0.084</td>
<td>0.153</td>
<td>+82 **</td>
</tr>
<tr>
<td>Co(II)</td>
<td>3.75 x 10^{-3}</td>
<td>0.097</td>
<td>0.097</td>
<td>0 **</td>
</tr>
<tr>
<td></td>
<td>2.93 x 10^{-3}</td>
<td>0.097</td>
<td>0.080</td>
<td>-18 **</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>7.64 x 10^{-5}</td>
<td>0.134</td>
<td>0.108</td>
<td>-19 ***</td>
</tr>
<tr>
<td></td>
<td>7.59 x 10^{-4}</td>
<td>0.134</td>
<td>0.039</td>
<td>-71 ***</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>3.27 x 10^{-5}</td>
<td>0.162</td>
<td>0.138</td>
<td>-15 ***</td>
</tr>
<tr>
<td></td>
<td>4.87 x 10^{-4}</td>
<td>0.162</td>
<td>0.020</td>
<td>-88 ***</td>
</tr>
</tbody>
</table>

*Int = Interference  
** Al(III) = 3.59 x 10^{-5} M  
*** Al(III) = 7.18 x 10^{-5} M
to Fe(II) and tie it up as a complex.

The same interferences were evaluated for the response of the sensor in the presence of beryllium. Al(III) was also tested as an interference. The major enhancement interference is Al(III) as shown in Table 5. In order to use the sensor to determine beryllium in the presence of Al(III), it would be necessary to complex the Al(III) with EDTA (29). Proper selection of excitation wavelength could also be used as mentioned earlier. The major quenching interferences are Fe(III) and Cu(II) as in the case of the aluminum sensor. The effect of Fe(III) is somewhat less. probably due to the higher pH of the system used for the Be(II) sensor. To use the sensor to determine beryllium in the presence of these interferences, EDTA could again be used to form complexes with Fe(III) and Cu(II) or tactics similar to those mentioned in the previous paragraph could be used.
Table 5:
Effect of Interferences on the Be(II) Response of the Immobilized Morin Sensor

<table>
<thead>
<tr>
<th>Interference</th>
<th>Concentration (M)</th>
<th>Signal</th>
<th>Signal w/Int*</th>
<th>%Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(II)</td>
<td>$3.39 \times 10^{-4}$</td>
<td>0.097</td>
<td>0.100</td>
<td>+3 **</td>
</tr>
<tr>
<td></td>
<td>$2.65 \times 10^{-3}$</td>
<td>0.097</td>
<td>0.094</td>
<td>-3 **</td>
</tr>
<tr>
<td>Mg(II)</td>
<td>$3.59 \times 10^{-4}$</td>
<td>0.135</td>
<td>0.145</td>
<td>+7 **</td>
</tr>
<tr>
<td></td>
<td>$2.80 \times 10^{-3}$</td>
<td>0.135</td>
<td>0.142</td>
<td>+5 **</td>
</tr>
<tr>
<td>Al(III)</td>
<td>$4.99 \times 10^{-5}$</td>
<td>0.148</td>
<td>0.187</td>
<td>+26 ***</td>
</tr>
<tr>
<td></td>
<td>$2.95 \times 10^{-4}$</td>
<td>0.148</td>
<td>0.224</td>
<td>+51 ***</td>
</tr>
<tr>
<td>Co(II)</td>
<td>$7.45 \times 10^{-5}$</td>
<td>0.134</td>
<td>0.123</td>
<td>-8 **</td>
</tr>
<tr>
<td></td>
<td>$2.91 \times 10^{-3}$</td>
<td>0.134</td>
<td>0.040</td>
<td>-70 **</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>$7.59 \times 10^{-5}$</td>
<td>0.123</td>
<td>0.077</td>
<td>-37 **</td>
</tr>
<tr>
<td></td>
<td>$1.12 \times 10^{-3}$</td>
<td>0.123</td>
<td>0.004</td>
<td>-97 **</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>$3.25 \times 10^{-5}$</td>
<td>0.105</td>
<td>0.077</td>
<td>-27 **</td>
</tr>
<tr>
<td></td>
<td>$1.28 \times 10^{-3}$</td>
<td>0.105</td>
<td>0.017</td>
<td>-84 **</td>
</tr>
</tbody>
</table>

*Int = Interference
**Be(II) = 4.75 \times 10^{-5} \text{M}
***Be(II) = 3.29 \times 10^{-5} \text{M}
CHAPTER V

CHARACTERIZATION OF IMMOBILIZED CALCEIN

Introduction

The properties of immobilized calcein (3,6-dihydroxy-2,4-bis(N,N'-di(carboxymethyl)aminomethyl)fluoran; Fluorexon; etc.) as a possible sensor for Cu(II), Co(II), or Ni(II) are described in this chapter.

In 1956 Diehl and Ellingboe (30) reported an indicator for the titration of calcium with EDTA prepared by condensing iminodiacetic acid, formaldehyde, and fluorescein. They designated the product calcein. Using calcein as an endpoint indicator, the titration of calcium could be performed in the presence of magnesium. Below pH 12, both the free indicator and the calcium complex of calcein are yellow-green, but above pH 12 the free indicator is brown while its calcium complex remains yellow-green. The endpoint was taken as the change from yellow-green to brown (30). Diehl and Ellingboe made no reference to changes in fluorescence with calcium binding, but fluorescence changes were later described by Tucker (31). The preparation of calcein, its acid-base and fluorescence characteristics, and its use for the ultramicro determination of calcium were described by Wallach, et al. in 1959 (32). The method was modified for fluorometric determination of calcium in serum by Kepner and Hercules (33) in 1963, Philips (34) in
Moser and Gerade (36) adapted the method to the "UNOPETTE" system in 1969. The linearity and reproducibility of the method was later studied by Bardrowski and Benson (34). Calcein has also been used for fluorometric determination of Al(III), alkaline earths, Co(II), Cu(II), Ni(II), Zn(II) (37), and Cd(II) (38).

When calcein is prepared by the condensation of iminodiacetic acid, formaldehyde, and fluorescein, a mixture of compounds results (35). Wallach, et al. have presented evidence that the product of their condensation results in the structure shown in Figure 40A. Calcein is available as calcein-W (Fisher) (39) and Fluorexon (Aldrich) (40). The structure given is shown in Figure 40B.

Calcein is analytically useful because it forms fluorescent complexes with metals such as Ca(II) and Cd(II) above pH 12 where the free indicator is nonfluorescent. The basis of the fluorescence behavior of calcein differs from other reagents used to form fluorescent metal chelates (e.g., 8-hydroxyquinoline, morin, 2,2'-dihydroxyazobenzene) in that the metal ion does not tie up nonbonding electrons associated with the aromatic system. Instead, it merely associates with the iminodiacetate group; however, this is sufficient to change the fluorescence behavior so that analysis for Ca(II) is possible (22). Calcein can also be used to determine heavy metals such as Co(II) and Cu(II) at neutral pH where the quenching of its fluorescence is
Figure 40: A: Structure of calcein according to Wallach (32).
   B: Structure of calcein-W or Fluorexon (40).
related to metal ion concentration.

In order to be used as a sensor for metal ions either by formation of fluorescent complexes or by quenching, calcein needs to be immobilized on a solid support. Because amino compounds are readily immobilized on solid supports, such as controlled pore glass, one approach to immobilized calcein would be to prepare an amino derivative of calcein. A three step synthesis to prepare an amino derivative of calcein is shown in Figure 41. The amino calcein prepared in this way can be immobilized to controlled pore glass or powdered cellulose in the same manner as fluoresceinamine. Alternatively, in order to avoid the preparation of an amino derivative of calcein, calcein-W can be immobilized on powdered cellulose via the phenolic group using cyanuric chloride as shown in Figure 12.

The fluorescence characteristics of calcein are dependent on pH and metal ion concentration. In order to use immobilized calcein as a sensor for metal ions, the solution must be buffered to a constant pH. Because calcein immobilized on cellulose will be hydrolyzed from the surface at high pH, calcein immobilized via cyanuric chloride can not be used to determine metals which form fluorescent complexes at high pH. However, at neutral pH immobilized calcein should be quenched by the heavy metal ions such as Cu(II), Co(II), and Ni(II). Metal ion concentration can be related to the extent of quenching.
Figure 41: Three step synthesis for preparation of an amino derivative of calcein.
Theory

This section develops the theory to describe the relative response of immobilized calcein as a function of metal ion concentration, and to determine equilibrium constants for metal binding to immobilized calcein. The treatment is similar to that in Chapter IV. The metals to be studied, such as Cu(II), Co(II), and Ni(II), when complexes to calcein should quench fluorescence. In this treatment, it is again assumed that the total number of immobilized calcein molecules, C, is much less than the number of metal ions in solution and that the equilibrium constant for complex formation is relatively small. Under these conditions the insertion of the sensor will not significantly affect the metal ion concentration in solution. The equilibrium constant for metal binding to immobilized ligand is represented in equation 4-1. A 1:1 complex is again assumed since calcein forms strong 1:1 complexes with Cu(II), Co(II), and Ni(II) (37). The number of immobilized ligand molecules, C, remains as in equation 4-2.

Since calcein is fluorescent by itself and the calcein-metal complex is nonfluorescent, the fluorescence signal will now depend on the amount of ligand uncomplexed:

\[ I = kL \tag{5-1} \]

where I is the fluorescence intensity and k is a proportionality constant relating fluorescence intensity to the
amount of uncomplexed ligand. It is assumed that intensity is proportional to the number of sites.

By substituting equation 4-2 into equation 4-1 and rearranging, it is possible to express \( L \) in terms of \( C, a_m \), and \( K \):

\[
L = \frac{C}{1 + K a_m}. \quad 5-2
\]

By substituting equation 5-2 into equation 5-1 an expression for fluorescence intensity as a function of metal ion activity is obtained:

\[
I = \frac{K C}{1 + K a_m}. \quad 5-3
\]

To test whether the experimental data fits the theory and to determine \( K \) for the immobilized calcein, a linear form of equation 5-3 can be used:

\[
\frac{1}{I} = \frac{K a_m}{kC} + \frac{1}{kC}. \quad 5-4
\]

A plot of \( 1/I \) versus \( a_m \) will be linear with a slope of \( K/kC \) and an intercept of \( 1/kC \) if this model applies.

Because iminodiacetic acid forms strong complexes with many metals including Cu(II), Co(II), and Ni(II) (41), and calcein contains two iminodiacetate groups, the assumption that the insertion of the sensor will not significantly affect the metal ion concentration may not be a valid one. Instead, the binding constant may be so large that the immobilized calcein will extract a large percentage of the metal ion from dilute solutions. In
this case it is not possible to determine the binding constant by plotting $1/I$ versus $a_m$. Instead, a method based on competitive binding may be used to obtain the desired binding constants. A ligand to use for competitive binding should have metal binding constants of approximately the same magnitude as immobilized calcine. Since the chelating part of calcine resembles iminodiacetic acid (IDA), IDA should be a good choice.

The equilibrium of interest and the equilibrium constant remain as in equation 4-1. When the competitive, auxiliary ligand IDA is added, other equilibria need to be taken into account. The metal complexes that IDA can form and the acid-base characteristics of IDA must be accounted for in developing an expression for the equilibrium constant for metal binding to immobilized calcine.

Since the addition of IDA will affect the metal in solution, equation 4-1 needs to be modified by considering the fraction metal not bound to calcine. The fraction of metal in all forms other than bound to calcine, $\beta$, can be represented by the following:

$$\beta = \frac{a_m}{c_m}$$

where $a_m$ is the free metal at equilibrium, and $c_m$ is the total concentration of metal in all forms.

When equation 5-5 is solved for $a_m$ and substituted in the equilibrium constant expression in equation 4-1, the following expression is obtained for $K$: 
\[ K = \frac{M}{L_{m}c_{m}}. \]

\( \beta \) can be calculated from the following expression (42):

\[ \beta = \frac{1}{1 + K_{1}[L^{-3}] + K_{2}[L^{-3}]^{2}} \]

where \([L^{-3}]\) represents the equilibrium concentration of IDA in the \(L^{-3}\) form and \(K_{1}\) and \(K_{2}\) are the formation constants for the IDA-metal equilibria (41).

In addition, the acid-base characteristics of IDA must be taken into account. This can be done by calculating the fraction of ligand in the \(L^{-3}\) form. At a known, fixed pH, \(\alpha\), the fraction of ligand in the \(L^{-3}\) form is given by:

\[ \alpha = \frac{[L^{-3}]}{c_{l}} \]

where \(c_{l}\) is the total ligand concentration not complexed to metal. If the pH is known, \(\alpha\) can be calculated by the following expression (42):

\[ \alpha = \frac{1}{1 + K_{a1}[H^{+}] + K_{a1}K_{a2}[H^{+}]^{2} + K_{a1}K_{a2}K_{a3}[H^{+}]^{3}} \]

where \(K_{a1}, K_{a2}, K_{a3}\) are the acid dissociation constants for IDA.

When equation 5-8 is solved for \(L^{-3}\), a new expression for \(\beta\) is obtained:

\[ \beta = \frac{1}{1 + K_{a1}c_{l}\alpha + K_{2}(c_{l}\alpha)^{2}}. \]

Thus, \(\beta\) can be calculated at any concentration of IDA \((c_{l})\) if the pH is fixed and known. It is assumed that the only
metal-ligand complexes which are important to consider are metal-calcein and metal-IDA. This is a reasonable assumption because the experiments can be carried out at pH 7 and below, and the hydrolysis constants for the metals used and the constants for metal binding to components of the buffer system used are relatively small in comparison to the constants for binding to IDA.

The fluorescence intensity from the immobilized calcein depends on the relative amounts in the complexed (nonfluorescent) and uncomplexed (fluorescent) forms. As the amount of competing ligand is increased, $\beta$ decreases causing a change in the conditional binding constant. (See equation 5-6.) Experimentally, the immobilized calcein is inserted into a solution of metal ion quencher. Fluorescence intensity is measured as a function of added IDA. The resulting data should be similar to the plot shown in Figure 42A. Fluorescence intensity increases when sufficient IDA is added to pull the quenching metal ion away from the immobilized calcein. At the midpoint in the curve, $ML = L$ and from expression 5-6, since $ML/L = 1$:

$$K = \frac{1}{c_m \beta}.$$  \hspace{1cm} 5-11

The equilibrium constant can be determined from the experimental plot using calculated values of $c_m$ and $\beta$ at the half way point. A linear method of finding the value of $\beta$ at the half way point can also be used and is illustrated in Figure 42B. When logarithms are taken of
Figure 42: A: Plot of the expected results of competitive binding expressed as relative fluorescence intensity as a function of \(-\log \beta\).

B: A linear method of finding \(\beta\) at the half way point represented as \(-\log ML/L\) as a function of \(-\log \beta\). (See equation 5-12.)
equation 5-6 and the expression is rearranged, the following expression results:

\[-\log \frac{ML}{L} = -\log K - \log \beta - \log c_m \quad 5-12\]

The relative amounts of ML and L are measured from the plot. When \(-\log \frac{ML}{L}\) is plotted versus \(-\log \beta\) a straight line should result and the x intercept will be the value of \(-\log \beta\) at the half way point since \(-\log \frac{ML}{L} = 0\) or \(ML/L = 1\).

Either method can be used to find the value of \(\beta\) at the half way point. Once this value is found, \(K\) can be calculated using the following form of equation 5-12:

\[\log K = -\log \beta - \log c_m \quad 5-13\]

The equilibrium constant determined in this way will still be a conditional constant, which will depend on the acid-base characteristics of immobilized calcein.

Experimental

Reagents

Calcein-W (indicator grade) and recorcinol were purchased from Fisher Scientific. Cyanuric chloride and 4-nitrophthalic acid (80%, remainder is 3-nitrophthalic acid) were purchased from Aldrich.

Attempted Synthesis of Aminocalcein

Preparation of nitrofluorescein. The preparation of nitrofluorescein was accomplished by a modification of a
previous procedure (43,44). Equal masses (4.00 g) of 4-nitrophthalic acid and recorcinol were mixed and heated in an oil bath at 200°C for 2 hours. The dark red-brown mass was cooled and chipped from the beaker and mixed with HCl (63 mL, 0.6 N) and boiled for 1 hour. The solution was decanted and the precipitate was washed with three 12 mL portions of hot 0.6 N HCl and then filtered using a Buchner funnel. The solid was dried in a 100°C oven for 1 hour yielding 4.26 g (0.011 mol, 60% yield) of product. Elemental analysis (C,H,N), melting points, and an IR spectrum of the product were obtained.

**Attempted preparation of nitrocalcein.** Preparation of nitrocalcein from nitrofluorescein was attempted by using a modification of the procedure for preparation of calcein from fluorescein (30,32). Nitrofluorescein (3.78 g) was dissolved in 10 mL of 95% ethanol, 3 mL 30% NaOH (w/v), and 3 mL deionized water. The mixture was stirred and chilled to 0°C in a 50 mL round bottom flask fitted with a reflux condenser. Iminodiacetic acid (disodium salt, 4.33 g) was dissolved in 4 mL 30% NaOH and 4 mL deionized water. The iminodiacetic acid mixture was added to the chilled nitrofluorescein mixture. The solution was then chilled to 0°C. Formaldehyde (2.43 g, 37%) was added dropwise with stirring. The temperature was slowly raised to 60-70°C and kept there for 6 hours. The mixture was cooled to room temperature for 1 hour, diluted to 100 mL deionized water. The precipitate was then heated with 60 mL 50% ethanol and stirred
for 2 hours while heated. The solution was filtered hot and the red-orange crystals which formed were filtered on a Buchner funnel and allowed to air dry. Elemental analysis (C, H, N), melting points, and an IR spectrum of the product were obtained.

**Attempted reduction of nitrofluorescein to aminofluorescein.** Two reduction methods (43) were used in an attempt to reduce nitrofluorescein to fluoresceinamine with the hope that the same procedure could be used to reduce the nitrocalcine product. Because the nitrocalcine product was a mixture of unknown composition, the reduction methods were not used on this product. In the first procedure nitrofluorescein (2.0 g) was refluxed with 100 mL 5% sodium dithionite for 24 hours. The solution turned from orange to deep red and was cooled to room temperature. The solution was acidified with glacial acetic acid (5 mL) and a dark red precipitate formed. The precipitate was filtered on a glass filter and then refluxed in 6% HCl and filtered hot. The solution was cooled and then reheated and allowed to cool overnight. A small amount of precipitate dropped out of solution. The solution was reheated to boiling, and allowed to cool. The solution was then acidified and then 30% NaOH was added until a precipitate formed. The precipitate was filtered and dried at room temperature. A melting point and IR spectrum of the product were obtained. This material was also immobilized on controlled pore glass as fluoresceinamine was in
Chapter III.

In the second method sodium sulfide (Na₂S·9H₂O, 1.15 g) and 20 mL deionized water were mixed and nitrofluorescein (0.5 g) was added. When the nitrofluorescein was dissolved, sodium sulfhydride (NaHS·xH₂O, 0.54 g) was added and the mixture was refluxed for 24 hours. The mixture was allowed to cool and was acidified with glacial acetic acid. The precipitate was filtered on a Buchner funnel and then was dissolved in 8 mL 6% hot HCl and filtered hot through a glass filter. The solution was boiled again and allowed to cool. Potassium hydroxide (1 M) was added and a precipitate formed which was filtered and dried at room temperature.

**Attempted Synthesis of Aminocalcein on Controlled Pore Glass**

Because of the apparent failure of the synthesis of nitrocalcein which made the completion of the synthesis of aminocalcein unfeasible, and the ease with which fluoresceinamine can be bound to controlled pore glass (see Chapter III) the reaction of fluoresceinamine immobilized on glass with iminodiacetic acid was attempted in order to produce aminocalcein on glass.

Fluoresceinamine immobilized on glass (10 mg) was added to a solution of iminodiacetic acid (disodium salt, dihydrate, 2.0 g) in deionized water (25 mL) and 65% ethanol (20 mL). The mixture was chilled to 4°C and formaldehyde (1.5 mL) was added dropwise. The temperature of the solution was increased to 70°C for 2 hours. The
solution was cooled and the glass was filtered, washed with 100 mL of deionized water, and dried by suction. To test the chelating ability of the product, its fluorescence was observed under UV light with and without Cd(II) and Co(II) at pH 12.

**Immobilization of Calcein-W on Cellulose**

Calcein-W was immobilized on powdered cellulose using cyanuric chloride using the same procedure as for fluoresceinamine and morin (Chapters III and IV). The concentration of the calcein soaking solution was 0.0583 g/20 mL acetone.

**Determination of Amount of Calcein Bound**

Calcein was stripped from the cellulose with KOH (1 M) and the absorbance measured using spectrophotometry following the procedure used for morin (Chapter IV). Standards ranging from 3.24 x 10^-6 to 1.08 x 10^-5 M calcein in 1 M KOH were made by diluting amounts of calcein stock solution (2.7 x 10^-5 M ethanol-water) in 25 mL volumetric flasks with 1 M KOH. Weighed amounts of the immobilized calcein (0.0127 and 0.0127 g) were soaked in 1 M KOH for 2½ hours. Standards and samples remained in contact with KOH for comparable amounts of time as the calcein absorbance degrades with time in strong base (38). The absorbance was measured at 500 nm.

**Fluorescence Spectra**

In order to determine the excitation and emission
maxima, fluorescence excitation and emission spectra were measured for calcein in solution on the Perkin-Elmer 204 spectrofluorometer at low, neutral, and high pH.

Fluorescence excitation spectra were measured for the immobilized calcein on the optic surface by using the SLM spectrofluorometer. The ratio mode was used in recording the spectra. The emission wavelength was selected with an interference filter with maximum transmittance at 520 nm. Spectra were measured at pH 1.26, 3.63, 5.20, and 7.16 using 0.1 M acetate buffers and Tris buffers, respectively, to control pH. Spectra were measured for calcein in solution for comparison at the same pH values as well as at pH 9.11, 11.27, and 12.61 by using 0.1 M borate buffer, phosphate buffer, and KOH, respectively, to control pH.

Response to Metals

The response to Cu(II), Co(II), and Ni(II) was determined for immobilized calcein in solution at pH 5.2 and 7.0 (0.1 M acetate and Tris buffers, respectively) using the SLM spectrofluorometer. The ratio mode was used to compensate for drift in source intensity. The excitation wavelength used was 485 nm. The emission wavelength was selected using an interference filter with maximum transmittance at 520 nm for the immobilized calcein. A long pass filter with maximum transmittance at 590 nm (87%), 1% transmittance at 500 nm, and 80% transmittance at 533 nm was used for solution measurements. The monochromator slit width was set at 8 nm for the immobilized calcein and
2 nm for calcein in solution. Measurements were made on immobilized calcein (1 mg) as the optic was immersed in buffer solution (15 mL) and microliter amounts of Cu(II) sulfate (0.00115 M), Co(II) nitrate (0.00113 M), and Ni(II) nitrate (0.00150 M) were added. Solution measurements were made by adding microliter amounts of the metal solutions to calcein (6.6 x 10^{-6} M) in buffer (3.5 mL) in a cuvette.

The response of immobilized calcein as an indirect sensor for Zn(II) was tested by adding Co(II) nitrate (25 µL, 0.113 M) to immobilized calcein in 15 mL Tris buffer (0.1 M) at pH 7.06. Fluorescence intensity was measured as microliter amounts of Zn(II) chloride (0.0101 M) were added. The fiber optic fluorometer was used for this study. The excitation and emission wavelengths were selected using interference filters with maximum transmittance at 488 nm and 520 nm, respectively.

**Determination of Binding Constants**

The constants for Cu(II), Co(II), and Ni(II) binding to both immobilized and dissolved calcein were determined by competitive binding using the SLM spectrofluorometer. The excitation and emission wavelengths and other instrumental parameters were the same as in the response experiments described previously. Fluorescence of immobilized calcein on the optic surface was measured with the optic immersed in 15 mL of buffer containing a known concentration of the metal, typically on the order of 10^{-5} M. Then stock iminodiacetic acid solution (0.300 M at the same pH
as the buffer) was added in microliter amounts and fluorescence intensity was measured after the response became constant. Solution measurements were made using calcein \((3.34 \times 10^{-6} \text{ M})\) in \(3.5 \text{ mL}\) of buffer solution in a cuvette in the presence of metal ion. Microliter amounts of iminodiacetic acid were then added.

**Immobilized Calcein as an Endpoint Detector**

The use of immobilized calcein as an endpoint detector in the EDTA titration of Cu(II) was illustrated by immersing the optic with immobilized calcein into a buffer (0.1 M Tris, pH 7, 15 mL) containing Cu(II) \((5.75 \times 10^{-6} \text{ mol})\). Fluorescence measurements were made using the SLM spectrofluorometer as EDTA \((0.16 \text{ M}, \text{pH 7.0})\) was added in microliter amounts. The excitation and emission wavelengths were set as in the previous experiments and the ratio mode was used. Fluorescence was measured as a function of volume of titrant added.

**Results and Discussion**

**Attempted Synthesis of Aminocalcein**

During the attempted synthesis of aminocalcein, it was found that Calcein-W, which was available commercially, could be bound to powdered cellulose via cyanuric chloride. Consequently, there was no need to continue with the preparation of aminocalcein. Although the synthesis was not pursued to completion, the synthesis of aminocalcein was not a complete failure.
The first step of the synthesis, preparation of nitrofluorescein, did succeed as results of elemental analysis and IR spectra show. Elemental analysis obtained was 59.20 %C, 3.75 %N, and 2.85 %H as compared with the theoretical 63.66 %C, 3.71 %N, and 2.93 %H ($C_{20}$H$_{11}$N$_{1}$). The product did not melt up to 330°C, but darkened upon heating. According to the literature, nitrofluorescein does not melt up to 350°C and darkens (44). The IR spectrum of the product differed considerably from the IR spectrum of the starting materials and after reduction was almost identical to that of fluoresceinamine. The spectrum of the product after reduction is shown in Figure 43. (For spectra of starting materials and fluoresceinamine see Sadtler Spectra (45).)

The product of the second step differs from nitrofluorescein, but it is not conclusively nitrocalcein. The IR spectrum of the product is shown in Figure 44. Elemental analysis shows that the product is not nitrofluorescein, but not pure calcein as the composition differs from what is predicted for pure calcein (Found: 62.43 %C, 4.00 %N, 3.13 %H; theoretical: 53.81 %C, 6.28 %N, 4.04 %H; $C_{30}O_{15}$H$_{27}$N$_{3}$). It appears that the product is not one pure compound, but a mixture. This could have been checked with HPLC or TLC if the synthesis was to be carried further. The apparent reason that a mixture was obtained is that the reaction did not go to completion, resulting in some nitrofluorescein with no iminodiacetate groups, some with one
Figure 43: IR spectrum of reduction product of nitrofluorescein.
Figure 44: IR spectrum of the product of the reaction of nitrofluorescein condensed with iminodiacetic acid and formaldehyde.
iminodiacetate group, and some with two iminodiacetate groups.

Because the product from step two was evidently a mixture, the synthesis was not carried further using this product. Two reduction methods (using sodium dithionite or sodium sulfhydrate) were applied to nitrofluorescein to produce fluoresceinamine in order that a procedure suitable for nitrocalcein be found. The first method, in which dithionite was used, appeared to be a success as the product has an IR spectrum similar to that of the expected product, fluoresceinamine. (See Figure 43.) The melting point of the product was 210-220°C (decomposes) as compared with the literature melting points 223°C (decomposes, Isomer I), 285°C (Isomer II) (40). The product was also successfully immobilized on controlled pore glass. The success of the second method was not determined.

The product of the synthesis of aminocalcein immobilized on controlled pore glass from immobilized fluoresceinamine was fluorescent when observed under UV light and remained fluorescent at pH 12. When Ca(II) was added the product remained fluorescent, but no increase in fluorescence was noted. The fluorescence of the product was quenched by Co(II). From these tests, the product did not appear to be immobilized calcein, since calcein should be nonfluorescent at high pH and fluorescence should increase when calcium is added. Another explanation is that immobilization changes the properties of calcein. Calcein
should be quenched by complexation with Co(II), but the quenching effect of Co(II) does not prove calcein was produced because Co(II) would also quench the fluorescence of fluoresceinamine to some extent.

Response to Metals

The effect of Cu(II), Co(II), and Ni(II) on the fluorescence of immobilized calcein at pH 5 and 7 is shown in Figure 45. All three metals quench the fluorescence of the immobilized calcein as expected. At pH 7 the fluorescence intensity is higher than at pH 5, which is expected because the fluorescence intensity increases with pH as shown in Figure 46 (37). For all three metals at pH 7, the relative quenching is greater than at pH 5. This is probably due to the fact that the conditional binding constant is larger at higher pH. The response curves for the three metals differ both in the amount of curvature and in the amount of residual fluorescence at high metal concentration.

Calcein in solution behaves in a similar manner as shown in Figure 47. However, in solution the signal drops off to a negligible value: with immobilized calcein significant residual signal remains when metal ion is in excess. This signal is most likely due to light scattered and reflected off the support since the excitation and emission wavelengths are fairly close together. (See Chapter III.) Residual fluorescence of the complex may also contribute. When the calcein sensor is immersed in
Figure 45: Response of immobilized calcein as a function of A) Cu(II), B) Co(II), and C) Ni(II) concentration at pH 5 (△) and pH 7 (□).
Figure 46: Variation of fluorescence of free calcein with pH (37).
Figure 47: Response of dissolved calcein as a function of A) Cu(II), B) Co(II), and C) Ni(II) concentration at pH 5 (△) and pH 7 (□).
EDTA, fluorescence is restored. However, it does not change when the sensor is placed in a fresh buffer, indicating that strong complexes are formed.

If immobilized calcein were to be used as a sensor, the range would be limited as seen in Figure 45. The rapid decrease in intensity with added metal indicates that the binding constants for the complexes formed are relatively large. A more quantitative determination of the binding constants follows.

**Determination of Binding Constants**

The fluorescence intensities obtained and metal ion concentrations are plotted in the form of the linear model using data for Cu(II) in Figure 48. (See equation 5-7.) The obvious lack of fit indicates that the model is not adequate to describe this system. The probable explanation is that the assumption that the insertion of the sensor does not significantly affect concentration of metal ion in solution is not valid. This confirms that the binding constants are relatively large and can not be determined by this method.

Representative results for the competitive binding experiments to determine the constants for Cu(II), Co(II), and Ni(II) binding to immobilized calcein at pH 5 and 7 are shown in Figure 49. Similar results for calcein in solution are shown in Figure 50. The constants which were calculated from the curves using equation 5-15 are listed in Table 6. The uncertainty in the values for the
Figure 48: A plot of the reciprocal of the relative fluorescence intensity as a function of Cu(II) concentration at pH 7 to test the theoretical model represented in equation 5-4.
Figure 49: A plot of representative results showing relative fluorescence intensity as a function of $-\log \beta$ for immobilized calcein in the presence of Cu(II) at pH 7.
Figure 50: A plot of representative results showing relative fluorescence intensity as a function of $-\log \beta$ for dissolved calcein in the presence of Co(II) at pH 7.
Table 6:
Values for the Conditional Binding Constants for Immobilized and Dissolved Calcein (Expressed as $\log_{10} K$).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Immobilized Calcein</th>
<th>Dissolved Calcein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.15</td>
<td>pH 6.95</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>9.4</td>
<td>12.4</td>
</tr>
<tr>
<td>Co(II)</td>
<td>5.9</td>
<td>8.0</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>6.6</td>
<td>11.1</td>
</tr>
</tbody>
</table>

*pH 5.05 and 7.05, respectively*
constants for immobilized calcein is due to experimental problems such as the difficulty with keeping the pH constant. A slight change in pH can slightly change the fluorescence intensity. Another problem is the large amount of IDA needed to obtain an increase in fluorescence to a level response. There is uncertainty in determining the midpoint of the curve from the graph. The linear method of finding the midpoint was tried and makes the determination easier, but the uncertainty remains.

As is evident by the results in Table 6, the constants are large enough so that immobilized calcein does not function as a true sensor. The constants for complexes formed by immobilized calcein are the same magnitude as those for calcein in solution. This indicates that the complexes formed are of the same type. The magnitude of the binding constants suggest the formation of chelates of the same type as those formed by IDA. IDA forms strong tridentate chelates with these metals (46). Calcein most likely forms tridentate chelates with these metals, with the possibility of terdentate chelates. Terdentate chelates would consist of an iminodiacetate group and a phenolic oxygen on the ring. The phenolic oxygen would be one not tied up in the immobilization procedure.

Although immobilized calcein can not be used as a true sensor, it can be used in other applications such as endpoint detection in titrations of metal ions (which will be discussed later) or as a chelating resin. The binding
constants for calcein-metal complexes are of the same magnitude as those for Chelex-100 (46). To be useful as a chelating resin a substance must have large binding constants. An advantage for immobilized calcein relative to Chelex-100 is that calcein will lose fluorescence when saturated with metal.

Determination of Amount of Calcein Bound

The number of immobilized calcein molecules/gram of cellulose is $6.1 \times 10^{-6}$ as determined by spectrophotometry. The absorption spectra for calcein and the product stripped from the cellulose are apparently identical and are shown in Figure 51. The calibration curve is shown in Figure 52. Since 1 mg of cellulose was typically used for fluorescence measurements, this represents $6.1 \times 10^{-8}$ mole of calcein on the optic surface when measurements were made.

Excitation Spectra

The excitation spectra of immobilized calcein at various pH values are shown in Figure 53 and are similar to the spectra for calcein in solution in Figure 54. The spectrum of the immobilized calcein changes with pH in the same manner as in solution. The differences between the spectra of the immobilized calcein relative to calcein in solution are that the spectra for immobilized calcein show a broadening and a slight red shift in excitation maxima. This was also evident in the morin-Al(III) system.
Figure 51: Absorbance spectra (in 1 M KOH) for calcein (A) and for calcein stripped from cellulose (B). (Absorbance is in arbitrary units and the spectra are placed above each other for comparison.)
Figure 52: A plot of absorbance as a function of calcein concentration for determination of amount of calcein stripped from cellulose. Experimental points for standards (*) are shown along with the Y predicted points for the samples and the least squares line.
Figure 53: Excitation spectra (corrected for variations in source intensity with wavelength) of immobilized calcein at pH 1.26 (△), pH 3.63 (□), pH 5.20 (☆), and pH 7.16 (◊).
Figure 54: Excitation spectra (corrected for variations in source intensity with wavelength) for dissolved calcein at A) pH 1.26 (△), pH 3.63 (□), pH 5.20 (★), pH 7.16 (○); B) pH 9.11 (△), pH 11.27 (□) and pH 12.61 (★).
Use of Immobilized Calcein as an Endpoint Detector

The use of immobilized calcein as an endpoint detector was demonstrated as an application of immobilized calcein. Cu(II) \((5.75 \times 10^{-6} \text{ mol})\) was titrated with EDTA at pH 7. The results which are presented as fluorescence intensity versus volume EDTA are shown in Figure 55. Fluorescence intensity increases slightly until around the calculated endpoint of 359 \(\mu\text{L}\). At this point the fluorescence increases sharply and continues to increase after the endpoint. It is not clear why the fluorescence intensity does not level off, but it could be a pH effect.

Response as an Indirect Sensor for Zn(II)

Zn(II) is reported to displace Co(II) from calcein in solution and cause an increase in fluorescence (37). For this reason the immobilized calcein-Co(II) complex was tested as a sensor for Zn(II). The response of the immobilized calcein-Co(II) complex to Zn(II) is shown in Figure 56. The fluorescence intensity increases as An(II) displaces Co(II), but the relative change is small. This is probably because the binding constant of calcein for Zn(II) is not substantially larger than it is for Co(II). The binding constants (expressed as \(\log K\)) for Zn(II) and Co(II) binding to IDA are 7.03 and 6.95, respectively (46). Because of the limited linear range and small change in signal, the system was not studied in detail.
Figure 55: A plot of relative fluorescence intensity as a function of EDTA concentration for immobilized calcein in the presence of Cu(II) at pH 7.
Figure 56: A plot of relative fluorescence intensity as a function of Zn(II) concentration for the immobilized calcein sensor in the presence of Co(II).
Immobilized calcein in the presence of a quencher ion could be used to determine ligands which may compete with calcein for the quencher.
CHAPTER VI

OTHER SYSTEMS STUDIED FOR USE AS SENSORS

Introduction

This chapter will briefly describe several systems which were studied as possible sensors, but were unsuccessful. These systems included 8-hydroxyquinoline, quinalizarin, and 2,2'-dihydroxyazobenzene whose structures are shown in Figure 57. All three are nonfluorescent and form fluorescent chelates with a number of metals (22). The objective in studying these systems is the same as for the systems already described: to immobilize the reagent and use the change in fluorescence behavior with metal ion as a means to determine the metal ion.

8-hydroxyquinoline is an attractive choice for a sensing fluorogenic reagent because it forms fluorescent chelates with many metals including Ag, Al, Mg, Ca, Cd, Zn, Sn, Sr, and Ba (22). 8-hydroxyquinoline has been immobilized via an azo linkage which kills fluorescence (17). If 8-hydroxyquinoline is immobilized using cyanuric chloride via the phenol group it will lose its complexing ability, since the phenol group is involved in complex formation. An approach to immobilization would be preparation of an amine derivative of 8-hydroxyquinoline for immobilization on controlled pore glass or powdered cellulose. A synthesis to form an amino derivative is shown in Figure 58.
Figure 57: Structures of A) 8-hydroxyquinoline, B) quinalizarin, and C) 2,2'-dihydroxyazobenzene.
Figure 58: Reaction sequence for synthesis of 5-amino-8-hydroxyquinoline.
Quinalizarin forms fluorescent chelates with metals such as Mg and Al (47,48). Quinalizarin, like morin (Chapter IV), can easily be immobilized on powdered cellulose via cyanuric chloride.

2,2'-dihydroxyazobenzene forms fluorescent chelates with metals such as Al, Ga, Se, Mg, In (220, and was immobilized on silica gel using cyanuric chloride by Dr. M. Ditzler at Holy Cross. A batch of immobilized reagent was obtained from his laboratory for study as a metal ion sensor.

Experimental

Reagents

8-hydroxyquinoline, sodium nitrite, and sodium di-thionite were purchased from Fisher. β-napthylamine hydrochloride was purchased from Baker. Quinalizarin was a product of Schering-Kahlbaum (A.G., Berlin).

Synthesis of 5-amino-8-hydroxyquinoline

β-napthylamine hydrochloride (8.975 g, 0.05 mol) was dissolved in 25 mL of water and cooled in an ice bath. After concentrated HCl (6 mL) was added the temperature was maintained at 5°C. Sodium nitrite (3.45 g, 0.05 mol) was added very slowly to the napthylamine mixture, keeping the temperature below 5°C. This took 1 hour. Extra sodium nitrite was added until starch iodine paper turned blue. A solution of 8-hydroxyquinoline (7.25 g, 0.05 mol/20 mL) in 6% NaOH was added. A purple product resulted which was
filtered using a Buchner funnel. Half of the product was mixed with 50 mL of 5% sodium dithionite and heated to 45°C for 10 hours. Since no change was noted, an additional 25 mL of 5% sodium dithionite was added and the mixture was heated to 45°C for 7 hours. Then the temperature was raised to 60°C for 1 hour. The liquid was removed and 30 mL of 5% sodium dithionite was added and heated to 80°C for 8 hours. The product changed from purple to brown. The liquid was removed and the product was filtered with a Buchner funnel, washed with 500 mL of water and dried at room temperature. The product should be a mixture, but no separation was attempted.

**Immobilization of Quinalizarin and Determination of Response to Al(III)**

Quinalizarin was immobilized on filter paper and powdered cellulose by the same procedure as for fluorescein-amine, morin, and calcein. The product was pink with orange fluorescence that was visible under UV light.

The response of quinalizarin immobilized on cellulose to Al(III) was determined using the fiber optic fluorometer with excitation and emission wavelengths of 460 and 550 nm selected by interference filters. The wavelengths were based on excitation and emission spectra for dissolved quinalizarin. Fluorescence intensity was measured as micro-liter amounts of aluminum sulfate (0.01 M) were added to the sensor in 15 mL distilled water.
Determination of the Response of Immobilized 2,2'-dihydroxyazobenzene to Al(III)

The response of 2,2'-dihydroxyazobenzene immobilized on silica gel was determined using the fiber optic fluorometer with excitation and emission wavelengths selected by interference filters with maximum transmittance of 505 and 550 nm, respectively. The selection of wavelengths was based on spectra of the the immobilized reagent which were obtained at Dr. Ditzler's laboratory. Fluorescence intensity was measured as microliter amounts of aluminum sulfate (0.01 M) were added to the sensor in 15 mL of distilled water.

Results and Discussion

All three of the systems studied were not useful as metal ion sensors. The synthesis of 5-amino-8-hydroxquinoline did not appear to be successful and was undertaken using other methods by another person in our research group, Robert Hudson, who was also unsuccessful (49). The use of 8-hydroxyquinoline as a sensor is still desirable. It has recently been immobilized as 8-hydroxyquinoline-5-sulfonic acid on anion exchange membrane and is being studied by another research group member, Zhang Zhujun.

Immobilized quinalizarin and 2,2'-dihydroxyazobenzene both have high background fluorescence and show a small relative increase in fluorescence with added Al(III) ion. The high background fluorescence could be due to impurities in the reagents or fluorescence of the ligand as a result.
of immobilization. Because immobilized morin responds more sensitively to Al(III) it was studied as a sensor for Al(III) instead of either quinalizarin or 2,2'-dihydroxyazobenzene. For this reason further study on these reagents was not undertaken. Either system may still serve as a sensor for other metal ions since, by proper selection of wavelengths, the background fluorescence may be decreased relative to the fluorescence of the complexes formed.
CHAPTER VII

CONCLUSIONS AND FUTURE WORK

The sensors based on immobilized fluoresceinamine and immobilized morin offer several advantages in relation to conventional solution fluorescence methods. The sensors do not perturb the sample and can be used for continuous sensing. Immobilization, however, results in some undesirable properties. The fluorescence intensity of fluorescein is lowered upon immobilization due in part to concentration quenching. This causes a lower sensitivity. The use of immobilized morin for the determination of Al(III) or Be(II) results in poorer detection limits than can be attained in solution. This is mainly due to variations in background fluorescence.

Immobilized calcein has been shown to form strong complexes with a number of metal and does not function as a true sensor. Immobilized calcein can be used for other applications which take advantage of calcein's strong affinity for metals and change in fluorescence with metal binding. One application is the use of immobilized calcein as a chelating resin for separation or concentration of metals. An expected advantage for immobilized calcein, which has binding constants similar to Chelex-100 (46), is that immobilized calcein's loss of fluorescence will indicate saturation of the resin with metal. Other applica-
tions for immobilized calcein which take advantage of the strong chelating ability are use as an endpoint detector in chelatometric titrations and use for determination of metal binding constants for ligands which compete with calcein for metals.

The support used for immobilization and its attachment to the fiber optic should be improved. Powdered cellulose was used as a support instead of controlled pore glass because of the higher intensity obtainable when similar amounts are immobilized to each support. A greater number of reagents can be immobilized on powdered cellulose than on controlled pore glass. Reagents immobilized on cellulose via cyanuric chloride, however, are subject to hydrolysis at high pH. Both powdered cellulose and controlled pore glass cause scattering, resulting in a relatively high background. The problem with attachment of these supports to the end of the optic have already been mentioned.

Solutions to these problems might involve choice of a support which is clear and easy to attach to the optic. The immobilized reagent should be stable to cleavage. A clear film which can be held in place on the end of the optic would offer advantages over supports which need to be glued to the surface. A support with the same refractive index as water would reduce scattering. A support which has these advantages is anion exchange membrane which has recently been used in our research group for immobilization of anionic fluorogenic reagents. Other alternatives are
immobilization on polyvinyl chloride, polyvinyl alcohol, cellulose dialysis tubing, or cellophane (which is transparent, flexible cellulose).

In future work the instrumentation can be modified for use with other fluorogenic reagents. Fused silica fiber optics which transmit in the UV widen the choice of fluorogenic reagents which can be used. A trifurcated fiber optic which has one silica and two glass arms has recently been acquired and is being used. A more intense source is desirable in some cases; this has also been purchased.

In general, the metal sensing fluorogenic reagents used in this research were nonfluorescent and became fluorescent with added metal ion (or were nonfluorescent and were quenched with added metal ion). For future work the choice of reagents need not be limited to this type. Any change in fluorescence behavior with pH or metal ion may be utilized. For example, a change in emission wavelength with changes in pH or metal ion concentration can be used if emission intensity is monitored at two appropriate wavelengths and a ratio of intensities is taken. This is currently being investigated by our research group.
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17. Pierce Catalog, 1979.


