Topics in Fluorescence Spectroscopy

Volume 10
Advanced Concepts in Fluorescence Sensing
Part B: Macromolecular Sensing

Topics in Fluorescence Spectroscopy

Edited by JOSEPH R. LAKOWICZ and CHRIS D. GEDDES

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Volume 10: Advanced Concepts in Fluorescence Sensing
Part B: Macromolecular Sensing

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Edited by

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PREFACE

Over the last decade fluorescence has become the dominant tool in biotechnology and medical imaging. These exciting advances have been underpinned by the advances in time-resolved techniques and instrumentation, probe design, chemical / biochemical sensing, coupled with our furthered knowledge in biology.

Ten years ago Volume 4 of the Topics in Fluorescence Spectroscopy series outlined the emerging trends in time resolved fluorescence in analytical and clinical chemistry. These emerging applications of fluorescence were the result of continued advances in both laser and computer technology and a drive to develop red/near-infrared fluorophores. Based on the advancements in these technologies, it was envisaged that small portable devices would find future common place in a doctor's office or for home health care.

Today, these past emerging trends in fluorescence sensing are now widely used as either standard practices in clinical assessment or commercialized health care products. Miniature lasers in the form of laser diodes and even light emitting diodes are widely used in applications of time-resolved fluorescence. Computer clock-speed is now not considered a hurdle in data analysis. Even our choice of fluorophores has changed dramatically in the last decade, the traditional fluorophore finding continued competition by fluorescent proteins and semi-conductor quantum dots, to name but just a few.

This volume "Advanced Concepts in Fluorescence Sensing: Macromolecular Sensing" aims to summarize the current state of the art in fluorescence sensing. For this reason we have invited chapters, encompassing a board range of macromolecular fluorescence sensing techniques. Chapters in this volume deal with macromolecular sensing, such as using GFP, Aptamers and fluorescent pebble nano-sensors. This volume directly compliments volume 9 of the Topics in Fluorescence Spectroscopy series, which deals with advanced concepts in small molecule fluorescence sensing.

While many of the changes in recent fluorescence have been well received, its continued growth in the world has created a challenge in trying to archive and document its use. Subsequently Chris D. Geddes has now become co-series editor of the Topics in Fluorescence Spectroscopy series. We have also recently launched the Reviews in Fluorescence series, which co-edited also by Dr's Geddes and Lakowicz and published annually, is meant to directly compliment the Topics in Fluorescence Spectroscopy series, with small chapters summarizing the yearly progress in fluorescence.

Finally we would like to thank all the authors for their excellent contributions, Mary Rosenfeld for administrative support and Kadir Aslan for help in typesetting both volumes 9 and 10.

Chris D. Geddes
Joseph R. Lakowicz
Baltimore,Maryland, US.
August 2004

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PROTEIN-BASED BIOSENSORS WITH POLARIZATION TRANSDUCTION

Richard B. Thompson[1]

1.1. INTRODUCTION

Fluorescence-based biosensors employing biological recognition molecules such as proteins offer unmatched selectivity and sensitivity for real time determination and imaging of analytes such as metal ions. In some cases the analyte can be quantitated by changes in fluorescence anisotropy (polarization) which offers all the advantages of classical ratiometric techniques, as well as certain optical advantages, especially for microscopy. This chapter introduces the principles of such sensors and displays some examples of the results which may be obtained by their use.

1.2. PRINCIPLES OF OPERATION

The operating principles of anisotropy-based sensors are fairly straightforward, which is perhaps unsurprising given the relatively simple physics of fluorescence polarization and its measurement (Joseph R. Lakowicz, 1999). Fluorescence emission may be polarized, the degree of which can be described quantitatively by the polarization, p, or the anisotropy, r. The polarization and anisotropy are calculated from observed fluorescence intensities through polarizer(s) oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the plane of polarization of the excitation **Figure 1.1**):

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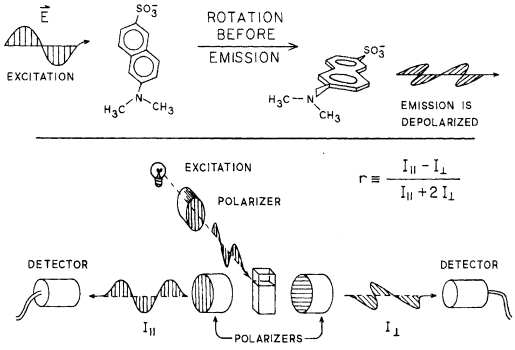

Figure 1.1. Principle of fluorescence anisotropy / polarization.

Anisotropy and polarization are calculated:

$$r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2\, I_{\perp}) = [(I_{\parallel} / I_{\perp}) - 1] / [(I_{\parallel} / I_{\perp}) + 2] \quad (1)$$

$$p = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp}) = [(I_{\parallel} / I_{\perp}) - 1] / [(I_{\parallel} / I_{\perp}) + 1] \quad (2)$$

The terms "polarization" and "anisotropy" are synonymous in this context and algebraically interconvertible, but the theory of polarized fluorescence is much simpler in terms of anisotropy than polarization, thus we shall employ the former term herein. Note that the term polarization appears in much of the older literature and currently in the high throughput screening and clinical immunoassay literature. Polarization of fluorescence is observed when fluorophores excited with plane polarized light do not rotationally diffuse significantly before emitting. Quantitatively, the degree of polarization may be predicted by the Perrin-Weber equation:

$$r_0 / r = 1 + (\tau / \theta_c) \quad (3)$$

where r_0 is the (excitation wavelength-dependent) limiting anisotropy in the absence of diffusion, r is the observed anisotropy, τ is the fluorescence lifetime, and θ_c is the rotational correlation time of the fluorophore, the inverse of the rotational rate. Rigorous treatments of the time-resolved decay of anisotropy, particularly of non-symmetrical fluorophores, are beyond the scope of this article and have been disclosed elsewhere (Joseph R. Lakowicz, 1999; Steiner, 1991). The rotational correlation time can be estimated:

$$\theta_c = \eta V / RT \quad (4)$$

where T is temperature, η is viscosity, R is the gas constant, and V is the volume of the

rotating unit. For more or less spherical macromolecules this can be approximated by the dimensions of the macromolecule, corrected for hydration. Thus a reasonably spherical 45,000 Dalton protein might exhibit roughly a 21 nsec rotational correlation time. For most of the assays it is unnecessary to know θ_c, rather one only need know it approximately to choose a fluorophore with the appropriate lifetime (see below).

To a first approximation, one can thus imagine assaying or detecting an analyte by changes in fluorescence anisotropy if the analyte somehow changes the rotational rate or lifetime of the fluorophore. While the shape and size of the fluorophore would appear to be properties of the molecule and therefore immutable, it is in fact commonplace to attach the fluorophore to a macromolecule and perturb the macromolecule's shape or size to change the anisotropy. Similarly, although the intrinsic lifetime of the fluorophore is a property of the molecule, many means are known for reducing the lifetime by quenching, and they result in measurable changes in anisotropy as well. The basic theory of the effects of changing either rotational or apparent lifetimes is presented below. Although fluorescence anisotropy has been used to look at "microviscosity" of lipid bilayers and micelles as a means of understanding their dynamics (Shinitzky & Barenholz, 1978), as well as assessing fetal lung maturity by the microviscosity of the surfactant, these essentially are studies of the bulk properties of the fluorophore's surroundings, and not of analytes *per se*, and will not be considered further. Similarly, there are many well-known enzyme assays based on fluorescence polarization, which usually rely on a change in size between substrate and product (e.g., protease assays) to provide signal. For the most part, these cannot provide a continuous readout of the level of the analyte, and also will not be considered further.

1.3. ADVANTAGES OF ANISOTROPY-BASED SENSING

Among fluorescence-based sensing approaches, anisotropy-based sensing has a unique portfolio of advantages. First, it is a steady-state (as opposed to a time-resolved) measurement, which requires only simple instrumentation. It can be readily configured in the "T-format"as a true ratiometric measurement (**see Figure 1.1**) as described by Weber nearly fifty years ago to avoid the noisy excitation sources then available (Weber, 1956)(Equations 1 and 2). The advantages of fluorescence ratiometric measurements are widely appreciated based on the use of ratiometric calcium and pH indicators. They include (to a first approximation) immunity from fluctuations in the light source, variations in indicator amount due to bleaching or washout (but see Dinely, et al., for an important caveat regarding indicator amounts) (Dinely, Malaiyandi, & Reynolds, 2002), variations in cell thickness or indicator distribution, and potentially facile calibration *in situ* (Nuccitelli, 1994). Several groups have constructed fluorescence polarization microscopes (Axelrod, 1989; Dix & Verkman, 1990)including confocal microscopes (Bigelow, Conover, & Foster, 2003)and demonstrated images with contrast based on variations in fluorescence polarization. While polarization can be measured merely by changing polarizers in a filter wheel apparatus, electrooptic devices offer the prospect of changing polarization at faster than video frame rates. An important potential advantage is that polarization requires no change of excitation

or emission wavelength, making it particularly well-suited for laser-excited microscopies such as confocal microscopy and multiphoton excitation microscopy. While few reports have appeared describing multiphoton excited fluorescence anisotropy sensing (Thompson, Maliwal, & Zeng, 2000), this technique could potentially be incorporated into an imaging application as well. While filtration to avoid scattered light is important, it is a further advantage that one may use the entire emission band for anisotropy measurements instead of a narrow band for wavelength ratiometric measurements. Finally, anisotropy-based sensing offers the possibility (in suitable cases) of an expanded dynamic range (see below)(Thompson, Maliwal, & Fierke, 1998).

One of the strengths of anisotropy-based sensing is its simplicity, since relatively few phenomena perturb the rotational diffusion rate of a molecule: changes in medium viscosity or temperature, as well as changes in molecular size or shape. It is usually trivial to control the temperature and bulk composition of the assay medium, or at least assure they do not change significantly during the short time of the assay. Similarly, few things can influence the lifetime of fluorescence, as this is an inherent property of the molecule. The important exception , as we will see, is fluorescence quenching, but under ordinary conditions most quenchers must be present at near millimolar concentrations to perturb the anisotropy of typical fluorophores with nanosecond lifetimes.

1.4. FLUORESCENCE POLARIZATION IMMUNOASSAY

The earliest example of determination of a chemical analyte by fluorescence anisotropy is the fluorescence polarization immunoassay introduced by Dandliker for antigens that are not macromolecules (Dandliker, Kelly, Dandliker, Farquhar, & Levin, 1973) In its simplest form this is configured as a competition assay wherein a sample containing an unknown amount of analyte is mixed with known amounts of antibody and a fluorescent-labeled analog of the analyte. The fluorescent-labeled analyte competes with the unlabeled for the binding site on the antibody; the bound fluorophores (molecular weight < 1000 Daltons) have their rotational diffusion substantially reduced by tight association with the relatively massive antibody (molecular weight of an IgG is c. 150,000 Daltons) **Figure 1.2**.

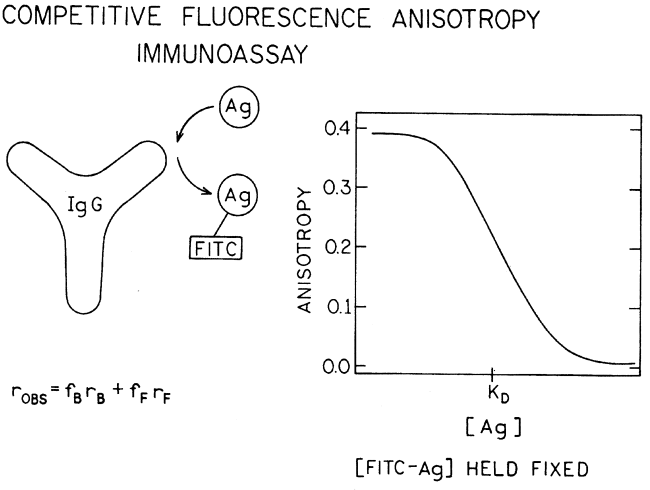

Figure 1.2. Principle of fluorescence polarization immunoassay. Antigen (Ag) in the sample competes with a fixed amount of fluorescent-labeled antigen (FITC-Ag) to bind to a fixed amount of antibody IgG.

For a label like fluorescein with roughly a four nanosecond lifetime, one can calculate using Equations 1 and 2 that the free labeled analyte should exhibit an anisotropy of roughly 0.05 and the bound form, 0.36. As the analyte concentration in the sample increases the proportion of bound fluorescent-labeled analyte decreases, and with it the anisotropy, which is just the arithmetic sum of the anisotropies of the labeled antigen in each form:

$$r_{obs} = r_f f_f + r_b f_b \tag{5}$$

where r_{obs} is the observed anisotropy; r_f and r_b are the anisotropies of the free and bound forms, respectively; and f_f and f_b are the fractions of fluorophore free and bound. If the apparent quantum yield changes upon binding, a small correction is necessary (Joseph R. Lakowicz, 1999).

The results of such an immunoassay are depicted in **Figure 1.3**. Evidently such analytes can be quantified by fluorescence polarization immunoassay at the micromolar level and below with good accuracy, if a fluorescent analog of the antigen can be made with similar affinity for the antibody. This technique is still in wide clinical use, particularly for analysis of drugs of abuse in urine specimens. The advantages of such immunoassays are that they are much faster than ELISA'a or RIA's because washing is unnecessary, that the same instrument can be used for a variety of analytes (if the fluorophores are spectrally similar), calibration is straightforward, and for analytes such as drugs of abuse it is difficult to adulterate specimens to produce a false negative. An important advantage is that no

modification or purification of the antibody is necessary, such that even antisera can be used, with significant cost savings.

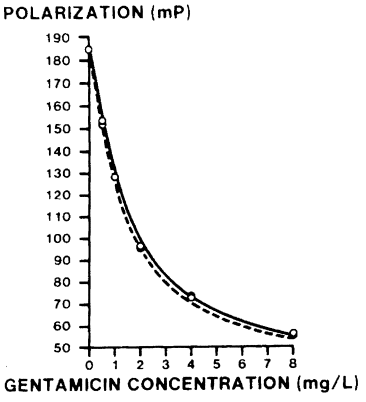

Figure 1.3. Fluorescence polarization immunoassay results for gentamicin; reproduced with permission from (Jolley et al., 1981).

Until recently, such immunoassays were unsuited for macromolecule antigens because the ratios of rotational correlation time to lifetime for free vs. bound antigen were not significantly different for typical nanosecond fluorophores **see Figure 1.4.** The figure depicts the expected anisotropies for a macromolecule antigen (serum albumin in this example, molecular weight 65,000) in the free form and bound to a high molecular weight antibody such as an IgM (molecular weight about one million) for two different label lifetimes. If the label lifetime is relatively short (4 nsec), the long rotational correlation time of the labeled antigen differs little in the free and antibody-bound states (0.02); however, if the label lifetime is longer (400 nsec), the anisotropy difference between free and bound is large enough (0.165) to be really useful. Evidently the anisotropy change will be significant only if the label lifetime is upwards of 100 nanoseconds. Fortunately, the Lakowicz group (Guo, Castellano, Li, & Lakowicz, 1998; Terpetschnig, Szmacinski, & Lakowicz, 1995) introduced the use of long-lived metal to ligand charge transfer (MLCT) probes to immunoassay, many of which have lifetimes approaching one microsecond. With such labels determination of macromolecule antigens can be done by fluorescence anisotropy immunoassay; the results of such an assay are depicted **in Figure 1.5**

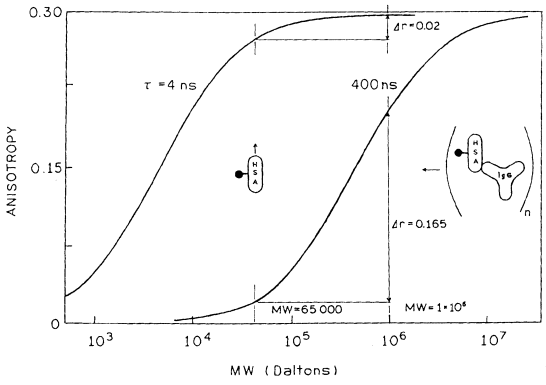

Figure 1.4. Simulated anisotropy as a function of molecular weight for two different fluorescence lifetimes: with a 4 nsec lifetime HSA exhibits a 0.02 anisotropy change upon binding to IgM; with a 400 nsec lifetime it exhibits a 0.165 change. Reproduced with permission from (Terpetschnig et al., 1995).

In this assay a solution of serum albumin labeled with a long lifetime (average lifetime in air = 2.75 microseconds) Re (I) complex is mixed with an anti-serum albumin IgG (mol. wt 150,000 Daltons), and challenged with samples containing varying concentrations of unlabeled serum albumin. The unlabeled antigen competes with the labeled antigen, effectively displacing it from the IgG, and resulting in an increasing proportion of the labeled antigen exhibiting the reduced rotational correlation time of the free form. The longer lifetime of this label and the smaller size of the IgG compared with the IgM in the simulation (**Figure 1.4**) results in reduced anisotropies of both forms, and more particularly a reduced difference (0.04) in the anisotropy between free and bound. Nevertheless, the difference in anisotropy is certainly usable, and these results indicate what the technique is capable of. .

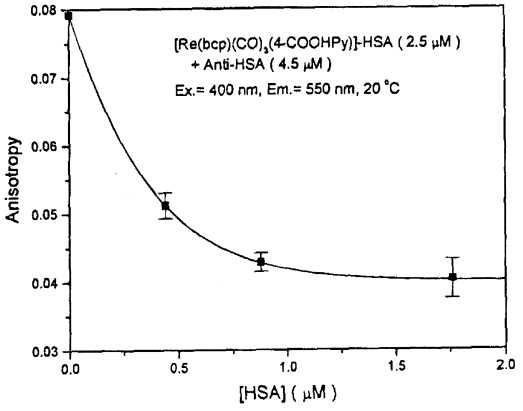

Figure 1.5. Fluorescence polarization immunoassay using a long-lived fluorescent label. Reproduced with permission from (Guo et al., 1998)

For applications requiring continuous measurements such immunoassays are not very useful, for two reasons. First, the fluorescent labeled analyte must be free to diffuse away from its binding site and diffuse rotationally. This makes it difficult to use in a continuous mode because of the risk of the small molecule diffusing away when the probe is exposed to the analyte. Also troublesome are the very slow dissociation rate constants of typical antibodies for their cognate antigens. The very slow off rate means that the binding reaction is often effectively irreversible. For instance, an antibody might have an affinity (K_d) for its cognate antigen of one picomolar (10^{-12} molar); the K_d is merely the ratio of association and dissociation rate constants for the antigen binding reaction. For a small molecule antigen (hapten) the association rate constant might be 10^7 to 10^8 M^{-1} sec^{-1} (nearly diffusion controlled) whereas for a macromolecule antigen, it might be 100-fold slower (10^5 M^{-1} sec^{-1}). Thus the dissociation rate constant in the former case would be 10^{-4} to 10^{-5} sec^{-1}, and for the macromolecule 10^{-7} sec^{-1} . The half-time of these antibody:antigen complexes is then just $0.693/k_{dissoc}$, or 1.9 to 19 hours for the hapten, and more than eleven weeks for the macromolecule antigen. One example exists of a recognition protein in a biosensor (carbonic anhydrase) being engineered to obtain more rapid association and dissociation constants to improve response times (Huang, Lesburg, Kiefer, Fierke, & Christianson, 1996), but to date we know of no antibody which has been molecularly engineered to improve binding kinetics.

1.5. ANISOTROPY-BASED METAL ION BIOSENSING

In general, it would appear difficult to determine the concentrations of most metal ions in solution by fluorescence anisotropy, since most metal ions of interest are not even photoluminescent in solution, and it clearly is difficult to raise antibodies to, for instance, free zinc or copper ions (but see (Darwish & Blake, 2002)). Fortunately, many metal ions can serve as quenchers by various mechanisms, and consequently can be made to report their presence by changes in fluorescence intensity (Fernandez-Gutierrez & Munoz de la Pena, 1985; White & Argauer, 1970), and in some cases, lifetime and anisotropy . For many classical metallofluorescent indicators such as hydroxyquinolines, interaction with metal ions may result in increases in quantum yield and lifetime (Szmacinski & Lakowicz, 1994). However, the lifetime of the indicator with either metal bound or in the absence of metal will typically be in the nanosecond range whereas its rotational correlation time is likely to be less than 100 psec, such that there is a negligible difference in anisotropy between the free and bound forms. Similarly, metallofluorescent indicators that detect analyte by collisional quenching exhibit decreased fluorescence lifetimes as well, but again the decline results in a negligible change in anisotropy for the same reason. Note that using an indicator with a very short lifetime confers no advantage because only very high concentrations of quencher are able to diffuse to and quench the indicator during its brief lifetime. Indicators which form a complex with the analyte are much more sensitive (Grynkiewicz, Poenie, & Tsien, 1985), but indicators of this sort which are quenched typically are completely quenched by the ligand, and consequently the bound form does not contribute to the emission.

We found that the issue of the rapid rotation of the fluorophore compared to its lifetime could be dealt with by attaching it to a macromolecule; moreover, the macromolecule could be chosen to bind particular metal ions with unmatched affinity and selectivity (Fierke & Thompson, 2001), and by judicious choice of the fluorophore, to provide high sensitivity, and even an enhanced dynamic range.

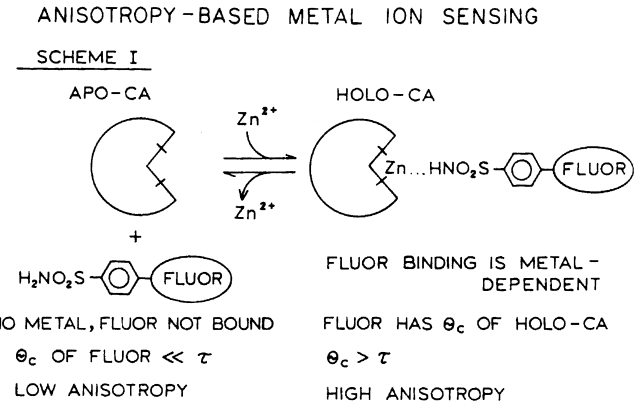

Figure 1.6. Schematic of zinc binding assay by fluorescence anisotropy. Binding of zinc promotes binding of a fluorescent aryl sulfonamide to the protein, increasing its rotational correlation time and anisotropy.

We have devised two methods for determining metal ions by fluorescence anisotropy. The first is based on metal-dependent binding of a fluorescent ligand to a protein, changing its rotational correlation time, the second by having the metal ion change the fluorescence lifetime of a fluorescent labeled protein. One can imagine elaborating either method to ligands other than metal ions. The first method takes advantage of the fact that aryl sulfonamide inhibitors of carbonic anhydrase exhibit much higher affinity for the protein in the zinc-containing holo form than the metal-free apo form. This is unsurprising inasmuch as much of the binding free energy comes from the (weakly acidic) aryl sulfonamide binding to the active site zinc ion as a fourth ligand, in the form of the sulfonamide anion (Maren, 1977) . Thus in the presence of zinc dansylamide binds relatively tightly (Kd ~0.8 uM) to holo carbonic anhydrase, but does not bind measurably to the apoprotein (Chen & Kernohan, 1967; Thompson & Jones, 1993). By binding to the protein the rotational diffusion of the fluorescent aryl sulfonamide is reduced and its anisotropy increases **Figure 1.6**.

Because the increase in rotational correlation time can be substantial (e.g., from perhaps 100 psec for a 500 Dalton inhibitor to 15 nsec for its complex with 30,000 Dalton protein), the increase in anisotropy can be dramatic: an example is shown for the sulfonamide BTCS **Figure 1.7**. In this case the fluorophore exhibits nearly a five-fold increase in its anisotropy upon binding, resulting in an anisotropy increase of 0.20. In terms of polarization this is 0.27, or 270 mP (a nomenclature used in describing polarization immunoassays). This is a substantial change, indicating that the assay can be very accurate. The detection limit

achieved for free zinc concentration in this example is a few picomolar, much better than has been achieved with small molecule indicators (Kimura & Aoki, 2001; Thompson et al., 2002; Walkup, Burdette, Lippard, & Tsien, 2000).

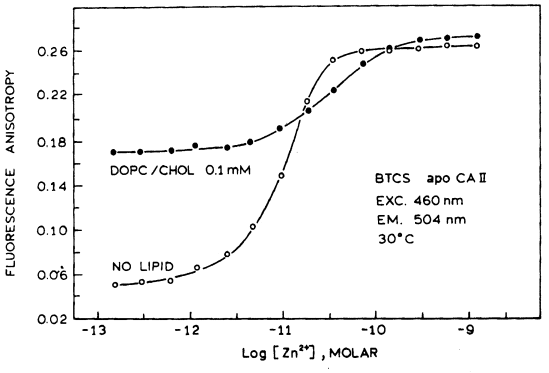

Figure 1.7. Zinc-dependent anisotropy of BTCS (benzothiazolyl coumarin sulfonamide) in the presence of apocarbonic anhydrase without (open circles) and with DOPC:Cholesterol unilammelar vesicles. Reproduced from (Thompson et al., 2000) with permission.

In some sense this approach has the defects of its virtues: of the metal ions which bind to the active site only Zn strongly promotes the binding of sulfonamides, which makes the assay quite specific. Cobalt(II) is much less effective in this regard (Sven Lindskog & Thorslund, 1968) and generally quenches most fluorophores, making it inaccessible for this type of assay. Other metals which bind to the protein do not promote sulfonamide binding, and cannot be measured by this approach; consequently, it is very specific for zinc.. Another issue is that several of the fluorescent aryl sulfonamides we and others (Chen & Kernohan, 1967) have developed for carbonic anhydrase-based Zn sensing exhibit large increases in their fluorescence lifetimes upon binding to the protein, which largely offset the increases in rotational correlation time, and therefore lead to only modest increases in anisotropy. Thus Dapoxyl sulfonamide exhibits a nearly twenty-fold increase in quantum yield and lifetime, which more than offsets the increase in rotational correlation time, leading to a negligible change in anisotropy. For best results the fluorescent sulfonamide should have only a small change in lifetime accompanying binding.

Some fluorescent aryl sulfonamides exhibit a substantial shift in their excitation or emission wavelengths, which allows the dynamic range of the assay to be expanded by choice of wavelength excited or observed (Thompson, Maliwal, & Fierke, 1998). Thus the sulfonamide ABD-M exhibits a sixty nanometer blue shift in its emission upon binding to the protein, enabling one to selectively observe the emission of a small proportion of the bound form in the presence of a large excess of the free form by looking on the blue side of the

emission, and *vice versa*. Therefore the range of analyte concentration which can be determined with a given level of accuracy may be adjusted by choice of excitation or emission wavelength. In the example illustrated **Figure 1.8**, free zinc concentrations ranging from 0.003 to 0.3 nM may be quantitated by observing emission at 450 nm, whereas concentrations from 0.2 to 3.0 nMmay be accurately quantitated at emission wavelength 590 nm. This is an advantage anisotropy-based sensing shares with lifetime-based sensing (Szmacinski & Lakowicz, 1993; Thompson & Patchan, 1995) which does not obtain with wavelength-ratiometric determinations.

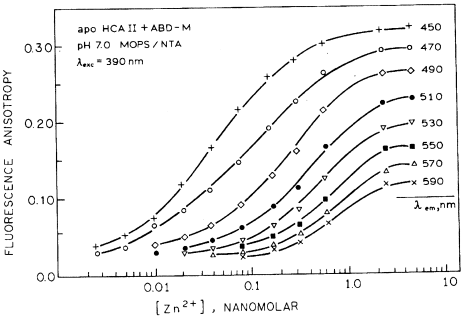

Figure 1.8. Zinc-dependent fluorescence anisotropies of apo-carbonic anhydrase plus ABD-N observed at different emission wavelengths. Reproduced from (Thompson, Maliwal, & Fierke, 1998)with permission.

One of the drawbacks of this particular approach is the difficulty of configuring these assays as sensors: e.g., capable of being inserted into a solution and *continuously* measuring the analyte. The issue arises in that the aryl sulfonamide and zinc ion must both be free to diffuse to and from their respective binding sites on the protein, but the zinc ion must come from the aqueous matrix in which the transducer is inserted. The problem is that whereas the analyte may diffuse into and out of the transducer vicinity, the sulfonamide may do the same thing, with irretrievable loss (Thompson & Jones, 1993). While ionophores capable of selectively transporting the zinc ion through membranes are known (Ammann, 1986), the ion flux through these membranes in the absence of an applied potential is modest. Attaching the sulfonamide to a macromolecule to co-entrap it within a dialysis membrane perforce reduces its rotational diffusion and increases its anisotropy, reducing or eliminating the anisotropy change upon binding to the protein.

A related approach which does not use a separate, diffusible molecule takes advantage of the fact that analyte-induced quenching of a fluorophore may reduce its lifetime, and thereby increase its anisotropy. If the fluorophore is attached to a macromolecule (which may also selectively bind to the analyte, for instance), the rotational correlation time of the macromolecule may more closely match the (nanosecond) lifetime of the fluorophore, resulting in a sizable change in anisotropy upon binding of the analyte (and quenching). **Figure 1.9**.

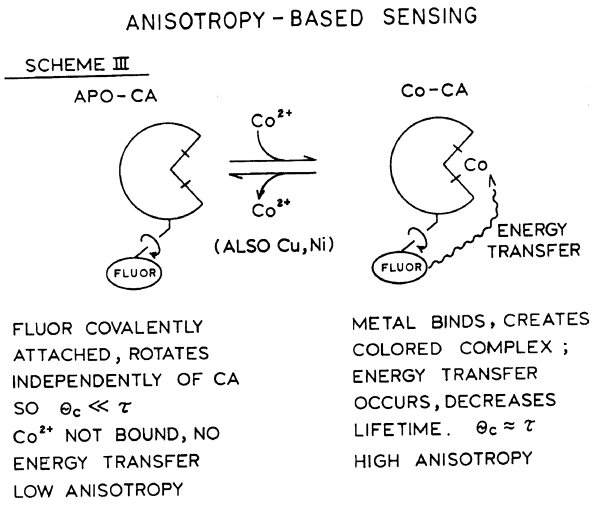

Figure 1.9. Principle of anisotropy-based sensing of metal ions employing quenching by the metal ion.

It is important that the quenching of the label emission not be quantitative; e.g., there must be some emission from the bound form or no change in the anisotropy will be observed. It is convenient that the quenching in these cases is by Förster energy transfer (Forster, 1948), which is a through-space interaction. For instance, when Co, Cu, or Ni bind to apocarbonic anhydrase, they exhibit weak d-d absorption bands in the visible and near-IR which can serve as energy transfer acceptors for fluorophores whose emission overlaps the absorption (S. Lindskog et al., 1971). Using the theory of Forster transfer, we can predict the change in lifetime of the fluorophore upon binding of the metal ion based on the known absorbance of the metal ion in the bound state and the position of the fluorescent label with respect to the metal center, and thus the anisotropy response of a sensor transducer given the (known) rotational correlation time (Thompson, Maliwal, Feliccia, Fierke, & McCall, 1998) . The results of such a prediction using carbonic anhydrase are shown in **Figure 1.10**. Evidently, very usable changes in anisotropy can be expected for a properly positioned fluorescent label (which may be accomplished by site-selective mutagenesis of the protein and selective labeling (Thompson, Ge, Patchan, & Fierke, 1996) with the right lifetime (in comparison to the rotational correlation time of the protein). We note that there is a tradeoff in response of the system due to efficient quenching: greater quenching efficiency upon analyte binding reduces the lifetime more and increases the change in anisotropy, but also the relative contribution of the bound form to the total emission decreases. A happy medium is about 75 % quenching, which in this case results in a 55 % increase in anisotropy, and which is controlled by the positioning of the label with respect to the metal center.

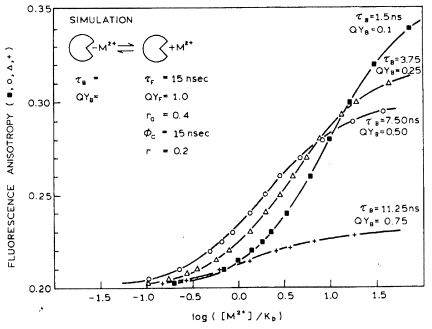

Figure 1.10. Simulated metal-dependent anisotropy of a 30 kD protein when binding of the metal results in 25% (+), 50 % (circles), 75% (triangles), or 90% (squares) decline in the lifetime of a 15 nsec lifetime label. Reproduced from (Thompson, Maliwal, Feliccia et al., 1998)with permission.

This approach works very well in practice, as illustrated in **Figure 1.11,** which illustrates the anisotropy response of a fluorescent-labeled apocarbonic anhydrase to a series of metal ions. The substantial changes in anisotropy indicate that the assay can be quite accurate. In this case the quenching of the fluorophore does not take place by energy transfer alone, as Cd and Zn do not exhibit d-d absorbance bands upon binding to the protein, but the quenching need only be partial and reduce the lifetime, it need not be by any particular mechanism. It is worthwhile noting that the selectivity and sensitivity of this sensor remains to be matched by any other (non-carbonic anhydrase-based) sensor.

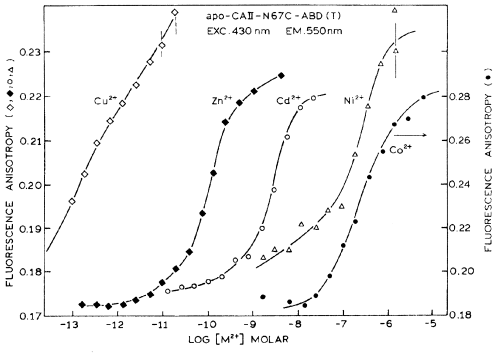

Figure 1.11. Cu- (open diamonds), Zn- (filled diamonds), Cd- (open circles), Ni- (triangles), and Co-dependent (filled circles) fluorescence anisotropies of apo-N67C-ABD-T. Reproduced from (Thompson, Maliwal, Feliccia et al., 1998) with permission.

It is also worth noting that the sensitivity and dynamic range of this (and other anisotropy-based approaches) is in part limited by the precision and accuracy with which anisotropy can itself be measured. For research-grade fluorometers and some microwell plate readers, accuracy and precision are usually within $\pm$ 0.002 . However, others have built instruments with much better precision and accuracy, and there seems no *ab initio* reason why the precision and accuracy of other instruments cannot be much better, particularly t-format instruments (Weber, 1956) or those using semiconductor sources and detectors.

Finally, we note that these approaches need not be limited to metal ions as analytes, although this has been the focus of our own interest. Thus the sulfonamide approach outlined above **in Figure 1.6** does not require the presence of a sulfonamide and is not necessarily limited to zinc or another metal, it only requires that the analyte's binding promote (or reduce) the binding of a fluorescent ligand to the macromolecule (which need not be a protein). Many examples of this are known or can be constructed (Kolb & Weber, 1975). Similarly, the second approach outlined in **Figure 1.9** does not require that the analyte be a metal or (evidently) quench by energy transfer. Energy transfer has the virtues of having a predictable, controllable response which is straightforward to engineer. Rather, the analyte (or a competitor in a competitive assay) need only partially quench the label on the macromolecule by binding to it. As interest grows in real time determination of a variety of analytes, particularly in imaging their concentrations within cells, the usefulness of these approaches will become more and more apparent.

1.6. ANISOTROPY-BASED SENSING OF OTHER ANALYTES USING PROTEINS AS TRANSDUCERS

The Lakowicz group has introduced enzymes and other proteins as anisotropy-based transducers for analytes other than metal ions, and developed a very clever approach that transduces the analyte level as a change in the observed fluorescence polarization of a device that occurs without a change in rotational diffusion or lifetime(J. R. Lakowicz et al., 1999). This approach effectively transduces an intensity change caused by the analyte to a fluorescent-labeled protein as a change in polarization by admixture of the emission from the protein with emission from a physically separate reference fluorophore having much higher anisotropy. The reference fluorophore is typified by an oblong fluorophore like Hoechst 33342 being entrapped in a polymer film which is stretched to orient the fluorophores, and which exhibits a polarization approaching 1.0. As the relative contribution of the fluorescence due to the analyte increases, the apparent net polarization declines. This is an effective approach, demonstrated using an iodo-anilinonaphthalene sulfonate-labeled variant of a glucose/galactose-binding protein (ANS-Q28C GGBP) isolated from *E. coli* to determine glucose: saturation with 6 uM glucose resulted in a 25 % decline in intensity in the ANS fluorescence, which induced a 0.15 increase in polarization. The value of this approach is that many examples of analytes or ligands inducing changes in fluorescent intensity of (for instance) labeled macromolecules are known, which could be converted to polarization based indicators by this method. In this sense it is akin to other external reference methods using

separate fluorophores emitting (or being excited at) different wavelengths. The drawback in these approaches is that when the reference fluorophore is not the same molecule as the transducing fluorophore (as it is in classical ratiometric indicators or the quenching-based anisotropy indicators described above), assorted influences such as bleaching, precipitation, or pH may affect the emission of the one and not the other, defeating the purpose. Most of these influences can be curtailed in macroscopic (cuvette-sized) embodiments, but this would appear harder to arrange in microscopic specimens.

This group has used a similar approach but with a different binding protein, lactate dehydrogenase noncovalently labeled with ANS, to measure lactate in the 0 to 240 uM range (normal clinical values are 1.0 to 1.8 mM). In this case saturation with lactate results in a 40% decrease in intensity (D'Auria, Gryczynski, Gryczynski, Rossi, & Lakowicz, 2000) . Noteworthy in this paper was the very simple apparatus they used to measure the apparent polarization of the sample essentially using a rotating polarizer to balance (null) the apparent intensity of the sample and a reference chamber containing the same labeled protein unexposed to lactate. This same nulling approach can even be performed by eye, with surprising accuracy. This improvement avoids the issue of the reference fluorophore behaving differentially to physical influences. More recently, the Lakowicz group used apoglucose dehydrogenase from a thermophilic organism with noncovalently bound ANS in a similar approach to measure glucose concentrations in the clinically relevant range (D'Auria, Cesare et al., 2000).

1.7. CONCLUSIONS

Based on the few results presented above, the field of anisotropy-based biosensors incorporating proteins as recognition molecules is obviously in its infancy. Yet the approach would appear promising, particularly for studies in cell biology where, in an approach akin to that which has proved so successful for calcium, one would wish to know the spatial and temporal distribution of an analyte within the cell. Powerful approaches have been developed for delivering to or expressing protein biosensors in various compartments of the cell (Schwarze, Ho, Vocero-Akbani, & Dowdy, 1999; Zelphati et al., 2001), so this no longer represents a barrier (R. Bozym and R. Thompson, unpublished results). A key issue for this class of sensors remains, however. While Hellinga has recently shown that binding proteins for small molecule analytes can be constructed almost at will(Looger, Dwyer, Smith, & Hellinga, 2003), the ability to design or modify the recognition molecule such that some nearby fluorescent label undergoes a usable change in its emission upon binding some arbitrary analyte remains beyond the state of the art. For spectroscopically active analytes such as certain metal ions, this is straightforward (see above); for the majority of small molecule analytes of biomedical interest which lack spectroscopic "handles", it is far from easy. The most universal approach has been the use of covalent and non-covalent derivatives of "solvent-sensitive" fluorophores such as the ANS family (Godwin & Berg, 1996; J. R. Lakowicz et al., 1999; Li & Cass, 1991; Marvin & Hellinga, 1998; Thompson & Jones,