Fluorescence Anisotropy Studies

Introduction

Polarized light striking a fluorescent molecule results in polarized fluorescence. This polarized emission gradually returns to unpolarized fluorescence depending on rotational diffusion and other factors. *Anisotropy* is directly related to the polarization, and is the ratio of the polarized-light component to the total light intensity. With optional polarizers installed in a spectrofluorometer, we define light intensities: I_{VV} is with excitation and emission polarizers mounted vertically; I_{HH} is for excitation and emission polarizers mounted horizontally. I_{HV} uses an excitation polarizer horizontal and the emission polarizer vertical; I_{VH} requires the excitation polarizer vertical and emission polarizer horizontal. The basic setup, called "L-format," is shown in Fig. 1.

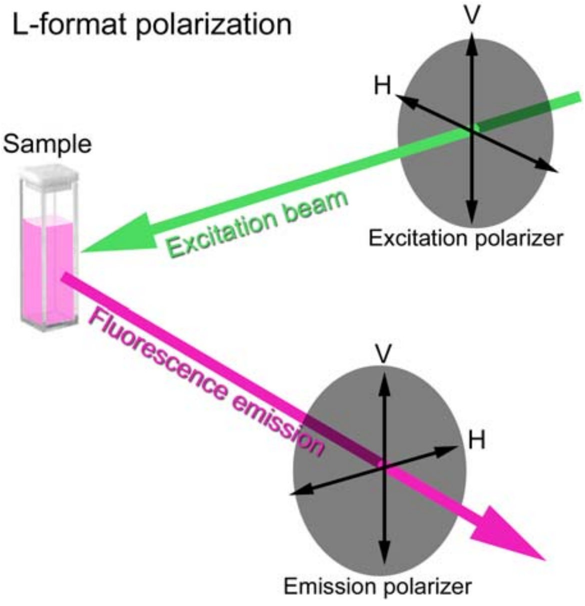

Fig. 1. *Diagram of L-format fluorescence polarization. Vertical (V) and horizontal (H) orientations of each polarizer are shown.*

Anisotropy, <*r*>, is defined as[1]

$$\langle r\rangle = \frac{I_{VV} - G * I_{VH}}{I_{VV} + 2 * G * I_{VH}} \qquad \text{Eq. 1}$$

where *G*, the "*G* factor," is

$$G = \frac{I_{HV}}{I_{HH}} \qquad \text{Eq. 2}$$

Conversion between <*r*> and polarization, *P*, is shown in Equation 3:

$$P = \frac{3\langle r\rangle}{2 + \langle r\rangle} \qquad \text{Eq. 3}$$

Four intensity measurements, corresponding to permutations of both polarizers' orientations, are needed to determine <*r*> or *P*.

Both Fluorolog® and FluoroMax® spectrofluorometers with a polarizer accessory can do L-format polarization measurements. The Fluorolog®'s modular design, however, introduces unequalled flexibility with optional "T-format" polarization. The T-format uses two emission polarizers, speeding up data-acquisition via simultaneous vertical and horizontal polarizer emission measurements. A diagram of the T-format method is shown in Fig. 2.

Anisotropy provides information on molecular size and shape, and local viscosities of a fluorophore's environment, as well as offering insight into changes in molecular sizes of polymers and other macromolecules. Protein-ligand interactions and binding assays

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[1] Joseph R. Lackowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed., New York, Springer, 2006, pp. 353–354, 361–364.

can be investigated, and fluorophore lifetimes can be determined.

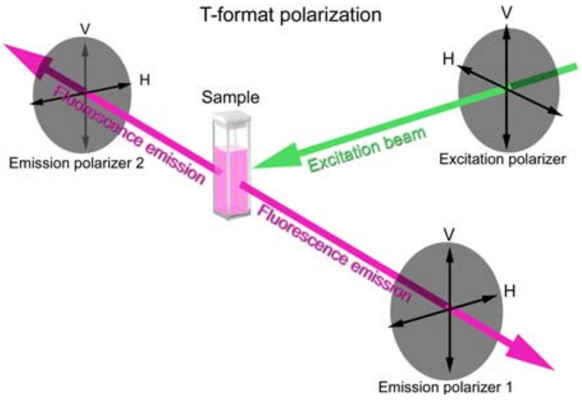

Fig. 2. *Diagram of T-format fluorescence polarization. Vertical (V) and horizontal (H) orientations of each polarizer are shown.*

Anisotropy of binding curves

Platelet-derived growth factor (PDGF) is a protein in platelets and elsewhere in the human body. Several isoforms exist (PDGF-AA, PDGF-AB, and PDGF-BB), made of PDGF-A and PDGF-B subunits. PDGF, especially the BB isoform, may promote cell-growth and division. PDGF-BB binds to two β-PDGF-R receptors on the cell membrane to activate phosphatidylinositol 3-kinase inside the cell, signaling cell growth. Some malignant tumors contain excess PDGF, so this protein may indicate cancer.

An aptamer (synthetic oligonucleotide with two hairpins) was designed to bind to PDGF, and contained fluorescein dye at one end. A cuvette with 0.1 µ*M* fluorescein-labeled aptamer was placed into a Fluorolog®-3 equipped with a polarizer accessory. The emission monochromator was parked at 490 nm, while the excitation monochromator was set to 514 nm. Integration time = 3.3 s, and 1 µ*M* PDGF-BB was injected into the cuvette (Fig. 3).

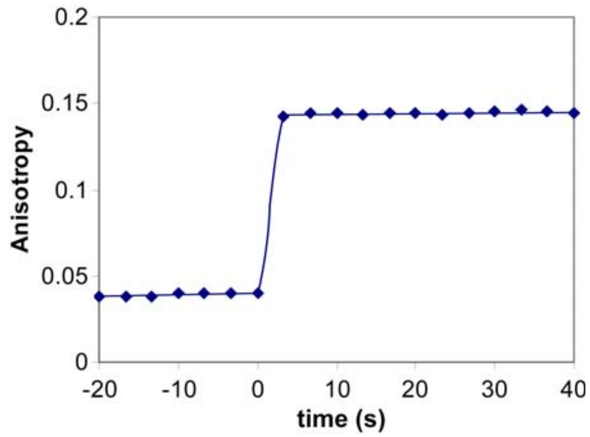

Fig. 3. *Anisotropy of 0.1 µ*M *labeled aptimer solution. PDGF-BB, 1µ*M, *was added at time = 0 s.*

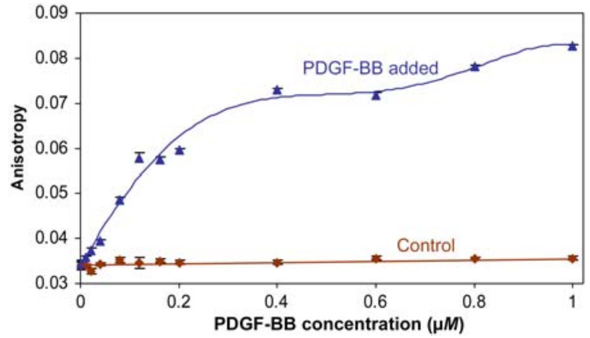

Fig. 4. *Anisotropy of 0.1 µ*M *labeled aptimer solution, with varying amounts of 1µ*M *PDGF-BB added. The brown curve is a control with only buffer added to the aptimer.*

The anisotropy change is detectable when PDGF is added to the solution. Binding characteristics between the aptamer and PDGF were studied. Fig. 4 shows a reproducible possible biphasic curve; perhaps two aptamers exist with different affinities to PDGF. As a control, buffer without PDGF was added to a second solution. The control shows no change in anisotropy as PDGF is added.

The detection limit via the anisotropy change was found using 2 n*M* aptamer, using a bandpass filter instead of an emission monochromator. PDGF-BB was added stepwise, and a linear least-squares fit was performed on the fluorescence-intensity data, giving a detection limit of ~0.22 n*M* PDGF-BB (Fig. 5).

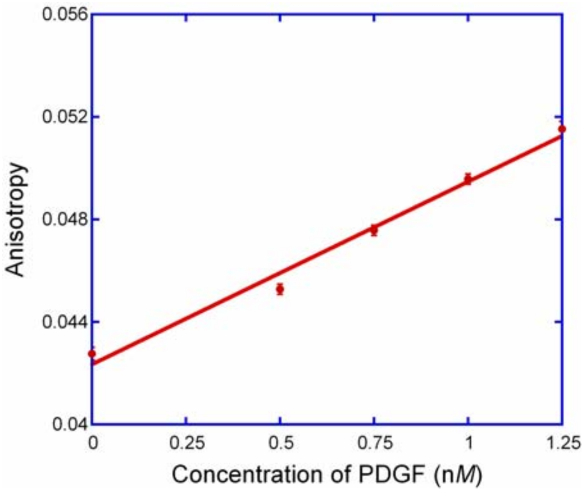

Fig. 5. *Anisotropy vs. [PDGF] in 2 n*M *aptamer solution. From the least-squares fit to the data, a detection limit of ~0.22 n*M *PDGF was found.*

Assay of protease activity

A ribonuclease (RNase) is an enzyme that hydrolyzes RNA into smaller molecules. Typical RNase probes present problems, because contamination can be difficult to identify. With sensitive fluoresence-polarization methods, results are easier to determine. An example was performed on a Fluorolog®-3 with double excitation monochromator. Fluorescein-labeled RNA (F-RNA) was digested for ≥ 1 h with RNase A at 37°C. The reaction was quenched with Tris-HCl at pH 8.0 in 0.125% sodium dodecyl sulfonate. The reaction is given below:

$$\underset{\text{high polarization}}{\text{F–RNA}} \xrightarrow{\text{RNase}} \underset{\text{low polarization}}{\text{F–nucleotides}} + \text{F–oligomers}$$

The RNase is expected to lower the anisotropy as the RNA gets digested into smaller, more freely-rotating fragments. Fig. 7 shows precisely this effect: as more RNase is added to the labeled RNA, the polarization falls, revealing the effects of digestion.

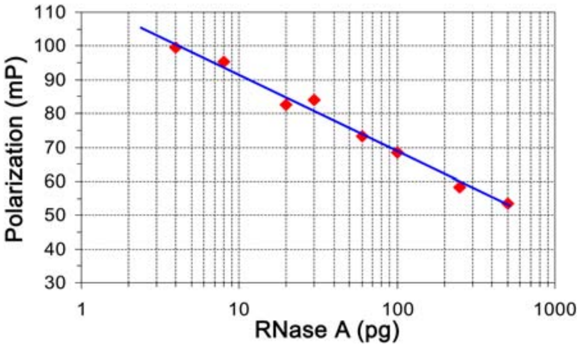

Fig. 7. *Polarization vs. amount of RNase added to 25 ng fluorescein-labeled RNA. Data were taken after ≥ 1 h for complete hydrolysis. The anisotropy falls as more RNase is added, indicating increased fragmentation of the RNA.*

Phase studies

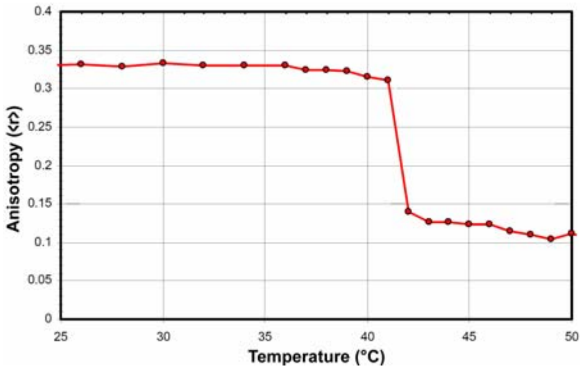

Fig. 8. *Anisotropy vs. temperature for a fluorophore in a lipid membrane. The anisotropy drops suddenly at the transition temperature, indicating freer movement of the fluorophore.*

A lipid membrane shows a phase transition with anisotropy data. As the temperature rises (Fig. 8), a sharp drop in anisotropy appears at 41–42 °C, indicating freer rotation of the fluorophore.

Immunoassays

Theophylline is a drug for myocardial stimulation, increased coronary blood-flow, and is a bronchiodilator. Fluorescence polarization of fluorescein-labeled theophylline is inversely related to drug concentration, as shown below.

Theophylline in pH 7.2 buffer, fluorescein-labeled theophylline (λ_{exc} = 483 nm; λ_{em} = 513 nm), and antiserum were mixed and incubated for 3 min. Polarization measurements (Fig. 9) were performed on a T-format Fluorolog® with polarizers. Free, labeled theophylline shows $P \approx 0$, while labeled theophylline bound to the antibody gives $P \approx 0.27$. Thus free and complexed drug can be distinguished.

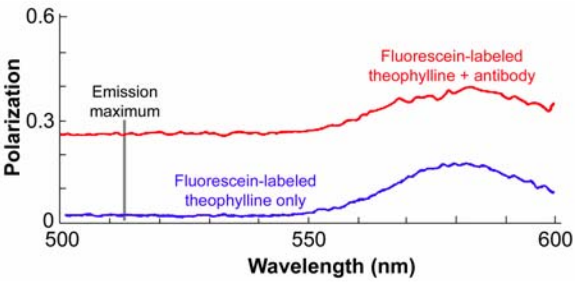

Fig. 9. *Polarization emission scan for labeled theophylline with (red) and without (blue) antibody (λ_{exc} = 485 nm). The emission maximum for labeled theophylline is marked by a gray line.*

A calibration curve (Fig. 10) for theophylline was generated by setting the monochromators to excitation and emission maxima. Eleven readings were averaged at each of six concentrations (0, 2.5, 5, 10, 20, and 40 µg/mL); integration time = 5 s.

To test the calibration curve, a trial sample was created as shown in Table 1. Three determinations of the sample gave $P = 0.122 \pm 0.002$, corresponding to [theophylline] = 18.0 ± 0.2 µg/mL on the calibration curve, agreeing with the original mixture's concentration.

Table 1. *Components of trial sample for testing the calibration curve.*

gentamicin	5 µg/mL	salicylate	220 µg/mL
theophylline	**17.8 µg/mL**	acetaminophen	217 µg/mL
tobramycin	6.5 µg/mL	meclofenamic	6.0 µg/mL
quinidine	2.6 µg/mL	phenobarbital	51.4 µg/mL
digoxin	2.2 ng/mL	lithium	1.2 meq/L
phenytoin	12 µg/mL		

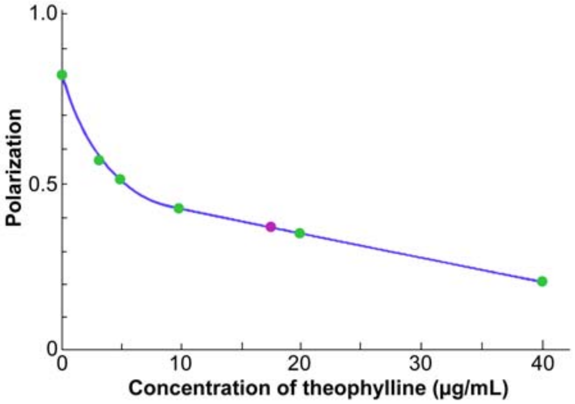

Fig. 10. *Calibration curve (blue line) for theophylline at various concentrations (green points). The purple point is a theophylline determination from the trial mixture in Table 1.*

Two advantages are clear for fluorescence immunoassay measurements: No interference appears from gentamicin, quinidine, salicylate, and acetaminophen, unlike in a normal fluorescence scan. Also, no separation step is required unlike a radioimmunoassay.

Conclusions

Polarization measurements using HORIBA Jobin Yvon spectrofluorometers are a sensitive tool for probing many biochemical interactions.

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