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Applications of Fluorescence Spectroscopy in Chiral Recognition

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ABSTRACT

Fluorescence spectroscopy is a useful tool in the study of chiral recognition. This paper highlights research on applications of fluorescence spectroscopy as a measure of chiral recognition. Research began by evaluating the theoretical basis of relating the ratio of the average anisotropy to temperature and the thermodynamic parameters ΔG, ΔH, and ΔS. It also evaluates the statement that the quantum yield is the same for the free and bound species of analyte, which was previously assumed in deriving the equation. Data obtained in this study showed that assumption to be false. Further testing on individual enantiomers was done to determine if the results were enantioselective.

INTRODUCTION

Neutral β-Cyclodextrin was used as a selector molecule throughout this study. This molecule has a molecular weight of 1134.987 and a chemical structure of $C_{42}H_{70}O_{35}$. β-Cyclodextrin is a host molecule composed of six D-glucose units linked head to tail to form a ring called cyclohexaamylose. It has a relatively inflexible doughnut-shaped structure where the top of the molecule has twelve hydroxyl groups and the bottom has six primary hydroxyl groups. The outside of the molecule has hydrophilic hydroxyl groups while the inside cavity of the molecule has rather hydrophobic C-O bonds. The fact that cyclodextrins have differences in internal and external hydrophobicities mean that cyclodextrins have the ability to accept smaller host molecules into their cavity.[3] Figure 1 is a structural representation of β-Cyclodextrin.

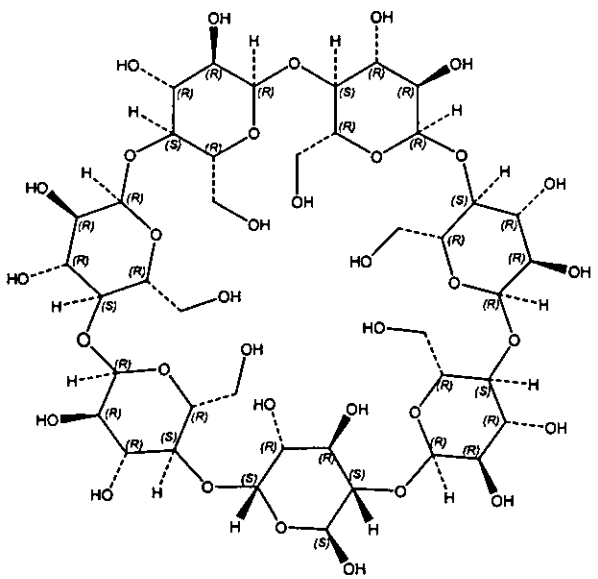

Figure 1: Structural representation of β -Cyclodextrin

Binapthyl-2,2'-diylhydrogenphosphate (BNP) was used as an analyte throughout this study. BNP has a molecular weight of 348.30 and a chemical formula of $C_{20}H_{14}O_4P$. Figure 2 shows the structure of BNP.

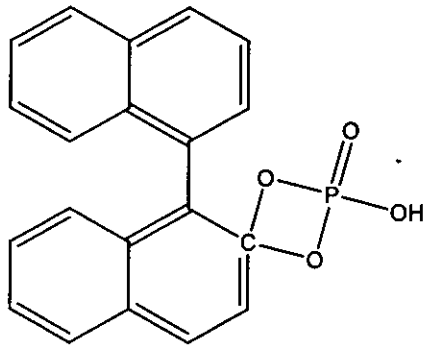

Figure 2: Structure of Binapthyl-2,2'-diylhydrogenphosphate (BNP)

Luminescence is the emission of light from any substance and occurs from electronically excited states. Luminescence is formally divided into two categories, fluorescence and phosphorescence, depending on the nature of the excited state. In excited singlet states, the electron in the excited orbital is paired (of opposite spin) to the second electron in the ground-state orbital. Consequently, return to the ground state is spin-allowed and occurs rapidly by emission of a photon.[1] Once a molecule has absorbed energy in the form of electromagnetic radiation, there are a number of routes by which it can return to ground state. The processes that occur between the absorption and emission of light are usually illustrated by a Jabłoński diagram, which displays these processes.[4] Figure 3 displays one form of a Jabłoński diagram.

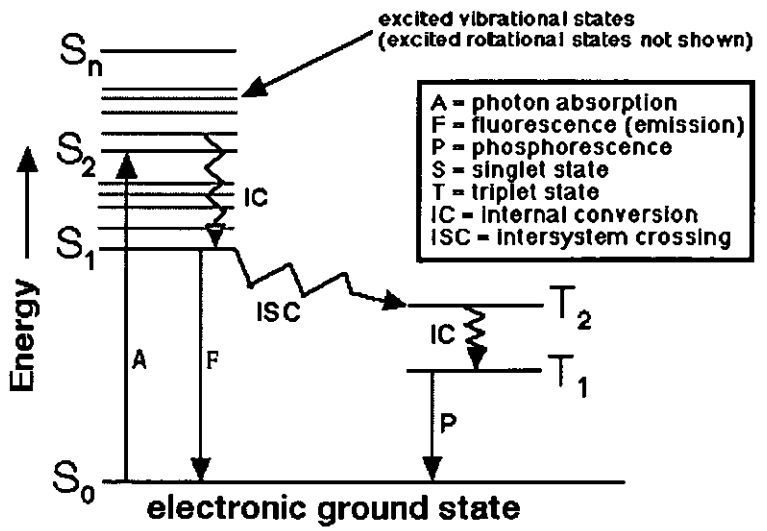

Figure 3: One form of a Jabłoński diagram.[4]

The emission rates of fluorescence are typically 10^8 s^{-1}, so that a typical fluorescence lifetime is near 10 ns (10×10^{-9} s). Fluorescence typically occurs from aromatic molecules. Fluorescence spectral data are generally presented as emission spectra. A fluorescence emission spectrum is a plot of the fluorescence intensity versus wavelength (nanometers).[1]

Fluorescence spectroscopy can be a useful tool in the study of chiral recognition. Fluorescence occurs due to an energy difference between absorbed and emitted photons within a molecule given off as molecular vibrations.

Some molecules or ions can act as quenchers of fluorescence. These molecules decrease the intensity of the emission. Such decreases in intensity are called quenching. The intensity of the fluorescence can be decreased by a wide variety of processes. For instance, fluorophores can form nonfluorescent complexes with quenchers. The fluorophore can be returned to the ground state during a reaction with the quencher. Common quenchers are iodide, oxygen, and acrylamide. [1]

Fluorescence anisotropy measurements can provide information on the size and shape of a moleule. Fluorescence anisotropy is a function of anisotropy (r), fluorescence lifetime (τ), and rotational correlation time (ϕ). The dependence of fluorescence anisotropy on molecular rotation can be described quantitatively with the Perrin equation (equation 1).[2]

$$r_0 / r = 1 + \tau / \phi \qquad (1)$$

The magnitude of anisotropy of an enantiomer can be related to the binding strength of an analyte to a chiral selector and is a measure of chiral recognition in a given system. Anisotropy ratios between enantiomeric configurations of an analyte represent the chiral selectivity between the two enantiomers.[2]

A recent publication[2] by Yafei Xu and Dr. Matt McCarroll identifies the following theoretical development of relating the ratio of the average anisotropy to various thermodynamic considerations. For a given host-guest complexation system, the association can be represented by,

$$A + S \leftrightarrow AS \qquad (2)$$

where A represents a guest molecule or analyte and S represents a host molecule or selector. The guest molecules may exist in the form of a bound complex (A-S) or the free species (A). The measured anisotropy (r_{avg}) is the weighted average of the anisotropy values of the bound (r_b) and the free analyte (r_f), where the contribution is determined by the fractional fluorescence intensity from the bound (f_b) and free (f_f) species.

$$r_{avg} = f_b r_b + f_f r_f \qquad (3)$$

Assuming fractional intensity corresponds to population distributions, the fraction of the bound species can be expressed in terms of the concentrations of the complexed and free forms of the analyte.

$$f_b = [AS] / [AS] + [A] \quad (4)$$

Considering the expression of the association constant of the complexation reaction,

$$K = [AS] / [A][S] \quad (5)$$

Equation 4 can be arranged to represent the fraction of the bound species in terms of K,

$$f_b = K[S] / K[S] + 1 \quad (6)$$

and,

$$f_f = 1 - (K[S] / K[S] + 1) \quad (7)$$

Incorporation of equations 6 and 7 into equation 3 results in,

$$r_{avg} = r_b (K[S] / K[S] + 1) + r_f [1 - (K[S] / K[S] + 1)] \quad (8)$$

which relates to the anisotropy of the bound species, the binding constant, and the concentration of the selector. Because r_f usually approaches zero, equation 8 can often be approximated by,

$$r_{avg} = r_b (K[S] / K[S] + 1) \quad (9)$$

In order to specifically examine the enantioselectivity, we can examine the ratio of the anisotropy values by,

$$\frac{r_{avg,R}}{r_{avg,S}} = \frac{r_{b,R}K_R[S]}{r_{b,S}K_S[S]} \frac{K_S[S]+1}{K_S[S]+1} \quad (10)$$

If the concentration of the free selector and the binding constant are relatively small, the term K[S] is typically much less than unity and equation 10 can be effectively reduced to,

$$\frac{r_{avg,R}}{r_{avg,S}} = \frac{r_{b,R}K_R}{r_{b,S}K_S} \quad (11)$$

It is well established that the Gibbs free energy for complex formation can be related to the binding constant by,

$$\Delta G^o = -RT\ln K \quad (12)$$

where ΔG° is the Gibbs free energy for the formation of the complex. Furthermore, it is known that the differential free energy of enantioselective binding is given by,

$$\frac{\Delta\Delta G^{\circ}_{R,S}}{-RT} = \ln \frac{K_R}{K_S} = \frac{-\Delta\Delta H^{\circ}}{RT} + \frac{\Delta\Delta S^{\circ}}{R} \qquad (13)$$

Evaluating the logarithm of equation 11 results in,

$$\frac{\Delta\Delta G^{\circ}_{R,S}}{-RT} = \ln \frac{K_R}{K_S} + \ln \frac{r_{b,R}}{r_{b,S}} \qquad (14)$$

which allows substitution of the $\Delta\Delta G^{\circ}$ term from equation 13, resulting in equation 15, the final form of the equation.

$$\ln \frac{\Delta\Delta G^{\circ}_{R,S}}{-RT} = \frac{-\Delta\Delta H^{\circ}}{RT} + \frac{\Delta\Delta S^{\circ}}{R} + \ln \frac{r_{b,R}}{r_{b,S}} \qquad (15)^2$$

The following study uses this equation as a basis of research and highlights applications of fluorescence spectroscopy as a measure of chiral recognition.

EXPERIMENTAL

Instrumentation

For all fluorescence measurements, a modular spectrofluorometer (Photon Technology International Inc., New Jersey) equipped with double monochromators and a photon counting PMT detector was used. The instrument is equipped with large aperture Glann-Thompson polarizers. A Xe lamp was used as an excitation source. Measurement temperature was controlled and adjusted using a thermocirculator (NESLAB Instruments, Inc., Newington, NH) and 1-cm quartz cuvettes were used. Measurements were taken with a two-turn slit width at a constant temperature of 25°C. Excitation and emission spectra were obtained.

Materials and Chemicals

β -Cyclodextrin was a gift from Cerestar USA, Inc (Hammond, IN). Binaphthyl-2,2'-diylhydrogenphosphate (BNP) was purchased from Aldrich Chem. Co. (Milwaukee, WI). Water used in all experiments was purified by a Milli-Q system (Millipore Inc. Milford, MA).

Solution Preparation

Solutions used in this study were prepared with aqueous phosphate buffer (50 mM, pH 6.9). Buffer was made from Na_2HPO_4 and NaH_2PO_4. Racemic, R, and S Binapthyl-2,2'-diylhydrogenphosphate (BNP) were used as analytes. BNP solutions were made by dissolving solid BNP in ethanol. Analyte concentration was held constant at 1 μM. β -

Cyclodextrin solutions were prepared by dissolving solid β -Cyclodextrin in phosphate buffer. Selector concentration ranged from 0 – 11 mM. Before measurement, all solutions were mixed by sonication for approximately 20 minutes then allowed to equilibrate.

RESULTS AND DISCUSSION

The first aspect of fluorescence spectroscopy looked at in this study dealt with the theoretical development of relating the ratio of the average anisotropy to various thermodynamic considerations. A previous publication identifies the theoretical development of relating the ratio of the average anisotropy to various thermodynamic considerations.[2] This derivation was based upon several assumptions. The first assumption was that the quantum yields are the same for the free and bound species of analyte. This assumption was tested and will be discussed at a later time. A second assumption made was that $K_s[S] << 1$. A third assumption made was that the anisotropy of the free species of analyte is equal to zero.

An equation relating the ratio of the average anisotropy to various thermodynamic considerations without making either of the two latter assumptions was derived. Equations 1 – 8 remain valid without assuming that $K_s[S] << 1$ or that the anisotropy of the free species of analyte is equal to zero. Making use of equation 12, the following expressions were derived. Equation 16 relates the average anisotropy ratio to temperature, anisotropy of the bound species, selector concentration, and ΔG. Given equation 17, equation 18 relates the average anisotropy ratio to temperature, anisotropy of the bound species, selector concentration, ΔH, and ΔS.

$$\frac{r_{avg,R}}{r_{avg,S}} = \frac{r_{b,R}}{[e^{\wedge}(-\Delta G_R/RT)[S]+1]} \frac{[e^{\wedge}(-\Delta G_S/RT)[S]+1]}{r_{b,S}} e^{\wedge}[-(\Delta G_R - \Delta G_S)/RT] \quad (16)$$

$$\Delta G = \Delta H - T\Delta S \quad (17)$$

$$\frac{r_{avg,R}}{r_{avg,S}} = \frac{r_{b,R}}{[e^{\wedge}-(\Delta H_R - T\Delta S_R/RT)[S]+1]} \frac{[e^{\wedge}(-(\Delta H_S - T\Delta S)/RT)[S]+1]}{r_{b,S}} e^{\wedge}[-(\Delta GR - \Delta GS)/RT] \quad (18)$$

These equations are only applicable within the liquid water phase boundaries between 273° and 373 ° K. Outside this range, the binding constant K is no longer valid.

Figure 4 is an example of the trend produced by Equation16. The graph presents the natural log of the average anisotropy ratio on the y-axis and 1/T (K) on the x-axis. For this example, the selector concentration has been set to millimolar range, the constant R is 8.314×10^{-3} KJ/mol, $r_{b,R}$ and $r_{b,S}$ have been set to 0.303 and 0.300 respectively, and ΔG_R and ΔG_S have been set to 6.2 and 5.9 respectively.

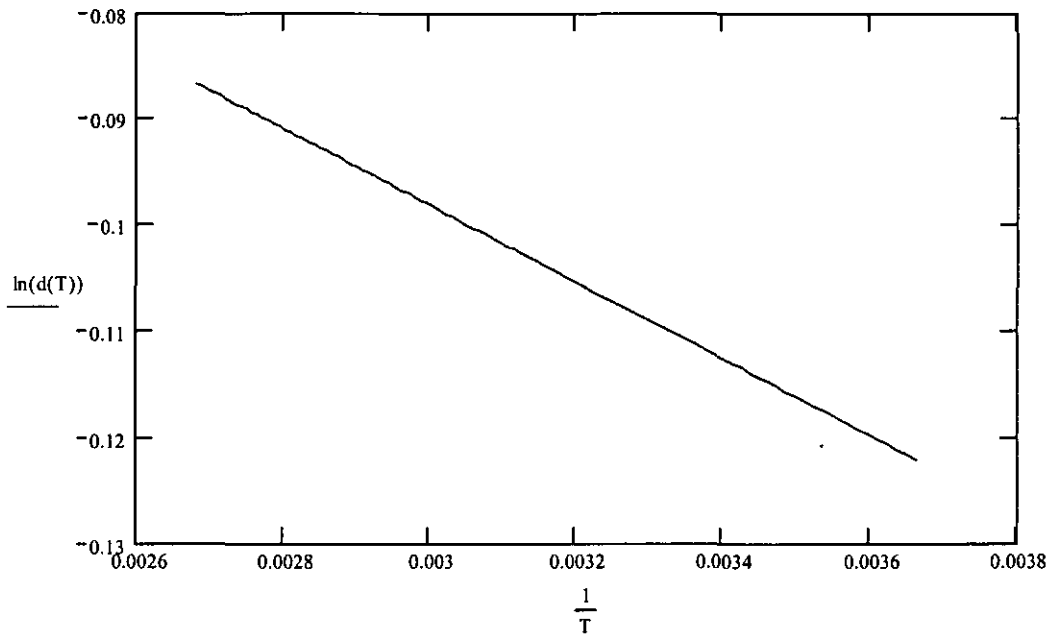

Figure 4: Graphical example of equation 16.

Figure 4 shows a graphical representation of equation 16. Typical numerical values have been used to show a general trend. As it appears that the line expressed on the graph is linear, it is in fact not completely linear. Assuming that the trend was linear may have led to error in past thermodynamic calculations.

The next step in this study dealt with an assumption that was made in order to derive the previously discussed equations. It was assumed that the quantum yields are the same for the free and bound species of analyte, as represented by equation 3. In order to test the validity of this assumption, a series of solutions will be tested for fluorescence intensity and plotted against selector concentration. The analyte used in this experiment was Binapthyl-2,2'-diylhydrogenphosphate (BNP) at a constant 1 μM and the selector was β –Cyclodextrin, 0 – 11 mM. Figures 5 and 6 display data obtained from that experiment.

Selector Concentration, mM	Fluorescence Intensity	Standard Deviation	Emission Maximum	Excitation Maximum
11	151600	966.5	375.7	306
10.725	153100	965.5	375.7	305
10.45	158200	978.3	376.3	305
10.175	160800	1055	375.4	305
9.9	163600	858	375.4	305
9.625	166100	1142	375.4	305
9.35	167400	860.2	376.6	305
9.075	169400	1056	376.6	305
8.8	169400	1109	376.1	306
8.525	171400	1078	376.1	306
8.25	173200	1110	376.1	306
7.975	175000	1156	375.7	306
7.7	177000	1151	376.3	306
7.425	178100	1072	376.5	306
7.15	178800	1687	376.8	306
6.875	181000	1141	376.4	306
6.6	181700	1389	376.4	306
6.325	183400	1224	376.4	306
6.05	184700	1294	376.4	306
5.775	186000	1026	376.4	306
5.5	187400	1343	376.4	306
5.225	187500	1023	376.4	306
4.95	188400	1131	376.4	306
4.675	189100	1082	376.6	306
4.4	192700	967.3	376.6	305
4.125	193300	1204	376.6	305
3.85	194700	1091	376.6	305
3.575	194300	1179	377	305
3.3	197500	1372	377	304
3.025	198600	1236	377	304
2.75	198900	1116	377	304
2.475	199700	1382	377.6	304
2.2	201500	1290	377.6	304
1.925	202500	2247	378	304
1.65	203600	1409	378.2	304
1.375	206000	2229	378.2	304
1.1	206100	1110	379.5	304
0.825	205800	1204	380.1	304
0.55	207200	1346	379.7	304
0.275	206900	1454	379.7	303
0	207200	1565	380	303

Figure 5: Data obtained in quantum yield experiment.

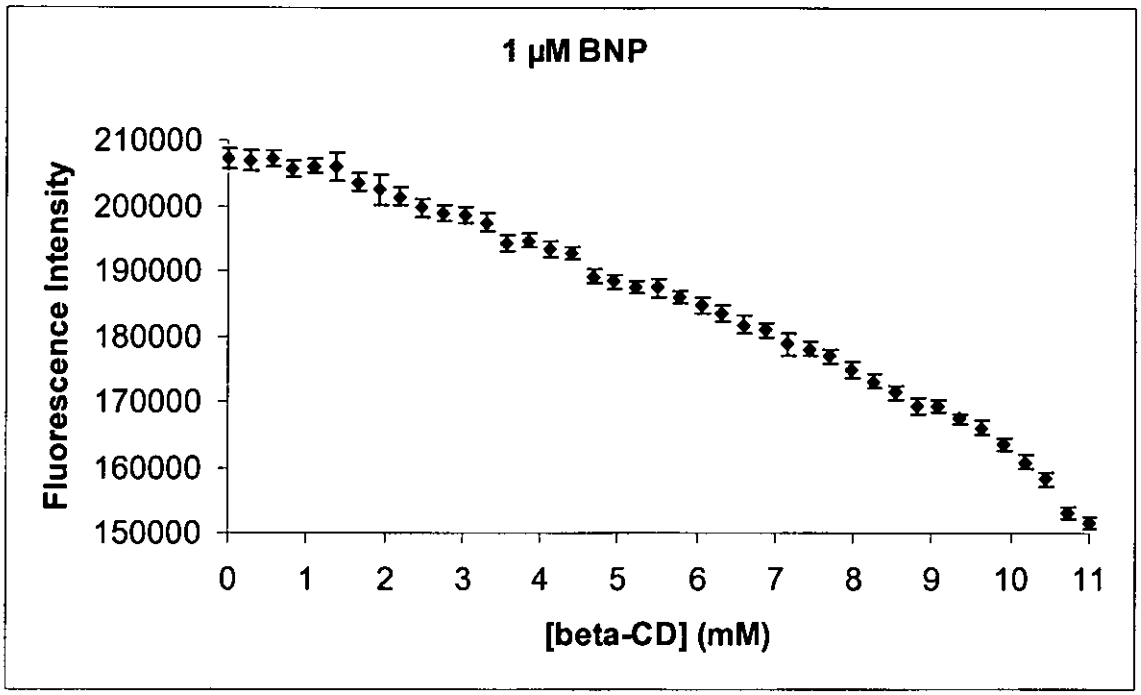

Figure 6: Graphical Representation of Figure 5.

In this example, there is approximately a 27% difference between the bound (11 mM) and the free (0 mM) species of analyte. The decreasing slope is indicative of a quenching effect.

Based on the previous results, an experiment was designed to show whether or not the quenching effect is enantioselective. For this experiment, a series of solutions will be tested for fluorescence intensity and plotted against selector concentration. Varying from the last experiment, the individual enantiomers, rather than the racemic mixture, will be tested. R and S-BNP solutions were prepared at 1 μM concentration. The selector was β –Cyclodextrin, 0 – 11 mM. Figures 7 and 8 display data obtained from the R-enantiomer.

Selector Concentration, mM	Fluorescence Intensity	Standard Deviation	Emission Maximum	Excitation Maximum
11	66870	930.6	377.1	304
9.625	67650	925.8	377.9	304
8.25	72730	951.3	377.4	305
6.875	73860	986.2	378.4	305
5.5	75050	902.1	378.4	305
4.125	76220	996.8	378.1	304
2.75	77530	932.1	378.1	304
1.375	78150	994.7	379.3	304
0	78330	922	381.5	303

Figure 7: Data obtained in enantioselective quenching experiment, R-enantiomer.

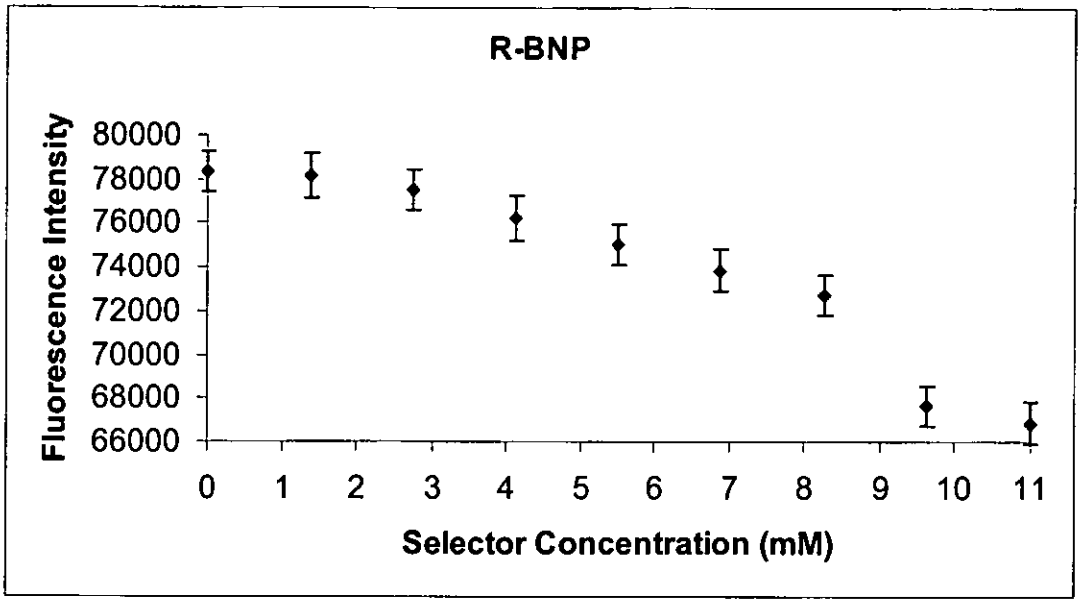

Figure 8: Graphical representation of Figure 7.

Similar to the 1 μM racemic BNP fluorescence intensity trend shown in Figures 5 and 6, the 1 μM R-BNP shows decreasing fluorescence intensity with increasing selector concentration, indicating a quenching effect. The range of intensities is not as great as that shown in the racemic solutions. The percent difference between the bound and free states is about 17%. Again, this value is smaller, but is consistent considering the smaller intensity range.

Figures 9 and 10 display data obtained from the S-enantiomer.

Selector Concentration (mM)	Fluorescence Intensity	Standard Deviation	Emission Max	Excitation Max
11	61180	316.2	374.5	305
9.625	60150	297.6	374.8	305
8.25	59470	286.3	375	305
6.875	59070	370.1	375.5	305
5.5	58670	236	375.8	305
4.125	58460	388.7	377.6	305
2.75	57530	344.5	377.5	305
1.375	57020	376.2	379.5	305
0	52660	258.1	380	305

Figure 9: Data obtained in enantioselective quenching experiment, S-enantiomer.

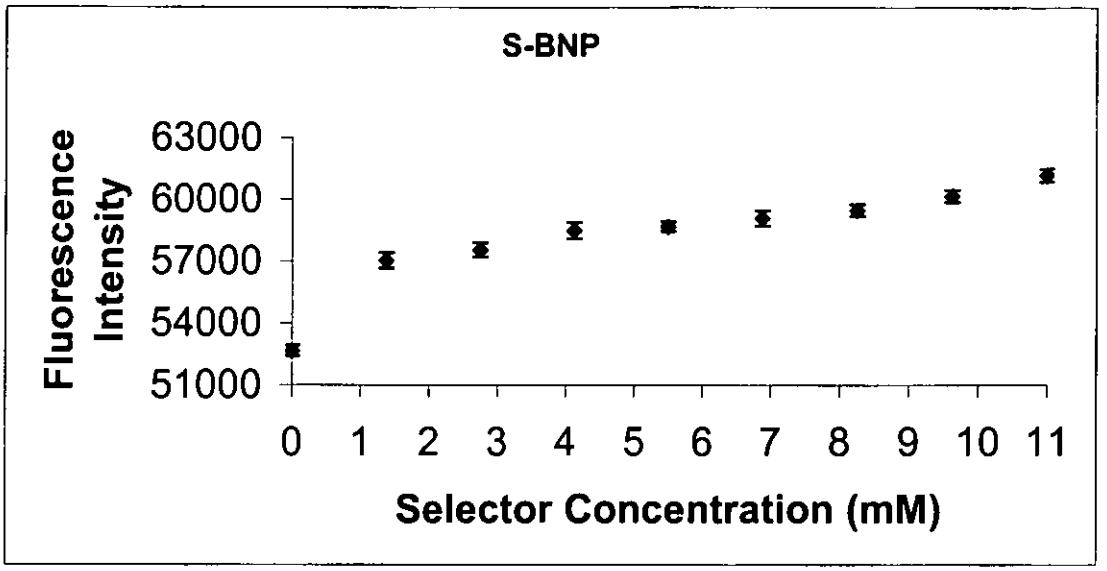

Figure 10: Graphical representation of Figure 9.

Differing from the 1 μM racemic and R-BNP fluorescence intensity trends, the 1 μM S-BNP shows increasing fluorescence intensity with increasing selector concentration. The range of intensities is not as great as that shown in the racemic solutions. The percent difference between the bound and free states is about 14%. Again, this value is smaller than the racemic solution but is consistent considering the smaller intensity range.

CONCLUSIONS

Several aspects of fluorescence spectroscopy were studied during this research. Each aspect dealt with testing an assumption that was previously made in the theoretical development of this topic. First, an evaluation of the theoretical basis of relating the ratio of the average anisotropy to temperature and the thermodynamic parameters ΔG, ΔH, and ΔS was done. Equations 16 and 18 are the final forms of those equations. Next, the statement that the quantum yields are the same for the free and bound species of analyte was evaluated. Data obtained in this study showed this to be false. The racemic mixture of BNP showed approximately a 27% difference between the free and bound states. The R-BNP showed a 17% difference and the S-BNP showed a 14% difference between the free and bound states. Further evaluation of this will lead to an additional parameter being added to equations 16 and 18. Furthermore, that testing showed there to be an enantioselectivity in the quenching effect. The racemic mixture showed decreasing fluorescence intensity with increasing selector concentration. The R-enantiomer also showed this trend. The S-enantiomer did not show this trend.

FUTURE RESEARCH

The next step in my research will be to continue to examine the quenching effect seen in the last experiment. An evaluation of the seen trend will lead to an additional parameter

being formulated and added to the overall equation relating the ratio of the average anisotropy to temperature and the thermodynamic parameters ΔG, ΔH, and ΔS.

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