Journal of Fluorescence, Vol. 2, No. 4, 1992

pH-Dependent Fluorescence and Singlet Energy Transfer in Water-Soluble Polymers Containing Eosin and Phenol Red Chromophores

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Received August 24, 1992; revised January 6, 1993; accepted January 15, 1993

The pH-dependent fluorescence and energy transfer properties of water-soluble polyacrylamide labeled with a donor (eosin) and acceptor (phenol red) were studied. Eosin emission intensity decreases with pH increases. This observation corresponds to the expectation that the fluorescence intensity of eosin should diminish with pH increases resulting from increased spectral overlap between dyes. The lower energy transfer efficiency compared to a pH sensor made with the same polymer, but with a cross-linker, can be explained by the larger distances between the two chromophores resulting from conformational flexibility and electrostatic repulsion of the chromophore ions.

KEY WORDS: pH; fluorescence; energy transfer; singlet; water-soluble polymers; eosin; phenol red; chromophores.

INTRODUCTION

Studies of polymer photophysics and photochemistry have become very active areas in recent years [1]. One aspect of this field has been the study of nonradiative energy transfer between chromophores attached to polymer chains [2,3]. It has been recognized that there are at least two "polymer effects" that differentiate this field from small-molecule energy transfer: (i) solvent effects on both the polymer-bound chromophore and the polymer coil itself and (ii) intracoil energy transfer and/or excited-state annihilation. In the former case, the polymer can be used to direct the photophysical process via electrostatic interactions. In the latter case, the polymer can be used as a photoharvesting agent, providing intracoil multiphoton processes [4]. The present study is related to the former one in that the effect of spectral overlap on the energy transfer efficiency causes a pH-dependent fluorescence change in a bichromophoric polymer indicator.

According to Förster resonance energy transfer theory [5], the efficiency of energy transfer is given by

$$E = [1 + (r/R_0)^6]^{-1} \quad (1)$$

$$R_0 = (9.79 \times 10^3)(K^2 n^{-4} \Phi_d J)^{1/6} \quad \text{(in Å)} \quad (2)$$

where r is the distance between donor and acceptor, J is the overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor, n is the refractive index of the medium, Φ_d is the quantum yield of the donor in the absence of acceptors, K^2 is a function of the mutual orientation of the donor and acceptor transition moments, and R_0 is the characteristic distance of the donor–acceptor pair for which the probability of excitation energy transfer is 50%. It is obvious from the above equation that the energy transfer process depends principally on (i) the distance r and (ii) the spectral overlap integral J. The energy transfer effi-

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1053-0509/92/1200-0231$06.50/0

ciency should increase with increasing spectral overlap, resulting in diminished fluorescence intensity of the donor.

In a previous paper [6], we reported the pH-dependent spectroscopic properties of a water-soluble polymer with pendent merocyanine and eosin moieties. Eosin emission intensity increased with pH increases. This observation conflicted with the expectation that the fluorescence intensity of eosin should diminish with pH increases resulting from increased spectral overlap between the dyes. The diminished fluorescence intensity at low pH levels was explained by the strong electrostatic attraction between the two chromophores, bringing them into proximity and resulting in efficient energy transfer even at very low polymer concentrations. In the present paper, the fluorescence intensities of water-soluble polyacrylamide labeled with eosin as donor and phenol red as acceptor are examined for changes in energy transfer efficiency at various pH's. The results are opposite those obtained with the eosin–merocyanine-labeled polymer system but are consistent with conventional treatments in which increased energy transfer occurs with increased spectral overlap. The polymer demonstrates the utility of coupling fluorescent indicators with absorbance indicators to prepare novel water-soluble polymeric indicators with useful spectral and analytical properties.

EXPERIMENTAL

Materials

5-Aminoeosin and 5-isothiocyanatoeosin were purchased from Molecular Probes (Eugene, OR). Ammonium persulfate was from Bio-Rad Laboratories (Richmond, CA). Dialysis membranes were from Spectrapor (Los Angeles, CA). All other chemicals used in this work were obtained from Aldrich Chemical Co. (Milwaukee, WI). All purchased reagents were used without further purification except for THF, which was freshly distilled from lithium aluminum hydride in a nitrogen atmosphere just prior to use.

N-(5-Eosinyl) acrylamide was prepared immediately prior to use by mixing dry acetone (1 ml) with 5-aminoeosin (10 mg), then adding acryloyl chloride (18 μl), after which the mixture was allowed to stand in the dark at room temperature overnight. The reaction was monitored by TLC using a methylene chloride/methanol/benzene solvent system [6:3:1 (v/v) R_f = 0.82]. ^{1}H NMR (DMSO-d_6): δ 5.7–6.6 (m, 3H), 7.0 (s, 2H), 7.7 (d, 1H), 8.1 (dd, 1H), 8.5 (d, 1H). The product was used in solution for the polymerization without purification.

Glass-on-glass optical fibers with a 200/250-μm diameter (core/clad) were cut into 1-m lengths, terminated with AMP connectors on one end, and polished on both ends. The stripped ends were cleaved squarely as verified by microscopic examination. The polished distal end was protected with a retractable capillary tube during all operations.

Copolymerization

A stock solution of acrylamide (5.64 *M*) was prepared by dissolving 40 g of acrylamide in 100 ml of 0.1 *M* phosphate buffer (pH 7.4). The polymerization medium was prepared by mixing the stock acrylamide solution (2 ml) with phenol red (32 mg). The resulting mixture was deoxygenated with nitrogen bubbling. To the solution was added with stirring the THF or acetone solution of *N*-(5-eosinyl) acrylamide (1 ml), ammonium persulfate (34 mg), and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) (20 μl). The mixture was allowed to stand at room temperature in a nitrogen atmosphere for 1 h until a soft gel formed. If gelation had not occurred within this time, heating (to approximately 50°C) brought about polymerization in about 1 h.

After polymerization, the polymers were purified by precipitating with methanol. The precipitates were dissolved in water and dialyzed at room temperature in a cellulose membrane (molecular weight cutoff, 2000; followed by 8000 and, finally, 15,000) against pure water, with daily water changes for 1 week to remove residual monomer.

The polymer solution of polyacrylamide tagged with eosin was made by mixing the acetone solution of *N*-(5-eosinyl) acrylamide (1 ml) and the stock acrylamide solution (2 ml). The procedure for polymerization was the same as above. Similarly, the polymer solution of polyacrylamide tagged with phenol red was prepared with phenol red (20.5 mg) and the acrylamide solution (2 ml). Both solutions were polymerized with the same amount of initiator as in the copolymerization.

Measurements

Steady-state emission and excitation spectra were measured by the two instrumentation systems designed for use with optical fibers. The first system was a 488-nm argon ion laser, Spectra-Physics Model 162A-04 (Mountain View, CA), used as the excitation source. The second system was a fluorimeter designed for use with an optical fiber [7]. It consists of four basic components: a variable-wavelength light source for excitation, an optical coupler for conducting light into the sensor

and to the detector, an emission detection system, and a computer control and data acquisition system. The excitation light source consists of a 75-W high-pressure xenon arc lamp (Osram Co.), which gives a continuous spectrum from 190 to 750 nm, and a Spex 1680 0.22-m double monochromator for selecting any specified excitation wavelength light.

Buffer solutions were prepared in the manner of McIlvaine, and the pH values were verified by measurement prior to use.

All fluorescence intensity measurements were observed at a fixed emission maximum wavelength. All pH response values were determined in replicate. Absorbance measurements for characterizing the dye concentrations were made by an IBM UV-visible 9420 double-beam spectrophotometer. All measurements were performed at room temperature.

RESULTS AND DISCUSSION

We developed a bichromophoric indicating system based on energy transfer from a pH-insensitive fluorophore, eosin, to a pH-sensitive absorber, phenol red. Phenol red was selected because, in the pH range 6.0–8.0, it absorbs in the same region that eosin fluoresces. Protonation of phenol red shifts its absorbance maximum to shorter wavelength and thus causes diminished absorptivity in the spectral overlap region (see Fig. 7 in Ref. 8). Since the extent of energy transfer is proportional to the spectral overlap integral, the amount of energy transfer should increase as the pH increases and will be detected as a decrease in eosin's emission intensity. Consequently, the efficiency of energy transfer should be dependent upon pH.

Peterson *et al.* [9] have reported that phenol red may be incorporated directly into acrylamide polymers without derivatization. We believe that the polymerization reaction of phenol red with acrylamide succeeds because of phenol red's quinone-like character. After polymerization, unpolymerized eosin, phenol red, and low molecular weight species were removed by exhaustive dialysis. The resulting linear polymer was water soluble. The absorption spectrum of the polymer showed that it contained both phenol red and eosin. The distribution of phenol red and eosin moieties is not known because their relative rates of incorporation into the acrylamide chain have not been investigated, however, we assume that their distribution in the polymer is random.

As controls, separate polymerizations of phenol red with acrylamide and eosin with acrylamide provided single dye-substituted linear polymers. After dialyzing to remove low molecular weight materials, the single dye-labeled polymers were mixed to provide a solution with an absorption spectrum identical to that of the two-dye copolymer. The mixture was then used for reference measurements. Aliquots of the stock polymer solutions were then added to different pH buffer solutions. When the polymers were added to buffers, they exhibited time-dependent fluorescence intensities. Measurements were performed using an optical fiber spectrofluorimeter to minimize inner filter effects caused by the longer path lengths of conventional instruments. Table I shows the time-dependent fluorescence data for various eosin–phenol red-labeled copolymer solutions. The fluorescence intensity gradually increased with time at near-neutral pH values (pH 5,6,7) and was almost constant in the basic buffer. This phenomenon may be related to the effect of pH on the polymer chain conformation [10]. In basic solutions the polymer chains tend to unravel due to repulsive interactions because both chromophores are negatively charged, giving longer distances between the two dyes, whereas in acidic buffers, entanglement occurs, resulting in a closer distance between chromophores, causing efficient energy transfer. The anomalous lower intensities at pH 5 may be due to aggregation of the neutral lactone forms of the two dyes in acid solution. This behavior also takes place in the polyeosin solution because of self-quenching (Table II). In order to make the polymer chain relax completely in the buffer solutions, solutions were allowed to equilibrate. After equilibration, the measurements of fluorescence intensities as a function of pH for both the copolymer and a mixture of the phenol red polymer with the eosin polymer were taken. After 1 day, the chain conformation tends to stabilize and further changes in fluorescence are not observed. The results are shown in Table III. The lower fluorescence ratios of the two dyes bound to the copol-

Table I. Time Dependence of Fluorescence Measurements for Copolymer Solutions

	Time (h)			
pH	0	2	6	20
5	63	69	76	120
6	72	105	123	190
7	106	109	107	150
8	80	83	81	113
9	70	74	73	101
10	58	60	60	86
Ratio (pH 10/pH 6)	0.81	0.57	0.49	0.45

Table II. Time Dependence of Fluorescence Measurements for Poly(Eosin)

pH	Time (h)			
	0	2	6	20
5	65	92	101	148
6	151	157	147	206
7	152	160	149	210
8	132	141	144	210
9	112	132	143	204
10	107	131	142	200
Ratio (pH 10/pH 6)	0.71	0.83	0.97	0.97

ymer are attributed to the existence of energy transfer from eosin to phenol red. In the eosin polymer/phenol red polymer mixture, there is only a small fluorescence quenching from pH 6 to 10 except at high concentrations. The decrease in fluorescence intensity for the mixture in basic buffers at high concentration is due primarily to inner filter effects [11]. It was observed further that the change in fluorescence intensity ratios for the different copolymer systems did not correlate completely with the starting concentrations of the two dye monomers in the polymerization. From this result, it can be deduced that the dyes are incorporated randomly into the polymer chain, thus average distances between the two dyes on the polymer chain are approximately constant.

It is interesting to note that although the concentration of the two dyes is the same for both the copolymer solution and the mixture, the absolute fluorescence signal in the mixture is much larger than that in the polymer solution (Table IV). The reduction in the quantum efficiency for the copolymer is attributed partly to the proximity of the two chromophores, resulting in extremely efficient energy transfer even in the absence of extensive spectral overlap. In addition, the measurements shown in Table IV suggest that the pK_a of the polymer dye system is higher than the pK_a for phenol red (about 7.6). This phenomenon is probably the result of a combination of both the intrinsic pK_a of phenol red and polymer conformation-induced energy transfer.

The much lower fluorescence intensity ratio from pH 10 to pH 6 at all concentrations of the copolymer solution compared to that of the polymer dye mixture solutions at the same concentrations confirm that energy transfer took place, but the ratio is higher (0.78 average from Table III) than the value obtained with the pH sensor prepared with a cross-linked polymer employing the same dyes [12]. This phenomenon can be explained by the difference in the distance between the two chromophores in these two systems. In the gel system, the distance between the two dye moieties is small and fixed as a result of cross-linking, thereby limiting the energy transfer changes to those resulting from spectral overlap changes. The smaller fixed distance between the donor and the acceptor gives more efficient energy transfer. In the linear polymer solution, the distance between the two moieties is uncertain, and only an average distance is assumed due to the polymer's conformational change in solution. This distance should be larger than that in the polymer gel because of the absence of cross-linker. Furthermore, flexibility in the linear polymer chain allows for electrostatic interactions of the chromophore ions on

Table III. Relative Fluorescence Signal Ratios (pH 10/pH 6) for Different Copolymers and Polymer Mixtures

Relative concentration (μl)[a]	Relative signal ratio (pH 10/pH 6) (±rel. SD)					
	Copolymer 1[b]		Copolymer 2		Copolymer 3	
	Copolymer	Mixture	Copolymer	Mixture	Copolymer	Mixture
10	0.851±0.047	0.985±0.021	0.822±0.024	0.945±0.020	0.812±0.059	0.973±0.016
30	0.817±0.019	0.940±0.027	0.789±0.033	0.937±0.033	0.806±0.048	0.959±0.029
50	0.799±0.012	0.895±0.024	0.779±0.032	0.892±0.010	0.785±0.045	0.955±0.023
70	0.780±0.034	0.867±0.041	0.742±0.027	0.877±0.005	0.771±0.045	0.939±0.012
100	0.757±0.034	0.848±0.035	0.718±0.018	0.863±0.005	0.764±0.031	0.913±0.013
150	0.751±0.013	0.782±0.027	0.711±0.017	0.795±0.011	0.749±0.040	0.890±0.015
200	0.728±0.013	0.770±0.022	0.689±0.021	0.753±0.005	0.738±0.046	0.841±0.017

[a]The relative concentration refers to the volume of polymer taken from the dialysis bag and added to 1 ml pH buffer. The concentration of phenol red in the stock solution is approximately 5×10^{-5} *M* as determined by its absorptivity.

[b]The concentration ratio of two dyes (eosin:phenol red) in the polymerizations: Sample 1, 1:2.86 (mg); Sample 2, 1:7.00 (mg); Sample 3, 1:2.25 (mg).

Table IV. Comparison of Fluorescence Intensities for Eosin–Phenol Red Polymers and Mixtures at the Same Concentrations (kcps)[a]

pH	Polymer	Mixture
6	164	278
7	162	279
8	153	268
9	136	258
10	123	260
Ratio (pH 6/pH 10)	0.75	0.935
λ_{ex} = 480 nm	λ_{em} = 545 nm	

[a]kcps, thousands of photon counts per second.

the chain. As pointed out in a previous paper [6], a strong attraction of opposite charges will bring the two chromophores into proximity, while the repulsion of like charges will cause the chain to expand, resulting in an increase in distance between the two chromophores. In the eosin–phenol red polymer solution, there is a repulsive interaction because both chromophores are negatively charged in basic buffers, resulting in a larger distance and less efficient energy transfer. Thus the overall effect is a combination of two competing effects—distance and spectral overlap.

CONCLUSION

Water-soluble polyacrylamides labeled with an eosin donor and phenol red acceptor were examined for changes in energy transfer efficiency at various pH's. The fluorescence intensity of eosin decreased with increased pH, corresponding to an increased energy transfer efficiency with increased spectral overlap. At the concentrations examined, no inner filter effects can occur due to the low absorptivity of the solution. The less efficient energy transfer compared to pH sensors with a polymer gel layer can be explained by the larger distances between the two chromophores resulting from polymer conformational changes and electrostatic repulsion of the chromophoric ions. These bichromophoric polymers possess pH sensitivity even at extremely dilute concentrations and can be used as new fluorescent indicators.

ACKNOWLEDGMENTS

Financial support for this work was generously provided by the Environmental Protection Agency through the Tufts Center for Environmental Management.

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