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pH-Dependent Fluorescence of Merocyanine–Eosin-Labeled Water-Soluble Polymers

Ping Yuan and David R. Walt*

Max Tishler Laboratory for Organic Chemistry, Department of Chemistry, Tufts University, Medford, Massachusetts 02155

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ABSTRACT: The pH-dependent fluorescence and energy-transfer properties of water-soluble polymers labeled with both a donor (eosin) and acceptor (merocyanine) were studied. 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 is attributed to strong electrostatic attraction bringing the two chromophores into proximity and resulting in efficient energy transfer even at very low polymer concentrations.

Introduction

In recent years there has been rapid growth in the field of polymer photophysics due to the proliferation of spectroscopic tools to elucidate polymer conformation and morphology[1,2] and to the design of polymers with novel photophysical characteristics.[3] One such class of polymers contains fluorophores that interact to exhibit conformation-dependent energy transfer.[4-6] These studies have been undertaken principally to examine the polymer's conformation in solution. The present paper describes the pH-dependent spectroscopic properties of water-soluble polymers with pendent merocyanine and eosin moieties and reports results concerning the association of ion pairs in these polymers. Our overall objectives in this study are (1) to clarify the structural parameters necessary to refine the use of bichromophores as fluorescent indicators, (2) to understand the effect of the polymer environment on the photophysical behavior of pendent chromophores, and (3) to reveal the effect of spectral overlap on the energy-transfer efficiency.

According to Förster resonance energy-transfer theory,[7] the efficiency of energy transfer is given by

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

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

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, and K^2 is a function of the mutual orientation of the donor and acceptor transition moments. 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 equations that the energy-transfer process depends on (1) the distance r and (2) the value of the spectral overlap integral J. The energy-transfer efficiency should increase with increasing spectral overlap, resulting in diminished fluorescence intensity of the donor. In the polymer system described below the reversed phenomenon was observed—the donor's fluorescence intensity increased with increasing spectral overlap. This result is attributed to two important factors: (1) pH effects on both the polymer-bound chromophores and the polymer chain itself and (2) ionization-induced changes in polymer chain density.

Experimental Section

Materials. All of the starting materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) except 2-bromoethyl methacrylate (Polysciences, Inc., Warrington, PA) and aminoeosin (Molecular Probes, Eugene, OR). 1-[2-(Methacryloyloxy)ethyl]-4-[2-(4-hydroxyphenyl)ethenyl]quinolinium bromide (MA-Mc) was synthesized by a two-step procedure as described in the literature[8] with some modifications.

In the first step, 4-[2-(4-hydroxyphenyl)ethenyl]quinoline was prepared by refluxing a solution of methanol (15 mL) containing *p*-hydroxybenzaldehyde (6.75 g), 4-methylquinoline (7.9 g), and piperidine (5 mL) for 12 h. Upon cooling, the reaction mixture was diluted with methanol (10 mL) and hexane (20 mL) and placed under refrigeration at 4 °C overnight. The resulting yellow solid was collected by filtration and dried in vacuo at room temperature. The structure of the solid product was confirmed by ^{1}H NMR.[8] In the second step, a solution of DMF (9 mL) containing 4-[2-(4-hydroxyphenyl)ethenyl]quinoline (0.5 g), freshly distilled 2-bromoethyl methacrylate (0.8 g), and hydroquinone (10 mg) was stirred at 100 °C for 24 h. Upon cooling, the reaction mixture was poured into a large excess of ether with vigorous stirring to precipitate the crude product. The solid was recrystallized from a mixture of methanol and ethyl acetate. The product is pH-sensitive, and the NMR spectrum is consistent with literature data.[8]

N-(5-Eosinyl)acrylamide was prepared immediately prior to use by mixing dry acetone (1 mL) with 5-aminoeosin (10 mg) and 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, 3 H), 7.0 (s, 2 H), 7.7 (d, 1 H), 8.1 (dd, 1 H), 8.5 (d, 1 H). The product was used in solution for the polymerization without purification.

Copolymerization. A stock solution of acrylamide (2.82 M) was prepared by dissolving 20 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 MA-Mc dye (32 mg) in methanol (0.5 mL) and the acetone solution of *N*-(5-eosinyl)acrylamide (1 mL). The resulting solution was deoxygenated with nitrogen and maintained in a nitrogen atmosphere. To the reaction were added with stirring ammonium persulfate (30 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. If gelation had not occurred within this time, heating (to ca. 50 °C) brought about polymerization in ca. 1 h.

The polymer solution of polyacrylamide tagged with eosin was made by mixing the acetone solution of *N*-(5-eosinyl)acrylamide (1 mL) and acrylamide solution (2 mL). The procedure for polymerization was the same as above. Similarly, the polymer solution of polyacrylamide tagged with merocyanine dye (PMc) was prepared by mixing the MA-Mc dye (21.3 mg) in methanol (0.5 mL) with the acrylamide solution (2 mL).

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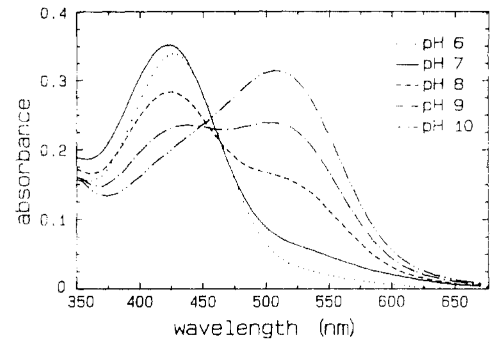


Figure 1. Absorption spectra of PMc at various pHs.

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 for 1 week to remove residual monomer.

The number-average molecular weights of the copolymers were estimated from their intrinsic viscosities, using the equation $[\eta] = 6.8 \times 10^{-4} M_n^{0.66}$.[9]

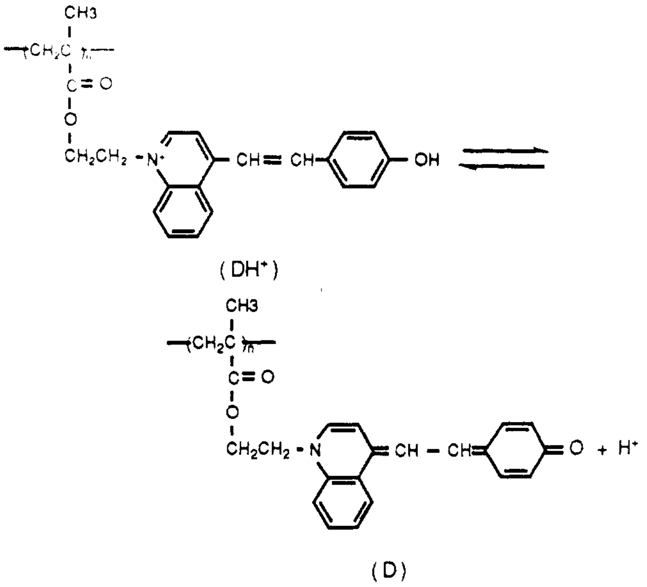
Measurements. Steady-state emission and excitation spectra were measured by a fluorometer system that was designed for use with an optical fiber.[10] The system consists of four basic components: a variable-wavelength light source of excitation, an optical coupler for conducting light into the sensor and to the detector, an emission detection system, and a computer control and data acquisition system. A bare optical fiber was immersed in the solution as a detection probe. The excitation light source consists of a 75-W high-pressure xenon arc lamp (Osram Co.) that 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. The emitted light returns through the same fiber, and the detection system is comprised of a second Spex 1680 0.22-m double monochromator with a 300 line/mm grating and an RCA 31034A-02 photomultiplier tube. The detected signal is then processed by a photometer and displayed on a PC's Limited 286 computer.

Absorbance measurements for characterizing the dye concentrations were made with an IBM UV-vis 9420 double-beam spectrophotometer. All measurements were performed at room temperature.

Results and Discussion

Figure 1 shows absorption spectra of PMc measured in aqueous buffer solutions at various pHs. The merocyanine dye exhibits a sufficiently large spectral change according to the acid-base equilibrium given in Scheme I and therefore can serve as a sensitive pH indicator.[11] The color changes from yellow (DH^+ form) to red (D form) with increasing pH. Figure 2 is the emission spectrum of an eosin-tagged polyacrylamide solution, which is pH-insensitive above pH 7. It can been seen that there is considerable overlap of the eosin emission and the Mc dye absorption spectrum in its D form, indicating that the donor-acceptor characteristics of these two chromophores are well matched. The excitation and emission spectra of PMc at various pHs are shown in Figures 3 and 4, respectively. In Figure 4 there is a broad emission centered at 552 nm, which is very close to the emission maximum of eosin (545 nm). Both chromophores were attached covalently to flexible chains which permit proximity even in very dilute solution; this proximity can lead to interaction between the chromophores of an energy-transfer type. For our donor-acceptor pair, the spectral overlap increases as the pH increases; the amount of nonradiative energy transfer should increase and a diminished fluorescence intensity of eosin (donor) with pH increase should be observed. The experimental observations directly conflicted with this expectation. As pH increased, the fluorescence intensity of eosin increased (Figure 5). To confirm that this phenomenon was related to the interaction of the pendent chromophores on the polymer chain, we made the same measurements for a mixture of two polyacrylamides tagged separately with the Mc dye and with eosin. The concentration of dyes in the mixture was matched to that of the copolymer solution by matching the UV-visible spectra of the two solutions. Figure 6 shows that the fluorescence intensities of the mixture remain almost unchanged with pH increases. Furthermore, PMc alone exhibits decreasing emission and excitation (λ_{em} = 440 nm) intensities with increasing pH. Therefore, PMc

Scheme I



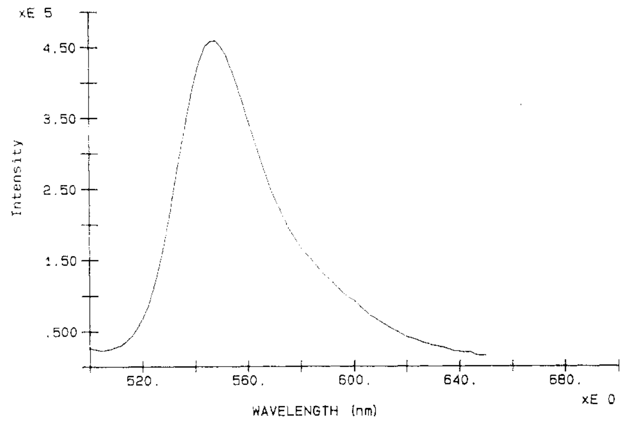


Figure 2. Emission spectrum of eosin. λ_{ex} = 480 nm.

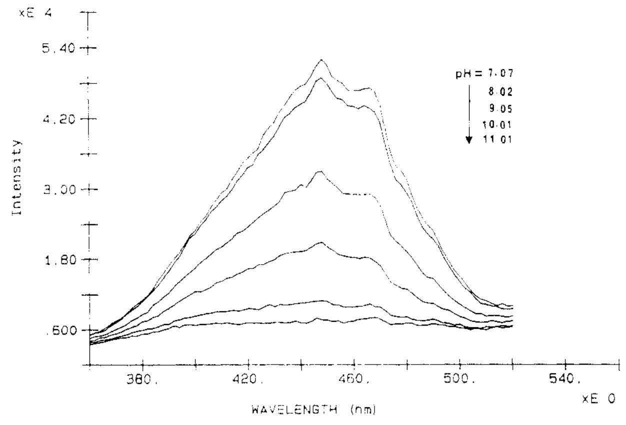


Figure 3. Excitation spectra of PMc at various pHs. λ_{em} = 580 nm.

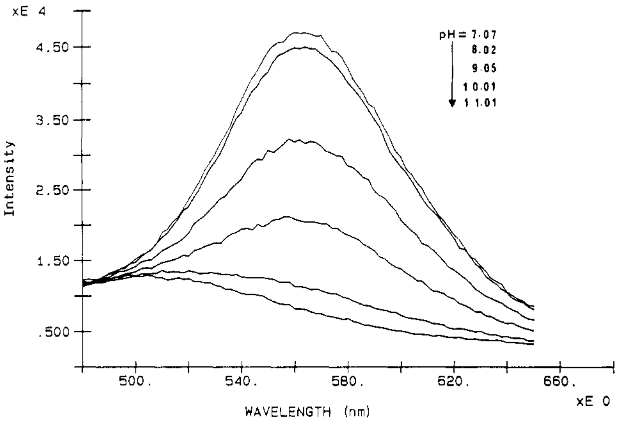


Figure 4. Emission spectra of PMc. λ_{ex} = 440 nm.

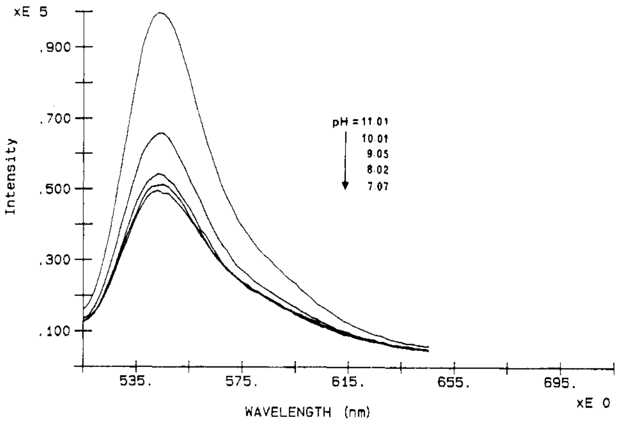


Figure 5. Emission spectra of eosin–Mc copolymer at various pHs. λ_{ex} = 480 nm. The concentration of the test solution is approximately 3 × 10^{-6} M.

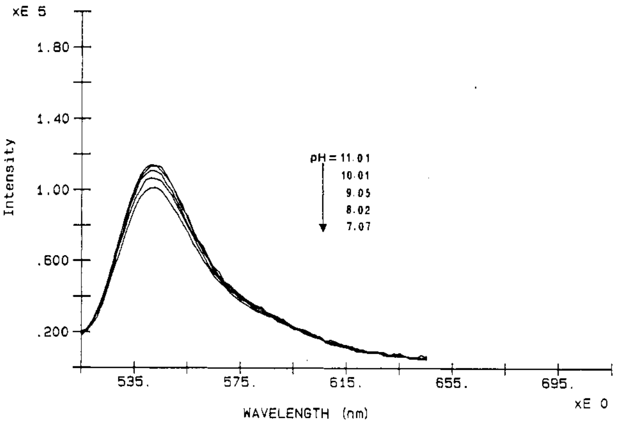


Figure 6. Emission spectra of eosin–Mc mixture at various pHs.

is nonfluorescent at basic pH values. These results eliminate the possibility that Mc is the energy donor or that its emission spectrum accounts for the pH dependence of the copolymer.

What is the mechanism for the intensity increase of eosin with pH increases? It is well-known that the photophysical behavior of polymer systems is sometimes complicated due to the long polymer chain and its conformational properties, including excimer (exciplex) formation, solvent-induced conformational changes, and ionization and association in solution. Nagata and Morawetz[12] observed an enhanced efficiency of energy transfer when the energy donor (naphthyl group) and the energy acceptor (anthryl group) were attached to polymers carrying charges of opposite sign. Also relevant was a study of interpolymeric associations of fluorescently labeled (hydroxypropyl)celluloses as monitored by energy transfer between energy donors (fluorene) and energy acceptors (pyrene) attached to different polymer chains.[13] Finally, an indicator for use as an ionic strength sensor based on the association of two water-soluble polymers using fluorescence energy transfer was reported.[14] In the present system, the two chromophores carry different charges at low pH (Figure 7). A strong attraction for these opposite charges in the polymer chain brings the two chromophores into proximity and leads to a more compact chain. Such charge effects on polymer chain size are not limited to our system. Aviram[15] has reported a gel that expands by 35% in linear dimensions when exposed to light, because the repulsion of the positive charges on the polymer side chains induced by photoionization caused the chains to expand.

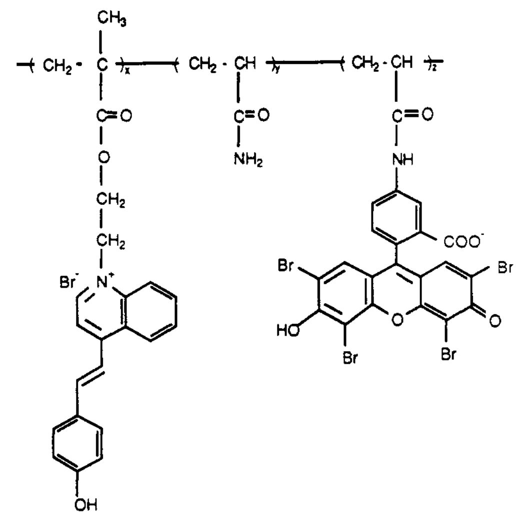


Figure 7. Structure of merocyanine–eosin-labeled polymer.

Table I
Relative Fluorescence Signal Ratios (F_7/F_{11}) for the Copolymer and Mixture Systems

rel concn,[a] μL	F_7/F_{11} copolymer	F_7/F_{11} mixture
50	0.45	0.95
100	0.39	0.92
200	0.36	0.87
300		0.84

[a] The relative concentration of the stock polymer solution refers to the amount taken from the dialysis bag and added to 1 mL of pH buffer.

In addition to intramolecular electrostatic interactions, intermolecular attraction between the two chromophores could also result in the interpenetration or overlap of polymer chains in solution. This intermolecular association would be negligible at very high dilution. Table I reports the ratio values of fluorescence intensities at pH 7 and pH 11 (F_7/F_{11}) for different relative concentrations of the mixture and the copolymer solutions. It appears that interchain association is favored at higher concentrations of the two chains; i.e., F_7/F_{11} decreases as the concentrations of both the mixed polymers and copolymers increase. At very low concentrations of the dye mixture (<10^{-6} M) the ratio is ca. 1, lending support to the hypothesis that only intrachain association occurs at very low concentrations. Both intra- and intermolecular associations cause the distances between the two chromophores to be so close that very efficient energy transfer can occur by either Förster long-range transfer or Dexter[16] exchange interaction transfer processes even with only small spectral overlap. It is obvious from the equations presented above that the distance r is a predominant factor on the energy-transfer

Table II
Comparison of Fluorescence Intensities[a] for the E–Mc Polymer and Mixture at the Same Concentrations[b]

pH	polymer	mixture
7	41	269
8	87	276
9	108	282
10	128	296
11	139	300
F_7/F_{11}	0.295[c]	0.897

[a] In thousands of photon counts per second. [b] λ_{ex} = 480 nm, λ_{em} = 545 nm. [c] Reference 18.

Table III
Fluorescence Intensity[a] Measurements for Different E–Mc Copolymers

pH	PEM-1	PEM-2	PEM-3
7	32	32	88
8	41	36	115
9	48	39	136
10	53	49	150
11	56	51	164
F_7/F_{11}	0.57	0.53	0.54

	eosin/Mc	
copolym	rel mol of starting dyes	dye concn in copolym, $\times 10^{-5}$ M
PEM-1	1/5	0.68/1.51
PEM-2	1/3	0.40/2.27
PEM-3	1.5/1	0.39/2.95

[a] In thousands of photon counts per second. [b] λ_{ex} = 480 nm, λ_{em} = 545 nm.

efficiency and that J is only a moderately important factor ($R_0 \propto J^{1/6}$). Figure 1 shows that there is a small but sufficient amount of overlap between the absorption spectrum of the acidic form of the Mc dye and the emission spectrum of eosin to allow efficient energy transfer at small r. Because of the relatively large value of ϕ_d (0.22) for eosin,[17] the electrostatically induced proximity of the two chromophores results in efficient energy transfer although the effective changes in J are negligible. As pH increases, the Mc dye is neutralized, resulting in diminished attraction and less efficient energy transfer, leading to the increased fluorescence intensities of eosin. Comparisons of the fluorescence intensities for the copolymer and mixture are shown in Table II. In this experiment, the concentrations of the separately immobilized dyes were matched to the dye copolymer as monitored by UV absorption. The intensities for the mixture are significantly higher than those of the copolymer. The reduced eosin intensities can be explained by the efficient energy transfer resulting from the small distance between the two chromophores on the polymer chain. Even after the intramolecular electrostatic interaction of the two dyes is relieved at high pH, the intrachromophore distance is still relatively small.

Three Mc–eosin copolymer preparations having different ratios of eosin and Mc dyes were examined. Figure 8 shows the absorptions of these polymer solutions in pH 7 buffer, indicating that the Mc dye is incorporated more efficiently in the polymer than eosin. The dye concentrations in the polymer solutions were estimated by UV absorption using experimentally determined values for the molar coefficients at λ_{max} = 420 nm for Mc dye ($\epsilon = 1.38 \times 10^4$) and 516 nm for eosin ($\epsilon = 5.72 \times 10^4$). The fluorescence intensity measurements and the dye contents for the polymers are shown in Table III. The molecular weights of these polymers are close (ca. 300 000) due to the same amount of starting acrylamide monomer. Although the relative dye concentrations in the polymer are different, the ratios of the fluorescence intensities at pH 7 to 11 are approximately the same. This result can be explained only if the average distance between donor and acceptor molecules is relatively invariant. In these systems, the average distance is determined both by dye distribution in the polymer and by the dynamic conformation changes of the polymer chain. This latter effect is apparently the predominant one in the present system.

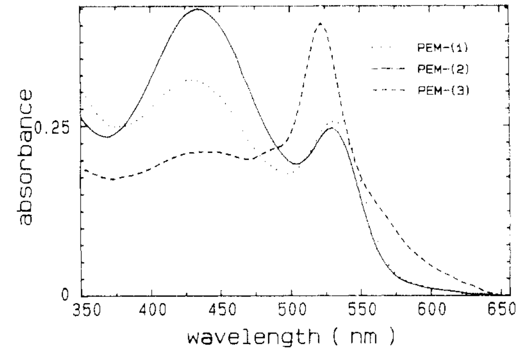


Figure 8. Absorption spectra of three different eosin–Mc copolymers.

Conclusion

Fluorescence intensities of water-soluble polymers labeled with both a donor (eosin) and an acceptor (merocyanine) were examined for changes in energy-transfer efficiency at various pHs. An enhanced fluorescence intensity of eosin with pH increases was observed. This phenomenon is explained in terms of the strong intramolecular and intermolecular electrostatic attractions which bring the two chromophores into proximity and result in efficient energy transfer even at very low polymer concentrations. The pH dependence was found to be the result of changes in the intrachromophore distance r rather than the spectral overlap integral J. The resulting bichromophore in the polymer is pH-sensitive and can be used as a new fluorescent indicator.

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(18) The smaller ratio for the polymer (0.295) compared to the values in Tables I and III (0.53) is due to the higher concentrations of polymer solution used in this experiment.

Registry No. MA–Mc, 111026-40-5; (MA–Mc)(H_2C=$CHCONH_2$)[*N*–(5–eosinyl)acrylamide] (copolymer), 129216-98-4; (MA–Mc)(H_2C=$CHCONH_2$) (copolymer), 111026-43-8; (H_2C=$CHCONH_2$)[*N*–(5–eosinyl)acrylamide] (copolymer), 129239-37-8; 4–[2–(4–hydroxyphenyl)ethenyl]quinoline, 789-76-4; *p*-hydroxybenzaldehyde, 123-08-0; 4–methylquinoline, 491-35-0; 2–bromoethyl methacrylate, 4513-56-8; *N*–(5–eosinyl)acrylamide, 123402-06-2; 5–aminoeosin, 129239-35-6; acryloyl chloride, 814-68-6.