

Supplementary Information for

Digital Fluorescent pH Probes: Polymer-Based Design, Fluorescence Response, Mechanism, Functional Tuning and Application to Logic Operation in Live Cells

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Experimental Section

Materials. *N*-[2-(Diethylamino)ethyl]acrylamide (DEAEAM),^{S1} *N*-(3-(Bis(2-hydroxyethyl)amino)propyl)acrylamide (HEAPAM)^{S2} and *N*-(2-morpholinoethyl)acrylamide (MEAM)^{S3} were obtained as previously reported.

Procedure for preparing S1–S11. NIPAM, a proton-binding monomer (DMPAM, MPAM, DEAEAM, APAM or HEAPAM) (0.5 M in total for NIPAM and proton-binding monomer, see Table S3 for feed ratios), DBD-AA (0.5 mM) and AIBN (5 mM) were dissolved in 1,4-dioxane (10 mL). The solution was bubbled with dry nitrogen or argon for 30 min to remove dissolved oxygen. The reaction mixture was polymerized at 60 °C for 4–8 h (4h for **S3–S6**, 6h for **S8–S11** and 8 h for **S1**, **S2** and **S7**), cooled to room temperature, and poured into diethyl ether (200 mL). The obtained copolymer was purified via reprecipitation using 1,4-dioxane (10 mL) and diethyl ether (200 mL) for **S1–S9** and by dialysis overnight for **S10** and **S11**. The yields are listed in Table S3. **S1–S11** were characterized as described in the main text and summarized in Table S3.

Table S1. Fluorescence lifetimes of **1** at the maximum emission wavelength (λ_{em}), various pH values and 50 °C in a Britton–Robinson buffer (0.01 w/v%) during excitation at 456 nm.

pH	λ_{em}/nm	τ_1/ns	$P_1^a/\%$	τ_2/ns	$P_2^b/\%$	τ_f^c/ns
3.97	604	1.19	45.1	3.50	54.9	2.46
6.00	602	1.24	41.8	3.56	58.2	2.59
6.98	605	1.13	37.2	3.59	62.8	2.67
7.46	603	1.46	44.0	4.03	56.0	2.90
7.80	603	1.66	50.4	4.46	49.6	3.05
7.99	604	1.47	44.3	4.43	55.7	3.12
8.20	601	1.38	36.6	4.39	63.4	3.29
8.38	600	1.90	50.7	6.17	49.3	4.01
8.59	583	2.10	32.4	9.20	67.6	6.90
8.80	577	2.92	23.5	9.83	76.5	8.21
8.99	573	4.18	32.6	10.65	67.4	8.54
9.22	573	3.31	24.6	10.15	75.4	8.46
9.40	574	3.55	25.1	10.47	74.9	8.73
9.72	575	3.15	22.5	10.17	77.5	8.59
10.06	575	3.74	26.9	10.47	73.1	8.66
10.99	574	3.19	23.0	10.24	77.0	8.62

^a Composition of τ_1 . ^b Composition of τ_2 . ^c Average fluorescence lifetime calculated using eqn (7).

Table S2. Fluorescence properties of DBD-IA in mixtures of water and 1,4-dioxane at 25 °C; the maximum absorption wavelength (λ_{abs}), molar absorption coefficient (ϵ), the maximum emission wavelength (λ_{em}), fluorescence quantum yield (Φ_f), fluorescence lifetime (τ_f), fluorescence rate constant (k_f), and nonradiative rate constant (k_{nr}).

Water/% ^a	D^b	λ_{abs}/nm	$\epsilon/cm^{-1}M^{-1}$	λ_{em}^c/nm	Φ_f	τ_f/ns	$k_f/10^7s^{-1}$	$k_{nr}/10^7s^{-1}$
100	78.48	453	10300	621	0.030	1.1 ^d	2.7	88.8
90	70.33	452	10300	614	0.042	1.6 ^e	2.7	60.6
80	61.86	452	10300	605	0.068	2.2 ^f	3.1	42.1
70	53.28	452	10200	603	0.098	3.1 ^g	3.2	29.4
60	44.54	452	10100	602	0.14	4.2 ^h	3.3	20.6
50	35.85	452	10200	597	0.19	5.5	3.5	14.5
40	27.21	451	10200	589	0.27	7.2	3.7	10.1
30	19.07	449	10300	584	0.36	9.1	4.0	7.0
20	11.86	450	10400	579	0.47	11.4	4.1	4.7
10	6.07	448	10300	572	0.63	14.5	4.3	2.6
0	2.21	446	10200	557	0.91	18.9	4.8	0.5

^a w/w. ^b Dielectric constant [ref. S4]. ^c Corrected. ^{d-h} Average fluorescence lifetimes calculated using double-exponential components and eqn (7): ^d 0.45 ns (15.0%) and 1.21 ns (85.0%), ^e 0.47 ns (7.3%) and 1.67 ns (92.7%), ^f 0.74 ns (6.6%) and 2.32 ns (93.4%), ^g 1.10 ns (8.0%) and 3.24 ns (92.0%), ^h 1.76 ns (7.9%) and 4.38 ns (92.1%).

Table S3. Characterization of the as-synthesized copolymers comprising thermoresponsive NIPAM, proton-binding, and fluorescent DBD-AA units.

No.	Proton-binding unit	Feed ratio ^a	Yield (%)	Actual unit ratio ^b	<i>M</i> _w ^c	<i>M</i> _n ^d	<i>M</i> _w / <i>M</i> _n
S1	DMAPAM	95 : 5 : 0.1	72	95.6 : 4.4 : 0.048	134,000	46,000	2.91
S2	DMAPAM	85 : 15 : 0.1	40	87.2 : 12.8 : 0.035	98,400	41,800	2.36
S3	DMAPAM	80 : 20 : 0.1	65	81.0 : 19.0 : 0.064	130,000	70,700	1.84
S4	DMAPAM	70 : 30 : 0.1	62	69.6 : 30.4 : 0.063	106,000	42,600	2.47
S5	DMAPAM	60 : 40 : 0.1	39	59.3 : 40.7 : 0.066	27,200	13,900	1.95
S6	DMAPAM	50 : 50 : 0.1	42	47.9 : 52.1 : 0.060	46,800	29,200	1.60
S7	MPAM	80 : 20 : 0.1	61	81.0 : 19.0 : 0.054	87,700	42,700	2.05
S8	DEAEAM	90 : 10 : 0.1	72	89.4 : 10.6 : 0.039	75,200	41,400	1.82
S9	MEAM	90 : 10 : 0.1	83	91.2 : 8.8 : 0.080	93,700	44,100	2.12
S10	APAM	90 : 10 : 0.1	46	93.6 : 6.4 : 0.10	53,600	25,500	2.10
S11	HEAPAM	90 : 10 : 0.1	51	83.1 : 16.9 : 0.097	36,100	17,300	2.08

^a NIPAM : monomer for proton-binding units : DBD-AA. ^b NIPAM unit : proton-binding unit : DBD-AA unit.

^c Weight-average molecular weight. ^d Number-average molecular weight.

Table S4. Fluorescence lifetimes of the synthesized copolymers in Britton-Robinson buffer solutions.

Copolymer	State	pH	<i>T</i> (°C)	λ_{em}/nm	τ_1/ns	<i>P</i> ₁ ^a /%	τ_2/ns	<i>P</i> ₂ ^b /%	τ^c/ns
1	Off	7	50	605	1.68	47.3	4.69	52.7	3.3
	On	11	50	574	4.04	17.8	11.02	82.2	9.8
2	Off	7	40	600	1.76	56.4	5.08	43.6	3.2
	On	11	40	570	4.03	19.7	12.00	80.3	10.4
3	Off	4	50	601	1.61	47.7	4.20	52.3	3.0
	On	8	50	570	4.17	15.5	10.78	84.5	9.8
4	Off	2	40	602	1.68	49.8	4.27	50.2	3.0
	On	6	40	571	4.21	15.5	11.17	84.5	10.1
5	Off	6	45	600	1.58	52.5	4.31	47.5	2.9
	On	2	45	560	5.58	10.1	13.58	89.9	12.8
6	Off	6	20	601	1.83	16.1	9.83	83.9	8.5
	On	2	20	553	6.47	6.8	19.08	93.2	18.2
7	Off	7	37	600	1.81	46.7	5.36	53.3	3.7
	On	11	37	568	4.20	15.4	11.29	84.6	10.2
9	Off	2	20	604	1.55	53.0	4.01	47.0	2.7
	Off	12	20	604	1.68	51.5	4.71	48.5	3.2
	Off	2	40	604	1.58	50.7	3.89	49.3	2.7
	On	12	40	580	3.30	14.5	9.10	85.5	8.3

^a Composition of τ_1 . ^b Composition of τ_2 . ^c Average fluorescence lifetime calculated using eqn (7).

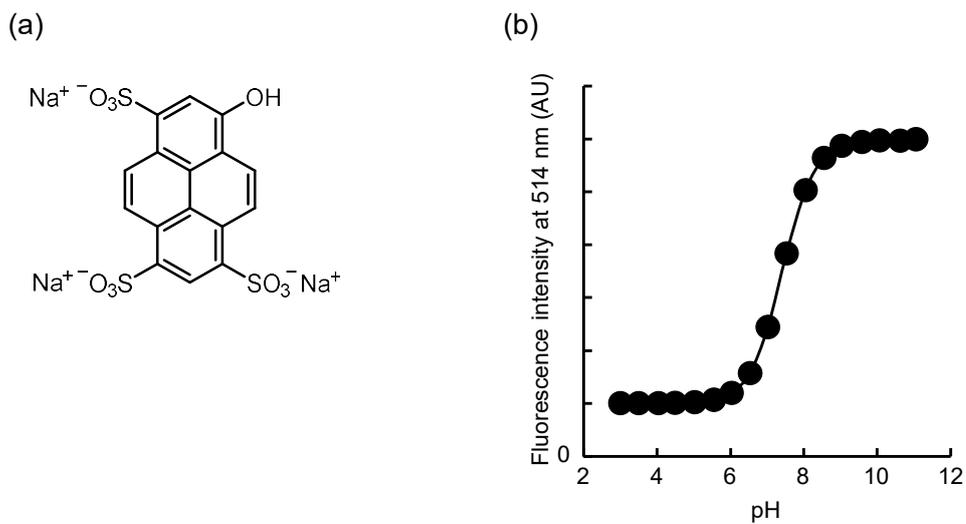


Figure S1. Conventional fluorescent pH probe (HPTS). (a) Chemical structure. (b) Fluorescence responses of HPTS ($1 \mu\text{mol L}^{-1}$) to pH variations in Britton–Robinson buffer solutions at $25 \text{ }^\circ\text{C}$. Samples were excited at 454 nm .

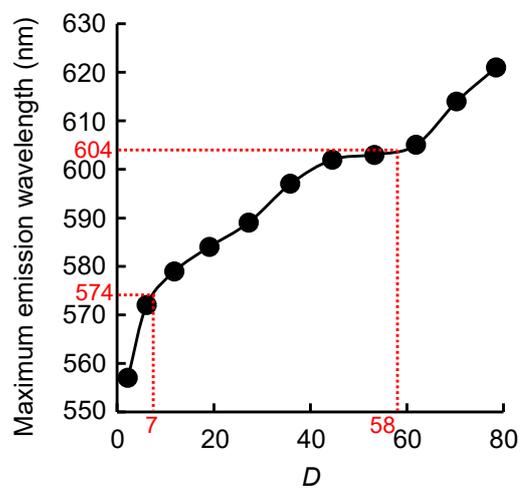


Figure S2. Relation between the maximum emission wavelength of DBD-IA and dielectric constant (D) of solvent. As indicated in red, the local D near 1 at 50 °C were evaluated from the maximum emission wavelengths of DBD-AA units (604 and 574 nm at acidic and basic regions) to be 58 and 7, respectively.

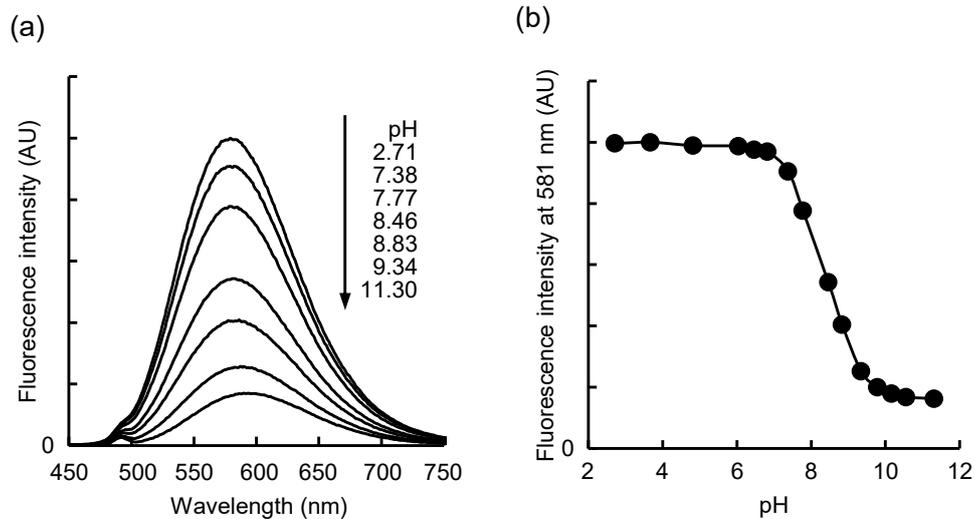


Figure S3. pH-dependent fluorescence behavior of DBD-DMAEA (10 μ M) in water. (a) Fluorescence spectra. (b) Relation between fluorescence intensity at 581 nm and pH. Samples were excited at 420 nm at 25 $^{\circ}$ C. pH of the solution was adjusted using HCl and NaOH.

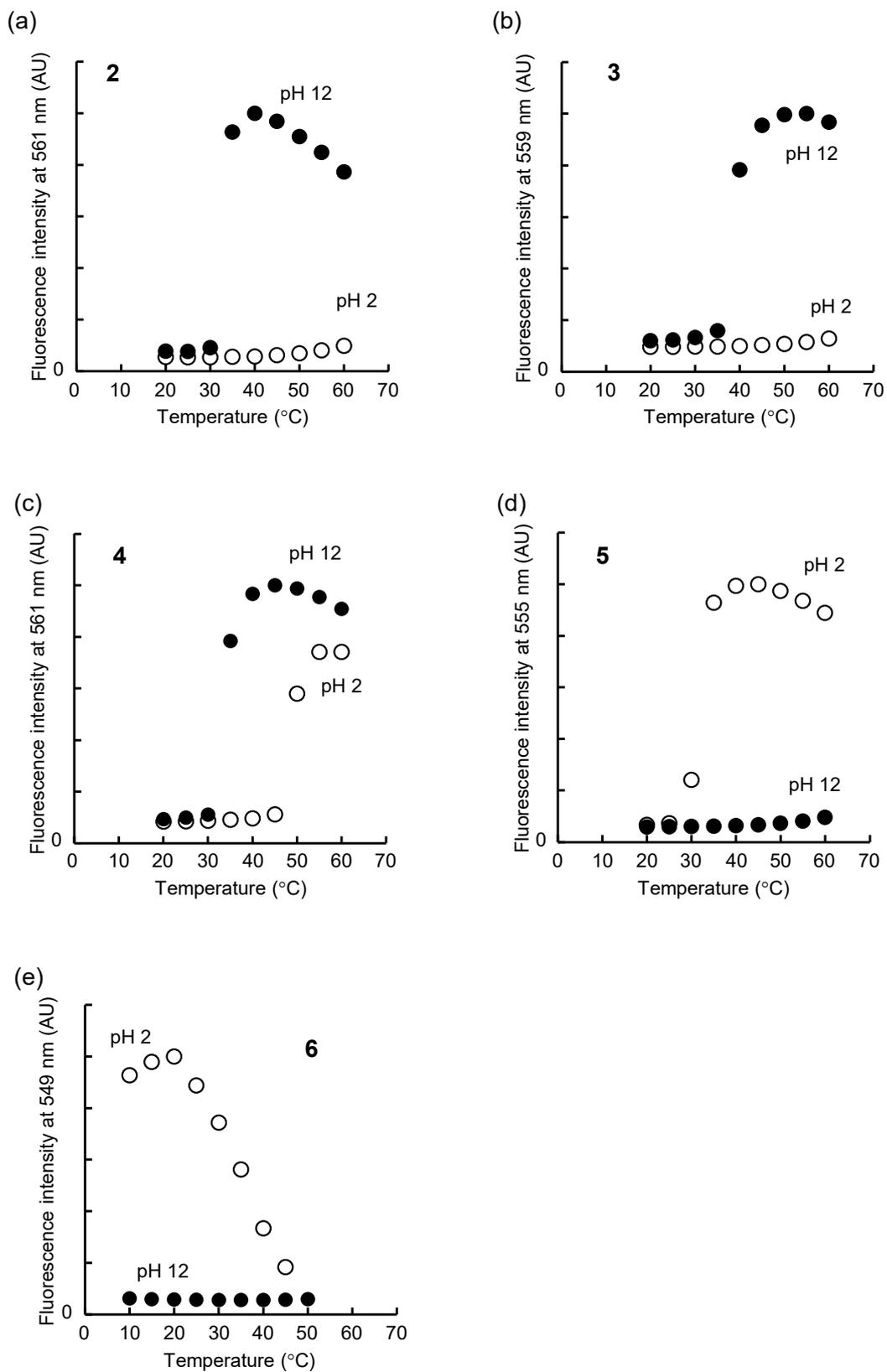


Figure S4. Temperature-dependent fluorescence intensity of copolymers 2–6. (a) **2**, (b) **3**, (c) **4**, (d) **5** and (e) **6** at pH 12 (closed) and 2 (open) in Britton–Robinson buffer solutions. Samples (0.01 w/v%) were excited at 450 nm.

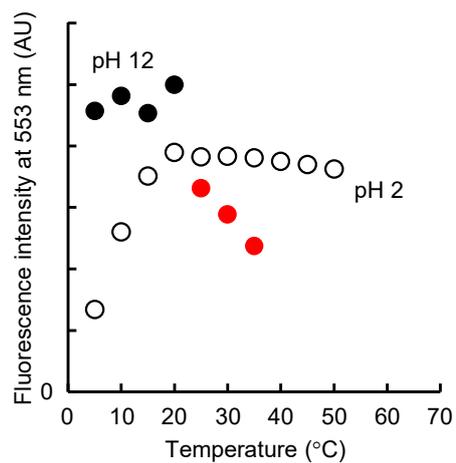


Figure S5. Temperature-dependent fluorescence intensity of copolymer 8. At pH 12 (closed) and 2 (open) in Britton–Robinson buffer solutions. Copolymer 8 precipitated at 25 °C and pH 12 (as indicated in red). Samples (0.01 w/v%) were excited at 450 nm.

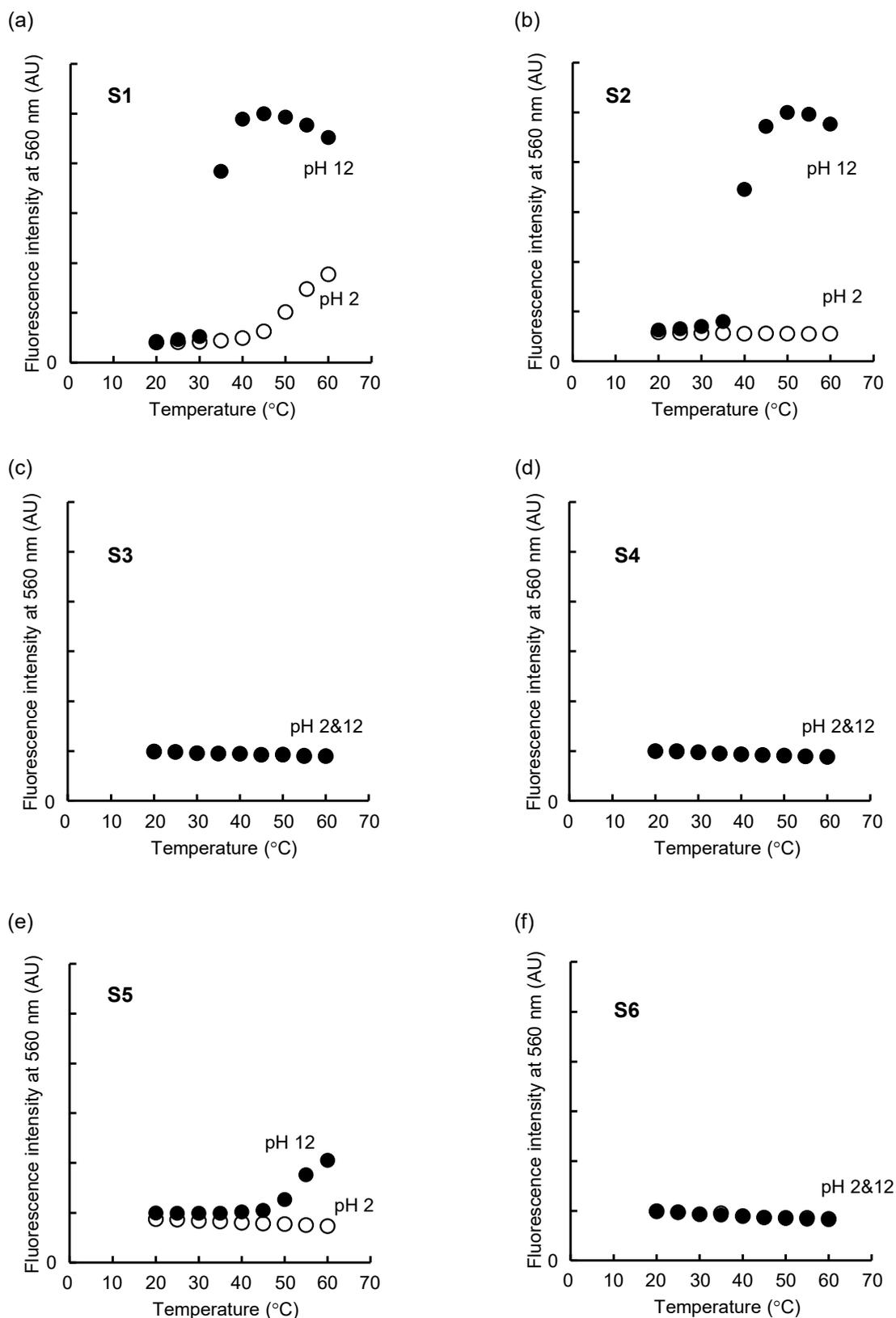


Figure S6. Temperature-dependent fluorescence intensity of copolymers S1–S6. (a) S1, (b) S2, (c) S3, (d) S4, (e) S5 and (f) S6 at pH 12 (closed) and 2 (open) in Britton–Robinson buffer solutions. In panels (c), (d) and (f), the data at pH 2 overlap with those at pH 12. A small increase in the fluorescence intensity of S5 at pH 12 over 50 °C was consistently observed across different lots. Samples (0.01 w/v%) were excited at 450 nm.

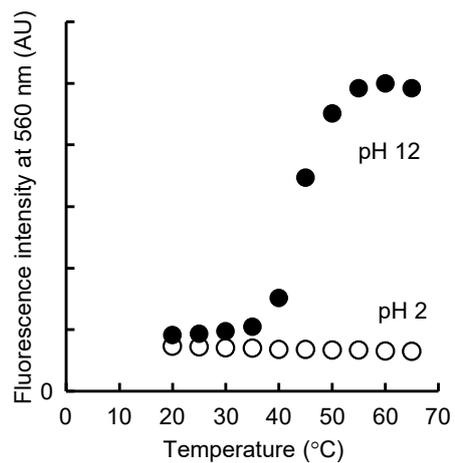


Figure S7. Temperature-dependent fluorescence intensity of copolymer S7. At pH 12 (closed) and 2 (open) in Britton–Robinson buffer solutions. Samples (0.01 w/v%) were excited at 450 nm.

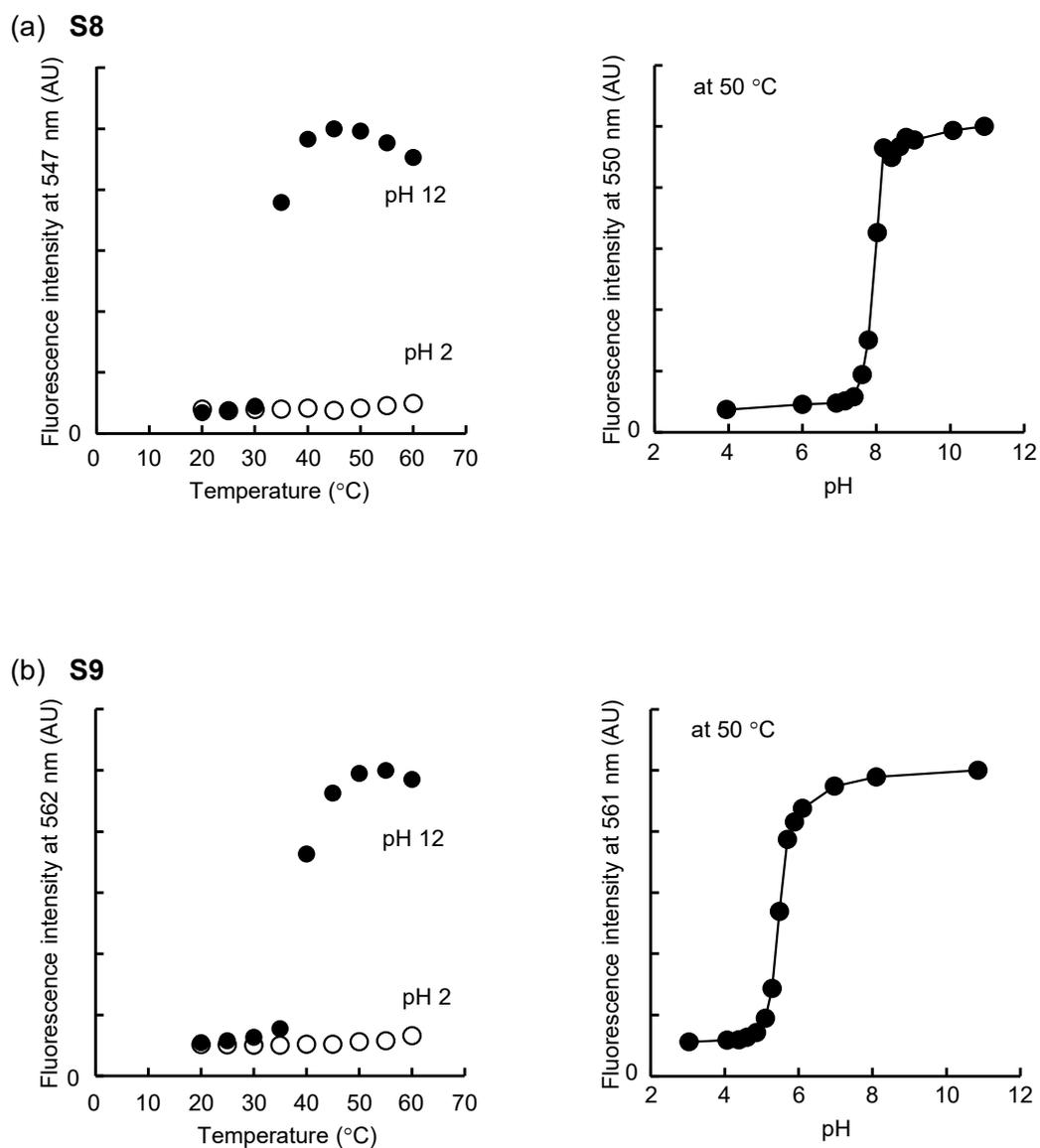


Figure S8. Fluorescence characteristics of copolymers S8 and S9 in Britton–Robinson buffer solutions. Temperature-dependent fluorescence intensities at pH 12 (closed) and 2 (open) (left) and pH-dependent fluorescence intensity responses to pH variations at 50 °C (right) for (a) **S8** and (b) **S9**. Samples (0.01 w/v%) were excited at 450 nm.

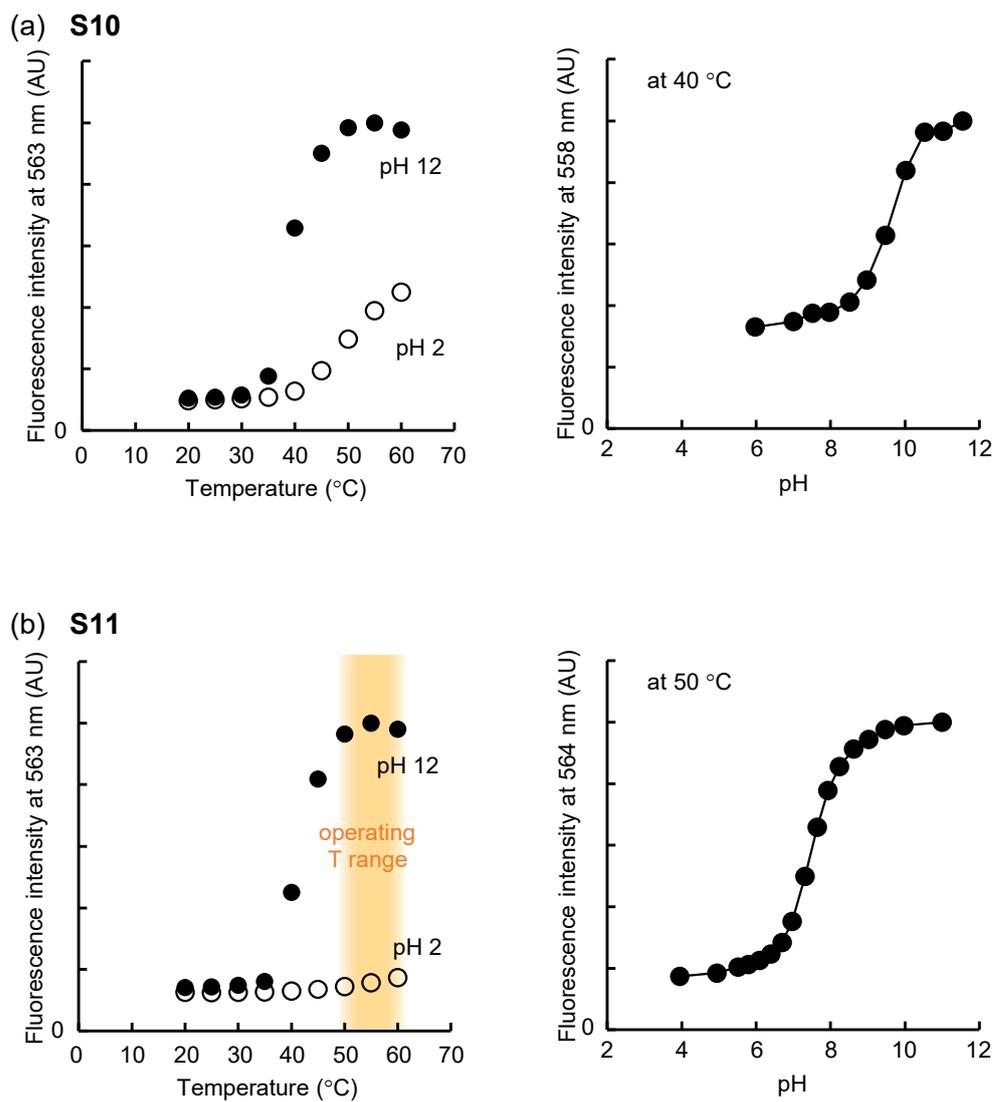


Figure S9. Fluorescence characteristics of copolymers S10 and S11 in Britton–Robinson buffer solutions. Temperature-dependent fluorescence intensities at pH 12 (closed) and 2 (open) (left) and pH-dependent fluorescence intensity responses to pH variations (right) for (a) **S10** at 40 °C and (b) **S11** at 50 °C. Samples (0.01 w/v%) were excited at 450 nm.

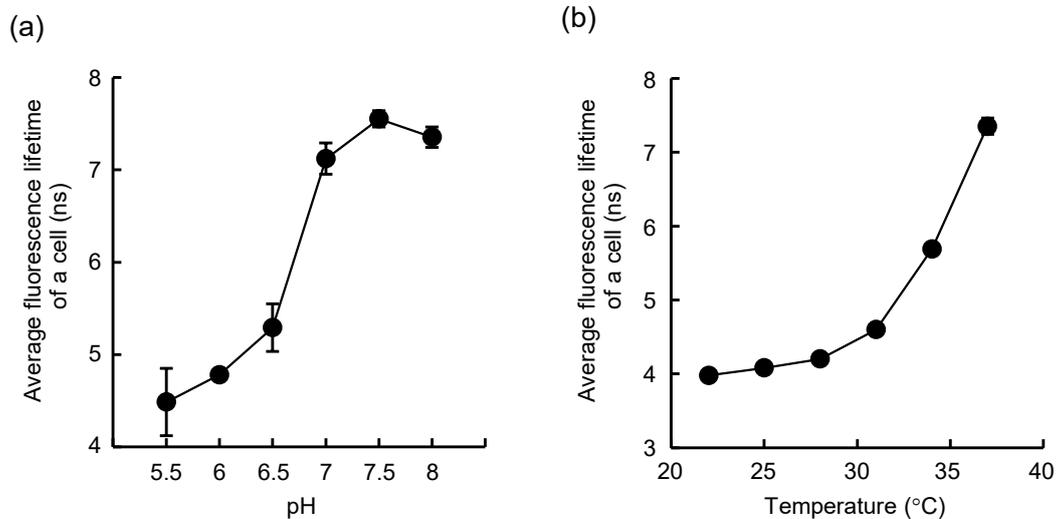


Figure S10. Fluorescence lifetime of 9 in living HeLa cells in phosphate buffer solutions. Effects of (a) pH at 37 °C and (b) temperature at pH 8. Average \pm s.d. for 3–8 cells. Samples were excited at 470 nm. From the data indicated in panel (a), the a and pK_a values were evaluated to be 2.46 and 6.68.

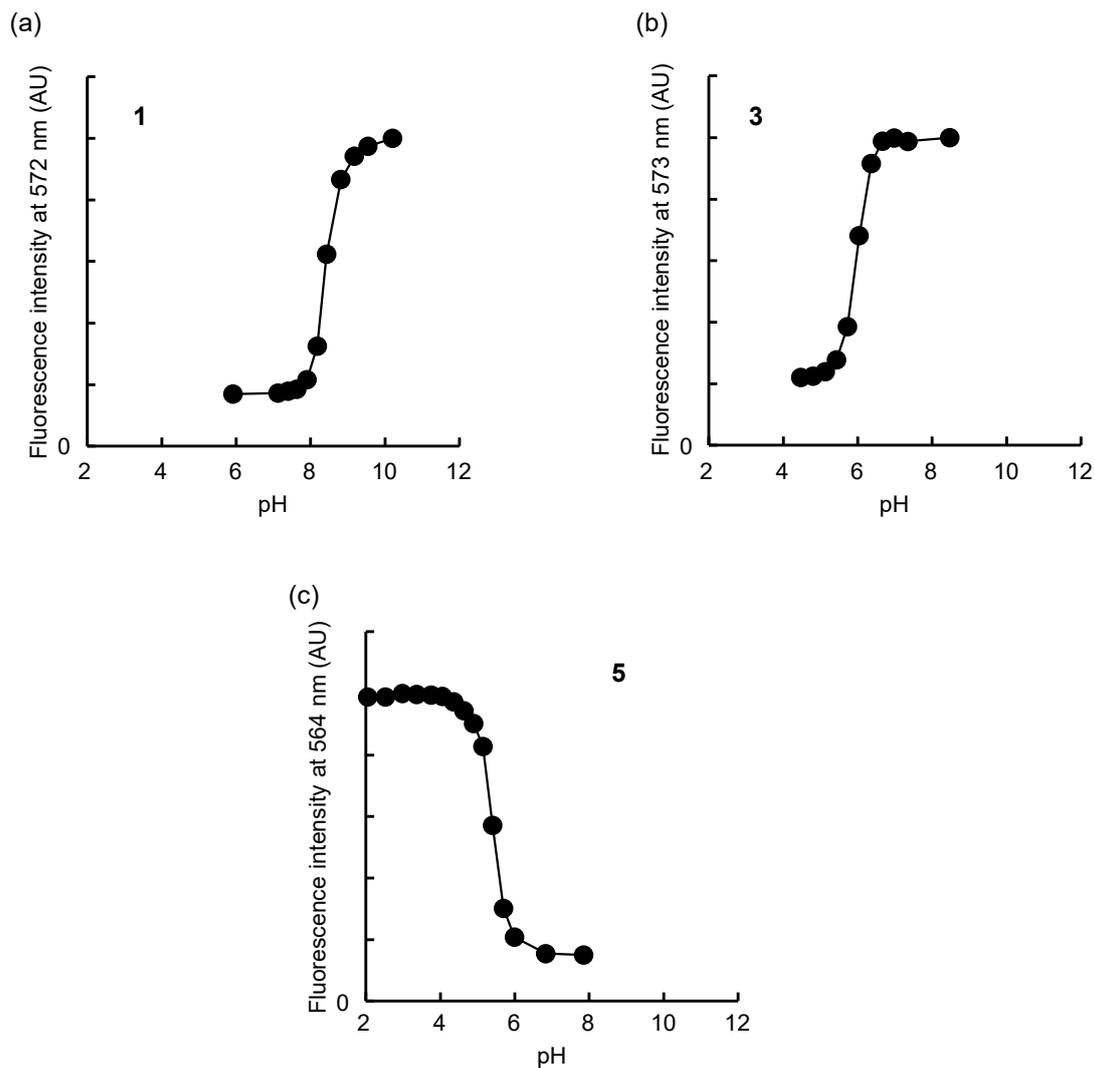
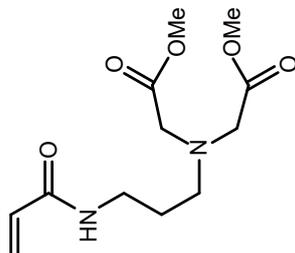


Figure S11. Fluorescence characteristics of copolymers 1, 3 and 5 in various buffer solutions. pH-dependent fluorescence intensity of (a) **1** in 0.1 M borate buffer solutions at 50 °C, (b) **3** in 0.2 M phosphate buffer solutions at 50 °C and (c) **5** in 0.2 M citrate buffer solutions 45 °C. Samples (0.01 w/v%) were excited at 450 nm.

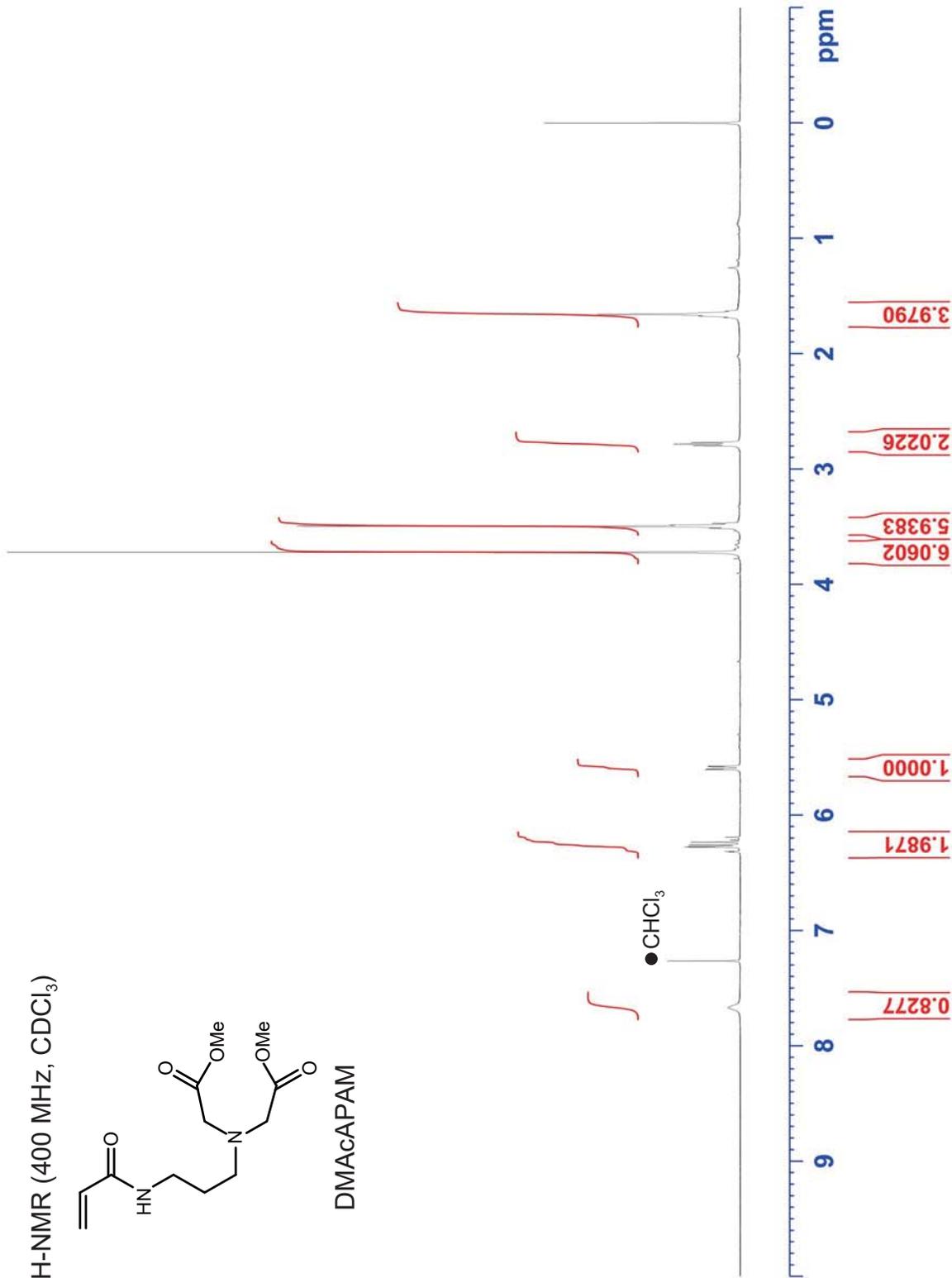


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¹H-NMR (400 MHz, CDCl₃)



DMAcAPAM



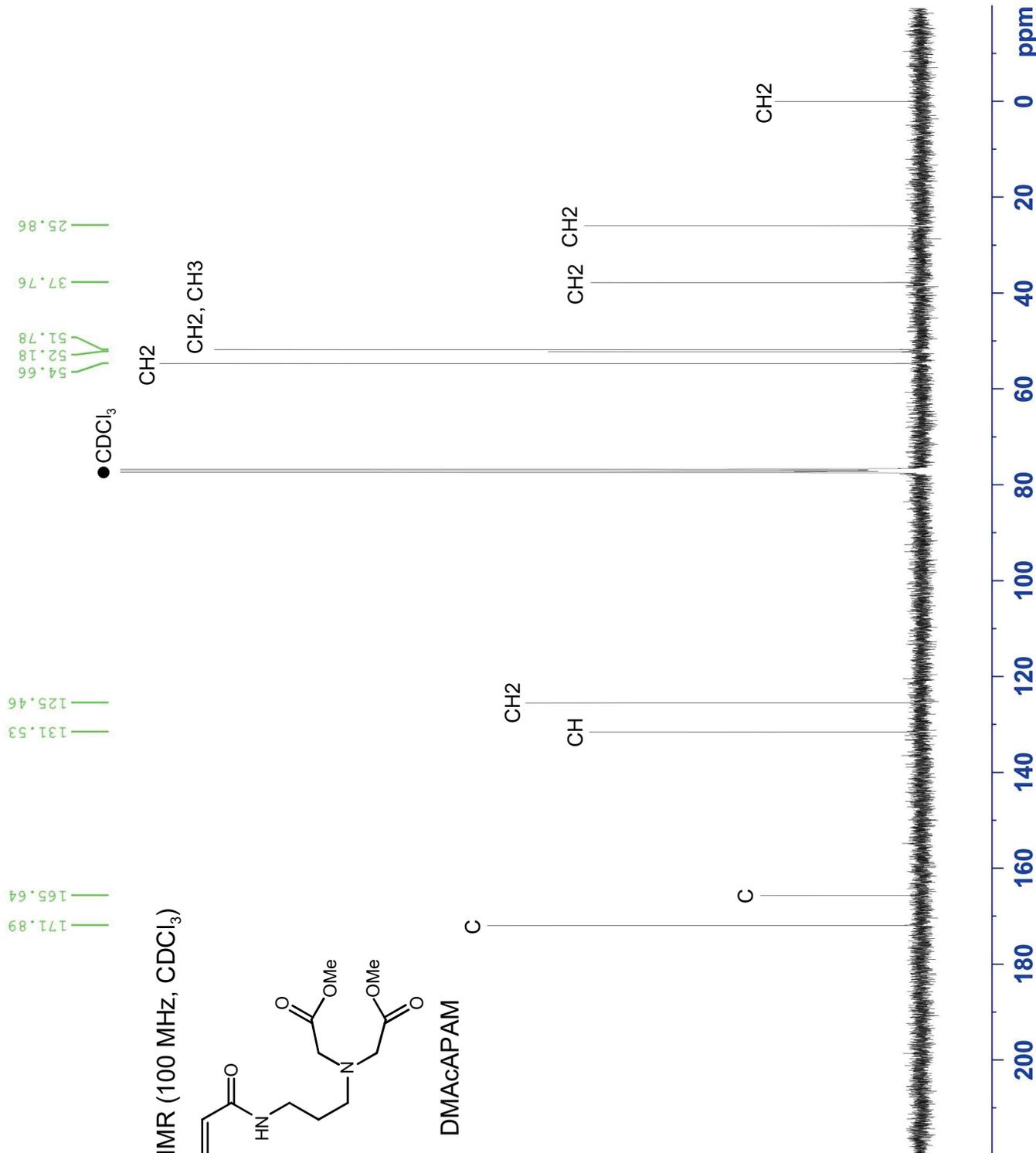
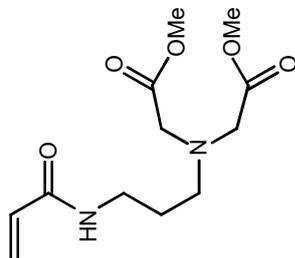
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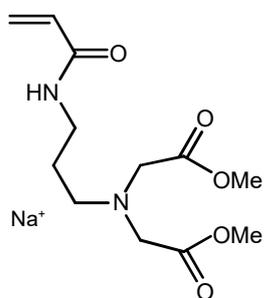
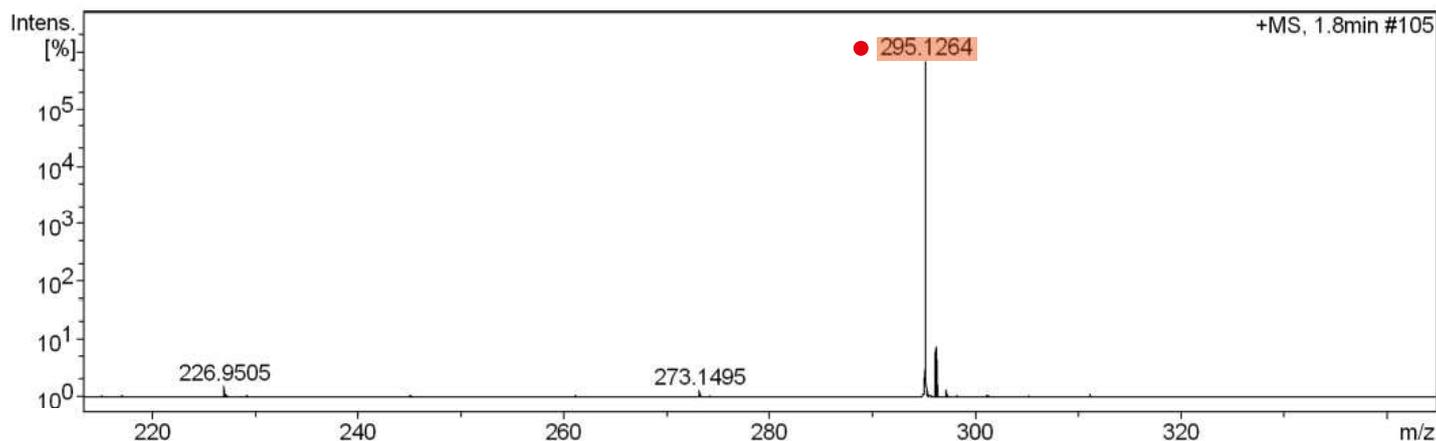
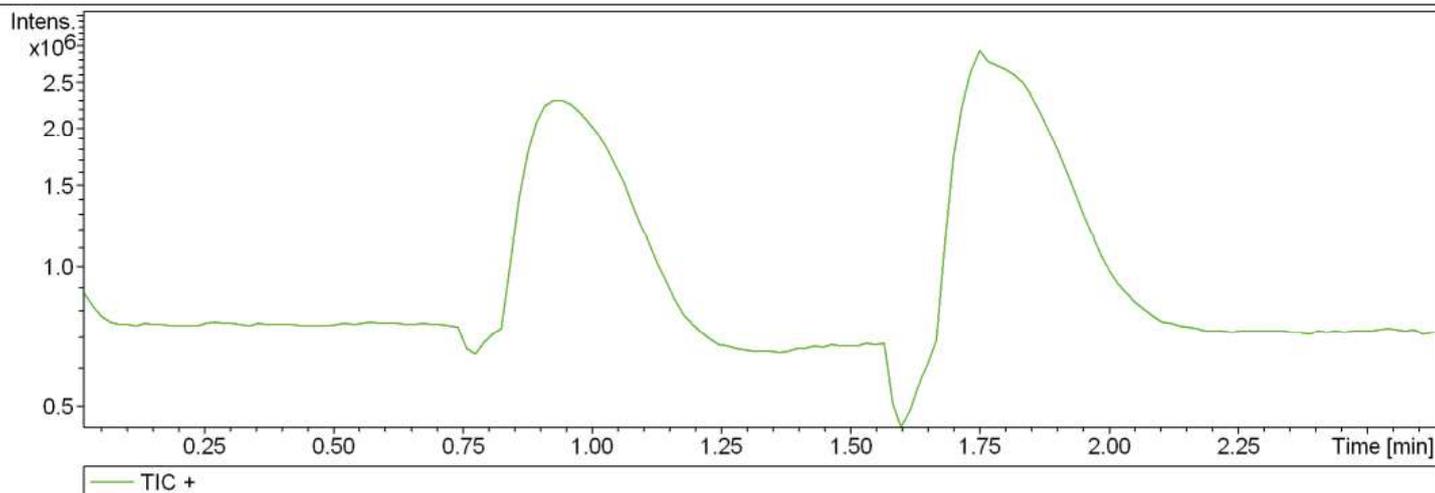
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[M+Na]⁺ calcd for
C₁₂H₂₀N₂NaO₅⁺: 295.1264

Supplementary References

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