Supporting Information

Design of Thermo-Responsive Polymers Toward Antibody Purification

Kana Nishimori[1], Masashi Maruyama[2,], Yuzuru Shimazaki[2,*], Makoto Ouchi[1,*], and Hiroshi Yoshida[2]*

[1]Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Katsura Nishikyo-ku, Kyoto 615-8510, Japan

[2]Research & Development Group, Hitachi Ltd., 7-1-1 Omika-cho, Hitachi City, Ibaraki 319-1292, Japan

*Correspondence to: masashi.maruyama.qn@hitachi.com

*Correspondence to: yuzuru.shimazaki.es@hitachi.com

*Correspondence to: ouchi@living.polym.kyoto-u.ac.jp

Contents

Experimental Section

Materials

N-isopropylacrylamide (NIPAM, TCI; purity >98%) was recrystallized twice in acetone before use. Tris[2-(dimethylamino)ethyl]amine (Me_6TREN, Sigma Aldrich; purity 97%) was degassed by bubbling dried nitrogen for 15 min. Cupper chloride (I) (CuCl, Wako; purity >99.9%), *N*-Boc-ethylenediamine (Sigma Aldrich; purity >98%), 2-chloropropanoyl chloride (TCI; purity >95%), trifluoroacetic acid (TFA, Wako; purity >98%), 1,4-dioxane (Wako, purity >99.5%), protein A mimetic ligand precursor **1** (intellim; purity >95%), human γ-globulin (NICHIYAKU; 450 mg/3mL), were used without purification. *tert*-Butylacrylate (TBA, Wako; purity >97%), triethylamine (Et_3N, TCI; purity >99%) were distilled before use.

Characterization

Molecular weight distribution (MWD) curves, number-average molecular weight (M_n) and M_w/M_n ratio of the polymers were measured by size exclusion chromatography (SEC) in DMF containing 10 mM LiBr at 40 ˚C (flow rate: 1 mL/min) on three linear-type polystyrene gel columns (Shodex KF-805L:exclusion limit = 4×10^6; particle size 10 μm; pore size = 5000 Å; 0.8 cm i.d. × 30 cm) that were connected to a Jasco PU-2080 precision pump, a Jasco RI-2031 refractive index detector and a Jasco UV-2075 UV/vis detector set at 270 nm. The columns were calibrated against 10 standard PMMA samples (Polymer Laboratories: M_p = 2,680-1,250,000; M_w/M_n = 1.02-1.09). ^{1}H NMR spectra was recorded on a JEOL JNM-ECA500 spectrometer, operating at 500 MHz. Temperature variable turbidity profiles were recorded in 5mM PBS buffer (pH = 7.4) on UV-1800 (Shimadzu, optical path length = 1.0 cm) at λ = 670 nm upon heating ([Polymer] = 0.5 to 1.0 mg/mL, heating speed = 1 ˚C/min). Chemical conversion of PNIPAM derivatives was monitored using high performance liquid chromatography (HPLC) equipped with a reverse-phase HPLC column (Nacalai Tesque, Cosmosil Protein-R) under a gradient elution condition (H_2O/CH_3CN with 0.1% trifluoroacetic acid). For the SPR measurement, self-assembled monolayer (SAM) of thiol-terminated poly (ethylene glycol) (PEG) was prepared on the gold surface. The PEGs (C445 and H354) were purchased from Dojindo Molecular Technologies, Inc. The adsorption and desorption of the polymers (P2 and P3) and IgG were monitored by a commercial SPR system (MP-SPR NAVI^{TM} 210A, BioNavis). 20 mM PBS buffer (pH = 7.4) was used as an elution buffer

and the flow rate was 20 μL/min. The flow of the SPR experiment was as follows. First, the solution of the polymer (0.02 wt%) was applied to the SAM-modified surface at 20 ˚C for 10 min, and then the surface was rinsed for 90 min at 20 ˚C. The adsorption and desorption were measured in the PBS buffer solution. Then, the PBS solution of IgG (1.0 μM) was applied to the surface at 20 ˚C or 36 ˚C for 10 min and the amount of adsorption of IgG was evaluated.

Supporting Data

Synthesis of the Initiator 2[12]

N-Boc-ethylenediamine (984 μL, 6.25 $\times 10^{-3}$ mol) and THF (20 mL) were added in a flask under dried argon atmosphere. Et_3N (0.91 mL, 6.6 $\times 10^{-3}$ mol) was added with stirring and the solution was cooled to -78 ˚C. Then, 2-chloropropanoyl chloride (0.653 mL, 6.6 $\times 10^{-3}$ mol) was added in a dropwise for 10 min and the solution was left stirring at room temperature for 18 h. The precipitate was removed by filter and the solution was evaporated under reduced pressure. The obtained white solid was dissolved in methanol and precipitated into saturated Na_2CO_3 aq. The solvent was removed by filter and the precipitate was dissolved in methanol. The solution was filtrated, evaporated under reduced pressure and dried overnight to yield white solid (670 mg, yield 43%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.39 (q, 1H, CH), δ 3.39 (m, 2H, CH_2), δ 3.32 (m, 2H, CH_2), δ 1.73 (d, 3H, CH_3), δ 1.45 (s, 9H, $C(CH_3)_3$). (Figure S2a)

Polymerization and Characterization

ATRP of NIPAM with the initiator **2** was performed in 2-propanol at 25˚C: $[NIPAM]_0$ = 3500 mM; $[\mathbf{2}]_0 = [CuCl]_0 = [Me_6TREN]_0$ = 35 mM.[13] The conversion of NIPAM reached 47% in 1 hour, and at this point 60 eq. (for polymer chain or initiator) of TBA was added into the solution. Herein, the amount of added TBA was roughly calculated from the remained amount of NIPAM and reactivity ratios (r_1 = 0.4, r_2 = 0.8 for *N*-propyl acrylamide (M_1) and butyl acrylate (M_2) as the model monomers for NIPAM and TBA, respectively) to make the copolymer segment of approximately 1:1 composition ratio. The polymerization was quenched in 20 min: TBA was slightly consumed ($Conv._{TBA}$ = 8%) and NIPAM was further consumed together with TBA ($Conv._{NIPAM}$ = 48%).

Detailed process is shown below.

Polymerization was carried out by the syringe technique under dry argon in baked glass tubes equipped with three-way stopcock. To Schlenk tube A, NIPAM (5.00 g, 4.44×10^{-2} mol, 100eq.), CuCl (43.73 mg, 4.44×10^{-4} mol, 1eq.) and stirrer bar were added in 8.33 mL 2-propanol and 0.20 mL tetralin. Then, Me_6TREN (0.119 mL, 4.44×10^{-4} mol, 1eq.) was added and the solution was stirred for 20 min at room temperature to form complex with CuCl and Me_6TREN completely. To another Schlenk tube B, initiator **2** (111.73 mg, 4.44×10^{-4} mol, 1eq.) was added in 4.20 mL 2-propanol. Then, all the solution in tube B was added to tube A at r.t.. After mixing, the tube was immediately placed in water bath at 25 ˚C. After 60 min, 8.88 mL of the solution was sampled by syringe under dry argon and the polymerization was terminated by bubbling with air to obtain **P4**. After sampling, degassed TBA (1.92 mL, 13.3×10^{-2} mol, 60eq. for polymer chain or initiator) was added immediately to the tube B. After 20 min, the three-way stopcock was removed and the copolymerization was terminated by bubbling with air to obtain **P1**. Monomer conversion was determined by ^{1}H NMR from the integrated peak area of the olefinic protons of the monomer with tetralin as an internal standard. The two monomer conversions (NIPAM/TBA) were 47%/0% at **P4** and 48%/8% at **P1** respectively. The SEC curve of the resultant polymer was unimodal (M_w/M_n = 1.10) and shifted to higher molecular weight than that for before the TBA addition, indicating that copolymerization of the two comonomers likely took place from the PNIPAM chain. Each polymer solution was evaporated *in vacuo* and the polymers were dissolved in methanol. Then, the solutions were dialyzed using membrane (Spectra/Por®7, MWCO 500) in methanol for 3 days to remove unreacted monomers and Cu catalysis. After dialysis, the solutions were evaporated and freeze-dried in 1,4-dioxane to yield white powder (**P4**: 600 mg, M_n = 8000, M_w/M_n = 1.08, **P1**: 800 mg, M_n = 9200, M_w/M_n = 1.10, Figure S1).

The polymer structure was evaluated by ^{1}H NMR after dialysis for removal of low molecular weight compounds. For the polymer before the TBA addition, peaks derived from initiator moieties (*a'*, *b'*, and *c'* in Figure S2b), whose positions were close to those for the initiator **2** (*a*, *b*, and *c* in Figure S2a), were observed along with those from PNIPAM (*f*, *g*, *h* in Figure S2b). The number-averaged polymerization degree (*m*) for the PNIPAM was estimated to be 71. The value is different from the ideal value (*m* = 48) calculated by the conversion (48%). Part of the initiator molecules likely suffered from irreversible side reactions at the initial stage and thus the number of actual initiator was decreased. For the polymer after TBA addition, the signal derived from the *tert*-butyl group (*i* in Figure S2c) were apparently observed in addition to the signals derived from

the PNIPAM before the TBA addition. each number-averaged polymerization degree ($DP_{n,NIPAM}$ and $DP_{n,TBA}$) was similarly calculated: $DP_{n,NIPAM} = 78$ and $DP_{n,TBA} = 6$. Thus, the polymer consists of homopolymer segment of NIPAM (m = 71) and random copolymer segment of NIPAM and TBA (n = 7, l = 6: n and l represent number-averaged polymerization degree of NIPAM and TBA in the copolymer segment, respectively).

Introduction of Ligand via Post Reaction in P1

In a flask equipped with three-way stopped cock, **P1** (723 mg) and stirrer bar were added in 15 mL dichloromethane. Then, 15 mL TFA was added in dropwise for 5 min at 0 ˚C and the solution was stirred at room temperature. After 5 h, 1.5 mL distilled water was added and the solution was stirred for 18 h. The solution was evaporated *in vacuo* and the residue was redissolved in water and Na_2CO_3 aq. was added to the solution to reach pH = 10. After dialysis in water for 3 days, the solution was evaporated *in vacuo* and the residue was freeze-dried in 1,4-dioxane to obtain **P2** (white powder). The signal derived from the NBoc and *tert*-butyl groups (*a"*, *i* in Figure S2c) disappeared, indicating the deprotection was completed (Figure S2d). Deprotection of NBoc and *tert*-butyl ester was also confirmed by HPLC (Figure S4).

In a flask equipped with three-way stopped cock, **P2** (500 mg, M_n = 9200, 5.4×10^{-5} mol) and ligand precursor **1** (190 mg, 5.0×10^{-4} mol) were dissolved in 17 mL 1,4-dioxane and 6 mL distilled water under dry argon. Then, Et_3N (1.2 mL) was added to the solution and the solution was heated in oil bath at 100 ˚C for 23 h. After cooling at r.t., the solution was evaporated *in vacuo* and the residue was purified by dialysis in ethanol for 1 week to remove unreacted ligand. After dialysis, the solution was evaporated and freeze-dried in 1,4-dioxane to yield **P3**, pale brown powder (355 mg). In Figure S2e, small signals were observed at lower magnetic field (around 6.0-9.0 ppm). The peaks are likely derived from the ligand, and the increase in the integration ratio supported quantitative introduction of the ligand. Induction of the ligand was also confirmed by HPLC (Figure S4).

Introduction of Ligand via Post Reaction in P4

In a flask equipped with three-way stopped cock, **P4** (548 mg) and stirrer bar were added in 11 mL dichloromethane. Then, 11 mL TFA was added in dropwise for 5 min at 0 ˚C and the solution was stirred at room temperature. After 5 h, 1.2 mL distilled water was added and the solution was stirred for 18 h. The solution was evaporated *in vacuo* and the residue was redissolved in water. Then, Na_2CO_3 aq. was added to the solution to reach pH = 10. After dialysis in water for 3 days, the solution was evaporated *in vacuo* and the residue was freeze-dried in 1,4-dioxane to obtain **P5** (white powder). Deprotection of NBoc was confirmed by ^{1}H NMR and HPLC (Figure S3 and S4).

In a flask equipped with three-way stopped cock, **P5** (400 mg, M_n = 8000, 5.0×10^{-5} mol) and ligand precursor **1** (85 mg, 2.2×10^{-4} mol) were added in 14 mL 1,4-dioxane under dry argon. Then, Et_3N (1000 μL) was added to the solution and the solution was heated in oil bath at 100 ˚C for 19 h. After cooling at r.t., the solution was evaporated *in vacuo* and the residue was purified by dialysis in ethanol for 1 week to remove unreacted ligand. After dialysis, the solution was evaporated and freeze-dried in 1,4-dioxane to yield **P6**, pale brown powder (240 mg). Introduction of the ligand was confirmed by ^{1}H NMR and HPLC (Figure S3 and S4).

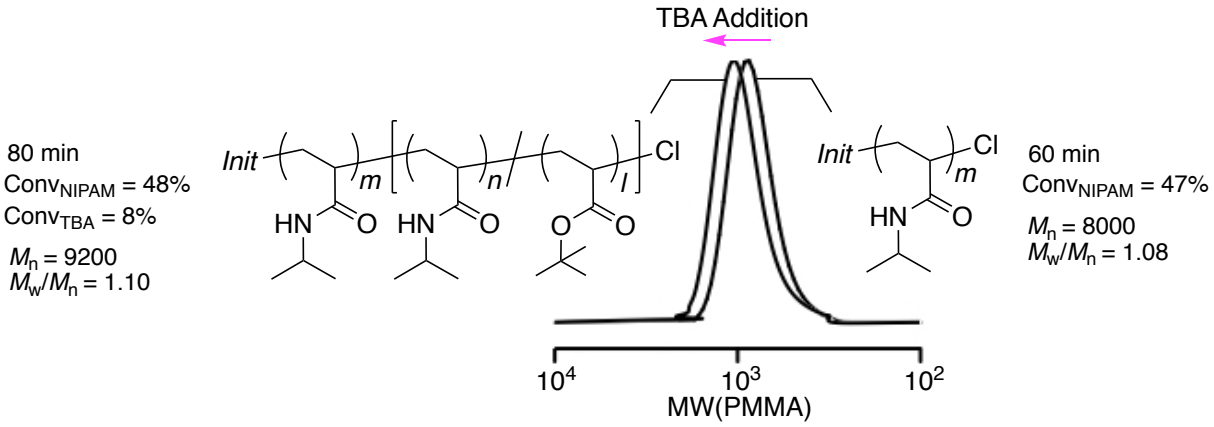

Figure S1. SEC curves of the copolymer **P1** and **P4**.

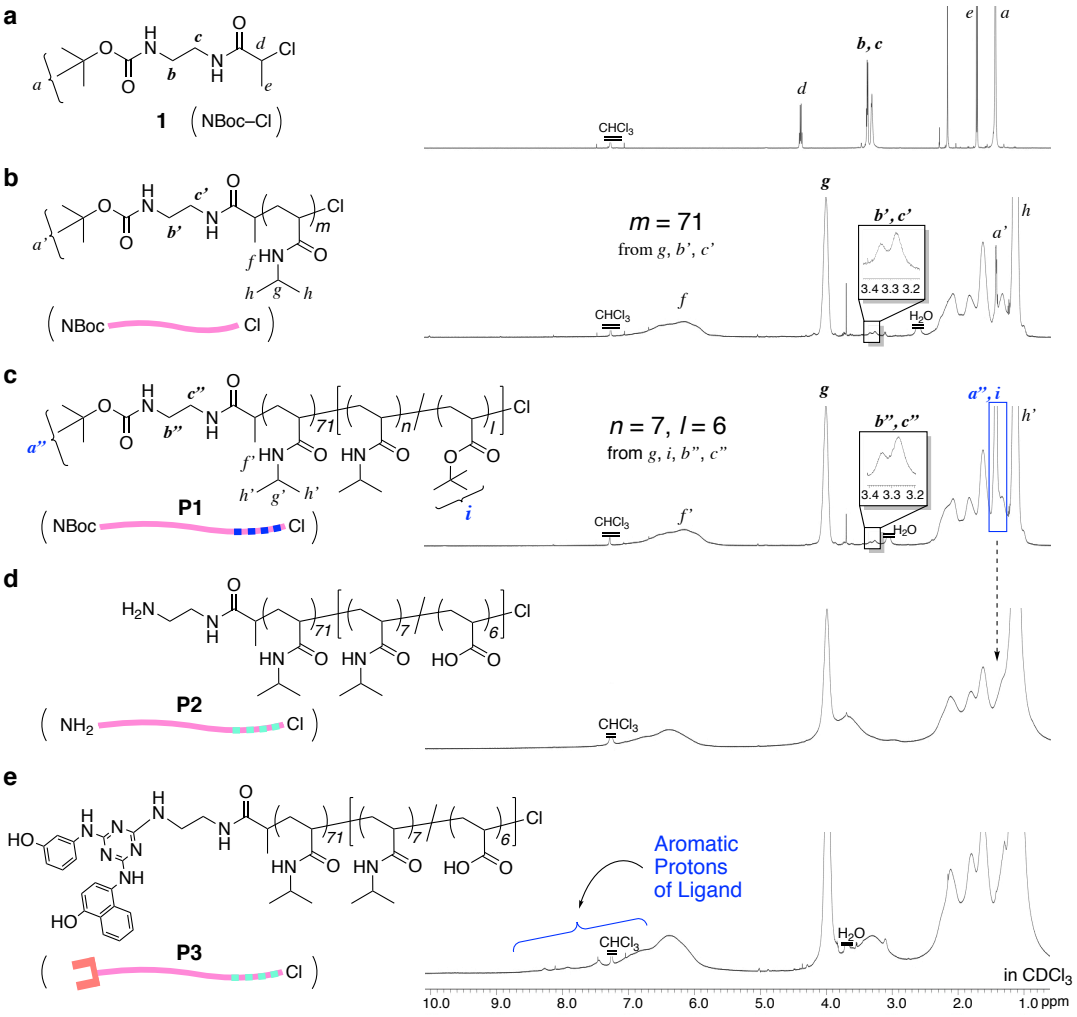

Figure S2. ^{1}H NMR spectra of initiator and obtained copolymers-s: (a) initiator, (b) the polymer before addition of TBA, (c) the polymer after addition of TBA (**P1**), (d) after deprotection (**P2**) and (e) after incorporation of ligand (**P3**). Polymerization: $[NIPAM]_0/[2]_0/[CuCl]_0/[Me_6TREN]_0$ = 3500/35/35/35 mM in 2-propanol at 25 ˚C, $[TBA]_{add}$ = 2100 mM.

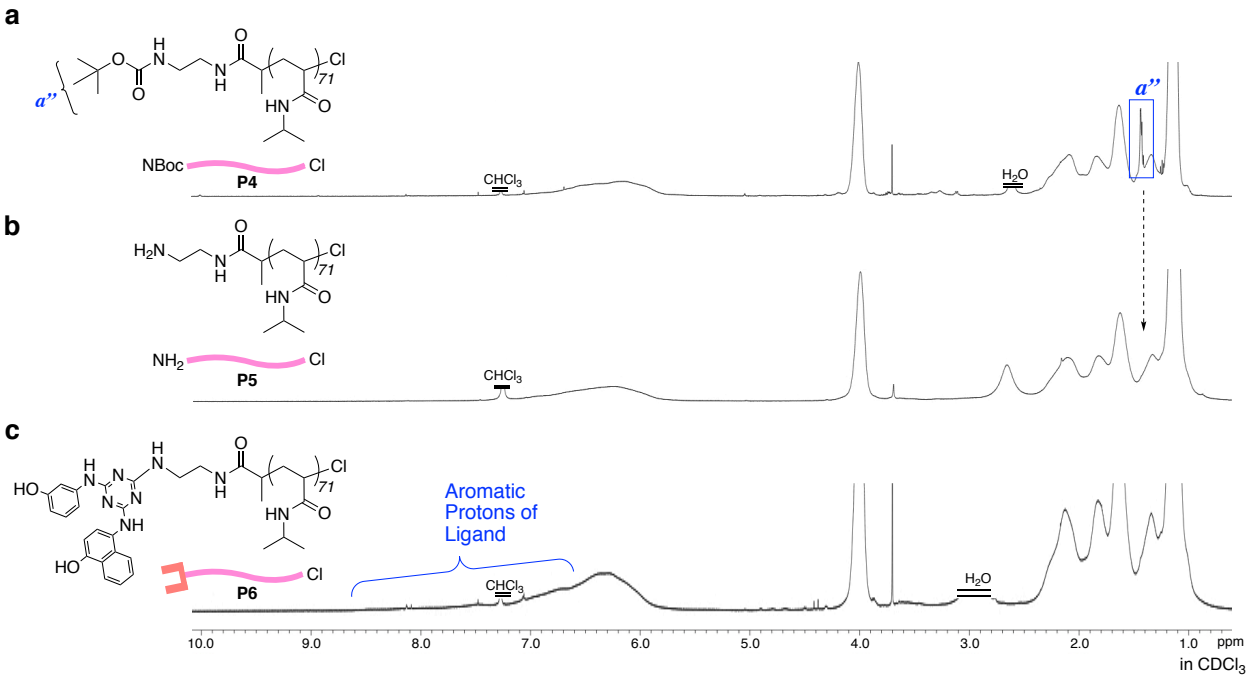

Figure S3. ^{1}H NMR spectra of the polymers: (a) **P4**, (b) **P5** and (c) **P6**.

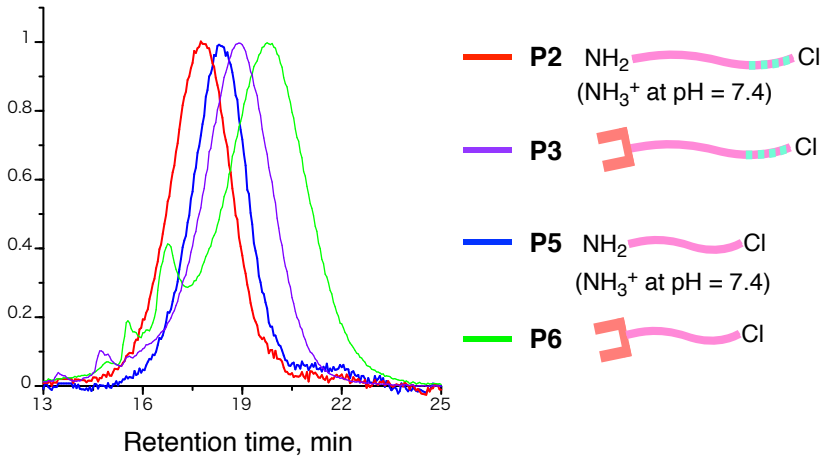

Figure S4. HPLC chromatograms of **P2** (red), **P3** (purple), **P5** (blue) and **P6** (green). Elution condition: H_2O/CH_3CN = 90/10 to 10/90 (linear gradient, 0~20 min) + 0.1%TFA, wave length = 220 nm.

Comment on Figure S4: The retention time obviously depended on the terminal groups of the copolymers: the copolymer carrying the ligand gave longer retention time than the precursor of amine terminal. The trend shows the hydrophobic feature of the ligand at the terminal.

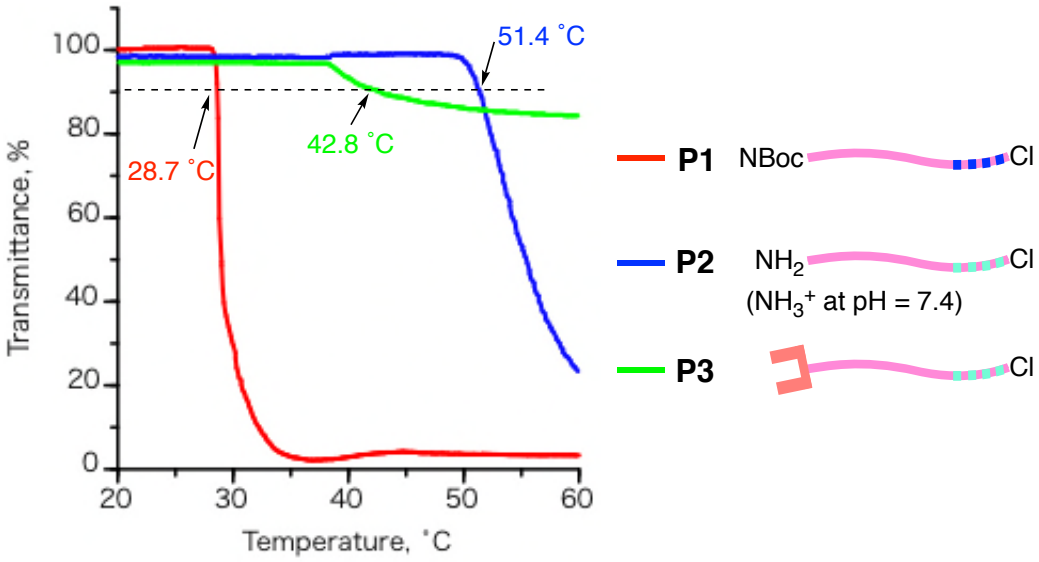

Figure S5. Temperature variable turbidity measurement for PBS buffer solutions of **P1** (red), **P2** (blue) and **P3** (green): [polymer] = 1.0 mg/mL in 5 mM PBS buffer (heating rate: 1 ˚C/min).