Article

pubs.acs.org/Biomac

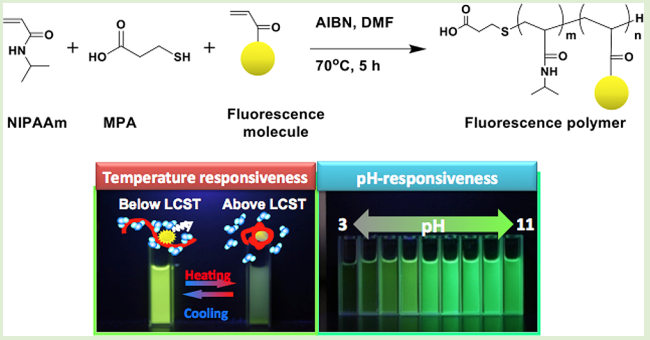
Design of Environmentally Responsive Fluorescent Polymer Probes for Cellular Imaging

Arisa Yamada, Yuki Hiruta, Jian Wang, Eri Ayano, and Hideko Kanazawa*

Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan

Supporting Information

ABSTRACT: We report the development of environmentally responsive fluorescent polymers. The reversible temperature-induced phase transition of copolymers composed of *N*-isopropylacrylamide and a fluorescent monomer based on the fluorescein (FL), coumarin (CO), rhodamine (RH), or dansyl (DA) skeleton was used as a molecular switch to control the fluorescence intensity. The poly(*N*-isopropylacrylamide) (PNIPAAm) chain showed an expanded coil conformation below the lower critical solution temperature (LCST) due to hydration, but it changed to a globular form above the LCST due to dehydration. Through the combination of a polarity-sensitive fluorophore with PNIPAAm, the synthetic fluorescent polymer displayed a response to external temperature, with the fluorescence strength dramatically changing close to the LCST. Additionally, the P(NIPAAm-*co*-FL) and P(NIPAAm-*co*-CO) polymers, containing fluorescein and coumarin groups, respectively, exhibited pH responsiveness. The environmental responsiveness of the reported polymers is derived directly from the PNIPAAm and fluorophore structures, thus allowing for the cellular uptake of the fluorescence copolymer by RAW264.7 cells to be temperature-controlled. Cellular uptake was suppressed below the LCST but enhanced above the LCST. Furthermore, the cellular uptake of both P(NIPAAm-*co*-CO) and P(NIPAAm-*co*-RH) conjugated with a fusogenic lipid, namely, L-α-phosphatidylethanolamine, dioleoyl (DOPE), was enhanced. Such lipid-conjugated fluorescence probes are expected to be useful as physiological indicators for intracellular imaging.



INTRODUCTION

Environmentally responsive polymers are well-known as valuable materials in biomaterial sciences and biomedical technologies.[1] Such polymers change their structures and physical properties rapidly and reversibly in response to subtle external stimuli such as temperature, pH, and light. Within this group of polymers, poly(*N*-isopropylacrylamide) (PNIPAAm) has been found to exhibit thermally reversible solubility changes in response to temperature changes across its lower critical solution temperature (LCST) at 32 °C.[2] In aqueous solution, PNIPAAm shows an expanded coil conformation below the LCST due to strong hydration, but it changes to a globular form above the LCST due to sudden dehydration. This unique property has allowed PNIPAAm to be widely applied in drug delivery systems,[3] cell culture substrates,[4] and separation systems.[5]

Recently, fluorescent polymers composed of environmentally responsive polymers and fluorescent molecules have received a great deal of attention. It is well-known that the wavelength and strength of a fluorescent molecule are sensitive to changes in the adjacent molecular environment, including density, temperature, and pH. Annaka et al. reported the use of a PNIPAAm-based fluorescent polymer to study the swelling behavior of grafted PNIPAAm hydrogels by fluorescence depolarization.[6] In addition, fluorescent thermometer beads on a PNIPAAm-based polymer have been studied extensively.[7−9] Although a range of fluorescent polymers has been developed, the majority allow only simple monitoring of the solution temperature in terms of emission intensity.

Recently, we developed a dual temperature/pH-responsive fluorescent polymer for application in cellular imaging.[10] The temperature and pH of all living cells are known to change during cellular events such as enzyme reactions, gene expression, and metabolism.[11] From a clinical viewpoint, pathological cells are warmer than normal cells due to their enhanced metabolic activity.[12] Abnormal intracellular pH values are therefore associated with inappropriate cell function and growth and are observed in a number of common diseases such as cancer[13] and Alzheimer's disease.[14] Monitoring intracellular temperature and pH, therefore, can provide critical information for studying physiological and pathological processes within living cells. We previously reported the synthesis of poly(*N*-isopropylacrylamide) (PNIPAAm) bearing a fluorescent molecule at the terminal end and confirmed its temperature- and pH-dependent cellular uptake.[15,16]

Received: May 1, 2015
Revised: June 17, 2015

DOI: 10.1021/acs.biomac.5b00591
Biomacromolecules XXXX, XXX, XXX−XXX

With the aim of implementation in cellular environmental imaging, we herein report the preparation of four fluorescent PNIPAAm-based copolymers and characterization of the effects of pH and/or temperature on their fluorescent behavior.

MATERIALS AND METHODS

Materials. *N*-Isopropylacrylamide (NIPAAm) was kindly provided by KJ Chemicals Corporation (Tokyo, Japan) and was purified by recrystallization from *n*-hexane and dried at 25 °C *in vacuo*. Fluorescein *o*-acrylate was purchased from Sigma-Aldrich (St. Louis, MO, USA). 7-(4-Trifluoromethyl)coumarin acrylamide, dansyl chloride, *N*,*N*-dimethylethylenediamine, acryloyl chloride, and triethylamine were purchased from Tokyo Kasei Industry (Tokyo, Japan). Methacryloxyethyl thiocarbamoyl rhodamine B was purchased from Funakoshi (Tokyo, Japan). 2,2′-Azobis(isobutyronitrile) (AIBN), 3-mercaptopropionic acid (MPA), and L-α-phosphatidylethanolamine, dioleoyl (DOPE) were purchased from Wako (Osaka, Japan). *N*-Hydroxysuccinimide (NHS) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were purchased from Kanto Chemical (Tokyo, Japan). All other reagents and solvents were of analytical grade.

Synthesis of *N*-[2-[[[5-(*N*,*N*-Dimethylamino)-1-naphthalenyl]sulfonyl]amino]ethyl]-2-propenamide. *N*-[2-[[[5-(*N*,*N*-Dimethylamino)-1-naphthalenyl]sulfonyl]amino]ethyl]-2-propenamide (DA) was prepared according to literature methods.[17] A mixture of dansyl chloride (200 mg, 0.74 mmol) and *N*,*N*-dimethylethylenediamine (445 mg, 7.42 mmol) in dichloromethane was stirred at 0 °C for 1 h to give the DA precursor, *N*-(2-aminoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide. After purification by column chromatography (silica gel), *N*-(2-aminoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide (120 mg, 0.40 mmol) was reacted with acryloyl chloride (45 mg, 0.49 mmol) in the presence of triethylamine (50 mg, 0.49 mmol) in THF at 0 °C for 24 h. The crude material was purified by column chromatography to give DA as a light green solid (82 mg, 0.23 mmol). δ ^{1}H NMR (600 MHz, $CDCl_3$): 8.56 (d, 1H, J = 8.5 Hz), 8.24 (m, 2H), 7.55 (m, 2H), 7.19 (d, 1H, J = 7.3 Hz), 6.20(d, 1H, J = 16.9 Hz), 5.91 (m, 2H), 5.61 (d, 1H, J = 10.2 Hz), 5.31 (s, 1H), 3.37 (m, 2H), 3.07 (m, 2H), 2.90 (s, 6H).

Synthesis of Poly(*N*-isopropylacrylamide-*co*-DA). Poly(*N*-isopropylacrylamide-*co*-DA) [P(NIPAAm-*co*-DA)] was prepared using radical polymerization, as shown in Scheme 1. NIPAAm (10.0 g, 88.4 mmol) and DA (30.5 mg, 0.09 mmol) were dissolved in *N*,*N*-dimethylformamide (20 mL). AIBN (58 mg, 0.35 mmol) and MPA (263 mg, 2.48 mmol), which act as the radical initiator and the chain transfer agent, respectively, were added to the solution. The reaction mixture was degassed by subjecting to freeze−thaw cycles and then heated at 70 °C for 5 h. The reaction solution was then poured into diethyl ether (1 L) to precipitate the polymers. The crude product was further purified by repeated precipitation using diethyl ether (1 L) from a solution of acetone (20 mL), filtered, and dried to give the desired polymer as a red solid (7.0 g). The molecular weight of the polymer was determined by GPC analysis.

Synthesis of Other Fluorescence Polymers. Poly(*N*-isopropylacrylamide-*co*-fluorescein *o*-acrylate) [P(NIPAAm-*co*-FL)], poly[*N*-isopropylacryamide-*co*-7-(4-trifluoromethyl)coumarin acrylamide] [P(NIPAAm-*co*-CO)], and poly[*N*-isopropylacrylamide-*co*-methacryloxyethyl thiocarbamoyl rhodamine B] [P(NIPAAm-*co*-RH)] were prepared from NIPAAm and either fluorescein *o*-acrylate, 7-(4-trifluoromethyl)coumarin acrylamide, or methacryloxyethyl thiocarbamoyl rhodamine B, respectively, according to the above procedure.

Analytical Techniques. ^{1}H NMR spectra were acquired on a JNM-EP600 spectrometer (600 MHz, JEOL, Tokyo, Japan) using tetramethylsilane as the internal standard. GPC was conducted on a TOSOH GPC-8020 system equipped with a differential refractive index detector, TSK guard column, and two TSK GEL α-M columns. The mobile phase was composed of 10 mM LiCl in DMF (at 40 °C, flow rate = 1.0 mL/min). Calibration was performed using near-monodisperse poly(ethylene glycol) standards obtained from TOSOH (Tokyo, Japan). The lower critical solution temperatures (LCSTs) of the polymers were determined by measuring the optical transmittance of their aqueous solution (0.5% w/v). The optical transmittance of each polymer solution was measured at 500 nm over a range of temperatures using a UV−vis spectrophotometer (V-630, JASCO, Tokyo, Japan). The temperature was controlled using an ETC-717 controller (JASCO) and a PT-31 Peltier system (Krüss, Hamburg, Germany); the heating rate was 0.1 °C/min. The LCST was determined at the temperature at which 50% optical transmittance of the polymer solution was achieved. Fluorescence spectra were measured using an FP-6300 spectrofluorometer (JASCO), and the temperature was controlled as described above. A quartz cuvette with a 1 cm path length was used. The maximum excitation wavelengths and measured emission wavelengths of the fluorescent polymers were as follows (0.5% w/v aqueous solution): P(NIPAAm-*co*-DA) λ_{ex} = 310 nm, λ_{em} = 500 nm; P(NIPAAm-*co*-FL) λ_{ex} = 440 nm, λ_{em} = 515 nm; P(NIPAAm-*co*-CO) λ_{ex} = 350 nm, λ_{em} = 460 nm; and P(NIPAAm-*co*-RH) λ_{ex} = 540 nm, λ_{em} = 590 nm. The effects of pH and temperature on the fluorescence intensity of P(NIPAAm-*co*-FL) and P(NIPAAm-*co*-CO) were evaluated in Briton−Robinson universal buffers (pH 3−11) between 25 and 35 °C.

Synthesis of the Lipid-Conjugated Polymers. The terminal carboxyl groups on P(NIPAAm-*co*-CO) and P(NIPAAm-*co*-RH) were esterified using NHS in the presence of DCC (molar ratio, 1:2.5:2.5) in dichloromethane prior to conjugation with DOPE. The activated esterified polymer (200 mg) was dissolved in anhydrous 1,4-dioxane (10 mL) and reacted with DOPE (25 mg) for 2 days at 25 °C. After evaporation of the reaction solution, the residue was dissolved in MeOH and subjected to dialysis, using a dialysis membrane with a 3500 amu cutoff (Spectra/Por, Spectrum Laboratories, CA, USA) at 4 °C for 3 days. After this time, evaporation of the solvent gave the desired product as a white ([P(NIPAAm-*co*-CO)-DOPE]) or pink−red solid ([P(NIPAAm-*co*-RH)-DOPE]).

Cell Culture. RAW264.7 cells (RIKEN BRC Cell Bank) were cultured as subconfluent monolayers in a 75 cm^2 culture flask with a vent cap using MEM, supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 μg/mL streptomycin, at 37 °C in a humidified incubator containing 5% CO_2. Subconfluent cells were dissociated using a cell scraper (30 cm, TPP, Switzerland) and plated in a flask for 2−3 days.

Detection of Cellular Uptake of the Polymer and Flow Cytometric Analysis. To detect cellular uptake of polymers, cells were seeded in a 60 mm dish at a density of 5.0×10^5 cells per dish, in 5 mL of medium. After overnight incubation, the cells were further incubated for 0.5, 1, 2, or 4 h with either P(NIPAAm-*co*-CO), P(NIPAAm-*co*-CO)-DOPE, P(NIPAAm-*co*-RH), or P(NIPAAm-*co*-RH)-DOPE at 37 or 27 °C in a humidified atmosphere containing 5% CO_2. The concentrations of P(NIPAAm-*co*-CO) and P(NIPAAm-*co*-CO)-DOPE at this point were calculated to be 400 μg/mL based on the culture medium, whereas those of P(NIPAAm-*co*-RH) and P(NIPAAm-*co*-RH)-DOPE were 200 μg/mL. After incubation, RAW264.7 cells were washed twice with PBS and harvested with trypsin/EDTA. Cells were resuspended in PBS, and their cell-associated fluorescence was detected using a flow cytometer (BD LSRII Flow Cytometer, BD Biosciences, San Jose, CA, USA).

Fluorescence Microscopy. RAW264.7 cells were seeded in 35 mm glass-bottomed dishes at a density of 2.0×10^5 cells per dish, in 2 mL of medium. After overnight incubation, the cells were further incubated for 0.5 or 2 h with either P(NIPAAm-*co*-RH) or P(NIPAAm-*co*-RH)-DOPE at 37 or 27 °C in a humidified atmosphere containing 5% CO_2. Following incubation, the RAW264.7 cells were rinsed twice with PBS and fixed with a 4% paraformaldehyde phosphate buffer solution for 20 min. The fixed cells were rinsed with PBS and incubated for 15 min with Hoechst 33258 (5 μg/mL in PBS) to stain their nuclei. After this time, the cells were then washed twice with PBS, and the RAW264.7 cells were visualized by fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan).

RESULTS AND DISCUSSION

Synthesis of Fluorescent Polymers. We first synthesized four fluorescent polymers via radical polymerization. The

DOI: 10.1021/acs.biomac.5b00591
Biomacromolecules XXXX, XXX, XXX−XXX

Scheme 1. Syntheses of Fluorescent Polymers

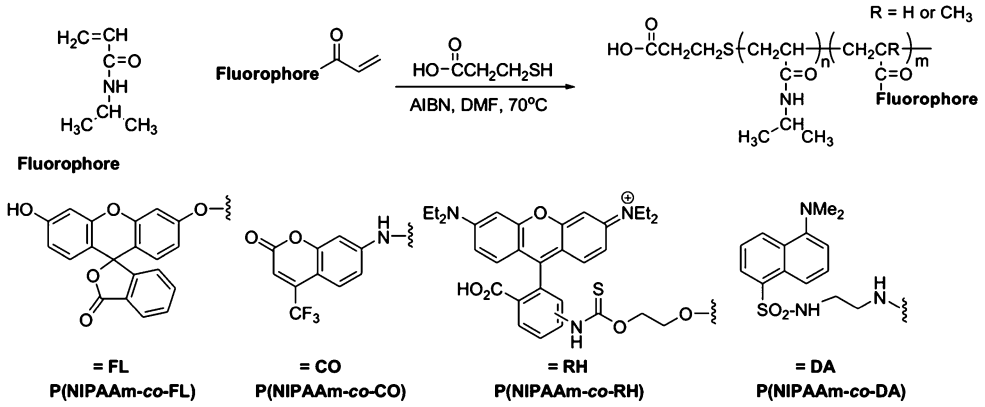


Table 1. Characterization of Synthetic Fluorescent Polymers

polymer	M_n[a]	M_w[a]	M_n/M_w	LCST (°C)[b]	λ_{ex} (nm)	λ_{em} (nm)
P(NIPAAm-*co*-FL)	26 779	52 994	1.979	30.5	490	515
P(NIPAAm-*co*-CO)	25 360	47 494	1.873	31.5	376	460
P(NIPAAm-*co*-RH)	22 631	45 905	2.028	32.4	540	588
P(NIPAAm-*co*-DA)	21 974	43 596	1.984	31.1	335	526

[a]Determined by GPC using DMF with 10 mM LiCl. [b]Determined by transmittance measured at 500 nm.

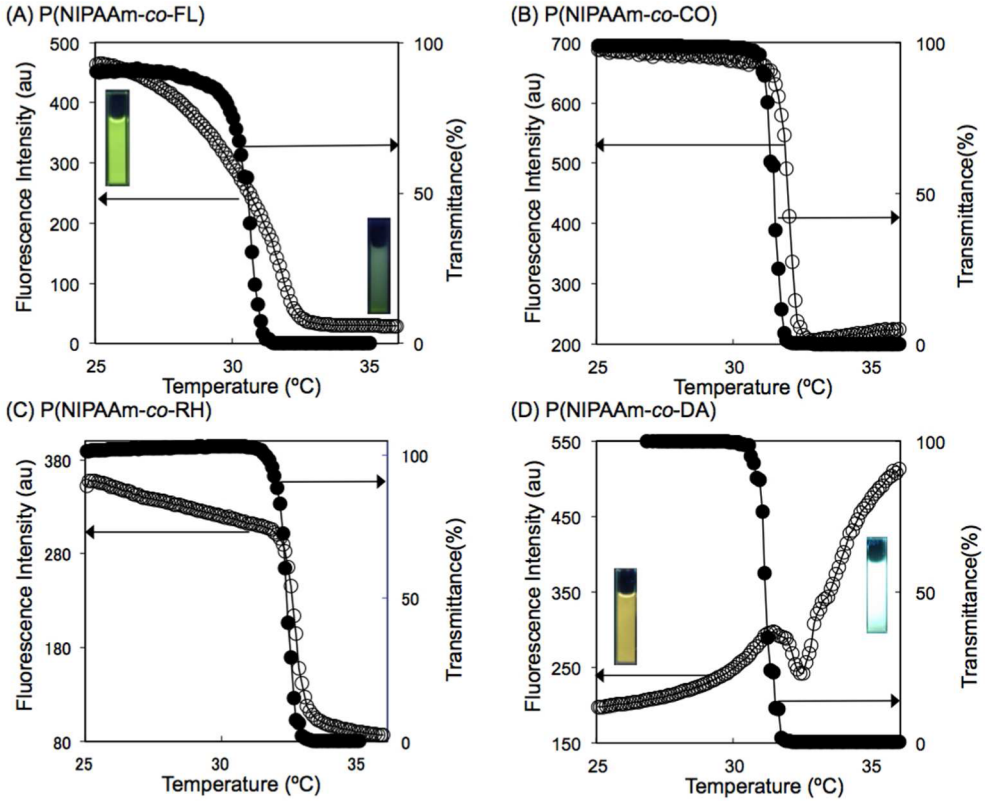


Figure 1. Temperature-responsive fluorescence spectral changes of (A) P(NIPAAm-*co*-FL) (λ_{ex} 440 nm), (B) P(NIPAAm-*co*-CO) (λ_{ex} 350 nm), (C) P(NIPAAm-*co*-RH) (λ_{ex} 540 nm), and (D) P(NIPAAm-*co*-DA) (λ_{ex} 310 nm) in aqueous solution (0.05% w/v) from 25 to 35 °C. Inset: temperature-dependent optical transmittance for the fluorescent polymers in aqueous solution (500 nm, 0.5% w/v).

synthetic scheme and structures of the target fluorescent polymers are shown in Scheme 1. For these preparations, we selected four fluorescent monomers, namely, fluorescein *o*-acrylate (FL), 7-(4-trifluoromethyl)coumarin acrylamide (CO), methacryloxyethyl thiocarbamoyl rhodamine B (RH), and *N*-[2-[[[5-(*N*,*N*-dimethylamino)-1-naphthalenyl]sulfonyl]amino]-ethyl]-2-propenamide (DA), which have been previously verified as being relatively temperature-resistant molecules. Using AIBN and 3-mercaptopropionic acid as the radical initiator and chain-transfer agent, respectively, *N*-isopropylacrylamide (NIPAAm) and the fluorescent monomer (molar ratio = 1000:1) were copolymerized in anhydrous DMF at 70

DOI: 10.1021/acs.biomac.5b00591
Biomacromolecules XXXX, XXX, XXX−XXX

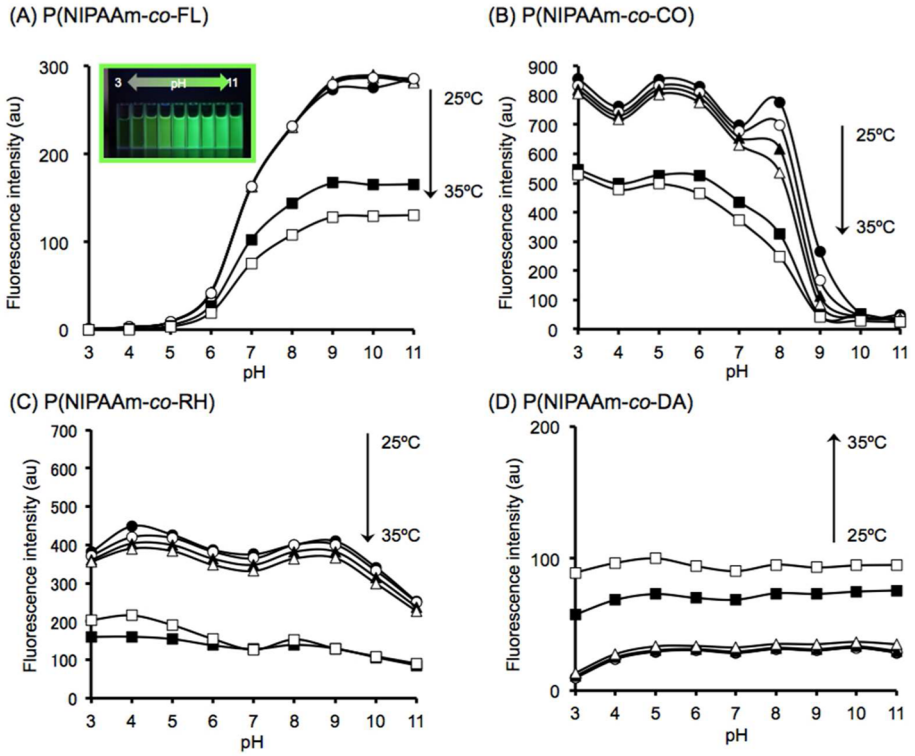


Figure 2. Fluorescence responses of (A) P(NIPAAm-*co*-FL) (0.05% w/v, λ_{ex} 440 nm, λ_{em} 515 nm), (B) P(NIPAAm-*co*-CO) (0.05% w/v, λ_{ex} 350 nm, λ_{em} 460 nm), (C) P(NIPAAm-*co*-RH) (0.05% w/v, λ_{ex} 540 nm, λ_{em} 590 nm), and (D) P(NIPAAm-*co*-DA) (0.05% w/v, λ_{ex} 310 nm, λ_{em} 500 nm) to pH variation in Britton−Robinson buffer from pH 3−11 at 25−35 °C. Key: Closed circles = 25 °C, open circles = 27 °C, closed triangles = 29 °C, open triangles = 31 °C, closed squares = 33 °C, open squares = 35 °C.

°C for 5 h. The successful introduction of the fluorescent moiety in the synthetic polymers was confirmed by ^{1}H NMR spectroscopy. The number- and weight-averaged molecular weights (M_n, M_w) were determined by gel permeation chromatography (GPC). All four polymers were found to have their LCST at approximately 31 °C and exhibit thermally reversible solubility changes in response to a temperature change in water. Detailed physicochemical data for the prepared synthetic polymers are shown in Table 1. In addition, as these polymers possess a single terminal carboxyl group, they have the potential to be further modified.[10,18]

Characterization of Fluorescent Polymers. We then chose to investigate the effect of temperature on the fluorescent behavior of our fluorescent polymers. The spectra of these polymers exhibited comparable fluorescent behavior to that of the parent fluorescent monomers (Figure S1). For example, the absorption and fluorescence maxima for P(NIPAAm-*co*-FL) were observed at 444 and 512 nm, respectively, which are comparable to those of the parent fluorescein (λ_{ex} = 492 nm, λ_{em} = 514 nm). The temperature-dependent fluorescence spectra and optical transmittance of P(NIPAAm-*co*-FL) in aqueous solution are shown in Figure 1A. The fluorescent intensity of P(NIPAAm-*co*-FL) was found to decrease with increasing temperature, with a sharp change in the intensity of this polymer being observed close to its LCST (30.5 °C). Below the LCST, P(NIPAAm-*co*-FL) assumes its hydrate form and swollen state driven by the native properties of PNIPAAm. As fluorescein displays a higher emission quantum yield in polar media, P(NIPAAm-*co*-FL) exhibited a strong fluorescence intensity under such conditions. In contrast, above the LCST, the polymer assumes its dehydrated globular state and, therefore, the fluorescein unit cannot yield strong fluorescence. Similar phenomena were observed for P(NIPAAm-*co*-CO) and P(NIPAAm-*co*-RH) (Figure 1B,C), as the fluorescent components coumarin and rhodamine display the same sensitivity as that of fluorescein in polar media. In contrast, the presence of the dansyl moiety in P(NIPAAm-*co*-DA) resulted in the opposite characteristics being observed. The fluorescence intensity of P(NIPAAm-*co*-DA) was stronger above the LCST (dehydrated state) than below the LCST (hydrated state) (Figure 1D). Unlike the other three fluorophores, the dansyl moiety displays strong emission in nonpolar media, and this fluorescent property is directly reflected in P(NIPAAm-*co*-DA). We could therefore conclude that the on/off emission responses of these polymers were driven by a combination of the properties of both PNIPAAm and the fluorescent molecules. In addition, these properties changed rapidly at temperatures close to the LCST of the individual polymers. Furthermore, to confirm that light scattering by the phase-separated polymer did not cause changes in the fluorescence intensity, we examined the optical transmittance and fluorescent spectra of the mixtures of each individual fluorophore with PNIPAAm. We observed that in all cases the fluorescence intensity of the mixed solutions remained largely unchanged, whereas the optical transmittance decreased above the LCST of PNIPAAm (i.e., 32 °C). These results confirmed that the increase in the fluorescence intensity of these fluorescent polymers was caused by the introduction of the fluorophores to PNIPAAm and not by light scattering alone.

We then investigated the influence of pH on the fluorescence intensity of the synthetic polymers. As previously described, pH

DOI: 10.1021/acs.biomac.5b00591
Biomacromolecules XXXX, XXX, XXX−XXX

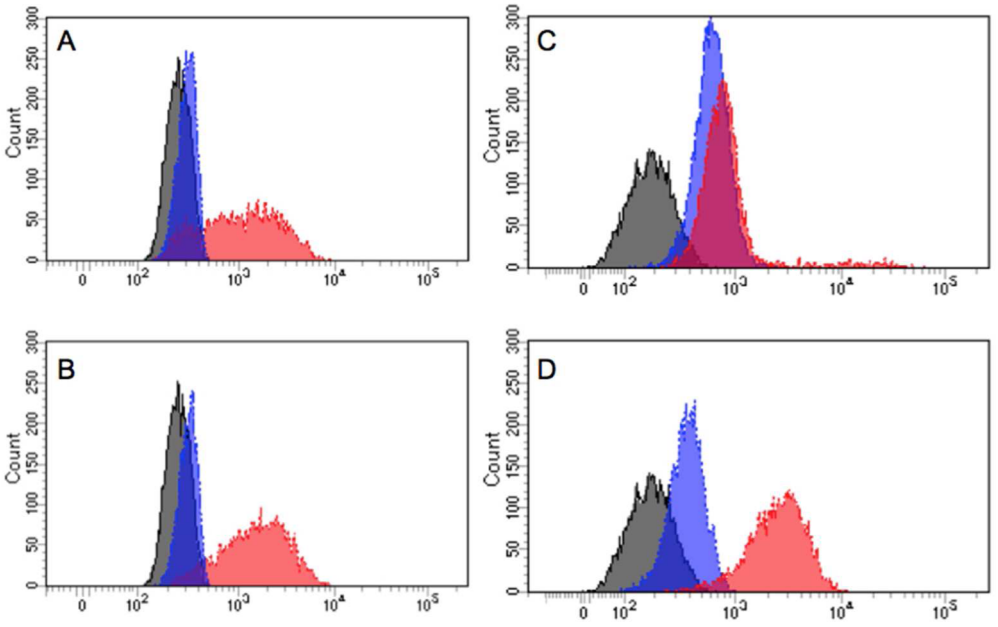


Figure 3. Effects of incubation temperature on polymer uptake by RAW264.7 cells. (A) P(NIPAAm-*co*-CO), (B) P(NIPAAm-*co*-CO)-DOPE, (C) P(NIPAAm-*co*-RH), and (D) P(NIPAAm-*co*-RH)-DOPE. Cells were incubated for 4 h (A, B) or 2 h (C, D) with the polymer at either 37 °C (red) or 27 °C (blue) in a humidified atmosphere containing 5% CO_2.

measurement inside living cells is important for monitoring cellular internalization pathways, such as receptor−ligand internalization. Figure 2 shows the fluorescence responses of the synthetic polymers to both pH (pH 3−11) and temperature (25−35 °C) in Britton−Robinson buffer. As with temperature dependence, P(NIPAAm-*co*-FL) also displayed a response to pH. The fluorescence intensity increased with basification, and the polymer showed complete loss of emission at acidic pH (<5) (Figure 2A). In addition, the fluorescence intensity rapidly increased between pH 6.0 and 7.0, which is the pH range corresponding to the pK_a of fluorescein (~6.5).[19] It is well-known that the emission of fluorescein is affected by pH. Under acidic conditions, fluorescein adopts the lactone conformation and displays low emission. Although P(NIPAAm-*co*-CO) has also been shown to display a pH response (Figure 2B), the opposite phenomenon was observed with P(NIPAAm-*co*-FL). P(NIPAAm-*co*-CO) exhibited strong fluorescence at low pH (<8), but it lost its emission at high pH. Comparison of the properties of CO with P(NIPAAm-*co*-CO) confirmed that both displayed comparable pH-dependent fluorescence behavior (data not shown). These results show that both P(NIPAAm-*co*-FL) and P(NIPAAm-*co*-CO) largely preserved the pH-responsiveness of their respective fluorophores and were confirmed to be dual temperature- and pH-responsive fluorescent polymers. In addition, as with the monomers RH and DA, the fluorescence intensity of polymers P(NIPAAm-*co*-RH) and P(NIPAAm-*co*-DA) was unaffected by pH.

Environmental-Dependent Cellular Uptake of Fluorescent Polymers. Following studies into the temperature- and pH-dependence studies, we chose to study the uptake of P(NIPAAm-*co*-CO) and P(NIPAAm-*co*-RH) into cultured macrophage cells (RAW 264.7) using both fluorescence microscopy and flow cytometry (FCM). To enhance cellular uptake, the fusogenic lipid, L-α-phosphatidylethanolamine, dioleoyl (DOPE), was conjugated to the fluorescent polymer. The fusogenicity of DOPE is derived from its tendency to form a nonlamellar phase (hexagonal phase) due to its cone-shaped geometry.[20] Figure 3 shows the effect of incubation temperature on the uptake of fluorescent polymers by RAW264.7 cells measured using flow cytometry. It was observed that cellular uptake of the fluorescent polymers did not take place below the LCST but increased drastically above the LCST. The large increase in cellular uptake of the fluorescent polymers above the LCST appeared to result from the dehydration of polymer chains, as observed in our previous studies.[15,16] As the hydrophilic PNIPAAm copolymer becomes hydrated below the LCST, the hydration layer around the polymer chain suppresses the interactions between the polymer chains and between the polymer and the cell membrane. Furthermore, after dehydration, the polymer chains were able to aggregate once again by means of hydrophobic interactions between the chains. This hydrophobic aggregation of polymer chains resulted in interactions with the cell membranes and thus cellular uptake was enhanced. Fluorescence microscopy images of P(NIPAAm-*co*-RH) and P(NIPAAm-*co*-RH)-DOPE uptake into RAW264.7 cells can be seen in Figure 4. The cellular uptake of fluorescent polymers was not observed below the LCST, regardless of whether DOPE was present. In addition, it was found that the LCSTs of P(NIPAAm-*co*-RH) and P(NIPAAm-*co*-RH)-DOPE were comparable (temperature-dependent optical transmittance curves are shown in Figure S2). Terminal modification of DOPE did not appear to affect the hydration layer around the polymer chain below the LCST; hence, it could be concluded that the fusogenicity of DOPE was not effective for cellular uptake below the LCST.

With an incubation time of 2 h, cellular uptake of the fluorescence polymers was also enhanced above the LCST. In addition, the DOPE-conjugated polymer was internalized more rapidly, with an incubation time of only 0.5 h (Figure 4). The time-dependent intracellular fluorescence intensity of both the DOPE-conjugated P(NIPAAm-*co*-CO) and P(NIPAAm-*co*-RH) fluorescent polymers, as observed by FCM, is shown in Figure 5. It can be seen that the modified fluorescent polymers internalized more rapidly and accumulated to a greater extent

DOI: 10.1021/acs.biomac.5b00591
Biomacromolecules XXXX, XXX, XXX−XXX

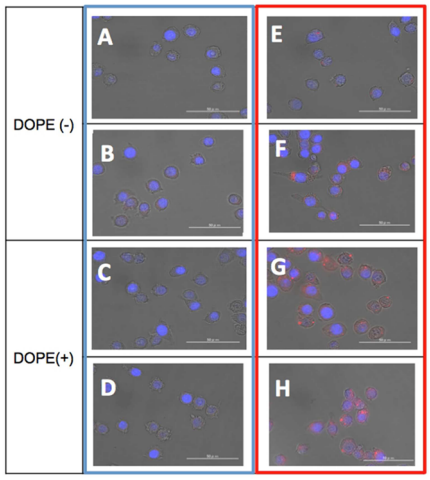


Figure 4. Effects of incubation temperature and polymer modification with DOPE to polymer uptake by RAW264.7 cells. Incubated with P(NIPAAm-*co*-RH) (A, B, E, F) and P(NIPAAm-*co*-RH)-DOPE (C, D, G, H). Cells were incubated for 0.5 h (A, C, E, G) or 2 h (B, D, F, H) with the polymer at 27 °C (A, B, C, D) or 37 °C (E, F, G, H) in a humidified atmosphere containing 5% CO_2.

inside the cells compared with that of the nonconjugated polymer. These results indicate that DOPE caused a strong preference for cellular uptake, likely due to its hydrophobicity, allowing DOPE to be rapidly internalized without the requirement for a delivery system.

CONCLUSIONS

We conclude that the development of environmentally responsive fluorescent polymers with four different fluorophores was successful. These PNIPAAm-based fluorescent copolymers were found to possess two specific characteristics of interest. First, they possessed sensitive temperature dependence derived from PNIPAAm and thermally reversible solubility changes in aqueous solution across the LCST. The polymer chains were found to display an expanded conformation below the LCST due to strong hydration and a compact globular form above the LCST due to dehydration. Second, the properties of the fluorescent monomers were reflected in the fluorescence polymers, despite a low NIPAAm-to-fluorescent monomer molar ratio (1000:1). As FL, CO, and RH have been found to display high quantum yields in polar solvents, their corresponding polymers, namely, P(NIPAAm-*co*-FL), P(NIPAAm-*co*-CO), and P(NIPAAm-*co*-RH), exhibited strong emission below their respective LCSTs (i.e., in the hydrated form). P(NIPAAm-*co*-FL) and P(NIPAAm-*co*-CO) were also found to display pH-dependent fluorescence behavior derived from the pH-responsive monomers, FL and CO. In cellular experiments, fluorescence polymers were successfully internalized above the LCST. Cellular uptake was enhanced by the conjugation of DOPE to the polymers. Studies into the development of multiresponsive fluorescent polymers and their applications in cellular imaging are currently underway and will be reported in due course.

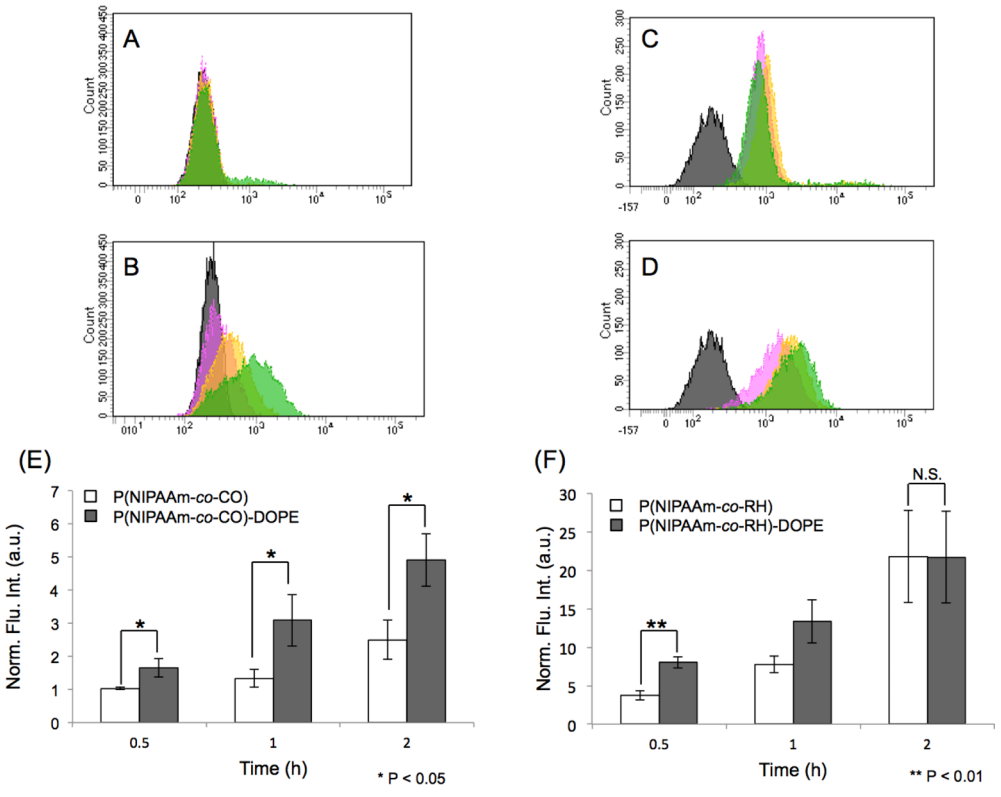


Figure 5. Effects of incubation time on polymer uptake by RAW264.7 cells. (A–D) Flow cytometry peaks of fluorescence intensity in cells treated with (A) P(NIPAAm-*co*-CO), (B) P(NIPAAm-*co*-CO)-DOPE, (C) P(NIPAAm-*co*-RH), and (D) P(NIPAAm-*co*-RH)-DOPE. Cells were incubated with the polymer for 0.5 h (pink), 1 h (yellow), and 2 h (green) at 37 °C in a humidified atmosphere with 5% CO_2. (E, F) Summary of mean fluorescence intensity in cells from flow cytometry over three independent runs.

DOI: 10.1021/acs.biomac.5b00591
Biomacromolecules XXXX, XXX, XXX−XXX

■ ASSOCIATED CONTENT

Supporting Information

Absorption and fluorescence spectra, temperature-dependent optical transmittance, and ^{1}H NMR spectra of fluorescence polymers. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.5b00591.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: kanazawa-hd@pha.keio.ac.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Part of this study was supported by a Grant-in-Aid for Exploratory Research (grant no. 25670081) from the Japan Society for the Promotion of Science (JSPS), Japan.

■ REFERENCES

(1) Gil, E. S.; Hudson, S. M. *Prog. Polym. Sci.* **2004**, *29*, 1173−1222.
(2) Heskins, M.; Guillet, J. E. *J. Macromol. Sci., Chem.* **1968**, *2*, 1441−1455.
(3) Yoshida, R.; Sakai, K.; Okano, T.; Sakurai, Y.; Bae, Y. H.; Kim, S. W. *J. Biomater. Sci., Polym. Ed.* **1992**, *3*, 155−162.
(4) Okano, T.; Yamada, N.; Okuhara, M.; Sakai, H.; Sakurai, Y. *Biomaterials* **1995**, *16*, 297−303.
(5) Kanazawa, H.; Sunamoto, T.; Matsushima, Y.; Kikuchi, A.; Okano, T. *Anal. Chem.* **2000**, *72*, 5961−5966.
(6) Annaka, M.; Tanaka, C.; Nakahira, T.; Sugiyama, M.; Aoyagi, T.; Okano, T. *Macromolecules* **2002**, *35*, 8173−8179.
(7) Uchiyama, S.; Kawai, N.; de Silva, A. P.; Iwai, K. *J. Am. Chem. Soc.* **2004**, *126*, 3032−3033.
(8) Gota, C.; Okabe, K.; Funatsu, T.; Harada, Y.; Uchiyama, S. *J. Am. Chem. Soc.* **2009**, *131*, 2766−2767.
(9) Wang, D.; Miyamoto, R.; Shiraishi, Y.; Hirai, T. *Langmuir* **2009**, *25*, 13176−13182.
(10) Kobayashi, H.; Nishikawa, M.; Sakamoto, C.; Nishio, T.; Kanazawa, H.; Okano, T. *Anal. Sci.* **2009**, *25*, 1043−1047.
(11) Lowell, B. B.; Spiegelman, B. M. *Nature* **2000**, *404*, 652−660.
(12) Karnebogen, M.; Singer, D.; Kallerhoff, M.; Ringert, R.-H. *Thermochim. Acta* **1993**, *229*, 147−155.
(13) Izumi, H.; Torigoe, T.; Ishiguchi, H.; Uramoto, H.; Yoshida, Y.; Tanabe, M.; Ise, T.; Murakami, T.; Yoshida, T.; Nomoto, M.; Kohno, K. *Cancer Treat. Rev.* **2003**, *29*, 541−549.
(14) Davies, T. A.; Fine, R. E.; Johnson, R. J.; Levesque, C. A.; Rathbun, W. H.; Seetoo, K. F.; Smith, S. J.; Strohmeier, G.; Volicer, L.; et al. *Biochem. Biophys. Res. Commun.* **1993**, *194*, 537−543.
(15) Hiruta, Y.; Shimamura, M.; Matsuura, M.; Maekawa, Y.; Funatsu, T.; Suzuki, Y.; Ayano, E.; Okano, T.; Kanazawa, H. *ACS Macro Lett.* **2014**, *3*, 281−285.
(16) Hiruta, Y.; Funatsu, T.; Matsuura, M.; Wang, J.; Ayano, E.; Kanazawa, H. *Sens. Actuators, B* **2015**, *207*, 724−731.
(17) Shea, K. J.; Stoddard, G. J.; Shavelle, D. M.; Wakui, F.; Choate, R. M. *Macromolecules* **1990**, *23*, 4497−4507.
(18) Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. *Bioconjugate Chem.* **1993**, *4*, 42−46.
(19) Martin, M. M.; Lindqvist, L. *J. Lumin.* **1975**, *10*, 381−390.
(20) Hamai, C.; Yang, T.; Kataoka, S.; Cremer, P. S.; Musser, S. M. *Biophys. J.* **2006**, *90*, 1241−1248.

DOI: 10.1021/acs.biomac.5b00591
Biomacromolecules XXXX, XXX, XXX−XXX