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Temperature-Responsive Fluorescence Polymer Probes with Accurate Thermally Controlled Cellular Uptakes

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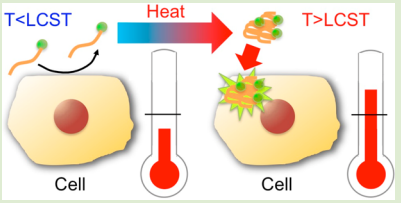
Supporting Information

ABSTRACT: Poly(*N*-isopropylacrylamide) (PNIPAAm)-based temperature-responsive fluorescence polymer probes were developed using radical polymerization, with 3-mercaptopropionic acid as the chain-transfer agent, followed by activation of terminal carboxyl groups with *N*-hydroxysuccinimide and reaction with 5-aminofluorescein (FL). The lower critical solution temperatures (LCSTs) of the resulting fluorescent polymer probes differed depending on the copolymer composition, and had a sharp phase-transition (hydrophilic/hydrophobic) boundary at the LCST. The cellular uptakes of the fluorescent polymer probes were effectively suppressed below the LCST, and increased greatly above the LCST. In particular, the cellular uptake of a copolymer with *N*,*N*-dimethylaminopropylacrylamide, P(NIPAAm-*co*-DMAPAAm2%)-FL (LCST: 37.4 °C), can be controlled within only 1 °C near body temperature, which is suitable for biological applications. These results indicated that the cellular uptakes of thermoresponsive polymers could be accurately controlled by the temperature, and such polymers have potential applications in discriminating between normal and pathological cells, and in intracellular drug delivery systems with local hyperthermia.



Fluorescence probes are powerful tools for understanding the dynamics and functions of living cells.[1−3] They are also used for labeling pathological cells such as tumor cells and enable detection of a single cell, even in vivo in real time.[4] Fluorescent probes have therefore attracted attention in a wide range of fields, including biological, pharmaceutical, and medical diagnosis.

Because most fluorescent probes lack specificity and have low target accumulations, the affinity and specificity based on antigen−antibody or ligand−receptor recognition processes are used for specific cell and organelle targeting. Fluorescent probes are covalently conjugated with antibodies or ligands and then used to label antigens or receptors in several living cells selectively and efficiently.[5−11]

Stimuli-responsive polymers have been developed for interesting applications in biomaterial science and technology.[12,13] Such polymers change their structure and physical characteristics in response to external stimuli such as temperature, pH,[14] ions,[15] saccharides,[16] and light.[17] Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of the most widely studied stimuli-responsive polymers; it exhibits a drastic temperature-dependent phase-transition in aqueous solution at a lower critical solution temperature (LCST) of around 32 °C.[18] Furthermore, the LCST of PNIPAAm can be precisely controlled to a specific temperature using the copolymer composition. Hydrophobic polymers such as butyl methacrylate (BMA) lower the LCST, and hydrophilic monomers such as *N*,*N*-dimethylaminopropylacrylamide (DMAPAAm) raise the LCST.[19−21] Stimuli-responsive polymers have therefore been widely used in drug-delivery systems,[22] cell culture substrates,[23] and separation systems.[24]

The discrimination of normal and pathological cells is attractive from a clinical viewpoint. Pathological cells might have higher temperatures than normal ones as a result of enhanced metabolic activities.[25] In addition, drug targeting based on a combination of thermoresponsive polymers and local hyperthermia has also attracted attention because it can synergistically enhance tumor cytotoxicity combined with chemotherapy.[26] It is therefore important to investigate the interactions between thermoresponsive polymers and living cells and tissues.

Wan and co-workers developed thermosensitive polymer-conjugated albumin nanospheres and observed the difference between adhesion on the surface of the cell membrane above the LCST and that below the LCST.[27] Furthermore, Akimoto and co-workers developed thermoresponsive polymeric micelles and observed temperature-induced intracellular uptake.[28] However, exact temperature control of intracellular uptake by thermoresponsive polymers has never been achieved.

In this work, we developed thermoresponsive fluorescence polymer probes based on PNIPAAm copolymers (Chart 1), with different LCSTs depending on the copolymer composition and composition ratio and evaluated the exact temperature-dependent cellular uptake. Temperature-responsive polymers

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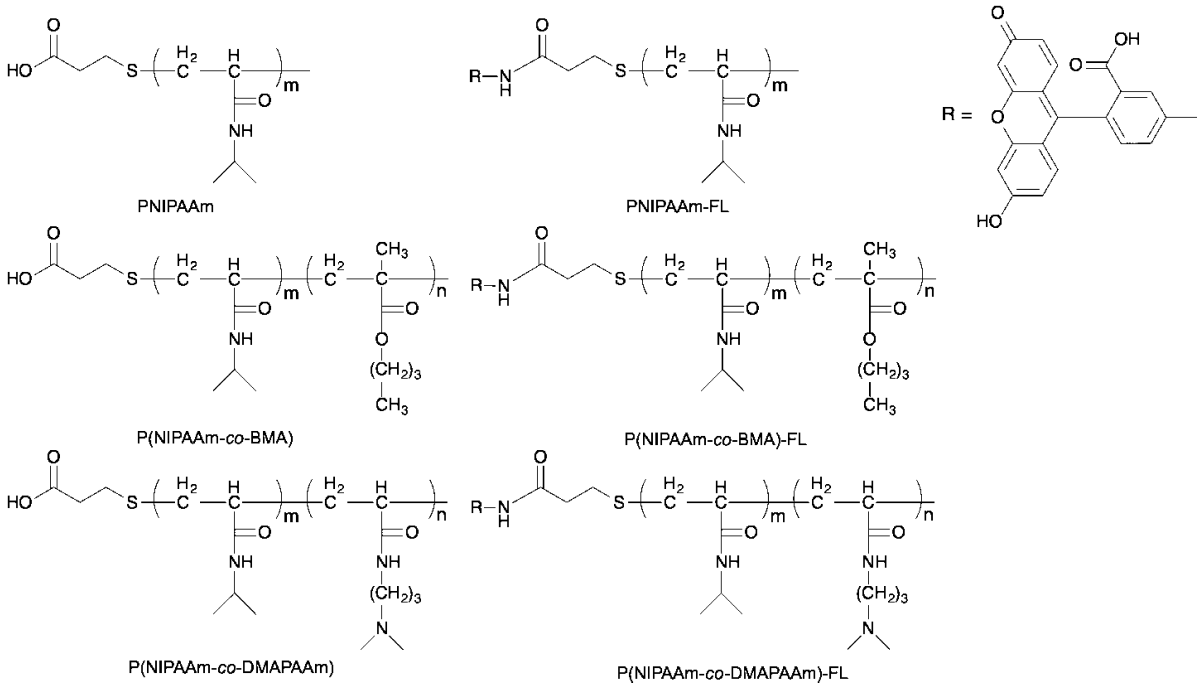
Chart 1. Structures of Thermoresponsive Polymers and Fluorescence Polymer Probes



with carboxyl end-groups, that is, PNIPAAm, P(NIPAAm-*co*-BMA), and P(NIPAAm-*co*-DMAPAAm), were synthesized by radical polymerization using 3-mercaptopropionic acid as a chain-transfer agent.[29] The LCSTs of the thermoresponsive polymers were adjusted by changing the copolymer composition ratio. Figure 1 shows the temperature-dependent optical transmittances of PNIPAAm, P(NIPAAm-*co*-BMA*x*%), and P(NIPAAm-*co*-DMAPAAm*x*%) in water. PNIPAAm exhibited a drastic phase-transition at 32.7 °C. P(NIPAAm-*co*-BMA*x*%) and P(NIPAAm-*co*-DMAPAAm*x*%) also exhibited phase transitions at their LCSTs. On increasing the BMA composition ratio, the LCST decreased via hydrophobization of the polymer chain, and the transmittance curves broadened, particularly for BMA ratios greater than 3%. In contrast, on increasing the DMAPAAm composition ratio, the LCST increased via hydrophilization of the polymer chain, and the transmittance curves remained sharp, except at a DMAPAAm ratio of 5%. Because P(NIPAAm-*co*-DMAPAAm2%) exhibited its LCST at 37.3 °C, near body temperature, which is suitable for biological applications, this polymer composition was used for fluorescein labeling. For comparison, PNIPAAm (LCST: 32.7 °C) and P(NIPAAm-*co*-BMA3%) (LCST: 27.2 °C) were also labeled with fluorescein. The terminal carboxyl groups of these polymers were activated with *N*-hydroxysuccinimide reacted with 5-aminofluorescein (FL). The physicochemical data for these polymers are given in Table 1. Fluorescein labeling did not greatly affect the LCST. With respect to PNIPAAm, P(NIPAAm-*co*-BMA3%), and P(NIPAAm-*co*-DMAPAAm2%), LCSTs were also measured by differential scanning calorimetry (DSC) shown in Figure S1 in the Supporting Information. LCSTs determined from DSC were consistent with those determined from optical transmittances.

Cellular uptake of nanoparticles generally depends on the particle size.[30,31] During thermally induced phase transitions of thermoresponsive polymers based on PNIPAAm, the hydrodynamic diameters also change. The temperature dependences of the hydrodynamic diameters of PNIPAAm-FL, P(NIPAAm-*co*-BMA3%)-FL, and P(NIPAAm-*co*-DMAPAAm2%)-FL in water were therefore measured (Figure 2). As the temperature increased, the hydrodynamic diameter gradually increased above the LCST. The increase in the hydrodynamic size was

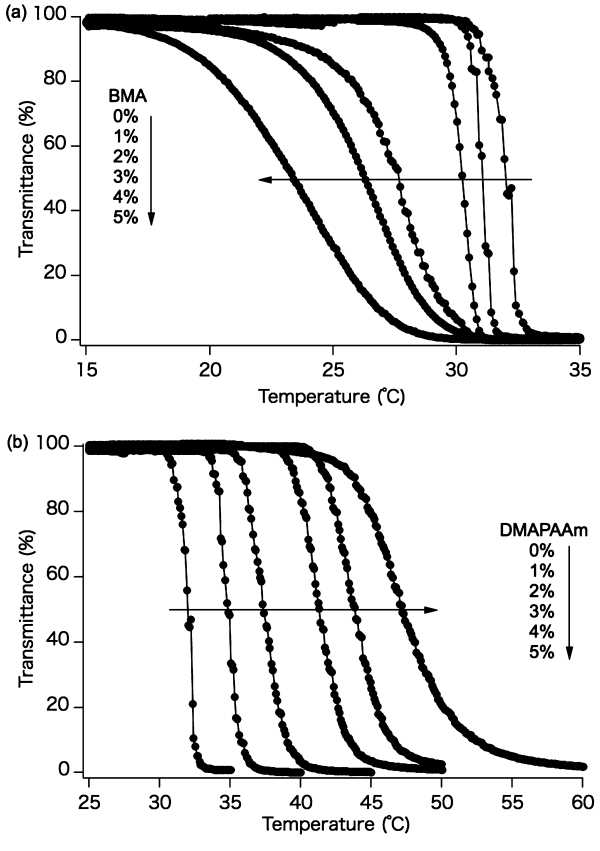


Figure 1. Temperature-dependent optical transmittances in aqueous solutions (0.5 w/v%): (a) P(NIPAAm-*co*-BMA*x*%) and (b) P(NIPAAm-*co*-DMAPAAm*x*%).

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Table 1. Physicochemical Characteristics of Thermoresponsive Polymers

polymer	M_n[a]	M_w[b]	M_w/M_n	LCST (°C)
PNIPAAm	7100	17800	2.50	32.0
PNIPAAm-FL				32.8
P(NIPAAm-*co*-BMA3%)	17500	45500	2.60	26.9
P(NIPAAm-*co*-BMA3%)-FL				27.7
P(NIPAAm-*co*-DMAPAAm2%)	8200	15500	1.89	37.3
P(NIPAAm-*co*-DMAPAAm2%)-FL				37.4

[a]Number-average molecular weight. [b]Weight-average molecular weight.

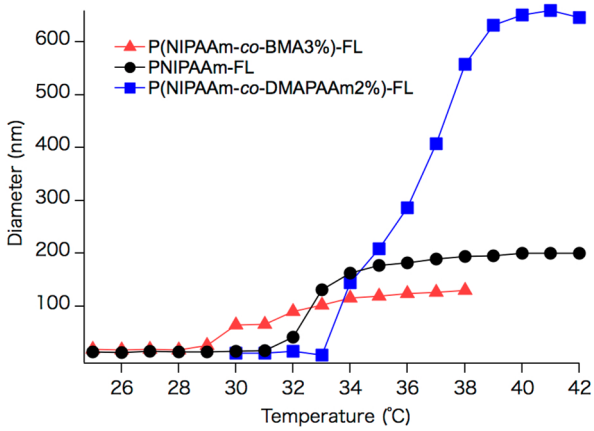

Figure 2. Temperature-dependences of hydrodynamic diameters for 0.5 w/v% aqueous solutions of fluorescence polymer probes.

caused by the temperature-responsive polymer chains changing from hydrophilic to hydrophobic, and subsequent aggregation by hydrophobic interactions between the polymer chains of the fluorescence polymer probes in water.

The temperature-dependent intracellular uptakes of the fluorescence polymer probes were investigated using macrophage-like cells (RAW 264.7 cells). PNIPAAm-FL, P(NIPAAm-*co*-BMA3%)-FL, and P(NIPAAm-*co*-DMAPAAm2%)-FL were each incubated at 25, 30, 34, 37, or 38 °C for 6 h. Before examination using fluorescence microscopy, the RAW 264.7 cells incubated with the fluorescence polymer probes were rinsed with Dulbecco's phosphate buffered saline without calcium chloride and magnesium chloride (DPBS(−), pH 7.4) to remove any fluorescence polymer probe adhered to the cell surfaces at 25 °C. Rinsing at 25 °C suppresses adsorption and aggregation of the fluorescence polymer probe by washing with solvent below the LCST, leading to thorough rinsing. In the fluorescence microscopy images, the green fluorescence derived from P(NIPAAm-*co*-BMA3%)-FL (LCST: 27.7 °C) was hardly observed at 25 °C. In contrast, this fluorescence can be clearly observed in other samples above 30 °C (Figure 3). Similarly, fluorescences derived from PNIPAAm-FL (LCST: 32.8 °C) and P(NIPAAm-*co*-DMAPAAm2%)-FL (LCST: 37.4 °C) were hardly observed below each LCST and can be clearly observed above each LCST (Figure 3). Low magnification images incubated with each fluorescence polymer probe at 38 °C for 6 h were shown in Figure S2 in the Supporting Information. Whole cellular uptake of fluorescence polymer probes was confirmed. The amount of fluorescence polymer probe internalized into the RAW 264.7 cells was measured from the fluorescence intensity, using a plate reader, and a flow cytometer. Figure 4 shows the

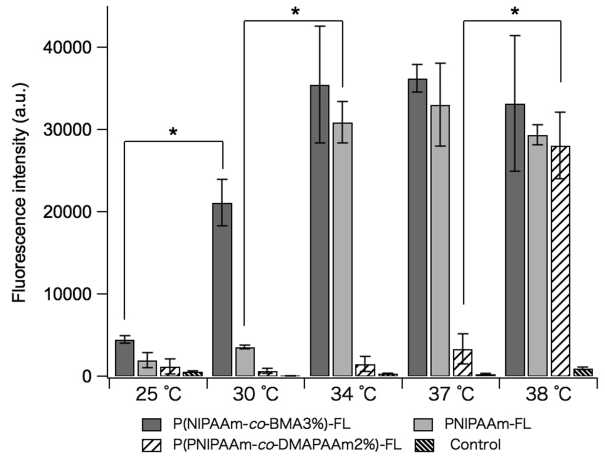

Figure 4. Fluorescence intensities of RAW264.7 cells incubated with P(NIPAAm-*co*-BMA3%)-FL, PNIPAAm-FL, and P(NIPAAm-*co*-DMAPAAm2%)-FL at different temperatures; Control means cultured cells were exposed to no polymer in DMEM/FBS; Error bars represent mean values ±1σ (n = 4); *P < 0.01.

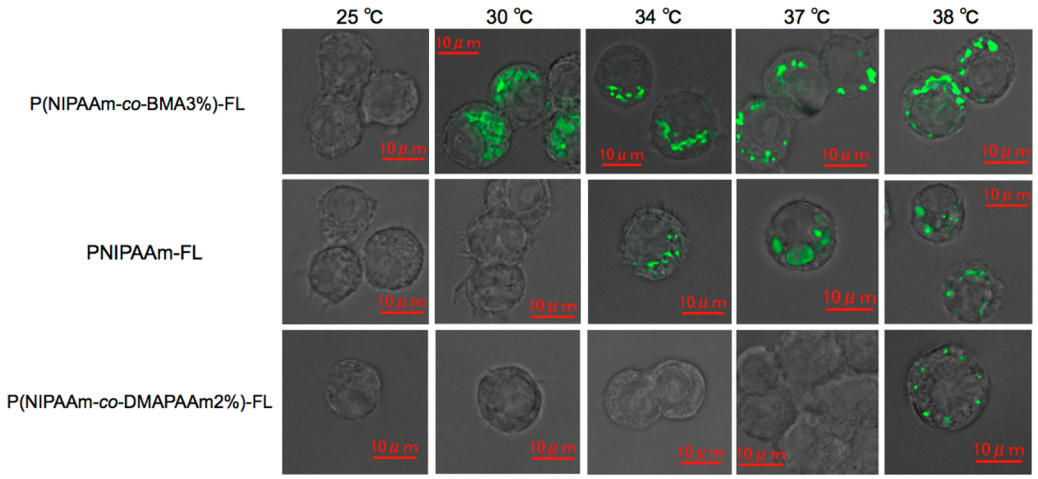

Figure 3. Fluorescence microscopy images of RAW264.7 cells incubated with P(NIPAAm-*co*-BMA3%)-FL, PNIPAAm-FL, and P(NIPAAm-*co*-DMAPAAm2%)-FL at different temperatures.

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fluorescence intensities of the fluorescence polymer probes depending on the temperature and polymer composition. The fluorescence intensity significantly increased at the LCST boundary ($P < 0.01$). Only 5-aminofluorescein was also incubated at each temperature, and fluorescence intensities were almost the same as control (data not shown). Flow cytometric fluorescence histograms of RAW 264.7 cells incubated with or without fluorescence polymer probes at 38 °C for 6 h were shown in Figure S3 in the Supporting Information. These results indicated that cellular uptake of the fluorescence polymer probe could be controlled by the copolymer composition and temperature. The large increases in cellular uptakes of the fluorescence polymers at the LCST boundaries seem to result from two factors. The first one is the changes in the hydrated/dehydrated structures of the polymer chains. Below the LCST, the polymer chain was hydrated, and had hydrophilic characteristics. The hydration layer around the polymer chain disrupted the interactions between the polymer chain and cell membranes, and cellular uptake of the fluorescence polymer probe was not observed. In contrast, above the LCST, the polymer chain was dehydrated and had hydrophobic characteristics. The hydrophobic interactions between the polymer chain and cell membranes increased, and the cellular uptake of the fluorescence polymer probe was enhanced. The second one is the changes in the hydrodynamic size of the polymer. At the LCST boundary, the hydrodynamic size of the polymer increased with increasing temperature, as shown in Figure 2. When the hydrodynamic size of the fluorescence polymer probe increased, interactions between aggregates of the fluorescence polymer probe and cell membranes increased, and, accordingly, the cellular uptake was enhanced. We confirmed cytotoxicity of fluorescence polymer probes (200 μg/mL) by means of WST-1 assay. No significant cytotoxicity with PNIPAAM-FL and P(NIPAAm-*co*-BMA3%) and Minor cytotoxicity (cell viability: 88 ± 11%, n = 6) with PNIPAAm-co-DMAPAAm2%)-FL (200 μg/mL) were observed (data not shown). Nontoxicity of PNIPAAm-based materials have been reported.[32,33] These results indicated that there might be practical use with fluorescence polymer probes for in vivo applications.

In conclusion, we successfully synthesized three types of PNIPAAm-based temperature-responsive fluorescence polymer probes, with LCSTs controlled by the copolymer composition and ratio. PNIPAAm-FL, P(NIPAAm-*co*-BMA3%)-FL, and P(NIPAAm-*co*-DMAPAAm2%)-FL exhibited LCSTs at 32.8, 27.7, and 37.4 °C, respectively, and phase transitions (hydrophilic/hydrophobic) and increases in hydrodynamic sizes across a prominent LCST were observed. The resulting fluorescence polymer probes were incubated with RAW264.7 cells at 25, 30, 34, 37, or 38 °C. Intracellular uptakes accelerated significantly above the LCST of each fluorescence polymer probe. Notably, the intracellular uptake of P(NIPAAm-*co*-DMAPAAm2%)-FL (LCST: 37.4 °C) was controlled within only 1 °C between 37 and 38 °C, near body temperature, which is suitable for biological applications. This exact temperature-selective cellular uptake with stimuli-responsive polymers enables slight differences between normal and pathological cells to be identified and has the potential to enable early detection of disease; it could also be used in intracellular drug-delivery systems with local hyperthermia.

■ ASSOCIATED CONTENT

Supporting Information

Materials, methods, detailed experimental procedures, supporting data, and ^{1}H NMR spectra of polymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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