A Polymer Micelle Responding to the Protein Kinase A Signal

Yoshiki Katayama,*,†,‡ Tatsuhiko Sonoda,† and Mizuo Maeda†

Department of Applied Chemistry, Faculty of Engineering, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan, and PRESTO, Japan Science and Technology Corporation, Japan

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ABSTRACT: New polymer−peptide conjugates, NIPAM−PEP and NIPAM−PEPEP, were designed and synthesized. These graft-type polymers contained a substrate peptide of protein kinase A (PKA), which forms one of the most important intracellular signals in cellular signal transduction. The NIPAM−PEP containing the *N*-isopropylacrylamide unit and the substrate peptide unit raised its lower critical solution temperature (LCST) from 36.7 to 40 °C in response to phosphorylation by activated PKA. The NIPAM−PEPEP containing another poly(ethylene glycol) unit formed a polymer micelle-type particle above LCST. This particle disintegrated in response to phosphorylation by activated PKA. Dansylaniline, which was encapsulated in the hydrophobic core of the particle, tended to be released with the particle disintegration occurring in response to the PKA signal. NIPAM−PEPEP, which is the first example of a polymer−peptide conjugate responding to an intracellular signal, offers the possibility of a novel, intelligent drug capsule communicating with cells to release its pharmacological activity.

Introduction

Living cells possess an intracellular signal transduction system that includes many biological reactions, the purpose of which is to allow cells to respond to their outer environment and thus to maintain themselves. Protein phosphorylations are one of the most important and versatile information-processing signals in the transduction system. The phosphorylation of the target proteins causes conformational changes in the proteins, thus activating or inactivating their physiological functions, based on one of three following factors: (i) enhancement of hydration around the phosphorylation site, (ii) the introduction of anionic charge, or (iii) the introduction of an additional hydrogen-bonding site. Such protein phosphorylations are accomplished via protein kinases. Of these protein kinases, cyclic AMP-dependent protein kinase (protein kinase A, PKA) is one of the most important kinases.[1−6] The kinase is activated with cyclic AMP, which is synthesized intracellularly in response to various extracellular signals such as hormones, cytokines, or neurotransmitters. The kinase regulates a wide variety of cellular functions such as gene transcription,[7] secretion,[8] neuronal plasticity,[9] cellular proliferation,[10] differentiation,[11] and death,[12] and this extraordinary activation of enzymes is closely related to various diseases, such as melanoma,[5] prostate tumor,[13] or colon cancer.[14] Thus, the enzymatic activity is an important criterion for the cellular condition. If any materials that release pharmacological activities responding to the extraordinary activating signal of PKA could be designed, it could constitute a novel, intelligent drug with sensing ability with regard to the cellular condition. However, such materials have not been reported. Here we report the first example of a polymer micelle responding to the PKA signal. For the development of such a PKA-responsive material, we have designed a new thermoresponsive polymer possessing selective PKA phosphorylation sites.[15,16] The polymer can form polymer micelle-type colloidal particles if poly(ethylene glycol) units are introduced. The polymer micelle changes its physical properties so that it disintegrates in response to phosphorylation by an activated protein kinase. Such a polymer micelle may be able to act as a novel, intelligent drug communicating with living cells to change cellular responses.

* Corresponding author. E-mail ykatatcm@mbox.nc.Kyushu-u.ac.jp.
† Kyushu University.
‡ PRESTO JST Co.

Experimental Section

Preparation of NIPAM−PEP and NIPAM−PEPEP. The *N*-methacryloylpeptide monomer (*N*-methacryloyl-GLR-RASLG) was synthesized with automatic peptide synthesizer by Fmoc chemistry using corresponding Fmoc amino acids and *N*-methacryloylglycine as the N-terminus amino acid. The *N*-methacryloylglycine was easily obtained from glycine (2 g, 26.7 mmol) and methacryloyl chloride (2.6 mL, 26.7 mmol) in THF/water (1/1 v/v) in the presence of an equimolar amount of NaOH at 0 °C followed by recrystallization from chloroform (white powder; yield 750 mg, 20%). The obtained peptide was purified with reverse-phase high-performance liquid chromatography (HPLC) using acetonitrile/0.1% TFA (20−80% v/v) and then lyophilized. The *N*-methacryloyl-PEG was prepared by overnight stirring of *N*-methacryloyloxysuccinimide (180 mg, 1 mmol) and α-methoxy-*ω*-aminopoly(ethylene oxide) (500 mg, MW 5000) in chloroform (6 mL) at room temperature. α-Methoxy-*ω*-aminopoly(ethylene oxide) was kindly gifted from Nippon Oil & Fats Co. Ltd. The obtained *N*-methacryloyl-PEG was purified by column chromatography on sephadex LH-20 using methanol as an eluting solvent. NIPAM-PEP was synthesized by ordinary radical copolymerization of *N*-isopropylacrylamide (40 mg, 0.35 mmol) and *N*-methacryloylpeptide (6 mg, 6.7 μmol) in water (4.5 mL) at room temperature for 1 h under nitrogen atmosphere using ammonium persulfate (3.1 mg, 2.9 mmol) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (4 μL, 5.8 mmol) as a redox initiator couple. The NIPAM−PEP was purified with dialysis using a semipermeable membrane bag (with a molecular weight cutoff of 10 000) against water overnight followed by lyophilizing. We obtained 31 mg of the polymer, and the peptide content in the polymer chain was 0.77 mol % calculated from the value of the elemental analysis. NIPAM−PEPEP was synthesized in a manner similar to that of NIPAM−PEP using *N*-methacryloyl-PEG (2 mg, 0.4 μmol), *N*-methacryloylpeptide (3 mg, 3.3 μmol), and *N*-isopropylacrylamide (20 mg, 0.18 mmol) to obtain 16 mg of a white powder. In this case, the semipermeable membrane bag (with a molecular weight cutoff of 50 000) was used for the dialysis.

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Monitoring of Phosphorylation of Materials with PKA. A determination of phosphate incorporation into NIPAM−PEP, NIPAM−PEPEP, or a substrate peptide was carried out by a coupled enzyme assay.[17] The oxidation of NADH, which can be monitored spectrophotometrically as an absorbance decrease at 340 nm, is coupled to the production of ADP using lactate dehydrogenase and pyruvate kinase. After the PKA C-subunit (50 units) was incubated in 2 mL of 50 mM MOPS buffer (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase at 25 °C, all reactions were initiated by the addition of the aqueous polymer solution (20 mg/mL) at a final concentration of 0.2 mg/mL. When the substrate peptide monomer itself, methacryloyl-GLRRASLG, was used, the peptide solution was added to initiate the reaction at a final concentration of 20 μM. Definitions of enzymes used here are as follows; In PKA, 1 unit is defined as the amount of the enzyme required incorporating 1 pmol of phosphate into casein in 1 min. In lactate dehydrogenase, it is defined as the amount of the enzyme causing the oxidation of 1 μmol of NADH per min at 25 °C and pH 7.4. In pyruvate kinase, unit definition is the amount of the enzyme that converts 1 μmol of phosphoenolpyruvate to pyruvate per min at pH 7.6, 37 °C in the presence of 1.0 mM fructose 1,6-diphosphate.

Laser Light Scattering. A modified commercial LLS spectrometer (DLS-7000, Ohtsuka Electronics Co. Ltd.) and an argon laser (output power ca. 20 mW at $\lambda = 488$ nm) were used. The incident light beam was vertically polarized with respect to the scattering plane. In the static light scattering experiment, the angular dependence of the excess absolute time-averaged scattering intensity, known as the Rayleigh ratio, was measured.

Fluorescent Measurement. Measurement of the fluorescent spectra was performed on an RF-5300PC spectrofluorophotometer (Shimazu Kyoto, Japan). Dansylaniline (final concentration 20 μM) was dissolved in 50 mM MOPS buffer solution (pH 7.0) containing 10 mM $MgCl_2$ and 0.2 mM ATP in the presence of 0.3 mg/mL NIPAM−PEPEP. The fluorescent spectra were then measured at an excitation wavelength of 340 nm at each temperature. For the experiment using phosphorylated NIPAM−PEPEP, the 12.5 U PKA C subunit was added to the above solution 2 h before the fluorescent measurements.

Results and Discussion

Our strategy for designing a PKA-responsive polymer was to use the substrate peptide of PKA as a receptor of phosphorylation to control the hydration around the poly(*N*-isopropylacrylamide) (PNIPAM) chain backbone of the thermosensitive polymer. PNIPAM is hydrophobic at the temperatures higher than ∼32 °C (LCST), while at lower temperatures it becomes water-soluble due to the enhancement of hydration around the polymer chain.[18] Since the controlling factor of this phase transition is the extent of hydration, it may be possible to make such a polymer hydrophilic above the LCST if many phosphate groups, which are highly water-soluble, are introduced into the polymer chain (Figure 1a). We therefore designed two types of copolymers, NIPAM−PEP and NIPAM−PEPEP, as protein kinase A (PKA)-responsive materials (Figure 1b). Both polymers are graft-type copolymers that were synthesized using each methacryloyl monomer with radical copolymerization. The NIPAM−PEP contains two functionalized units: a *N*-isopropylacrylamide unit, which forms the typical thermoresponsive polymer, and a peptide pendant unit, which acts as a phosphate receptor site that is selectively phosphorylated with PKA. The peptide contains a consensus amino acid sequence, RRXSL, for the selective phosphorylation site of PKA.[15,16] NIPAM−PEPEP contained another hydrophilic poly(ethylene oxide) unit. The peptide contents in NIPAM−PEP and NIPAM−PEPEP were estimated to be 0.77 and 1.76 mol %, respectively, using the results of elemental analysis and ^{1}H NMR spectra. The PEG unit content in NIPAM−PEPEP was determined as 0.32 mol % in a similar manner.

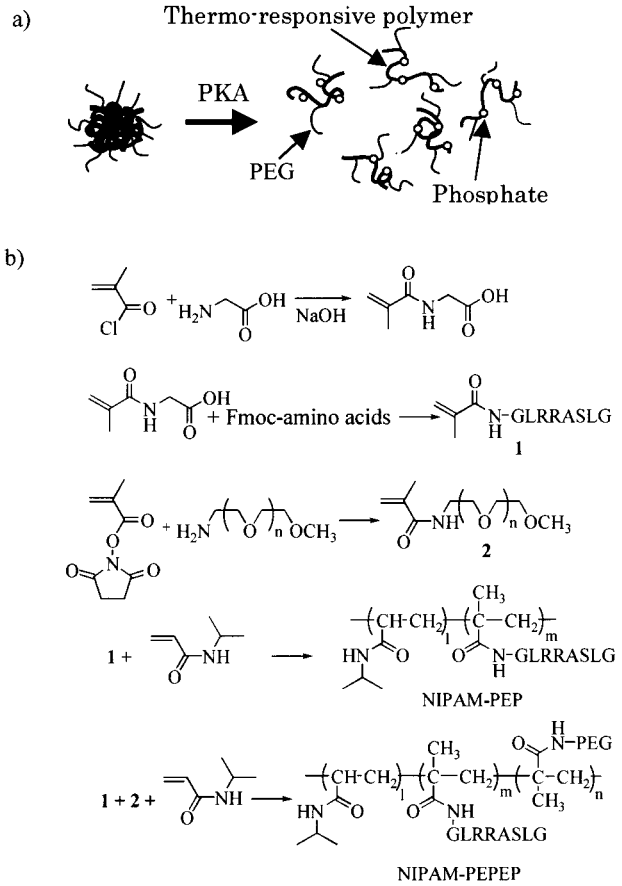

Figure 1. Strategy for the preparation of NIPAM−PEP and NIPAM−PEPEP. (a**)** A diagram of the suggested mechanism for the disintegration of the NIPAM−PEPEP polymer micelle in response to phosphorylation with activated protein kinase A. (b**)** Synthetic schema of NIPAM−PEP and NIPAM−PEPEP. Both materials were synthesized by radical copolymerization of appropriate monomers. The peptide monomers were prepared using automatic peptide synthesizer after *N*-methacryloylglycine was synthesized. PEG monomer was prepared by the reaction between methacryloyloxysuccinimide and α-methox-ω-amino-PEG.

For the first experiment, we investigated whether the peptide sequences still acted as a PKA substrate after being incorporated into the polymer backbone. Phosphorylations of NIPAM−PEP and NIPAM−PEPEP were monitored with a spectrophotometric coupled-enzyme assay reported by Cook et al.[17] (Figure 2). In the assay, the production of ADP, which is derived from ATP as a byproduct of the peptide phosphorylation, finally brings about an oxidation of NADH using lactate dehydrogenase and pyruvate kinase. The phosphorylation can thus be monitored as correlative to the decrease in absorbance at 340 nm. The polymer chain backbone slightly depressed the rate of phosphorylation for the substrate peptides with PKA, probably due to the steric hindrance of the polymer chain, but NIPAM−PEP and NIPAM−PEPEP were still good substrates for the kinase. In all cases investigated, the phosphorylation

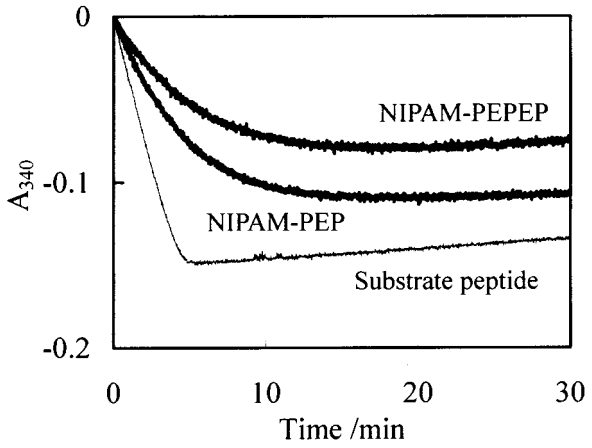

Figure 2. Time-dependent decrease in the absorbance at 340 nm based on the oxidation of NADH, which was initiated by PKA phosphorylation of the NIPAM−PEP, NIPAM−PEPEP, and substrate peptide in the coupled enzyme assay. The measurement was performed with 20 μM of the substrate peptide or 0.2 mg/mL of NIPAM−PEP or NIPAM−PEPEP in 50 mM MOPS buffer (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase at 25 °C.

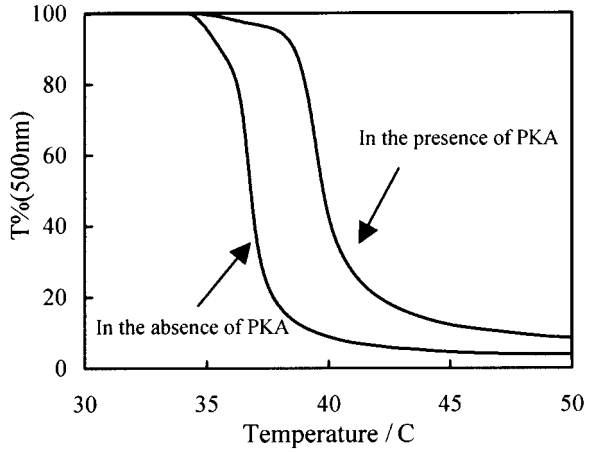

Figure 3. Temperature dependence of the light transmittance of the 0.3 mg/mL NIPAM−PEP solution before and after phosphorylation with activated PKA (12.5 U). The experiment was performed in 50 mM MOPS (pH 7.0) containing 0.2 mM ATP and 10 mM $MgCl_2$. In the case of phosphorylated NIPAM−PEP, light transmittance was measured after the 2 h incubation of the solution with the PKA C subunit (12.5 U).

reactions practically finished within 10 min using 50 U of PKA under LCST.

For the next experiment, we investigated the phosphorylation effects on the LCST of NIPAM−PEP. Figure 3 shows the temperature dependence of the light transmittance (absorbance at 500 nm) of the NIPAM−PEP/ MOPS buffer solution (pH 7.0) before and after phosphorylation. The polymer was found to be water-soluble at temperatures lower than 33 °C. However, the solution gradually became turbid with increases in temperature, and the polymer became completely insoluble in water above 38 °C (LCST: 36.7 °C). In contrast, the LCST rose to 40 °C following phosphorylation with PKA due to the enhancement of hydration around introduced phosphate groups. These results suggest that the polymer changed from water-insoluble to water-soluble with phosphorylation at 38 °C.

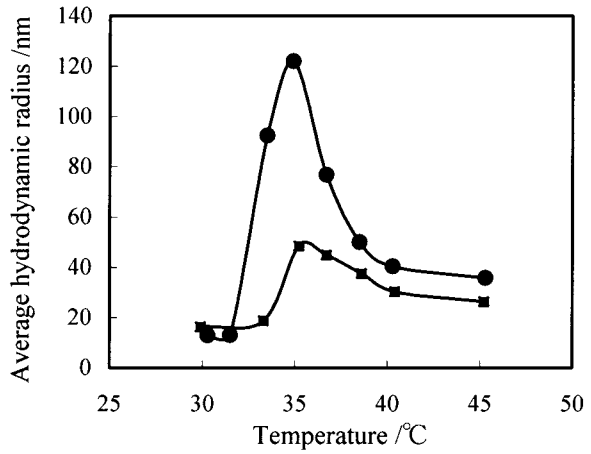

Figure 4. Temperature dependence of the average hydrodynamic radius of NIPAM−PEPEP (0.3 mg/mL) before (●) and after (■) phosphorylation with the 35 U PKA C-subunit in 50 mM MOPS buffer (pH 7.0) containing 0.2 mM ATP and 10 mM $MgCl_2$. The light-scattering intensity of the phosphorylated NIPAM−PEPEP was measured 2 h after the addition of activated PKA (35 U). The completion of phosphorylation was confirmed with the coupled enzyme assay.

Since it became clear that the introduction of phosphate groups into the polymer dramatically changed the polymer's physical properties, we then prepared NIPAM−PEPEP, because PNIPAM grafted with poly(ethylene oxide) chains forms core−shell-type colloidal particles above the LCST.[19,20] Dynamic light scattering (DLS), differential scanning calorimetry, and light-transmittance measurements at various temperatures indicated that NIPAM−PEPEP formed narrowly distributed colloidal particles in which the mean hydrodynamic radius was 100 nm above 36 °C at a concentration of 0.3 mg/mL in 50 mM MOPS buffer solution (pH 7.0). Figure 4 shows the temperature dependence of the mean hydrodynamic radius of the NIPAM−PEPEP buffer solution before and after phosphorylation with PKA in the DLS experiment.

In all these experiments, the mean particle size of the phosphorylated polymer micelle of NIPAM−PEPEP was smaller than that of the unphosphorylated particle. In addition, the polymer formed relatively large-sized particles at around 36 °C, but the particle size gradually decreased with further temperature increases.

Static light scattering experiments gave an apparent molecular weight of 160 000 for NIPAM−PEPEP in a random coiled state, while this steeply increased to 10 000 000 above 36 °C, indicating that this polymer micelle-type particle consists of an aggregate with 62−63 polymer chains. The second shrinkage process with regard to particle size that was observed with further increases in temperature, as seen in Figure 4, did not decrease the apparent molecular weight, indicating that the intrachain coil-to-globule transition is dominant in this shrinking process. Probably, the smaller size of the phosphorylated particles observed in the DLS study was due to the smaller number of aggregated polymer chains. Figure 5 shows the time dependence of the mean hydrodynamic radius of the colloidal particle with PKA phosphorylation at 38 °C. The particle size decreased gradually with time in the presence of activated PKA (C subunit), and the polydispersity increased at the same time, clearly indicating that this type of polymer micelle is disintegrated by gradual dissociation of the phosphorylated polymer chains in response to the PKA

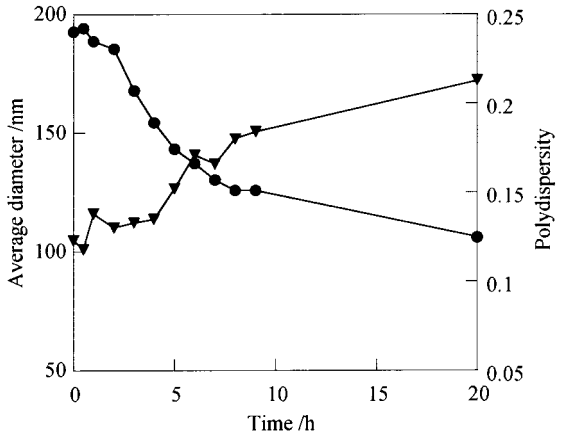

Figure 5. Time course of the change in the average hydrodynamic radius (●) and polydispersity (▼) of the NIPAM−PEPEP particle with the phosphorylation by PKA (35 U). NIPAM−PEPEP (0.3 mg/mL) was dissolved in 50 mM MOPS (pH 7.0) containing 0.2 mM ATP and 10 mM $MgCl_2$. The light-scattering intensity was monitored at 36 °C with a 90° detecting angle. Other experimental conditions were similar to those described in Figure 4.

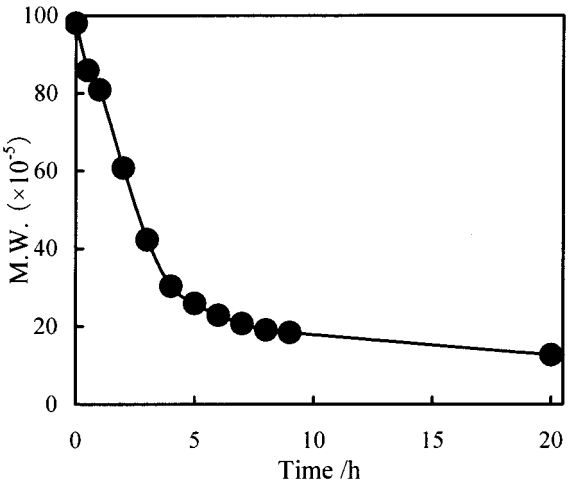

Figure 6. Time course of the change in the apparent molecular weight of the NIPAM−PEPEP particle with the phosphorylation by PKA (35 U). NIPAM−PEPEP (0.3 mg/mL) was dissolved in 50 mM MOPS (pH 7.0) containing 0.2 mM ATP and 10 mM $MgCl_2$. The light-scattering intensity was monitored at 36 °C. Other experimental conditions were similar to those described in Figure 4.

signal (Figure 5). The apparent molecular weight of the particle was also decreased with time from 10 000 000 to 2 000 000 in the presence of activated PKA (Figure 6). This means that the aggregation number of the polymer chain in the colloidal particle changed from 62 to 12 with the phosphorylation of the polymer chain to dissociate the phosphorylated polymer chains.

For the last experiment, dansylaniline, which gives a fluorescent spectrum sensitive to environmental hydrophobicity, was mixed with NIPAM−PEPEP at various temperatures (Figure 7). When the temperature was gradually raised, the fluorescent maximum shifted from 550 to 512 nm, and its intensity was increased, indicating that the dye molecules were located in a more hydrophobic environment. A comparison of the temperature dependence of the fluorescent spectra before and after phosphorylation indicates that the environment of the total fluorescent dye molecules is more hydrophilic in the phosphorylated polymer probably due to an increase in the numbers of dye molecules in the aqueous

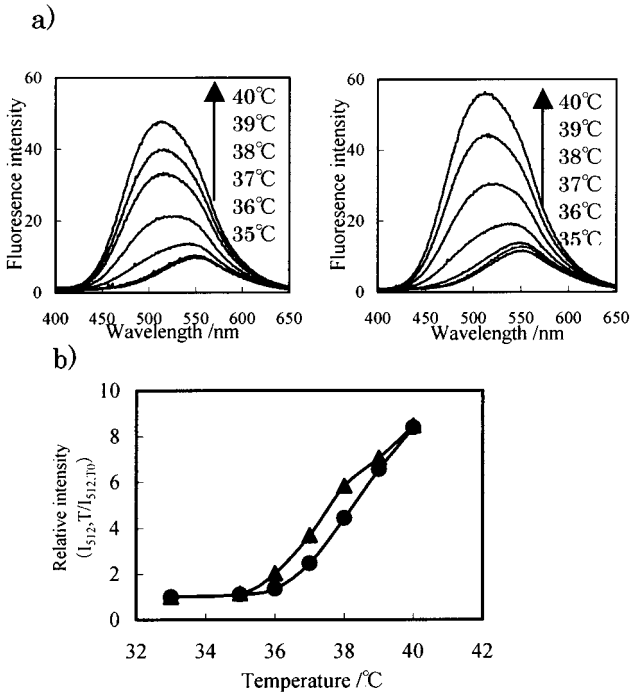

Figure 7. Temperature dependence of fluorescent spectra (a) and the change in the fluorescent intensity at 512 nm (b) of dansylaniline in the presence of NIPAM−PEPEP with (right panel in a and ● in b) or without (left panel in a and ▲ in b) phosphorylation. The change in the fluorescent intensity at 512 nm was estimated as the ratio of the intensity at each temperature to that at 33 °C. *N*-Dansylaniline (10 μM as a final concentration) was dissolved in 50 mM MOPS (pH 7.0) containing 0.3 mg/mL of NIPAM−PEPEP, 0.2 mM ATP, and 10 mM $MgCl_2$. The phosphorylation was performed with an addition of 12.5 U of the PKA C-subunit.

phase rather than in the hydrophobic core of the particle. When the fluorescent intensity at 512 nm was monitored in the presence of the polymer micelle and activated PKA at 38−39 °C, we observed that the intensity decreased linearly with time, indicating a gradual release of fluorescent molecules (data not shown).

Conclusion

We report herein the first example of a polymer micelle that disintegrates in response to a kinase signal. We used protein kinase A (PKA) activated by cyclic AMP as a target signal. This kinase is one of the key kinases controlling many cellular events in the course of intracellular signal transduction. However, there are many kinds of kinases in intracellular space, and if these kinases become abnormally activated, the cell will fall into a disordered state.[21−30] Our strategy reported here could be applied to any other kinase-responsive polymers by changing only the substrate peptide sequence for the target kinase. Our polymer micelle-type particle would release an encapsulated drug only in such disordered cells. This characteristic makes it potentially useful in the design of a new type of intelligent drug capsules that can communicate with cells to release its pharmacological activity. This concept may provide a novel strategy for realizing selective drug dosage in response to cellular conditions.

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