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# Separation of phosphorylated peptides utilizing dual pH- and temperature-responsive chromatography

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ABSTRACT

The phosphorylation of a peptide is considered to be one of the most important post-translational modification reactions that can alter protein function in mammalian cells. To separate and purify, we developed a dual temperature- and pH-responsive chromatography based on terpolymer composed of *N*-isopropylacrylamide, *N*,*N*′-dimethylaminopropylacrylamide and butylmethacrylate. The property of the surface of the terpolymer-grafted stationary phase altered from hydrophilic to hydrophobic, and from changed to non-charged by changes in the temperature and the pH, respectively. In addition, it was possible to appear and hide ion-exchange groups on the polymer chain surface by temperature changes. These phenomena resulted from changes in the charge and the hydrophobicity of the pH- and temperature-responsive polymer on the stationary surface by controlling the temperature. In the developed environmental-responsive chromatographic system, the ionizable dimethylamino group of *N*,*N*′-dimethylaminopropylacrylamide in terpolymer played a key role for the separation. We applied the developed chromatographic system to the separation of phosphorylated compounds, such as phospho-tyrosine, phosphopeptide and oligonucleotides. At a low column temperature, the electrostatic interaction plays a predominant role for retain anionic phosphorylated compounds, because of the strong interaction between the cationic dimethylamino group in the stationary phase and the anionic phosphoric group in the analyte. On the contrary, the hydrophobic interaction became predominant upon increasing the temperature. The results showed that both the electrostatic and the hydrophobic interactions became controllable with a temperature change during the chromatographic process. Dual pH- and temperature-responsive chromatography would be very useful for biomacromolecules separation and purification.



## 1. Introduction

In the post-genomic era, the separation and identification of the bioactive-protein has been becoming increasingly important for the comprehensive analysis of biological processes and life science. In the life action, phosphorylation, glycosylation, methylation and acetylation dramatically enhance the diversity of genetically encoded proteins, and a play key role in regulating the function of target proteins [1]. Among these modifications, phosphorylation represents a most important post-translational modification of proteins. Reversible protein phosphorylation plays a major role in many signal-transduction processes, docking with other proteins, cell-cycle progression, energy metabolism and cell apoptosis [2–4]. Because phosphoproteins comprise only a small fraction of the total protein in a cellular lysate, strenuous efforts have been focused on the development of efficient separation methodologies. At the same time, the analysis of the quality of oligonucleotides is critical in all applications of genomic studies, because synthetic nucleotides have significantly increased due to their application for PCR, genotyping and microarrays. The methodology of their purification is still relies on polyacrylamide gel electrophoresis and HPLC. In the latter case, the reversed-phase HPLC (RP-HPLC) method is often combined with ion-exchange HPLC. Though the combination two HPLC methods offer a product of high quality, it is laborious to operators.

Recently, various polymers that respond to external stimuli have been developed, and these polymers change their functions and structure reversibly, in response to the surrounding conditions, such as light, pH, temperature, electric field and chemical substances. Poly(*N*-isopropylacrylamide) (PNIPAAm), which is one of these polymers, exhibits a thermally reversible soluble–insoluble change in the response to temperature changes across a lower critical solution temperature (LCST) at 32 °C in aqueous solution [5]. PNIPAAm undergoes a sharp phase separation and coil globule tran-

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sition of the polymer chain structure in the vicinity of its LCST. In an aqueous solution, PNIPAAm exhibits an expanded conformation below the LCST due to strong hydration, and a change to a compact form above the LCST by sudden dehydration. Based on its unique property, PNIPAAm has been used for drug-delivery systems [6], cell culture dishes, cell sheets [7,8] and bio-imaging [9].

Aside from these, we have been applying PNIPAAm to using a thermally responsive stationary phase in a chromatographic system [10–13]. Hydrophobic interaction, between analyte and the PNIPAAm grafted stationary phase are the driving force for the separation of several hydrophobic steroids [10]. In addition, a thermoresponsive modified copolymer composed from butylmethacrylate (BMA) and a NIPAAm grafted stationary phase was used for phenyl thiohydantoin amino acid analysis [11]. Furthermore, we separated ionizable compounds, such as nonsteroidal anti-inflammatory drugs, melatonin and related compounds by introducing an ionizable group into PNIPAAm stationary phase [12,13]. The temperature-responsive chromatographic system is simple and user-friendly, because elution can be controlled only by adjusting the column temperature, and merely an aqueous solution is used as the mobile phase. Temperature is known to play a significant role in some chromatographic system [14], but its influence on the separation of small molecules in conventional RP-HPLC is much less important. In addition, the proposed analytical method has some advantages compared with the conventional RP-HPLC method. First, the avoidance of complicated mobile phase preparation was possible, and only temperature changing could regulate the retention time ($t_R$) of the analyte. Second, by using water as the mobile phase instead of an organic solvent, it is an ideal analytical method according to green chemistry.

In the present study, we prepared and characterized a pH- and temperature-responsive polymer, which is composed from NIPAAm as the temperature-sensitive component, and *N*,*N*′-dimethylaminopropylacrylamide (DMAPAAm) as the pH-sensitive component. Next, we applied the terpolymer grafted stationary phase to analyze the phospho-amino acid, phosphopeptide and oligonucleotides.

## 2. Experimental

### 2.1. Materials and chemicals

*N*-Isopropylacrylamide (NIPAAm) was kindly provided by KOHJIN (Tokyo, Japan), and was purified by recrystallization from *n*-hexane and dried at 25 °C *in vacuo*. DMAPAAm was purchased from KOHJIN. BMA and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-Mercaptopropionic acid (MPA), *N*,*N*-dimethylformamide (DMF) and *N*,*N*′-methylenebisacrylamide (MBAAm) were purchased from Kanto Chemical (Tokyo, Japan). 4,4′-Azobis (4-cyanovaleric acid) (ACV; Wako) and 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ; Tokyo Chemical Industry, Tokyo, Japan) were used without further purification. *O*-Phospho-L-tyrosine was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Protein tyrosine phosphatase (PTPase) substrate (Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys) [Insulin Receptor fragment 1142–1153 human, $C_{72}H_{107}N_{19}O_{24}$, MW 1622.73] and PTPase substrate monophosphate (Thr-Arg-Asp-Ile-Tyr[$PO_3H_2$]-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys) ([$pTyr^{1146}$]-Insulin Receptor fragment 1142–1153, MW 1702.8) were purchased from Sigma–Aldrich (St. Louis, USA). Synthetic oligonucleotides, containing 2, 3, 4, 5, 6, 10, 15, and 20-mers of poly(dT) [$p(dT)_2$, $p(dT)_3$, $p(dT)_4$, $p(dT)_5$, $p(dT)_6$, $p(dT)_{10}$, $p(dT)_{15}$ and $p(dT)_{20}$ were purchased from Espec Oligo Service Corp. Japan. Water used for sample preparation and the LC mobile phase was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All other reagents and solvents were of analytical grade. Aminopropyl silica beads (average diameter, 5 μm; pore size, 120 Å) were purchased from Nishio Industry (Tokyo, Japan).

### 2.2. Synthesis of linear polymer

The synthesis of semitelechelic poly(NIPAAm-*co*-BMA-*co*-DMAPAAm) (Terpolymer is abbreviated as IBD) was carried out as previously reported [11,15]. After NIPAAm (21.89 g, 193.44 mol), BMA (1.44 g, 10.13 mmol) and DMAPAAm (1.667 g, 10.67 mmol) were dissolved in DMF (50 mL), AIBN (0.15 g, 0.91 mmol) and MPA (0.64 g, 6.03 mmol), which act as a radical initiator and chain transfer reagent, were added to the solution. The reaction mixture was degassed by subjecting to freeze–thaw cycles and heated at 70 °C. After a reaction for 5 h, the reaction solution was poured into diethyl ether to precipitate the polymer. The crude product was further purified by repeated precipitation from an acetone solution into diethyl ether, and then dried to give a white solid.

### 2.3. Transmittance measurement

The LCST of the IBD terpolymer was determined by measuring the optical transmittance in an aqueous solution (0.5 w/v%). The transmittance changes at 500 nm of the IBD terpolymer were measured at various temperatures using a V-630 spectrophotometer (JASCO, Tokyo, Japan). The LCST defined the temperature at the 50% optical transmittance of the IBD terpolymer aqueous solution.

### 2.4. Introduction of terpolymer hydrogel to silica bead surfaces

Aminopropyl silica beads were used as a base support for modification with thermoresponsive cationic polymers. A polymerization initiator, ACV, was covalently immobilized on aminopropyl silica beads through amide bond formation. A detailed procedure for ACV immobilization and surface-controlled PNIPAAm polymerization has been previously reported [16]. Crosslinked terpolymer hydrogel comprising NIPAAm, DMAPAAm, and BMA was prepared by radical copolymerization at the surfaces of initiator-immobilized silica beads in the presence of the crosslinker, MBAAm. A typical procedure was as follows; NIPAAm:BMA:DMAPAAm (mol ratio; 90:5:5) and MBAAm were dissolved in 200 mL of ethanol in a glass ampule. ACV-immobilized silica beads (4.0 g) were added to the solution of a monomer. The reaction mixture was then bubbled with $N_2$ gas for 1 h, and polymerization was carried out at 70 °C for 5 h under a $N_2$ gas atmosphere. IBD terpolymer hydrogel-modified silica beads were collected by filtration, followed by washing three times with ethanol to remove unreacted monomers and unbound polymers, then dried for 12 h under a vacuum at 25 °C.

### 2.5. Environmental-responsive chromatographic analysis of phosphorylated compounds

The poly(NIPAAm-*co*-BMA-*co*-DMAPAAm) hydrogel-modified silica bead support was packed into a stainless-steel column (50 mm × 4.6 mm I.D.), and the column was connected to a Hitachi HPLC system (Hitachi L-7100 intelligent pump, L-7405 UV detector and D-7000 integrator, Tokyo, Japan). The column oven was an Aqua Way Gradient (CellSeed, Tokyo, Japan). The column was used at a flow rate of 1.0 mL/min. Other analytical conditions were as follows: *O*-phospho-L-tyrosine; 10 mM $CH_3CO_2NH_4$ (pH 6.0, mobile phase), 215 nm (detection wavelength), phosphopeptides; 10 mM $CH_3CO_2NH_4$ (pH 6.0 mobile phase), 215 nm (detection wavelength), oligonucleotides; 1/15 M dihydrogen phosphate/disodium hydrogen phosphate buffer (pH 6.4 or 6.5, mobile phase), 265 nm (detection wavelength). Standard

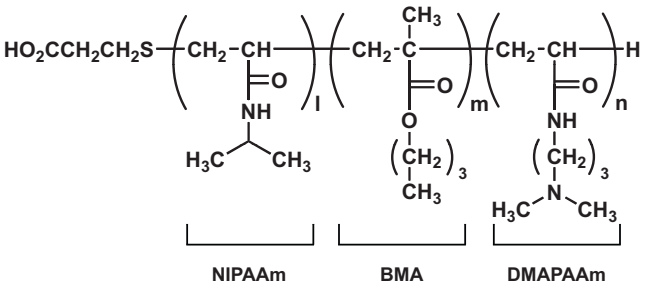


**Fig. 1.** Structure formula of the poly(NIPAAm-*co*-BMA-*co*-DMAPAAm) terpolymer.

solutions of *O*-phospho-L-tyrosine and phosphopeptides were dissolved in the mobile phase at initial concentrations of 0.5 mg/mL. Standard solutions of oligonucleotides were prepared with $p(dT)_2$ (25.11 μg/mL), $p(dT)_3$ (41.25 μg/mL), $p(dT)_4$ (48.42 μg/mL), $p(dT)_5$ (56.21 μg/mL and 10.76 μg/mL), $p(dT)_{10}$ (47.83 μg/mL), $p(dT)_{15}$ (47.83 μg/mL), $p(dT)_{20}$ (47.83 μg/mL) by dissolving in the mobile phase; the injection volume was 10 μL.

The retention factor, *k*, for each peptide at a given temperature was determined by using:

$$k = \frac{t_R - t_0}{t_0}$$

where $t_0$ is the retention time of deuterium oxide ($D_2O$), used as a standard since it elutes at the column void volume, and $t_R$ is the retention time for each peptide and oligonucleotides.

## 3. Result and discussion

### 3.1. Environmental-responsive polymer

Because a terpolymer IBD-modified silica column was utilized in this study, we synthesized and evaluated the physicochemical property of a linear polymer of IBD. The chemical structure of the semitelechelic IBD terpolymer is shown in Fig. 1. The IBD terpolymer was soluble in water below its LCST and on precipitate above the LCST because of a speedy hydrophilic–hydrophobic change. Furthermore, the LCST of IBD terpolymer is dependent on the pH or the ionic strength of the solution with a dimethylamino group of DMAPAAm, which acts as the ion-exchange group. The LCSTs of the IBD terpolymer have a function of pH, as shown in Fig. 2. The LCST of the terpolymer decreased with increasing pH indicating that charge may be the dominant factor influencing the LCST. In the low-pH condition, the dimethylamino groups were the protonated form, and the IBD terpolymer showed a more hydrophilic property compared with at high-pH condition (decreasing charge of DMAPAAm). These results show that the IBD terpolymer is a dual-temperature- and pH-responsive polymer, and has possibilities to be applied to the bioseparation of phosphopeptides and oligonucleotides, which have an ionic functional group.

There are some reports about the effect of the pH on the LCST of the PNIPAAm copolymer. Hoffman and co-workers researched the copolymer, which consists of NIPAAm and acrylic acid [17]. This polymer showed an increase of the LCST with an increase of the pH in the used buffer solution. Because the carboxylic group ($pK_a$ 4–5) is deprotonated in the above condition, the copolymer showed a more hydrophilic condition followed by LCST increase. Aside from this, Kim and co-workers reported on the pH responsibility of the LCST of a copolymer containing NIPAAm and (diethylamino)ethyl methacrylate [18]; their polymers showed a decrease of the LCST with an increase of the pH in the used buffer solution. This phenomenon is quite contrary to the effect of the pH on the LCST of the PNIPAAm copolymer.

### 3.2. Temperature-responsive analysis of phospho-tyrosine

As described above, phosphorylation of the peptide is considered to be one of the most important post-translational modification reactions that can alter the protein function in mammalian cells. Phosphorylation mainly occurs on serine, threonine and tyrosine residues in the substrate protein. Among three amino acids, tyrosine is the most important target of tyrosine kinase which is an active site of a molecular-targeting drug for cancer [19]. At first, we applied an IBD hydrogel modified column to analyze the phospho-tyrosine using the 10 mM $CH_3CO_2NH_4$ buffer (pH 4.5 and 6.0) as the mobile phase. Chromatograms of phospho-tyrosine are shown in Fig. 3 at different column temperatures (from 10 °C to 50 °C). This result shows that the retention time decreased with increasing the column temperature and the pH of the mobile phase. The $pK_a$ values of the primary phospholic and carboxylic groups in phospho-tyrosine are ~2, and 2.2, respectively; they are dissociated forms under this pH condition (pH 4.5) [20]. Furthermore, the dimethylamino group of DMAPAAm in the IBD terpolymer is protonated, because the $pK_a$ value of this functional group is 10.4. On the IBD-modified column, the retentions of the phospho-tyrosine increased below the LCST (the LCST value of polymer is 38.9 in a pH 4.5 buffer solution), and decreased the retention above it. Because the phosphoric group is in charged forms at pH 4.5, the electrostatic interactions between the phosphoric group and the amino group of the DMAPAAm on the IBD stationary phase should cause increased

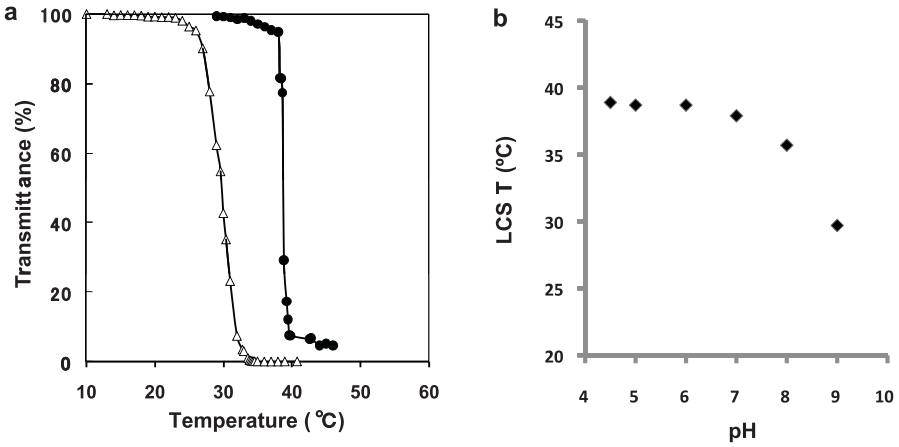


**Fig. 2.** LCST of the poly(NIPAAm-*co*-BMA-*co*-DMAPAAm) terpolymer as a function of the pH. (a) The optical transmittance at 500 nm of a solution of terpolymer versus temperature (△, pH 9.0; ●, pH 5.0). (b) LCST of terpolymer at various pH values.

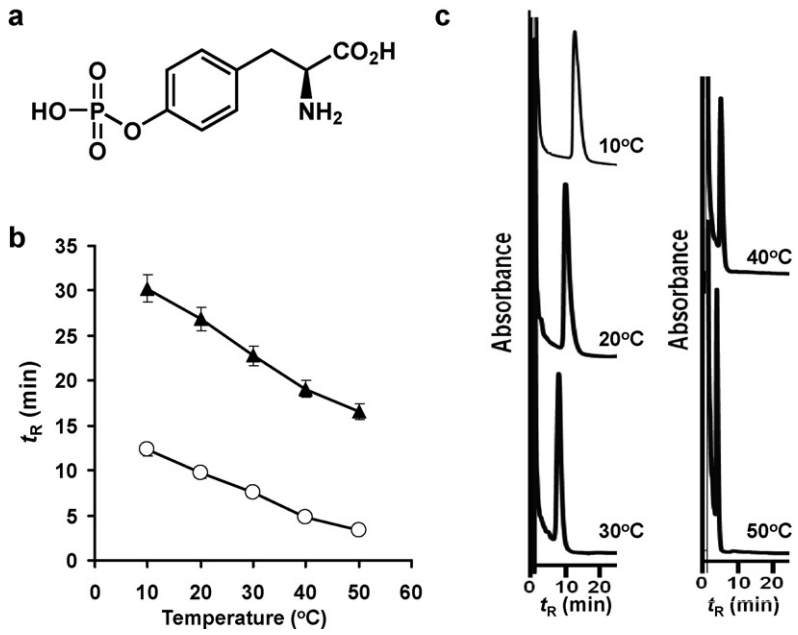


**Fig. 3.** Analysis of phospho-tyrosine by environmental-responsive chromatography. Structure of phospho-tyrosine (a). Change in the retention time [▲, pH 4.5; ○, pH 6.0] (b) and chromatograms (c) of phospho-tyrosine on IBD hydrogel modified column (50 mm × 4.6 mm I.D.) [HPLC condition: 10 mM $CH_3CO_2NH_4$ (pH 6.0, mobile phase), 215 nm (detection wavelength), 1.0 mL/min (flow rate), column temperature (10–50 °C)].

retention below the LCST. By raising the column temperature, the electrostatic interactions were reduced by hiding the charge. As a result, it was observed that the retention of phospho-tyrosine was decreased. According to changing the column temperature, IBD terpolymer was extended, and showed a hydrophilic property at low analytical temperatures. This is because the electrostatic interaction between the positively charged stationary phase (under lower pH condition than p$K_a$ value of polymer) and the negatively charged analyte became strong. On the contrary, in high column temperature condition above the LCST of IBD terpolymer, the retention of the analyte decreased. Because the IBD polymer shrunk and buried its charged functional group at high temperature, the electronic density of the stationary phase decreased, and the interaction between the analyte and the polymer wakened. However, the retention of the non-phosphorylated tyrosine hardly changed under changing the column temperatures (data not shown). Upon changing the pH and the temperatures under analytical condition, the retention of the phospho-amino acid was well controlled. The precision of the method was established from 5 assays and coefficient of variation (CV) values of the retention times were within 1.4%. These results promoted us to analyze the phosphopeptide utilizing environmental-responsive chromatography.

### 3.3. Analysis of phosphopeptide by environmental-responsive chromatography

Next, we applied the developed chromatographic system to a phosphopeptide analysis. Protein phosphorylation has an outstanding role in signal transduction pathways and are thus of significant importance in disease-related studies. A protein tyrosine phosphatase substrate and its monophosphorylated-peptide were used as the analyte [21]. These peptides were composed of twelve amino acids, and only one tyrosine was phosphorylated. Fig. 4 shows a comparison of the retention time of both the intact- and monophosphorylated-peptide utilizing environmental-responsive chromatography, while under the 10 mM $CH_3CO_2NH_4$ buffer (pH 6.0) as a mobile phase at a flow rate of 1.0 mL/min. Under this analytical condition, because the pH value of the mobile phase was set at 6.0, the dimethyamino group of the stationary phase was protonated, and showed cationic property. The effect of the temperature on the retention time had the same tendency as a phospho-tyrosine analysis. Though both retentions of the intact- and monophosphorylated-peptides are shortened with increasing column temperature, there is an obvious difference between them. Remarkably, the magnitude of the retention is quite different between the phospho- and non-phosphopeptide, especially at a low column temperature. The change of the retention time of non-phosphopeptide is slight from 10 °C to 50 °C for the column temperature. On the other hand, the retention time at 10 °C is about five-times longer than that at 50 °C in a phosphopeptide analysis. These results show that the magnitude of the retention is quite

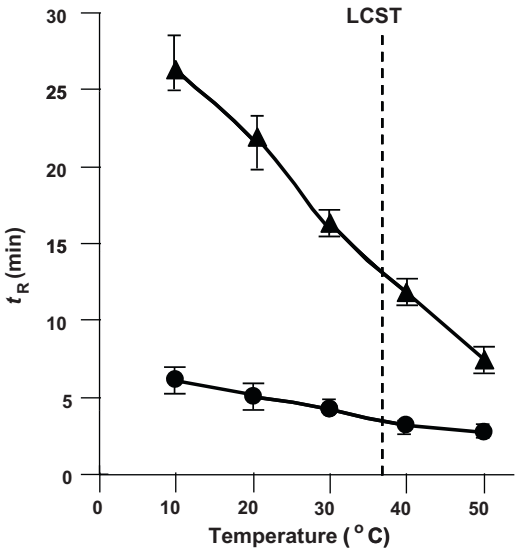


**Fig. 4.** Analysis of monophosphorylated- and intact-peptide by environmental-responsive chromatography. Change in the retention time on an IBD hydrogel modified column (50 mm × 4.6 mm I.D.) [▲, monophosphorylated-peptide; ●, intact-peptide] [HPLC condition; 10 mM $CH_3CO_2NH_4$ (pH 6.0, mobile phase), 215 nm (detection wavelength); 1.0 mL/min (flow rate); column temperature, 10–50 °C].

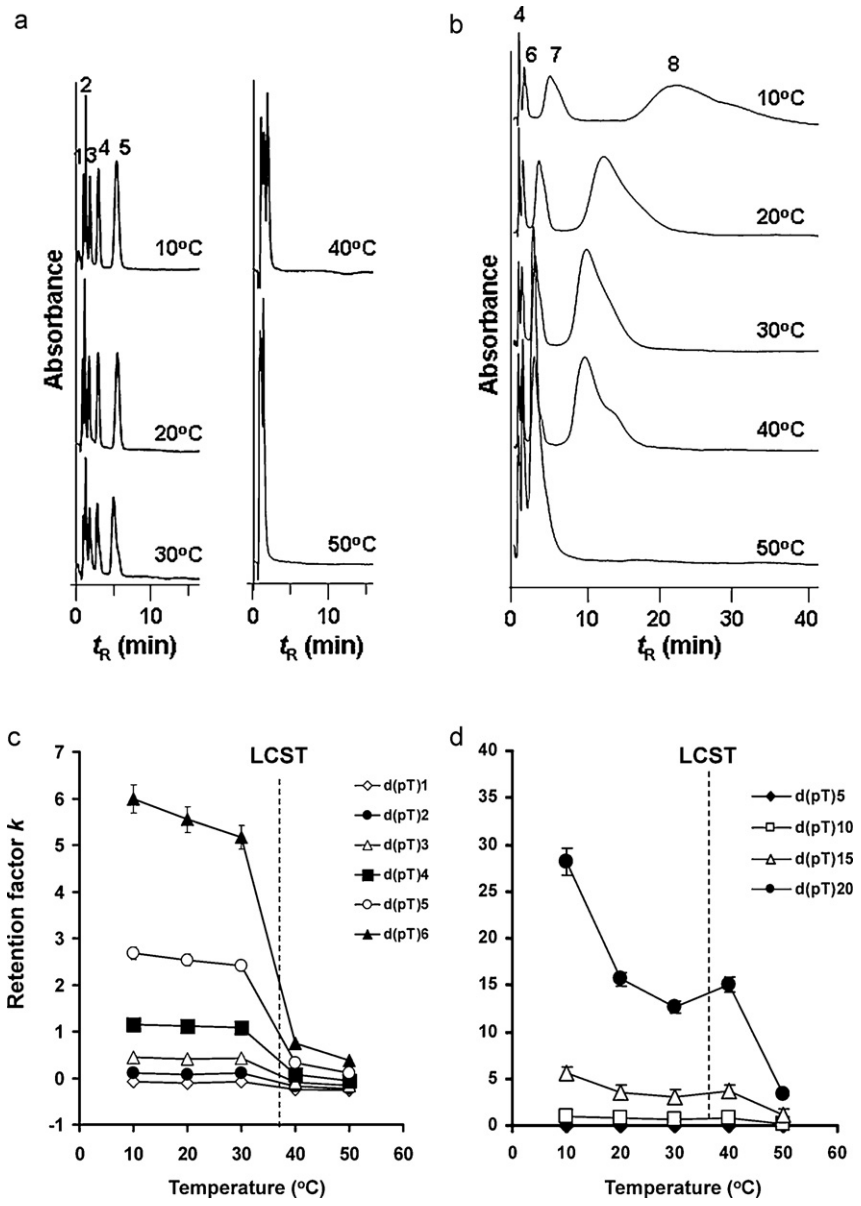


**Fig. 5.** Analysis of oligonucleotides [p(dT)] by environmental-responsive chromatography on IBD hydrogel modified column [15 mm × 4.6 mm I.D. (a), (c) and 50 mm × 4.6 mm I.D. (b), (d)] [HPLC condition; 1/15 M sodium dihydrogen phosphate/disodium hydrogen phosphate buffer[pH 6.4 (a), (c) and 6.6 (b), (d) mobile phase], 265 nm (detection wavelength) 1.0 mL/min (flow rate), column temperature (10–50 °C)]. (a) 1: $p(dT)_2$, 2: $p(dT)_3$, 3: $p(dT)_4$, 4: $p(dT)_5$, 5: $p(dT)_6$ and (b) 7: $p(dT)_{10}$, 8: $p(dT)_{15}$, 9: $p(dT)_{20}$.

different between the phospho- and non-phosphopeptide, especially at a low column temperature. As described in Section 3.2, the electrostatic interaction between the positively charged stationary phase and negatively charged analyte works strongly in such a condition. In conventional HPLC, the separation of these phosphopeptides is performed using an acetonitrile gradient with TFA or acetic acid as a counter-ion [22,23]. In contrast, the present method was achieved by merely an isocratic aqueous mobile phase without an organic solvent. Additionally, these results show that the IBD terpolymer has the specific ability to capture the phosphoric group, because both analytes have another ionic side chain in their structure, such as a carboxylic group (asparagic acid and glutamic acid), amino group (lysin) or guanidyl group (arginine). Further research to elucidate this interesting phenomenon is now progressing in our laboratory. We designed and synthesized a thermo-responsive polymer carrying an amino acid ester residue as a side chain. The poly (acryloyl-L-proline methyl ester)-modified stationary phase showed greater affinity for hydrophobic aromatic amino acids than the PNIPAAm-modified surface [24]. Under a high column temperature condition, the retention of phosphopeptide decreased promptly. Because the IBD terpolymer hides its cationic group and shows only the hydrophobic property above the LCST, the electronic density of the stationary phase decreased, followed by a static interaction between the analyte and the polymer was waken.

### *3.4. Environmental-responsive chromatographic separation of oligonucleotides*

Single-nucleotide polymorphisms (SNPs) are single base-pair variations in DNA that can provide valuable information on the genetic variation within a population. They can also help to identify the genes that cause certain human diseases [25]. In general, the syntheses of sequences of single-stranded DNA containing 20–30 nucleotides are used as primers in polymerase chain reactions. Though there are several techniques for separat-

ing oligonucleotides, these analytical methods are mainly based on the utilizing the molecular size (molecular length) or the charge in the analyte. In a previous study, we demonstrated that dual pH- and temperature-responsive chromatography is useful for 4-mer oligonucleotides [26]. In a present study, we tried to separate lager molecular-weight oligonucleotides that contained 2, 3, 4, 5, 6, 10, 15 and 20-mers. In the separation of the shorter oligonucleotides in the range 2- to 5-mer length, the column length on 150 mm was used. As retention of the longer oligonucleotides on IBD column was increased, the column length of 50 mm was used for the separation of the longer oligonucleotides (5-, 10-, 15- and 20-mer). Chromatograms of oligonucleotides are shown in Fig. 5. In an analytical phosphate buffer solution (pH 6.4 and 6.6), the phospho group of oligonucleotides is deprotonated and indicates the anionic property. Any molecular sizes of oligonucleotides show more retention on the stationary phase at low analytical temperatures compared with that in high temperatures. The electrostatic interactions between the oligonucleotides and the charged moiety on the stationary phase should cause increased retention below the LCST. By raising the column temperature above the LCST, charged groups may be immersed in the hydrophobic polymer chain, and the reduced electrostatic interaction causes decreased retention times. These phenomena were the same as phospho-amino acid or phosphopeptide analysis derived from an electrostatic interaction between the cationic stationary phase and the anionic phospho group in the oligonucleotides. Furthermore, there is a good resolution in the analyte at low column temperatures. The elution order depended on the molecular size of the analyte.

## 4. Conclusion

We developed a dual-temperature- and pH-responsive, so to speak, environmental-responsive chromatography based on NIPAAm, BMA and DMAPAAm, which work as an ion-exchange functional group. The IBD terpolymer changed from a hydrophilic phase to hydrophobic phase transition reversely, and from charged to non-charged in response to a change in the column temperature and the pH value of the mobile phase. In this work, we demonstrated an effective separation of the phosphoric group containing biomolecules, such as phospho-tyrosine, phosphopeptide and oligonucleotides by environmental-responsive chromatography. Temperature- and pH-dependent elution profiles were clearly observed. At a low analytical temperature, the electrostatic interaction plays an important role to retain the anionic analyte. The results indicate that the modulation of charged or hydrophobic interaction modes is achieved by only changes in the column temperature below or above the surface polymer phase transition temperature. Further research about the separation of biomacromolecules and pharmaceuticals utilizing environmental-responsive chromatography is now progress in our laboratory.

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