Mechanistic insights into the novel glucose-sensitive behavior of P(NIPAM-co-2-AAPBA)

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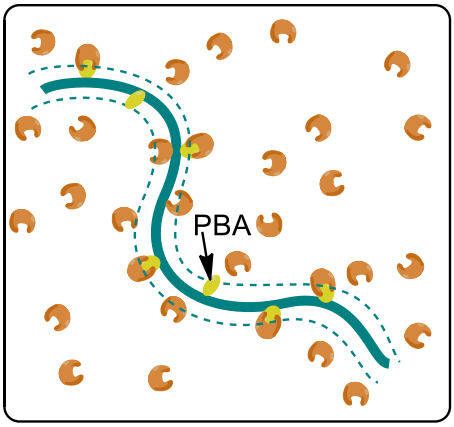
Table of contents graphic:



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Abstract: A glucose-sensitive polymer, poly(N-isopropylacrylamide-co-2-acrylamidophenylboronic acid) (P(NIPAM-co-2-AAPBA)), was synthesized by RAFT copolymerization. Addition of glucose results in reduced solubility and hence increased turbidity, rather than the normal increase in solubility (decreased turbidity) observed for other PBA-based glucose-sensitive polymers. The novel glucose-sensitive behavior is explained by a new mechanism, in which glucose acts as an additive and depresses the lower critical solution temperature (LCST) of the polymer, instead of increasing solubility by increasing the degree of ionization of the PBA groups. Experimental and theoretic analysis for the influence of glucose on the thermal behavior of P(NIPAM-co-2-AAPBA) reveals that glucose depresses the LCST of P(NIPAM-co-2-AAPBA) copolymers in a two-stage manner, a fast decrease at low glucose concentrations followed by a slow decrease at high glucose concentrations. For low glucose concentrations, the binding of glucose with PBA groups on the polymer chain increases the number of glucose molecules proximal to the polymer which influences the thermal behavior of the polymer, causing a rapid decrease in LCST. Importantly, the transition occurs at a glucose concentration equal to the reciprocal of the binding constant between PBA and glucose, thus providing a novel method to determine the binding constant. Other saccharides, including mannose, galactose and fructose, also depress~~es~~ the LCST of P(NIPAM-co-2-AAPBA) copolymer in the same way.

Keywords: Glucose-sensitive mechanism, phenylboronic acid, thermosensitive, binding constant, lower critical solution temperature, polymers

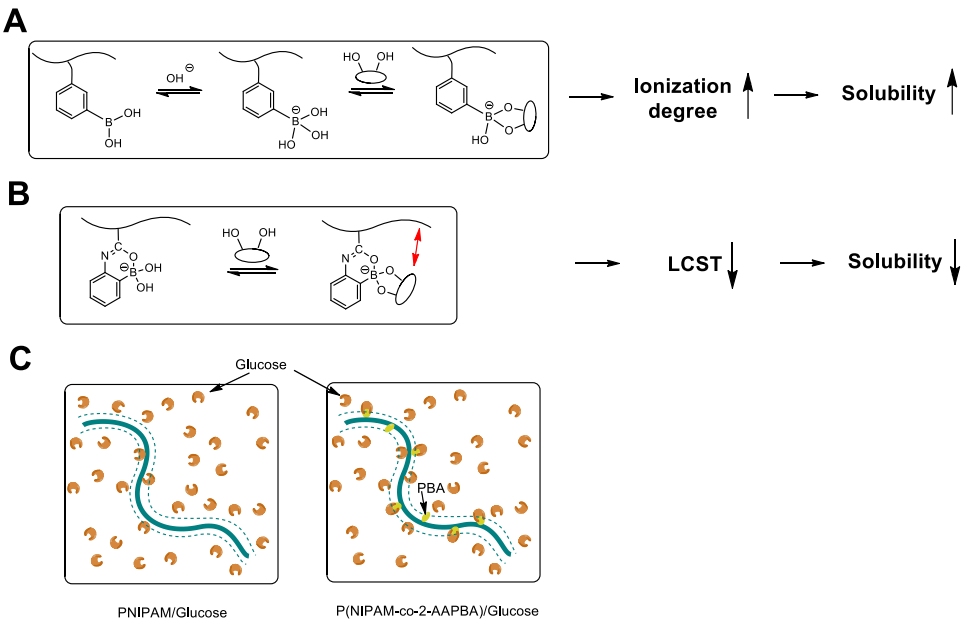
1 Introduction

Phenylboronic acid (PBA) has been extensively exploited as recognition element for diols.[1-3] Particularly numerous glucose-sensitive PBA-containing polymers were designed for the construction of glucose sensors[4-10] and self-regulated insulin release systems,[11-13] both of which are crucial in the treatment of diabetes. As a synthetic glucose reporter, PBA has the advantage of high reliability and long-term storability over other glucose reporters, such as glucose oxidase[14, 15] and concanavalin A.[16, 17] In addition, this reporter allows the design of various forms of glucose-sensitive materials, including linear polymers,[18-20] micelles,[21-23] thin films,[5, 24] microgels,[25-29] and bulky gels.[8, 9, 13]

PBA can be used as glucose reporter because it usually exists in aqueous solutions in equilibrium between the neutral, undissociated form and the negatively charged, dissociated form, and both forms react reversibly with 1,2- and 1,3-diols, including glucose, to form phenylboronate esters.[13] The esters from the undissociated form are unstable and highly susceptible to hydrolysis, in contrast, the ones from the dissociated form are thermodynamically more favourable. Therefore the binding of glucose with PBA will increase the fraction of charged form of PBA groups, making the PBA-containing polymers bear more negative charges and become more hydrophilic.[13] In the case of linear PBA-containing polymers, the increased ionization degree will lead to an increased solubility of the polymers.[18-20] (Scheme 1A) When they are engineered into other forms, the increased ionization degree will lead to the disassembly of micelles, [21-23] and the swelling of hydrogels[8, 13] and microgels.[25-29].

Here we report a new PBA-containing linear polymer, poly(N-isopropylacrylamide-co-2-acrylamidophenylboronic acid) (P(NIPAM-co-2-AAPBA)) with very different glucose-sensitive

behaviors. This polymer uses a 2-substituted PBA as glucose reporter instead of the commonly used 3- or 4-substituted PBAs. Unlike the previously reported linear PBA-containing polymers whose solubility increases upon addition of glucose,[18-20] addition of glucose reduces the solubility of this polymer. The abnormal behavior of this polymer certainly cannot be explained by the commonly-used glucose-sensitive mechanism, however it can be well explained by a new mechanism, i.e., glucose can depress the lower critical solution temperature (LCST) of the thermosensitive polymer and therefore addition of glucose is equivalent to heating the polymer solution.(Scheme 1B) In this work the influence of glucose on the thermal behavior of the polymer was studied in details. A two stage depression on LCST of the polymer was revealed. A significantly enhanced depression on LCST at the low glucose concentration range was found which is attributed to the binding of glucose with PBA groups, making glucose molecules close to the polymer chain and therefore influence the thermal behavior of the polymer more effectively. The mechanism not only explains the abnormal glucose sensitive behaviors of the polymer, but also provides a novel method to determine the apparent binding constant between PBA and saccharides.



Scheme 1. (A) Commonly used glucose-sensitive mechanism: glucose binding increases the ionization degree of PBA groups and thus increases the solubility of the polymer. (B) New glucose-sensitive mechanism for P(NIPAM-co-2-AAPBA): the binding of glucose with PBA group draws glucose molecules close to the polymer chains, allows it lower the LCST of the polymer efficiently, and thus decreases its solubility. (C) Comparison PNIPAM/glucose system and P(NIPAM-co-2-AAPBA)/glucose system.

2 Experimental details

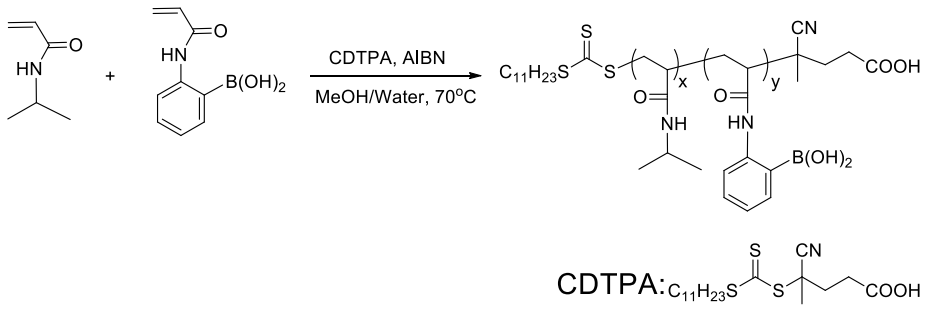
2.1 Materials

N-Isopropylacrylamide (NIPAM) was purchased from Tokyo Chemical Industry Co. 2-Aminophenylboronic acid (2-APBA) was purchased from Shanghai Bi De Pharmaceutical Technology Co., Ltd. 3-Aminophenylboronic acid (3-APBA) was purchased from Meryer (Shanghai) Chemical Technology Co., Ltd. Acryloyl chloride was purchased from Heowns Biochem LLC. 2, 2′-Azobis(2-methylpropionitrile) as purchased from Tianjin Chemical

Company. D-Fructose, D-xylose and D-glucose were purchased from local providers. NIPAM were purified by recrystallization from a hexane–acetone mixture(10:1 v/v) and dried under vacuum. Acryloyl chloride was distilled under reduced pressure. 2, 2′-Azobis(2-methylpropionitrile) was recrystallized from ethanol before use. Other reagents were used as received.

2-(Acrylamido)phenylboronic acid (2-AAPBA) and 3-(acrylamido)phenylboronic acid (3-AAPBA) were synthesized from acryloyl chloride and 2-APBA or 3-APBA according to ref[30]. The RAFT agent, 4-cyano-4-(dodecylsulfanylthiocarbonyl)sulfanyl pentanoic acid (CDTPA) was synthesized according to ref[31].

2.2 RAFT synthesis of poly(N-isopropylacrylamide-co-2-acrylamidophenylboronic acid) (P(NIPAM-co-2-AAPBA)) copolymer



Scheme 2. The RAFT synthesis of the copolymer P(NIPAM-co-2-AAPBA).

Random linear copolymer of NIPAM and 2-AAPBA, i.e., P(NIPAM-co-2-AAPBA), with a narrow molecular weight distribution was synthesized by reversible addition−fragmentation chain transfer (RAFT) polymerization of NIPAM and 2-AAPBA using CDTPA as RAFT agent and AIBN as initiator at 70 ℃ in 95% methanol/5% water (Scheme 2). P(NIPAM-co-2-AAPBA) copolymers with different molecular weights and 2-AAPBA contents were synthesized by altering the feeding ratio of monomers, RAFT agent and initiator. The synthesis and properties of the copolymers were collected in Table S1. The copolymers were named according to their feeding

ratio of 2-AAPBA. As an example, the synthesis of P(NIPAM-co-2-AAPBA)-15% was carried out as follows. In a 25 mL Schlenk flask 1.13 g of NIPAM (10 mmol), 0.34 g of 2-AAPBA (1.8 mmol), 12.0 mg of CDTPA (0.03 mmol) and 1.0 mg of AIBN (0.006 mmol) were dissolved in 5 mL of methanol/water. The solution was thoroughly degassed via three freeze-pump-thaw cycles under a N_2 atmosphere and then stirred at 70°C for 24 h to perform the polymerization. The polymerization was quenched by immersion in an ice-water bath and exposure to the air. The copolymer was precipitated in cold diethyl ether, collected by three precipitation/filtration cycles and then dried at 30 °C under vacuum. The yield was ~60%. The weight average molecular weight (Mw, Mn) and PDI were measured by GPC after pinacol protection to be 4.43×10^4 g/mol, 6.10×10^4 g/mol and 1.37, respectively. The content of PBA group in the copolymer was determined to be 14.5 mol % by ^{1}H NMR spectrum. RAFT synthesis of P(NIPAM-co-3-AAPBA), the random linear copolymer of NIPAM and 3-AAPBA, was carried out in the same way.

2.3 Characterizations

^{1}H NMR analysis was performed on a Bruker Avance III 400 MHz NMR spectrometer using D_2O as the solvent. The apparent molecular weights and polydispersities of the polymers were determined by GPC characterizations after pinacol protection. The GPC equipped with a Hitachi L-2130 HPLC pump, three Shodex columns (5000–5K, 400–0.5K, and 5–0.15K molecular Ranges), and a Hitachi L-2490 refractive index detector. In GPC measurements, DMF containing 10 mM LiBr was used as the mobile phase, and the flow rate was 1.0 mL/min. Monodisperse poly(methyl methacrylate)s with Mw of 6.47×10^3, 1.92×10^4, 7.72×10^4, 2.12×10^5 and 5.73×10^5 were used for calibration. The lower critical solution temperature (LCST) of the copolymers was determined using turbidity analysis at 550 nm on a TU 1810PC UV−vis spectrophotometer equipped with a thermo-regulator (±0.1 °C) at a heating rate of 1°C/ 5 min, in which the LCST

was determined at starting change in the absorbance. Dynamic light scattering was performed on a Brookhaven 90Plus laser particle size analyzer. All measurements were carried out at a scattering angle of 90°. The sample temperature was controlled using a built-in Peltier temperature controller. Zeta potential was measured on a Zetasizer Nano ZS90 (Malvern, Southborough, MA).

3 Results and discussion

3.1 Synthesis of P(NIPAM-co-2-AAPBA) copolymers

The new glucose-sensitive polymer, P(NIPAM-co-2-AAPBA), was synthesized via RAFT copolymerization of NIPAM and 2-AAPBA employing a trithiocarbonate, CDTPA, as the RAFT agent and AIBN as initiator. By varying the [NIPAM]/[2-AAPBA] ratio, a series of copolymers with different PBA contents were synthesized. The composition and molecular weight of the polymers were characterized by ^{1}H NMR, GPC and FTIR (Figure S1). The results were summarized in Table S1. For comparison, a copolymer of NIPAM and 3-AAPBA, P(NIPAM-co-3-AAPBA), was also synthesized via RAFT copolymerization.(Table S1) The copolymers were labelled according to their PBA contents. For example, P(NIPAM-co-2-AAPBA)-15% means its PBA content is 15 mol%.

3.2 Thermo- and glucose-sensitive behaviors

Like PNIPMA, the P(NIPAM-co-2-AAPBA) copolymers are thermosensitive. As an example, Figure 1 shows that the turbidity of a solution of P(NIPAM-co-2-AAPBA)-15%, represented as the absorbance at 550 nm, is low at a low temperature, suggesting water is a good solvent for the polymer under the conditions. A sharp increase in turbidity was observed when the

solution was heated above a critical temperature, suggesting water becomes a poor solvent for the polymer above this temperature. Therefore the polymer chains aggregate and the turbidity of the solution increases. Similar phenomena were observed for PNIPAM and P(NIPAM-co-3-AAPBA).(Figure 1) The lower critical solution temperature (LCST), i.e., the critical temperature at which the transition starts, was determined to be ~32℃, ~33 ℃, and ~20 ℃ for PNIPAM, P(NIPAM-co-2-AAPBA)-15%, and P(NIPAM-co-3-AAPBA)-15%, respectively. One can see the introduction of 3-subtituted PBA groups significantly shifts the LCST to lower temperature, because a large fraction of the group exists as the neutral, undissociated form under the experimental conditions, which is highly hydrophobic. In contrast, as Lowe et al[30] demonstrated, the 2-subtituted PBA groups exist dominantly in a negatively-charged, tetrahedral structure, because this structure can be stabilized by the intramolecular B–O coordinated interaction.(Scheme 1B) Therefore the LCST of P(NIPAM-co-2-AAPBA)-15% is close to that of PNIPAM.

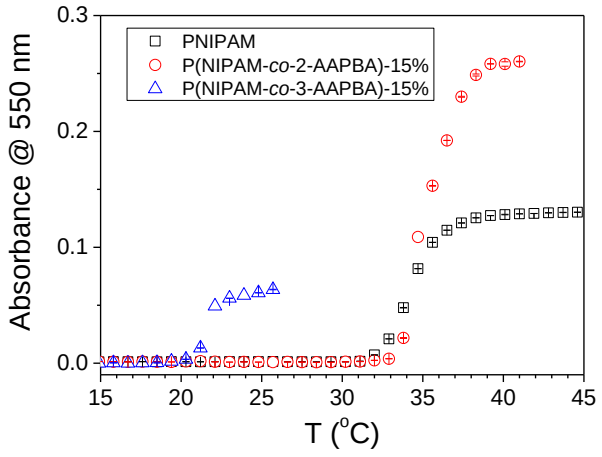


Figure 1. Change in turbidity, represented as absorbance at 550 nm, of PNIPAM, P(NIPAM-co-2-AAPBA)-15% and P(NIPAM-co-3-AAPBA)-15% solution upon heating at a rate of 1 ℃/5 min.

The concentrations of the polymers were all 0.1 mg/mL. PNIPAM and P(NIPAM-co-2-AAPBA)-15% was dissolved in 20 mM pH7.4 phosphate buffer. P(NIPAM-co-3-AAPBA)-15% was dissolved in 20 mM pH8.5 phosphate buffer.

More importantly, the P(NIPAM-co-2-AAPBA) polymers are also glucose-sensitive. As an example, when glucose was added into the solution of P(NIPAM-co-2-AAPBA)-15% which was incubated at 31℃, a significant increase in turbidity was observed.(Figure 2A) The change can even be observed directly by visual inspection. As shown in Figure 2C, the solution is originally transparent, but becomes slightly turbid after the introduction of glucose. DLS study further reveals that the polymer chains dissolve well in water in the absence of glucose (particle size ~20 nm), but aggregate into larger particles with a size ~700 nm when glucose is added.(Figure 2E)

It is noteworthy that the glucose-sensitive behaviors of P(NIPAM-co-2-AAPBA) are right the opposite of the previously synthesized PBA-containing polymers.[18-23] As an example, Figure 2B, 2D and 2F show the glucose-sensitive behavior of P(NIPAM-co-3-AAPBA)-15%. Addition of glucose increases its solubility in water, resulting in a reduced turbidity (Figure 2B and 2D) and reduced aggregate size (Figure 2F). This kind of glucose-sensitive behaviors has been widely observed previously[18-23] and can be well-explained by an increased ionization degree of the PBA groups as a result of glucose binding.(Scheme 1A) Apparently the abnormal behaviors of P(NIPAM-co-2-AAPBA) cannot be explained by the same mechanism. Instead, we suggest that the unique behaviors could be explained by the fact that the P(NIPAM-co-2-AAPBA) polymer is thermosensitive, and its LCST can be lowered by adding glucose. Therefore adding glucose is equivalent to elevating temperature, and thus cause the aggregation of the polymer chains and hence increased turbidity.

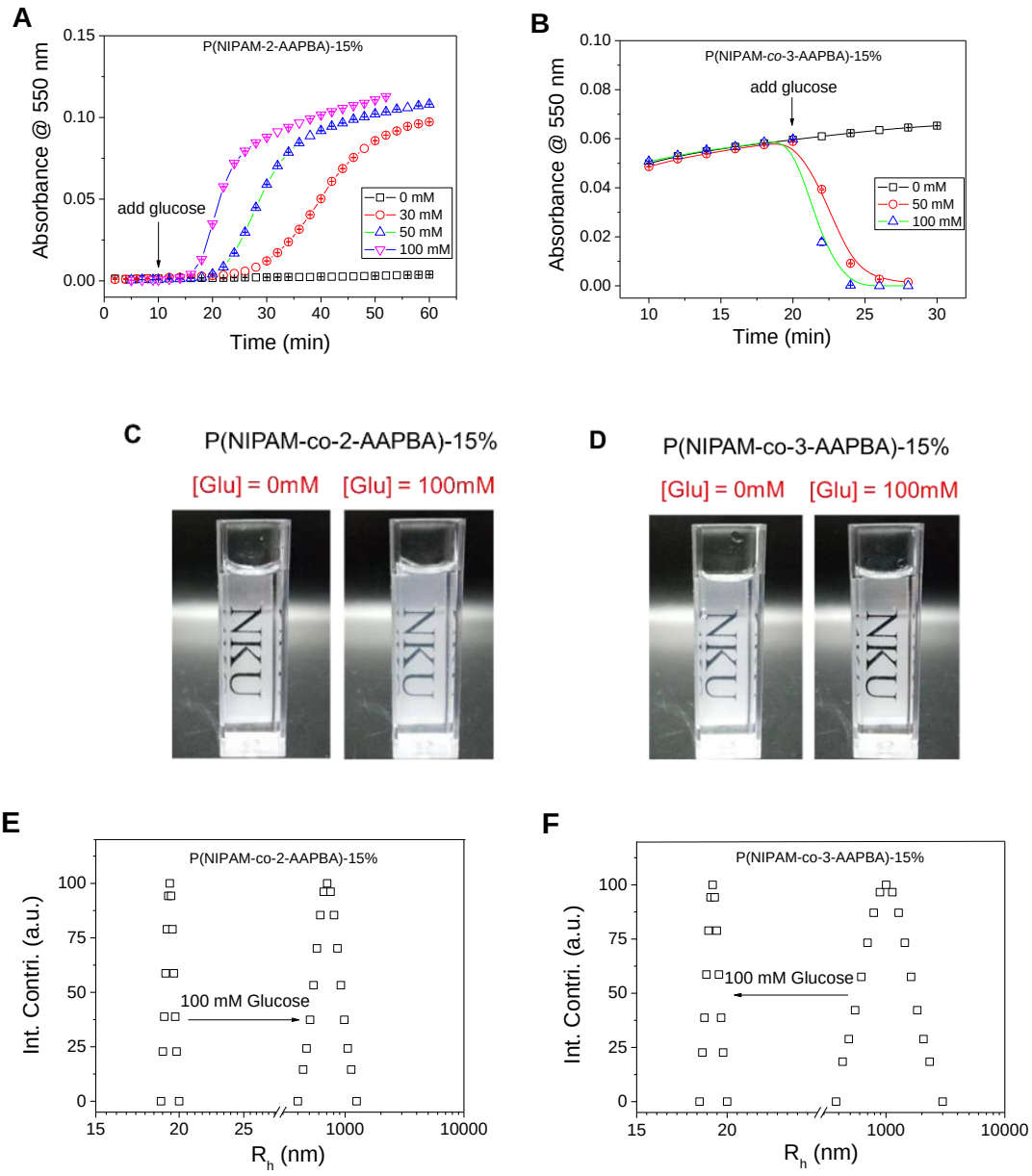


Figure 2. (A, B) Change in turbidity, represented as absorbance at 550 nm, of a P(NIPAM-co-2-AAPBA)-15% solution (A) and a P(NIPAM-co-3-AAPBA)-15% solution (B) upon addition of various concentrations of glucose. Concentration of the solutions: 0.1 mg/mL. P(NIPAM-co-2-AAPBA)-15% was in 20 mM pH 7.4 phosphate buffer at 31°C. P(NIPAM-co-3-AAPBA)-15% was in 20 mM pH 8.5 phosphate buffer at 24°C. (C, D) Appearance of a P(NIPAM-co-2-AAPBA)-

15% solution (C) and a P(NIPAM-co-3-AAPBA)-15% solution (D) before and after glucose addition. Concentration of the solutions: 0.1 mg/mL. P(NIPAM-co-2-AAPBA)-15% was in 20 mM pH 7.4 phosphate buffer at 33℃. P(NIPAM-co-3-AAPBA)-15% was in 20 mM pH 8.5 phosphate buffer at 25℃. (E, F) Aggregate size in a P(NIPAM-co-2-AAPBA)-15% solution (E) and a P(NIPAM-co-3-AAPBA)-15% solution (F) before and after glucose addition. P(NIPAM-co-2-AAPBA)-15% was in 20 mM pH 7.4 phosphate buffer at 30℃. P(NIPAM-co-3-AAPBA)-15% was in 20 mM pH 8.5 phosphate buffer at 22℃. Concentration of the solutions: 0.1 mg/mL.

3.3 Glucose-induced LCST depression

The thermosensitivity of P(NIPAM-co-2-AAPBA) has been demonstrated in Figure 1. To test if glucose can lower the LCST of P(NIPAM-co-2-AAPBA), various amount of glucose was added into P(NIPAM-co-2-AAPBA)-15% solution. As Figure 3A shows, addition of glucose shifts the LCST of P(NIPAM-co-2-AAPBA)-15% gradually to a lower temperature. When [Glu] increases from 0 to 100 mM, LCST drops from ~33 to ~29℃. When further increasing [Glu] to 1000 mM, an even lower LCST (~20℃) was observed.

Glucose-induced change in LCST of PBA-functionalized polymers was previously reported. However usually an increased LCST was observed.[18, 19] For example Kataoka and Miyazaki synthesized a copolymer of N,N-dimethylacrylamide and 3-AAPBA and found its LCST increases by ~15℃ when 16.7 g/L glucose is added.[18] Later the same group synthesized the copolymer of NIPAM and a 4-substituted PBA, 4-(1,6-dioxo-2,5-diaza-7-oxamyl) phenylboronic acid. Again a higher LCST was observed in the presence of glucose.[19] The increased LCST was

well-explained by an increased ionization degree of the PBA groups as a result of glucose binding (Scheme 1A).[18, 19]

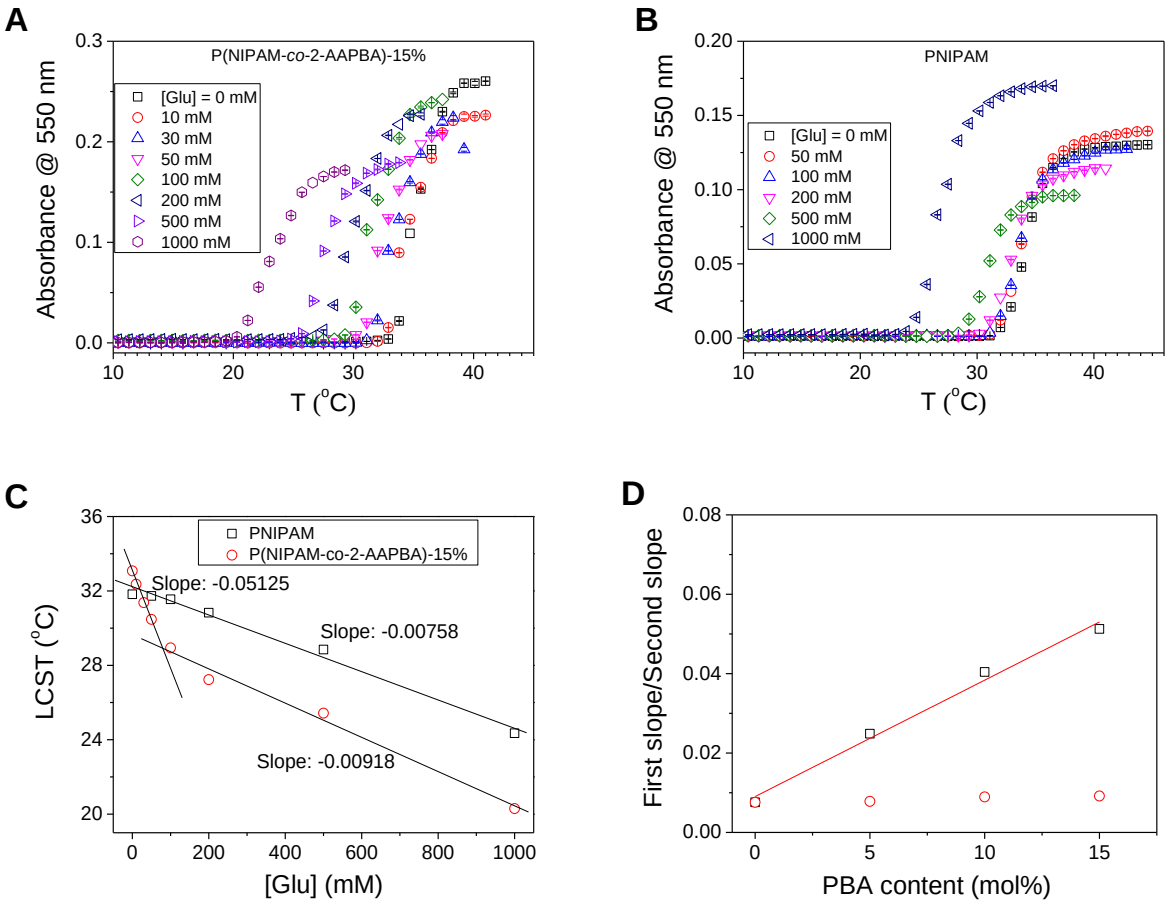


Figure 3. (A, B) Temperature-induced phase transition of the P(NIPAM-co-2-AAPBA)-15% (A) and PNIPAM (B) in the presence of various concentrations of glucose. The polymer concentration is 0.1 mg/mL. Measured in 20 mM pH 7.4 phosphate buffer. (C) LCST of P(NIPAM-co-2-AAPBA)-15% and PNIPAM as a function of glucose concentration. (D) First (red circle) and second slope (black square) of the LCST-$[Glu]_0$ plot of P(NIPAM-co-2-AAPBA) polymers as a function of PBA content in the polymer.

The lowered LCST of P(NIPAM-co-2-AAPBA) in the presence of glucose could not be explained by a change in the ionization degree of PBA groups. As mentioned above, the PBA groups in P(NIPAM-co-2-AAPBA) exist dominantly in a negatively-charged, tetrahedral structure.[30] Therefore addition of glucose will not significantly change the ionization degree.(Figure S2) In addition, glucose-binding can only increase ionization degree, which will increase LCST, not decrease LCST. The phenomenon could not be explained by the change in the hydrophilicity/hydrophobicity of the polymer either. Since glucose is hydrophilic, binding with glucose will lead to an increased hydrophilicity of the polymer, and therefore a higher LCST, not a lower LCST as observed here.

A reasonable explanation is that glucose lowers the LCST of P(NIPAM-co-2-AAPBA) as an additive. It is well-known that a lot of additives, including salt,[32, 33] saccharides,[34-37] and surfactants, can change the LCST of thermosensitive polymers. Particularly previous studies demonstrated that addition of glucose can lower the LCST of a lot of linear thermosensitive polymers, for example, Pluronics,[34] poly(organophosphazenes),[35] and PNIPAM.[34, 36, 37] Our own results also confirm the ability of glucose to lower LCST of PNIPAM. As shown in Figure 3B, with increasing glucose concentration in the solution, the LCST of PNIPAM shifts gradually to lower temperature.[34]

Although glucose can lower the LCST of both PNIPAM and P(NIPAM-co-2-AAPBA)-15%, a larger change in LCST was observed for the latter.(Figure 3C) For example, at [Glu] = 1000 mM, the LCST of P(NIPAM-co-2-AAPBA)-15% was lowered by ~12.8℃, but only ~7.5℃ for PNIPAM. The difference in the low glucose concentration range is even remarkable. For example, addition of 100 mM glucose only slightly decreases the LCST of PNIPAM by ~0.3℃, but significantly decreases the LCST of P(NIPAM-co-2-AAPBA)-15% by ~4.1 ℃. The larger

LCST depression of P(NIPAM-co-2-AAPBA)-15% can be attributed to the binding of glucose with the PBA groups on the polymer chain, which shortens the distance between the additive and the thermosensitive polymer and therefore amplifies its ability to influence the thermal behavior of the polymer.(Scheme 1C)[38-40]

Previous studies revealed that many additives such as glucose and NaCl do not interact directly with the polymer, instead they influence the thermal behavior of PNIPAM by affecting the solvent quality.[41] According to a generally accepted theory about the LCST behaviors, PNIPAM dissolves in water because water molecules form "ice-like structure" around the hydrophobic isopropyl groups and lead to their hydrophobic hydration. However, heating breaks down the "ice-like structure", exposes the hydrophobic isopropyl groups and thus leads to the collapse and aggregation of the polymer chains.[42, 43] Additives can reduce the structured water around hydrophobic isopropyl groups, therefore in their presence, heating-induced phase transition will occur at a lower the temperature.[34, 37, 43] The mechanism implies that, only when the additive molecules are in close proximity to the polymer chains can they influence the thermal behavior of PNIPAM.[38-40]

In the case of PNIPAM solution, when glucose is added, the glucose molecules will be distributed homogenously in the solution. Only a very small fraction of the additive molecules will be close enough to the PNIPAM chain to influence its thermal behavior.(Scheme 1C) The number of glucose molecules close enough to the polymer chain increases linearly with increasing glucose concentration in the solution and can be written as $e \cdot [Glu]_0$, and hence the LCST of the polymer at a glucose concentration [Glu] can be written as

$$LCST = LCST_0 + a_{Glu}[Glu]_0 \quad (1)$$

where $LCST_0$ is the LCST in the absence of glucose, $[Glu]_0$ is the total concentration of glucose in the solution, and a and e are constants.

In the case of P(NIPAM-co-2-AAPBA), some glucose molecules will be close to the polymer chain for the same season in the case of PNIPAM. In addition, some other glucose molecules will be drawn close to the polymer chain because they form phenylboronate ester with the PBA groups on the polymer chain.(Scheme 1C) Therefore the total number of glucose molecules close enough to influence LCST will be $e\cdot[Glu]_0+\theta\cdot\chi_{PBA}$, where χ_{PBA} is the molar ratio of 2-AAPBA unit on the polymer chain, and θ the fraction of PBA groups bound with glucose.

The reaction equilibrium between glucose and PBA group can be written as follows (because of a low $[PBA]_0$ in the system, [Glu] can be replaced by $[Glu]_0$):

$$K_e = \frac{[Glu-PBA]}{[Glu][PBA]} \approx \frac{[PBA]_0 - [PBA]}{[Glu]_0[PBA]}$$

From the relationship, θ can be calculated to be:

$$\theta = \frac{[Glu-PBA]}{[PBA]_0} = \frac{K_e[Glu]_0}{1+K_e[Glu]_0}$$

Therefore the LCST of the polymer at a glucose concentration $[Glu]_0$ will be:

$$\begin{aligned} LCST &= LCST_0 + a(e[Glu]_0 + \theta\chi_{PBA}) \\ &= LCST_0 + a[Glu]_0(e + \frac{K_e\chi_{PBA}}{1+K_e[Glu]_0}) \end{aligned} \quad (2)$$

When $[Glu]_0$ is low, $K_e[Glu]_0 << 1$, therefore (2) can be rewritten to be:

$$LCST = LCST_0 + a[Glu]_0(e + K_e\chi_{PBA}) \quad (3)$$

Since e, K_e and χ_{PBA} are all constant, Eq (3) indicates a linear relationship between LCST and $[Glu]_0$ at low $[Glu]_0$, and the slope is

$$m = \alpha g(e + K_e \chi_{PBA})$$

When $[Glu]_0$ is high, θ will be close to 1. Therefore (2) can be rewritten to be:

$$LCST = LCST_0 + \alpha g e g[Glu]_0 + \alpha g \chi_{PBA} \quad (4)$$

Eq (4) indicates at high glucose concentration range, LCST still changes linearly with $[Glu]_0$, and the slope, n, is

$$n = \alpha g e$$

The theoretical analysis can explain well the experimental results collected in Figure 3C. From Eq. (1), one can predict that the LCST of PNIPAM will decrease linearly with increasing $[Glu]_0$. From Eq. (3) and (4) the LCST of P(NIPAM-co-2-AAPBA) will first decrease at a fast rate with increasing $[Glu]_0$ and then decrease with increasing $[Glu]_0$ at a slow rate. Linear relationship between LCST and $[Glu]_0$ can be found at both stages. In addition, the slope of the second stage of P(NIPAM-co-2-AAPBA) is equal to that of PNIPAM. All these predictions were well confirmed by the results collected in Figure 3C.

In addition, from Eq. (3) it can be predicted that the first slope of the LCST-$[Glu]_0$ plot of P(NIPAM-co-2-AAPBA) is proportional to χ_{PBA}, i.e., the molar ratio of 2-AAPBA unit on the polymer chain. To test the prediction, the influence of glucose on the thermal behavior of other P(NIPAM-co-2-AAPBA) polymers, i.e., P(NIPAM-co-2-AAPBA)-5%, P(NIPAM-co-2-AAPBA)-10% was also studied. The results were collected in Figure 4. As expected, a two-stage

behavior was observed from both polymers. The second slopes are also close to the slope of PNIPAM. More importantly, the first slope increases linearly with increasing PBA content in the polymer.(Figure 3D) These results confirm again the reliability of the proposed mechanism.

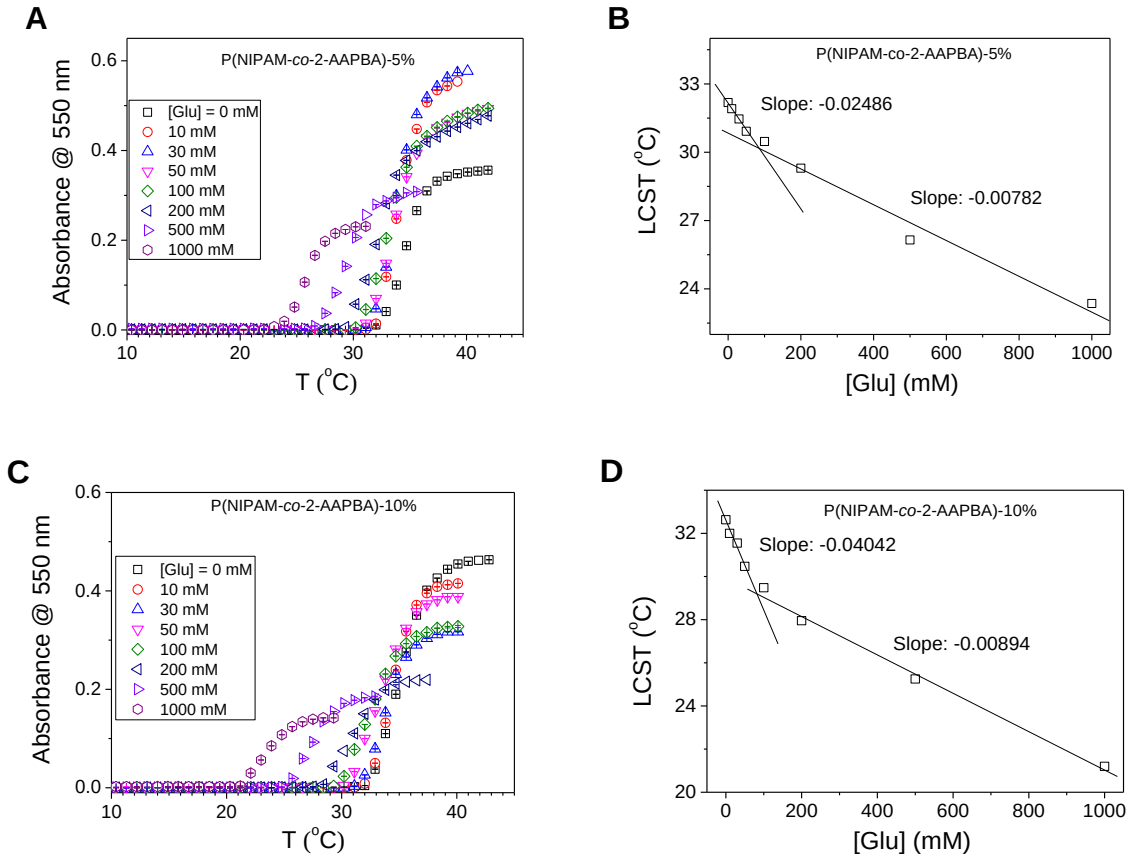


Figure 4. (A, C) Temperature-induced phase transition of the P(NIPAM-co-2-AAPBA)-5% (A), P(NIPAM-co-2-AAPBA)-10% (C) in the presence of various concentrations of glucose. The polymer concentration is 0.1 mg/mL. Measured in 20 mM pH 7.4 phosphate buffer. (B, D) LCST of P(NIPAM-co-2-AAPBA)-5% (B), P(NIPAM-co-2-AAPBA)-10% (D) as a function of glucose concentration.

From the LCST-$[Glu]_0$ plots, one can further determine the binding constant between PBA group and glucose, K_e, a very important constant for PBA as glucose reporter. In the LCST-$[Glu]_0$

plots, the two fitted lines crossover at a certain glucose concentration, $[Glu]_c$. Combined Eq. (3) and (4), one will get the following relationship:

$$[Glu]_c = \frac{1}{K_e} \tag{5}$$

Since K_e is a constant, this relationship indicates that the two fitted lines will crossover at the same glucose concentration for P(NIPAM-co-2-AAPBA) polymers with different PBA contents. Indeed, for all 3 P(NIPAM-co-2-AAPBA) polymers, the two fitted lines all crossover at a glucose concentration ~80 mM.(Figure 3C, and Figure 4B, 4D) In turn, from $[Glu]_c$, one can calculate the apparent binding equilibrium constant between glucose and PBA, K_e:

$$K_e = \frac{1}{[Glu]_c} \tag{6}$$

In this way, K_e was determined to be 12.5 M^{-1}.

3.4 LCST depression by other saccharides and determination of binding constant

Besides glucose, the influences of some other saccharides, including mannose, galactose and fructose, on the thermal behavior of P(NIPAM-co-2-AAPBA) polymers were also studied. The results were collected in Figure S3, S4, and S5. One can see the influence of these saccharides is very similar to that of glucose. The LCST of PNIPAM decreases linearly with increasing saccharide concentration. For P(NIPAM-co-2-AAPBA) polymers, however, a two-stage behavior was observed. The LCST of the copolymers first decreases at a fast rate with increasing saccharide concentration, followed by a decrease with a slow rate. In addition, the slope of the first stage

increases linearly with increasing PBA content in the copolymer, and the slopes of the second stage are close to the slope of PNIPAM. (Figure S3I, Figure S4I, Figure S5I) Like glucose, these saccharides can also act as additive to lower the LCST of PNIPAM.[34, 36, 37] In addition, they can bind with PBA groups and thus be drawn close to the copolymer chain. Therefore their ability to depress the LCST is significantly amplified.

The apparent binding constants between PBA and the saccharides were then determined. Like glucose, for each saccharide studied here, on the LCST-$[Saccharide]_0$ plots, the two fitted lines crossover at almost the same saccharide concentration, which is ~72.2 mM for mannose, ~59.6 mM for galactose, and ~12.5 mM for fructose. From these values, the corresponding K_e values were calculated. (Table 2) The K_e value decreases in the order fructose > galactose > mannose > glucose. It is interesting that the same order was previously reported, [10, 44] in spite that the values were determined using different methods, different PBAs, and under different conditions. However we do not think the K_e values reported here have high accuracy. Like other methods, errors may be introduced in every steps of the method, particularly when determining the LCSTs of the polymer. However we believe the new method developed here is valuable because it allows the direct determination of the important constant in polymer with minimal instrument requirement.

Table 2. Apparent binding constant between PBA and saccharides (M^{-1}).

Saccharide	This work	Ref[44]	Ref [10]
D-glucose	12.5 ± 0.3	4.6	87.18 ±1.80
D-mannose	14.2 ± 0.4	13	92.48 ±3.79

D-galactose	16.8 ± 0.3	15	221.4 ±8.05
D-fructose	80.3 ± 4.0	160	1381.7 ±41.80

4 Conclusions

In conclusion, a new glucose-sensitive polymer, P(NIPAM-co-2-AAPBA), was synthesized by RAFT copolymerization. Different from PBA-based glucose-sensitive polymers synthesized previously, addition of glucose decreases the solubility of P(NIPAM-co-2-AAPBA) in water and causes an increased turbidity. The abnormal glucose-sensitive behavior cannot be explained by the commonly used glucose-sensitive mechanism. Instead it is explained by a new mechanism, in which glucose acts as additive and depresses the LCST of the polymer, instead of changing the ionization degree of PBA groups.

To support the new mechanism, the influence of glucose on the thermal behavior of P(NIPAM-co-2-AAPBA) copolymers was studied both experimentally and theoretically. Addition of glucose will lower the LCST of P(NIPAM-co-2-AAPBA) copolymers in a two-stage manner. At both stages, LCST decreases linearly with increasing glucose concentration. The fast depression on LCST at the low glucose concentration range is attributed to the binding of glucose with PBA groups on the polymer chain, leading to an increased amount of glucose molecules close enough to influence the thermal behavior of the polymer. The slope of the first stage increases linearly with increasing PBA content in the copolymer. The transition occurs at the same glucose concentration, which equals to the reciprocal of the binding constant between PBA and glucose. Other saccharides, including mannose, galactose and fructose, also depresses the LCST of P(NIPAM-co-2-AAPBA) copolymer in the same way. These observations not only explain the

abnormal glucose sensitive behaviors of P(NIPAM-co-2-AAPBA) copolymer, but also provide a novel method to determine the apparent binding constant between PBA and saccharides.

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Conflict of interest The authors declare that they have no conflict of interest.

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