Saccharide Effect on the Lower Critical Solution Temperature of Thermosensitive Polymers

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ABSTRACT: Effects of low molecular weight saccharides in the presence and absence of salts on the lower critical solution temperature (LCST) of typical thermosensitive polymers such as Pluronics, poly(*N*-isopropylacrylamide), and *N*-isopropylacrylamide copolymers with ionizable groups were examined. Both a monosaccharide, glucose, and a disaccharide, maltose, were used in this study. The LCSTs of the polymers were significantly influenced by the polymer concentration. The addition of saccharides into aqueous polymer solutions decreased the LCSTs of all polymers. As the polymer concentration increased, saccharide effects became more pronounced. A monosaccharide, glucose, was more effective than a disaccharide, maltose, in lowering the LCST, especially in Pluronic solutions.

Introduction

Polymers and hydrogels which show phase separation in water when the temperature is raised above a critical point, termed a lower critical solution temperature (LCST), have been investigated for applications such as drug delivery systems, enzyme and cell immobilization, solute separation, immunodiagnostic assays, and purification and partitioning in biotechnology.[1–5]

These polymers have been considered as a simple model to study the behaviors of biopolymers, particularly proteins, in an aqueous system, since the effects of additives (e.g., inorganic salts) on the LCSTs of the polymers are closely related to the salt effects on protein conformational transitions.[6]

It has been widely accepted that the relative effectiveness of various salts in stabilizing or destabilizing biopolymers and synthetic polymers in aqueous solution follows the classical Hofmeister series.[7,8] In a simple experimental way their effectiveness can be compared by determining the change in transition temperatures of the macromolecules. Depending on the characteristics of the salt ions, salting-in or biopolymer destabilizing salts decrease a folding–unfolding or order–disorder transition temperature, called a melting temperature (T_m) (stabilize unfolded, denatured structure), while salting-out or stabilizing salts increase it (stabilize folded, native structure). On the other hand, for synthetic polymers showing LCST behaviors, salting-out salts tend to lower the LCST (stabilize insoluble phase), while salting-in salts increase the LCST (stabilize soluble phase). The ranking of salts in order of effectiveness was found to be approximately equal in both biopolymers and synthetic polymers.[7]

For biochemical separations of macromolecules and cells, the most commonly used aqueous polymer two-phase systems are composed of poly(ethylene oxide) (PEO) and dextran, a polyglucose.[9] The physical chemical mechanistic basis for phase separation of the PEO/dextran system is the polymer incompatibility or simple coacervation, resulting from repulsive interaction and the small entropy of mixing per monomer unit in polymers.[10] Also, sucrose, a disaccharide of glucose and fructose, has been known to stabilize proteins against thermal denaturation.[11] As a result, sucrose stabilizes the native, folded conformation of proteins and therefore increases their T_m. The effects of sucrose on proteins are closely related to those on synthetic polymers, leading to a decrease in the LCST of poly(vinyl methyl ether) in the presence of sucrose.[12] One simple experimental approach to the mechanistic understanding of polysaccharide-induced phase separation and saccharide-induced thermal stabilization is to study how the LCST of thermosensitive synthetic polymers having relatively simple structures is influenced by the presence of various glucose mono- and dimers.

Also, in the pharmaceutical area, significant efforts and advances on the development of glucose responsive polymeric systems have been made. For a better treatment of diabetes, it is important to construct a modulated system where insulin is released in a reversible and pulsitile manner responding to plasma glucose concentration, therefore being passive under a normal condition but being active by a change in glucose level. Several approaches for the development of a self-regulating insulin delivery system have been investigated. One approach is to utilize competitive binding of Con A with glucose and glycosylated insulin.[13] Another approach is based on pH changes by the formation of gluconic acid resulting from the glucose–glucose oxidase reaction.[14] When self-regulating systems are combined with systems triggered by changes in the environment such as temperature, pH, or their combination, well-controlled and improved insulin therapies can be achievable. We have reported that linear pH and temperature sensitive polymers used in the present study had a potential as insulin carriers in terms of efficient aqueous phase loading and preservation of the conformation and bioactivity of insulin.[15] Therefore, the study on the effect of glucose on the LCST of thermosensitive polymers was necessary to control the polymer swelling responding to the external glucose concentration and thereby achieve an optimum release rate of insulin.

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The LCST phenomenon of water soluble polymers arises from a balance of hydrophilicity and hydrophobicity of the polymers.[16,17] This balance could be obtained either from the repeat units of a homopolymer, from random copolymers of monomeric units with different degrees of hydrophilicity, or from block copolymers of hydrophilic and hydrophobic blocks. The LCST can also be adjusted by adding a third component (additive) such as salts,[18-20] surfactants,[21] and nonelectrolytes.[12]

An elevated temperature weakens hydrogen bonding, which may disturb hydrophobic hydration (iceberg-like water structure around hydrophobic moieties of polymers) and therefore enhance hydrophobic interaction. The salt and nonelectrolyte effects on the LCST have been explained in terms of changes in the water structure in bulk or the polymer–water interface[12,17-19] or changes in the polymer conformation.[22-24] Most of the ionic surfactants as additives have been known to have different effects. They adsorb onto the hydrophobic segments of the polymers and disguise the polymer as dangled with a hydrophilic portion of the surfactant, resulting in the elevation of the LCST.[21]

In the present study, saccharide effects in the presence and absence of inorganic salts on the LCST behaviors of typical thermosensitive polymers of which the LCSTs are around ambient temperature were examined. The tested polymers include ABA type block copolymers of poly(ethylene oxide) and poly(propylene oxide) (Pluronics), poly(*N*-isopropylacrylamide), and *N*-isopropylacrylamide copolymers with ionizable groups. The ionizable group in the copolymer increases the LCST due to its strong hydrophilicity rather than charge effect.[17] The incorporation of small amounts of ionizable repeat units will vary the LCST. This may allow us to test how the ionizable group in the polymer and added saccharides are mutually compromised.

Materials and Methods

Materials. Pluronic L31, L62, F68LF, and L92 were purchased from BASF Wyandotte Corp. (Parsippany, NJ) and used as received. *N*-Isopropylacrylamide (NiPAAm), obtained from Eastman Kodak Co. (Rochester, NY), was recrystallized from hexane. Acrylic acid (AA), purchased from Aldrich Chemical Co. (Milwaukee, WI), was purified by vacuum distillation at 39 °C/10 mmHg. Butyl methacrylate (BMA), obtained from Polysciences Inc. (Warrington, PA), was purified by vacuum distillation at 57 °C/17 mmHg. 2, 2'-Azobis(isobutyronitrile) (AIBN), purchased from Eastman Kodak Co. (Rochester, NY), was recrystallized from methanol. Butyl peroxyoctanoate (BPO) was purchased from Polysciences Inc. (Warrington, PA). Glucose and maltose were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Polymer Synthesis. The synthesis of linear terpolymers composed of NiPAAm, BMA, and AA with varying feed ratios (NiPAAm/BMA/AA mole ratio = 90/10/0, 89/10/1, 88/10/2, 87/10/3, or 85/10/5) was carried out in dioxane (300 mL) with AIBN as a free radical initiator (7.41×10^{-3} mol of AIBN/mol of monomer). Dried N_2 gas was bubbled through the solution for 20 min to remove dissolved oxygen. The solution was polymerized overnight at 60 °C under a N_2 atmosphere. The synthesized terpolymers were recovered by precipitation in *n*-hexane (3 L) and purified by dissolving in tetrahydrofuran (THF, 100 mL) and reprecipitation in diethyl ether (2 L). The polymers were filtered and dried in vacuum overnight.

Linear poly(NiPAAm) was synthesized in dioxane with BPO as a free radical initiator (1.13×10^{-2} mol of BPO/mol of monomer). The solution was polymerized at 80 °C for 3 h, and the precipitation and purification procedure was the same as for linear terpolymers.

The molecular weight and molecular weight distribution were determined by gel permeation chromatography using polystyrene standards. The number-average molecular weights were in the range 20 000–26 000 and the polydispersity was around 2.5.

Table 1. Effect of Pluronic Concentration on the LCST of Pluronic Solution in Distilled Water

pluronic	average MW[a]	PEO content (% wt)[a]	LCST (°C) 10% w/v	LCST (°C) 1% w/v
L31	1100	10	31	45
L62	2500	20	23, 45[b]	33
F68LF	7700	80	22	36
L92	3650	20	16	30

[a] From BASF technical bulletin. [b] Double LCSTs.

Table 2. LCSTs of Pluronic L62 as a Function of Concentration in Distilled Water

L62 concentration (% w/v)	LCST 1 (°C)	LCST 2 (°C)
0.5	35	
1.0	32	
2.5	28	
5.0	26	40
7.5	24	43
10.0	23	45

LCST Determination. The LCST was determined by the cloud point measurement. The polymer solutions were prepared in either distilled water (dw) or phosphate buffered saline (PBS; 0.013 M $NaH_2PO_4 \cdot H_2O$, 0.053 M Na_2HPO_4, and 0.075 M NaCl), pH 7.4. To these solutions, various amounts of saccharides (glucose as a monosaccharide and maltose as a disaccharide) were added. The temperature of the solutions was raised at a constant rate (0.4 °C/min) and the absorbance at 450 nm was measured using a Perkin-Elmer Lamda 7 UV/vis spectrophotometer. The LCST was defined as the temperature at the inflection point in the normalized absorbance versus temperature curve.

Results and Discussion

As shown in Table 1, the LCSTs of Pluronics studied were significantly influenced by the polymer concentration. As the concentration of Pluronics increased, the LCSTs decreased. Interestingly, while the other Pluronics had one, Pluronic L62 showed double LCSTs at 10% and a single LCST at 1%. When the LCST of Pluronic L62 in distilled water was measured as a function of the polymer concentration (Table 2), double LCSTs were obtained at higher than 5% concentration. As the polymer concentration was increased, the lower LCST was decreased, while the higher LCST increased. The origin of double LCSTs is not clear yet, but it may be due to the presence of the impure compound containing a relatively large portion of PEO. Also, it was found that the LCSTs of Pluronics were more sensitive to saccharides with increasing polymer concentrations (data not given).

Figures 1–4 show the effect of saccharides on the LCST of Pluronic L31, F68LF, L92, and L62, respectively. The Pluronic concentration was fixed at 10% w/v, and added amounts of saccharides were increased depending on the saccharide/Pluronic ratio. In the absence of saccharides, LCSTs of Pluronic solutions prepared in PBS, pH 7.4 were lowered by 3–5 °C than those in dw. Since these Pluronics are nonionic polymers and therefore LCSTs are independent of the solution pH, the decreased LCST by PBS is due to the salting-out effect of ions present in this buffer (i.e., sodium cation and phosphate and chloride anions, $I = 0.247$). The LCST changes of synthetic polymers affected by salts have been found to follow the classical Hofmeister or lyotropic series, representing the relative

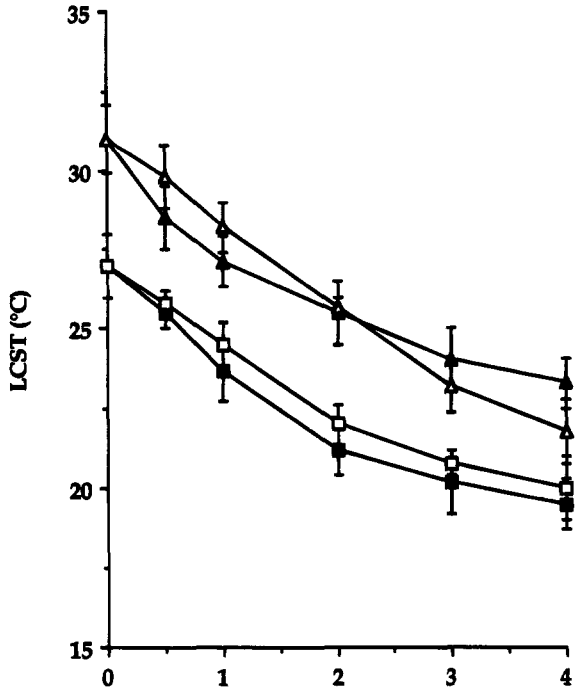

Figure 1. Effect of saccharides on the LCST of Pluronic L31. The polymer solutions containing glucose (closed symbols) and maltose (open symbols) were prepared in either distilled water (triangles) or PBS, pH 7.4 (squares). The polymer concentration was fixed at 10% w/v. $N = 3$; mean ± sd.

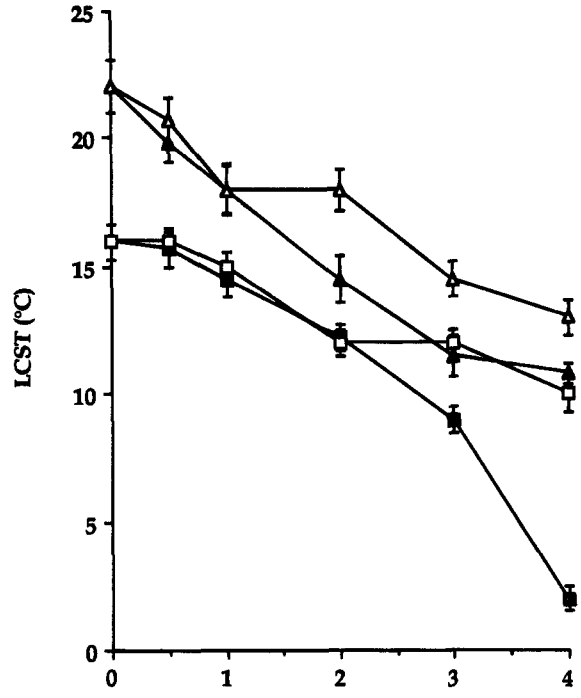

Figure 2. Effect of saccharides on the LCST of Pluronic F68LF. The polymer solutions containing glucose (closed symbols) and maltose (open symbols) were prepared in either distilled water (triangles) or PBS, pH 7.4 (squares). The polymer concentration was fixed at 10% w/v. $N = 3$; mean ± sd.

effectiveness of ions in altering protein configurations.[7,8,25] Florin et al.[22] explained salt effects as due

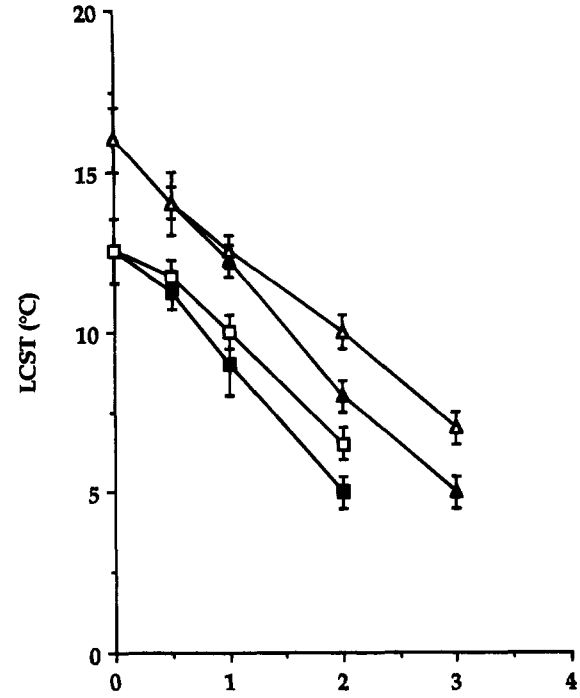

Figure 3. Effect of saccharides on the LCST of Pluronic L92. The polymer solutions containing glucose (closed symbols) and maltose (open symbols) were prepared in either distilled water (triangles) or PBS, pH 7.4 (squares). The polymer concentration was fixed at 10% w/v. $N = 3$; mean ± sd.

to the existence of a hydration shell with enhanced structuring of water as well as a salt-deficient zone surrounding the polymer chains. Small, highly charged ions with positive viscosity B coefficients (a qualitative measure of the effects of salts or ions on the water structure) are classified as water structure makers.[12] These ions would be depleted from the poorly polarizable polymer chain due to repulsive image charge forces (the effective repulsion action between ions and low-dielectric interfaces) and therefore increase the size of a salt-deficient zone. This zone provides an attractive force between polymer chains since the overlap between two zones liberates water to the bulk salt solution where it has a lower chemical potential. As a result, small, highly charged ions decrease the LCST.

As shown in Figure 1, the LCST of Pluronic L31 was decreased by addition of glucose and maltose. However, there was no significant difference between glucose and maltose in lowering the LCST. This may be due to the low MW and small PEO content of Pluronic L31, leading to a low sensitivity to saccharide addition. In the case of Pluronic F68LF and L92 shown in Figures 2 and 3, the effects of saccharides were more pronounced, as compared to Pluronic L31. At relatively low saccharide concentrations, both saccharides decreased LCSTs to the same extent. However, as the saccharide concentration was increased, there was a significant difference between the two saccharides and glucose as a monosaccharide decreased the LCSTs more than maltose as a disaccharide. As mentioned above and can be seen in Figure 4, Pluronic L62 at 10% showed double LCSTs in dw and PBS, and both LCSTs were decreased by the saccharide addition. Glucose was more effective than maltose in lowering the LCST.

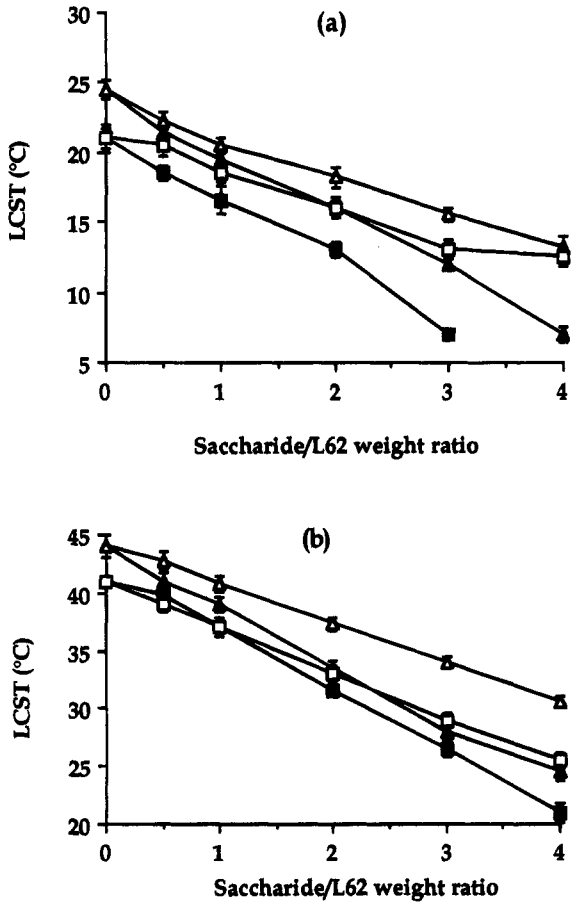

Figure 4. Effect of saccharides on the LCSTs of Pluronic L62. The polymer solutions containing glucose (closed symbols) and maltose (open symbols) were prepared in either distilled water (triangles) or PBS, pH 7.4 (squares). The polymer concentration was fixed at 10% w/v. At this concentration, this Pluronic showed two LCST: (a) lower LCST and (b) higher LCST. $N = 3$; mean ± sd.

Few selected experiments were performed with linear poly(NiPAAm) and its copolymers to study the effect of saccharides as additives on their LCSTs. Due to the solubility problem of these polymers in the presence of glucose, the polymer concentrations were lowered to make clear solutions containing large amounts of glucose.

The decrease in the LCST by addition of glucose to 5% poly(NiPAAm) solution can be seen in Figure 5. The LCST of poly(NiPAAm) was decreased by 10 °C with a glucose/polymer w/w ratio of 4. Figure 6 shows the effect of the polymer concentration on the LCST and glucose sensitivity of poly(NiPAAm-*co*-BMA-*co*-AA) containing 1 mol % of AA. When the polymer concentration was increased, the LCST was decreased and the effect of glucose decreasing the LCST was more pronounced. The effect of glucose on the LCSTs of poly(NiPAAm-*co*-BMA-*co*-AA) solutions containing various mole percents of AA is shown in Figure 7. In the absence of glucose, as the AA content in the polymer was increased, the LCST was increased. Increased polymer–water interaction by the strong hydrophilicity of the charged AA group in PBS, pH 7.4 would interfere with the hydrophobic interaction between polymer chains and thus shift the LCST to a higher temperature with increasing AA content. As a result of increased hydrophilicity due to the presence of the charged AA group, the LCSTs of these polymers were decreased by glucose to a lesser extent than those of nonionic polymers, poly(NiPAAm) and Pluronics. However, it should be considered that the concentration of poly(NiPAAm-*co*-BMA-*co*-AA) (2.5%)

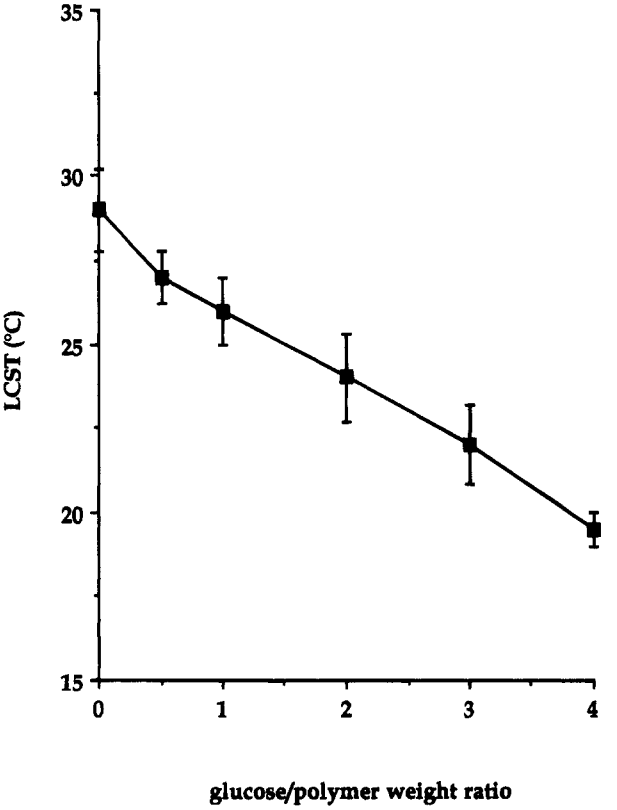

Figure 5. Effect of glucose on the LCST of poly(NiPAAm) in PBS, pH 7.4. The polymer concentration was 5% w/v. $N = 3$; mean ± sd.

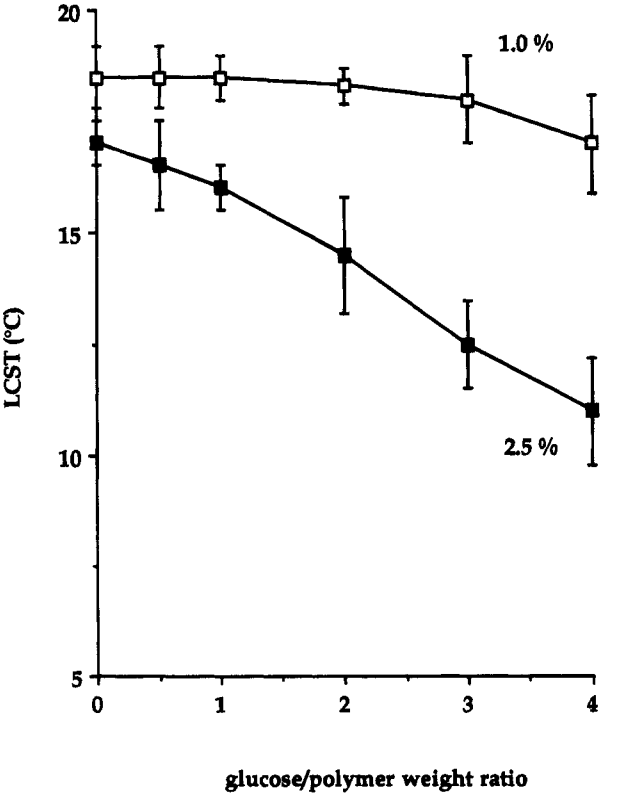

Figure 6. Effects of the polymer concentration and glucose on the LCST of poly(NiPAAm-*co*-BMA-*co*-AA) containing 1 mol % of AA in PBS, pH 7.4. Two polymer concentrations were studied: 1% w/v (open squares) and 2.5% w/v (closed squares). $N = 3$; mean ± sd.

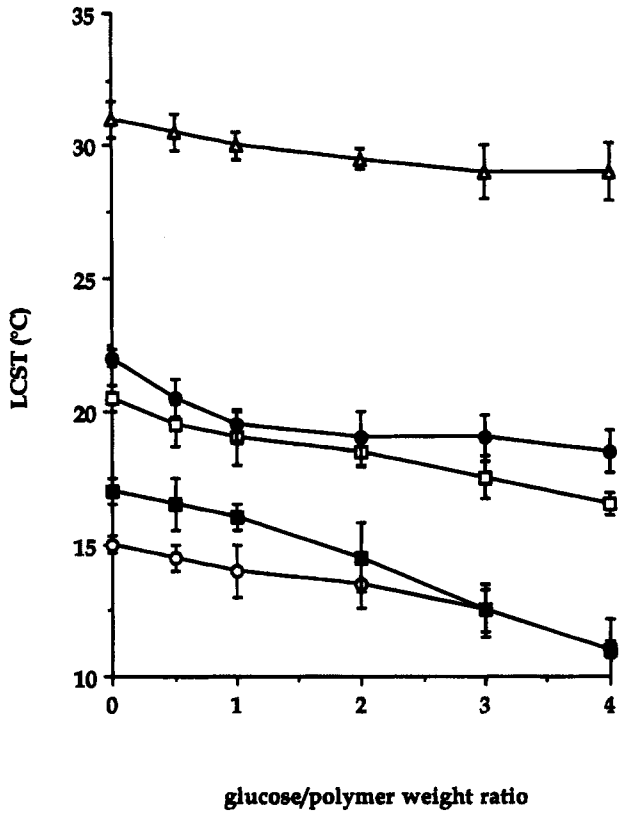

Figure 7. Effect of glucose on the LCST of poly(NiPAAm-*co*-BMA-*co*-AA) containing various amounts of AA in PBS, pH 7.4. From top: 5 mol % AA, 3 mol % AA, 2 mol % AA, 1 mol % AA, and 0 mol % AA. The polymer concentration was fixed at 2.5% w/v. $N = 3$; mean ± sd.

was much lower than those of poly(NiPAAm) (5%) and Pluronics (10%).

The experimental results indicate that (i) LCSTs of the polymers studied were significantly influenced by the polymer concentration, (ii) the addition of saccharides significantly decreased the LCSTs of all polymers studied, (iii) as the polymer concentration increased, saccharide effects became more pronounced, and (iv) a monosaccharide, glucose, was more effective than a disaccharide, maltose, in lowering the LCST, especially in Pluronic solutions. Hydrophobic interactions have been known to play an important role in the phase separation of LCST polymers in aqueous solution. When the phase separation takes place, the reduced entropy of the polymer chain is compensated for by a gain in entropy due to the release of structured water around the hydrophobic group on the polymer, which is supported by an endothermic heat of phase separation obtained from the DSC experiment.[17,26] Therefore, saccharide effects on the LCST may be explained by considering the effect of saccharides on the structured water around the polymer. However, whether additives increase or decrease the amount of structured water around hydrophobic polymer side groups, which is a driving force for the increased hydrophobic interactions between polymers, is still unknown.[17,26,27] Saccharide may enhance the hydrophobic interaction between the polymer chains either by immobilizing water molecules near them and consequently weaken hydrophobic hydration or by increasing local order of water molecules around the polymer and consequently increasing the driving force for hydrophobic interaction. The strong water structure-making power of low molecular weight saccharides is represented by the large positive value of the viscosity B coefficient (e.g., the value of sucrose is 0.8786).[28]

In addition to the structuring of water, the presence of saccharides may stabilize and increase the population of nonpolar polymer conformations in aqueous solution, which has been proposed as a model for the temperature-induced phase separation of aqueous PEO and Pluronic solutions[22,23,29] and for the saccharide effect on the LCST of the PEO solution.[24] Whether poly(NiPAAm) and its copolymers possess different local conformations due to the temperature change and the presence of saccharides remains to be studied.

The effect of saccharides on the LCST for an aqueous PEO solution is found in the literature.[5,24] The phase diagram of a mixture of glucose with PEO and water showed that when the system separated into two phases, the glucose distributed predominantly to the polymer poor phase. Therefore, it was suggested that the decrease in the LCST by glucose was the result of the strong repulsive interaction between PEO and glucose, thus inducing predominant partitioning of glucose into the water phase, lowering the chemical potential of water, and facilitating the phase separation at a low temperature. On the basis of this interpretation, the driving force for the effect of saccharides inducing hydrophobic interaction of the polymers may be a simple incompatibility between the LCST polymers and saccharides, resulting from the strong H-bonding of saccharides with bulk water. Possible changes in the water structure near the hydrophobic moiety of the polymer and in the local polymer conformation may be secondary effects. Synergistic effects of strong salting-out salts (i.e., phosphate and sulfate salts) and these saccharides in lowering the LCST are expected, since these salts have been known to decrease the LCST and partition predominantly to the polymer poor phase (incompatible with the polymer) in the PEO–water system.[10] On the other hand, strong salting-in salts (i.e., thiocyanate and perchlorate salts), which are more compatible with the LCST polymers than salting-out salts, and saccharides may have opposite effects on the LCST.

Timasheff et al.[30–32] have studied the effects of additives on the stability and solubility of protein in view of preferential interactions. Solvent additives such as guanidine hydrochloride, urea, salting-in salts, and some organic salts showed preferential binding to some proteins, which could be related to their destabilizing effect on proteins. On the other hand, preferential hydration (i.e., preferential exclusion) was observed in aqueous salting-out salts, amino acids, sugars, and glycerol solutions, resulting in an unfavorable free energy change. This unfavorable free energy change was correlated with the increase in the surface tension of water, resulting from the increase in cohesive force between these additives and water. This preferential hydration was related to the protein stabilization by these additives. Preferential hydration in the presence of low molecular weight saccharides seems to be closely related to the strong water structure-making power of saccharides and the predominant distribution of saccharides to the polymer poor phase in an aqueous PEO–saccharide system, which were described above to explain saccharide effects on the LCST of thermosensitive polymers. Whether the preferential hydration mechanism can be applied to synthetic thermosensitive polymers remains to be studied.

A 1,4-linked disaccharide (maltose) has a possibility of making an intramolecular H-bond.[24] This means that maltose is less able to interact with bulk water than glucose, making it less effective than glucose in the water structure-making power or less incompatible with polymers than glucose. This is a tentative explanation of why maltose is less effective than glucose in lowering LCSTs.

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