Articles

Effects of Polyols, Saccharides, and Glycoproteins on Thermoprecipitation of Phenylboronate-Containing Copolymers

Alexander E. Ivanov,[†] Koichiro Shiomori,[†,‡] Yoshinobu Kawano,[‡] Igor Yu. Galaev,[†] and Bo Mattiasson*[,†]

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden, and Department of Applied Chemistry, Miyazaki University, Miyazaki 889-2192, Japan

Received March 17, 2005; Revised Manuscript Received February 3, 2006

The copolymer of 3-(acrylamido)phenylboronic acid and *N*-isopropylacrylamide (82:18, $M_n = 47000$ g/mol) was prepared by free radical polymerization. The copolymer showed typical thermoprecipitation behavior in aqueous solutions; its phase transition temperature (T_P) was 26.5 ± 0.2 °C in 0.1 M glycine−NaOH buffer containing 0.1 M NaCl, pH 9.2. Due to specific complex formation of the pendant boronates with sugars, T_P was strongly affected by the type of sugar and its concentration at pH 9.2. Fructose, lactulose, and glucose caused the largest increase in T_P (up to 4 °C) at 0.56 mM concentration, attributed to the high binding affinity of the sugars to borate and phenylboronate. Among the sugars typical of nonreducing ends of oligosaccharides, *N*-acetylneuraminic acid had the strongest effect on T_P (ca. 2 °C at 0.56 mM concentration and pH 9.2), while the effects of other sugars are well expressed at the higher concentrations (16 and 80 mM) and decreased in the order xylose ≈ galactose ≥ *N*-acetyllactosamine ≥ mannose ≈ fucose ≫ *N*-acetylglucosamine. The effect exerted on the phase transition by glycoproteins was the strongest with mucin from porcine stomach and decreased in the series mucin > horseradish peroxidase > human γ-globulin at pH 9.2. As a first approximation, the weight percentage and/or the number of oligosaccharides in glycoproteins determined the character of their interaction with the pendant phenylboronates and, therefore, the effect on the copolymer phase transition.

Introduction

Reversible complex formation of borate and phenylboronate ions with mono- and oligosaccharides in aqueous solution has been well-known for many years.[1−3] These interactions were employed in separation[4−6] and detection[7−10] of sugars, glycoproteins,[11−13] nucleotides,[14] and nucleic acids[15] and synthesis of sugar-responsive materials.[16] Equilibrium constants of the complex formation (K_{ass}) have been reported,[1−3] including those relevant to borate interaction with particular diols in the saccharide molecules.[17] Values of the constants vary from 6 to 2000 M^{-1} for different sugars,[3] suggesting high selectivity of borate interaction with glycoproteins and carbohydrates bound to cells. Recognition of carbohydrates by lectins and antibodies is involved in many important processes in nature, including cell−cell interaction,[18] adhesion of bacteria to animal tissues,[19] and others. One can suppose, therefore, that synthetic, sugar-sensitive polymers may act as effective regulators of the above bioadhesion phenomena.

Recently, some efforts were made to synthesize water-soluble copolymers containing phenylboronic acid and to study their lectin-like interactions with cells.[20,21] The selectivity of binding to the cell carbohydrates can be, however, different between the pendant and free phenylboronate, either due to multipoint chelation of carbohydrate residues by pendant phenylboronates or for any sterical reasons. Interaction of thermoresponsive boronate-containing copolymers with some carbohydrates and polyols was recently studied by us[22] in a comparative way: owing to the hydrophilic properties of the associating molecules, their binding to the copolymer led to a shift of its phase transition temperature (T_P),[22,23] which might be easily quantified.[22] Different reactivities of carbohydrates with phenylboronic acid immobilized on an insoluble carrier has also been demonstrated in a chromatographic mode.[12] The aim of the present work is to characterize interactions of the boronate-containing copolymers[22] with sugars typical of the nonreducing ends of oligosaccharides carried by glycoproteins: mannose, galactose, xylose, fucose, *N*-acetylneuraminic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetyllactosamine. We attempted to correlate the shift of T_P to the known equilibrium constants of sugars and polyols binding to borate and phenylboronate and thus to get a tentative method for evaluation of the copolymer reactivity toward these substances. We have also studied thermal precipitation of the copolymer in the presence of some glycoproteins exhibiting glycans with different sugar residues at their nonreducing ends.

Experimental Section

Materials. 3-Aminophenylboronic acid hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). Acryloyl chloride was purchased from Fluka Chemie AG (Buchs, Switzerland). *N*-Isopropylacrylamide (NIPAM) was obtained from Eastman Kodak Co. (New York). 2,2′-Azobis(2-methylpropionitrile) (AMPN) was purchased from

* To whom correspondence should be addressed. Fax: +46 46 222 4713. E-mail: Bo.Mattiasson@biotek.lu.se.
[†] Lund University.
[‡] Miyazaki University.

10.1021/bm050208i CCC: $33.50
Published on Web 03/08/2006

Acros Organics (Geel, Belgium). 1,4-Dioxane, diethyl ether, sodium hydroxide, sodium chloride, hydrochloric acid, D-fructose, D-glucose, *N*-acetyl-D-glucosamine (>99% purity), *N*-acetyl-D-galactosamine, L-arabinose, D-sorbitol, sucrose, raffinose, mucin from porcine stomach (type III), and horseradish peroxidase (type VI-A) were products of Sigma Chemical Co. *N*-Acetylneuraminic acid (type IV-S, minimum 95% purity) was a product of Sigma-Aldrich Chemie GmbH (Steinheim, Germany). D-Mannose (>99% purity) and L-(−)-fucose were purchased from Fluka Chemie AG. D-Galactose obtained from Merck (Darmstadt, Germany) contained <0.5% water and <0.1% glucose. γ-Globulin was a product of Pharmacia & Upjohn AB (Stockholm, Sweden). Synthesis of *N*-acryloyl-3-aminophenylboronic acid (NAAPBA) was performed as described previously.[22]

Copolymerization of NAAPBA with NIPAM. NIPAM (2.14 g, 19 mmol), NAAPBA (0.19 g, 1 mmol), and AMPN (20 mg, 0.1 mmol) were dissolved in 20 mL of 1,4-dioxane. The reaction mixture was placed in a two-necked flask with a cooling condenser. Free radical polymerization was started by heating the reaction mixture to 70 °C under nitrogen bubbling and carried out for 6 h. The obtained solution having a high viscosity was added dropwise to 150 mL of diethyl ether for precipitation of the copolymer and its separation from the unreacted monomers. The precipitate was collected by filtration with a paper filter, washed with diethyl ether, and dried in air under vacuum. The yield of the copolymer was 67%.

Characterization of the NAAPBA−NIPAM Copolymer. The ^{1}H NMR spectrum of the copolymer was recorded with a 500 MHz Bruker DRX500 spectrometer using DMSO-d_6 as solvent. Two parallel samples were used for the determination of the phenylboronate group content. Size-exclusion HPLC of the copolymer was performed on a Shodex GPC KF-804L column using detection at 210 nm. The column was eluted with tetrahydrofuran at 0.5 mL/min. The column was calibrated with standard polystyrenes. All analyses by HPLC were performed at ambient temperature.

Thermoresponsive Properties of Polymers. The copolymer was dissolved at a concentration of 2 mg/mL (2.8 mM PBA groups) in 0.1 M glycine−NaOH buffer, pH 9.2, containing 0.1 M NaCl. The obtained solution (0.25 mL, pH 9.2) was combined with a sugar solution (1 mL) of 0.7−100 mM concentration dissolved in the same buffer to obtain a sugar concentration in the range of 0.56−80 mM. Otherwise, the copolymer solution (0.25 mL) was combined with a glycoprotein solution (1 mL, 5 mg/mL) dissolved in the same buffer and dialyzed against the buffer before the experiment. The optical density of the polymer aqueous solutions at various temperatures was measured at 500 nm using a spectrophotometer,[15,22] after 8 min of incubation at every given temperature (Ultrospec 1000, Pharmacia Biotech, Sweden). For estimation of the phase transition temperature (T_P), the linear, low-temperature parts of the thermoprecipitation curves were extrapolated to zero optical densities using the linear regression program available in Microsoft Excel 2000, so that their crossing points with the abscissa gave T_P; see Interpretation of Thermoprecipitation Curves in the Results and Discussion.

To choose the optimal incubation time, separate experiments with NAAPBA−NIPAM copolymer were performed. The optical density of NAAPBA−NIPAM copolymer solution (0.4 mg/mL) in 0.1 M glycine−NaOH buffer, pH 9.2, containing 0.1 M NaCl was studied as a function of incubation time at temperatures of 26.5 and 27 °C, or as a function of temperature varied from 25.5 to 28 °C. During the first experiment, a spectrophotometric cuvette with the copolymer solution was kept in a thermostating water bath, the optical density being estimated at certain time intervals, as illustrated by Figure 3. The cuvette was taken out of the bath to make a measurement and returned to the bath after the measurement. The temperature change in the sample during its exposure to room temperature (23 °C) was within 0.1 °C at the moment of measurement. During the second experiment, the copolymer solution was first kept in the bath at 25.5 °C for 10 min and then for 1, 2, 4, 8, 16, or 24 min at each higher temperature, the temperature being increased stepwise by 0.5 °C before the next incubation. The measurements of the optical density were performed as described above. The T_P values were obtained by linear regression of the first experimental points situated above the baseline and below an optical density of ca. 0.6, i.e., in the lower part of the curves (see Figure 4).

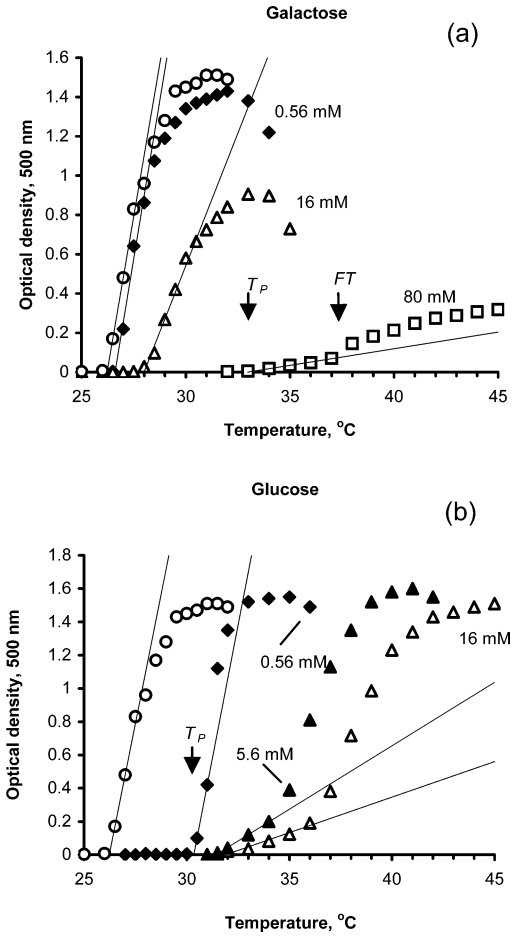

Figure 1. Temperature dependence of the optical density of the NAAPBA−NIPAM copolymer (0.4 mg/mL) in the absence (○) and presence of galactose (a) and glucose (b) at different concentrations of the sugars. Buffer: 0.1 M glycine, containing 0.1 M NaCl, pH 9.2.

Results and Discussion

Synthesis and Characterization of the NAAPBA−NIPAM Copolymer. The copolymer of NAAPBA and NIPAM was obtained by free radical polymerization. The ^{1}H NMR spectrum of the NAAPBA−NIPAM copolymer used in the present study was reported elsewhere,[22] the content of phenylboronic acid in the copolymer being 18 mol %. The number- and weight-average molecular masses (M_n and M_w) of the NAAPBA−NIPAM copolymer were 47000 and 183000 g/mol, respectively, and M_w/M_n was 3.9, as determined from the SEC chromatogram (see the Experimental Section). The molecular mass at the peak top in SEC (M_p) was 224000 g/mol.

Poly(NAAPBA−NIPAM) exhibits a thermoprecipitation behavior typical of polyNIPAM and many NIPAM copolymers, which are soluble at lower temperatures and undergo phase transitions when temperature increases;[25] see Figure 1. Due to ionization of the phenylboronate group (pK_a = 8.6 for *N*-propionyl-*m*-aminophenylboronic acid,[23] a structural analogue of the monomer unit), see Figure 2, the phase transition temperature (T_P) of the copolymer (1.0 mg/mL) in 0.1 M NaCl varied from 23 °C at pH 6.5 to 32 °C at pH 9.7.[22] In the present study we have chosen the buffered reaction medium with pH 9.2, because most of the pendant phenylboronate groups would exist in the

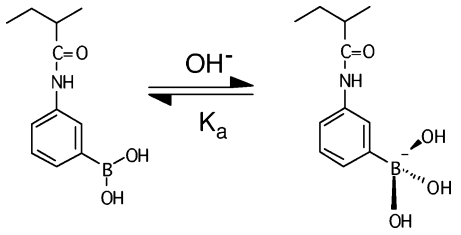

Figure 2. Equilibrium of the phenylboronic acid group between an uncharged trigonal form and a charged boronate anion.

reactive, charged form under these conditions. Besides, the buffered reaction medium prevents lowering of the pH typical of borate−sugar interactions[1] and, therefore, dehydroxylation of the charged, tetrahedral boronate anions. The constant pH of the reaction medium allows for more reliable interpretation of the shifts in the phase transition temperature (ΔT_P).

Interpretation of Thermoprecipitation Curves. Phase transition phenomena induced by temperature in an aqueous solution of polyNIPAM can be arbitrarily subdivided into three steps. In the range of temperatures from 20 to 30.5 °C the polymer chains gradually decrease in their dimensions due to increasing hydrophobic intrachain interactions (step 1).[26] They approach Θ conditions at 30.6 °C[26] in water. Above this point the radius of gyration and the hydrodynamic radius of polymer coils decrease sharply,[27] their hydrophobicity increases, and spatial fluctuations of polymer concentrations (Δc) appear due to increasing chain association[28] (step 2). Thus, the conformational transition of the polymer results in its phase transition, i.e., the formation of a fine suspension. This process can be registered by static light scattering[28] as the scattered intensity (I) is proportional to the fluctuations: $I \approx \langle \Delta c^2 \rangle$.[29] The phase transition of polyNIPAM in water was also studied by differential microcalorimetry, the calorimetric endotherm being centered at 32.2 °C, and agreed well with the light scattering measurements.[28] Optical density is less sensitive to the formation of the new phase as compared to light scattering: the maximum of the endotherm coincides with the initial, low-temperature part of the thermoprecipitation curve obtained by measurements of transmitted light.[28] At temperatures higher than the phase transition temperature, flocculation of the polymer suspensions takes place, resulting in the formation of large particles (step 3). The inflection points of the thermoprecipitation curves obtained by this method can be called, therefore, flocculation temperatures (FTs).[30] Recently, FTs of boronate-containing polyNIPAM submicrometer particles were reported to fit the range of 32−35 °C.[30]

In the present work we extrapolated the low-temperature parts of the curves to zero optical density to get the phase transition temperatures (T_P; see Figure 1) at given concentrations of sugars. To illustrate better the technique used and to justify the time interval (8 min) chosen for thermostating the reaction mixtures, the time dependencies of the optical density of the NAAPBA−NIPAM copolymer solution (0.4 mg/mL) were studied at two different temperatures (see Figure 3). The optical density monotonically increased with incubation time, a steep increase being observed during the first 2−4 min of the incubation**.** A constant value of optical density has not been attained, however, even after 1 h of incubation at 26.5 or 27 °C (data not shown). The question, therefore, arose of whether T_P could be estimated in reasonable time and what the error of T_P estimation was. To answer this question, the optical density of the copolymer solution was studied as a function of temperature varied from 25.5 to 28 °C, at different incubation times at each temperature (see Figure 4). The obtained values of T_P fit a range of ca. 0.25

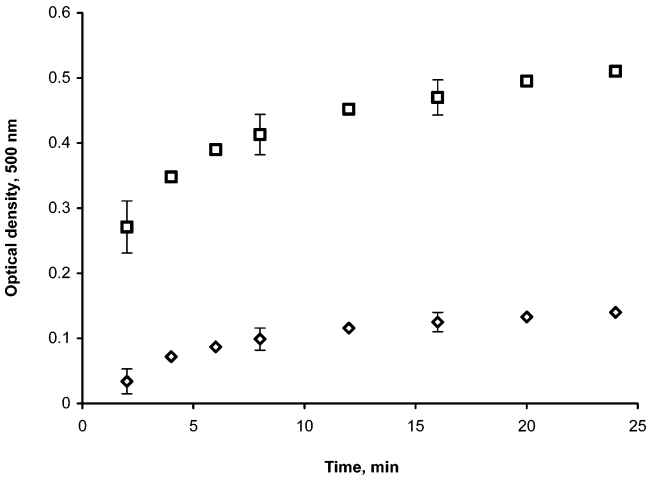

Figure 3. Optical density of the NAAPBA−NIPAM copolymer (0.4 mg/mL), pH 9.2, as a function of incubation time at 26.5 °C (◇) and 27 °C (□). The experimental points are averages obtained in three independent measurements. Error bars designate the standard deviation.

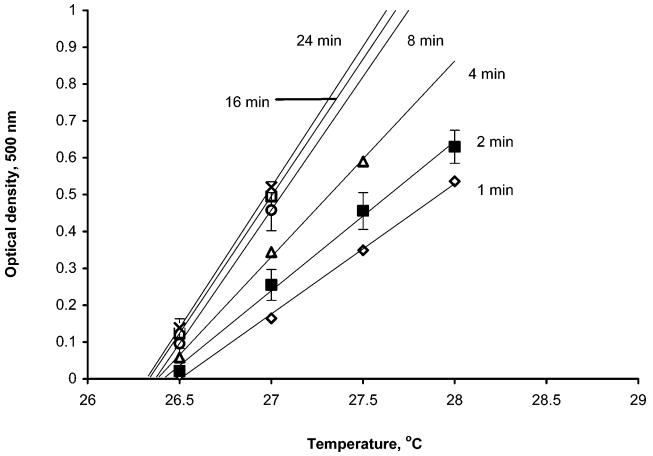

Figure 4. Linear regression of thermoprecipitation curves of the NAAPBA−NIPAM copolymer (0.4 mg/mL), pH 9.2, obtained at different incubation times at temperatures from 26.5 to 28 °C. The experimental points obtained at 2, 8, and 16 min incubations are averages of three independent measurements. Error bars designate the standard deviation.

°C and slightly decreased with incubation time increasing from 1 to 24 min (see Figure 3). For incubation times of 4, 8, 16, and 24 min the obtained values of T_P were very similar (within ca. 0.1 °C) and fitted the limits of experimental errors obtained during measurements of optical density. Despite the changes in the optical density with time (see Figure 3), estimation of T_P was possible with reasonable accuracy, with an incubation time of 8 min. An example of a heating rate described in the literature for the estimation of T_P of thermoresponsive, boronate-containing copolymers in the presence of sugars is 0.2 °C/min. [31]

It is worth noting that thermoprecipitation curves can exhibit maxima in their right, high-temperature parts; see Figure 1. The maxima are due to aggregation of the large particles followed by their sedimentation and withdrawal from the optical path. At a high concentration of sugars (for example, 80 mM galactose; see Figure 1a) the complex formation with the NAAPBA−NIPAM copolymer is well expressed, because the thermoprecipitation curve is strongly shifted to higher temperatures. The macromolecules, as well as suspended particles of the copolymer, acquire high hydrophilicity and display a low tendency to aggregate and/or sediment. The T_P, FT, and positions

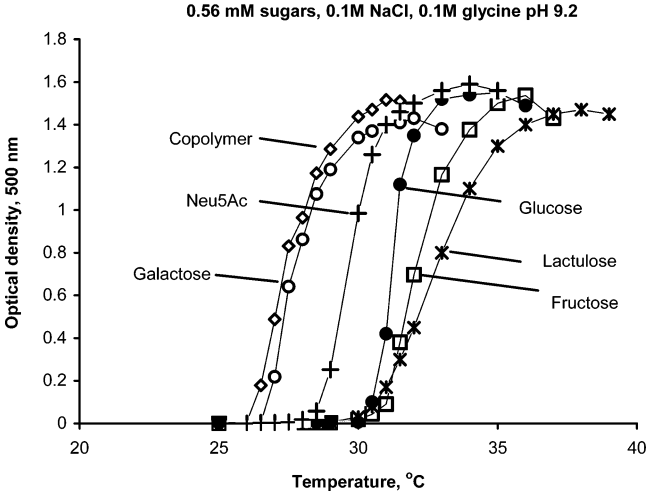

Figure 5. Temperature dependence of the optical density of the NAAPBA−NIPAM copolymer in the absence (◇) and presence of various sugars at 0.56 mM concentration. Neu5Ac = *N*-acetylneuraminic acid. Buffer: 0.1 M glycine, pH 9.2, containing 0.1 M NaCl.

of maxima shift to the higher temperatures, depending on the type and concentration of the sugar.

Effect of Sugars on Thermoprecipitation of the NAAPBA−NIPAM Copolymer. The thermoprecipitation behavior of the copolymer in 0.1 M glycine−NaOH buffer containing 0.1 M NaCl at pH 9.2 was studied in the presence of various sugars at 0.56 mM concentration, which is close to the concentration of the pendant phenylboronates in the reaction mixture; see the Experimental Section. T_P of the copolymer in the absence of sugars was equal to 26.5 ± 0.2 °C. This value is different from that reported earlier[22] because of the different composition of the reaction medium and the different method for T_P calculation used in the present study; both the above-mentioned factors resulted in a decrease of the T_P value. Fructose, lactulose, and glucose caused a large increase in T_P (ΔT_P) equal to ca. 4 °C; see Figure 5. The shift of the phase transition temperature presumably reflected the amount of sugar bound to the copolymer and thus the association constant K_{ass} between the sugar and pendant phenylboronates; see eq 1. The values of ΔT_P are listed in Table 1 for different sugars together with their association constants with borate and phenylboronate obtained from the literature.[1,3,31]

The effects of fructose and lactulose on T_P agree well with the high binding affinity of the two sugars to borate and phenylboronate; see Table 1. Although the binding affinity of glucose to phenylboronate (log K_{ass} = 2.0[3]) is much lower than that of fructose (log K_{ass} = 3.6[3]), the effects of both sugars on T_P of the boronate-containing copolymer appeared to be comparable. The reason may be chelation of glucose by two pendant phenylboronates: complexes of one glucose molecule with two phenylboronate ions were earlier shown to form.[1] The apparent log K_{ass} for monovalent interaction of glucose with pendant phenylboronates of the NAAPBA−NIPAM copolymer was found by us to significantly exceed 2.8.[22]

In the presence of galactose, arabinose, mannose, or *N*-acetyllactosamine the copolymer exhibited only a slight ΔT_P < 1 °C. No effect of *N*-acetylgalactosamine, *N*-acetylglucosamine, sucrose, raffinose, and glycerol on T_P of the copolymer was observed, even if the concentration of sugar was raised 10-fold (up to 5.6 mM). Mannose and fucose produced similar effects ($\Delta T_P \approx 1$ °C) at 5.6 mM concentration. The further increase in concentration (up to 16 mM) resulted in increasing ΔT_P due to

Table 1. Increase in the Thermoprecipitation Temperature of the NAAPBA−NIPAM Copolymer in the Presence of 0.56 mM Sugars and Polyols (ΔT_P) and the Association Constants of Borate and Phenylboronate with Sugars and Polyols (log K_{ass}) As Reported in refs 1 and 3

		log K_{ass}	
compound	ΔT_P	borate[3]	phenylboronate[1]
D-fructose	4.5	2.82	3.6
lactulose	3.6	2.91	
mannitol	4.0	3.3[a]	3.4
D-glucose	4.2	1.80	2.0
L-arabinose	0.4	2.14	2.6
D-xylose	0.4	2.2[b]	
D-galactose	0.4	1.99	2.4
D-mannose	0.3	2.01	2.2
L-fucose	0.3		
N-acetylneuraminic acid	2.0		1.3−1.5[c]
N-acetylgalactosamine	<0.2		
N-acetylglucosamine	<0.2		
sucrose	<0.2	0.86	
raffinose	<0.2	1.35	
glycerol	<0.2	1.2[a]	1.3

[a] Taken from ref 1. [b] Taken from ref 17. [c] Calculated from data in ref 32.

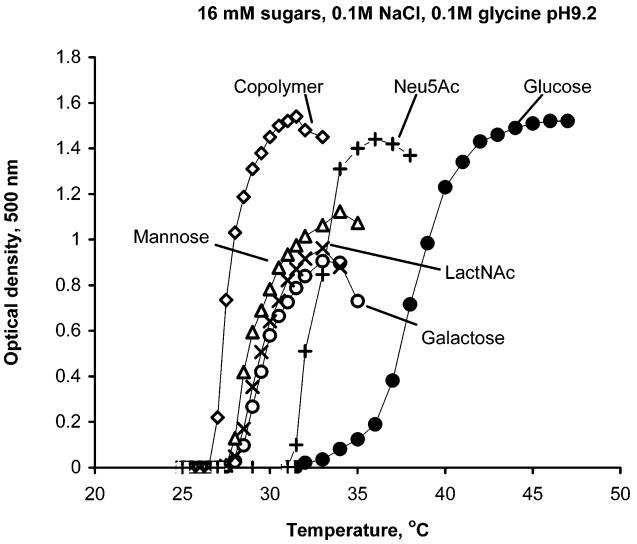

Figure 6. Temperature dependence of the optical density of the NAAPBA−NIPAM copolymer in the absence (◇) and presence of various sugars at 16 mM concentration. Neu5Ac = *N*-acetylneuraminic acid, and LactNAc = *N*-acetyllactosamine. Buffer: 0.1 M glycine, pH 9.2, containing 0.1 M NaCl.

interaction of the copolymer with galactose (see Figure 1a), mannose, *N*-acetyllactosamine, and *N*-acetylneuraminic acid; see Figure 6. This can be ascribed to the higher amount of sugar bound to the pendant phenylboronates at 16 mM sugar concentration compared to 0.56 mM concentration, according to the following equilibrium model.

The simplest case of monovalent reversible boronate−sugar complex formation can be described by the equations

$$K_{ass} = [BS]/[B][S] \quad (1)$$

$$[B]_0 = [B] + [BS] \quad (2)$$

$$[S]_0 = [S] + [BS] \quad (3)$$

where [B] and [S] are the equilibrium concentrations of boronate

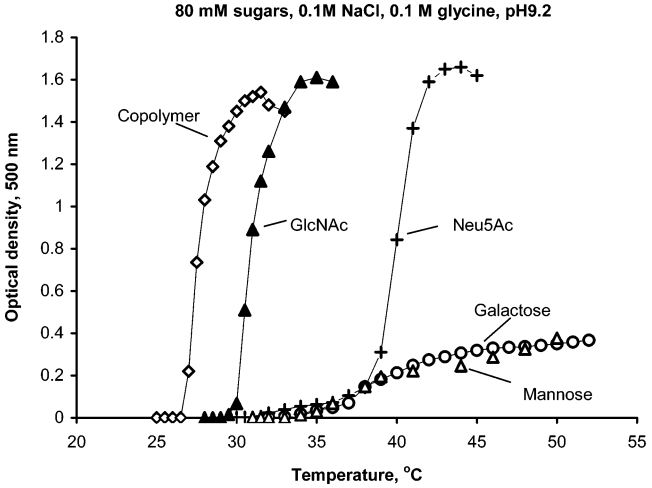

Figure 7. Temperature dependence of the optical density of the NAAPBA−NIPAM copolymer in the absence (◇) and presence of various sugars at 80 mM concentration. Neu5Ac = *N*-acetylneuraminic acid, and GlcNAc = *N*-acetylglucosamine. Buffer: 0.1 M glycine, pH 9.2, containing 0.1 M NaCl.

and sugar, respectively, $[B]_0$ and $[S]_0$ are the initial concentrations of the same reagents, [BS] is the equilibrium concentration of sugar−boronate complexes, and K_{ass} is the association constant. Taking into account a large molar excess of sugars at 5.6 and 16 mM concentration over reactive boronates ($[B]_0 \approx$ 0.56 mM; see the Experimental Section), one can develop the following equation for the relative amount of boronate−sugar complexes, $[BS]/[B]_0$:

$$[BS]/[B]_0 = K_{ass}[S]_0/(1 + K_{ass}[S]_0) \quad (4)$$

Obviously, at $K_{ass}[S]_0 \gg 1$ (the case of fructose and glucose) almost all reactive boronates will be populated with sugar molecules ($[BS]/[B]_0 \approx 1$). Thus, the values of T_P exhibited by the copolymer in the presence of 5.6 and 16 mM glucose turned out to be similar; see Figure 1b. Probably, most of the reactive boronates were saturated by glucose at concentrations as low as 0.56 mM.

In the case of weaker interactions displayed, for example, by mannose ($\log K_{ass} = 2.2$, $K_{ass} = 160\ M^{-1}$; see Table 1) at 16 mM concentration, one can expect $[BS]/[B]_0 \approx 0.7$ and, therefore, incomplete saturation of the pendant boronates with sugar and a further possible increase of T_P with concentration. Indeed, T_P becomes larger at 80 mM concentration of mannose as well as galactose; see Figures 1a, 6, and 7. At 80 mM concentration the effect displayed on T_P by *N*-acetylglucosamine also becomes noticeable. Although the association constants of free and pendant phenylboronate with the same sugar can be different, the above observations allow a tentative conclusion. The influence of sugar concentration on T_P basically conforms to the simple equilibrium scheme of their complex formation with the pendant phenylboronates. Roughly speaking, one could expect a significant (>1 °C) shift of T_P in the presence of sugars, if their concentrations were about $1/K_{ass}$ or higher, where K_{ass} is the equilibrium constant of association between the sugar and low molecular weight phenylboronate or borate. At such concentrations the majority of reactive pendant phenylboronates can form complexes with sugar molecules; see eq 4.

An important exception from this tendency was represented (besides glucose discussed above) by *N*-acetylneuraminic acid (Neu5Ac). Among the sugars typical of nonreducing ends of oligosaccharides,[18] *N*-acetylneuraminic acid had the strongest effect on T_P, while the effects of the others decreased in the order xylose ≈ galactose ≥ *N*-acetyllactosamine ≥ mannose ≈ fucose ≫ *N*-acetylglucosamine. The significant ΔT_P caused by Neu5Ac at low 0.56 mM concentration (ca. 2 °C; see Figure 5) can be partially explained by the highly hydrophilic nature of the sugar containing carboxyl and acetamide functions as well as five hydroxyl groups. On the other hand, the increase in Neu5Ac concentration from 16 to 80 mM did not result in a big change of ΔT_P (see Figured 6 and 7), unlike the effects produced by galactose and mannose. Presumably, the pendant boronates were close to saturation with Neu5Ac already at 16 mM, which indicated a higher association constant compared to those of the above sugars.

Binding of Neu5Ac to 3-propioamidophenylboronic acid (PAPBA; the low molecular weight analogue of the NAAPBA monomeric unit) was recently studied in great detail.[32] Unlike mannose, galactose, and glucose, Neu-5Ac was found to form relatively stable complexes with PAPBA at rather low pH from 4 to 7. The authors explained this effect by the formation of complexes between the *neutral* form of PAPBA and Neu5Ac stabilized by intramolecular interaction of the boron atom with the *N*-acetyl group of Neu5Ac.[32] In contrast to borate and boronate anions, the neutral forms of boric and phenylboronic acid are known to form unstable complexes with *cis*-diols and polyols.[33] The ^{11}B NMR spectra presented in ref 32 testified, however, to the complex formation of PAPBA with Neu5Ac even at pH 4 ≪ pK_a of PAPBA = 8.6. A possible reason for the relatively strong effect of Neu5Ac on T_P could deal with the reactivity of both the neutral and anionic forms of the pendant phenylboronic acid toward Neu5Ac.

An important practical consequence of the phenylboronate−Neu5Ac interaction could be preferential binding of the NAAPBA-containing copolymers to biological surfaces populated with end groups of Neu5Ac. Those could be, for example, mucosal surfaces[34] including those containing tumor-associated carbohydrate antigens, such as sialyl-Le^a or sialyl-Tn antigens.[35] Reversible interaction of the NAAPBA−NIPAM copolymer with mucin and other glycoproteins can be employed in biomedical applications and, therefore, was investigated as described below.

Thermoprecipitation of the NAAPBA−NIPAM Copolymer in the Presence of Glycoproteins. On the basis of the above series of sugar reactivity toward pendant phenylboronic acid, it seems attractive to predict the reactivity of phenylboronate-containing copolymers toward oligosaccharides contained in glycoproteins. One can foresee, however, a principal limitation for this approach: the stability of carbohydrate complexes with borate and phenylboronate is often determined by the presence of free glycosidic hydroxyl groups. For example, complexes of borate with maltose, which consists of two glucose moieties, are weaker than those of glucose itself, and the complexes of lactose are weaker than those of its two constituents, glucose and galactose.[3] Broadly speaking, the reactivity of glycans in glycoproteins cannot be predicted on the basis of the reactivity of the corresponding reducing sugars.

On the other hand, galactose and *N*-acetyllactosamine, a disaccharide containing galactose on its nonreducing end, displayed similar effects on T_P; see Figure 6. Owing to the very low reactivity of *N*-acetylglucosamine, the second sugar in *N*-acetyllactosamine, one can ascribe the reactivity of the latter to a slightly reduced but basically retained reactivity of galactose. This result seems to contradict the view of galactose as a sugar capable of complex formation with borate[36] and phenylboronate,[37] only in its furanose form. Interaction of boronic acids with α-galactopyranose via its 3,4-diol was

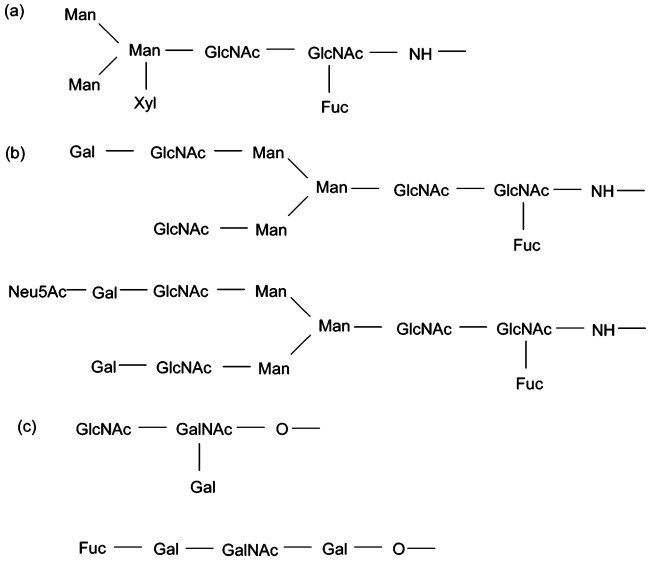

Figure 8. Structures of oligosaccharides from glycoproteins: (a) horseradish peroxidase,[40] (b) human γ-globulin,[42] (c) mucin from porcine stomach.[34,35,39] GlcNAc = *N*-acetylglucosamine. GalNAc = *N*-acetylgalactosamine, Fuc = fucose, Man = mannose, Gal = galactose, Neu5Ac = *N*-acetylneuraminic acid, and Xyl = xylose.

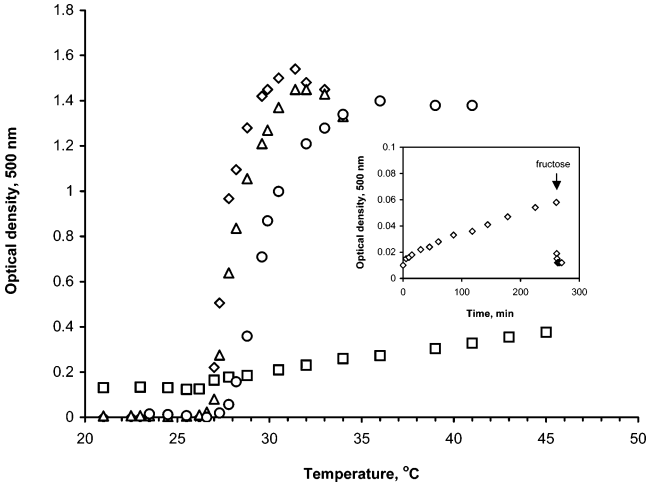

Figure 9. Temperature dependence of the optical density of the NAAPBA−NIPAM copolymer (0.4 mg/mL) in the presence of glycoproteins at 4 mg/mL concentration: γ-globulin (△), peroxidase (○), and mucin (□). The inset shows the development of optical density in the mixture of mucin and the NAAPBA−NIPAM copolymer. The arrow indicates addition of 0.1 M fructose (0.125 mL) to the reaction mixture. Buffer: 0.1 M glycine, pH 9.2, containing 0.1 M NaCl. The volume of the reaction mixture was 1.25 mL.

proposed in ref 37. Although α- and β-methylgalactopyranosides failed to show complexation with *m*-nitrophenylboronic acid,[37] interaction of α-methylgalactopyranoside with borate was registered by ^{11}B NMR.[33] It is relevant to note that "addition of galactose to saccharose, to form raffinose, increases the complex stability".[3] There exists some other evidence for the possibility of complex formation between galactopyranosides and borate. Specific binding of *p*-nitrophenyl glycosides of glucose, galactose, mannose, and xylose to the adamantyl derivative of PBA have been reported, and the transport of glycosides via liquid organic membrane assisted by either PBA or the adamantyl-modified PBA has been studied.[38] Among the glycosides used, it was *p*-nitrophenyl galactoside which exhibited the fastest transport due to its strongest binding to both PBA and its adamantyl derivative. Binding of borate to 4,6-diols of galactose moieties of galactomannan has been demonstrated by ^{11}B NMR.[33] One can suppose, therefore, that galactose as the end group of glycans will be probably involved in the complex formation with phenylboronate. The same seems to be true for Neu5Ac, the sugar, which reacts with phenylboronic acid via the glycerol chain,[32] so that the sugar attachment via its glycosidic hydroxyl should not strongly affect the complex formation.

We have studied thermoprecipitation of the NAAPBA−NIPAM copolymer (0.4 mg/mL) in the presence of three different glycoproteins: horseradish peroxidase, human γ-globulin, and mucin from porcine stomach at pH 9.2 and a glycoprotein concentration of 4 mg/mL. Detectable changes in the thermoprecipitation behavior of the copolymer could be observed at a 10-fold excess of the glycoproteins over the copolymer by weight. Although the carbohydrate compositions of all three glycoproteins exhibit wide variations (especially in the case of mucin), the typical carbohydrate structures of their *O*- and *N*-glycans can be summarized as shown in Figure 8.

Mucin from porcine stomach displayed the strongest effect on the phase transition pattern of the copolymer; see Figure 9. Mucins are known to contain ca. 80 wt % carbohydrates, most of them being O-linked oligosaccharides.[34] The length of the O-linked carbohydrate chains may vary from one to more than twenty sugars.[35] *N*-Acetylgalactosamine, fucose (ca. 9 wt %), and sialic acid (ca. 1 wt %) typical of the nonreducing ends of A- and H-blood group antigens are known to be present in the glycans of pig gastric mucus glycoprotein.[39]

Combination of the mucin and NAAPBA−NIPAM copolymer solutions resulted in gradually increasing turbidity of the mixture at 22 °C, i.e., below T_P, due to intermolecular cross-linking and formation of insoluble aggregates; see the inset in Figure 9. The soluble fraction of the copolymer underwent the phase transition at a temperature close to T_P of the pure copolymer, while thermally induced flocculation was strongly reduced, probably due to a relatively low concentration of the copolymer in the soluble fraction. The specific character of cross-linking and aggregation at 22 °C was proven by immediate dissolution of the aggregates in 10 mM fructose, a strong competitor for mucin binding to phenylboronates.

Horseradish peroxidase (HRP) taken at 10-fold weight excess over the copolymer produced a ca. 1 °C shift of T_P, i.e., similar to that produced by mannose at 5.6 mM concentration; see above. HRP is known to contain eight N-glycosylation sites occupied by oligosaccharides and ca. 21 wt % carbohydrates.[40] All the oligosaccharides contain one or two mannose residues at their nonreducing ends;[40] see Figure 8. The major oligosaccharide, as well as some others, contains a residue of xylose. Using the molecular mass of the major oligosaccharide (1189 g/mol[40]), one can calculate that the reaction mixture of HRP and the copolymer (see the Experimental Section) contained ca. 0.7 mM oligosaccharide or, in total, ca. 2 mM end groups of mannose and xylose. Thus, the effect of protein-bound sugars on T_P was obtained at their concentrations ca. 3-fold lower than that of the same sugars in solution and indicated high reactivity of the end groups of oligosaccharides. Interfacial recognition of yeast mannan by a PBA-containing self-assembled monolayer[41] is in agreement with our findings.

The effect of human γ-globulin on T_P was minimal if noticeable at all. IgG, the main constituent of γ-globulin, contains two oligosaccharides bound to the F_c-region of its molecule and ca. 2 wt % carbohydrate by weight.[34] A portion of the oligosaccharides have residues of Neu5Ac as the end groups (their content was found to be 0.46 mol/mol of protein in human IgG), while the other oligosaccharides may have

residues of galactose as the end groups.[42] The weak effect of γ-globulin on T_P can be explained by the low content of carbohydrates in the protein molecule. It is worthwhile to note that the F_c-bound glycans of IgG have extensive contacts with the protein surface,[43] being presumably less accessible to the phenylboronates of NAAPBA−NIPAM. As a first approximation, however, the weight percentage and/or the number of oligosaccharides in glycoproteins determine the character of their interaction with the pendant phenylboronates and, therefore, the effect on the copolymer phase transition. On the other hand, the example of γ-globulin shows that protein molecules by themselves are rather inert with respect to binding to NAAPBA−NIPAM copolymers and their effects exerted on T_P are most probably due to interaction of the oligosaccharide moieties with the phenylboronates.

Conclusion

The thermally induced phase transition of the phenylboronate-containing copolymer of *N*-isopropylacrylamide was studied in aqueous solutions of sugars, polyols, and glycoproteins and allowed detection of their association with the copolymer. As a rough approximation, one can expect a significant shift of the phase transition temperature of the copolymer ($\Delta T_P > 1$ °C) at sugar concentrations of about $1/K_{ass}$ or higher, where K_{ass} is the equilibrium constant of association between the sugar and low molecular weight phenylboronate or borate. Two exceptions, glucose and *N*-acetylneuraminic acid, produced strong effects on T_P at their concentrations much lower than $1/K_{ass}$. The effect exerted on the phase transition by glycoproteins was the strongest with mucin from porcine stomach and decreased in the series mucin > horseradish peroxidase > human γ-globulin, at pH 9.2. The specific character of mucin interaction with the phenylboronate-containing polymer was proven by facile dissolution of the reaction product in 10 mM fructose. A significant shift of T_P in the presence of horseradish peroxidase testifies to a high reactivity of mannose-containing oligosaccharide chains of the glycoprotein with the copolymer.

Acknowledgment. This research was supported by the Swedish Foundation for Strategic Research (area of Chemistry for Life Sciences), the Swedish Competence Center for Bioseparation (CBioSep), the Carl Tryggers Foundation (Project CTS 02:82), Swedish Research Council (Project 50360401), and INTAS (Projects 0673 and 4813).

References and Notes

(1) Lorand, J. P.; Edwards, J. O. Polyol complexes and structure of the benzeneboronate on. *J. Org. Chem.* **1959**, *24*, 769−774.
(2) Evans, W. J.; McCourtney, E. J.; Carney, W. B. A comparative analysis of the interaction of borate ion with various polyols. *Anal. Biochem*. **1979**, *95*, 383−386.
(3) Verchere, J. F.; Hlaibi, M. Stability constants of borate complexes of oligosacchraides. *Polyhedron* **1987**, *6*, 1415−1420.
(4) Di Luccio, M.; Smith, B. D.; Kida, T.; Borges, C. P.; Alves, T. L. M. Separation of fructose from a mixture of sugars using supported liquid membranes. *J. Membr. Sci.* **2000**, *174*, 217−224.
(5) Kida, T.; Furue, D.; Matsuyama, A.; Nakatsuji, Y.; Ikeda, I. Selective transport of saccharides through a bulk liquid membrane using reversed micelle carriers. *Chem. Lett*. **1996**, *9*, 733−734.
(6) Takeushi, M.; Koumoto, K.; Goto, M.; Shinkai, S. Efficient glucoside extraction mediated by boronic acid with intramolecular quaternary ammonium ion. *Tetrahedron* **1996**, *52*, 12931−12940.
(7) James, T. D.; Shinkai, S. Artificial receptors as chemosensors for carbohydrates. *Top. Curr. Chem.* **2002**, *218*, 159−200.
(8) Zhao, J.; Davidson, M. G.; Mahon, M. F.; Kociok-Köhn, G.; James, T. D. An enantioselective fluorescent sensor for sugar acids. *J. Am. Chem. Soc.* **2004**, *126*, 16179−16186.
(9) Badugu, R.; Lakowisz, J. R.; Geddes, C. D. Boronic acid fluorescent sensors for monosaccharide signaling based on the 6-methoxyquinolinium heterocyclic nucleus: progress toward noninvasive and continuous glucose monitoring. *Bioorg. Med. Chem.* **2005**, *13*, 113−119.
(10) Cannizzo, C.; Amigoni-Gerbier, S.; Larpent, C. Boronic acid-functionalized nanoparticles: synthesis by microemulsion polymerization and application as a re-usable optical nanosensor for carbohydrates. *Polymer* **2005**, *46*, 1269−1276.
(11) Larsson, E. L.; Linné-Larsson, E.; Jungvid, H.; Galaev, I. Yu.; Mattiasson, B. Affinity chromatography of neoglycoproteins. *Bioseparation* **2001**, *9*, 315−323.
(12) Larsson, E. L.; Linné-Larsson, E.; Jungvid, H.; Galaev, I. Yu.; Mattiasson, B. Shielding of protein-boronate interactions during boronate chromatography of neoglycoproteins. *J. Chromatogr., A* **2001**, *909*, 137−145.
(13) Liu, X.-C.; Scouten, W. H. Studies on oriented and reversible immobilization of glycoprotein using novel boronate affinity gel. *J. Mol. Recognit.* **1996**, *9*, 462−467.
(14) Elmas B.; Omur M. A.; Senel S.; Tuncel A. Thermosensitive N-isopropylacrylamide-vinylphenyl boronic acid copolymer latex particles for nucleotide isolation. *Colloids Surf., A* **2004**, *232*, 253−259.
(15) Uguzdogan E.; Denkbas E. B.; Tuncel A. RNA-sensitive N-isopropylacrylamide/vinylphenylboronic acid random copolymer. *Macromol. Biosci.* **2002**, *2*, 214−222.
(16) Hisamitsu, I.; Kataoka, K.; Okano, T.; Sakurai, Y. Glucose-responsive gel from phenylboronate polymer and poly(vinyl alcohol): Prompt response at physiological pH through the interaction of boronate with amino group in the gel. *Pharm. Res*. **1997**, *14*, 289−293.
(17) van den Berg, R.; Peters, J. A.; van Bekkum, H. The structure and local stability constants of borate esters of mono- and di-sacchrides as studied by ^{11}B and ^{13}C NMR Spectroscopy. *Carbohydr. Res*. **1994**, *253*, 1−12.
(18) Murray, R. K.; Granner, D. K.; Mayes, P. A.; Rodwell, V. W. *Harper's Biochemistry*, 24th ed.; Prentice Hall International: London, Sydney, 1996; pp 648−666.
(19) Wenneraas, C.; Neeser, J.-R.; Svennerholm, A.-M. Binding of the fibrillar CS3 adhesion of enterotoxigenic *Escherichia coli* to rabbit intestinal glycoproteins is competitively prevented by GalNAc 1-4 Gal-containing glycoconjugates. *Infect. Immun.* **1995**, *63*, 640−646.
(20) Uchimura, E.; Otsuka, H.; Okano, T.; Sakurai Y.; Kataoka, K. Totally synthetic polymer with lectin-like function: Induction of killer cells by the copolymer of 3-acrylamidophenylboronic acid with N,N-dimethylacrylamide. *Biotechnol. Bioeng*. **2001**, *72*, 307−314.
(21) Winblade, N. D.; Nikolic, I. D.; Hoffman, A. S.; Hubbel, J. A. Blocking adhesion to cell and tissue surfaces by the chemisorption of a poly-L-lysine-graft-(poly(ethylene glycol); phenylboronic acid) copolymer. *Biomacromolecules* **2000**, *1*, 523−533.
(22) Shiomori, K.; Ivanov, A. E.; Galaev, I. Yu.; Kawano, Y.; Mattiasson, B. Thermosensitive properties of sugar sensitive copolymer of N-isopropylacrylamide and 3-(acrylamido)phenylboronic acid. *Macromol. Chem. Phys.* **2004**, *205*, 27−34.
(23) Kataoka, K.; Miyazaki, H.; Okano, T.; Sakurai, Y. Sensitive glucose-induced change of the lower critical solution yemperature of poly-[N,N-dimethylacrylamide-co-3-(acrylamido)phenylboronic acid] in physiological saline. *Macromolecules* **1994**, *27*, 1061−1062.
(24) Eggert, M.; Baltes, T.; Garret-Flaudy, F.; Freitag, R. Affinity precipitation−an alternative to fluidized bed adsorption? *J. Chromatogr., A* **1998**, *827*, 269−280.
(25) Galaev, I. Yu.; Mattiasson, B., Smart polymers and what they could do in biotechnology and medicine. *Trends Biotechnol.* **1999**, *17*, 335−340.
(26) Kubota, K.; Fujishige, S.; Ando, I. Single-chain transition of poly-(N-isopropylacrylamide) in water. *J. Phys. Chem*. **1990**, *94*, 5154−5158.
(27) Fujishige, S.; Kubota, K.; Ando, I. Phase-transition of poly(N-isopropylacrylamide) and poly(N-isopropylmetacrylamide). *J. Phys. Chem*. **1989**, *93*, 3311−3313.
(28) Schild, H. G.; Tirrell, D. A. Interaction of poly(N-isopropylacrylamide) with sodium n-alkyl sulphates in aqueous solution. *Langmuir* **1991**, *7*, 665−671.
(29) Kazakov, S. V.; Chernova, N. I. In *Light scattering and photon-correlation spectroscopy*; Pike, E. R., Abbiss J. B., Eds.; NATO ASI Series; Kluwer Academic: Dordrecht, The Netherlands, 1997; pp 401−422.
(30) Elmas, B.; Onur, M. A.; Senel, S.; Tuncel, A. Temperature-controlled RNA isolation by N-isopropylacrylamide-vinylphenyl boronic acid copolymer latex. *Colloid Polym. Sci.* **2002**, *280*, 1137−1146.

(31) Matsumoto, A.; Ikeda, S.; Harada, A.; Kataoka, K. Glucose-responsive polymer bearing a novel phenylboronate derivative as a glucose-sensing moiety operating at physiological pH conditions. *Biomacromolecules* **2003**, *4*, 1410−1416.
(32) Otsuka, H.; Uchimura, E.; Koshino, H.; Okano, T.; Kataoka, K. Anomalous binding profile of phenylboronic acid with N-acetylneuraminic acid (Neu5Ac) in aqueous solution with varying pH. *J. Am. Chem. Soc.* **2003**, *125*, 3493−3502.
(33) Pezron, E.; Richard, A.; Lafuma, F.; Audebert, R. Reversible gel formation induced by ion complexation. 1. Borax-galactomannan interactions. *Macromolecules* **1988**, *21* (4), 1121−1125.
(34) Horton, H. R.; Moran, L. A.; Ochs, R. S.; Rawn, J. D.; Scrimgeour K. G. *Principles of Biochemistry*; Prentice Hall: Upper Saddle River, NJ, 2002; pp 256−257.
(35) Aksoy, N.; Akinci, O. F. Mucin macromolecules in normal, adenomatous and carcinomatous colon: Evidence for the neotransformation. *Macromol. Biosci*. **2004**, *4*, 483−496.
(36) Chapell, S.; Verchere, J.-F. A ^{11}B and ^{13}C NMR determination of the structures of borate complexes of pentoses and related sugars. *Tetrahedron* **1988**, *44*, 4469−4482
(37) Nicholls, M. P.; Paul, P. K. C. Structures of carbohydrate-boronic acid complexes determined by NMR and molecular modelling in aqueous alkaline media. *Org. Biomol. Chem.* **2004**, *2*, 1434−1441.
(38) Morin, G. T., Paugam, M.-F., Hughes, M. P., Smith, B. D. Boronic acid mediate glycoside transport through a liquid organic membrane via reversible formation of trigonal boronate esters. *J. Org. Chem.* **1994**, *59*, 2724−2728.
(39) Mantle, M.; Allen, A. Isolation and characterization of the native glycoprotein from pig small-intestinal mucus. *Biochem. J*. **1981**, *195*, 267−275.
(40) Gray, J. S. S.; Yang, B. Y.; Montgomery R. Heterogeneity of glycans at each N-glycosylation site of horseradish peroxidase. *Carbohydr. Res.* **1998**, *311*, 61−69.
(41) Kanayama, N.; Kitano, H. Interfacial recognition of sugars by boronic acid-carrying self-assembled monolayer. *Langmuir* **2000**, *16*, 577−583.
(42) Shantha Raju, T.; Briggs, J. B.; Borge, S. M.; Jones, A. J. S. Species-specific variations in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology* **2000**, *10* (5), 477−486.
(43) Wormald, M. R.; Dwek, R. A. Glycoproteins: glycan presentation and protein-fold stability. *Structure* **1999**, *7*, R155−R160.

BM050208I