Biochemical and Biophysical Research Communications 443 (2014) 562–567

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

BSA–boronic acid conjugate as lectin mimetics

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A R T I C L E I N F O

Article history:
Received 20 November 2013
Available online 8 December 2013

Keywords:
Boronic acid
Bovine serum albumin
Carbohydrate
Lectin
Alizarin Red S
Surface plasmon resonance

A B S T R A C T

We report bovine serum albumin (BSA)–boronic acid (BA) conjugates as lectin mimetics and their glyco-capturing capacity. The BSA–BA conjugates were synthesized by amidation of carboxylic acid groups in BSA with aminophenyl boronic acid in the presence of EDC, and were characterized by Alizarin Red S (ARS) assay and SDS–PAGE gel. The BSA–BA conjugates were immobilized onto maleimide-functionalized silica beads and their sugar capturing capacity and specificity were confirmed by ARS displacement assay. Further, surface plasmon resonance (SPR) analysis of the glyco-capturing activity of the BSA–BA conjugates was conducted by immobilizing BSA–BA onto SPR gold chip. Overall, we demonstrated a BSA–BA-based lectin mimetics for glyco-capturing applications. These lectin mimetics are expected to provide an important tool for glycomics and biosensor research and applications.

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1. Introduction

Carbohydrate recognition is a crucial event in many biological processes [1]. For example, cell surface carbohydrates, existing as glycoproteins [2], glycolipids [3], or proteoglycans [4], are involved in cell–cell signaling [5], immune recognition events [6], pathogen/host interactions [7], tumor metastasis [8] as well as other cellular events. Therefore, carbohydrate recognition has come to the forefront of biological scientific research aiming to uncover the molecular mechanisms of many physiological and pathological processes and explore potential therapeutic targets or diagnostic mechanisms for various diseases, including viral infections, autoimmune diseases, cancer, and cardiovascular disorders [9]. In the past decade, lectins, the sugar-binding proteins, have been conventionally used to determine the structure and function of glycoproteins [10]. In particular, microarrays using panels of lectins on a single chip have been demonstrated as useful tools for glycomic analysis [11–15]. Though these lectin microarrays were claimed to be rapid and sensitive for glycomic study, the number of available lectins is still limited compared with diversity of glycan structures. In addition, some lectins can also be cross specific and binding multiple glycan structures and some even introduce some variability in their binding affinities dependent on their purification [16]. Therefore, exploring new lectins or lectin mimetics is highly demanded. Boronic acid (BA)-containing compounds have unique properties for carbohydrates since they form cyclic esters with diols of sugars in aqueous solution. BA conjugates have been employed as an artificial carbohydrate receptors [17], membrane transport agents [18], cell surface carbohydrate recognition ligands [19], and as protective agents in synthesis of carbohydrates as well [20]. Most recently, boronic acid-containing macroligands such as nanoparticles [21–23], silica gel [24,25], magnetic beads [26], and polymers [27–35] etc. have been explored for glyco-capturing applications.

Bovine serum albumin (BSA) is a most abundant plasma protein and is very stable and soluble both *in vitro* and *in vivo*. It has been commonly used in laboratories for various biological assays. For example, BSA can be used to block nonspecific binding sites in many immunochemical experiments such as ELISA, immunoblotting and immunohistochemical studies. It is also widely used as carrier or supporter protein for vaccine and affinity ligand engineering. Herein, we envision that BSA–BA conjugate can be used as lectin mimetics for glycomic applications. First, the BSA–BA conjugates were synthesized by amidation of carboxylic acid groups in BSA with aminophenyl boronic acid. Then, BSA–BA conjugates were immobilized onto maleimide-functionalized silica beads and surface plasmon resonance (SPR) chip and their carbohydrate binding affinity and specificity were evaluated by column and SPR technique, respectively. The BSA–BA-based lectin mimetics are expected to provide an important tool for glycomics and biosensor research and applications (Fig. 1).

Abbreviations: ARS, Alizarin Red S; BSA, bovine serum albumin; BA, boronic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; SPR, surface plasmon resonance.

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http://dx.doi.org/10.1016/j.bbrc.2013.12.006

2. Materials and methods

2.1. Chemicals and reagents

Bovine serum albumin (BSA), Aminophenylboronic acid, Alizarin Red S (ARS) and Sugars (Galactose, Glucose, Fructose, Fucose, Mannose, Lactose, Sialic acid, *N*-acetyl glucosamine) were purchased from Aldrich–Sigma Co. Bi-carbonate buffer (pH 8.3) was prepared using 0.1 M $NaHCO_3$ and 0.5 M NaCl, and then adjusted to pH 8.3. PBS buffer (pH 7.4) was prepared using NaCl, KCl, Na_2HPO_4, KH_2PO_4 and adjusted to pH 7.4.

2.2. Synthesis of BSA–boronic acid with conjugates

BSA (100 mg, 1.5 μmol) and aminophenyl boronic acid (20 mg, 130 μmol) were dissolved in 5 mL of 0.05 M MES buffer (2-(*N*-morpholino)ethane sulfonic acid) at pH 6.0. To this mixture 10 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) HCl was added at constant stirring and was allowed to react for 2 h at room temperature and the pH was adjusted to 7.0 and left overnight at room temperature. The mixture was then subjected to Sephadex G-25 column for purification and lyophilized. BSA–BAs of different densities were prepared using same procedure as above by changing the ratios of BSA to BA.

2.3. Immobilization of BSA–BA onto silica beads

BSA–BA (45 mg) was dissolved in 3 mL of PBS (pH 7.4) buffer, to this mixture 250 mg of maleimide-functionalized silica beads were added and reacted for 4 h at room temperature. This mixture was then subjected to centrifugation to remove the unreacted BSA–BA and washed 3 times for 10 min in PBS (pH 7.4) buffer and dried overnight under vacuum. Same procedure was followed for the immobilization of BSA–BA2 and BSA–BA3 onto maleimide functionalized silica beads.

2.4. Characterization of BSA–BA on silica beads

BSA–BA modified silica beads (15 mg) were incubated with ARS (4×10^{-4} M) in PBS (pH 7.4) buffer for about 30 min and centrifuged to remove the unreacted ARS solution. These beads were washed for 3 times with PBS (pH 7.4) buffer to remove any unreacted or loosely bound ARS. 1 mL of 0.1 M fructose sugar in PBS (pH 7.4) buffer was added and incubated for 30 min. Displaced ARS solution was removed by centrifugation and subjected to fluorescence spectroscopy.

2.5. Quantification of BSA–BA on silica beads

Different concentrations of ARS solutions were prepared and subjected to UV–vis spectroscopy to obtain a calibration curve. To BSA–BA1, BSA–BA2 and BSA–BA3 modified silica beads (15 mg) 0.5 ml of ARS (4×10^{-4} M) solution was added and reacted for 30 min. The mixture was centrifuged and supernatant liquid was subjected to UV–vis spectroscopy to quantify the amount of BSA–BA immobilized onto silica beads.

2.6. Sugar binding specificity to BSA–BA–SB at pH 7.4 and pH 8.3

Maleimide functionalized silica beads, BSA modified Silica beads, and BSA–BA modified silica beads (15 mg) were incubated with ARS solution (4×10^{-4} M) for 30 min and centrifuged to remove the unreacted ARS solution. Silica beads were washed 3 times with PBS (pH 7.4) to remove any unreacted or loosely bound ARS followed by incubation of these silica beads with sugar solutions (Lactose, Fructose, Fucose, Glucose, Galactose, Mannose, Sialic acid, *N*-acetyl glucosamine) in PBS (pH 7.4) (0.1 M, 1 mL) and $NaHCO_3$ (pH 8.3) buffer (0.1 M, 1 mL) for 30 min, respectively. Displaced ARS solutions were removed by centrifugation and subjected to fluorescence spectroscopy.

2.7. Sugar binding specificity to BSA–BA by SPR

The specific binding of sugars to immobilized BSA–BA was analyzed with SPR with a BI 2000 biosensor system (BI Biosensing Instruments). To prepare the sensing surface, the commercial SPR gold chip (BI Biosensing Instruments) was rinsed with piranha solution of sulfuric acid and hydrogen peroxide (1:1, v/v), followed by water and ethanol (3 times) and dried under nitrogen. BSA–BA3 was covalently immobilized onto gold chip surface by flowing the BSA–BA3 solution (0.375 mg/mL, PBS (pH 7.0) buffer) at a flow rate of 7 μL/min for 600 s.

Then, a series of sugar solutions (Lactose, Fructose, Fucose, Glucose, Galactose, Mannose, Sialic acid, *N*-acetyl glucosamine, 0.1 nM) in PBS running buffer (pH 7.4) were injected over the immobilized BSA–BA3 at a flow rate of 15 μL/min for 200 s. The association and dissociation constants of the sugar binding to immobilized BSA–BA3 were determined by standard BI 2000

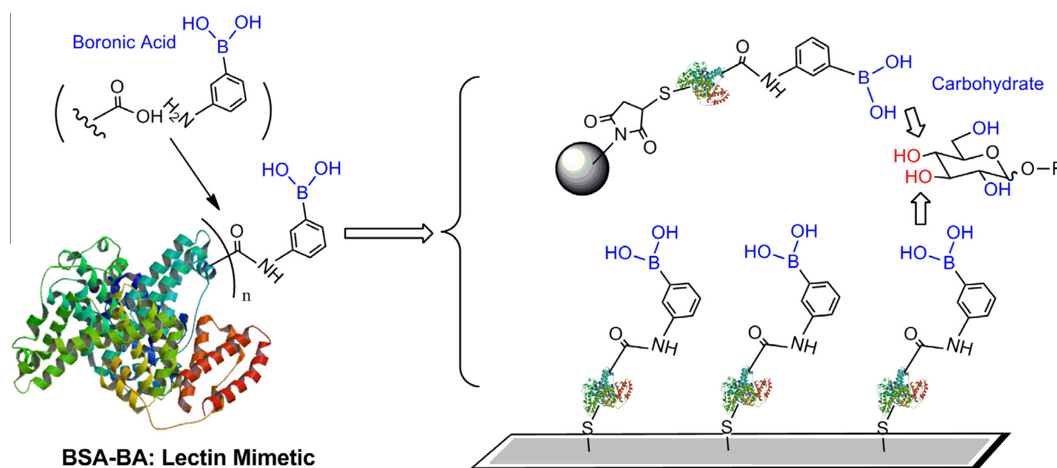

Fig. 1. BSA–boronic acid (BA) conjugate as lectin mimetics and its immobilization onto silica gel beads and SPR chip for glycomics and biosensor applications.

(scrubber) evaluation software. For studying the sugar binding at $NaHCO_3$ (pH 8.3) buffer same procedure was followed as above.

3. Results and discussion

3.1. Synthesis and characterization of bovine serum albumin (BSA) boronic acid conjugates (BSA–BA)

The BSA–BA conjugates were synthesized by amidation of carboxylic acid groups in BSA with aminophenyl boronic acid in the presence of EDC in MES buffer at pH 7.0 overnight, followed by purification on Sephadex G-25 column. Different densities of BSA–BA were synthesized by altering the ratio of APBA to BSA (Table 1). The resultant BSA–BA conjugates were characterized by Alizarin Red S (ARS) assay and SDS–PAGE gel. When ARS binds to boronic acid a dramatic change in color, UV, and fluorescence intensity was observed, thus, it has been used extensively to quantify the boronic acid and also to determine the carbohydrate binding affinity to boronic acid ligands [36–38]. Briefly, ARS shows a color change from pink to yellow when bound to BSA–BA and shifts the UV absorption wavelength from around 510 to 460 nm in PBS (pH 7.4) buffer. When adding high concentration (1 M) of fructose, the fructose–boronic acid complex forms and releases ARS with the color changing from yellow to pink and the wavelength shifting back to 510 nm (Fig. 2A). Further, the BSA–BA conjugates were characterized by SDS–PAGE (Fig. 2B), where the BSA–BA conjugates was observed with increase in molecular weight. Three BSA–BA conjugates of different BA densities were synthesized and quantified by ARS assay monitored through fluorescent spectroscopy (Table 1). As a result, a linear increase in the conjugation of boronic acid to BSA was observed with the BA/BSA ratio increase in the reactions.

Silica gel has been widely used as small, rigid particles for high performance affinity chromatography as it is capable of withstanding high flow rates and/or pressures. Recently, surface functionalization of silica gel has received vast attentions for affinity chromatography applications [27,39]. In the present study, BSA–BA conjugate was immobilized onto silica gel and its carbohydrate binding affinity and specificity was investigated. First, BSA–BA was dissolved in PBS (7.4 pH) buffer and added to commercially available maleimide functionalized silica beads (Sigma), allowed to react for 4 h, followed the un-reacted BSA-BA was removed by washing the silica beads with PBS (pH 7.4) buffer 3 times to afford the BSA–BA functionalized silica gel beads (SB–BSA–BA). The resultant SB–BSA–BA was characterized by IR spectroscopy by comparing with BSA modified silica beads and un-treated maleimide functionalized silica beads, respectively. In particular strong amide bond absorptions (1600 cm^{-1}) were observed for both BSA–BA and BSA modified silica beads, while strong hydroxyl group absorptions (3700 cm^{-1}) were observed for BSA–BA modified silica beads by comparing to un-treated maleimide functionalized silica beads (Supporting Information Fig. S1).

Table 1
Quantification of BSA–BA conjugates based on ARS assay.

Protein–BA	*Reaction*: APBA/Protein (mol)	*Product*: BA/Protein (mol)
BSA–BA1	21/1	9/1
BSA–BA2	32/1	17/1
BSA–BA3	42/1	26/1

The carbohydrate binding affinity and specificity of the SB–BSA–BA was confirmed by ARS displacement assay. First, SB–BSA–BA (15 mg) was incubated with ARS (0.1 μM) in PBS (pH 7.4) buffer at room temperature for 30 min, then the unreacted ARS was removed by pipetting the supernatant out and washing the silica beads with PBS (pH 7.4) buffer 3 times. As a result, SB–BSA–BA incubated with ARS solution showed strong ARS binding compared to unmodified silica gel beads and BSA modified silica beads incubated with the same ARS solution (Fig. 3A1–5). Among the BSA–BA conjugates used, the BSA–BA3 modified silica beads displayed highest binding of ARS as it has higher density of BA compared to BSA–BA2 and BSA–BA1, respectively (Table 1). Further, the ARS bound SB–BSA–BA was incubated with fructose (1 M) in PBS (pH 7.4) buffer at room temperature for 30 min to displace the bound ARS from the SB–BSA–BA. The displaced ARS was subjected to fluorescent spectroscopy. Again, solution from the BSA–BA3 modified silica beads showed the highest fluorescent intensity (Fig. 3D3) compared to BSA–BA2 and BSA–BA1 modified silica beads (Fig. 3D4 and D5), while BSA modified silica beads and silica gel beads alone showed no ARS released (Fig. 3D1 and D2). All these data indicated that SB–BSA–BA has BA-density-dependent carbohydrate binding capacity.

Different sugars have special binding specificities to boronic acid and it is highly pH dependent. In the present study, we investigated the specificity of eight common carbohydrates to BSA–BA3 modified silica gel under two different pH conditions, 7.4 and 8.3 by ARS displacement assay, respectively. Briefly, BSA–BA3 modified silica beads, BSA modified silica beads and maleimide functionalized silica beads were incubated with ARS solution (0.1 mM PBS (pH 7.4) buffer) for 30 min at room temperature, then centrifuged and the unreacted ARS was removed by washing the silica beads with PBS (pH 7.4) buffer. Next, sugar solutions (Lactose, Fructose, Fucose, Glucose, Galactose, Mannose, Sialic acid, *N*-acetyl glucosamine, 0.1 mM) in PBS (pH 7.4) buffer and $NaHCO_3$ (pH 8.3) buffer were added and incubated for 30 min, respectively. ARS displaced by each sugar was subjected to fluorescence spectroscopy. As a result, at pH 7.4, the binding specificities were shown from

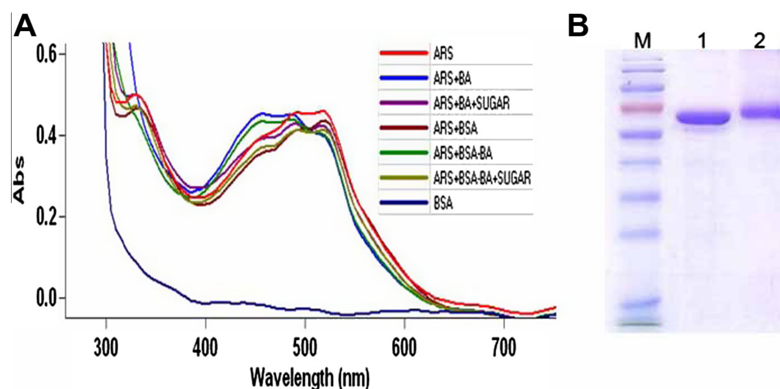

Fig. 2. Characterization of BSA–BA: (A) Alizarin Red S assay and (B) SDS–PAGE (M. Marker, 1. BSA, 2. BSA–BA).

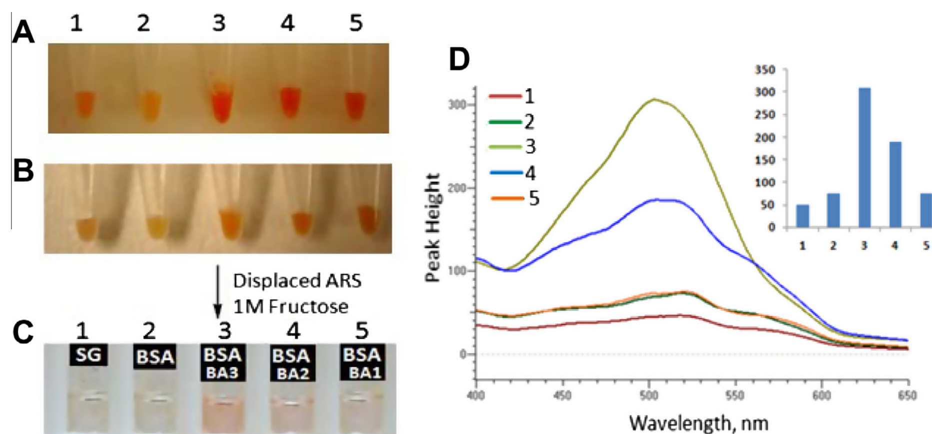

Fig. 3. ARS displacement assay: (A) ARS bound to maleimide functionalized silica beads (1), BSA modified silica beads (2), BSA–BA3 modified silica beads (3), BSA–BA2 modified silica beads (4), and BSA–BA1 modified silica beads (5); (B) beads 1, 2, 3, 4, and 5 after ARS displaced with fructose; (C) ARS solutions released from beads 1, 2, 3, 4, and 5 after incubation with fructose in PBS (pH 7.4) buffer; (D) fluorescence spectra of ARS solutions released from beads 1, 2, 3, 4, and 5 after incubation with fructose in PBS (pH 7.4) buffer.

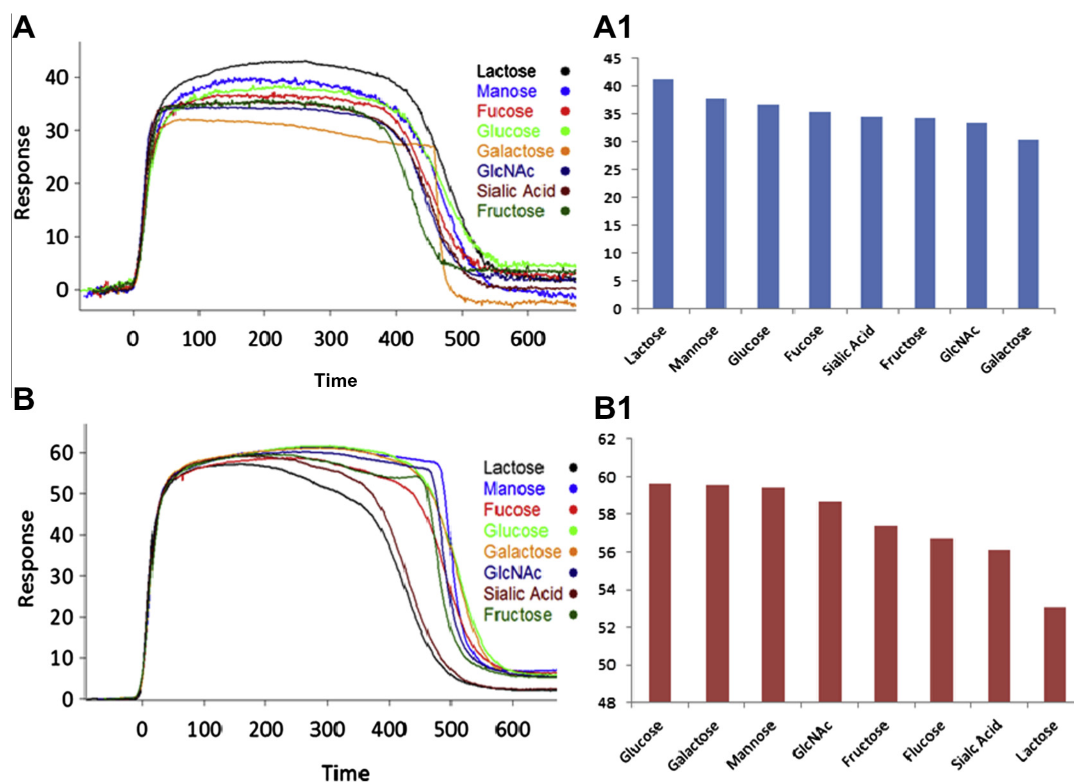

Fig. 4. SPR Sensorgrams of sugars binding to BSA–BA: (A) sugars binding to BSA–BA at pH 7.4; (A1) steady state response of sugars binding to BSA–BA at pH 7.4; (B) sugars binding to BSA–BA at pH 8.3; (B1) steady state response of sugars binding to BSA–BA at pH 8.3.

highest to lowest as Fructose > Galactose > Mannose > Fucose > Lactose > Sialic Acid > GlcNAc > Glucose, while pH at 8.3, was Fructose > Fucose > Lactose > Galactose > Mannose > GlcNAc > Sialic Acid > Glucose (Supporting Information Fig. S2). Also, the sugar binding capacity are higher at pH 8.3 than at 7.4 as the all fluorescent intensities of ARS released by all sugars at pH 8.3 are higher than that in pH 7.4 (Supporting Information Fig. S2).

3.2. Determination of sugar binding specificity of BSA–BA by surface plasmon resonance (SPR)

For further confirmation of sugar binding to BSA–BA, SPR technique was employed. BSA–BA3 was immobilized on the gold chip *via* Au – thiol coupling in PBS (pH 7.4) buffer for 10 min at flow rate 7 µL/min. Followed by injecting the sugar solutions (0.1 µM) at flow rate of 15 µL/min for 3 min in PBS (pH 7.4) buffer (Fig. 4A). The same SPR experiment was performed by using $NaHCO_3$ (pH 8.3) buffer for studying sugar binding specificities (Fig. 4B). All the sugars exhibited approximately similar binding response between 30 RU and 40 RU at pH 7.4 (Fig. 4A), while the binding response of all the sugars increased to around 60 RU at pH 8.3 (Fig. 4B). Interestingly, the binding specificities of all sugars were different from that in the ARS assay. However, the binding capacity of the sugars was higher at pH 8.3 than that in pH 7.4, which was the same as observed in ARS assay. Further, the association constant of almost all sugars was observed to be higher at pH 8.3 than

Table 2
Binding kinetics of sugars to immobilized BSA–BA in SPR assays.

	7.4 pH			8.3 pH		
	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (M)	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (M)
Lactose	2.22×10^5	8.91×10^{-3}	40.07×10^{-8}	6.27×10^5	1.65×10^{-2}	26.37×10^{-8}
Mannose	2.09×10^5	1.11×10^{-2}	53.29×10^{-8}	3.81×10^5	5.63×10^{-3}	14.78×10^{-8}
Fucose	2.63×10^5	1.06×10^{-2}	40.40×10^{-8}	4.48×10^5	6.71×10^{-3}	14.99×10^{-8}
Glucose	2.27×10^5	8.34×10^{-3}	36.74×10^{-8}	3.62×10^5	6.11×10^{-3}	16.88×10^{-8}
Galactose	3.25×10^5	1.29×10^{-2}	39.73×10^{-8}	3.78×10^5	6.30×10^{-3}	16.66×10^{-8}
GlcNAc	4.33×10^5	1.21×10^{-2}	28.10×10^{-8}	4.07×10^5	6.75×10^{-3}	16.57×10^{-8}
Sialic acid	3.79×10^5	1.28×10^{-2}	33.85×10^{-8}	4.75×10^5	1.44×10^{-2}	30.41×10^{-8}
Fructose	3.87×10^5	1.85×10^{-2}	47.87×10^{-8}	4.05×10^5	7.52×10^{-3}	18.56×10^{-8}

at pH 7.4 and the dissociation of all sugars except lactose was observed to be almost 2-fold lower at pH 8.3 than at pH 7.4 (Table 2) indicating that the sugar bindings are much stronger at pH 8.3 than at pH 7.4.

4. Conclusion

In this work, we demonstrated a BSA–boronic acid conjugate as lectin mimetics. The conjugates were synthesized by conjugation of amino phenyl boronic acid to bovine serum albumin (BSA). The boronic acid conjugated proteins were immobilized onto commercially available maleimide functionalized silica gel *via* thiol–maleimide coupling and on the gold chip *via* thiol–thiol coupling, which were used to study the sugar binding specificity of several sugars by ARS displacement assay and SPR, respectively. The binding specificity and capacity of different carbohydrates to immobilized BSA–BA were investigated at different pH conditions, proving that the carbohydrate specificity and capacity to the boronic acid are pH dependent. These lectin mimetics will provide an important tool for future glycomics and biosensor research and applications.

Acknowledgments

The authors acknowledge the Ohio Department of Development (ODOD) for supporting this research through research fund at the Center for Gene Regulation in Health and Disease (GRHD) at Cleveland State University. This work was supported in part by Grants from National Science Foundation MRI Grant (CHE-1126384). This work was partially supported by Grants from The National Natural Science Foundation of China (31328006).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.006.

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