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Molecular imprinting of fructose using a polymerizable benzoboroxole: Effective complexation at pH 7.4

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ABSTRACT

Covalent molecularly imprinted polymers against D-fructose employing 5-methacrylamido-2-hydroxymethylphenylboronic acid as functional monomer and trimethylpropane trimethacrylate (TRIM) as the crosslinking agent were prepared by a conventional radical bulk polymerization (MIP-BX(Fru)). Batch binding studies for fructose in aqueous buffers containing 10% methanol revealed that the binding capability of MIP-BX(Fru) is paramount compared to a MIP prepared with vinylphenylboronic acid MIP-BA(Fru). Especially, at the biological important pH-value of 7.4 the rebinding of fructose to the MIP-BX(Fru) is with 60 nmol per mg polymer about 3.2 higher compared to the MIP-BA(Fru). A pinacol imprinted polymer was also investigated and showed in case of MIP-BX still an imprinting of 1.7 at pH 7.4 whereas MIP-BA did not show a difference. Cross-reactivity studies at pH 7.4 show the shape-selectivity of the MIP-BX(Fru) in the order of L-fructose, sorbitol, glucose and sucrose.

1. Introduction

Molecular recognition of saccharides in water at physiological pH-values is one prominent problem in (bio-)organic chemistry [1–6]. Proteins and nucleic acids exhibit a variety of different functional groups combined with different structural conformations, which makes their molecular recognition comparatively easier [7–10]. In contrast, unsubstituted saccharides differ just in their intrinsic geometrical orientation of hydroxyl groups and especially in aqueous media the competition between the sugar's hydroxyl groups and hydroxyl groups from water molecules is a major challenge [11,12]. One method of producing selective materials in a robust and easy way is the molecular imprinting approach [13–15].

In general, molecular imprinting offers the possibility to insert tailor-made artificial binding sites on a molecular level into a 3-dimensional polymeric network. For that a polymerization is carried out in the presence of the later analyte which is called the 'template'. Due to the employment of functional monomers that are able to interact with the template molecule in a covalent, non-covalent or metal-coordinated way, a template-functional monomer complex is formed and copolymerized with a crosslinking agent. Affinity maybe tuned by the choice of the functional monomer – template interaction whereas selectivity can be obtained by the polymer itself a highly crosslinked and therefore rigid. After the polymerization, the template is extracted, and, in principle, the polymer bearing artificial binding sites is able to (re-) bind the analyte. Molecularly imprinted polymers are described for a variety of different analytes ranging from small molecules including saccharides, proteins to even whole cells [16]. These artificial receptor units are used for solid phase extraction, chromatography or sensor applications.

Molecularly imprinted polymers for monosaccharides and derivatives thereof were synthesized making use of covalent, non-covalent or metal-coordinated interactions between the saccharide and the functional monomer. A problem (to be addressed for aqueous media) lies in the competition of hydroxyl groups attached to the carbohydrate scaffold and the most abundant ones from water. Therefore, in many cases organic media or alkaline conditions had to be employed for saccharide rebinding. For example, Mosbach et al. described a non-covalent molecularly imprinted polymer with p-nitrophenyl-α-D-galactoside in ethylene glycol dimethacrylate (EGDMA) crosslinked polymers [17]. Recently sucrose was imprinted with MAA and EGDMA for aqueous batch rebinding [18]. Metal coordination is also a well-known method to bind saccharides and to polymerize copper-ligands into a molecularly imprinted polymer as functional monomer [19].

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The use of aryl boronic acids for saccharide recognition presents several advantages [20,21]. They are able to bind cis-diol containing compounds forming a covalent, reversible cyclic boronic acid ester. This boronic ester is easily formed in alkaline media because an additional hydroxyl ion can coordinate to the boron and saturates its electron deficiency – combined with that the resulting tetrahedral boron changes its hybridization state from sp^2 to sp^3. The optimal binding pH is dependent on the pK_a of the boronic acid employed and the specific cis-diol compounds. In general, electron withdrawing groups attached to the boronic acid are able to lower the binding pH. Another principle firstly described by Wulff and co-workers is to provide a hemilabile ligand in ortho-position to the boron [22]. Different hemilabile ligands such as amine- or carbonyl-containing residues are described since the electron lone pairs of these groups can coordinate intramolecularly to the boron [23]. Recently, Hall and co-workers screened a variety of ortho-substituted aryl boronic acids for their ability to bind glycopyranosides and found that an *ortho*-hydroxylmethyl group is effective at promoting the covalent complexation of diol molecules [24,25]. This type of benzoboroxole was described as an effective binding agent for saccharide recognition at pH 7.4 [26]. Consequently, the benzoboroxole was used in different approaches as binding agent for the detection of glycoproteins such as the TF-antigen or the gp120 of HI virus [27,28]. Benzoboroxole-containing molecularly imprinted polymers are described for monoalcohols or steroids but so far not for the recognition of unprotected monosaccharides such as fructose [29,30].

Herein, we report the synthesis of benzoboroxole-containing covalently imprinted polymers using fructose as a model template due to its high binding affinity to aryl boronic acids. As the functional monomer 5-methacrylamido-2-hydroxymethylphenylboronic acid **3** was synthesized and employed due to its known ability to bind saccharides at a physiological pH-value. For comparison purposes, the appropriate pinacol-imprinted (MIP-BX(Pin)) and also the fructose (MIP-BA(Fru)) and pinacol (MIP-BA(Pin)) imprinted polymers with 3-vinylphenylboronic acid **4** as functional monomer were synthesized. Accordingly, batch binding experiments were performed at pH 11.4, 8.7 and 7.4 to show the binding behavior of these molecularly imprinted polymers at different pH-values. Moreover, the binding of fructose to a control polymer was also tested in order to assess the unspecific binding. The shape-selectivity of the MIP-BX(Fru) was investigated by competition between D-fructose and L-fructose, glucose, sucrose or sorbitol.

2. Materials and methods

2.1. Chemicals

3-Vinylphenylboronic acid, pinacol (2,3-dimethyl-2,3-butanediol), methacryloylchloride (97%), trimethylolpropane trimethacrylate (techn.) (TRIM), Glucose, Sucrose and Sorbitol were purchased from Sigma–Aldrich, 5-amino-2-hydroxymethylphenylboronic acid as a dehydrated HCl salt, from Combi-Blocks, D-fructose from Merck, D-fructose [^{3}H-(G)] from Biotrend and AIBN from Fluka. As scintillation liquid Rotiszint from Carl Roth was purchased. L-fructose was purchased by TCI Europe. Solvents for polymerization were dried before use or purchased as anhydrous grades. All other substances were used without further purification.

2.2. Synthesis of 5-methacrylamido-2-hydroxymethylphenylboronic acid **3**

1.5 g (10.9 mmol) 5-amino-2-hydroxymethylphenylboronic acid **1** and 1.75 g (43.8 mmol) sodium hydroxide were dissolved in a small amount water to yield an almost saturated solution which was stirred for 10 min at 0 °C. Then 1.6 mL (22 mmol) of methacryloylchloride **2** was slowly added via a syringe pump and vigorous stirring within 1 h maintaining the 0 °C. After 4 h the solution was acidified very slowly using concentrated HCl avoiding unwanted polymerization. The pale yellow precipitate which appeared during the acidification was filtered off yielding **3** as pale yellow crystals which were pure by 1H NMR. Yield: 79–82% ^{1}H NMR (300 MHz, DMSO): δ [ppm] = 8.06 (m, 1H, Ar–H); 7.67 (m, 1H, Ar–H); 7.34 (m, 1H, Ar–H); 5.80 (s, 1H, 13-H); 5.50 (s, 1H, 13-H); 4.95 (s, 2H, 7-H); 1.96 (s, 3H, 12-H). ^{13}C NMR (300 MHz, DMSO): δ [ppm] = 166.71, 148.97, 140.33, 137.67, 123.52, 122.26, 121.25, 119.78, 69.68, 18.69. MS (ESI+): m/z = 217.12 (M^+), 218.10 ($M + H^+$), 240.10 ($M + Na^+$).

2.3. Synthesis of fructose ester **5a** and **5b**

Fructose ester synthesis (**5a** and **5b**) was carried out as described by Wulff et al. [31] D-fructose (2.5 mmol) was esterified with the desired boronic acid 3 or 4 (5 mmol) in 80 mL of dioxane solution in presence of nitrobenzene (10 μL) under argon atmosphere. The water generated was removed by azeotropic distillation (88 °C) from the reaction mixture. The residual solvent was removed in vacuo. Purification was performed by dissolution of the ester in DCM.

Analytical data **5a**: Yield: 80.5% ^{1}H NMR (300 MHz, CDCl3): δ [ppm] = 8.31–7.51 (m, 6H, Ar–H), 5.79 (s, 2H, 19-H, 31-H), 5.45 (s, 2H, 19-H, 31-H), 5.03 (s, 4H, 14-H, 26-H), 2.04 (s, 6H, 18-H, 30-H). As discussed in the Results and Discussion part, strong peak broadening was observed. ^{13}C NMR (300 MHz, CDCl3): δ [ppm] = 167.22, 166.92, 149.80, 140.56, 136.85, 129.25, 126.83, 123.82, 123.45, 120.14, 103.60, 73.25, 72.56, 72.22, 71.12, 70.80, 66.04, 61.58, 18.68. IR: n 3276 cm^{-1} (s), 2927 cm^{-1} (m), 1655 cm^{-1} (m), 1614 cm^{-1} (m), 1524 cm^{-1} (m), 1398 cm^{-1} (s), 1214 cm^{-1} (w), 1054 cm^{-1} (s), 980 cm^{-1} (s), 914 cm^{-1} (m), 822 cm^{-1} (s), 754 cm^{-1} (s) MS (ESI^-): m/z = 577.27 ($M + H^+$)

Analytical data **5b**: Yield: 82% ^{1}H NMR (500 MHz, CDCl3): δ [ppm] = 7.89 (m, 2H, Ar–H), 7.73 (m, 2H, Ar–H), 7.57 (m, 2H, Ar–H), 7.38 (m, 2H, Ar–H), 6.74 (dd, 2H, 14-H, 22-H, J = 10.9, 17.6 Hz), 5.81 (ddd, 2H, 15-H, 23-H, J = 0.4, 7.5, 17.6 Hz), 5.28 (dd, 2H, 15-H, 23-H, J = 8.5, 11.1 Hz), 5.12 (dd, 1H,4-H, J = 2.4, 8.4 Hz), 4.85 (d, 1H, 3-H, J = 2.4 Hz), 4.71 (dd, 1H, 5-H, J = 1.6, 8.4 Hz), 3.98(d, 1H, 6-H, J = 13.7 Hz), 3.85 (dd, 1H, 1-H, J = 5.1, 12.1 Hz), 3.78 (dd, 1H, 6-H, J = 2.1, 13.8 Hz), 3.67 (d, 1H, 1-H, J = 12.1 Hz). ^{13}C NMR (500 MHz, CDCl3): δ [ppm] = 137.23, 137.09, 136.56, 136.38, 134.56, 134.34, 133.11, 132.89, 130.08, 129.52, 128.28, 128.16, 114.46, 114.21, 105.01, 72.69, 72.60, 72.43, 65.99, 61.88. MS (ESI+): m/z = 405, 40 ($M + H^+$), 427.19 ($M + Na^+$), 443, 18 ($M + K^+$).

2.4. Synthesis of pinacol ester **6a** and **6b**

Pinacol esters (**6a** and **6b**) were synthesized analogous. Pinacol (590 mg, 5 mmol) was esterified with the equimolar boronic acids **3** or **4** in 180 mL of toluene in presence of nitrobenzene (10 μL) under argon atmosphere. Generated water was removed by azeotropic distillation (84 °C) and finally solvent was removed in vacuo.

Analytical data **6a**: Yield: Pale yellow solid, 90% 1H NMR (300 MHz, CDCl3): δ [ppm] = 8.24 (m, 1H, 8-H), 8.01–7.48 (m, 3H, Ar–H), 5.79 (s,1H, 12-H), 5.46 (s, 1H, 12-H), 4.67 (s, 2H, 7-H), 2.06 (s, 12H, 15-H, 16-H, 17-H, 18-H). 13C NMR (300 MHz, CDCl3): δ [ppm] = 166.44, 143.45, 140.76, 136.72, 129.63, 129.24, 127.66, 122.92, 119.81, 117.74, 84.43, 65.44, 24.79, 18.68. MS (ESI+): m/z = 340.20 ($M + H^+ + Na^+$).

IR: n 3310 cm^{-1} (m), 2979 cm^{-1} (m), 2931 cm^{-1} (w), 1664 cm^{-1} (m), 1529 cm^{-1} (s), 1343 cm^{-1}(s), 1142 cm^{-1} (s), 1068 cm^{-1} (m), 967 cm^{-1} (w), 855 cm^{-1} (w).

Analytical data **6b**: Yield: 70–78%, oil ^{1}H NMR (300 MHz, CDCl3): δ [ppm] = 7.84 (s, 1H, 2-H); 7.70 (m, 1H Ar–H); 7.52 (m, 1H Ar–H); 7.34 (m, 1H Ar–H); 6.73 (dd, 1H, 7-H, J = 10.9, 17.6 Hz); 5.79 (dd, 1H, 8-H, J = 0.9, 17.6 Hz); 5.24 (dd, 1H, 8-H, J = 0.9, 10.9 Hz); 1.36 (s, 12H, 11-H, 12-H, 13-H, 14-H). ^{13}C NMR (500 MHz, CDCl3): δ [ppm] = 136.86, 136.76, 134.18, 132.72, 128.85, 127.91, 113.86, 83.83, 24.86. MS (ESI+): m/z = 230.19 (M^+), 231.19 ($M + H^+$).

2.5. Synthesis of polymers

For polymer synthesis a modified approach was applied where 2 mmol of the desired boronic acid ester **5a**, **5b**, **6a** or **6b** and thermal initiator AIBN (295 mg, 1.8 mmol) were dissolved in 9 mL THF for **5a**/**6a** or 9 mL acetonitrile/toluene 1:1 for **5b**/**6b** [32]. After addition of crosslinker TRIM (9 g, 8.5 mL), mixture was mixed well and purged with argon for 10 min. Polymerization was initiated and carried out at 65 °C for 48 h and afterward increased up to 95 °C for 24 h. The synthesized polymer monoliths were crushed and ground in a ball mill (MM200, Retsch) for 2 min at 30 Hz. Polymer powder was wet sieved (mesh 25 μm) using acetone. Template molecules fructose and pinacol were removed from polymers by washing with methanol and water. The polymer particles were dried in vacuo and stored at room temperatures.

2.6. Batch rebinding studies

Batch rebinding experiments were accomplished at three different pH-values: pH 11.4 (0.1 M sodium carbonate solution + 10% methanol), pH 8.7 and 7.4 (0.1 M phosphate buffer + 10% methanol respectively). Fructose stock solutions of 10.0 mM were prepared with each of these buffers and doped with ^{3}H-fructose. Finally, to 10 mg of polymer, fructose stock solution and buffer was added to yield 1 mL fructose solutions of 0.1–5 mM. The mixtures were incubated over night at 20 °C and centrifuged (9000 g) for 10 min. 500 μL of the supernatant were discharged to a separate vessel and 5 mL of scintillation solution (Rotiszint® eco plus) were added. Radioactivity was measured in Scintillation counter LS 650 (Beckmann Coulter) and unbound fructose could be calculated with a calibration curve.

2.7. Competitive binding

Competitive binding studies were done with MIP-BX(Fru) at pH 7.4 with different sugars/sugar alcohol: L-fructose, sorbitol, glucose and sucrose. The same solutions as for batch binding experiments were used, completed with different 50 mM sugar solutions. For the experiments, fructose stock solution, buffer solution and 50 μL of the desired sugar solution were added to 10 mg of polymer to yield a 1 mL reaction mixture. Fructose concentrations varied from 0.1 to 4.5 mM and the competitive sugar/sugar alcohol had constant concentrations of 2.5 mM. Incubation, centrifugation and detection were done the same way as for simple binding experiments.

2.8. Adsorption/desorption measurements

The porosity of the MIPs was determined by nitrogen adsorption/desorption porosimetry on a Fisons Sorptomat 1900.

3. Results and discussion

3.1. Synthesis

Since benzoboroxole was described as a potential binding agent for monosaccharides in aqueous media at pH 7.4 a corresponding polymerizable derivative **3** was synthesized (Scheme 1). The synthesis was performed starting from a commercially available 5-amino-2-hydroxymethylphenylboronic acid **1**, which reacted with methacryloylchloride **2** at alkaline pH-values yielding the polymerizable benzoboroxole **3** after slow acidification.

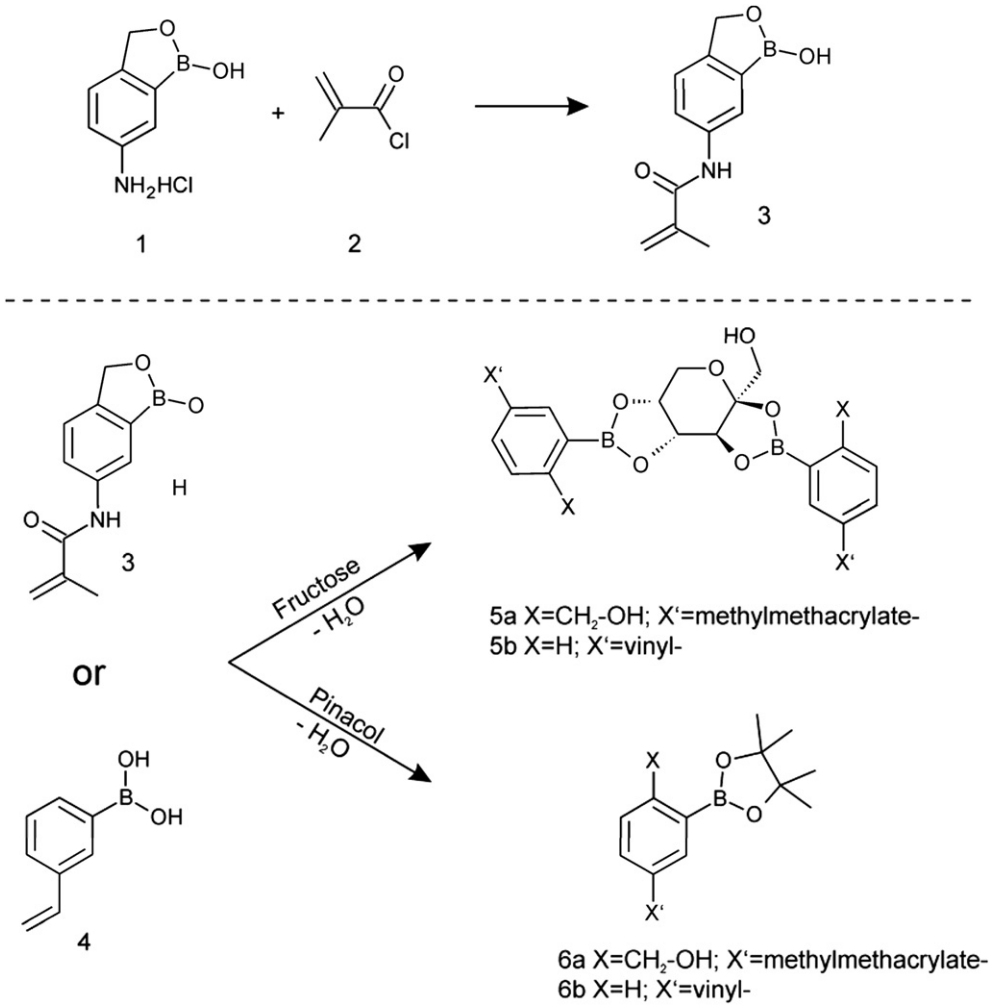
For the covalent imprinting approach it is necessary to synthesize the functional monomer – template complex before its polymerization. In the case of saccharide imprinting using aryl boronic acids the particular boronic acid ester has to be synthesized. Depending on the saccharide under investigation different binding complexes between the aryl boronic acid and the saccharide are revealed and proposed in the literature [33,34]. In our case, the binding of fructose to aryl boronic acids has a high binding strength due to the syn-periplanar arrangement of its hydroxyl groups and was investigated by means of NMR spectroscopy. Norrild and Eggert studied the esterification in solution between fructose and p-tolylboronic acid varying the ratio between fructose and the boronic acid derivative. By applying a 2:1 ratio between boronic acid and fructose in DMSO they found different 1:1 (60%) binding complexes and one 2:1 complex (33%) [33]. Since a stoichiometric pure 2:1 binding complex for fructose is envisaged. Therefore, azeotropic distillation in anhydrous dioxane was chosen for the synthesis of the fructose-benzoboroxole ester **5a** and fructose vinylphenylboronic acid ester **5b** since the removal of the released water forces the reaction into the direction of the 2:1 boronic acid ester. Using this ester as functional monomer template complex in molecular imprinting is believed to result in higher binding constants and superior selectivity also described by other authors. A conversion of about 80% in each case was reached. Furthermore the synthesis was started with the desired 2:1 ratio between the aryl boronic acid derivative and fructose avoiding the removal of unreacted boronic acid. The pinacol aryl boronic acid esters **6a** and **6b** were also prepared by azeotropic distillation.

The characterization of the products was easy in case of the phenyl boronic acid esters and the analytical data matched data already reported in Ref. [32]. In contrast, the characterization of the 5-methacrylamido-2-hydroxymethylphenylboronic acid ester **5a** is more complicated. As reported earlier, the esterification of benzoboroxole with different substituted saccharides leads to a peak broadening in the aromatic region due to either slow exchange times (which cannot be revealed by standard ^{1}H NMR spectroscopy) or due to the formation of different possible binding complexes [25]. Thus, NMR experiments with D-fructose and benzoboroxole were performed in deuterated phosphate buffer at pH 7.4 and at equimolar concentration (Fig. 1).

Beside new peaks at 7.2 and 7.4 ppm which can be assigned to the formed benzoboroxole ester also huge peak broadening could be observed. In line with these results, the synthesized polymerisable benzoboroxole ester **5a** also showed a peak broadening which leads to the assumption that the ester was formed. In combination with mass spectrometry and IR data it is concluded that the fructose – benzoboroxole ester was synthesized.

The synthesized template-functional monomer complexes were then polymerized with trimethylolpropane trimethacrylate (TRIM) as the crosslinking agent. Four polymers were synthesized, fructose-imprinted polymers with either esters **5a** or **5b** and pinacol imprinted polymers with either **6a** or **6b** as control polymers (Fig. 2). It was not possible to synthesize a polymer without the template molecule since the solubility of the bare aryl boronic acid derivatives in the pre-polymerization mixture was not sufficient. Thus, a bare control polymer without any boronic acid content was synthesized to evaluate the interaction of the polymeric backbone with the targeted substances.

After the polymerization in a two temperature process the template was removed by simple washing in MeOH/water mixtures until no further fructose was detected in the washing solution using a colorimetric anthrone assay (data not shown) [35].



Scheme 1. Synthesis of functional monomer and functional monomer–template complex.

The ground and sieved polymer particles in the size of 25–50 μm (which were also used for batch binding studies) were characterized by means of nitrogen sorption measurements. Here, the specific surface area (BET) and the pore volumes of either mesopores (BJH) or micropores (HK) were analyzed. The data are found in Table 1. For comparison also a control polymer containing just crosslinker was analyzed.

The values obtained for the surface area and pore volumes are differing between the boronic acid containing polymers and the control polymer devoid of it. The control polymer shows the

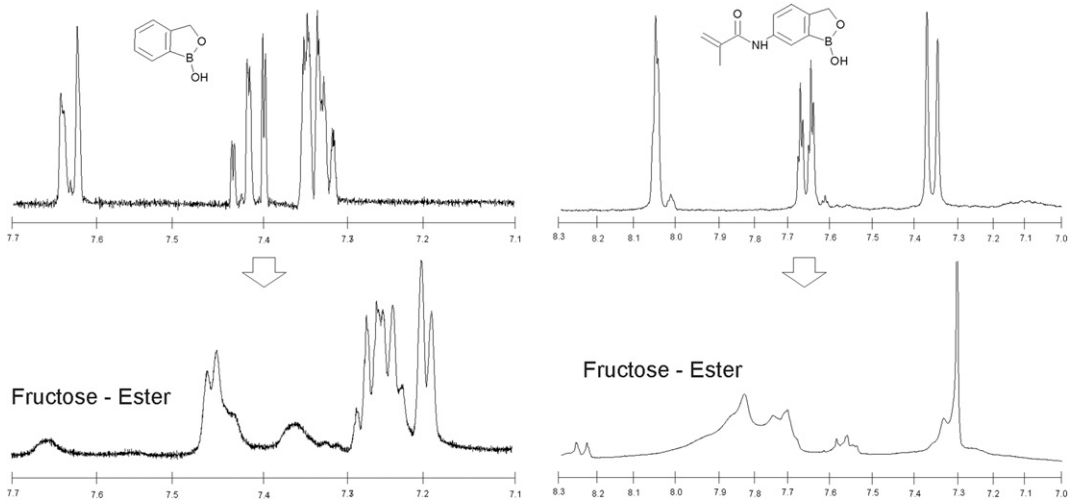


Fig. 1. NMR comparison of neat benzoboroxole derivatives and their fructose ester.

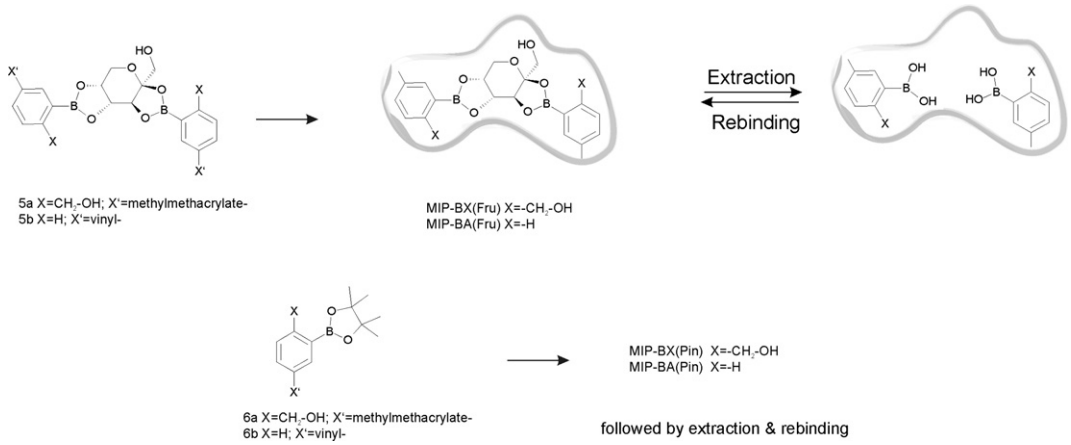


Fig. 2. Synthesis of MIP-BX(Fru), MIP-BA(Fru), MIP-BX(Pin) and MIP-BA(Pin).

highest BET surface area of 482 m^2/g whereas the surface areas of the imprinted polymers are between 332 and 436 m^2/g. The values reflect the synthetic procedure since the overall monomer concentration is slightly lower for the control polymer. The polymeric structure in the mesopore regime shows also the same trend. The control polymer which consists just of crosslinker shows with 0.42 mL/g the highest pore volume which is up to two times higher compared to the imprinted polymers. In terms of pore size the imprinted polymers exhibits a pore size around 2 nm whereas the control polymer shows a value of 4.7 nm. Thus, a mass transfer of solvent and saccharides can be present in both cases but due to the size slightly favored in the control polymer. The values for the HK-measurements displaying the micropores show that there is no huge difference between the polymers.

3.2. Medium optimization

Before starting batch binding experiments the medium was especially optimized for the recognition of fructose at pH 7.4 (Fig. 3).

A medium optimization was conducted and the MIP-BX(Fru) compared to the MIP-BX(Pin)was tested with different buffer compositions at pH 7.4. In a first step the type of *co*-solvent was compared. 10% of either methanol or acetonitrile were added to 0.1 M phosphate buffer. The experiments were performed with 2 mM initial fructose concentration. The rebinding capacity of 2 mM fructose was 23 nmol/mg polymer and thus, inferior in the case of acetonitrile compared to methanol with almost 30 nmol. Two hypotheses can explain this behavior: (i) acetonitrile is able to interact with the polymer and could be responsible for a morphology change of the polymer; (ii) the binding of one fructose releases in case of a 2:1 (boronic acid:fructose) binding four methanol molecules which favors the reaction entropically. Therefore, methanol was used as co-solvent in the subsequent buffer optimization. Beside phosphate buffers consisting of substituted sulfonic acids – namely, MOPS, HEPES and ACES were also chosen. Moreover, to show the effect of possible nitrogen coordination, a phosphate buffer containing 1% tetramethylethylenediamine (TEMED) was used [20]. In general, the binding of fructose in sulfonic acid containing buffers is lower as compared with binding in phosphate buffer. Among the sulfonic acid buffers the binding in ACES is higher compared to MOPS and HEPES due to the possible coordination of the primary amide. The binding of fructose to the imprinted polymers is comparable if either a neat or a TEMED containing phosphate buffer was used. Since the imprinting factor (IF) – the difference between the MIP-BX(Fru) *vs.* MIP-BX(Pin) – is in both cases about 1.9, just the neat phosphate buffer was used for the following binding studies at pH 7.4.

Table 1
Summarized pore volumes obtained by nitrogen sorption measurements (BET).

Polymer	BET surface area/m^2/g	BJH-mesopore volume/mL/g	Poresize BJH/nm	HK-micropore volume/mL/g	Poresize HK/nm
MIP-BX(Fru)	436	0.21	1.9	0.21	1
MIP-BX(Pin)	360	0.24	2	0.18	1
MIP-BA(Fru)	332	0.29	1.8	0.16	1.2
MIP-BA(Pin)	360	0.28	1.9	0.18	0.9
CP	482	0.42	4.7	0.23	0.9

3.3. Binding studies

The batch binding studies of fructose were performed in 0.1 M carbonate solution pH 11.4 with 10% methanol according to earlier works in our group [32]. Furthermore, the binding pH was lowered down to pH 8.7 and pH 7.4 to show the ability of the MIP-BX(Fru) to bind at neutral pH-values as well.

The batch binding studies at pH 11.4 revealed high capacities of the synthesized MIP-BX(Fru) and MIP-BA(Fru) of 135.6 and 114.7 nmol/mg polymer, respectively (Fig. 4A). The theoretical binding capacities of both fructose-imprinted MIP polymers are about 200 nmol/mg polymer. Therefore, about 60% of the possible binding pockets are accessible at an initial fructose concentration of 5 mM. The binding behavior of both MIP-BX(Fru) and MIP-BA(Fru) is comparable and can be explained by the overwhelming concentration of hydroxyl ions in solution at this alkaline environment

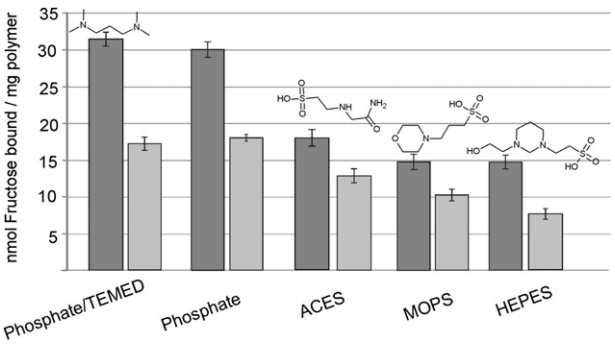


Fig. 3. Media optimization for 2 mM fructose at pH 7.4 with 10% MeOH; MIP-BX(Fru) (dark bars); MIP-BX(Pin) (light bars).

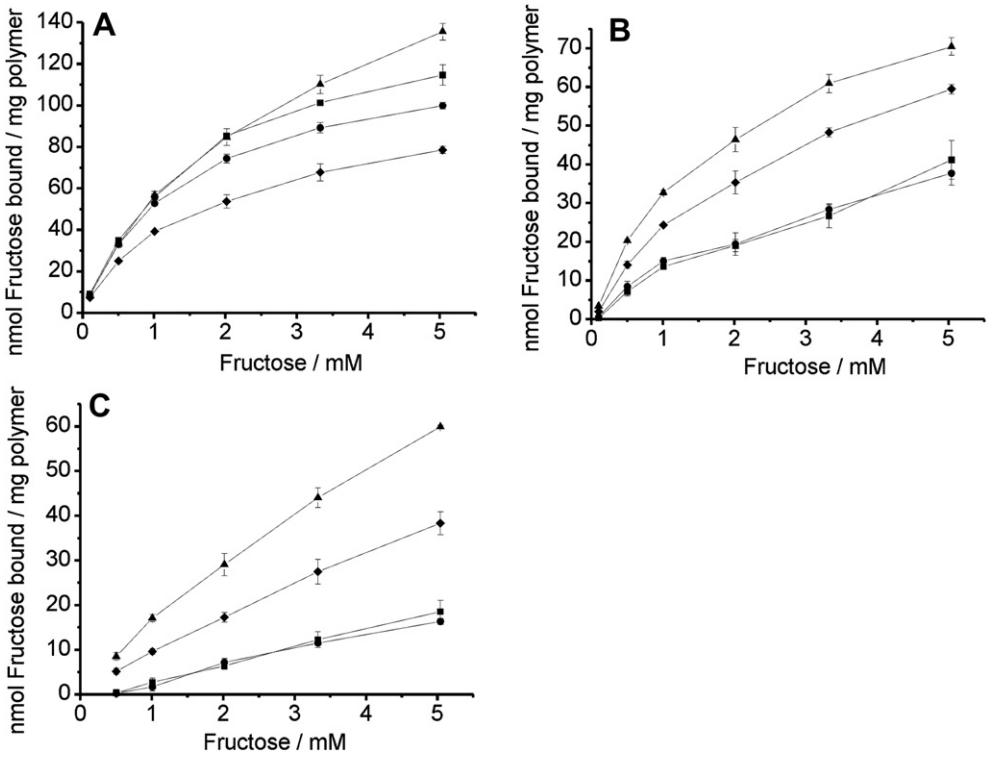


Fig. 4. Batch binding experiments for different fructose binding MIPs at different pH-values. (A–C): Concentration dependency for fructose binding to MIP-BX(Fru) (▲), MIP-BA(Fru) (■), MIP-BX(Pin) (◆) and MIP-BA(Pin) (●); (A): carbonate solution, pH 11.4, 10% MeOH; (B): phosphate buffer, pH 8.7, 10% MeOH; (C): phosphate buffer, pH 7.4, 10% MeOH.

which strongly favors fructose complexation regardless of the boron monomer structure. Consequently, the methylhydroxylgroup in the benzoboroxole derivative plays a negligible role in terms of binding strength. Nevertheless, there is a pronounced difference between the MIP-BX(Pin) and MIP-BA(Pin). In case of the MIPs with vinylphenylboronic acid the difference is (with 15 nmol/mg polymer) comparably small. In contrast, the difference in capacity of the MIP-BX(Fru)and the MIP-BX(Pin) is four times higher (about 60 nmol/mg) showing that imprinting efficiency is higher. One possibility could be that the methylhydroxylgroup increases the affinity due to steric hindrances within the smaller binding pocket obtained for the MIP-BX(Pin).

The binding to the control polymer synthesized just with TRIM as the crosslinker shows negligible binding to fructose (at different pH-values) at all concentrations studied. This is in line with the literature since TRIM is a favored crosslinking agent for the recognition of saccharides. The two times higher pore size of the control polymer shows that the binding of fructose is not dominated by the polymer morphology. Taking into account the lower binding of fructose to the MIP-BX(Pin) it can rather be explained by the presence of boronic acid entities and their right arrangement showing the desired imprinting effect.

The rebinding of fructose at pH 8.7 shows (Fig. 4B) a clear difference between MIP-BX(Fru) and MIP-BA(Fru). The capacity at an initial concentration of 5 mM is about 70 nmol/mg polymer for MIP-BX(Fru) whereas for the MIP-BA(Fru) a binding of only 40 nmol/mg polymer is reached. Here, most likely a mixture between intra- and inter-molecular coordination of the boron is present. Two findings support this assumption. Firstly, the rebound concentration of fructose to the MIP-BX(Fru) is decreasing with decreasing pH-value. This could be due to a lower coordination of hydroxyl ions from the solution to the boron. Secondly, MIP-BX(Fru) exhibits a higher binding capacity compared to MIP-BA(Fru) which shows that an intramolecular coordination of the hydroxyl group to the boron occurs.

Furthermore, in the case of the MIP-BX(Fru) and MIP-BX(Pin) there is still a significant difference between both polymers for fructose binding. The difference in capacity at 5 mM initial fructose concentration is about 12 nmol/mg polymer. The binding isotherms of fructose to the MIP-BA(Fru) and MIP-BA(Pin) at pH 8.7 are almost similar and show no difference.

At pH 7.4 the advantage of intramolecular coordination provided by the benzoboroxole units becomes evident (Fig. 4C). The polymers bearing vinylphenylboronic acid exhibit a small binding ability with just about 18.5 nmol/mg polymer at 5 mM initial fructose concentration and no differentiation between the fructose and pinacol imprint was seen. At this concentration the MIP-BX(Fru) is still able to bind 60 nmol/mg polymer which is a factor of 3.2 compared to the MIP-BA(Fru). The MIP-BX(Pin) binds 40 nmol/mg polymer at 5 mM fructose resulting in a difference of about 20 nmol/mg polymer. The decreasing difference in capacity between the MIP-BX(Fru) and MIP-BX(Pin) with decreasing pH-values underlines the effect of the possible free hydroxyl group in *ortho*-position to the boron which can lead to an additional hydrogen bonding to the saccharide. Since this effect could also be present during the polymerization, it is just possible in the MIP-BX(Fru).

3.4. Cross reactivity

To verify the selectivity, the affinity between boronic acids and different saccharides in solution has to be considered. In general, the affinity is dependent on the pKa of the aryl boronic acid derivative as well as of the structure of the saccharide. Moreover, in case of aqueous environment the tautomeric form of the saccharide is also one of the key factors regarding binding strength. The

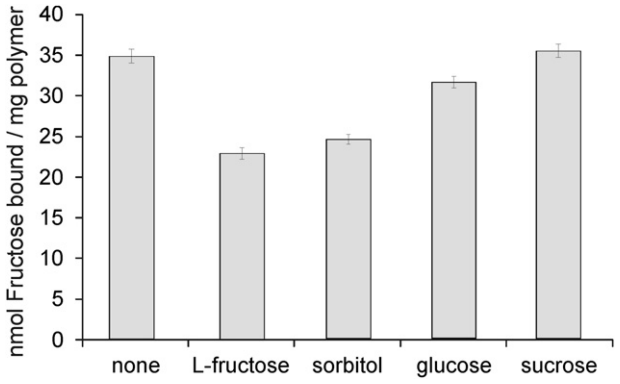


Fig. 5. D-fructose binding in presence of competitors at equimolar concentration.

binding strength of fructose is comparably high due to the high presence of about 25% of the β-D-fructose furanose species (water, 31 °C). The binding to this form is preferred due to the presence of a syn-periplanar hydroxyl pair (C2–C3). Compared to glucose the binding strength is much higher because the binding of the pyranose-isomer (α-D-glucofuranose) is just present with 0.14% in deuterated water at 27 °C. Taking these limitations into account, the cross reactivity was evaluated with L-fructose since the discrimination of enantiomers is described for many different molecular imprints. Beside L-fructose, D-glucose, sucrose and sorbitol were used as competitors (Supporting information; at equimolar concentration shown in Fig. 5). Therefore, the difference of the binding with and without competitor reflects the displacement of fructose showing the degree of cross reactivity.

As expected, the degree of competition is dependent on strength of the saccharide – boronic acid interaction (Fig. 5). D-glucose and sucrose influence the binding of D-fructose to the MIP-BX(Fru) slightly whereas L-fructose and sorbitol have a higher impact. It is noteworthy that the extent of competition between sorbitol and L-fructose are comparable even their binding constants to aryl boronic acids vary by a factor of two. More than 90% of D-fructose is still bound in the presence of glucose or sucrose (more than 95%) as competitors. In contrast, sorbitol and L-fructose act as competitors since less D-fructose could bind to the polymer. 36 nmol fructose per mg polymer are bound without competitor, whereas around 24 nmol fructose are bound with equimolar addition of either L-fructose or sorbitol. Consequently, 2/3 of the possible binding cavities are occupied by D-fructose and just 1/3 by either L-fructose or sorbitol at equimolar concentrations. Moreover, at smaller D-fructose concentrations than the concentration of the competitor also high amounts of D-fructose are bound to the MIP-BX(Fru).

The separation factor α which describes the interaction of a targeted molecule with a stationary phase compared to a competitor gives a quantitative value (equation: [2]). The values for L-fructose and sorbitol are 2 and 2.5 at an equimolar concentration of D-fructose to the competitor, respectively. These values show that L- and D- fructose can be distinguished in a normal batch binding experiment, which is equal to one plate in terms of chromatography. The α-values for D-glucose and sucrose are 10.7 and 71 underlining the small degree of competition with these saccharides.

[2] $\alpha = (n_{\text{bound\_fructose}} \bullet n_{\text{free\_saccharide}})/(n_{\text{free\_fructose}} \bullet n_{\text{bound\_saccharide}})$.

4. Conclusion

For the first time a molecularly imprinted polymer with 5-methacrylamido-2-hydroxymethylphenylboronic acid as functional monomer for the binding of fructose was prepared. In comparison to earlier reports for fructose recognition, the binding pH could be lowered and a favored rebinding in comparison to the MIP-BX(Pin) analog could be observed. It was shown that the MIP-BX can be used for saccharide recognition at pH 7.4 in aqueous environment which was so far not reported for unprotected saccharides such as fructose.

Appendix. Supporting information

Supplementary data related to this article can be found online at doi:10.1016/j.polymer.2011.04.002

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