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Toward an alternative for specific recognition of sulfated sugars. Preparation of highly specific molecular imprinted polymers

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Abstract—Sulfated sugars are a class of complex naturally occurring compounds some of which play central biological roles in mammals. Among them, heparan sulfates are multifunctional cell regulators, whose biological activities are related to their sulfation pattern. Determination of fine structures of these sulfated sugars is a prerequisite for understanding their biological roles. We investigated the applicability of molecular imprinting technology for recognition of the biologically relevant 6-*O*-sulfate substitution on sugars by using glucose-6-*O*-sulfate as model. Our results show that molecular imprinted polymers can specifically recognize sulfated sugars by the introduction of primary amines at the polymer side. Imprinted polymers showed excellent selectivity with regard to the sulfate position, the sugar configuration, and the presence of *N*-acetyl groups. These factors are essential for specific recognition of heparan sulfates' sequences. Molecular imprinting technology promise a significant contribution to the selection of sulfated sugar fragments of biological relevance.

1. Introduction

Sulfated sugars are a class of complex compounds some of which are naturally present in mammals wherein they play important biological roles. The best example of this class of compounds is the glycosaminoglycan (GAG) family of linear polysaccharides that includes heparan sulfates (HS), definitely recognized as a new class of multifunctional cell regulators.[1,2] HS, whose biological activities have been largely related to their specific sulfation patterns, bind to complementary proteins known as 'heparin-binding proteins' (HBP).[3,4] One of the most studied HBP member is the fibroblast growth factor 2 (FGF-2), which possess a mitogenic activity known to be regulated by a specific HS sequence containing 2-*O*- and 6-*O*-sulfated sugars.[5] It is now well established that determination of fine structural characteristics of these complex sulfated sugars is a prerequisite to elucidate the interactions with matrix effective proteins and, therefore, essential in understanding their biological functions.[6–8] Although a number of antibodies that recognize sulfated sugars as HS have already been developed, few is known for their specificity at the fine structural level.[9–11] Furthermore, sequencing, analytical, and chemical methods in this area are extremely laborious and complex, and require approaches that should include the development of new recognition entities that are capable to detect and even quantify particular and well characterized fractions of sulfated sugars in biological extracts.

Molecular imprinting is a technology receiving increasing attention as potential tool for specific molecular recognition. A variety of drug enantiomers, hormones, toxins, pesticides, peptides, proteins, or nucleic acids have been selectively recognized using this technology.[12,13] Molecular imprinting makes possible the introduction of recognition sites into highly cross-linked polymers via template-directed assembly of functionalized monomers in a polymer-forming mixture. This enables the formation of discrete cavities with the precise spatial arrangement of functional groups to provide specific interactions with the template when rebinding.[14,15]

In this report, we investigated the applicability of molecular imprinting technology to the specific recognition of sulfated sugars. To validate the feasibility of this approach, we considered the most extensively studied aspect of relationship between HS fine structure and growth factor signaling that involves 6-O-sulfation for the activation of FGF-2 mitogenic

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activity.[16] Although construction of imprinted polymers has never been reported with sulfated templates, compounds containing sulfonic acid groups have been used as functional monomers to afford non-covalent bond interactions with amine bearing templates.[17,18] It has also been reported that the introduction of amidinium-type groups as functional monomers in imprinted polymers allows the binding of carbonates, phosphate, or phosphonate esters through stoichiometric non-covalent interactions.[19,20] Here, we show that molecular imprinted polymers (MIPs) can be engineered to specifically recognize sulfated sugars by the introduction of primary amines in the polymer with excellent selectivity against HS related sugars with regard to the position of the sulfate group, the isomeric sugar configuration, and the presence of *N*-acetyl groups. These factors are essential for selective and specific recognitions of HS key sequences.

2. Results and discussion

Several biological activities of sulfated sugars have been related to their sulfation pattern.[3,4] Since the effect of polyamines on blood coagulation and fibrinolysis in the presence of GAG was recently demonstrated based on the interaction of heparin with spermine,[21] we assumed that negative charges on this sulfated polysaccharide could efficiently be complemented by positively charged amines. On these basis, a series of novel MIPs designed for selective glucose-6-*O*-sulfate (Glc-6S) recognition and their related controls, namely non-imprinted polymers (NIPs), were prepared according to the composition showed in Table 1.

Table 1. Monomer/template mixture compositions (mmol) used to prepare Glc-6S imprinted polymers in DMSO. Corresponding non-imprinted polymers were prepared under the same conditions but excluding Glc-6S from the reaction mixture

Polymer	Glc-6S	VBTA (R_4N^+)	DEM (R_3N)	AEM (RNH_2)	IMA (RCONHR)	MAM ($RCONH_2$)	EGDMA
MIP-1	0.1	0.1	—	—	—	0.7	4.0
MIP-2	0.1	0.1	—	—	—	0.7	2.0
MIP-3	0.1	—	0.1	—	—	0.7	4.0
MIP-4	0.1	—	—	0.1	—	0.7	4.0
MIP-5	0.1	—	—	—	0.1	0.7	4.0

The sulfate group on Glc-6S was targeted by a non-covalent complexation with different amine bearing acrylates including the quaternary amine (ar-vinylbenzyl)trimethylammonium chloride (VBTA), the tertiary amine 2-(dimethylamino)ethyl methacrylate (DEM), or the primary amine 2-aminoethyl methacrylate (AEM). Although use of a single functional monomer is the simplest approach to non-covalent imprinting, it has been assumed that using a combination of them gives MIPs the possibility to better interact with the template leading to functional receptor sites.[14] Thus, we considered the additional introduction of the neutral monomer methacrylamide (MAM) to provide, in addition to sulfate–amine interactions, a supplementary binding via hydrogen bonding between hydroxyl and amide groups. In a control experiment, we tested the inability of amides alone to form efficient Glc-6S recognition sites by using a combination of MAM and *N*-isopropylmethacrylamide (IMA).

Selection of ethylene glycol dimethacrylate (EGDMA) as cross-linker was based on previously reported anomeric and epimeric recognitions of non-sulfated sugars obtained by molecular imprinting,[22] while selection of DMSO as solvent and porogen was a compromise between reagents solubility and the polarity required to promote interaction.[18] It has been well established that in non-covalent imprinting a complex and highly dynamic interaction system is formed between templates and functional monomers producing a high heterogeneity of receptor sites.[23] Hence, the multifunctional character of sulfated sugars must allow the formation of multiple interaction sites increasing the complexity on the reaction mixture during polymerization. Likewise, the proportion between templates and functional monomers should also be an important parameter to system complexity.

To first compare the capacity of the different nitrogen bearing groups to efficiently bind sulfated sugars, we used the amine-functionalized monomers in stoichiometric proportions to the Glc-6S template. **MIP-1** and **MIP-2** were synthesized with the quaternary ammonium salt VBTA, **MIP-3** with the tertiary amine DEM, and **MIP-4** with the primary amine AEM. As stated above, MAM was added into the reaction mixtures in excess. Hence, to test the ability of amides to form efficient Glc-6S recognition sites, **MIP-5** was prepared as a control with the secondary amide IMA in the presence of MAM. Formation of highly cross-linked polymers was assured by EGDMA incorporation at more than 80% of all polymerizable components corresponding to 40 equiv relative to template,[14,24] with exception of **MIP-2**, prepared with only 20 equiv. Reactions were carried at 50 °C by free-radical polymerization with 2,2′-azobis(isobutyronitrile) (AIBN) as initiator (see Section 4).

Once washed to eliminate the template from the formed cavities, synthesized MIPs and NIPs were tested for their capacities to bind Glc-6S. Binding tests were performed in equilibrium conditions (see Section 4) with 1 mL of Glc-6S solution (1 mg/mL, 3.3 mM) in the presence of increasing amounts of the corresponding polymer in different milieus including DMSO, pure water, acidified or basified water (pH 3, 6, and 9), and in an ammonium acetate buffer (pH 5). Significant and highly specific Glc-6S rebinding capabilities were only detected with **MIP-4**, which contains a primary amine in the polymeric matrix. This polymer bound 80% of template in DMSO when 125 mg of polymer mass was used while the corresponding control **NIP-4** did not show any appreciable binding over all the range of polymer mass essayed. Moreover, when experiments took place at different pHs in aqueous environments, neither **MIP-4** nor **NIP-4** bound to the template. On the other hand, **MIP-1** and **NIP-1**, containing a quaternary ammonium salt in the polymeric matrix, showed high but non-specific binding since both, MIP and NIP, presented exactly the same affinity to bind with the template. This avidity was strongly diminished in all aqueous media. These results reveal that use of quaternary ammonium salts for sulfated sugar complexation favors non-specific binding. Very low or no binding was observed in acetate buffer or in basified water suggesting a competition between the different anionic species (sulfate, acetate, or hydroxide group) for interaction with the cationic salt. Similarly, competition might exist in acidic conditions between the positive charges at the polymer side and the free

ammonium ion or the free protons in the acidic solution. Accordingly, **MIP-3**, prepared with a tertiary amine, completely failed to display any affinity for the template neither in DMSO nor in any of the different pH aqueous solutions essayed. Thus, the ammonium salt would be responsible for non-specific binding in DMSO while in aqueous environments, even if the amine becomes charged, competition with other ions would avoid this binding. Moreover, this data show the incapacity of the amide function to afford detectable template binding. This was clearly confirmed with polymers **MIP-5** and **NIP-5**, prepared with the amides IMA and MAM, by the absence of binding observed in all the solvents assayed. Besides, reduction of the cross-linker participation to 40% in **MIP-2** resulted in the total elimination of non-specific binding.

These results indicate that only the primary amine moiety at the polymer side allows convenient formation of specific Glc-6S recognition sites on the **MIP-4** cavities. Table 2 shows ^{1}H NMR chemical shifts of the AEM amine protons in the presence of various sugars.

Table 2. Chemical shifts (δ and $\Delta\delta$) of the AEM amine protons after addition of different sugars. Effect of temperature on the AEM/Glc-6S complex (300 MHz, DMSO-d_6)

Added sugar[a]	T (°C)	δ (ppm)	$\Delta\delta$
None	25	8.25	—
Glucose (1.0 equiv)	25	8.25	0.00
Glucuronic acid (1.0 equiv)	25	8.25	0.00
Glc-6S (0.4 equiv)	25	8.08	−0.17
Glc-6S (0.2 equiv)	25	8.15	−0.10
Glc-6S (0.2 equiv)	35	8.13	−0.12
Glc-6S (0.2 equiv)	45	8.11	−0.14

[a] Prior to analysis, each sugar was added to a 80 mM solution of AEM at the indicated stoichiometry.

At the used concentrations, only Glc-6S was able to complex the AEM protons indicating that the sulfate group is responsible for the interaction between these compounds in DMSO. Based on these results, we first assumed the formation of a Schiff base between the primary amine and the non-reducing carbon on the sugar side. However, any imine signal could be observed by ^{1}H and ^{13}C NMR studies realized under the reaction conditions but in the absence of the initiator. Moreover, when experiments took place at different pHs in aqueous environments, neither **MIP-4** nor **NIP-4** bound to the template. This is in contradiction with the known formation of the Schiff base in aqueous media. Thus, the resulted binding and the chemical shift variations of the amine proton on the Glc-6S/AEM complex (Table 2) indicate that the MIP–template interaction might be done through hydrogen bonding as represented in Figure 1.

Moreover, when water was added to the NMR tube containing the sugar/AEM complex, the amine proton signal became very broad (result not shown). Since specific binding of Glc-6S to **MIP-4** occurred only in DMSO and not in any aqueous media it can be admitted that water readily competes with hydrogen bonding formation between the sulfate and the primary amine. This observation is in agreement with the molecular modeling docking studies performed with HS monosaccharides and endostatin. In these studies, the presence of hydrogen bonds between the sulfate groups

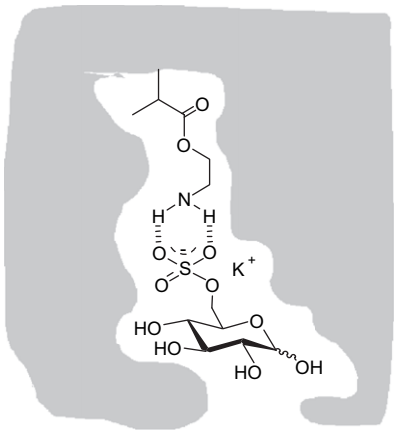

Figure 1. Schematic representation of the main interaction between Glc-6S and the primary amine from AEM in DMSO. Under these conditions, the amine in **MIP-4** cavities should be conveniently positioned to bind the sulfate group of the template without ionic or steric hindrance.

at the monosaccharide side and the amino groups on the arginine residues was established.[25]

After selection of AEM as the functional monomer enabling specific recognition of the sulfated sugar we examined the influence of MAM addition on the efficacy of imprinted polymers to selectively recognize Glc-6S. It is known that the extent of specific recognition of polyfunctionalized templates can be improved by adapting the proportion of functionalized monomers on MIPs.[23] Thus, a series of MIPs and their respective NIPs were prepared. As considered before, the molar proportion of EGDMA was conserved at about 80% of molar total polymerizable components. This results in the variation of the template/polymer ratio as seen in Table 3. Under these conditions, and taking as a reference **MIP-4**, enlarged contributions of AEM (**MIP-6** and **MIP-7**) and suppression of the co-monomer MAM (**MIP-8** and **MIP-9**) were studied.

Titration experiments with increasing amounts of these polymers and a fixed amount of Glc-6S (1 mg/mL, 3.3 mM) were conducted in DMSO, water, or ammonium acetate buffer (pH 5). As expected, DMSO was the only solvent where all MIPs showed clear template binding with binding isotherms exhibiting dissimilar shapes as shown in Figure 2a.

Under the test conditions, none of the control NIPs bound to the template. Compared to polymer **MIP-4**, prepared by using 1 equiv of AEM, an apparent decrease in binding was observed with **MIP-6** and **MIP-7**, respectively, prepared

Table 3. Monomer/template mixtures (mmol) used to prepare imprinted polymers of Glc-6S in DMSO using AEM as functional monomer

Polymer	Glc-6S	AEM	MAM	EGDMA[a]	Template/polymer[b]
MIP-4	0.1	0.1	0.7	4.0	0.11
MIP-6	0.1	0.4	0.7	5.4	0.08
MIP-7	0.1	0.8	0.7	7.3	0.06
MIP-8	0.1	0.1	—	0.5	0.85
MIP-9	0.1	0.4	—	2.0	0.22

[a] EGDMA molar proportion with regard to all polymerizable components was 83%.
[b] The ratio template par milligram of polymer is expressed in μmol/mg.

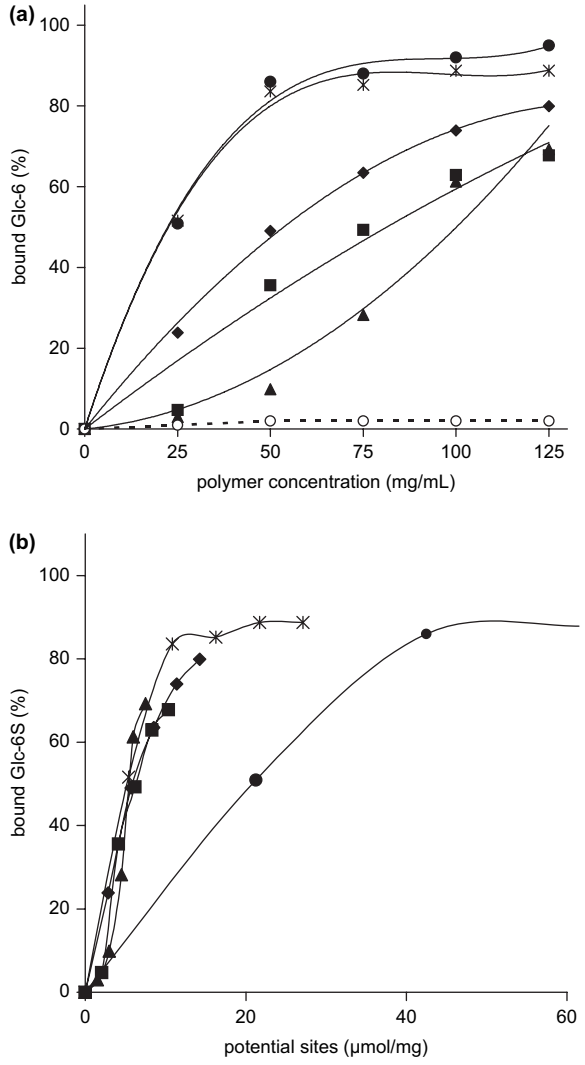

Figure 2. Binding properties of polymers **4-6-7-8-9**. (a) Binding isotherms of Glc-6S are represented as a function of polymer concentration. (b) Glc-6S bound to the different polymers is represented as a function of the total number of potential sites in the polymer used for binding experiments. **MIP-4** (diamonds), **MIP-6** (squares) **MIP-7** (triangles), **MIP-8** (circles), **MIP-9** (asterisks), and **NIP-4-6-7-8-9** (empty circles).

with 4 and 8 equiv of amine. Binding seemed to increase when MAM was eliminated from the MIP composition (**MIP-8** and **MIP-9**). This is reflected in the polymer concentration required to adsorb 50% (P_{50}) of Glc-6S, whereas only 25 mg of **MIP-8** and **MIP-9** were needed to adsorb 50% of template, the corresponding values for **MIP-4**, **MIP-6**, and **MIP-7** were, respectively, 50, 75, and 85 mg (Fig. 2a). **MIP-9** exhibited a slight decrease in rebinding with respect to **MIP-8**, which is in agreement with the results found above when increasing the number of equivalents of the amine AEM in the polymer composition.

However, with the exception of **MIP-8**, all MIPs showed similar responses when the bound Glc-6S was plotted against the total number of potential sites in the mass of polymer used (Fig. 2b). This number of potential sites was obtained as the product of the polymer mass used for the binding test and the number of micromole of template per milligram of polymer determined as given in Table 3. This amount corresponds to the theoretical maximum of binding sites formed during polymerization considering that one template molecule is able to form one recognition site. Thus, compared to other MIPs, each potential individual binding site in **MIP-8** are less active (Fig. 2b). This is in agreement with the non-covalent character of the pre-polymerization mixture and with the assumption that a combination of functional monomers allows the formation of receptor like sites.[14] However, and although **MIP-8** presented lower capacity relative to the number of theoretical sites, the overall capacity per milligram of polymer was highest, as seen in Figure 2a, as it contains more sites per mass unit. The enhanced binding of Glc-6S by **MIP-8** over the other polymers was confirmed by the binding isotherms of the sulfated sugar (1.34–6.70 mM, DMSO) recorded at the corresponding polymers P_{50} (Fig. 3). A clearly increased binding ability is displayed by **MIP-8** and **MIP-9** since they needed 2–3.4 times less polymer for binding nearly three folds more template, consequently they have an about 10 times higher capacity.

Concerning the selectivity, it has been recognized that one particular difficulty associated to HS like sugars' recognition will certainly be related to their regioselective rebinding. Thus, the recognition particles prepared with the molecular imprinting technology are expected to discriminate, to some extent, different sugar moieties, in particular glucosamine and galactosamine derivatives, main constitutive isomers of the different GAGs (HS and chondroitin sulfate, respectively). Selectivity based on the sulfate group position was obviously considered of importance as well as the discriminatory recognition of other substitutions as the *N*-acetyl group at the C-2 position, are also present in these natural sulfated sugars. On these basis, binding experiments were performed in DMSO using **MIP-4**, **MIPs-6** to **-9**, and their corresponding NIPs with galactose-6-sulfate (Gal-6S), glucose-3-sulfate (Glc-3S), *N*-acetyl-glucosamine-6-sulfate (GlcNAc-6S), and glucose (Fig. 4).

As expected, all MIPs prepared for Glc-6S recognition showed negligible affinities for Glc-3S, Gal-6S, or GlcNAc-6S (Fig. 5). Interestingly, only **MIP-9** bound to Glc-3S but with lower affinity than the template (Fig. 5b). This indicates that an increased participation of functional monomer AEM (4 equiv) and the absence of co-monomer MAM in the polymer constitution of **MIP-9** induce a reduced discrimination of sugar derivatives. On the other hand, the

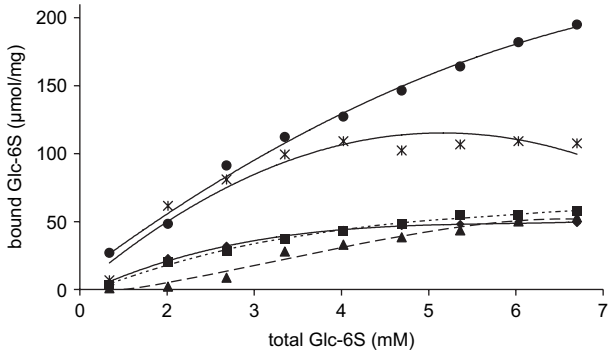

Figure 3. Binding isotherms of Glc-6S as a function of Glc-6S concentration to P_{50} mass of polymers. **MIP-4** (diamonds), **MIP-6** (squares), **MIP-7** (triangles), **MIP-8** (circles) and **MIP-9** (asterisks).

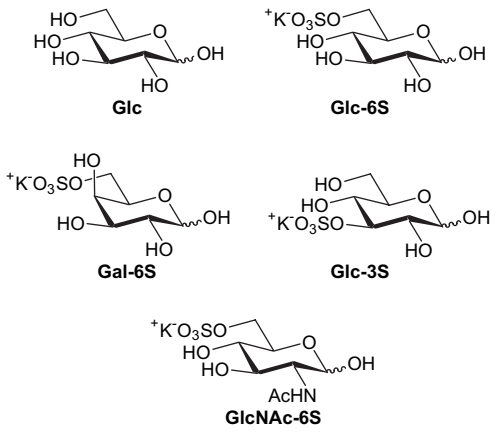

Figure 4. Structure of Glc-6S and related saccharides used to evaluate the selectivity of MIPs.

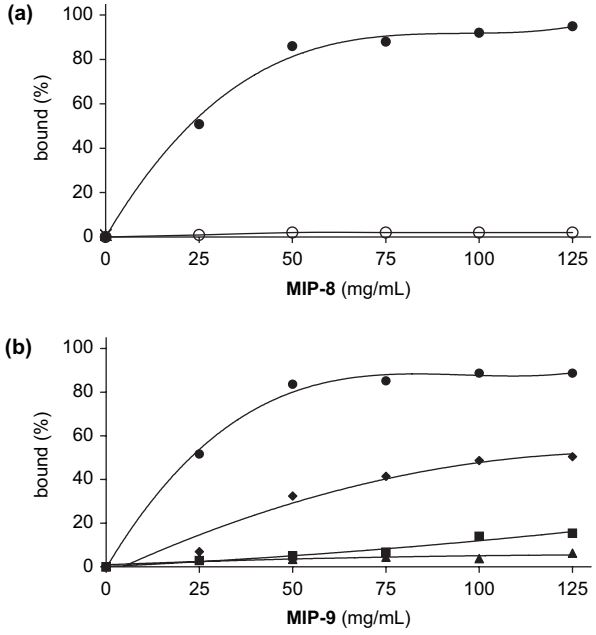

Figure 5. Binding isotherms of **MIP-8** and **MIP-9** with different sugars. (a) **MIP-8** binds Glc-6S (circles), Glc-3S, Gal-6S, or GlcNAc-6S (empty circles). (b) **MIP-9** binds Glc-6S (circles), Glc-3S (diamonds), Gal-6S (squares), or GlcNAc-6S (triangles).

presence of MAM (**MIP-5** to **7**) or a stoichiometric contribution of AEM (**MIP-8**) seems to enhance the differences in binding among the saccharides.

The results suggest that the binding sites formed in the polymer matrix discard same-sized Glc-6S isomers as Glc-3S or Gal-6S, as well as other larger analogs (GlcNAc-6S). At the same time, these binding sites still presented adequate cavities for smaller molecules, like glucose.

3. Conclusions

Our results show that MIPs could readily be prepared to specifically recognize Glc-6S with discriminating selectivity against its isomers and related molecules. Contrarily to our initial assumption that the use of permanently charged monomers would allow the formation of specific imprinted sites for sulfated sugars, it appeared that hydrogen bonding interactions between the sugar sulfate groups and the primary amine groups in the polymer side were the key element for specific binding in DMSO as a solvent. Addition of an amide bearing functional monomer MAM into the pre-polymerization mixture did not affect the template binding ability of most MIPs, with only one exception concerning **MIP-8** in which a decrease of binding by potential template binding site was observed. Moreover, the excellent regioselectivity obtained with structurally related sugars, shows that specific MIPs can be generated with highly selective recognition sites with regard to the position of the sulfate group, the isomeric sugar form, and the absence of recognition of *N*-acetylated sugars. These factors are essential for the construction of recognition entities that are able to selectively and specifically recognize HS related sugars. The high water solubility of these types of compounds, imprinting and recognition studies in aqueous media could also be considered. Molecular imprinting technology has the potential to significantly contribute to the selection of HS fragments with identified biological activities. We are currently working in this area.

4. Experimental section

4.1. General

(Vinylbenzyl)trimethyl-ammonium chloride (VBTA), 2-(dimethylamino)ethyl methacrylate (DEM), 2-aminoethyl methacrylate (AEM), methacrylamide (MAM), *N*-isopropylmethacrylamide (IMA), and ethylene glycol dimethacrylate (EGDMA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMSO was obtain from Fischer and 2,2′-azobis(isobutyronitrile) (AIBN) from Fluka (Fluka–Sigma–Aldrich, St. Louis, MO, USA). Sodium acetate, acetic acid, and all other reagents were of the highest analytical grade from Merck (Darmstadt, Germany). Deionized H_2O was prepared before use in a milliQ system (Waters, USA). Polymerization reactions were carried out in an electric oven (Fisher Scientific Labosi, France). Temperature inside the oven was controlled by a digital temperature controller. NMR spectroscopic data were colleted and recorded on a Brucker DMX300 spectrometer. The HPLC pump system used was from Knauer (Berlin, Germany). Aqueous mobile phases were filtered through cellulose 0.22 μm filter (Millipore, USA).

4.2. Typical procedure of polymer synthesis

4.2.1. Preparation of MIP-4. Potassium D-glucose-6-sulfate of 0.03 g (0.10 mmol), 0.60 g of MAM (0.70 mmol), 0.18 g (0.10 mmol) of AEM (hydrochloride, 90%), 0.76 mL of EGDMA (4.00 mmol), and 8.21 mg (0.05 mmol) of AIBN were placed in a glass tube and dissolved in 1.16 mL of DMSO. The solution was then degassed with nitrogen for 15 min, and the tube was sealed and heated at 50 °C for 24 h. After polymerization, the bulk polymer was wet-crushed in water with a mortar and pestle. The milled polymer was washed widely on a G4-glass filter funnel with acetone (200 mL), methanol (200 mL), and deionized H_2O (200 mL). Then the template was extracted with water until it could no longer be detected in the washing solution by

HPLC. Thereafter, the fine polymer particles were dried at 40 °C under vacuum. Control non-imprinted polymer (NIP-4) was prepared in the same way without the addition of the template; 0.84 g of MIP-4 (97% yield) and 0.85 g of NIP-4 (98% yield) were obtained.

4.3. Binding experiments

Saturation studies of the MIP polymer particles were carried out to estimate their binding capacity. Increasing amounts of polymer (25, 50, 75, 100, and 125 mg) were incubated on a rocking table with 1 mg/mL (3.3 mM) solution of analyte (in DMSO, pure water, basified water, acidified water, or ammonium acetate buffer pH 5) and allowed to reach equilibrium. After 24 h, the particles were sedimented by centrifugation and the supernatants were analyzed by HPLC system including a ProPac PA1 Analytical Column (Dionex) eluted with NH_4OAc (0.1 M, pH 5) at a flow rate of 0.5 mL/min and detected with an Evaporative Light Scattering system (Chromachem®, France). The amount of analyte bound to the polymer was determined by subtracting the peak area of the unbound analyte to the peak area of the standard solution under the limits of a calibration curve (1.34–6.70 mM, r^2=0.998).

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