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Molecular imprinting technology for specific recognition of heparan sulfate like disaccharides

Dominique Singabraya [a], Laurent Bultel [b], Fernando Siñeriz [c], Mouna Mothéré [a], David Lesur [b], José Kovensky [b,*], Dulce Papy-Garcia [a,**]

[a] Laboratoire CRRET EAC 7149, Université Paris Est Créteil, 61, Av. du Général de Gaulle, 94010 Créteil Cedex, France
[b] Laboratoire des Glucides, FRE 3517, Université de Picardie, 33 Rue Saint-Leu, 80039 Amiens Cedex, France
[c] OTR3 SAS, 4, Rue Française, 75001 Paris, France

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ABSTRACT

Iinteractions of biologically active proteins with sulfated glycans, particularly heparan sulfates (HS), are dependent on factors involving amounts and positions of the sulfate groups in the sugars chains. Although the importance of knowing the exact positions of the sulfate groups in particular HS sequences is well recognized, at present, approaches in this area are complex and still considered as a challenge. Here, we investigated the applicability of the 'Molecular Imprinting Technology' for the generation of imprinted polymers able to specifically recognize a model HS-like disaccharide. In order to advance on the applicability of this technology to the recognition of these complex sugars, we prepared a library of imprinted polymers to investigate the impact of the polymerization reaction conditions and stoichiometry on the generation of binding sites able to specifically recognize the model sulfated sugar. Our results show that imprinted polymers able to specifically bind HS-like saccharide can readily be obtained. This constitutes a suitable option for developing novel strategies directed to study fine sulfated sugars structures.

1. Introduction

During the last decades, substantial efforts have been directed to the development of new technologies or strategies allowing the study of sulfated glycans because of accumulated evidences showing the importance of these complex molecules in the regulation of a large number of biological processes [1,2]. However, even if huge advances have been possible in this area, heparan sulfates (HS) remains as the most important saccharides for which existing technologies are not yet sufficient to depict, in an accessible approach, the structural information that they contain [3]. HS are highly complex glycans formed of repeating disaccharide building blocks made of a glucuronic acid (GlcA) linked to an *N*-acetyl glucosamine (GlcNAc). The presence of sulfate groups irregularly positioned in the several hydroxyl and amino groups on the sugar backbone results in a considerable diversity of structures that distinctively and specifically interact and regulate the activity of several proteins, known as 'heparin binding proteins' (HBP) [4]. Because the fine structural characterization of HS sequences is essential to understand the biological mechanisms of action of these compounds [5], a number of technologies for glycans structural analysis, as disaccharide compositional analysis and/or various spectrometric assisted sequencing strategies, have been developed. However, all these methodologies are extremely laborious, complex and limited by the requirement of highly purified HS fragments hard to be obtained from biological fluids or tissues [1,6–9]. In the other side, a number of anti-HS antibodies have been developed, but the poor characterization of the sulfated sequences that they recognize limits the retrieval of fine structural data [10–14]. Thus, it is accepted that advancing in this area requires new approaches that should include the development of new materials able to recognize specific HS structures.

Molecular imprinting is a technology that makes possible the construction of cavities shaped to specifically bind a target molecule, called template, inside a polymeric material called MIP, for 'Molecular Imprinted Polymer'. These cavities are formed by polymerization of functional monomers and cross-linkers around the target molecule, which, after been extracted from the resulting polymer, provides template shaped sites for specific rebinding [15–18]. Interestingly, the interactions between the functional monomer and the template can be non-covalent, allowing versatility, fast, and reversible binding of the template [19]. Moreover, this technology allows straightforward and inexpensive production of polymers characterized by very good thermal and mechanical

* Corresponding author. Tel.: +33 322827567; fax: +33 322827568.
** Corresponding author. Tel.: +33 145177081; fax: +33 145171816.
E-mail addresses: jose.kovensky@u-picardie.fr (J. Kovensky), papy@u-pec.fr (D. Papy-Garcia).

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stabilities [20]. Thus, MIPs have been successfully used for separation strategies in chromatography or solid-phase extraction [21,22], in reaction catalysis [23], and as biomimetic sensors [24,25].

Because of the potential value of the Molecular Imprinting Technology in the glycobiology domain, we recently proposed the applicability of imprinted polymers to the specific recognition of HS-like sugars based on data showing that specific and selective recognition of simple monosulfated sugars was possible by using 2-aminoethyl methacrylate (*AEM*) as a functional monomer on the polymer side [26]. However, when a sulfated disaccharide was used as template, its specific recognition failed and very modest specificity was only observed by using MIP post-synthetic strategies [27]. The non recognition observed in that study could be due (i) to a non optimization of the polymer synthesis, limited by the low availability of template, and/or (ii) to the presence of a carboxylate in the template structure, which recognition conditions should further be studied. Here, we focused our attention to investigate if modulating the stoechiometry of functional monomers and cross-linkers during the polymer synthesis could result in the production of MIPs able to directly and specifically recognize a template disaccharide possessing, at this stage, only sulfate groups. We thus synthesized the novel methyl 6,6′-di-O-sulfonato-β-D-maltoside (*MSGG*) as a model disaccharide template. This compound was chosen because of its straightforward and simple synthesis able to render the product available at the gram scale required for the research of optimal conditions for MIPs synthesis and template rebinding. *MSGG* was used for the synthesis of a library of MIPs and of their corresponding 'Non Imprinted Polymers' (NIPs). Studies on various reaction conditions finally showed that controlling the reagents steochiometry during the polymerization reaction can profoundly affect the polymers recognition capabilities and that efficient MIPs can effectively be obtained under optimized conditions. This reinforces the interest of the imprinting technology as a potential tool in glycomics and points out the importance of fine investigations required for the efficient preparation of these tools.

2. Material and methods

Maltose, glucose-3-O-sulfate, 2-aminoethyl methacrylate (AEM), ethylene glycol dimethacrylate (EGDMA), azobisisobutyronitrile (AIBN), and crystalline phenol were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Saccharose, sodium hydroxide and sulfuric acid were from Labosi/Thermo Fischer Scientific (Illkirch, France). Sodium acetate and all solvents were from Prolabo VWR International (Fontenay sous Bois, France). HS disaccharide α-ΔUA2S-[1→4]-GlcNS6S and α-ΔUA-[1→4]-GlcNAc6S were purchased from Dextra laboratories (Reading, UK). H_2O employed was milliQ quality (Millipore SAS, France). For synthetic procedures, TLC was carried out on pre-coated aluminum plates (0.1 mm) of silica gel 60F-254; detection was performed by exposure to UV light and by spraying the plate with 5% (v/v) H_2SO_4 in EtOH followed by heating. All reactions were carried under nitrogen atmosphere unless other indication. For ^1H, ^{13}C nuclear magnetic resonance (NMR) spectra, chemical-shifts are reported in parts per million relative to tetramethylsilane or a residual solvent peak ($CHCl_3$: ^1H: $\delta = 7.26$ ppm, ^{13}C: $\delta = 77.2$ ppm). Assignments of ^1H and ^{13}C were assisted by 2D ^1H COSY and 2D ^1H–^{13}C CORR experiments. High resolution mass spectra HRMS where obtained by Electrospray Ionization (ESI). Optical rotations were measured in a 1 cm cell in the stated solvent; $[\alpha]_D$ values are given in $10^{-1}{}^\circ\,cm^2\,g^{-1}$ (concentration *c* given as g/100 mL).

2.1. Synthesis of template

Methyl 2,2′,3,3′,4′-penta-O-benzyl-β-D-maltoside (**2**). Trityl chloride (16.4 g, 59 mmol) was added to a solution of methyl β-D-maltoside (1) [28,29] (10 g, 28 mmol) in pyridine (300 mL) and the reaction mixture was stirred for 12 hours (h) at 80 °C. The solvent was then evaporated and the residue dissolved in CH_2Cl_2 (200 mL). Water (200 mL) was added and the organic phase was separated, dried with Na_2SO_4 and concentrated to sirup. The sirup was dissolved in DMF (125 mL) and added drop-wise to a suspension of sodium hydride (4.76 g, 119 mmol) in dry DMF (300 mL) previously cooled at 4 °C. Benzyl bromide (BnBr, 14.1 mL, 119 mmol) was slowly added to the reaction mixture. After stirring during 12 h at room temperature (rt), MeOH (15 mL) was added and the mixture was concentrated to a sirup. The sirup was dissolved in 95:5 CH_2Cl_2–water (200 mL) and a trifluoroacetic acid solution (10% in CH_2Cl_2, 200 mL) was added. After stirring 20 min at rt, the reaction mixture was diluted with CH_2Cl_2 and treated with saturated aqueous $NaHCO_3$ (400 mL). The organic phase was separated and washed with water, dried ($MgSO_4$) and concentrated to a sirup. Silica gel chromatography (1:1 AcOEt–cyclohexane) afforded disaccharide 2 (12.5 g, 55% yield) as a colorless sirup: $[\alpha]_D^{20} = +30$ (*c* 0.1, acetone). ^1H RMN ($CDCl_3$): δ 7.29 (m, 25H, Ph), 5.81 (d, 1H, $J_{1',2'}$ 3.5 Hz, H-1′), 5.08–4.56 (m, 10H, CH_2Ph), 4.44 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1), 4.17 (t, 1H, $J_{4,5}$ 8.7 Hz, H-4), 4.00 (t, 1H, $J_{3',4'}$ 9.6Hz, H-3′), 3.96 (m, 3H, H-6, H-6a′), 3.89 (t, 1H, $J_{3,4}$ 8.7 Hz, H-3), 3.81 (m, 1H, H-5′), 3.70 (m, 1H, H-6b′), 3.63 (s, 1H, OCH_3), 3.54 (m, 3H, H-5, H-2, H-2′), 3.48 (t, 1H, $J_{4',5'}$ 9.6 Hz, H-4′). ^{13}C RMN ($CDC1_3$): δ 138.8–137.9 (C ipso), 128.5–126.5 (Ph), 104.7 (C-1), 96.8 (C-1′), 84.7 (C-3), 82.4 (C-2), 81.9 (C-3′), 79.3 (C-2′), 78.2 (C-4′), 75.5, 75.2 (CH_2Ph), 74.6 (C-5, CH_2Ph), 73.9, 73.4 (CH_2Ph), 72.3 (C-5′), 71.4 (C-4′), 62.1 (C-6′), 61.3 (C-6), 57.2 (OCH_3). HRMS: Calcd for $C_{48}H_{54}O_{11}Na$ $[M+Na]^+$: m/z 829.3564; found: 829.3565.

Methyl 6,6′-di-O-sulfonato-β-D-maltoside, disodium salt (*MSGG*). To a solution of disaccharide **2** (5.3 g, 6.57 mmol) in dry DMF (200 mL), SO_3–NMe_3 complex (9.5 g, 65.7 mmol) was added. After 48 h at 55 °C, the solvent was evaporated and the crude product was purified on a Sephadex G-25 M column eluted with MeOH–CH_2Cl_2 (1:1) to give a sirup that was dissolved in 9:1 MeOH–water (20 mL). $Pd(OH)_2$ (700 mg) was then added and the reaction mixture was stirred 24 h under H_2 atmosphere (50 bar), the mixture was filtered and then stirred with an Amberlite IR120 (Na^+) resin for 30 min. After filtration, the solvent was evaporated to afford the disulfated disaccharide *MSGG* (3.5 g, 95% yield) as a foam: $[\alpha]_D^{20} = +37$ (*c* 0.4, water). ^1H RMN (CD_3OD): δ 5.42 (d, 1H, $J_{1',2'}$ 3.0 Hz, H-1′), 4.34 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1), 4.25 (dd, 2H, $J_{5',6'b}$ 1 Hz, $J_{6'a,6'b}$ 11.8 Hz, H-6'a and H-6'b), 4.14 (dd, 2H, $J_{5,6b}$ 3.9 Hz, $J_{6a,6b}$ 11.4 Hz, H-6a and H-6b), 3.82 (t, 1H, $J_{3',4'}$ 9.6 Hz, H-3′), 3.66 (m, 4H, H-4′, H-5′, H-3, H-5), 3.49 (m, 5H, H-4, OCH_3, H-2′), 3.23 (t, 1H, $J_{2,3}$ 8.3 Hz, H-2). ^{13}C RMN (CD_3OD): δ 103.0 (C-1), 98.9 (C-1′), 76.2 (C-3), 75.4 (C-4′), 72.9 (C-2), 72.5 (C-5), 72.3 (C-5′), 71.4 (C-4), 70.5 (C-3′), 68.6 (C-2′), 66.9 (C-6), 66.4 (C-6′), 57.2 (OCH_3). HRMS: Calcd for $C_{13}H_{23}O_{17}S_2$ $[M-H]^-$: m/z 515.0377; found: 515.0374.

2.2. Synthesis of MIPs and NIPs

Several MIPs (*MIP-1A* to *-8H*) and NIPs were prepared by following reaction stoichiometry and conditions (Table 1). In a typical procedure, *MIP-2A* was prepared by first dissolving 4.7 mg of the disulfated disaccharide *MSGG* (8.4 μmol) and 2.8 mg of the functional monomer AEM (16.9 μmol) 4.7 mL of DMSO and 8 μL of cross-linker EGDMA (105.8 μmol) in a glass tube. After complete dissolution of the solid reagents, 0.1 mg of AIBN (148 μmol, 10 μL of a 10 mg/mL DMSO solution) were added

Table 1
Monomer compositions (mM) of imprinted polymers of template in DMSO.

Polymer	[*E*/*A*][a] [*E*/*E*+*A*][b]	**A** 2.5 (71.4%)	**B** 3.0 (75.0%)	**C** 3.5 (77.8%)	**D** 4.0 (80.0%)	**E** 4.7 (82.6%)	**F** 6.0 (85.7%)	**G** 7.0 (87.5%)	**H** 9.0 (90.0%)
	AEM (mM)	**EGDMA** (mM)							
NIP[c]	3.6	9.0	10.8	12.6	14.4	17.1	21.6		
MIP-1	3.6	9.0	10.8	12.6	14.4	17.1	21.6	25.2	32.4
MIP-2	9.0	22.5	27.0	31.5	36.0	42.8	54.0	63.0	81.0
MIP-3	18.0	45.0	54.0	63.0	72.0	85.5	108.0	126.0	162.0
MIP-4	27.0	67.5	81.0	94.5	108.0	128.3	162.0	189.0	243.0
MIP-5	36.0	90.0	108.0	126.0	144.0	171.0	216.0	252.0	324.0
MIP-6	45.0	112.5	135.0	157.5	180.0	213.8	270.0	315.0	405.0
MIP-7	54.0	135.0	162.0	189.0	216.0	256.5	324.0	378.0	486.0
MIP-8	63.0	157.5	189.0	220.5	252.0	299.3	378.0	441.0	567.0

Template (*MSGG*, 1.8 mM); functional monomer, 2-aminoethyl methacrylate (*AEM*); cross-linking monomer, ethylenglycoldimethacrylate (*EGDMA*). Reactions were carried out in DMSO as porogen.

[a] [*E*/*A*] ratio between *EGDMA* and *AEM*.
[b] [*E*/*E*+*A*] percentage of cross-linking.
[c] NIPs are prepared without template and only cross-linking degree changed for them. *AEM* (3.6 mM).

to the reaction mixture, the solution was immediately degassed by flushing N_2 during 3 min and the tube sealed. The reaction mixture was heated at 50 °C for 24 h. After polymerization, the bulk solid polymer was crushed with a cryogenic impact grinder (Spex Certiprep 6750 Freezer/Mill). The milled polymer was transferred to a G4-glass filter funnel and washed sequentially with acetone, MeOH and water. Washing solutions were recovered and analyzed by HPLC as described below for detection of template until complete elimination. Thereafter, the polymer particles were dried at 40 °C under vacuum. Elemental analysis for sulfur content was performed in the dried polymers; non-sulfur traces were detected in any of the washed polymers. Control non-imprinted polymers (*NIP-A* to *-H*) were prepared in the same way but without the addition of the sulfated disaccharide *MSGG* to the reaction mixture.

2.3. Rebinding experiments with HPLC analysis of supernatants

For rebinding experiments, 5.0 mg of polymer (MIPs or NIPs) were suspended in 0.25 mL of a DMSO solution of *MSGG* (0.18 mM), or other molecule when indicated. The rebinding mixture was incubated at rt with gentle agitation during 24 h. Then, the polymer particles were sedimented by centrifugation and the supernatants analyzed by liquid chromatography to determine the amount of template unbounded to the polymer. Chromatography was performed using a Dionex ICS 3000 HPLC system including an auto-sampler module connected to a pre-column and to a Dionex Ion Pac AS-11-HC column (4×250 mm^2), followed by a pulsed amperometric detector (Dionex). Mobile phase solutions A (100 mM NaOH) and B (100 mM NaOH, 500 mM CH_3COONa) were degassed with helium. The column was equilibrated with solvent A for 10 min before each sample run. Supernatants (10 μL) were automatically injected through the system and eluted at a flow rate of 1 mL/min with a multi-step gradient of solvents A and B as follows: 0–20 min for 0–100% of solvent B, and then 20–25 min for 0–100% of solvent A. A calibrating curve of the template at concentrations ranging from 45 to 357 μM was constructed with a linearity of response characterized by a $R^2=0.9968$. The amount of template uptaked by the polymer was obtained by subtracting the peak area of the unbound template to the peak area of the initial 'before-uptake' solution. Results, are expressed in % of template uptaked by the polymer, and are the average of 3 binding experiments ($n=3$) performed for each point.

2.4. Carbohydrate colorimetric analysis on supernatants from rebinding experiments

In order to confirm results obtained from the HPLC analysis of template in supernatants from rebinding experiments, a colorimetric phenol-method for total glycan quantification was adapted for simultaneous multi-sample analysis on a 96-wells ELISA type micro-plate [30]. Briefly, 50 μL of supernatants solution from rebinding experiments were added to wells followed by addition of 150 μL of a concentrated sulfuric acid solution (95%) and 30 μL of a 5% phenol solution. Samples were heated at 90 °C during 30 min. Coloration appeared in the wells and absorbance was measured at 490 nm in a micro-plate reader Infinite M1000 (TECAN, Switzerland). A calibrating curve, treated and analyzed in the same set of experiments, was constructed for each standard compound (1 to 25 μg). Response linearity was characterized by a minimum $R^2=0.9950$.

3. Results and discussion

It has been recognized that the interaction of HS with biological relevant proteins depends on the presence, at the protein side, of particular amino acid sequences, and at the glycan side, of particular sugar sequences characterized by specifically positioned sulfate groups [4]. At present, regardless of the recognized importance of sulfated glycans in biology, glycomics has not yet resolved the rapid and efficient identification of specific sulfated sequences, as proteomics or genomics do for determining specific amino acid or nucleotides sequences. Currently, the approaches used to extract and/or study sulfated sugar sequences in biological samples are still extremely complex. Here, as an attempt to develop new strategies allowing advances in this area, we investigated the potential applicability of the 'Molecular Imprinting Technology' for the generation of imprinted polymers able of specifically recognize HS-like sulfated sugars.

3.1. Synthesis of disulfated disaccharide MSGG

Because of the difficulty to rapidly and efficiently produce sufficient amounts of pure HS saccharides, which are typically available at the milligram scale, the novel 6-O-disulfated maltose derivative *MSGG* (Fig. 1) was synthesized at the gram scale and used as a model template to investigate the applicability of the Molecular Imprinting Technology to the specific recognition of

sulfated sugars. It is well known that 6-O-sulfation is one of the most common features conferring to HS particular biological activities [4]. Thus, the sulfated template *MSGG* was designed to hold two sulfate groups at the position 6 of each sugar unit. Scheme 1 resumes the chemical synthesis of the disulfated *MSGG* from methyl-maltoside **1**. In this synthesis, secondary hydroxyl groups in maltoside **1** were selectively substituted through a protection/deprotection strategy to afford the benzyl derivative **2** (53% yield, 3 steps). The two hydroxyl groups in C-6 of the disaccharide **2** were thus available for sulfation with the SO_3–DMF complex and quantitatively furnished a benzyl protected disulfated disaccharide. Benzyl groups in this compound were then deprotected by $Pd(OH)_2$ catalyzed hydrogenolysis (50 bar, 24 h) to give *MSGG* in a quantitative yield. Structure of the completely deprotected disulfated disaccharide was confirmed by ^1H, ^{13}C NMR and mass spectrometry.

3.2. MSGG imprinted polymers synthesis and recognition specificity

In molecular imprinting technologies it is known that the imprinting effect results from the formation of specific cavities created during the pre-polymerization assemblage of the template with the functionalized monomers [15]. Besides, literature data suggest the importance of the selection and proportion of functional monomers and cross-linkers in the reaction mixture during the polymer preparation [31]. In this work, we used 2-aminoethyl methacrylate (AEM) as functional monomer to conveniently target sulfate groups in polymerizable mixtures as we previously described [26]. Thus, a series of MIPs and NIPs were newly synthesized by varying the proportion of this amine and that of the cross-linker ethylene glycol dimethacrylate (EGDMA), regarding to the template amount in the reaction mixture (Table 1). Since template was not present during NIPs synthesis, only one NIP of defined EGDMA/AEM ratio was prepared for each family of MIPs. This could be justified from preliminary calculations to prepare a different NIP for each corresponding MIP. For instance, for NIP-2A, NIP-3A, or NIP-8A synthesis, the same EGDMA/AEM molar ratio is obtained and thus the 3 NIPs will be identical. However, to prepare MIPs, the presence of template is considered and so different MIPs are obtained for only one corresponding NIP. Reactions were carried out in DMSO by free-radical polymerization with AIBN as initiator. Under these conditions, a library of 64 different MIPs and their 8 corresponding NIPs (Table 1) was prepared by adapting reactions stoechiometries to study the influence of functional monomer (polymers 1 to 8) and cross-linker contents (polymers A to H) on the MIP's template sorption capability and specificity. Sorption capability was defined as the percentage of template uptaked by the polymer from the initial template solution used in the binding test [32,33]. Specificity was defined as the sorption capability of a particular MIP compared to that of the corresponding NIP (MIP/NIP) [32,33]. Results in Fig. 2 shows the effect of the functional monomer (AEM) content in the sorption capabilities and specificities of two groups of polymers, the first (Fig. 2a) corresponds to polymers of the group B prepared at low cross-linker content (75%) and the second (Fig. 2b) corresponds to polymers of the group H, prepared at high cross-linker content (90%). In both groups, polymers prepared with a 9 mM AEM concentration (5 eq of AEM/template) showed the higher sorption capabilities and very good specificities, as observed when comparing to the corresponding NIPs. Interestingly, uptake and specificity decreased in polymers prepared with higher AEM concentrations, until 45 mM (25 eq of AEM/template); while increased sorption capabilities and specificities were again observed at higher concentrations, from 54 mM (30 eq of AEM/template), although in lower extent. These effects were also observed on other polymers groups although with considerable lower uptakes and specificities (data not shown). The loss of sorption capability and specificity at AEM concentrations from 9 to 45 mM (5 to 25 eq of AEM) can be explained by the increasing amine density which can generate monomer

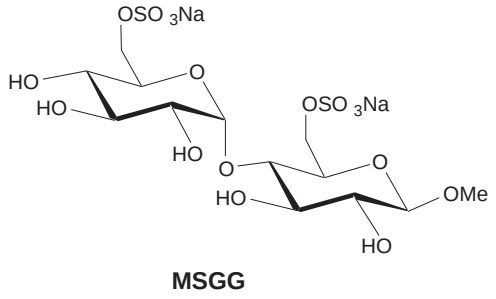
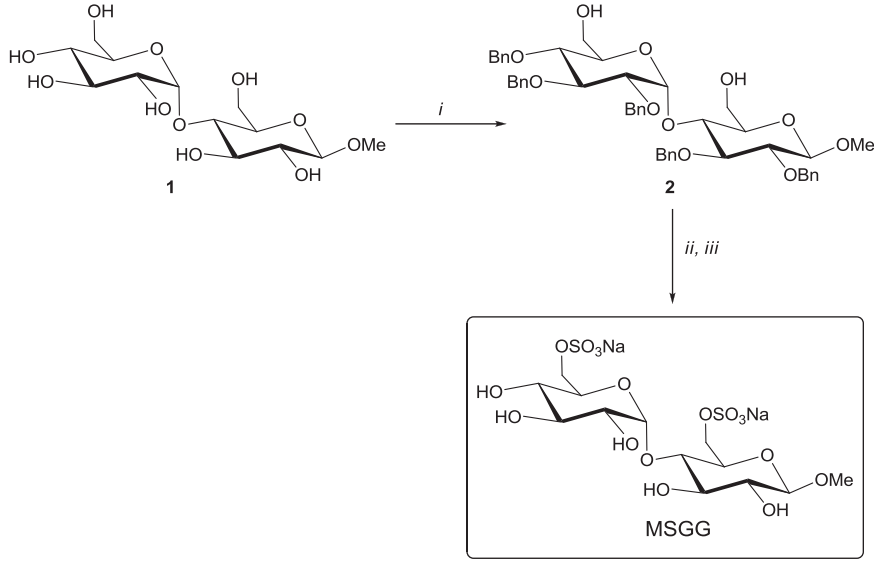


Fig. 1. Methyl 6,6′-di-O-sulfonato-β-D-maltoside (*MSGG*).



Scheme 1. . Synthesis of the disulfated disaccharide *MSGG*. Reagents and conditions: i. (a) TrCl, pyridine, 80 °C, 12 h, (b) NaH, BnBr, DMF, rt, 12 h, (c) 10% TFA in CH_2Cl_2, rt, 20 min (53%, 3 steps); ii. SO_3–NMe_3, DMF, 55 °C, 48 h (quantitative); iii. $Pd(OH)_2$ (10%), $MeOH/H_2O$ (9:1), H_2 (50 bar), rt, 24 h (quantitative).

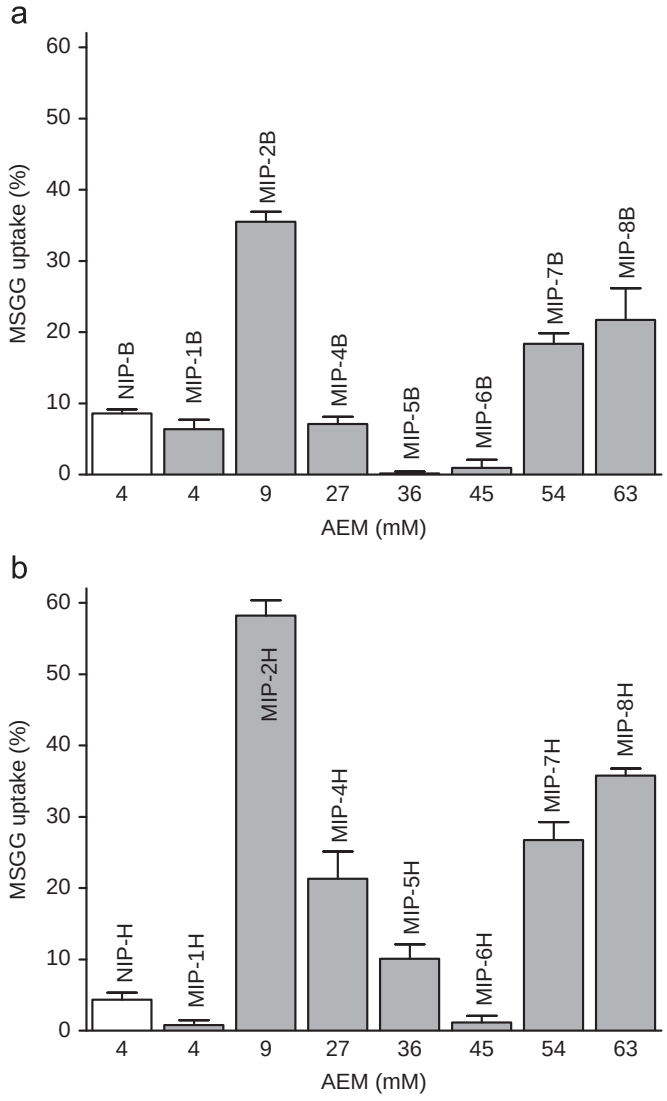


Fig. 2. Variation on the polymer template uptake (%) in function of the *AEM* concentration used in the polymerization reaction mixture for (a) *MIP*-1*B* to *MIP*-8*B* (75% cross-linking), and (b) *MIP*-1*H* to *MIP*-8*H* (90% cross-linking). Polymers (5 mg) were incubated with a *MSGG* solution (0.18 mM, 0.25 mL) at rt. After 24 h, sorption capability, defined as the percentage of template bond to polymer, was calculated as the difference between the amount of template present in the supernatant after incubation compared to that before incubation. Results are the average of 3 different experiments ($n=3$) for each point. Bars correspond to standard deviations.

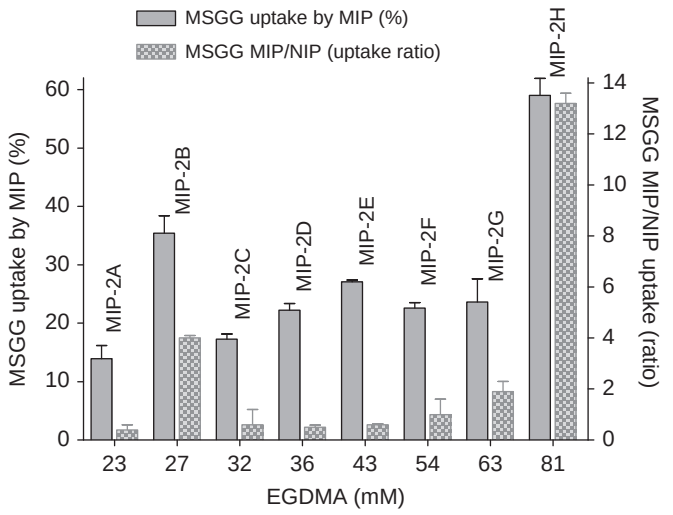


Fig. 3. Polymers *MSGG* sorption capabilities (% of MSGG uptake) and specificities (MIP/NIP uptakes) as a function of the *EGDMA* cross-linker content at 9 mM of *AEM*. Polymers (5 mg) were incubated with 0.25 mL a 0.18 mM *MSGG* template solution for 24 h at rt. Left grey bars represent the *MSGG* percentage of uptake calculated as the difference between the amount of template in the supernatant after incubation compared to that before incubation. Right dotted bars represent the specificity of binding defined as the amount of *MSGG* uptaked by a MIP divided by that uptaked by the corresponding NIP. Results are the average of 3 different experiments ($n=3$) for each point. Bars correspond to standard deviations.

dimerization, which reduce formation of effective binding sites [34,35]. In the other hand, the increased template binding observed on polymers prepared with AEM from 54 to 63 mM (30 to 35 eq) could be the result of a new spatial arrangement due to very high amount of amine in the pre-arrangement step before polymerization occurs.

It has been widely recognized that, in addition to the influence of functional monomer chemistry and stoichiometry, imprinted polymers should be highly cross-linked to allow the formation of cavities able to retain their shape after removal of the template [31]. In the other side, it has also been suggested that high reticulation can interfere with a certain degree of polymer chain's flexibility, which provides rapid equilibration with template when re-binding [19]. Hence, the MIP sorption capabilities and specificities were investigated depending on the amount of the cross-linker EGDMA in the polymerization mixture (Fig. 3). Our results show that the polymer's *MSGG* sorption capabilities and specificities are clearly affected by the EGDMA content in the polymerization mixture. *MIP*-2*H* and *MIP*-2*B*, both prepared at 81 mM EGDMA (90% cross-linker), showed the best *MSGG* binding capabilities with the best specificities among all the synthesized polymers. The lower binding capabilities observed on the low cross-linked MIPs can be explained by their high flexibility due to their low reticulation. This may allow template binding in a non-specific way with specificity loss [35]. Accordingly, the higher *MSGG* binding capability was observed at the higher EGDMA proportion present in *MIP*-2*H* (90% cross-linking), which showed a negligible binding to the corresponding *NIP-H* (90% cross-linking). This can be the result of the formation of cavities with an ideal spatial shape and a right number of binding sites obtained to fit-match template in a ligand/receptor like manner under optimized reaction conditions and stoichiometry. The good specificities obtained for polymers *MIP*-2*B* and *MIP*-2*H* were confirmed by their binding isotherms compared to those of their respective NIPs (*NIP-B* and *NIP-H*) (Fig. 4). These results show that, by controlling polymerization reaction conditions and stoichiometry, MIPs can effectively be prepared to recognize sulfated sugars with good binding capabilities and specificities.

In additional experiments, selectivity [32,33] was examined by performing *MIP*-2*H* binding to a series of template analogs and other sulfated and unsulfated saccharides. Compounds used for these experiments included two unsulfated disaccharides: the methyl maltoside **1** (un-sulfated template) and saccharose (**3**); two monosulfated sugars: glucose-3-O-sulfate (**4**) and the HS derivative α-ΔUA-[1→4]-GlcNAc6S (**5**); one trisulfated disaccharide: the HS derivative ΔUA2S-[1→4]-GlcNS6S (**6**); and the oversulfated maltose (**7**). Binding of these sugars to *MIP*-2*H* and to their respective *NIP-H* are shown in Fig. 5. The MIP and NIP uptakes of these compounds were analyzed by HPLC or by the colorimetric phenol/sulfuric acid assay. For each compound, a calibrating curve was prepared (data not show). Results in Fig. 5 show that any of the unsulfated compounds (**1** or **3**) could bind to the polymers. Concerning the monosulfated products, an attempt was performed to examine the influence of the sulfate position in recognition by using glucose-3-O-sulfate (**4**) in the binding

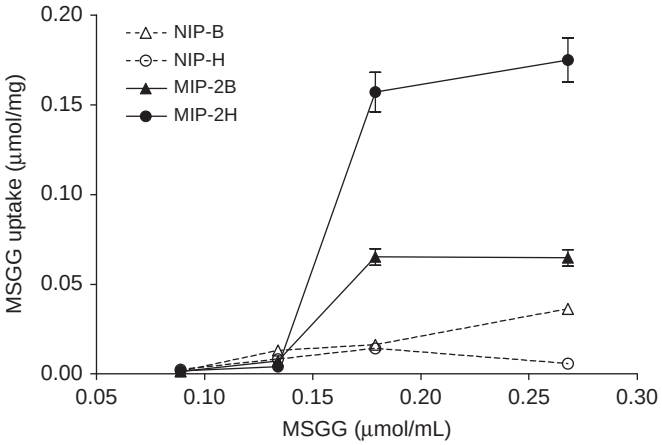


Fig. 4. Binding isotherm of *MSGG* to *MIP-2B* and *MIP-2H* compared to those of their respective NIPs. Polymers (5 mg) were incubated with *MSGG* template solutions (0.25 mL) at the indicated concentrations for 24 h at rt. *MSGG* uptake was calculated as the difference between the amount of template in the supernatant after incubation with the polymer compared to that in solution before incubation and reported to mg of polymer. *MIP-2B* (full triangles), *MIP-2H* (full circles), *NIP-B* (empty triangles), *NIP-H* (empty circles). Results are the average of 3 different experiments ($n=3$). Bars correspond to standard deviations.

experiments. Remarkably, the monosulfated product was not recognized by the MIP possibly because of the presence of a sulfate in the discriminating C3 position instead of the C6 imprinted in the polymer cavity. Nonrecognition was neither observed with the monosulfated HS derivative **5** in where, even if a sulfate group in a C6 was present, the global shape of the molecule possibly avoided binding to the imprinted specific sites. These results suggest that the Molecular Imprinting Technology can avoid crossed recognition among differently sulfated or differently shaped sugars. This is of particular importance since cross recognition represents an important drawback when using existing antibodies. However, binding experiments with highly sulfated compounds, as the trisulfated HS derivative **6** and the over-sulfated template analog **7**, showed low specificity traduced from a considerable template binding to the NIPs but not to MIPs. Interestingly, it was only observed with the trisulfated derivatives. Thus, the over-sulfated template analog **7** could not at all be uptaked by *MIP-2H* while it was highly uptaked by its corresponding NIP. From these results, it can be assumed that the imprinted polymer could discriminate the fully sulfated compound because of a possibly well-organized localization of the amine binding groups in specific binding cavities hardly accessible by hindered compounds. This suggests that MIPs can

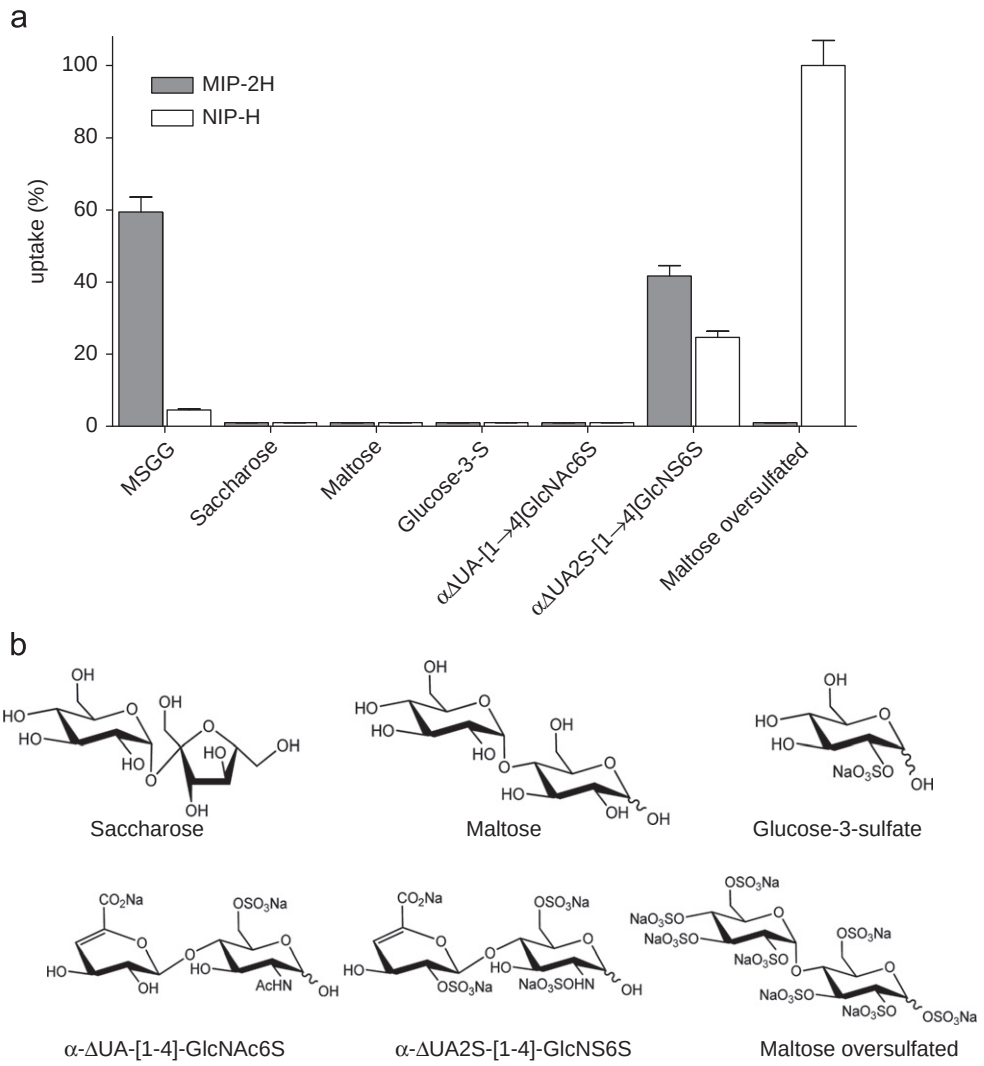


Fig. 5. Selectivity of *MIP-2H* (a) uptake (%) of *MSGG* and analogs (100 μg/mL) for *MIP-2H* and *NIP-H*. (b) Chemical structures of *MSGG* analogs and other sugars used in the study. Results are the average of 3 different experiments ($n=3$). Bars correspond to standard deviations.

selectively discriminate template related sulfated sugars even if the corresponding NIP shows non-specific binding. This assumption is not in disagreement with the high uptake of the oversulfated derivative **7** by *NIP-H*, in where the absence of specific binding cavities could result in a random amine group distribution through the polymer allowing non-specific binding. Although our results suggest that increasing sulfation levels in the template analogs enhances non-specific binding to NIP, the disaccharide **6** could also be partially uptaked by *MIP-2H,* possibly because a not enough big compound size allowing the non-specific binding due to the high negative charge. Because of these assumptions, differently sulfated compounds have to be synthesized to explore the selectivity of MIPs on the binding of other template related sugars.

4. Conclusion

In this work we have prepared a library of MIPs and their corresponding NIPs in order to establish the polymerization conditions and stoichiometry required for the production of imprinted polymers able to specifically recognize the disulfated disaccharide *MSGG*. Our results show that fine optimization of the polymer synthesis is required to guaranty good recognition specificity and sorption capability, explaining, at least in part, the no specific binding observed in previous work [27]. The good MIPs specificity obtained under the optimized polymerization conditions gives to the Molecular Imprinting Technology a potential place among tools in glycomics as a new approach for the regio-selective recognition of sulfate groups in small glycanic fragments. However, as for other strategies, the final goal has still to be attained and new studies are still required to continue advancing in the selective recognition of complex sulfated HS saccharides.

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