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New glycopolymers as multivalent systems for lectin recognition

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A new type of glycopolymers has been designed and evaluated as multivalent system for lectin recognition. They were prepared by living Anionic Ring-Opening Polymerization (AROP) of disubstituted cyclopropane-1,1-dicarboxylates and thiol-ene post-modification. Fully modified oligomers with single or geminal sugar substituents located on every third carbon alongside the macromolecular scaffold were obtained under mild conditions. The resulting glycopolymers have shown efficient binding potency towards Concanavalin A.

In the last decade glycopolymers received much attention in polymer chemistry [1-4], because of their ability to mimic the multivalent presentation of carbohydrates at the cell surface. [5,6]. In particular, glycopolymers are excellent candidates to recognize lectins through multivalent interactions [4]. As demonstrated with all multivalent glycoconjugates, the strength and selectivity of interaction not only depend on the number of sugars but is also closely related to the structural parameters of glycopolymers such as architecture, molecular weight and sugar distribution on the polymeric scaffold [7-10].

Due to the involvment of carbohydrates in many biological events, the development of efficient synthetic methodologies to prepare glycopolymers in good yields and reproducibility remains highly challenging for numerous applications going from anti-adhesive therapy to glycan microarrays. [11-13]. Until now, the synthesis of glycopolymers by post-modification using "click" chemistry such as the copper catalyzed azide-alkyne cycloaddition (CuAAC) has been largely explored [14,15]. To avoid the use of Cu(I) catalyst that maybe difficult to removed, bioconjugation using thiol-based reactions have attracted attention in the last five years [16]. In particular, the photo-induced free-radical thiol-ene coupling (TEC) has been shown recently useful in carbohydrate chemistry because of its high efficiency, ease of use, short reaction times and mild reaction conditions. For example, post-modification of poly[2(-3-isobutenyl)-2-oxazoline] with acetylated thioglucose has been performed quantitatively by TEC in less than 24h [17]. Other studies based on the glycosylation of copolymers were reported such as statistical copolymers [18,19] or block copolymer with one block bearing carbohydrate side chain functionality [20,21]. Their enhanced binding affinity and the possibility to generate micellar structures make them interesting models for targeted drug carrier and or to investigate carbohydrate–protein interactions.

Recently, we developed a novel type of thiol-ene clickable polymers dubbed OCDs [22]. These polymers were obtained by the anionic ring-opening polymerization of diallyl cyclopropane-1,1-dicarboxylate with controlled molecular weights and narrow molecular weight distributions. The obtained polymers have a unique structure displaying geminated allyl groups on every third carbon alongside the macromolecular backbone. The coupling of several mercaptans (2-mercaptoethanol, 11-mercaptoundecanol, 3-mercaptopropionic acid and 2,2-dimethyl-1,3-dioxolan-4-yl methanethiol) with the allyl C=C double bonds has been performed leading to a range of functional OCDs and appear to be fully quantitative despite the high density of allyl side groups. Winnick and co-authors used this new multivalent polymeric scaffold to prepare Metal-Chelating Polymers to be used in multiplexed immunoassays based on mass cytometry [23]. Their preparation required the TEC reaction of the OCD scaffold with cysteamine hydrochloride. Here we present the synthesis of two OCDs scaffolds **P1** and **P2** differing by the number of allyl groups per repeat unit (Scheme 1). The coupling of both monomers and polymers with glucose (Glc) and *N*-acetylglucosamine (GlcNAc) has been performed by photochemically induced TEC reactions. This is the first time glycopolymers are obtained from OCDs scaffolds. The interaction of glucosilated polymers with the lectin Concanavalin A (ConA) has been studied by Enzyme-Linked Lectin Assay (ELLA).

Experimental section

Materials and methods

Phosphazene base *t*-BuP_4 (0.8 mol.L-1 hexane solution, Aldrich), thiophenol (≥ 99%, Aldrich), hydrochloric acid (37%, Aldrich), allyl alcohol (> 99%, Aldrich), malonyl chloride (97%, Aldrich), ethyl malonyl chloride (> 99%, Aldrich), 2,2-dimethoxy-2-phenyl-acetophenone (DPAP) (99%, Aldrich), K_2CO_3 (> 99%, Aldrich) and 1,2-dibromoethane (≥ 98%, Fluka) were used without purification. THF was dried with sodium-benzophenone and then distilled. HRP-labelled concanavalin A, BSA and SIGMA FAST OPD were purchased from Sigma-Aldrich. Glycosyl thiols β-D-GlcSH and β-D-GlcNAcSH were prepared from the corresponding 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide [24] and 2-deoxy-2-acetamido-3,4,6-tri-O-acetyl-α-D-glucopyranosyl chloride [25] by treatment with potassium thioacetate (KSAc) [26] followed by de-*O*-acetylation under standard conditions. Chemical analysis as ^{1}H NMR, ESI and other spectroscopic data are in good agreement with what previously reported by Davis and co workers. [27]

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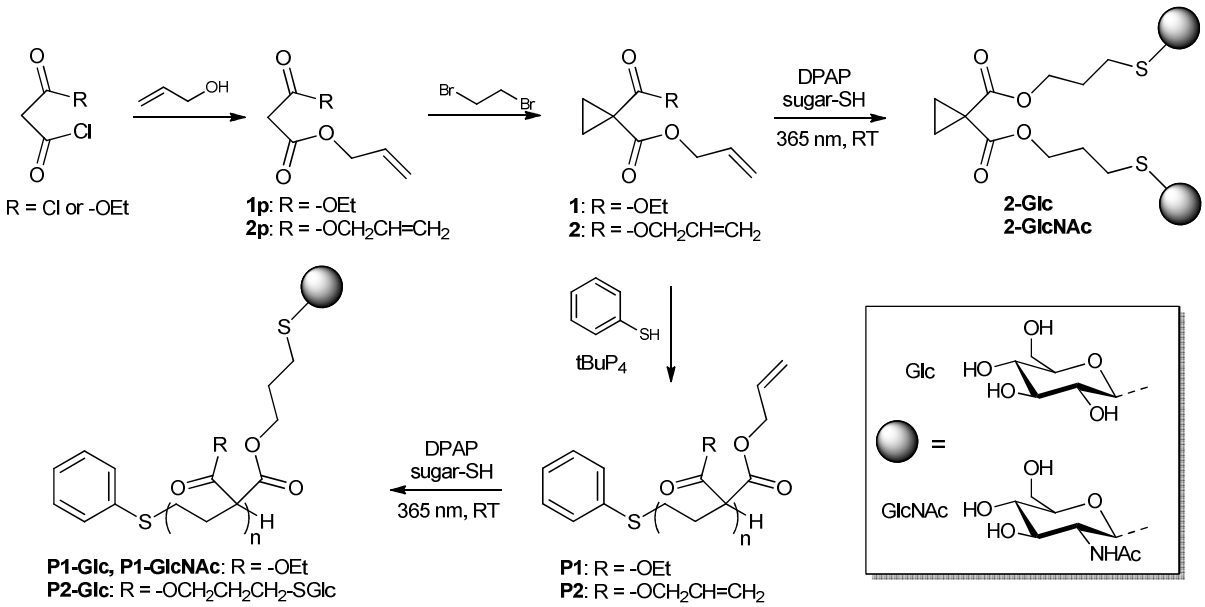

Scheme 1. Synthesis pathway for the preparation of glycopolymers.

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, CD_3COCD_3, D2O, or MeOD using a Bruker 400 MHz NMR spectrometer. Size Exclusion Chromatography (SEC) experiments were performed in THF (1 mL.min^{-1}) at room temperature, using a Spectra Physics P100 pump, and two PLgel Polymer Laboratories linear columns (5 µm Mix-C, separation range 200 to 2 x 106). A Wyatt Technology Optilab Rex interferometric refractometer (690 nm laser) was used as a detector. Molecular weights were obtained using a polystyrene calibration. IR spectra were obtained with a Thermo-Fischer IS10 spectrometer (ATR mode). The MALDI mass spectra were performed with an Autoflex Speed TOF/TOF spectrometer (Bruker Daltonics) equipped with a 355 nm Smartbeam II laser. Spectra were acquired in the linear positive mode, with an accelerating voltage of 19.5 kv. Calibration was performed externally using a solution of peptide calibration standard and protein calibration standard I (1:1) (Bruker daltonics) mixed with the DHB matrix. Samples were spotted on a MTP 384 polished steel target plate. Mass spectra were processed with Flex Analysis software (Bruker Daltonics). ESI analysis were performed on aEsquire 3000+ Bruker apparatus (normal mode).

Monomers synthesis and their polymerization

Compounds **2** and **P2** were synthesized as described in reference 22.

Synthesis of **1**

*Synthesis of ethyl allyl malonate **1p***

Allyl alcohol (1.95 g, 0.0337 mol) was added dropwise to a solution of ethyl malonyl chloride (5.07 g, 0.0337 mol) in toluene (20 mL) at 55 °C. After evaporation under vacuum, the residue was distilled (4.6 mBar, bp 72°C) to provide a colorless liquid with a yield of 74%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 1.21 (t, 3H, CH_3); 3.33 (s, 2H, $CH_2(CO_2R)_2$), 4.12-4.17 (q, 2H, C**H**$_2$-CH_3), 4.57, 4.59 (d, 2H, C**H**$_2$CH=CH_2), 5.17 – 5.29 (m, 2H, CH_2CH=C**H**$_2$), 5.80 - 5.90 (m, 1H, CH_2C**H**=CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) : δ (ppm) = 14.0 (CH_3), 41.5 (**C**$H_2(CO_2R)_2$),61.4 (COO**C**H_2CH_3), 66.0 (COO**C**H_2CH=CH_2), 118.6 (CH_2CH=**C**H_2), 131.5 (CH_2**C**H=CH_2), 166.3 (CH_2(**C**$O_2R)_2$). IR (ν, cm^{-1}): 3092, 2987, 1720; 1650, 1447, 1413, 1370, 1329, 1270, 1146, 1097, 992, 935,845, 784, 682.

*Synthesis of ethyl allyl cyclopropane-1,1-dicarboxylate **1***

A mixture of di-n-allyl malonate **1p** (4.26 g, 0.0248 mol), 1,2-dibromoethane (56.4 g, 0.0495 mol), anhydrous potassium carbonate (20.6 g, 0.149 mol), and DMSO (25 mL) was stirred very vigorously for 3 days at room temperature. The solution is then filtrated and 20 mL of water is added. The obtained solution is extracted with diethylether (5 x 10 mL). The combined ether extracts were dried over sodium sulfate. The ether was evaporated and the residue distilled under vacuum (4.5 mBar, bp 76°C) to provide a colorless liquid with a yield of 70%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 1.29 (t, 3H, CH_3), 1.47 (s, 4H, CH_2 cyclopropyl), 4.19-4.24 (q, 2H, C**H**$_2CH_3$), 4.65, 4.66 (d, 2H, C**H**$_2$CH=CH_2), 5.25- 5.39 (m, 2H, CH_2CH=C**H**$_2$), 5.88-5.98 (m, 1H, CH_2C**H**=CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) = 14.0 (CH_3), 16.4 (CH_2 cyclopropyl), 28.1 ((CH_2)$_2$**C**$(CO_2R)_2$), 61.3 (COO**C**H_2CH_3), 65.8 (COO**C**H_2CH=CH_2), 118.2 (CH_2CH=**C**H_2),131.8 (CH_2**C**H=CH_2),169.5 (C(**C**$O_2R)_2$). IR (ν, cm^{-1}): 3091, 2962, 1721, 1650, 1447, 1419, 1370, 1316, 1172; 1128, 1028; 991, 933; 857; 753. ESI : 221 (**1** + H), 237 (**1** + Na)

Polymerization of **1**

Filling of the polymerization tube with the reagents prior to its closure was carried out in a glove-box. **1** (0.5 g, 2.53 mmol) was introduced under argon into a polymerization tube fitted with a

Rotaflo®. THF (0.5 mL), thiophenol (12.8 µL, 0.126 mmol, [1]0/[PhSH]0 = 20) and t-BuP$_4$ (2005 µL, 0.16 mmol) were successively added at room temperature. After careful closure of the reaction tube, the mixture was stirred at 60°C for 24h. The reaction was quenched with a large excess of a 12 mol.L^{-1} HCl aqueous solution (20 equivalents with respect to thiophenol). The polymer was recovered by dissolution of the final mixture in chloroform and precipitation in methanol (yield: ~60%). The white powder obtained after centrifugation was dried under vacuum at room temperature for 24 h. ^{1}H NMR (CD_3COCD_3, 400 MHz): δ (ppm) = 1.27 (CH_3), 1.81 (s, 4H, -C**H**$_2$C**H**$_2$C(CO_2R)$_2$), 4.20-4.25 (m, 2H, C**H**$_2$-CH_3), 4.67, 4.68 (d, 2H, C**H**$_2$CH=CH_2), 5.25- 5.39 (m, 2H, CH_2CH=C**H**$_2$), 5.91-6.02 (m, 1H, CH_2C**H**=CH_2). ^{13}C NMR (CD_3COCD_3, 100 MHz): δ (ppm) = 13.6 (CH_3), 25.9 (-**C**H$_2$**C**H$_2$C(CO_2R)$_2$), 56.6 ((CH_2)$_2$**C**(CO_2R)$_2$), 61.3 (O**C**H$_2$$CH_3$), 65.8 (-**C**H$_2$CH=$CH_2$), 118.2 ($CH_2$CH=**C**H$_2$), 129.0 (phenyl end group), 132.1 (CH_2**C**H=CH_2), 170.2 (C(**C**O$_2$R)$_2$). IR (ν, cm^{-1}): 3092, 2979, 1723, 1650, 1449, 1368, 1276, 1159, 1094, 1041, 1024, 990, 941, 859, 795, 723.

Thiol-ene coupling

Allyl compound, the selected thiol (3-5 equivalents per allyl) and 2,2-dimethoxy-2-phenylacetophenone (DPAP; 0.1thiol eq) were dissolved in dry DMF. After 10 min of argon bubbling, the stirred mixture was irradiated by UV light at 365 nm (Mgc typ838 150 W, 8 tubes) for a given time (See Table 1). All polymers were recovered by dialysis against water (1000 Da).

P1 modified with Glc

^{1}H NMR (D_2O, 400 MHz): δ (ppm) = 1.22 (3H, CH_3), 1.74 (4H, -C**H**$_2$C**H**$_2$C(CO_2R)$_2$), 1.96 (2H, CH_2C**H**$_2$$CH_2$S), 2.74 (2H, CH_2-S); 3.27-3.80 (Glc), 4.19-4.26 (4H, COOCH_2), 4.47 (1H, anomeric H).

P2 modified with Glc

^{1}H NMR (D_2O, 400 MHz): δ (ppm) = 1.78 (4H, -C**H**$_2$C**H**$_2$C(CO_2R)$_2$), 2.01 (4H, CH_2C**H**$_2$$CH_2$S), 2.78 (4H, CH_2-S), 3.33-3.88 (Glc), 4.30 (4H, COOCH_2), 4.52 (1H, anomeric H).

Enzyme-linked lectin assay (ELLA)

Nunc-Immuno plates (Maxi-Sorp) were coated with polymeric glycosylated compounds **P1-Glc** or **P2-Glc** (100 µL per well of serial twofold dilutions in PBS diluted from a starting concentration of 600 µg mL^{-1}) for 1h at 37°C. The wells were then washed with T-PBS (3x100 µL well^{-1}, PBS containing 0.05% (v/v) Tween 20). The washing procedure was repeated after each incubation throughout the assay. The wells were then blocked with BSA in PBS (3% w/v, 100 µL well^{-1}) at 37°C for 1h. After washing, the wells were filled with 100 µL of peroxydase-labelled concanavalin A (Con A-HRP; 15 µg mL^{-1}) diluted in PBS containing 0.1 mM Ca^{2+}, 0.1 mM Mn^{2+} (pH 7.4) and BSA (0.3% w/v) and were incubated for another hour at 37°C. The plates were then washed with T-PBS (3x100 µL well^{-1}) and the OPD substrate was added (100 µL per well). Colour development was stopped after 10 min by adding H_2SO_4 (30% v/v, 50 µL per well) and the absorbances were measured at 490 nm using a microtitre plate reader (SPECTRAmax, model PLUS384, Molecular Devices).

Result and discussion

Monomers synthesis and modification with sugar thiols. Two disubstituted cyclopropane-1,1-dicarboxylate monomers **1** and **2** bearing one and two allyl groups, respectively, were prepared according to a two-step procedure and purified by distillation. ^{1}H and ^{13}C NMR spectra and ESI analysis confirmed the chemical structure of both monomers. Monomer **2** was modified by reacting the allyl groups with GlcSH and GlcNAcSH (Scheme 1). Dondoni *et al.* have found previously that the concentration is a critical parameter for the reaction of sugar thiols with a fourth generation di-allyl malonate functional dendrimer [28]. That is why the reaction was performed under high allyl concentration (Table 1). After 90 min, ESI analysis of the crude reaction media were performed. For both reactions the expected mass was observed (i.e. 602 + 23 for **2-Glc** and 684+23 for **2-GlcNAc**). Neither trace of monomers nor monosubstituted product was observed. Reactions are complete despite the steric hindrance on the malonate.

Table 1. Coupling of sugar thiols (4eq/allyl) to **2**, **P1** and **P2**, dry DMF, DPAP 0.1 thiol eq, 365 nm.

Sample	Sugar (eq/allyl)	Allyl compound concentration	Cv	time
2-Glc	GlcSH (4)	(1) 0.63M	100	1h30
2-GlcNAc	GlcNAcSH (4)	(2) 0.53M	100	1h30
P1-Glc	GlcSH (4)	P1[a]	100	8h15
P1-GlcNAc	GlcNAcSH (5)	P1[a]	85	6h45
P2-Glc	GlcSH (4)	P2[a]	100	3h

[a] 4 w% polymer solution

Table 2. Polymerization of **1** and **2**, 60°C, THF. Characterization by Size Exclusion Chromatography in THF and ^{1}H NMR in CD_3COCD_3.

Samples	$M_{n,th}$	$M_{n,exp}$ (x 10^{-3})		M_w/M_n
	x10^{-3}	^{1}H NMR	SEC-RI	SEC-RI
P1	4	3.8	3.8	1.1
P2	5.2	7.1	7.4	1.2

Glycopolymers synthesis. Allyl group bearing polymers **P1** and **P2** were prepared by Anionic Ring-Opening Polymerization of monomers **1** and **2** following the methodology developed earlier [29]. This method uses phosphazene *t*-BuP$_4$ to generate the polymerization initiator *in situ*, i.e. thiophenolate in the present case (Scheme 1). The resulting polymers have monomodal distributions with low polydispersity indexes as expected for a living polymerization (Table 2). Moreover, the molecular weights were evaluated by SEC and ^{1}H NMR (Figure 1) using the signals corresponding to the proton of the phenyl end group as an internal reference (7.2-7.4 ppm) (Table 2). Analytical results were consistent with the expected molecular weights.

P1 and **P2** were next reacted with GlcSH. For **P1**, MALDI analysis was performed after 45 min, 1h30 and 3h to determine the reaction time necessary to reach complete reaction. As shown in Figure 2, the reaction was found to be complete after 45 min. Moreover, the obtained polymer has a molecular weight around 6700 g/mol, compared to 3800 g/mol for **P1**. The shift towards higher molecular weights confirms the coupling reaction between GlcSH and allyl groups of **P1**occurred according to the photo-induced free-radical thiol-ene addition [30]. The MALDI analysis also confirmed the formation of the desired product with the expected repeat units of 395. However, we also detected the existence of a second repeat unit of 186 which appears after the polymerization in both polymers **P1** and **P1-Glc** as previously observed with other systems [31]. After the TEC, the solutions were dialyzed in water for 3 days to remove the excess of thiol glycoside using a membrane with a cut-off of 1000 Da. The glycopolymers **P1-Glc** and **P2-Glc** were obtained after lyophilisation as white powders and were perfectly soluble in water.

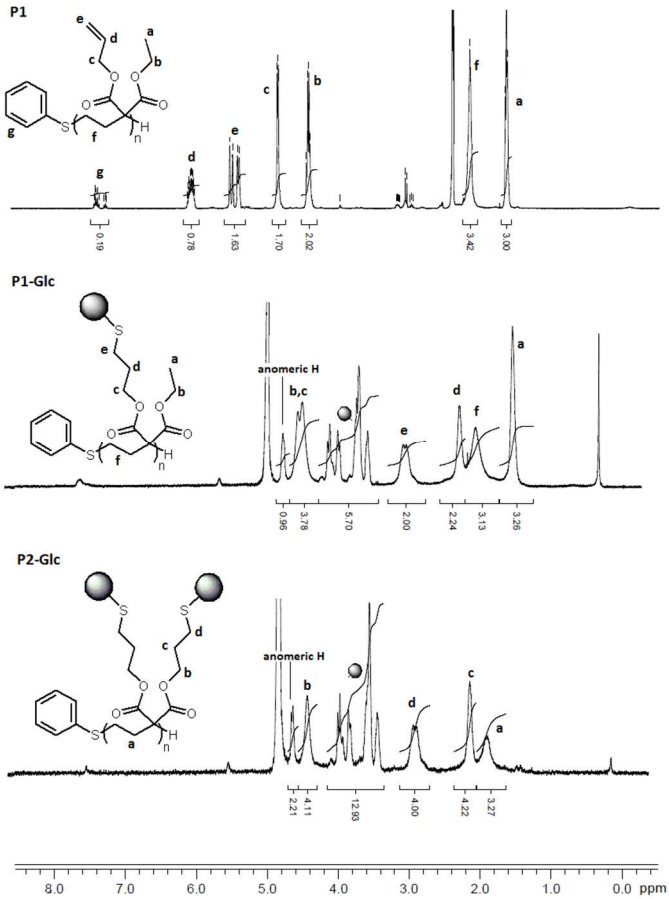

Figure 1. ^{1}H NMR spectra of **P1** in CD_3COCD_3, **P1-Glc** and **P2-Glc** in D_2O (400MHz).

A typical ^{1}H NMR spectrum of **P1** modification with glucose thiol is given in Figure 1. New signals between 3.3 ppm and 3.9 ppm corresponding to the carbohydrate protons were observed, together with the anomeric proton at 4.8 ppm as confirmed by COSY experiments. Moreover a new signal at 2.75 ppm corresponding to the CH_2S protons confirmed the effective addition of the thiol sugar onto the polymeric scaffold (Figure 1). Allyl protons, present at 5.25, 5.28, 5.35 and 5.39 ppm for CH_2-CH=C**H**$_2$ and between 5.91 and 6.02 ppm for CH_2-C**H**=CH_2 for **P1** and **P2** were not observed on NMR spectra of glycopolymers, suggesting that the functionalization of polymers was quantitative. Finally, the signal around 5.7 ppm which correlates with the carbohydrate protons seems to reveal the presence of traces of glucose dimerized by disulfide bond.
In contrast to glucose, the modification of **P1** with GlcNAc-SH was found incomplete (85% of functionalization after 6h45) as protons of the allyl group were still visible in the ^{1}H NMR spectrum of the isolated glycopolymer (see supporting information). This difference of reactivity could be explained by the presence of the *N*-acetyl group that may decrease the nuleophilicity of the thiol group.

This is the first time glycopolymers are made using an OCD scaffold. These novel glycopolymers display unprecedented structural features with one (**P1-Glc**) or two (**P2-Glc**) sugar moieties located on every third carbon alongside the macromolecular backbone. This structure is unique and differs from others structures obtained by polymerization of acrylates [32], methacrylates [11] or acrylamides [19, 33] that lead to glycopolymers with different sugar distribution.

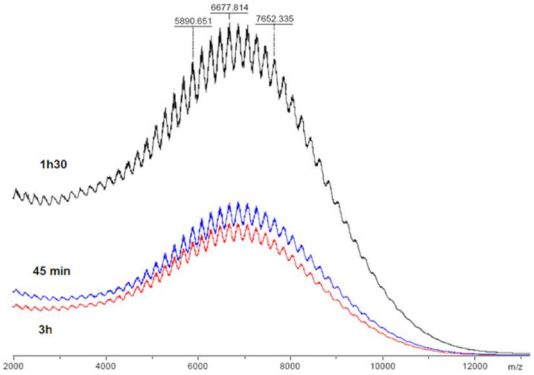

Figure 2. MALDI spectra recorded at different times for the reaction of **P1** with Glc in DMF. Matrix: DHB.

Interaction of P1-Glc and P2-Glc with Con A. The binding potency of the glucosylated polymers **P1-Glc** and **P2-Glc** was next studied with a model lectin from *Canavalia ensiformis* (ConA) which is specific for mannose and glucose. The glycopolymers were immobilized in microtiter plates using serial dilutions of P1-Glc and P2-Glc. After washings and incubation of ConA labelled with horseradish peroxidase, we observed variable coloration in each well. This result confirms that the lectin binds to the glycopolymer and that the interaction is closely dependent on the quantity of immobilized glycopolymer. Moreover, no interaction was observed with unglycosylated polymer, suggesting that the binding is due to the presence of glucose units rather than the polymer framework itself.

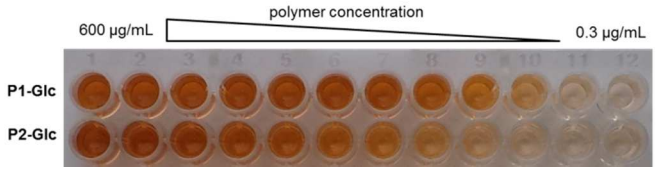

Figure 3. Binding assay with ConA (lectin concentration: 15 µg. mL^{-1}).

Conclusion

In summary, we report a new class of glycopolymers with original distances between pendant glucose moieties. The polymerization chemistry allows for the control of the molecular weights with narrow molecular weights distributions and represents an elegant method to modulate the density of sugars. Glycopolymers are made from two polymeric multivalent scaffolds differing by the number of allyl pendant groups per repeat unit. Both scaffolds were fully modified with glucose by metal-free thiol–ene click reaction and glucosylated polymers have shown promising binding potency with a model lectin. This strategy is currently used in our laboratory to prepare glycopolymers with antiadhesive properties towards bacterial lectins.

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Notes and references

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Electronic Supplementary Information (ESI) available: ^{1}H NMR and IR specta of (**1p**) and (**1**), ^{13}C NMR, MALDI, IR spectra and SEC analysis of **P1**, ESI analysis for **2** coupling with sugar thiols, ^{1}H NMR spectrum of **P1-GlcNAc**, COSY spectra of **P1-Glc** and **P2-Glc** and plots of the absorbance signal for ELLA assays . See DOI: 10.1039/c000000x/

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