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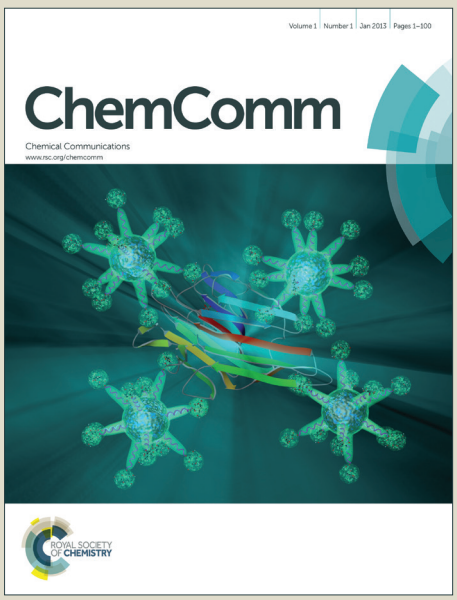

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Glyco-stereoisomerism effect on hydrogelation of interacting polymers *via* dynamic covalent bond

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This work explores, for the first time, the stereoisomerism effect of sugar units of glycopolymers on hydrogelation. Three glycopolymers with the identical main chain but different pendent sugar stereoisomers are employed. Hydrogelation of the glycopolymers occurs driven by the dynamic covalent bonds between the sugar units and benzoboroxole(BOB)-containing polymer. We conclude that the gelation ability of the glycopolymers differs obviously as shown in the sequence of Man > Gal > Glc due to their corresponding difference in sugar-BOB interaction ability.

Glycopolymers have been developed as a kind of simplified but powerful building block to mimic various glyco-conjugates in nature, which include polysaccharide, proteoglycan, glycolipid and glycoproteins[1]. In this respect, using glycopolymers shows obvious advantages: 1) nowadays polymer scientists are able to design and synthesis various polymeric sugars with well-defined structures and rather high molecular weight via controlled polymerization; 2) the target glycopolymers could self-assemble into desired nano-structures and even bulky materials.

Artificial hydrogel is one kind of important materials for many biomedical applications[2]. Hydrogels composed of glycopolymers are normally prepared via in situ polymerization of glyco-monomer with chemical crosslinker, where the glyco-units do not show any significant roles as they are not involved in the crosslinking. In the current study aiming at the stereoisomerism effect of sugar units on hydrogelation, the well-known dynamic covalent bond between phenylboronic acid and sugars is introduced to crosslink the glycopolymer chains[3]. As far as we know, there is only one example in literature used well-controlled glycopolymers to prepare hydrogels by crosslinking via boron-sugar bond[4], but without paying attention on the structural effect of the sugar units on hydrogelation. Furthermore, in the work the presence of open chain form of sugars might compete with pyranose sugars binding to the boron.

In nature, slight structural variation of sugars is capable of inducing significant change of the property of bulk material. For example, α-(1-4) linked glucopyranoside forms amorphous amylose, while β-(1-4) linked glucopyranoside does crystalline cellulose. This shows the tremendous effect of the difference in chirality of the anomeric center of the constituent sugar units. It is also well known that the stereoisomers of sugar may show contrast binding behavior to lectins[5], e.g. α-mannopyranoside binds to lectin Concanavalin A while α-galactopyranoside does not[6]. Recently, we demonstrated the effect of glyco-regioisomerism on the pathways of nanoparticles after cellular uptake[7], i.e. nanoparticles containing 1-Gal (anomeric linkage) reached lysosome while those with 6-Gal (linked via primary hydroxyl group at 6 position) only reached early endosome. However, we noticed that such important effect of sugar structure has not been explored in hydrogelation of glycopolymers. No doubt deciphering this effect would not only benefit creation of new glyco-based hydrogel materials for various applications but also promote our understanding of the relationship between the detailed structures and functions of sugars in nature.

Herein, three different glycopolymers with the same molecular weight (M_w) and polydispersity (PDI) containing respective monosaccharide stereoisomers, i.e. α-mannopyranoside, α-galactopyranoside and α-glucopyranoside are synthesized and employed (Figure 1). The gelation of the glycopolymers was performed by their further interacting with benzoboroxole (BOB)-containing polymer (Figure 1).

Three glycopolymers are prepared via post polymerization modification, which ensures the structural identity of the polymer backbones (Figure 1, Scheme S1). Briefly, poly(glycidyl methacrylate) (PGMA) prepared via Atom Transfer Radical Polymerization (ATRP) was characterized as $M_{n,GPC} = 1.33 \times 10^4$ by GPC (DMF as eluent, PEG as standard (Figure S1), $M_{n,NMR} = 3.33 \times 10^4$ by ^{1}H NMR in Figure S2). The subsequent ring opening of the pendent epoxide group of PGMA with NaN_3 afforded the product polymer PGMA-N_3 bearing one azide on

each repeating unit (characterization in Figure S1 and S3). PGMA-N_3 was further clicked with 1-(2'-propargyl)-α-D-galactoside, 1-(2'-propargyl)-α-D-mannoside and 1-(2'-propargyl)-α-D-glucoside, affording glycopolymers **PGal**, **PMan** and PGlc respectively (^{1}H NMR and FT-IR spectra in Figure S4-S7). Benzoboroxole (BOB)-containing polymer PNIPAm-co-PBOB (**PBOB**) was prepared via free radical polymerization of monomer *N*-isopropylacrylamide (NIPAm) and 5-acrylamidobenzoboroxole (AABOB) with feed ratio of 19:1 (^{1}H NMR in Figure S8). The small proportion of AABOB was adapted in the copolymer because it serves as polymeric crosslink in the study. AABOB was prepared according to our previous reported procedures[8] (Scheme S2). The polymer **PBOB** was characterized as M_n = 8600 by GPC (Figure S9).

The phenylboronic acid (PBA) family is known for forming reversible boron-diol bonds with sugars. This binding is more obvious when α,β-diol is in the open chain form[9]. It is also widely accepted that fructose as a furanose sugar gives a higher binding ability than the common pyranose sugars do and reducing monosaccharides which can undergo reversible equilibrium between cyclic form and open-chain form, bind PBA and its derivatives. However the non-reducing derivatives of monosaccharides with substitutes at anomeric position bind PBA weakly or even not do so entirely. In our study, the sugars are linked from their anomeric position to polymer chain, thus the traditional PBA does not bind to these glycopolymers. Therefore BOB is selected as a derivative of PBA[4], because of its distinctive binding ability to non-reducing sugars[10]. Moreover, in this case, the binding takes place at neutral pH, which is another advantage of BOB compared to the traditional PBA.

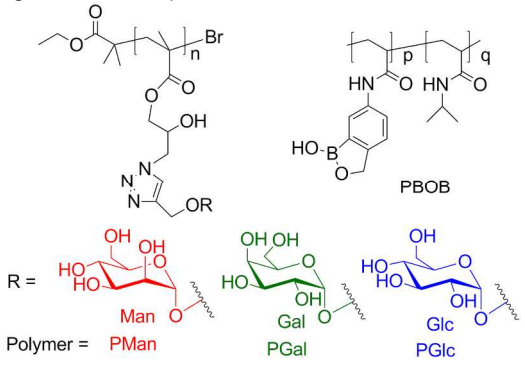


Figure 1. Chemical structures of **PBOB**, **PMan**, **PGal** and **PGlc**.

Then hydrogels are prepared simply by mixing 1 mL glycopolymer (100 mg/mL in PBS buffer (pH 7.4, salt concentration: 100 mM) with 1 mL **PBOB** (100 mg/mL in H_2O, pH 8.0) at room temperature, so the mixture has a total weight content of 10% with equal content of the two polymers. The obtained three hydrogels or viscous solutions are coded **Gal-10**, **Glc-10** and **Man-10**, respectively. Three mixtures with a total concentration of 5% were also prepared. At a high concentration (solid content 10%), the three glycopolymers gave hydrogels as proved by tube-inversion assay. The gelation proceeded quite fast with the slowest one within 5 min. However, frequency sweeps (Figure 2a) in rheology study at room temperature demonstrate the apparent difference of gelation in detail among them. The elastic (G') and viscous (G") moduli of the **Man-10** exhibit characteristic features of a solid material with G' > G" over the frequency range. Moreover, G' of **Man-10** gel reaches 10^3 Pa, much higher than the moduli of gels **Glc-10** and **Gal-10**. In low frequency region, for both **Gal-10** and **Glc-10**, G" was higher than G', indicating the intrinsic liquid property of the samples. As frequency increased, a cross point of G' = G" was observed showing the gelation. When the frequency further increased, the solid property (G' > G") becomes obvious. Such significant difference of **PMan** hydrogel to **PGal** and **PGlc** hydrogels was also observed at 5% solid content (Figure 2b, S10). Here G' and G" of **Man-5** are about two manifolds higher than those of **Glc-5** and **Gal-5**, where the moduli of **Glc-5** are too small to be measured accurately. Meanwhile, viscosity of the gels measured in the same dynamic rheology test exhibits the similar tendency to that of the moduli, i.e. **Man-10** > **Gal-10** > **Glc-10** and the same for the mixtures of 5% (Figure 2c,d).

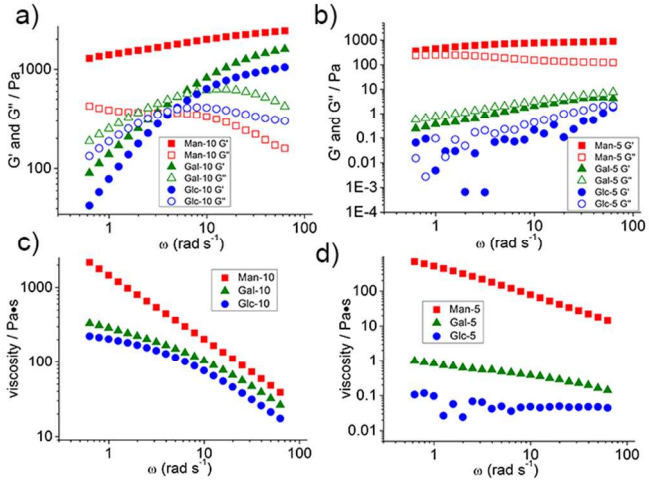


Figure 2. Dynamic rheology measurement of G', G" (a) and viscosity (c) of **Man-10**, **Gal-10**, **Glc-10** and G', G" (b) and viscosity (d) of **Man-5**, **Gal-5**, **Glc-5** as a function of angular frequency (ω) at 20 °C (strain 1%).

The temperature effect on hydrogelation was also measured by rheology. As shown in Figure 3a, to the samples of **Gal-10** and **Glc-10**, the increase of temperature brought an overlapping of G' and G", resulting in G' slightly larger than G" when the temperature was higher than 30 ºC. This phenomenon can be explained by the temperature-induced phase transition of PNIPAm, forming new physical crosslinking domains of the collapsed PNIPAm[11]. Moreover, the moduli of **Gal-10** were higher that those of **Glc-10** at elevated temperatures. Again, **Man-10** showed very different behavior with temperature from those of **Glc-10** and **Gal-10**. For **Man-10**, both G' and G" increased during heating and G' kept larger than G" over the whole temperature range, while those of **Gal-10** slightly decreased and those of **Glc-10** decreased significantly. This result is understandable because unlike **Gal-10** and **Glc-10**, a well-organized gelation network already exists inside **Man-10** at low temperature, which could not be affected by the aggregation of PNIPAm. Very similar trend was also observed for those at

lower concentration, as shown in Figure 3b, only **Man-5** gel exhibited solid properties during heating with its G' always higher than G". **Gal-5** sample exhibited the property of a viscous liquid with its moduli two manifolds lower than those of **Man-5**, while the moduli of **Glc-5** cannot be measured accurately. Similar to the property of **Gal-10**, the moduli of **Man-5** slightly decreased during the heating process. The evolution of viscosity measured during the same dynamic rheology measurements of the hydrogels at 10 wt% and 5 wt% was similar to that of the moduli, which are shown in Figure S11.

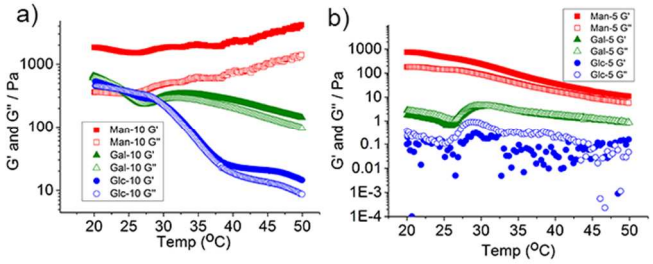


Figure 3. Dynamic rheology measurement of G' and G" of (a) **Man-10**, **Gal-10**, **Glc-10** and (b) **Man-5**, **Gal-5**, **Glc-5** as a function of temperature (constant shear frequency: 1 Hz).

The strength sequence of the hydrogels mentioned above could be attributed to the possible binding sequence of bonding ability of the pendent sugar units to **PBOB**. This idea found supports from two series of fluorescent experiments exploring the interaction between **PBOB** and the different glycopolymers. First, a small amount of (0.83 %) fluorescent moiety 7-nitro-2,1,3-benzoxadiazole (NBD) was introduced to the polymer chain of **PBOB** by copolymerization of NBD-containing monomer with NIPAm and AABOB (**N-PBOB**, Scheme S2). After the same amount of glycopolymers was added into the copolymer solution at a concentration of 0.25 mg/mL, relative fluorescent intensity of **N-PBOB** was increased to 1.60 for **PMan** and 1.24 for **PGal**, but kept unchanged for **PGlc**. In the **N-PBOB** solution, fluorescent quenching of chromophore NBD induced by self-aggregation was expected, thus the observed fluorescent increase can be attributed to the interaction of **N-PBOB** with the glycopolymer, which dissociates this aggregation. Thus the interaction sequence between **N-PBOB** and glycopolymer is **PMan** > **PGal** > **PGlc**. This conclusion was confirmed by the experiments of fluorescence resonance energy transfer (FRET). Here the NBD group in **N-PBOB** was utilized as a donor, while fluorescent acceptor rhodamine B (RhB) was attached to the main chain of glycopolymers as a pendant group, by click reaction of alkyne modified RhB to PGMA-N_3 with propargyl sugars (Scheme S2). As shown in Figure 4b, in the solution of the equal amount of the donor polymer and acceptor polymer, the ratios of the fluorescent intensity of the acceptor to that of the donor are 1.88, 1.71 and 1.14, for **PMan**, **PGal** and **PGlc**, respectively. It means that the intimateness between the polymer chains caused by the dynamic covalent bond is in the sequence of **PMan** > **PGal** > **PGlc**. In short, the consistent results from the two fluorescent experiments conclude that the difference in forming hydrogel of the glycopolymers stems from the different binding ability of **PMan**, **PGal** and **PGlc** to **PBOB**.

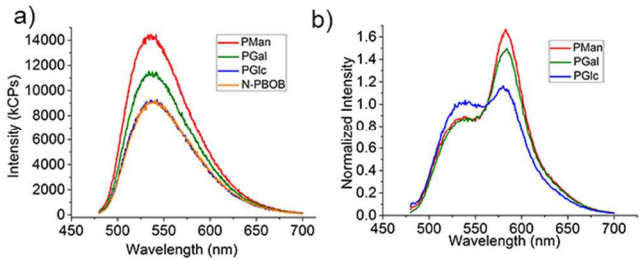


Figure 4. Fluorescent intensity of **N-PBOB** donor with (a) glycopolymers **PMan**, **PGal** and **PGlc** and (b) the corresponding glycopolymer acceptors. (excitation wavelength of 466 nm for NBD group)

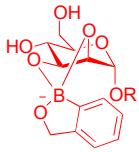
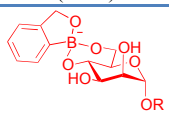
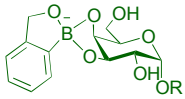
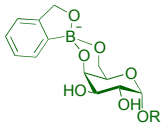
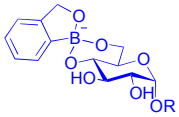
Different from the intimateness sequence showing in glycopolymers to **PBOB** mentioned above, the association constants measured between the small molecules by the Alizarin Red S (ARS) three-component essay in literature showed no significant difference among the three sugars. Association constants (K_a, M^{-1}) of sugar to BOB are measured as 22 (methyl α-D-glucopyranoside), 29 (methyl α-D-galactopyranoside) and 24 (methyl α-D-mannopyranoside), which are slightly lower than that of reducing α-D-glucose (31 M^{-1}). In other words, the binding constants would not show any significant impact on hydrogel strength. In literature, that binding constants of different metal-ligand pairs also did not show any significant contribution to hydrogel strength was reported by Craig *et al*[12].

Hall *et al*[10a] investigated the interaction between BOB and common α-D-pyranosides by ^{1}H NMR and proposed the possibility of different interacting mode at small molecular level. For example, on the galactopyranose ring, the possibilities of the interaction of BOB with *cis-3,4-diol* and *4,6-diol* are much higher than that with *trans-2,3-diol*, because high strain exists in the five-membered ring containing boronic ester formed by the latter unfavorable, which will be ignored in our discussion. Moreover, the interacting possibility of BOB with *cis-3,4-diol* of galactoside is higher than that of *4,6-diol.* Thus based on the BOB-binding possibility trend of galactopyranoside *cis-3,4-diol* > *4,6-diol* >> *trans-2,3-diol*, we draw the possible binding modes between **PBOB** and the different glycopolymers in Table 1.

As shown in Table 1, both of **PMan** and **PGal** bind to BOB in two possible structures, while **PGlc** does it only in one. Moreover, in **PGal**, as 4-OH participates both of the *4,6-diol* mode and the *cis-3,4-diol* one, the two binding modes cannot exist on the same monosaccharide at the same time. However, in the case of **PMan**, both of the *cis-2,3-diol* one and the *4,6-diol* one can be adapted on the same mannopyranoside. This fact does not bring any difference at small molecular level, because the binding stoichiometry of BOB with different monosaccharides were proved as 1:1[10a]. However, when the sugars are grafted to polymer chain, local concentrations of **PBOB** and/or glycopolymer vary due to limited diffusion, thus more binding

sites mean higher binding possibility. Thus **PMan** binds to **PBOB** much stronger and more efficient than **PGal** and **PGlc** do. This effectiveness of **PMan** is further supported by the fact that its hydrogelation still took place when the solid content is as less as 1% (Figure S12), while **PGal** and **PGlc** could not do so even if the solid content is around 5% (Figure S10). Moreover, when the three glycopolymers were mixed with a higher content of **PBOB** (i.e. weight ratio 1:3), instead of the previous 1:1 at the total concentration of 10%, although all of them could pass the vial inversion test, only that formed by **PMan** and **PBOB** was stable enough to be a real "hydrogel", the other two samples did not hold water effectively within 10 min (Figure S13).

Table 1. Possible binding modes of **PMan**, **PGal** and **PGlc** to **BOB**.

	cis-diol (strong)	logic relationship	*4,6-diol* (weak)
PMan		AND	
PGal		OR	
PGlc	/	/	

Last but not least, the responsive property of the hydrogels showed sugar-dependence as well. All of the hydrogels **Man-10**, **Gal-10** and **Glc-10** showed pH responses, i.e. when acid (aqueous 1 M HCl) was added to tune the pH around 2.0, *gel-to-sol* transition occurred and then the resultant sol returned to gel when pH reached 7.4 as base (aqueous 1 M NaOH) was added. Hydrogels **Gal-10** and **Glc-10** were responsive to excess of free glucose, which led *gel-to-sol* transition at a glucose concentration of 30 mg/mL (1.34 equiv. sugar moieties), as a result of the competition between the free glucose and the sugar units of the glycopolymer in binding to **PBOB**. However, glucose was not capable of transforming the *gel* of **Man-10** to *sol* even at a concentration as high as 100 mg/mL (4.47 equiv. sugar moieties). In fact, such sol-to-gel transformation of **Man-10** could be realized by addition of fructose (30 mg/mL), which was found to be the only effective free monosaccharide. Moreover, the **Man-10** has been also proved more stable than **Gal-10** and **Glc-10**, during a long period of time (Figure S14). In addition, very nice 3D network was observed from freeze-dried gel **Man-10** under Scanning Electron Microscope (SEM) with the diameter of pores around 2 μm (Figure S15-20). Last but not least, our cell cytotoxicity evaluation of hydrogel PBOB/PGal with MTT assay indicated its very low cytotoxicity (Figure S21). Considering all these features and the related bioapplications reported in literature[13], our materials might have a promising future in biomedical applications, e.g. cell incubation at a rather high glucose concentration.

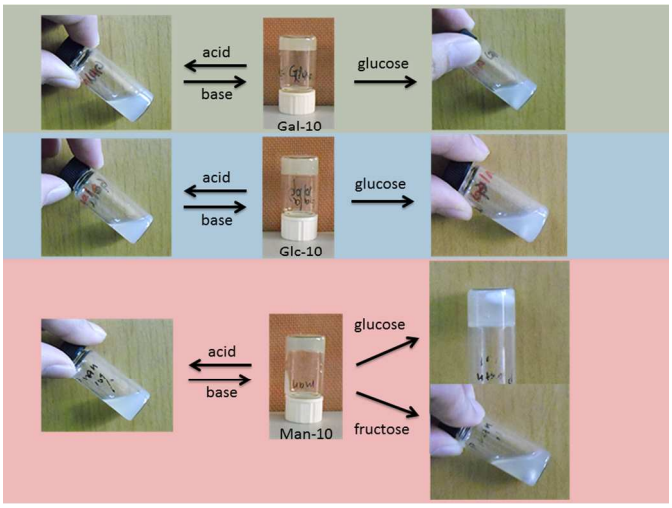


Figure 5. Various responses of different hydrogels **Glc-10**, **Gal-10** and **Man-10** (pH 7.4, Glucose 30 mg/mL for Glc-10 and Gal-10 and 100 mg/mL for Man-10; Fructose 30 mg/mL for Man-10).

Notes and references

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