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The glyco-stereoisomerism effect on hydrogelation of polymers interacting *via* dynamic covalent bonds†

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This work explores, for the first time, the stereoisomerism effect of sugar units of glycopolymers on hydrogelation. Three glycopolymers with an 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 the benzoboroxole (BOB)-containing polymer. We conclude that the gelation ability of the glycopolymers differs obviously as shown in the sequence, Man > Gal > Glc due to the corresponding difference in their sugar–BOB interaction ability.

Glycopolymers have been developed as simplified but powerful building blocks to mimic various glycoconjugates in nature, which include polysaccharides, proteoglycans, glycolipids and glycoproteins.[1] In this respect, using glycopolymers shows obvious advantages: (1) nowadays polymer scientists are able to design and synthesise various polymeric sugars with well-defined structures and rather high molecular weights *via* controlled polymerization; (2) the target glycopolymers can self-assemble into desired nanostructures and even bulky materials.

Artificial hydrogels are important materials for many biomedical applications.[2] Hydrogels composed of glycopolymers are normally prepared *via in situ* polymerization of glycomonomers with chemical crosslinkers, where the glyco-units do not show any significant role as they are not involved in the crosslinking. In the current study aiming at determining 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 the literature in which well-controlled glycopolymers were used to prepare hydrogels by crosslinking *via* boron–sugar bonds,[4] but no attention was paid to the structural effect of the sugar units on hydrogelation. Furthermore, in the work the open chain form of sugars might compete with pyranose sugars in binding to boron.

In nature, a slight structural variation in sugars is capable of inducing a significant change in the properties of bulk materials. For example, α-(1-4) linked glucopyranoside forms amorphous amylose, while β-(1-4) linked glucopyranoside forms 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 sugars may show contrasting 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 lysosomes, while those with 6-Gal (linked *via* a primary hydroxyl group at the 6 position) only reached early endosomes. However, we noticed that such an important effect of the sugar structure has not been explored in hydrogelation of glycopolymers. No doubt, deciphering this effect would not only benefit the formation 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, were synthesized and employed (Fig. 1). The gelation of the glycopolymers was performed by their subsequent interaction with the benzoboroxole (BOB)-containing polymer (Fig. 1).

Three glycopolymers were prepared *via* post polymerization modification, which ensured the structural identity of the polymer backbones (Fig. 1, Scheme S1, ESI†). Briefly, poly(glycidyl methacrylate) (PGMA) prepared *via* atom transfer radical polymerization (ATRP) was characterized as $M_{n,GPC}$ = 1.33×10^4 by GPC (DMF as eluent and PEG as standard (Fig. S1, ESI†)) and $M_{n,NMR}$ = 3.33×10^4 by ^{1}H NMR (Fig. S2, ESI†). The subsequent ring opening of the pendent epoxide group of PGMA with NaN_3 afforded the product polymer PGMA-N_3

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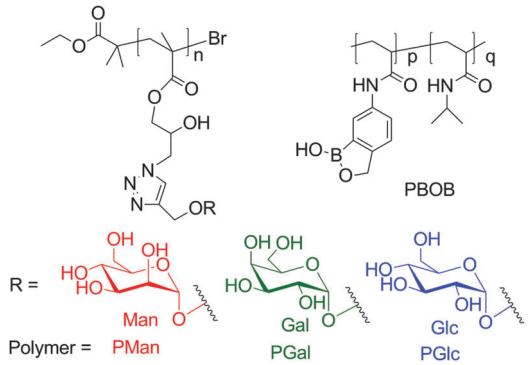


Fig. 1 Chemical structures of **PBOB**, **PMan**, **PGal** and **PGlc**.

bearing one azide on each repeating unit (characterization in Fig. S1 and S3, ESI†). 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 Fig. S4–S7, ESI†). The benzoboroxole (BOB)-containing polymer, PNIPAm-*co*-PBOB (**PBOB**), was prepared *via* free-radical polymerization of monomer *N*-isopropylacrylamide (NIPAm) and 5-acrylamidobenzoboroxole (AABOB) with a feed ratio of 19 : 1 (^{1}H NMR in Fig. S8, ESI†). A small proportion of AABOB was adapted in the copolymer because it serves as a polymeric crosslink in the study. AABOB was prepared according to our previously reported procedures[8] (Scheme S2, ESI†). The polymer **PBOB** was characterized as M_n = 8600 by GPC (Fig. S9, ESI†).

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 exhibits a higher binding ability than the common pyranose sugars do, and reducing monosaccharides which can undergo reversible equilibrium between the cyclic form and the open-chain form bind PBA and its derivatives. However, the non-reducing derivatives of monosaccharides with substitutes at the anomeric position bind PBA either weakly or do not bind at all. In our study, the sugars are linked to polymer chains from their anomeric positions, thus the traditional PBA does not bind to these glycopolymers. Therefore, BOB is selected as a derivative of PBA,[4] because of its distinctive ability to bind 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.

Then hydrogels were prepared simply by mixing 1 mL of glycopolymer (100 mg mL^{-1} in PBS buffer, pH 7.4, salt concentration: 100 mM) with 1 mL of **PBOB** (100 mg mL^{-1} in H_2O, pH 8.0) at room temperature, so the mixture has a total weight content of 10% with an equal content of the two polymers. The obtained three hydrogels or viscous solutions are coded as **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

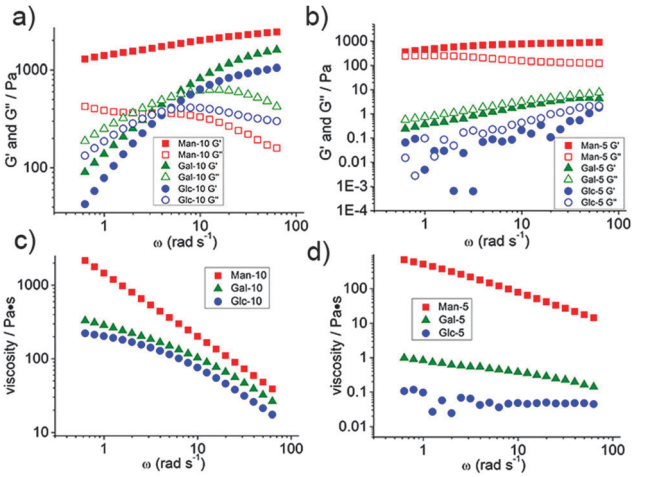


Fig. 2 Dynamic rheology measurements of G', G'' (a) and viscosity (c) of **Man-10**, **Gal-10**, and **Glc-10** and G', G'' (b) and viscosity (d) of **Man-5**, **Gal-5**, and **Glc-5** as a function of angular frequency (ω) at 20 °C (strain 1%).

sweeps (Fig. 2a) in the rheological study at room temperature demonstrated the apparent difference in gelation among them in detail. The elastic (G') and viscous (G'') moduli of **Man-10** exhibit characteristic features of a solid material with $G' > G''$ over the frequency range. Moreover, G' of the **Man-10** gel reaches 10^3 Pa, much higher than the moduli of **Glc-10** and **Gal-10** gels. In the low frequency region, for both **Gal-10** and **Glc-10**, G'' was higher than G', indicating the intrinsic liquid properties of the samples. As the frequency was increased, a cross point of $G' = G''$ was observed showing the gelation. When the frequency was further increased, the solid properties ($G' > G''$) became obvious. Such a significant difference between the **PMan** hydrogel and **PGal** and **PGlc** hydrogels was also observed at 5% solid content (Fig. 2b, Fig. S10, ESI†). Here G' and G'' of **Man-5** are about two-fold 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 a similar tendency to that of the moduli, *i.e.* **Man-10** > **Gal-10** > **Glc-10** and the same for the mixtures of 5% (Fig. 2c and d).

The temperature effect on hydrogelation was also measured by rheology. As shown in Fig. 3a, in the samples of **Gal-10** and **Glc-10**, the increase of temperature caused an overlapping of G'

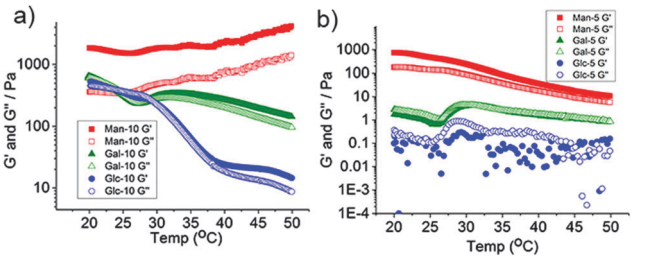


Fig. 3 Dynamic rheology measurements of G' and G'' of (a) **Man-10**, **Gal-10**, and **Glc-10** and (b) **Man-5**, **Gal-5**, and **Glc-5** as a function of temperature (constant shear frequency: 1 Hz).

and G'', resulting in G' being slightly larger than G'' when the temperature was higher than 30 °C. 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 than those of **Glc-10** at elevated temperatures. Again, behavior of **Man-10** upon temperature variation was very different from those of **Glc-10** and **Gal-10**. For **Man-10**, both G' and G'' increased during heating and G' remained larger than G'' over the whole temperature range, while those of **Gal-10** slightly decreased and those of **Glc-10** decreased significantly. These results are 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. A very similar trend was also observed for glycopolymers at the lower concentration, as shown in Fig. 3b, only the **Man-5** gel exhibited solid properties during heating with its G' always higher than G''. The **Gal-5** sample exhibited the properties of a viscous liquid with its moduli two-fold lower than those of **Man-5**, while the moduli of **Glc-5** could not be measured accurately. Similar to the moduli 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 Fig. S11 (ESI†).

The strength sequence of the hydrogels mentioned above could be attributed to the possible sequence of binding ability of the pendent sugar units to **PBOB**. This idea was supported by two series of fluorescence experiments exploring the interactions between **PBOB** and the different glycopolymers. First, a small amount of (0.83%) fluorescent moiety 7-nitro-2,1,3-benzoxadiazole (NBD) was introduced into the polymer chain of **PBOB** by copolymerization of NBD-containing monomers with NIPAm and AABOB (**N-PBOB**, Scheme S2, ESI†). Upon addition of the same amount of glycopolymers into the copolymer solution at a concentration of 0.25 mg mL^{-1}, relative fluorescence intensity of **N-PBOB** was increased to 1.60 for **PMan** and 1.24 for **PGal**, but remained unchanged for **PGlc**. In the **N-PBOB** solution, fluorescence quenching of chromophore NBD induced by self-aggregation was expected, thus the observed fluorescence 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 the glycopolymer is **PMan** > **PGal** > **PGlc**. This conclusion was confirmed by fluorescence resonance energy transfer (FRET) experiments. 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 a click reaction of alkyne modified RhB to PGMA-N_3 with propargyl sugars (Scheme S2, ESI†). As shown in Fig. 4b, in the solution of the equal amount of the donor and acceptor polymers, the ratios of the fluorescence 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 association between the polymer chains caused by the dynamic covalent bond is in the following sequence: **PMan** > **PGal** > **PGlc**. In short, from the consistent results of the two fluorescence experiments it can be concluded that the

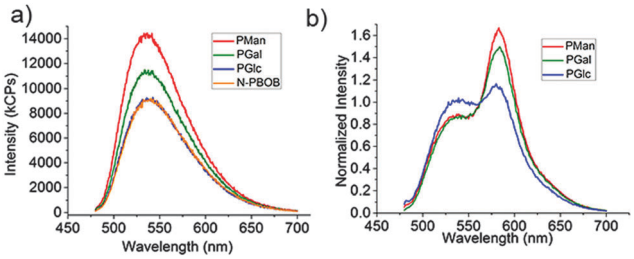


Fig. 4 Fluorescence intensity of the **N-PBOB** donor with (a) glycopolymers **PMan**, **PGal** and **PGlc** and (b) the corresponding glycopolymer acceptors. (Excitation wavelength of 466 nm for the NBD group).

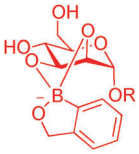
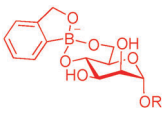
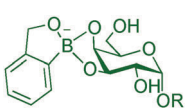
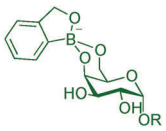
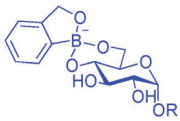
difference in hydrogel formation of the glycopolymers stems from the different binding abilities of **PMan**, **PGal** and **PGlc** to **PBOB**.

Different from the association sequence of glycopolymers to **PBOB** mentioned above, the association constants of the small molecules measured by the Alizarin Red S (ARS) three-component assay reported in the literature showed no significant difference among the three sugars. Association constants (K_a, M^{-1}) of sugars to BOB were measured to be 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 did not show any significant impact on the hydrogel strength. Craig *et al.* reported that binding constants of different metal–ligand pairs also do not show any significant contribution to the hydrogel strength.[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 modes at the small molecular level. For example, on the galactopyranose ring, the possibility of the interaction of BOB with *cis*-3,4-diol and 4,6-diol is much higher than that with *trans*-2,3-diol, because high strain existing in the five-membered ring containing boronic ester formed by the latter is unfavorable, which has been ignored in our discussion. Moreover, the possibility of interaction of BOB with *cis*-3,4-diol of galactoside is higher than that with 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 have drawn the possible binding modes between **PBOB** and the different glycopolymers, as shown in Table 1.

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

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	—	—	

PBOB more strongly and more efficiently than **PGal** and **PGlc**. The effectiveness of **PMan** was further supported by the fact that its hydrogelation still took place when the solid content was as less as 1% (Fig. S12, ESI†), while **PGal** and **PGlc** could not do so even if the solid content was around 5% (Fig. S10, ESI†). Moreover, upon mixing the three glycopolymers with a higher content of **PBOB** (*i.e.* weight ratio 1 : 3), instead of the previous 1 : 1 ratio, at the total concentration of 10%, although all of them passed the vial inversion test, only the one 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 (Fig. S13, ESI†).

Last but not least, the responsive properties of the hydrogels showed sugar-dependence as well. All of the hydrogels **Man-10**, **Gal-10** and **Glc-10** showed pH responsiveness, *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 upon addition of base (aqueous 1 M NaOH). Hydrogels **Gal-10** and **Glc-10** were responsive to excess of free glucose, which led the *gel-to-sol* transition at a glucose concentration of 30 mg mL^{-1} (1.34 equiv. sugar moieties), as a result of the competition between the free glucose and the sugar units of the glycopolymer for binding **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^{-1} (4.47 equiv. of sugar moieties). In fact, such a sol-to-gel transformation of **Man-10** could be realized by addition of fructose (30 mg mL^{-1}), which was found to be the only effective free monosaccharide. Moreover, **Man-10** has also been proved to be more stable than **Gal-10** and **Glc-10**, during a long period of time (Fig. S14, ESI†). In addition, a very nice 3D network was observed for freeze-dried gel **Man-10** under a scanning electron microscope (SEM) with the diameter of pores around 2 μm (Fig. S15–S20, ESI†).

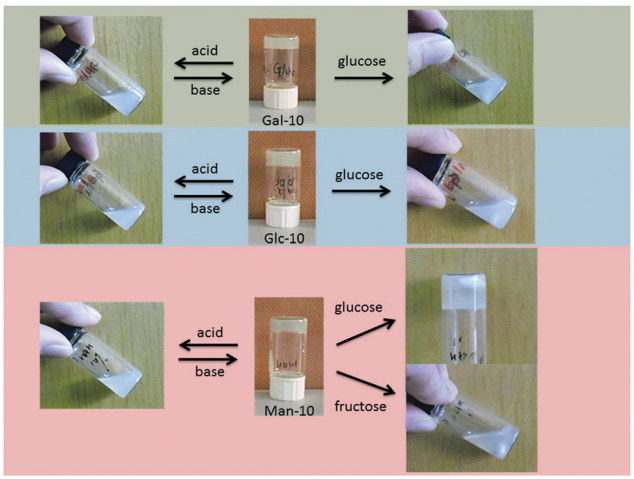


Fig. 5 Various responses of different hydrogels **Glc-10**, **Gal-10** and **Man-10** (pH 7.4, glucose 30 mg mL^{-1} for **Glc-10** and **Gal-10** and 100 mg mL^{-1} for **Man-10**; fructose 30 mg mL^{-1} for **Man-10**).

Last but not least, our cell cytotoxicity evaluation of hydrogel **PBOB**/**PGal** by the MTT assay indicated its very low cytotoxicity (Fig. S21, ESI†). Considering all these features and the related bioapplications reported in the literature,[13] our materials might have a promising future in biomedical applications, *e.g.* cell incubation at a rather high glucose concentration (Fig. 5).

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