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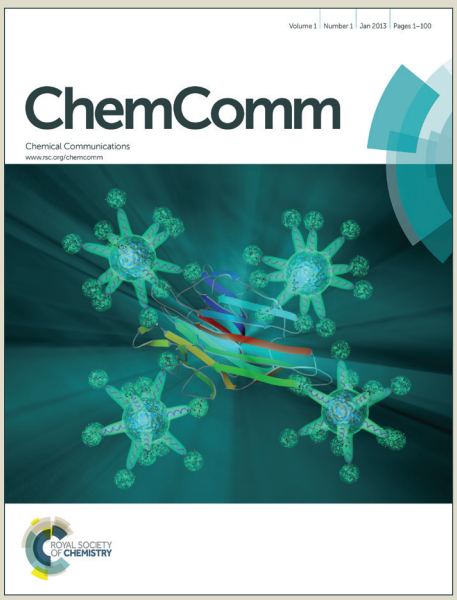

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Synthesis of Clickable Amphiphilic Polysaccharides as Nanoscopic Assemblies

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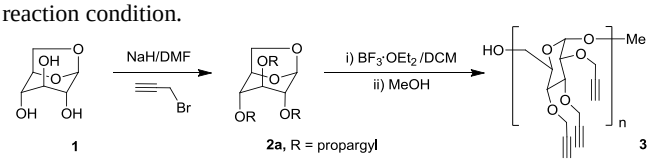
The synthesis of clickable polysaccharides was achieved by using alkynylated 1,6-anhydro glucopyranose as a monomer and $BF_3{\cdot}OEt_2$ as an effective catalyst. Subsequent click conjugation with polyethylene glycol (PEG) afforded PEG-grafted polysaccharides in nearly quantitative efficiency.

Polymeric nanoparticles (NPs), with the capability and versatility to package and deliver payloads to targets, are emerging as promising delivery systems in drug delivery.[1-4] However, despite much progress, few NP delivery systems are able to make it to clinical studies. Among the many issues, poor biocompatibility and poor degradability remain as major concerns when considering clinical safeties. To address these concerns, efforts have been invested in pursuing safe polymers and materials. In light of this, polysaccharides have attracted special attention because of their inherent biocompatibility and degradability.[4-6] However, the use of natural polysaccharides is facing many challenges and limitations, including the need of extensive purification; batch-to-batch variations in structures, molecular weights and polydispersities; as well as possible contamination with biological toxins; etc. Notably, the major limitation is the lack of a method to functionalize the sugars. In order to serve as a NP delivery system, chemical conjugations, either with a drug or with a hydrophobic segment (to form amphiphilic polymers), are required. This is especially true when considering that over 40% of currently marketed drugs are highly hydrophobic.[7] Unfortunately, chemical modifications on naturally occurring polysaccharides are extremely challenging, due to their low solubility in organic solvents and the presence of multiple –OH groups. As a result, the conjugations were often tedious with low yields.[8-11] To circumvent these limitations and challenges, we pursued a polymerization method that allows for manipulating the polymer chemistry at the monomer level, thereby achieving a functionalized polymer upon successful polymerization. The polymerization should also offer mechanistic control of the structure and composition in the polymer, imparting desired chemistry and functionalities for further modification and conjugation. Currently, two polymerization methods, including polycondensation using a di-functional AB-monomer, and ring-opening polymerization using an anhydro sugar (ROPAS), have been reported. While the polycondensation method can only produce oligosaccharides with molecular weights of a few kDa,[12-14] the ROPAS method can often lead to molecular weights of dozens of kDa.[15-20] Our aim was therefore to design a functionalized monomer for ROPAS, thereby achieving functionalized polysaccharides for further conjugation. Among the many conjugation methods, copper-catalysed azide-alkyne cycloaddition (click chemistry) is arguably the most efficient for macromolecules.[21] With these considerations, we hypothesized that a post-polymerization click conjugation may allow for the synthesis of amphiphilic polymers,[22,23] which could be used for the construction of nanoparticles. Herein, we report the synthesis of clickable polysaccharides through ROPAS and their subsequent conjugation with PEG to give an amphiphilic polymer, which was then used to construct polysaccharide-based NPs.

With the aim to achieve efficient click conjugation, multiple alkyne groups should be installed. Therefore, an alkyne-containing monomer **2a** (Scheme 1) was designed, and then synthesized from commercially available 1,6-anhydro glucopyranose (**1**). The reaction with propargyl bromide in the presence of NaH/DMF went smoothly with excellent yield. The NMR studies (Figure 3A and ESI) indicated that the anhydro structure was not affected under this reaction condition.



1 2a, R = propargyl 3

Scheme 1. The synthesis of monomer **2a** and its ROPAS to give polysaccharide **3**.

With the successful synthesis of the monomer, we then attempted the polymerizations under varying reaction conditions. In ROPAS, the Lewis acid catalyst is a critic al component, which initiates the polymerization, affects the α/β stereochemistry of the glycosyl linkages between each sugar unit, and determines the molecular weights. Several Lewis acids, including $TiBr_4$,[24] $SbCl_5$[25] and ZnI_2[26] have been reported. Unfortunately, these catalysts did not produce polymers in our case. Both TLC and NMR studies indicated that the monomer largely remained intact. Possibly, these transition metal-based Lewis Acids have chelated with the alkyne functionality and were thereby inactivated.

Table 1: Conditions and results of ROPAS using **2a** as a monomer and $BF_3{\cdot}OEt_2$ as a catalyst

entry[a]	catalyst mol%	t (h)	conv.	M_n (kDa)[b]	PDI[b]	yield
1	5%	0.5	21%	8.3	1.8	n/a[c]
2	5%	1	33%	13.6	1.7	n/a[c]
3	5%	2	44%	33.5	1.6	n/a[c]
4	5%	3	49%	35.4	1.5	n/a[c]
5	5%	4	52%	36.1	1.4	43%[d]
6	10%	4	78%	34.3	1.7	40%[d]
7	2.5%	4	40%	37.3	1.4	34%[d]

a. all polymerizations were performed at rt using dichloromethane as a solvent.
b. measured by gel permeation chromatography (GPC) using THF as an eluent. The GPC was calibrated using polystyrene standards.
c. not isolated and purified.
d. measured after purification by precipitation in cold methanol.

The polymerization was then performed using a non-transition metal catalyst---$BF_3{\cdot}OEt_2$. To our delight, this catalyst was highly effective. With only 5mol% catalyst, the polymerization gave a polymer with M_n^{GPC} = 8.3 kDa and PDI = 1.8 (Table 1, entry 1) at 0.5 h. As the polymerization progressed, the molecular weight continued to increase, reaching 13.6, 33.5, 35.4 and 36.1 kDa after 1, 2, 3 and 4 h reaction, respectively (Table 1, entries 2-5). Thereafter, an extension of the reaction time did not increase the molecular weights significantly. The kinetic studies, by means of parallel experiments, indicated that the conversions and the molecular weights increase sharply within the first 2 h, but thereafter gave a nearly-flat curve (Figure 1A and 1B). The initial increase of conversion is understandable, because in ROPAS, the initiator is the monomer itself. Therefore, at the beginning, many polymer chains were formed for propagation, resulting in fast consumption of the monomers. The fast increase in the molecular weight was a result of effective initiation and high polymerizability of the monomer. However, after about 2 h, the conversion and the molecular weight did not increase significantly, largely due to the increase of steric hindrance when the molecular weights reached a certain high value.

Overall, the conversions were relatively low. At 5mol% catalyst, the conversion was only about 52% after 4 h reaction. The low conversion is possibly due to the "site reaction" of the Lewis acid catalyst, which also chelated with the many oxygen atoms on the monomers, oligomers and polymers in the reaction mixture. To improve the conversion, we then increased the amount of catalyst to 10mol%, which led to the increase of the conversion to 78%. However, PDI increased sharply without noticeable increase in the molecular weight (Table 1, entry 6). On the other hand, a decrease of the amount of $BF_3{\cdot}OEt_2$ to 2.5mol% led to lower conversion (40%) and slightly higher molecular weight (Table 1, entry 7). These results are in general agreement with results from previous studies.[16,19,27] Nonetheless, we were pleased that the ROPAS allowed for efficient polymerization of the alkyne-functionalized monomer, giving polymer **3** with molecular weights of 36.1 kDa, as measured by GPC (Figure 1).

The ^{1}H-NMR studies showed that the polymers possessed α-anomeric protons resonating at 5.03 ppm (Figure 2A), and the ^{13}C-NMR studies showed the α-C1s resonating at 96.79 ppm (see ESI. All NMR signals were assigned based on 2D COSY and HMQC NMR studies). These studies indicated the exclusive formation of α-1,6 glycosyl linkages between each sugar unit. The NMR studies also indicated that the alkyne-functionality was intact during the polymerization, with alkyne protons resonating at *ca.* 2.5 ppm and 4.4 ppm. The successful synthesis of polysaccharides with multiple pendent alkyne groups[+] may allow for click conjugation with an azide-functionalized molecule and/or a polymer.

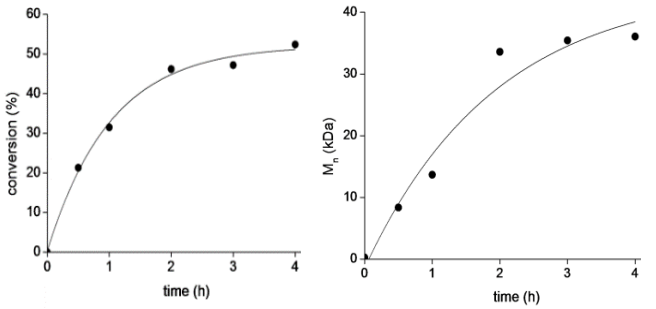


Fig. 1 Kinetic plots of conversion versus time (left) and molecular weight versus time (right). The conversions were obtained from ^{1}H-NMR analyses, while the molecular weights were determined by GPC analyses.

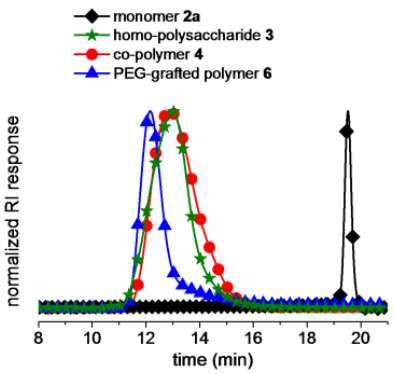


Fig. 2 The GPC curves of monomer **2a**, homo-polymer **3**, co-polymer **4** and PEG-grafted polysaccharide **6**. All polymers were measured after purification by precipitation using cold methanol.

In order to adjust the conjugation density, we then studied the co-polymerization of **2a** with another monomer of distinct structure. Methylated 1,6-anhydro glucopyranose (**2b**, Scheme 2) was chosen as the second monomer because methylated polysaccharides have demonstrated excellent biocompatibility.[28] Monomer **2b** was synthesized in similar manner as that of **2a** (see ESI). With this monomer in hand, we then performed a co-polymerization using a monomer ratio of **2a**:**2b** = 1:7 (Scheme 2) to obtain co-polymer **4** with molecular weights of 34.5 kDa, as measured by GPC (Figure 1). It is fortunate that the H-1s derived from monomer **2a** are resonating slightly more up-field than those derived from **2b** (Figure 3B); allowing for the measurement of the ratio of the two monomers incorporated into the co-polymer. The integration ratio is roughly 1:7, indicating that the two monomers have similar polymerizability under this catalytic condition. Based on the GPC and NMR studies, it can be estimated that each polymer chain contains *ca.* 17 units of monomer **2a** and 122 units of monomer **2b**, giving *ca.* 51 alkyne functionalities on each polymer chain. Studies using different monomer ratios (**2a**:**2b** = 1:2 and **2a**:**2b** = 1:15) were also performed and similar molecular weights and PDIs were obtained (see ESI).

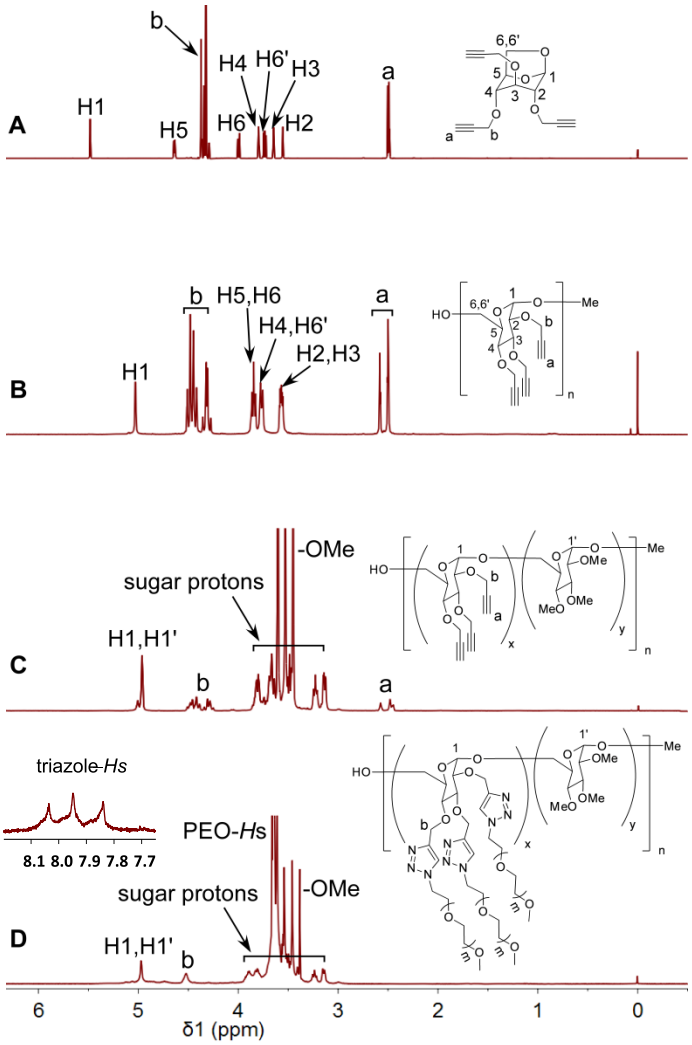
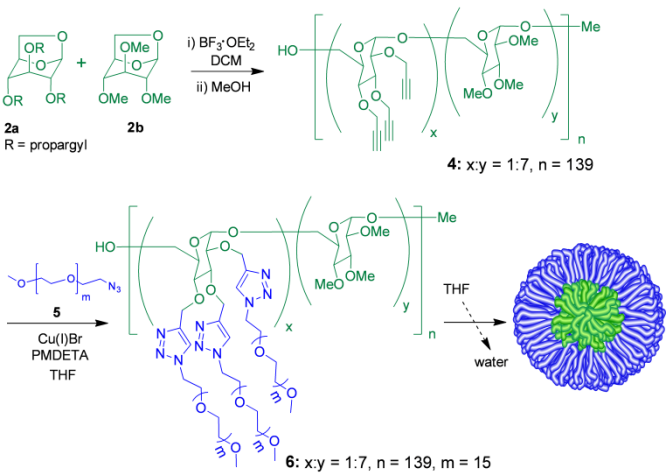


Fig. 3 The ^{1}H-NMR spectra of monomer **2a** (A), homo-polymer **3** (B), co-polymer **4** (C) and PEG-grafted polysaccharide **6** (D).



Scheme 2. The synthesis of co-polymer **4**, the subsequent click conjugation with PEG to give PEG-grafted polysaccharide **6**, and the construction of NPs through self-assembly.

For click conjugation, azide-functionalized PEG (M_n = 750) (**5**)[29] (see ESI) was employed, which may serve as a hydrophilic segment to form an amphiphilic polysaccharide. The conjugation was performed using Cu(I)Br as a catalyst and N,N,N',N',N''-pentamethyldiethylenetriamine (PMDETA) as a chelating agent.[30] The reaction went efficiently with nearly 100% conversion. The NMR analyses indicated that all alkyne groups (resonating at *ca*. 2.5 ppm) were consumed, and the emerging tri-azole protons resonate at *ca*. 7.8-8.1 ppm (Figure 3D). FT-IR studies also indicated the complete disappearance of the alkyne groups upon successful click conjugation (Figure 4). The GPC measurement gave M_n^{GPC} = 51 kDa (Figure 2), which is very close to the theoretical value (M_n^{theo} = 58 kDa).

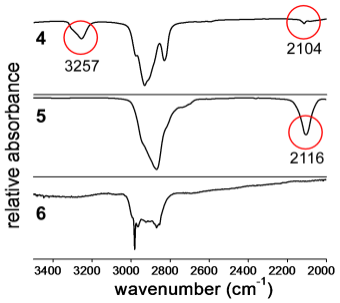


Fig. 4 The FT-IR spectra of co-polymer **4**, azide-functionalized PEG **5** and PEG-grafted polysaccharide **6**.

The PEG-grafted polysaccharide **6** was then used to construct nanostructures through a self-assembly process. The polymer was first dissolved in a good solvent (THF), upon transitioning to aqueous solution, self-assembly occurred to give NPs with TEM sizes of 94 ± 15 nm (Figure 5, left) and hydrodynamic light scattering (DLS) sizes D_h (intensity) = 226 ± 35 and D_h(volume) = 245 ± 66 nm (Figure 5, right). The increase of the DLS sizes over TEM sizes is the result of the hydration of the nanoparticles in aqueous solution.[31] It should be noted that the reported structure here has the capability to tune the density of PEG, and therefore the size and morphology of the nanoparticles[29] to suit specific biomedical applications.

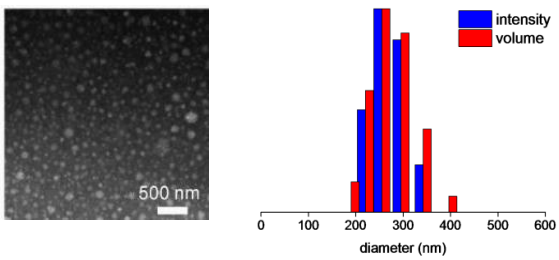


Fig. 5 TEM image (left) and the DLS sizes (right) of the NPs derived from PEG-grafted polysaccharides **6**.

The NPs, with polysaccharide backbone and PEG side chains, are expected to have low cytotoxicity. Indeed, when the cell viability studies were performed using HCT116 cells, the polysaccharide-based NPs showed minimal toxicity to the cells (Figure 6). The percentage of cell viability is even higher than that using media only, suggesting that these polysaccharide-based NPs exhibit excellent biocompatibility. The cell viability studies were also performed

using another cell line---HEK293 cells. Similarly, the polysaccharide-based NPs showed minimal cell toxicity (see ESI).

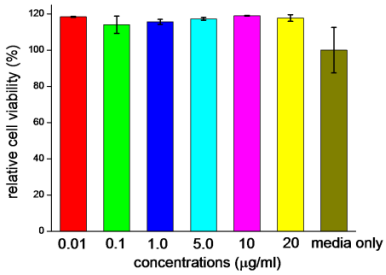


Fig. 6 The cell viability studies using HCT116 colon cancer cells.

In conclusion, we reported a facile synthesis of alkyne-functionalized α-1,6-glycosylated polysaccharides through ROPAS. With multiple alkyne groups on the polymers, click conjugation with PEG was achieved to give amphiphilic grafted polysaccharides, which can be used to construct polysaccharide-based NPs. Herein we only demonstrated the high conjugation efficiency using azide-functionalized PEG as a model polymer. However, with multiple alkyne groups on the polysaccharide backbone, a mixture of azide-functionalized drugs and PEG (or other polymers) at varying ratios can be clicked simultaneously, giving drug conjugated PEG-grafted polymers and nanostructures, as demonstrated previously.[32] The cell viability studies indicated that such NPs exhibit minimal toxicity to those cells. The successful syntheses of clickable polysaccharides can effectively address many issues associated with current methods of modification of naturally occurring polysaccharides, allowing for convenient access polysaccharides for conjugation and functionalization. Considering the high efficiency of click chemistry, and a large number of azide-containing functional molecules/ polymers, the work presented herein may allow for the synthesis of a vast number of polysaccharide-based polymers and biomaterials for many biological and biomedical studies. Current studies, including the conjugation with many azide-functionalized drugs and polymers,[32] as well as *in vitro* drug delivery, are underway in our laboratory.

Notes and references

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†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

+It should be noted that the polymer, with multiple alkyne groups, was susceptible to crosslinking when exposed to light and/or to air. Therefore, care should be taken during storage.

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