Supramolecular glycopolymers with thermo-responsive self-assembly and lectin binding†

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Incorporating monomers into sequence-defined synthetic macro-molecules endows them to mimic nature which results in key residues being anchored in the molecular recognition pattern. Developing controlled carbohydrate sequences has critical importance in understanding the multivalent binding motifs of oligosaccharide and sequence-controlled glycopolymers to various lectins. Here, we describe the development of thermo-responsive copolymer scaffolds bearing adamantane groups that enable the formation of an inclusion complex with mono and hepta mannosylated-cyclodextrin molecules through host–guest interaction. We have demonstrated the synthesis of a triblock copolymer via RAFT polymerization, the complexation of the adamantane containing a thermo-responsive copolymer and α-β-mannose-CD, as well as their interactions with Con A.

Introduction

Biomacromolecules, such as proteins, nucleic acids and polypeptides, are largely responsible for the complexity and diversity of biological materials mainly due to their precisely controlled secondary and tertiary structures. Controlling the monomer sequence is a key strategy in synthetic polymer science towards mimicking biomacromolecules. In recent years, there is an increasing tendency towards the preparation of well-defined sequence-controlled polymers.1–3 Precise positioning of monomer units along a polymer chain allows the generation of precisely tailored materials through self-assembly,4 with control over nanoscale-domain geometry, packing symmetry and chemical composition. Recently, new methodologies have emerged for multi-block copolymer synthesis based on one-pot sequential addition of monomers while targeting near full conversion per block, thus removing the need for intermediate purification steps.5,6 Perrier and co-workers demonstrated that reversible addition–fragmentation chain transfer (RAFT) polymerization is very useful in making macro-molecules featuring a large number of block sequences in a controlled fashion. Importantly, the authors nicely demonstrated that the livingness of a RAFT process depends on the knowledge of polymerization kinetics.7–9

Similarly, stimuli-responsive or smart behavior can also be imparted to synthetic polymers by incorporation of functional groups that can undergo changes in physical properties, which allow the conjugation of other molecules, such as peptides, carbohydrates, targeting vectors or drugs, onto the polymer backbone. Interest in smart polymers has remained high over many decades, displaying great promise in numerous nanotechnological and biomedical applications.10,11 Smart polymers show rapid and reversible changes in response to small changes to their environment (e.g., pH, temperature, salt concentration, and co-solvent).12–15

The most commonly investigated stimulus has been temperature, which is achieved by simply incorporating thermo-responsive groups in the polymer structure that exhibit LCST (lower critical solution temperature) behavior. Thermo-responsive polymers, such as poly(N-isopropyl acrylamide) (PNIPAM), undergo a reversible phase transition from hydrophilic to hydrophobic when their solution temperature is raised above their LCST, changing from an extended chain conformation below LCST into a collapsed chain above LCST.16–19 Aqueous solutions of PNIPAM exhibit LCST behavior around 32 °C. In general, the LCST of PNIPAM can be influenced by several factors, e.g. molecular weight (Mw), polydispersity, end groups, topology and incorporating hydrophilic or hydrophobic characteristics by copolymerization with hydrophobic or hydrophilic comonomers.20–23 According to these studies, well-defined aqueous solutions of high molecular weight PNIPAM samples with precise end groups, show a dramatic decrease in the cloud point due to the reduced entropy of mixing with increasing Mw. Moreover, by increasing the hydrophilic nature of the polymers, the overall hydrogen...
bonding ability of the macromolecules is increased, which leads to higher transition temperatures. Incorporating hydrophobic groups causes a disruption to the structure of water around the macromolecules and lowers the cloud point. This enhances the interaction of hydrophobic species, further facilitating aggregation. With the same approach, Ritter and co-workers investigated the LCST behavior of both hydrophobic and hydrophilic comonomers after copolymerization with NIPAM and further through supramolecular interactions of these comonomers with cyclodextrins (CD).24

The assembly of new polymeric structures by non-covalent interactions, such as hydrogen bonding, ligand–metal coordination or “host–guest” inclusion complexation, has recently attracted considerable interest.25–28 Cyclodextrin and its derivatives are of great importance due to their well-known ability to incorporate hydrophobic molecules yielding inclusion complexes. One of their potential applications is the use of these host/guest complexes in drug delivery systems.29

Adamantane is one of the most important guests of β-CD due to its effective inclusion entrapment and high binding affinity. Among all the investigated pairs, β-CD and adamantane have been frequently employed due to their high association constant \((10^{7}–10^{8} \text{ M}^{-1})\) in water.28

Glycopolymers are synthetic polymers with sugar moieties attached and have great potential for mimicking biopolymers.30,31 The recognition event of carbohydrates and lectins is critical in a wide variety of intercellular processes.6,32–37 In general, the interaction of a lectin with a carbohydrate is quite weak, but can be dramatically enhanced by the multivalent effect of glycopolymers, which is known as the “glycocluster

Scheme 1 Synthesis of the triblock copolymer by sequential RAFT polymerization and its host–guest interaction with self-assembly behaviour. (a) Schematic representation of the synthesis of the triblock copolymer by RAFT in a one pot sequential fashion. (b) Molecular structure of the synthesized triblock copolymer P1 and its self-assembly micelle formation in water. For simplicity, these structural formulae display only the average DMA, Adac composition of the polymers and do not give information about the localization of the Adac units in the chains. (c) Schematic representation and molecular structures of β-CD derivatives. (d) Self-assembly micelle formation of the P1 triblock copolymer and equimolar β-CD derivatives in water.
effect. The multivalency has a significant effect on carbohydrate-lectin binding through intermolecular crosslinking through lectin receptors. Parameters, such as the density of sugar units, the type of sugar unit and the architecture of the glycopolymers, have an even larger impact on the binding. Although some advanced techniques, such as isothermal titration calorimetry (ITC), quartz crystal microbalance (QCM), and surface plasmon resonance (SPR) have been employed to explore the lectin–glycopolymer interactions, traditional and more widely employed methods (quantitative precipitation, turbidimetry, fluorescence quenching assays, etc.) have also been used to understand these interactions. Recently, extensive reports have been published on this topic, emphasizing the synthesis of different thermo-responsive glycopolymers with a variety of shapes and compositions. Furthermore, as a result of the thermo-responsive behavior of these kinds of polymers, different conformations of the polymers below and above the cloud point temperature influenced the lectin–polymer interaction. Another interesting example was demonstrated by Seeberger et al. on the preparation of multiple multivalent carbohydrate ligands by supramolecular assembly wherein they have investigated the binding interactions between the carbohydrate epitopes and lectins.

In this study, we describe the preparation of a triblock copolymer architecture that has precisely positioned random DMA/adamantane blocks within the first and the third, while the middle block has thermo-responsive PNIPAM that enables the formation of a self-assembled micellar structure above the cloud point. Moreover, we have prepared mono or hepta mannosylated cyclodextrin compounds via a copper catalyzed cyclo-addition reaction. The complexation of the adamantane containing triblock copolymer and cyclodextrin compounds with different amounts of α-β-mannose groups has been performed and characterized in detail. Finally, we have investigated the binding properties of these supramolecular glycopolymers to Con A using turbidimetry (Scheme 1).

Results and discussion

In order to synthesize sugar decorated β-CD derivatives from azide functionalized β-CD derivatives via CuAAC, the preparation of sugar aldehydes is required. Since the discovery of Fischer glycosylation, sulfuric acid immobilized on silica was used as a catalyst for the preparation of alkyl glycosides from free sugar. Thus, alkynyl functionalized α-β-mannose was synthesized according to the literature. The preparation of β-CD-OTs and β-CD-N₃ was well described by Ritter and the same route was adopted to synthesize mono-azide functional CD (β-CD-N₃). In the ¹H NMR spectrum of β-CD-OTs two peaks at 7.42 and 7.75 ppm are assigned to the phenyl protons. The ratio of these two peaks and the peaks corresponding to 2,3- and 6-positioned hydroxyl groups of the β-CD ring approaches 1:1:7, suggesting that only one TsCl is reacted with the 6-positioned hydroxyl group of the β-CD ring. In further reactions, the characteristic phenyl protons in β-CD-OTs disappear, which verifies the complete replacement of the para-toluene-sulfonyl group by the azide group (ESI Fig. S1 and S2†). Hepta-azide functional CD (β-CD-(N₃)₇) was prepared according to our previously published paper. The ratios of all the hydroxyl groups and CH₂Br or CH₃-N₃ protons in ¹H NMR spectra indicate the quantitative functionalization of end-groups (ESI Fig. S3 and S4†). The CuAAC of β-CD-N₃ and β-CD-(N₃)₇, with alkynyl functionalized α-β-mannose was carried out using a CuBr/MesoTREN catalyst system in DMSO as the solvent to yield the β-CD-mannose (CD₄) and β-CD-(mannose)₂ (CD₂) glycosyclusters, respectively. The ¹H NMR spectra for both CD substances showed the presence of a triazole proton at approximately 7.9 ppm and CD residues at 6.0 ppm (OH) and 5.2 ppm (H-1) indicating the success of the CuAAC reaction (ESI Fig. S5 and S6†). FT-IR spectra (ESI Fig. S8 and S9†) confirmed the disappearance of azide functionalities at 2100 cm⁻¹ following the click reaction. DMF SEC analysis (ESI Fig. S10†) revealed the shift of elution traces after the reaction due to the change in the hydrodynamic volume.

Recently, Perrier and coworkers were able to synthesize one-pot, sequence-controlled multi-block copolymers using the RAFT methodology. They focused on acrylamide monomers because acrylamide monomers show a high propagation rate (high kₚ) despite the low initiator concentration to maintain the number fraction of dead chains at minimum. Junkers and coworkers mapped end-group compositions of RAFT polymers obtained from consecutive polymerizations. Consecutive chain extensions of methyl acrylate polymers were carried out under variation of initiator concentrations and investigated in detail via ESI-MS to carefully map the end-group composition evolution. Consequently, this procedure appears to be very efficient for the polymerization of both acrylamide and high kₚ acrylate monomers.

In our study, adamantane modified thermo-responsive tri-block copolymer (P1) was synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization in a sequential addition fashion. We focused on three different monomers, two of which are commercially available, N,N-dimethylacrylamide (DMA) and N-isopropylacrylamide (NIPAM), and the third one is adamantane acrylate (Adac), which is used after modification of 1-adamantane methanol with acryloyl chloride.

The optimized polymerization conditions were chosen as 65 °C in dioxane, and experiments revealed an optimal monomer concentration of 1 M for the first and second blocks and 0.4 M for the third block, which enables rapid polymerization at moderate viscosity of solution.

The contribution of this study is the preparation of a triblock copolymer (P1) which was synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization in a sequential addition fashion. The necessary number of dead chains at minimum, was minimized. The [CTA]₀/|AIBN|₀ ratio was fixed at 130:1 to avoid dead-end polymerization, which is a polymerization where all initiators are decayed before full conversion is reached. When the AIBN...
concentration is lowered, the polymerization eventually requires a longer reaction time before reaching full conversion. Therefore, increasing reaction times were applied based on lower AIBN concentrations, so that in all cases almost full conversions are reached (in practice ≥91–99% was achieved as measured by gas chromatography). DMA and Adac showed similar conversion rates, increasing linear relation with time (Table S1† for the quantity of reagents needed for the triblock copolymer). To obtain full monomer conversion, the reaction time was increased, but the conversion and molecular weight were kept constant.

Thermal phase transition temperature of the polymer solution, expressed as LCST, was measured as a function of temperature at a specific wavelength (λ) 500 nm using a UV-Vis spectrometer. In order to obtain a clear solution, the polymer concentration was chosen as 2.5 mg ml⁻¹. Polymer P1 showed a distinct heat-induced phase transition with a 50% transmittance point at about 24 °C (Fig. 1a). By increasing the hydrophilic nature of the polymers, the overall hydrogen bonding ability of the macromolecules is increased, which leads to higher transition temperatures. Incorporating hydrophobic groups lowers the LCST. Furthermore, the addition of hydrophobic groups causes a disruption of the structure of water around the macromolecules. This enhances the interaction of hydrophobic species, further facilitating aggregation. Compared to the literature value of 32 °C reported for high-molecular weight linear PNIPAM, the decreased LCST of P1 can be attributed to both the rather low molecular weight and the presence of hydrophilic (DMA) and hydrophobic (Adac) units along the backbone. The measured hydrodynamic diameters of this ‘hydrophobic’ conjugated copolymer below its LCST indicate that it exists as unimers in solution, suggesting that the expected increase in LCST is as a consequence of the formation of micelles (Fig. 1b).

Analysis of the molecular weight distributions by size-exclusion chromatography (SEC) revealed monomodal distributions with a clear shift to higher molecular weights after each monomer addition. However, in all cases molecular weights are low and do not differ significantly from each other as should be expected (Fig. 1c). Low molecular-weight tailing was observed after each block extension, ascribed to the accumulation of initiator-derived chains. In each step, the dispersity continuously increased from 1.1 to 1.3. This increase in dispersity could be caused by the nature of different monomers used in the copolymerization (Table S2†). Two different monomers were used for the first and third blocks. Vinyl protons over-
lapped with each other and the monomer conversions could not be calculated by $^1$H NMR spectra. Therefore, we have used GC to obtain more accurate conversion values. The $^1$H NMR spectrum was used to identify the obtained copolymer qualitatively after purification (Fig. 1d).

Adamantyl-functionalized thermo-responsive triblock copolymer (P1) provided supramolecular host–guest interactions with sugar decorated and non-decorated β-CD derivatives. Dynamic light scattering (DLS) is a versatile tool to investigate the complex formation in solution. We employed DLS to obtain the hydrodynamic diameter ($D_h$) of both β-CD complexed thermo-responsive triblock copolymer coils in solution. Heating of the CD-complexed and non-complexed thermo-responsive triblock copolymers showed reversible temperature-induced transition from solution to micelle. That is, under the cloud point ($T_c$), the PNIPAM chain of the polymer remained water soluble, and no micelle was formed. As the temperature increased, the PNIPAM chains dehydrated and aggregated through hydrophilic–hydrophobic interactions, thus acting as a hydrophobic core for the micelle formation. Firstly, $D_h$ of around 6 nm for P1, after heating above the $T_c$ aggregates are formed with $D_h$ around 240 nm (Fig. 1b and 2a). A strong host–guest interaction above the LCST causes the formation of smaller particles. All β-CD complexed thermo-responsive triblock copolymers show similar $D_h$ above the LCST (ESI Fig. S11†). For DLS measurements, the LCST is identified by a sudden change in the particle size. Cloud points of all β-CD complexed thermo-responsive triblock copolymers were determined by DLS. DLS measurements show in almost all cases an increased cloud point of the β-CD complexed copolymers compared to the adamantyl-functionalized copolymer P1 (Fig. 2a), which is an expected behavior of supramolecular block copolymers with a thermoresponsive block.55,56 A possible explanation for such a behavior might be the shielding of the hydrophobic end groups by the CD moiety, increasing the water solubility of adamantyl-functionalized copolymers. The other possible explanation of low $D_h$ values can be the steric hindrance that reduces the micelle formation on the β-CD complexed thermo-responsive triblock copolymers. As it is noted, in general, incorporating β-CD derivatives causes the host–guest interaction and alters the micelle sizes. Furthermore, this effect was also confirmed by SEC chromatograms, upon complexation polymers shifted against the obtained $D_h$ (Fig. 2b and Table S3†). Moreover, to investigate the supramolecular complex formation and its effect on the micelle size, the host:guest ratio was monitored by DLS (ESI Fig. S12†). It

Fig. 2 Characterization of the β-CD complexed triblock copolymer. (a) Temperature responsive behavior of the adamantyl functionalized building block (P1) and its complexes with equimolar β-CD derivatives (P1-CD0, P1-CD6, and P1-CD7) at 1 mg mL$^{-1}$ in H$_2$O. (b) SEC traces for the adamantyl functionalized building block (P1) and its complexes with equimolar β-CD derivatives over several hours in H$_2$O at 25 °C using a RI detector in DMF at 50 °C. (c–d) 2D NOESY NMR spectrum of a 1:1 molar mixture of P1 and β-CD (CD0) in D$_2$O (20 mg mL$^{-1}$) at 25 °C and 70 °C, respectively.
was observed that less β-CD molecules increase the micelle size that allows the penetration of more polymer chains for micelle formation.

2D NOESY NMR was utilized to prove the molecular nature of the complex formation, which is a well-suited tool to study host/guest complex formation. While DLS experiments probe the molecular nature of the triblock copolymers, 2D NOESY NMR was performed to demonstrate the formation of the inclusion complex between the adamantyl moieties and β-CD molecules. The 2D NOESY NMR spectrum shows cross-correlation peaks that can be assigned to the signals of the adamantyl moiety at 1.5, 1.7, and 2.1 ppm and the inner protons of β-CD between 3.7 and 3.8 ppm thus proving that a supramolecular complex between β-CD and adamantyl-CD is present. The inclusion complexation was shown to be reversible, as heating to 70 °C leads to an expulsion of the adamantyl- and β-CD and cooling to 25 °C leads to reformation of the complex (Fig. 2c and d).

Lectin binding assays of all the three β-CD and α-D-mannose decorated β-CD complexed thermo-responsive triblock copolymers (P1-CD$_0$, P1-CD$_1$, and P1-CD$_7$) with concanavalin A (Con A), a lectin which has a good affinity for α-D-mannose, were then carried out to fully assess and compare binding efficiencies based on their structures and sugar densities. A UV-Vis spectrophotometer is a simple and valuable tool for analyzing protein–carbohydrate complex formation. Kiesling and coworkers investigated Con A to the glucose-containing glycopolymer binding rate by a turbidimetric assay and measured absorbance changes at 420 nm in HEPES buffer.$^{39}$ In this study, the rate of binding of Con A to glycopolymers P1-CD$_0$, P1-CD$_1$, P1-CD$_7$ was assessed by a turbidimetric assay, measuring changes of the absorbance at 420 nm of appropriate solutions of the lectin and polymer in HEPES buffer at pH 7.4, using a UV-vis spectrophotometer both below (12 °C) and above (40 °C) the cloud point (Fig. 3a and b). In the preliminary turbidity assay, we found that the temperature had an
influence on the interaction of Con A and mannosylated polymer residues. As shown in Fig. 3a, at low temperature, below LCST, glycopolymers P1-CD7 and P1-CD8 do not show a sharp increase in activity with increasing sugar functionalization, nor would such an increase be expected because these unassembled mannosylated triblock copolymers, the unimers, are not large enough to span multiple binding sites of Con A. Above LCST, micelles formed from the assembly of mannosylated triblock copolymers were encouraging. With the interaction between Con A and glycopolymers, the micelles increase in size causing significant scattering, thus leading to a turbid solution, which is an indication of the presence of binding events. When glycopolymers P1-CD1 and P1-CD7 were mixed with Con A in a buffer solution, the turbidity of the solution increased over time. This result is consistent with the formation of higher order, cross-linked complexes in the presence of ligands, and soon a precipitate was deposited. Precipitation can be rationalized on the basis of cross-linkage between the tetrameric Con A molecule and the multivalent polymer molecule carrying α-D-mannose residues in each repeating unit. It was clearly seen that, an elevated temperature accelerated the reaction significantly, where the non-active DMA groups were hidden inside and the α-D-mannose functionalized triblock copolymer in the precipitation assay was formed at a significantly higher concentration of Con A. However, the precipitation assay was performed at 40 °C, which is above the LCST. The precipitation assay suggests that the surface of the glycopolymer micelle is saturated with Con A. However, the precipitation assay was performed at a significantly higher concentration of Con A. In one α-D-mannose containing experiments (P1-CD1), the ratio of the α-D-mannose functionalized triblock copolymer in the precipitates to Con A quickly increased with an increasing amount of the α-D-mannose functionalized triblock copolymer.

Since the concentration of Con A was high enough, the glycopolymer caused the precipitation of the lectin. The ratio of glycopolymer to Con A at the point where maximum precipitation of Con A is observed is considered to be the maximum stoichiometry of Con A to the glycopolymer. In seven α-D-mannose containing experiments (P1-CD7) a maximum was reached, and then the ratio steadily decreased. This decreasing behavior was likely to be caused due to the ionic strength of the solution. The salt content influences sugar binding to Con A. A high salt content in the assay buffer can lower the affinity of the protein to α-D-mannose, which might disrupt the sugar-polymer cross-links and reduce the amount of precipitate observed in the precipitation assays. Con A concentration has not changed during the precipitation assays of both glycopolymers P1-CD1 and P1-CD7, and the Con A concentration was...
high enough for binding with glycopolymer P1-CD1, whereas it was insufficient for binding with a high sugar content of P1-CD7. When the sugar concentration and the ionic strength of the solution are high enough, soluble glycopolymer–protein complexes may occur and only small aggregates would form under these conditions. The precipitation assays suggest that we can independently attenuate lectin binding affinity and lectin clustering.

Conclusions

In summary, we discuss the synthesis and characterization of a novel adamantane-containing ABA type triblock copolymer as well as its self-assembly properties in water. The triblock copolymer is composed of two similar arms of poly(N,N-dimethylacrylamide) (PDMA) and hydrophobic poly(adamantane-acrylate) (PAdac) and the mid-block of poly(N-isopropylacrylamide) (PNIPAM). By carefully selecting the polymerization degree of PDMA and PAdac, and consequently the hydrophobic/hydrophilic ratio, a specific amphiphilic polymer has been identified that forms stable micelles in water as is clearly revealed by DLS. In these polymers, two different carbohydrates are involved in establishing noncovalent interactions: β-cyclodextrin operates as a receptor for adamantane whereas α-D-mannose acts as a ligand for Con A. The addition of lectins induces agglutination of the micelles without disrupting the micelle bilayer. Lectin precipitation requires high carbohydrate density at the micelle surface. Furthermore, micelle agglutination is reversed by the addition of competing binders for either of the noncovalent binding sites (cyclodextrin and lectin). In conclusion, polymer chemistry provides a versatile toolbox to allow the creation of various bioactive glycopolymer structures, which enable the careful fine-tuning of the interaction between lectins and glycopolymers.

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Notes and references


