Synthesis of well-defined glycopolymers and some studies of their aqueous solution behaviour

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Well-defined polymers with carbohydrate residues pendant to the main chain (glycopolymers) were prepared by reversible addition fragmentation chain transfer (RAFT) polymerisation. Excellent control over molecular weight and narrow polydispersities (1.1–1.2) were achieved over a range of molecular weights. In addition, efficient synthesis of block copolymers by sequential monomer addition with both hydrophilic and hydrophobic non-carbohydrate blocks was demonstrated. The aqueous solution behaviour of amphiphilic block glycopolymers was investigated, revealing the formation of multivalent carbohydrate-bearing aggregates in solution with the capability for the solubilisation of hydrophobic species (a water-insoluble dye). One such amphiphilic glycopolymer shows by TEM the formation of a worm-like micelle phase. Further investigations of these novel bioactive macromolecular assemblies are underway.

Introduction

It is now widely recognized that carbohydrates play a key role in a range of biological processes, including (but not limited to) fertilisation, the inflammatory cascade, viral particle docking and entry and signal transduction. These processes are initiated by carbohydrates (glycans), which are attached to cell surface lipids and proteins, binding noncovalently to receptor proteins known as lectins. The pattern of carbohydrates on cell surfaces is often referred to as the 'glycocode'; decoding this language and elucidating the role of carbohydrates in biological processes is a thriving new discipline known as 'glycomics'.

Interactions between individual sugars and lectins are unusually weak; association constants (K_A) being of the order of 10^3 M^{-1}. The high specificity of a lectin for a given carbohydrate is brought about through a multivalent binding process known as the 'cluster glycoside' effect,[1] where many copies of the same sugar are presented to the lectin in the correct orientation, leading to much higher apparent K_A values (10^9–10^{12} M^{-1}). High avidity synthetic multivalent ligands such as glycodendrimers[2] and glycopolymers[3] have been prepared in order to exploit the cluster glycoside effect and study the glycocode. A number of studies of the binding of such multivalent ligands to receptors have been carried out,[4–6] however, a clear picture of the nature of multivalent binding is still lacking. While all studies indicate enhanced

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binding between multivalent ligand and receptor, the levels of enhancement vary enormously. For example, the avidity of a tetravalent glycoconjugate for *Dioclea grandiflora* lectin (DGL) was found to be around 50 times higher than that for the monovalent ligand,[5] whereas truly enormous binding enhancements (e.g. 10^{10} fold) have been found for binding between a trivalent vancomycin derivative and a trivalent D-Ala-D-Ala receptor, in which all three ligands bind simultaneously to the three binding sites.[7] The reasons for these vastly different avidities in multivalent systems are not clear.

In order to further our understanding of multivalent binding events, and thus develop new therapeutic[8] and diagnostic technologies,[9] there is a need for simple synthetic methodologies to prepare multivalent ligands (glycoconjugates) of well-defined architecture. Using controlled polymerisation strategies, a carbohydrate-based monomer can be converted to a multivalent glycopolymer[6,10–13] or, alternatively, carbohydrates can be attached to a reactive prepolymer.[14] In both cases, exquisite control over chain length and carbohydrate density along the backbone can be achieved through the use of techniques such as atom transfer radical polymerisation (ATRP) or reversible addition fragmentation chain transfer (RAFT) polymerisation. Furthermore, a wealth of macromolecular architectures, including block copolymers and stars, are available, and from these, larger nanostructures such as micelles and vesicles can be constructed through self-assembly. In this article, our efforts to prepare well-defined glycopolymers in aqueous solution using RAFT polymerisation are described. Excellent control over chain length is achieved and block copolymers can easily be prepared. Amphiphilic block glycopolymers are shown to be able to self-assemble in aqueous solution to form micellar aggregates that can solubilise a water-insoluble dye. Evidence of the formation of worm-like micelles is presented.

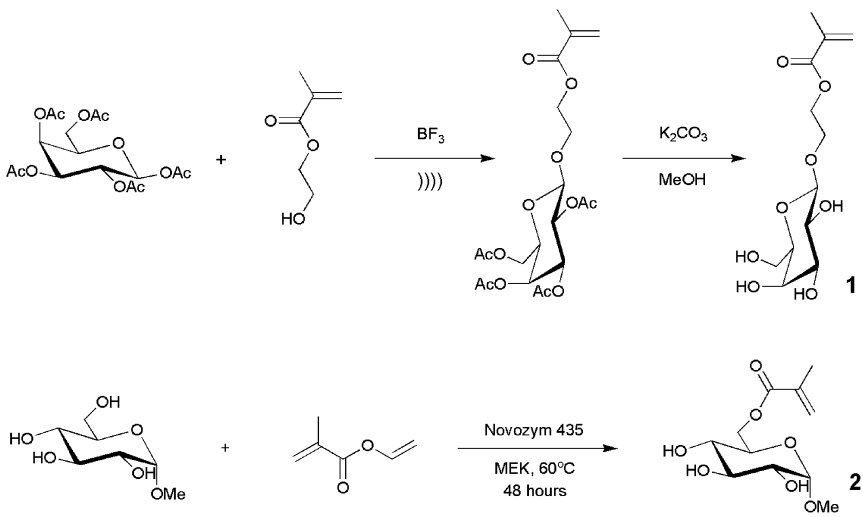
Experimental

β-D-Galactose pentaacetate (98%), 2-hydroxyethyl methacrylate (HEMA, >98%), cation exchange resin DOWEX × 50W × 2–200 (H^+), sodium (99%), methanol (99.8%) and sodium borohydride (98%) were purchased from Aldrich. Boron trifluoride diethyl etherate (purum, dist.) and 4,4′-azobis(4-cyanopentanoic acid) (>98%) were purchased from Fluka. Methyl β-D-galactopyranoside and methyl β-D-glucopyranoside were purchased from Sigma. Novozym 435 was kindly donated by Novozymes A/S. Before use, boron trifluoride diethyl etherate was distilled *in vacuo*; all other chemicals were used without further purification. Automated flash chromatography was carried out using a Biotage SP1 system with automatic fraction collector and on-board UV detector, using chloroform–methanol (8 : 2) as the eluent. TLC analysis was performed on glass-backed silica-gel plates (60 Å, 5–17 μm, Macherey-Nagel); following solvent evaporation, the developed plates were immersed in a 20% H_2SO_4–ethanol solution and heated at 110 °C for 15 min for spots detection. Dialysis purifications were performed at room temperature against purified water (100–150 times the volume of the sample) using Slide-A-Lyzer Dialysis Cassettes (3–13 mL, 3.5 kDa MWCO, Pierce Biotechnology); during the process, the water was changed twice (typically after 4 and 23 hours) and the sample was kept in the dark. (4-Cyanopentanoic acid)-4-dithiobenzoate (CPADB)[15] was prepared according to published methods and its structure was confirmed *via* ^{1}H and ^{13}C-NMR. Degassing of polymerization solutions was performed *via* four freeze–evacuate–thaw cycles (ultimate pressure 6×10^{-2} mbar).

NMR spectra were recorded using a Varian Inova 500 spectrometer at 499.87 (^{1}H) and 125.67 MHz (^{13}C) (^{1}H decoupled at 500 MHz) or using a Bruker Avance 400 at 400.13 MHz (^{1}H). NMR spectra were analyzed using the Varian VNMR 6.1C software. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer by casting a film on NaCl plates from either DCM or MeOH. Mass spectra were acquired using a Micromass LCT spectrometer using ES+ and ES− modes of ionization. Elemental analyses were obtained using an Exeter Analytical Inc. CE-440

Elemental Analyzer. Aqueous SEC was performed using a triple detection method (with angular correction) employing a Viscotek TDA 301 triple detection SEC fitted with two (300 × 7.5 mm) GMPWxl methacrylate-based mixed bed columns with an exclusion limit of 5×10^7 g mol^{-1}, having refractive index, viscometer and RALLS detectors. The eluent used was an aqueous solution of $NaNO_3$ (0.2M) and NaH_2PO_4 (0.1M), at a flow rate of 1.0 ml min^{-1} and at a constant temperature of 30 °C. Calibration (for detector response) was achieved using a single narrow PEO standard (Polymer Labs) of 82 500 g mol^{-1} and a d*n*/d*c* value of 0.133 ml g^{-1}. Molecular weights were determined using the Omnisec 4.0 for Windows software with a d*n*/d*c* value of 0.153 ml g^{-1} for poly[2-(β-D-galactosyloxy)ethyl methacrylate] (calculated using the multipoint RI method2). DMF SEC was performed using an instrument consisting of a GBC LC1110 HPLC pump, a Viscotek VE5111 manual injector port, and a Viscotek TriSEC Model 302 triple detector array comprising a 90° angle laser light scattering detector and a differential refractometer operating at the same wavelength ($\lambda = 670$ nm). The system was equipped with a 50 × 7.5 mm guard column and three 300 × 7.5 mm linear columns (PLgel 500, 10^3 and 10^4 Å pore size; 5 μm particle size; Polymer Laboratories). *N*,*N*-Dimethylformamide (0.1% w/v LiBr, 0.05% w/v 2,6-di-*tert*-butyl-4-methylphenol) was used as eluant at a flow rate of 1 mL min^{-1} while the columns' temperature was maintained at 60 °C. Polymer solutions (2–3 mg mL^{-1} in DMF eluant) were injected in 100 μL volumes. SEC traces were analyzed with OmniSEC 4.0 software (Viscotek). A d*n*/d*c* value of 0.090 was used for molecular weight calculations that was determined from repeated injections of pure poly(methyl-6-*O*-methacryloyl-α-D-glucoside) dithiobenzoate solutions of known concentration (M_n (SEC) 20,400, PDI (SEC) 1.05). TEM images were obtained from a Philips CM100 instrument. Aqueous solutions of glycopolymer were prepared by the film rehydration method. The sample (5 μl) was adsorbed onto a freshly glow-discharged grid for 1 minute before the excess was removed by blotting. The grid was then dipped in a drop of uranyl formate solution for 20 seconds and the excess was removed by blotting. Alternatively, phosphomolybdic acid solution (0.5 mg ml^{-1}) was used as a staining agent.

Monomers 2-(β-D-galactosyloxy)ethyl methacrylate (GalEMA; **1**)[13] and methyl 6-*O*-methacryloyl-α-D-glucoside (MAMGlc; **2**)[12] were prepared using previously-described procedures, with some minor modifications. In the case of **1**, the first step was conducted with sonication of the reaction mixture using an ultrasonic bath (Scheme 1), resulting in complete conversion (TLC) after 1 hour. In the



Scheme 1 Synthesis of glycomonomers.

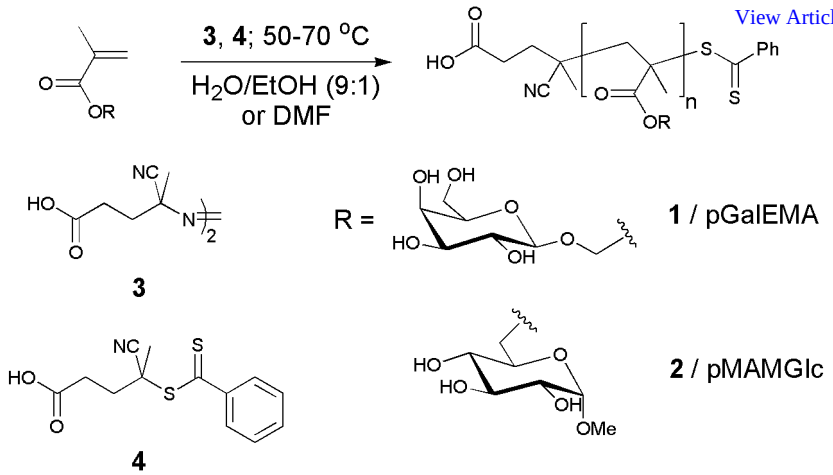
synthesis of **2**, the use of butanone as reaction solvent together with a reaction temperature of 60 °C reduced the reaction time to 48 hours from 7 days. Polymerisations were conducted as described previously, in either an aqueous ethanol solution[13] (homopolymerisation of **1** and block copolymerisation with DMAEMA) or in DMF[10] (block copolymerisations of **1** and **2** with *n*-butyl methacrylate and *n*-butyl acrylate). A typical example procedure is as follows: To a solution of **1** (100 mg, 0.348 mmol) in UHQ water was added (4-cyanopentanoic acid)-4-dithiobenzoate (**2**, 3.5 μmol) and 4,4′-azobis(4-cyanopentanoic acid) (**3**, 1.75 μmol) as 8 mgml^{-1} solutions in absolute ethanol. The solution was degassed by three freeze–pump–thaw cycles and purged with nitrogen before sealing. The solution was stirred at 70 °C for 3 h then dialyzed and lyophilized to yield pGalEMA as a hygroscopic pink solid (80 mg, 80%). IR (NaCl plates)ν/cm^{-1} 3446 (OH), 1718 (C=O of methacrylate ester), 1052 (C=S); δ_H: (500 MHz; D_2O) 0.93–1.09 (3H, br, CH_3), 1.89–2.13 (2H, br, CH_2), 3.58–4.5 (10H, br, carbohydrate and methylene side chain protons), 4.39 (1H, br, anomeric H). M_n (SEC) 24,100; M_w/M_n (SEC) 1.09; $M_{n,th}$ 23,751 at 80% conversion (theoretical number average molecular weight, $M_{n,th} = (x \times ([M]_0/[RAFT]_0) \times M_m) + M_{RAFT}$, where x = fractional conversion, M_m = monomer molecular weight and M_{RAFT} = RAFT agent molecular weight). Polymerizations for kinetics studies were performed in NMR tubes fitted with a Young's valve, and conversions were monitored *via in situ* ^{1}H NMR spectroscopy. In a typical run, 1.00 mL of a solution of monomer in D_2O (c = 0.863M) was mixed with a calculated amount of radical initiator and RAFT agent, both as EtOH solutions. About 0.6 mL of the resulting mixture (D_2O–EtOH 9 : 1) was transferred to the NMR tube and degassed. The magnet was shimmed on a dummy sample containing the monomer and initiator at the same concentrations as in the real sample. Subsequently, the sample was lowered into the magnet cavity and allowed to equilibrate for about 1 min, after which additional shimming was carried out to optimize the system fully. Data acquisition was started 3–5 min after the sample had been inserted into the magnet, the exact time being noted. A pulse program lasting 36 s (2.3 μs (40°) pulse width, 2 s acquisition time) was repeated at 5 min intervals for the duration of the experiment. The beginning of each pulse program was taken as the time point for conversion *vs.* time plots. The progress of the reaction was monitored by the disappearance of the vinyl protons' signals. Conversions (x) were estimated from the ratio between the area (A) of the vinyl proton peak H_E-10 [12] and that of the glycoside anomeric proton peak (H-1, internal standard) according to the formula:

$$x = 1 - \frac{A_{H_E10}}{A_{H1}} \quad (1)$$

where $A_{H_E}10/A_{H1}$ for the monomer (zero conversion) was 1.0.

Results and discussion

The glycomonomers 2-(β-D-galactosyloxy)ethyl methacrylate (GalEMA; **1**) and methyl 6-*O*-methacryloyl-α-D-glucoside (MAMGlc; **2**) were synthesized following procedures based on those described in the literature, with some aspects optimised (Scheme 1). In the case of **1**, the ultrasonication method described by Deng *et al.*[16] has been employed to reduce drastically the time required for the first step (glycosidation), from 36 hours to 1 hour. Investigation of the conditions for the enzymatic coupling step to produce **2** indicated that a higher boiling solvent (butanone instead of acetone) coupled with a higher temperature (60 °C) cuts the time of reaction from 7 days to 48 hours as well as increasing yield to around 85%. Thus, pure, multigram batches of both glycomonomers could be obtained in relatively short times (1 day for **1**, 3 days for **2**).



Scheme 2 Synthesis of glycopolymers by RAFT polymerisation.

Monomers **1** and **2** were then subjected to polymerisation by RAFT in homogeneous solution, employing 4,4′-azobis(4-cyanopentanoic acid) (ACPA; **3**) as initiator and (4-cyanopentanoic acid)-4-dithiobenzoate (CPADB; **4**) as chain transfer agent (Scheme 2). The RAFT polymerisation of monomer **2** has been studied previously in some detail. For instance, it has been shown that this monomer can be polymerised efficiently to produce well-defined, narrow polydispersity glycopolymers with a range of molecular weights, remarkably down to as low as $DP_n = 10$ without loss of control. However, glycopolymers obtained from **2** are not able to participate in binding to lectins due to substitution of the 6-*O* position on the glucopyranoside ring. Thus, it is important to demonstrate that monomer **1** is also able to be polymerised efficiently by RAFT. A previous communication described the successful polymerisation of **2** by RAFT, but only one molecular weight was targeted and block copolymer preparation was not described.

The kinetics of polymerisation of **1** by RAFT in homogeneous aqueous solution were investigated by *in situ* NMR spectroscopy; the results are shown in Fig. 1 for three different targeted number average degrees of polymerisation ($DP_n = 25$, 50

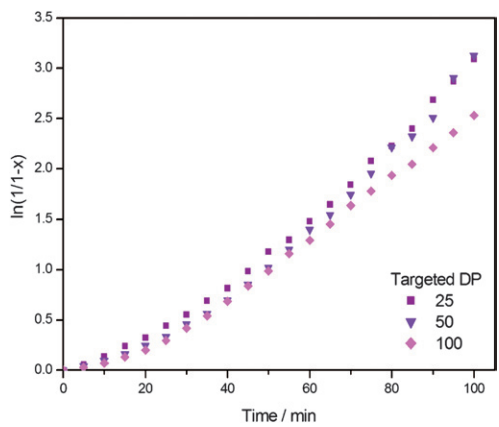


Fig. 1 Kinetics of RAFT polymerization of **1** for different target DP_n values. Polymerisation temperature = 70 °C; [**3**] : [**4**] = 0.5.

and 100). As is frequently observed in RAFT polymerisations, there is an initial non-steady state (retardation) period in each case, the exact cause of which is still the subject of debate.[17] In agreement with our previous findings,[10] this period is relatively short (*ca.* 35 min) for the longest targeted chain length and increases as target DP_n decreases. In fact, it is arguable whether the polymerisation with the smallest monomer : CTA ratio ever achieves steady state.

The evolution of molecular weight and polydispersity with conversion was also investigated for the RAFT polymerisation of **1** (Fig. 2). The polymerisation reaction was sampled periodically and the samples analysed by aqueous GPC with triple detection. Fig. 2a) indicates a linear evolution of M_n with up to high conversion. In addition, good agreement between M_n determined by GPC and that predicted on the basis of the initial monomer : RAFT agent ratio (depicted by the line in Fig. 2a) is also shown. In the upper plot of Fig. 2a, the polydispersity values obtained from both triple detection (TriSEC)[18] and conventional calibration are given. The values for TriSEC are lower than would be expected for a controlled radical polymerisation process (<1.05 from 55% conversion onwards), conversely the values from conventional calibration appear rather high. To determine which set of values is more realistic, the polydispersity of a PEO standard (Polymer Labs, $M_n = 28\,700$ g mol^{-1}, dn/dc=0.133 ml g^{-1}) was determined using the same calibrations. In this case, polydispersities were calculated as 1.02 and 1.19 by TriSEC and conventional calibrations, respectively, compared to a value of 1.04 quoted by the manufacturer. Thus, although the values of M_w/M_n from TriSEC are lower than expected, they appear to be the more realistic. It is not obvious why the TriSEC should produce lower than expected results, but one possible cause is the value for dn/dc of PEO used in the calibration, which is that of the polymer in pure water. The cause of the elevated values from conventional calibration is unknown.

To probe the range of glycopolymer molecular weights available by RAFT, experiments were conducted to prepare polymers of different number average degree of polymerisation (DP_n). In RAFT polymerisations, this is easily achieved by varying the initial monomer : RAFT agent ratio. Polymers with target DP_n values of 25, 50, 100, 150 and 200 were thus prepared and analysed by SEC (Fig. 3). For each molecular weight targeted, relatively good agreement between experimental and theoretical M_n was observed and polydispersities were quite narrow. SEC traces in addition were all found to be monomodal. Thus, it can be seen that polymers with a wide range of chain lengths, at least up to 200 monomer units, can be prepared in a controlled manner.

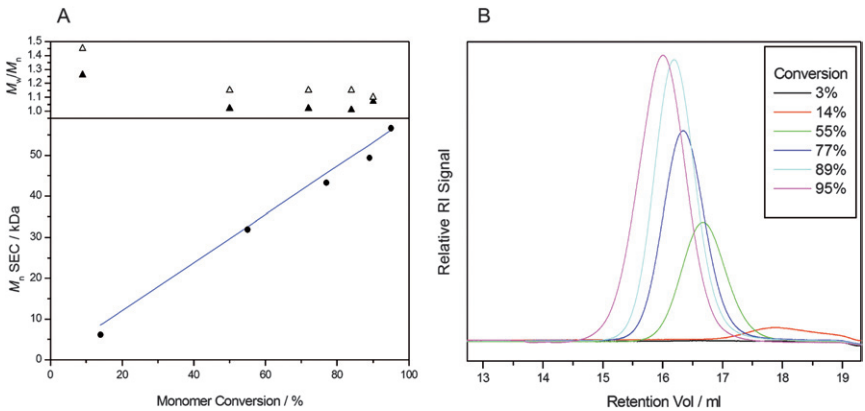


Fig. 2 (a) Evolution of number-average molecular weight (M_n) and polydispersity (M_w/M_n) with conversion for the RAFT polymerization of **1** (upper: polydispersity values taken from RI (open triangles) and RALLS (closed triangles) detectors; lower: line shows predicted evolution of M_n with conversion); (b) corresponding SEC traces (conversion increases right to left).

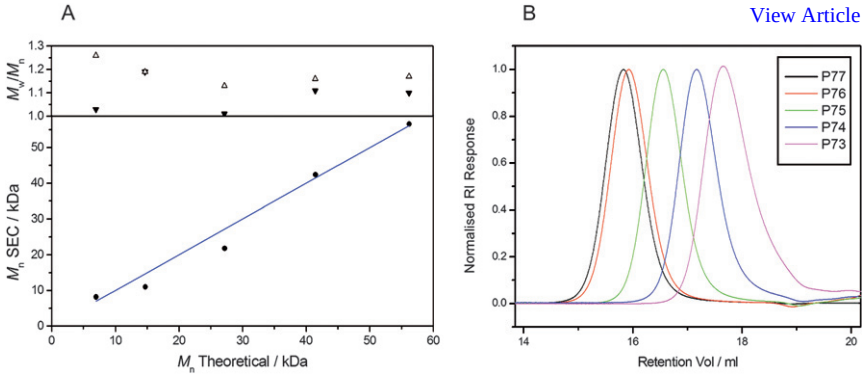


Fig. 3 (a) Molecular weight data for RAFT polymerization of **1** with different targeted values of DP_n (upper: polydispersity values taken from RI (closed triangles) and RALLS (open triangles) detectors; lower: agreement between SEC (circles) and predicted (line) number-average molecular weight (M_n) values for different target DP_n; (b) corresponding SEC traces (target DP_n increases right to left).

Next, the ability of the resulting polymers to act as macroRAFT agents for the preparation of block copolymers was studied. Thus, polymers derived from both **1** and **2** were subjected to chain-extension: **1** with *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA) and *n*-butyl acrylate (BA) and **2** with *n*-butyl methacrylate (BMA). The molecular weight data of the resulting block copolymers are shown in Table 1. In general, blocking efficiency is high; the DP_n of the second block is close to the target value at the obtained conversion and the polydispersity remains narrow. The SEC traces of two of the block copolymers, together with the starting glycopolymers, are shown in Fig. 4. In both cases, a complete shift in the SEC trace to lower retention volume is found, indicating good blocking efficiency. The SEC trace of the amphiphilic glycopolymer (Fig. 4b) shows evidence of dead polymer produced from irreversible bimolecular termination, however the PDI of the main peak is still low (1.06).

The solution behaviour of the amphiphilic block copolymer p(MAMGlc-*b*-BMA) was investigated (Fig. 5). Dynamic light scattering (Fig. 5a) indicates the formation of aggregates with a mean diameter of around 40 nm, together with the presence of unimers (diameter *ca.* 6 nm). The addition of an ethyl acetate solution of the water-insoluble dye Sudan III causes the formation a population of aggregates with a mean diameter of around 30 nm together a smaller population at around 200 nm. Optically, the resulting almost-clear solution has a red coloration due to the presence of solubilised dye (Fig. 5b).

This amphiphilic block copolymer was further investigated for its ability to act as a macromolecular surfactant (Fig. 6). An aqueous solution of glycopolymer was mixed with increasing quantities of the same ethyl acetate solution of Sudan III as

Table 1 Block copolymer molecular weight data

Polymer	Target composition	2nd block conversion (%)	$M_{n,SEC}$/kg mol^{-1}	M_w/M_n	Actual composition
p(GalEMA-*b*-DMAEMA)	50–50	50	18.2	1.21	50–25
p(GalEMA-*b*-BA)	25–56	80	11.0	1.22	25–45
p(MAMGlc-*b*-BMA)	35–30	100	14.9	1.05	35–28

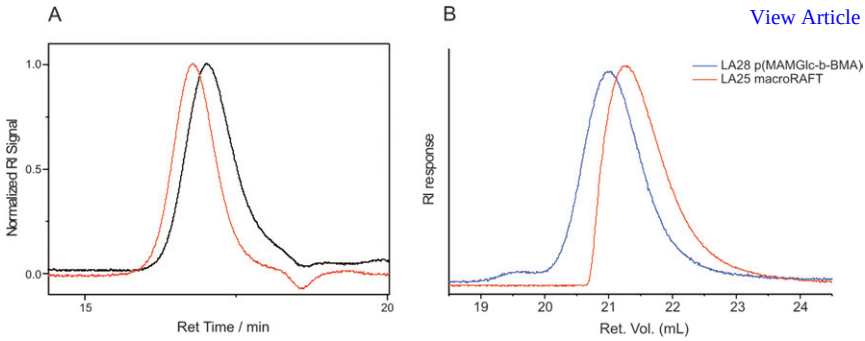


Fig. 4 SEC traces of block glycopolymers: (a) p(GalEMA$_{50}$-*b*-DMAEMA$_{25}$); (b) p(MAMGlc$_{35}$-*b*-BMA$_{28}$). The higher retention time/vol. traces in (a) and (b) represent the initial glycopolymer macroRAFT agent.

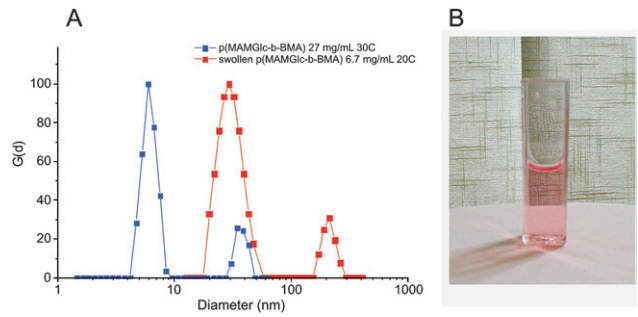


Fig. 5 a) Dynamic light scattering (DLS) traces of aqueous solutions of p(MAMGlc$_{35}$-*b*-BMA$_{28}$) (blue trace) and p(MAMGlc$_{35}$-*b*-BMA$_{28}$) in the presence of an ethyl acetate solution of Sudan III (red trace); (b) digital photograph of the solution producing the red trace in (a).

was used in the preparation of solutions shown in Fig. 5, and the resulting mixture shaken vigorously. Images were then taken immediately after shaking and also after standing for 10 minutes. In all cases, solubilisation ability was demonstrated qualitatively by the presence of a red colouration, the intensity of which increased with increasing dye solution concentration (Fig. 6a). On standing, some dye was observed to drop out of solution but some remained solubilised by the amphiphilic polymer. Thus, the glycopolymer has an ability to solubilise water insoluble species in aqueous solution.

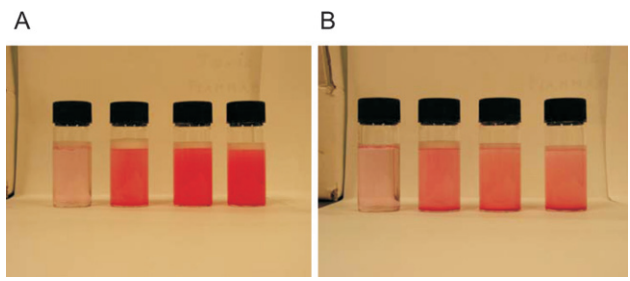


Fig. 6 Emulsification of a dichloromethane solution of Sudan Red (0.5 mg mL^{-1}) by aqueous solutions of p(MAMGlc$_{35}$-*b*-BMA$_{28}$) (1 mg mL^{-1}): (a) immediately and (b) 10 min after shaking. The quantity of added Sudan Red solution increases left to right: 0.05, 0.06, 0.075 and 0.1 mL.

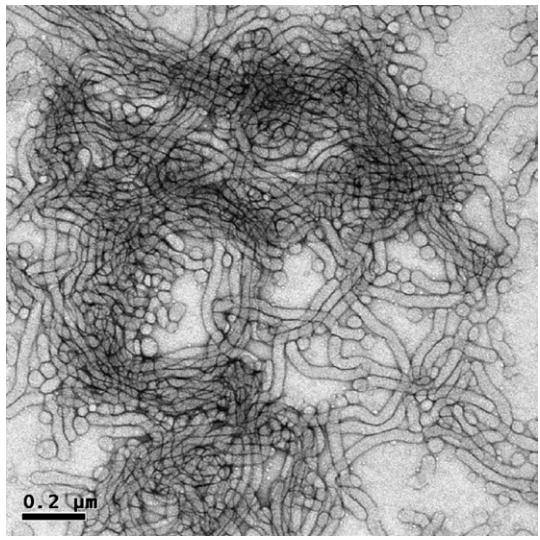


Fig. 7 TEM image of assemblies formed by p(GalEMA$_{25}$-*b*-BA$_{100}$).

The morphology of aggregates formed by the amphiphilic glycopolymer p(GalEMA$_{25}$-*b*-BA$_{100}$) was investigated by TEM (Fig. 7). A solution of the polymer was dried onto a TEM grid and stained with uranyl formate, then imaged by TEM. The resulting micrograph clearly shows that this glycopolymer forms a phase characterised by a majority of worm-like micelles. The hydrophilic volume fraction, f, of this copolymer is calculated to be 0.35, which is a little below the minimum value at which wormlike micelles are normally observed ($f = 0.40$). However, this classification was obtained based on data from poly(ethylene glycol) (PEG) block copolymers, which have a very different architecture from the glycopolymers studied here.

Conclusions

Well-defined glycopolymers, including diblock copolymers, can be prepared by RAFT polymerisation. The polymerisation process is efficient and polymers of predetermined molecular weight and low polydispersity are obtained. The method is versatile and can be applied to different glycomonomers and non-carbohydrate comonomers. Amphiphilic glycopolymers so prepared have been shown to form aggregates in aqueous solution that are capable of solubilising a water-insoluble dye. TEM of one such glycopolymer with a hydrophilic volume fraction of 0.35 indicates a phase that consists of a majority of worm-like micelles. Further work is underway to study in more detail the properties and phase behaviour of such polymers together with their ability to bind to lectins.

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