SUPPORTING INFORMATION

Cooling rate uncovers epimer-dependent supramolecular organization of carbohydrate amphiphiles

Vânia I. B. Castro,[a,b] Yuting Gao,[a,b,c] Alexandra Brito,[a,b] Jie Chen,[c] Rui L. Reis,[a,b] Iva Pashkuleva[a,b,*] and Ricardo A. Pires[a,b,*]

[a] 3B's Research Group, I3Bs – Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Ave-Park, Parque de Ciência e Tecnologia, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal
[b] ICVS/3B's–PT Government Associate Laboratory, Braga/Guimarães, Portugal
[c] Department of Chemical Engineering, School of Environmental and Chemical Engineering, Shanghai University, Shangda Road 99, Shanghai 200444, P. R. China
* Corresponding authors: Ricardo A. Pires, rpires@i3bs.uminho.pt; Iva Pashkuleva, pashkuleva@i3bs.uminho.pt

Experimental Section

Materials

All reagents were used as received without further purification. D-galactosamine hydrochloride (D-GalN) was acquired from Carbosynth with a purity of 99.0 %. D-glucosamine hydrochloride (D-GlcN) and D-mannosamine hydrochloride (D-ManN) were purchased from Fisher Scientific with a purity of 98.0 % and 99.6 %, respectively. 9-Fluorenylmethoxycarbonyl chloride (Fmoc-Cl) was acquired from Bachem with a purity of 98.9 %.

Synthesis and purification of Fmoc-sugars

The amino sugar hydrochloride (D-GalN, D-GlcN and D-ManN) (1.15 mmol) was dissolved in water (7 mL) with 2.8 equiv of $NaHCO_3$ (Riedel-de Haen). In a separate flask, Fmoc-Cl (2.2 equiv) was dissolved in dioxane (7 mL, Sigma) and added dropwise to the previously prepared sugar solution. The solution was stirred at room temperature (RT) under a nitrogen atmosphere during 24 h. The reaction was followed by thin layer chromatography (TLC, ethyl acetate:methanol:water, 7:2:1) and stopped after 24 h because the spot of the amino sugar hydrochloride disappeared and the spot of the product with higher Rf emerged.

The reaction mixture was freeze-dried and the free Fmoc-Cl was removed using liquid-liquid extraction (water-diethyl ether). The water fraction was again freeze-dried. The purity of the synthetized Fmoc-sugars was assessed by high-performance liquid chromatography (HPLC) (Smart Line, Knauer) using a reverse-phase C18 Atlantis column (5 µm, 250 × 4.6 mm, Waters). We used a flow of 1 mL/min, and water (A) and acetonitrile (B), both supplemented with trifluoroacetic acid (TFA, Sigma-Aldrich) were used as eluents. Gradient: an initial step of 4 min under isocratic flow of 80% of A, followed by a gradient to 80% of B at 31 min; this gradient was maintained for 5 min and followed by a gradient to 80% of A for 2 min and an isocratic elution to 80% of A during 4 min. The Fmoc-sugars were characterized using nuclear magnetic resonance (NMR, 400.13 MHz Avance III spectrometer, Bruker, Germany) in DMSO-d_6, and mass spectroscopy (Quattro Micro API, Waters Corporation, U.K.) in positive-ion mode.

Hydrogel preparation

The Fmoc-sugars' powders were suspended in water (Milli-Q) at the respective concentration (6, 8, 10, 12, 14, 16 and 18 mM). The temperature of the mixture was increased to 363 K and kept at this temperature until complete dissolution and formation of a clear solution (apx. 3 – 5 min). The warm solution was cooled to RT. In the CD, fluorescence spectroscopy, and mechanical experiments a slow cooling rate of 5 K/min or a fast cooling rate of 40 K/min was applied using

the equipment temperature control. In the experiments without temperature control, namely NMR, fast cooling was achieved by immersing the recipient with the heated (363 K) CA solution in an ice bath for a quick cool down or by keeping the warmed solution at room temperature for a gradual temperature decrease (slow cooling), thereby inducing self-assembly and gel formation.

Fluorescence emission spectroscopy

Fluorescence emission spectra were acquired between 300-600 nm, using an excitation wavelength at 295 nm, a bandwidth of 2.5 nm, a 50 msec response time and a 2.5 nm data pitch on a Jasco FP-8500 spectrofluorometer (Jasco Corporation, Japan) at a scanning speed of 200 nm/min. The measurements were performed in a 1 mm path-length quartz cell at 5 K intervals in the temperature range between 343 and 283 K, with an equilibration stage in each temperature. The fluorescence emission spectra were recorded at different concentrations of Fmoc-sugars (6, 8, 10, 12, 14, and 16 mM).

Circular Dichroism (CD) Spectroscopy

CD spectra were acquired on a Jasco J1500 spectropolarimeter (Jasco Corporation, Japan) using a quartz cuvette with a 1 mm pathlength cell. The gel was formed within the cell, each sample was subject to a heating cycle of 363 at 283 K at a rate of 5 K/min and the ramp measurements were performed between 240-320 nm. CD spectra of Fmoc-sugar at different concentrations (6, 8, 10, 12, 14, and 16 mM) were recorded at a scan speed of 200 nm/min with a data pitch of 0.5 nm and a bandwidth of 2.0 nm. For all the conditions, a triple acquisition was performed, and the solvent background was subtracted from the obtained spectra.

Oscillatory Rheology

Dynamic frequency sweep experiments were conducted on a strain-controlled rheometer (Kinexus Pro, Malvern, U.K.) equipped with 20 mm parallel-plate geometry, and using a gap of 0.40 mm. Hydrogels were prepared *in situ*, i.e., the warm solution was placed on the warmed plate (353 K) of the rheometer, and the cooling was performed at 5 K/min (slow) or 40 K/min (fast) to 293 K. At this temperature, the measurements were performed. The linear viscoelastic range (LVER) of the hydrogels was determined, and amplitude sweeps at a frequency of 1 Hz and 0.1 − 2% strain were applied. Taking into consideration the LVER results, measurements of storage modulus (G′) and loss modulus (G″) were acquired at a frequency of 1 Hz and a strain of 1%. All measurements were performed in triplicate, and data are reported as average values ± standard deviations.

Scanning electron microscopy (SEM)

High-resolution field emission scanning electron microscope (SEM, Auriga Compact, Zeiss) was used to observe and image the helicity of nanofibers assembled from the different Fmoc-glycosamines. Freshly cleaved mica sheets were rinsed with deionized water and dried under a nitrogen flow. The Fmoc-glycosamines' hydrogels were placed in contact with the mica during 3 - 4 min to allow the transfer of the fibers from the gel to the mica. The fibers on the mica sheets were washed three times with deionized water and dried under a stream of nitrogen. Samples were sputter-coated with a layer of gold (EM Leica ACE600). The SEM analysis was carried out using an accelerating voltage of 2.5 kV, with magnifications of x30k and x50k.

Characterization of synthetized carbohydrate amphiphiles

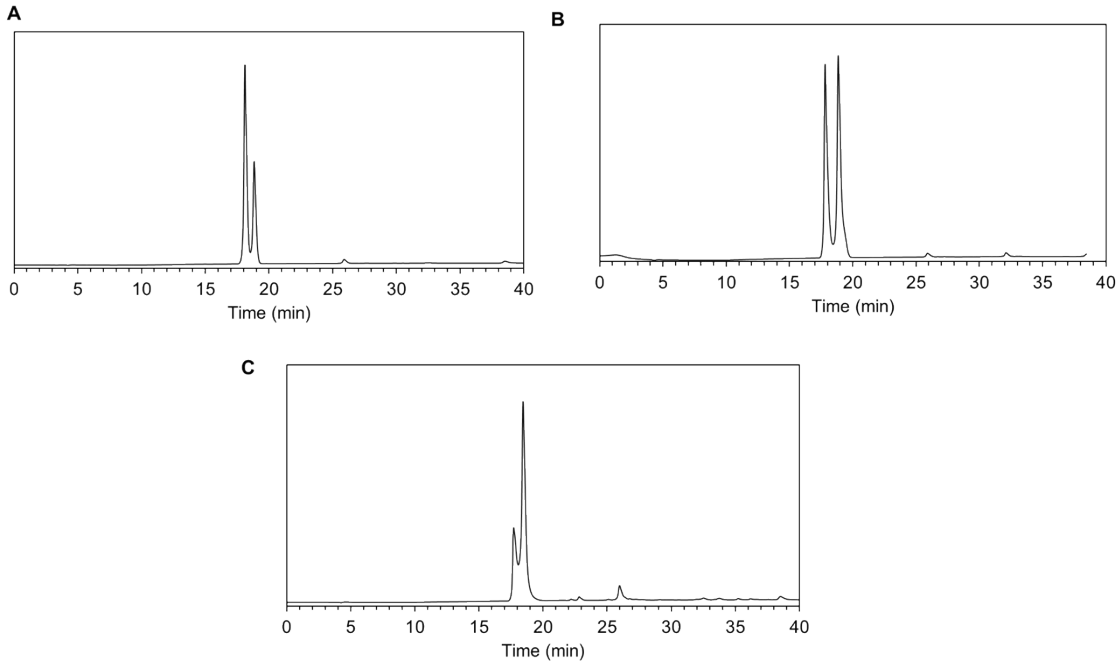


Figure S1. HPLC chromatograms of the purified compounds: Fmoc-GlcN (**1**, A), Fmoc-GalN (**2**, B) and Fmoc-ManN (**3**, C), showing the α and β anomers.

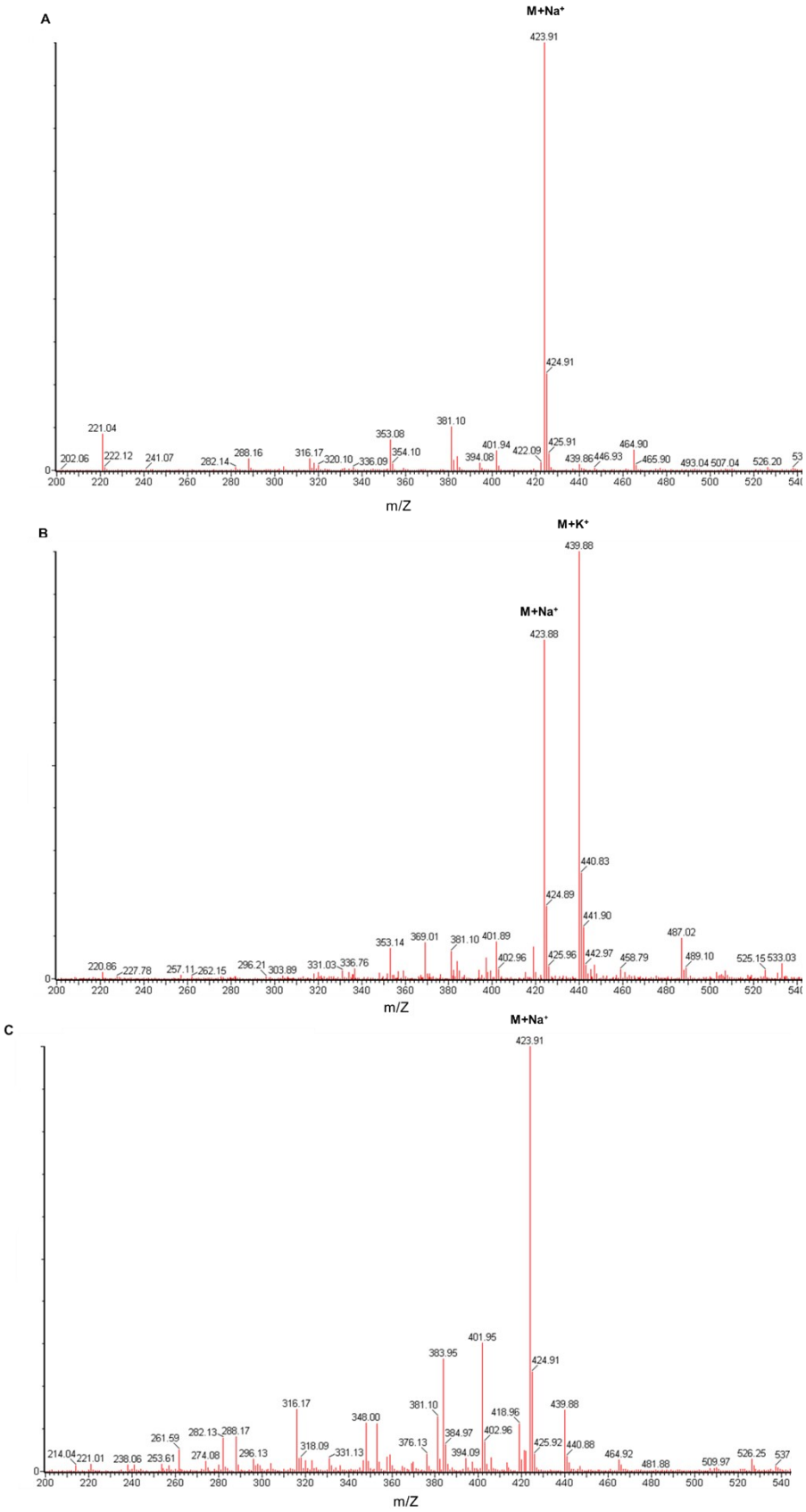
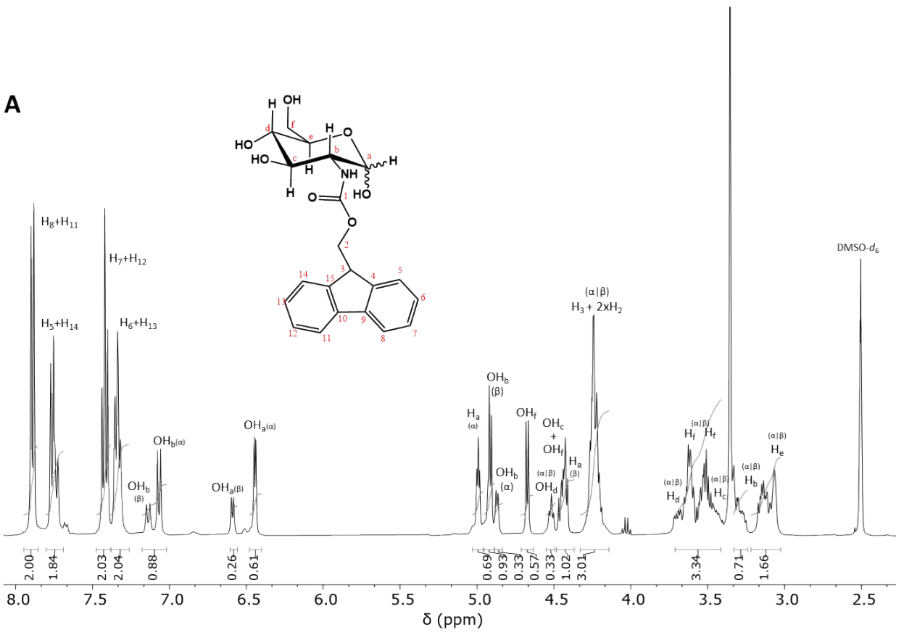
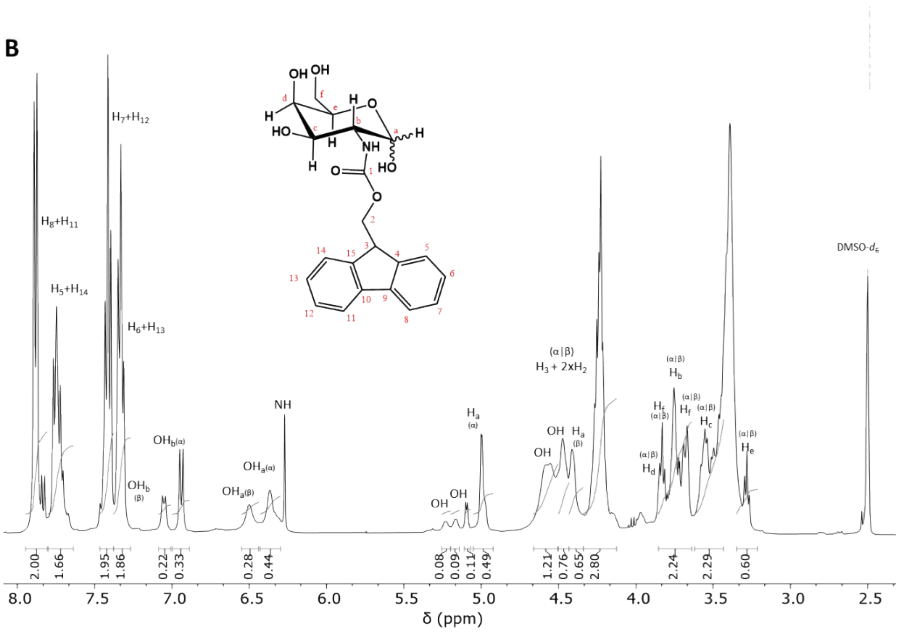


Figure S2. Electrospray ionization mass spectroscopy (ESI-MS) of the purified compounds, Fmoc-GlcN (**1**, A, m/z $[M+Na]^+$ = 424), Fmoc-GalN (**2**, B, m/z $[M+Na]^+$) = 424, $[M+K]^+$) = 440) and Fmoc-ManN (**3**, C, m/z $[M+Na]^+$ = 424).

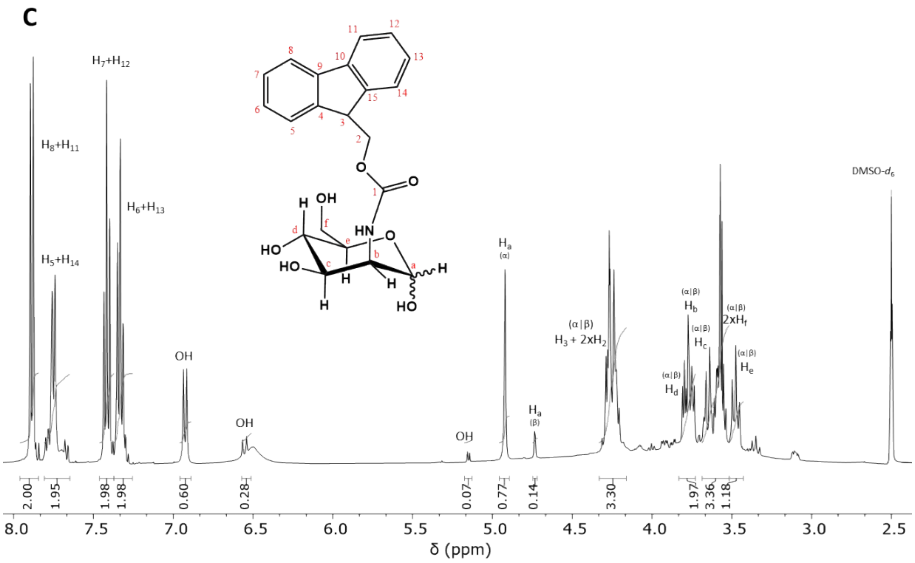


δ_H (ppm) 3.04-3.20 (m, 2H, H_e + H_b anomer α|β), 3.39-3.75 (m, 4H, H_c + H_d anomer α|β + 2xH_f anomer α|β), 4.16-4.32 (m, 3H, H_3 and 2xH_2), 4.37-4.48 (m, 2H, H_a anomer β and OH_c), 4.51 (t, 1H, OH anomer α|β, J = 5.6 Hz), 4.66 (d, 1H, OH_f anomer α|β, *J* = 5.2 Hz), 4.87 (d, 1H, OH_b anomer α), *J* = 4.7 Hz), 4.91 (d, 1H, OH_b anomer β), *J* = 6.4 Hz), 4.99 (t, 1H, H_a anomer α, *J* = 4.0 Hz), 6.45 (d, 1H, OH_a anomer α, *J* = 4.8 Hz), 6.59 (d, 1H, OH (OH_a anomer β), 7.04 (d, 1H, OH_b anomer α, *J* = 9.2 Hz) 7.11 (d, 1H, OH_b anomer β, *J* = 9.6 Hz), 7.34 (td, 2H, H_6 + H_{13} , *J* = 1.3 Hz and 7.6 Hz), 7.41 (t, 2H, H_7 + H_{12}, *J* = 7.2 Hz), 7.68-7.76 (m, 2H, H_5 + H_{14}), 7.86 (d, 2H, H_8 + H_{11}, *J* = 7.6 Hz).



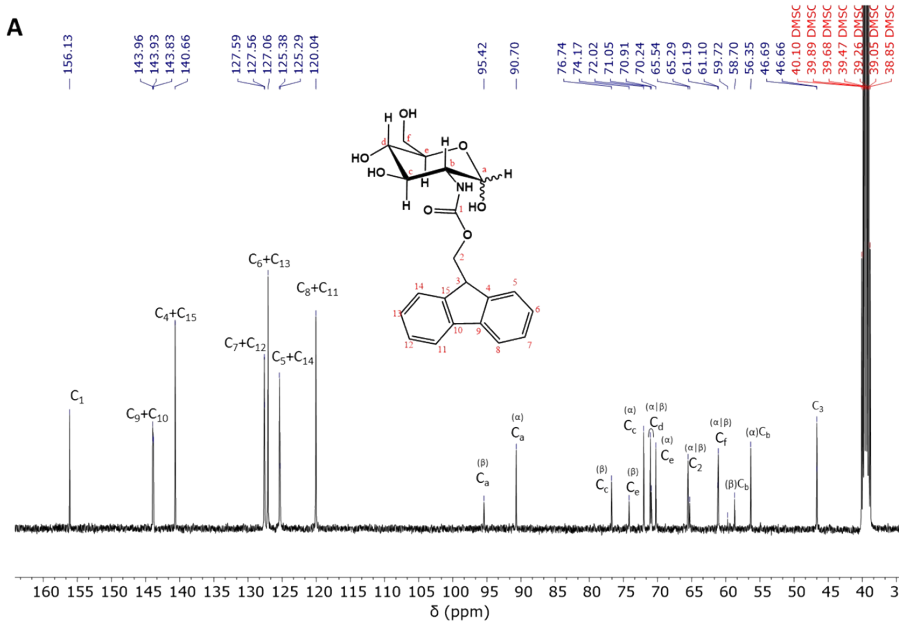
δ_H (ppm) 3.26-3.31 (m, 1H, H_e anomer α|β), 3.44-3.88 (m, 1H, H_c anomer α|β), 3.62-3.88 (m, 4H, H_b + H_d anomer α|β + 2xH_f anomer α|β), 4.17-4.29 (m, 3H, H_3 and 2xH_2), 4.35-4.66 (m, 3H, H_a anomer β and 2xOH), 4.99 (d, 1H, H_a anomer α, *J* = 2.4 Hz), 5.09 (d, 1H, OH, *J* = 4.4 Hz), 6.26

(s, 1H, NH), 6.36 (broad singlet, 1H, OH_a anomer α), 6.49 (broad singlet, 1H, OH_a anomer β), 6.93 (d, 1H, OH_b anomer α, *J* = 9.6 Hz), 7.05 (d, 1H, OH_b anomer β, *J* = 8 Hz), 7.33 (t, 2H, H_6 and H_{13}, *J* = 7.4 Hz) 7.41 (t, 2H, H_7 and H_{12}, *J* = 7.4 Hz), 7.68-7.79 (m, H_5 and H_{14}), 7.88 (d, 2H, H_8 and H_{11}, *J* = 7.2 Hz).

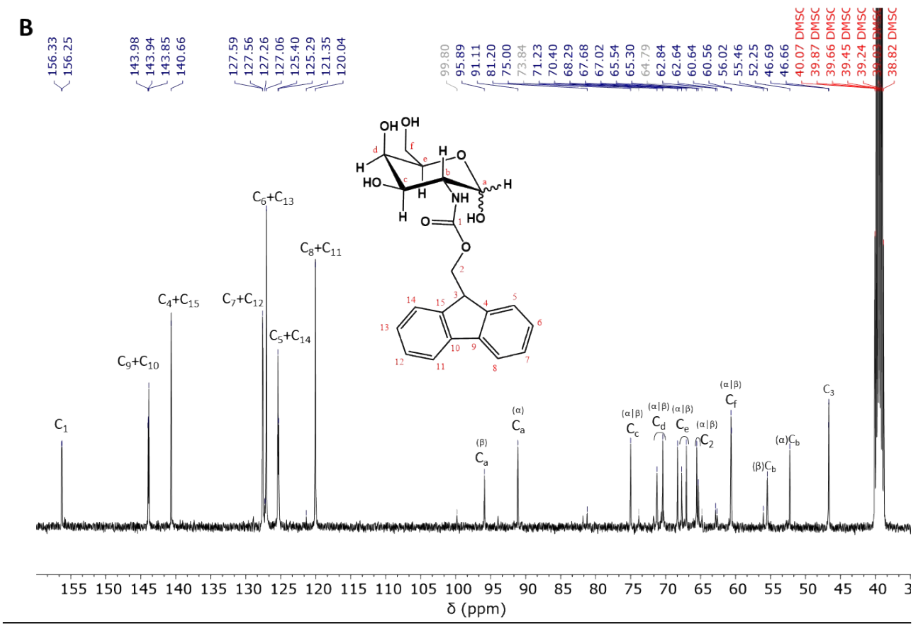


δ_H (ppm) 3.45-3.50 (t, 1H, H_e anomer α|β, *J* = 8.8 Hz), 3.50-3.69 (m, 3H, H_c anomer α|β + 2xH_f anomer α|β), 3.72-3.81 (m, 2H, H_b + H_d anomer α|β), 4.18-4.31 (m, 3H, H_3 and 2xH_2), 4.73 (broad singlet, 1H, H_a anomer β), 4.92 (broad singlet, 1H, H_a anomer α), 6.54 (d, 1H, OH, *J* = 11.5 Hz), 6.93 (d, 1H, OH, *J* = 9.6 Hz), 7.30 -7.36 (td, 2H, H_6 and H_{13}, *J* = 0.9 Hz and 7.6 Hz), 7.41 (td, 2H, H_7 and H_{12}, *J* = 1.1 Hz and 7.6 Hz), 7.75 (d, H_5 and $H_{14,}$ *J* = 8 Hz), 7.88 (d, 2H, H_8 and H_{11}, *J* = 7.2 Hz).

Figure S3. ^{1}H NMR spectra of Fmoc-GlcN (**1**, A), Fmoc-GalN (**2**, B) and Fmoc-ManN (**3**, C) in DMSO-d_6.

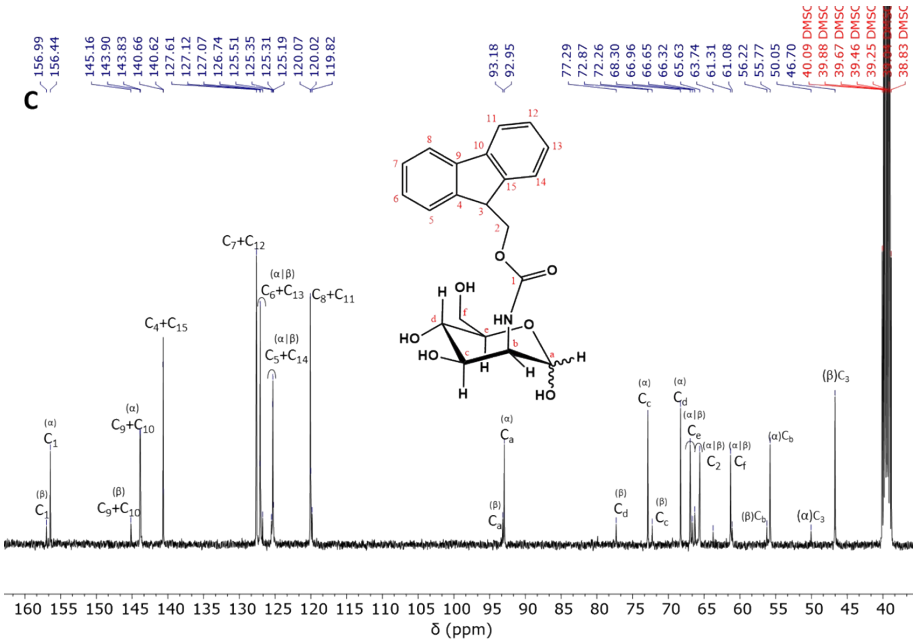


δ_C (ppm) 46.66, 46.69 (C_3 anomer α|β), 56.35 (C_b anomer α), 56.35 (C_b anomer β), 61.10, 61.19 (C_f anomer α|β), 65.29, 65.54 (C_2 anomer α|β), 70.24 (C_e anomer α), 70.91, 71.05 (C_d anomer α|β), 72.02 (C_c anomer α), 74.17 (C_e anomer β), 76.74 (C_c anomer β), 90.70 (C_a anomer α), 95.42 (C_a anomer β), 120.04 (C_8 and C_{11} anomer α|β),125.29, 125.38 (C_5 and C_{14} anomer α|β), 127.06 (2C, C_6 and C_{13} anomer α|β), 127.56, 127.59 (C_7 and C_{12} anomer α|β), 140.66 (C_4 and C_{15} anomer α|β), 144.83, 143.96 (C_9 and C_{10} anomer α|β), 156.31 (C_1 anomer α|β).



δ_C (ppm) 46.66, 46.69 (C_3 anomer α|β), 52.25 (C_b anomer α), 55.46 (C_b anomer β), 60.56, 60.64 (C_f anomer α|β), 65.30, 65.54 (C_2 anomer α|β), 67.02, 67.68 (C_e anomer α|β), 70.40, 71.23 (C_d anomer α|β), 75.00 (C_c anomer β), 91.11 (C_a anomer α), 95.89 (C_a anomer β), 120.04 (C_8 and C_{11} anomer α|β),125.29, 125.40 (C_5 and C_{14} anomer α|β), 127.06 (C_6 and C_{13} anomer α|β), 127.56,

127.59 (C_7 and C_{12} anomer α|β), 140.06 (C_4 and C_{15} anomer α|β), 143.85, 143.94 (C_9 and C_{10} anomer α|β), 156.25, 156.33 (C_1 anomer α|β).



δ_C (ppm) 46.70 (C_3 anomer α|β), 55.77 (C_b anomer α), 56.22 (C_b anomer β), 61.98, 61.31 (C_f anomer α|β), 65.53 (C_2 anomer α|β), 66.65, 66.96 (C_e anomer α|β), 68.30 (C_d anomer α), 72.26 (C_c anomer β), 77.29(C_d anomer β), 92.85 (C_a anomer α), 93.18 (C_a anomer β), 120.02, 120.07 (C_8 and C_{11} anomer α|β), 125.19, 125.31, 125.35 (C_5 and C_{14} anomer α|β), 127.07, 127.12 (C_6 and C_{13} anomer α|β), 127.61 (C_7 and C_{12} anomer α|β), 140.62, 140.66 (C_4 and C_{15} anomer α|β), 143.83, 143.90 (C_9 and C_{10} anomer α|β), 156.44, 156.99 (C_1 anomer α|β).

Figure S4. ^{13}C NMR spectra of Fmoc-GlcN (**1**, A), Fmoc-GalN (**2**, B) and Fmoc-ManN (**3**, C) in DMSO-d_6.

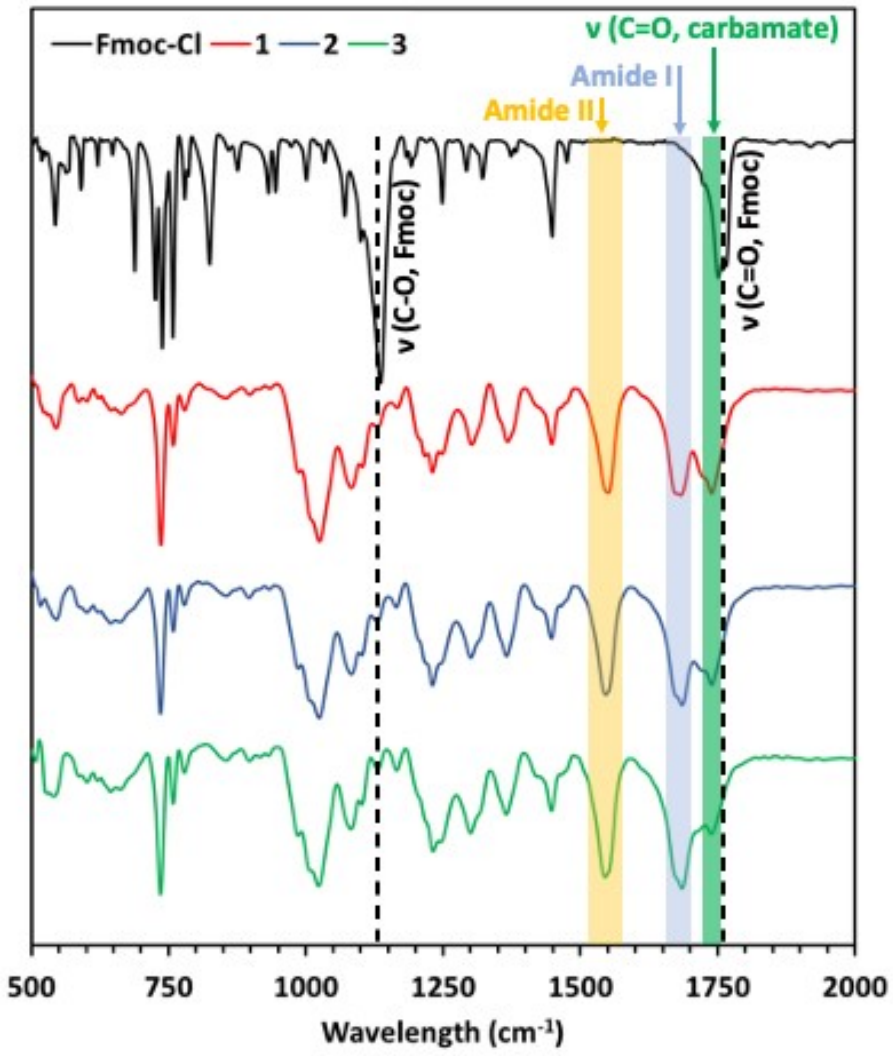


Figure S5. FTIR spectra of Fmoc-Cl (black line), Fmoc-GlcN (**1**, red line), Fmoc-GalN (**2**, blue line) and Fmoc-ManN (**3**, green line), showing the formation of the carbamate upon reaction of Fmoc-Cl with the aminosugars (stretching vibration from C=O, as well as the characteristic Amide I and Amide II vibrations).

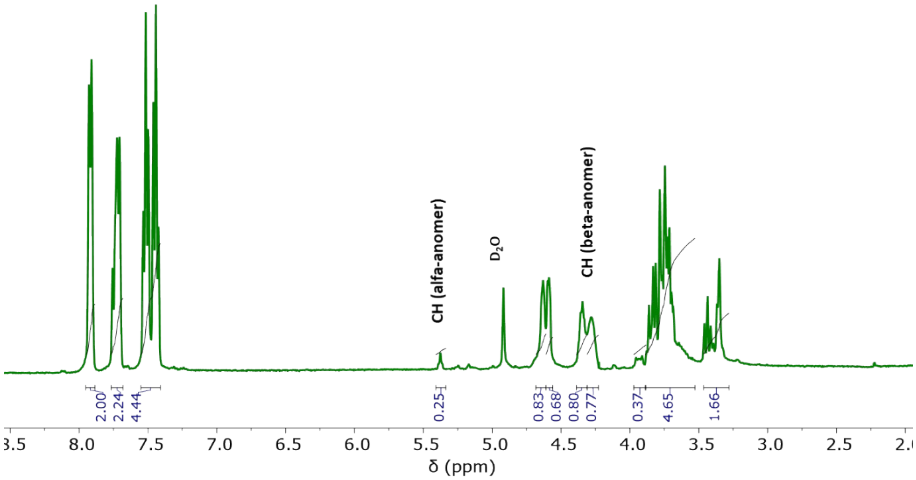


Figure S6. ^{1}H NMR spectra of Fmoc-ManN (**3**, C) in D_2O at ~80°C.

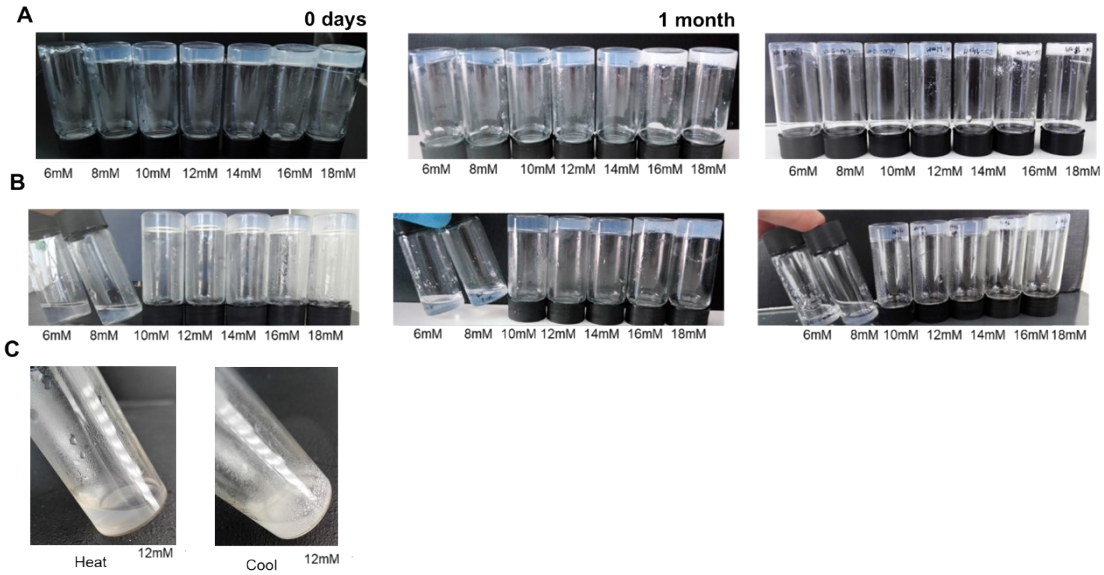


Figure S7. Macroscopic images of the hydrogels/solutions generated by cooling Fmoc-GlcN (**1**, A), Fmoc-GalN (**2**, B) and Fmoc-ManN (**3**, C) at the time of preparation and over 1 and 2 months of storage.

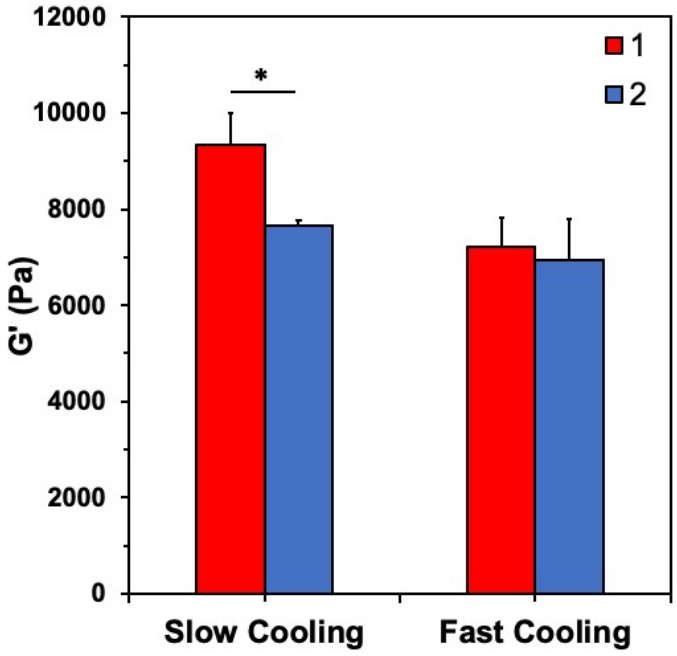


Figure S8. Rheology data for the elastic modulus (G') of the hydrogels generated from 12mM Fmoc-GlcN (**1**) and Fmoc-GalN (**2**) at slow and fast cooling. Statistically significant differences between the G' of the hydrogels generated by **1** *vs* **2**, are marked with * $p < 0.05$.