.0000000000.-Supporting information-

Solvent-free synthesis of polysaccharide derivatives via heterogeneous Schiff base chemistry

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Materials

Potassium Hydroxide (KOH) pellets, Sodium Bicarbonate, anhydrous methanol, isopropyl alcohol (IPA), ethanol, and glacial acetic acid were purchased from Bio-lab (Jerusalem, Israel). Chitosan (MW~890 kDa, 100-300 cps) was obtained from Glentham Life Sciences (Corsham, UK). Monochloroacetic acid and 4-methoxybenzaldehyde were purchased from Alfa Aesar (MA, USA). Citral (mixture of isomers) and pyrene were purchased from Sigma Aldrich (Missouri, USA). Dextran standards were purchased from PSS Polymer (Mainz, Germany). Ammonium Bicarbonate was purchased from Honeywell (Seelze, Germany). Benzaldehyde, hexanal, 4-chlorobenzaldehyde, and trans-cinnamaldehyde were purchased from Acros Organics (Geel, Belgium). Deuterated water (D_2O) was obtained from Tzamal-D-Chem (Petah Tikva, Israel). Aquastar 1% water standard, Aquastar combisolvent Keto and Aquastar CombiTitrant 5 Keto were purchased from Merck (Dermstadt, Germany). All reagents and solvents were used without further purification.

Methods

Synthesis of Carboxymethyl Chitosan (CMCS)

CMCS synthesis was based on Chen's method with mild modifications.[1] 10 g of chitosan (62.1 mmol, mass divided by molecular weight of glucosamine monomer 161 gr/mol) was suspended in 200 ml of 1:9 (v/v) distilled water: IPA at room temperature (RT) under magnetic stirring for 10 min in a 600 ml beaker, followed by the addition of 18.9 g crushed KOH (336.8 mmol, 5.4 eq) pellets. Then the mixture was cooled to 4 °C and magnetic stirred for one hour until a thick slurry was formed. 15 g of monochloroacetic acid (158.7 mmol, 2.55 eq) was dissolved in 20 ml IPA and added dropwise by addition funnel for 10 min to CMCS slurry while magnetic stirring at 4 °C . The mixture was left to stir for 12 h at 4 °C . After 16 h, 200 ml of 70 % aqueous ethanol solution was added to the mixture and stirred for 10 min to quench the reaction. Then the mixture was filtered under vacuum and rinsed three times with 200 ml of 70 % aqueous ethanol solution. The potassium salt of CMCS was freeze-dried under vacuum. Prolonging the reaction time at low temperature resulted in a complete solubility of the CMCS product without unreacted chitosan. CMCS was characterized by ATR-FTIR and ^{1}H-NMR, confirming the formation of the desired product (98.4%

yield) (Figure S1) CMCS degree of substitution (DS) was calculated to 96% using a previously reported method[2].

Synthesis of CMCS-aldehyde derivatives using heterogeneous procedure

CMCS-aldehyde derivatives were synthesized from solid particles of CMCS potassium salt that were sieved to a size range of under 0.2 mm (80 mesh) by a manual test sieve. 250 mg of CMCS (1.0 mmol, mass divided by molecular weight of potassium salt of carboxylated glucosamine monomer 252 g/mol with calculated DS of 0.96), 5 ml of the selected aldehyde (the volume was determined to achieve proper mixing of the solid particles in the reaction medium), and 125 µl of glacial acetic acid (2.5%) were added to a 15 ml centrifuge tube. The tube was vortexed for 1 min to ensure complete suspension of solid particles in the reaction mixture. The suspension was placed in an orbital shaker at 300 RPM with different temperatures and reaction times for each aldehyde (Table S1). After the reaction was finished, the solid product was separated by filtration and dried under vacuum.

Table S1. Reaction conditions for each synthesis of the CMCS-derivatives.

Aldehyde	Reaction time [h]	Temperature [°C]	Yield** [%]
Hexanal	1	45	28.8
Citral	1	45	5.4
Benzaldehyde	2	45	10.4
4-chlorobenzaldehyde	2	50*	4.2
4-methoxybenzaldehyde	2	45	6.5
Trans-cinnamaldehyde	2	45	12.1

* 4-chlorobenzaldehyde was heated to 50 °C in order to reach its melting point (47 °C).

** Yield was calculated based on substituted CMCS based on DS/100% substituted CMCS×100.

Synthesis of Carboxymethyl chitosan-N-benzylidene, 3', using homogenous procedure

500 mg (2.0 mmol) of CMCS was dissolved in 100 ml of pre-heated DW at 45 °C; the solution pH was adjusted to 4.5 with glacial acetic acid. To the solution, 1 ml of benzaldehyde (9.8 mmol, 4.9 eq) diluted with 10 ml of ethanol was added dropwise by addition funnel, followed by additional 5 ml of ethanol. The reaction mixture was stirred by a magnetic stirrer under heating. After 4 h, the solution's pH was elevated and neutralized with a 5% ammonium bicarbonate solution (w/v). The product was

precipitated with the addition of ethanol having three times the reaction mixture volume. The carboxymethyl chitosan-N-benzylidene (**3'**) product was filtered and dried under vacuum (0.9% yield).[3]

Continuous reaction for the synthesis of carboxymethyl chitosan-N-benzylidene

Carboxymethyl chitosan-N-benzylidene **(3)** was selected as a model to test the continuous reaction procedure. The procedure was similar to the heterogeneous reaction with few adjustments. Once the first reaction cycle was finished, the reaction mixture was centrifuged at 6000 RPM for 1 min to precipitate the product as solid particles, then the supernatant containing the aldehyde and acetic acid was collected by a syringe, 4.9 ml of the reacting medium was collected presenting 93.3% collection yield (volume of collected medium/initial volume×100%) , the supernatant was added to a new 15ml centrifuge with ,233 mg of CMCS (the same ratio of CMCS/aldehyde+acid) vortexed, and reacted again. Once the cycle is finished, the reaction medium was collected and added to the new portion of polymer reagent, completing four reaction cycles.

Characterizations of the prepared materials

Attenuated Total Reflectance Fourier Transforms Infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectroscopy was performed using a Thermo Scientific Nicolet iS5 FTIR spectrometer. CMCS and CMCS-derivatives powder was subjected to 32 scans at a 0.5 cm^{-1} resolution between 500 to 4000 cm^{-1}.

Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H spectra were recorded using a Bruker Avance III NMR spectrometer at 400 MHz (9.4Tesla). Chemical shifts *(δ)* are reported in ppm, while coupling constants *(J)* are expressed in Hz. Multiplets are denoted as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br). ^{1}H-NMR spectra were calibrated to the solvent residual peak (HOD at 4.79 ppm). All NMR samples were prepared using D_2O as a solvent, at 298 K. The diffusion measurements were performed as 1D STE (stimulated echo) with bipolar gradients and were performed with SMQS10.100 shaped gradients. The duration of each gradient in the bipolar block was 2ms (total of 4ms) with an amplitude of 40 Gauss/cm. The diffusion time was 60 ms, and the number of scans was 64 with a

relaxation delay of 2s. The DS of CMCS was calculated based on the previously reported method.[2] The DS of CMCS-derivatives was defined by relying on the ratio of the integral value between methyl peaks of **1** and **2**, and with the H peak of the aromatic imine at **3-6** to the methyl protons of the acetylated amine (deacetylation percentage provided by the supplier of the original Chitosan 90%) as a reference.[4]

Gel Permeation Chromatography (GPC).

Number-average molecular weights of CMCS and CMCS-derivatives were calculated using gel permeation chromatography (GPC). Waters' Alliance system e2695 separations module was used (Waters, United States), for the study. It si equipped with a refractive index detector, model blue 2414. The mobile phase used was 0.1 M sodium bicarbonate solution under isocratic elution for 30 min at a flow rate of 0.7 mL/min. The injection volume was 100 µL, and the temperature of both the detector and columns was 30 °C. Analyses were carried out using an Ultrahydrogel column, 1000 Å, 12 µm, 7.8 mm × 300 mm, 2−4000 kDa (Waters, United States). The molecular weights were calculated relative to standard dextran known molecular weight with Mn (the number-average molecular weight) range of 5.2-668.0 kDa. The data provided by the GPC system was collected and analyzed with the Empower 3 personal dissolution software. The polymer's powders were dissolved in the mobile phase to give a final concentration of 1 mg/mL. The solutions were filtered through a 0.45 µm nylon syringe filter.

Mechanical properties

Young's modulus (YM), Tensile strength (TS), and elongation break (EB) were determined using an Instron 3345 instrument with an Instron force transducer load cell (Norwood, MA, USA). Tests were performed at a speed of 1 mm/min. TS was expressed in [MPa] and was calculated by dividing the maximum load [N] by the cross-sectional area [m^2]. EB was calculated by dividing the extension at the moment of rupture by the initial gauge length of the samples and multiplying by 100. YM was expressed in [MPa] and was determined by the stress ratio along an axis over the strain along that axis in the range of stress. All measurements were performed in triplicate for each film.

Water Contact Angle (WCA)

Water contact angles for the film samples were measured at ambient conditions using a Kruss Drop Shape Analyzer instrument, model DSA100S. Angles were measured within 1 s of contact after placing 5 μL individual drops of deionized water on the film surface. For each sample, contact angles of three different positions on the surface were measured in triplicate and calculated by the sessile drop method, using the Advance software to consider the average value.

Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed on Perkin-Elmer TGA 8000 (TA Instruments). Ceramic crucibles were loaded with 4-10 mg of each sample and heated from 50 °C to 800 °C with a heating rate of 20 °C/min under a flow of N_2 (20 mL/min).

Differential scanning calorimetry (DSC)

DSC measurements were conducted with a Perkin-Elmer DSC 6000 instrument calibrated using Indium and Zinc standards. Thermograms of each sample were obtained from the second heating run up to 440 °C, after the first run of heating up to 200 °C and cooling to 30 °C at a constant rate of 20 °C/min, under N_2 purge of 20 mL/min. Aluminum crucibles with pierced lids were loaded with 2-5 mg of each sample.

Dynamic Light Scattering (DLS)

All measurements were recorded on a Zeta Sizer (3000HSa, Malvern Instruments Ltd., UK) equipped with a 50 mW laser at an operating wavelength of 532 nm. All measurements were conducted at 23 °C (refractive index, 1.330; viscosity, 0.890 cP for water) with an angle detection (θ) of 173°. CMCS and CMCS-derivatives samples were prepared by dissolving 2.5 mg/mL in DW. Each sample was measured three times, and the average size values were calculated.

Electrokinetic properties (ζ-potential)

ζ-Potential were determined by a Zeta Sizer (3000HSa, Malvern Instruments Ltd., UK). ζ-Potential values of CMCS and CMCS-derivatives were measured at 2.5 mg/mL aqueous solution at pH 7.5 maintained by ammonium bicarbonate. The measurements were done in triplicates, and the obtained values represent mean ± standard error.

Critical aggregation concentration (CAC)

Critical aggregation concentration (CAC) of the prepared conjugates polymers was studied using pyrene as a fluorescent probe. Its fluorescent emission spectrum comprises vibronic peaks that strongly depend on the solvent's polarity. The ratio between two specific peaks (i.e., I_3 ~383 nm and I_1 ~373 nm) in pyrene's spectrum was used to measure its microenvironment's polarity. Any change in the surrounding polarity, such as when pyrene is encapsulated from an aqueous environment by a hydrophobic-cored aggregate, is expressed in a significant change of this ratio value.[5] Pyrene was first dissolved in absolute ethanol to obtain a 2.4 mM stock solution and then further diluted with various water-based modified biopolymer solutions at varying concentrations, always yielding a final concentration of 1.2 μM. Samples were prepared from the original solutions at varying dilutions and vortexed before measurements. Fluorescence spectra of modified biopolymer samples were measured. The excitation wavelength for pyrene was 340 nm with a slit width of 3 nm, and the emission band recorded was 360–400 nm with a slit width of 3 nm, at increments of 0.5 nm. All samples measured were kept at 23 ± 1 °C. All samples were made in triplicates, and each triplicate was scanned twice. Spectra were not corrected for instrumental bias and may differ slightly in position and intensity from spectra collected on other instruments. However, any deviations from accurate spectra due to our instrumental configuration are consistent across all samples within this dataset. CAC values were calculated as the intersection between two linear lines depicting aggregate formation dependent on concentration in solution.[5]

Atomic Force Microscopy (AFM)

Topographic imaging was performed using Innova AFM with a NanoDrive Controller (Bruker, Camarillo, CA USA) operating in the tapping mode (in air at room temperature). Surface images, using scan width of 5 x 5 μm, and a scan rate of 0.5 Hz was acquired at fixed resolution (512 x 512 pixels). Bruker 0.01-0.025 Ohm-cm Antimoni (n) doped silicon probes (model: RTESPA MPP-11120-10) were used. The roughness parameter, such as the root mean square (Rq), was calculated for the scanned area (5 x 5 μm) using NanoScope Analysis software after 2nd order image flattening. All measurements were done in 5 different locations for each film type, and the average values are presented.

Karl Fischer (KF) titration method

The water content of the reaction medium was measured by the Karl Fischer titration method, using Titroline 7500 KF, (SI Analytics, Germany). The KF titrator was washed with anhydrous methanol and calibrated by Aquastar 1% water standards measured 1.0358 %. Samples from the reaction medium were measured before the 1st reaction cycle and after each reaction cycle. The water content was measured by standard procedure using Aquastar combisolvent Keto for sample dissolution and Aquastar CombiTitrant 5 Keto for the water titration.[6]

Stability studies

Imine bond stability (Schiff base) was tested in different pH's and temperatures (Figure 3). The stability of the bond was represented by a color change, as a result of the reaction in the acetylacetone test with free aldehyde in the mixture. The test was performed by Kamimura's method[7] with mild alterations. 2 mg/ml solution of each derivatives was prepared by dissolving the CMCS-derivatives powder in solution at pH 3, 5-9 and 11 maintained by 37% hydrochloric acid and NaOH pellets. Then the samples were divided according to the test conditions. The heated samples were heated to 60^0C by magnetic plate equipped with a water bath. Once finished, 0.5 ml of each polymer's solution was reacted with 0.5 ml of the acetylacetone reagent for 30 min at 60^oC.

Green Metrics Analysis

Green metrics analysis was done by calculating the environmental factor (E-factor) for **3** and **3'** (Eqn S1) using the method reported by Sheldon.[8]

Equation S1. E-factor equation for **3** and **3'**

$$E-factor = \frac{\mathrm{m(waste)}}{m(products)} = \frac{\Sigma m(raw\ materials) + \Sigma m(reagents) - m(products)}{m(products)}$$

For continuous reaction, waste was considered as 6.7% (aldehyde and acetic acid that are lost in one cycle upon separation of the product from the reaction mixture)."

CMCS synthesis

CMCS was synthesized from chitosan in a heterogeneous reaction based on Chen method with mild changes.[1] By prolonging the reaction time to 12 h, no unreacted chitosan was remained. The product was purified and characterized by ATR-FTIR (Figure S1) and ^{1}H-NMR (Figure S2). ATR-FTIR scans demonstrate increased specific COOK frequencies at 1598 cm^{-1}. ^{1}H-NMR scans revealed the successful modification, demonstrating the peak at 4.03 ppm that belongs to 3', 6' O-Carboxymethyl substitution, and peak at 3.43 ppm that belongs to N-Carboxymethyl substitution.[1]

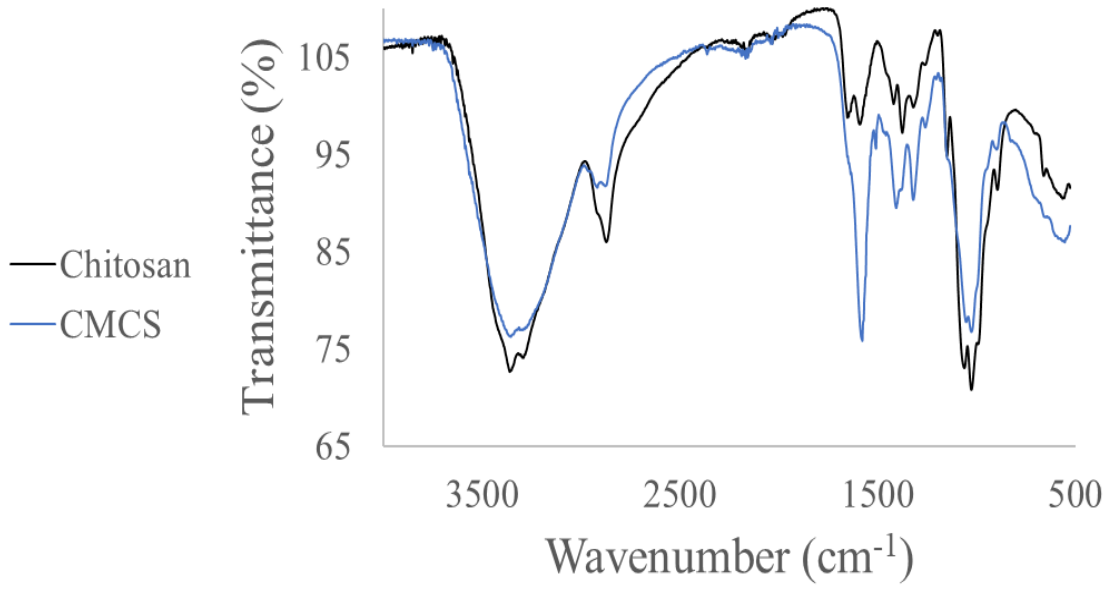

Figure S1. ATR-FTIR spectra of chitosan and CMCS.

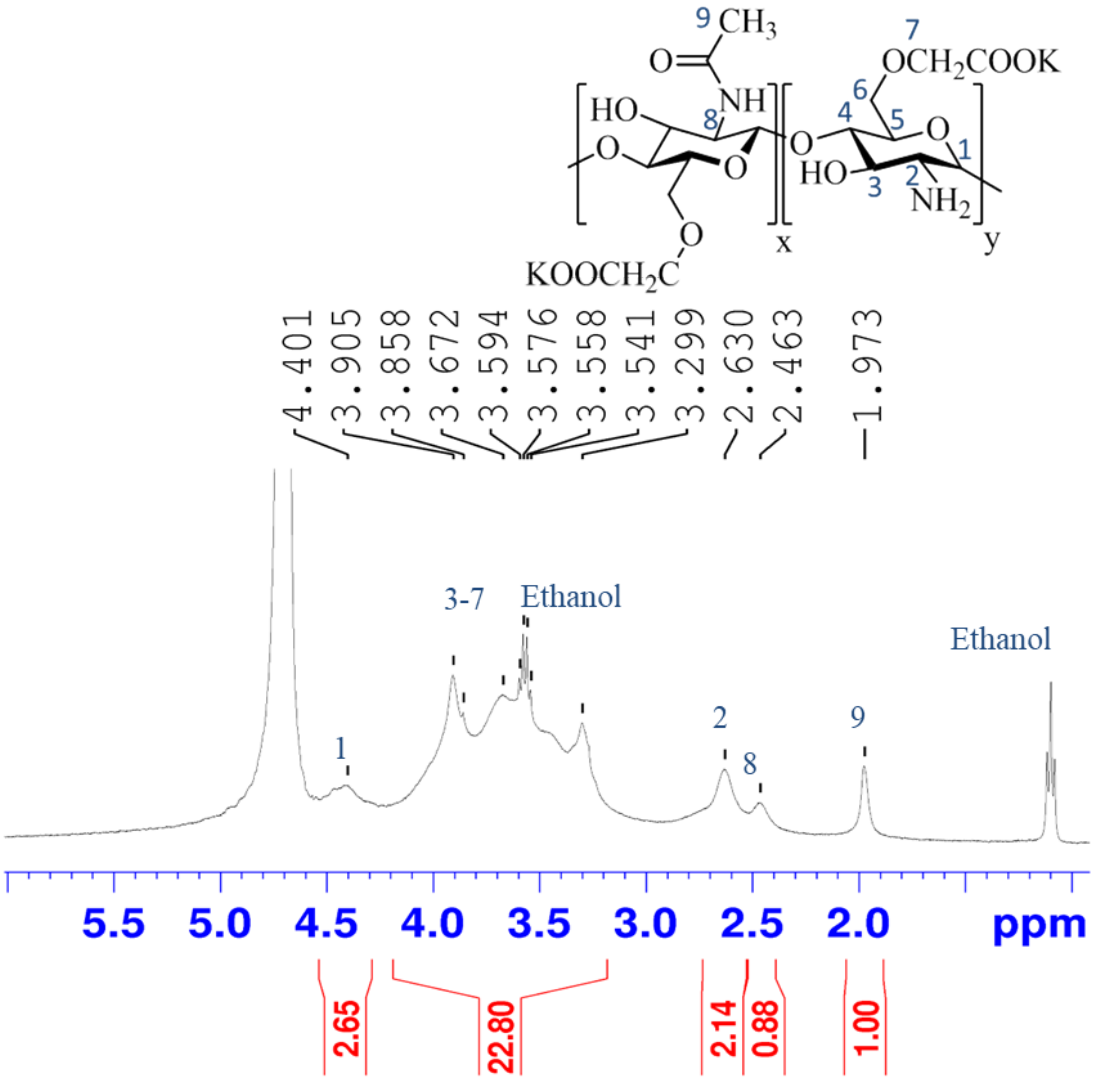

Figure S2. [1]H NMR (400 MHz, D2O) of CMCS, δ 4.40 (br, 1H), 3.30-3.91 (br, 7H) 2.63 (br, 1H), 2.46 (br, 1H), 1.97 (s, 3H).[2]

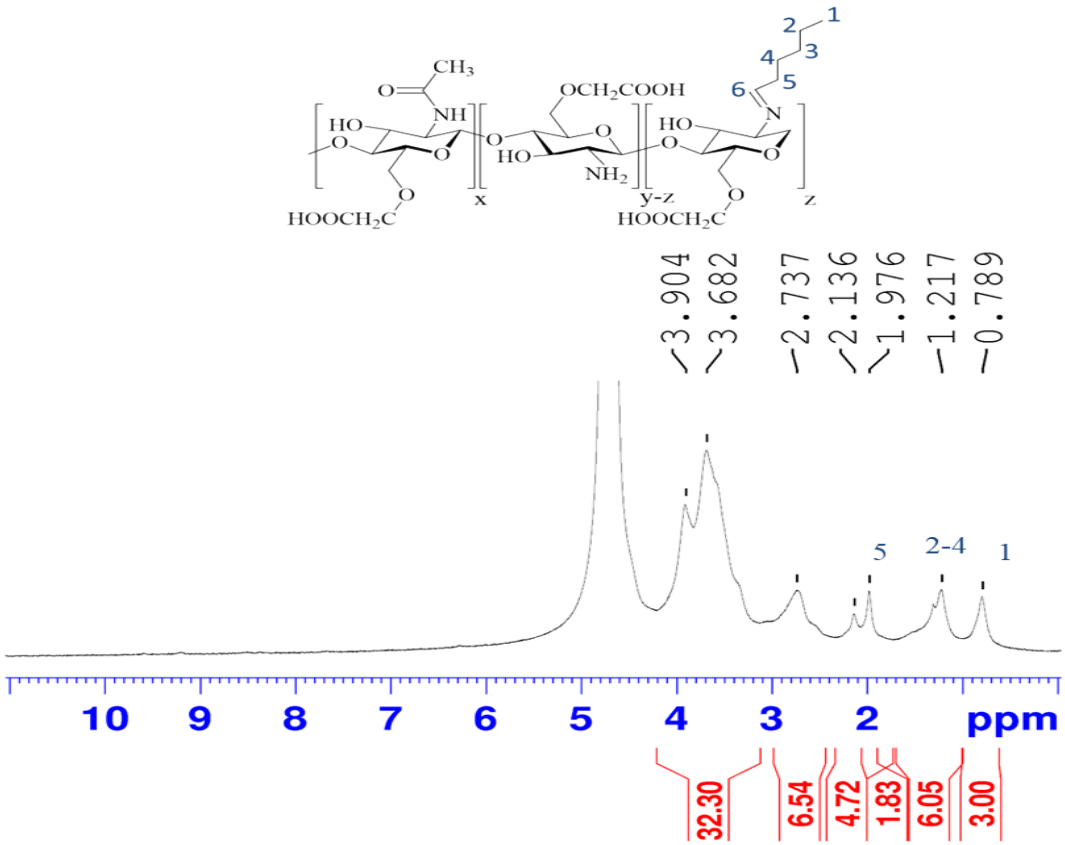

Figure S3. [1]H NMR (400 MHz, D2O) of CMCS-N-hexylidene, **1**. δ 2.13 (br, 2H), 1.21 (br, 6H), 0.79 (br, 3H).[9]

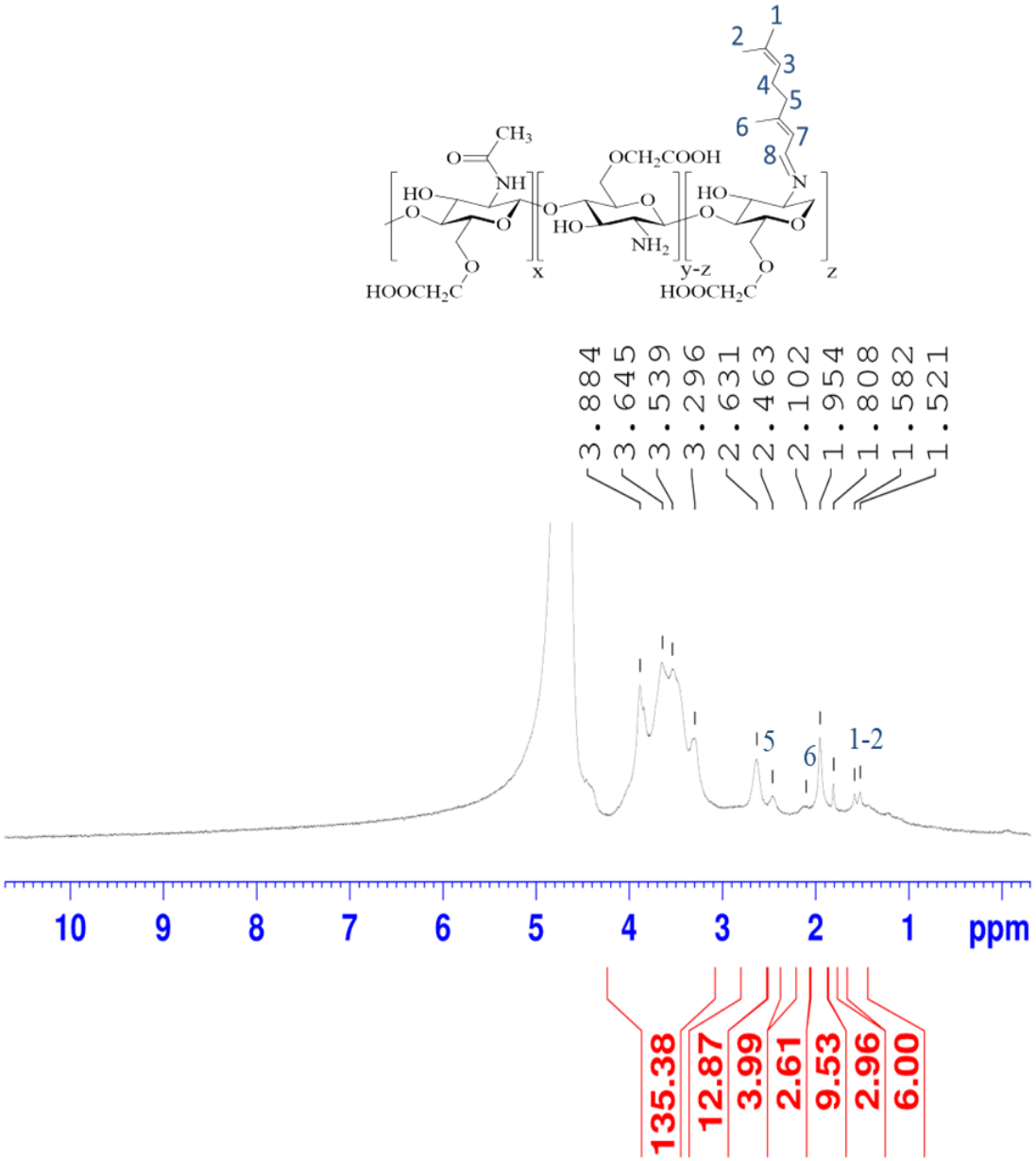

Figure S4. [1]H NMR (400 MHz, D2O) of CMCS-N-(3,7-dimethylocta-2,6-dien-1-ylidene), **2**. δ 2.1 (br, 2H), 1.81 (s, 3H), 1.52-1.58 (ds, 6H) 3,4,7,8 peaks are masked by CMCS sugar ring and the water peaks.[10]

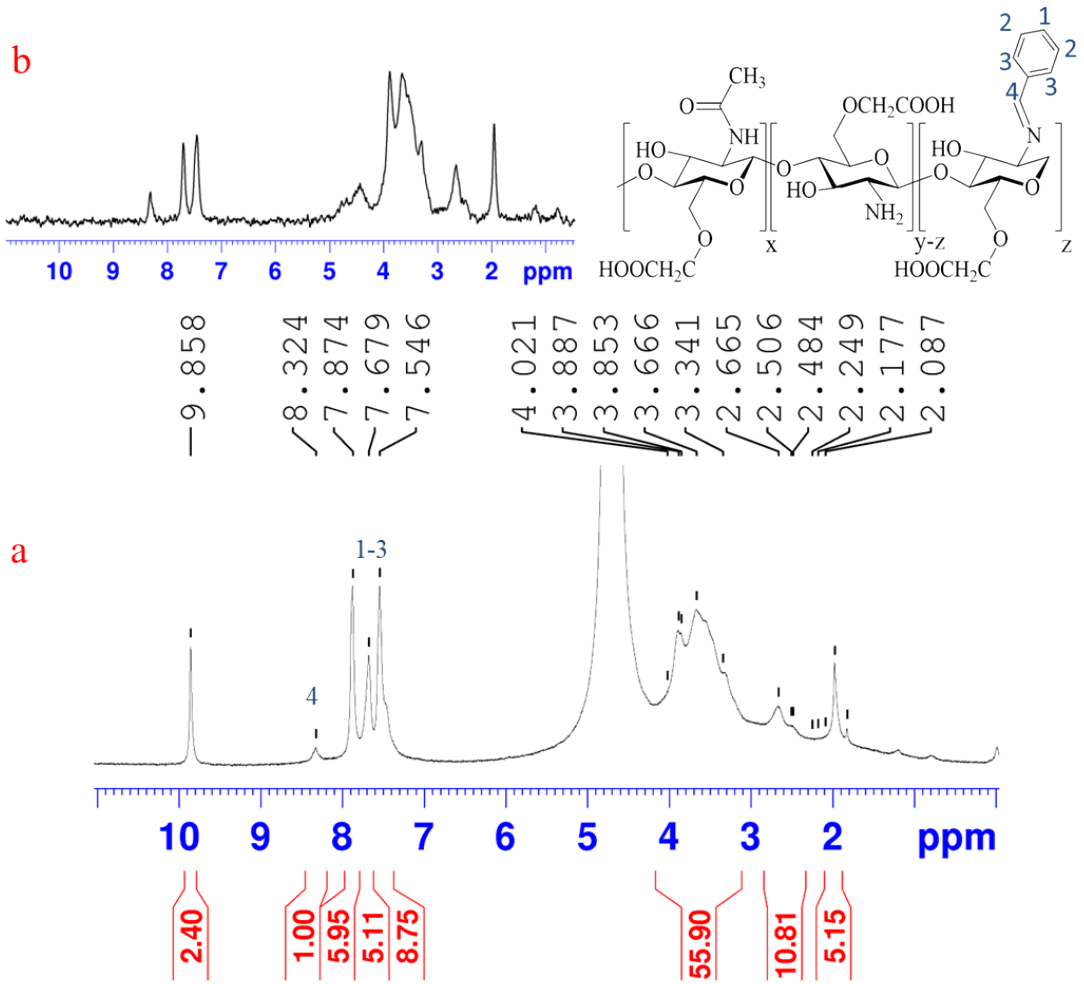

Figure S5. (a) ^{1}H NMR (400 MHz, D2O) of CMCS-N-benzylidene, **3**. δ 8.32 (s, 1H), 7.55-7.68 (br, 5H). (b) 1D diffusion test of CMCS-N-benzylidene.

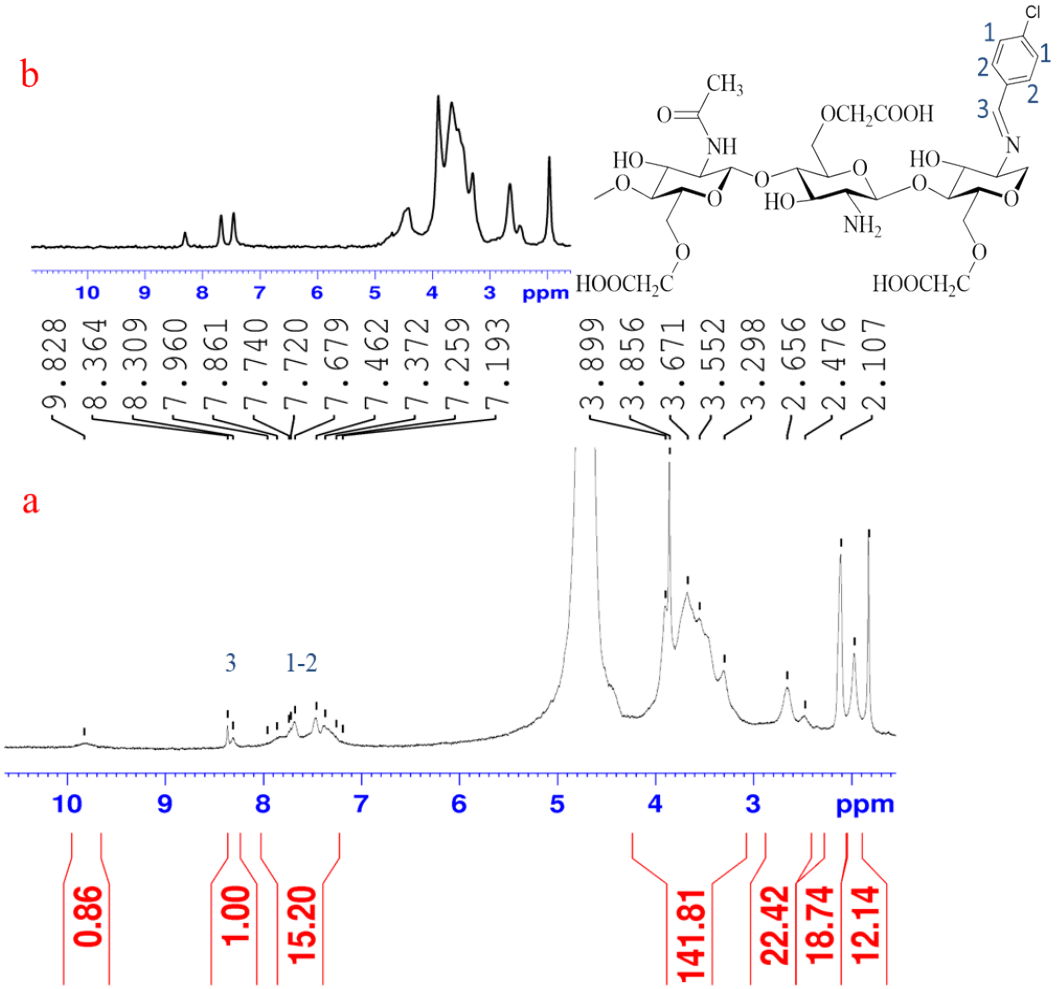

Figure S6. (a) ^{1}H NMR (400 MHz, D2O) of CMCS-N-(4-chlorobenzylidene), **4**. δ 8.36 (s, 1H), 7.68-.7.46 (br, 4H). (b) 1D diffusion test of CMCS-N-(4-chlorobenzylidene)

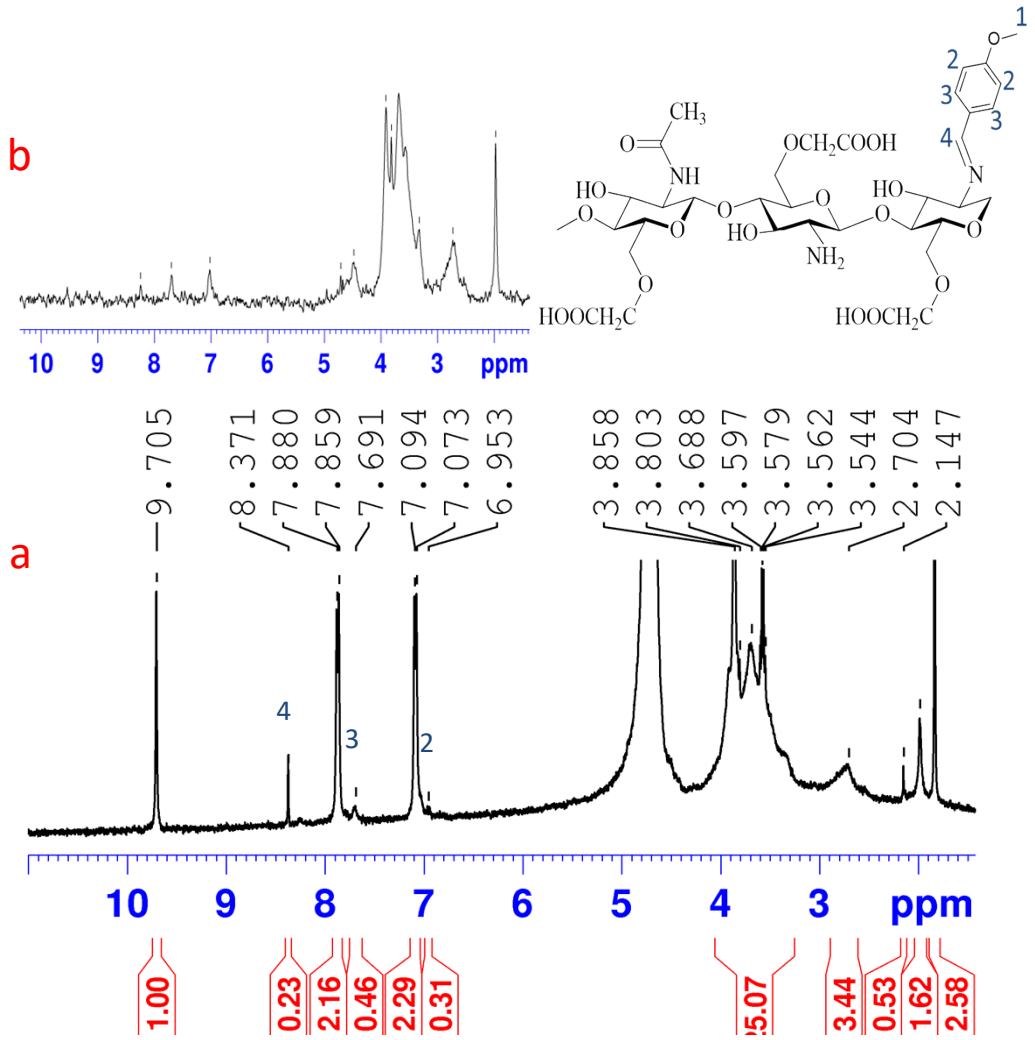

Figure S7. (a) ^{1}H NMR (400 MHz, D2O) of CMCS-N-(4-methoxybenzylidene), **5**. δ 8.37 (s, 1H), 7.69 (br, 2H), 6.95 (d, 2H), 1 masked by CMCS sugar ring backbone. (b) 1D diffusion test of CMCS-N-(4-methoxybenzylidene).

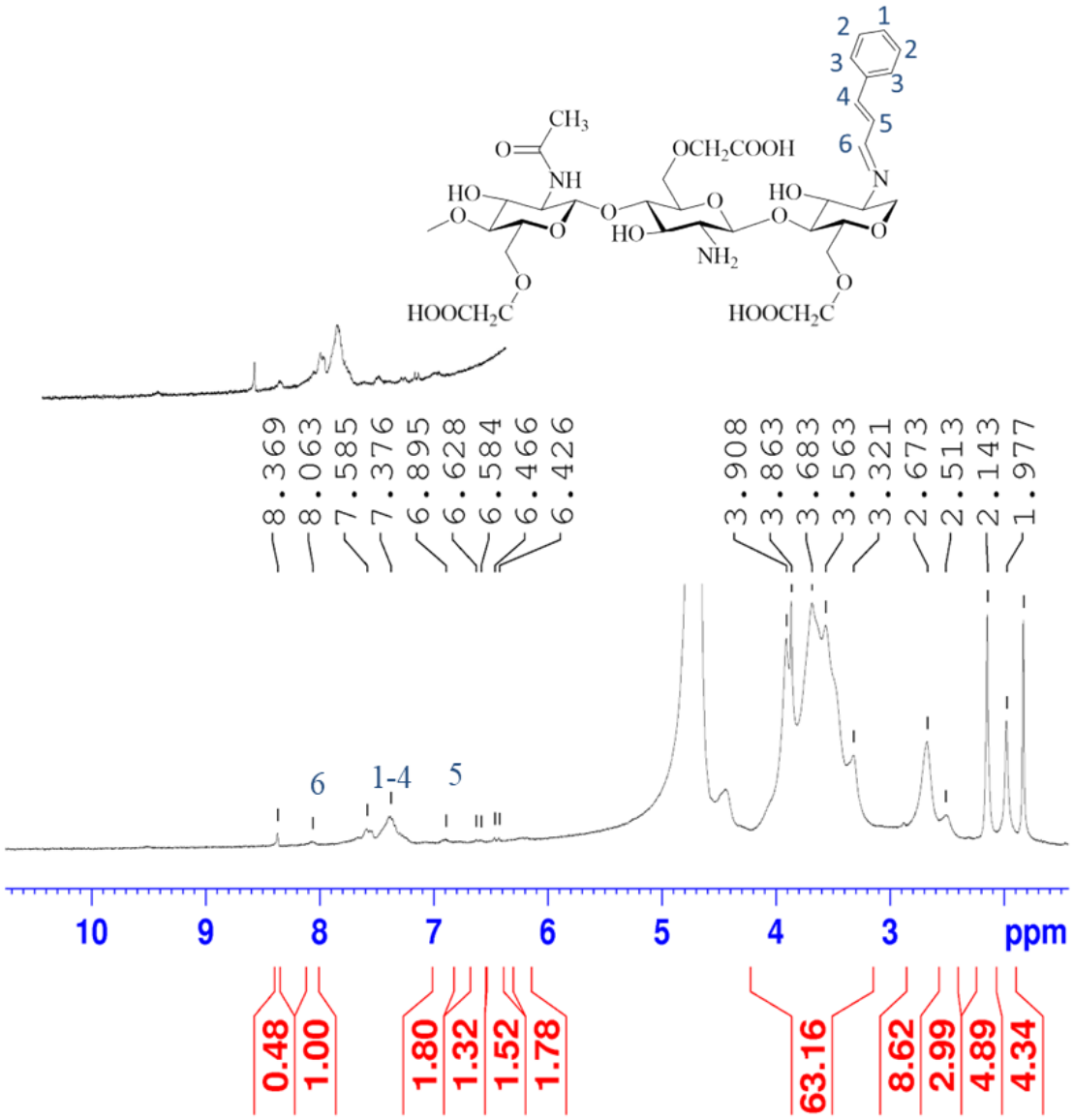

Figure S8. [1]H NMR (400 MHz, D2O) of CMCS-N-(3-phenylallylidene), **6**. δ 8.06 (br, 1H), 7.39-7.59 (br, 6H), 6.90 (br, 1H).[11]

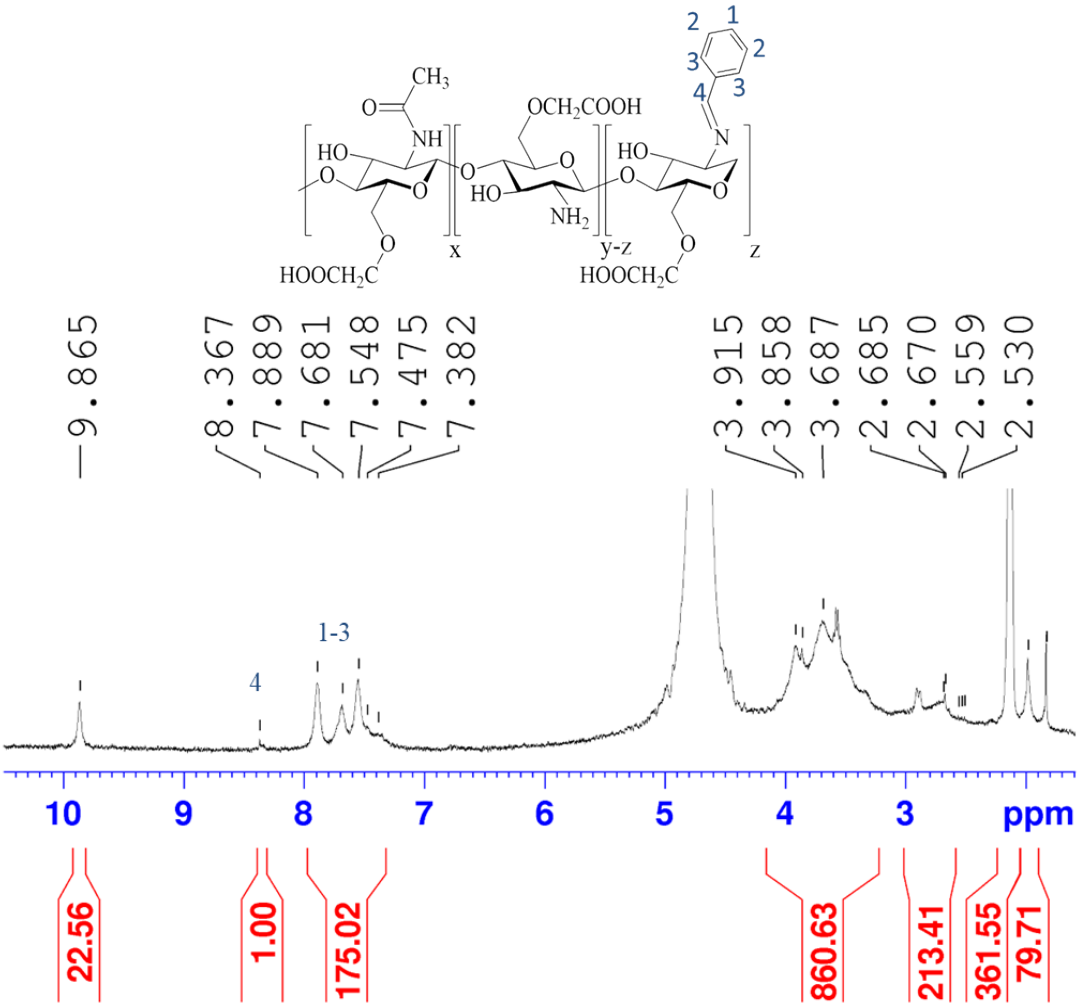

Figure S9. [1]H NMR (400 MHz, D2O) of CMCS-N-benzylidene (homogenous synthesis product) **3'** δ 8.36 (s, 1H), 7.38-7.88 (br, 5H).

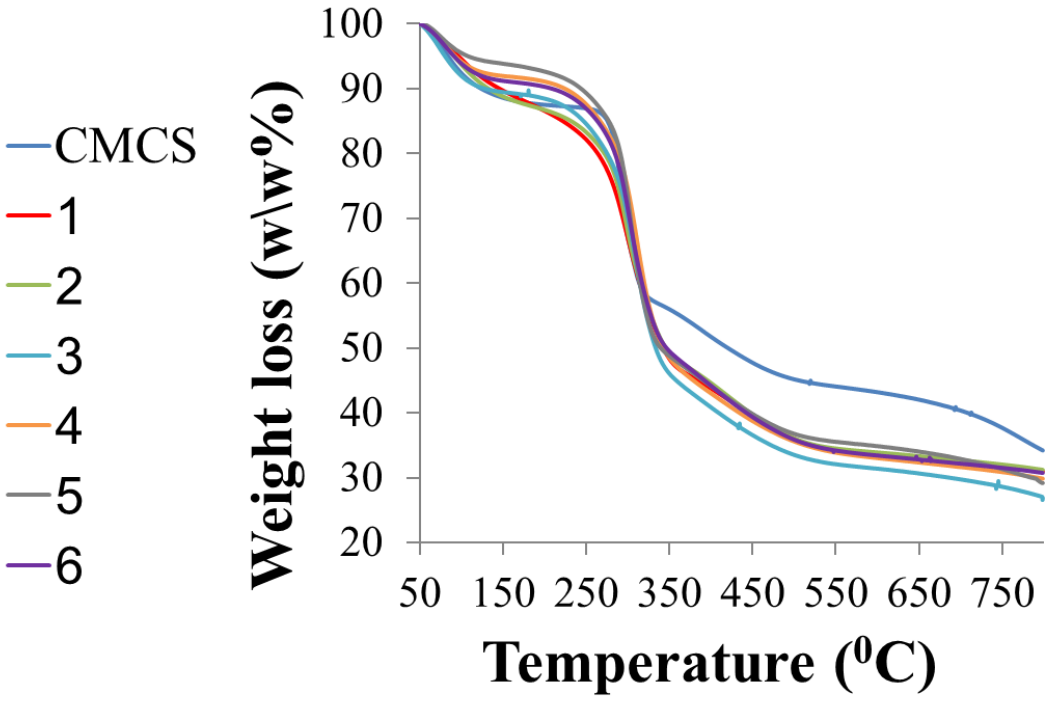

Figure S10. TGA spectra of CMCS and CMCS derivatives.

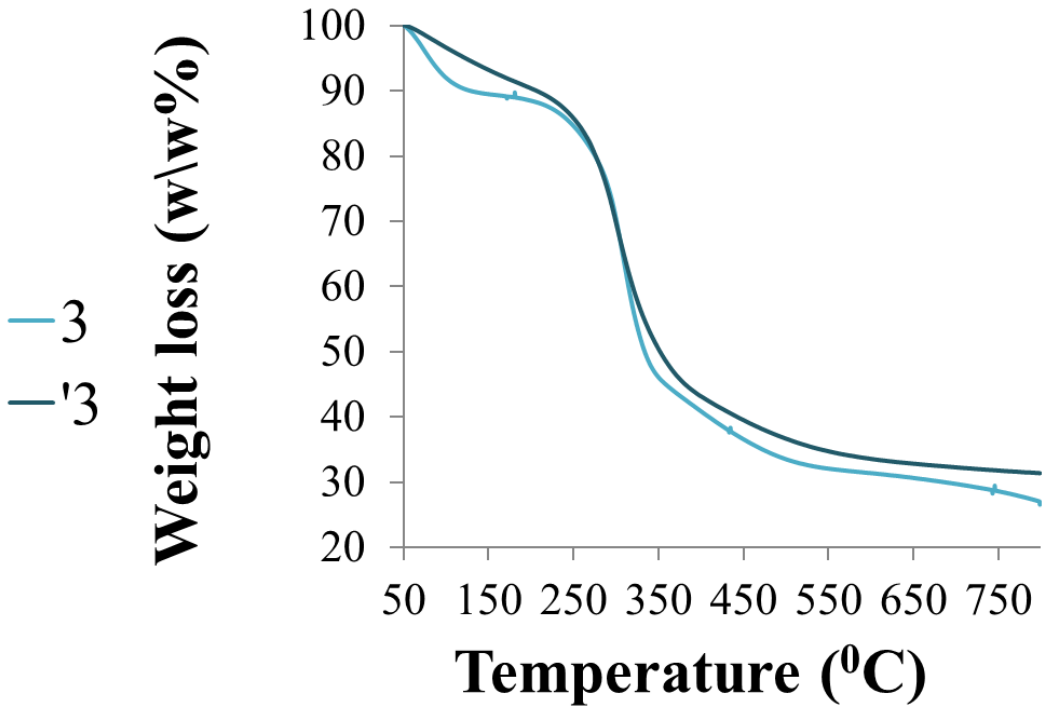

Figure S11. TGA spectra of **3** and **3'**.

Table S2. Main pyrolytic events for the CMCS-derivatives.

Polymer	Main pyrolytic event [ºC]
CMCS	291
1	299
2	297
3	308
4	311
5	303
6	302

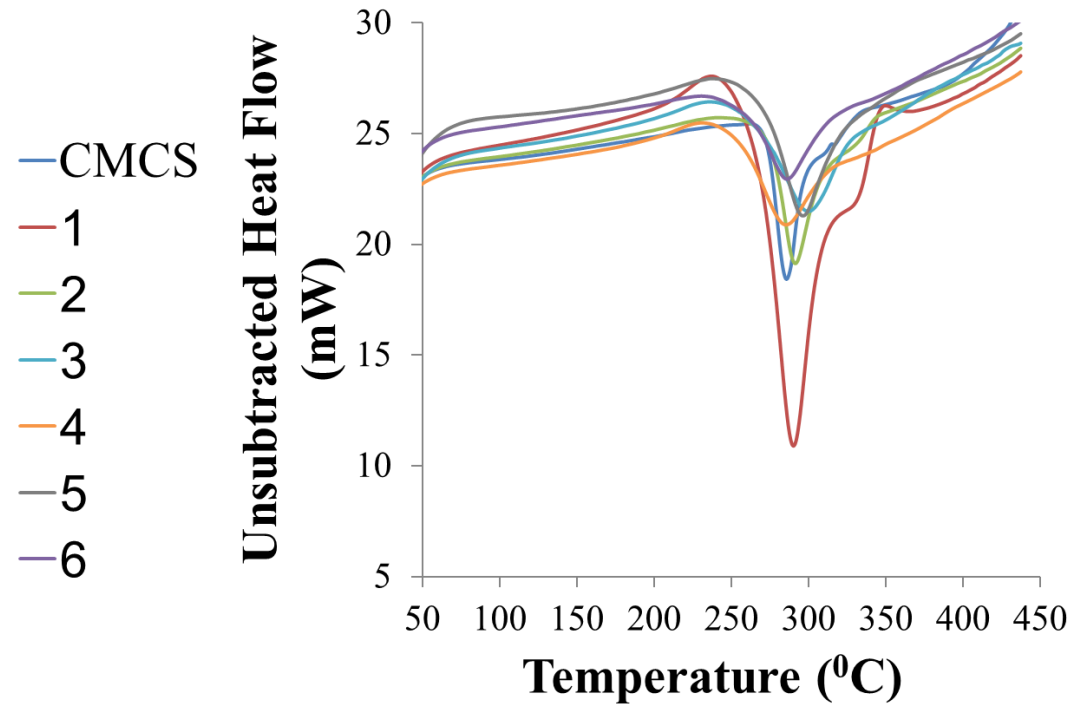

Figure S12. DSC spectra of CMCS and CMCS-derivatives.

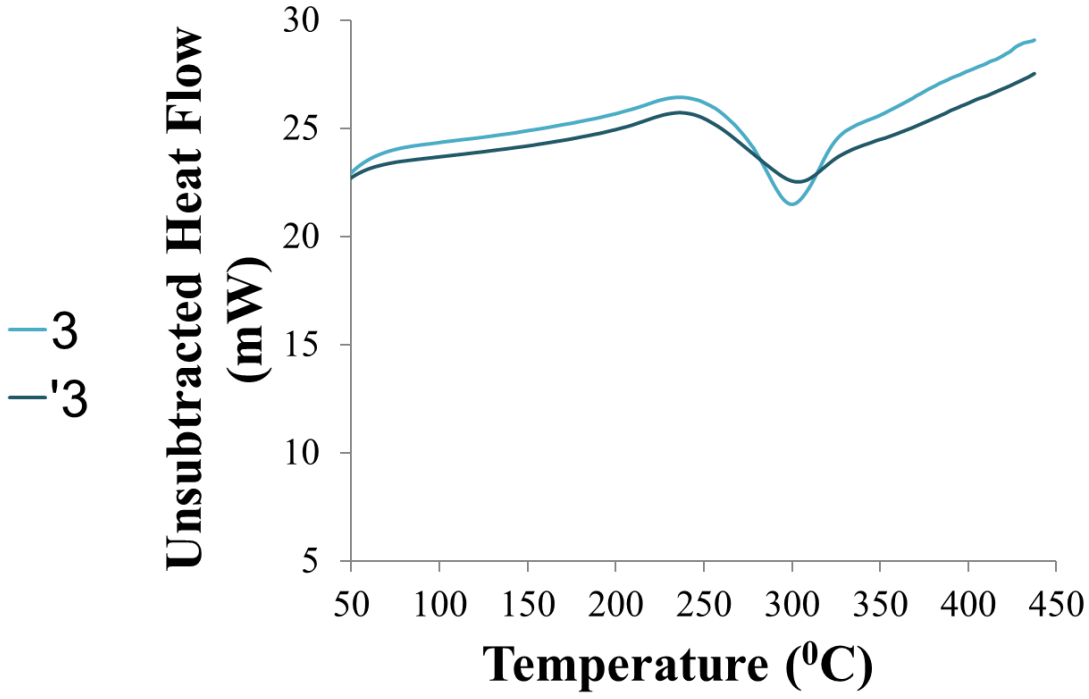

Figure S13. DSC spectra of **3** and **3'**.

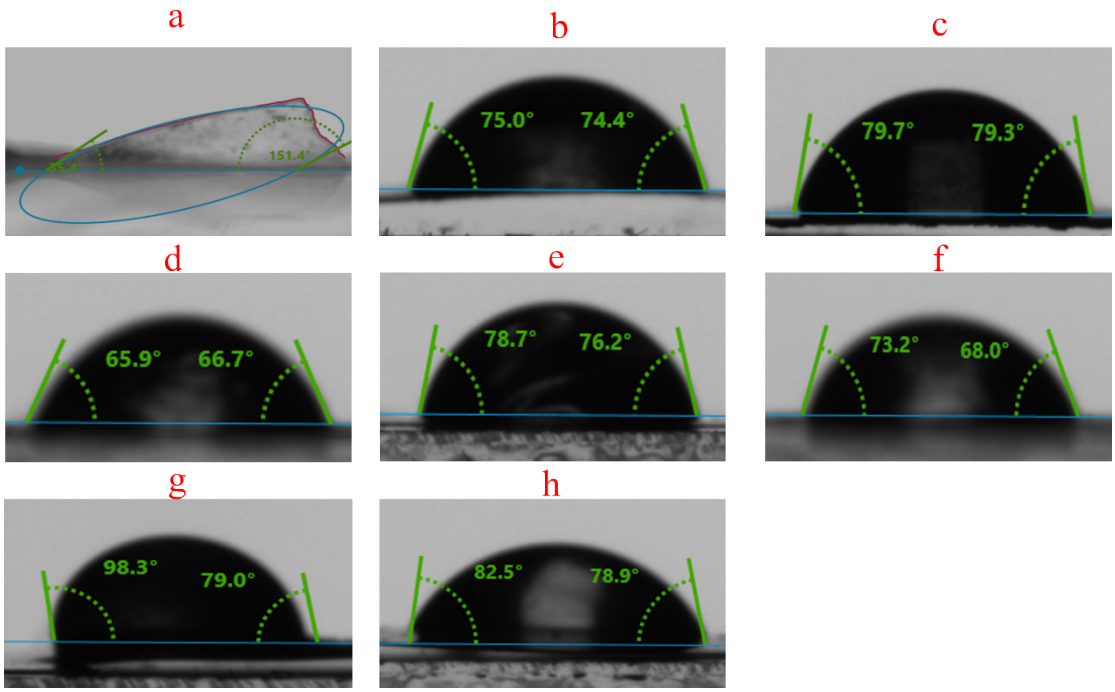

Figure S14 Water contact angle measurements of (a) CMCS. (b) **1**. (c) **2**. (d) **3**. (e) **4**. (f) **5**. (g) **6**. (h) **3'**.

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