Supporting Information for:

Disclosing a NMR-Invisible Fraction in Chitosan and PEGylated Copolymers and Its Role on the Determination of Degrees of Substitution

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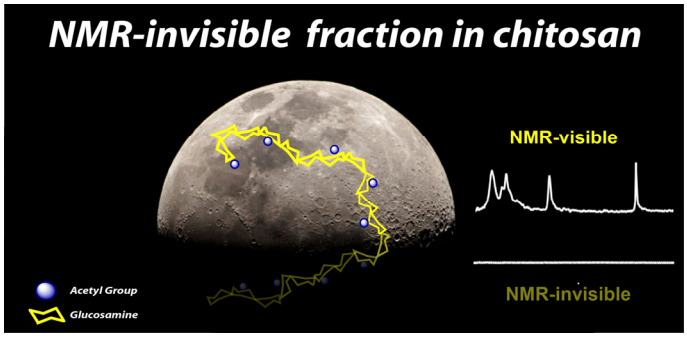

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1. PFG NMR experiments of CS.

Table S1. Diffusion coefficients (D) of CS in 350 mM CD_3CO_2D/135 mM NaOD.

C (g/L)	D (cm^2/s)·10^{-8}			
	CS_{1200-1}	$CS_{1200-70}$	CS_{360-1}	CS_{360-70}
0.2	20	22	53	56
1	6.1	8.7	22	31

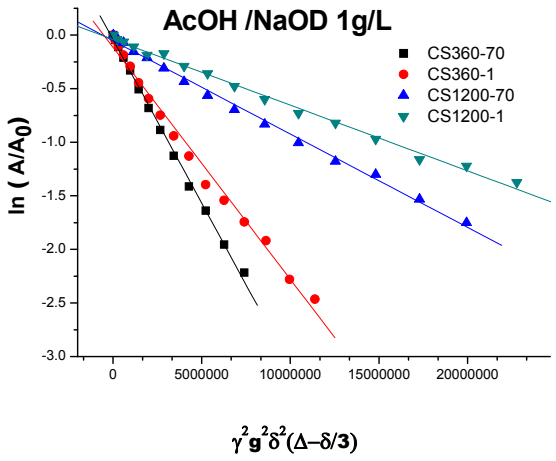

Figure S1. Stejskal-Tanner plots of CS as a function of DP and DA in 350 mM CD_3CO_2D/135 mM NaOD at 1 g/L (δ = 1 ms, Δ =1 s).

2. Preparation of CS-*g*-PEG.

Table S2. Conditions for the preparation of CS-*g*-PEG (EDC, NHS).

entry	PEG (mol %)	EDC (mol %)	NHS (mol %)	DS[a]	Coupling yield (%)
1	5.75	5.0	0	0.22	4
2	5.75	25.0	0	0.38	6
3	5.75	0	0	0.86	15
4	5.75	25	25	1.40	24
5	5.75	125	25	5.40	94
6	5.75	146	5.75	4.90	85
7	5.75	146	5.75	3.50	61[b]
9	2.90	73.0	2.90	2.20	76
10	1.17	29.5	1.15	0.71	61
11	0.57	14.6	0.57	0.29	50
12	0.68	30.0	3.50	0.55	80
13	1.20	71.5	6.00	1.10	92

[a] DS determined in 2% DCl after heating at 343 K for 8 h.
[b] pH 6.0

Table S3. Conditions for the preparation of CS-*g*-PEG (EDC, HOBt, pH 4.5).

entry	PEG (mol %)	EDC (mol %)	HOBt (mol %)	DS[a]	Coupling yield (%)
1	10	100	100	1.4	15
2	10	150	50	4.0	40
3	10	250	50	8.5	85
4	0.6	15	3	0.45	73
5	3	75	15	2.8	93
6	15	375	75	11.5	77
7	20	500	100	14	70
8	40	1000	200	23	60
9	80	2000	400	33	40

[a] DS determined in 2% DCl after heating at 343 K for 8 h.

3. ^{1}H NMR characterization of CS-*g*-PEG and experimental procedures for the determination of DS.

Table S4. ^{1}H T_1 (s) of CS-*g*-PEG (10 g/L, 500 MHz) in different solvents and temperatures.

Solvent	DS	Temp (K)	H2 GlcN	NAc (AcOH)	H4 AH-Man[a]
D_2O	0.5	278	1.77	1.92	-
		298	1.42	1.68	-
		323	1.37	1.70	-
		343	1.40	1.66	-
	2.8	278	1.78	1.83	-
		298	1.30	1.21	-
	4.2	298	Over.[b]	1.51	-
	14	298	Over.	1.49	-
2% DCl[c]	5	298	1.71	1.31 (2.6)	-
20% DCl[d]	5	343	2.68	(11.3)	-
0.2% DCl + $NaNO_2$[e]	0.5	298	1.82	1.57	1.46

[a] AH-Man (Anhydro-Man) = terminal anhydromannose.
[b] Over. = Overlapped.
[c] NMR after depolymerization by heating at 343 K for 8 h.
[d] NMR after depolymerization by heating at 343 K for 4 h.
[e] NMR after depolymerization with $NaNO_2$ for 5 min at 343 K.

Figure 6c illustrates an increase in the apparent DS from 3.5 to a value close to 28 in the pD range 1.2-10.7 (sharper variation at pD ~ 7) in agreement with deprotonation of the amino groups and concomitant reduced solubility of CS (reduced T_2). As for the effect of C, it was surprising to see an increase of apparent DS upon dilution (from 10 to 0.5 g/L, Figure 6d), which probably results from an increased rigidity of the CS backbone (reduction of ^{1}H T_2 for H2 GlcN from 17 to 13 ms) in agreement with previous viscosity results by Dong and coworkers.[1]

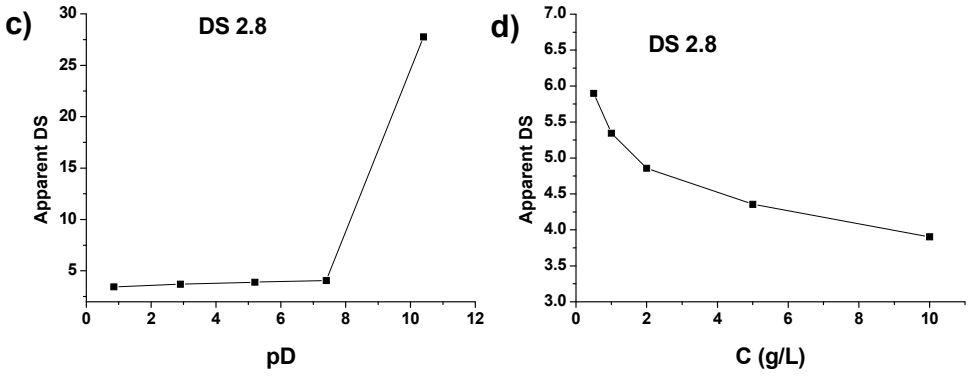

Figure 6cd. Apparent DS for CS-*g*-PEG·HCl (DS 2.8, 10 g/L in D_2O, 400 MHz) determined as a function of the pD and concentration. Lines are guides for the eye.

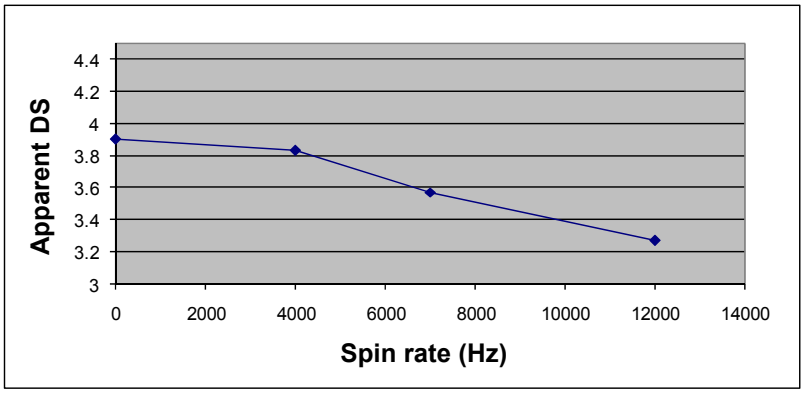

Figure S2. Apparent DS for CS-*g*-PEG·HCl (DS 2.8) obtained by HR-MAS (D_2O, 100 µL rotor, 500 MHz) using 10 s of recovery delay and spin rates 4000, 7000, and 12000 Hz.

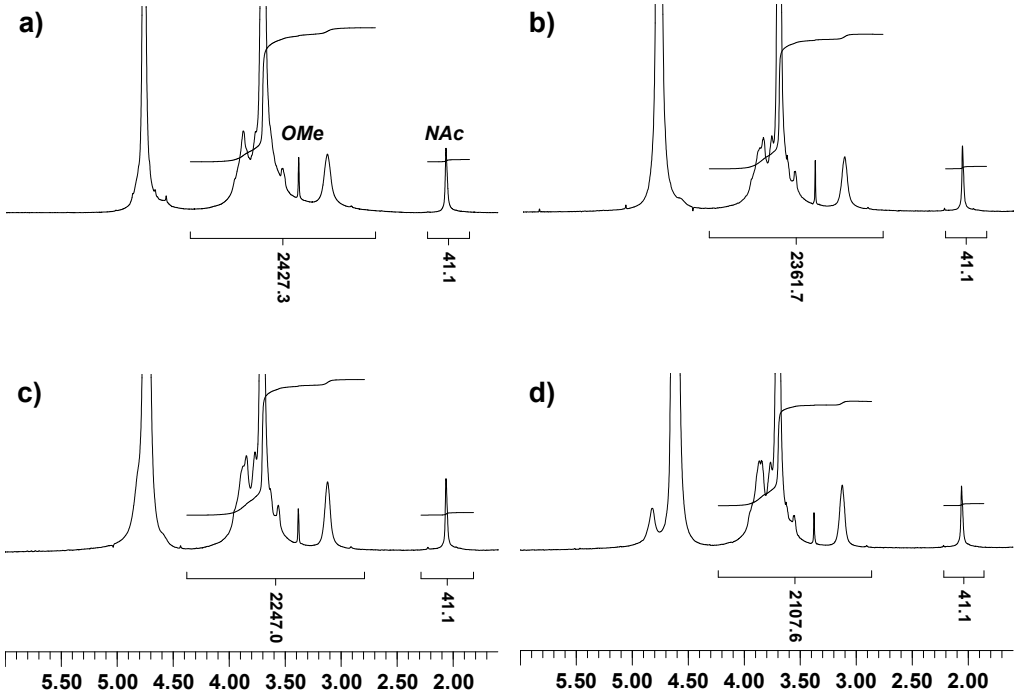

Figure S3. (a) ^{1}H NMR spectrum of CS-*g*-PEG·HCl (DA 14, DS 2.8) and HR-MAS spectra (100 mL rotor) recorded at spin rates (b) 4000, (c) 7000, and (d) 12000 Hz (10 s recovery delay, D_2O, 500 MHz). Clearly, as the spin rate increases, the intensity of the OMe group (3.4 ppm, PEG signal) decreases relative to the acetyl group.

Table S5. Apparent PEG mol % (relative to CS monomers) of CS/PEG mixtures determined by ^{1}H NMR under different experimental conditions (10 g/L, 400 MHz).

PEG mol %	D_2O (298 K)	2% DCl (298 K), depolymerization 1 h, 343 K	2% DCl (298 K), depolymerization 8 h, 343 K	2% DCl (298 K), depolymerization 16 h, 343 K	20% DCl (343 K), depolymerization 4 h, 343 K
1.0	1.0	1.0	1.0	1.0	1.0
3.6	4.7	3.6	3.6	3.6	3.6
5.0	7.4	5.4	5.1	5.0	5.1
10	16	12	10	11	10
20	35	25	20	21	20

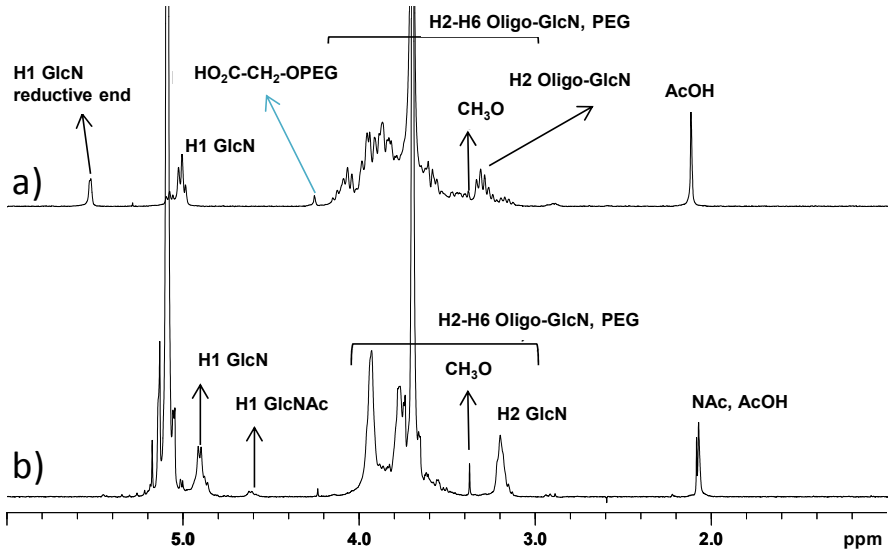

Figure S4. [1]H NMR spectra of CS-*g*-PEG (DS 2.8, 10 g/L, 400 MHz) in 20% DCl (343 K) after 4 h of heating at 343 K (**a**) and in 2% DCl (298 K) after 8 h of heating at 343 K (**b**).

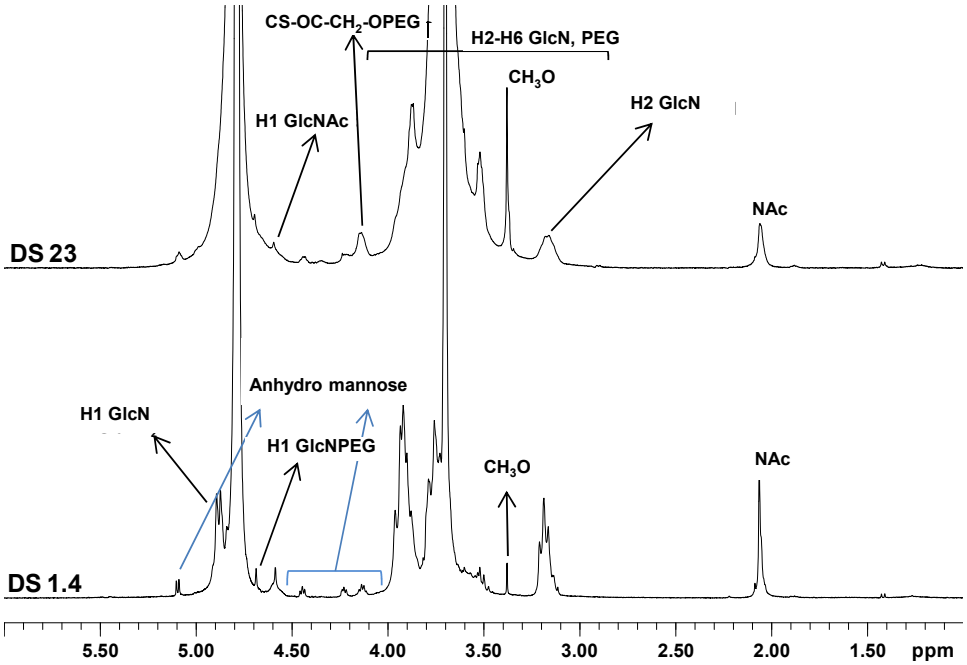

Figure S5. [1]H NMR spectra of CS-*g*-PEG DS 23 and DS 1.4 in 0.2% DCl (298 K) after heating for 5 min at 343 K in the presence of 0.05M $NaNO_2$ (400 MHz).

^{1}H NMR in 2% DCl. CS-*g*-PEG was suspended in 2% DCl (10 g/L) and heated at 343 K (variable times) before acquisition of the ^{1}H NMR spectra. ^{1}H NMR spectra were acquired at 298 K with a relaxation delay of 13 s. Detailed description of CS-*g*-PEG with DS 2.8 is included as a representative example. CS-*g*-PEG DS 2.8: ^{1}H NMR (400 MHz) δ: 2.08 (s, 18.4H, CH_3 GlcNAc), 2.09 (s, 22.7H CH_3 AcOH), 3.10-3.25 (m, 80H, H2 GlcN), 3.25-4.10 (m, 1805H, CH_3O, H2-H6 CS), 4.10-4.19 (m, CS-OC-C*H*$_2$OPEG), 4.24 (s, HO_2C-C*H*$_2$OPEG), 4.55-4.72 (m, H1 GlcN, H1 GlcNPEG), 4.83-4.95 (m, H1 GlcNAc) 5.43-5.50 (m, 3.4H, H1 reductive end GlcN).

^{1}H NMR in 20% DCl. CS-*g*-PEG was suspended in 20% DCl (10 g/L) and heated at 343 K for 4 h before acquisition of the ^{1}H NMR spectra. ^{1}H NMR spectra were acquired at 343 K with a relaxation delay of 60 s. Detailed description of CS-*g*-PEG with DS 5 is included as a representative example. CS-*g*-PEG DS 5: ^{1}H NMR (400 MHz) δ: 2.13 (s, 41.1H, CH_3 AcOH), 2.80-4.20 (m, 3005H, H2-H6 CS), 4.24 (s, 9.7H, HO_2C-C*H*$_2$OPEG), 4.98-5.15 (m, 58.1H, H1, GlcN), 5.43-5.50 (m, 39.9H, H1 reductive end GlcN).

^{1}H NMR in 0.2% DCl supplemented with $NaNO_2$. CS-*g*-PEG was suspended in 0.2% DCl (10 g/L) and heated at 343 K for 10 min. Afterwards, $NaNO_2$ (20 μL, 0.05 M in D_2O) was added and the sample was further heated at 343 K for 5 min. ^{1}H NMR spectra were acquired at 298 K with a relaxation delay of 13 s. Because of signal overlapping with the HOD signal, integral values of the anomeric protons are omitted.

CS-g-PEG DS 0.45: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.18 (t, *J* = 9.08 Hz, 82H, H2 GlcN), 3.25-4.10 (m, 807H, CH_3O, H2-H6 CS), 4.13 (dd, *J* = 9.85, 5.09 Hz, 3H, H5 AH-Man), 4.20-4.26 (m, 3H, H4 AH-Man), 4.41-4.48 (m, 3.4H, H3 AH-Man), 4.58 (br s, H1 GlcN), 4.68 (br s, H1 GlcNPEG), 4.83 (br s, H1 GlcNAc), 5.09 (d, *J* = 5.42 Hz, 3.4H, H1 AH-Man).

CS-g-PEG DS 1.4: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.18 (t, *J* = 9.08 Hz, 80H, H2 GlcN), 3.25-4.10 (m, 1176H, CH_3O, H2-H6 CS), 4.13 (dd, *J* = 9.78, 4.80 Hz, 6.1H, H5 AH-Man), 4.20-4.26 (m, 4.4H, H4 AH-Man), 4.41-4.48 (m, 4.4H, H3 AH-Man), 4.58 (br s, H1 GlcN), 4.68 (br s, H1 GlcNPEG), 4.83 (br s, H1 GlcNAc), 5.09 (d, *J* = 5.42 Hz, 4.5H, H1 AH-Man).

CS-g-PEG DS 2.8: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.18 (t, *J* = 9.08 Hz, 80H, H2 GlcN), 3.25-4.10 (m, 1832H, CH_3O, H2-H6 CS), 4.09-4.18 (m, 5.5H, H5 AH-Man), 4.20-

4.26 (m, 4.6H, H4 AH-Man, HO_2C-CH_2OPEG), 4.41-4.48 (m, 4.3H, H3 AH-Man), 4.58 (br s, H1 GlcN), 4.68 (br s, H1 GlcNPEG), 4.83 (br s, H1 GlcNAc), 5.09 (d, *J* = 5.42 Hz, 4.5H, H1 AH-Man).

CS-g-PEG DS 4.2: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.18 (t, *J* = 9.08 Hz, 79H, H2 GlcN), 3.25-4.10 (m, 2476H, CH_3O, H2-H6 CS) , 4.09-4.18 (m, 5.9H, H5 AH-Man), 4.20-4.26 (m, 4.5H, H4 AH-Man, HO_2C-CH_2OPEG), 4.41-4.48 (m, 3.5H, H3 AH-Man), 4.58 (br s, H1 GlcN), 4.68 (br s, H1 GlcNPEG), 4.83 (br s, H1 GlcNAc), 5.09 (d, *J* = 5.42 Hz, 3.5H, H1 AH-Man).

CS-g-PEG DS 8.5: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.18 (t, *J* = 9.08 Hz, 74H, H2 GlcN), 3.25-4.10 (m, 4478H, CH_3O, H2-H6 CS), 4.09-4.18 (m,7H, H5 AH-Man, CS-OC-CH_2OPEG), 4.20-4.26 (m, 2.7H, H4 AH-Man, HO_2C-CH_2OPEG), 4.45 (br s, 2.3H, H3 AH-Man), 4.58 (br s, H1 GlcN), 4.68 (br s, H1 GlcNPEG), 4.83 (br s, H1 GlcNAc), 5.09 (d, *J* = 5.42 Hz, 3.5H, H1 AH-Man).

CS-g-PEG DS 14: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.13-3.25 (m, 62H, H2 GlcN), 3.25-4.10 (m, 7070H, CH_3O, H2-H6 CS), 4.09-4.18 (m,13H, H5 AH-Man, CS-OC-CH_2OPEG), 4.20-4.26 (m, 5.8H, H4 AH-Man, HO_2C-CH_2OPEG), 4.41-4.48 (m, 3.6H, H3 AH-Man), 4.58 (br s, H1 GlcN), 4.68 (br s, H1 GlcNPEG), 4.83 (br s, H1 GlcNAc), 5.09 (d, *J* = 5.42 Hz, 4H, H1 AH-Man).

CS-g-PEG DS 23: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.13-3.25 (m, 46H, H2 GlcN), 3.25-4.10 (m, 10941H, CH_3O, H2-H6 CS), 4.09-4.18 (m, 23.8H, H5 AH-Man, CS-OC-CH_2OPEG), 4.20-4.26 (m, 3.9H, H4 AH-Man, HO_2C-CH_2OPEG), 4.41-4.48 (m, 3.9H, H3 AH-Man), 4.58 (br s, H1 GlcN), 4.68 (br s, H1 GlcNPEG), 4.83 (br s, H1 GlcNAc), 5.09 (d, *J* = 5.30 Hz, H1 AH-Man).

CS-g-PEG DS 32: ^{1}H NMR (400 MHz) δ: 2.06 (s, 41.1H, CH_3 GlcNAc), 3.13-3.25 (m, 39H, H2 GlcN), 3.25-4.10 (m, 15355H, CH_3O, H2-H6 CS), 4.09-4.18 (m, 14.2H, H5 AH-Man, CS-OC-CH_2OPEG), 4.20-4.26 (m, 14.3H, H4 AH-Man, HO_2C-CH_2OPEG), 4.41-4.48 (m, 3.4H, H3 AH-Man), 4.58 (s, H1 GlcN), 4.68 (s, H1 GlcNPEG), 4.83 (s, H1 GlcNAc), 5.09 (d, *J* = 5.30 Hz, H1 AH-Man).

4. Reference.

[1] Deng, L.; Qi, H.; Yao, C.; Feng, M.; Dong, A. *J. Biomater. Sci. Polymer Edn.* **2007**, *18*, 1575.