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

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ABSTRACT: An unexpected ^{1}H NMR invisible fraction (IF) for chitosan (CS) and CS-*g*-PEG is reported. The presence of this IF is remarkable considering that solution NMR is recognized as the method of choice for studying structural modifications in CS, including the degrees of acetylation (DA) and substitution (DS). In spite of IF figures as high as 50%, this IF does not interfere in the correct determination of the DA by ^{1}H NMR, pointing to a homogeneous distribution of acetyl groups along the visible and invisible fractions. Quite in contrast, the IF negatively biases the determination of the DS in CS-*g*-PEG, with relative errors as high as 150% in a broad range of temperatures, pH values, and concentrations. This fact raises concerns about the accuracy of previously reported DS data for CS-*g*-PEG and many other CS copolymers. Efficient user-friendly conditions have been developed for the correct determination of the DS of CS-*g*-PEG by depolymerization by nitrous acid.

Keywords: NMR-invisible fraction, chitosan, PEG, degree of substitution, transverse relaxation time

Introduction

Chitosan (CS, Figure 1) is the major cationic polysaccharide in nature. It is composed by a variable proportion of glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) linked through β(1-4) glycosidic bonds. CS is considered as a renewable, inexpensive, and biocompatible polycation with applications in drug delivery, food industry, and water purification.[1,2,3] The copolymerization of CS at the amino/hydroxyl groups has also rendered materials of interest in drug delivery, metal absorption, cell culture, or antibacterial membranes and films.[4] CS-*graft*-PEG (CS-*g*-PEG, Figure 1) constitutes the most relevant CS copolymer for biomedical purposes, with numerous reports for the delivery of nucleic acids, proteins and peptides.[5,6] As the majority of the usages of CS relate to its tendency to aggregate in solution, much effort has been devoted to analyze its aggregation by different techniques.[7,8,9,10,11] Recently, we have performed a study by pyrene fluorescence and rheology as a function of the degrees of acetylation (DA) and polymerization (DP) showing that the viscosity of CS solutions increases with DP and decreases with DA.[12]

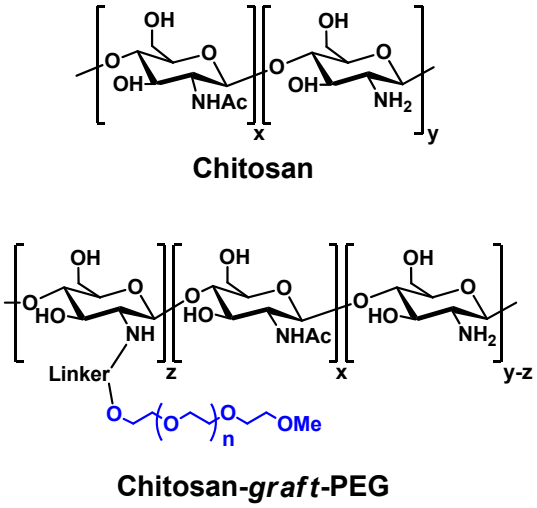

Figure 1. Chemical structure of chitosan (CS) and CS-*g*-PEG.

Nuclear magnetic resonance (NMR) is a powerful tool to analyze the dynamics and aggregation of polymers in solution. In a recent contribution, we have reported a ^{1}H NMR relaxation study on the dynamics of CS that revealed its semirigid character [transverse relaxation times (T_2) *ca.* 20 ms] and an increased flexibility of the chain at higher DA (reduced electrostatic repulsion between protonated amino groups).[13] Herein we report that pulsed field gradient (PFG) NMR experiments performed at concentrations (C) higher than the overlap concentration (C*) afford inconsistent diffusion behaviors as a function of DA, which point to a ^{1}H NMR-invisible fraction (IF) for CS in the semidilute regime. Confirmation of this IF for CS and CS-*g*-PEG was obtained by quantitative NMR experiments using depolymerized samples. A detailed study on the extent of the IF and its dependence on structural (DA, DP) and experimental parameters (C, temperature, pH, magnetic field) is also included. In addition, the influence of the IF on the determination of the DA of CS and the degree of substitution (DS) of CS-*g*-PEG by ^{1}H NMR is addressed. The lack of previous reports on the NMR IF of CS is remarkable considering that solution NMR is recognized as the method of choice for studying structural modifications in CS, and for the determination of its DA[13,14,15,16] and DS of its copolymers.[17,18,19,20,21,22,23,24]

Experimental Section

Materials. Four commercial CS samples with different DP and DA were purchased and denoted as $C_{x\text{-}y}$, where x represents the DP, and y the DA. Samples $CS_{360\text{-}7}$ and $CS_{480\text{-}14}$ were obtained from FMC BioPolymer as hydrochloride salt: Protasan Cl 110 (batch number 310-490-01) and Protasan Cl 113 (batch number FP-110-02), respectively. Samples $CS_{730\text{-}17}$ and $CS_{1200\text{-}20}$ were obtained from Fluka: CS low MW (catalog number 22741, lot 407568/1) and CS high MW (catalog number 22743, lot 371936/1), respectively. These

commercial samples were purified, and then acetylated/deacetylated as previously described.[13] MeO-PEG-OCH_2CO_2H was synthesised from a commercially available MeO-PEG-OH (M_n 5055, M_w 5088, by MALDI-TOF) according to known procedures.[25] All other reagents were obtained from Sigma-Aldrich.

General procedure for the synthesis of CS-*g*-PEG. $CS_{480\text{-}14}$, *N*-hydroxysuccinimide (NHS) or 1-hydroxybenzotriazole (HOBt), and MeO-PEG-OCH_2CO_2H were dissolved in H_2O. Then, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) was added in four portions every 30 min. The resulting solution was stirred at rt for 22 h, and then ultrafiltered (YM30) with 10^{-3} M HCl and H_2O, and lyophilized to afford CS-*g*-PEG hydrochloride salt (CS-*g*-PEG·HCl) as a white foam. Details on the relative amounts of reactants are included in the Supporting Information (SI).

NMR spectroscopy. NMR experiments were acquired on Bruker DRX-500, AMX 500, DPX-250, and Varian Inova 400 and 750 spectrometers. Chemical shifts are reported in ppm (δ) and referred to internal sodium 3-trimethylsilylpropane sulfonate. Mnova software (Mestrelab Research) was used for spectral processing and OriginPro 7.5 software (Originlab) to perform exponential/linear fittings on relaxation times and PFG experiments.

^{1}H PFG NMR experiments were acquired on a 750 MHz spectrometer (Varian INOVA) equipped with a triple gradient shielded probe. The gradient was varied linearly along 16 experiments from 2 to 65 G·cm^{-1} to obtain the diffusion dimension. The pulse sequence used includes the bipolar-gradient pulse pairs and stimulated echo experiment with an additional delay time for longitudinal eddy current compensation (LED-BPPSTE).[26] The diffusion time (Δ) was set at 1 s for solutions of CS at 0.2 and 1 g/L, and at 3 s for 8 g/L. The duration of the gradients (δ) in the sequence was 1 ms for 0.2 and 1 g/L, and 2 ms for 8

g/L, which was followed by a stabilization delay period of 0.2 ms. The recovery delay was set at 13 s.

Measurement of T_1 and T_2. Longitudinal relaxation times (T_1) were determined using a standard inversion-recovery pulse sequence. The recovery delay (τ) between the inversion pulse and the read pulse was varied along 16 values. Transverse relaxation times (T_2) were determined using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence using 16 values of τ, with a delay between π pulses of 140 μs. A recovery delay between scans higher than 5 times the highest T_1 in the sample was set for all ^{1}H NMR experiments.

Quantification of the ^{1}H NMR IF of CS. The combined integral of the H2-H6 protons of GlcN and GlcNAc was compared to an external reference (maleic acid, 10 g/L) placed in a coaxial capillary inside the NMR tube. Spectra were acquired with a recovery time 5 times longer than the highest T_1 in the sample.

Determination of the DS in CS-*g*-PEG by ^{1}H NMR. ^{1}H NMR spectra of CS-*g*-PEG were acquired at 10 g/L in: D_2O, 2% and 20% DCl in D_2O, and 0.2% DCl supplemented with $NaNO_2$ as depolymerizing agent.[27] DS were determined by integration of the singlet at 2.06 ppm (corresponding to the acetyl/AcOH signal) and the multiplet between ~3.1-4.3 ppm (corresponding to the PEG and H2-H6 signals of CS) according to equation [1]. All experiments fulfilled the condition of a recovery delay between scans higher than 5 times the longest T_1 in the sample (delay 13 s for experiments at 298 K and 60 s for 343 K). NMR and depolymerization conditions, as well as relaxation times for CS-*g*-PEG are included in the SI.

$$DS_{PEG} = \frac{I_{H2-H6,HPEG} - \frac{6I_{CH_3}}{DA\times 3}\times 100}{HPEG\frac{I_{CH_3}}{DA\times 3}\times 100}\times 100 \qquad [1]$$

Results and Discussion

A NMR invisible fraction of CS in solution. Pulsed field gradient (PFG) is a common NMR technique to study the diffusion of polymers,[28,29] which in addition to single chains is able to detect the diffusion of slower modes caused by aggregation.[30,31,32] With the aim of studying the aggregation of CS, PFG experiments were recorded on four samples ($CS_{360\text{-}1}$, $CS_{360\text{-}70}$, $CS_{1200\text{-}1}$, $CS_{1200\text{-}70}$) selected on the basis of their significantly different aggregation abilities.[12] Measurements were carried out at different C (0.2-8.0 g/L) in acetate buffer (pD 4.5, 350 mM CD_3CO_2D/135 mM NaOD in D_2O). Stejskal-Tanner plots for the four samples at 0.2 g/L, below the overlap concentration (C* *ca.* 1 g/L depending on DA and DP),[10] revealed linear diffusive components associated to the polymer chain self-diffusion (Figure 2). As expected, larger diffusion coefficients (D) were observed for the smaller DP 360 samples. Also, slightly increased D were seen for the DA 70 samples because of their relatively higher flexibility and smaller dimension (Table S1).[13,33] Upon increasing C to 1.0 g/L, single diffusive components were still observed for all samples, but important reductions of D revealed a transition between the dilute and semi-dilute regimes (Figure S1 and Table S1). At even higher C values, Stejskal-Tanner plots showed a clear deviation from linearity, consistent with the presence of additional diffusive processes caused by aggregation (acetate buffer and 0.056 M TFA-*d*, Figure 2).[8,9] More importantly, the expected reduction of D on lowering DA (because of increased viscosity)[12] was not observed,[32,34] pointing to a ^{1}H NMR IF for CS in the semidilute regime. Indeed, the observation of ^{1}H signals in high resolution NMR spectroscopy entails nuclei with T_2 over the 1-10 ms range. If this condition were not fulfilled, the inverse proportionality between T_2 and the half-height width of the peaks will result in signals so broad after Fourier transform that remain embedded in the base line of the spectra and hence, invisible for

detection.[35] This is for example the case of liquid crystalline systems,[36] cross-linked polymer gels,[37] macromolecular aggregates,[38] or fairly rigid polysaccharides with ordered conformations like, pectin,[39] hyaluronic acid,[40] and carrageenan.[35] In these examples, the existence of an IF renders the direct analysis of NMR spectra unsuitable for the determination of relevant NMR data such as, diffusion coefficients, relative integrals, or relaxation times. In the case of CS, we hypothesize the existence of an IF related to nuclei with ^{1}H T_2 very low due to aggregation.

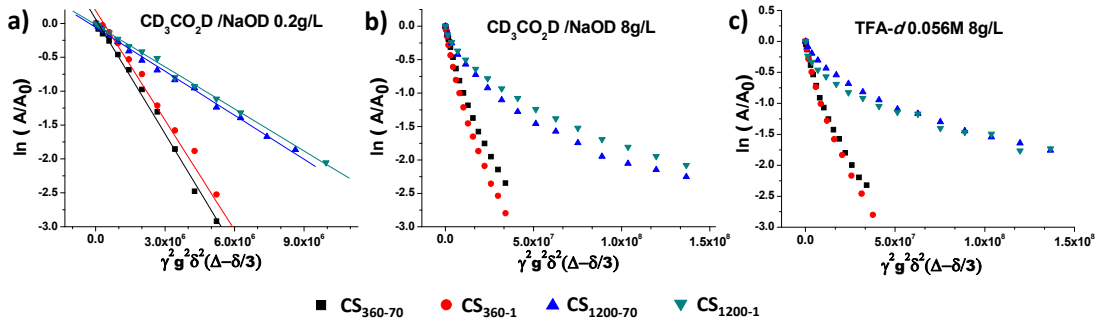

Figure 2. Stejskal-Tanner plots (750 MHz) for CS in pD 4.5 acetate buffer (0.2 and 8.0 g/L) and in 0.056 M TFA-*d* (8 g/L). The diffusion time (Δ) was set at 1 s for 0.2 g/L, and 3 s for 8 g/L. The duration of the gradients (δ) was 1 ms for 0.2 g/L, and 2 ms for 8 g/L.

To confirm the existence of an IF for CS, we analyzed the effect produced by depolymerization on the ^{1}H integrals of the polymer. The objective was to obtain oligomers with lower aggregation ability[12] and longer T_2[13] (reduced probability to participate in an IF). With this aim, sequential additions of $NaNO_2$ (cleavage of glycosidic bonds)[27] were done to solutions of CS in NMR tubes ($CS_{360\text{-}1}$, $CS_{360\text{-}70}$, $CS_{1200\text{-}1}$, $CS_{1200\text{-}70}$; 0.2-8.0 g/L in 0.056 M TFA-*d*; 500 MHz), till the integral of the H2-H6 multiplet relative to an external reference (maleic acid) showed no variation. The IF was then calculated as the percentage of integral increase compared to the original spectra (Figures 3 and 4). The presence of a

NMR IF was revealed for all CS samples at C ≥ 1 g/L, which points to aggregation and a concomitant reduction of ^{1}H T_2,[13] at the origin of this IF. Confirmation of this point came also from the increased percentage of IF for the most aggregating samples of higher DP and lower DA values.[12]

Taking into account the known dependence of T_2 on the magnetic field,[41,42] it was of interest to check the variation of the percentage of NMR IF for CS (various DA and DP, 8.0 g/L) at different fields. As seen in Figure 4, on going from 250 to 500 MHz, an absolute 15-25% decrease of IF was revealed for all samples. This result agrees with an expected increase of T_2 with the field and a concomitant lower proportion of nuclei with ^{1}H T_2 so low to remain in the base line of the spectra at 500 MHz. It is important to stress at this point that the percentage of NMR IF must not be regarded as percentage of aggregated chains. Thus, the IF does not mirror a two-component model (long T_2 "visible", short T_2 "invisible"), but rather a continuous distribution of relaxation times,[35] which depending on sample structural parameters (DA, DP) and experimental conditions (C, magnetic field), adopts a certain visible/invisible ratio.

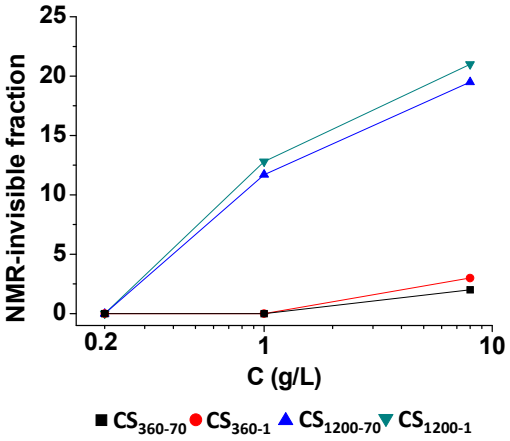

Figure 3. NMR-invisible fraction (IF) of CS as a function of the concentration (pD 4.5 acetate buffer, 500 MHz). Lines are guides for the eye.

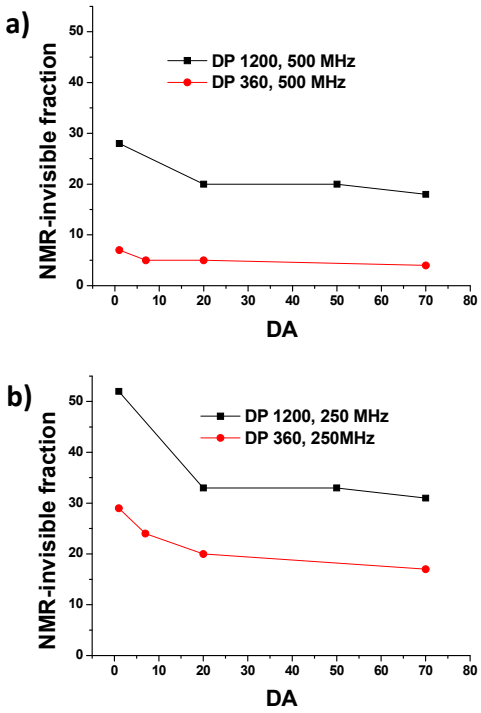

Figure 4. NMR IF of CS (8 g/L) as a function of DP and DA in 0.056 M TFA-*d* (D_2O) at 500 MHz (**a**) and 250 MHz (**b**). Lines are guides for the eye.

Influence of the NMR invisible fraction of CS in the determination of the degree of acetylation. Since solution ^{1}H NMR is the method of choice for determining the DA of CS, we decided to check any potential undesired bias of the IF in the process. With this aim, the DA of a series of 14 CS samples with varying DA (1-70) and DP (360, 730, 1200) was determined (250 and 500 MHz) following standard NMR conditions for this goal: i) 2% DCl at 298 K (Fernandez-Megia/Riguera),[14] ii) 20% DCl at 343 K (Shigemasa),[16] iii) 0.056 M TFA-*d* at 298 K (Fernandez-Megia/Riguera; the only conditions tested where no depolymerization takes place),[13] and iv) 0.056 M TFA-*d* supplemented with $NaNO_2$ at 298 K.[27] In addition, DA values have been determined by UV spectrophotometry.[43] As seen in

Table 1, only small differences in DA (within the experimental error) were observed among the various conditions, regardless of their depolymerizing strength and magnetic field. This result clearly points to the existence of a homogeneous distribution of acetyl groups along the visible and invisible fractions not influencing the determination of the DA by ^{1}H NMR. As the DA determined by analysis of the visible fraction represents the DA of the whole sample, DA data reported in the literature must be regarded as correct.

Table 1. DA of CS determined by ^{1}H NMR under different experimental conditions (8 g/L) and UV spectrophotometry.

	UV (0.1M HCl)	**2% DCl (298 K), depolymerization 1 h, 343 K**	**20% DCl (343 K), depolymerization 4 h, 343 K**	**0.056 M TFA-*d* (298 K)**		**0.056 M TFA-*d* (298 K) + $NaNO_2$**	
Sample		**500 MHz**	**500 MHz**	**250**	**500**	**250**	**500**
CS_{360-1}	0	0.7	-	1.0	0.9	0.8	0.8
CS_{360-7}	6	7.0	-	6.8	6.5	7.2	7.1
CS_{360-20}	-	22	-	22	22	22	22
CS_{360-70}	72	-	69	70	68	69	68
CS_{730-0}	0	0	-	0	0	-	-
CS_{730-6}	6	6.2	-	5.7	6.1	-	-
CS_{730-17}	16	17	-	19	19	20	21
CS_{730-27}	-	27	-	27	28	-	-
CS_{730-39}	40	39	39	39	39	-	-
CS_{730-65}	66	-	65	66	65	-	-
CS_{1200-1}	-	0.9	-	1.0	0.8	1.0	-
$CS_{1200-20}$	18	20	-	21	18	21	20
$CS_{1200-50}$	-	-	48	49	49	49	50
$CS_{1200-70}$	67	-	69	70	70	69	69

A NMR invisible fraction in CS copolymers: The case of CS-*g*-PEG. Considering the importance of CS copolymers in numerous applications and the relevance of a precise determination of their DS for the correct interpretation of structure-activity relationships,[4] we turned our attention to unveil an IF in the ^{1}H NMR spectra of CS-*g*-PEG, the most relevant CS copolymer within the biomedical field.[5,6] CS-*g*-PEG also represents an excellent test attending to the large differences in ^{1}H T_2 between the CS (*ca.* 20 ms, 8 g/L, 298 K, 500 MHz) and PEG chains (0.62 s, 5 g/L, 298 K, 500 MHz). To this end, a collection of CS-*g*-PEG samples with DS up to 32 (PEG weight fraction 91%) was prepared by carbodiimide mediated amide formation (EDC, NHS or HOBt) from $CS_{480\text{-}14}$ and MeO-PEG_{5000}-OCH_2CO_2H.[44] The relative proportions between CS, PEG, EDC, and NHS/HOBt were optimized in order to maximize the coupling yield. Improved conditions involved an excess of EDC (2500 mol %) and NHS/HOBt (500 mol %) relative to PEG at pH 4.5 (Tables S2 and S3). A representative ^{1}H NMR spectrum of CS-*g*-PEG is depicted in Figure 5.

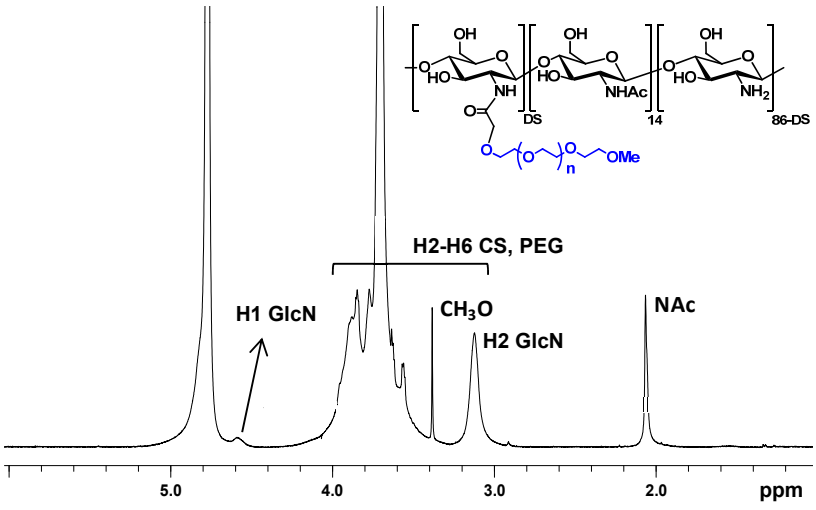

Figure 5. ^{1}H NMR spectrum of CS-*g*-PEG·HCl (DS 2.8) in D_2O (10 g/L, 400 MHz).

To ascertain the existence of a NMR IF in CS-*g*-PEG, quantitative ^{1}H NMR spectra of two samples (DS 0.45 and 2.8, *vide infra* for their correct DS determination) were recorded at various temperatures, pH, and C (^{1}H T_1 of CS-*g*-PEG included in Table S4). Apparent DS obtained by integration at 298 K revealed figures larger than the correct values in agreement with the presence of an IF strongly affecting the determination of the DS in CS-*g*-PEG (Figure 6). While increasing the temperature allowed to correctly determine the DS value for the DS 0.45 sample (faster dynamics of the CS chain, larger ^{1}H T_2), neither variations of the temperature nor pH or C, afforded the correct DS value for the more PEGylted sample (DS 2.8) (Figure 6 and commentary in the SI). Overestimated DS were also obtained by HR-MAS experiments even at spin rates at high as 12000 Hz (Figures S2 and S3).

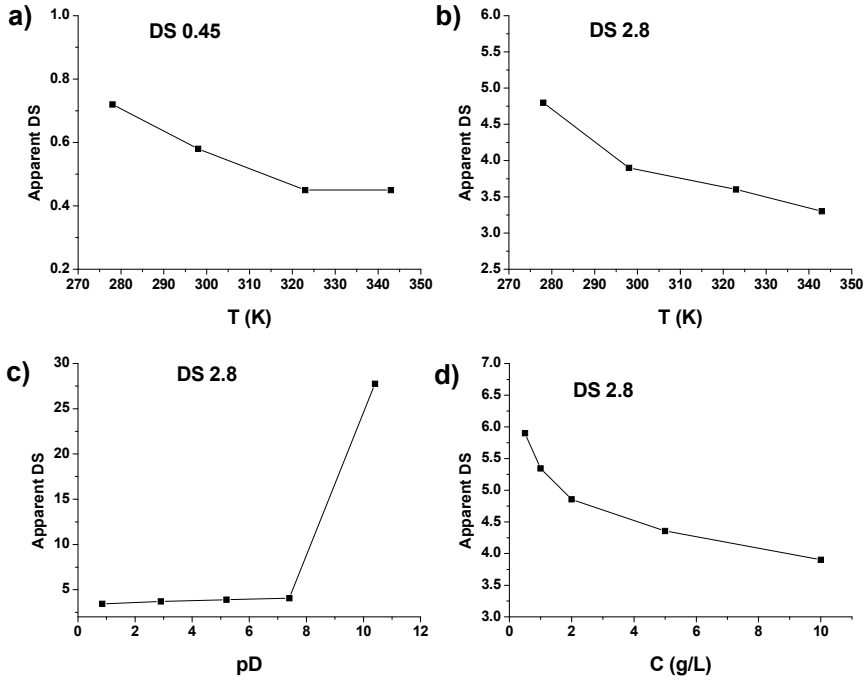

Figure 6. Apparent DS for CS-*g*-PEG·HCl (10 g/L in D_2O, 400 MHz) determined as a function of the temperature (**a** and **b**, for DS 0.45 and 2.8), pD and concentration (**c** and **d**, for DS 2.8). Lines are guides for the eye.

As previously done for CS, correct DS values for CS-*g*-PEG were pursued by integration of samples depolymerized under acidic conditions (cleavage of amide and glycosidic bonds). CS-*g*-PEG of varying DS were treated with 2 and 20% DCl and heated at 343 K until constant DS values were obtained: 8 h for 2% DCl, and 4 h for 20% DCl (Tables 2 and S4). Relative differences of 20-30% between the DS obtained in D_2O and the correct values were found for samples with DS 0-3, which increased up to 150% in the case of the more PEGylated copolymers. Confirmation of the resulting DS being correct was obtained by control depolymerization experiments on $CS_{480\text{-}14}$/MeO-PEG_{5000}-OCH_2CO_2H mixtures with different molar ratios (apparent PEG:CS ratios reached values coincident with the correct ones after depolymerisation periods similar to those for the copolymers, Table S5). The great differences between apparent and correct DS values for CS-*g*-PEG raise concerns about the accuracy of previously reported DS data for CS-*g*-PEG[17-20] and other CS copolymers (*i.e.* PEI,[21] PNIPAM-co-PAA,[22] PNVCL,[23] POEGMA[24]), obtained by integration of NMR spectra recorded after simply dissolving the samples in D_2O.

Table 2. Apparent DS of CS-*g*-PEG determined by ^{1}H NMR under different conditions (10 g/L, 400 MHz).

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 36 h, 343 K	20% DCl (343 K), depolymerization 4 h, 343 K	0.2% DCl (298 K) + $NaNO_2$
0.25	0.21	0.19	0.17	0.19	-
0.60	0.53	0.52	-	0.50	-
1.1	0.90	0.85	-	0.90	-
3.3	-	2.5	-	2.5	-
7.4	6.2	4.9	4.9	4.9	-
0.55	-	0.44	-	-	0.45
1.9	-	1.4	-	-	1.4
3.9	-	2.7	-	-	2.8
6.5	-	4.0	-	-	4.2
14	-	8.3	-	-	8.5
30	-	14	-	-	14
36	-	23	-	-	23
82	-	33	32	-	32

Optimal routine conditions for the determination of the DS in CS-*g*-PEG. Having confirmed the IF negatively influences the determination of the DS in CS-*g*-PEG, we have optimized the procedure for its correct determination in the presence of nitrous acid.[27] With this aim, $NaNO_2$ (20 μL, 0.05 M in D_2O) was added to the copolymer dissolved in 0.2% DCl (10 g/L) and heated at 343 K. A clear reduction of viscosity was evident after 2-5 min, leaving the sample ready for NMR acquisition (298 K, Figure S5 and SI). As seen in Table 2, DS determined by integration according to equation [1] were coincident with those

obtained after acidic depolymerization, while benefiting from a much shorter sample preparation. This user friendly procedure is envisaged with general application for CS copolymers other than CS-*g*-PEG.

Conclusions

Herein we demonstrate the presence of an unexpected ^{1}H NMR invisible fraction (IF) for CS in the semidilute regime, which increases with the concentration and the aggregation ability of the sample (high DP, low DA), and reduces with the magnetic field. The presence of IF as high as 50% has been unveiled which is remarkable considering that solution NMR is recognized as the method of choice for determining structural modifications in CS, including the DA and DS of copolymers. In spite of these large figures, the IF does not interfere in the correct determination of the DA by ^{1}H NMR, which points to a homogeneous distribution of acetyl groups along the visible and invisible fractions. Quite in contrast, the IF negatively biases the determination of the DS in CS-*g*-PEG, with relative errors as high as 150% in a broad range of temperatures, pH values, and concentrations. The great differences between apparent and correct DS values raise concerns about the accuracy of previously reported DS data for CS-*g*-PEG and other CS copolymers. We are currently studying the presence of unexpected IF in many other natural and synthetic polymeric systems.

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Supporting Information Available. Stejskal-Tanner plots and diffusion coefficients of CS, details on the synthesis of CS-*g*-PEG, and ^{1}H NMR characterization and determination of the DS of CS-*g*-PEG. This material is available free of charge via the Internet at http://pubs.acs.org.

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