Study of the acetylation pattern of Chitosan by pure shift NMR

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SUPPORTING DATA

EXPERIMENTAL SECTION

Reagents

Commercial chitin (C9213, batch #061M0110V), obtained from shrimp shells (*Pandalus borealis*) from Iceland, was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Deuterium oxide (99.98%), DCl (30%), and NaOH pellets were obtained from Merck KGaA (Darmstadt, Germany). The water used was MilliQ purified (Millipore, Billerica, MA, USA). All reagents used were of analytical grade.

Sample preparation:

Chitosan samples (Chi1 – Chi20) with different degrees of acetylation (F_A from 0.001 to 0.419) were prepared by alkaline deacetylation of chitin as follow:

Chi-1 – Chi-11: 3 g of chitin were suspended in 90 mL 50% (w/w) NaOH solution and heated to 90°C with stirring at 300 rpm. A portion of the reaction mixture was removed for analysis after 9 min (Chi-1), 10 min (Chi-2), 15 min (Chi-3), 20 min (Chi-4), 25 min (Chi-5), 30 min (Chi-6), 35 min (Chi-7), 40 min (Chi-8), 60 min (Chi-9), 150 min (Chi-10), and 300 min (Chi-11). Chi-12 sample was prepared under the same conditions, removed after 9 min.

Chi-13 – Chi-20: To 3 g of chitin, placed in a round bottom flask and left under vacuum for 24 h, 90 mL of a 50 % NaOH aqueous solution were added. The mixture was then heated to 120°C with stirring at 300 rpm under nitrogen atmosphere. A portion of the reaction mixture was removed for analysis after 7 min (Chi-13), 25 min (Chi-14), 35 min (Chi-15), 40 min (Chi-16), 55 min (Chi-17), 100 min (Chi-18), 145 min (Chi-19), and 175 min (Chi-20).

The material removed in all cases was immediately washed with MilliQ water to pH 7, dried at 50°C for 3 days, and kept in a closed low humidity chamber (11% RH) until analysis. The NMR samples were prepared by re-dissolving 10 mg of the recovered dried solid in 0.82 mL of a 0.3% (v/v) DCl/D_2O.

NMR acquisition parameters

^{1}H-SAPPHIRE-PSYCHE experiments were acquired in a pseudo 3D manner with 16 transients, 2 K complex data points, 5 KHz spectral width, 8 SAPPHIRE interferogram in F2, and 32 Pure Shift interferogram with 39.063 Hz spectral width in F1. Total experimental time, 4 h 55 min.

^{1}H-^{1}H-PSYCHE-TOCSY experiment was acquired with 8 transients, 2 K * 64 complex data points, 5 kHz * 4 kHz spectral width in F2 and F1 respectively, 16 Pure Shift interferogram with 64 points per block in F3, and 80 ms TOCSY mixing time. Total experimental time, 12 h 52 min. Adiabatic excitation of ^{1}H-SAPPHIRE-PSYCHE and ^{1}H-^{1}H-PSYCHE-TOCSY was performed with a 20° flip angle double saltire CHIRP pulse, 30 ms duration, 10 KHz sweep-width combined with a weak field gradient of 1.08 Gauss/cm.

Pure shift 2D ^{1}H-^{13}C-HOBS-HSQC-TOSCY were recorded with 16 transients, 2 K * 256 complex points, 5 kHz * 20 kHz spectral width in F2 and F1 respectively, and 80 ms TOCSY mixing time. Homonuclear decoupling was performed in real-time acquisition manner using HOBS scheme. We performed two 2D ^{1}H-^{13}C-HOBS-HSQC-TOSCY experiments: one, using 4 ms Rsnob selective inversion with 5.13 ppm carrier position for $H1_A$ and $H1_D$ homonuclear decoupling and, the second one, using 6 ms Rsnob selective inversion with 3.59 ppm carrier position for $H2_D$ homonuclear decoupling. Total experimental time, 13 h 42 min.

Pure shift ^{1}H-^{13}C-PS-HSQC was acquired with 16 transients, 2 K * 256 complex points, 5 kHz * 20 kHz spectral width in F2 and F1 respectively. Homonuclear decoupling was performed in real-time acquisition manner using BIRD scheme. Total experimental time, 11 h 47 min.

SUPPORTING FIGURES

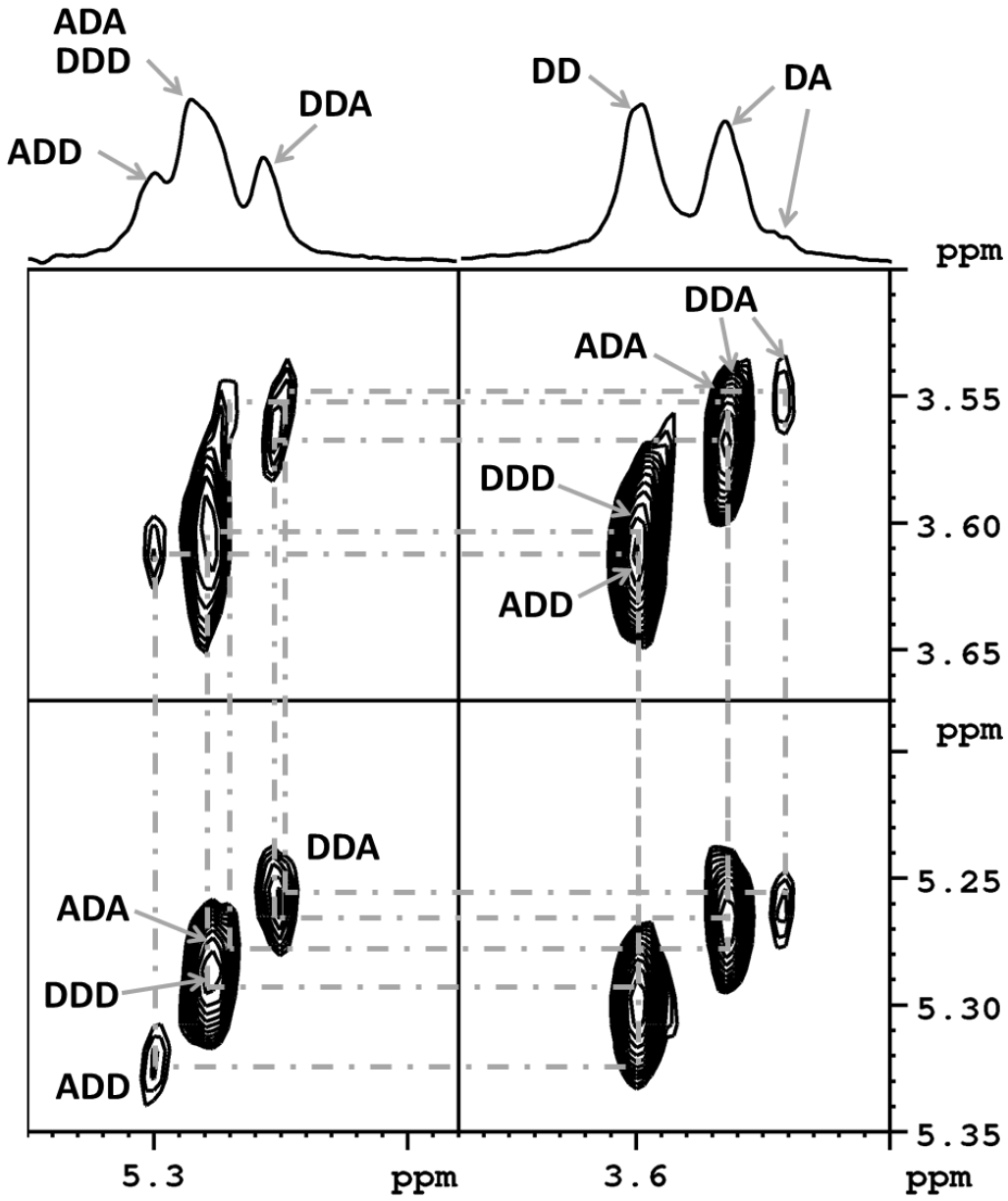

Figure S1. $H1_D$ and $H2_D$ region of ^1H-^1H-PSYCHE-TOCSY spectrum (sample Chi-1). The correlation between $H1_{DDA}$ resonance (5.26 ppm) and $H2_D$ resonance (small signal at 3.54 ppm) shows that it is related to DDA triads.

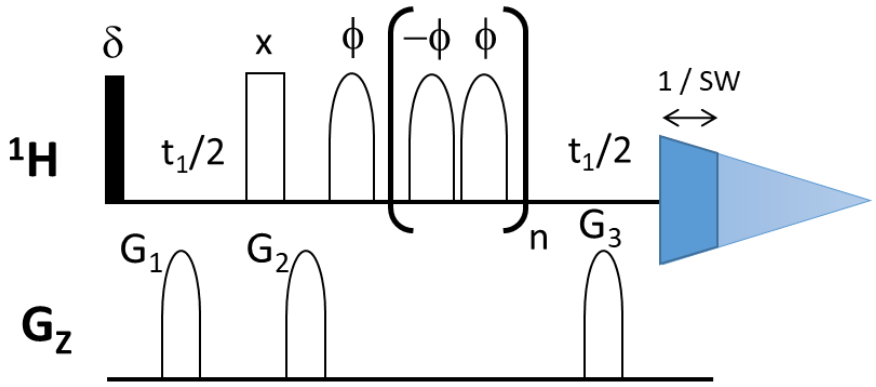

Figure S2. Modified interferogram ^{1}H-HOBS pulse sequence used to measure relaxation during the selective pulse. Filled and empty rectangles indicate 90° and 180° pulses, respectively. Semi-ellipses indicate a 180° selective pulse. Pulses inside the brackets are repeated n times. Pulsed field gradients G_1, G_2, and G_3 are applied along the z-axis (G_z). The G_1, G_2, and G_3 relative strength ratio were set according to $G_3 = G_1$-G_2. Phase cycling: δ = x, x, -x, -x; ϕ = x, y, x, y; ϕrec = x, -x, -x, x. Interferogram acquisition was achieved by acquiring part of the FID with duration 1/SW for each t1 increment.

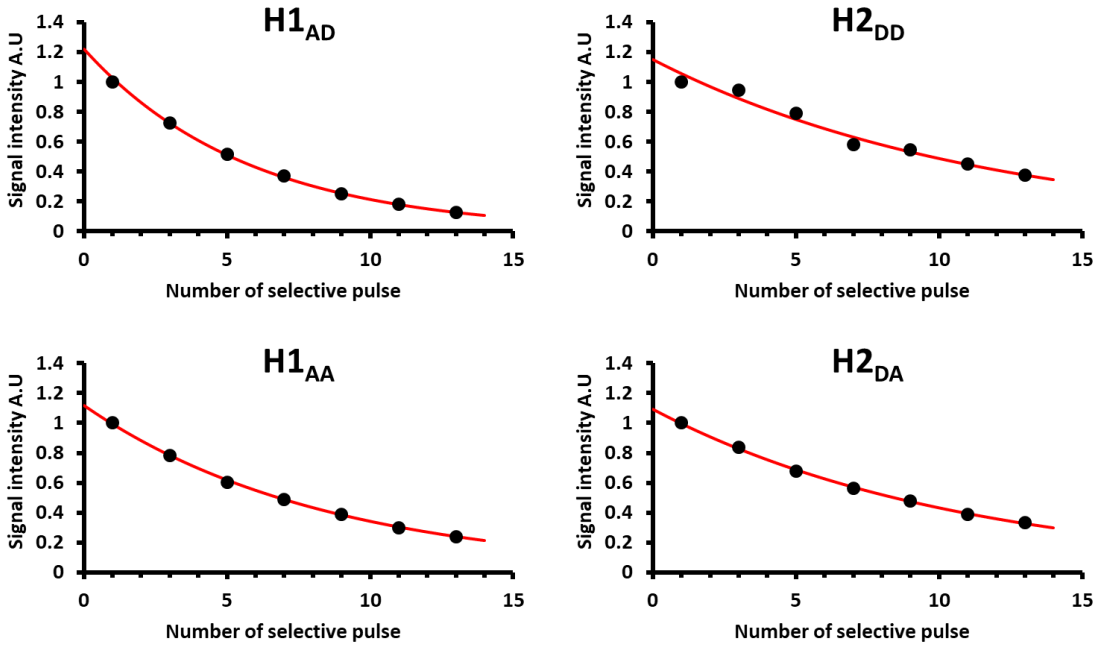

Figure S3. Relaxation during the selective pulse measured in sample Chi-3 using the pulse sequence described in Supporting Figure 2. The data was fitted according to $\mathbf{I = I_0 * Exp(-R_{Rsnob} * (2n + 1))}$, where 2n+1 = total number of selective pulses. Relaxation values (R_{Rsnob}) were obtained for $H1_{AD}$ (0.1741), $H1_{AA}$ (0.1184), $H2_{DD}$ (0.0855), and $H2_{DA}$ (0.0924).

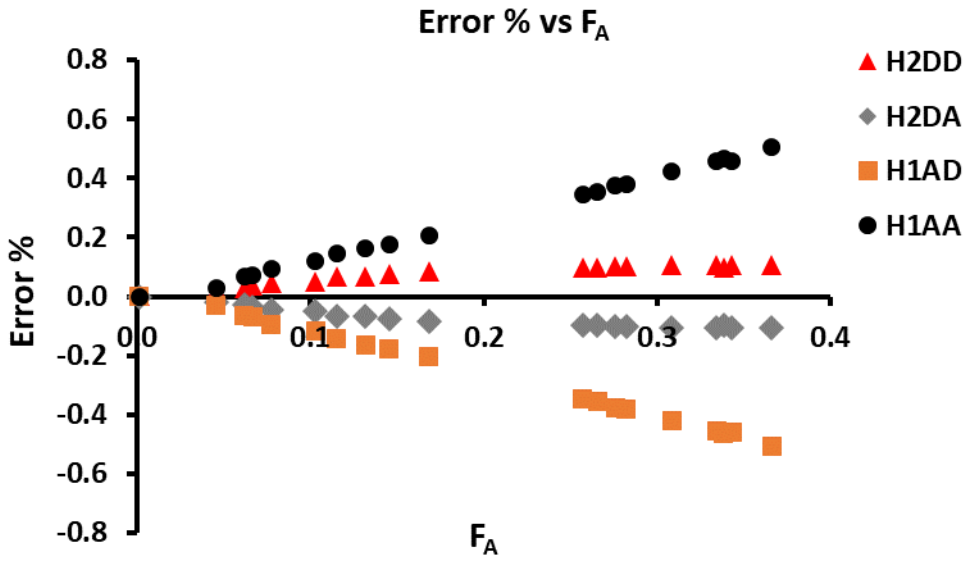

Figure S4. Error induced by the relaxation on the diads frequencies values. Experiments were recorded in interferogram manner with 8 transients, 6 kHz spectral width, and 32 Pure Shift interferograms with 64 complex point per block and a total relaxation time of 10.34 s. Selective inversion was achieved by a 20 ms Rsnob composite pulse. We performed two ^{1}H-HOBS experiments with different carrier position: 4.98 ppm for $H1_A$ and 3.59 ppm for $H2_D$. The relaxation corrected diads frequencies values were calculated using the following formulas.

^{1}H-HOBS experiment with 4.98 ppm carrier position:

$$\mathbf{F_{AD}} = (\mathbf{F_A} * \mathbf{I_{1AD}} * \mathbf{C_{1AD}}) \,/\, (\mathbf{I_{1AD}} * \mathbf{C_{1AD}} + \mathbf{I_{1AA}} * \mathbf{C_{1AA}})$$

$$\mathbf{F_{AA}} = (\mathbf{F_A} * \mathbf{I_{1AA}} * \mathbf{C_{1AA}}) \,/\, (\mathbf{I_{1AD}} * \mathbf{C_{1AD}} + \mathbf{I_{1AA}} * \mathbf{C_{1AA}})$$

^{1}H-HOBS experiment with 3.59 ppm carrier position:

$$\mathbf{F_{DA}} = ((\mathbf{1} - \mathbf{F_A}) * \mathbf{I_{2DA}} * \mathbf{C_{2DA}}) \,/\, (\mathbf{I_{2DD}} * \mathbf{C_{2DD}} + \mathbf{I_{2DA}} * \mathbf{C_{2DA}})$$

$$\mathbf{F_{DD}} = ((\mathbf{1} - \mathbf{F_A}) * \mathbf{I_{2DD}} * \mathbf{C_{2DD}}) \,/\, (\mathbf{I_{2DD}} * \mathbf{C_{2DD}} + \mathbf{I_{2DA}} * \mathbf{C_{2DA}})$$

Spectra deconvolution were performed using Topspin software. Correction factors C were calculated by: $\mathbf{C} = \boldsymbol{e}^{-\mathbf{R_{Rsnob}}}$. The figure shows a strong dependency between the error and the F_A values. The error in all cases was less than 1%.

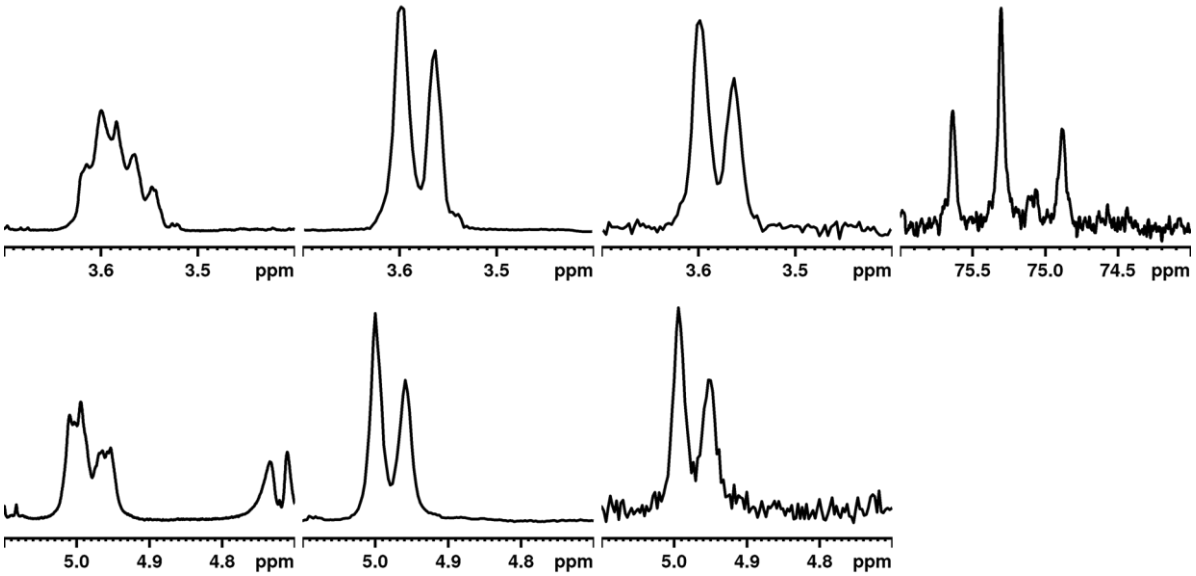

Figure S5. ^{1}H-NMR, ^{1}H-iHOBS and ^{13}C-NMR spectra sensitivity comparison acquired on Chi-12 sample. ^{1}H-NMR on 12.2 mg/mL sample: $H1_D$ (top left) and $H1_A$ (bottom left); 1H-iHOBS on 12.2 mg/mL sample: $H1_D$ (top middle left) and $H1_A$ (bottom middle left); 1H-iHOBS on 0.45 mg/mL sample: $H1_D$ (top middle right) and $H1_A$ (bottom middle right) and ^{13}C-NMR on 12.2 mg/mL sample: C5 (top right). ^{1}H-NMR and ^{1}H-iHOBS experiments were acquired with 90° flip angle excitation, 8 scans, total relaxation time of 10.34 s and 32 increments for iHOBS. ^{13}C-NMR spectra was acquired using 30° flip angle, 16384 scans, 1.1 s acquisition time, and relaxation delay of 2 s.

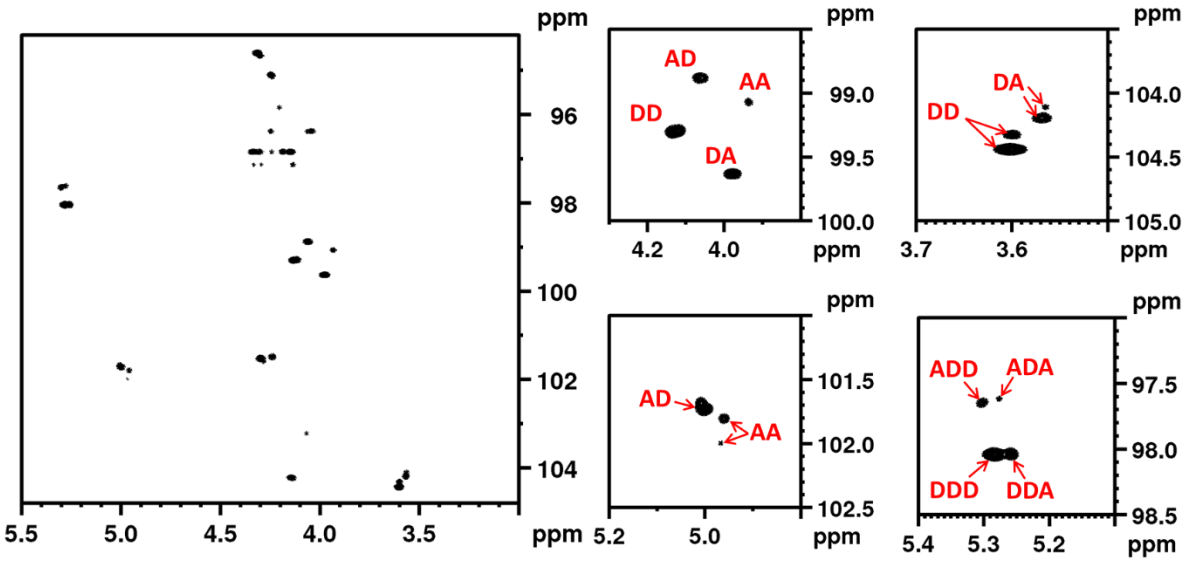

Figure S6. Showing ^{1}H-^{13}C-SAPS-HSQC (left) plus H5-C5 (top middle), deacetylated H2-C2 (top right), acetylated H1-C1 (bottom middle) and deacetylated H1-C1 (bottom right) expansions.

The indirect dimension enhanced resolution (11.8Hz) results in an excellent separation of deacetylated H1-C1triads (bottom right). Likewise, a signal splitting belonging to deacetylated H2-C2 (top right), acetylated H1-C1 (bottom middle) diads is now observed. This separation is not observed in the regular ^{1}H-^{13}C PS-HSQC, nevertheless those better resolved signals could not be assigned to any specific triad or tetrad.

SUPPORTING TABLES

Table S1. Signal to Noise (S/N) and diads frequencies measured from supporting figure 5 spectra.

	Conc (mg/mL)	S/N_{DD}	S/N_{DA}	S/N_{AD}	S/N_{AA}	F_{DD}	F_{DA}	F_{AD}	F_{AA}	P_A
^{13}C-NMR	12.2	14.9	8.6	7.2	3.1	0.376	0.235	0.257	0.131	1.05
^{1}H-iHOBS	12.2	759.0	611.4	394.1	267.2	0.349	0.232	0.241	0.178	0.975
	0.45	31.7	23.0	13.8	8.9	0.356	0.224	0.236	0.183	0.950
^{1}H-NMR	12.2	427.0		214.8						

Table S2. Fraction of acetylation (F_A), diads frequencies and pattern of acetylation (P_A) of twenty chitosan samples (Chi-1-Chi-20) determined by standard ^{1}H-NMR, ^{1}H-iHOBS, and ^{13}C-NMR methodologies.

	^{1}H[b]	^{13}C[c]	^{1}H[d]				^{13}C[c]				^{1}H[d]	^{13}C[c]
	F_A	F_A	F_{DD}	F_{DA}	F_{AD}	F_{AA}	F_{DD}	F_{DA}	F_{AD}	F_{AA}	P_A	P_A
Chi-12	0.419	0.382	0.349	0.232	0.241	0.178	0.376	0.235	0.257	0.131	0.975	1.05
		0.369	0.350	0.234	0.239	0.177	0.429	0.202	0.227	0.142	0.975	0.935
Chi-1	0.366	0.382	0.376	0.258	0.200	0.166	0.377	0.241	0.209	0.173	0.958	0.939
		0.355	0.376	0.258	0.191	0.175	0.409	0.236	0.211	0.144	0.935	0.962
Chi-2	0.343	0.359	0.408	0.249	0.208	0.135	0.426	0.215	0.201	0.158	0.988	0.896
		0.344	0.395	0.262	0.204	0.139	0.417	0.239	0.196	0.149	0.998	0.936
Chi-13	0.338	0.367	0.451	0.211	0.191	0.148	0.430	0.203	0.208	0.159	0.884	0.887
		0.321	0.445	0.217	0.185	0.153	0.528	0.152	0.160	0.160	0.879	0.721
Chi-3	0.334	0.320	0.431	0.234	0.194	0.141	0.458	0.222	0.200	0.121	0.935	0.950
		0.352	0.432	0.234	0.186	0.149	0.459	0.190	0.231	0.121	0.913	0.950
Chi-4	0.309	0.263	0.464	0.227	0.177	0.132	0.537	0.201	0.144	0.119	0.909	0.834
		0.281	0.459	0.233	0.174	0.134	0.503	0.216	0.159	0.122	0.910	0.877
Chi-5	0.282	0.313	0.503	0.215	0.167	0.115	0.490	0.197	0.185	0.128	0.900	0.880
		0.337	0.493	0.225	0.168	0.115	0.512	0.151	0.184	0.153	0.916	0.769
Chi-6	0.276	0.308	0.522	0.203	0.160	0.115	0.484	0.208	0.192	0.116	0.870	0.926
		0.292	0.517	0.207	0.158	0.118	0.516	0.192	0.161	0.131	0.868	0.828
Chi-7	0.265	0.322	0.539	0.196	0.160	0.105	0.514	0.164	0.179	0.143	0.877	0.796
		0.243	0.526	0.209	0.152	0.113	0.576	0.181	0.144	0.099	0.871	0.842
Chi-8	0.257	0.258	0.547	0.196	0.154	0.103	0.575	0.167	0.135	0.123	0.870	0.758
		0.242	0.531	0.212	0.154	0.103	0.561	0.197	0.130	0.111	0.897	0.822
Chi-9	0.168	0.162	0.685	0.147	0.115	0.053	0.672	0.165	0.116	0.046	0.871	0.925
		0.131	0.654	0.177	0.121	0.048	0.718	0.151	0.087	0.044	0.944	0.872
Chi-14	0.146	0.109	0.727	0.127	0.100	0.046	0.793	0.099	0.099	0.010	0.849	1.017
		0.145	0.697	0.158	0.103	0.043	0.716	0.139	0.145	0.000	0.910	1.165
Chi-15	0.132	0.173	0.754	0.115	0.089	0.043	0.729	0.098	0.133	0.040	0.821	0.879
		0.202	0.730	0.138	0.093	0.039	0.677	0.120	0.168	0.035	0.884	0.981
Chi-16	0.115	0.178	0.774	0.111	0.077	0.038	0.715	0.107	0.104	0.075	0.817	0.714
		0.247	0.751	0.133	0.080	0.035	0.640	0.112	0.091	0.156	0.876	0.530
Chi-17	0.103	0.115	0.815	0.082	0.074	0.029	0.783	0.102	0.095	0.020	0.816	0.943
		0.130	0.800	0.097	0.075	0.028	0.736	0.133	0.070	0.060	0.851	0.751
Chi-18	0.078	0.050	0.853	0.069	0.054	0.024	0.918	0.032	0.034	0.015	0.787	0.718
		0.109	0.837	0.085	0.054	0.023	0.791	0.101	0.078	0.031	0.826	0.845
Chi-19	0.067	0.082	0.876	0.058	0.050	0.016	0.882	0.036	0.074	0.007	0.824	0.941
		0.135	0.846	0.087	0.051	0.016	0.783	0.082	0.080	0.055	0.887	0.689
Chi-20	0.062	0.123	0.895	0.043	0.046	0.016	0.792	0.085	0.096	0.027	0.785	0.875
		0.070	0.865	0.073	0.045	0.017	0.875	0.055	0.045	0.025	0.837	0.717
Chi-10	0.046	0.000	0.924	0.030	0.040	0.006	0.999	0.001	0.000	0.000	0.890	0.554
		nd[e]	0.856	0.099	0.034	0.011	nd[e]	nd[e]	nd[e]	nd[e]	0.930	nd[e]
Chi-11	0.001	0.000	0.998	0.000	0.001	0.000	1.000	0.000	0.000	0.000	1.001	1.000
		nd[e]	0.999	0.000	0.001	0.000	nd[e]	nd[e]	nd[e]	nd[e]	1.001	nd[e]

[a] Chi sample data was deconvoluted using Topspin software (first row on each) and Mestrenova (second row on each). [b] F_A values were calculated using classical ^{1}H-NMR spectra.[14,15] [c] Inverse gate ^{13}C-NMR spectra[10] were used for F_A and P_A determinations. [d] ^{1}HOBS methodology implemented in this study was used to calculate diads fractions and P_A values. [e] nd cannot be determined.

Table S3. Fraction of acetylation and diads frequencies of chitosan samples (Chi3, Chi-9, Chi-12 and Chi-15) determined by ^{1}H-NMR, ^{1}H-^{13}C-SAPS-HSQC, ^{1}H-iHOBS, and ^{13}C-NMR methodologies.

	^{1}H	**SAPS-HSQC**				**^{1}H-iHOBS**				**^{13}C**			
	F_A	F_{DD}	F_{DA}	F_{AD}	F_{AA}	F_{DD}	F_{DA}	F_{AD}	F_{AA}	F_{DD}	F_{DA}	F_{AD}	F_{AA}
Chi-12	0.419	0.385[a] 0.361[b]	0.211[a] 0.219[b]	0.261[a] 0.245[b]	0.143[a] 0.175[b]	0.349	0.232	0.241	0.178	0.376	0.235	0.257	0.131
Chi-3	0.334	0.526[a] 0.493[b]	0.172[a] 0.246[b]	0.216[a] 0.188[b]	0.086[a] 0.073[b]	0.431	0.234	0.194	0.141	0.458	0.222	0.200	0.121
Chi-9	0.168	0.717[a] 0.676[b]	0.112[a] 0.132[b]	0.107[a] 0.122[b]	0.064[a] 0.070[b]	0.685	0.147	0.115	0.053	0.672	0.165	0.116	0.046
Chi-15	0.132	0.796[a] 0.770[b]	0.071[a] 0.099[b]	0.081[a] 0.079[b]	0.030[a] 0.052[b]	0.754	0.115	0.089	0.043	0.729	0.098	0.133	0.040

^{1}H-^{13}C-SAPS-HSQC quantification is obtained by direct integration of the signal H5-C5 or by combining integrations from acetylated H1-C1 and deacetylated H2-C2 signals.

[a]Diads frequencies determined ^{1}H-^{13}C-SAPS-HSQC by integrating H5-C5 signals:

$$\mathbf{F_{DA} = I_{5DA} \,/\, (I_{5DA} + I_{5AD} + I_{5DD} + I_{5AA})}$$
$$\mathbf{F_{AD} = I_{5AD} \,/\, (I_{5DA} + I_{5AD} + I_{5DD} + I_{5AA})}$$
$$\mathbf{F_{DD} = I_{5DD} \,/\, (I_{5DA} + I_{5AD} + I_{5DD} + I_{5AA})}$$
$$\mathbf{F_{AA} = I_{5AA} \,/\, (I_{5DA} + I_{5AD} + I_{5DD} + I_{5AA})}$$

[b]Diads frequencies determined ^{1}H-^{13}C-SAPS-HSQC by integrating deacetylated H2-C2 and acetylated H1-C1 signals:

$$\mathbf{F_{DA} = ((1 - F_A) * I_{2DA}) \,/\, (I_{2DD} + I_{2DA})}$$
$$\mathbf{F_{DD} = ((1 - F_A) * I_{2DD}) \,/\, (I_{2DD} + I_{2DA})}$$
$$\mathbf{F_{AD} = (F_A * I_{1AD}) \,/\, (I_{1AD} + I_{1AA})}$$
$$\mathbf{F_{AA} = (F_A * I_{1AA}) \,/\, (I_{1AD} + I_{1AA})}$$

;Selective pulse Relaxation reset_HOBS_1d-R

;avance-version (14/08/29)

;pseudo 2D sequence

;band selective homodecoupling using a HOBS element

;

;

;$CLASS=HighRes

;$DIM=2D

;$TYPE=

;$SUBTYPE=

;$COMMENT=

#include <Avance.incl>

#include <Grad.incl>

#include <Delay.incl>

"d11=30m"

"d12=20u"

"in0=dw*l31"

"d0=3u"

"p2=2*p1"

"DELTA2=l31*2*dw"

"DELTA3=p16+d16+d0*2+de+dw*2*l30+5u"

"l29=2*l31*td1"

1 ze

2 d11

3 d12

d1 pl1:f1

50u UNBLKGRAD

(p1 ph3):f1

d0

p16:gp1*0.5

d16

(p2 ph5):f1

p16:gp1*-0.5

d16

DELTA3

(p47:sp34 ph2):f1

4 (p47:sp34 ph12):f1

(p47:sp34 ph2):f1

lo to 4 times l11

```
 5u
 p16:gp1*-1.0
 d16
 d0

 4u BLKGRAD
 go=2 ph31

 d11 mc #0 to 2
   F1QF(caldel(d0, +in0))

 DELTA2
exit



ph2=0 1
ph3=0 0 2 2
ph4=0
ph5=0
ph12=2 3
ph31=0 2 2 0



;pl1 : f1 channel - power level for pulse (default)
;sp30: f1 channel - shaped pulse 180 degree (Bip720,50,20.1)
;sp34: f1 channel - shaped pulse 180 degree for Zangger-Sterk element
;p1 : f1 channel -  90 degree high power pulse
;p16: homospoil/gradient pulse
;p44: f1 channel - 180 degree shaped pulse for refocussing
```

```
;d0 : incremented delay (2D)                    [3 usec]
;d1 : relaxation delay; 1-5 * T1
;d11: delay for disk I/O                        [30 msec]
;d12: delay for power switching                 [20 usec]
;d16: delay for homospoil/gradient recovery
;l11: n 180 degree shaped pulse
;l29: total number of points in reconstructed FID
;l30: number of complex points at the beginning not to be included
;       in reconstruction
;l31: number of complex points along the acquisition dimension per block
;       block length about 8 to 10ms
;in0: = dw*l31
;ns: 1 * n, total number of scans: NS * TD0
;ds: 4
;FnMODE: QF


;for z-only gradients:
;gpz0: 2%
;gpz1: 19%

;use gradient files:
;gpnam0: RECT.1
;gpnam1: SMSQ10.100



;use AU-program proc_reset to process data
```