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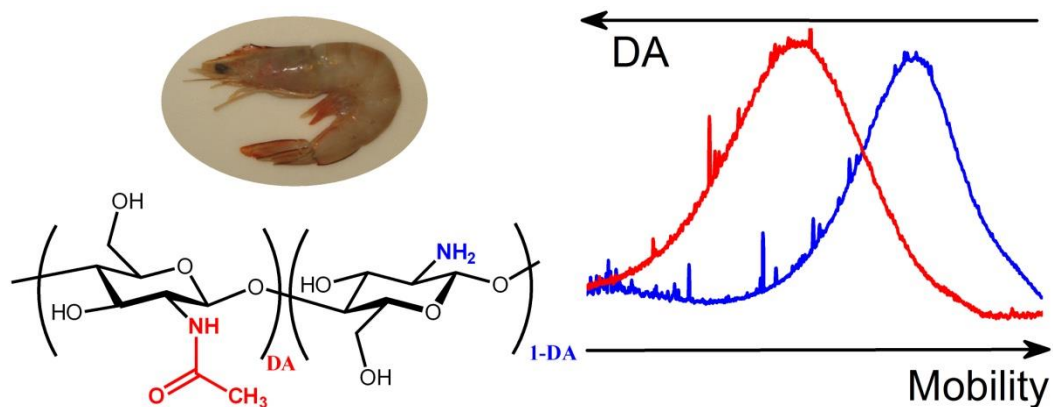
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### Online Abstract Figure



Separation of chitosan molecules by their composition with free-solution capillary electrophoresis

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# Separation of Chitosan by Degree of Acetylation using Simple Free Solution Capillary Electrophoresis

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## ABSTRACT

Chitosan is a biopolymer of increasing significance, as well as a renewable and sustainable material. Its main molecular characteristics are molar mass and degree of acetylation (composition). Precise average degrees of acetylation were measured by quantitative <sup>1</sup>H solution-state NMR spectroscopy. While number-average degree of acetylation had already been determined by <sup>1</sup>H NMR spectroscopy, weight-average degree of acetylation are also determined and may be more relevant for some properties, such as mechanical properties. We report the first separation of chitosan according to its degree of acetylation using free solution capillary electrophoresis. Capillary electrophoresis separates chitosan in the ‘critical conditions’: the molar mass plays little role and the separation is by the degree of acetylation. It characterizes the heterogeneity of chitosan samples in terms of composition (dispersity of the distribution of degrees of acetylation). This heterogeneity (broad distribution of degrees of acetylation) cannot be neglected contrary to a common assumption found in the literature. This fast and easy separation will allow establishing structure-property relations.

**KEYWORDS:** *capillary electrophoresis, chitosan, degree of acetylation, critical conditions, NMR spectroscopy.*

## Introduction

Chitin is the second most abundant polymer in the world [1]. This waste of the seafood industry is a renewable but also sustainable biopolymer source, whose partial deacetylation yields a copolymer: chitosan (Fig. S-1) including N-acetyl-D-glucosamine as well D-glucosamine units [1]. The copolymer composition is also named the degree of acetylation (DA) in the case of chitosan. Chitosan is biocompatible, biodegradable, antimicrobial and antifungal. The great variety of chitosan properties is determined by the polysaccharide's chemical structure [1, 2], and this chemical structure changes with the source of origin and the extraction process. The natural variability of chitosan makes the characterization of its chemical structure very important. The DA, the degree of polymerization (DP, or the molar mass MM) and their distributions are among the most critical parameters that govern the properties of chitosan [1, 2] (see Fig. S-1 for a description of DP and DA).

Different methods are used to estimate or quantify an average DA. Some are based on indirect measurements such as chemical / enzymatic analysis or liquid chromatography [2]. Further methods are based on direct measurements with techniques such as  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  NMR spectroscopy in solution and in the solid state.  $^1\text{H}$  solution-state NMR spectroscopy provides the most precise and accurate measurement of the average DA for chitosan [2], as it takes advantage of the high sensitivity of the  $^1\text{H}$  nucleus in NMR. The measurement is quantitative if parameters are set up to allow full relaxation between scans (see Electronic Supplementary Material). Quantitative  $^1\text{H}$  solution-state NMR was used in this work to quantify the average DA of the investigated chitosan samples. The literature never mentions whether number-average or weight-average DA are measured, although this distinction is nowadays the norm for molar masses in the literature.

Very little has been done to characterize the distribution of DAs. The distribution of DAs affects properties important for biomedical applications, such as solubility and biodegradability: samples with the same average DA (and the same distribution of molecular weights) are expected to exhibit different solubility and

biodegradation behaviours. It has been shown recently for the first time using fractionation by preparative size-exclusion chromatography (SEC/GPC) coupled offline to  $^1\text{H}$  NMR spectroscopy that chitosan had a significant distribution of DAs [3]. While the results of this SEC×NMR characterization are invaluable, the method is expensive and time-consuming. Furthermore, SEC does not separate by the DA (or molar mass) but by the hydrodynamic volume [4]. The most common alternative is liquid chromatography at the critical adsorption point (LC-CC). LC-CC does not separate the compound of interest by its molar mass and can thus separate complex polymers by their structure, including separation of copolymers by their composition [5]. The critical conditions in LC are however tedious to determine and recovery is poor (see Electronic Supplementary Material). We are proposing free-solution capillary electrophoresis (CE) as an alternative.

Free solution CE is used to separate DNA and proteins. Applications of free solution CE to other polymers have been successful [6], but applications to chitosan have been very limited (see Electronic Supplementary Material). No separation of chitosan by DA has ever been published (by CE or by any other separation method). In the case of polymers, no separation by molar mass is observed in free solution CE of single-stranded or double-stranded DNA, poly(styrene sulfonate) and a few other polyelectrolytes [6, 7]. This effectively corresponds to the “critical conditions” sought in LC-CC. Free solution CE technique can be used as a method to separate polyelectrolytes by their microstructure, such as composition or branching. In analogy with LC-CC, we named the method CE in the critical conditions (CE-CC), as the absence of separation by molar mass allows separation by the microstructure [8, 9]. Critical conditions in CE do not correspond to free-draining, but rather quasi-parallel increase of the nominal charge and the friction with the degree of polymerization (see Electronic Supplementary Material). The aim of this work is to explore the potential of NMR spectroscopy and CE-CC for the characterization of the heterogeneity of the DA in chitosan (dispersity of its distribution of degrees of acetylation) and for the fast separation of chitosan by its DA.

## Results and discussion

Details of the materials,  $^1\text{H}$  solution-state NMR spectroscopy and capillary electrophoresis experimental conditions are given in Electronic Supplementary Materials.

### Average degrees of acetylation measured by quantitative NMR spectroscopy

While measurements of average DAs at 25 °C and 90 °C are both precise, the high-temperature measurement is more accurate (see Electronic Supplementary Material, including Fig. S-2 and S-3), and the resulting  $DA_n^{\text{NMR}}$  was used in this work. The trend in  $DA_n^{\text{NMR}}$  values is the same as the trend in DA values provided by the supplier,  $DA^{\text{supp}}$ , for each supplier. However, it is not the case between suppliers, presumably due to different experimental methods being used by different suppliers to determine DA. Note that the suppliers do not specify what method they use to determine DA, or if they determine a number-average (Equation S-4) or a weight-average (Equation S-5) DA.

In the case of molecular weight, the number-average is widely used to relate to the kinetics of polymerization, while the weight-average is widely used for different purposes, for examples to relate to the polymer properties. The relationship between  $DA_n$  and  $DA_w$  can easily be derived (see Electronic Supplementary Material, Equations S-4 to S-12):

$$DA_w = \frac{DA_n \cdot M_A}{DA_n \cdot M_A + (1 - DA_n) \cdot M_D} \quad (1)$$

Equation (1) is valid for any copolymer (in the particular case of chitosan, see Equation (S-12)).  $DA_w$  has never been used in the literature, but its correlation with different properties can be easily tested. Equation (S-12) was used to calculate  $DA_w$  for the chitosan samples analysed in this work (Table S-1). It is to be noted that the weight-average degrees of acetylation are systematically higher than the number-average degrees of acetylation, and do not exactly correspond to the DA value given by the supplier. Like in the case of  $DA_n^{\text{NMR}}$  mentioned above, the trend in  $DA_w$  values is the same as the trend in  $DA^{\text{supp}}$ , for each supplier, but it is

not the case between suppliers. The value of the dispersity from these averages correlates with  $DA_n$ :

$$D = \frac{DA_w}{DA_n} = \frac{1}{M_D/M_A + (1 - M_D/M_A)DA_n} \quad (2)$$

This dispersity thus does not bring any information on the heterogeneity (in terms of degree of acetylation) of the chitosan polymer chains. This is due to the fact that NMR is a local method determining the acetyl groups at the monomer unit's level. In order to get information about the heterogeneity of the polymer chains, separation by DA is required.

### **Separation by the degree of acetylation using CE in the critical conditions**

Fused-silica capillaries were used since they lead to no significant chitosan adsorption onto their surface at low pH (see Electronic Supplementary Material). Capillaries used had a 50  $\mu\text{m}$  inner diameter, a 360  $\mu\text{m}$  outer diameter, a total length of 64, 61 or 40 cm, corresponding to a length to the detector of 55.5 cm, 51.5 cm or 31.5 cm, respectively; a fused silica capillary with extended light-path detection window was also used, with a 64 cm total length. Phosphate buffer at pH 2 was used previously to determine the amount of chitosan (it is transparent in UV contrary to acetic acid). In this work, we do not only want to determine chitosan but also to separate it by its DA. Electrophoretic mobilities of chitosan are the same at pH 3 and 3.5 indicating complete ionization at pH 3, which was thus preferred to avoid any degradation. In order to minimize or prevent chitosan adsorption and interaction on the capillary wall, a high concentration phosphate buffer is required. The separations are fast, taking less than 10 min (Fig. 1a), repeatable and reproducible (Fig. S-5 to S-7). Seven different chitosan samples were successfully separated by their DA (Fig. 1) and the separation reproduced by another operator (Fig. S-4). The shoulder observed at low migration times of Fig. 1a for the chitosan with 16.5 % average DA might be due to some aggregation (see Electronic Supplementary Material).

Derivatization or indirect detection was not necessary in this work to obtain sufficient sensitivity since polymers and not oligomers are studied. The sensitivity of the UV detection (at 200 nm) is high enough especially using a modern

instrument. A high-sensitivity capillary can further increase the sensitivity (Fig. S-7). At 200 nm, the acetyl groups of the chitosan chains are detected, therefore the samples with higher DAs appear with larger peak areas when injected in similar concentrations (see Fig. S-8 in Electronic Supplementary Material). The separations do not reveal the presence of oligomers: no sharp contiguous peaks are observed [7]. The molar masses of the samples are not known, but they are high enough to be in the critical conditions. This is also consistent with the random order of migration in terms of viscosity: 55; 55; 570; 590; 320 and then 32 cps with increasing migration time. This CE-CC method is currently using the free solution CE technique, although most free solution CE separations currently published rely on different separations mechanisms than critical conditions. Note that CE-CC does not have to be restricted to the free solution CE technique.

### **Electrophoretic mobility according to the degree of acetylation**

The lower the DA is, the higher the number of free amino groups is, which subsequently results in a higher cationic charge of the molecule and thus in a higher electrophoretic mobility. An equivalent behavior has already been observed for pectins and carboxymethyl cellulose. The electrophoretic mobility of pectins increases with the pectin's charge. Furthermore in an electrolyte solution the net charge of polyelectrolytes with a high charge density is not proportional to their charge density due to the condensation of counter ions onto the polymer backbone [10]. Counter ion condensation is a critical factor responsible for a nonlinear relationship between the degree of substitution (DS) and mobility of the carboxymethyl cellulose with a high charge density (DS) [10]. Fig. 2a represents the relation between the  $DA_w$  or the  $DA_n$  and the electrophoretic mobility. Both give a relatively poor linear correlation (regression coefficient of 0.86 with both  $DA_w$  and  $DA_n$ ) in the case of chitosan in this work. The relation between mobility and DA may thus also best be described by a nonlinear behavior. A lower selectivity for degrees of acetylation lower than about 17 % is consistent with Manning's condensation of the counter-ions (see Electronic Supplementary Material). The non-linear behavior of the mobility with DA was thus expected. The electrophoretic mobility has been empirically observed to be inversely proportional to the degree of substitution of pectins (the migration time has been



observed to linearly correlate with the degree of substitution). In the case of chitosan, the correlation of the inverse electrophoretic mobility with either  $DA_n$  or  $DA_w$  leads to correlation coefficients poorer than the correlation of the mobility with the  $DAs$  (see Fig. 2b). We do not observe with our chitosan samples the same correlation as observed in the literature with pectins. This may be due to a broader distribution of substitution / acetylation in the case of chitosan or to solubility issues, which will be the subject of future investigations. The calibration of the electrophoretic mobility by the  $DA_w$  or the  $DA_n$  will allow the determination of the distribution of  $DAs$ , similarly to the distribution of molecular weights. Fig. 1 shows that a distribution of  $DAs$  exists for each sample: none seems to be monodisperse and they have different dispersities: as a crude estimate the full width at half maximum (fwhm) can be used. The samples with larger average  $DA$  values exhibit a higher dispersity of their distribution of  $DAs$  (see Fig. S-9 in Electronic Supplementary Material).

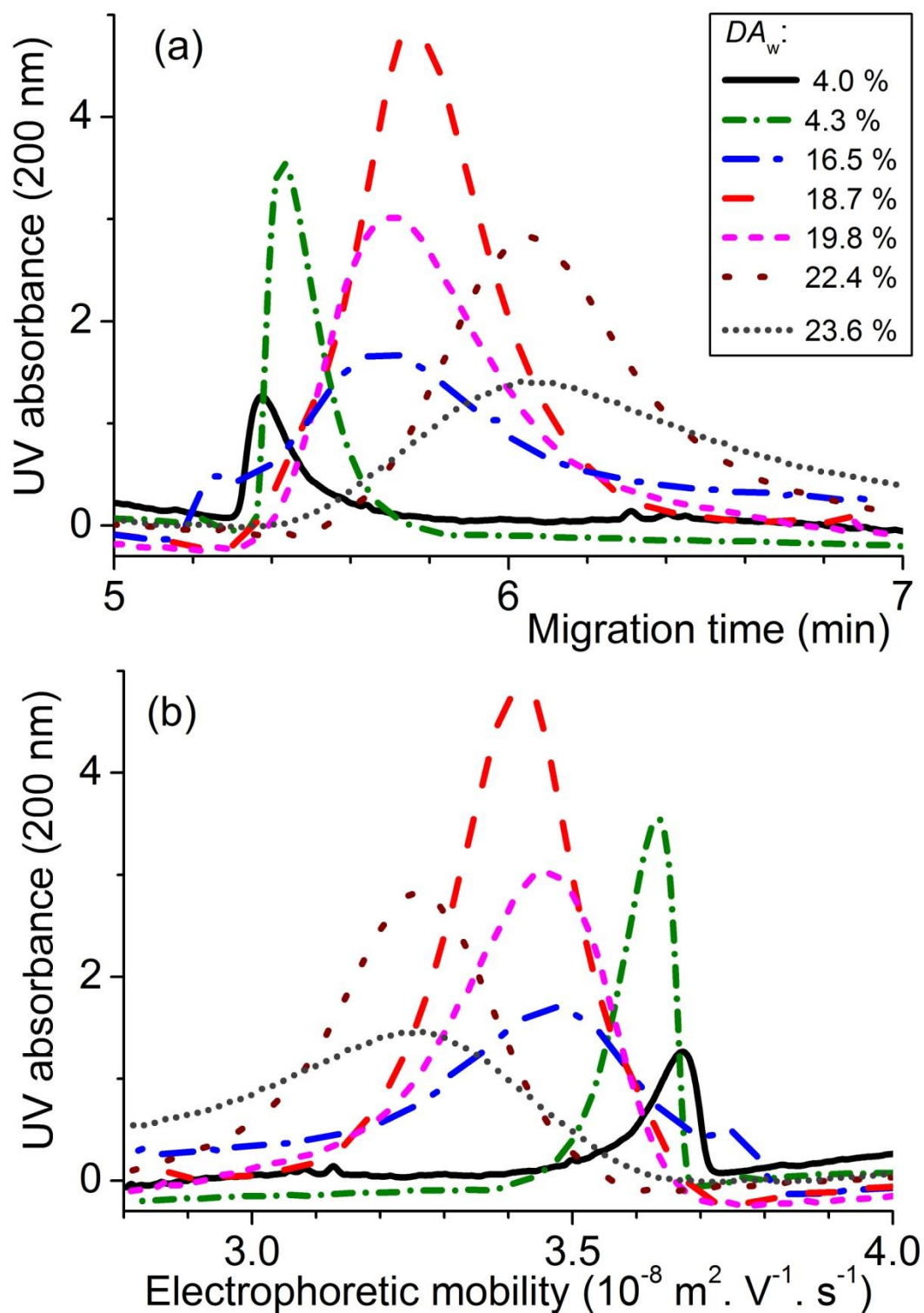
## Conclusions

Chitosan's exceptional biological properties will enable it to achieve its potential as biomaterial with proper separation and characterization methods. We have shown that the existence of a distribution of degrees of acetylation ( $DA$ ) is of importance (as is the distribution of molar masses). NMR spectroscopy is usually only used to determine number-average  $DAs$  ( $DA_n$ ), but weight-average  $DAs$  ( $DA_w$ ) can also be determined without any additional experimental work. In analogy to molar mass, the weight-average  $DA$  may be the most relevant parameter to correlate for example with rheological properties. Separation according to the distribution of  $DA$  is possible with simple free solution CE: CE in the critical conditions, CE-CC. CE-CC does not separate by molar mass but only by  $DA$ . The method is thus complementary to size-exclusion chromatography (SEC or GPC), the most popular separation method for polysaccharides. SEC separates by hydrodynamic volume that depends on both on  $DA$  and molar mass. By separating solely by  $DA$ , CE-CC can shed a light on the distribution of  $DA$  but also on the accuracy of the distribution of molar masses determined by SEC.

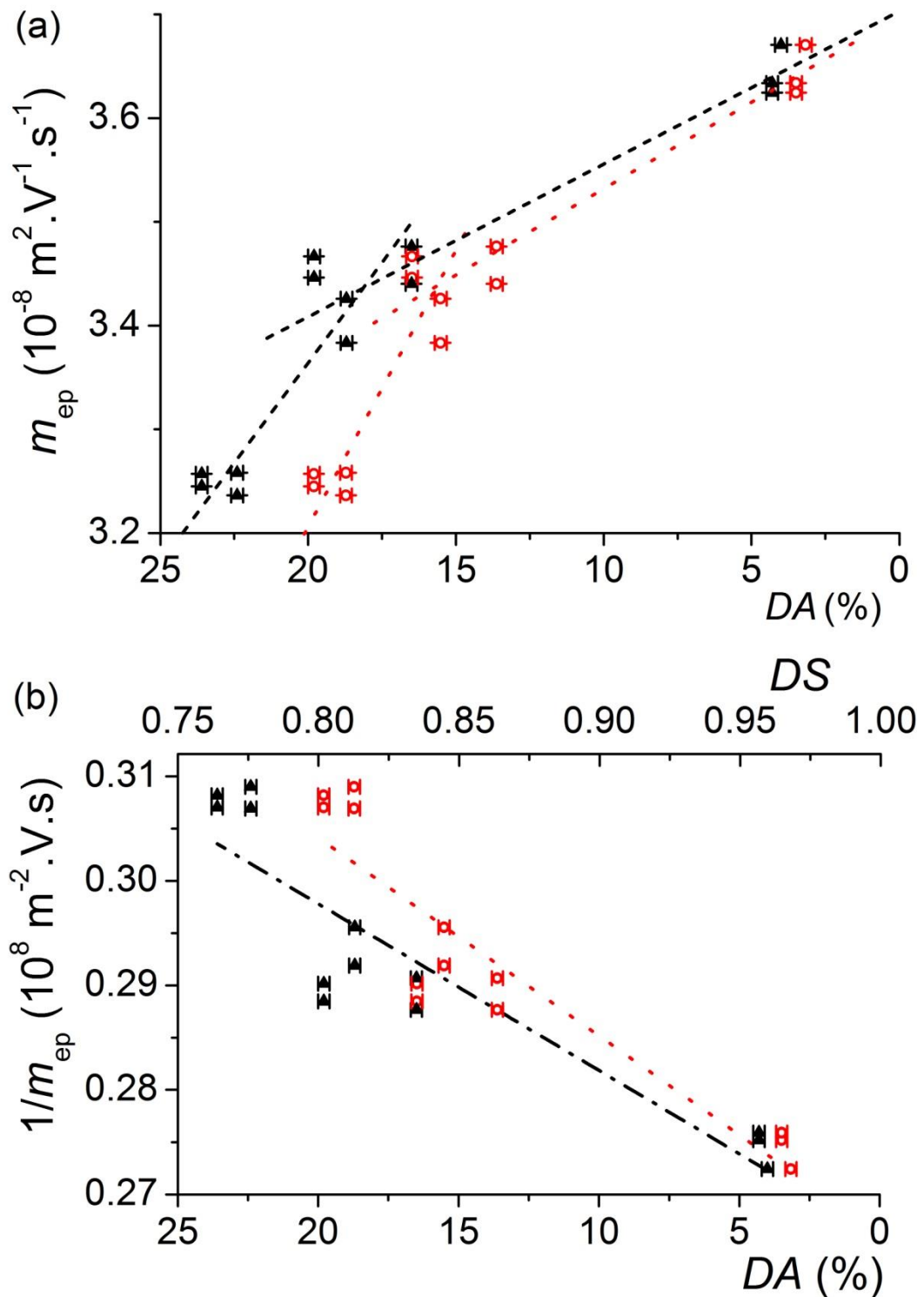
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**Fig. 1** Separation of chitosan samples by their DA with CE: electropherogram shown (a) as a function of migration time, and (b) as a function of apparent electrophoretic mobility. Samples AKbioD1, AKbioV2, AKbioV3, MedMW, AKbioD2, AKbioD3 and chitAL are shown. Conditions: 64 cm capillary total length (55.5. cm effective length), 30 kV voltage, sodium phosphate buffer (pH 3, 100 mM) as background electrolyte



**Fig. 2 a** Electrophoretic mobility against  $DA_n$  (red circles, linear fit not shown,  $R^2=0.861$ ; the red dotted lines are a guide to the eye) and  $DA_w$  (black triangles, linear fit not shown,  $R^2=0.856$ ; the black dashed lines are a guide to the eye); **b** Inverse electrophoretic mobility against  $DA_n$  (linear fit shown as red dotted line:  $R^2=0.837$ ) and  $DA_w$  (linear fit shown as black dashed-dotted line:  $R^2=0.831$ ); mobility and DA were measured respectively by CE and NMR for chitosan samples AKbioD1, AKbioV2, AKbioV3, MedMW, AKbioD2, AKbioD3 and

chitAL; the error bars along the X axis were estimated from NMR signal-to-noise ratio (see Electronic Supplementary Material)

# Electronic Supplementary Material

by

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for

## **Separation of Chitosan by Degree of Acetylation using Simple Free Solution Capillary Electrophoresis**

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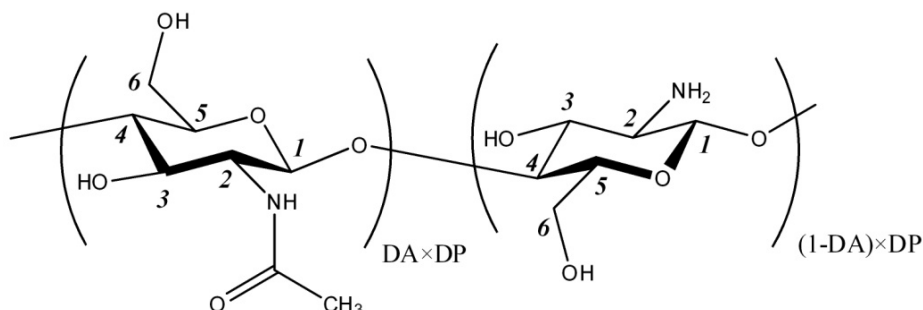
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This file contains the description of chitosan and of chitosan samples, the detailed experimental conditions for quantitative <sup>1</sup>H solution-state NMR measurements, the determination of average DA from <sup>1</sup>H solution-state NMR measurements, the derivation of the relationship between number-average and weight-average degrees of acetylation, a description of the critical conditions for polymer separation in liquid chromatography and in capillary electrophoresis, the detailed experimental conditions for capillary electrophoresis, the calculation of the apparent electrophoretic mobility and the electroosmotic mobility, the separation of chitosan according to the degree of acetylation, repeatability and reproducibility studies, as well as the calculation of the Bjerrum length.

## Chitosan and chitosan samples

Chitin is a homopolymer of  $\beta$ -(1 $\rightarrow$ 4)-2-acetamido-D-glucose (or N-acetyl-D-glucosamine), insoluble in common aqueous or organic solvents, whose deacetylation leads to chitosan[1, 2] shown on Figure S-1.



**Fig. S-1** Chemical structure of a chitosan chain with a degree of polymerization DP and a degree of acetylation DA. Chitin corresponds to DA=1. Note that a chitosan sample contains a wide distribution of chains with different DP and different DA values. The glucosamine and N-acetyl glucosamine units are not arranged in a block copolymer structure as could be inferred from the figure, but have a more complex arrangement along the polymer chain (see introduction of the manuscript). The numbers from 1 to 6 are used for the signal assignment of the NMR spectra shown in Fig. S-2 and S-3

Chitosan samples were purchased from Aldrich, Sydney, Australia (medium molar mass, MedMW, batch number 03318AJ), and from AK Biotech, Jinan, China (samples AKbioV2, AKbioV3, AKbioD1, AKbioD2, AKbioD3), and were used as received. A further chitosan sample, chitAL, was obtained from Mahtani Chitosan, Veraval, India and was deacetylated using a previously published method [3].

**Table S-1** Characteristics of investigated chitosan samples

Sample	$DA^{\text{supp}}$ (%) <sup>a</sup>	$Visc^{\text{supp}}$ (cps) <sup>b</sup>	$DA_n^{\text{NMR}}$ (%) <sup>c</sup>	$DA_n^{\text{NMR, RT}}$ (%) <sup>d</sup>	$DA_w$ (%) <sup>e</sup>	$D^f$	$C_{\text{inj}}$ (mg.mL <sup>-1</sup> ) <sup>g</sup>
MedMW	20	590 <sup>h</sup>	15.5	16.7	18.7	1.21	1.5
AKbioD1	13.9	35 <sup>i</sup>	19.8		23.6	1.19	1.4
AKbioD2	9.8	55 <sup>i</sup>	13.6		16.5	1.21	1.2
AKbioD3	4.4	55 <sup>i</sup>	3.5		4.3	1.24	1.3
AKbioV2	14	320 <sup>i</sup>	18.7	16.9	22.4	1.20	1.4
AKbioV3	14	570 <sup>i</sup>	16.5		19.8	1.20	1.5
chitAL	2.5		3.2	2.7	4.0	1.24	1.3

<sup>a</sup> Average degree of acetylation (DA) as given by the supplier, analytical method not specified. <sup>b</sup> Viscosity as given by the supplier. <sup>c</sup> Number-average DA measured in this work with <sup>1</sup>H NMR at 90 °C. <sup>d</sup> Number-average DA measured in this work with <sup>1</sup>H NMR at 25 °C. <sup>e</sup> Weight-average DA determined in this work from  $DA_n^{NMR}$  and Equation (1). <sup>f</sup> Dispersity calculated as the ratio of  $DA_w$  to  $DA_n^{NMR}$ . <sup>g</sup> Injection concentration used in the CE experiments. <sup>h</sup> Viscosity of 1% chitosan solution in a 1% acetic acid solution. <sup>i</sup> Viscosity measured at 20 °C.

## Quantitative <sup>1</sup>H solution-state NMR measurements

### Background:

A high measurement temperature (70 °C to 90 °C) yields a higher resolution [4], while room temperature yields shorter measurement time and could be less damaging to the sample [5].

### Experimental conditions

The samples were dissolved at 1 to 1.5 g·L<sup>-1</sup> in 50 mM DCl in D<sub>2</sub>O (same concentrations as in Table S-1) and heated at 65 °C for 45 min. Solution-state <sup>1</sup>H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at a Larmor frequency of 400 MHz for <sup>1</sup>H and equipped with a 5 mm BBO probe, using a 23 μs 90° pulse at 90 °C and a 21 μs 90° pulse at 25 °C. Quantitative <sup>1</sup>H NMR spectra were recorded with a repetition delay of 10 s at 90 °C and of 6 s at 25 °C (see below for the determination of these repetition delays). At least 64 transients were accumulated. The chemical shift scale was calibrated with the HOD signal at 4.72 ppm at 25 °C and at 4.10 ppm at 90 °C [6]. Spectra were processed with Topspin 3.1 (Bruker Biospin).

Care was taken to ensure that the magnetization was fully recovered between pulses for all signals of interest, in order to determine true relative signal integrals [7]. To ensure full magnetization recovery between pulses, <sup>1</sup>H NMR spectra must be recorded with a delay between pulses longer than 5 times the longitudinal relaxation time ( $T_1$ ) for each signal of interest. One-dimensional inversion-recovery  $T_1$  relaxation measurements (1D  $T_1$ ) were conducted, each with a single delay in the ‘indirect dimension’, and corresponding delays between pulses of  $5 \times 1.44$  times that value. Phasing the spectra the same way as a conventional <sup>1</sup>H spectrum recorded in the same conditions, each signal is negative for short delay values, positive for long delay values, with a zero crossing occurring at  $T_1 / 1.44$ .

At 90 °C, delays ranged from 1.042 to 3.47 s. for a delay of 1.389 s, the chitosan signals of interest changed from negative to positive, and thus their  $T_1$  relaxation time is less than  $1.389 \times 1.44 = 2$  s. Measuring all subsequent <sup>1</sup>H solution-state NMR spectra at room temperature with a repetition delay of than  $5 \times 2 = 10$  s thus ensured that full magnetization recovery took place and the <sup>1</sup>H spectra were quantitative.



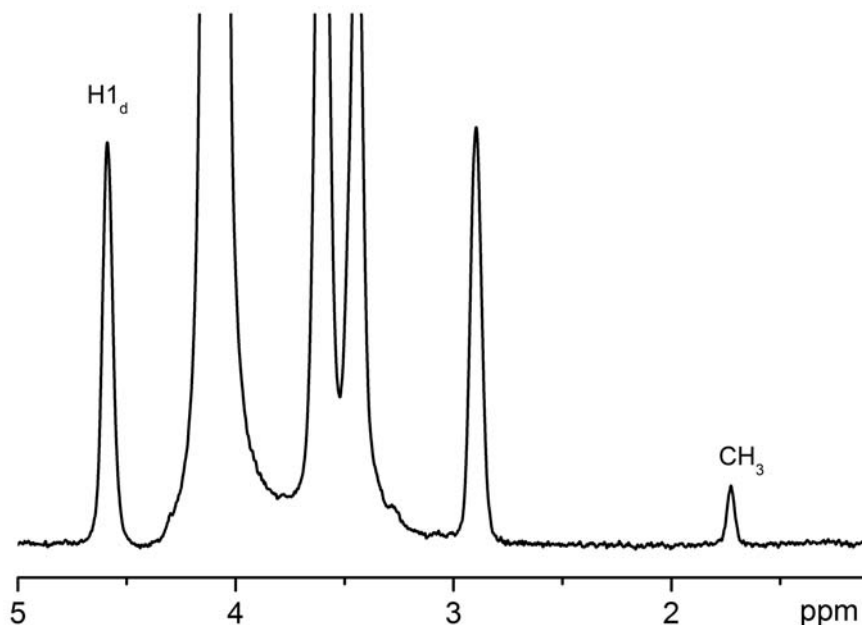
At room temperature, delays ranged from 0.695 to 1.042 s. For a delay of 0.833 s, the chitosan signals of interest changed from negative to positive, and thus their  $T_1$  relaxation time is less than  $0.833 \times 1.44 = 1.2$  s. Measuring all subsequent  $^1\text{H}$  solution-state NMR spectra at room temperature with a repetition delay of than  $5 \times 1.2 = 6$  s thus ensured that full magnetization recovery took place and the  $^1\text{H}$  spectra were quantitative.

## Determination of average DA from $^1\text{H}$ solution-state NMR measurements

For the quantitative  $^1\text{H}$  solution-state NMR spectra recorded at  $90^\circ\text{C}$ , the number-average DA was calculated from Equation (S-1):

$$DA_n^{NMR}(\%) = \frac{100 \times I(\text{CH}_3) / 3}{I(\text{H1}_d) + I(\text{CH}_3) / 3} \quad (\text{S-1})$$

where  $I(\text{CH}_3)$  is the integral of the signal of the methyl group of the acetyl group at 1.7 ppm (corresponding to 3 protons of the acetylated units only), and  $I(\text{H1}_d)$  is the integral of the signal of the anomeric proton of the deacetylated unit at 4.6 ppm (corresponding to one proton of the deacetylated units only). A typical spectrum is shown on Fig. S-2.

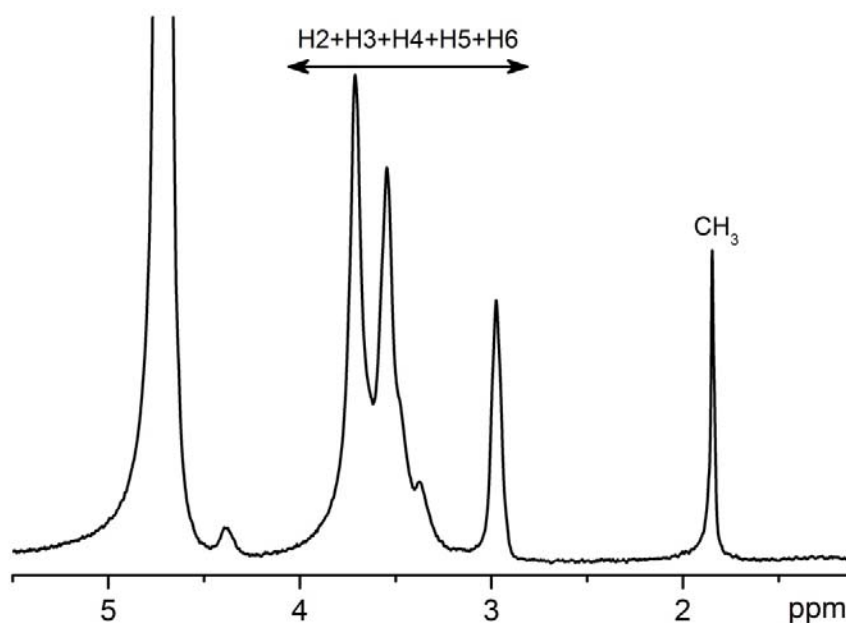


**Fig. S-2** Typical quantitative  $^1\text{H}$  NMR spectrum of chitosan at  $90^\circ\text{C}$  (sample AKbioV3 is shown)

For the quantitative  $^1\text{H}$  solution-state NMR spectra recorded at 25 °C, the average DA was calculated from Equation (S-2):

$$DA_n^{NMR,RT}(\%) = \frac{100 \times I(\text{CH}_3) / 3}{I(\text{H2} + \text{H3} + \text{H4} + \text{H5} + \text{H6}) / 6} \quad (\text{S-2})$$

where  $I(\text{CH}_3)$  is the integral of the signal of the methyl group of the acetyl group at 1.8 ppm (corresponding to 3 protons of acetylated units only), and  $I(\text{H2} + \text{H3} + \text{H4} + \text{H5} + \text{H6})$  is the integral of the signals of the protons H2 to H6 between 2.8 and 4.2 ppm (corresponding to 6 protons of all units, see Fig. S-1 for assignment). A typical spectrum is shown on Fig. S-3.



**Fig. S-3** Typical quantitative  $^1\text{H}$  NMR spectrum of chitosan at 25 °C (sample AKbioV2 is shown)

$DA_{ns}$  measured at 25°C and 90 °C are shown in Table S-1. The relative experimental error SD originating solely in the limited signal-to-noise ratio was calculated from Equation (S-3) [8], where SNR is the signal-to-noise ratio for the  $\text{CH}_3$  signal [8]:

$$SD(\%) = \frac{238}{SNR^{1.28}} \quad (\text{S-3})$$

It was estimated to about 0.2 for all measurements. The trend of the average DA values is the same at both temperatures; however, in some instances the difference between the values measured at both temperatures is significantly larger than twice 0.2. This can be explained by the fact that the experimental error does not only

originate in limited signal-to-noise ratio, but could also result from imprecise data treatment (phase correction, baseline correction) or imprecise integration due to overlap of the signals of interest with other signals. The robustness of phase correction and baseline correction was checked through the obtainment of identical results by two different operators. Upon closer examination, the spectra recorded at 90 °C showed resolution to the baseline for both integrated signals at 1.7 and 4.6 ppm (see Fig. S-2), while on the spectra recorded at 25 °C, there is not a complete return to the baseline between the massif integrated from 2.8 to 4.2 ppm, and the strong residual solvent signal at 4.7 ppm (see Fig. S-3), leading to a significant error which cannot be quantified. It is concluded that while both measurements are precise, the high-temperature measurement is more accurate. The possible sample degradation at high temperature is considered negligible as the total measurement time at 90 °C was about 15 minutes (including parameters optimization). Note that the time gained by measuring at 25 °C was less than a factor of 2. It was decided to measure all samples by quantitative <sup>1</sup>H NMR at 90 °C, to yield the most accurate and precise DA values for all the samples,  $DA_n^{\text{NMR}}$ , and to use that average DA value in this work.

## Number-average versus weight-average degree of acetylation

Usual methods used to quantify the DA in chitosan, namely FT-IR and NMR spectroscopy, rely on the detection of individual monomer units via their relative numbers. Therefore they measure a number-average degree of acetylation  $DA_n$  on the whole sample (not related to single polymer chains, but to the monomer units):

$$DA_n = \frac{n_A}{n_A + n_D} \quad (\text{S-4})$$

where  $n_A$  is the number of acetylated monomer units,  $n_D$  is the number of deacetylated monomer units.

The weight-average degree of acetylation  $DA_w$  of the whole sample can be defined as:

$$DA_w = \frac{n_A \cdot M_A}{n_A \cdot M_A + n_D \cdot M_D} \quad (\text{S-5})$$

where  $M_A$  is the molar mass of an acetylated monomer unit,  $M_D$  is that of a deacetylated monomer unit.

$$\text{(S-4) can be rearranged into } \frac{1}{DA_n} = 1 + \frac{n_D}{n_A} \quad (\text{S-6})$$

$$\text{from which follows } \frac{n_D}{n_A} = \frac{1}{DA_n} - 1 \quad (\text{S-7})$$

$$\text{(S-5) can be rearranged into } DA_w = \frac{1}{1 + \frac{n_D \cdot M_D}{n_A \cdot M_A}} \quad (\text{S-8})$$

$$\text{Combining (S-8) with (S-7): } DA_w = \frac{1}{1 + \left(\frac{1}{DA_n} - 1\right) \cdot \frac{M_D}{M_A}} \quad (\text{S-9})$$

$$\text{From which follows: } DA_w = \frac{1}{1 + \left(\frac{1-DA_n}{DA_n}\right) \cdot \frac{M_D}{M_A}} \quad (\text{S-10})$$

$$\text{And finally: } DA_w = \frac{DA_n \cdot M_A}{DA_n \cdot M_A + (1-DA_n) \cdot M_D} \quad (\text{S-11})$$

The molar mass of the monomer units are  $M_A = 203 \text{ g}\cdot\text{mol}^{-1}$  for the acetylated monomer unit and  $M_D = 162 \text{ g}\cdot\text{mol}^{-1}$  for the (positively charged) deacetylated monomer unit. Thus (S-11) can be re-written as:

$$DA_w = \frac{DA_n}{DA_n + 0.798(1-DA_n)} \quad (\text{S-12})$$

## **Critical conditions: Liquid chromatography at the critical point of absorption (LC-CC) and capillary electrophoresis in the critical conditions (CE-CC)**

Liquid chromatography at the critical point of adsorption (LC-CC)[9] is a powerful method to separate polymers according to their structure. It is mainly applied to copolymers (a polymer derived from more than one species of monomer), for example block copolymers[10], but LC-CC also applies to branched polymers[11, 12]. The critical conditions are a separation mechanism and refer to the absence of separation of a homopolymer by its molar mass. For example, critical conditions for a copolymer such as chitosan implies that chitosan homopolymers ( $DA=0$ ) with different molar masses all elute at the same time. The molar mass thus becomes effectively “invisible”. If copolymers based on chitosan were injected, the separation would then take place only according to the other comonomer. These critical conditions thus allow decomposing a complex problem in a series of simpler ones. The principle of the method is excellent and has attracted a lot of enthusiasm. LC-CC has however been typically plagued by low sample recovery and critical conditions that are very tedious to establish and maintain.[13, 14] On the contrary, we have shown that critical conditions in free-solution CE are very robust and that capillary electrophoresis in the critical conditions (CE-CC) can be used to investigate colloidal systems in which molecules are aggregating in solution, as is the case of the polysaccharide gellan gum.[15] CE-CC allows to determine the electrophoretic mobility, which is the proportionality constant between the velocity of the molecule under an electric field and this electric field. The electrophoretic mobility of a polyelectrolyte is independent of its molar mass[16-18] (“critical conditions”), but changes with the structure of the polyelectrolyte (such as copolymer composition, or branched structure). Critical conditions in CE do not correspond to free-draining as defined by Flory [19], but rather to a quasi-parallel increase of the nominal charge and the friction with the degree of polymerization [20, 21]. It applies not only to chitosan but also to polyelectrolyte architectures in general.[15, 22]

## Capillary electrophoresis: experimental part and determination of mobilities

CE experiments were carried out using an HP<sup>3D</sup> (Hewlett Packard, now Agilent Technologies, Australia) and an Agilent 7100 CE systems (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD). Polyimide-coated fused silica capillaries (50  $\mu\text{m}$  inner diameter, 360  $\mu\text{m}$  outer diameter, from Polymicro, USA) were used. Fused silica capillaries thermostated at 25  $^{\circ}\text{C}$  had a total length of 64 cm (55.5 cm effective length) or 60 cm (51.5 cm effective length) or 40 cm (31.5 cm effective length). A fused silica capillary with extended light-path detection window (Agilent) was also used (64 cm total length). The capillary was pretreated by flushing for 10 min with 1 M NaOH, for 5 min with 0.1 M NaOH, MQ water and 200 mM borate buffer. Three different running buffer concentrations corresponding to 50, 100 and 500 mM were tested. A 500 mM concentration results in too high currents around 290  $\mu\text{A}$ , leading to unstable baselines and currents. Concentrations of 50 mM and 100 mM resulted in high but reasonable currents around 100  $\mu\text{A}$  for capillaries total length ( $L_t$ ) of 40 cm and 60 cm, respectively. For capillaries with  $L_t$  of 64 cm total length the current was around 103  $\mu\text{A}$ . Data analysis was carried out using the software *Origin 8*.

Equation (S-13) was used to calculate the apparent electrophoretic mobility  $m_{\text{app}}^i$  (in  $\text{m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ ) [23].

$$m_{\text{app}}^i = \frac{v_i}{E} = \frac{L_d \cdot L_t}{t_m \cdot V} \quad (\text{S-13})$$

where  $v_i$  is the velocity,  $E$  is the electric field,  $L_d$  is the length of capillary to the detector (m),  $L_t$  is the total length of the capillary (m),  $t_m$  is the migration time (s), and  $V$  is the electric field (V).

Equation (S-14) was used to calculate the electroosmotic mobility  $m_{\text{eof}}$ :

$$m_{\text{eof}} = \frac{L_t \cdot L_d}{V \cdot t_{\text{eof}}} \quad (\text{S-14})$$

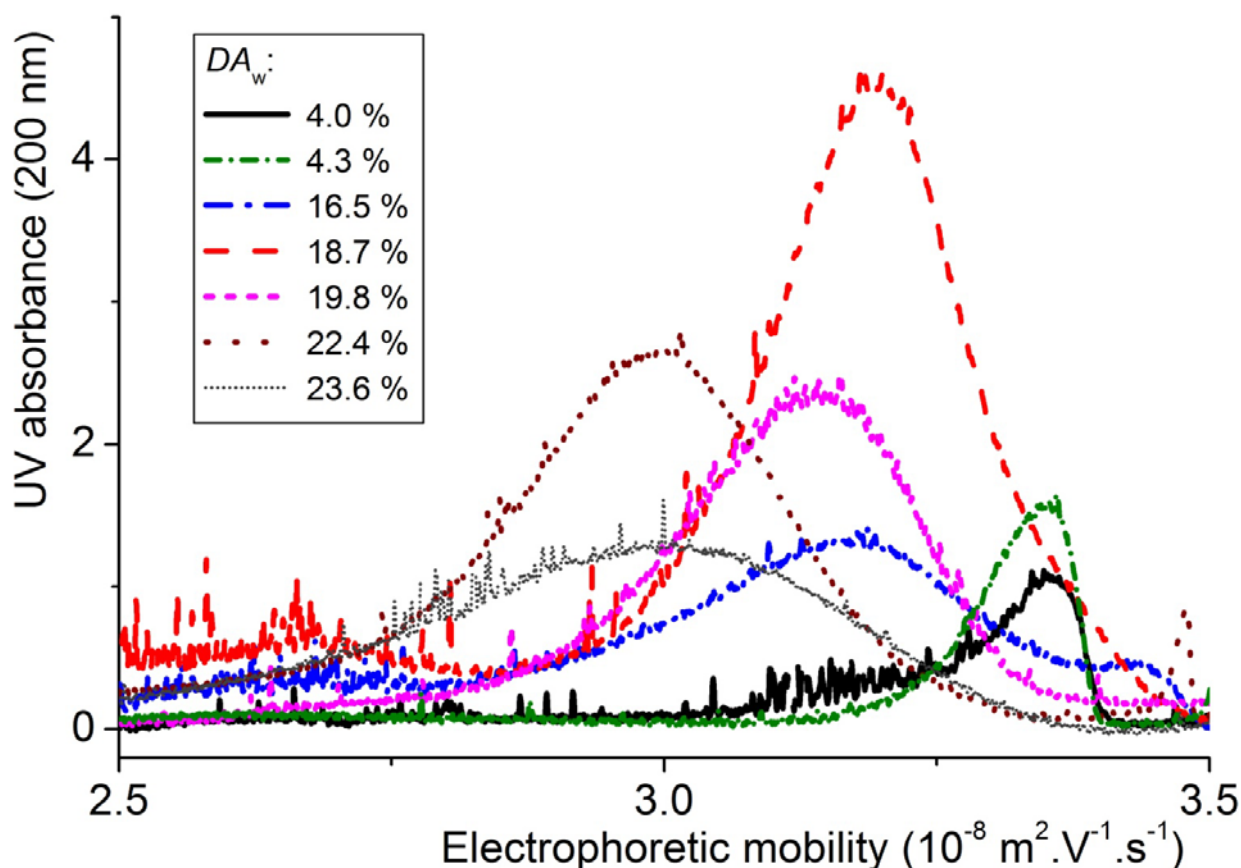
where  $t_{\text{eof}}$  is the migration time of the electroosmotic flow.  $t_{\text{eof}}$  was measured to be 40 min.

Electrophoretic mobilities are presented rather than migration times for two reasons. First the separation is according to the electrophoretic mobility, and electrophoretic mobility can be used to understand and calibrate the separation [24]. Second, the electrophoretic mobility is a molecular property of the samples while migration time is not [25].

## Separation by degree of acetylation by capillary electrophoresis

Free-solution CE proved successful without derivatization to determine chitosan [26], to separate chitosan from conjugates [27], to demonstrate successful chemical modification [28], or separate oligomers according to their DP [29] (oligomers were also separated using indirect detection [30] or after derivatization [31, 32] in CE or earlier by polyacrylamide gel electrophoresis [33]). Fused-silica capillaries were used since they proved to lead to no significant chitosan adsorption onto their surface at low pH [26] (although adsorption has been reported for oligomers [30]). Phosphate buffer at pH 2 was used previously to determine the amount of chitosan [27]. In this work, we do not only want to determine chitosan but also to separate it by its DA. A pH of 2 was not used to avoid degradation. A phosphate buffer at pH 3.01 was chosen for separations, as it protonates the amine groups of chitosan molecules and it is transparent in UV contrary to acetic acid, which is widely used as a mobile phase in LC [34]. Electrophoretic mobilities are the same at pH 3 and 3.5 indicating complete ionization of the chitosan at pH 3 [30].

The separation by DA presented in Fig. 1 was successfully reproduced by another operator using another capillary (conventional instead of high sensitivity) and another CE instrument (HP<sup>3D</sup> instead of 7100), shown on Fig. S-4. The same migration order with DA was observed.



**Fig. S-4** Separation of chitosan samples by their DA with CE using HP<sup>3D</sup> CE. Samples AKbioD1, AKbioV2, AKbioV3, MedMW, AKbioD3, AKbioD2 and chitAL are shown

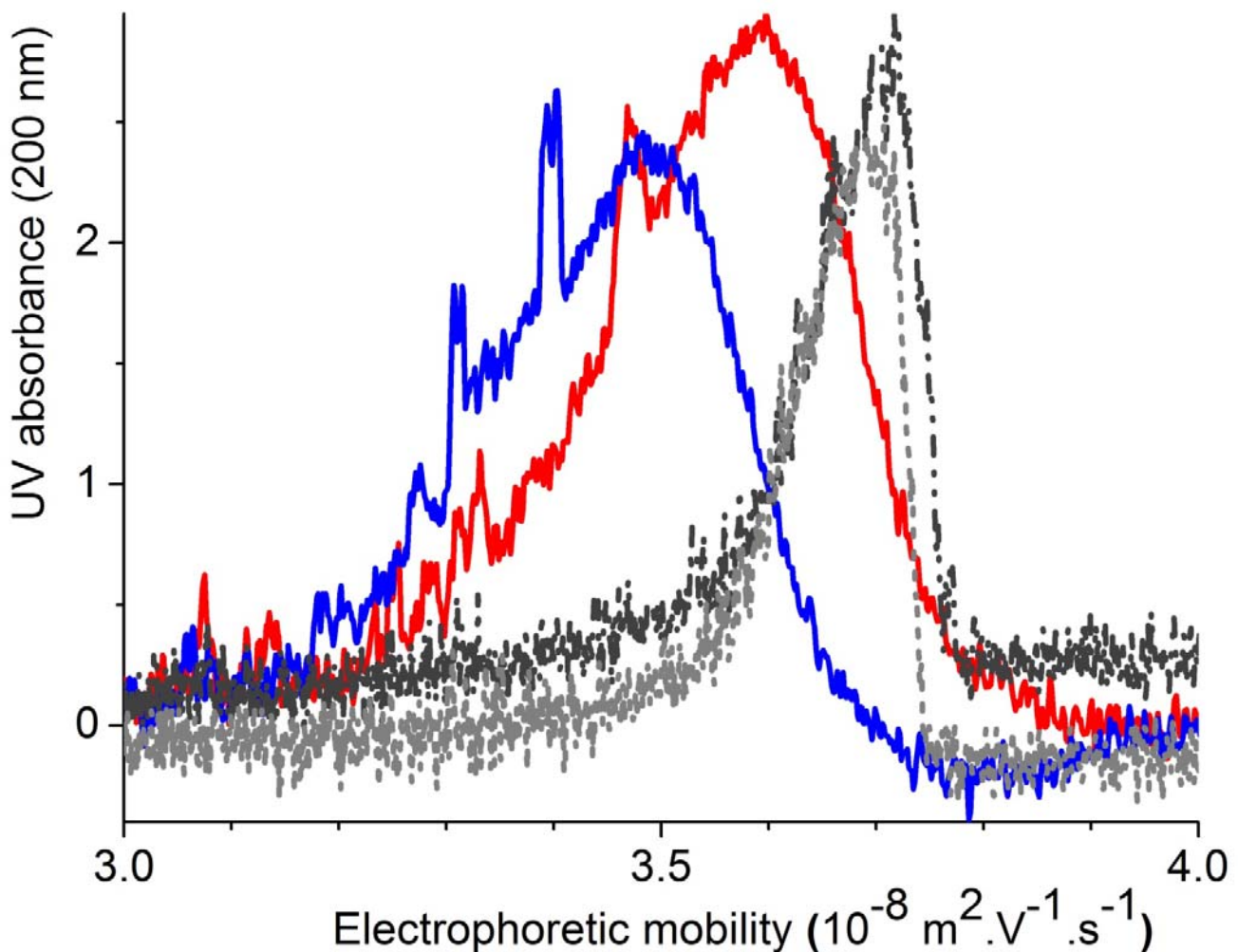
The electroosmotic flow and the electrophoretic mobility are in the same direction. Comparing the different samples, it is clear that higher degrees of acetylation correspond to lower apparent mobilities. The electroosmotic mobility was measured around  $5 \cdot 10^{-9} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$  (See Equation S-14) which is one order of magnitude lower than the apparent mobility. It is constant and it is negligible in a first approximation compared to the electrophoretic mobility. The apparent mobility is thus considered in this work to be equal to the electrophoretic mobility of chitosan in a first approximation (an internal standard could be added to improve the precision of the electrophoretic mobility values).

A shoulder is observed on the high mobility side of the electropherogram of the chitosan sample with an average DA 16.5 %. This might be due to some limited aggregation. Chitosan is known to be prone to aggregate in solution.[35]

Guillotin and coworkers [24] characterized high methyl-esterified pectins by their charge density and concluded that the electrophoretic mobility increases with the pectin charge. The electrophoretic mobility has been empirically observed to be inversely proportional to the degree of substitution of pectins (the migration time has been observed to linearly correlate with the degree of substitution) [36]. In the case of chitosan, the correlation of the inverse electrophoretic mobility with either  $DA_n$  or  $DA_w$  (Fig. 2a) leads to similar correlation coefficients, respectively 0.84 and 0.83. These correlation coefficients are poorer than the ones for correlation of the mobility with the  $DA$ s (Fig. 2bS-5).

## Repeatability of the capillary electrophoresis separations

A good repeatability was obtained when using a 5 min wash with 10 mM phosphoric acid before each injection (See Fig. S-5): the mobility values are poorly repeatable without the 5 min wash with 10 mM phosphoric acid. Two capillaries were tested corresponding to the total length ( $L_t$ ) of 40 cm (31.5 cm effective length) and 60 cm (51.5 cm effective length). The short capillary exhibited good repeatability and faster migration time and mobility while good repeatability was obtained using the long capillary with longer migration time and lower mobility. Washing 60 cm capillary with phosphoric acid has improved repeatability dramatically in contrast to 40 cm capillary which was not washed previously (Fig. S-5).

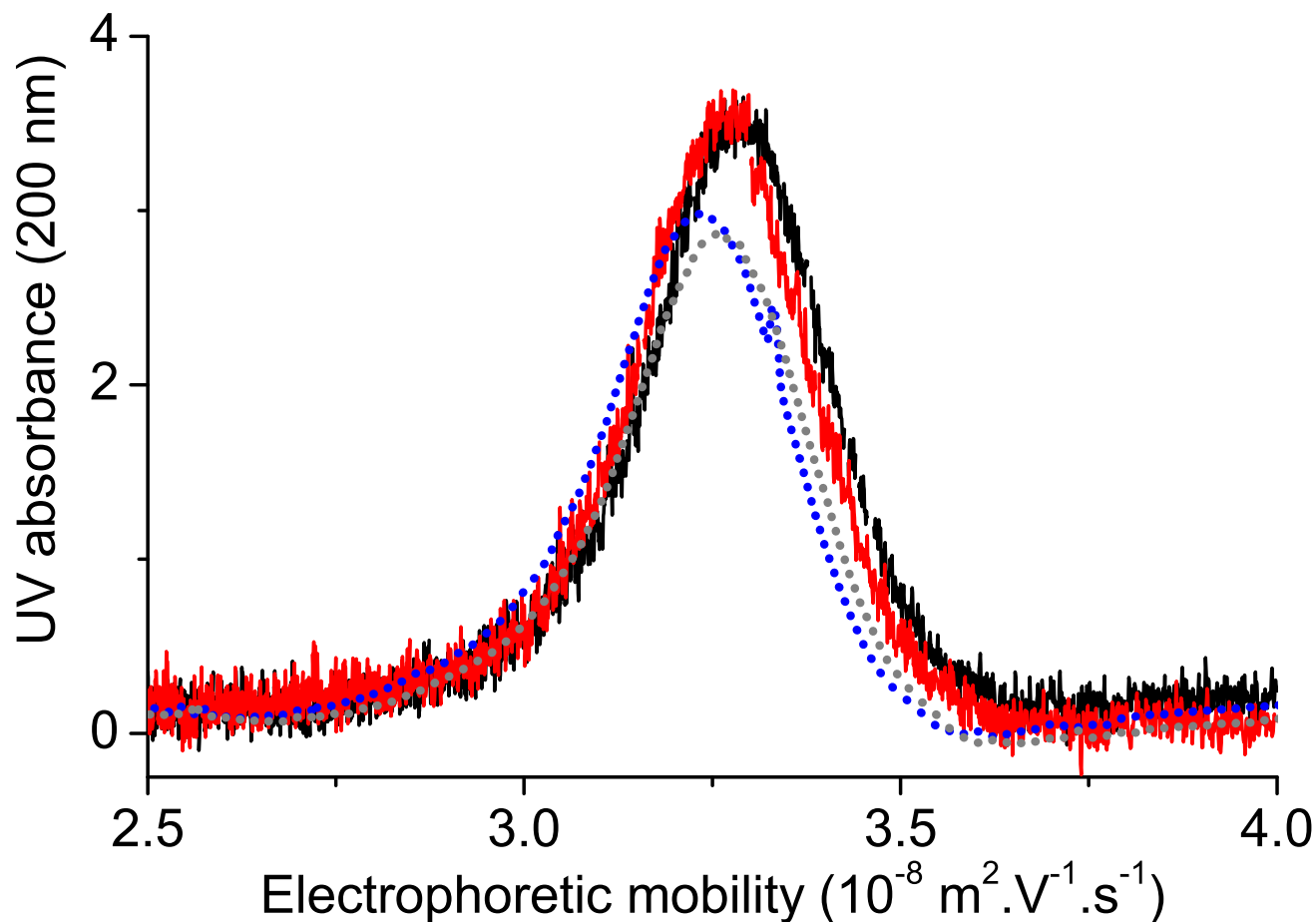


**Fig. S-5** The repeatability of the separation of chitosan sample AKbioD3 ( $DA_w = 4.3\%$ ) using two different capillaries of total length ( $L_t$ ), 40 cm (blue and red solid lines) and 60 cm (dark grey and grey dashed lines), respectively without (blue and red) and with (black and grey) preconditioning with phosphoric acid

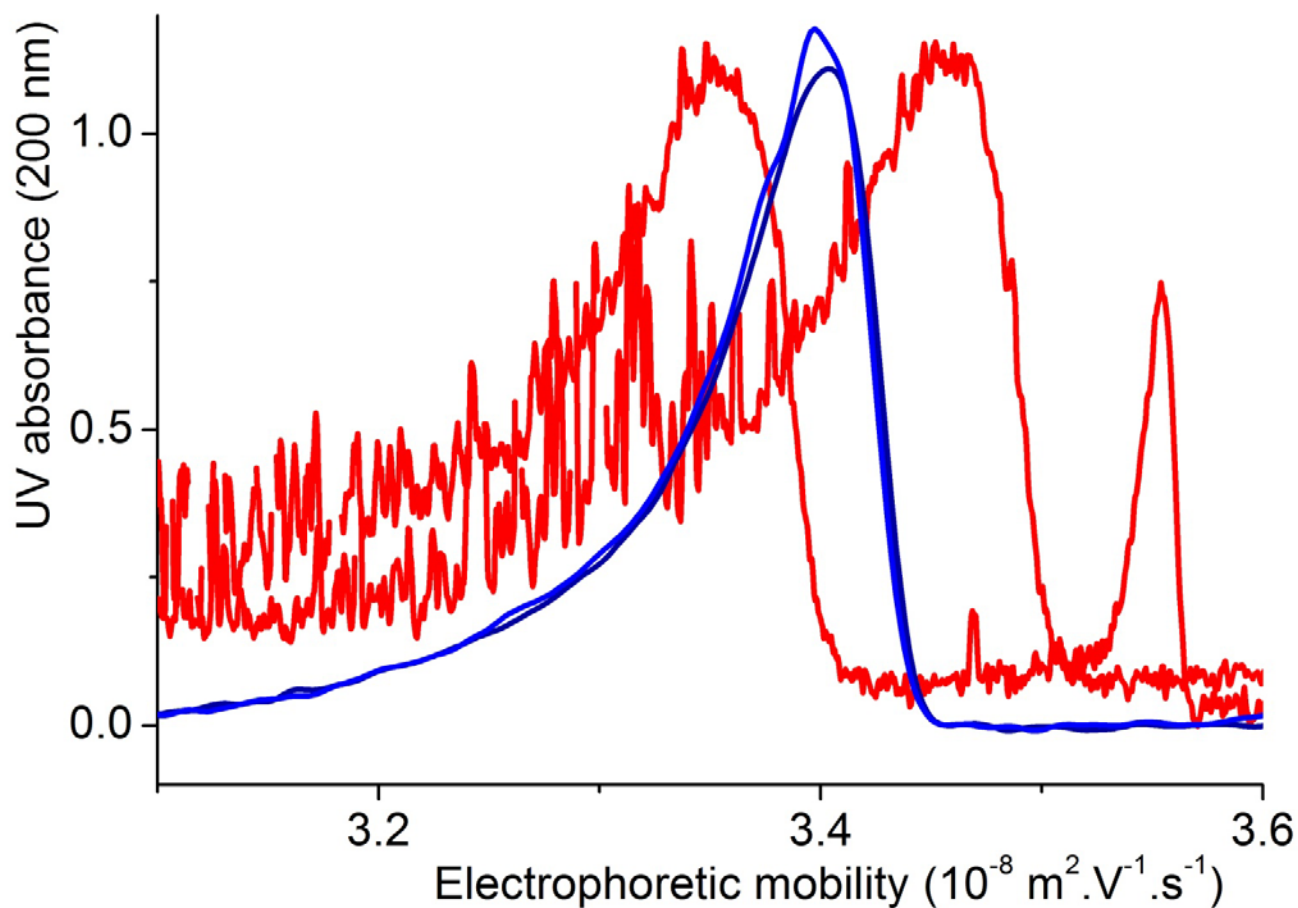


## Reproducibility

The reproducibility of this separation method is also good as shown on Fig. S-6 in the case of sample AKbio V2 ( $DA_w$  of 22.4 %) using two capillary electrophoresis instruments operated by different analysts on different days, employing different capillaries (see also Fig. S-7).



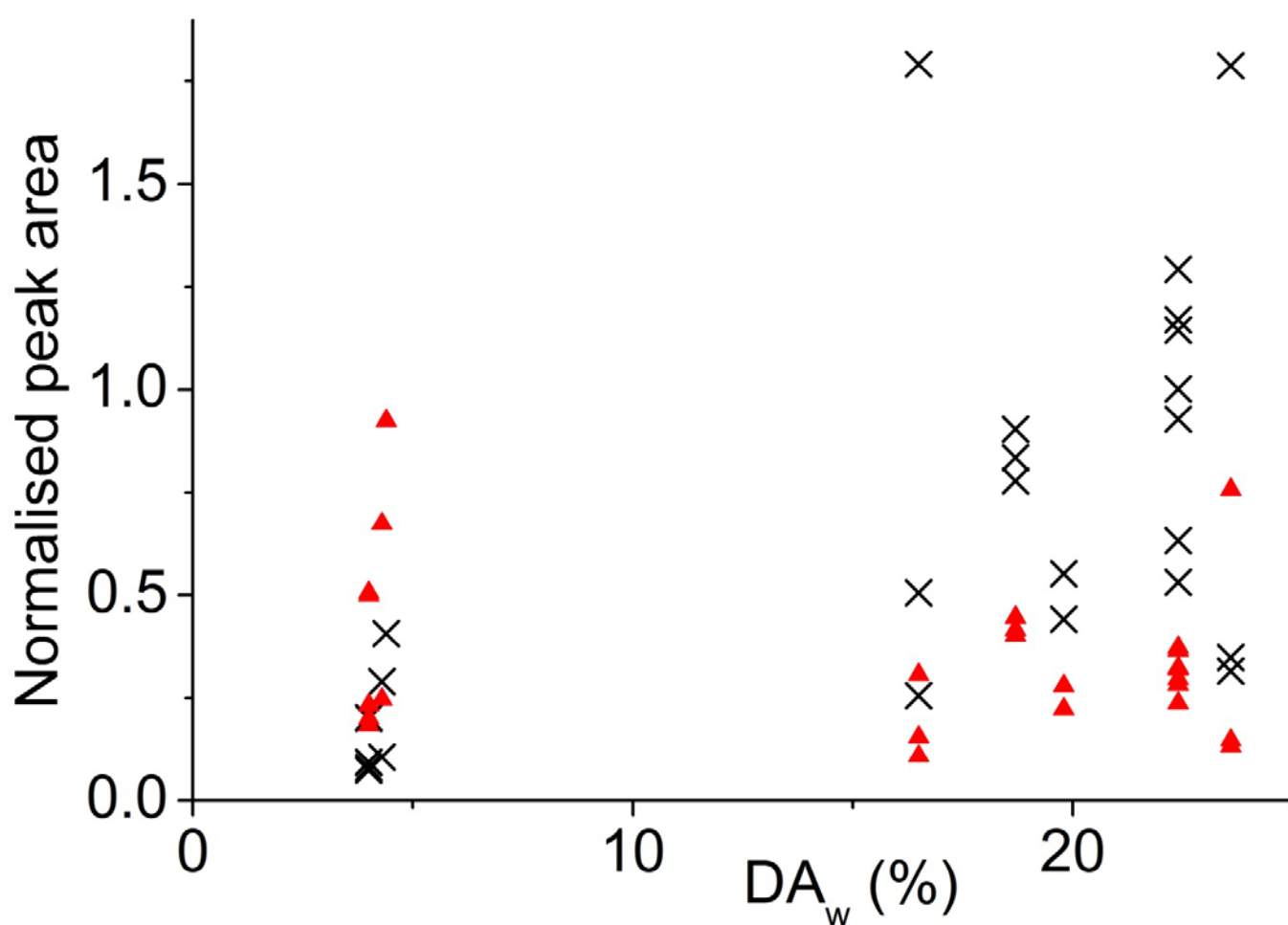
**Fig. S-6** Reproducibility of the CE separation method studied by different analysts on HP<sup>3D</sup> (black and red solid lines) and Agilent 7100 CE (blue and grey dotted lines) systems using capillaries with  $L_t$  of 64 and 60 cm. All injections were hydrodynamic (30 mbar), injection time was 10 s



**Fig. S-7** Reproducibility of the separation of chitosan chitAL ( $DA_w^{NMR} = 4.0\%$ ). Samples were previously prepared by two analysts, then injected at  $0.98\text{ g L}^{-1}$  using HP<sup>3D</sup> CE with conventional capillary and buffers (red lines) or using 7100 CE with high sensitivity capillary (blue lines)

## Influence of the degree of acetylation on the detection sensitivity

The samples were all injected with the same injection volume, in similar concentrations ranging from 1.2 to 1.5 mg·mL<sup>-1</sup>, but they strongly differ by their DAs, ranging from 4 to 23.6 % (see Table S-1). The observed peak areas for the different samples were plotted as normalized with the sample concentration, and showed no constant behaviour or systematic variation (see Figure S-8). The UV detection is at 200 nm. At this wavelength, it is expected that the acetyl group of the acetylated monomer units are predominantly detected. Therefore, the detection is expected to exhibit a higher sensitivity for the chains with a higher DA. The observed peak areas for the different samples were plotted as normalized with the DA (and with the sample concentration), and a smaller scatter of the data was observed (see Figure S-8). This is in agreement with a higher detection sensitivity at 200 nm for the polymer chains with a higher DA.



**Fig. S-8** Normalised peak areas observed on the electropherograms of chitosan samples (when plotted as a function of electrophoretic mobility in  $\text{m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ ): peak areas normalized with respect to the injection concentration in  $\text{mg} \cdot \text{mL}^{-1}$  (black crosses, values multiplied by  $10^8$ ) and peak areas normalized with respect to  $DA_w$  and to the injection concentration in  $\text{mg} \cdot \text{mL}^{-1}$  (red triangles, values multiplied by  $10^9$ )

## Counter-ion condensation and DA

The Bjerrum length,  $l_B$ , is calculated as [37]:  $l_B = \frac{e^2}{4\pi\epsilon_0\epsilon_r k_B T}$  (S-15)

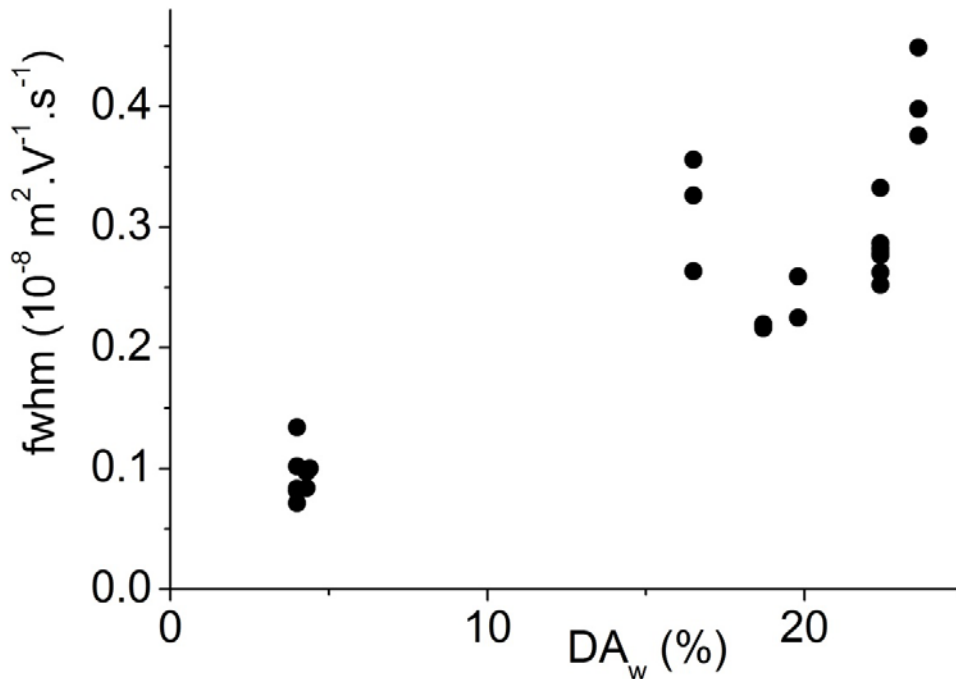
where  $e$  is the elementary charge, taken as  $1.60 \times 10^{-19}$  C,  $\epsilon_0$  is the vacuum permittivity, taken as  $8.85 \times 10^{-12}$  F·m<sup>-1</sup>,  $\epsilon_r$  is the dielectric constant taken as 80 (water),  $k_B$  is the Boltzmann constant taken as  $1.38 \times 10^{-23}$  m<sup>2</sup>·kg<sup>-1</sup>·s<sup>-2</sup>, and  $T$  is the temperature taken as 298 K.

It leads to:  $l_B = 7.0 \text{ \AA}$  (S-16)

If ones assumes a distance between monomer units of 8.5 Å on average for glucosamine and N-acetyl glucosamine, and a typical DA of 17 % (charge density of 83 %), then the distance between charges is 7.1 Å. This is thus consistent with counter-ion condensation for DA below 17 %.

## Estimation of the dispersity of the distribution of DAs

One aim of the CE-CC method is to allow a separation of the chitosan samples to reveal the dispersity of the distribution of degrees of acetylation for the first time. The dispersités of the distributions of DAs shown on Figure 1b were estimated through the full width at half maximum, fwhm (Figure S-9). The samples with higher average DA values exhibit a higher dispersity of their distribution of DAs.



**Fig. S-9** Full width at half maximum, fwhm, observed on the electropherograms of chitosan samples (when plotted as a function of electrophoretic mobility in m<sup>2</sup>·V<sup>-1</sup>·s<sup>-1</sup>)

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