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Note

Limitations of pH-potentiometric titration for the determination of the degree of deacetylation of chitosan

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Abstract—The degree of deacetylation (DDA) of chitosan determines the biopolymer's physico-chemical properties and technological applications. pH-Potentiometric titration seems to offer a simple and convenient means of determining DDA. However, to obtain accurate pH-potentiometric DDA values, several factors have to be taken into consideration. We found that the moisture content of the air-dry chitosan samples can be as high as 15%, and a reasonable fraction of this humidity cannot be removed by ordinary drying. Corrections have to be made for the ash content, as in some samples it can be as high as 1% by weight. The method of equivalence point determination was also found to cause systematic variations in the results and in some samples extra acid as high as 1 mol % of the free amino content was also identified. To compensate for the latter effect, the second equivalence point of the titration has to be determined separately and the analytical concentration of the acid be corrected for it. All the corrections listed here are necessary to obtain DDA values that are in reasonable agreement with those obtained from ^{1}H NMR and IR spectroscopic measurements. The need for these corrections severely limits the usefulness of pH-metry for determining accurate DDA values and thus potentiometry is hardly able to compete with other standard spectroscopic procedures, that is, ^{1}H NMR spectroscopy.

Keywords: Chitosan; Chitin; Polysaccharides; pH-Potentiometry; Titration; ^{1}H NMR spectroscopy; IR spectroscopy; Thermogravimetry; Degree of deacetylation

Chitosan is a derivative of one of the most abundant glycans, chitin, a member of the family of β-(1→4)-linked polysaccharides. Chitosan is obtained by partial (or in some rare cases complete) deacetylation of chitin and consists of D-glucosamine (D-GlcN) and *N*-acetyl-D-glucosamine (D-GlcNAc) monomeric units.[1] The most important parameter for characterising a given chitosan specimen is the degree of deacetylation (DDA), which reflects the balance between the two kinds of monomeric residues and is defined as

$$\mathrm{DDA} = 100\frac{n_{\text{D-GlcN}}}{n_{\text{D-GlcN}} + n_{\text{D-NGlcNAc}}} \quad (1)$$

where $n_{\text{D-GlcN}}$ and $n_{\text{D-GlcNAc}}$ represent the average number of D-GlcN and D-GlcNAc units within the macromolecule, respectively. The DDA influences the physical, chemical and biological properties of chitosan, such as acid–base and electrostatic characteristics,[2] biodegradability,[3] self-aggregation,[4] sorption properties,[5] and the ability to chelate metal ions.[6]

The DDA allows the definition of the terms chitosan and chitin, that is, chitosan is usually defined as the derivative that is soluble in dilute acidic solutions. The lowest DDA corresponding to chitosan varies in the literature and ranges from 40%,[2] through 50%[7] up to 60%.[8] The majority of the commercial chitosan samples has average DDA's of 70–90%, and their DDAs are always lower than 95%.[5] For some special biomedical applications, chitosan with DDA of >95% may be prepared via further deacetylation steps, which not only increases the cost of the preparation, but also often results in partial depolymerization.[9]

Accurate determination of DDA is one of the key issues of chitin chemistry. Over the last 50 years, several methods have been employed including IR-spectroscopy,[7,10]

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^{1}H NMR spectroscopy,[11–14] pH-potentiometric titration,[15–17] UV-spectroscopy,[6,18] CD-spectroscopy,[19] colloidal titration,[20] and enzymatic methods.[21] The most common methods are IR and NMR spectroscopy and pH-potentiometric titration.

The advantage of IR spectroscopy is that it is rapid, does not require aqueous solutions, is suitable for samples with any DDA and that it is relatively insensitive to most inorganic and organic impurities. It suffers, however, from systematic errors associated with the arbitrary definition of the baseline and more importantly the need of extremely well dried samples. The best achievable accuracy of DDA measurements, even with the most carefully controlled experimental conditions, is around ±2%. ^{1}H NMR method is mainly used for water soluble chitin derivatives; there are, however, some successful attempts of using solid state CP/MAS NMR for this purpose.[12,22,23] The best achievable accuracy of the NMR technique (determined by the accuracy of peak integration) is usually in the range of ±1%, which makes it superior to IR; however, the relatively high costs associated with the technique somewhat limit its applicability for serial measurements.

pH-Potentiometric titration, suggested first by Broussignac,[15] is one of the simplest methods and it has been used by the chitosan industry for many years due to its low reagent and equipment cost. With relatively simple instrumentation and evaluation techniques, by using pH-potentiometry, in general, the concentrations of acids and bases can routinely be determined with a precision of ±0.5%, but with further polishing even ±0.2% can be achieved. During chitosan titration, the biopolymer is dissolved in a known excess of HCl and the solution is then titrated with NaOH, while the pH of the solution is monitored with a calibrated pH-sensitive glass electrode. The procedure results in a titration curve with two inflexion points: the first corresponds to the excess of HCl, while the second to the protonated chitosan. The difference between the two inflexion points yields the moles of H^+ required for the protonation of the free (deacetylated) amino groups and results in the amount of D-GlcN in the titrant solution. Assuming that the rest of the sample is D-GlcNAc, the DDA value of the specimen can readily be obtained. To derive reliable DDA values from these measurements, the chitosan has to be appropriately purified and dried prior to accurate weighing. Such sample pretreatments are often not feasible, in these cases for the non-chitosan impurities, like moisture or organic/inorganic impurities, the weighed polymer mass has to be corrected.

It is well documented in the literature that the DDA values determined for the same chitosan specimen were found to be almost always method dependent and very often unacceptably large variations (sometimes even tens of percents) are found between the results of various techniques. Certainly, such differences are associated with systematic errors, which distort the results of various techniques in a method dependent way. For example, it was stated that the results obtained from pH-potentiometric method were found to depend on the method of equivalence point determination,[16,17] and are always largely different from the data obtained from other techniques, that is, IR, ^{1}H NMR, first derivative UV-spectroscopies, and elemental analyses or nynhidrin test.[6,24] With pH-potentiometric titrations one would expect more reliable DDA's, in particular for specimens with DDA >85%.[25] Therefore, an investigation was undertaken to identify and assess the factors causing such systematic errors in the DDA values obtained from pH-potentiometric titrations. For this the DDA of a series of commercially available chitosan samples were determined by pH-potentiometric titrations, and, for comparison, by ^{1}H NMR and IR spectroscopic techniques. The measurements were supplemented by thermogravimetric analysis (to account for the moisture content of the samples) and ash content determination (to take into consideration the non-combustable impurities of the specimen).

Thermogravimetry. A typical thermogravimetric curve is shown in Figure 1 (lowest curve). The initial weight loss in the T <150 °C region is likely to be associated with the evaporation of the water in the samples. Based on this assumption, the moisture content of the various specimens has been calculated (Table 1) and, as can be seen, it can be as high as 15%, even though the samples appeared to be air dry.

As the water content is problematic for both pH-potentiometry and IR measurements, an attempt was made to completely eliminate the moisture content in the samples. For this, the samples were stored in vacuo, over P_2O_5 for several days. As can be seen, after 24 h of such drying, the sample still contained significant amount of moisture (ca. 2.6% by weight, middle curve

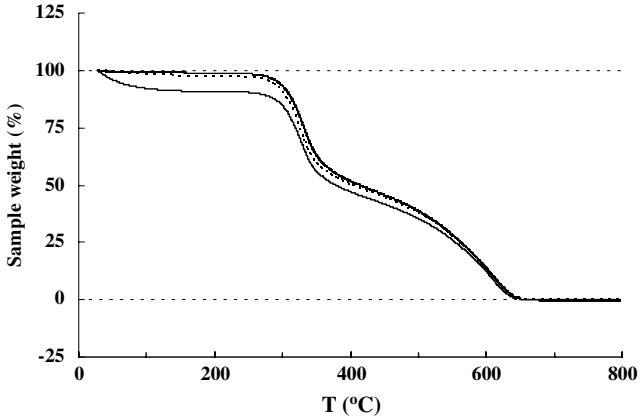

Figure 1. Thermogravimetric curves of chitosan sample no. 6, as received (lower curve) and after 1 and 6 days of drying (middle and upper curve, respectively); drying was performed at room temperature, in vacuo in a vacuum desiccator, over P_2O_5.

Table 1. Properties of the chitosan samples studied

Sample no.	Supplier	Description	Moisture content[a] (%)	Ash content[a] (%)
1	Sigma	Tech. grade	14.20	0.64
2	—	Sample no. 1 purified[b]	6.60	0.10
3	Aldrich	Tech. grade, high M_w	15.30	0.76
4	—	Sample no. 3 purified[b]	5.43	0.58
5	Fluka	Low viscosity	5.10	0.0
6	Fluka	Medium M_w	9.36	0.29
7	—	Sample no. 5 fully deacetylated[c]	6.03	0.08

[a] Determined thermogravimetrically for samples, as received.
[b] Purification performed according to Ref. 26.
[c] Deacetylation performed according to Ref. 9.

in Fig. 1), and even after six days of such treatment, the TG measurements still indicated about 0.8% by weight of water (upper curve in Fig. 1). It should be noted that the dried samples were exposed to the air only for a very short period of time (i.e., a few ten seconds in transferring them from the desiccator to the thermogravimetric instrument). Therefore, it is possible that the moisture content obtained (or some part of it) resulted from absorption of moisture in the air. Regardless of the origin, ordinary drying is not suitable for the complete elimination of hydrating water. It seems that accurate determination of moisture content is critical in particular for DDA determinations that aim at accuracies better than 1%. Given the hygroscopic nature of chitosan, to obtain highly accurate DDA values it seems vital always to couple pH-potentiometric measurements with thermogravimetric moisture determinations.

Ash content. The ash contents of the samples were also found to be significant, in particular for technical grade chitosan specimens (Table 1). However, its effect on the accuracy of DDA determinations is less than the moisture content, being always less than 1% by weight. Purification (i.e., repeated dissolution, filtration and precipitation) always decreased the ash content indicating that it is likely to be associated either with small amounts of insoluble organic matter or with some inorganic salts.

pH-Potentiometric titrations. Typical pH-potentiometric titration curves (three parallels) are shown in Figure 2. The usual reproducibilities of the measurements between the parallel runs were found to be within 1 mV in the well-buffered region. At around a 70% degree of titration, precipitation occurred in the solutions and in this range the response of the glass-electrode became sluggish. However, after the second equivalence point, a reasonably fast electrode response was achieved, again indicating that proton-exchange processes including chitosan are already over in this pH-range.

The average degree of deprotonation (i.e., the average number of protons bound by one deacetylated amine

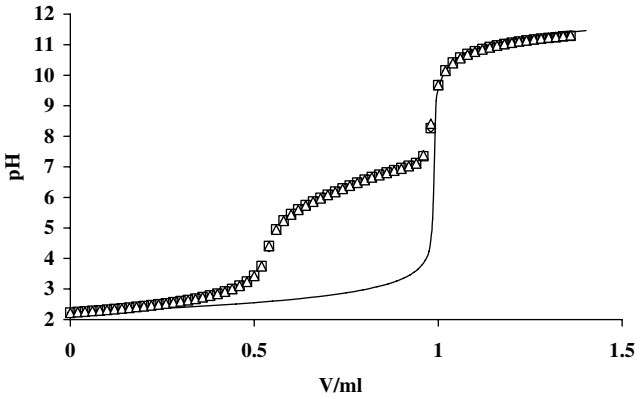

Figure 2. A typical set of pH-potentiometric titration curves of chitosan (sample no. 5), three parallel measurements, and the background acids' titration curve without the polymer. For experimental details, see text.

group of the molecule, H) as a function of pH has been calculated for all samples studied (Fig. 3). As it can be seen, the curves are practically superimposable at the $H \geqslant 0.3$ range, that is, in homogeneous systems, and they start to diverge only when the polysaccharide is already (at least partially) precipitated from the solution. Given the relatively narrow DDA range of the chitosan samples studied, in homogeneous solutions these functions are expected to be superimposable,[2] and the divergence observed at low degree of protonation is likely to be due to the presence of slowly deprotonating precipitate in the solution.

The two equivalence points on the titration curves were calculated by using derivative method (i.e., the location of the equivalence point equals to the maximum of the first derivative of the $\mathrm{pH} = f(V_{\mathrm{NaOH}})$ function) and Gran-type linearization, as described in the literature[16] (Table 2). As it is seen from the data of Table 2,

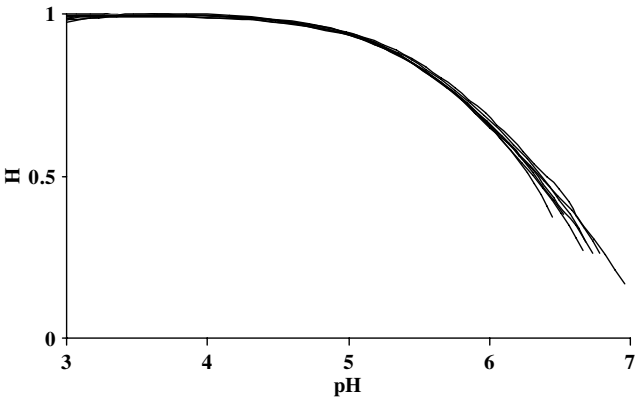

Figure 3. The average degree of protonation (H) of the various chitosan samples investigated as a function of the solution pH. The solutions become turbid in the range of $H = 0.2$–0.3, and the H versus pH functions have been plotted only up to the point of the appearance of visible precipitate.

Table 2. DDA values obtained from pH-potentiometric titrations

Sample no.	c[a] (mg/100 mL)	ΔV_1[b] (mL)	ΔV_2[c] (mL)	$\Delta V_2/\Delta V_1$	DDA_1[d] (%)	DDA_2[e] (%)	$DDA_{av} \pm STD$[f]
1	101.4	0.457	0.475	1.039	88.6	91.4	91.0 ± 0.5
		0.464	0.469	1.011	89.6	90.5	
		0.457	0.473	1.039	88.6	91.0	
2	107.0	0.513	0.540	1.052	84.5	88.1	88.2 ± 1.2
		0.527	0.533	1.010	86.4	87.1	
		0.534	0.550	1.030	87.3	89.4	
3	99.6	0.454	0.472	1.041	88.3	91.3	91.5 ± 0.3
		0.458	0.475	1.038	88.9	91.7	
		0.481	*0.395*	*0.822*	*92.6*	*78.8*	
4	106.0	0.487	0.489	1.004	84.4	84.7	84.9 ± 0.2
		0.485	0.490	1.010	84.1	84.8	
		0.485	0.494	1.019	84.1	85.3	
5	50.9	0.235	0.246	1.047	82.9	86.1	86.6 ± 1.4
		0.239	0.244	1.021	84.1	85.5	
		0.237	0.253	1.068	83.5	88.1	
	158.4	0.691	0.703	1.017	80.0	81.2	81.1 ± 0.1
		0.693	0.701	1.012	80.2	81.0	
		0.686	0.702	1.023	79.6	81.1	
	102.5	0.452	0.455	1.007	80.8	81.2	81.8 ± 0.5
		0.449	0.459	1.022	80.3	81.8	
		0.449	0.462	1.029	80.3	82.3	
6	101.5	0.443	0.441	1.004	83.6	83.9	85.1 ± 1.2
		0.437	*0.488*	*1.117*	*82.6*	*90.5*	
		0.464	0.464	1.011	85.4	86.2	
7	107.2	0.573	0.579	1.011	96.3	97.2	97.2 ± 0.5
		0.572	0.576	1.007	96.2	96.7	
		0.572	0.584	1.020	96.2	97.8	

Data written in italics are outlying values and were omitted from the calculations.
[a] Concentration of the polymer.
[b] The difference between the equivalence points determined from derivative method (for details see text).
[c] The difference between the equivalence points as determined by using the Gran method according to Jiang et al.[16]
[d] DDA value determined from ΔV_1.
[e] DDA values determined from ΔV_2.
[f] Recommended value for the DDA of the various samples and their standard deviation.

the ΔV values of the parallel runs agree within the expected value of ±0.5%. However, the ΔV values (and thus the DDA values) obtained from the derivative method are systematically and significantly lower than those obtained from linearization. The most likely reason for this is that around the first equivalence point, at the steepest section of the $pH = f(V_{NaOH})$ function the Chitosan is already partly (~2%) dissociated and thus the first derivative method (under the experimental conditions employed in these studies) overshoots this equivalence point. On the basis of this, the use of the method of Jiang et al.[16] is strongly recommended and the DDA values obtained from it can be considered as 'best' values.

For sample 5, DDA values were determined at three different polymer concentrations. As can be seen from Table 2, the STD values are much higher at $c_{chitosan}$ ~0.5 g/L than at higher ones; this is most likely due to the uncertainties associated with the low ΔV values at these low polymer concentrations. On the other hand, at $c_{chitosan}$ >1 g/L the drastically increasing solution viscosity appeared to cause severe technical problems: the accurate pipetting and the efficient stirring and purging of the titrand became more and more difficult. Therefore, the use of polymer concentrations of approximately 1 g/L (and around 0.010 M strong acid concentrations) seems to be optimal for performing such titrations accurately and conveniently.

In three specimens (1, 3 and 5), we observed that the second equivalence point on the titration curve was beyond that expected from the analytical concentration of the HCl added to the system. The only way of explaining such an effect is the presence of extra acid in the dry chitosan sample. For example, some residual acetic acid, which is often used during the purification of the product during manufacturing, might have remained in the specimen, or the manufacturer would have obtained the solid chitosan in partly protonated form during precipitation. In some samples, this extra acid content can be as high as 1 mol % of the free chitosan base. There-

Table 3. DDA values for the chitosan samples studied, obtained from various experimental techniques (Diff denotes the difference between the DDA values obtained from pH-potentiometry and ^{1}H NMR)

Sample no.	IR	^{1}H NMR	pH-Potentiometry	Difference
1	89	87.8	91.1	+3.3
2	83	86.5	88.2	+1.7
3	—	87.7	91.5	+3.8
4	85	86.3	84.9	−1.4
5	82	83.4	81.8	−1.6
6	—	85.6	85.1	−0.5
7	94	96.6	97.2	+0.6

fore, for accurate pH-potentiometric DDA values to be obtained, it is necessary to determine both equivalence points independently.

Comparison of DDA values with other experimental methods. The DDA values obtained from pH-potentiometry and ^{1}H NMR and IR spectroscopy are summarized in Table 3. It seems that there are no systematic method-dependent deviations in the various DDA values obtained. It is remarkable that the DDA values from pH-potentiometry and ^{1}H NMR agree well and are within 2% for almost all samples. Given the large number of corrections that had to be taken into consideration for DDA values obtained from pH-potentiometry and the uncertainties associated with all of them individually, this agreement can be considered reasonable. Unsatisfactory agreement was, however, obtained for the two technical grade samples (1 and 3), where the pH-potentiometric DDA value was found to be higher by almost 4% than that obtained from ^{1}H NMR. This difference is likely to be associated with the non-specific nature of pH-potentiometry. In technical grade samples proton-active impurities (such as proteins) are most likely to be present, and unlike for the specific ^{1}H NMR method, such impurities are non-distinguishable from the protonating fraction of chitosan for pH-potentiometry. This argument is further supported by the fact that purification of these samples by repeated recrystallization significantly diminished the difference between the results of the two methods to acceptable levels (+1.6% and −1.7%, respectively). This observation suggests that pH-potentiometric titration can only be used to accurately determine DDA values for sufficiently pure samples, which contain only negligible amounts of proton active impurities.

Conclusions. For accurate pH-potentiometric DDA determinations of chitosan, the moisture content of the samples has to be determined and (particularly for the low purity technical grade samples) their ash content has to be measured as well. Further possible complications, which are associated with the mathematical treatment of the titration curves, have been identified: the method of equivalence point determination (both linearization and second derivative method) causes systematic variations in the DDA values obtained and in some samples extra acid content (in some cases, 1 mol % of the free amino content) was observed. From these observations, the drawbacks of pH-potentiometry are as follows:

(i) due to solubility constraints, pH-potentiometry can only be reliably performed with chitosan samples of narrow range of DDA >75%;
(ii) the samples require either careful drying or independent thermogravimetric analysis;
(iii) technical grade samples are definitely not adequately measured by this method;
(iv) in some samples, the second end point and the acid content have to be determined and necessary corrections have to be introduced.

From these results, one has to conclude that pH-potentiometric titration can yield accurate DDA values only when a series of corrections (analysed in detail in the current study) are made. In terms of accuracy, pH-potentiometry can only compete with spectroscopic techniques, such as ^{1}H NMR spectroscopy, when titrations are not only supplemented by additional measurements, but also when rigorous data processing protocols are employed. These additions to the easy-to-perform and low-cost pH-potentiometric measurements make the technique rather circuitous, slow and labour intensive and make it inferior to standard spectroscopic procedures described in the literature.

1. Experimental

1.1. Materials

The chitosan samples used for the measurements are listed in Table 1. Unless otherwise stated, the samples were stored in closed, airtight containers at room temperature. Purification of the technical grade samples 1 and 3 was carried out as previously described.[26] A further deacetylation step was performed on sample 5 according to the procedure described in the literature,[9] yielding sample 7. For solution preparations, MilliQ-Millipore water was used throughout. All the other chemicals used were of analytical grade.

1.2. Methods

1.2.1. Thermogravimetry. Thermogravimetric measurements were performed on a Perkin–Elmer PGA-7 precision thermogravimetric analyzer, in air stream, in the temperature range of 20–800 °C, and at 4 °C/min heating rate. In the TG curves, the initial weight loss, appearing at 20 °C $\leqslant T \leqslant$ 130 °C, was attributed to the moisture content of the samples. The moisture content of the various specimens was determined graphically, from the plateau appearing between 130 and 200 °C on the TG curves. At around 250 °C, burning of the

polysaccharide commences. Moisture determinations were performed twice, firstly after receiving the samples and secondly a half year later. The difference between the two sets of measurements was within the limit of the expected reproducibility of TG measurements (±0.2%), indicating that storage of the samples in air-tight containers for extended period does not cause significant variation in the moisture content (the values of which are shown in Table 1.) After the complete burning of the polymer, the ash content of the specimen was estimated. As the ash content of the samples was found to be less than 1% by weight, thermogravimetry could give only a first approximation. Therefore, a more accurate method was employed for this purpose.

Ash contents were determined gravimetrically. A ca. 200 mg part of each sample was accurately (±0.1 mg) weighed in a porcelain container, which was stored overnight over P_2O_5. The triplicate samples were placed in a furnace (800 °C). After the complete combustion, the jars were allowed to cool, were dried and their weight was re-determined.

1.2.2. pH-Potentiometry. pH-Potentiometric titrations were carried out in aqueous solutions at constant ionic strength (0.1 M NaCl), using an automatic titration set including a Dosimat 665 (Metrohm) autoburette (stated precision 0.002 mL), an Orion 710A precision digital pH-meter and an IBM compatible PC.[27] The Orion 8103BN semimicro pH glass electrode was calibrated via the modified Nernst equation,

$$E = E_0 + K\log[H^+] + J_H[H^+] + J_{OH}\frac{K_w}{[H^+]} \qquad (2)$$

where J_H and J_{OH} are fitting parameters in acidic and alkaline media for the correction of experimental errors of the glass electrode; $K_w = 10^{-13.75}\,M^2$ is the ionic product of water at 0.1 M NaCl ionic strength. The parameters of Eq. 2 were calculated by a non-linear least squares method.

Both calibrations and titrations were carried out in a thermostatted double-walled cell, at a temperature of $T = 298.0 \pm 0.1$ K, in an inert atmosphere (N_2 gas bubbling). The titrant solutions ($[HCl]_T = 0.010$ M) were prepared from BDH standard ampoules. During the pH-metric titrations, a total volume of 10 mL of initially acidic solution was titrated with NaOH ($c_{NaOH} \approx 0.1$ M, standardized against KH-phthalate, carbonate content minimized via washing off the surface carbonate with distilled water). Potential readings were recorded when the displayed pH value was stable for several minutes in the third decimal place (i.e., within ±0.1 mV).

Chitosan stock solutions used for pH-potentiometric titrations were prepared via accurate weighing of usually ca. 100 mg solid sample, which was transferred to a 100 mL volumetric flask. A calculated amount of standard HCl solution was then accurately pipetted to the system, which makes up the analytical concentration of HCl to 0.010 M. About 50 mL distilled water was added to the system, and the suspension was allowed to stand for several hours (usually overnight), providing sufficient time for the polymer to get hydrated. Poor reproducibilities were obtained between the parallel runs if shorter time was allowed most likely due to incomplete dissolution of the polymer. When the chitosan specks or flakes completely dissolved in the acidic medium, a calculated amount of solid NaCl was added to the flask, and the solution was brought to volume. From the homogenized solution, exactly 9.981 mL aliquots were pipetted manually in the titration vessel. Usually three parallel measurements were performed with each stock solution, and 50–100 titration points were collected in each titration.

1.2.3. ^{1}H NMR spectroscopy. During the ^{1}H NMR measurements, approximately 5 mg of the chitosan sample was placed in an Eppendorf tube, and 1 mL of D_2O (99.9%, Aldrich) plus 20 μL of DCl_{cc} (ca. 35% m/m, Aldrich) dissolved in D_2O was added to it from a microsyringe. The tubes were closed (to minimize exchange with the atmospheric H_2O) and the samples were allowed to stand until complete dissolution of chitosan. Measurements were performed on a Bruker Avance DRX500 instrument, operating with 11.7 T magnetic field strength, and at 500.13 MHz. Measurements were performed and evaluated as described in the literature,[13] except for solvent signal suppression, which was found to result in distorted ^{1}H NMR signals (in particular, those which were close to the HDO signal situating at 4.80 ppm), and erroneous, sometimes even chemically meaningless integrations. DDA values were estimated from the integrated intensity ratios of the appropriate D-GlcN and D-GlcNAc protons, as described in Ref. 13.

1.2.4. IR spectroscopy. FT-IR measurements were carried out on a Bio-Rad FTS-65A/869 (Digilab Division) Fourier transform infrared spectrometer. The spectra were recorded in KBr, in the 400–4000 cm^{-1} range, by using DTGS detector. The spectral resolution was 4 cm^{-1} and 128 scans were averaged. Data were processed by using the software GRAMS 386.3 (Galactic Corporation). Samples were stored in a vacuum oven, over P_2O_5 at 60 °C for at least a day, to minimize their moisture content. This was necessary because IR bands of the amide groups and water overlap and therefore result in faulty DDA values. KBr pellets were prepared in a dry box, which was filled in and permanently soaked with dry air. DDA values were derived from the comparison of the intensity of the amide band (1320 cm^{-1}) with the broad composite bands situating at 3450 and 1420 cm^{-1} and the intensity of which is directly proportional to the total number of monomer units within the polymer.[7]

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