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Poly-acid properties of biosynthetic hyaluronan studied by titration

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ABSTRACT

In this study the poly-acid properties of biosynthetic hyaluronan produced by fermentation of *Bacillus subtilis* have been investigated. Potentiometric titration as well as ^{1}H NMR titration have been used to determine the dissociation constants of the carboxylic group on hyaluronic acid. The intrinsic pK_a and p$K_{a,\ \alpha=0.5}$ were determined in the presence of 0.1 M salt to be 2.99 and 3.37, respectively. The p$K_{a,\ \alpha=0.5}$ was found to be unaffected by variations in the ionic strength which is in good agreement with the fact that at $\alpha = 0.5$, 50% of the carboxylic moieties on the hyaluronan are charged. On the other hand the intrinsic pK_a was found to be dependent on the ionic strength until the Debye-Hückel screening length approaches the length of repeating disaccharide unit of hyaluronic acid.

Our findings are in good agreement with previously determined dissociation constants for other sources of hyaluronan. We have also shown that ^{1}H NMR spectroscopy is the preferred method for polyelectrolyte titration because of the ability to isolate the contribution of several ionisable groups on a polymer on molecular level.

1. Introduction

Hyaluronan is a linear unbranched biopolymer naturally abundant in mammalian tissues. The chemical structure consists of two alternating monosaccharide units, *N*-acetyl glucosamine and glucuronic acid, connected by β-(1 → 3) and β-(1 → 4) glycosidic bonds, respectively. It is the negative charge on the repeating disaccharide which is the cause on many of the unique properties of this polyacid in solution. HA produces highly viscous solution in water (Lapcik, Lapcik, De Smedt, & Demeester, 1998; Gibbs, Merrill, Smith, & Chabrecek, 1968). Due to the alternating β-backbone, the viscosity of HA is strongly dependant on ionic strength (Smidsrød & Haug, 1971; Hayashi, Tsutsumi, Norisuye, & Teramoto, 1996). As the concentrations of salts increase, the repulsion between negatively charged disaccharide units decrease and the polymer change from a rod-like towards the flexible confirmation of the random coil (Furlan, La Penna, Perico, & Cesaro, 2005; Tanford, 1961). A human adult contain approximately 15 g hyaluronic acid. Hyaluronan is present in the eyes, skin (both in the epidermis and dermis) and synovial fluid (Lepperdinger, Fehrer, & Reitinger, 2004). Further, hyaluronan makes up the backbone of the proteoglycan aggregates being a main component of sinew and cartilage (Heinegård, Björnsson, Mörgelin, & Sommarin, 1998). The polysaccharide has a high turnover in the body, 7 g/day, i.e. half of all HA in the body is exchanged every day (Lepperdinger et al., 2004). The molecular weight strongly depends on the biological origin (Lapcik et al., 1998). When produced by microbial fermentation the molecular weight ranges from just below 1×10^6 up to 4×10^6 g/mol (Armstrong & Johns, 1997). When prepared by extraction, molecular weights up to 6×10^6 have been reported (Lee & Cowman, 1994).

Today, two main microbial sources are exploited for hyaluronan production, *Streptococcus* strains (Sutherland, 1990) and recombinant *Bacillus subtilis* (Widner et al., 2005). In *Streptococcus*, hyaluronan is produced as part of the capsule bound to the cell membrane, making it challenging to isolate. Hyaluronan from *Bacillus*, on the other hand, is excreted extracellularly and can therefore conveniently be isolated as a high purity product (Widner et al., 2005).

There are two main challenges when studying the poly-acid properties of hyaluronic acid. First, to assure a pure substance free of matrix components such as proteins and other glucosaminoglycans that can interfere. Secondly, the polyelectrolyte properties of the polysaccharide must be taken into consideration during the titration. Preston, Davies, and Ogston (1965) used specific viscosity measurement on a crude hyaluronan from bovine synovial fluid while Park and Chakrabarti (1978a) used optical rotation to study the dissociation phenomena in a hyaluronan prepared from rooster comb. Cleland (1982, 1984) and Cleland, Wang, and Detweiler (1982) studied a crude sample of hyaluronan obtained from bovine vitrous humor using potentiometric titration. The problem with the hyaluronan extracted from eukaryotic organisms (rooster comb etc.) is that it is difficult to remove all remnants of proteins and other charged compounds such as other glucosaminoglycans.

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In this work we combine the successful NMR approach to poly-acid titration with the pure, biosynthetic hyaluronan from a *B. subtilis* engineered to excrete a minimum of bi-products. Further, this strain is cultivated on a chemically defined minimal media, free of any peptides (as opposed to streptococcal fermentations) (Widner et al., 2005). To the authors' knowledge, no work has previously been presented using such a pure and well characterised hyaluronan sample.

2. Experimental

2.1. Materials

Commercially available sodium hyaluronate ($[\eta]$ = 1700 ml/g, M_w = 1.0 MDa) prepared by fermentation of *B. subtilis*, Novozymes Biopolymer A/S. Sodium deuterium oxide (NaOD) solution (30%) in 99.5% D_2O from MERCK (Schuchardt, Germany), deuterium chloride (DCl) solution (35%) in 99% D_2O were obtained from Sigma–Aldrich (St. Louis, MO, USA), 99.9% deuterium oxide (D_2O) containing 0.05% trimethylsilyl propionate (TSP-d_4) from Sigma–Aldrich (St. Louis, MO, USA), bovine testicular hyaluronidase (100 U/mg, type VIII, CAS number 37326-33-3) were obtained from Sigma–Aldrich (St. Louis, MO, USA), All salts were analytical grade. Dialysis tubes were from Spectra por 4 (MWCO 12–14 kDa).

2.2. Preparation of hyaluronan fragments

Hyaluronan (3 g, 1.0 MDa) was dissolved on stirring overnight at room temperature in buffer (500 ml; 0.15 M sodium chloride and 0.1 M sodium acetate in de-ionized water, pH 5.2). Testicular hyaluronidase (100 mg, total 10,000 U) was added and digestion proceeded for 24 h at 37 °C. After digestion, the solution was heated to 95 °C for 30 min before the denatured protein was removed by filtration. The filtrate was further purified by dialysis against de-ionized water (3 × 3 h) at 4 °C (regenerated cellulose, Spectra Por 4, MWCO 12–14 kDa) until conductivity outside dialysis bags was <5 μS/cm. The solution was then frozen and lyophilized. The resulting degraded hyaluronan was analyzed by ^{1}H NMR spectroscopy (10 mg/ml sample was dissolved in 0.7 ml D_2O with addition of 5 μl of a stock solution of 1% TSP (trimethyl-silyl propionate-d_6) as internal standard and analyzed at 30 and 80 °C, using a Varian 400 MHz, 128 scans). In addition, the initial sample as well as the enzymatically degraded hyaluronan were analyzed by SEC-MALLS-VISC-RI. The set up of the system was a Waters alliance HPLC with four TSK columns (2500, 4000, 5000, 6000 PW_{XL}) with the light scattering detector DAWN EOS, a Optilab rEX for refractometric index determination and a ViscoStar for viscosity determination. All detectors were obtained from Wyatt Technology Corp. (Santa Barbara, CA, USA).

Sample (0.1–1%) dissolved in buffer (50 mM HaH_2PO_4 and 150 mM NaCl) was injected using a 500 μl injection loop and a flow rate of 0.5 ml/min.

2.3. Potentiometric titration using pH electrode

Hyaluronan was titrated by using 0.10 M HCl. The pH of the HA solution (100 ml, concentration 10 mg/ml) was adjusted to pH of 8.99 by use of NaOH. The 0.10 M HCl solution was added in increments of 100 μl (corresponding to 10 μmol) while stirring. The pH was measured after each addition. This procedure was continued until pH 1.50 (almost 2 pH units below expected pK_a of HA) was reached.

2.4. Titration of acidic groups using ^{1}H NMR spectroscopy

Samples were dissolved in D_2O containing 0.05% sodium 3-(trimethylsilyl)-propionate-d_4 (TSP-d_4) and NaCl (10, 50 or 100 mM) before transferring to 5 mm NMR tubes. The pH* (uncorrected value measured by pH electrode in D_2O solution) was adjusted using 10, 50 or 100 mM DCl/NaOD, respectively, so that the ionic strength was maintained constant for all three sample series. All chemical shifts were determined relative to the internal standard, TSP-d_4 as previously described (Whisart et al., 1995). The NMR spectra were acquired on a Varian Mercury VX 400 MHz with a 4-nucleus auto-switchable probe and the WNMR software (ed. 6.1). All results were obtained at 30 °C using 128 scans.

3. Results and discussion

Weak acidic groups on charged polymers differ from the corresponding mono-acids. This is due to the electrostatic co-operative interaction between the moieties along the polymer chain. The strength of this interaction depends on several factors, e.g., the distance between charges and the ionic strength of the solution (Smidsrød & Haug, 1971; Tanford, 1961). Further, the presence of other poly-ions (i.e., peptides, proteins, polysaccharides, RNA and DNA), their chain-length and charge distribution as well as the presence of multivalent counter-ions will influence this interaction (Katchalsky, 1954; Alexandrowicz & Katchalsky, 1963). Therefore, particular considerations must be taken into account when studying weak poly-acids and their dissociation behavior. The aim of this work has hence been to analyze the poly-acid properties of hyaluronan without the interference of secondary inter-ionic phenomena.

3.1. Preparation of hyaluronan fragments

A LMW fragment of hyaluronan was prepared by enzymatic degradation. After the purification, the material was analyzed using ^{1}H NMR spectroscopy and SEC-MALLS-VISC-RI. The results are shown in Figs. 1 and 2, respectively. The peaks in the ^{1}H NMR spectrum were assigned according to Siciñska, Adams, and Lerner (1993) and Tawada et al. (2002). The α and β reducing ends of GlcA can be seen at 5.2 and 4.6 ppm, respectively. This is the only reducing end observed, as expected for a hyaluronan hydrolase which specifically degrades the β-(1 → 3) glycosidic bond between GlcA and GlcNAc. The weight average molecular weight

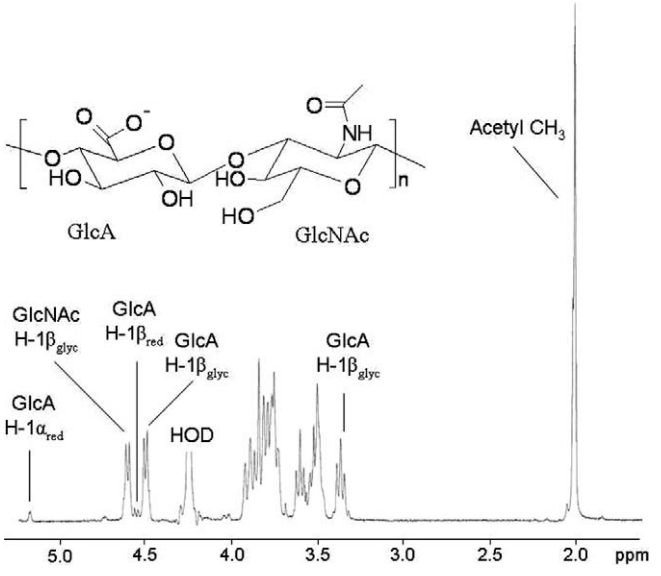

Fig. 1. ^{1}H NMR spectrum of hyaluronan fragment prepared by enzymatic degradation. The assignment of the spectrum is based on Siciñska et al. (1993) and Tawada et al. (2002).

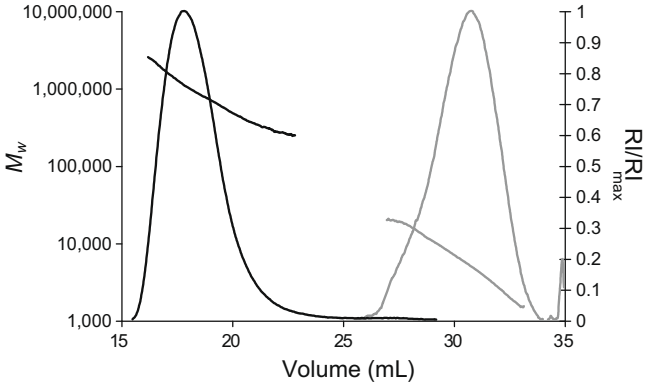

Fig. 2. SEC-MALLS-VISC-RI of starting hyaluronan (black) and obtained fragment after enzymatic degradation (grey).

(M_w) was determined to be 4830 g/mol with a rather narrow polydispersity (1.38) using SEC-MALLS-VISC-RI. Also, the native HA starting material was characterised using this method and the results are presented together with that of the HA fragment in Fig. 2 and Table 1. Evaluating the ^{1}H NMR spectrum and the SEC chromatogram, no remnants of enzyme or other peptide impurities could be observed.

3.2. Potentiometric titration

The low molecular weight hyaluronan fragment was analyzed by potentiometric titration in 0.1 M NaCl and $NaNO_3$ at constant ionic strength using 0.1 M HCl and NaOH. The resulting data are presented in Fig. 3. These were treated using the theory of weak poly-acids by Katchalsky (1954) stating that due to interaction between the charged groups along a polymer chain, the dissociation constant will depend on both ionic strength in the solution (degree of shielding) and the distance to the next charged group on the polymer. Katchalsky (1954) established the following relationship; $pH = pK_0 + \log[\alpha/(1-\alpha)] - 0.434\varepsilon\Delta\psi/kT$, where pK_0 is the intrinsic pK_a, α is the degree of dissociation, $-0.434\varepsilon\Delta\psi/kT$ is also denoted by ΔpK. This term is the contribution of the polyelectrolyte field to the standard free energy of ionization of a single group, in addition to the free energy of ionization in surroundings in which no such field is acting (pK_0). According to this theory, the dissociation constant, when 50% of the carboxylic groups are protonated, $pK_{a,\ \alpha=0.5}$, can be determined by plotting pH as function of $\log[(1-\alpha)/\alpha]$ (Fig. 4). Further, by plotting the apparent dissociation constant, $pK_{a,\ apparent}$ as function of α according to the following; $pK_{a,\ apparent} = pH - \log[\alpha/(1-\alpha)]$ one can determine the intrinsic dissociation by extrapolation to $\alpha = 0$. The intrinsic dissociation constant is the dissociation constant of a single acidic group when all neighbouring acidic groups are without charge. The plot is presented in Fig. 5. All results are summarized in Table 2. Gatej, Popa, and Rinaudo (2005) described how the dynamic viscosity of HA solutions increase as pH is lowered to 2.5 due to increase in formation of hydrogen bonds. Therefore, we decided to do the titration with both NaCl and $NaNO_3$, since the latter has a weakening effect on the hydrogen bonding between polysaccharides in solution and reduce viscosity (Lazarus, 1983). A difference can be seen between the two salts below $\alpha < 0.2$ in Fig. 5, but without influencing the resulting dissociation constants. This is due to that NO_3^- decrease the strength of the hydrogen-bonds inter- and intra-molecularly for HA in solution.

No formation of precipitates where observed during the titration over the entire pH range studied 1.50–8.99 for neither the HA solution containing 0.1 M $NaNO_3$ or the one with 0.1 M NaCl.

Table 1
Properties of hyaluronan starting material and prepared degraded sample as determined by SEC-MALLS-VISC-RI.

	M_w (10^3 g/mol)	M_w/M_n	$R_{g,z}$(nm)	$R_{h,w}$(nm)	$[\eta]_w$ (ml/g)
Native bHA	1020	1.35	120	64	1700
Degraded bHA	4.83	1.38	11	1.8	7.7

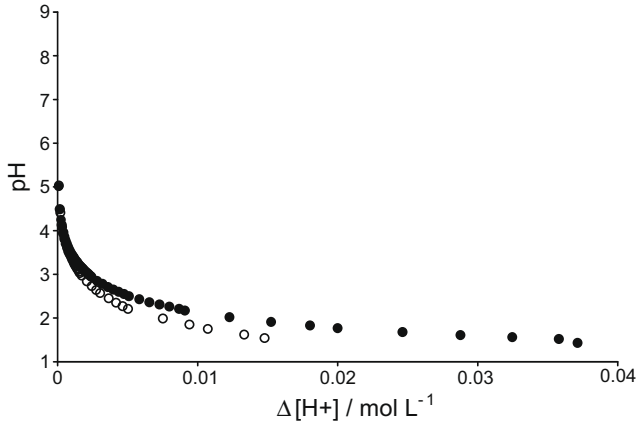

Fig. 3. Titration curve (pH as function of $\Delta[H^+]$) for HA in the presence of 0.1 M NaCl (closed circles) and 0.1 M $NaNO_3$ (open circles).

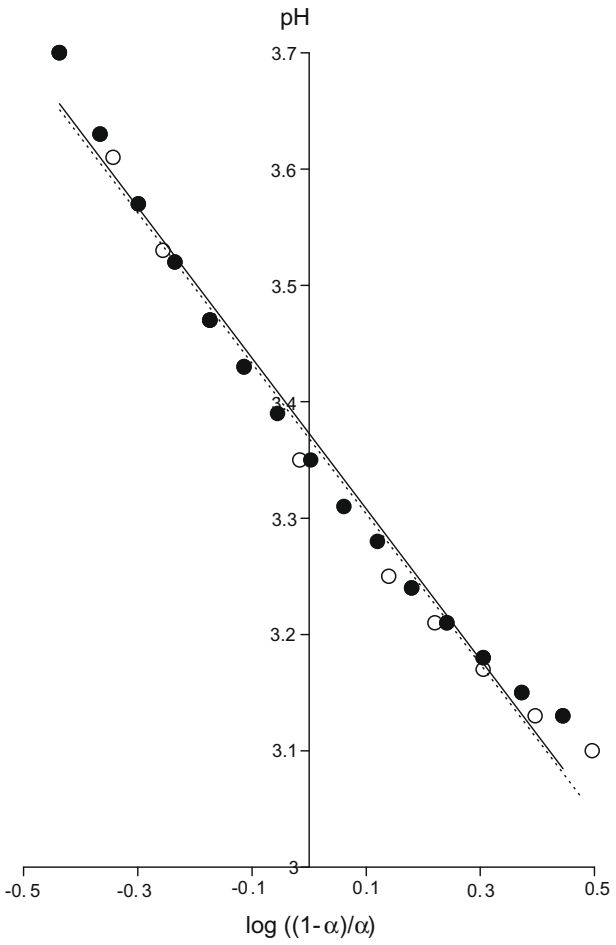

Fig. 4. pH as function of $\log[(1-\alpha)/\alpha]$ in the presence of 0.1 M NaCl (closed circles) or 0.1 M $NaNO_3$ (opencircles). $pK_{a,\ \alpha=0.5}$ is determined when $\log[(1-\alpha)/\alpha]$ is zero.

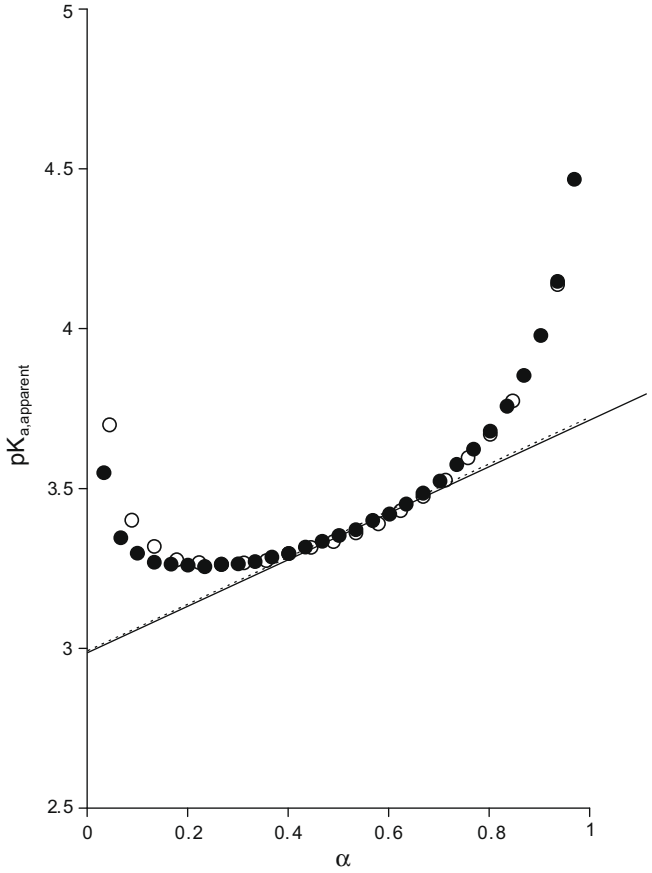

Fig. 5. $pK_{a,\ apparent}$ as function of $\log[\alpha/(1-\alpha)]$ in the presence of 0.1 M NaCl (closed circles) and 0.1 M $NaNO_3$ (open circles). The intrinsic pK_a ($pK_{a,\ int}$) is determined when α is extrapolated to zero.

Table 2
Intrinsic and apparent dissociation constant ($pK_{a,\ int}$ and $pK_{a,\ \alpha=0.5}$) determined by potentiometric titration of hyaluronan (ionic strength, [*I*] = 0.1 M).

Salt	$pK_{a,\ int}$	$pK_{a,\ \alpha\ =\ 0.5}$
NaCl	2.99	3.37
$NaNO_3$	2.99	3.37

This observation is in agreement with Gatej et al. (2005), studying aqueous solutions of HA over the pH range 1.6–12.6.

3.3. Influence of ionic strength on the dissociation constants of hyaluronan using titration with ^{1}H NMR spectroscopy

By use of ^{1}H NMR spectroscopy, it is possible to study how equilibriums change as the chemical environment is modified. It is possible to do titrations on the molecular level by following how the chemical shifts of protons close to the functional group change with change in the solution. H-1 of GlcA was chosen to monitor the chemical shift change with pH since it has a largest change in chemical shift as function of ΔpH^* due to it being physically very close to the carboxylic acid moiety of the GlcA unit. A further advantage of choosing H-1 of GlcA, is that the peak is well separated from the two neighbouring peaks; H-1 of GlcNAc (δ = 4.60 ppm) and the water peak (HOD) over the entire pH range covered during the titration experiment (see Fig. 1). Even at the lowest pH^* value, when the H-1 of GlcA and H-1 of GlcNAc are as closest, there was still baseline separation between them ensuring correct evaluation of the chemical shift of H-1 GlcA.

Three titration experiments were performed varying the ionic strength; 10, 50 and 100 mM NaCl. By following the change in chemical shift as function of pH^*, titration curves were obtained (Fig. 6), and the data is plotted as; pH^* vs. $\log[(1-\alpha)/\alpha]$ and $pK^*_{a,\ apparent}$ vs. α in Figs. 7 and 8, respectively. The resulting dissociation constants are summarized in Table 3. The Debye-Hückel screening length (κ^{-1}), defined by; $\kappa^{-1} = [1000/(8\pi l_b N_A I)]^{1/2}$ (Dautzenberg et al., 1994) is given in Table 3 for the three ionic strengths. The screening length gives the distance, at a certain ionic strength, where the interaction between two neighbouring charged groups on the polymer becomes negligible. It would be expected that the influence by the neighbouring charged group on the dissociation constant will be seen only when the distance between the charges on the polymer is shorter than κ^{-1}, i.e. when the weak acid groups are influenced by the neighbouring carboxylic acid groups. The length of a disaccharide unit of HA has previously been determined to be about 1 nm (Cleland, 1984; Gatej et al., 2005). When studying the determined dissociation constants in Table 3, we see that the $pK_{a,\ \alpha=0.5}$ is apparently unaffected by the change in ionic strength. The intrinsic pK_a, on the other hand, decrease with the increasing ionic strength. The effect is most pronounced when going from 10 to 50 mM, and there is only a slight further decrease when going from 50 to 100 mM NaCl. This can be explained by the changes in the ionic environment experienced by the weakly acidic groups on the HA molecule. The $pK_{a,\ \alpha=0.5}$ is determined when 50% of the carboxylic acid moieties are charged. At this degree of dissociation, the polymer in itself is contributing to the ionic environment experienced by the weak acid group. Hence, the $pK_{a,\ \alpha=0.5}$ is found to be unaffected by variations in the surrounding ionic strength. The intrinsic pK_a is determined when the number of charges on the polymer goes towards asymptotic value of zero. Therefore, the polymer does not contribute in itself to the ionic surrounding of the weak acid group. The intrinsic dissociation constant is therefore seen to be very much dependant on the ionic strength of the solution. As previously mentioned, the largest decrease in $pK_{a,\ int}$ is seen when going from 10 to 50 mM NaCl. Under the same conditions the Debye-Hückel screening length (κ^{-1}) goes from 3.04 to 1.36 nm. Only a negligible change is seen in the dissociation constant when going from 50 to 100 mM, i.e. κ^{-1} of 1.36 and 0.96 nm, respectively. Since the length of repeating disaccharide unit of HA has been determined to 1 nm (Gatej et al., 2005), this is also the distance between the weak acidic groups on the glucuronic acid units. Therefore, when κ^{-1} is approaching 1 nm, the distance between neighbouring charges,

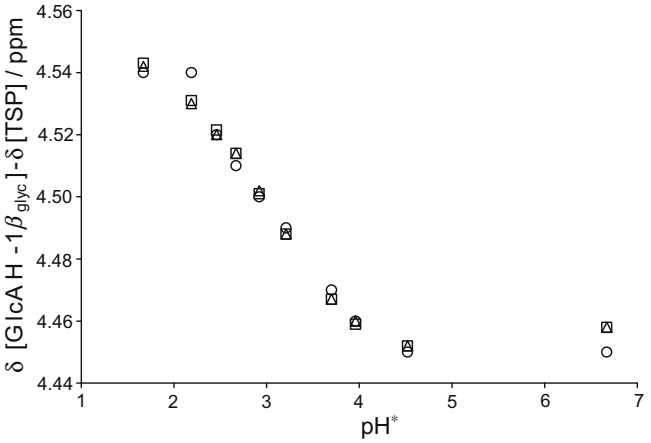

Fig. 6. Titration curves obtained by following the chemical shift of GlcA H-1β_{glyc} relative to sodium 3-(trimethylsilyl)-propionate-d_4 (TSP-d_4) as function of pH^* at constant ionic strengths. Legend: 10 (open circle), 50 (triangle) and 100 (square) mM NaCl.

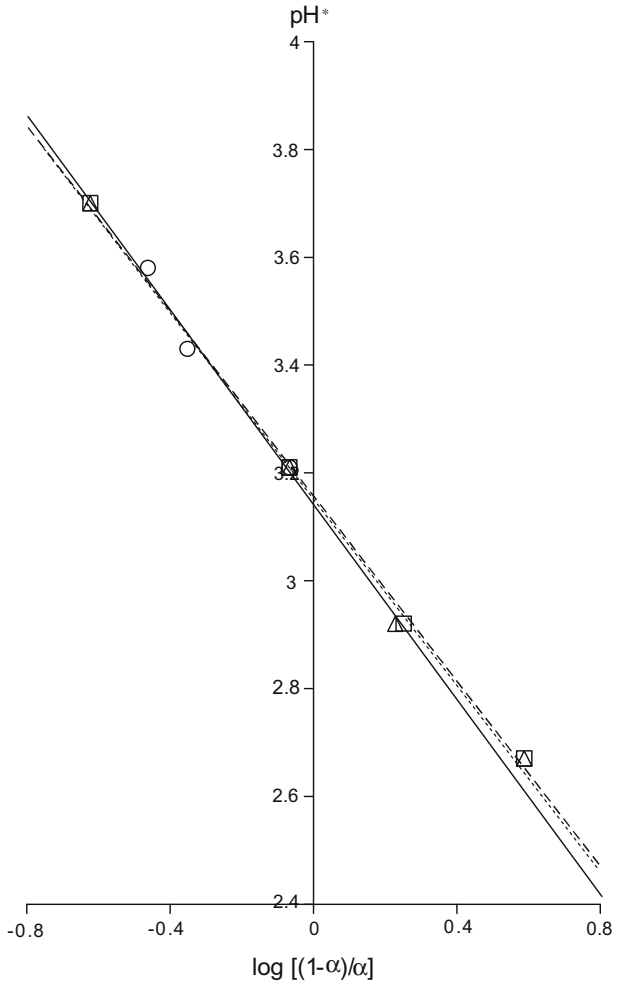

Fig. 7. pH* as function of. log[(1 − α)/α] in the presence of 10 (circle), 50 (triangle) and 100 (square) mM NaCl. The plot is based on the titration curves in Fig. 6.

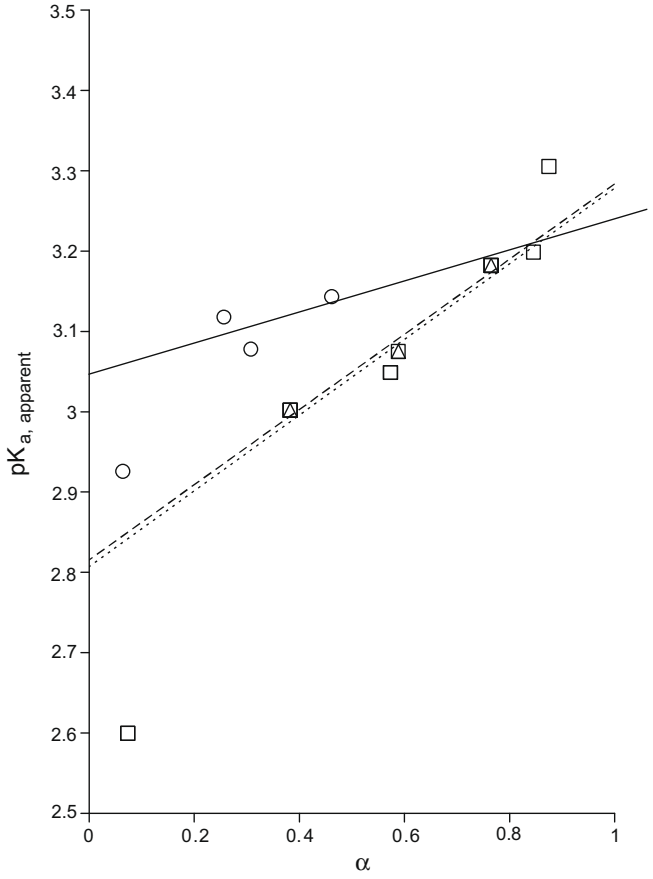

Fig. 8. pK_a^*, apparent as function of α in the presence of 10 (open circle), 50 (triangle) and 100 (square) mM NaCl. The plot is based on the titration curves in Fig. 6.

the intrinsic dissociation constant will become unaffected by the ionic strength in the solution.

3.4. Potentiometric titration vs. ^{1}H NMR spectroscopy

The determined dissociation constants ($pK_{a,\ \alpha=0.5}$ and $pK_{a,\ int}$) from the potentiometric titration and the ^{1}H NMR titration are presented in Tables 2 and 3, respectively, and are discussed individually in the previous sections. Rueda, Arias, Galera, López-Cabarcos, and Yagüe (2001) determined the $pK_{a,\ int}$ to 3.02–3.10 and $pK_{a,\ \alpha=0.5}$ = 2.9–3.0 (depending on the HA concentration varying from 2×10^{-4} to 2×10^{-2} %w/v) in 0.1 M NaCl using potentiometric titration. Park and Chakrabarti (1978a, 1978b) used optical dichroism to determine the $pK_{a,\ \alpha=0.5}$ to 3.36 in 0.1 M NaCl. Cleland (1982, 1984) and Cleland et al. (1982) determined the $pK_{a\ int}$ to 2.9 ± 0.1, pK_a 3.23 ± 0.02 for a crude hyaluronan by potentiometric titration at constant ionic strength (0.1 M NaCl). Wang, Duraikkannu, and Lindhardt (1991) determined the pK_a in aqueous solution for α-D-GlcA to 2.93 and β-D-GlcA to 2.83, respectively. Kohn and Kovac (1978) determined the pK_a of a mixture of α/β-D-GlcA to 3.28 by titration in water. It is clear that our determinations are in agreement with those determined previously by various methods.

Comparing results from potentiometric titration and NMR titration in this study we see that there is a certain deviation in the values of the dissociation constants determined by the two methods. This can be explained by the difference in ability to associate with negative charges for deuterium ions compared to protons. This will give a shift in the equilibrium constants determined in D_2O compared to H_2O. This has previously been observed for determinations of equilibrium constants by use of ^{1}H NMR spectroscopy (Wang et al., 1991; Strand, Tømmeraas, Vårum, & Østgaard, 2001; Tømmeraas et al., 2002). The shift observed in equilibrium constants has been seen to be systematic between different D_2O/H_2O ratios. The comparison of determined equlibria constants is valid as long as they are determined in the same solvent system (i.e., D_2O/H_2O ratio) (Strand et al., 2001). In general, the deviation between dissociation constants determined in D_2O compared to H_2O (Glasoe & Long, 1960; Kohn & Kovac, 1978; Jencks, 1989; Rueda et al., 2001; Tømmeraas et al., 2002); pH = pH* + 0.2 (i.e. pK_a = pK_a^* + 0.2), where pH* (pK_a^*) is the value determined using a pH electrode without correcting for the deuterium effect. Comparing the dissociation constants in 100 mM NaCl in H_2O and D_2O (see Fig. 9), we see a deviation of about 0.2 i.e. in agreement with the above mentioned relationship.

Further, the potentiometric titration is a macroscopic analysis dependant on all ionisable molecules present in the solution

Table 3
Intrinsic and apparent dissociation constant ($pK^*_{a,\ int}$ and $pK^*_{a,\ \alpha=0.5}$) determined by potentiometric titration for hyaluronan using NMR spectroscopy (in D_2O). The Debye-Hückel screening length (κ^{-1}) is calculated from the equation $\kappa^{-1} = [1000/(8\pi l_b N_A I)]^{1/2}$ (Dautzenberg et al., 1994).

[I] (mM NaCl)	$pK_{a,\ int}^*$	$pK_{a,\ \alpha\ =\ 0.5}^*$	κ^{-1} (nm)
10	3.05	3.14	3.04
50	2.82	3.16	1.36
100	2.81	3.15	0.96

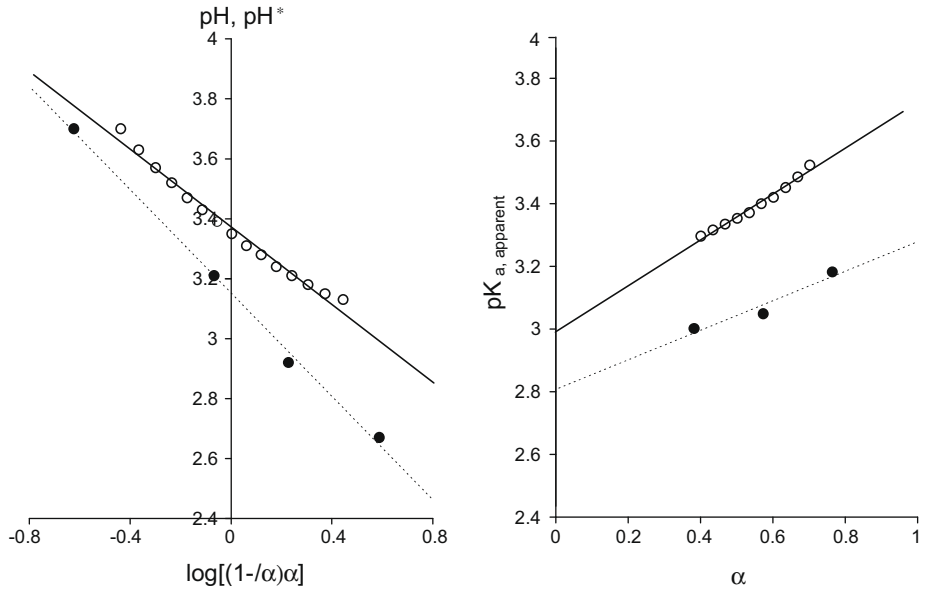

Fig. 9. Comparison of results for potentiometric (open circles; in H_2O) and ^1H NMR spectroscopy (closed circles; in D_2O) titration at the same ionic strenght (0.1 M NaCl).

(carbonate, phosphates, etc.). When using ^1H NMR spectroscopy, on the other hand, one is specifically observing the actual protonisable group of interest, or several in parallel (Tømmeraas et al., 2002), as function of changes in the solution. This analysis will be unaffected by additional weak acids/bases. Therefore, ^1H NMR spectroscopy gives the possibility for studying several parallel equilibria on the molecular level ongoing at the same time in the solution.

4. Conclusion

Hyaluronan produced by fermentation of *B. subtilis* was studied by potentiometric titration (macroscopic level) as well as ^1H NMR spectroscopy (molecular level). The dissociation constants were found to be in agreement with those previously presented for other sources of hyaluronic acid.

Therefore, it could be argued that the polyelectrolyte properties and therefore the chemical structure are identical. We have also shown that ^1H NMR spectroscopy is the preferred method for polyelectrolyte titration because of the ability to isolate the contribution of several ionisable groups on a polymer on molecular level.

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