Ionization and Solubility of Chitosan Solutions Related to Thermosensitive Chitosan/Glycerol-Phosphate Systems

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Chitosan is a linear cationic biopolymer composed of glucosamine and N-acetyl-glucosamine that is only soluble in acidic aqueous solutions and precipitates when neutralized. However, it was recently discovered that chitosan dissolved in solutions containing glycerol phosphate was soluble at near neutral pH and produced a sol−gel transition when heated. Understanding this unique thermogelling system requires improved characterization of the ionization and solubility behaviors of chitosan, in particular dependencies on temperature, salt, chitosan concentration, and \( f_0 \), where \( f_0 \) is the fraction of glucosamine monomers (deacetylated monomers) in chitosan. In the current study we performed temperature-controlled titration and dilution experiments on chitosan solutions with \( f_0 \) of 0.72, 0.85, and 0.98 at concentrations ranging from 1.875 to 30 mM of its glucosamine monomer and with 0 to 150 mM added salt. Light transmittance measurements were performed during titration to indicate precipitation. We found the apparent proton dissociation constant of chitosan, \( pK_{ap} \), to (1) decrease strongly with increased temperature, (2) increase strongly with increased salt, (3) increase strongly with increased chitosan concentration in low-salt conditions, and (4) decrease weakly with increasing \( f_0 \). All of the above influences on chitosan \( pK_{ap} \) were accurately predicted using a mean-field Poisson−Boltzmann (PB) cylindrical cell model where the only adjustable parameter was the temperature-dependent chitosan intrinsic monomeric dissociation constant \( pK_d(T) \). The resulting chitosan \( pK_0 \) values at 25 °C were in the range from 6.63 to 6.78 for all chitosans and salt contents tested. The temperature dependence of chitosan ionization was found to strongly reduce \( pK_d(T) \) by 0.023 units per °C, for example, resulting in a reduction of chitosan \( pK_d(T) \) from 7.1 at 5 °C to 6.35 at 37 °C for \( f_0 \) of 0.72 in 150 mM salt. A similar temperature-dependent reduction of the \( pK_0 \) of the glucosamine monomer was found (−0.027 units per °C) while the \( pK_0 \) of glycerol phosphate did not change significantly with temperature. The latter result suggested that chitosan solutions heated in the presence of glycerol phosphate will become partly neutralized by transferring protons to glycerol phosphate and thereby allow attractive interchain forces to form a physically cross-linked gel under the appropriate conditions. Additionally, the degree of ionization of chitosan when it precipitates upon addition of a strong base was measured to be in the range from 0.25 to 0.55 and was found to (1) be insensitive to temperature, (2) increase strongly with increased salt, and (3) increase strongly with \( f_0 \). The salt effect was accounted for by the PB model, while the influence of \( f_0 \) appeared to be due to acetyl groups impeding attractive chain-to-chain association to increase solubility and require reduced ionization levels to precipitate.

Introduction

Chitosan is a linear cationic polyelectrolyte derived by alkaline deacetylation of chitin in crustacean shells1 and is composed of glucosamine and N-acetyl-glucosamine monomers linked by \( \beta-(1\rightarrow4) \) glycosidic bonds. The fraction of monomers that are glucosamine is defined as \( f_0 \) (fraction of deacetylated monomers). Chitosan possesses beneficial biological properties including biodegradability2 and low toxicity3,4 and can be used in drug delivery, gene delivery,5−7 and wound healing.8,9 Recently, a thermosensitive gelling system based on chitosan solutions buffered with glycerol phosphate (chitosan/GP)10 was discovered and has been successfully applied to improve repair of lesions in articular cartilage.11 Nonetheless, a satisfactory understanding of chitosan/GP solution properties, solubility, and thermosensitive characteristics is lacking. To fill this void, studies are required to examine chitosan ionization12−20 and solubility behavior,17,18,21 including dependencies on ionic strength, chitosan concentration and degree of deacetylation, and temperature.

Potentiometric titration is widely used to investigate polyelectrolyte behavior in solution. Previous studies have reported titration12−19 and precipitation20,21 behavior of chitosan during neutralization. However, no molecular scale theoretical model was proposed or tested to predict chitosan titration nor has work to date investigated the particular influence of temperature on chitosan ionization. The objective of the current study was therefore to examine chitosan ionization and solubility behavior as a function of ionic strength, \( f_0 \), chitosan concentration, and temperature. A second objective was to assess the ability of the nonlinear Poisson−Boltzmann cylindrical cell model to predict the apparent dissociation constant of chitosan at finite concentration, its \( pK_{ap} \), and the \( pK_{ap} \) dependence on chitosan ionization, level of deacetylation, concentration of chitosan, medium ionic strength, and temperature. Use of this model to predict the polyelectrolyte titration behavior was pioneered by Kotin and Nagasawa.22 We performed titration and dilution experiments simultaneously measuring laser light transmittance...
to detect precipitation of chitosan solutions at different but constant temperatures ranging from 5 to 37 °C. Solutions of chitosan with $f_D$ ranging from 0.72 to 0.98 and with the monovalent salt concentration ranging from 0 to 150 mM were titrated, and the resulting data were analyzed using a nonlinear Poisson–Boltzmann cylindrical cell model. We hypothesized that (i) increased ionic strength would dampen ionization-dependent behavior via electrostatic screening and decrease chitosan solubility, (ii) that increased $f_D$ would increase solubility and reduce apparent $pK_a$ via increased electrostatic repulsion, (iii) that increasing the concentration of chitosan in solution would increase apparent $pK_a$ by flattening intermolecular electrostatic potential profiles, and (iv) that increasing temperature would expel protons from chitosan and thereby reduce chitosan $pK_a$.

We also performed temperature ramp experiments with simultaneous pH measurement on solutions of simple electrolytes including glucosamine (the ionizable subunit of chitosan) and GP, the anionic buffer present in chitosan/GP solutions. The goal here was to evaluate the contribution of the temperature-dependent ionization behavior of the monomer (glucosamine) toward behavior of the polymeric form (chitosan) and to examine the influence of temperature on GP ionization. We hypothesized that GP ionization may be relatively insensitive to temperature such that GP may act as an efficient acceptor of protons released from chitosan when chitosan/GP solutions are heated. Such a heat-induced transfer of protons from chitosan to GP would reduce the degree of ionization of chitosan and, therefore, chitosan intermolecular electrostatic repulsion, allowing attractive hydrophobic and hydrogen bonding interchain forces to initiate physical cross-linking, resulting in the formation of a gel in sufficiently concentrated solutions.

Theory

A cylindrical cell model representation of chitosan in solution was used to obtain a molecular electrostatic potential by solving the nonlinear Poisson–Boltzmann (PB) equation within the cylindrical cell. ¹ The model was developed for solutions that contain the cationic polyelectrolyte, its anionic counterion (Cl⁻), a strong base titrant (NaOH), and a monovalent salt (NaCl). The molecular electrostatic potential profile from a numerical solution was then used to predict titration pH curves as described below.

Structural Parameters of the Cylindrical Cell Model. Chitosan is composed of two distinct monomers: a fraction $f_D$ of ionizable glucosamine and a fraction $1 - f_D$ of non-ionizable N-acetyl-glucosamine (Figure 1A). Chitosan is represented as an infinitely long impermeable cylinder of radius $a$ (Figure 1B) where discrete charge sites are smeared out to form a uniform surface charge density $\sigma$

$$\sigma = \frac{eaf_D}{2\pi al}$$  \hspace{1cm} (1)$$

where $e$ is the elementary charge, $\alpha$ is the degree of ionization of the polycation ($\alpha = 0$ is neutral and $\alpha = 1$ is fully ionized), and $l$ is the length of the monomer that is set to $l = 0.52$ nm following structural data. ² ³ ⁴ The radius of the inner cylinder representing chitosan is taken as $a = 1.3$ nm, even though this value is greater than what structural data suggest (0.42 nm), ² ⁴ an assumption that is elaborated upon below in the Discussion. Each polymer chain is located at the center of a cylindrical cell whose radius $b$ (Figure 1B) is determined from the monomer

![Figure 1](Image)

$Figure 1.$ (A) A four monomer segment of chitosan represented with two protonated monomers, a neutral monomer, and a non-ionizable N-acetyl-glucosamine monomer. Each monomer has length $l$. (B) Cylindrical cell model showing the inner cylinder with radius $a$, representing a chitosan molecule that is contained in its electrolyte envelope extending to radius $b$, which is determined by chitosan concentration (eq 2). Representative profiles of electrostatic potential $\psi(\rho)$, counterion concentration, $c_-$, and co-ion concentration, $c_+$, are shown for the case of $c_{NaCl} = 15$ mM at $\alpha = 0.75$ and $f_D = 1.00$. The circle indicates the electrostatic potential at the surface of the polyelectrolyte, $\psi(r=a)$, concentration $c_p$ (including both glucosamine and N-acetyl-glucosamine) and monomer length $l$, according to

$$b = \left( \frac{1}{\pi l c_p N_A} \right)^{1/2}$$  \hspace{1cm} (2)$$

where $N_A$ is Avogadro’s number.

Counterion and Co-ion Molecular Scale Concentration Profiles, Average Macroscopic Values, and Activities. The polycation is surrounded by mobile ions in the region $a < r < b$. Using the mean-field approximation, ²³ these ions are assumed to follow a Boltzmann distribution at equilibrium, resulting in a concentration profile $c_i(r)$ for mobile ion $i$ about the polyion that is a function of radial position $r$ and electrostatic potential $\psi(r)$

$$c_i(r) = c_i^0 \exp \left( -\frac{z_i e \psi(r) kT}{kT} \right)$$  \hspace{1cm} (3)$$

where $z_i$ is the valence of the mobile ion $i$, $T$ is the temperature, and $k$ is Boltzmann’s constant. The value of $c_i^0$ is related to the mean concentration of positive and negative electrolyte ions, $\bar{c}_\pm$, in the volume of the cylindrical cell according to

$$\bar{c}_\pm = c_i^0 \int_{b}^{a} 2\pi r e^{\frac{z_i e \psi(r) kT}{kT}} dr$$  \hspace{1cm} (4)$$

where a mono-monovalent electrolyte, $z_i = +1$ or $-1$, is considered. $\gamma_\pm = b^2 \int_{a}^{b} 2\pi r e^{\frac{z_i e \psi(r) kT}{kT}} dr$ are the mobile ion activity coefficients in the cylindrical cell and $\gamma_\pm c_\pm = c_i^0$ are ion activities ($\alpha_\pm = \gamma_\pm c_\pm = c_i^0$) as derived previously ²³ for this particular mean-field theory.

Poisson–Boltzmann Equation. The electrostatic potential, $\psi(\rho)$ ($a < r < b$), can be found from the solution to the Poisson–Boltzmann equation ²⁶–²⁸ in cylindrical coordinates
subject to boundary conditions from Gauss’ law
\[
\frac{d\psi(r)}{dr}igg|_{r=a} = -\frac{\sigma}{\epsilon} = -\frac{e\sigma 0}{2\pi a \epsilon} \quad \text{and} \quad \frac{d\psi(r)}{dr}igg|_{r=b} = 0 \tag{6}
\]
where \( \epsilon \) is the permittivity of water and \( \rho(r) \) is the spatially varying charge density. The solution to eq 5 must also satisfy eq 4 since the polyelectrolyte solution is contained in a closed volume with known total mobile ion concentrations rather than in contact with an infinite bath.

We now demonstrate that the choice of reference electric potential is arbitrary. If we add a constant \( C \) to a solution, \( \psi_{\text{sol}}(r) \), of eqs 4–6, then this new function, \( \psi_{\text{osol}}(r) + C \), clearly satisfies the boundary conditions of eq 6 since only derivatives of the potential appear in eq 6. However, to satisfy eq 4, the mobile ion activities, i.e., \( c_0^+ \) and \( c_0^- \), must change to \( e_0^+ \) and \( e_0^- \), respectively. Finally, inserting \( \psi_{\text{osol}}(r) + C \) into eq 5 written for a mono-monovalent electrolyte and using \( e_0^+ \) and \( e_0^- \) for \( c_0^+ \) and \( c_0^- \) we find

\[
\frac{d^2(\psi_{\text{osol}}(r) + C)}{dr^2} + \frac{1}{r} \frac{d\psi_{\text{osol}}(r) + C}{dr} = e_0^+ e^{-e_0^+ CRRT} e^{e_0^- (\psi_{\text{osol}}(r) + C)RT} - e_0^- e^{-e_0^- (\psi_{\text{osol}}(r) + C)RT} - \frac{e}{e_0^-} e^{e_0^- (\psi_{\text{osol}}(r) + C)RT} - \frac{e}{e_0^+} e^{-e_0^+ (\psi_{\text{osol}}(r) + C)RT} \tag{7}
\]

showing that \( \psi_{\text{osol}}(r) + C \) satisfies the PB equation and boundary conditions as well as the known content of mobile ions in the closed solution, as long as \( e_0^+ \) and \( e_0^- \) become \( e_0^+ e^{-e_0^+ CRRT} \) and \( e_0^- e^{-e_0^- CRRT} \), respectively. This result also reveals the interesting conclusion that there always exists a reference potential (value of \( C \)) for which mobile ion activities are equal, \( e_0^+ = e_0^- \).

In the context of this study, we consider three types of mobile ions, namely, the counterion Cl\(^-\) (from the solvent HCl and NaCl salt added), the co-ion Na\(^+\) (from the dissociation of NaOH and NaCl), and protons (H\(^+\)). Hydroxyl ions (OH\(^-\)) are neglected since only acidic solutions are considered. To facilitate numerical solution of eq 5, we choose to equate total counterion and co-ion activities

\[
c_0^+ = c_0^+ \quad \text{and} \quad c_0^- = c_0^- \tag{8}
\]
which simply implies a particular value of reference potential, or \( C \), as described above. Note that Na\(^+\) and H\(^+\) can be grouped together as they are both monovalent cations following the same Boltzmann distribution. Equation 5 can then be rewritten in terms of \( \epsilon_0^+ \) alone, using eq 8, to obtain

\[
\frac{d^2\psi(r)}{dr^2} + \frac{1}{r} \frac{d\psi(r)}{dr} = \frac{2\epsilon_0^+}{\epsilon} \sinh \left( \frac{\epsilon\psi(r)}{kT} \right) \tag{9}
\]

For a given \( \alpha \) that defines the polyelectrolyte surface charge \( \sigma \) according to eq 1, and a given polyelectrolyte monomer concentration \( c_p \), which defines the outer cell radius \( b \) according to eq 2, we numerically solved eq 9 such that the boundary conditions of eq 6 are satisfied. An initial guess for \( \epsilon_0^+ \) was taken, and the solution was then iterated with different values of \( \epsilon_0^+ \) until the right-hand side of eq 4 converged to the experimentally known average ion concentrations, \( \tilde{c}_i \). In this way the Poisson–Boltzmann equation was solved for a closed volume of polyelectrolyte solution at finite concentration that is not in equilibrium with an external bath. The numerically obtained solution for \( \psi(r) \) was then finally adjusted by subtracting from it the value found for \( \psi(b) \) to redefine the reference potential as \( \psi(b) = 0 \), which has no physical consequence, but conforms to the reference potential used to derive eq 11 below in Appendix 1 of the Supporting Information. This reference potential also conveniently redefines \( c_0^+ \) as the ion concentrations at \( r = b \), in addition to the ion activity \( a_\circ \) in the polyelectrolyte fluid phase.

**Theoretical Dependence of pH on \( pK_{ap} \), \( \alpha \), and \( \psi|_{r=\infty} \).** The pH is related to proton activity, \( a_\circ \), in the fluid phase via

\[
pH = -\log_{10} \frac{a_\circ}{a_\circ} - \frac{\epsilon\psi|_{r=\infty}}{kT \ln 10} \tag{10}
\]

where the rightmost term contains the proton concentration, \( a_\circ \), at the cylindrical cell boundary, \( r = b \), where \( \psi(r = b) = 0 \) and \( d\psi(r = b)/dr = 0 \), allowing these protons to behave ideally. By equating the chemical potential of protons in the fluid phase to that of the polyelectrolyte chain, a theoretical relationship for pH as a function of \( pK_{ap} \), \( \alpha \), and \( \psi|_{r=\infty} \), the electrostatic potential at the surface of the polyelectrolyte in the Poisson–Boltzmann cylindrical cell model was derived (eq 35 in Appendix 1 of the Supporting Information; see also Marcus\(^\text{23}\) for a similar derivation) as

\[
pH = pK_{ap}(T) + \log_{10} \frac{1 - \alpha}{\alpha} - \frac{\epsilon\psi|_{r=\infty}}{kT \ln 10} \tag{12}
\]

that includes two contributions, the first representing the intrinsic monomeric dissociation constant \( pK_\circ(T) \) and the second containing the polyelectrolyte surface potential \( \psi|_{r=\infty} \) which can be found by solving the Poisson–Boltzmann equation. Note that for simple acid–base electrolytes \( \psi|_{r=\infty} = 0 \) in the current model so that the apparent \( pK_a \) (\( pK_{ap} \)) and \( pK_a \) become identical \( pK_{ap}(T) = pK_a(T) = pK_a(T) \).

**Electroneutrality and the Poisson–Boltzmann Equation** Determine \( \alpha \) and \( \psi|_{r=\infty} \). The degree of ionization, \( \alpha \), is required to calculate pH from eq 11 and to initiate solution of the eq 9 to find \( \psi|_{r=\infty} \), which is also required in eq 11. To determine \( \alpha \), we used the condition of macroscopic electroneutrality, again neglecting hydroxyl ions

\[
\tilde{c}_\text{Cl} - \tilde{c}_\text{Na} - \tilde{c}_\text{H} - c_g^+ = 0 \tag{13}
\]

where \( c_g^+ \) is the concentration of ionized glucosamine monomers

\[
c_g^+ = \alpha f_p c_p \tag{14}
\]

Substituting eqs 10 and 14 into eq 13 we find
and the corresponding \( \psi_{T \text{ref}} \) were determined for each particular experimental pH in titration and dilution experiments. In most cases, the proton concentration, \( 10^{-pH} \), is negligible, and \( \alpha \) is simply determined from the known ion and monomer concentrations (taking into account any dilution from the cumulative titrant addition). For cases where proton concentration must be considered, \( 10^{-pH} \gamma_+ \) can be estimated by using the experimental pH and assuming ideality for the protons \( \gamma_+ = 1 \), thus avoiding the need to simultaneously solve the Poisson Boltzmann equation to find \( \alpha \).

**Determination of \( \text{pK}_a(T) \) and of the Temperature Dependence of \( \text{pK}_a \).** Once the values of \( \psi_{T \text{ref}} \) and \( \alpha \) for each pH in a given titration curve are found as described above (for pH values in the solvable range of titration experiments), we subsequently insert these \( \psi_{T \text{ref}} \) and \( \alpha \) values into eq 11 and fit eq 11 to pH data by adjusting \( \text{pK}_a(T) \) to find the best fit value of \( \text{pK}_a(T) \) for each temperature. Temperature-induced changes in \( \text{pK}_a \) were directly derived from temperature-induced pH changes using the following relationship derived in Appendix 2 of the Supporting Information

\[
dpK_a = \frac{\alpha (1-\alpha) (c_{\text{H}^+} + c_{\text{OH}^-})}{c_{\text{H}}^0 - c_{\text{g}}^0} \cdot dpH - \frac{c_{\text{OH}^-}}{c_{\text{g}}^0} \cdot dpK_{\text{water}}
\]

where \( c_{\text{H}^+} \) and \( c_{\text{OH}^-} \) are proton and hydroxyl ion concentrations, \( c_{\text{g}}^0 = f_{\text{H}^+} c_g \) is the total glucosamine monomer concentration and \( dpK_{\text{water}} \) is the dissociation constant of water. Equation 16 can be simplified to

\[
dpK_a \approx dpH
\]

if

\[
\frac{c_{\text{H}^+}}{c_{\text{g}}^0} \ll 1 \quad \text{and} \quad \frac{c_{\text{OH}^-}}{c_{\text{g}}^0} \ll 1
\]

and \( \alpha \) is not too close to 0 or 1. These conditions were valid in our experiments where \( c_g > 3 \text{mM} \) and pH > 5 (corresponding to \( \alpha < 0.75 \)) or pH > 5.3 (corresponding to \( \alpha < 0.90 \)) for cases with 0 or 150 mM added salt, respectively. Under these conditions, the temperature-induced change in \( \text{pK}_a \), \( \Delta pK_a(T) = pK_a(T) - pK_a(T_{\text{ref}}) \), with respect to that of an arbitrary reference temperature \( T_{\text{ref}} \), can be determined from the corresponding pH difference via

\[
\Delta pK_a(T) = pK_a(T) - pK_a(T_{\text{ref}}) = pH(T) - pH(T_{\text{ref}})
\]

**Experimental Methods**

**Reagents and Solutions.** Ultrapure chitosans with \( f_d \) ranging from 0.72 to 0.98 were provided by BioSyntech (Table 1). These polymers have a number average molecular weight (\( M_n \)) ranging from 100 to 550 kDa and a polydispersity index (PDI = \( M_w/M_n \)) of 1.6–2.3. NaOH 1 N (Aldrich, catalogue no. 31951-1) and HCl 1 N (Aldrich, catalogue no. 31894-9) were used to prepare the titrant solution and to dissolve chitosan, respectively. Chitosan solutions with precise concentrations were prepared from powders dried at 60 °C for 2 days using a heated centrifugal vacuum concentrator (Savant Speedvac, model SS110) and kept in a desiccator unit until use.

**Table 1. Characteristics of Chitosan**

<table>
<thead>
<tr>
<th>( f_d )</th>
<th>( M_n ^a ) (kDa)</th>
<th>PDIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.72</td>
<td>553</td>
<td>2.3</td>
</tr>
<tr>
<td>0.85</td>
<td>226</td>
<td>1.7</td>
</tr>
<tr>
<td>0.98</td>
<td>103</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\( ^a \) Number average molecular weight (\( M_n \)) by triple detector gel permeation chromatography.\(^b \) Polydispersity index (PDI = \( M_w/M_n \)) using weight average molecular weight (\( M_w \)) obtained by gel permeation chromatography, both provided by the manufacturer.

For titration experiments, chitosan was dissolved to obtain a 3 mM concentration of its glucosamine monomer in a 500 mL volumetric flask, and HCl was added to a molar ratio of HCl/glucosamine of 1:1 resulting in \( \alpha = 0.97 \) according to electroneutrality (eq 13) and using the resulting pH of the solution of about 4. To make these solutions, dried chitosan was added to deionized water, stirred to disperse the powder prior to adding HCl, and then stirred overnight to ensure complete dissolution. The NaCl concentration was adjusted by adding appropriate amounts of 5 M NaCl (Fisher Scientific, catalogue no. S271-1) resulting in a maximum dilution of glucosamine monomer and HCl to 2.91 mM at the highest level of added salt used of \( c_{\text{NaCl}} = 150 \text{mM} \).

In experiments where chitosan was diluted rather than titrated to obtain concentration-dependent behavior, chitosan solutions corresponding to 30 mM glucosamine monomer were prepared in a manner similar to that described above but to obtain molar ratio of HCl/glucosamine of 0.75:1. The NaCl concentration was subsequently adjusted by adding appropriate amounts of 5 M NaCl. Chitosan solutions were then diluted while monitoring pH as described below from the initial 30 mM glucosamine monomer up to a dilution corresponding to 1.875 mM glucosamine monomer. Dilution was performed using each of the following conditions: (1) Chitosan without added salt was diluted with deionized water, (2) chitosan with excess salt of \( c_{\text{NaCl}} = 300 \text{mM} \) was diluted with deionized water, and (3) chitosan with \( c_{\text{NaCl}} = 300 \text{mM} \) was diluted with 300 mM NaCl. The variation of \( \text{pK}_a \) during these dilution experiments corresponds to the recorded pH variation similar to eq 19, but with temperature replaced by concentration, since \( \alpha = 0.75 \) is constant throughout these experiments and proton concentrations are negligible.

Monomeric glucosamine (non-polyelectrolyte) solutions were also prepared by adding 12.9 mg d-(+)-glucosamine hydrochloride (Sigma, catalogue no. G1514) to 20 mL of distilled and deionized water to obtain 3.00 mM d-(+)-glucosamine with 3.00 mM Cl− counterion. Further addition of 0.3 mL of 0.01 N NaOH solution produced a solution with \( \alpha = 0.95 \) that was used for temperature ramp tests described below. (Equation 17 is satisfied since \( pH > 5.8 \), and \( dpK/dpH = 1.00 \pm 0.01 \).) Glycerol phosphate solutions at a concentration of 50 mM with \( \alpha = 0.5 \) were prepared by adding 297 mg of GP (Sigma, catalogue no. G9891) to 20 mL of distilled and deionized water followed by addition of 0.5 mL of 1 N HCl. (Equation 17 is satisfied since \( pH = 6.2 \) and \( dpK/dpH = 1.00 \pm 0.01 \).)

**Experimental Apparatus.** A custom apparatus (Figure 2) allowed simultaneous measurement of pH and laser light relative transmittance (\( L_o \)), the latter used to detect precipitation of chitosan solutions. We used this apparatus for titration and dilution experiments of chitosan and to measure pH and \( \text{pK}_a \) of d-(+)-glucosamine, chitosan, and GP solutions during temperature ramp tests. Solutions were continuously stirred inside a 50 mL reaction jacketed beaker (Kontes, catalogue no. 317000-0050) with the jacket coupled to a heating circulating bath (Neslab, model RT-111) to control temperature via an automatic temperature compensation (ATC) probe (Accumet, Fisher Scientific catalogue no. 13-620-16) immersed in the tested solution. The pH electrode (Orion, model no. 8115 connected to the Accumet, model 20 pH meter) was calibrated with National Institute of Standards and Technology standards at room temperature, and the automatic ATC probe was corrected for the temperature dependence of the pH electrode. Addition of titrant was performed using an automatic burette (Schott,
Titronic Universal 20 mL), which added 0.3 mL increments of 0.01 N NaOH every 2 min for titration experiments; for dilution experiments either deionized water or 300 mM NaCl were added as described above. To detect chitosan precipitation, laser light relative transmittance, $L_T$, was measured throughout titration by passing a 635 nm diode laser beam (Coherent, 5 mW, 31-0128) through the solution and walls of the beaker with detection by a photo detector (Coherent, Laser-Q VIS, 33-0241 connected to a multimeter Fluke, model 45 dual display). The point of precipitation was characterized by a sharp decrease of $L_T$ following injection of the titrant (as in Figure 3). The value of $R_p$ at which these $L_T$ values decreased to indicate precipitation was termed $R_p$. The computer controlled the titration burette and bath temperature in addition to acquiring pH, temperature, and $L_T$ data.

**Statistical Analysis.** In this study, when presenting experimental data with error bars, the mean value with a confidence interval of one standard deviation (SD) and the number of independent measurements ($n$) are reported. For example, mean ± SD, $n = 3$, indicates that the mean of three measurements with a confidence interval of one standard deviation is reported. In the case where fit parameters are reported, the above also applies except that the SD now corresponds to the standard deviation of each fit parameter obtained with $n$ independent fits, one for each measurement.

**Results**

**Influence of Ionic Strength and Fraction of Deacylation ($f_D$) on Titration ($pK_{ap}$ and $pK_0$) and Precipitation ($R_p$) Behavior of Chitosan.** In regions where the chitosan ionization state is relatively high ($\alpha > 0.5$), increasing ionic strength significantly increased chitosan $pK_{ap}$ almost by a full unit upon addition of 150 mM salt (Figure 4). On the contrary, similar measurements of monomeric glucosamine displayed only a minimal increase in $pK_a$, indicating that the salt dependence of $pK_{ap}$ of chitosan is due to modulation of the surface potential in the polyelectrolyte term, $-\psi_j\sigma / (kT \ln 10)$ of eq 12. This conclusion was also supported by the PB model predicting a strong decrease of the chitosan surface potential $\sigma$ as ionic strength increased (Figure 5A) and by the ability of this model to capture titration behavior (Figure 4). The degree of ionization of chitosan at the point of precipitation, $R_p$, increased with increasing ionic strength (Table 2), presumably due to increased electrostatic screening (Figure 5A) allowing for readier inter-chain association.

Increasing $f_D$ produced only a slight reduction in $pK_{ap}$ (Figure 6) that was also consistent with the PB model showing a slight
increase in the electrostatic potential for higher $f_D$ (Figure 5B). This reduction in $p_{K_0}$ with increasing $f_D$ is larger without added salt (Figure 6). In contrast to the relatively slight effect of $f_D$ on $p_{K_0}$, a much stronger influence of $f_D$ on the degree of ionization at precipitation, $\alpha_p$, was observed where chitosan with higher levels of deacetylation precipitated at higher degrees of ionization (Table 2). For example, for solutions without added salt, chitosan with $f_D = 0.98$ precipitated at $\alpha_p = 0.50$ while chitosan with $f_D = 0.72$ precipitated at $\alpha_p = 0.25$.

Table 2. Degree of Ionization of Chitosan at Precipitation, $\alpha_p$, as well as $p_{K_0}$ and the Slope of $p_{K_0}$ vs $\alpha$, $\Delta p_{K_0}/\Delta \alpha$ or $C_{NOCl}$, Measured at 25 °C for Different $f_D$ Values and with Different NaCl Concentrations, $C_{NaCl}$

<table>
<thead>
<tr>
<th>$C_{NaCl}$ (mM)</th>
<th>$f_D$</th>
<th>$p_{K_0}$</th>
<th>$\Delta p_{K_0}/\Delta \alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.72</td>
<td>0</td>
<td>6.40</td>
<td>0.25 (0.01)</td>
</tr>
<tr>
<td>0.85</td>
<td>0.25</td>
<td>0.30</td>
<td>0.43 (0.008)</td>
</tr>
<tr>
<td>0.98</td>
<td>0.30</td>
<td>0.40</td>
<td>0.48 (0.009)</td>
</tr>
</tbody>
</table>

$^a$ Calculation of $\alpha_p$ from experimental measurements ($n = 3$ with error of ±0.05 due to measurement accuracy). Similar values were obtained at 5 and 37 °C with $n = 3$. $c_{NOCl}$ are values of $p_{K_0}$ obtained using $a = 1.3$ nm via the PB fit described in the "Determination of $p_{K_0}$ (T) and of the Temperature-Induced Change in $p_{K_0}$" subsection. The error is represented as the standard deviation of the $p_{K_0}$ values (n = 3). $\Delta p_{K_0}/\Delta \alpha$ is a quality of fit parameter and corresponds to the root-mean-square of the difference between the experimental and fit (PB) $p_{K_0}$ values for the non-precipitated region and with $a = 0.90$. The error is represented as the standard deviation of the $p_{K_0}$ values (n = 3). $\Delta p_{K_0}/\Delta \alpha$ are slope values obtained by a linear fit of the theoretical PB calculation in the non-precipitated region and with $a = 0.90$ using $a = 1.3$ nm. The value in parentheses is a quality of fit parameter and corresponds to the root-mean-square of the difference between the experimental and fit (PB) $p_{K_0}$ values for the non-precipitated region. $p_{K_0}$ are values of $p_{K_0}$ obtained using a linear fit of the experimental $p_{K_0}$ values in the non-precipitated region and with $a = 0.90$. The error is represented as the standard deviation of the $p_{K_0}$ values (n = 3).

The intrinsic monomeric dissociation constant $p_{K_0}$ at 25 °C obtained from linear fits of titration data (Figure 6) revealed a slight dependence on the concentration of added salt and $f_D$ ($p_{K_0}$ in Table 2). The lowest $p_{K_0}$ (6.40 at 25 °C) was found for chitosan ($f_D = 0.98$) without added salt, and the highest ($p_{K_0}$ = 6.71 at 25 °C) for the same chitosan ($f_D = 0.98$) with 150 mM added salt.

The intrinsic $p_{K_0}$ obtained from PB model fits (Figure 4), $p_{K_0}$, at 25 °C displayed less dependence than $p_{K_0}$ on $f_D$ and $C_{NaCl}$. We found no influence of $f_D$ on $p_{K_0}$ for a given amount of added salt while the values of $p_{K_0}$ at $C_{NaCl} = 150$ mM were slightly higher than those at 0 or 15 mM added salt. An exception to these trends was seen with $f_D = 0.98$, where $C_{NaCl}$ = 0 mM, where the PB fit was not as good as that for other conditions (Figure 4). We found that a larger chitosan rod radius of $a = 1.5$ nm rather than 1.3 nm for $f_D = 0.98$, $C_{NaCl} = 0$ mM, resulted in a better fit and a value of $p_{K_0}$ of 6.69, which is more consistent with $p_{K_0}$ found for other conditions.

Nonlinearity and Convexity of the PB Model of $p_{K_0}$(α). The PB model (Figure 5) indicated that the normalized surface potential was typically greater than unity (with the exception of $C_{NaCl} = 150$ mM or small values of $\alpha$) so that linearization...
Therefore a nonlinear variation of the potential with α is expected, particularly for low levels of added salt (0 and 15 mM). When using the PB model to calculate the surface potential $\psi|_{\text{m}}$ to obtain pH (eq 11) and $pK_{ap}$ (eq 12) we found $\psi|_{\text{m}}$ to be a concave function of α such that its negative in the rightmost term of $pK_{ap}$ (eq 12) was a convex function of α (Figure 7). The nonlinearity and convexity of $pK_{ap}(\alpha)$ was particularly pronounced for low-salt cases and when the counterion was present (Figure 7A, 0 mM salt, no co-ions). The nonlinearity of $pK_{ap}(\alpha)$ was also visible in experimental curves at $c_{\text{NaCl}} = 0$ and 15 mM where we can see a slight convexity of titration curves, more easily observed for $f_D$ of 0.72 and 0.85 since they have more data in the non-precipitated range (Figure 4). An important consequence of the nonlinearity of $pK_{ap}$ versus α in our study was its ability to account for the discrepancy between $pK_0$ found by the nonlinear PB model ($pK_{0(\text{PB})}$) versus that found by linearly extrapolating experimental $pK_{ap}$ from the non-precipitated region to $\alpha = 0$ ($pK_{0(\text{lin})}$). The linearly extrapolated $pK_0$ ($pK_{0(\text{lin})}$) values were always lower than the $pK_0$ values from the PB model ($pK_{0(\text{PB})}$) (Figure 7). This difference was larger in the absence of added salt and when $f_D$ was high and also when extrapolation was performed using a small number of experimental points, as expected because of the higher curvature of theoretical $pK_{ap}$ curves at large $f_D$ and low added salt (Figure 7). As an example, the largest difference was found between $pK_0(\text{PB})$ and $pK_{0(\text{lin})}$ (Table 2) when $f_D = 0.98$ and $c_{\text{NaCl}} = 0$ mM, with $pK_0(\text{PB}) = 6.78$ versus $pK_{0(\text{lin})} = 6.40$, respectively, while an excess of salt with $c_{\text{NaCl}} = 150$ mM produced similar $pK_0(\text{PB})$ and $pK_{0(\text{lin})}$ values of 6.75 and 6.71.

**Influence of Chitosan Concentration on $pK_{ap}$**

The effect of chitosan concentration on chitosan $pK_{ap}$ at 25 °C was tested by performing dilution experiments using a chitosan with $f_D = 0.72$ at $\alpha = 0.75$. The $pK_{ap}$ decreased significantly, by more than 0.4 units when diluting chitosan with deionized water from 30 to 3 mM glucosamine, whether salt was initially present or not (Figure 8A). On the contrary, adding excess salt to chitosan and diluting with excess salt at the same concentration had no effect on chitosan $pK_{ap}$ (Figure 8A). Thus chitosan $pK_{ap}$ depends strongly on chitosan concentration when either no salt is present or when changing chitosan concentration changes salt concentration due to the diluting solution. These results were also well described by PB model (Figure 8B) since dilution of chitosan increases surface potential $\psi|_{r = \alpha}$ when salt is not in excess (Figure 5C) and thus expels protons from the chain to reduce pH, or equivalently $pK_{ap}$. Interestingly, the surface potential calculated from the PB model without added salt was linearly dependent on the logarithm of the glucosamine concentration in the range of 1–30 mM (inset in Figure 8B), consistent with previous observations.31

**Temperature Dependence of $pK_a$ of Glycerol Phosphate and Glucosamine and of $pK_{ap}$ and $\alpha_p$ of Chitosan**

Temperature ramps that heated solutions from 5 to 40 °C were performed for two simple electrolytes, d-(+)-glucosamine and glycerol phosphate, as well as on chitosan solutions. $pK_{ap}$ versus temperature was obtained from the measured pH according to eq 19 with $T_{ref} = 5$ °C (Figure 9). d-(+)-Glucosamine exhibited a strong variation of $pK_a$ with a temperature of $-0.027 \pm 0.001$ pK unit/°C (mean ± SD, $n = 3$), representing nearly a full unit decrease from 5 to 40 °C. In contrast the $pK_a$ of glycerol phosphate was nearly invariant in the same temperature range. A similarly strong temperature dependence of glucosamine $pK_a$ was observed previously by Neuberger32 while the temperature-
Temperature ramp tests revealing temperature dependence of pK\textsubscript{a} of glycerol phosphate was observed previously by Fukada.\textsuperscript{33}

**Figure 8.** Dependence of chitosan pK\textsubscript{a} at 25°C on chitosan concentration (ΔpK\textsubscript{a}(\text{chitin}) = pK\textsubscript{a}∞(\text{chitin}) − pK\textsubscript{a}c(chitin) = 30mM) represented as glucosamine monomer concentration. (A) Dilution of chitosan (f\textsubscript{D} = 0.72) at a molar ratio of HCl/glucosamine monomer of 0.75:1. The PB model is shown as continuous lines. (B) PB model with f\textsubscript{D} = 0.72 for chitosan with a molar ratio of HCl/glucosamine monomer of 0.75:1 and then diluted with various concentrations of NaCl, where the initial NaCl concentration in the chitosan solution was identical to that used for dilution.

**Figure 9.** Temperature ramp tests revealing temperature dependence of pK\textsubscript{a} of glycerol phosphate (α = 0.5) and glucosamine (α = 0.95) and pK\textsubscript{a} of chitosan (0.01 N NaOH added to obtain α = 0.75), the latter with f\textsubscript{D} = 0.72 and c\textsubscript{NaCl} = 150 mM (from eq 19 with T\textsubscript{ref} = 5°C) without added salt. The gray squares are PB model values of ΔpK\textsubscript{a} = pK\textsubscript{a}(T) − pK\textsubscript{a}(T\textsubscript{ref}) obtained from the PB model fits of pK\textsubscript{a} versus α titration measurements of chitosan at constant but different temperatures of 5, 25, and 37°C (Figure 10) in the non-precipitated region and where α ≤ 0.90. pK\textsubscript{a} of D-(+)-glucosamine and pK\textsubscript{a} of chitosan displays a significant decrease upon heating while pK\textsubscript{a} of glycerol phosphate remained constant.

Chitosan solutions with f\textsubscript{D} = 0.72 and 150 mM NaCl at α = 0.75 were also exposed to temperature ramps from 5 to 40°C and chitosan pK\textsubscript{a} versus temperature obtained from the measured pH according to eq 19 with T\textsubscript{ref} = 5°C (Figure 9). The observed temperature dependence of chitosan pK\textsubscript{a} was slightly lower than that for glucosamine with a slope of −0.0232 ± 0.0003 pK unit/°C (mean ± SD, n = 3), indicating that this temperature-dependent ionization of chitosan arises mainly from the temperature dependence of its intrinsic monomeric dissociation constant pK\textsubscript{d}(T) in eqs 11 and 12. This conclusion was further confirmed by the negligible effect of temperature on the normalized potential (e\textsubscript{q}/kT) profile in the PB model (Figure 5D), eliminating any temperature-dependent contribution of the electrostatic contribution to chitosan pK\textsubscript{a} (i.e., −e\textsubscript{q}|\textsubscript{m}/kT ln 10 in eq 12 is temperature-independent). It is noteworthy that the temperature independence of the normalized potential in the PB model (Figure 5D) was due to the temperature dependence of water permittivity, which decreases with increasing temperature, resulting in a linearly increasing electrostatic potential and therefore a potential (e\textsubscript{q}/kT) normalized to temperature that was temperature-independent.

Chitosan titration experiments performed at different but constant temperatures (5, 25, and 37°C in Figure 10) also showed a strong temperature dependence of chitosan pK\textsubscript{a} that was independent of α since increasing temperature vertically shifted pK\textsubscript{a} versus α and uniformly so for all values of α in the non-precipitated regions. The pK\textsubscript{a} values obtained from PB model fits of these latter titration measurements were used to calculate ΔpK\textsubscript{a} = pK\textsubscript{d}(T) − pK\textsubscript{d}(T\textsubscript{ref}) with T\textsubscript{ref} = 5°C (gray squares in Figure 9) and revealed a temperature dependence similar to pK\textsubscript{a} measured by temperature ramp tests of chitosan. In contrast to the strong temperature dependence of chitosan pK\textsubscript{a}, the degree of ionization of chitosan at precipitation, α\textsubscript{p}, did not appear to depend on temperature according to experiments performed at 5, 25, and 37°C with f\textsubscript{D} of either 0.72 or 0.98 since α\textsubscript{p} values at 25°C were indistinguishable from those obtained at 5 and 37°C (Table 2).

**Discussion**

The primary objective of the current study was to examine chitosan ionization and precipitation behavior as a function of ionic strength, fraction of deacetylation (f\textsubscript{D}), chitosan concentration, and temperature and to assess the ability of the nonlinear

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**Figure 10.** Chitosan (f\textsubscript{D} = 0.72) pK\textsubscript{a} vs α (mean ± SD, n = 3) at constant but different temperatures of 5, 25, and 37°C without added salt. Data obtained in the precipitated region are shown in gray, and PB model predictions as continuous lines.
Poission–Boltzmann cylindrical cell model to predict chitosan solution properties at finite chitosan concentration. We found that increasing salt \((c_{NaCl})\) strongly increased \(pK_{ap}\) (Figures 4 and 6) since increased electrostatic screening facilitated chain protonation\(^{14}\) by reducing the chitosan surface potential and the magnitude of the polyelectrolyte term \(-\psi_j / (\epsilon_0 kT \ln 10)\) in eq 12. As also expected, chitosans with higher levels of deacetylation (higher \(f_0\)) bore a slightly increased surface charge density (eq 1) and therefore possessed slightly lower \(pK_{ap}\) values (Figures 5C and 6) due to an increased surface potential in eq 12. This lower chitosan \(pK_{ap}\) at higher \(f_0\) was similar to that observed in one prior study titrating chitosan in 100 mM KClO\(_4\),\(^{14}\) but not as strong as that reported in another.\(^{12}\) Both the level of deacetylation (\(f_0\)) and concentration of added salt \((c_{NaCl})\) were found to influence the degree of chitosan ionization at which precipitation occurs (\(\alpha_p\)), where \(\alpha_p\) was increased for either an increase in \(f_0\) or \(c_{NaCl}\) (Table 2). Temperature had no discernible effect on \(\alpha_p\). Our finding that increased levels of deacetylation increased the degree of ionization at which precipitation occurs was opposite to our initially proposed hypothesis since we expected higher charge densities along the chain to increase electrostatic repulsion and impede interchain association to inhibit precipitation. However it appears that increasing the fraction of monomers bearing acetyl groups is effective at blocking interchain associations, possibly due to steric hindrance via these side groups and/or a more difficult alignment of the polymer chains with increasing amounts of acetyl groups, as suggested previously.\(^{21}\) In any case, this reduced solubility of chitosan at higher \(f_0\) has been reported previously by several authors.\(^{12,14,21}\)

We found that intrinsic monomeric dissociation constants, \(pK_{0}\), obtained from linear extrapolations of \(pK_{ap}\), using data in the soluble range of the chitosan degree of ionization (\(\alpha > 0.3\) to 0.6 depending on \(f_0\) or \(c_{NaCl}\), Figure 4); i.e., \(pK_{0}^{lin}\) ranged from 6.4 to 6.7, while those obtained from PB model fits, i.e., \(pK_{0}^{PB}\), ranged from 6.63 to 6.78. The larger range in the linearly extrapolated values was due to the linear extrapolation failing to follow the convexity and nonlinearity of the PB model for \(pK_{ap}\) versus \(\alpha\) (Figure 7). Nonetheless, this nonlinearity and convexity of the PB model was eliminated in the presence of excess salt (150 mM) such that linearly extrapolated \(pK_{0}^{lin}\) agreed with the PB model \(pK_{0}^{PB}\) (Figure 7) to provide \(pK_{0}\) in the range from 6.70 to 6.76 and independent of \(f_0\). This range of values of chitosan \(pK_{0}\) (6.70–6.76) agrees with a previously reported \(pK_{0}\) of 6.74 that was extrapolated from titrations performed on chitosans oligomers of increasing lengths.\(^{16}\) The latter method bears the advantage of oligomer solubility over the entire range of ionization. In contrast to the close agreement between our \(pK_{0}\) values and those of Tsukada,\(^{16}\) there is significant variability of the previously reported chitosan \(pK_{0}\) values, ranging from 6.0 to 9.0.\(^{12,14,19}\) The two studies that reported chitosan \(pK_{0}\) near \(9.15,19\) are not compatible with our results nor with those of several other studies,\(^{12,14,16–18}\) possibly due to a sign error in the \(pK_{ap}\) equation that was used, i.e., \(pK_{ap} = pH - \log_{10}(\alpha(1 - \alpha))^{19}\) or \(pK_{ap} = pH + \log_{10}((1 - \alpha)/\alpha)\)\(^{18}\) rather than \(pK_{ap} = pH + \log_{10}(\alpha/1 - \alpha)\) where \(\alpha\) is the fraction of protonated glucosamine monomer on the chain. We estimated that correction of the sign error in refs 19 and 15 would result in chitosan \(pK_{0}\) values in the range of 6.5 to 7.0.

Another previous study\(^{17,18}\) found chitosan \(pK_{0}\) values to be 6.0 in both acetic acid and hydrochloric acid. This value appears low compared to our range from 6.70 to 6.76 and may be inaccurate due to pH measurements occurring at a low degree of ionization where chitosan is partly insoluble. Our range of obtained chitosan \(pK_{0}\) values of 6.70–6.76 in 150 mM NaCl is however relatively close to those of Sorlier et al.\(^{14}\) and Domard\(^{12}\) who found \(pK_{0}\) values of 6.5 for chitosans with \(f_0\) values similar to those tested in our study. We found salt to have a slight effect on \(pK_{ap}^{PB}\) where values at \(c_{NaCl} = 0\) and 15 mM were slightly lower by \(\sim 0.1\) unit than at \(c_{NaCl} = 150\) mM (Table 2). However, \(pK_{0}^{PB}\) values were independent of \(f_0\) (Table 2), suggesting that intramolecular interactions have negligible effects on \(pK_{0}\). One possible exception to the latter is the case \(f_0 = 0.98\), \(c_{NaCl} = 0\) mM, where \(pK_{0}^{PB} = 6.78\) was slightly higher than that for other \(f_0\) values (Table 2) but for which the PB fit using \(\alpha = 1.3\) nm was also not as good as that for the other conditions (Figure 4).

We found that the accuracy of the potentiometric titration of chitosan was improved by taking the following precautions: (1) The chitosan used should be of high purity and thoroughly dried, or the water content should be precisely known (2) \(f_0\) should also be precisely known and should preferably be measured by \(^1\)H NMR\(^{34}\) (3) the occurrence of precipitation during titration should be detected by a sensitive method, rather than by visual inspection, to ensure valid analysis of the results that depend on solution homogeneity and (4) the determination of \(pK_{0}\) of chitosan by linear extrapolation in solutions without added salt should be avoided. A particular limitation of our study was a variable molecular weight (MW) between chitosans with different \(f_0\) values. Ideally all of our chitosans would have had the same MW. However, even with the variable MWs of the three chitosans used, it is reasonable to assume that all are sufficiently long (length \(> \sim 600\) monomers) so as to not allow size to influence the surface potential of the polymer in a soluble state. However, the MW could possibly influence the charge state at which precipitation occurs, as it is expected that at constant \(f_0\) precipitation of higher MW chitosan could occur at a higher \(\alpha\) than for a shorter chain.

A novel finding in our study was the significant influence of chitosan concentration on chitosan \(pK_{ap}\) without added salt while for excess added salt no influence of chitosan concentration on chitosan \(pK_{ap}\) was observed (Figure 8A). These effects were accurately predicted by the PB model (Figure 8A) and were similar to those reported previously by Nitta and Sugai\(^{41}\) and by Katchalsky et al.\(^{33}\) for other polyelectrolytes. The influence of chitosan concentration on ionization behavior should be kept in mind, particularly when reporting \(pK_{ap}\) values of chitosan solutions without added salt.

We discovered in the current study that increasing temperature strongly reduced the apparent \(pK_{a}\) of chitosan, \(pK_{ap}\), by \(\approx 0.023\) \(pK_{a}\) units per °C, in a manner that is very similar to the temperature dependence of \(pK_{0}\) of monomeric glucosamine (Figures 9 and 10). This similar behavior for \(pK_{ap}\) of chitosan and \(pK_{0}\) of glucosamine strongly suggested this effect to be mediated by the intrinsic monomeric dissociation constant of chitosan \(pK_{d}(T)\) in eq 12 rather than the polyelectrolyte term \(-\psi_j / (\epsilon_0 kT \ln 10 kT)\) in eq 12. This conclusion was supported by the PB model, which predicted a negligible variation of the normalized electrostatic potential with temperature (Figure 6A) when the dependence of water permittivity on temperature was included.

The Poisson–Boltzmann cylindrical cell model was remarkably successful in providing molecular level insight and interpretation of all of the above findings. We found that this PB model using a polyelectrolyte radius \(a\) of 1.3 nm accurately predicted the dependence of chitosan \(pK_{ap}\) on \(f_0\), salt, chitosan concentration, and temperature (Figures 4, 6, 8, 9, and 10). While polyelectrolyte titrations have been modeled using several approaches\(^{32,36–40}\) (see Ullner\(^{41}\) for a review), we used the mean-
field Poisson—Boltzmann cylindrical cell model, since the chitosan persistence length is about 10–15 nm, suggesting that the chain is stiff enough to apply this model. We set the radius of the impenetrable inner cylinder to \(a = 1.3\) nm, which is greater than the crystallographic structural parameter (0.42 nm), yet appears most appropriate since all \(\theta\) curves obtained at 25 °C could be described well using \(a = 1.3\) nm. Furthermore, a value of the rod radius that is greater than the structural value is often used in the cylindrical PB model to describe polyelectrolyte data. It should be noted that PB model fits under low-salt conditions were more sensitive to the choice of the \(a\) value since much higher electrostatic potentials occur. Even though the PB model is generally nonlinear, a useful simplification of eq 7 that reasonably predicts \(\theta\) at least in the non-precipitated range is \(\theta = \theta_0 - C_{\alpha}(T - T_{ref}) - C_{\alpha NaCl}\alpha\) where we found \(C_{\alpha} = 0.023/°C\) (Figures 9 and 10) to be independent of \(c_{\alpha NaCl}\) and \(f_\theta\) while \(C_{\alpha NaCl}(C_{\alpha NaCl}f_\theta)\) and \(\theta_0\) do depend on the concentration of added salt \((c_{\alpha NaCl})\) and \(f_\theta\) as shown in Table 2 at \(T_{ref} = 25\) °C. For temperature-dependent measurements, the ability of the pH probe to compensate for temperature is important and care should be taken to test pH accuracy versus temperature. Also, results obtained in this paper show that \(\alpha\) is a more appropriate parameter than pH to characterize precipitation or solubility, as suggested by Siegel and Cornejo-Bravo, since \(\alpha\) is the ionization state of the polyelectrolyte and is a primary determinant of solubility. In contrast pH can be strongly influenced by temperature and the presence and dissociation of other solution components that may or may not influence precipitation.

For the temperature range from 5 to 40 °C, we found that the \(K\) of glucosamine and the \(K\) of chitosan decrease significantly with temperature by \(-0.027\) and \(-0.023\) \(\pH\) units/°C, respectively, while the \(K\) of glyceral phosphatase was found to be temperature-independent. Gurney proposed the following relation for the temperature variation of proton dissociation constants of noncharged and negatively charged acids

\[
\log_{10}(K) = -C(A + \exp(T/\theta))
\]

(20)

where \(C\) and \(A\) are characteristic of the acid and \(\theta\) is a parameter that depends only on the solvent. For positively charged acids such as chitosan and glucosamine, proton dissociation is free from any work necessary to separate two charged species in solution, and the contribution of the \(\exp(T/\theta)\) term, which contains the effects of the dielectric constant of the solvent, is negligible. Thus, chitosan and glucosamine are expected to show a linear variation of \(\theta\) versus \(1/T\), and the relationship of Gurney is simply

\[
\theta = \frac{CA}{T} = \frac{D}{T}
\]

(21)

Using a \(K\) of 6.6 (Table 2) for chitosan and a \(K\) of 7.8 for glucosamine at \(T = 298\) K in eq 21, we found that \(D = 1967\ K^{-1}\) for chitosan and \(D = 2324\ K^{-1}\) for glucosamine. For chitosan, calculation of the \(K\) at 278 and 313 K gives 7.08 and 6.28, respectively, corresponding to an average variation of \(-0.0227\) \(\pH\) units/°C for the range of 5–40 °C. For glucosamine, the same calculation gives an average variation of \(-0.0267\) \(\pH\) units/°C for the range of 5–40 °C. These calculated values are very close to the experimental values found in our study of \(-0.0232 \pm 0.0003\) \(\pH\) units/°C for chitosan and \(-0.027 \pm 0.001\) \(\pH\) units/°C for glucosamine.

The marked influence of temperature on chitosan \(\theta\) values (Figures 9 and 10) and the corresponding temperature insensitivity of the \(K\) values of glyceral phosphate (Figure 9) sheds light on the molecular mechanism of heat-induced gelation of these systems that has been reported previously. Heating of chitosan significantly reduces its \(\theta\) value, thus producing a tendency for chitosan amine groups to release protons and become neutralized, as would occur if base were added. However, chitosan can release protons and become neutralized upon heating only if a proton acceptor is present in solution. Glyceral phosphate is an ideal proton acceptor since its proton equilibrium is not affected by heating, and its \(\theta\) is close to the chitosan \(\theta\).

We therefore expect a net transfer of protons from chitosan to glyceral phosphate to reduce \(\alpha\) with heating, thereby neutralizing chitosan in solution in a spatially uniform manner that allows for bulk gelation in sufficiently concentrated solutions. One would expect multiple parameters to influence this gelation process including: (1) the amount of HCl present where lower amounts reduce the initial \(\alpha\) to gel at lower temperature, as observed previously, and (2) the amount of glyceral phosphate where higher amounts will also reduce the initial \(\alpha\) and more efficiently accept protons such that chitosan will gel at lower temperature, also as observed previously.

An additional critical factor expected from our study is the degree of deacetylation, which strongly affects solubility via its influence on \(\alpha\) (Table 2). Studies are ongoing where the current model is elaborated to explicitly account for other titratable species in solution, such as glyceral phosphate, and to include the dependence of the degree of ionization of chitosan at precipitation \((\alpha_\theta)\) on ionic strength and degree of deacetylation to provide a complete model able to predict gelation behavior and design specific systems with desired solubility, ionization, and thermosensitive characteristics.

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Supporting Information Available. Derivation of the \(\pH\) dependence on the electrostatic potential (eq 11) and derivation of \(d\theta = d\pH\) (eq 16). This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

APPENDIX I

Titration of a Cationic Polyelectrolyte

The protonation of ionisable sites of the polycation is described at equilibrium by $AH^+ \rightleftharpoons H^+ + A$, where $A$ is the protonatable group on the polycation ($A = NH_2$ for chitosan). Equilibrium of this proton exchange between the polycation and the solution phase occurs when the proton chemical potential is equal in the two phases,

$$\mu^F_{\text{H}^+} = \mu^P_{\text{H}^+}$$

(22)

where $\mu^F_{\text{H}^+}$ is the proton chemical potential in the fluid electrolyte phase ($a < r < b$) and $\mu^P_{\text{H}^+}$ is the proton chemical potential in the form bound to the polycation ($AH^+$) at $r = a$. The proton chemical potential in the fluid phase is based on the mixing entropy of protons in the solvent, subject to correction for non-ideal behavior, via an activity coefficient $\gamma^0$, i.e.

$$\mu^F_{\text{H}^+} = \mu^0_{\text{H}^+} + kT \ln a_{\text{H}^+} = \mu^0_{\text{H}^+} + kT \ln \gamma_{\text{H}^+} \bar{c}_{\text{H}^+} = \mu^0_{\text{H}^+} + kT \ln c^0_{\text{H}^+}$$

(23)

where $\mu^0_{\text{H}^+}$ is the proton standard chemical potential, $a_{\text{H}^+}$ is the proton activity, $\gamma_{\text{H}^+} = \gamma_+^0$ is the proton activity coefficient (given by Eq 4), $\bar{c}_{\text{H}^+}$ is the average proton concentration in the fluid electrolyte phase of the polycation solution and $c^0_{\text{H}^+}$ is the proton concentration at the periphery of the cylindrical cell boundary at $r = b$, where $\psi(r = b) = 0$ and $d\psi(r = b)/dr = 0$. The pH is the related to proton activity via

$$\text{pH} = -\log_{10} a_{\text{H}^+}$$

(24)

which in light of Eq 10 becomes simply related to the proton concentration at the cylindrical cell boundary where the absence of an electric field, due to Eq 6, allows protons at this location to behave ideally

$$\text{pH} = -\log_{10} c^0_{\text{H}^+}$$

(25)
Since the pH depends on proton activity that in turn depends on Eq 22 describing proton equilibrium between the two phases, we require an expression for the proton chemical potential on the polycation that can be derived from the Helmholtz free energy, $F$, of one polycation chain in the cylindrical cell as

$$\mu_H^p = \frac{\partial F}{\partial n_H^p} \bigg|_{r}$$ 

(26)

where $n_H^p$ is the number of protonated sites on the polycation. The Helmholtz free energy of the polycation chain in the cylindrical cell is taken as

$$F = E_e - TS_{\text{mix}} + n_H^p \mu_{\text{AH}}^0 + \left(m - n_H^p\right) \mu_{\Lambda}^0$$

(27)

where $E_e$ is the electrostatic energy of the polycation, $T$ is the temperature, $S_{\text{mix}}$ is the entropy of mixing of protonated and unprotonated monomeric sites on the polycation, $m$ is the number of monomers on the chain, and $\mu_{\text{AH}}^0$ and $\mu_{\Lambda}^0$ are standard chemical potentials of a protonated site and a non-protonated site on the polycation, respectively. The electrostatic energy of the polycation

$$E_e = n_H^p e \psi \bigg|_{r=a}$$

(28)

varies with proton number according to

$$\frac{\partial E_e}{\partial n_H^p} \bigg|_{m,T} = e \psi \bigg|_{r=a}$$

(29)

The entropy of mixing of protonated and unprotonated sites on the polycation, $S_{\text{mix}}$, is identical to that of any two state system with non-correlated sites and is given by

$$S_{\text{mix}} = k \ln \frac{m!}{n_H^p! (m-n_H^p)!} = -km \left[ \alpha \ln \alpha + (1-\alpha) \ln (1-\alpha) \right]$$

(30)

where $n_H^p = m \alpha$ has been used. The entropy variation with proton number on the polycation is then

$$\frac{\partial S_{\text{mix}}}{\partial n_H^p} \bigg|_{m,T} = \frac{1}{m} \frac{\partial S_{\text{mix}}}{\partial \alpha} \bigg|_{m,T} = -k \left[ \ln \alpha + 1 - \ln (1-\alpha) - 1 \right] = -k \ln \frac{\alpha}{1-\alpha}$$

(31)

Differentiating the last two terms of the Helmoltz free energy (Eq 27) provides
\[
\frac{\partial (n_H^p \mu_{AH^+}^0 + (m - n_H^p) \mu_A^0)}{\partial n_H^p} = \mu_{AH^+}^0 - \mu_A^0
\]  

(32)

We find the proton chemical potential on the polycation by substituting Eqs 32, 31, 29 and 27 into Eq 26

\[
\mu_H^0 = e \psi \bigg|_{\alpha = \alpha} + kT \ln \frac{\alpha}{1 - \alpha} + \mu_{AH^+}^0 - \mu_A^0
\]  

(33)

Equalizing the proton chemical potentials in the two phases to obtain equilibrium is achieved by substituting Eq 33 and Eq 23 into Eq 22 to obtain

\[
e \psi \bigg|_{\alpha = \alpha} + kT \ln \frac{\alpha}{1 - \alpha} + \mu_{AH^+}^0 - \mu_A^0 = \mu_H^0 + kT \ln c_H^0
\]  

(34)

Substituting the proton activity from Eq 34 into the definition of pH in Eq 22 provides

\[
pH = -\log c_H^0 = pK_0(T) + \log_{10} \frac{1 - \alpha}{\alpha} - \frac{e \psi \bigg|_{\alpha = \alpha}}{kT \ln 10}
\]  

(35)

where

\[
pK_0(T) = \frac{\Delta \mu^0}{kT \ln 10} = \frac{\mu_H^0 + \mu_{AH^+}^0 - \mu_A^0}{kT \ln 10}
\]  

(36)

Note that the cationic nature of the polyelectrolyte is seen in the rightmost term of Eq 35, \(\frac{e \psi \bigg|_{\alpha = \alpha}}{kT \ln 10}\), where increasing electrostatic potential and energy (Eq 27) of a proton on the polyelectrolyte chain will increase proton concentration in solution, thereby decreasing pH.
APPENDIX II

Derivation of $\text{dp}K_{ap} \approx \text{dpH}$

The apparent dissociation constant of the glucosamine monomer ($A = \text{NH}_2$ for chitosan) $\text{AH}^+ \rightleftharpoons H^+ + A$, of the cationic polyelectrolyte is written

$$K_{ap} = \frac{c_{H^+}c_A}{c_{AH^+}} = \frac{c_{H^+}c_g^n}{c_g^c} = \frac{c_{H^+}(1-\alpha)}{\alpha} \Rightarrow \ln K_{ap} = \ln(1-\alpha) - \ln \alpha + \ln c_{H^+}$$

(37)

where $c_g^+$ is ionized glucosamine monomer, $c_g^c$ is neutral glucosamine monomer, $c_g' = c_g^+ + c_g^c = f_D c_p$ is total glucosamine monomer and $\alpha = \frac{c_g^+}{c_g'}$ is the degree of ionization. Dissociation of water is described as

$$K_w = c_{H^+}c_{OH^-} \Rightarrow \ln K_w = \ln c_{H^+} + \ln c_{OH^-}$$

(38)

Solution electroneutrality in the presence of a strong base and/or acid like NaOH or HCl is

$$\sum_i z_i c_i = c_{Na^+} + \alpha c_g^+ + c_{H^+} - c_{Cl^-} - c_{OH^-} = 0$$

(39)

Taking $c_{Na^+}$ and $c_{Cl^-}$ as invariant and using the relation

$$d\log_{10}(X) = \log_{10}(e) d \ln X = \log_{10}(e) \frac{dX}{X}$$

(40)

we find that the total differential of Eqs 37, 38 and 39 as:

$$\text{dp}K_{ap} = \text{dpH} + \log_{10}(e) \left( \frac{d\alpha}{1-\alpha} + \frac{d\alpha}{\alpha} \right) = \text{dpH} + \log_{10}(e) \frac{d\alpha}{\alpha(1-\alpha)}$$

(41)

$$\text{dp}K_w = \text{dpH} + \text{dpOH}$$

(42)

$$c_g' d\alpha + dc_{H^+} - dc_{OH^-} = 0$$

(43)

From Eq 43 and by using again Eq 40, we have
\[
\frac{d\alpha}{\alpha} = \frac{d\ln c_{OH}^i}{c_g^i} = \frac{d\ln c_{H^+}^i}{c_g^i} = \ln 10 \left( \frac{c_{H^+}^i dpH - c_{OH}^i dpOH}{c_g^i} \right)
\]

(44)

Using Eqs 42 and 44 in Eq 41, we then obtain

\[
dpK_{ap} = dpH + \frac{1}{\alpha(1-\alpha)} \left( \frac{c_{H^+}^i}{c_g^i} + \frac{c_{OH}^i}{c_g^i} \right) dpH - \frac{c_{OH}^i}{c_g^i} dpK_w
\]

(45)

Eq 45 can be simplified to

\[
dpK_{ap} \approx dpH
\]

(46)

if \( \frac{c_{H^+}^i}{c_g^i} \) and \( \frac{c_{OH}^i}{c_g^i} \) << 1 and \( \alpha \) is not close to 0 or 1. The above relation Eq 46 is used in our study for experiments that satisfy these conditions.