Competitive Ion Complexation to Polyelectrolytes: Determination of the Stepwise Stability Constants. The Ca^{2+}/H^{+}/Polyacrylate System

Calin David,[†] Encarnació Companys,[†] Josep Galceran,[†] Josep Lluís Garcés,[†] Francesc Mas,[‡] Carlos Rey-Castro,[†] José Salvador,[†] and Jaume Puy*,[†]

Departament de Química, Universitat de Lleida (UdL), Av. Rovira Roure 191, E-25198 Lleida, Spain, and Centre Especial de Recerca de Química Teòrica (CeRQT, PCB) and Departament de Química Física, Facultat de Química, Universitat de Barcelona (UB), C/ Martí i Franquès 1, E-08028 Barcelona, Spain

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This work presents a new methodology aimed at obtaining the stepwise stability constants corresponding to the binding of ions (or other small molecules) to macromolecular ligands having a large number of sites. For complexing agents with a large number of sites, very simple expressions for the stepwise stability constants arise. Such expressions are model-independent; that is, they allow the determination of the stepwise stability constants without making any previous assumption of the detailed complexation mechanism. The formalism is first presented for a single complexing ion and further extended to competitive systems where the competing ions can display, in general, different stoichiometric relationships. These ideas are applied to the analysis of experimental titrations corresponding to competitive binding of calcium ions to poly(acrylic acid) for different pH values and ionic strengths. Intrinsic stability constants were estimated from the stepwise stability constants (by removing the corresponding statistical factor), and split into specific and electrostatic contributions (by means of the Poisson−Boltzmann equation). After this treatment, the specific proton binding energies showed almost no dependence on the coverage and ionic strength. Likewise, for the range of concentrations studied, the specific component of the intrinsic stability constants of the calcium ions, calculated assuming bidentate binding of Ca to neighboring groups of a linear chain, is almost independent of the calcium and proton coverage and ionic strength.

1. Introduction

The description of binding equilibria of ions and other small molecules to macromolecules is a crucial task in the understanding of many chemical, biological, and environmental systems. For instance, the interaction of small molecules and ions with biological macromolecules, such as proteins and DNA, is in many cases responsible for the regulation of their biological activity.[1,2] In supramolecular chemistry, the assembling of complex supramolecular architectures is controlled by the interactions of heavy metal ions with specific organic ligands.[3,4] The importance of macromolecular binding equilibria can also be recognized in the field of environmental chemistry, where the complexation of heavy metals to natural polyelectrolytes, such as fulvic or humic acids, plays a key role in the regulation of their toxicity and bioavailability.[5,6]

Binding equilibria are usually studied by measuring the average number of bound metal ions (or other small inorganic molecules) as a function of the free metal concentration, for a fixed amount of macromolecular ligand.[2,7−14] The resulting plot, the so-called binding curve, can then be analyzed by using different theoretical tools. The description of the binding phenomena can be made by means of the fundamental equilibrium equations of the stepwise complexation processes. Stepwise species are characterized by the number of occupied sites, regardless of the specific sites to which the complexing agent is bound.[15] The equilibrium constants of the processes corresponding to the occupation of an increasing number of sites are called stepwise stability constants. The resulting set of stepwise stability constants (as many as macromolecular sites) can later be interpreted, if wished, in terms of microscopic, site-specific binding parameters, by using the appropriate statistical mechanics techniques.[16−18] Up to now, stepwise constants have been mainly used in the description of binding to ligands with a reduced number or sites.[19]

However, many macromolecules of interest have a very large number of complexing sites, which introduces additional complications to the study of such systems because of the physicochemical phenomena involved (heterogeneity, positive and negative cooperativity, polyelectrolytic behavior, chelate binding, conformational changes, etc).[5,16] Much work has been done in order to understand such phenomena. For instance, heterogeneity of the binding sites has been undertaken by means of the affinity spectrum formalism, both for monocomponent and competitive systems.[20−24] Although unable to account for chelate binding (i.e., when proton and metals have different stoichiometric relationships), the concept of the affinity spectrum has been helpful and widely used, especially in environmental chemistry. On the other hand, the polyelectrolytic effect has also been undertaken, for instance, by means of the extended use of the Poisson−Boltzmann equation.[18,25,26] In recent years, new approaches have been developed with the aim of understanding conformational transitions and chelate complexation in polyelectrolytes.[24,27−31]

A second difficulty arises from the fact that, when the number of sites becomes large, the nonlinear fitting involved in the finding of the stepwise stability constants can become unstable

* Corresponding author. E-mail: jpuy@quimica.udl.es.
[†] Universitat de Lleida (UdL).
[‡] Universitat de Barcelona (UB).

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and strongly dependent on the experimental error. Therefore, the knowledge of these constants could appear as a cumbersome task in a system that contains a ligand with a high number of sites. However, for systems with only one complexing agent, it was shown that the finding of the stepwise stability constants can be performed in a very simple way, by taking advantage of the use of the thermodynamic limit conditions. Actually, by applying the classical Darwin−Fowler method, very simple expressions for the stepwise stability constants have been presented.[32]

It is the aim of the present work to show how this method can be easily extended to cases with competitive binding. The new basic ideas are developed in section 2. After the Experimental Section (section 3), the methodology presented is exemplified, in section 4, for a monocomponent system, by determining the stepwise stability constants of the proton binding to polyacrylate (PAA). In section 5, we determine the stepwise stability constants for the competitive binding system Ca^{2+}/H^{+}/PAA. This system is particularly interesting in showing how the formalism here presented can be used in dealing with different stoichiometric relationships for the bound ions. The need for theoretical frameworks, able to deal with competitive binding with different stoichiometric relationships, is especially relevant, since exchange relationships among metal ions and protons higher than 1 have been experimentally reported.[8,12] Finally, in all cases, the correction for the electrostatic contribution to the binding energy, by means of the Poisson−Boltzmann equation, allows us to draw some conclusions about the electrostatic and specific contributions to the binding of these ions to PAA.

2. Methodology: Determination of the Stepwise Stability Constants for a Macromolecular Ligand with a Large Number of Sites

2.1. Theoretical Background: Stepwise and Intrinsic Stability Constants. Let us represent with M_iP the set of macromolecular species, usually called stepwise or stoichiometric species, consisting of the macromolecular ligand P (with a total number of complexing sites, s) with i attached metal ions, independently of the particular occupied sites. The sequential complexation of metals to the macromolecule can, then, be schematized as

$$M_{i-1}P + M \overset{K_i}{\rightleftharpoons} M_iP \tag{1}$$

where K_i is defined as

$$K_i = \frac{c_{M_iP}}{c_{M_{i-1}P} c_M} \tag{2}$$

with c_{M_iP} being the concentration of the stepwise species and c_M the free ion concentration. From the fundamental equilibrium relationship, eq 2, the average number of bound ions, ν (a quantity which is experimentally available if the total concentration of macromolecules is known), can be expressed as a function of the free ion concentration

$$\nu = \frac{\sum_{i=1}^{s} i\beta_i c_M^i}{\sum_{i=1}^{s} \beta_i c_M^i} \tag{3}$$

where $\beta_i = K_1 \cdot K_2 \cdot \ldots \cdot K_i$ is the so-called "overall stability constant", corresponding to the stability constant of the process of binding i ions to the naked macromolecule. The standard procedure would consist of fitting eq 3 to the experimental binding data and obtaining the set of stepwise equilibrium constants, K_i.[15,19,33] If necessary, techniques of statistical mechanics can be used to express the stepwise constants in terms of the number of ions, i, and the microscopic parameters (binding and interaction free energies, conformational properties, etc).[17,31] For complexation to identical and independent sites, β_i and K_i adopt the very simple forms

$$\beta_i = \binom{s}{i} k^i \tag{4}$$

and

$$K_i = \frac{\beta_i}{\beta_{i-1}} = \frac{s - i + 1}{i} k \tag{5}$$

where k is the stability constant associated to the complexation of a particular site. The factor $(s - i + 1)/i$ is called the "statistical factor", and is an entropic contribution to the free energy due to the change in the number of microstates of the macromolecule in passing from $i - 1$ to i bound ions.[1]

In a general complexation model, the dependence of K_i on i and on the microscopic parameters is not trivial. In this general case, one can still use the ideal complexation model as a reference and introduce the intrinsic stability constants in terms of the stepwise constants and the ideal entropic factor as

$$K_i = \frac{s - i + 1}{i} K_{i,\text{int}} \tag{6}$$

Obviously, for the ideal complexation model, $K_{i,\text{int}}$ becomes independent of i ($K_{i,\text{int}} = k$), while, for a general complexation case, the computation of $K_{i,\text{int}}$ can be used to quantify the divergence of our system from the ideal complexation model by looking at the dependence of $K_{i,\text{int}}$ on i.

Notice that the entropic factor appearing in expressions 5 and 6 comes from the assumption that the complexation is monodentate. Clearly, the corresponding entropic factor will be different if chelate complexation is assumed. Indeed, in the general case, we have to introduce the intrinsic stability constants as

$$K_i = g_i K_{i,\text{int}} \tag{7}$$

where g_i accounts for the change in the number of microstates arising from the process of binding a new metal to the macromolecule. For instance, if in a given system one ion is attached to m neighboring identical and independent functional groups of a linear macromolecule (of a total of n groups), the number of species differing in the specific sites occupied with i bound metals can be obtained as the number of permutations without repetitions of the bound metals (i) plus the empty functional groups ($n - im$). The resulting expressions for β_i and K_i are[34,35]

$$\beta_i = \frac{(n - im + i)!}{i!(n - im)!} k^i \tag{8}$$

and

$$K_i = \frac{\beta_i}{\beta_{i-1}} = g_i k = \frac{(n - im + m)\cdots(n - im + 1)}{i(n - im + i + m - 1)\cdots(n - im + i + 1)} k \quad (9)$$

and, accordingly, for a general case where the sites can exhibit different affinities for the metal, the stepwise constant can be split in terms of its intrinsic stability constant and the entropic factor as

$$K_i = \frac{(n - im + m)\cdots(n - im + 1)}{i(n - im + i + m - 1)\cdots(n - im + i + 1)} K_{i,\mathrm{int}} \quad (10)$$

In particular, for multidentate binding to m identical and neighboring functional groups of a linear chain, eq 10 leads to $K_{i,\mathrm{int}} = k$ and eq 9 is recovered.

In general, $K_{i,\mathrm{int}}$ can be seen as an average of the site-specific constants. For instance, for independent nonidentical sites, $K_{i,\mathrm{int}}$, as defined in eq 7, can be physically interpreted as the average of the microscopic (site-specific) stability constants corresponding to the free sites of the stepwise species.[35] $K_{i,\mathrm{int}}$, thus, offers a more transparent interpretation of the binding properties of the system than K_i, something which will be used in the next sections.

2.2. The Stepwise Stability Constants for a Macromolecule with a Large Number of Sites. In many macromolecules of interest, the number of complexing sites, and thus the number of stepwise constants, can be very large. As a consequence, the nonlinear fitting of eq 3 becomes a cumbersome task, and the result of the fitting is very unstable and highly dependent on the experimental error.[33] However, in a previous paper, it was shown that the finding of the stepwise stability constants can be performed in a very simple way. Using the Darwin−Fowler method,[36] a technique originally employed in the solving of some formal aspects of statistical mechanics, it was shown[32] that, for large values of the number of sites, s, K_i can be expressed as

$$\log K_i = -\int_i^{i+1} \log c_{\mathrm{M}}\, \mathrm{d}\nu - \frac{1}{2}\log\left\{\left(\frac{[c_{\mathrm{M}}]_{\nu=i}}{[c_{\mathrm{M}}]_{\nu=i+1}}\right)^2 \frac{\left(\frac{\mathrm{d}\nu}{\mathrm{d}\log c_{\mathrm{M}}}\right)_{[c_{\mathrm{M}}]_{\nu=i+1}}}{\left(\frac{\mathrm{d}\nu}{\mathrm{d}\log c_{\mathrm{M}}}\right)_{[c_{\mathrm{M}}]_{\nu=i}}}\right. \quad (11)$$

where $[c_{\mathrm{M}}]_{\nu=i}$ indicates the ion concentration when $\nu = i$. Notice that the right-hand side of eq 11 only depends on information which can be directly obtained from the binding curve, and it is independent of the details of the binding processes. In this sense, one can say that eq 11 is model-independent.

Furthermore, it was shown in ref 32 that eq 11 adopts, in the limit $s \to \infty$, the extremely simple form

$$\lim_{s\to\infty} \log K_i = -[\log c_{\mathrm{M}}]_{\nu=i} \quad (12)$$

Expression 12 provides a direct and simple way of finding the value of the stepwise stability constants, which we depict in Figure 1: when replotting the binding curve using ν as the abscissa axis and $-\log c_{\mathrm{M}}$ as the ordinate, one can directly identify the value of the ordinate when $\nu = i$ as $\log K_i$.

How can we explain, in physical terms, the result expressed by eq 12? Let us recall eq 2, the definition of the stepwise stability constants. One can see, by simple inspection, that

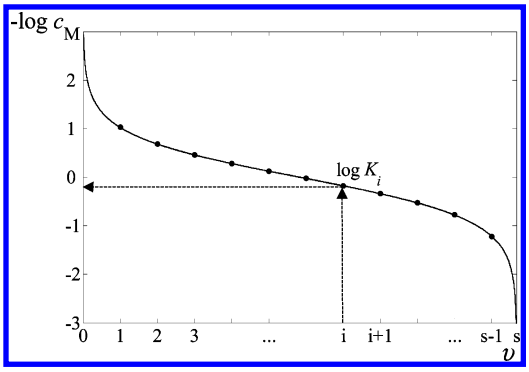

Figure 1. Plot of pM $= -\log c_{\mathrm{M}}$ vs ν. The value of pM at integer values of $\nu = j$ directly yields, in the limit $s \to \infty$, $\log K_j$ (see eq 12). This property provides a very simple method to find the stepwise stability constants for macromolecules with a large number of sites.

expression 12 can be obtained from eq 2 assuming that $c_{\mathrm{M}_i\mathrm{P}} \approx c_{\mathrm{M}_{i-1}\mathrm{P}}$ when $\nu = i$. That is, eq 12 is equivalent to accepting that, independently of the complexation model (the Darwin−Fowler method is model-independent), the concentrations $c_{\mathrm{M}_i\mathrm{P}}$ and $c_{\mathrm{M}_{i-1}\mathrm{P}}$ tend to collapse for $\nu = i$ when the number of sites, s, increases.

This behavior is illustrated for the particular ideal complexation model (identical and independent sites) in Figure 2. For this simple model, the concentration of the stepwise species is given by[37,38]

$$c_{\mathrm{M}_i\mathrm{P}} = c_{\mathrm{T,P}} \binom{s}{i} \left(\frac{\nu}{s}\right)^i \left(\frac{s-\nu}{s}\right)^{s-i} \quad (13)$$

where $c_{\mathrm{T,P}}$ indicates the total concentration of polymer P, which can be taken as $c_{\mathrm{T,P}} = 1$ M, without loss of generality. $c_{\mathrm{M}_i\mathrm{P}}$ shows, once plotted versus ν, a peaked behavior with the maximum value located at $\nu = i$. Notice, by comparing parts a, b, and c of Figure 2, that, for a given integer abscissa value, i, the ordinates corresponding to the concentration of $\mathrm{M}_i\mathrm{P}$ and $\mathrm{M}_{i-1}\mathrm{P}$ tend to merge as the total number of sites increases. (The convergence for the particular case $i = 2$ is highlighted in the figure).

Finally, we highlight that, although $s \to \infty$ can be seen as a very restrictive condition, for several complexation models[32] (heterogeneous systems and systems with interaction between occupied sites), it has been shown that the application of eq 11 is quite accurate for systems where s is of the order of 10, while eq 12 can be accurately used when $s \geq 20$. Certainly, this is a very low number of sites for many polyelectrolytes of interest, with a characteristic number of sites of the order of 10^2-10^4.

2.3. Extension to Competitive Metal Complexation. Competitive binding is the common situation in metal binding to polyelectrolytes, since metal ions are usually bound to sites with acid/base properties. As a consequence, the competition between protons and metal ions is in most cases unavoidable. Let us extend the methodology outlined in the previous sections to competitive complexation between several ions.

Consider a macromolecule with s_{H} sites for the protons and s_{M} sites for the metal ions. s_{H} and s_{M} do not need to be equal, so that different stoichiometric relationships (chelate binding) can be included in our formalism. Under the new situation, the stepwise species have to be defined by the number of metals and protons bound to the skeleton. The set of stepwise constants can now be labeled by using two subscripts, i and j

$$\mathrm{H}_i\mathrm{M}_{j-1}\mathrm{P} + \mathrm{M} \overset{K_{i,j}}{\rightleftharpoons} \mathrm{H}_i\mathrm{M}_j\mathrm{P} \quad (14)$$

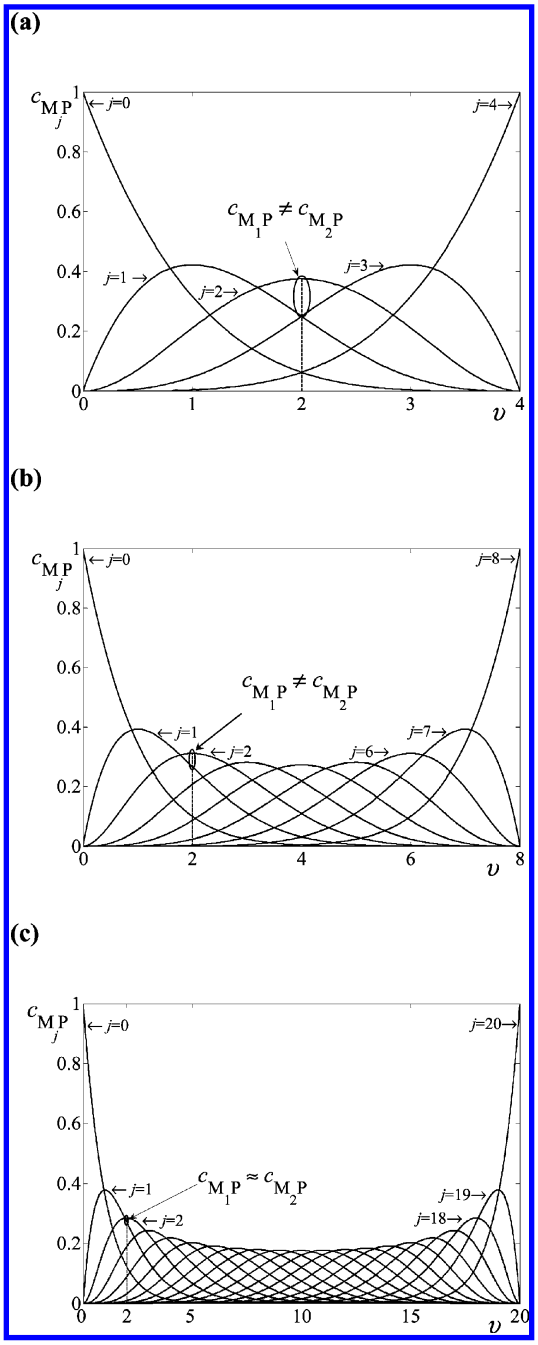

Figure 2. Curves from left to right represent, respectively, the concentrations of stepwise species P, MP, M_2P, ... , M_sP vs the average number of complexed ions, ν. All macromolecules have a fixed maximum number, s, of independent and identical sites. The number of sites is $s = 4$ (panel a), $s = 8$ (panel b), and $s = 20$ (panel c). Each concentration, c_{M_jP}, shows a maximum when the average number of complexed ions is $\nu = j$, except for c_P and c_{M_sP}. The concentrations c_{M_jP} and $c_{M_{j-1}P}$ at $\nu = j$ tend to converge when the number of sites, s, increases. As an example, we have highlighted in the panels how c_{MP} and c_{M_2P} converge to the same value for $\nu = 2$ going from panel a to panel c. The macromolecule concentration, $c_{T,P}$, has been taken, without loss of generality, as 1 M (see eq 13).

with

$$K_{i,j} = \frac{c_{H_iM_jP}}{c_{H_iM_{j-1}P}c_M} \tag{15}$$

The first subscript, i, indicates the number of bound protons and the second one, j, the number of bound metal ions.

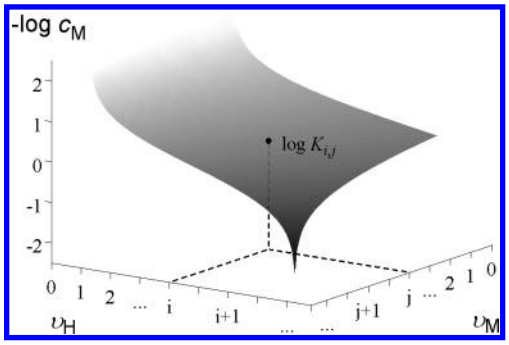

Figure 3. Plot of $pM = -\log c_M$ vs ν_H and ν_M. The value of pM at integer values of $\nu_H = i$ and $\nu_M = j$ yields, in the limit $s \to \infty$, directly $\log K_{i,j}$. This procedure extends the finding of the stepwise constants, depicted in Figure 1, to competitive complexation systems.

In the Appendix, we show that, by applying the maximum term theorem of statistical mechanics,[32,39] the central result (eq 12) can be straightforwardly extended to competitive complexation. We obtain

$$\lim_{\substack{s_M \to \infty \\ s_H \to \infty}} \log K_{i,j} = [-\log c_M]_{\nu_H = i, \nu_M = j} \tag{16}$$

The proposed procedure is depicted in Figure 3. We plot $pM = -\log c_M$ as a function of ν_M and ν_H, and the value of the ordinate at the point $\nu_M = j$ and $\nu_H = i$ provides directly $\log K_{i,j}$. We can check this expression by considering the particular case of identical and independent sites (ideal complexation). Let us label the stability constant for the overall reaction of adding i protons and j metals to the macromolecular skeleton P as $\beta_{i,j}$, and let us assume $s_H = s_M = s$. Then, $\beta_{i,j}$ is given by

$$\beta_{i,j} = \frac{c_{H_iM_jP}}{c_P c_H^i c_M^j} = \binom{s}{i}\binom{s-i}{j} k_H^i k_M^j \tag{17}$$

where k_H and k_M are the stability constants corresponding to the elementary process of binding of a proton or a metal, respectively, to a particular site of the macromolecular skeleton. The combinatorial term in eq 17 comes from the number of ways of distributing i protons among s sites and j metals among the remaining free sites after the binding of the protons. The stepwise constant, $K_{i,j}$, for ideal complexation can be written as

$$K_{i,j} = \frac{\beta_{i,j}}{\beta_{i,j-1}} = \frac{s-i-j+1}{j} k_M \tag{18}$$

Notice that the result of eq 18 is identical to that corresponding to noncompetitive systems (see eq 5) but with replacement of the number of sites s by $s - i$ (i sites are occupied by protons).

Alternatively, we can apply the limiting expression 16 to try to recover eq 18 from the multicomponent Langmuir isotherm (which, as known, corresponds to a model of identical and independent sites)

$$\nu_H = s\frac{k_H c_H}{1 + k_H c_H + k_M c_M} \tag{19}$$

$$\nu_M = s\frac{k_M c_M}{1 + k_H c_H + k_M c_M} \tag{20}$$

Replacing ν_H and ν_M by i and j, respectively, we obtain $k_H c_H = i(1 + k_M c_M)/(s - i)$ from eq 19. Once this value is replaced in

eq 20, the metal concentration for a mean metal occupation of $\nu_M = j$ is given by

$$K_{i,j} = \left[\frac{1}{c_M}\right]_{\nu_H=i,\nu_M=j} = \frac{s-i-j}{j}k_M \approx \frac{s-i-j+1}{j}k_M \quad (21)$$

given that $s \gg 1$. We have, thus, recovered the same expression for $K_{i,j}$ given by eq 18 by using a completely different method based on eq 16.

3. Experimental Section

3.1. Materials. Poly(acrylic acid) (PAA), with an average molecular weight of 250 kD, was purchased from Aldrich and used without further purification. KNO_3 (Fluka, TraceSelect) was used as a supporting electrolyte. The titrating solutions were 0.1 M standard aqueous solutions of HNO_3 and KOH (Merck Titrisol) and 0.0333 M $Ca(NO_3)_2$ solutions prepared from the solid product (Merck, analytical grade). Ultrapure water (Milli-Q plus 185 System from Millipore) was employed in all of the experiments. Purified-water-saturated nitrogen N_2(50) was used for deaeration.

3.2. Methods. The potentiometric measurements were carried out with a Wallingford Titrator, a computer-controlled titration system.[40] The samples were placed in a double-wall potentiometric plexiglass cell thermostatized at 25 °C. N_2 bubbling and soda lime traps were used throughout to prevent CO_2 contamination. The potential between the ion selective electrode (pH or Ca electrode) and a Ag/AgCl reference electrode was measured with a high impedance voltmeter (Microlink PH4) and recorded after a drift criterion of <0.1 mV/min was achieved. The ion selective electrodes were calibrated at each ionic strength by performing several titrations with standard calcium and HNO_3 acid solutions in the absence of PAA. The potential versus free ion concentration data were then used to fit a Nernst-type equation. The titrations of PAA with KOH were carried out at a polymer concentration of 2.5 mmol/L (on monomer basis) in the presence of 0.01, 0.05, 0.1, and 0.5 mol/L KNO_3, respectively. The calcium titrations were carried out at the same polymer concentration throughout pH-static experiments (by addition of convenient amounts of KOH and/or HNO_3) in 0.05, 0.1, and 0.5 mol/L KNO_3. At least three different fixed pH values within the range $6 < pH < 10$ were chosen for the experiments at each ionic strength. The pCa values measured ranged between 3 and 5.

The mean occupation numbers of calcium and/or proton are calculated from mass balance considerations. Indeed, ν_{Ca} straightforwardly follows from the measurement of the free calcium concentration as a function of the total amount of metal added. The proton occupation number, ν_H, can be calculated from the volumes of acid and base added along the titration using

$$\nu_H = s - \frac{1}{c_{T,P}}\left(c_H - \frac{K_w}{c_H} + c_{KOH} - c_{HNO_3}\right) \quad (22)$$

This equation comes from the mass and charge balances, where c_{HNO_3} and c_{KOH} are the overall concentrations of HNO_3 and KOH added at each titration point. K_w is the ionic product of water, s is the total number of sites, and c_H and $c_{T,P}$ are the proton and the total polymer concentration, respectively. An alternative procedure for the calculation of ν_H, with greater accuracy than eq 22, is discussed in section 5 for the case of calcium titration at constant pH.

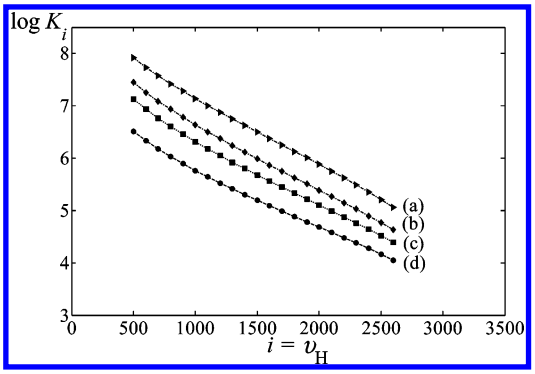

Figure 4. Stepwise protonation constants of PAA obtained from experimental data at different salt levels: [KNO_3] equal to 0.01 M (a), 0.05 M (b), 0.1 M (c), and 0.5 M (d). The values of K_j were calculated using eq 12. Parameters: total number of carboxylic groups in the PAA molecule, 3469; concentration of PAA, 2.5×10^{-3} M in carboxylic groups.

Several limitations arise in the experimental work: on one hand, the detection limit of the calcium electrode determines the lowest free calcium concentration that can be measured with accuracy, which also depends on pH and ionic strength.[41] On the other hand, calcium precipitation can occur at high metal concentrations and high pH values. Both factors determine the range of experimental conditions probed in this work, that is, the range of calcium and proton coverage measured for each ionic strength. The highest coverages of calcium and proton are obtained at the lowest ionic strength studied, where the screening effect of the background electrolyte is also the weakest.

4. Application to H^+ Binding to PAA. Chemical and Electrostatic Contributions to Binding

As a first application of the methodology outlined in section 2.1, let us obtain the stepwise constants corresponding to proton binding to PAA. According to the molar mass of the PAA used in this work (see the Experimental Section) and the molar mass of the PAA monomers, 3469 proton binding sites are present in this macromolecule. Therefore, we are under excellent conditions to apply the limiting expression (eq 12) derived from the consideration of thermodynamic limit conditions.

The application of this expression to the experimental data leads to the results depicted in Figure 4, where the stepwise constants depend on the ionic strength and on the stoichiometric step. The decreasing trend of K_i as i increases is expected from the entropic contribution to the Gibbs binding energy. As i increases, the number of available sites for the proton binding decreases, and so does the entropic contribution to the Gibbs energy of the corresponding stoichiometric binding process. As commented above, from a physical point of view, it is convenient to calculate the intrinsic stability constants which can be done by applying eq 6 (corresponding to taking ideal complexation as a reference) to the stepwise constants. The resulting values at the different ionic strengths are depicted in Figure 5. It can be observed that the slope of the curves is noticeably smaller than in Figure 4. However, $K_{i,int}$ values are still dependent on i, thus showing a deviation from the behavior expected for ideal complexation.

The decreasing behavior of $K_{i,int}$ with i can be justified by the decreasing contribution of the electrostatic energy involved in the proton binding as the number of protonated sites increases. Actually, the electrostatic energy decreases when the net charge

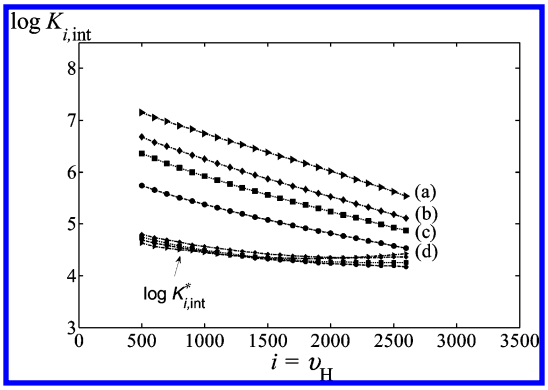

Figure 5. Intrinsic protonation constants of the PAA, $K_{i,int}$, at different concentrations of KNO_3, the background electrolyte. The concentration of KNO_3 is equal to 0.01 M (a), 0.05 M (b), 0.1 M (c), and 0.5 M (d). Intrinsic constants are obtained from the stepwise constants shown in Figure 4 after removing the ideal entropic factor (see eq 6). At the bottom, intrinsic constants after removing the electrostatic contribution, $K^*_{i,int}$, obtained with eq 23, once Ψ_S is calculated using the Poisson−Boltzmann equation under cylindrical geometry and cylinder radius $r_0 = 5.5 \times 10^{-10}$ m. Parameters: distance between functional groups, 2.5×10^{-8} m; number of carboxylic groups as in Figure 4.

TABLE 1: Average of the Intrinsic Constants after Removing the Electrostatic Component for the Proton, k_H, and Calcium, k_M, Binding to PAA[a]

$[KNO_3]$/M	0.5	0.1	0.05	0.01
$\log(k_H/M^{-1})$	4.4	4.4	4.4	4.41
$\log(k_M/M^{-1})$	0.88	0.87	1.27	

[a] The maximum deviation of the $\log K^*_{i,j,int}$ values with respect to the average is 0.5 pK units.

of the macromolecule is reduced as more protons are bound. The electrostatic contribution will also be responsible for the decreasing $K_{i,int}$ values observed at increasing ionic strengths, due to the more effective screening of the background electrolyte.

A standard way to estimate the electrostatic contribution to the binding consists in using the Poisson−Boltzmann equation. Assuming cylindrical geometry for the macromolecule with a radius[26] of $r_0 = 5.5 \times 10^{-10}$ m (which can be accepted as a good approximation along a persistent length of the polyelectrolyte, as was reported in ref 42), we can estimate the electrostatic potential at the surface of the cylinder where the binding sites are assumed to be located, ψ_S. The intrinsic stability constants free from electrostatic effects, $K^*_{i,int}$, can then be computed as

$$K_{i,int} = K^*_{i,int} e^{-F\psi_S/RT} \tag{23}$$

where Ψ_S is the estimated electric potential at the macromolecular surface.[43] Figure 5 also shows the resulting $K^*_{i,int}$. Details of the numerical procedure used to solve the Poisson−Boltzmann equation when cations of charge 1 and 2 are present in the system are given in ref 26. A reasonable convergence of all the values corresponding to different ionic strengths is observed, this indicating the suitability of the Poisson−Boltzmann equation in describing the electrostatic contribution. Additionally, the mild dependence of $K^*_{i,int}$ on i indicates that the intrinsic binding of the proton to PAA is close to that predicted by the ideal model (i.e., $K^*_{i,int} = k_H$, where k_H is the stability constant corresponding to the protonation of a particular site; see eq 5). The average values for k_H are shown in Table 1, at the different ionic strengths. We point out the remarkable convergence of the different values obtained, in agreement with data previously obtained using different methodologies.[26,42] We highlight that such convergence, although not perfect, is obtained despite the important approximations involved in the Poisson−Boltzmann approach. Only slight differences (less than 0.3 pK units) are observed at very large and very small i values.

5. Ca^{2+} Binding to PAA

Let us now consider Ca^{2+} binding to PAA. As detailed in the Experimental Section, free and bound calcium concentrations in equilibrium at different fixed pH values have been recorded along several titrations of solutions containing PAA and background electrolyte. Figure 6 gathers the raw experimental binding data. The application of eq 16 requires as a previous step the determination of ν_H from the experimental binding curves.

The use of mass and charge balances (eq 22) can lead to a significant error in the calculation of ν_H (due to the high number of small volume additions needed for pH adjusting) at the relatively high pH values at which the calcium titration experiments were carried out. For this reason, we have applied an alternative procedure to obtain the values of ν_H along a titration with calcium at a fixed pH. Let us start from the limiting point corresponding to the absence of Ca^{2+} in the solution. In this point, ν_H is given by the value obtained in the acid/base titration for this pH value. When adding Ca^{2+}, the occupation of protons decreases when more Ca^{2+} ions get bound. It can be shown that, at any point of the calcium binding curve, ν_H can be determined by the equation

$$\nu_H(c_H,c_{Ca}) = \nu_H(c_H,0) + \int_{-\infty}^{\ln c_{Ca}} \left(\frac{\partial \nu_H}{\partial \ln c_{Ca}}\right)_{\ln c_H} d \ln c_{Ca} \tag{24}$$

where the quantity $(\partial\nu_H/\partial \ln c_{Ca})_{\ln c_H}$ in the integral can be calculated by using the thermodynamic consistency relationship[2,17]

$$\left(\frac{\partial \nu_H}{\partial \ln c_{Ca}}\right)_{\ln c_H} = \left(\frac{\partial \nu_{Ca}}{\partial \ln c_H}\right)_{\ln c_{Ca}} \tag{25}$$

The derivative $(\partial\nu_{Ca}/\partial \ln c_H)_{\ln c_{Ca}}$ can be computed by comparing titration curves at different pH values for the same Ca concentration.

Once the values of ν_H and ν_M are known, we can compute the values of the stepwise stability constants, $K_{i,j}$, by using eq 16. The values of $K_{i,j}$, for three different ionic strengths, are plotted in Figure 7 as continuous 3D surfaces, which were obtained by interpolation from the resulting experimental data. The range covered by the surfaces shown here corresponds to the values of calcium and proton coverages available within the limitations of the experimental techniques and procedures, as explained in section 3. Since such ranges strongly depend on the ionic strength, we have plotted, for clarity, our results in different figures ($[KNO_3]$ equal to 0.05 and 0.1 M in Figure 7a; $[KNO_3]$ equal to 0.1 and 0.5 M in Figure 7b).

Again, it can be observed that the values of the stepwise constants decrease with the occupation numbers of the complexing ions, due, as happened with noncompetitive proton binding, to both the decreasing entropic contribution to the free energy of binding and to the electrostatic effects. Compared with the monocomponent case, however, the ranges of ion coverages available in the competition experiments are narrower, and therefore, the change in $\log K_{i,j}$ is relatively small.

As done in section 4 for proton binding, we are going to compute the intrinsic stability constants, $K_{i,j,int}$, by eliminating

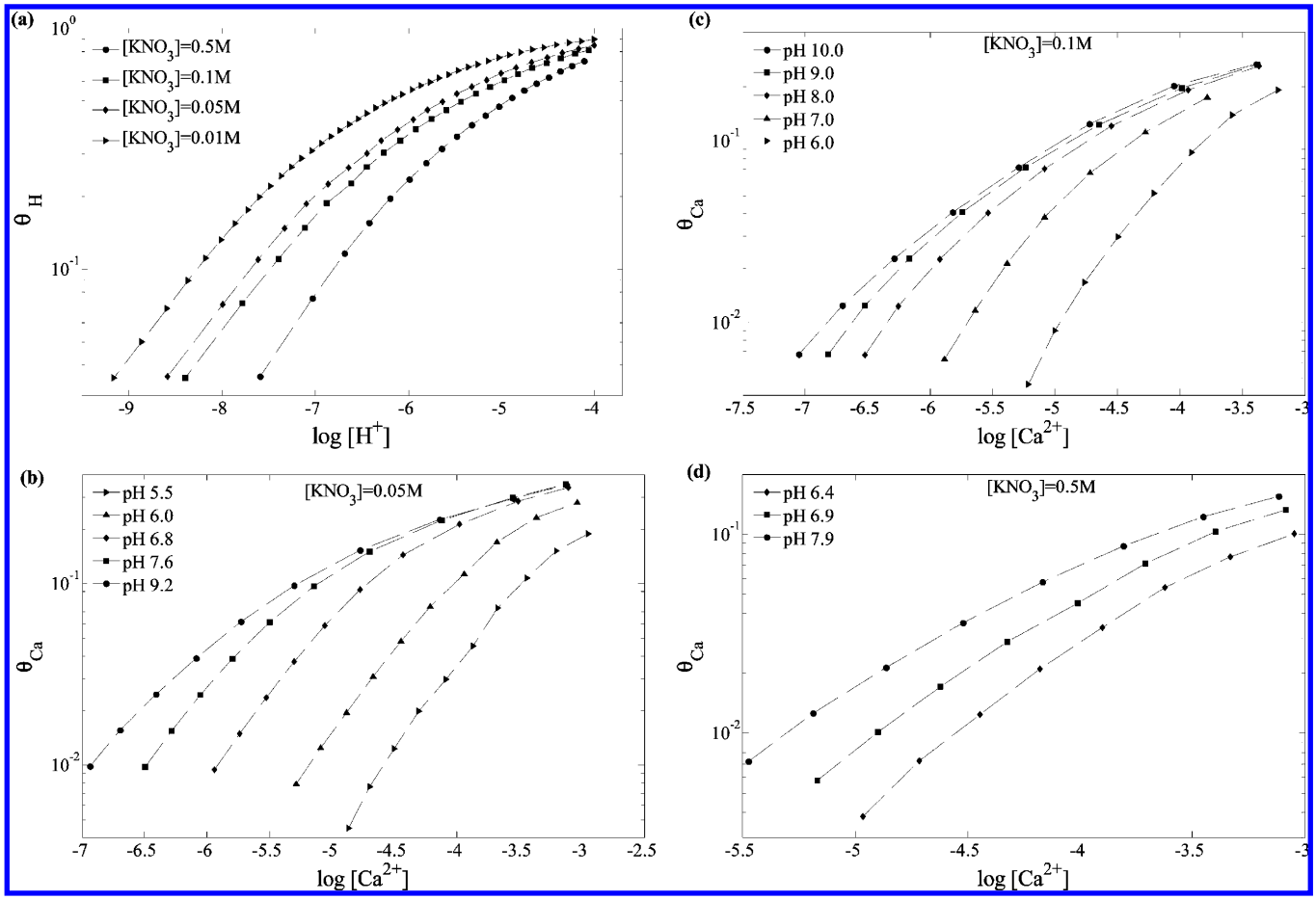

Figure 6. Proton and calcium binding curves to PAA. In panel a, markers depict the experimental proton coverage, θ_H, vs proton concentration. Rest of panels: markers depict experimental θ_{Ca} vs pCa at different constant pH and salt levels. The concentrations of KNO_3, the background electrolyte, are 0.05 M (panel b), 0.1 M (panel c), and 0.5 M (panel d).

the entropic contribution to binding. This needs the previous knowledge of the stoichiometry of Ca binding to PAA. Indeed, as commented in section 2, the entropic factors depend on stoichiometric binding relationships, since they determine the number of microstates of the stepwise species. We shall assume here, from simple steric and electrostatic considerations, that the Ca^{2+} ions bind to PAA through a bidentate chemical bond to neighboring sites. A stoichiometric factor of 2 will then be assumed in agreement with previous works.[44−46] Additional support to this assumption can be obtained by plotting the exchange ratio

$$r_{ex} = -\left(\frac{\partial \nu_H}{\partial \nu_{Ca}}\right)_{\ln c_H} \tag{26}$$

as a function of the Ca^{2+} concentration. The exchange ratio provides the average number of protons released by a Ca^{2+} ion at each point of the metal titration carried out at constant pH. Figure 8 depicts this information for two salt levels (0.05 and 0.1 M). The obtained exchange ratios are higher for the lowest concentration of the background electrolyte. Indeed, for the lowest ionic strength, both the number of bound protons and the electrostatic effects following the calcium binding justify the highest proton release. Notice the relevance of the electrostatic effects at low ionic strengths: the decrease of the macromolecular charge resulting from the Ca^{2+} binding decreases the electrostatic binding energy of the protons and facilitates the release of protons. Accordingly, Figure 8 also shows that, in general, r_{ex} tends to reach the largest values at the lowest free Ca^{2+} concentrations, where the electrostatic effect is, in relative terms, more important. For both ionic strengths, the exchange ratio reaches values higher than 1 at the lowest Ca^{2+} loading. This result is consistent with the use of a stoichiometric factor of 2 for the competitive calcium/proton binding.

By applying the same procedure used in deriving eq 8, the overall stability constants for multidentate competitive complexation can be written as

$$\beta_{i,j} = \frac{(n - mj + j)!}{i!j!(n - mj - i)!} k_H^i k_M^j \tag{27}$$

where n is the total number of monomers present in the macromolecule and m indicates the stoichiometry of the metal ions. The stoichiometry of protons is taken as 1:1. The entropic factor in eq 27 arises from the number of combinations without repetition of the $n - mj - i$ free monomers, the i bound protons, and the j bound metals. In the particular case $m = 2$ (bidentate metal binding), one can compute the stepwise constants, identify the corresponding entropic factor, and define the intrinsic stability constants as

$$K_{i,j} = \frac{(n - 2j - i + 2)(n - 2j - i + 1)}{j(n - j + 1)} K_{i,j,int} \tag{28}$$

with $K_{i,j,int} = k_M$ (a value independent of the stoichiometric step)

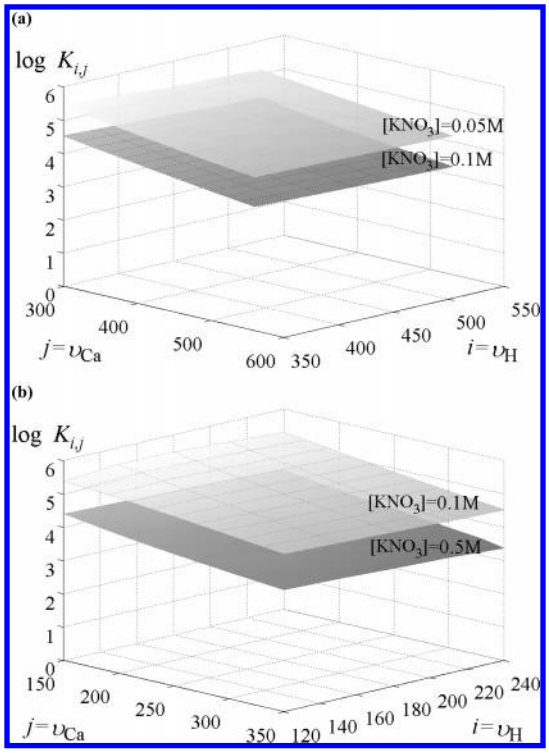

Figure 7. Stepwise stability constants of the Ca^{2+} binding to PAA vs the proton and metal occupation numbers, at three different salt levels. The values were calculated by using eqs 24 and 16 on the raw binding data depicted in Figure 6. The concentrations of KNO_3, the background electrolyte, are 0.05 and 0.1 M (panel a); 0.1 and 0.5 M (panel b).

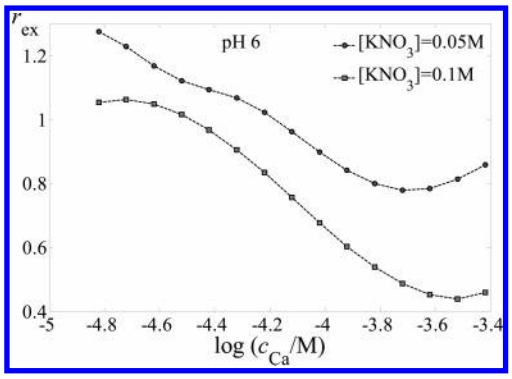

Figure 8. Values of the exchange ratio, $r_{ex} = -(\partial\nu_H/\partial\nu_{Ca})_{\ln c_H}$, vs the free calcium concentration at pH 6, for two different concentrations of KNO_3: 0.05 and 0.10 M. Values of the exchange ratio larger than 1 can support the assumption of a stoichiometric factor of 2 for the Ca^{2+} ion.

for identical functional groups and the absence of interactions between bound ions.

The use of eq 28 to calculate the intrinsic stability leads to the surfaces depicted in Figure 9, for the three ionic strengths.

Finally, we can split the intrinsic binding energy into a chemical or specific component and an electrostatic one. Notice that, since calcium is a divalent ion, the Boltzmann factor that relates the calcium concentrations in the bulk solution and at the macromolecular surface is given by $e^{-(2F/RT)\psi_S}$, so that

$$K_{i,j,\mathrm{int}} = K^*_{i,j,\mathrm{int}} e^{-(2F/RT)\psi_S} \qquad (29)$$

where ψ_S, the electrostatic potential at the surface of the macromolecular species, has again been computed by solving the Poisson−Boltzmann equation assuming cylindrical geometry for the macromolecule and using the same cylinder radius as

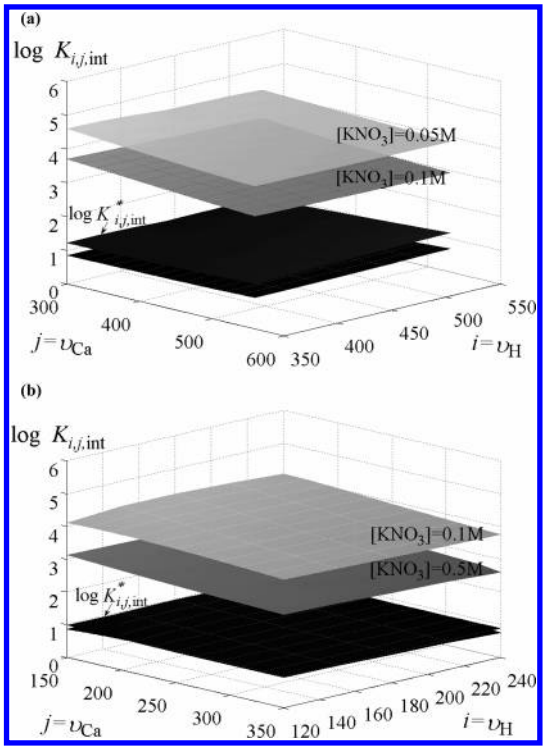

Figure 9. Intrinsic stability constants corresponding to Ca^{2+} binding to PAA at different ionic strengths, as a function of ν_H and ν_M. These data were obtained from the results shown in Figure 7 after removing the corresponding entropic factor (see eq 28). The merged surfaces at the bottom were obtained from the upper ones after correction for electrostatic contribution, eq 29, using the Poisson−Boltzmann equation. Parameters as in Figure 5.

that reported in Figure 5 to describe the proton binding. The resulting $K^*_{i,j,\mathrm{int}}$ values lead to the surfaces depicted in parts a and b of Figure 9 (bottom). Notice the remarkable collapse of the surfaces (especially those corresponding to the $[KNO_3]$ equal to 0.1 and 0.5 M), this indicating the successful evaluation of the electrostatic contribution by means of the Poisson−Boltzmann equation.

Since the remaining dependence of $K^*_{i,j,\mathrm{int}}$ on i and j (the number of bound proton and calcium ions) is certainly small (less than 0.5 pK units), it is interesting to calculate the average value of the set of $pK^*_{i,j,\mathrm{int}}$ (obtained by averaging over i and j). These values are shown in Table 1, for the different ionic strengths. As happened in the analysis of proton binding, the convergence is remarkable given the approximate character of the Poisson−Boltzmann equation when applied to divalent ions, the simple entropic factors used, the presence of some remaining heterogeneity in the binding energies, the conformational changes in the macromolecule, and so forth. This convergence suggests that the binding of calcium to PAA is not far from being homogeneous and independent for the range of coverage studied. However, using k_H and k_M values shown in Table 1, we inverted the above process until recovering the theoretically expected binding data. Experimental and recovered values (data not shown) agree only semiquantitatively. The ideal complexation model is, thus, just a rough first approximation. The small divergences between the log $K^*_{i,j,\mathrm{int}}$ surfaces in Figure 8 and a flat surface have still an important effect in the detailed quantitative description of the calcium binding to the PAA.

6. Conclusions

It has been shown that stepwise stability constants can be, for systems with a large number of sites, easily obtained from

the binding curve and used in describing macromolecular complexation. The inverse of the free ion concentration taken at integer values of the average number of bound ions provides the corresponding stepwise stability constant. This simple result has been extended to competitive systems in a straightforward manner.

The methodology presented is first applied to proton binding to PAA, and the corresponding stepwise stability constants are determined. In a second step, entropic contributions to binding are removed and the intrinsic stability constants are computed. Once the electrostatic contribution to the binding is taken into account by means of the Poisson−Boltzmann equation, the resulting intrinsic constants, free from the electrostatic contribution, indicate the homogeneous nature of proton/PAA binding.

These ideas have been extended to the Ca^{2+}/H^+ competitive binding to PAA. In this case, the exchange ratio, r_{ex}, reaches values higher than 1 at salt levels of 0.05−0.1 M and pH values close to 6. This means that more than one carboxylic group of the PAA is involved in the binding site of a calcium ion, and supports a stoichiometry factor of 2 between Ca^{2+} and the carboxylic groups of PAA.

The stepwise and intrinsic constants (these latter by using the entropic factors that arise from the binding to two neighboring functional groups of a linear chain) for the binding of calcium ions to PAA molecules with different protonation degrees have been obtained. Once the intrinsic constants are split into a specific (or chemical) and an electrostatic contribution, a remarkable convergence of the specific contribution to the binding was obtained, independently of the ionic strength of the medium. In effect, the intrinsic binding constants of Ca^{2+} to PAA are not far from those expected in homogeneous and independent complexation.

We highlight that the methodology here presented is fundamentally different from that used in the literature. Instead of checking different models, searching the one which fits the experimental data, we have used the model-independent eq 16 which allows, via the intrinsic stability constants, one to retrieve information on the involved binding events.

Finally, we point out that, although different stoichiometric factors are expected in the binding of metal ions to macromolecular ligands, most theoretical frameworks used in the literature only account for 1:1 macromolecular binding. The use of the stepwise constants, here developed, allows an explicit description of systems for which the stoichiometric factors of the competing binding ions are different.

Appendix: Derivation of an Expression for the Stepwise Constants Corresponding to Competitive Systems with Ligands Having a High Number of Sites

For a general complexation model, the proton and the metal occupation degrees can be obtained as[2]

$$\nu_H = \left(\frac{\partial \ln \Xi}{\partial \ln c_H}\right)_{c_M} \tag{A-1}$$

$$\nu_M = \left(\frac{\partial \ln \Xi}{\partial \ln c_M}\right)_{c_H} \tag{A-2}$$

where

$$\Xi = \sum_{i,j} \beta_{i,j} c_H^i c_M^j \tag{A-3}$$

is the macrocanonical partition function where $\beta_{i,j}$ are the overall stability constants (corresponding to the process of attaching j ions and i protons to the macromolecule) and ν_H and ν_M are the mean occupation numbers of protons and metal ions bound per macromolecule. The summation over the indices i and j is extended up to their possible maximum values, which are s_H and s_M, respectively. Notice that both quantities can be different, that is, $s_H \neq s_M$, since chelate complexation is possible. A very usual technique in statistical mechanics, valid only for large values of s_H and s_M, is the so-called method of the maximum term, which consists in assuming that, at a given proton and metal concentration, only one term (the maximum one) contributes to the partition function.[39] As a consequence, the values of i and j corresponding to the maximum term, say i_{max} and j_{max}, can be identified with ν_H and ν_M, respectively. Taking derivatives of $\log(\beta_{i,j} c_H^i c_M^j)$ with respect to the indices i and j, the condition of maximum leads us to the equations

$$\left[\frac{\partial \log \beta_{i,j}}{\partial i} + \log c_H\right]_{\substack{i=i_{max}=\nu_H \\ j=j_{max}=\nu_M}} = 0 \tag{A-4}$$

$$\left[\frac{\partial \log \beta_{i,j}}{\partial j} + \log c_M\right]_{\substack{i=i_{max}=\nu_H \\ j=j_{max}=\nu_M}} = 0 \tag{A-5}$$

Notice that, for large values of s_H and s_M, $\beta_{i,j}$ can be considered as a continuous function of the indices i and j. Hence, one can assume that

$$\frac{\partial \log \beta_{i,j}}{\partial j} = \frac{\log \beta_{i,j} - \log \beta_{i,j-1}}{\Delta j} = \log\left(\frac{\beta_{i,j}}{\beta_{i,j-1}}\right) = \log K_{i,j} \tag{A-6}$$

and expression A-5 becomes

$$\log K_{i_{max},j_{max}} + [\log c_M]_{\nu_H=i_{max},\nu_M=j_{max}} = 0 \tag{A-7}$$

where one can recognize eq 16. This derivation of eq 16 (see expressions A-4 and A-5) requires that $\beta_{i,j}$ is "a continuous and derivable function of i and j". This is equivalent to saying that our complexing system can be somehow "modeled" by taking advantage of its "regularity". This is especially reasonable for linear polyelectrolytes such as PAA. However, although not detailed in this work, one can alternatively derive eq 16 without performing any derivative of $\beta_{i,j}$, by using the Darwin−Fowler method (indeed, the most rigorous, although involved, way to do it; see ref 32). Therefore, result 12 is more general and not subject to the limitations involved in steps A-4 and A-5 of the present derivation.

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