

On the Molecular Mechanism of the Calcium-Induced Gelation of Pectate. Different Steps in the Binding of Calcium Ions by Pectate

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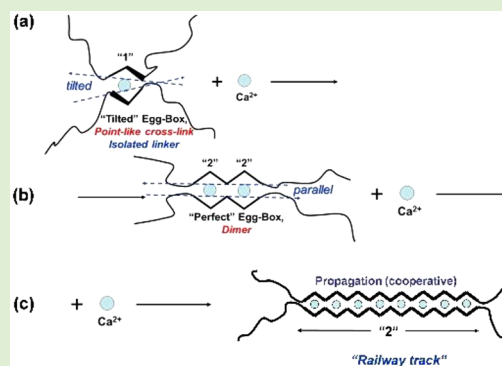


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Supporting Information

ABSTRACT: Pectic acid/sodium pectate is one of the most widespread hydrocolloid used in the food industry. It is able to form strong ionotropic gels by the addition of ions, in particular, calcium ions. The initial steps of binding Ca^{2+} ions to a sample of sodium pectate with a composition close to 90% of ideal Na^+ -poly(galacturonate) were investigated by means of circular dichroism (CD), microcalorimetry, dilatometry, viscosity, and membrane osmometry, as a function of increasing R_p , R_j being the ratio of the molar concentrations of Ca^{2+} and pectate repeating units. Data were collected in aqueous NaClO_4 at 25 °C. The key instrument of interpretation has been the counterion condensation theory (CCT) of linear polyelectrolytes, modified to include the presence of both specific affinity of the divalent counterion for the polysaccharide (“territorial binding”), and, very importantly, strong chemical bonding (not a covalent bonding, though) of Ca^{2+} on conformationally well-defined sites on the polymer, with local charge annihilation. Intrinsic viscosity and number-average molar mass data as a function of R_j showed that calcium bonding brings about chain association right from the beginning of addition to pectate. The analysis of the microcalorimetric curve using the modified CCT revealed two types of bonding. In the order of development as a function of R_p , the first mode (type 1) could be reconciled with the “tilted egg-box” type, recently proposed for Ca^{2+} binding to alginate and the second mode (type 2) with the “shifted egg-box” proposed for calcium pectate on the basis of conformational analysis investigation. Likewise, the two types of bonding turned out to be superimposable with similar bonding categories proposed for alginate and low-methoxyl pectin (LMP), on the one side, and for the association of semiflexible polyelectrolytes, on the other. The analysis allowed us to obtain standard Gibbs free energy, enthalpy, entropy, and volume molar values both for the affinity and the chemical bonding processes. Interestingly, the analysis of the dependence of the gelation temperatures, T_g , of LMP upon increasing additions of calcium ions provided the values of T_g and standard Gibbs free-energy of calcium-to-pectate association coinciding with those obtained from calorimetry for the type-2 bonding process. This finding corroborated previously reported evidence on the enthalpic nature of the elasticity of Ca^{2+} -pectate gels. Finally, comparative analysis of different techniques, but of CD in particular, enabled proposing a “loose- 2_1 -helix” as the starting conformation of sodium pectate in aqueous solution.



1. INTRODUCTION

This paper is at the crossroad of three lines of investigation, namely, (a) the elaboration of the counterion condensation (CC) theory of polyelectrolytes, mostly developed by Manning^{1–3} and later developed to include more “chemical” aspects such as those connected with mixtures of counterions of different valencies,^{4,5} specific affinity of one counterion for the polymer backbone,⁶ and even charge annihilation;^{6–9} (b) the application of the theory developed by Borukhov *et al.* on the association of two semiflexible polyelectrolytes by interchain linkers,¹⁰ and (c) the possibility that calcium binding by polyuronates, in particular by alginates, follows a two-step mechanism, with the modulation of the popular “egg-box” model (EBM),^{11–16} to foresee an initial “tilted egg-box” mode of binding.¹⁷

The specific case of convergence of the above approaches is that of the first stages of the interaction of increasing amounts

of calcium ions with sodium pectate, that is, the sodium salt of poly(galacturonic acid), which eventually leads to gel formation. This polymer is the (ideal) homopolymeric ionic component of the homogalacturonan (HG) structural element (also identified as the “smooth” region) of the class of pectins and pectinates, fundamental constituent of the vegetal tissue, presenting minor and variable amounts of neutral sugars in the main chain as well as variable amounts of ester methylation at the uronic groups;¹⁸ moreover, pectins rank among the

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commercially most important hydrocolloids.¹⁹ An extensive literature has been collected over several decades covering various aspects of the Ca^{2+} -pectate interaction at the root of its ionotropic gelation.^{20–25} Still, a full thermodynamic description of the different contributions associated with the interaction of calcium with pectate and of the steps leading to chain association and gelation is lacking. Reference 26 made an important step in that direction, both experimentally and from the standpoint of the interpretation. The system variable chosen as the independent one to trigger the association process was the concentration of calcium ions added [as $\text{Ca}(\text{ClO}_4)_2$] to a diluted (about 0.08% w/w) pectate solution in the presence of a constant concentration of the supporting (nongelling) 1:1 electrolyte (NaClO_4), providing a practically constant value of a solution ionic strength of 0.05 M. The investigated calcium ion concentration ranged from 0 to about 1.1 mM, covering the range of Ca^{2+} concentration experimentally determined in various vegetal tissues.²⁷ It corresponds to steps I and II as indicated by Fang *et al.*²⁸ Circular dichroism (CD) data allowed determining the ratio, θ , of strongly bound calcium ions over their maximum value (bonding site saturation fraction or, more in general, the reaction coordinate of the bonding process), which enabled the further interpretation of the calorimetric and dilatometric results; the main features/conclusions can be summarized as follows:

- i the theoretical model described in ref 2 can be consistently applied to describe the interaction of calcium ions with pectate in aqueous solution.
- ii Two modes of binding are present at the same time: a territorial affinity of the divalent ions for both the isolated single chain and the chain dimer, on one side, and a strong chemical bonding (although not a covalent bonding) of calcium ions by (within) the chain dimer, which brings about an annihilation of the fixed charges of the coordinating COO^- groups, on the other side.
- iii Strong chemical bonding is associated with a conformational transition of the polysaccharide chains, similar to the most significant cases of ionic polysaccharides (eventually leading to gel formation as its necessary molecular precondition).^{20,21,24,29}
- iv The minimization procedure using the experimental chiro-optical and microcalorimetric data provides constant values for free energy, enthalpy (and hence entropy) changes of the affinity of calcium as a function of the molar ratio of total calcium over polymer. The favorable affinity interaction is all due to a large and positive change of entropy, whereas the enthalpy change is positive.
- v The values of the free energy of chemical bonding significantly depend on the molar ratio of (total) calcium over polymers, being numerically very close to the corresponding affinity term for a low calcium content (built up by favorable contributions of both enthalpy and entropy) and rapidly increasing toward less favorable values on increasing the concentration of added calcium. The enthalpy change of chemical bonding (an intensive property) is always negative: initially decreasing in a sigmoid fashion to reach eventually a constant value.
- vi Both modes of binding are accompanied by substantial, positive volume changes, that accompanying chemical

bonding is larger than that stemming from affinity interactions.

The task of the present paper is to build over the achievements of ref 26, trying to unveil details of the Ca^{2+} /pectate interaction according to the initial approaches listed under (b) (Borukhov's model) and (c) (the tilted EBM), ambitiously aiming at proposing a consistent frame of description of the calcium-induced gel formation by polyuronates.

2. MATERIALS AND METHODS

2.1. Materials. The sample of sodium pectate used in ref 26 (and to which the present analysis refers) is the same as that described in ref 29. The main features are summarized here: the equivalent weight was 222.8 g/(mole of the charged repeating unit—*i.e.*, unsubstituted galacturonic acid)⁻¹, the nonionic component (methyl esterification of galacturonic groups and/or neutral sugars) was 10.6%, and the number of constituent water molecules per repeating unit was 1.2, determined by Karl Fischer titration after extensive dehydration on P_2O_5 to constant weight. The microcalorimetric and the dilatometric data (Carlsberg dilatometers) of mixing the carbohydrate polyelectrolyte with the divalent ion of choice were treated according to a more than 40-year long experience in microcalorimetry and in dilatometry;^{29,30} particular attention was given to the proper correction for the dilution effects of both the added counterion (calcium) and polymer. No arbitrary interpretation model was used to determine the molar enthalpy change of mixing, $\Delta\bar{H}^{\text{mix}}(R_i)$ (*e.g.*, commercial routine software). The dilatometric data were treated in the same way.³⁰

2.2. Experimental Methods. All experimental data used in this paper have been previously obtained and published by this laboratory. A description of the experimental details is reported in Section 1 of the Supporting Information.

2.3. Determination of the Relative Molar Mass of Na^+ Pectate. The molar mass of the sample used in the previous work²⁶ whose results are at the root of the present paper had been determined and reported in ref 24. By definition, the relative molecular mass (a dimensionless quantity) and the molar mass (in $\text{g}\cdot\text{mol}^{-1}$) of the sample used numerically coincide as follows: the notation “MW” will occasionally be used for either quantity, just for practical convenience; any ambiguity will be avoided by the clear context of the wording. The results of membrane osmometry²⁴ provide the value of the number-average MW, \bar{M}_n : $\bar{M}_n = 2.1 \times 10^4$. Given the value of $M_{\text{r.u.}}$, that is, the relative molecular mass of the repeating unit, r.u. ($M_{\text{r.u.}} = 222.8^{29}$), one gets the number-average number of charged r.u./chain, \bar{N}_n^{GalH} , from $\bar{N}_n^{\text{GalH}} = \bar{M}_n/M_{\text{r.u.}}$: $\bar{N}_n^{\text{GalH}} = 94$. It is this value which is of interest in all calculations concerning whole-chain polyelectrolyte properties [the actual number-average degree of polymerization, \bar{DP}_n , *i.e.*, number of linkages, is 10.6% larger than \bar{N}_n^{GalH} (to account for the uncharged r.u.) minus 1, *i.e.*, $\bar{DP}_n = 104 - 1 = 103$].

Replotting of the original data of ref 29, obtained under both neutral and acidic conditions, is reported in Figure S1 of the Supporting Information. Besides showing excellent agreement as to \bar{M}_n for both sets of conditions and providing the corresponding values of the second virial coefficient for the polymer in the two conformations, they experimentally prove the intramolecular character of the pH-induced conformational transition of pectate that had been for the first time reported and analyzed experimentally in ref 29.

2.4. Data Minimization and Fitting. Minimization procedures and curve fitting of experimental data have been performed using the built-in procedure of program Origin by OriginLab Co., MA, USA (<https://www.originlab.com/index.aspx?go=PRODUCTS/Origin>).

3. RESULTS AND DISCUSSION

3.1. On the Molecularity of the Calcium Bonding by Pectate for Low Values of Calcium Addition. For more

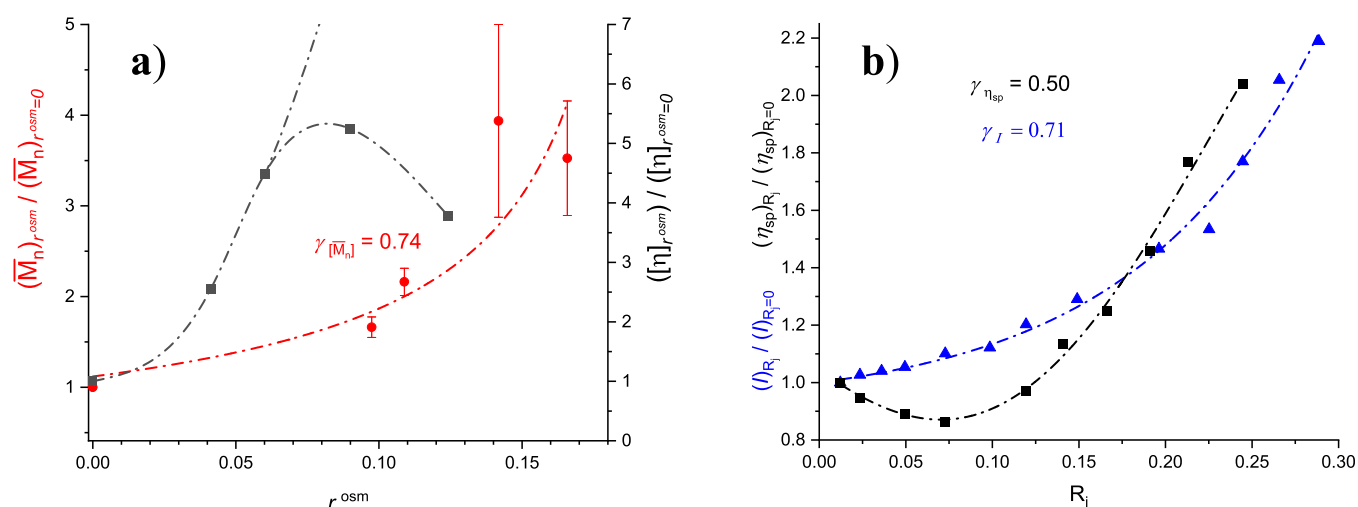


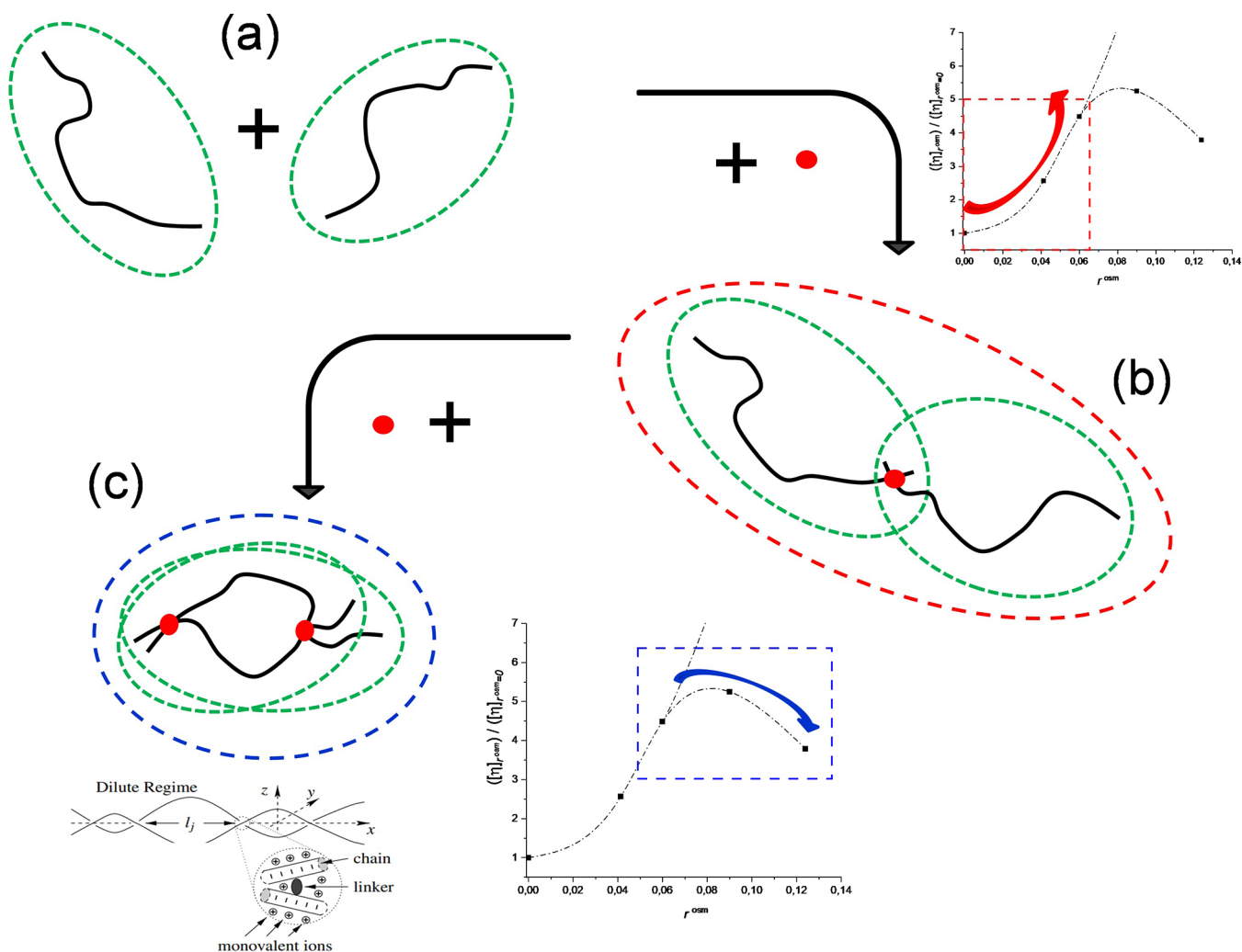
Figure 1. (a) Dependence of the relative number-average molar mass, $(\bar{M}_n)_{r^{\text{osm}}} / (\bar{M}_n)_{r^{\text{osm}}=0}$, (red symbols, l.h.s. scale)²⁴ and of the relative intrinsic viscosity $([\eta])_{r^{\text{osm}}} / ([\eta])_{r^{\text{osm}}=0}$ (black symbols, r.h.s. scale)⁴¹ as a function of the “total bound” calcium counterions, r^{osm} , for Na⁺-pectate in aqueous NaClO₄ 0.1 M at 25 °C and (b) dependence of the relative scattering intensity at 90°, $(I)_{R_j} / (I)_{R_j=0}$, (blue symbols) and of the relative specific viscosity $(\eta_{\text{sp}})_{R_j} / (\eta_{\text{sp}})_{R_j=0}$ (black symbols) as a function of the molar ratio of added calcium counterions, R_j , of pectate (degree of esterification < 2%) in aqueous salt-free solution at 25 °C.^{44,46}

than 30 years, the accepted model of calcium binding to alginate and pectate has been that so-called EBM,^{11–16,31–33} albeit with some structural variations.²⁵ This mode of binding, largely focused on detailed stereochemical arrangement at the binding site, had always been supposed to hold for all values of the extent of binding. In the more recent years, both theoretical approaches^{34,35} and advanced experimental ones^{36,37} introduced a slightly different perspective, essentially looking at a wider scale, whole-chain, approach. Nevertheless, the older point-of-view did not lose interest and applications. Starting in 2004, a series of papers^{9,28,38–41} introduced the hypothesis that the first step of calcium binding is intramolecular (“monocomplexation”) (step I), followed by the traditional step of calcium-driven chain dimerization for larger values of the concentration of added calcium (step II), and eventually massive lateral association (step III). It should be noted that in the first paper of the series,⁹ the model was set forth essentially based on the results of poly(acrylic acid) (PAA) with Mn²⁺ (and less so with Ca²⁺), whereas the extension of the model to the alginate/Ca²⁺ and pectin/Ca²⁺ systems was purely speculative as no direct experiment was reported for those systems. It is worth mentioning that PAA, at variance with those polyuronates, has a much larger linear charge density at the same pH and, most important of all, a tremendously higher chain flexibility due to its polyolefin backbone. The original investigated samples were alginates of different compositions and pectin samples with a moderate content of ionic galacturonate groups (around 50%), very different from the ideal structure of poly(galacturonic acid). The paper which really consolidated the idea of the monocomplex formation in the first step (step I) of calcium binding by alginate was that by Fang *et al.*³⁸ Therein, the only direct experimental evidence of the monocomplex hypothesis in step I was the observed initial decrease of the relative viscosity (*i.e.*, of the specific viscosity at a given, finite, value of the concentration of calcium over that of the calcium-free case, η_r^N) upon increasing the concentration of added calcium. The

calculated negative value of the binding enthalpy in step I was considered as a supporting evidence just because the comparison with the reported enthalpy for the monocomplex formation of the carboxylic group with Ca²⁺ was encouraging.⁴² All successive papers,^{28,39–41} including those which extended the model to the pectin/Ca²⁺ case, were essentially repetitions of the approach of ref 38.

Further work on alginate was devoted to elucidate this debated point, paralleling the use of light scattering (which is inherently by far more sensitive to association than turbidity) to viscosity, fluorescence quenching, and CD.¹⁷ The latter techniques revealed that the well-known conformational transition accompanying the formation of calcium–alginate complexes¹⁶ followed a bimodal evolution upon increasing the calcium concentration; the initial increase of chain ordering (in the range of added calcium encompassing the alleged monocomplexation) accounted for only about 50% of the total change at larger values of calcium concentration. The scattering data unequivocally demonstrated the intermolecular nature of the calcium–alginate complexes, starting from the very first step; fluorescence quenching showed that already the very first addition of calcium to alginate provoked a notable chain association, at variance with what produced the addition of the nonassociating Mg²⁺ ions. Equally important, the analysis of the polymer concentration dependence of some key viscosity parameters further supported the light-scattering evidence, fully in line with theoretical and experimental evidence on associating systems. The latter results were also an elegant demonstration of how it may be risky to rely upon a single feature of a given technique to derive a generalized conclusion. The decrease of the values of η_r^N in the calcium concentration range encompassing the alleged monocomplexation had been taken as a proof of the intramolecular character of the first step of calcium binding by alginate. Unfortunately, viscosity is primarily a measure of the hydrodynamic value of the polymer, and hence only indirectly also of molar mass, but certainly not of molar mass alone in a biunivocal relation. The

Scheme 1. Schematic representation of the steps in the association process of pectate chains driven by point-like cross-linking calcium ions^a



^a(a) Calcium-free isolated sodium pectate chains; the hydrodynamic volumes of two pectate chains (represented as black curves) have been drawn as green dotted ellipses. (b) Upon the addition of calcium ions (as red dots), the initial association is maximizing the distance of the associated species with an ensuing increase of the total hydrodynamic volume of the extended dimeric associate, represented by a red dotted ellipse. This step is revealed by the first rapid increase of $[\eta]_{r^{osm}}^N$, marked by a curved red arrow inside the dotted red rectangle drawn in the top right inset. (c) Further addition of calcium ions gives rise to folding of the associated chains to produce multiple single-point linkers separated by chain segments repelling from each other for electrostatic repulsion and a significant decrease of the hydrodynamic volume (within the blue dashed ellipse). This step is revealed by the rapid decrease of $[\eta]_{r^{osm}}^N$, marked by a curved blue arrow inside the dotted blue rectangle drawn in the bottom center inset. Redrawing with the adaptation of the original figure by Borukhov *et al.*¹⁰ to represent the system state (c) is on bottom left of the Scheme. Reprinted with permission from Borukhov, I.; Lee, K.-C.; Bruinsma, R. F.; Gelbart, W. M.; Liu, A. J.; Stevens, M. J. Association of Two Semiflexible Polyelectrolytes by Interchain Linkers: Theory and Simulations. *J. Chem. Phys.* **2002**, *117* (1), 462–480], with the permission of AIP Publishing

simplest example of partial lateral association of two chains (except—in part—for the peculiar case of a perfect head-to-tail association) clearly brings about a doubling of the molar mass but not of the hydrodynamic volume, whose increase of value—for the dimer—is only a fraction of that of the individual single chain.

Nevertheless, before analyzing the experimental data and applying a theoretical model (the polyelectrolyte model) which is strongly dependent on the molecularity of the polymer system (better to say: on the “charge-per-unit-length” of the species in equilibrium), in spite of repeated statements in the literature that “calcium pectate molecules exist initially as dimers”,^{43,44} it was considered as mandatory to apply the stringent analysis that led to exclude the intramolecular

binding in alginate also to the present case of poly(galacturonic acid). Osmotic pressure²⁴ and viscosity experiments⁴⁵ carried out on the pectate sample of ref 29 (which is also the present one) provided number-average molar mass values, \bar{M}_n , and intrinsic viscosity values, $[\eta]$, as a function of the fraction of osmotically inactive (“total bound”) ions, r^{osm} (see the next paragraph). To minimize the Donnan effect, it had been decided to use a larger value of the ionic strength, I , which was 0.1 M with NaClO_4 (the choice of the perchlorate anion was to ensure maximum transparency in the 200–250 nm region for the parallel CD measurements). Careful dialysis equilibrium, at all given values of r^{osm} , provided the “solvent” for dilution of the calcium-pectate solutions for extrapolation to infinite dilution, granting a constant chemical potential of the diffusible

species. Incidentally, to the best of our knowledge, these data are among the very few ones (if not the only ones) pertaining to “intrinsic” properties of calcium-pectate systems, namely, \bar{M}_n , and $[\eta]$. The results have been reported in panel a of Figure 1a. The r^{osm} profile of the relative intrinsic viscosity ($([\eta]_{r^{\text{osm}}})/([\eta]_{r^{\text{osm}}=0})$)—or $[\eta]_r^N$ —shows an immediate increase upon the addition of calcium, clearly indicating a large increase of the hydrodynamic volume of the polymer solute brought about by the binding of calcium, which is not compatible with an intramolecular process. This result finds a full counterpart in what Fang *et al.* concluded from the analogous relative viscosity results on $\text{Ca}^{2+}/\text{LMP}$ (low-methoxy pectin): “ $\eta_r^N > 1$ for LMP at $R < 0.25$ suggests an increase in molecular size, and it totally conflicts with an interpretation based on mono-complex formation. The appreciable increase in molecular size can only arise from an interchain crosslinking or dimerization of the LMP chains”.²⁸ The steep initial increase then flattens down to reach a maximum; the $[\eta]_r^N$ curve then starts decreasing, with a trend which has been observed for other $\text{Ca}^{2+}/\text{polyuronate}$ systems, such as low G alginate (LGA) (*e.g.*, see Figure 6 of ref 28). The data points of the relative number-average molar mass, ($(\bar{M}_n)_{r^{\text{osm}}}/(\bar{M}_n)_{r^{\text{osm}}=0}$) or $(\bar{M}_n)_r^N$ —as a function of r^{osm} can be well fitted using the equation proposed by Thibault and Rinaudo^{44,46} applying the fundamental equation of the gelation theory (after adapting their symbols to the present case): $(\bar{M}_n)_r^N \propto (r_{\text{crit}}^{\text{osm}} - r^{\text{osm}})^{-\gamma}$. γ is the critical exponent in the power law between the degree of chain association (presently as $(\bar{M}_n)_r^N$) and the amount of cross-linking calcium ions (presently as r^{osm}).

The best-fit (represented as the dash-dotted red curve) for the $(\bar{M}_n)_{r^{\text{osm}}}/(\bar{M}_n)_{r^{\text{osm}}=0}$ data of Figure 1a returned the following values: γ (herein: $\gamma_{\bar{M}_n}$) = 0.74 and $r_{\text{crit}}^{\text{osm}} = 0.2$, indicating that also the osmotic pressure data depict $\text{Ca}^{2+}/\text{pectate}$ as an associating system from the very beginning of the calcium additions, with a trend that will eventually lead to gel formation. It may be of interest to reproduce the relative viscosity data and the relative scattering (at 90°) data by Thibault and Rinaudo^{44,46} obtained by adding CaCl_2 to a dilute solution of pectate in the absence of added monovalent salt (panel b) of Figure 1. Such salt-free conditions, taken together with the data of panel a ($I = 0.1 \text{ M}$), demonstrate the generality of the conclusions on the association process over a very wide range of ionic strength values. Both sets of experimental data followed the proposed power-law dependence: the corresponding γ values have been reported in panel b of Figure 1; interestingly, the value of γ_I (scattering: $\gamma_I = 0.71$) was very close to the one presently found from osmotic pressure measurements ($\gamma_{\bar{M}_n} = 0.74$).

In conclusion, it is safe to postulate that calcium addition to sodium poly(galacturonate) produces an association of chains from the very first step, in line with the accepted view in the literature. The analysis of the CD and of the microcalorimetric data using an approach based on the CC theory of linear polyelectrolytes will start based on this result to get a deeper insight into the complex process of calcium crosslinking of pectate.

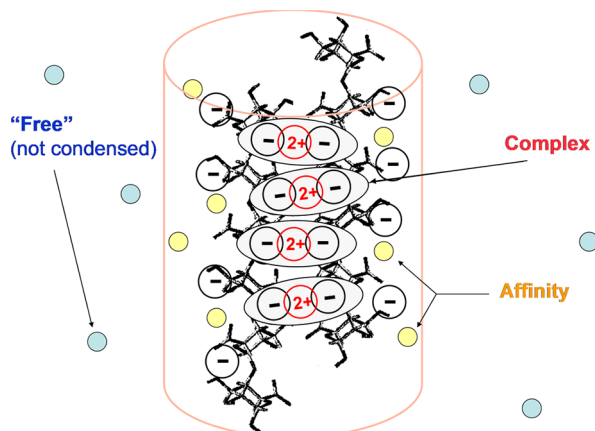
One of the theoretical models which have been used in the interpretation of the calcium/pectate bonding data is that of Borukhov *et al.*;¹⁰ it foresees that from the very beginning of the addition of linkers to a system of semiflexible polyelectrolytes, chain association takes place due to the

formation of multiple “point-like cross-links”^{39,40} generating loops of chain segments of same charge, repelling from each other (see the reproduction of original drawing from ref 8 in Scheme 1, bottom left). However, the profile of the intrinsic viscosity as a function of r^{osm} (see Figure 1a) suggests a bimodal mechanism, as depicted in Scheme 1. In the very first part of the $[\eta]$ versus r^{osm} curve, the marked increase of $[\eta]$ must be accompanied by a significant increase of the hydrodynamic volume of the polymeric solutes (in Scheme 1, it corresponds to the passage from a to b and to the red curved arrow in the top right inset). For $r^{\text{osm}} > \text{about } 0.06$, the sudden ending of viscosity growth and the beginning of a decrease of $[\eta]$ are necessarily attributed to a decrease of the hydrodynamic volume of the pectate chains. In Scheme 1, this second step is indicated as the passage from (b) to (c) and by a blue curved arrow in the inset on bottom left. During this bimodal process, all the other (site-related) properties—such as the molar ellipticity or the intrinsic thermodynamic parameters—keep the same value or the same trend of values: only a whole-chain property such as the intrinsic viscosity can be diagnostic of important changes in the hydrodynamic behavior of the associating system.

The above-described distinction of association modes corresponding to different variations of the hydrodynamic volumes of the polymeric solutes does not bear any consequence as to the mode of the underlying calcium bonding. In the following treatment, the same and one concept of point-like cross-link of polyuronate chains will be applied throughout the range of r^{osm} (or of similar variables) over which intrinsic viscosity data reveal two modes of chain association.

3.2. Polyelectrolytic Aspects of the Calcium Binding by Pectate. Complex formation of Ca^{2+} ions with pectate chains in aqueous solution has been consistently described by the model of ref 26. The model proposed that two modes of Ca^{2+} ion binding are present at the same time: (i) a territorial association (counterion condensation), driven by both non-specific polyelectrolytic interactions and a significant affinity (ion-specific) interaction of the divalent ions for both the isolated single chain and the chain dimer, on one side, and, on the other side, (ii) a strong chemical bonding (although NOT a covalent bonding) of calcium ions to well-defined sites in between two facing, ordered pectate chains (according to the generalized EBM). The latter bonding brings about an annihilation of the fixed charges of two coordinating COO^- groups (“complex formation”). Definitions such as “ionic bonding” or “inner sphere complexes” could be valid representations. Scheme 2 pictorially summarizes the different counterion states around the pectate macromolecular dimer.

The key parameter in the above derivation is the fraction of occupied sites (by chemically bonded Ca^{2+} ions), $\theta \leq \theta \leq \theta_{\text{MAX}} = 1$. θ measures the fractional occupation of bonding sites, not of the total number of bonding sites per polymer repeating unit: the latter value is given by σ , being $\sigma = \theta \cdot \sigma^0$.²⁶ In the following, σ will be substituted by σ_{tot} in relation to the presence of two subpopulations, σ_1 and σ_2 ($\sigma_{\text{tot}} = \sigma_1 + \sigma_2$). σ^0 is the complex (“calcium-to-polymer”) stoichiometric ratio, which for the “egg-box” type of bonding is taken as equal to 0.25 (1:4),¹⁶ as corroborated by the results illustrated in Figure 2 of ref 26. The total concentration of added calcium, $[\text{Ca}^{2+}]$, is conveniently expressed using the molar ratio R_j , with $R_j = [\text{Ca}^{2+}]/C_p$, C_p being the molar concentration of the (charged) repeating units of pectate and subscripts “i” and “j” referring to

Scheme 2. Polyelectrolyte model^a

^aSchematic representation of a tract of junction between two pectate chains, assumed to present a 2_1 symmetry; each negative charge on each alternate galacturonate unit—pointing to the external side of the junction—keeps its structural integrity but for the formal reduction due to condensed counterions. Only the condensed calcium ions—in an amount larger than that expected on pure stoichiometric grounds, thanks to a favorable specific affinity for the polymer—are represented as yellow circles; sodium ions are not indicated to avoid overcrowding. The condensed ions are free to move within the condensation volume, V_p , depicted as a cylinder whose axis coincides with the axis of the chain dimer;^a they can leave V_p only if an equal amount of free counterions enters V_p . Some calcium ions are strongly bonded on specific sites in between the parallel poly(galacturonate) chains. Their long residence time on the binding site as well as their close intimacy with the charged groups pointing to the core of the junction produce a net annihilation of the charge of the complex [ideally depicted in the pink ellipse containing two uronate negative charges (black circles) and the 2+ charge of a calcium ion—red circle]. Free sodium and calcium ions (the latter depicted as light-blue circles) can move everywhere in the bulk of the solution, though excluded from the condensation volume, which contains the polymer, the condensed counterions, and the strongly bonded calcium ions. Representation of the shifted pectate chain has been reprinted with permission from ref 25 (Braccini, I.; Pérez, S. Molecular Basis of Ca^{2+} -Induced Gelation in Alginates and Pectins: The Egg-Box Model Revisited. *Biomacromolecules* 2001, 2 (4), 1089–1096. Copyright 2011). ACS Publications.

monovalent and divalent ions, respectively. θ increases with R_j with a Langmuir-type dependence (see Figure 2a), which is the replotting of Figure 1 of ref 26, with a clear monomodal behavior, at variance with the bimodal behavior reported for the parallel case of alginate.¹⁷ A detailed description of the fitting of the CD data of Figure 2a is given in Section 2 of the Supporting Information. To facilitate model interpretation and to focus on the process of junction formation, in the present analysis, θ will often be used as the independent variable instead of R_j , given the known dependence of θ on R_j .

With reference to Scheme 2, it is useful to clarify the aspects related to the definition of “bound” (divalent) counterions depending on the experimental techniques used; the frame of the present discussion is that of a system in which divalent counterions (e.g., Ca^{2+}) strongly bind to a negatively charged polymer (e.g., a polyuronate) producing an (parallel) association of chain segments, within the junctions of supramolecular associations of macromolecules, eventually leading to gel formation.

CD is sensitive to perturbations of the environment of the (chiral) transition of the chemical group involved in intimate bonding: it is probably the “method-of-choice” for determining the fraction of (occupiable) sites occupied by chemically bonded counterions, θ .

In an equilibrium dialysis experiment, the fraction of all nondiffusible divalent counterions, r^{osm} , is determined; it corresponds to the sum of the strongly bonded counterions and of the territorially bound counterions. Using the notation of ref 26, it amounts to

$$\begin{aligned} r^{\text{osm}} &= (1 - \theta) \cdot r_C + \theta \cdot (1 - z_j \cdot \sigma^0) \cdot r_D + \sigma^0 \cdot \theta \\ &= C + D + \sigma_{\text{tot}} = P^{\text{cond}} + \sigma_{\text{tot}} \end{aligned} \quad (1)$$

where r_C is the fraction of divalent counterions condensed on the (single) chain fraction of the polymer repeating units ($1 - \theta$), r_D is the fraction of divalent counterions condensed on the dimer of the chain fraction of the polymer repeating units θ , z_j is the charge of the divalent counterion, and C and D are the symbols used in ref 26; P is the upper case Greek letter rho. All fractions refer to the total number of charged repeating units. In eq 1, as well as in panels a of Figure 1 and b of Figure 2, the subscript “j” was omitted in the notation of r_C , r_D , and $(r_{\text{cond}})^{\text{tot}}$ pertaining to calcium ions for simplicity. The variable θ is always without subscript “j”, having assumed that only divalent counterions can give rise to chemical bonding.

Erroneously but not infrequently, the reduction of the single-ion activity coefficient of, for example, calcium ion is entirely attributed to binding, thus overestimating the number of bound counterions

$$f^{\text{bound}} \equiv \left(1 - \frac{(\gamma_{\text{Ca}})_{\text{pol}}}{(\gamma_{\text{Ca}})_{\text{ref}}} \right) \quad (2)$$

being $((\gamma_{\text{Ca}})_{\text{pol}}/(\gamma_{\text{Ca}})_{\text{ref}})$ the relative single-ion activity coefficient in the presence of a polyelectrolyte over that in the absence of a polymer (e.g., in CaCl_2 solutions, as the reference). Instead, the correct expression reads

$$\begin{aligned} \frac{a_{\text{Ca}^{2+}}^{\text{pol}}}{a_{\text{Ca}^{2+}}^{\text{ref}}} &= \frac{(\gamma_{\text{Ca}})_{\text{pol}}}{(\gamma_{\text{Ca}})_{\text{ref}}} = \left(\frac{(\gamma_{\text{Ca}})_{\text{pol}}}{(\gamma_{\text{Ca}})_{\text{ref}}} \right)^{\text{free}} \cdot \frac{[\text{Ca}^{2+}]^{\text{free}}}{[\text{Ca}^{2+}]^{\text{total}}} \\ &+ \left(\frac{(\gamma_{\text{Ca}})_{\text{pol}}}{(\gamma_{\text{Ca}})_{\text{ref}}} \right)^{r^{\text{osm}}} \cdot \frac{[\text{Ca}]^{r^{\text{osm}}}}{[\text{Ca}^{2+}]^{\text{total}}} = \left(\frac{(\gamma_{\text{Ca}})_{\text{pol}}}{(\gamma_{\text{Ca}})_{\text{ref}}} \right)^{\text{free}} \\ &\cdot (1 - r^{\text{osm}}), \left(\frac{(\gamma_{\text{Ca}})_{\text{pol}}}{(\gamma_{\text{Ca}})_{\text{ref}}} \right) = \left(\frac{(\gamma_{\text{Ca}})_{\text{pol}}}{(\gamma_{\text{Ca}})_{\text{ref}}} \right)^{\text{free}} \cdot (1 - r^{\text{osm}}) \end{aligned} \quad (3)$$

being $[(\gamma_{\text{Ca}})_{\text{pol}}/(\gamma_{\text{Ca}})_{\text{ref}}]^{r^{\text{osm}}} = 0$ and $[\text{Ca}^{2+}]^{\text{free}}/[\text{Ca}^{2+}]^{\text{total}} = 1 - [\text{Ca}]^{r^{\text{osm}}}/[\text{Ca}^{2+}]^{\text{total}} = [\text{Ca}^{2+}]^{\text{total}} \cdot (1 - r^{\text{osm}})$. The erroneous assumption often is $((\gamma_{\text{Ca}})_{\text{pol}}/(\gamma_{\text{Ca}})_{\text{ref}})^{\text{free}} \equiv 1$, then leading to the wrong result of eq 2. On the contrary, given the strong electrostatic field around the polyelectrolyte, the activity of “free” counterions will be strongly reduced with respect to that of the polymer-free solution, thus being $[(\gamma_{\text{Ca}})_{\text{pol}}/(\gamma_{\text{Ca}})_{\text{ref}}]^{\text{free}} < 1$.^{1–3} Use of eq 2 clearly overestimates the fraction of bound counterions.

It is of interest to analyze the R_j profile of r^{osm} , $(1 - \theta) \cdot r_C$ (P^C), $\theta \cdot r_D$ (P^D), and σ_{tot} (data in panel b of Figure 2, replotted from Figure 4 of ref 26). All fractions are given per mole of the polymer repeating unit. Panel b of Figure 2 indicates that in

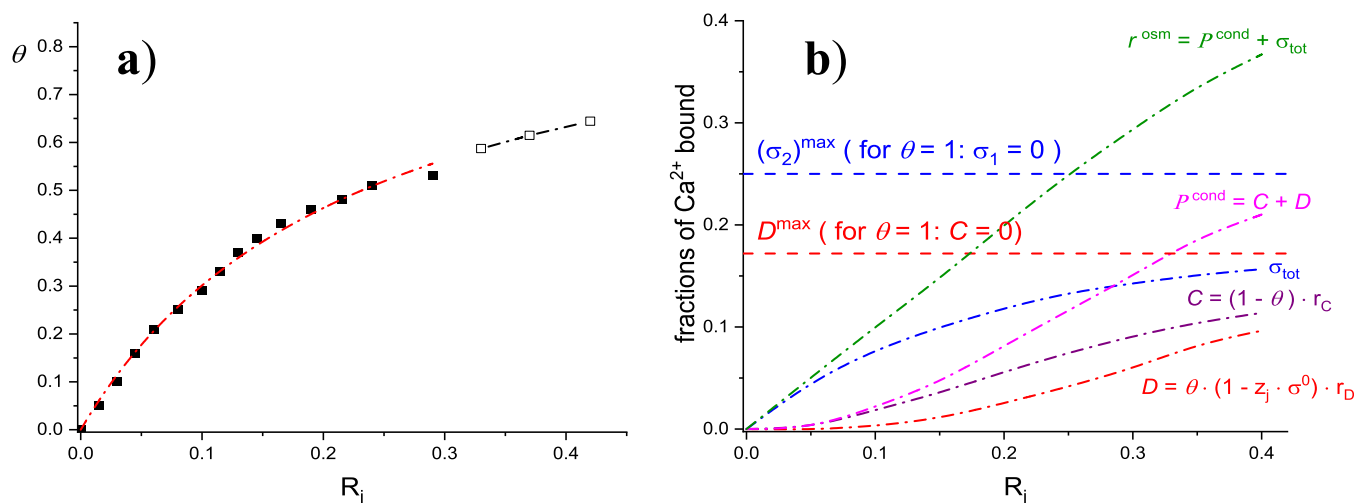


Figure 2. (a) Dependence of the fraction of bonded sites over the total number of sites able to chemically bond calcium ions, θ , on the molar ratio of added calcium over the polymer repeating unit, R_j , for pectate as determined by CD data. Replotting of the results of Figure 1 of ref 26. The red line is the best fit using the Langmuir equation; details are given in Section 2 of the Supporting Information. The same fitting curve was used to calculate the θ values corresponding to the R_j values investigated in the microcalorimetric experiments (filled square and dotted black curve). (b) Dependence on R_j of the various fractions of bound calcium ions to pectate; for definitions, see text.

the investigated range of R_j which corresponds to the initial formation of the calcium-pectate complexes, r^{osm} and σ_{tot} show a linear and a Langmuir-type of increase, respectively, the former being essentially dominated by the latter for a significant part of the initial range of R_j . In fact, up to $R_j = 0.25$, σ_{tot} represents more than 50% of the total amount of osmotically inactive calcium counterions (σ_{tot} and P^{cond} equalize at $R_j \approx 0.29$) and even >80% from $R_j = 0$ to $R_j = 0.1$. The total fraction of condensed divalent counterions, P^{cond} , [i.e., $(1 - \theta) \cdot r_C + \theta \cdot r_D$] starts to contribute significantly to r^{osm} only for $R_j > \approx 0.1$ to grow then very rapidly. The “C” component is always larger than the “D” component in the investigated range of R_j , but an inversion is expected to take place at larger values of R_j . This is reflected by the two indicated asymptotes; in fact, for $R_j \rightarrow \infty$ the following limits hold: $\lim_{R_j \rightarrow \infty} \theta(R_j) = 1$, $\lim_{R_j \rightarrow \infty} \sigma_{tot}(R_j) = \sigma^0 = 0.25$, and

$\lim_{R_j \rightarrow \infty} [(1 - \theta) \cdot r_C + \theta \cdot (1 - z_j \cdot \sigma^0) \cdot r_D] = (r_D)_j^{max} = 1/2 \cdot (1/2 \cdot (1 - 1/2 \cdot \xi_D)) = 0.17$, that is, the theoretical value for the pure divalent salt form of the polymer (in an infinite excess of divalent salt), taking into account the charge annihilation. ξ_D is the dimensionless linear charge density parameter of the “D” polymer stretches, given by: $\xi_D = l_b/b$, where l_b is the Bjerrum length (l_b is equal to 7.135 Å for water at 298.15 K) and b is the average value of the distance between the projections of the polycarboxylate charges on the chain axis [for poly(galacturonate) in the 2_1 helical conformation $b = 4.43$ Å and $\xi_D = 1.61$].²⁶

3.3. Isothermal Calorimetry, Dilatometry, and Determination of the Thermodynamic Data of Bonding. The approach chosen in ref 26 made use of (i) the two sets of experimental data from CD and microcalorimetry as a function of R_j , namely, the values of $\theta(R_j)$ and of the total enthalpy change of interaction (“mixing”) calcium ions and sodium pectate, $\Delta \bar{H}^{total}(R_j)$, respectively; (ii) the equation for the total free energy of “mixing” based on the formalism of the CC theory of polyelectrolytes,^{1–3} as modified to include effects stemming from both “specific affinity” (aff) interactions between the territorially bound calcium counterions (con-

densed) and the carbohydrate polyelectrolyte⁶ and from chemical bonding (bond), besides the purely electrostatic contributions of polyelectrolyte nature (el);⁷ and, finally, (iii) minimization of the total free energy with respect to the fitting parameters $\Delta \bar{G}^{aff}$ and $\Delta \bar{H}^{aff}$, namely, the free energy and enthalpy of specific affinity, respectively. It should be stressed that the concept of “affinity” as herewith applied does not pertain to the isolated, theoretically modeled interaction of a counterion of one type with the polyion⁴⁷ but, rather, to the enhanced selectivity in mixed counterion systems, as revealed by a wealth of different experimental techniques and treated theoretically in ref 6. It amounts to assuming Na^+ -pectate as the “reference” state as to the specific affinity interactions. Details are given in the Supporting Information. Keeping this distinction in mind, all the convincing conclusions of the quoted paper by Perić *et al.*⁴⁷ seem to be the best description of the territorially bound calcium counterions as defined in the present work. The use of the “integral” (or cumulative) values of the enthalpy of mixing, after proper correction for the dilution effects of both calcium salt and the polyelectrolyte, has been for long time the traditional way of plotting microcalorimetric data;⁴⁸ the same treatment has been used for the volume of mixing as to dilatometric data.⁴⁹ It is especially convenient in the case of experiments dealing with (bio)-polymeric systems, in which cooperative effects are more the rule than the exception, therefore providing the unbiased calorimetric evolution profile as a function of the chosen independent variable (e.g., the concentration of the added counterion), avoiding approaches based on a priori oversimplifying data-treatment routines. The microcalorimetric data from Figure 4 of ref 22 (which had been used in Figure 5 of ref 26) have been replotted in Figure 3 below. The data points show the same sigmoid behavior as that of the Ca^{2+} /alginate system at the same temperature, ionic strength, and nature of the ionic species.²² Incidentally, a very similar behavior is also apparent from the data of Figure 5 of ref 28 for the calorimetric data of the interaction of calcium with two alginate samples with different monomer compositions and two pectins with different methoxyl contents, as well as from those of Figure 2 of ref 39 for the isothermal titration

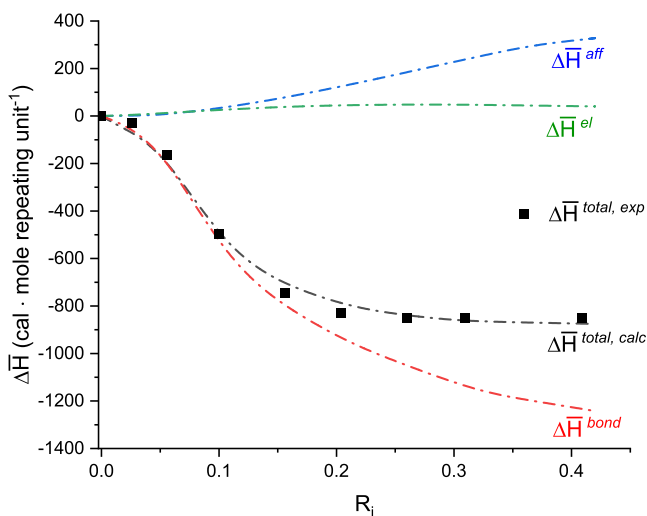


Figure 3. Dependence on R_j of the different components to the total enthalpy of mixing, $\Delta\bar{H}^{\text{total,calc}}$, according to the procedure of ref 26: affinity, $\Delta\bar{H}^{\text{aff}}$ (blue), electrostatic, $\Delta\bar{H}^{\text{el}}$ (green), and bonding, $\Delta\bar{H}^{\text{bond}}$ (red); all plots have been plotted as dash-dotted curves. The experimentally determined enthalpic values, $\Delta\bar{H}^{\text{total,exp}}$, have been represented as black squares. All experiments were carried out in aqueous NaClO_4 at 25 °C.

calorimetry study of the binding of Ca^{2+} and Zn^{2+} by LMP. More in general, the behavior of calcium/pectate is strongly reminiscent of that of galactans undergoing a conformational transition by the addition of gelling monovalent ions: for example, K^+ with ι -carrageenan⁵⁰ and Cs^+ with κ -carrageenan.⁵¹

The result of the process indicated above from (i) to (iii) allows deriving the thermodynamic parameters of specific affinity (i.e., $\Delta\bar{G}^{\text{aff}}$ and $\Delta\bar{H}^{\text{aff}}$, which were both assumed to be independent of R_j based on previous evidence⁶) and of chemical bonding (i.e., $\Delta\bar{G}^{\text{bond}}(R_j)$ and $\Delta\bar{H}^{\text{bond}}(R_j)$), which will be altogether discussed in the following paragraph. Calculations also provide the values of the fractions of condensed counterions, both mono- and divalent, for the two separate

cases considered of the dimer of chains, D (sandwiching the chemically bonded calcium ions), and for the (single) chain, C, connecting the stretches of repeating units in the D conformation; they have been reported in Figure 2b. Finally, the procedure also returned the values of the (molar) volumes of condensation for the C and the D conformations, $\bar{V}_C^p(R_j)$ and $\bar{V}_D^p(R_j)$, respectively.

The availability of the latter parameters from the fitting of the calorimetric data made it simpler the subsequent fitting of the dilatometric data, also performed as a function of R_j . Extensive literature data demonstrate that counterion–polyion interactions always bring about significant volume changes, which, almost always, are positive and can be traced back to desolvation of the charged interacting species. Linderstrøm-Lang (“Carlsberg”) dilatometers were used to measure volume changes following the procedure of Begala and Straus.⁴⁹ All measurements were carried out at 25 °C, with the bath temperature held constant to ± 0.001 °C. The above procedure allowed determining the volume changes of affinity, $\Delta\bar{V}^{\text{aff}}$, and of chemical bonding, $\Delta\bar{V}^{\text{bond}}(R_j)$, according to the previously described treatment.⁵¹

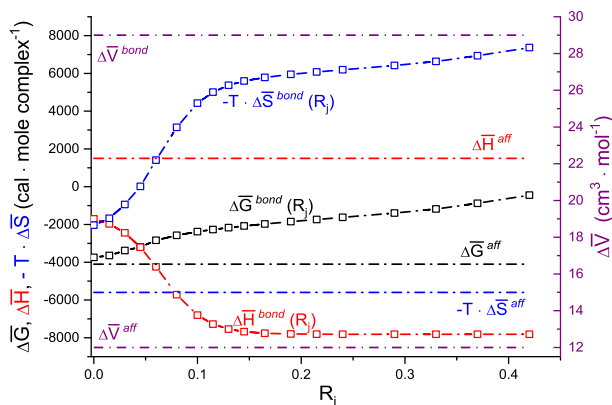
The affinity (aff) and bonding (bond) thermodynamic parameters are intensive properties pertaining to the bound counterion (“complex”), either in an affinity-modulated territorial binding (which can be regarded as a “transfer” from the “free” phase to the “condensed” phase) or in a strong chemical bonding, respectively

$$\Delta\bar{H}^{\text{total}}(R_j) = \Delta\bar{H}^{\text{el}}(R_j) + (r_{\text{cond}})^{\text{tot}}(R_j) \cdot \Delta\bar{H}^{\text{aff}} + \sigma^0 \cdot \theta(R_j) \cdot \Delta\bar{H}^{\text{bond}}(R_j) \quad (4)$$

$$\Delta\bar{G}^{\text{total}}(R_j) = \Delta\bar{G}^{\text{el}}(R_j) + (r_{\text{cond}})^{\text{tot}}(R_j) \cdot \Delta\bar{G}^{\text{aff}} + \sigma^0 \cdot \theta(R_j) \cdot \Delta\bar{G}^{\text{bond}}(R_j) \quad (5)$$

$$\Delta\bar{S}^{\text{total}}(R_j) = \Delta\bar{S}^{\text{el}}(R_j) + (r_{\text{cond}})^{\text{tot}}(R_j) \cdot \Delta\bar{S}^{\text{aff}} + \sigma^0 \cdot \theta(R_j) \cdot \Delta\bar{S}^{\text{bond}}(R_j) \quad (6)$$

a)



b)

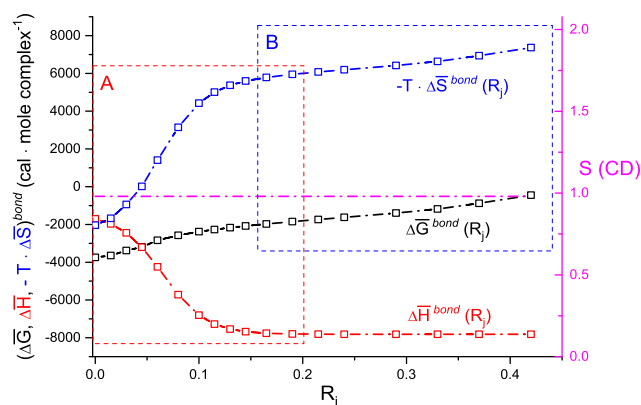


Figure 4. (a) Dependence of the thermodynamic functions of the bonding process (per mol of the bonded Ca^{2+} ion) on the molar ratio of added calcium, R_j ; for definition, see text. (b) Dependence (dash-dotted curves and symbols) on R_j of the fractional variation of the molar ellipticity, $S(\text{CD})$, (r.h.s. scale, magenta) and of $\Delta\bar{G}^{\text{bond}}$ (black), $\Delta\bar{H}^{\text{bond}}$ (red), and $-T \cdot \Delta\bar{S}^{\text{bond}}$ (blue) with the identification of two domains: (A) R_j range in which the variation of the bonding functions is sigmoid (red dashed rectangle) and (B) R_j range in which the variation of the bonding functions $\Delta\bar{G}^{\text{bond}}$ and $-T \cdot \Delta\bar{S}^{\text{bond}}$ is monotonously increasing, whereas $\Delta\bar{H}^{\text{bond}}$ is constant (blue dashed rectangle).

$$\Delta\bar{V}^{\text{total}}(R_j) = \Delta\bar{V}^{\text{el}}(R_j) + (r_{\text{cond}})^{\text{tot}}(R_j) \cdot \Delta\bar{V}^{\text{aff}} + \sigma^0 \cdot \theta(R_j) \cdot \Delta\bar{V}^{\text{bond}}(R_j) \quad (7)$$

The result of the fitting procedures of the calorimetric data, reported as a dash-dotted black curve in Figure 3 ($\Delta\bar{H}^{\text{total,calc}}$) through the experimental data (black squares: $\Delta\bar{H}^{\text{total,exp}}$) as the replotting of Figure 5 of ref 26, is really very encouraging. The results of the calculations pertaining to the different thermodynamic parameters are reported in Figure 4, panel a (for entropy: as $-T \cdot \Delta\bar{S}^{\text{bond}}(R_j)$). From the first glance, it appears that a very good fitting can be obtained assuming that all thermodynamic parameters pertaining to the affinity process are independent of R_j ; likewise, both the aff and bond volume change parameters are independent of R_j . The situation is quite different for the enthalpy and entropy bonding parameters: whereas $\Delta\bar{H}^{\text{bond}}(R_j)$ is independent of R_j for $R_j > 0.2$ and for $R_j < 0.2$ it does depend on R_j , both $\Delta\bar{G}^{\text{bond}}(R_j)$ and $\Delta\bar{S}^{\text{bond}}(R_j)$ depend on R_j over the whole investigated range of R_j . To focus on the peculiar features of these varying values, the thermodynamic data of bonding have been replotted in panel b of Figure 4. Therein, two specific domains have been identified: domain A, in which $\Delta\bar{G}^{\text{bond}}(R_j)$, $\Delta\bar{H}^{\text{bond}}(R_j)$, and $-T \cdot \Delta\bar{S}^{\text{bond}}(R_j)$ all show a peculiar sigmoid trend, and domain B, in which $\Delta\bar{H}^{\text{bond}}(R_j)$ is constant, whereas both $\Delta\bar{G}^{\text{bond}}(R_j)$ and $-T \cdot \Delta\bar{S}^{\text{bond}}(R_j)$ show a monotonic increase with R_j . Panel b also reports the curve of the absolute value of the fractional variation of the molar ellipticity, $S(\text{CD})$, as a function of R_j

$$S(\text{CD})(R_j) = \left| \frac{[\theta](R_j) - [\theta]_{R_j=0}}{[\theta]_{R_j=0}} \right| = \Delta B_{[\theta]} \cdot \theta(R_j) \quad (8)$$

where $[\theta]_{R_j}$ and $[\theta]_{R_j=0}$ are the molar ellipticity as a function of R_j and at $R_j = 0$, respectively, and $\Delta B_{[\theta]}$ is the (absolute) fractional change of molar ellipticity of the elementary conformational process. The absolute value enables one to focus on the extent of the variation only: in the UV region, the values of the molar ellipticity of polyuronates can show either positive or negative signs, and the change upon calcium addition can be, in turn, positive or negative, producing a complex variety of combinations.

Interestingly, two intensive parameters pertaining to the bonding process are independent of R_j over its total investigated range (see panel a), namely, $\Delta\bar{V}^{\text{bond}}$ and $S(\text{CD})$. It means that the desolvation effects accompanying the bonding of calcium by pectate are localized at the bonding sites, not involving neighboring groups and the rest of the chain. This was already observed for the (intrinsic) volume change of protonation of pectate, which is independent of the degree of dissociation, α , of the weak polyacid (*i.e.*, the charge density), and did not show any extra value related to the intramolecular conformational transition brought about by the addition of the H^+ ion.²⁹ Correspondingly, the constant value of $S(\text{CD})$ (see panel b) means that the change of conformational ordering produced by the strong binding of the Ca^{2+} ion to sodium pectate is the same throughout all the investigated ranges of R_j . This is at variance with the case of alginate, which shows a clear bimodal behavior of $S(\text{CD})$ with R_j ; in the range of R_j corresponding to the proposed “tilted egg-box” bonding mechanism, the value of $S(\text{CD})$ of alginate amounts to about the half of that for the “perfect egg-box” bonding.¹⁷

In the following, it was decided to analyze, sequentially, the behavior of the thermodynamic functions of bonding in domain A and then in domain B, as identified in Figure 4b.

3.4. Sigmoid Behavior of the Thermodynamic Parameters of Bonding. Two Modes of Bonding. As already pointed out, the values of the free energy, enthalpy, and entropy of chemical bonding of calcium by pectate significantly depend on the independent system variable R_j and hence on θ (see domain A), which for (bio)macromolecules (and for polyelectrolytes in particular) is more the rule than the exception. For the first strongly bonded Ca^{2+} ions (*i.e.*, at $R_j \rightarrow 0$), the free energy change is $(\Delta\bar{G}^{\text{bond}})_{R_j=0} = -3.74$ kcal (mole complex or bonded calcium ion)⁻¹, built up by favorable contribution of both enthalpy: $(\Delta\bar{H}^{\text{bond}})_{R_j=0} = -1.70$ kcal (mole complex or bonded calcium ion)⁻¹ and entropy: $(\Delta\bar{S}^{\text{bond}})_{R_j=0} = +6.8$ cal (mole complex or bonded calcium ion)⁻¹ K⁻¹.²⁶ $(\Delta\bar{G}^{\text{bond}})_{R_j=0}$ is very close to (albeit lower than) the corresponding affinity term: $\Delta\bar{G}^{\text{aff}} = -4.1 \pm 0.3$ kcal (mol of the condensed calcium ion)⁻¹ and then rapidly increases toward less favorable values on increasing R_j . The enthalpy change of chemical bonding (an intensive property) is always negative: in the range $0 < R_j < \sim 0.2$, it decreases in a sigmoid fashion to reach a constant value after that value of R_j : $(\Delta\bar{H}^{\text{bond}})_{R_j>0.2} \equiv \Delta\bar{H}_{\text{MAX}}^{\text{bond}} = -7.82$ kcal (mole complex or bonded calcium ion)⁻¹.

This behavior of $\Delta\bar{H}^{\text{bond}}$ might resemble that of a (cooperative) transition^{24,51} between two different conformational states taking place upon the increase of the system variable R_j ; however, this interpretation would be incorrect. In a study of the proton dissociation process of weak polyacids, it was demonstrated that a sigmoid variation of the intensive thermodynamic property (in that case, $\text{p}K_a$) with the system variable (in that case, α) might be incorrectly attributed to the presence of a pH-induced conformational transition. On the contrary, it was shown that the overall value of $\text{p}K_a$ showed such a sigmoid shape due to the presence of two—different—intensive properties (*e.g.*, $\text{p}K_a^1$ and $\text{p}K_a^2$), if the calculation treated the system ignoring this duality.⁵²

Much in the same way, the sigmoid profile of $\Delta\bar{H}^{\text{bond}}(R_j)$ can be attributed to the weighted sum of two contributions

$$\begin{aligned} \Delta\bar{H}^{\text{bond}}(R_j) &= f^{\text{initial}}(R_j) \cdot \Delta\bar{H}_{R_j=0}^{\text{bond}} + f^{\text{final}}(R_j) \cdot \Delta\bar{H}_{\text{MAX}}^{\text{bond}} \\ &= f_1(R_j) \cdot \Delta\bar{H}_1 + f_2(R_j) \cdot \Delta\bar{H}_2 \end{aligned} \quad (9)$$

where the subscripts “1” and “2” refer to two different processes of bonding that will be referred to as type 1 (or “1”) and type 2 (or “2”), respectively. The corresponding values are $\Delta\bar{H}_1 \equiv \Delta\bar{H}_{R_j=0}^{\text{bond}} = -1.70$ kcal·(mole calcium bonded)⁻¹ and $\Delta\bar{H}_2 \equiv \Delta\bar{H}_{\text{MAX}}^{\text{bond}} = -7.82$ kcal·(mole calcium bonded)⁻¹.

It holds

$$f_1(R_j) = \frac{n_1}{n_1 + n_2} = 1 - f_2(R_j) = 1 - \frac{n_2}{n_1 + n_2} \quad (10)$$

being n_1 and n_2 the number (bonded) sites of type “1” and “2”, respectively, (both functions of R_j) and

$$n_1 + n_2 = n_{\text{bond,tot}} \quad (11)$$

with $n_{\text{bond,tot}}$ the number of total sites that have bonded calcium ions in the “egg-box” structure, that is, $f_1(R_j)$ and $f_2(R_j)$ are

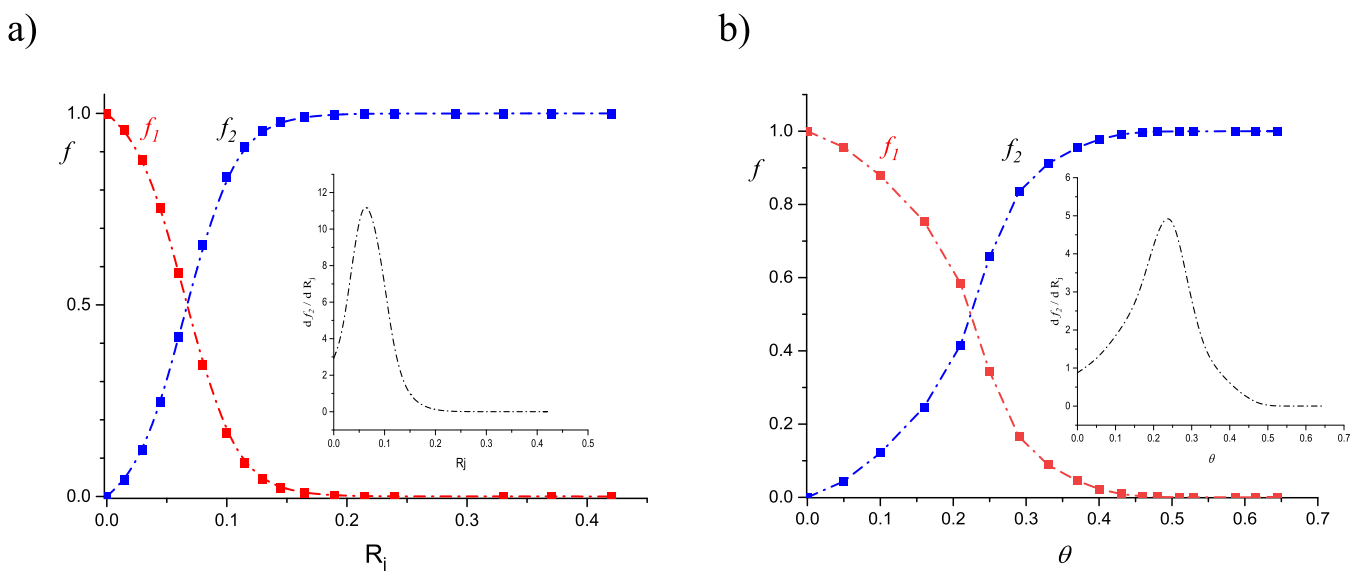


Figure 5. Dependence of the fraction of the bonded site of type 1, f_1 (red squares) and of the fraction of bonded site of type 2, f_2 (blue squares), on the molar ratio of added calcium/polymer, R_j , (l.h.s., a) and on the fraction of occupied bonding sites, θ (r.h.s., b). Dashed-dotted curves in both panels have been drawn to guide the eye. The inset panels represent the first derivative of f_2 with respect to R_j (l.h.s., a) and of θ (r.h.s., b), respectively, to highlight the “rounded” character of the phase transition from bonding mode “1” to “2”.¹⁰

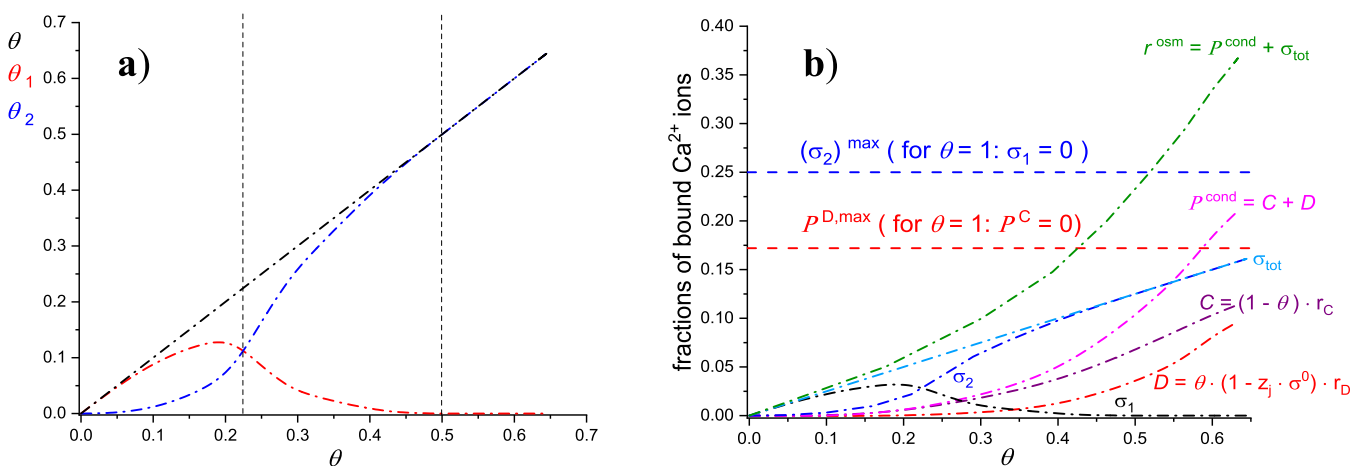


Figure 6. (a) Dependence of $\theta_1(\theta)$ (red full circles and dash-dotted curve) and $\theta_2(\theta)$ (blue full-squares and dash-dotted curve), as well of the total calcium bonded, θ (black open circles and dash-dotted curve), on the degree of bonding, θ . (b) Dependence on θ of the various fractions of bound calcium ions to pectate; for definitions, see text.

normalized not to the whole chain but only to the part strongly bonding calcium.

Then, at any value of the variable R_j , the degree of attainment of, for example, the second mode of bonding, $f_2(R_j)$, can be calculated as the fractional change (ratio) of $\Delta\bar{H}^{\text{bond}}(R_j)$ with respect to the maximum value of this function

$$f_2(R_j) \equiv (\Delta\bar{H}^{\text{bond}}(R_j) - \Delta\bar{H}_{R_j=0}^{\text{bond}}) / (\Delta\bar{H}_{\text{MAX}}^{\text{bond}} - \Delta\bar{H}_{R_j=0}^{\text{bond}}) \quad (12)$$

The dependence of f_1 and f_2 on R_j and on the degree of conversion (or the fraction of occupied bonding sites), θ , is reported in Figure 5a,b, respectively.

Figure 5 shows that for θ values larger than about 0.5 (i.e., $R_j > 0.2$), all bonded divalent ions will be present in type-2 complexes. Altogether, the two panels of Figure 5 can be looked upon as describing a “rounded transition”, that is, a true phase transition from a dilute linker phase to a dense linker

phase.¹⁰ Obviously, a “perfect” first-order transition would show a discontinuity at the “exact” transition point.

It is straightforward to calculate the fractions of occupied type-2 sites, $\theta_2(\theta)$, and that of “initial” or type-1 sites, $\theta_1(\theta)$, using the total fraction θ of occupied bonding sites, as provided by the CD experiments (see Figure 2a)

$$\theta_2(\theta) = f_2(\theta) \cdot \theta = f_2(\theta) \cdot (\sigma_{\text{tot}}(\theta) / \sigma^0) = \sigma_2(\theta) / \sigma^0 \quad (13)$$

and

$$\begin{aligned} \theta_1(\theta) &= f_1(\theta) \cdot \theta = \theta - \theta_2(\theta) = f_1(\theta) \cdot (\sigma_{\text{tot}}(\theta) / \sigma^0) \\ &= \sigma_1(\theta) / \sigma^0 \end{aligned} \quad (14)$$

respectively. $\sigma_{\text{tot}}(\theta)$, $\sigma_1(\theta)$, and $\sigma_2(\theta)$ are the fractions of bonded sites over the total number of bonding sites per polymer repeating unit and the corresponding fractions for type-1 and type-2 sites.

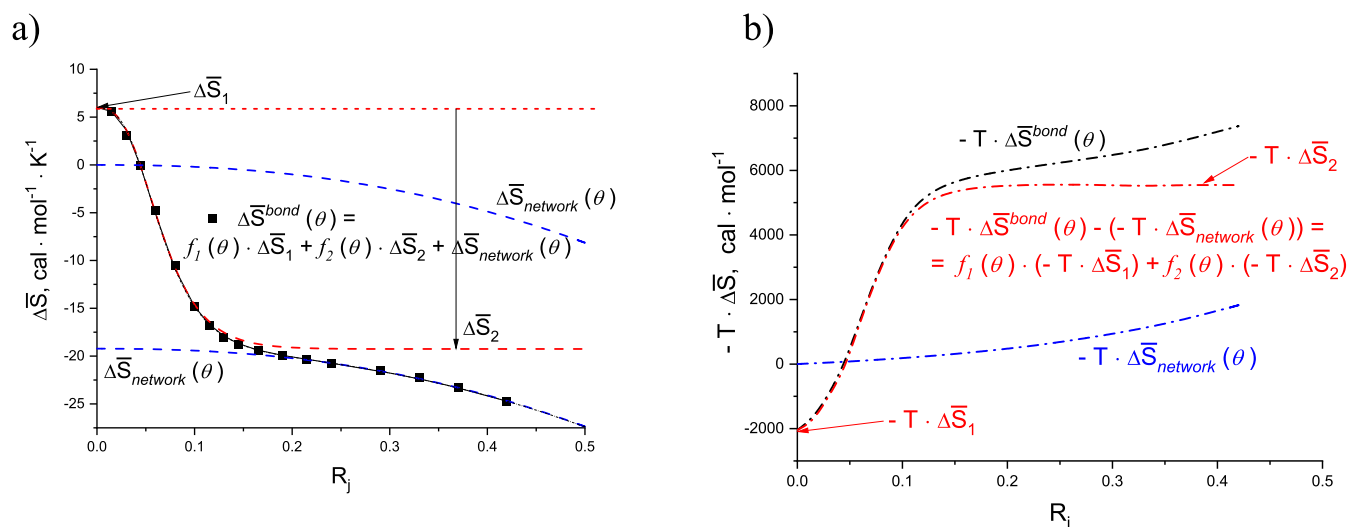


Figure 7. Dependence of $\Delta\bar{S}^{\text{bond}}$ and of its $\Delta\bar{S}_1$, $\Delta\bar{S}_2$, and $\Delta\bar{S}_{\text{network}}$ components (a) and of the corresponding free energy contributions, as $-T \cdot \Delta\bar{S}$, (b) on the molar ratio of calcium-to-polymer, R_j .

The results (full symbols) are reported in Figure 6a. It can be easily seen that at the beginning of the binding process, most (or even all) calcium ions distribute in structures of type 1, showing a maximum for $\theta \approx 0.2$ and then rapidly decreasing. The two fractions equalize at $\theta = 0.225$. Conversely, the fraction of coverage of type-2 sites shows a sigmoid increase with θ to merge with the diagonal (*i.e.*, $\theta_1(\theta) \approx 0$ and then $\theta_2(\theta) \approx \theta$) at around $\theta = 0.5$. In Figure 6a, the position of these two significant values of θ has been marked with vertical segments. They could mark the transition between the dilute to the crossover regime and from the crossover to the dense regime, respectively, according to the treatment by Borukhov *et al.*¹⁰ The data of Figure 2b have been replotted as a function of θ in Figure 6b, together with those derived from Figure 6a, with the definition given in eq 1. It tells us that strong bonding is dominant up to about $\theta \approx 0.225$, in particular being the majority of bonding according to type 1 up to $\theta \approx 0.15$. It is to note that r^{osm} grows with a power larger than 1 upon increasing θ due to a strong increase of the fraction of territorially bound ("condensed") calcium ions whose number equalize that of the strongly bonded counterions at $\theta \approx 0.55$. The theoretical limits indicate that for full bonding ($\theta = 1$), the amount of chemically bonded calcium counterions will exceed that of the condensed ones by about 38%, in all cases onto type-2 sites, due to the disappearance of the condensed counterions on single chain.

All in all, the result of the interpretation of the calorimetric data with the approach of the CC theory, modified to include specific affinity and chemical bonding, has allowed revealing the presence of two types of strong bonding of calcium by pectate, with a very high similarity to the case of alginate. The results of CD alone failed to provide such a result.

3.5. Residual Variation of Entropy and Free Energy of Bonding. After having consistently explained the sigmoid behavior of the enthalpy of bonding as a function of R_j , the task at hand is to analyze the source of variation of the entropy of Ca^{2+} ion bonding, $\Delta\bar{S}^{\text{bond}}(R_j)$, as $-T \cdot \Delta\bar{S}^{\text{bond}}(R_j)$, and of the related curve of free energy, $\Delta\bar{G}^{\text{bond}}(R_j)$. The shape of the curve (see Figures 4 and 7) is different from that of $\Delta\bar{H}^{\text{bond}}(R_j)$, since, after an initially similar sigmoid shape, at high values of R_j , there is no leveling off of the curve but, rather, $\Delta\bar{S}^{\text{bond}}(R_j)$ starts decreasing more and more.

This evidence induces to postulate that in the case of the entropy function, two trends are present: the former paralleling that of enthalpy, likely stemming from a common molecular root, and the latter, peculiar of $\Delta\bar{S}^{\text{bond}}(R_j)$ only, manifesting a process which becomes increasingly more important upon increasing levels of calcium binding. In the following, it will then be assumed that the observed decrease of $\Delta\bar{S}^{\text{bond}}(R_j)$ on increasing R_j stems from the sum of two negative contributions: a sigmoid trend mirroring that of $\Delta\bar{H}^{\text{bond}}(R_j)$ and an additional contribution which depends on R_j with a monotonously decreasing function (see Figure 7a).

$\Delta\bar{S}^{\text{bond}}(R_j)$ will then be factorized into three terms: much like the case of $\Delta\bar{H}^{\text{bond}}$, treated in eq 9, into (i) a term pertaining to type-1 bonding, $\Delta\bar{S}_1$, with $\Delta\bar{S}_1 \equiv \Delta\bar{S}_{R_j=0}^{\text{bond}}$, which also in this case is considered as the common, underlying process of the initial, single Ca^{2+} ion, bonding (type-1) and into (ii) a second term stemming from type-2 mode of binding ($\Delta\bar{S}_2$). In addition, and specifically for the entropic component only, (iii) a third term which is related to the build-up of the extensive three-dimensional network

$$\Delta\bar{S}^{\text{bond}}(R_j) = f'_1(R_j) \cdot \Delta\bar{S}_1 + f'_2(R_j) \cdot \Delta\bar{S}_2 + \Delta\bar{S}_{\text{network}}(R_j) \quad (15)$$

$\Delta\bar{S}_2$ is supposed to stem from the same, short-range, molecular processes as $\Delta\bar{H}_2$, being modulated by a similar factor $f'_2(R_j)$ to follow a similar sigmoid behavior; $\Delta\bar{S}_{\text{network}}(R_j)$ (Figure 7a) is the R_j -dependent entropy decrease whose origin is fully attributed to whole-chain, long-range interactions producing an extended network due to increasing chain association.

The following procedure has been used to resolve the $\Delta\bar{S}^{\text{bond}}(R_j)$ curve into its components. The assumption that $f'_2(R_j) \cdot \Delta\bar{S}_2$ parallels the R_j -dependence of $f_2(R_j) \cdot \Delta\bar{H}_2$ allows assuming that for $R_j > 0.2$ also the value of $f'_2(R_j)$ is constant, equal to 1, thereafter ascribing all the R_j -dependence of $\Delta\bar{S}^{\text{bond},0}(R_j)$ to $\Delta\bar{S}_{\text{network}}(R_j)$. A polynomial fitting of that part of the curve allowed determining the whole R_j -dependence of $\Delta\bar{S}_{\text{network}}(R_j)$. The difference between the $\Delta\bar{S}^{\text{bond}}(R_j)$ curve and that of $\Delta\bar{S}_{\text{network}}(R_j)$ not only trivially provided the value of $\Delta\bar{S}_2$ but also, excitingly, returned a profile of $f'_2(R_j)$ perfectly superimposable with that ($f_2(R_j)$) calculated from the calorimetric curve (see Figure 5a).

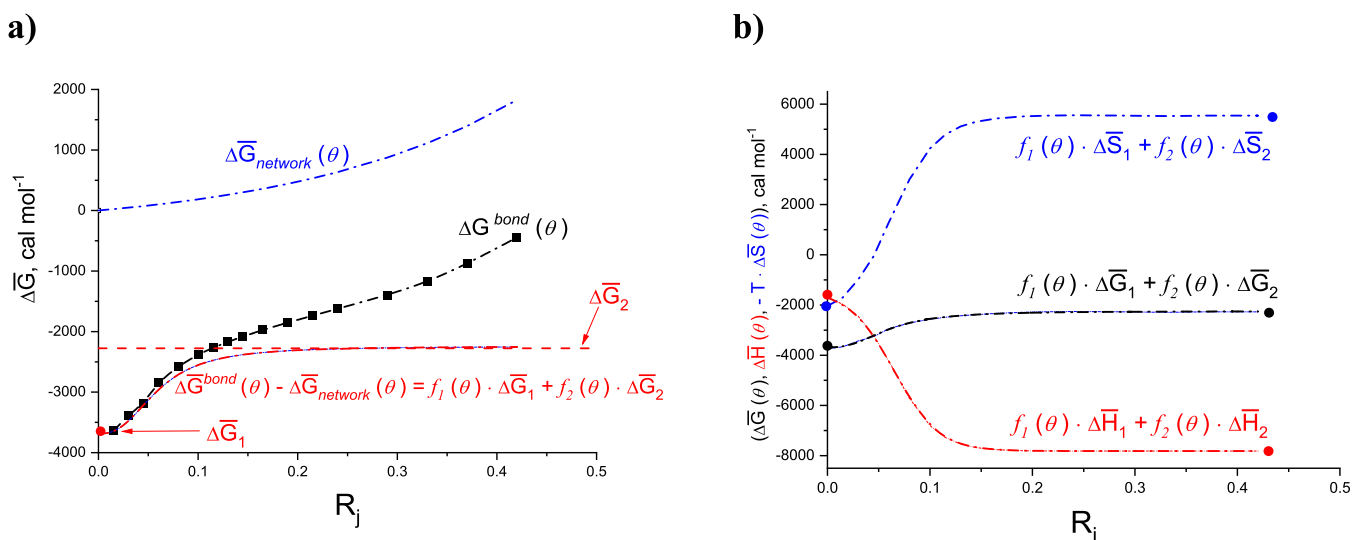


Figure 8. (a) Dependence of $\Delta\bar{G}^{bond}$ and of its $\Delta\bar{G}_1$, $\Delta\bar{G}_2$, and $\Delta\bar{G}_{network}$ components and (b) dependence of the thermodynamic parameters for the first and for the second mode of bonding weighted for the corresponding $f_1(R_j)$ and $f_2(R_j)$ fractions on the molar ratio of calcium-to-polymer, R_j .

Table 1. Thermodynamic Parameters of the Fundamental Short-Range Processes (i.e., Type 1 and Type 2) Accompanying Calcium Bonding and Network Formation by Sodium Pectate

TD param. Interaction	$\Delta\bar{G}^a$	$\Delta\bar{H}^a$	$T \cdot \Delta\bar{S}^a$	$\Delta\bar{S}^b$	$\Delta\bar{V}^b$	n_w^c	T_m	K	log(K)
	cal·(mol complex) ⁻¹	cal·(mol complex) ⁻¹	cal·(mol complex) ⁻¹ ·K ⁻¹	cal·(mol complex) ⁻¹ ·K ⁻¹	cm ³ ·(mol complex) ⁻¹	mol of released water·(mol complex) ⁻¹	K	e ^{-ΔḠ/R·T}	
affinity	-41 ₃₅	15 ₀₀	-56 ₃₅	18 ₉	12	6 ₇	79 ± 7	1074	3.03
type 1, "1"	-37 ₄₄	-17 ₀₄	-20 ₄₀	6 ₈	29	16 ₁	n.a.	555	2.74
type 2, "2"	-22 ₇₆	-78 ₁₇	55 ₄₁	-18 ₆	29	16 ₁	421 ± 25	47	1.67
"2"-"1"	14 ₆₉	-61 ₁₃	75 ₈₁	-25 ₄	0	0	240 ± 11	0.084	-1.08

^aThe probable error is ± 300 cal·(mol complex)⁻¹ for $\Delta\bar{G}$, ± 100 cal·(mol complex)⁻¹ for $\Delta\bar{H}$, and ± 320 cal·(mol complex)⁻¹ K⁻¹ for $-T \cdot \Delta\bar{S}$. ^bThe probable error is ± 1.1 cal·(mol complex)⁻¹·K⁻¹ for $\Delta\bar{S}$ and ± 0.5 cm³·(mol complex)⁻¹ for $\Delta\bar{V}$. ^cCalculated according to eqs 16 and 17.

It could be hard to imagine a better demonstration of the common origin of the enthalpic ($\Delta\bar{H}_1$ and $\Delta\bar{H}_2$) and of the corresponding entropic parameters ($\Delta\bar{S}_1$ and $\Delta\bar{S}_2$). The result is graphically reported in Figure 7a; the fitting parameters were $\Delta\bar{S}_1 = 6.8 \pm 0.3$ cal (mol calcium bound)⁻¹ K⁻¹ and $\Delta\bar{S}_2 = -18.6 \pm 0.3$ cal (mol calcium bound)⁻¹ K⁻¹. At $T = 298.15$ K, these values, combined with the corresponding enthalpic terms, give $\Delta\bar{G}_1 = -3.74$ kcal·(mol calcium bound)⁻¹ and $\Delta\bar{G}_2 = -2.28$ kcal·(mol calcium bound)⁻¹. The contributions to the Gibbs free energy of bonding, stemming from the different components, are reported in Figure 8a. The R_j -dependence of free energy, enthalpy, and entropy (as $-T \cdot \Delta\bar{S}$) of the type-1 and type-2 modes of the bonding (modulated by the $f_2(R_j)$ profile) has been reported in Figure 8b. All the results pertaining to the short-range interactions are summarized in Table 1.

The results of the fitting shed a new light on the role of strong chemical bonding in Ca²⁺-pectate association, leading to gelation: this is a process characterized, at the level of short-range interactions, by two molecular events, "1" and "2", both accompanied by favorable $\Delta\bar{H}$ changes, about 5 times more for type 2 with respect to type 1.

However, the main difference pertains to the entropy change: whereas it is moderate, but favorable, for type 1, it becomes very negative for type 2, determining a net endergonic difference, as $-T \cdot (\Delta\bar{S}_2 - \Delta\bar{S}_1)$, of about +7.5

kcal/mole complex. The ensuing Gibbs free-energy change is such that at its full development at room temperature, it is reduced by about 40% from the favorable value brought about by the initial, single-ion event of Ca²⁺ bonding ("1") [namely, $\Delta\bar{G}_2 = -2.28$ vs $\Delta\bar{G}_1 = -3.74$ kcal·(mol calcium bound)⁻¹, respectively] while still making $\Delta\bar{G}^{bond}$ favorable (see Figure 8a,b). In other words, there is a cost to pay for substituting a bonded calcium ion of type 1 with one of type 2, which can be completely traced back to the very large, unfavorable, entropy loss. The contribution of $\Delta\bar{S}_2$ is even more unfavorable than that of $\Delta\bar{S}_{network}$ for the stability of the strong bonding. In fact, it is easy to quantify that at around $\theta > \approx 0.45$, the hampering effect of networking ($\Delta\bar{S}_{network}$) is not dominating the total entropy loss, accounting only for about 25% of the total loss, being $\Delta\bar{S}_2$ the dominant one.

Desolvation of the interacting species is very important for both the specific affinity-driven territorial binding and the strong chemical bonding, amounting to 12 cm³·(mole complex)⁻¹ for $\Delta\bar{V}^{aff}$ and to 29 cm³·(mole complex)⁻¹ for $\Delta\bar{V}^{bond}$, respectively. Begala and Strauss proposed to estimate the amount of water molecules, n_w , released from the condition of electrostriction around cations and polyanions, taking the value of 1.1 g·(cm³)⁻¹ as the density of electrostricted water, $\rho_w^{electrostricted}$ ⁴⁹

$$n_w = \frac{\Delta\bar{V}}{\Delta\bar{V}_w^{liberated}} \quad (16)$$

being

$$\begin{aligned}\Delta\bar{V}_W^{\text{liberated}} &= (\bar{M}_W/\rho_W^{\text{free}}) - (\bar{M}_W/\rho_W^{\text{electrostricted}}) \\ &= (18/1.0) - (18/1.1)\end{aligned}\quad (17)$$

and therefore, $\Delta\bar{V}_W^{\text{liberated}} = 1.8 \text{ cm}^3 \cdot (\text{mole released water})^{-1}$. The calculated values for the affinity and the bonding processes then will be equal to 6.7 and 16.1 (mole released water) \cdot (mole complex) $^{-1}$, respectively. Such quite large values can be considered as extremely important for the binding of calcium and the stability of the bonded complex. Large, positive values of volume change are always accompanied by large, positive variations of entropy due to the acquired freedom of the water molecules liberated from the electrostriction shells around the separate ionic components. At the same time, these processes are accompanied by an endothermic variation ($\Delta\bar{H} > 0$) stemming from the disruption of ion–dipole bonds, still the unfavorable enthalpic component being overwhelmed by the very negative value of $-T \cdot \Delta\bar{S}$. All in all, one should then expect that both the enthalpic and the entropic terms pertaining to bonding, for both type-1 and type-2 modes, should turn out to be even more negative, when corrected for the desolvation contributions. Among the possible contributions to such $\Delta\bar{H}$ and $\Delta\bar{S}$ values $\ll 0$ one could name a conformational transition of the poly(galacturonic) backbone, a strong coordination of calcium by the carboxylated OH-rich uronate repeating unit, and the formation of strong hydrogen bonding among the facing pectate chains. On more general grounds, the observed diversity in sign of the two entropic terms of the bonding modes “1” and “2” (at variance with the identity of the enthalpic ones) points to conclude that different contributions contribute to modulate the overall thermodynamic parameters of the two modes of calcium bonding by pectate. Based on purely hypothetical grounds, a tentative identification and separation of such different contributions to the calculated thermodynamic parameters are given in Section 3 of the Supporting Information part.

As an additional consideration on the topic of the thermodynamics of the short-range interactions, it was resorted to compare the logarithm of the association constant of calcium by the two opposing disaccharides in the type-2 complex (which is the dominating mode over a wide R_f range) with literature data pertaining to the bonding of calcium by dianionic chelators, namely, carboxylic acids at neutral pH,⁵³ in particular including hydroxy-carboxylic acids in analogy with the abundance of OH groups on pectate. (EXAFS results clearly demonstrated that the coordinated calcium ion in hydrated pectate gels is involved in six Ca–O bonds.)⁵⁴ The choice of dianions is consistent with the assumption of charge neutralization for the type-2 complex, together with the assumed 2_1 symmetry of the “egg-box” structure. In fact, only one carboxylate group per disaccharide is assumed to point to the inner part of the egg-box to coordinate calcium, in doing so making the inner core of the facing disaccharides at the chelation site a (hydroxylated) dianion. The result is very encouraging. The ($\log C_2$) values by Cannan and Kibrick range from 1.06 to 1.80 (see their Table VIII), perfectly encompassing the value of $\log K_{\text{ass}}$ for the “2” complex of calcium, namely, 1.67 (see Table 1). The corresponding value of the type-1 site ($\log C_2 = 2.74$) is larger, as expected for the absence of the unfavorable entropic contributions which characterize the type-2 mode of bonding.

All the above discussion has been focused on the analysis of the contribution of short-range interactions to bonding; still, this treatment surprisingly allowed identifying a long-range hindering effect, of totally entropic nature, which increasingly affects the calcium-bonding capacity of pectate. The curves of Figure 8 tell that both short-range ($\Delta\bar{G}_2 - \Delta\bar{G}_1$) and long-range interactions ($\Delta\bar{G}_{\text{network}}$) produce an increase of $\Delta\bar{G}^{\text{bond}}$ upon increasing R_f , thus making bonding increasingly less favored. In particular, bonding of type 1 should be always favored over type 2, being its association constant (K_1) about 12 times larger than K_2 . To resolve the puzzle, the correct approach needs considering all free-energy terms, that is, also the electrostatic ones and those related to the condensation of calcium counterions with the correlated specific affinity, to adequately describe the process of progressive calcium binding. In fact, any short-sighted view, limiting the attention to the short-range bonding interactions only, would erroneously predict that the fraction of $\theta_1(\theta)$ sites should be increasingly more favored over $\theta_2(\theta)$. However, the experiment demonstrates that the opposite is true: by increasing R_f , $\theta_1(\theta)$ decreases in favor of an increase of $\theta_2(\theta)$, that is, the system shifts toward the increasing presence of type-2 bonding. Considering all free-energy contributions to the system, properly weighted and hence normalized for the total concentration of galacturonate repeating units is very revealing; the result is shown in Figure 9 (which replots the

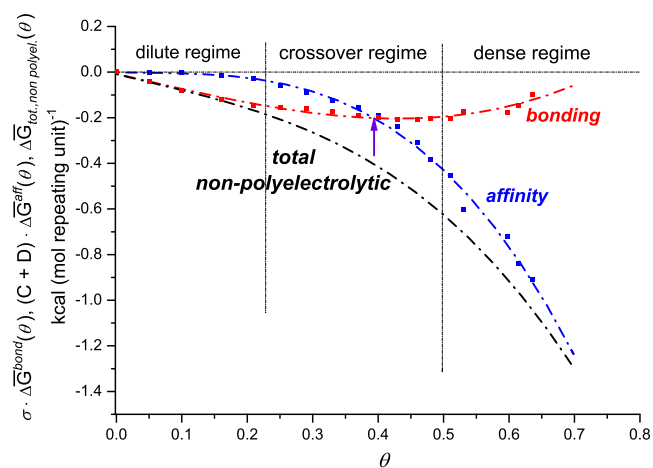


Figure 9. Total nonpolyelectrolytic free energy change as a function of the fraction of (total) bonded sites on pectate, θ (black curve), and its “bonding” (red curve) and “affinity” (blue curve) components.²⁶ “C” and “D” are the fractions of condensed calcium ions around the “single-chain” (C) polymer stretches connecting the chain dimers (D) held together by the “egg-box” structures, respectively. Values are extensive quantities, expressed in $\text{kcal} \cdot (\text{mol repeating unit})^{-1}$. The blue arrow marks the value of θ corresponding to isoenergetic (Gibbs) conditions between the “bonding” and the “affinity” components. The three indicated regimes follow the proposed partition from Figure 6a.

data of Figure 7 of ref 26). Three domains can be identified: the first one is in the range of θ from 0 to 0.225, in which the dominant driving force to binding is given by the free energy of chemical bonding. A crossover domain extends from $\theta = 0.225$ to $\theta = 0.5$, in which the contribution of territorial binding, modulated by a specific affinity of the condensed counterions for the polyelectrolyte, increases, equalizes that of bonding (at about $\theta = 0.4$), and then becomes even more important.

Finally, for $\theta > 0.4$, the contribution of bonding not only stops contributing favorably but even becomes deleterious to calcium binding. Meanwhile, the affinity term becomes the absolute key player in the increasing reduction of the free energy of calcium binding. This determining role of the affinity term is already underlined in ref 26 as “the significant contribution to the stabilization of the system arising from territorial condensation of calcium counterions”. Highlighting the role of the different contributions to the polyelectrolyte free energy of calcium pectate will be the matter of a forthcoming paper.

3.6. Relevance of the Equilibrium Thermodynamics Data for the Interpretation of the Rheological Behavior of Calcium-Pectate Gels. Starting with two papers by Blanshard and Mitchell in 1976,^{52,55} evidence accumulated over 20 years to make the idea progressively accepted that the major contribution to the elasticity of alginate and pectate gels formed with divalent counterions—and in particular with Ca^{2+} —was enthalpic.^{56–59} More specifically, it was agreed upon that the strength of the gels had to be traced back to the stability of the calcium-mediated interchain cross-links, the so-called “egg-box” sequences according to the definition by Rees and coworkers.^{15,16,20} Without totally dismissing the possibility of an entropic contribution to elasticity, the latter was confined to more flexible stretches connecting the cross-links, like in the case of mannuronic-rich segments in alginate. Within that time interval, two papers from our laboratory provided a strong evidence for the enthalpy-driven elasticity of gels of polyuronates with divalent ions. The signs of the temperature (T) dependence of the storage modulus buildup of gels made according to the four combinations of two different polymers (alginate and pectate) and two different divalent counterions (Ca^{2+} and Cu^{2+}) were exactly the same as those of the corresponding enthalpy changes accompanying the bonding of the divalent counterions by the polyuronates in dilute solution. The results are reported in Table 2. It should be underlined

Table 2. Comparison between the Signs of the Enthalpy Change upon Divalent Ion Binding from Calorimetric Measurements (“Calorimetry”) and of the Enthalpy Change upon Gel Junction Formation (upon Cooling) from Rheological Measurements (“Rheology”) ^a

	calcium		copper	
	calorimetry	rheology	calorimetry	rheology
alginate	– ₂₂	– ₆₀	+ ₆₁	+ ₆₀
pectate	– ₂₄	– ₆₂	– ₂₄	– ₆₀

^aReferences are given in each box.

that for determining the role of enthalpy in the strength of the elastic modulus (or in the rupture strength of gels, which is a different property), it is the sign that counts, inasmuch as already in 1980 Mitchell underlined the very wide range of numerical values reported in the literature (“from 5 to 65 kcal/mol”), due to the too many variables affecting the final numerical results.⁵⁶

The results of Table 2 are really significant inasmuch as they do not pertain to a single case, for which one might invoke serendipity, but to four different cases, which include the change of sign both upon changing ion (alginate: Ca^{2+} vs Cu^{2+}) and upon changing polymer (copper: alginate vs pectate). (The “anomalous” negative sign of the enthalpy change of

pectate with Cu^{2+} in comparison with possibly all known polycarboxylates—both natural and synthetic—will be addressed later on in the Conclusions section.) In an attempt at finding in the literature systematic rheological data on the calcium/pectate system to quantitatively compare the detailed thermodynamic data of Table 1, the paper by Lootens *et al.* turned out to be particularly interesting.⁶³ The authors “studied the influence of the calcium ion concentration, $[\text{Ca}^{2+}]$, and the pH on the storage (G') and loss (G'') shear modulus at 1 Hz of LMP solutions and gels. Upon lowering the temperature in the presence of Ca^{2+} , G' and G'' increase immediately, followed by a further slow logarithmic increase with time. The immediate response increases with increasing $[\text{Ca}^{2+}]$ and decreasing temperature. The “gel” temperature (T_g) where G' and G'' cross increases with increasing $[\text{Ca}^{2+}]$ (Figure 4). However, G' and G'' have a universal temperature dependence at all $[\text{Ca}^{2+}]$ if plotted as a function of $T - T_g$.⁶³ Both their G' and G'' curves (in Figure 3 of reference 63) were strongly reminiscent of the T -profile of the physical chemical properties of systems undergoing a conformational transition—in turn depending on the system conditions—such as specific counterions and/or ionic strength. For example, the T -profile of the optical activity of both κ -⁵¹ and ι -carrageenan⁶⁴ follows this behavior. Such systems are characterized by negative values of both enthalpy and entropy of conformational transition buildup, which implies the presence of a “melting” temperature, T_m : $T_m = \Delta\bar{H}/\Delta\bar{S}$. The dependence of the gel temperature (T_g) on the concentration of added calcium ions for their sample S1 resulting from the study of G'_0 and G''_0 was reported in Figure 4 of ref 63. The curve had the typical Langmuir-like behavior of a physical property depending on the concentration of a ligand (here: $[\text{Ca}^{2+}]$) associating/adsorbing on a lattice (here: LMP). It was decided to fit these data using the general Langmuir-Hill equation, which, as compared with the original Langmuir equation, can additionally reveal the presence of cooperativity

$$Y(x) = Y^{\max} \frac{x^n}{x^n + k^n} \quad (18)$$

For the present case, it reads

$$T_g([\text{Ca}^{2+}]) = T_g^{\max} \frac{[\text{Ca}^{2+}]^n}{[\text{Ca}^{2+}]^n + k^n} \quad (19)$$

For the case study, T_g^{\max} is the theoretical value of T_g for the complete saturation of the calcium-bonding sites. In the Langmuir-Hill equation, k is the elementary dissociation constant; it is possible to calculate the more familiar overall (or macroscopic) association constant, K , as $K = 1/(k^n)$.

The results are reported in Figure 10, showing the excellent fit of the rheology-derived T_g results ($R^2 = 0.99646$). The fitting parameters are $T_g^{\max} = 148 \pm 49$ °C, $n = 0.924 \pm 0.116$, and $k = 0.01056 \pm 0.0066$. The value of n indicates that the system is very slightly negatively cooperative but being close to 1 suggests that this effect is small indeed, if any. The result is $K = 67$, and then, $\Delta\bar{G}$ ($\Delta\bar{G} = -R \cdot T \cdot \ln(K)$) is $-24_{91} \pm 4_{01}$ cal·mol calcium⁻¹. The mode of calcium bonding which is considered the fundamental one in the cooperative structures holding two chains of pectate together (the “gel junction”) is obviously type 2, which is granted a thermodynamic value of $\Delta\bar{G}_2 = -22_{76} \pm 3_{00}$ cal mol calcium⁻¹ and, possibly more interesting, a value of $T_m = 421$ K, that is, 148 ± 25 °C (see Table 1). The latter value derives from the large negative values of both $\Delta\bar{H}$ and

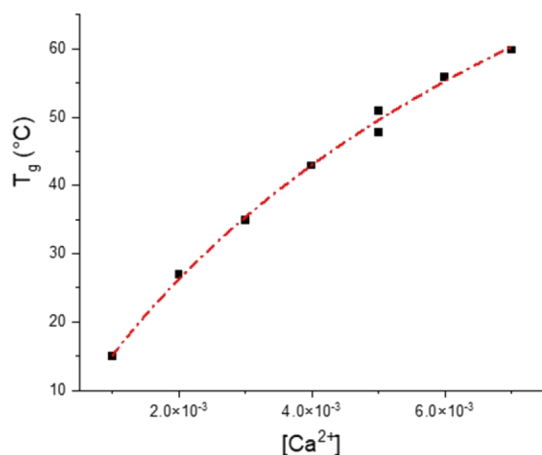


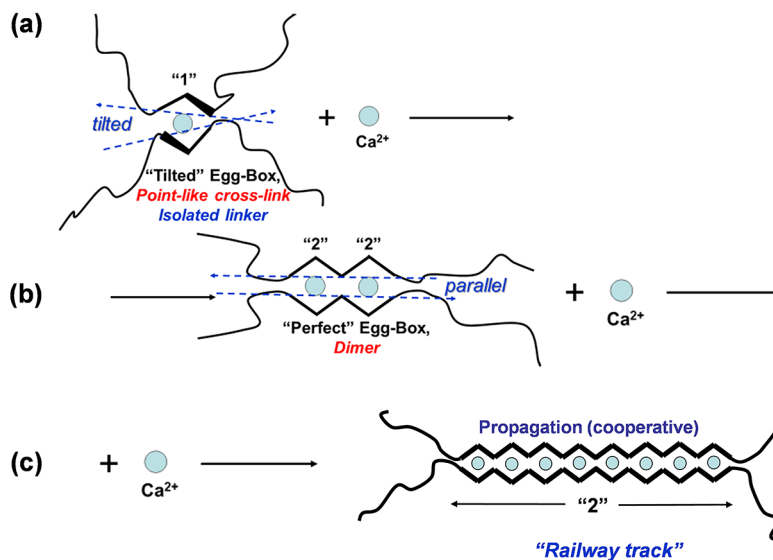
Figure 10. Dependence of the gel temperature on the Ca^{2+} concentration. T_g is defined as the temperature where $G'_0 = G''_0$. Data taken from Figure 4 of ref 63.

$\Delta\bar{S}$. The striking similarity of the values of both $\Delta\bar{G}$ and T_m from calorimetry and from rheology is a clear indication that the process of gel formation—followed monitoring the development of both G'_0 and G''_0 as a function of temperature for increasing concentrations of Ca^{2+} —finds its molecular basis in the formation of stretches of type-2 bonding sites. They are characterized by a large, favorable value of $\Delta\bar{H}_2$ ($\Delta\bar{H}_2 = -78_{17}$ cal·mol calcium⁻¹), whose sign had been identified as equal to

that of the rheological process already more than 30 years ago, a significant confirmatory evidence of the enthalpic nature of the elasticity of polyuronate gels.^{60,62}

3.7. Modeling of the Ca^{2+} Bonding by Pectate. Over several decades, various models have been proposed for the ionotropic or for the thermotropic gelation of polysaccharides on the basis of various experimental data (and often of molecular modeling), sometimes conflicting in their molecular details. One of the earliest schemes was the so-called EBM set forth by Grant *et al.*¹⁶ to describe the calcium-induced association of alginate chains, fundamental step—as the interchain link—in the ionotropic gelation of this algal polysaccharide. A few years later, the same authors proposed to extend the EBM to describe the (similar) association of pectate induced by Ca^{2+} , also invoking some conformational resemblance of the trans 1 → 4 diaxially linked α -L-guluronate in alginate with the trans 1 → 4 diaxially linked α -D-galacturonate in pectate, both responsible for strong calcium binding.²⁰ The EBM convincingly assumed that both polyuronates undergo a disorder-to-order conformational transition to accommodate calcium ions in between regular, facing 2_1 helical stretches. EBM gained a wide acceptance, albeit with some important modifications proposed for the poly(galacturonate) case, that is, the shifted EBM.²⁵ The effect on chain association and gelation of important structural details, such as the monomeric composition (*i.e.*, the mannuronate vs guluronate content in alginate and the degree of methyl esterification or amidation in pectins), monomer

Scheme 3. The "egg-box" model for calcium-pectate revisited^a



^a(a) Each bonding site ("Junction") is made of four galacturonate units, represented by a dotted or by a full black segment, and a Ca^{2+} ion (light blue circle); only four polymer repeating units are involved, with the formation of the initial bonding entity, type 1 or "1" (point-like cross-link,³⁹ "tilted egg-box",¹⁷ and isolated linker¹⁰), with a simultaneous conformational change. The disaccharides assume a tilted relative position, in order to maximize the distance of the four chain stretches originating from the junction to minimize the electrostatic repulsions: the "tilted egg-box". (b) If eight galacturonate units (four facing disaccharides) and two calcium "linkers" are involved, then over and above the initial bonding but with no additional conformational change there is further structure formation, with a nearest-neighbor effect (clusterization) and additional enthalpy and entropy changes: the second type of bonding entity, type 2 or "2" (dimer,³⁹ "perfect egg-box" structure,¹⁷ crossover situation from the "dilute" regime to the "dense" regime¹⁰). (c) Any further bonding of calcium ions will produce a cooperative elongation ("propagation") of the type-2 structures (formation of the—disordered—"railway rack"¹⁰). The segments depicting the disaccharide structures in both the type-1 structures and in the type-2 ones share the same increase of conformational ordering with respect to sodium pectate. The drawing, though, is unable to render the mutually shifted position of the two facing 2_1 helical stretches in the "shifted egg-box model"²⁵

distribution (*i.e.* block wise, alternating, or random), or the MW and the MW distribution, received particular attention. The study of the sequence of events accompanying the increasing addition of calcium received attention from a more limited number of investigations. Calorimetric and dilatometric studies were among those.^{24,45} Starting with alginate³⁸—and then extending the model to pectate²⁸—the already quoted (see *paragraph 3.1*) series of papers had the merit of systematically tackling the problem of the modes of sequential binding of calcium upon its increasing addition to the polyuronates. Among the relevant ones to the present case of calcium pectate, some papers proposed an initial step of formation of point-like cross-links³⁹ (step I), followed by the formation of dimers of parallel chains bridging the calcium ions according to the EBM (step II).^{39,40} In 2013, on the basis of converging evidence from different experimental techniques, the formation of a “tilted egg-box” structure as the first step of calcium binding was proposed, which did not differ much from the earlier proposed point-like cross-link.¹⁷ For larger values of R_1 , a subsequent dimerization according to a “perfect egg-box” pairing was proposed. A few years before and with no explicit reference to the case of the calcium-induced association of pectate but, rather, to the well investigated case of the polymerization of actin (F-actin), a model had set forth to describe the aggregation of two highly charged semiflexible polyelectrolytes in the presence of generalized linkers.¹⁰ The authors foresee “a rounded phase transition from a dilute linker gas where the chains form large loops between (single) linkers to a dense disordered linker fluid connecting parallel chains (the “railway track”). The onset of chain pairing occurs within the rounded transition”.¹⁰ Moreover, “at very large interlinker separations, the electrostatic repulsion dominates the bending energy and the crossing angles saturate to $\theta = 90^\circ$ ”.¹⁰ The latter point was actually at the root of the proposed tilted EBM.¹⁷

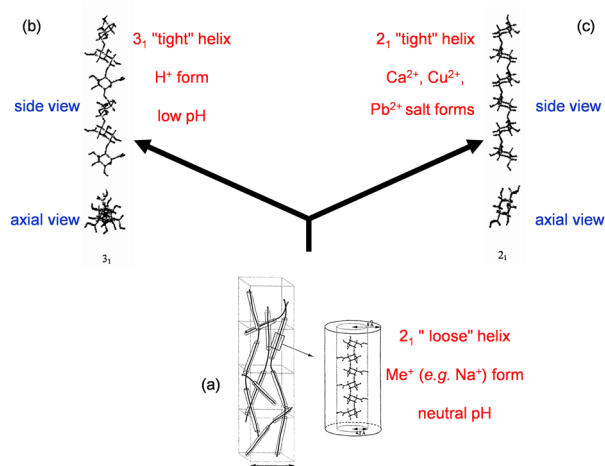
In our opinion, the three above-described models are fully compatible not only among them but also with the experimental evidence so far reported, in particular with those of the present paper. A schematic representation is given in *Scheme 3*, where the nomenclature of each of the three models has been properly attributed to type-1 and type-2 modes of bonding, as the result of the previous paragraphs. In a forthcoming paper, a further elaboration will be carried out on some details of the bonding process: from now on, the concepts (and even the wording) of the above-mentioned three models will be used interchangeably to highlight one or another aspect.

3.8. On the Conformations of Pectate in Aqueous Solution. In 1789, Antoine Lavoisier in collaboration with the mathematician Pierre Simon de La Place built the first calorimeter.⁶⁵ In spite of not being one of the most fashionable novel techniques, the great potential of calorimetry, coupled with a sound theoretical polyelectrolytic approach (CC), was once more demonstrated to be able to identify and describe chemical processes and interactions. The enthalpy data succeeded in providing evidence of a bimodal mode of bonding calcium by pectate, that the otherwise sensitive and accurate CD technique, in this particular case, failed to reveal. It should be strongly stressed that the method used for calculating the population fractions, that is, $\theta_1(\theta)$ and $\theta_2(\theta)$ (*Figure 6a*), of the present paper is based on the elaboration of the bimodal intensive enthalpic quantities (with the data from *Figures 4* and *5* and the use of *eqs 4–7*). The bimodal

(alginate)¹⁷ versus monomodal (pectate) CD behavior (see *Figure 2a*) highlights a profound difference among the two polyuronates. Alginate shows only a partial increase of chain ordering in the first bonding of calcium (“tilted egg-box”). The more stable type-2 mode characterizing the cooperative Ca^{2+} -linked interchain junctions is accompanied by a substantial further increase of molar ellipticity that tells us of a (only) finally achieved 2_1 ordered chain conformation. At variance, pectate seems to be more prone to immediate full chain ordering, even in the initial type-1 bonding mode. As a consequence, no difference among the two modes is detectable from the CD data and only microcalorimetry allows one to separate mode “1” from mode “2” on the basis of their (internal) energy characteristics. In this respect, it is very interesting to read the comment of the authors on the calorimetric behavior of the Cu^{2+} -pectate system: “in the case of mixing pectate with copper ions, the exothermic enthalpy of interaction is absolutely unique in this class of compounds. In fact, all known polyuronates,^{61,66} as well the monomeric galacturonate molecule,⁶⁷ have a positive enthalpy of interaction, irrespective of the stereochemistry of the sugar residue(s) and of the ability of gel formation. Most importantly, a positive enthalpy has been found also for the system copper-polyguluronate,⁶⁶ that is, the polymer which has a behavior very similar to polygalacturonate and differs from the latter only for the stereochemistry of C(3). It is therefore mandatory to ascribe the reported exothermic behavior of pectate with Ca^{2+} and Cu^{2+} ions to the very specific change of conformation of the polygalacturonate chain”.²⁴ Another anticipating sentence of that paper pertains to the apparent discrepancy between the evolution of the CD and of the calorimetric curves upon increasing R_1 : “the degree of linearity of the CD changes and the enthalpy changes for the Cu^{2+} titration are pretty similar, likely indicating a common origin for the two phenomena. In the case of Ca^{2+} , a slight difference in the dependence of the two functions on R is noticed. More information needs to be gained before a safe explanation can be inferred therefrom; for example, one might propose that the perturbation occurs at a local level only or that the progressive binding of calcium occurs onto an already ordered conformation of pectate induced by a very little amount of calcium ions”.²⁴ The present work (i) reconciles the apparent discrepancy recognizing to calorimetry a superior diagnostic ability with respect to CD in the case of pectate; however, at the same time, (ii) it suggests briefly revisiting the limiting chain conformations of pectate from a parallel reconsideration of the CD effects accompanying the exothermic interaction of pectate with both Cu^{2+} and Ca^{2+} (and with H^+ and Pb^{2+} as well).^{22,24} There is large convergence in accepting two (ideal) limiting ordered conformations for the poly(galacturonate) backbone: a 3_1 helix and a 2_1 helix.^{21,25,31} The former limiting form is usually attributed to that taken by pectate under acidic conditions [*i.e.*, that of poly(galacturonic acid)].^{21,25,29,31,68} The 2_1 helix is largely accepted as the conformation of (each of the two) pectate chains in the chelation of calcium in the so-called “egg-box” structure, confirmed also in the more recently proposed shifted EBM.²⁵ The conformation of sodium pectate (*i.e.*, under neutral pH conditions, *i.e.*, a salt form with a nongelling counterion) seems to deserve some comments. Ravanat and Rinaudo originally proposed the 2_1 helix also for Na^+ -pectate [“the data [...] lead us to conclude that two conformations exist: (1) a sodium or calcium form in a twofold screw conformation, with the binding of calcium being

stronger than that for sodium without a conformational change. (2) Another conformation for the acidic form which perhaps exhibits a threefold screw conformation.”²¹ However, this view was modified by the very anticipating sentence of ref 68: “the results suggest that three conformations exist, one in an acidic form, perhaps corresponding to a helix with a threefold symmetry, one in a sodium form, and another in a calcium form which may be reasonably described by a helix with a twofold symmetry.” The results of Figure 2a may be of help to resolve the issue. The result of the fitting indicates that the (absolute) fractional change of molar ellipticity of the elementary conformational process, $\Delta B_{[\theta]}$, is 0.98.²⁶ A similar fitting for *L.hyp.* sodium alginate on the data of Figure 1b of ref 17 for the total change returns the value of $\Delta B_{[\theta]} = 2.0$, namely, as twice as large as that of sodium pectate in Figure 2a. It is to be strongly stressed that $\Delta B_{[\theta]}$ values are the intrinsic values of the absolute value of change in molar ellipticity, unable to say anything on the initial or final values of $[\theta]$ nor, even more so, on the molecular conformations involved. Nevertheless, they can be even more useful as diagnostic tools for comparative purposes. Instead of focusing on the final conformation compatible with the CD data, it is useful to consider the possibility that the initial (average) conformation of Na⁺-alginate and Na⁺-pectate in solution is substantially different. The NMR work by Catoire *et al.*⁶⁹ provides the picture of sodium pectate for which “the optimum motional model could be described as anisotropic reorientation of twofold helical segments”, in full agreement with the statement by Pérez *et al.*⁷⁰ that “the HG macromolecule therefore tends to adopt a pseudohelical conformation even in its disordered state.” At this point, it seems reasonable to borrow from the work of Guenet *et al.* the concept of “loose-helix” to describe that of Na⁺-pectate. It was introduced to account for the small-angle neutron scattering curve obtained from the sol state of agarose in water at 70 °C:⁷¹ “a near-helical structure for which atomic position correlations vanish rapidly compared with those for a tight helix”.⁷² All in all, three limiting conformations could then be proposed to describe the behavior of pectate under different conditions (they have been sketched in Scheme 4): (i) a 3₁ “tight-helix” for pectic acid in acidic pH (whose expected rigidity is further corroborated by the negative value of the second virial coefficient, A_2 , at pH = 3.5, as reported in Section 2 of the Supporting Information part of this paper, at variance with the large and positive A_2 value at pH = 6.5); (ii) a 2₁ “tight-helix” as the molecular component of the “shifted egg-box” structure upon the chelation of various divalent ions, calcium ions being the most important from both a biological and an industrial standpoint; and (iii) a 2₁ “loose-helix” for the sodium salt form at neutral pH. The latter would then exhibit an already significant residual conformational ordering, at significant variance with alginate, so that upon the addition of calcium, the shift from “loose” to “tight” chain ordering would imply a variation of ellipticity of only 50% if compared to that of alginate. This explains very simply the significant “conformational ordering propensity” of sodium pectate,²⁴ which in the modeling work by Perić *et al.* was interpreted as “the possibility of forming an appropriate chelating environment (involving a local compensation of the counter-ion charges and thus the effective removal of interionic repulsions) by the association of a second polyuronate chain is likely to represent the leading driving force (in addition to presumably weaker direct chain–chain interactions) permitting chain stiffening, cation immobilization, and chain–chain association”.⁴⁷ As soon as a divalent

Scheme 4. Representations of the three proposed limiting conformations of the (single) poly(galacturonate) chain: (a) the “loose-2₁-helix”, (b) the “tight-3₁-helix”, and (c) the “tight-2₁-helix”^a



^aThe corresponding pictures have been taken with permission from ref 69 [for (a)] and from ref 73 [for (b) and (c)]; Scheme 4a: Reprinted from *Carbohydr. Res.*, **300** (1), Catoire, L.; Derouet, C.; Redon, A.-M.; Goldberg, R.; Hervédu Penhoat, C., An NMR Study of the Dynamic Single-Stranded Conformation of Sodium Pectate, **1997**, 19–29. Scheme 4b,c: Republished with Permission of Elsevier Science & Technology Journals from Conformational and Configurational Features of Acidic Polysaccharides and Their Interactions with Calcium Ions: A Molecular Modeling Investigation, Braccini, L.; Grasso, R. P.; Pérez, S. *Carbohydr. Res.* **1999**, **317** (1), 119–130; Permission Conveyed through Copyright Clearance Center, Inc.

cation with specific coordination ability (*i.e.*, “bonding”: Ca²⁺, Cu²⁺, and Pb²⁺—but neither Zn²⁺ nor Cd²⁺ which are more similar to Mg²⁺)²² condenses in close proximity of the “loose-2₁-helix” of Na⁺ pectate, each of the two poly(galacturonate) chains involved responds by easily acquiring the “tight-2₁-helix” conformation and forming a point-like cross-link (type-1 bonding, the “tilted egg-box”). Longer cooperative sequences made of neighboring bound ions (type-2 bonding) can thereafter be formed without any further increase of conformational ordering, having already acquired its maximum possible value (*i.e.*, the $\Delta B_{[\theta]}$ value associated to the type-1 to type-2 transition is zero). Only the associated enthalpy changes are different in both steps (while both being negative), becoming an excellent diagnostic tool to parallel CD or other conformation-sensitive techniques. Apparently, such a “conformational ordering propensity” is not shared neither by GG nor by MG dyads in sodium alginates.^{17,22}

4. CONCLUSIONS

The work described in this paper was inspired by the lines of investigation listed in the Introduction: the elaboration of the CC theory of polyelectrolytes,^{1–9,26} the theory by Borukhov *et al.* on the association of semiflexible polyelectrolytes,¹⁰ and the tilted EBM of initial binding of calcium by alginates.¹⁷ However, at the same time, it turned out to strengthen and correlate these theories and models, with a consistent interpretation of experimental results on the calcium/pectate system. All in all, the two-step model of calcium interaction with polyuronates, successfully put forth to explain the bimodal behavior of alginates,^{17,28} has found a solid, supporting

extension to the case of poly(galacturonate). This paper collectively treated experimental results obtained on the same sample but over different times and conditions. One limitation pertains to the different values of the ionic strength, I , at which some data were collected. In particular, both osmotic pressure and intrinsic viscosity experiments were carried out at $I = 0.1$ M for the obvious reason to reduce the Donnan effect as much as possible. Since the microcalorimetric, dilatometric, and CD experiments were carried out at $I = 0.05$ M, some bias—albeit a small one—cannot be excluded as to a perfect overlap of the compared properties when investigated as a function of added calcium ions (*i.e.*, vs R_j) or as a function of chemically bonded calcium ions (*i.e.*, θ or σ_{tot}). Ionic strength is a well-known fundamental parameter affecting the solution behavior of polyelectrolytes, and sodium pectate is no exception. However, sometimes it is practically impossible to cope with the requirements of simultaneously controlling ionic strength, concentration of specific monovalent and/or divalent ions, and the monovalent/divalent concentration ratio determining the local concentration of the territorially bound (condensed) ions. Moreover, the amount of experimental work enormously grows in parallel. As an example, the large number of experiments of intrinsic viscosity reported in Figure 1a had to be carried on both as a function of total calcium ion concentration and, for each value of r^{osm} , also as a function of polymer concentration (for the Huggins/Kraemer extrapolation). In addition, it should be recalled that for each point on the r^{osm} scale, dilution of the initial polymer solution had to be made with the outer compartment solvent in a dialysis equilibrium after equilibration to assure constant chemical potential of the diffusible species. Unfortunately, the lack of more data points at a larger value of r^{osm} , where the trend of $[\eta]$ is expected to increase again after the shrinkage at the transition from the dilute to the crossover regime, is likely to be attributed to the described laborious procedures.

In spite of the described (after all, small) limitations, the wide-angle view of the bonding process where the large number of different techniques provided rich of potential is still to be exploited. On the basis of these encouraging results, in a forthcoming paper, some in-depth understanding of these theoretical aspects will be presented, aiming at unveiling some important details of the response of the semiflexible galacturonate polyelectrolyte to the addition of the Ca^{2+} linkers, such as the cooperativity of the interactions and the size and number of the growing egg-box structures.¹⁰

On a wider perspective, the use of calorimetric results, shown to be extremely useful in this paper, will very likely produce also for alginates a wealth of information on this mode of interaction, once they will be elaborated according to the procedure herewith reported.

■ ASSOCIATED CONTENT


SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biomac.1c00958>.

Experimental details and more on thermodynamics of binding (PDF)

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Notes

The authors declare no competing financial interest.

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This paper is no. 4 of the series “Specific interactions versus counterion condensation”; paper nos. 1, 2, and 3 of the series are refs 6 7, and 26 in the present manuscript, respectively.

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■ ADDITIONAL NOTE

^aThis model^{1–3} was more recently confirmed for different polyuronates including poly(galacturonate) in the work by Perić *et al.*⁴⁷ “in all cases, the counter-ion atmosphere is almost entirely confined to a cylindrical region around the chain axis.”

■ REFERENCES

- (1) Manning, G. S. Limiting Laws and Counterion Condensation in Polyelectrolyte Solutions I. Colligative Properties. *J. Chem. Phys.* **1969**, *51*, 924–933.
- (2) Manning, G. S. Limiting Laws and Counterion Condensation in Polyelectrolyte Solutions: V. Further Development of the Chemical Model. *Biophys. Chem.* **1978**, *9*, 65–70.
- (3) Manning, G. S. Limiting Laws and Counterion Condensation in Polyelectrolyte Solutions II. Self-Diffusion of the Small Ions. *J. Chem. Phys.* **1969**, *51*, 934–938.
- (4) Benegas, J.; Paoletti, S.; Cesàro, A.; van den Hoop, M. A. G. T.; van Leeuwen, H. P. Limiting-Laws of Polyelectrolyte Solutions. Ionic Distribution in Mixed-Valency Counterions Systems. II. A Comparison of Conductometric Data and Theoretical Predictions. *Biophys. Chem.* **1992**, *42*, 297–303.
- (5) Paoletti, S.; Benegas, J.; Cesàro, A.; Manzini, G.; Fogolari, F.; Crescenzi, V. Limiting-Laws of Polyelectrolyte Solutions. Ionic Distribution in Mixed-Valency Counterions Systems. I: The Model. *Biophys. Chem.* **1991**, *41*, 73–80.
- (6) Donati, I.; Cesàro, A.; Paoletti, S. Specific Interactions versus Counterion Condensation. 1. Nongelling Ions/Polyuronate Systems. *Biomacromolecules* **2006**, *7*, 281–287.
- (7) Donati, I.; Benegas, J. C.; Cesàro, A.; Paoletti, S. Specific Interactions versus Counterion Condensation. 2. Theoretical Treatment within the Counterion Condensation Theory. *Biomacromolecules* **2006**, *7*, 1587–1596.
- (8) Porasso, R. D.; Benegas, J. C.; van den Hoop, M. A. G. T.; Paoletti, S. Chemical Bonding of Divalent Counterions to Linear Polyelectrolytes: Theoretical Treatment within the Counterion Condensation Theory. *Phys. Chem. Chem. Phys.* **2001**, *3*, 1057–1062.
- (9) Siew, C. K.; Williams, P. A.; Young, N. W. G. New Insights into the Mechanism of Gelation of Alginate and Pectin: Charge Annihilation and Reversal Mechanism. *Biomacromolecules* **2005**, *6*, 963–969.
- (10) Borukhov, I.; Lee, K.-C.; Bruinsma, R. F.; Gelbart, W. M.; Liu, A. J.; Stevens, M. J. Association of Two Semiflexible Polyelectrolytes

by Interchain Linkers: Theory and Simulations. *J. Chem. Phys.* **2002**, *117*, 462–480.

(11) Rinaudo, M.; Ravanat, G.; Vincendon, M. NMR Investigation on Oligo- and Poly(Galacturonic Acid)s; Gel Formation in the Presence of Ca^{2+} Counterions. *Makromol. Chem.* **1980**, *181*, 1059–1070.

(12) Kohn, R. Ion Binding on Polyuronates - Alginate and Pectin. *Pure Appl. Chem.* **1975**, *42*, 371–397.

(13) Kohn, R.; Luknár, O. Intermolecular Calcium Ion Binding on Polyuronates-Polygalacturonate and Polyguluronate. *Collect. Czech. Chem. Commun.* **1977**, *42*, 731–744.

(14) Kohn, R.; Luknár, O. Calcium and Strontium Ion Activity in Solutions of the Corresponding Pectinates and Its Dependence on Their Degree of Esterification. *Collect. Czech. Chem. Commun.* **1975**, *40*, 959–970.

(15) Rees, D. A. Polysaccharide Shapes and Their Interactions - Some Recent Advances. *Pure Appl. Chem.* **1981**, *53*, 1–14.

(16) Grant, G. T.; Morris, E. R.; Rees, D. A.; Smith, P. J. C.; Thom, D. Biological Interactions between Polysaccharides and Divalent Cations: The Egg-box Model. *FEBS Lett.* **1973**, *32*, 195–198.

(17) Borgogna, M.; Skjåk-Bræk, G.; Paoletti, S.; Donati, I. On the Initial Binding of Alginate by Calcium Ions. The Tilted Egg-Box Hypothesis. *J. Phys. Chem. B* **2013**, *117*, 7277–7282.

(18) Rombouts, F. M.; Thibault, J. F. Sugar Beet Pectins: Chemical Structure and Gelation through Oxidative Coupling. *Chemistry and Function of Pectins*; ACS Symposium Series; American Chemical Society, 1986; Vol. 310, pp 5–49.

(19) “The global pectin market is estimated to be valued at USD 1 billion in 2019 and is projected to reach USD 1.5 billion by 2025, recording a CAGR of 6.5%.” (<https://www.globenewswire.com/news-release/2019/09/30/1922332/0/en/Worldwide-Pectin-Market-Analysis-2019-2025-with-Ingredient-Cargill-DowDuPont-and-Kerry-Group-Dominating.html/#>).

(20) Morris, E. R.; Powell, D. A.; Gidley, M. J.; Rees, D. A. Conformations and Interactions of Pectins: I. Polymorphism between Gel and Solid States of Calcium Polygalacturonate. *J. Mol. Biol.* **1982**, *155*, 507–516.

(21) Ravanat, G.; Rinaudo, M. Investigation on Oligo- and Polygalacturonic Acids by Potentiometry and Circular Dichroism. *Biopolymers* **1980**, *19*, 2209–2222.

(22) Cesàro, A.; Delben, F.; Paoletti, S. Interaction of Divalent Cations with Polyuronates. *J. Chem. Soc., Faraday Trans. 1* **1988**, *84*, 2573–2584.

(23) Cesàro, A.; Delben, F.; Paoletti, S. Thermodynamic Properties of the Polyuronates- Ca^{2+} Ions Interaction in Aqueous Solution. *Proceedings of the Seventh International Conference on Thermal Analysis-Vol. II*; Miller, B., Ed.; Wiley Heyden Publication, 1992; pp 815–821.

(24) Paoletti, S.; Cesaro, A.; Delben, F.; Ciana, A. Ionic Effects on the Conformation, Equilibrium, Properties, and Rheology of Pectate in Aqueous Solutions and Gels. *Chemistry and Function of Pectins*; ACS Symposium Series; American Chemical Society, 1986; Vol. 310, pp 7–73.

(25) Braccini, I.; Pérez, S. Molecular Basis of Ca^{2+} -Induced Gelation in Alginates and Pectins: The Egg-Box Model Revisited. *Biomacromolecules* **2001**, *2*, 1089–1096.

(26) Donati, I.; Benegas, J. C.; Paoletti, S. Polyelectrolyte Study of the Calcium-Induced Chain Association of Pectate. *Biomacromolecules* **2006**, *7*, 3439–3447.

(27) Cataldi, T. R. I.; Margiotta, G.; Del Fiore, A.; Bufo, S. A. Ionic Content in Plant Extracts Determined by Ion Chromatography with Conductivity Detection. *Phytochem. Anal.* **2003**, *14*, 176–183.

(28) Fang, Y.; Al-Assaf, S.; Phillips, G. O.; Nishinari, K.; Funami, T.; Williams, P. A. Binding Behavior of Calcium to Polyuronates: Comparison of Pectin with Alginate. *Carbohydr. Polym.* **2008**, *72*, 334–341.

(29) Cesàro, A.; Ciana, A.; Delben, F.; Manzini, G.; Paoletti, S. Physicochemical Properties of Pectic Acid. I. Thermodynamic Evidence of a pH-Induced Conformational Transition in Aqueous Solution. *Biopolymers* **1982**, *21*, 431–449.

(30) Crescenzi, V.; Delben, F.; Paoletti, S.; Skerjanc, J. Thermodynamics of Polycarboxylate Aqueous Solutions. I. Dilatometry and Calorimetry of Protonation and Copper(II) Binding. *J. Phys. Chem.* **1974**, *78*, 607–611.

(31) Gilseman, P. M.; Richardson, R. K.; Morris, E. R. Thermally Reversible Acid-Induced Gelation of Low-Methoxy Pectin. *Carbohydr. Polym.* **2000**, *41*, 339–349.

(32) Morris, E. R.; Rees, D. A.; Sanderson, G. R.; Thom, D. Conformation and Circular Dichroism of Uronic Acid Residues in Glycosides and Polysaccharides. *J. Chem. Soc., Faraday Trans. 2* **1975**, *1418–1425*.

(33) Morris, E. R.; Rees, D. A.; Thom, D. Characterisation of Alginate Composition and Block-Structure by Circular Dichroism. *Carbohydr. Res.* **1980**, *81*, 305–314.

(34) Hecht, H.; Srebnik, S. Structural Characterization of Sodium Alginate and Calcium Alginate. *Biomacromolecules* **2016**, *17*, 2160–2167.

(35) Agulhon, P.; Markova, V.; Robitzer, M.; Quignard, F.; Mineva, T. Structure of Alginate Gels: Interaction of Diuronate Units with Divalent Cations from Density Functional Calculations. *Biomacromolecules* **2012**, *13*, 1899–1907.

(36) Urbanova, M.; Pavelkova, M.; Czernek, J.; Kubova, K.; Vyslouzil, J.; Pechova, A.; Molinkova, D.; Vyslouzil, J.; Vetchy, D.; Brus, J. Interaction Pathways and Structure–Chemical Transformations of Alginate Gels in Physiological Environments. *Biomacromolecules* **2019**, *20*, 4158–4170.

(37) Brus, J.; Urbanova, M.; Czernek, J.; Pavelkova, M.; Kubova, K.; Vyslouzil, J.; Abbrent, S.; Konefal, R.; Horský, J.; Vetchy, D.; Vyslouzil, J.; Kulich, P. Structure and Dynamics of Alginate Gels Cross-Linked by Polyvalent Ions Probed via Solid State NMR Spectroscopy. *Biomacromolecules* **2017**, *18*, 2478–2488.

(38) Fang, Y.; Al-Assaf, S.; Phillips, G. O.; Nishinari, K.; Funami, T.; Williams, P. A.; Li, L. Multiple Steps and Critical Behaviors of the Binding of Calcium to Alginate. *J. Phys. Chem. B* **2007**, *111*, 2456–2462.

(39) Assifaoui, A.; Lerbret, A.; Uyen, H. T. D.; Neiers, F.; Chambin, O.; Loupiac, C.; Cousin, F. Structural Behaviour Differences in Low Methoxy Pectin Solutions in the Presence of Divalent Cations (Ca^{2+} and Zn^{2+}): A Process Driven by the Binding Mechanism of the Cation with the Galacturonate Unit. *Soft Matter* **2015**, *11*, 551–560.

(40) Huynh, U. T. D.; Lerbret, A.; Neiers, F.; Chambin, O.; Assifaoui, A. Binding of Divalent Cations to Polygalacturonate: A Mechanism Driven by the Hydration Water. *J. Phys. Chem. B* **2016**, *120*, 1021–1032.

(41) Ventura, I.; Jammal, J.; Bianco-Peled, H. Insights into the Nanostructure of Low-Methoxyl Pectin–Calcium Gels. *Carbohydr. Polym.* **2013**, *97*, 650–658.

(42) Christensen, T.; Gooden, D. M.; Kung, J. E.; Toone, E. J. Additivity and the Physical Basis of Multivalency Effects: A Thermodynamic Investigation of the Calcium EDTA Interaction. *J. Am. Chem. Soc.* **2003**, *125*, 7357–7366.

(43) Thibault, J. F.; Rinaudo, M. Interactions of Mono- and Divalent Counterions with Alkali- and Enzyme-Deesterified Pectins in Salt-Free Solutions. *Biopolymers* **1985**, *24*, 2131–2143.

(44) Thibault, J. F.; Rinaudo, M. Chain Association of Pectic Molecules during Calcium-Induced Gelation. *Biopolymers* **1986**, *25*, 455–468.

(45) Gamini, A.; Civitarese, G.; Cesàro, A.; Delben, F.; Paoletti, S. Gelation Mechanism of Ionic Polysaccharides. *Makromol. Chem., Macromol. Symp.* **1990**, *39*, 143–154.

(46) Thibault, J.-F.; Rinaudo, M. Gelation of Pectinic Acids in the Presence of Calcium Counterions. *Br. Polym. J.* **1985**, *17*, 181–184.

(47) Perić, L.; Pereira, C. S.; Pérez, S.; Hünenberger, P. H. Conformation, Dynamics and Ion-Binding Properties of Single-Chain Polyuronates: A Molecular Dynamics Study. *Mol. Simul.* **2008**, *34*, 421–446.

(48) Crescenzi, V.; Delben, F.; Quadrioglio, F.; Dolar, D. Comparative Study of the Enthalpy of Ionization of Polycarboxylic Acids in Aqueous Solution. *J. Phys. Chem.* **1973**, *77*, 539–544.

- (49) Begala, A. J.; Strauss, U. P. Dilatometric Studies of Counterion Binding by Polycarboxylates. *J. Phys. Chem.* **1972**, *76*, 254–260.
- (50) Rochas, C. Calorimetric Study of Galactans. *Food Hydrocolloids* **1987**, *1*, 215–225.
- (51) Paoletti, S.; Delben, F.; Cesaro, A.; Grasdalen, H. Conformational Transition of κ -Carrageenan in Aqueous Solution. *Macromolecules* **1985**, *18*, 1834–1841.
- (52) Mitchell, J. R.; Blanshard, J. M. V. Rheological Properties of Pectate Gels. *J. Texture Stud.* **1976**, *7*, 341–351.
- (53) Cannan, R. K.; Kibrick, A. Complex Formation between Carboxylic Acids and Divalent Metal Cations. *J. Am. Chem. Soc.* **1938**, *60*, 2314–2320.
- (54) Alagna, L.; Prospero, T.; Tomlinson, A. A. G.; Rizzo, R. Extended X-Ray Absorption Fine Structure Investigation of Solid and Gel Forms of Calcium Poly(+ α -D-Galacturonate). *J. Phys. Chem.* **1986**, *90*, 6853–6857.
- (55) Mitchell, J. R.; Blanshard, J. M. V. Rheological Properties of Alginate Gels. *J. Texture Stud.* **1976**, *7*, 219–234.
- (56) Mitchell, J. R. The Rheology of Gels. *J. Texture Stud.* **1980**, *11*, 315–337.
- (57) Andresen, I.-L.; Smidsrød, O. Temperature Dependence of the Elastic Properties of Alginate Gels. *Carbohydr. Res.* **1977**, *58*, 271–279.
- (58) Moe, S. T.; Draget, K. I.; Skjåk-Bræk, G.; Simdsrød, O. Temperature Dependence of the Elastic Modulus of Alginate Gels. *Carbohydr. Polym.* **1992**, *19*, 279–284.
- (59) Clark, A. H.; Evans, K. T.; Farrer, D. B. Shear Modulus-Temperature Meltdown Profiles of Gelatin and Pectin Gels. A Cascade Theory Description. *Int. J. Biol. Macromol.* **1994**, *16*, 125–130.
- (60) Lapsin, R.; Paoletti, S.; Zanetti, F. *Rheological Investigation of the Formation of Ionic Polysaccharide Gels BT-Progress and Trends in Rheology II*; Giesekus, H., Hibberd, M. F., Eds.; Steinkopff: Heidelberg, 1988; pp 422–424.
- (61) Paoletti, S.; Cesaro, A.; Ciana, A.; Delben, F.; Manzini, G.; Crescenzi, V. Thermodynamics of Protonation and of Copper(II) Binding in Aqueous Alginate Solutions. *Solution Properties of Polysaccharides*; ACS Symposium Series; American Chemical Society, 1981; Vol. 150, pp 26–379.
- (62) Lapsin, R.; Priel, S.; Paoletti, S.; Zanetti, F. Novel Rheological Model for the Gelation Kinetics of Ionic Polysaccharides. *J. Appl. Polym. Sci.* **1990**, *41*, 1395–1410.
- (63) Lootens, D.; Capel, F.; Durand, D.; Nicolai, T.; Boulenger, P.; Langendorff, V. Influence of pH, Ca Concentration, Temperature and Amidation on the Gelation of Low Methoxyl Pectin. *Food Hydrocolloids* **2003**, *17*, 237–244.
- (64) Bongaerts, K.; Paoletti, S.; Deneff, B.; Vanneste, K.; Cuppo, F.; Reynaers, H. Light Scattering Investigation of *t*-Carrageenan Aqueous Solutions. Concentration Dependence of Association. *Macromolecules* **2000**, *33*, 8709–8719.
- (65) Meschel, S. V. A Brief History of Heat Measurements by Calorimetry with Emphasis on the Thermochemistry of Metallic and Metal-Nonmetal Compounds. *Calphad* **2020**, *68*, 101714.
- (66) Cesaro, A.; Paoletti, S.; Delben, F.; Crescenzi, V.; Rizzo, R.; Dentini, M. Thermodynamics of Ionic Saccharide Polymers in Aqueous Solution: The Calorimetric Approach. *Gazz. Chim. Ital.* **1982**, *112*, 115–121.
- (67) Aruga, R. Structure of the Galacturonate and Glucuronate Complexes with Copper(II) in Aqueous Solution. A Calorimetric Study. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 1233–1235.
- (68) Thibault, J. F.; Rinaudo, M. Interactions of Counterions with Pectins Studied by Potentiometry and Circular Dichroism. *Chemistry and Function of Pectins*; ACS Symposium Series; American Chemical Society, 1986; Vol. 310, pp 6–61.
- (69) Catoire, L.; Derouet, C.; Redon, A.-M.; Goldberg, R.; Hervédu Penhoat, C. An NMR Study of the Dynamic Single-Stranded Conformation of Sodium Pectate. *Carbohydr. Res.* **1997**, *300*, 19–29.
- (70) Pérez, S.; Mazeau, K.; Hervé du Penhoat, C. The Three-Dimensional Structures of the Pectic Polysaccharides. *Plant Physiol. Biochem.* **2000**, *38*, 37–55.
- (71) Paoletti, S.; Gamini, A.; Vetere, A.; Benegas, J. C. Metal Ion – Ionic Polysaccharide Interactions: Theoretical Developments and Experimental Results. *Macromol. Symp.* **2002**, *186*, 141–146.
- (72) Guenet, J. Factors Influencing Gelation versus Crystallization in Cooling Polymer Solutions. *Trends Polym. Sci.* **1996**, *4*, 6–11.
- (73) Braccini, I.; Grasso, R. P.; Pérez, S. Conformational and Configurational Features of Acidic Polysaccharides and Their Interactions with Calcium Ions: A Molecular Modeling Investigation. *Carbohydr. Res.* **1999**, *317*, 119–130.