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Structural studies on polygalacturonate gels: an EXAFS investigation combined with molecular modelling

M. Bosco[a], S. Miertus[b], S. Turchini[c], T. Prosperi[c], I. Ascone[d], R. Rizzo[e,*]

[a]*POLYtech S.C.ar.l., Area Science Park, Padriciano 99, 34012 Trieste, Italy.*
[b]*International Centre for Science and High Technology, Area Science Park, Padriciano 99, 34012 Trieste, Italy.*
[c]*ICMAT-CNR, Area della Ricerca, C.P. 10, 00016 Monterotondo Stazione, Italy.*
[d]*LURE, Université Paris-Sud, Bat. 209, 91045 Orsay Cedex, France.*
[e]*Dipartimento di Biochimica, Biofisica e Chimica de Macromolecole, Universita di Trieste, Via Licio Giorgieri 1, 34127 Trieste, Italy.*

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Abstract

Gels and powders of Cu(II) and Zn(II) polygalacturonate have been investigated using X-ray absorption spectroscopy to obtain coordination distances and coordination numbers. The analysis of the near edge zone of the spectra showed that, for a given cationic species, gels and powders exhibited the same coordination number. The analysis of the extended X-ray absorption fine structure (EXAFS zone) revealed that Cu(II) and Zn(II) complexes were characterised by different coordination numbers. Cu(II) ions resulted to be pentacoordinate with four coordination distances at 1.92 Å and one further distance at 2.25 Å, whilst Zn(II) was hexacoordinate exhibiting a coordination distance of 2.00 Å for all the ion-to-oxygen bonds. Both experimental coordination numbers and coordination distances were used as constraints for molecular modelling calculations. Different polymeric chain-packing configurations and the possibility to include water molecules into the coordination shells were taken into account. Possible detailed molecular models of cation complexation sites between ordered segments of polygalacturonate chains are reported. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Polygalacturonate gels; Divalent cation complexation; EXAFS spectroscopy; Molecular modelling

1. Introduction

The widely accepted molecular model for polysaccharidic aqueous gels is based on the following two main features. On one hand, both secondary structure stretches and conformationally disordered segments are present along the polymeric chains. On the second hand, ordered domains, belonging to different chains, interact with each other through van der Waals interactions and hydrogen bonding, as occurring in neutral polysaccharides (e.g. agar). Moreover, in ionic macromolecules (e.g. alginate and polygalacturonate) ion-bridges further stabilise the ordered domains. The above features give rise to an extended multichain network where the solvent can occupy the resulting empty spaces (Rees, Morris, Thom & Madden, 1982). The so-called "egg-box" model (Grant, Morris, Rees, Smith & Thom, 1973) for the Ca^{++}-induced gelation of alginates and polygalacturonate exhibits the above depicted structural features and, in addition takes into account conformational and configurational characteristics specific for these carboxylate polysaccharides.

Up to now, structural details of ordered domains of gelling-networks were obtained from solid state X-ray fibre diffraction studies (see Millane, 1990; Walkinshaw & Arnott, 1981, for polygalacturonate gels) and circular dichroism of solutions and films (Morris, Rees, Thom & Boyd, 1978; Ravanat & Rinaudo, 1980; Thom, Grant, Morris & Rees, 1982). The possibility of performing Extended X-ray Absorption Fine Structure (EXAFS) experiments directly on gels using synchrotron radiation, prompted us to use this technique for the investigation of the geometrical features of the binding sites of calcium atoms in polygalacturonate gels (Alagna, Prosperi, Tomlinson, & Rizzo, 1986). In this paper the complexation geometry of Cu(II) and Zn(II) was investigated. For the sake of comparison, EXAFS spectra of both gels and powders, the latter obtained from gels after freeze-drying, were acquired. Finally, the structural data obtained were used as the boundary conditions in molecular mechanics calculations in order to obtain detailed three-dimensional molecular models for the ordered domains of the gel structure.

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[*] Corresponding author. Fax: +390-406-763-691.
E-mail address: rizzor@bbcm.univ.trieste.it (R. Rizzo).

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2. Materials and methods

2.1. Sample preparation

A commercial sample of polygalacturonic acid (PGAL) (Sigma Co., USA) was used after purification. 2.0 g. of PGAL were suspended in 100 ml of double distilled water and the pH was increased to 8 using 1 M NaOH; the solution was then filtered through a glass syntherised funnel (n.4) and precipitated with 75% isopropyl alcohol/water (v/v). The precipitate was centrifuged and re-dissolved in water, the solution was then extensively dialysed, filtered and freeze-dried; 1.65 g of purified polymer were obtained.

Gel samples for EXAFS measurements were obtained using standardised solutions of $Cu(ClO_4)_2$ and $Zn(ClO_4)_2$; perchloric salts were chosen to avoid the presence of efficient anionic ligands in the coordination sphere of divalent cations. Solutions of sodium polygalacturonate (3.5 ml; polymer concentration = 0.087 mol of COO^-/dm^3) were dialysed against 100 ml of a solution of either copper or zinc perchlorate. Three cation-to-carboxylate equivalent ratio (0.4, 0.25 and 0.1) were used. After four days, the external solution was substituted with an equimolar $NaClO_4$ solution, in order to remove the excess of free divalent cations from the gel.

In order to check the presence of free divalent cations, Cu-gels were solubilised by addition of EDTA-NaOH and the total metal content was measured by spectrophotometric procedures (Cesàro, Delben & Paoletti, 1988). The polymer concentration (expressed as equivalent of carboxylate groups) always exceeded the metal cation molar content.

Polygalacturonate powders of either Cu(II) or Zn(II) salts were obtained by freeze-drying the gel and grinding it in a mortar under liquid nitrogen. The ground material was then mixed with boron nitride (BN) and pressed in order to obtain homogeneous disks.

Samples of standard compounds (acetates, carbonates, and oxides of either Cu(II) or Zn(II)) were investigated in the form of BN disks or as a deposit on Millipore filters (FHLP02500, 0.5 μm pore size) and used as references in the EXAFS data analysis.

Spectra of polygalacturonate gels were collected locating the dialysis bags, where gels were formed, directly in the monochromatic beam.

2.2. EXAFS and data analysis

EXAFS spectra were obtained on the beam-line XAS2 at DCI-LURE Synchrotron facility (Laboratoire pour l'Utilisation de la Radiation Electromagnetique, University of Paris-Sud at Orsay, France. Energy 1.8 GeV; stored current 300 mA). Data were collected at the exit of a double-crystal Si(111) monochromator. Fluorescence mode detection, with a plastic scintillator, was used for EXAFS data collection on gel samples. The energy calibration was obtained by means of a Cu foil by setting the Cu absorption edge at 8980 eV. Seven spectra were added together for a single run in order to improve the signal-to-noise ratio. Steps of 2 eV were used both in the pre-edge region and in the EXAFS region, whilst 0.5 eV steps were employed in the edge region.

EXAFS is a spectroscopic technique which can provide structural information independent of the aggregation state of the sample, and therefore suitable for gels. EXAFS refers to the oscillation of the X-ray absorption coefficient on the high-energy side of an absorption edge of core electrons. These oscillations are due to the interference between the photoelectron wave outgoing from the absorbing atom and that back-scattered by the neighbouring atoms. The normalised oscillatory part of the absorption coefficient, well above the absorption threshold, is obtained from the experimental results by applying a polynomial function to the data for pre-edge background and the subsequent subtraction of the smooth atomic absorption by means of a polynomial spline function. The general parametric expression for EXAFS is given by Eq. (1) (Gurman, 1990; Stern, Bunker & Heald, 1980):

$$\chi(\kappa) = \sum_s \frac{N_s A_s(\kappa)}{\kappa R_{as}^2} \mathrm{e}^{-(2R_{as}/\lambda(\kappa))} \mathrm{e}^{-2k^2\sigma_{as}^2} \sin(2\kappa R_{as} + \phi_{as}(\kappa)) \tag{1}$$

where $\chi(\kappa)$ is the fractional modulation in the absorption coefficient above the edge, κ is the photoelectron wavevector, N_s is the number of scatterers, R_{as} is the distance between the absorbing atom $a$and the s-th backscattering atom and σ_{as}, the so called Debye–Waller factor, is the variation in the R_{as} distance about its mean. Finally, λ is the photoelectron mean free-path and ϕ_{as} is the phase shift experienced by the ejected photoelectron when crossing the different potential surfaces of the absorber and scatterer atoms.

In order to obtain distances and coordination numbers from the experimental data, it is crucial to have information on the phase shift, the backscattering amplitude, the Debye–Waller factor and the electron mean free-path. This information can be achieved either by extracting the needed parameters from known molecular structures and inserting them in the EXAFS general equation or by resorting to tabulated values of backscattering amplitudes and phase shifts. We analysed the data by means of EXCURV 90 software package (Binsted, Campbell, Gurman & Stephenson, 1990) testing theoretical amplitudes and phases on model compounds (cupric and zinc oxides, acetates and carbonates). Finally the refined parameters were inserted into Eq. (1) to obtain the unknown structural parameters of the gels.

The extraction of the EXAFS signal from the extended absorption spectrum was carried out using a polynomial Victoreen function for the pre-edge background subtraction and a third-order polynomial spline for the smooth background removal. A fitting procedure of the experimental EXAFS spectrum with the parametric expression (Eq. (1))

was then carried out. The significance of the fit was assured taking into account a number of parameters, to be refined in the least-square procedure (N_{par}), below the number of independent points (N_{ind}), calculated as recommended by the International Committee on Standards and Criteria in EXAFS (Lytle, Sayers & Stern, 1988):

$$N_{\mathrm{ind}} = \frac{2\Delta\kappa\Delta R}{\pi}$$

where $\Delta\kappa$ is the κ-space Fourier transform range and ΔR is the R-space Fourier anti-transform range according to the Nyquist sampling rate of independent points. Figures for Zn(II) salts were: lyofilised samples $N_{\mathrm{ind}} = 11$ and $N_{\mathrm{par}} = 7$; gel samples $N_{\mathrm{ind}} = 10$ and $N_{\mathrm{par}} = 7$. For Cu(II) salts: lyofilised samples $N_{\mathrm{ind}} = 17$ and $N_{\mathrm{par}} = 10$; gel samples $N_{\mathrm{ind}} = 15$ and $N_{\mathrm{par}} = 10$. The error associated to a fitting parameter was evaluated by doubling the value of the least-square fit at the minimum, keeping constant all the remaining parameters.

2.3. Molecular modelling

All calculations were performed by means of molecular mechanics methods using NMR-Graph (version 3.1) software package (NMR-graph, Molecular simulation, Burlington, Massachusetts, 1991) which is provided with Dreiding II force field (Mayo, Olafson & Goddard, 1990). The starting structures were assembled by means a carbohydrate builder which is part of the NMR-graph package. The conformational maps relative to the α-D-Gal*Ap*-(1 → 4)-α-D-Gal*Ap* dimer were calculated using the grid $\Delta\psi = \Delta\phi = 20°$ and allowing the relaxation of all the geometrical parameters. The structure of gel chain–chain junction zones was simulated by means of oligogalacturonate octamers. Moreover, a limited (ca. 400) number of water molecules were considered around the oligosaccharide–cation complexes. An effective dielectric constant equal to three was used. However, computations carried out with different dielectric constant values led to very similar conclusions.

The general form of the non-bonding energy-potential functions relative to ion interactions was obtained from the software library. The only difference between Zn(II) and Cu(II) functions was the selection of the metal-to-oxygen distance relative to the energy-minimum position (2.04 Å for copper and 2.06 Å for zinc) which was obtained as the sum of the ionic radius and the oxygen van der Waals radius (Weast & Astle, 1980).

3. Result and discussion

PGAL is the chemically homogeneous fraction of pectins (Fishman & Jen, 1986; Harris, 1990). The accepted molecular model for polygalacturonate gel is currently indicated as the egg-box model. According to this scheme, two polysaccharidic chains are facing each other assuming an ordered 2_1-symmetry conformation and divalent cations (generally, calcium ions) are acting as ion-bridge formers being complexed between two chains (Thom et al., 1982).

An EXAFS study on Ca-PGAL gels, performed years ago (Alagna et al., 1986), confirmed some features of the egg-box model. However, calcium is not the best element to be investigated by means of EXAFS spectroscopy mainly because its spectrum in the pre-edge region is a featureless signal and its coordination number is variable, depending on the chemical environment. The calcium coordination number usually ranges between 8 and 10, and this feature necessarily involves a rather large number of water molecules into the complexation shell which could be difficult to model (Walkinshaw & Arnott, 1981). Therefore, we selected gel-forming divalent cations (Cu(II) and Zn(II)) able to give complexes exhibiting better-defined geometrical characteristics. EXAFS data might also contribute to define the type of atoms in the complexation shells. However, in the present study only oxygen atoms, belonging either to the solvent (water) or to the saccharidic moieties (hydroxyl groups, carboxylate groups), can be part of the coordination shells.

3.1. X-ray absorption spectroscopy

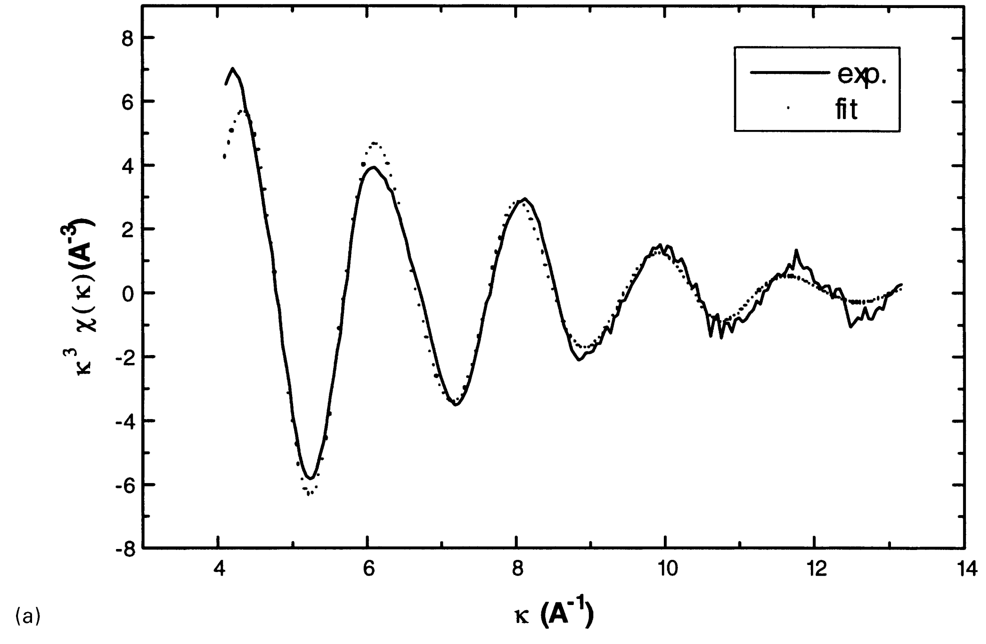
The earlier EXAFS investigation carried out on both Ca-polygalacturonate powders and gels (Alagna et al., 1986) showed that the two phases exhibited different calcium coordination numbers. This characteristic was assigned to a different symmetry in the secondary structure of polygalacturonate gel and powders. Contrary to this, near-edge absorption spectra (XANES) of copper and zinc complexes indicated that, for each cation investigated, the coordination number in gel and in the solid state was identical (data not shown).

The EXAFS data for zinc and copper complexes are hereafter discussed separately.

3.1.1. EXAFS spectra of zinc polygalacturonate

The κ^3-weighted EXAFS spectra for both solid state and gel forms of a polygalacturonate sample having a zinc-to-carboxylate equivalent ratio equal to 0.1 are reported in Fig. 1a and b. Spectra relative to higher equivalent ratios were practically identical. The results of the fitting procedures are also reported in Fig. 1. The high-energy noise for the gel sample was expected because of the low density of absorber atoms with respect to powders. The Fourier transformed EXAFS signals are shown in Fig. 2a and b.

The experimental findings for both powders and gel are reported in Table 1, where errors relative to shells at a distance higher than 2.5 Å were not evaluated due to a low signal-to-noise ratio. Both lyophilised and gel samples exhibited a coordination number equal to six for the first shell hinting to a standard octahedral coordination for zinc (Stouff & Boulegue, 1988).



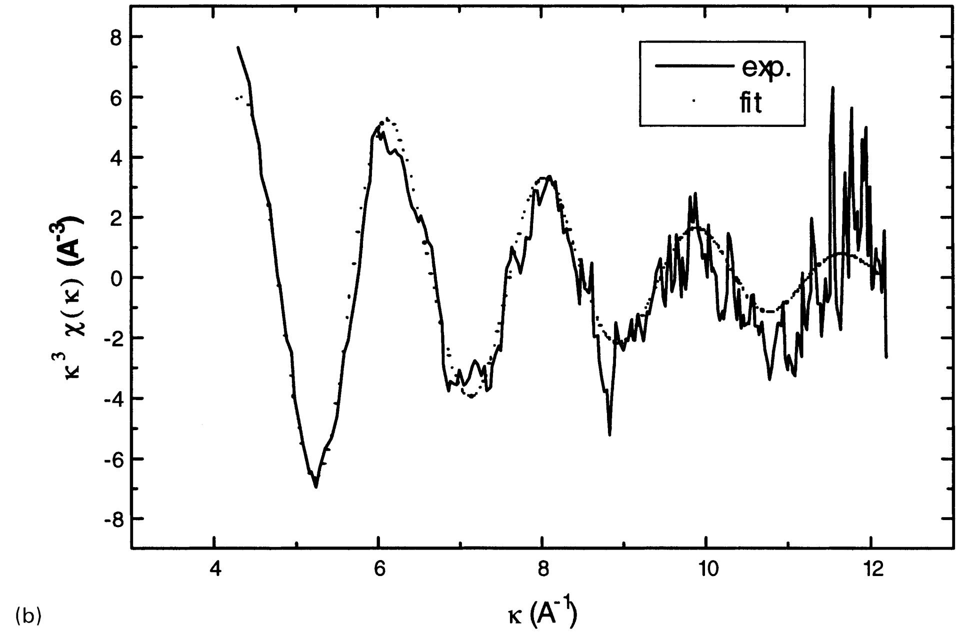


Fig. 1. Extracted EXAFS spectra: (a) Zn-polygalacturonate powders; (b) Zn-polygalacturonate gel.

The fitting procedure allowed multiple shells to be generated. Two shells were necessary both for the lyophilised samples and for the gel system where the noise at the high-energy end of the spectrum prevented a larger *k*-window.

3.1.2. EXAFS spectra of copper polygalacturonate

The κ^3-weighted raw EXAFS spectra for both solid state and gel forms of a polygalacturonate sample having a copper-to-carboxylate equivalent ratio equal to 0.1 are reported in Fig. 3a and b, together with the results of the fitting procedure. As for zinc complexes, spectra relative to higher equivalent ratios were practically identical and the spectrum of the gel sample showed the usual high noise at high *k*-values. The Fourier-transformed EXAFS signals are shown in Fig. 4a and b, for the lyophilised sample and gel, respectively.

The fitting procedures needed more parameters (shells) than those adopted for zinc complexes in order to converge to a reasonable goodness of fit values as is indicated in

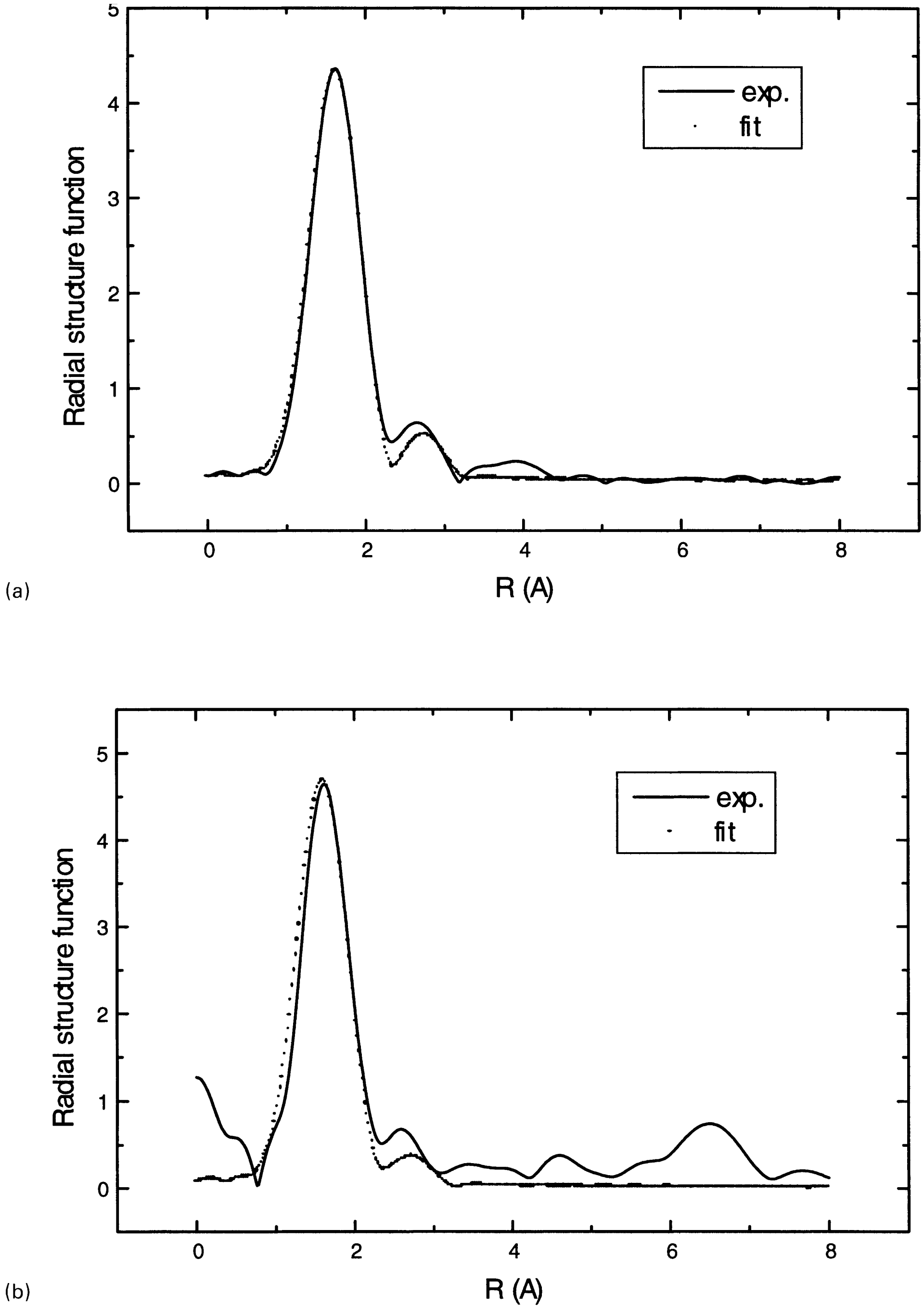


Fig. 2. Fourier transform moduli: (a) Zn-polygalacturonate powders; (b) Zn-polygalacturonate gel.

Table 2, where the experimental coordination numbers and the radial distances are reported. As for zinc, errors were evaluated only for shells at distances lower than 2.5 Å.

The experimental structural data for copper complexes indicated a penta-coordination characterised by four distances at 1.92 Å and one longer distance at 2.25 Å. The geometry of this coordination might be assumed to be a square-base pyramid as found in many copper binding sites (Adams, Bailey, Rodriguez de Barbarin, Fenton & He, 1995; Adams, Bailey, Campbell, Fenton & He, 1996).

3.2. Molecular modelling

The EXAFS experimental findings were used as constraints in molecular modelling calculations in order to obtain a more detailed description of the cation-induced association of the ordered segments of PGAL in the gel structure.

Two oligogalacturonate segments, composed of eight residues each, were computer-generated and let to interact in a parallel fashion. According to the available description

Table 1
Experimental structural data on Zn–polygalacturonate as obtained from EXAFS spectroscopy for lyophilised samples and gel. The divalent cation-to-carboxylate molar ratio was 0.1 for both samples

Sample	Shells	Coord. Number	Radial dist. (Å)	σ^2(Å^2)[a]
Zn lyoph.	1st	6.0±1	2.00±0.02	0.011±0.003
	2nd	2.0	3.18±0.09	0.02
Zn gel.	1st	6.0±2.0	2.00±0.03	0.010±0.004
	2nd	2.0	3.17±0.09	0.02

[a] σ was defined in Eq. (1).

of the secondary structures of PGAL (Morris, Powell, Gidley & Rees, 1982; Ravanat & Rinaudo, 1980), two conformations with different symmetry were selected as starting secondary structures: the 3_1 symmetry and the 2_1 symmetry. As a first step, each PGAL octamer, either in the 3_1 or in the 2_1 symmetry, was minimised optimising both bond lengths and bond angles, in the presence of monovalent ions as counterions.

When investigating divalent cation complexes, two saccharidic octamers and four divalent cations, being them either copper or zinc located between the two octamers,

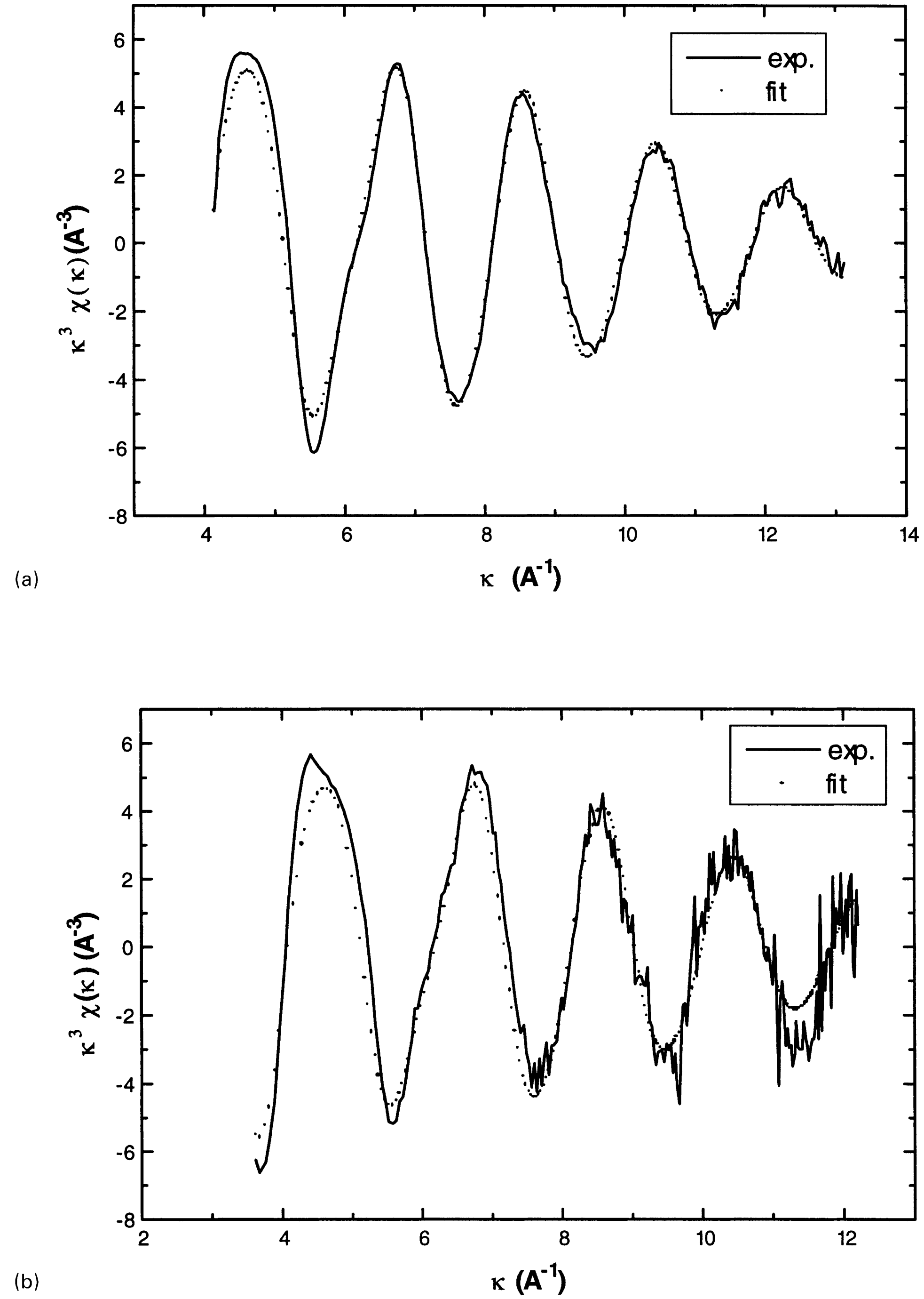


Fig. 3. Extracted EXAFS spectra: (a) Cu-polygalacturonate powders; (b) Cu-polygalacturonate gel.

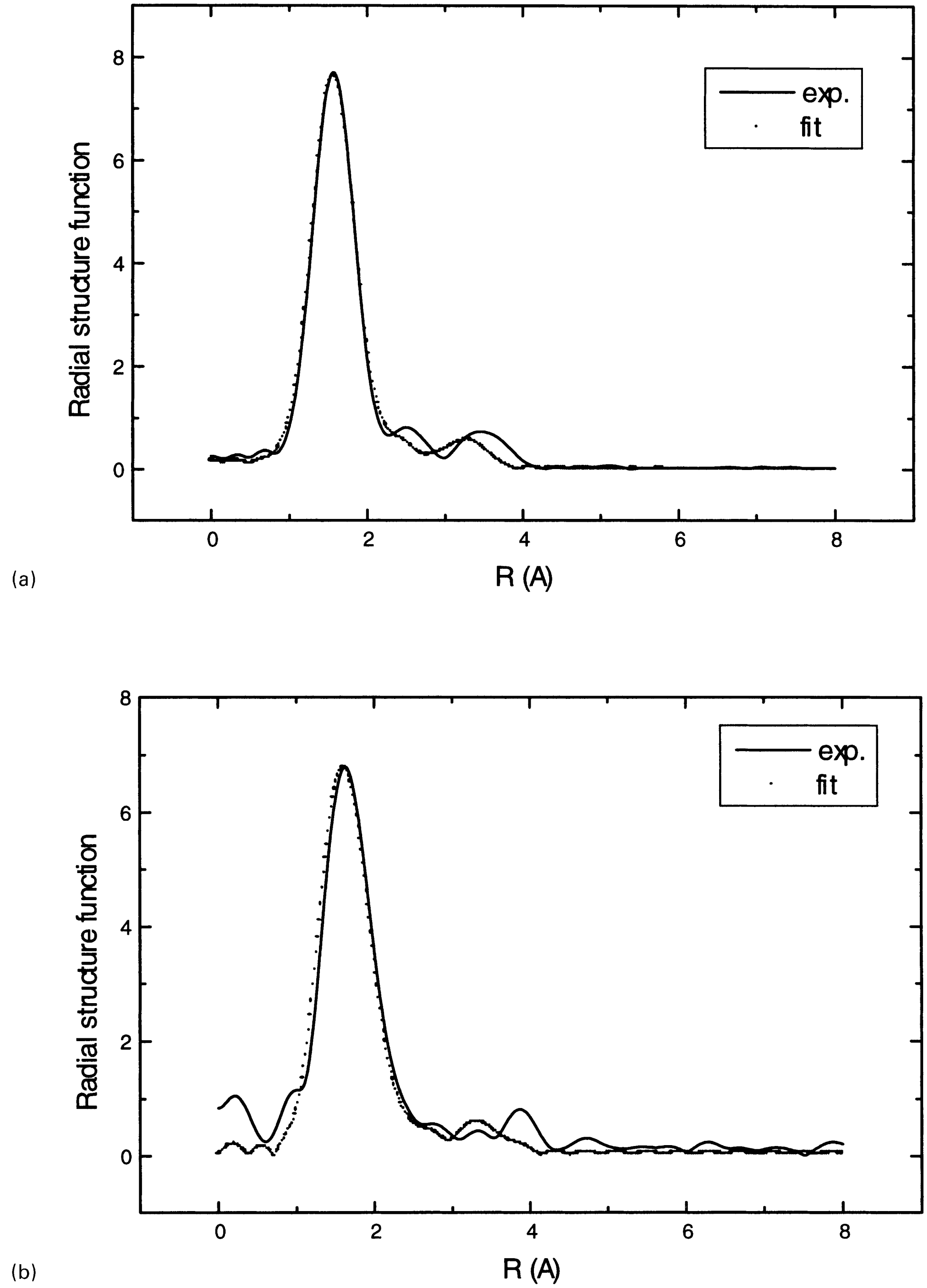


Fig. 4. Fourier transform moduli: (a) Cu-polygalacturonate powders; (b) Cu-polygalacturonate gel.

were considered. In addition to this, eight monovalent cations, saturating the remaining carboxylate negative charges, were added and randomly distributed in the outer shell of the overall structure.

A preliminary study of the divalent cation-mediated packing of two octamers indicated that, when two 3_1-symmetry structures were considered, the optimisation of both ions-octamer and octamer-octamer interactions drove the system so to transform the saccharidic chain symmetry into the 2_1 type. It has to be noted that indeed the 3_1 and the 2_1 structures are near each other in the conformational space. This preliminary result was in agreement with the generally accepted gel-structure model of PGAL and, therefore, we restricted the packing analysis to 2_1 symmetry structures.

Two chain association models were taken into account in the cation-mediated chain packing simulation. They were derived from the consideration that, assuming a 2_1 symmetry, the polysaccharidic chain is similar to a ribbon exhibiting flat faces and rims. The first geometry, which will be hereafter referred as “face-to-face” (FF) mode,

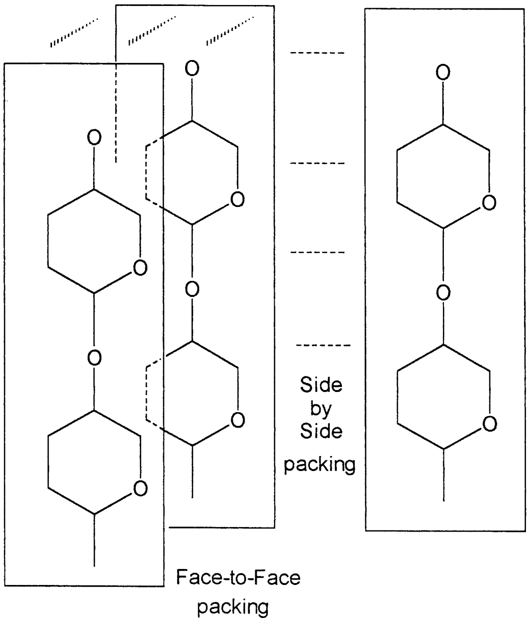


Fig. 5. Schematic representation of SS and FF chain association packing.

was characterised by two saccharidic chains facing each other with the flat face of the ribbons. In the second one, which will be hereafter referred as "side-by-side" (SS) mode, the two octamers were packed together through contacts between the rims of the ribbons (Fig. 5). In addition to this, the SS configuration could give rise to two slightly different types of association geometry, where the second one is obtained from the first one shifting one of the two oligosaccharidic chains of one residue along the chain axis. These two possibilities will be hereafter referred as SS$_1$ and SS$_2$, respectively. Finally, the packing association can be either of the parallel type (FF$_p$ or SS$_{\#p}$) or of the anti-parallel type (FF$_a$ or SS$_{\#a}$). In both FF and SS structures, the selected divalent cations were placed between the two oligosaccharides in order to be able to act as ion-bridge makers.

Two different molecular mechanics calculations were performed for each packing model: one without water molecules into the metal co-ordination sphere and the second one including two water molecules into each co-ordination sphere. These water molecules were placed directly between the two interacting octamers; however, it was paid attention not to locate them at co-ordination distances from the divalent cation. Their position was then allowed to change according to the fully unconstrained energy minimisation pathway. Some calculations were also performed in the presence of ca. 400 molecules of solvent (water) surrounding the solute system. As a matter of fact, during the minimisation procedures these solvent molecules always remained in the outer space with respect to the central interacting octamers, so that they were unable to perturb the situation present at the complexing sites. The structural findings obtained by means of molecular mechanics calculations are shown in Table 3.

Table 2
Experimental structural data on Cu–polygalacturonate as obtained from EXAFS spectroscopy for lyophilised samples and gel. The divalent cation-to-carboxylate molar ratio was 0.1 for both samples

Sample	Shells	Coord. Number	Radial dist. (Å)	σ^2(Å^2)[a]
Cu lyoph.	1st	4.0±0.5	1.92±0.01	0.005±0.001
	2nd	1.0±0.2	2.25±0.04	0.005±0.003
	3rd	1.0	2.71	0.010
	4th	2.0	3.70	0.009
Cu gel.	1st	3.8±0.7	1.92±0.02	0.006±0.002
	2nd	1.0±0.6	2.25±0.06	0.005±0.004
	3rd	1.0	2.71	0.010
	4th	2.0	3.70	0.009

[a] σ was defined in Eq. (1).

When considering the FF$_p$ and FF$_a$ packing schemes without water in the coordination shell, the maximum coordination number found was 2, where one oxygen atom from each of the two carboxylate groups, belonging to two facing galacturonic residues, was at a distance suitable for complexation and in rough agreement with the EXAFS findings. Fourfold co-ordination was obtained only in the presence of two water molecules into the first complexation shell. Any effort aimed at increasing the co-ordination number, either involving hydroxyl groups or considering more water molecules into the co-ordination sphere, was unsuccessful. Therefore, the FF association was considered a suitable model only for hydrated Cu(II) complexation.

When considering the SS packing scheme, a co-ordination number equal to 4 was easily obtained without considering water molecules into the co-ordination sphere. This figure rose up to 6 when considering them. Therefore, the SS model was regarded as a suitable model both for copper and zinc complexation, provided that, in the latter case, two water molecules were included into the co-ordination shell. It is worth mentioning that zinc acetate exists in two forms: an anhydrous one with fourfold coordination and a dihydrate one with sixfold coordination (Grady, Floyd, Genetti, Vanhoorne & Register, 1999). It is also interesting that both the SS$_{1p}$ and the SS$_{1a}$ packing configuration were able to give a penta-coordination (see Table 3), where the second shell distance was in very good agreement with the experimental one found for copper complexation. Actually, the fivefold coordination in copper complexes has been found in several systems, like in copper-lactate (Prout, Armstrong, Carruthers, Forrest, Murray-Rust & Rossotti, 1968) and in pyridine/phenol complexes (Adams et al., 1995, 1996).

Concerning the possibility to have either a parallel or an antiparallel chain association, it was found that the antiparallel association exhibited always a lower overall conformational energy. In addition to this, although the models described are a rough approximation of real systems and molecular mechanics calculations have an approximate character, the SS association total energy was lower than that of the FF$_a$ packing mode.

Table 3

Results of the Molecular mechanics calculations obtained for different chain packing models (the data reported refer to Cu^{++} complexes; correspondent data for Zn^{++} ions resulted in slightly longer distances and slightly higher energies)

Packing model	With eight water molecules			Without water molecules		
	Dist. (Å):COO^-–M H_2O–M	Coord. No. # ^-OOC[a] # H_2O	Total energy[b]	Dist. (Å): COO^-–M ROH–M	Coord. No. # ^-OOC[a] # ROH	Total energy[b]
FF_p	1.86–1.88 1.94–2.12	2 2	−223	1.84–2.40	2	−88
FF_a	1.86–1.881 1.94–2.12	2 2	−225	1.84–1.86	2	−96
SS_{1p}	1.90–1.94 2.14–2.25	4 1 ROH[c]	−326	1.91–1.93 2.09–2.12	4 1 ROH[c]	−194
SS_{1a}	1.91–1.96	4	−308	1.91–1.93	4	−200
	2.05–2.20	1 ROH[c]		2.1–2.2 (2.1–2.4)	1 ROH[c] (2 ROH)[c]	
SS_{2p}	1.90–1.92 2.10–2.20	4 2	−366	1.89–1.91 (3.6)	4 (1 ROH)[c]	−215
SS_{2a}	1.90–1.92 2.10–2.20	4 2	−375	1.89–1.91	4	−220

[a] The figure refers to the number of oxygen atoms belonging to carboxylate moieties.

[b] kcal/mole.

[c] This figure indicates the possibility that saccharidic OH groups are accommodated in the complexation shell.

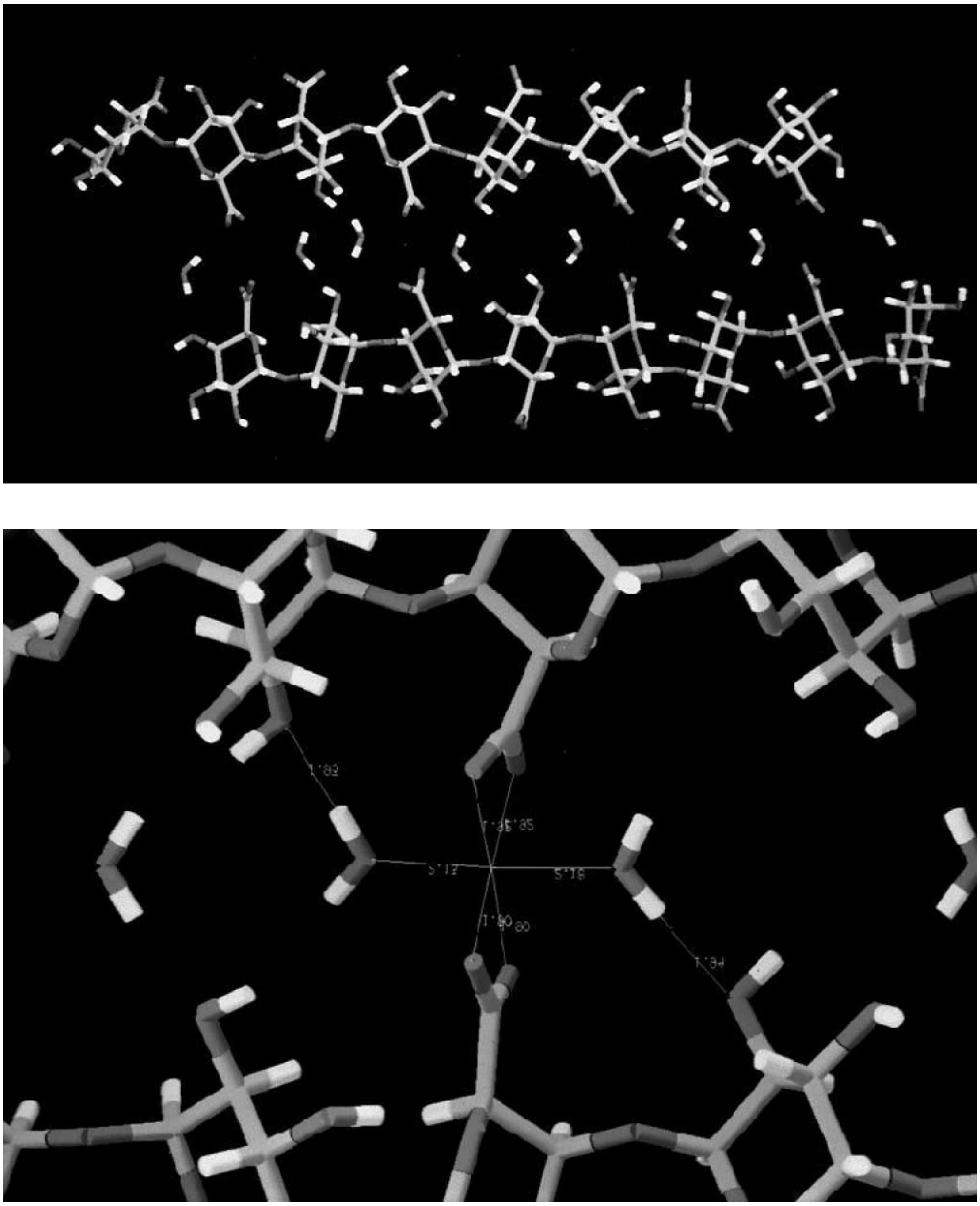


Fig. 6. (a) Sixfold coordination in Zn-oligogalacturonate complexes. Upper: general view; lower: detail of the complexation site. (b) Fivefold coordination in Cu-oligogalacturonate complexes. Upper: general view; lower: detail of the complexation site

The results of the modelling calculations clearly indicated that the SS model is flexible enough to accommodate a tetra-, penta- and hexa-coordination thus satisfying the experimental findings. The pictures of the SS packing structures as obtained by molecular modelling in the presence of divalent cations are shown in Fig. 6a and b.

4. Conclusions

The investigation described in this paper showed that, taking into account structural experimental data obtained directly on gel systems, it is possible to obtain reasonable molecular models of the gel structure. The SS packing model was found to better fit the experimental findings for both copper and zinc complexation, whereas the FF model was able to account only for the copper complexation.

Taking into account the spatial configuration of carboxylic groups, a multichain extension of the divalent cation-induced packing geometry may occur without major distortions of the polymer chain secondary structure. The above consideration could be used as an indication that junction zones in polygalacturonate gels can be considered as mini-crystallite domains.

In principle, it is possible to postulate that different cations might induce different chain association configurations; however, due also to the structural similarities between copper and zinc complexes, it can be concluded

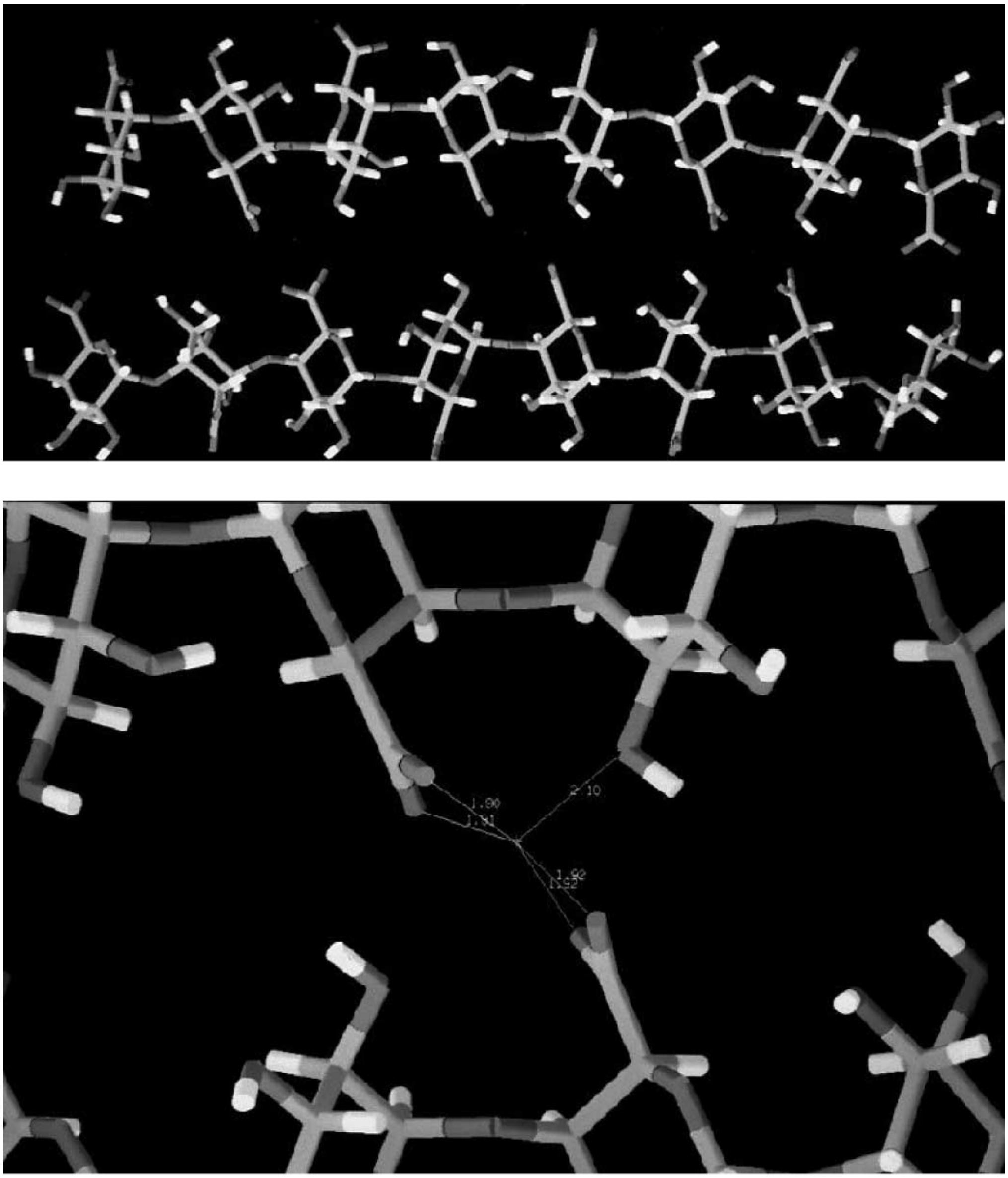


Fig. 6. (*continued*)

that the chain packing association for the two complexes was the same. Nevertheless, the flexibility of the SS model was such to accommodate the different coordination geometry of the two cations.

Finally, concerning the more familiar Ca^{2+}-polygalacturonate gel, it has to be stressed that the models discussed in this paper are valid for copper- and zinc-induced gelation, whereas calcium complexation, which is characterised by a larger complexation radius, up to 2.7–2.9 Å (Alagna et al., 1986), could, in principle, lead to a different structure.

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