

Phase separation involving ordered biopolymer systems

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Abstract. The formation of a gel phase is very common in the biopolymer field and is at the basis of many important biological and industrial processes. Among the several known gelling systems, those occurring by the local association of conformationally ordered chains are the most interesting for their peculiar reversibility. A structural and thermodynamic characterisation of these processes is presented, in particular, for three categories of biopolymers. A regular non-ionic microbial polysaccharide is shown to exhibit a very cooperative phase transition which is the prerequisite for a thermo-reversible gelation process (even at very low polymer concentration, down to 0.1g/L) and its behaviour is discussed in view of the supramolecular structures formed. The second example is given by the ionic and temperature dependence of the gelling properties of the sulphated algal polysaccharides, in which the interactions between ordered chains are dominated by the ionic interactions. Finally, the thermodynamic and kinetic characterisation of a new gel phase formed in a semi-concentrated solution of the microbial polyester, poly(D-(-)- β -hydroxybutyrate, is reported.

Introduction

Over the last few years the peculiar phenomenological behaviour of polymeric gel phases has provoked an increasing scientific and practical interest in the fundamental aspects of the structure and the formation mechanism of these intriguing systems. As a matter of fact, even the definition of gel needs some caution (1,2). The common definition of cross-linked polymeric gels excluded the reversible gels which are so important in biological systems and which are also well represented in the synthetic polymers. The interest in physical gels and on their formation mechanism lies mainly in the "quasi"-equilibrium state and the coexistence of "quasi-ordered" regions surrounded by a "quasi-fluid" system, and, therefore, on the possibility of tuning composition and properties. The insistence of the term "quasi" stresses the ill-defined gel structural and phase characteristics.

Probably, the property which univocally defines a gel is its viscoelastic behaviour. In fact, the viscoelastic spectra obtained by rheological measurements can provide the rank of the interactions from the entangled state to the weak-gel or strong-gel phase (2). The further differentiation between weak and strong gels is given by the large deformation behaviour; strong gels break and can be reformed only after a new gelation cycle, whereas weak gels are tixotropic and reform after a standing time. Given the role of the interactions in solution (3), which eventually determine the topological constraints at the basis of the gel properties, it is of fundamental importance to study and understand the molecular origin of the structural and dynamic features of the polymeric chains in this supramolecular organisation. This paper aims at providing some thermodynamic and structural (i.e., conformational) flavour to the problem of the formation of gel phases, presenting three cases of biopolymer gels. The ultimate aim of present research would be to deduce semi-empirical "selection rules" in order to predict the conditions for the gel formation and the gel properties.

In the particular case of the polysaccharide family, a number of empirical rules have already been assessed on the solubility and behaviour of these polymers in aqueous solution. The relevant structural features are reported in scheme I, where the example cases are reported for comparison. The hidden rule at the basis of this scheme is that the solubility depends heavily on the energetic efficiency of the packing of the polymeric chain in the solid state as opposed to the solution state. However, given the conformational polymorphism of many polymers in the solid (semi-crystalline) state, the thermodynamic definition, if any, of solubility is itself ambiguous.

Scheme I. Role of structural and conformational features on solubility of polysaccharides

Structural features	Case-polymers	Solution behaviour
<i>chain linkage</i>	<i>cellulose</i>	<i>stiff and insoluble</i>
	<i>dextran, pullulan</i>	<i>flexible and soluble</i>
<i>side chains</i>	<i>cellulose, curdlan</i>	<i>linear, insoluble</i>
	<i>scleroglucan</i>	<i>branched, soluble</i>
<i>non-sugar substituents</i>	<i>deacetylated gellan</i>	<i>gel with Ca, Mg</i>
	<i>native gellan (deacetylated)</i>	<i>soluble with Ca, Mg</i>
<i>ionic groups (e.g., carboxyl)</i>	<i>galactans, amylose</i>	<i>neutral, insoluble</i>
	<i>pectic, oxy-amylose</i>	<i>ionic, soluble, pH-depend.</i>

*The complex gelling behaviour of the capsular polysaccharide from *Rhizobium trifolii* TA1*

The most dramatic solution property exhibited by TA1-CPS is, by far, its ability to form aqueous thermo-reversible gels in a wide range of polymer concentrations (down to 0.1 g/L). In particular, due to the non-ionic character of the polysaccharide chain, gels can be formed in the absence of ionic co-solutes, and show remarkable gel strength (4,5). Such a gel exhibits melting-setting thermal cycles with hysteresis, as shown in Fig. 1, where the temperature dependence of the percentage change of scattering intensity and of optical rotation is reported (4). Melting and gelling temperatures, derived from the mid-point of the scattering light changes upon heating and cooling, are independent of the polymer concentration in the range of concentration between 0.2-2 g/L. Much more interesting than this is the unusual profile of the optical activity as a function of temperature, which is also a function of both the wavelength and the polymer concentration. This fact was properly ascribed in the early work (4) to the presence of dissymmetrical ordered aggregates at low temperatures. The supermolecular structures are therefore chiral aggregates of opposite chirality of the ordered conformation taken by the soluble chains.

Calorimetric experiments have been carried out with several high-sensitivity dsc instruments (in different laboratories) under similar conditions (6). The polysaccharide was repeatedly heated and cooled, and the thermal curves were not only almost completely reproducible, but also the responses of the different instruments were in satisfactory agreement. The dsc results (Fig. 1) showed a very sharp transition at around 47°C. The transition is reversible, sharp but slightly asymmetrical at low temperatures. The calorimetric heat of transition, $\Delta_M H$, is of 22.2±0.4 J/g (polymer concentration of 4 g/L) and the van't Hoff enthalpy is 1250 kJ/mol (of repeat units), based on the chemical structure characterised by a trisaccharide in the chain backbone which possesses two branches on the same glucosidic residue (Fig. 2). According to

literature procedures, the length of the cooperative unit was estimated to be about 57 repeat units, or in other units by a mass of about 57000. The concentration dependence inferred from the data at different concentrations clarifies the energy contribution of the chain-chain interaction and provides some information about the structure formation of the gel phase.

Although the poor quality of the original diffraction pattern of the TA1-CPS did not make a good resolution of its structure possible, a pseudo-double-helix (2-fold symmetry) has recently been stated with a chain repeat axis of 1.01 nm per repeating unit (7). This work does not substantially change the previous value of 0.98 nm proposed by the same authors but with a different structure. From these data, a length of about 60 nm for the cooperative segments can be assumed, if this is the only cooperative structure which stabilises the ordered helical conformation in the gel structure.

A number of other experimental observations (4,5), in particular on the thermal and rheological behaviour of TA1-CPS in the presence of co-solutes (urea, salt, or sucrose), suggest that at least three different levels of structure may be involved in the process of aqueous gel formation (8,9). The first level has been referred to as local chain ordering and corresponds to the process with 12.6 J/g. The second level has been thought to involve "intermolecular ordering between conformationally ordered segments" and may correspond to an enthalpy change of about 2.6 J/g. This second level of structure has been shown to resist shear and such denaturants as urea. The last level of structure provides the three-dimensional gel network and is labile under moderate shear and in concentrated urea solution: it involves supramolecular aggregation.

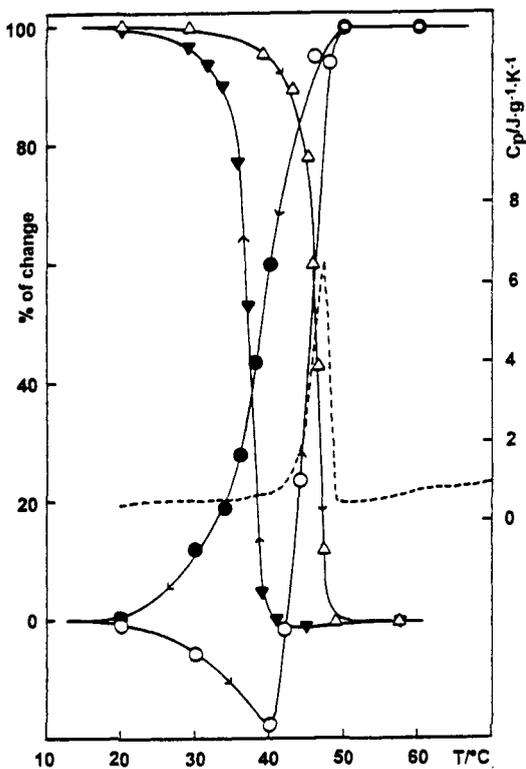


Figure 1. Temperature dependence of the change of scattering intensity (Δ) and of optical rotation (o), and dsc thermogram (dotted line) of CPS TA1 (1g/L) in water (open symbols: heating; full symbols: cooling).

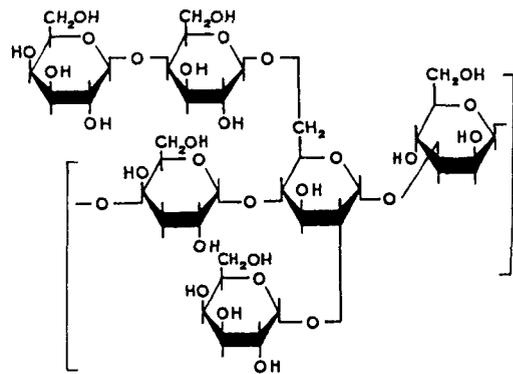


Figure 2. Schematic drawing of the chain repeat unit of the capsular polysaccharide from *Rhizobium trifolii*, strain TA 1.

Evidence showing a complex aggregation in the development of the gel structure has also been accumulated by experimental work carried out independently by Gidley et al. (5). In particular, both the typical hysteresis in the melting-setting thermal cycles and the temperature dependence of the rigidity (storage) modulus in water and in aqueous urea solution support the presence of an intermediate step for the formation of aggregate structures. Experimental results on the effect of the side chain modification have invariably demonstrated that any perturbation on the side chain integrity produces weaker gels and greater thermal instability of the ordered phase in the gel network (9,10).

A possible explanation for such complex gelling behaviour is that the stereo-regular non-ionic sidechains of CPS, which eventually determine the formation of the gel, maintain a helical conformation in water, which is very likely to be the same found for CPS fibres by means of X-ray diffraction (7). The gel network is then stabilised by an array of energetically favourable overlaps between the side chains of different molecules (9). Given the trend of the temperature dependence of chiral properties, the helical dissymmetry of the isolated chain must be of the opposite sign of the dissymmetry which arises from the aggregation of the chain. In the absence of a clear picture of the molecular model for the gel junctions, it is not possible to speculate further on the structure of the gel and on the meaning of the cooperative length found through calorimetric experiments. This number is related to the size of the thermodynamic domains of the polymer undergoing the transition, but it is not possible to state whether it is associated to a pair or to more than two chains. Some literature data (10) could provide additional evidence for this interpretation of the energetics of the gel, after making the proper corrections to the reported calorimetric values.

The polyelectrolyte effect on the carrageenan gels.

A thermodynamic model has been outlined for the ion-induced conformational transition (11), with the aim of finding correlation between the calculated changes of thermodynamic quantities and the experimental results. The purpose has also been to provide some understanding of compatibility between the conformational properties and the energetics of the κ -carrageenan chains under different conditions. These algal polysaccharides form thermally-reversible gels of industrial importance as a function of temperature, salt concentration and type of cation. The typical phase diagram showing the correlation between the temperature of transition (T_m) and the ionic strength of the solution (I_m) has been shown to be linear for different salts. On the basis of this linear and parallel trend shown by the phase diagram, the assumptions made in the thermodynamic treatment turned out to be appropriate (12). The change of electrostatic ΔG for the conformational transition was therefore calculated for different cations at each given temperature T_m . Given the constancy of the slope with the temperature, the stabilising (or destabilising) factor is of entropic origin and is associated to the character of the individual counterions (12,13).

The secondary structure of κ -carrageenan is easily recognised as being of the random-coil type at high temperature and low salt concentration, while an ordered helical conformation is adopted at low temperature and/or high ionic strength (14). Many experimental data on the ordered phase are in favour of a dimeric structure, nonetheless there are some controversial interpretations concerning whether the most stable conformation, as that deduced from molecular mechanics (15), implies a co-axial duplex (double helix), or a duplex of single-strands. Optical rotation measurements as a function of the temperature on segments of κ -carrageenan also show a concentration-dependent cooperative character, the ordering process appearing to be a prerequisite for the subsequent aggregational step (12).

For both the theoretical and experimental aspects, it is worth mentioning (13) another peculiar characteristic of κ -carrageenan which is its ability to bind anions, particularly iodide. The binding produces similar ordering phenomena as detected by optical rotation and NMR data, without observing an increase of molecular weight (14) as also shown by previous extensive and accurate macromolecular investigations, suggesting, therefore, that the transition is intramolecular from a disordered coil to an ordered single helix.

This intriguing problem has also been tackled theoretically within the polyelectrolytic theory (13) with the aim of giving a coherent thermodynamic description of the process of anion-polyanion binding and of conformational change. Indeed, anion binding to polyanions is not a common phenomenon, given the unfavourable change of the electrostatic free energy. The most reasonable assumption is that of a favourable contribution from enthalpic interaction terms (e.g. of the ion-dipole and/or dipole-dipole type), which must therefore be detected experimentally as negative enthalpy changes of interaction.

The changes of several properties on mixing the TMA salt of κ -carrageenan as a function of the ionic strength (CsCl solutions) clearly indicate the two-step process at lower temperatures (Fig. 3). This behaviour was associated to a disorder-order transition and to the subsequent cooperative aggregation, with a non-ionic enthalpy of transition of -5.9 kJ/mol (for the coil-helix transition) and of about -20 kJ/mol (for the transition with formation of dimers of helices). Given this value of about -6 kJ/mol for the enthalpy change from the disordered coil and the ordered single helix of κ -carrageenan, a more negative value of ΔH upon helix formation by I^- binding is expected. The experimental findings give a value of -13 kJ/mol for the full conformational transformation, therefore accounting for the high stability of the I^- binding helix at low temperatures and proving that a selective binding is present and not a mere "solvent effect". The resulting modification of the counterion distribution in the polyelectrolyte domain is being studied.

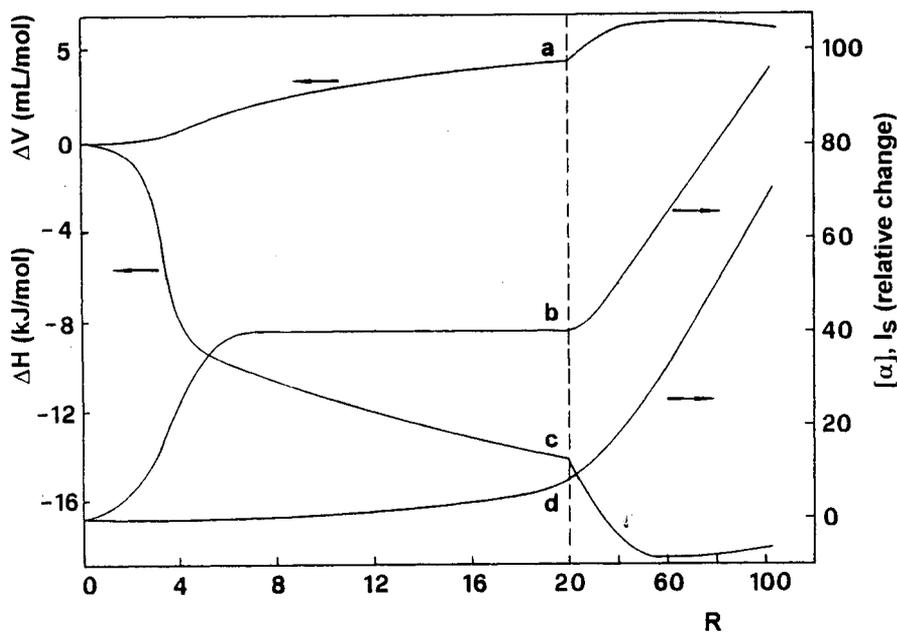


Figure 3. Experimental change of volume (a) and of enthalpy (c) on mixing CsCl with tetramethylammonium κ -carrageenan (10^{-3} M) at 283.2 K, and increment in optical rotation (b) and scattering intensity (d). The abscissa R is the molar ratio of salt to polymer concentration.

Kinetics and thermodynamics of gel formation of Poly(D-(-)- β -hydroxybutyrate).

The conformation in the crystalline and amorphous states is one important characteristic and determines the morphology and other physical properties of these stereo-regular polymers belonging to the family of biodegradable polyesters. The crystalline structure of poly(D-(-)- β -hydroxybutyrate), PHB, has been elucidated, showing a 2_1 helical conformation (16) in the monoclinic crystals. The crystallisation of PHB has recently been studied in some detail, while the conformation of amorphous polymer (solid or in solution) has been studied to a relatively smaller extent. Conformational analysis and statistics have been carried out on a portion of PHB chain in the isotactic configuration according to methods of molecular mechanics and by using Monte Carlo methods (17). The potential energy of the torsional angles have been evaluated as a function of torsional variables with the contribution of non-bonding and coulombic potentials. While the energy profile for the -O-CH- rotation is characterised by two narrow minima distorted from the trans and gauche (-) position, some larger conformational freedom arises from the rotation about the -CH-CH₂ and that about the -CH₂-CO- bonds, although no minima are found for the trans conformation of the latter rotation. These findings provide reasonable interpretation for the solid state helical conformation, but also suggest an intrinsic tendency of the solvated polymer to adopt some preferential local conformations (18) which may resemble the most stable one in the solid state (Fig. 4).

In addition to these studies on the conformational and solution properties, out of mere serendipity, fluorescence bands of PHB were detected which were a function of the monomer composition in the co(HB,HV) samples (19). The spectroscopic aspects of this phenomenon concern the conformation, the solvent interactions and other molecular characteristics of the polyhydroxyalkanoates (PHA). In fact, the preliminary observation was that chloroform, the most common solvent for PHA, is not able to generate fluorescence properties, whereas the polymers are fluorescent in dimethylsulfoxide (DMSO), dimethylformamide (DMF) and trifluoroethanol (TFE). PHB seems to be fluorescent in solvents where chain stiffness and/or aggregation is thought to be increased, as detected, for example, by solution viscosity. Only the spectral results in TFE have been unambiguously interpreted in terms of an intrinsic property of the co-polymer chemical composition (19), since the polymer forms aggregates in other solvents investigated, such as DMF and DMSO. In these solvents PHB is slightly soluble, it forms aggregates at high concentration and eventually undergoes a gel phase which may be considered as an interference with the fluorescence phenomenon. The common fluorescent behaviour in all three solvents is proof that it derives from a property of the polymer itself and not from the gel phase.

Here we wish specifically to report some phenomenological features of the gel formation of PHB, for which some enlightening aspects, not previously reported in the literature, have been found. The characterisation of the gel phase has been carried out with several experimental approaches aiming at elucidating the kinetics and the thermodynamics of this process (20). Solutions of PHB in DMF were prepared by heating at temperatures above 110°C. On cooling at room temperature, the optical activity of the solution (0.1%) deviated toward negative values of the rotation with a sigmoidal shape, monitoring the ordering of the chains. The formation of structures was also shown by the change in the viscosity as a function of time measured on rapidly cooled solutions. An increase of more than three times is observed in the relative viscosity of polymer solutions at low concentration. The turbidity of the solution is reversibly changed with the temperature (Fig. 5) and strongly depends on the wavelength used in the experiments. Quantitative analysis of the kinetics of gel formation has been carried out by measuring the increase of scattered light (fixed angle) at several temperatures (25°- 60°). The plot of the scattered intensity as a function of time

(logarithm scale) follows the principle of superimposition of the curves by "time-temperature shifting". A constant and unambiguous value of 4 has been found for the exponential coefficient of the kinetic law analysed according to the empirical Avrami equation. The suggestion of a specific mechanism of gel formation needs to be verified by other independent observations (e.g., by electron microscopy).

The second relevant result is given by the heat associated with the disruption of the gel phase which has been measured by differential scanning calorimetry. Several scanning cycles were shown to be reproducible, provided that the thermal history of the solution is cancelled. However, samples with different thermal history or concentration have shown two endothermic peaks of different size but with a constant total heat. In Fig. 5 two different heating thermograms are compared (a and b), in addition to a cooling curve (c), for which the hysteresis phenomenon is evident. The binodal "fusion" is also shown by the dependence of the turbidimetry at two wavelengths and is an intriguing result not found previously in other gels from synthetic polymers. Furthermore, on cooling it is evident that part of the heat of formation is slowly recovered, since the heat of melting is always greater than the heat of formation, even for subsequent heatings. Many of these features place the PHB biogels in a category which is quite different from the other biopolymeric gels studied so far (2).

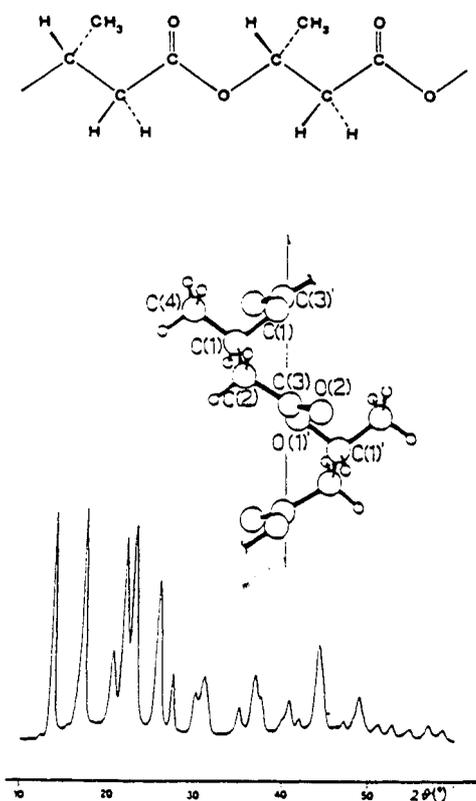


Figure 4. Chemical structure (a), powder X-ray diffraction profile (b) and crystal structure (c) of poly(D-(-)- β -hydroxybutyrate), PHB.

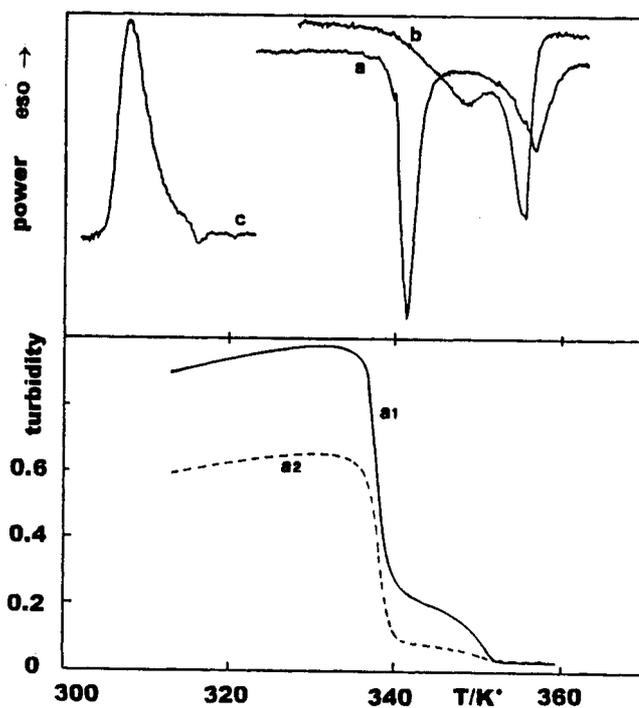


Figure 5. Dsc thermograms (a, b and c: first, second heating and cooling) of the gel phase of PHB in DMF (10g/L), and temperature dependence of turbidity at 350nm (a1) and at 650 nm (a2).

Some conclusive remarks

The results illustrated here are representative for three classes of behaviours with different complexities. The most common model for the polysaccharide gel-network is given by the carrageenan family and is treated with a more sophisticated approach which, taking into account the polyelectrolytic interactions, can be easily extended to include the binding of ions of similar sign. The structural features of a capsular polysaccharide provide a more complex pattern of interactions involving the side chains of this non ionic polymer. The peculiar optical properties and the calorimetric data have been interpreted on the basis of the role of the side chain integrity on the supermolecular structure of the gel. Even larger domains of stereo-regular conformations are detectable for the gel formation of PHB in which the high content of crystalline material can only be ascribed to a lamellar organisation of the chains. The rationale for the three categories is that the larger size of junction zones is directly related to the simplicity and the regularity of the chain structure, while chemical heterogeneity and disordered conformations provide swelled gel structures.

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