Absolute Determination of the Gelling Point of Gelatin under Quasi-thermodynamic Equilibrium

Franco Bellini, Ivana Alberini, María G. Ferreyra, and Ignacio Rintoul

Abstract: Thermodynamic studies on phase transformation of biopolymers in solution are useful to understand their nature and to evaluate their technological potentials. Thermodynamic studies should be conducted avoiding time-related phenomena. This condition is not easily achieved in hydrophilic biopolymers. In this contribution, the simultaneous effects of pH, salt concentration, and cooling rate (Cr) on the folding from random coil to triple helical collagen-like structures of gelatin were systematically studied. The phase transformation temperature at the absolute invariant condition of Cr = 0 °C/min ($T_T^{\mathrm{Cr}=0}$) is introduced as a conceptual parameter to study phase transformations in biopolymers under quasi-thermodynamic equilibrium and avoiding interferences coming from time-related phenomena. Experimental phase diagrams obtained at different Cr are presented. The $T_T^{\mathrm{Cr}=0}$ compared with pH and $T_T^{\mathrm{Cr}=0}$ compared with [NaCl] diagram allowed to explore the transformation process at Cr = 0 °C/min. The results were explained by electrostatic interactions between the biopolymers and its solvation milieu.

Keywords: biopolymers, gel, gelation, kinetics, phase transitions

Practical Application: This study is useful to establish an absolute method with no time interference to determine gelling properties of gels.

Introduction

Gelatin is widely used in the food, cosmetic, and pharmaceutical industries. Strong efforts are carried out to understand this material in the context of the emerging field of self-assembled fibrillar networks, with special attention on food for controlled nutrient delivery and biomaterials (Djabourov and others 1988; Fadda and Lairez 2001; Schrieber and Gareis 2007).

The versatility of this material results from its ability to form stable, physically reversible gels. The properties of these gels depend on the gelatin material itself, the milieu conditions, and the history of the system. The gelatin is defined by the primary source of collagen, the type (acidic or basic) and conditions of hydrolyzation, the amino acid sequence in the collagen molecules, the resulting spatial structure, and the molecular weight distribution (Veiss 1964; Ward and Courts 1977; Bohidar and Jena 1993). The milieu conditions may be defined in terms of concentration, water interactions, pH, salt concentration, and reaction with or the presence of other components (Bohidar and Jena 1994; Eliav and Navon 2002; Zhang and others 2007; Raman and others 2008). The history of the system refers to the level of shear during transformation, the temperature, and the time to dissolve the gelatin, the temperature and the time of gelation, and the cooling or heating rates of the gelatin system to pass from liquid solution to gel and vice versa (Slade and Levine 1987; Cuppo and others 2001; Fonkwe and others 2003).

The structure and kinetics of gelation have been extensively studied. The authors recommend the excellent works of Djabourov and others (1984), Josse and Harrington (1964), and Harrington (1964) for further details. Briefly, the 1st model proposed by Flory and Weaver (1960) and improved by Harrington and Rao (1970) describes the phase transformation process, including nucleation, and growing and annealing steps. More recent approaches propose a continuously living state where local disordered segments are slowly transformed into ordered helical fragments and then growing into larger ones (Eichinger 2000; Vernon and others 2001; Del Gado and others 2002).

Experimentally, the solution to gel transformation is characterized by the gelling time and the gelling temperature. Rheology and mechanical responses, changes in the spin–spin relaxation times, shifting of peaks in infrared spectra, optical rotation patterns, differential scanning, and isothermal titration microcalorimetry can be used to study gelation (Joly-Duhamel and others 2002a; Segtnan and Isaksson 2004; Zandi and others 2007; Privalov and Dragan 2007). However, the results presented in the literature are intrinsically affected by the history of the system. More clearly, the same gelatin solution may present very different gelation times and temperatures, and the resulting gel structure may result with different properties when the gelation process is carried out under different conditions (Fonkwe and others 2003). This particular behavior is the reason for the establishment of very strict protocols to carry out the gelation process always in the same manner. A good example is the Bloom measurement where even a slight modification of the containing vessel can introduce great variations in the resulting Bloom value (Schrieber and Gareis 2007). The conclusions of these studies are only valid within the frame of the specified protocol. Von Hippel and Wong (1962) tried to eliminate the kinetic factors using very low gelatin concentrations, very slow transformation time, very small solution volumes, and hypothesizing the absence of temperature gradients and instant thermal equilibrium. After this work, many researchers have studied the gelation process using different techniques. However, all they needed was long gelation times and small volume to avoid time-related noise in the measurements. This situation is certainly far from gelatin application conditions. In view of such restrictions,

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Table 1–Experimental conditions. Common conditions for all experiments: Type I B gelatin, [Gel] = 2 wt% in H_2O, cooling rate applied to each solution: 0.5, 1.0, 1.5, and 2.0 °C/min.

Nr	[NaCl] (wt%)	pH	Nr	[NaCl] (wt%)	pH	Nr	[NaCl] (wt%)	pH
A1	0	2.5	B1	1	2.5	C1	3	2.5
A2	0	3.5	B2	1	3.5	C2	3	3.5
A3	0	5.5	B3	1	5.5	C3	3	5.5
A4	0	7.5	B4	1	7.5	C4	3	7.5
A5	0	8.5	B5	1	8.5	C5	3	8.5
A6	0	10.0	B6	1	10.0	C6	3	10

the search of features of phase transformation, which refer only to the biopolymer properties and its solvation conditions, is imperative (Sobral and Habitante 2001; Joly-Duhamel and others 2002b).

The aim of this paper is to introduce a concept and an associated methodology useful to study the phase transformation process independently of the history of the system. The concept depends on the gelatin molecule and its solvation conditions only. The influence of the history of the system and size of the solution volume are formally eliminated from the concept. Phase diagrams showing the phase transformation process will be presented. The dimensions of the diagrams correspond to transformation temperature (T_T), pH, salt concentration ([NaCl]), and cooling rate (Cr) of the gelatin solution. The concept and the associated methodology may be applied to other biopolymers with the ability to gel (Blanco and others 2007). In addition, the concept can be used in computational rheology models and molecular dynamics approaches.

Materials and Methods

Materials

Technical-grade type I B gelatin (alkaline conditioned gelatin derived from bovine skin collagen) with Bloom 180-260 (ANMAXFG3 PB Leiner, Sauce Viejo Argentina) was selected as case study material. Sodium hydroxide (Cicarelli, San Lorenzo Argentina), hydrochloric acid, HCl (Cicarelli, San Lorenzo Argentina), and sodium chloride (Metraquimica, San Martin Argentina) were used for pH and salt concentration adjustments. All reactants were of proanalysis A.C.S. quality. The water was of Millipore quality (18.2 MΩ/cm).

Experimental set-up

The experimental set-up consisted of a double-jacketed glass reactor (Fermatec, Santa Fe Argentina) connected to a heating–cooling thermostat (PolyScience, Pasadena U.S.A.) with temperature control within 0.1 °C. The reactor permitted to measure simultaneously the dynamic viscosity, pH, and temperature of the gelatin solution during the early stages of the gelation transformation. The viscosity measurements were performed using a viscometer (Canon Instruments Model2020, Canon Instruments, State College U.S.A.) with spindle Nr 61 (18.85 mm in diameter) spinning at 100 rpm. The shear rate was set at 5.1/s according to the Nguyen equation (Billon 1996). The pH and the temperature were measured using a pH/ORP meter (Hanna instruments HI 98160, Hanna Instruments, Woonsocket U.S.A.) connected to a PC with online data acquisition (Hanna instruments HI92000 507).

Sample preparation and experimental conditions

Aqueous gelatin solutions (2 wt%) were prepared at different pH values and salt contents. For the preparation of each gelatin solution, the following procedure was applied. First, 8 g gelatin was swelled in 10 g H_2O at room temperature during 30 min. Second, the swelled gelatin was completely dissolved in 378 g H_2O at moderate stirring for 40 min at $T_0 = 50$ °C. Subsequently, the pH and the salt content of the solution were adjusted. The solution was transferred to the glass reactor. The temperature of the gelatin solution was kept at 50 °C for 30 min to ensure the absence of temperature gradients. Then the solution was cooled at a defined Cr until the observation of a sharp increase of its viscosity. The viscosity, pH, and temperature of the system were followed online during this process. For each procedure, a new fresh gelatin solution was used. Four Cr were applied to each combination of pH and salt content in the gelatin solution. The Cr were 0.5, 1.0, 1.5, and 2.0 °C/min. Table 1 presents all conditions. In addition, replicas of the procedures were performed recycling the same gelatin solution during the 4 cycles of cooling. The 1st gelled solution was heated to 50 °C and kept at this temperature for 30 min prior to a new cooling cycle at a different Cr.

Determination of the gelling point

The viscosity and the temperature of the system were plotted as function of time. Figure 1 shows a typical plot of temperature (T) and viscosity (η) against time (t). These data were obtained for all experiments.

The left part of the plots corresponds to the solution stage of the system. Here, the system behaves as a liquid solution whose viscosity gradually increases with the decrease of the temperature. The right part of the plots corresponds to the gel structuring stage of the system. Here, the viscosity increases sharply with the decrease of the temperature due to the building up of the gel microstructure. The gelling point occurs in the limit between these 2 stages.

The criteria for the determination of the gelling point are the subject of constant discussion in the scientific community. We

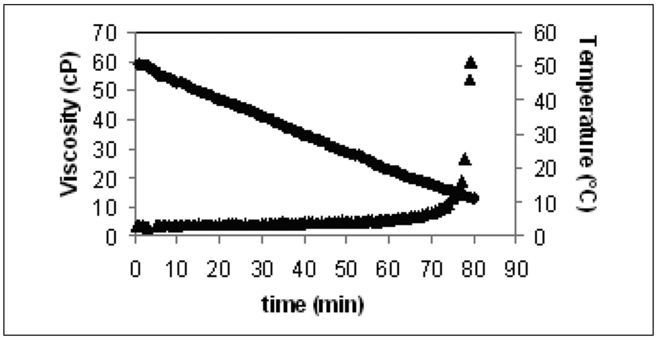

Figure 1–Plot of viscosity–temperature compared with time. Conditions: 2 wt% aqueous solution of bovine type IB gelatin, [NaCl] = 0 wt%, pH = 3.83, and cooling rate = 0.5 °C/min (procedure Nr A2). Viscosity: (▼); temperature: (●).

Table 2–Experimental results. T_T of 2 wt% aqueous gelatin solution at different pH, [NaCl], and cooling rate (Cr).

Nr	[NaCl] wt%	pH	Cr °C/min	T_T °C	Nr	[NaCl] wt%	pH	Cr °C/min	T_T °C	Nr	[NaCl] wt%	pH	Cr °C/min	T_T °C
A1	0.0	2.8	1.8	2.3	B1	1.0	2.5	2.1	9.5	C1	3.0	2.6	2.1	11.6
			1.2	2.9				1.4	8.4				1.3	12.6
			0.9	4.2				0.9	11.7				0.9	12.6
			0.5	8.3				0.5	12.9				0.5	13.9
A2	0.0	3.8	2.0	9.3	B2	1.0	3.7	2.3	9.3	C2	3.0	3.6	1.7	12.4
			1.3	11.4				1.4	9.3				1.3	12.1
			0.9	12.5				0.9	13.3				0.9	13.5
			0.5	12.7				0.6	14.5				0.5	15.0
			0.2	13.6	B3	1.0	5.7	2.1	10.5	C3	3.0	5.7	2.1	11.0
A3	0.0	5.9	2.1	14.5				1.5	10.5				1.3	12.6
			1.3	15.8				0.9	13.1				1.0	12.6
			1.0	16.8				0.5	15.7				0.6	14.5
			0.5	18.1	B4	1.0	7.3	2.2	12.1				0.5	14.7
A4	0.0	7.5	1.6	11.4				1.3	13.1	C4	3.0	7.5	2.1	10.5
			0.9	12.3				0.9	14.9				1.4	11.9
			0.6	14.6				0.5	16.6				1.1	12.5
			0.3	16.9	B5	1.0	8.9	2.4	8.6				0.5	14.1
A5	0.0	8.5	2.0	7.8				1.6	11.1	C5	3.0	8.6	2.1	9.6
			1.3	11.4				1.0	13.7				1.3	11.2
			1.0	12.0				0.5	15.3				0.9	12.3
			0.5	15.1	B6	1.0	10	2.2	8.7				0.5	13.2
A6	0.0	10.1	1.8	9.0				2.0	9.0	C6	3.0	10.0	2.0	8.5
			1.1	10.4				1.2	11.6				1.2	10.3
			0.9	10.2				1.1	12.1				0.8	11.4
			0.5	12.1				0.5	13.5				0.5	12.7

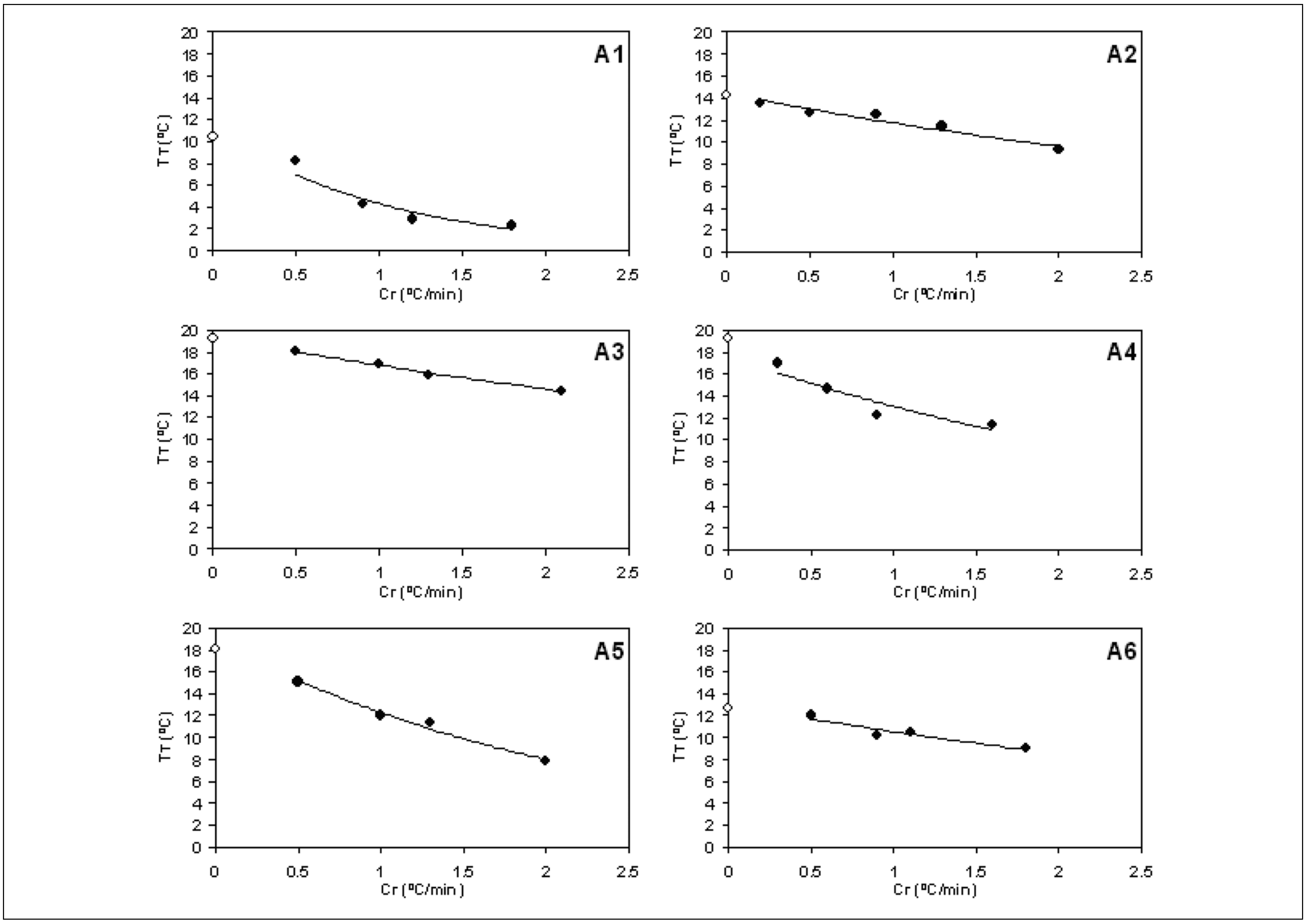

Figure 2–T_T compared with Cr of 2 wt% gelatin aqueous solution and [NaCl] = 0 wt%. A1: pH = 2.77, A2: pH = 3.83, A3: pH = 5.86, A4: pH = 7.47, A5: pH = 8.45, A6: pH = 10.12. Extrapolation to Cr = 0 °C/min (○).

Table 3–Exponential fitting coefficients for T_T(Cr).

	$T_T = T_T^{\mathrm{Cr}=0} \exp(\beta\ \mathrm{Cr})$		
Nr	$T_T^{\mathrm{Cr}=0}$ °C	β	R^2
A1	10.2	−0.96	0.988
A2	14.4	−0.20	0.959
A3	19.3	−0.14	0.987
A4	18.9	−0.30	0.987
A5	17.5	−0.43	0.981
A6	12.0	−0.21	0.991
B1	13.7	−0.22	0.968
B2	15.3	−0.27	0.986
B3	16.8	−0.26	0.984
B4	17.0	−0.18	0.991
B5	16.7	−0.18	0.995
B6	15.0	−0.24	0.990
C1	15.8	−0.10	0.970
C2	16.2	−0.17	0.980
C3	16.1	−0.18	0.940
C4	15.8	−0.18	0.999
C5	15.6	−0.20	0.999
C6	15.4	−0.20	0.990

Table 4–Polynomial fitting coefficients for $T_T^{\mathrm{Cr}=0}$(pH).

	$T_T^{\mathrm{Cr}=0}(\mathrm{pH}) = a\,(\mathrm{pH})^2 + b\,(\mathrm{pH}) + c$			
[NaCl] (wt%)	a	b	c	R^2
0	−0.632	8.4	−8.37	0.9994
1	−0.188	2.6	8.43	0.9989
3	−0.029	0.3	15.40	0.9982

applied the following criteria: The left and the right parts of the viscosity compared with time plot were fitted with exponential equations according to the Andrade's empirical equation: $\eta(t) = A \exp(-\alpha\ t)$. Here, A and α are empirical constants determined by least squares. The correlation value (R^2) resulted higher than 0.99 in all cases. The gelling time, also called the transformation time (t_T), was taken at the intersection between the resulting fits for $\eta^{\mathrm{sol}}(t)$ and $\eta^{\mathrm{gel}}(t)$. Here, the superscripts "sol" and "gel" indicate the fits for the solution and gel parts of the viscosity compared with time plot. Then t_T was introduced as input in the temperature compared with time plot to obtain the gelling temperature, also called the T_T, at a given condition of pH, [NaCl], and Cr. The intersection between $\eta^{\mathrm{sol}}(t)$ and $\eta^{\mathrm{gel}}(t)$ can be physically understood as the end of the viscous solution stage and the starting point for the building up of the gel microstrusture. Is important to be mentioned that several formulas for fitting were evaluated including the Ostwald de Waele power law model. However, the best correlation value was obtained with the Andrade's empirical formula.

Experimental reproducibility

The reproducibility of experimental results was evaluated. The procedure as described above was repeated 6 times. Minor

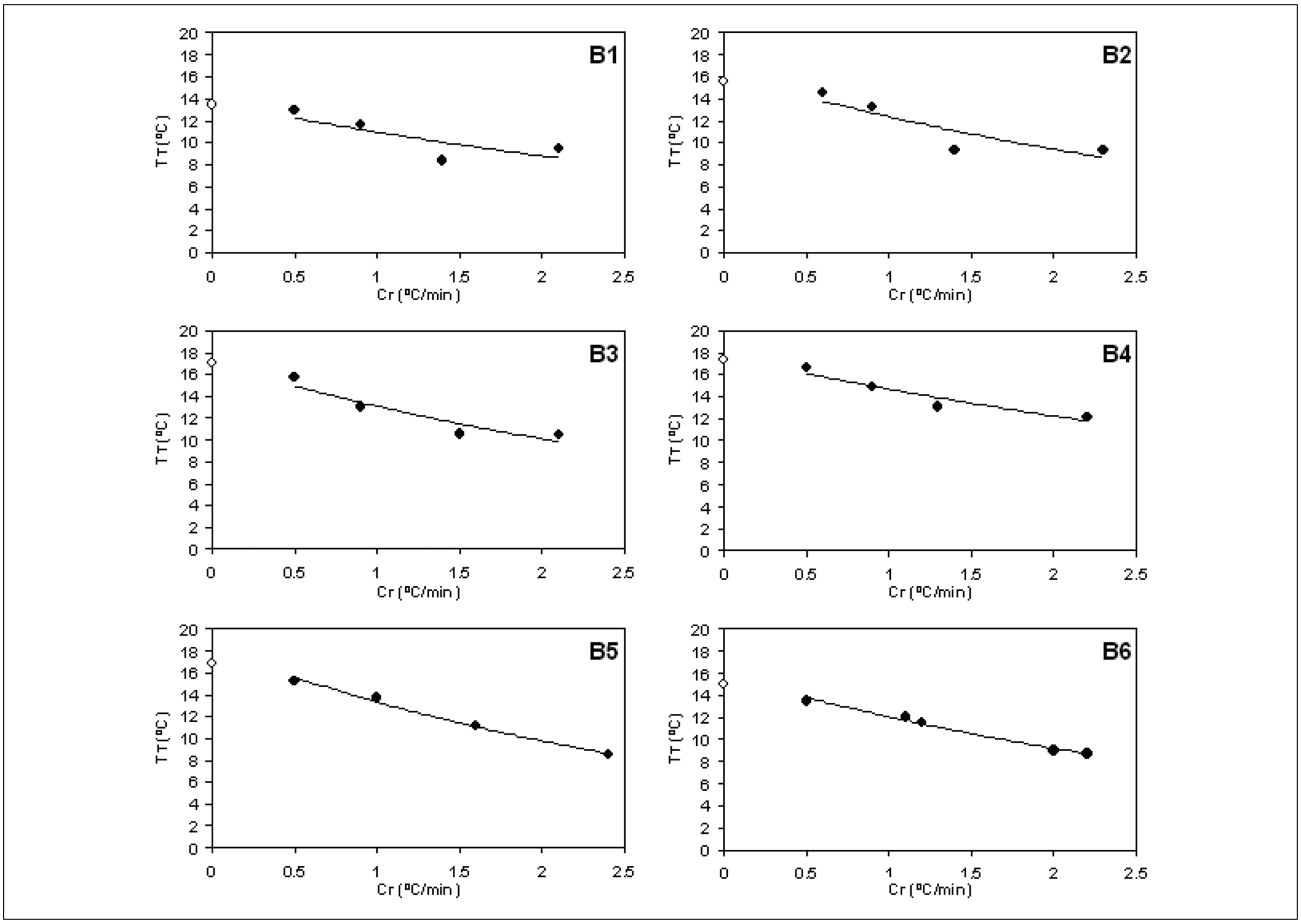

Figure 3–T_T compared with Cr of 2 wt% gelatin aqueous solution and [NaCl] = 1 wt%. B1: pH = 2.45, B2: pH = 3.67, B3: pH = 5.68, B4: pH = 7.31, B5: pH = 8.96, B6: pH = 10.00. Extrapolation to Cr = 0 °C/min (○).

variations between the plots were observed. The variations resulted in less than 2% for temperature–time plots and in nearly 5% for viscosity–time plots, thus demonstrating good experimental reproducibility. In addition, no significant variations in the obtained results were observed for the gelation behavior of fresh and recycled gelatin solutions.

Results and Discussion

T_T and t_T were experimentally determined for each condition detailed in Table 1. T_T and t_T varied with the Cr, the pH, and [NaCl] of the gelatin solution. Table 2 summarizes all results.

Mathematically, $t_T = (T_0 - T_T)/\text{Cr}$. Clearly, $t_T \rightarrow \infty$ when, $\text{Cr} \rightarrow 0$ and $T_T \rightarrow T_T^{\text{Cr}=0}$. This situation, justify the paper focus on temperature analysis rather than time analysis. Set and actual values for pH and Cr were slightly different. Set values will be used in general explanations and discussion to facilitate comprehension and actual values will be used in tables and figures to preserve accuracy.

The experimental T_T of gelatin solutions at different pH and [NaCl] were plotted against the corresponding Cr. Figure 2 to 4 may be useful to visualize the variation of the T_T with [NaCl] between 0 and 3 wt%, pH between 2.5 and 10 and, Cr between 0.5 and 2 °C/min.

The analysis of the tendency plots served to describe the general behavior of the system. All plots show the tendency of T_T to decrease with the increment of the Cr. Moreover, the T_T decreasing rate resulted faster for Cr in the range of 0.5 to 1.0 °C/min than for Cr in the range of 1.0 to 2.0 °C/min. At [NaCl] = 0 wt% and pH = 2.77, T_T decreased from nearly 8.3 to 4.2 °C and from 4.2 to 2.3 °C as the Cr varied from 0.5 to 0.9 °C/min and from 0.9 to 1.8 °C/min, respectively (Figure 2A1). This effect was more pronounced at low salt concentration and low and high pH. The pH affected T_T in other ways. For all conditions, it was observed that T_T increased from pH about 2 to 4.5, reaching a maximum value at pH 4.5 to 5.5. Afterwards, T_T decreased from pH of about 5.5 to 10. The increment of [NaCl] diminished the effect of Cr and pH on T_T. Interestingly, the increment of the [NaCl] raised T_T at low and high pH and diminished T_T at a pH of about 5. At a pH of about 2.5 and Cr of about 2 °C/min, T_T increased from 2.3 °C to 11.6 °C with the increment of [NaCl] from 0 to 3 wt%. Similar, but weaker, behavior could be observed at high pH. At a pH of about 5 and Cr approximately 2 °C/min, T_T decreased from 14.5 to 12.6 °C as the [NaCl] varied from 0 to 3 wt%. Table 2 and Figure 2 to 4 may be useful for further analysis.

$T_T^{\text{Cr}=0}$ values at different [NaCl] and pH were obtained by fitting the curves in Figure 2 to 4 with $T_T = T_T^{\text{Cr}=0} \exp(\beta\ \text{Cr})$. Here, $T_T = T_T^{\text{Cr}=0}$, when Cr = 0 °C/min. Table 3 and Figure 5 present the dependency of $T_T^{\text{Cr}=0}$ on the pH and the [NaCl]. At [NaCl] = 0 wt%, $T_T^{\text{Cr}=0}$ rapidly increased from nearly 10.5 to almost 19.5 °C with the increment of pH from 2.5 to 7. Subsequently, $T_T^{\text{Cr}=0}$ decreased sharply from 19.5 to near 15.5 °C with the increment of pH from 7 to 10. At [NaCl] = 1 wt%, the increase–decrease behavior was maintained within the same pH

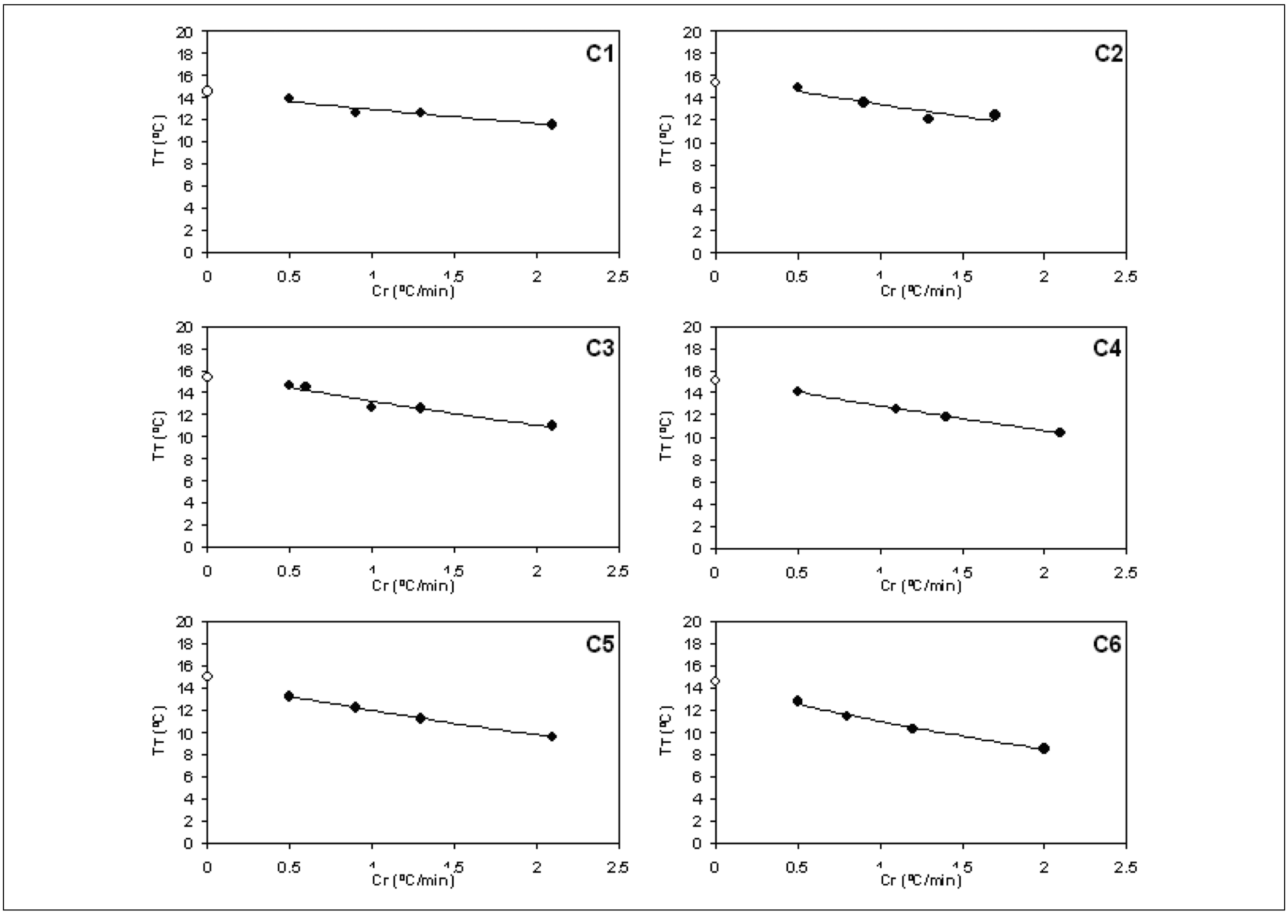

Figure 4–T_T compared with Cr of 2 wt% gelatin aqueous solution and [NaCl] = 3 wt%. C1: pH = 2.58, C2: pH = 3.65, C3: pH = 5.68, C4: pH = 7.48, C5: pH = 8.64, C6: pH = 10.00. Extrapolation to Cr = 0 °C/min (○).

ranges. However, the variations of $T_T^{Cr=0}$ were smaller than at [NaCl] = 0 wt%. $T_T^{Cr=0}$ resulted in about 13.5 °C at pH around 2.5, reached a maximum of 17.5 °C at a pH of about 7, and decreased to nearly 15 °C at pH = 10. Finally, at [NaCl] = 3 wt%, the influence of pH on $T_T^{Cr=0}$ was even smaller than the pH effect at [NaCl] = 1 wt%. $T_T^{Cr=0}$ increased from 15.5 to 16.5 °C as the pH increased from nearly 2.5 to nearly 7. Then $T_T^{Cr=0}$ decreased from 16.5 to 15.5 °C as the pH increased from 7 to 10.0.

The functionality of $T_T^{Cr=0}$ with pH at different [NaCl] was fitted using a grade 2 polynomial empirical relation. The polynomial coefficients and the corresponding correlation value (R^2) are listed in Table 4.

$T_T^{Cr=0}$ analysis

The $T_T^{Cr=0}$ parameter is proposed as a history independent parameter for the evaluation of phase transformation mechanisms in biopolymers. $T_T^{Cr=0}$ is proposed to depend on the properties of the biopolymers and its solvation conditions only. In the "Introduction" section is described how sensitive the transformation of gelatin is to the history of the system. The evaluation of the gelling temperature at Cr = 0 °C/min, that is by hypothetically infinite slow cooling and being at such temperature the transformation temperature, permits us to obtain information about the transformation mechanism without any noise introduced by time-related phenomena. Therefore, the transformation can be considered to occur under thermodynamic equilibrium.

The study of transformations under thermodynamic equilibrium is simpler than under non-equilibrium conditions. In this case, the usual hypothesis of absence of thermal, pressure, and chemical gradients became formally valid, and calculations of the free energy, entropy, enthalpy, and other thermodynamic potentials, used to define a transformation phenomenon, reach the nearest to real values. Knowledge of these thermodynamic potentials is useful as input data for molecular dynamics, statistical thermodynamics, and quantum physical–chemical calculations and models.

Influence of the Cr

The decreasing of T_T with the increment of the Cr can be explained in terms of homogeneous nucleation during the phase transformation process. The conformation transformation of the biopolymer from the random coil to the folded triple helix implies the apparition of a triple helix nucleus during the earliest stages of transformation. A nucleus is formed by the thermal spontaneous arrangement of molecules. Once formed, the nuclei grow and anneal, transforming sections of biopolymer from random coil to triple helix conformations. An interphase can be assumed to exist between random coil and triple helix sections of the biopolymers. The presence of such interphase implies the need of extra energy. Normally, the faster the Cr, the higher is the need for undercooling to compensate this extra interphase energy. This undercooling is experimentally observed as a decrease of the T_T. Furthermore, the increment of the Cr favors nucleation at the expense of growing and annealing processes. Gels obtained at high Cr are more disordered than gels obtained at low Cr.

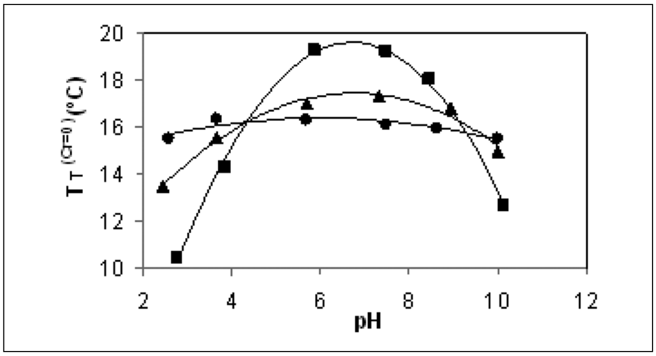

Figure 5–T_TCr=0 compared with pH and [NaCl]. [NaCl] = 0 wt% (■); [NaCl] = 1 wt% (▲); [NaCl] = 3 wt% (●).

Influence of pH

Gelatin is a polyampholyte. It means that positive and negative charges are present in the solvated biopolymer (Dobrynin and others 2004). The variation of the pH milieu may alter the distribution and charge balance in the biopolymer. At low and high pH, the polypeptide acquires a net positive and negative charge, respectively. At pH between 5 and 7, the biopolymer reaches the isoelectric point. Net positively or net negatively charged biopolymers in solution would tend to separate from each other by electrostatic repulsion. Such repulsion diminishes the probability for spontaneous arrangement of a triple helix nucleus. Consequently, higher thermal undercooling is necessary to compensate for the electrostatic repulsion. At the isoelectric point, the net charge is minimal and spontaneous nucleation is more probable. Finally, at low and high pH, the transformation from random coil to triple helix occurs at lower temperature than at pH between 5 and 7.

Influence of [NaCl]

The presence of extra ions in solution plays an interesting role. On the one hand, at pH lower than 5 and pH higher than 8, the salt masks the repulsion effect of the net positive and net negative charge of the biopolymer, allowing the biopolymers to approach and to rearrange in the new phase form. On the other hand, the ions in solution seem to impair the transformation when pH is between 5 and 8. As a result, the higher the salt concentration; the lower is the transformation temperature of gelatin at pH near the isoelectric point of the biopolymer. The formation of an ion layer on the surface of the biopolymer is proposed to be responsible for this effect. Such layer may be stable in the random coil conformation, but unstable in the triple helix conformation. The extra energy needed to remove the ion layer from the surface of the biopolymer prior to its transformation to triple helix may justify the increased undercooling for phase transformation at higher salt content near the isoelectric point.

Conclusions

The sol–gel transformation of type IB gelatin under different conditions was extensively examined. In particular, the conditions for gelation were modified by varying pH, [NaCl], and Cr. This investigation provides a clear comparison of the simultaneous effects of pH, [NaCl], and Cr on the sol–gel transformation of gelatin. Modification of the charge balance, clouding of the net charge, and the formation of structure-dependent ion layers around the biopolymer are proposed to explain effects of pH and [NaCl] on the T_T of gelatin in solution. In addition, Cr seems to modify the nucleation mechanism of new triple helix structures within preexisting random coil conformations of the biopolymers. A new concept, and an associated methodology to determine it are introduced here for the study of phase transformation in biopolymers. The $T_T^{Cr=0}$ parameter may permit us to study the transformation process under thermodynamic equilibrium and avoidance of noise coming from time-related phenomena.

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Author Contributions

F. Bellini assembled the experimental set-up and conducted the experiments. I. Alberini characterized the gelatin. M.G. Ferreyra collected test data and drafted the manuscript. I. Rintoul designed the study and interpreted the results.

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