Structure and rheology of gelatin gels: recent progress*

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This paper reviews recent progress in the study of gelatin gels. For example, it is now established that gel junction zones do not have a large cross-sectional radius of gyration, and both the modulus and the absolute optical rotation appear to increase slowly, but without limit, even when plotted on a log time axis. Such data are not consistent with substantial side-by-side aggregation following the initial renaturation of the collagen-like triple helix. The rheological consequences of this are discussed. At very low concentrations, atypical effects are seen, indicating the existence of a transient weak network.

(Keywords: gelatin; collagen; structure–property relations; rheology; gel; viscosity; gelation; scattering; microscopy)

INTRODUCTION

The structure and mechanical behaviour of gels from the polypeptide gelatin have been widely studied in the past[1,2], and indeed the term 'gel' (attributed to Thomas Graham) is derived from gelatin. In our recent review of the structural and mechanical properties of biopolymer gels[3], we categorized the wide range of different modern techniques into 'molecular', 'macromolecular' and 'supramolecular'. Each of these probe essentially different distance scales, and furnish information of a complementary nature. The respective length scales are defined as: 0.1–10 nm (molecular), probed e.g. by chiroptical techniques[4]; $10–10^4$ nm (macromolecular), e.g. light scattering; and $>10^4$ nm (supramolecular), particularly classical rheological methods. In the present paper we will describe recent progress that has led to an improved understanding of the behaviour of gelatin gels in these terms. Most of the work described here was carried out using essentially the same sample, a high-molecular-weight ossein supplied by Rousselot.

MOLECULAR STRUCTURE OF GELATIN SOLS AND GELS

Gelatin is derived by hydrolytic degradation of collagen, the principal protein component of white fibrous connective tissue (skin, tendon, bone, etc.). The fundamental molecular unit of collagen is the tropocollagen rod, a triple helical structure composed of three separate polypeptide chains (total molecular weight $\approx 330\,000$, persistence length ≈ 180 nm). The precise amino acid content and sequence varies, but always consists of large amounts of proline, hydroxyproline and glycine. The proline content is particularly important, as it tends to promote formation of the polyproline II helix, which ultimately determines the form of the tropocollagen trimer.

Gelatins normally dissolve in warm water ($\gtrsim 40°C$), and above this temperature the polypeptide exists as flexible single coils. On recooling, transparent gels are formed (provided the concentration is greater than some critical concentration, C_0, typically 0.4 to 1.0%). These are now generally accepted to contain extended physical crosslinks or 'junction zones' formed by a partial reversion to 'ordered' triple helical collagen-like sequences, separated along the chain contour by peptide residues in the 'disordered' conformation. The main evidence for this has come from optical rotation measurements, since the sign and magnitude of rotation of plane-polarized light transmitted through a solution can be correlated, at least semi-empirically, with the torsion angle specifying the relative orientation of adjacent peptide residues. Measurements of the specific rotation of tropocollagen and of 'denatured' gelatin at high temperatures can readily be made, and the specific rotation of cooled gelatin sols (and gels) then allows an estimate of the relative amount of helix—the proportion of peptide residues in the triplex[5]—to be made.

In most biopolymer systems, including DNA and a number of polysaccharides (agarose, the carrageenans), the coil to double helix naturation occurs very fast[6] (even very close to the transition temperature) and resembles a true first-order phase transition. For gelatin, however, it has long been known that there is an initial phase, lasting several hours, followed by a much slower process that appears to continue for very long times. For this reason the helix nucleation step is believed to be slow, but the subsequent propagation is even slower.

Mechanism of coil to helix renaturation

Because of this, it is very difficult to access the equilibrium state, so that temperature scanning studies are difficult to interpret and many authors have focused

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on isothermal renaturation. For gelatin Flory and Weaver[7] proposed a simple first-order kinetic scheme, whilst Harrington and coworkers[8,9] reviewed several first- and second-order models. For all of these, it is obligatory to include limiting values, and to neglect the slow, long-time change. A best fit proposed by Djabourov[10] combined first-order and logarithmic time processes, whilst Weidner and coworkers[11] using relaxation measurements found a continuous spectrum of times, again confirming the complexity of the process. From d.s.c. results Godard and coworkers[12] have proposed that gelatin renaturation may be described by Avrami kinetics, and suggested the analogy between renaturation and crystallization. The Avrami exponent is close to unity, so that this approach is identical to first-order kinetics. Recent studies by Djabourov and Papon[13] and by Durand and coworkers[14] confirm that simple kinetics can only be observed in the early stages of renaturation.

Until recently it was thought that the gelatin triple helices involved three separate intermolecularly wound peptide chains, as in the original tropocollagen helix, and each chain participates in several such junction zones. The topological consequences of this on subsequent helix formation and gelation are very significant, and perhaps for that reason are scarcely ever discussed! However, on the basis of the concentration-dependent order of kinetics (first order at low concentrations, increasing to just greater than second order at higher) observed by chiroptical techniques, it was proposed that helix nucleation was a *bimolecular* process, involving an intramolecular β-turn (a metastable 'hairpin' promoted by adjacent glycine and/or proline residues) and another gelatin macromolecule[15,16]. When a third segment meets a 'kink' with the correct orientation, the triple helix can be initiated. *Figure 1* illustrates the alternative hypotheses. Stainsby has recently suggested how this postulate may be tested by biochemical methods[17]. In particular, the hairpin bend requires a triple peptide sequence with only one imino (proline or hydroxypryoline) residue, and with suitably flexible neighbouring sequences. This is very different from earlier suggestions, which require that adjacent proline-rich sequences are required. An investigation of homogeneous chain fragments of known sequence should then enable the alternative hypotheses to be tested. He also notes how such studies would allow the future development of 'designer' gelatins with improved properties.

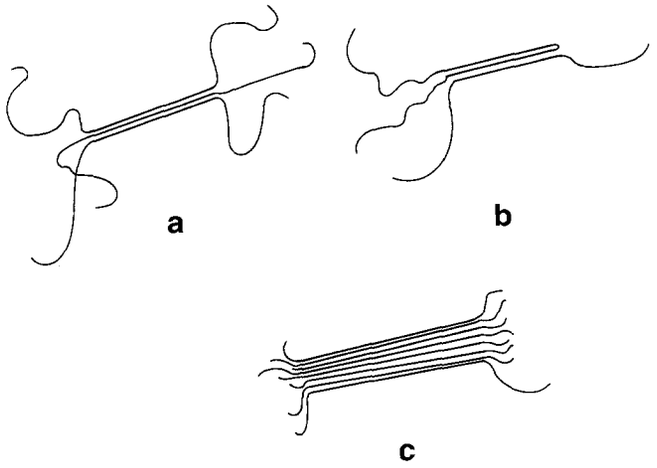

Figure 1 (a) Intermolecular triple helix. (b) Bimolecular triple helix with hairpin bend as proposed in refs. 15 and 16. These are represented as parallel lines, although the real structure consists of three interwound strands. (c) Side-by-side aggregation of helices as originally suggested to describe gelatin junction zones

The critical helix nucleation length

The nuclei are, of course, not stable unless a critical minimum size is reached, which depends on the temperature, and corresponds to the balance between an initial loss of entropy and the enthalpic stabilization due to helix formation. The critical size has usually been estimated from observations on very low-molecular-weight gelatin samples, since these cannot renature at all. Various estimates have been made from 40–80 peptide residues[18] to 20–30[8]. Recently Busnel and coworkers reported a lower limit of about 20 residues using narrow fractions obtained by preparative g.p.c.[16], whilst Weidner *et al.* showed that a 36 residue peptide can renature[11]. For such short oligopeptides there is no real possibility of intramolecular refolding, and we assume that at low temperatures the nucleus is formed by three strands of about 20 residues. The size of the nucleus should be[7] $\propto 1/(T_f - T)$, where T_f is the melting point of collagen (36°C). Below 20°C the nucleus remains only a few per cent of the primary chain length.

It is also now thought that the coil to triple helix propagation rate is limited by the presence of *cis*-proline residues in the backbone[19]. Reversion to the *trans* form allows the helix to extend only gradually, so that the overall growth rate is typically 4–6 orders of magnitude slower than for double helical systems. The very long-time behaviour is then associated with a slow 'shuffling of partners' as the peptide chain achieves a more ordered state. Again biochemical methods may be useful to test this idea.

Isolated helices vs. side-by-side aggregation

It was often believed that the initial helix formation was followed by substantial lateral aggregation leading to extended 'quasi-crystalline' junctions, as envisaged in the 'fringed micelle' model of crystallization, and as seems to be the case with many thermoreversible gels of biological (agarose, carrageenan) and synthetic (poly(vinyl chloride) (PVC), tactic polystyrene (PS)) origin[20]. However, a number of studies have challenged this model, and suggested that junction zone aggregation is not widespread, rather the helices 'shuffle' at long times to increase the proportion of peptides in the ordered conformation. Indeed, much of the evidence is against side-by-side aggregation. Even though they are not highly charged (cf. carrageenan) gelatin gels are generally quite transparent, so the degree of aggregation cannot be great. Further, since long-time measurements of the optical rotation increase slowly, but apparently without limit (even when plotted on a log time axis), then the proportion of residues in the ordered helical conformation must also be increasing. This suggests a considerable degree of conformational flexibility, even post-gel, and is rather unlikely to occur if the junction zones are formed of rigid crystallites.

To investigate the degree of side-by-side aggregation, the technique of small-angle X-ray/neutron scattering seems ideal since the cross-sectional mean-square radius, R_c, of the isolated macromolecule should be ca. 0.3 nm. Such measurements have recently been reported by

Djabourov and her coworkers using SANS[21]. They found that in the sol state R_c was 0.32 ± 0.1 nm, a value in good agreement with the calculated side-group extension of a collagen chain, whereas in a relatively concentrated gel (5%), R_c was 0.43 ± 0.1 nm, in agreement with the non-aggregated triple helix model. By contrast SAXS measurements for agarose, which is thought to be substantially side-by-side aggregated, give $R_c \approx 0.26 \pm 0.08$ nm for the coil, and an apparently bimodal population with $R_c \approx 1.0 \pm 0.1$ and 2.9 ± 0.3 nm in the cold set gel—both populations being much 'thicker' than the hot form[22].

The critical degree of helix formation

One particular series of experiments by Djabourov and by Durand and their respective coworkers are worthy of mention. The former group were able to show that at the gel point (determined viscometrically) the *proportion* of residues in the helical conformation at the gel point, h_c, monitored by optical rotation, was $\sim 1/15$, independent of the temperature[23], whilst the latter workers established that there was a linear relation between h_c and reciprocal concentration[24]. This observation is quite consistent with competition between inter- and intramolecular helix formation.

MACROMOLECULAR STRUCTURE

Quasi-elastic and inelastic light scattering

One of the active groups in this area, ter Meer *et al.*[24] comment that 'it comes as a surprise that hardly any experimental evidence for the intermediate macromolecular range ... has been presented'. In their isothermal experiments they investigate the time-dependent molecular-weight increase (actually the normalized second moment of the cluster distribution, M_2) on quenching sub-gelling concentrations using integrated light scattering, together with concomitant optical rotation studies. Results from the former are discussed in terms of a kinetic branching model, whilst simultaneous q.e.l.s. measurements allow the apparent fractal (Hausdorff) dimension d of the clusters to be estimated from a plot of $\log(1/M_2)$ vs. $\log(R_h)$, with R_h the Stokes radius. The value of d is very close to 1, suggesting a predominantly linear cluster growth. Of course, some branching must then take place, and the authors are careful to point out that this result only applies for the early stages of growth and for their sub-gelling concentrations.

Herning *et al.* have employed q.e.l.s. to investigate the conformation of gelatin sols (50°C) in dilute and semi-dilute solution[25]. In semi-dilute solutions they have analysed the two diffusive modes observed, the so-called 'slow' and 'fast' modes. The fast mode exponent is consistent with marginal solvent behaviour (χ for the 'aqueous' gelatin system is known to be just less than 0.5). The slow mode is more equivocal, but suggests the presence of clusters, as have been observed in a number of physical gel systems; these could be suppressed by adding sodium dodecyl sulphate (SDS) surfactant.

Electron microscopy

A number of investigations have been carried out using this technique but care has to be taken to ensure that artefacts do not intrude. Early work[26,27] has suggested that at least some of the junctions are composed of aggregated triple helices. More recent pictures by Favard and coworkers[28] using the technique of quick-freeze, deep-etch rotary replication, in which only a very thin layer of sample (5 μm) is fast frozen and vitrified, show a thin fibrillar network, and estimates of the minimum filament breadth give 1.5–2 nm, larger than from SANS, a result still consistent with isolated triple helices, since the Pt/C coating applied thickens the filaments[23].

SUPRAMOLECULAR STRUCTURE—VISCOELASTIC MEASUREMENTS

Viscoelastic measurements on gelatin gels are exemplified by those carried out by te Nijenhuis[29,30] and by Djabourov and coworkers[10,23]. They have used the small controlled strain oscillatory shear technique to monitor gel formation for solutions quenched rapidly to fixed temperatures. Curves of G' and G'' vs. time were obtained for a range of concentrations and frequencies. For systems allowed to gel under a well defined thermal regime, and allowed to achieve a limiting degree of crosslinking, there is a characteristic 'cure' curve of $\log(G', G'')$ against time[31]. This has an initial lag time, and both G'' and G' increase, but with G' increasing faster than G'' so that at a given time there is a 'cross-over'. Subsequently G' continues to increase, and, for gelatin, there is seemingly no final value of the modulus since the 'shuffling' of helix partners allows $\log(G')$ to increase indefinitely when plotted against log time. The value of G'' usually passes through a parabolic maximum, and then decreases to zero, an effect associated with the relaxation of 'dangling chain ends'[32]. *Figure 2* shows data from our own measurements[33] on a 3.8% gelatin sol/gel. Although initially $G' > G''$, this is an experimental artefact due to low torque signal, and allowing for this, the traces of G' and G'' have the expected profiles. *Figure 3* shows G^*, the complex modulus, vs. time for a range of concentrations from 1.1 to 6.6%. As C increases, the gel time t_c decreases (here this can be judged from the time when $G^* > 0.1$ Pa), and the 'final' modulus increases.

There is much interest currently in the more precise determination of gel times from cure curves, following work by Winter and coworkers[34] on poly(dimethyl siloxane) (PDMS) networks. They assert that t_c corresponds to that time when $G' = G''$ over a wide range of frequencies, i.e. both moduli are proportional to ω^x, and

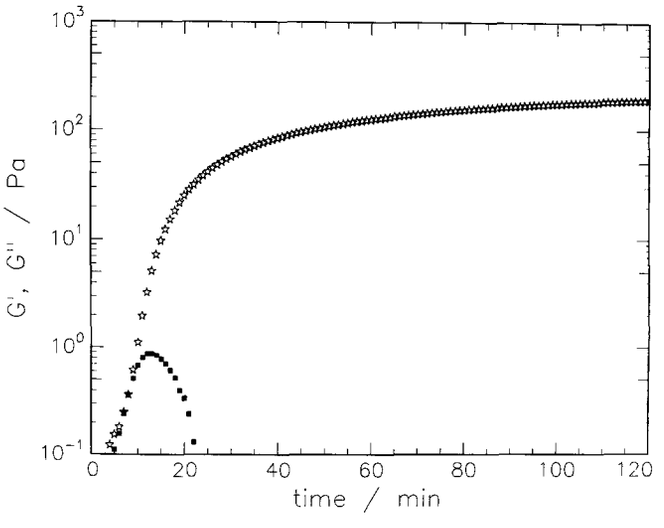

Figure 2 Cure curve: G' and G'' vs. time, for a 3.8% gelatin sol/gel; experimental details as in ref. 33

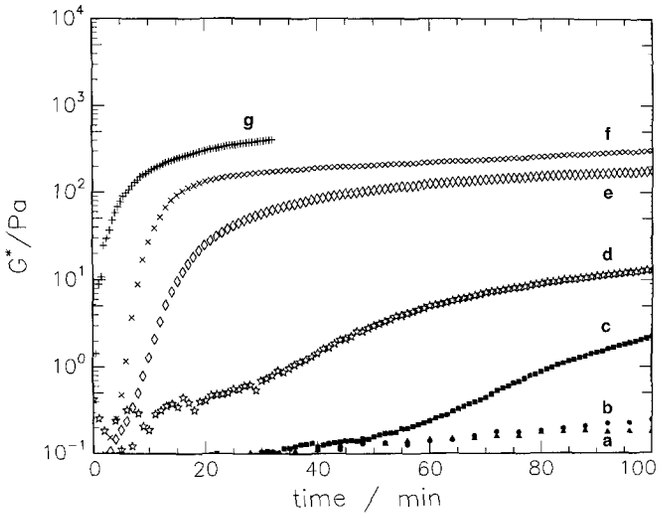

Figure 3 Plot of G^* vs. time for the sample as in *Figure 2*. Nominal percentage concentrations: 1.1% (a), 1.5% (b), 2.1% (c), 2.8% (d), 3.8% (e), 4.5% (f), 6.6% (g)

the exponent x is constant. Te Nijenhuis and Winter have applied the method to physical gels[35], by analysing spectra at different temperatures for PVC plastisols, but this method seems difficult to apply for low-concentration gelatin gels because it dictates that good data can be obtained well before the gel point[33]. Since frequency sweeps are performed 'on the fly', it also requires that t_c is substantially longer than the time required to perform a sweep, otherwise there will be a distortion from the ongoing gelation process.

Even just after the gel time the storage modulus G' of gelatin gels is almost independent of frequency down to frequencies lower than 10^{-2} rad s^{-1}—'a real rubbery network has been formed'[36] and 'mechanical spectra', $\log(G', G'')$ vs. $\log(\omega)$, show that $G''(\omega)$ is lower than but parallel to $G'(\omega)$, as expected for such a network. For highly swollen gels (concentration of gelatin $< 10\%$ w/w, say) there is sometimes a minimum in the mechanical spectrum, centred around 1 rad s^{-1} (0.16 Hz), which is presumably associated with relaxation processes occurring over much longer timescales. Nevertheless, over typical oscillatory frequencies (say $> 10^{-2}$ s^{-1}) there is no indication of 'terminal flow', at least for concentrations above say $4C_0$, and reasonably below the gel melting temperature. Gelatin gels are therefore much more solid than typical entangled melts of linear chains, where motion of large regions of chain (e.g. reptation) lead to substantial reduction of stress on equivalent timescales; such experiments leave open the question of the exact behaviour at very long times.

Creep measurements

This aspect was addressed in our recent constant-stress time-domain (creep) measurements[37]. In these the creep phase viscosity η was found to be $\propto C^{1.1}$, and the 'instantaneous' creep compliance $J \propto C^{-1.7}$. The latter is easy to understand, since for gelatin the literature shows that the low-frequency elastic modulus G (or E) is proportional to C^2 (or some slightly higher exponent such as 2.25). Since $J \approx 1/G$, we would expect $J \propto C^{-2}$, and the difference between -2 and -1.7 is not very significant.

For a network of non-interacting (phantom) chains, G will be proportional to the number of crosslinks. If the rate of formation of crosslinks is an equilibrium process, and the rate of formation (renaturation) is second order in concentration (from chiroptical measurements), $G \propto C^2$. Alternatively, we could consider the effect of entanglements and interactions between non-bonded chains. These would also give $G \sim$ number of contacts $\sim C^2$; the plateau modulus of entanglement systems is indeed usually proportional to $\sim C^2$. Finally if we adopt the 'osmotic scaling' hypothesis, the modulus of a gel is related to the concentration dependence of the osmotic pressure. For good solvents this gives an exponent 2 (or 9/4 if critical fluctuations are included[38]). Although the exponents in each of these cases are not very different, they are deduced from rather different assumptions. Also each neglects details of the specificity of the gelatin system, illustrating how difficult it may be to choose between different models precisely on the basis of the power-law exponent. In any case, close to C_0 much higher exponents, n, are seen (in the limit it can be argued that $n \to \infty$ as $C \to C_0$)[1,39].

As far as the $\eta \propto C^{1.1}$ behaviour is concerned, after very long times only the junction zones (and any 'permanently' trapped entanglements) will support a stress. If the junctions are themselves transient, we expect to reach a terminal flow regime with a creep rate governed by the making and breaking of junctions[40]. Unfortunately, the precise behaviour will probably depend upon the relative contributions of different mechanisms to this rate. In the absence of a back-reaction for helix-coil equilibrium, the mean *length* of helical junction zones should be a decreasing function of concentration, because the rate of helix initiation increases with concentration, whereas the rate of helix growth will be approximately independent of concentration. Because of this, the lifetime of a helix will *increase* rapidly with length, and therefore should *decrease* with concentration. It can then be argued[37] that each junction should contribute *less* to the viscosity as the concentration is increased. Unfortunately, evidence from d.s.c. does not support this argument; rather it seems to suggest that, although smaller helices are formed at low temperatures, they also appear more quickly[15]. It may be that junction zone annealing via melting helices determines the creep behaviour. A further complication is that the number and length of junction zones is related to the temperature of formation of the gel, because the rate of initiation and of propagation of the triple helix will depend upon temperature. At low temperatures the number of helical units formed per unit time will be greater, but the number of such units per junction zone may also be reduced, so that the number of junction zones per chain is increased.

The above discussion affirms that the molecular rheology of gelatin networks is intrinsically rather difficult, because the *potential* number of junction zones per primary chain, the 'functionality' f, and the extent (molecular weight) of the junction along the chain profile can be estimated only indirectly. All these factors must influence the *actual* number of physical crosslinks and consequently the modulus and relaxation behaviour of the final gel. Since the modulus in particular will reflect both entropic (rubber-like elasticity) and enthalpic contributions, an *a priori* description of the mechanical response is bound to involve a number of approximations. Nonetheless a simple phantom network model for G can

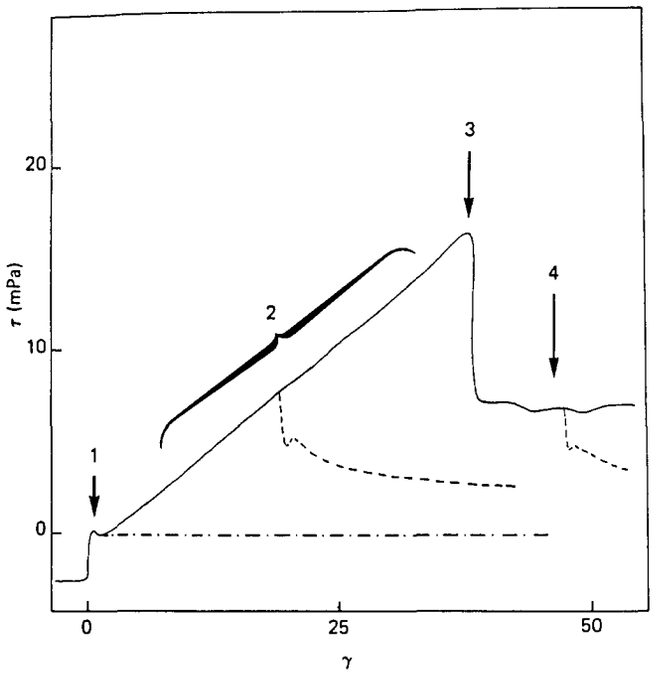

Figure 4 Stress–strain curve for a dilute gelatin solution during renaturation. Concentration 0.1% shear rate 0.277 s^{-1}, ageing time 30 min at 5°C. (1) Small shear overshoot peak. (2) Linear increase of stress vs. strain. (3) 'Rupture' of structure. (4) Viscous response—stress independent of strain. (– – –) Relaxation curves; (— · —) solvent curve. In this case the stress peak extends to ~38 strain units. From ref. 45

give remarkable agreement with molecular parameters[41]. This may merely reflect the unexpected fact that gelatin gels have an elastic front factor close to unity[39]. At the same time, since any particular chain presumably consists of regions of essentially rod-like helical chain (the persistence length, q, of triple helical collagen is $q \approx 180$ nm) and more flexible polypeptide chain ($q \approx 2$ nm), further development of more rigorous models, such as those describing 'reel-chains' and 'rod-chains'[42,43], is essential.

Low-concentration behaviour

As the concentration of gelatin gels approaches and goes below 'C_0'* the viscoelastic behaviour appears to deviate from that expected. There is evidence[44,45] to suggest that pre-critical clustering occurs down to very low concentrations (~100× below that where a true gelation occurs). This is exemplified by solid-like behaviour detected in constant-strain-rate measurements, as shown in *Figure 4*, which breaks down at very large strains. Such behaviour suggests that there is microphase incompatibility, where the sol–gel line crosses the binodal[46]. This gives two concomitant phases, one a small volume fraction of gel, and the other a larger volume of dilute sol (this is not the same as saying that phase separation 'drives' gelation, since aggregation in the one-phase region will radically shift the position of the binodal). Indeed, for gelatin the situation will be even more complex, because the binodal for the coil conformation should resemble that for a flexible polymer[47], and be quite different to that of the helix, which will presumably approach that for rigid rods[48]. Concomitantly the depth of quench may induce spinodal decomposition. Indeed this mechanism, originally suggested for agarose gelation by Prins and coworkers[49], has recently received support from work by San Biagio *et al.*[50,51]. For very dilute systems they suggest that even the 'concentrated' phase may no longer be percolative (i.e. no longer gives a gel). The dilute solution viscosity anomalies for gelatin seem very similar.

* We now use apostrophes to indicate that, for physical gels, there is no sharp 'critical' concentration

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