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Segregative phase separation in agarose/whey protein systems induced by sequence-dependent trapping and change in pH

Lita Katopo[a], Stefan Kasapis[a,*], Yacine Hemar[b]

[a] School of Applied Sciences, RMIT University, City Campus, Melbourne, Vic 3001, Australia
[b] Department of Chemistry, University of Auckland, Private Bag 92019, Auckland, New Zealand

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ABSTRACT

The structural properties and morphology of mixed gels made of aqueous preparations of agarose and whey protein were modified by changing thermal treatment and pH. The conformationally dissimilar polymers phase separated and this process was followed by small-deformation dynamic oscillation in shear, differential scanning calorimetry and environmental scanning electron microscopy. Experimental protocol encourages formation of a range of two-phase systems from continuous agarose matrices perforated by liquid-like whey protein inclusions to phase inverted preparations where a soft protein matrix suspends hard agarose-filler particles. These distinct morphologies have widely different mechanical moduli, which were followed by adapting a theoretical analysis (isostress–isostrain and Lewis–Nielsen blending laws) from the literature in synthetic block polymers and polyblends. Based on this framework of thought, reasonable predictions of the elastic moduli in the composite gels were made that led to patterns of solvent partition between the two polymeric networks. It was shown that proteins, in mixture with polysaccharide, exhibit favorable relative affinity (*P*-factor) for water molecules at a pH above their isoelectric point. This is an unexpected outcome that adds to the central finding of a single *P* value for the distribution of solvent between the continuous matrix and discontinuous inclusions of binary gels. It was thus proposed that phase continuity and solvent distribution in agarose/whey protein systems are under kinetic control that can be heavily governed by pH changes in the aqueous environment.

1. Introduction

Agarose is a structural polysaccharide occurring in different species of red seaweed (*Rhodophyceae*). Its primary structure comprises an alternating repeating sequence of 1,3-linked β-D-galactopyranose and 1,4-linked (3→6)-anhydrogalactopyranose (A and B units, respectively). The 3,6-anhydro units are in the L form, which is reflected in a left-handed helix geometry (Normand et al., 2003). Furthermore, the anhydride bridge may be absent in a proportion of the 4-linked residues, which changes the geometry of the sugar ring to a form that is sterically incompatible with incorporation in the ordered double helix. The presence of this "kinking" residue is essential for the development of gel networks, as it terminates the ordered junction zone association and allows each chain to participate in more than one intermolecular junction zones (Barrangou, Daubert, & Foegeding, 2006).

Agarose molecules are insoluble in cold water but soluble in hot. Their solutions are heat-stable with essentially constant viscosity in neutral pH and give gels even at concentrations of 0.1% or lower. Agarose gels exhibit thermal hysteresis and the melting temperature is dependent on the concentration used (Fujii, Yano, Kumagai, & Miyawaki, 2000). The gel finds a wide range of industrial applications especially as a moulage material capable of reproducing fine details with great accuracy thus being used in dentistry, plastic surgery, criminology, etc. (Puertolas et al., 2011). In USA, it is mostly used in microbiology at liquid media concentrations of 0.007–0.08%. Even at such low concentrations, agarose is able to prevent the entry of oxygen into liquid media, making the cultivation of anaerobes feasible in air-exposed broths (Yokoyama, Kishida, Uchimura, & Ichinole, 2006). Agarose is also used in a wide range of processed food products to improve texture and stability (yoghurts, cream cheeses), as an antitackiness or antistaling ingredient (cookies, cakes, pie fillings, meringues), and in vegetarian and health food products (cereals, meat/fish substitutes) (Barrangou, Daubert, et al., 2006; Barrangou, Drake, Daubert, & Foegeding, 2006).

Traditionally the term "whey protein" has described those milk proteins remaining in the serum after precipitation of the caseins at pH 4.6 and about 20 °C. The major families of proteins included in this class are the β-lactoglobulins, α-lactalbumins, serum albumins and immunoglobulins (Fitzsimons, Mulvihill, & Morris, 2007).

* Corresponding author. Tel.: +61 3 9925 5244; fax: +61 3 9925 5241.
E-mail addresses: stefan.kasapis@rmit.edu.au (S. Kasapis), Y.Hemar@auckland.ac.nz (Y. Hemar).

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The primary amino acid sequence of β-lactoglobulin B consists of 162 amino acid residues with a molecular weight of 18,277, with the corresponding primary structure characteristics of the major α-lactalbumin being 123 and 14,174, respectively. In the pH range from 5.2 to 7.5, β-lactoglobulins exist primarily as dimers, which consist of two spheres with radii of 17.9 Å and a distance from centre to centre of 33.5 Å joined as to possess a dyad axis of symmetry. On the acid side of the isoelectric point, especially below pH 3.5, the dimer dissociates into monomers, with the extent of dissociation increasing as the pH is lowered (Chamani et al., 2006). α-Lactalbumin, on the other hand, exists primarily in neutral and alkaline media as a near spherical, compact globular monomer of about 2.2 nm × 4.4 nm × 5.7 nm. At pH values below the isoelectric point, α-lactalbumin associates to form dimers and trimers, which lead to a polymeric network formation (Nakamura et al., 2010).

There is only a small amount of β-lactoglobulins and α-lactalbumins in whey (about 0.6%), which is a sidestream product of cheese or casein manufacture. Upon concentration, whey powder is produced with an approximate composition of 76% lactose, 13% protein, 10% ash and 1% fat. To make a 35 or 50% whey protein concentrate (WPC), the dilute solution of whey is concentrated by ultrafiltration (UF) that physically separates the whey protein and fat from the lactose and minerals. Spray drying concentrates the whey protein solution from about 35 to 95% total solids, and control of the dryer and atomization of the liquid allows low-temperature drying to maintain product solubility (Thomas, Scher, Desobry-Banon, & Desobry, 2004). The manufacture of 80% WPC or whey protein isolate (WPI) is essentially the same as for 35 and 50% WPC products but it includes a diafiltration step to further concentrate the system from about 60 to 80% protein. This enhances the whey protein purity and ingredient functionality as well as controlling the final composition.

Commercial development of the functional product concept saw the use of polysaccharide-protein mixtures as multifunctional agents in material processing (Narchi, Vial, & Djelveh, 2009). Agarose–whey protein preparations are among the basic tools of achieving the required properties in industrial formulations with superior structural and nutritional functionality. Heating of the whey protein solution disrupts the native conformation and induces aggregation followed by gelation at a high enough temperature. This is distinct from the cold setting behaviour of gelatin whose phase morphology in mixture with polysaccharides (dietary fibre or various starches) has been studied frequently in the past (Nickerson et al., 2006).

The present work examines how the differences between the network of associated agarose double helices and the aggregation of thermally unfolded whey-protein molecules effect changes in the phase behaviour of their binary mixtures. It uses a protocol of physicochemical analysis to examine the structural properties of the composite gels with emphasis on solvent partition between the two polymeric phases. Besides the sequence-dependent thermal treatment, distinct network structures and steric exclusion patterns are induced by change in pH, which appears to be a necessary parameter to establish the identity of phase separation in these systems.

2. Experimental protocol

2.1. Materials

Agarose (Type 1-B) was purchased from Sigma–Aldrich, Gillingham, UK. The material has an off-white colour and high gel strength as recorded for the 1.0% (w/w) preparation in Fig. 1a of this investigation ($G' \sim 52$ kPa at 5 °C and pH 7.0). Using aqueous size exclusion chromatography, the supplier determined the number average molecular weight of this agarose sample ($M_n \sim 120$ kDa). Moisture, ash and sulfate contents were of less than 5.0%, 0.25% and 0.10% (w/w), respectively. Furthermore, the pH test of 1.5% (w/w) agarose gel was 7.16.

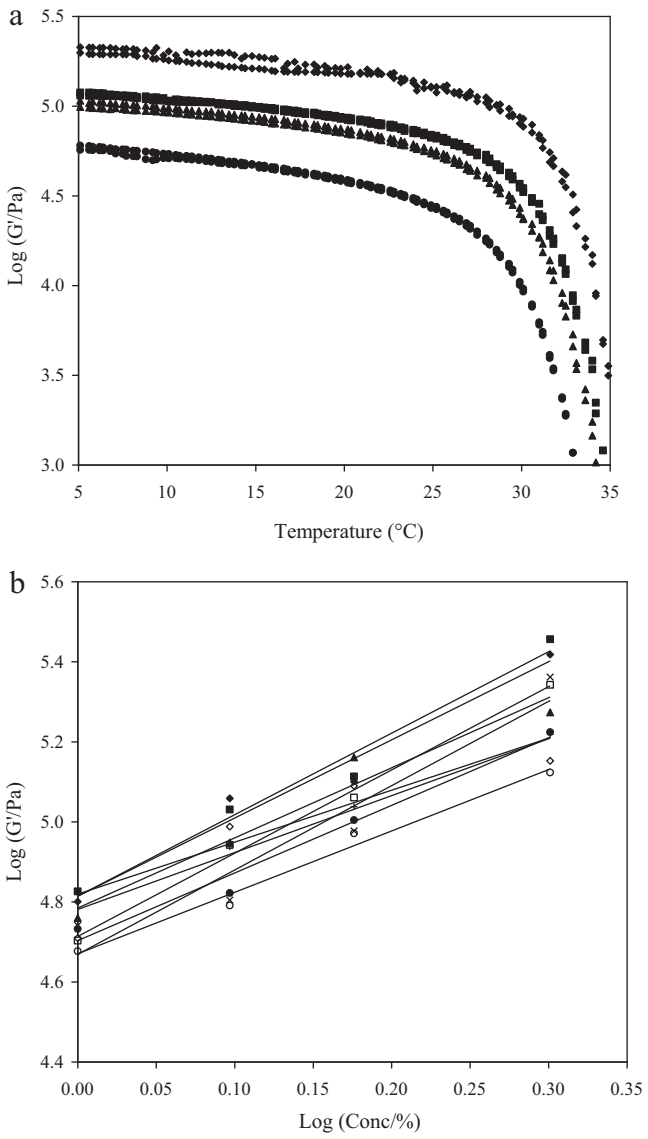

Fig. 1. (a) Cooling profiles of G' for 1.0 (●), 1.25 (▲), 1.5 (■) and 2.0% (♦) agarose at pH of 4.0 and 7.0 for each polymer concentration (scan rate of 1 °C/min), and (b) calibration curves of G' at 5 °C as a function of agarose concentration at pH values of 4.0 (♦), 4.5 (■), 5.0 (▲), 5.5 (+), 6.0 (○), 6.5 (●), 7.0 (◊), 7.5 (X) and 8.0 (□).

The sample of whey protein was supplied in the form of a creamy white powder by Fonterra, Auckland, New Zealand. It is a protein isolate commercially available as WPI Instantized 894. Kjeldahl analysis produced a protein content ($N \times 6.38$) of 93.0% (w/w; dry basis), which consists of β-lactoglobulin (71.0%; ratio of monomer to dimer is 5:1), α-lactalbumin (l8.0%) and 6.0% bovine serum albumin (BSA). Molecular weights of the proteins are 18.6 (monomer), 14.2 and 66.0 kDa, respectively (Mercade-Prieto & Gunasekaran, 2009). The fat, ash and moisture contents are 0.93%, 3.3%, and 4.83% (w/w), respectively, with the pH being around 6.9. The isoelectric pH (*p*I) ranges from 4.8 for α-La and 5.1 for BSA to 5.3 for β-Lg (Kinsella & Whitehead, 1989). The method of production involves cross flow micro-filtration (MF) and ultra-filtration (UF) through cellulose acetate membranes at ambient temperature, which leaves the final product in the native conformation.

This possesses good dispersibility/solubility for the water-holding modeling of the present work.

2.2. Sample preparation

Agarose solutions (1.0, 1.25, 1.5 and 2.0%, w/w) were prepared by dissolving the powder in deionized water at 80 °C using vigorous agitation for 20 min. For each sample, the pH was then adjusted to specific values (from 4.0 to 8.0 with 0.5 increments) by adding either NaOH (0.01 M) or HCl (0.01 M).

Whey protein solutions were readily prepared by dispersing the powder in deionized water at ambient temperature to produce concentrations of 15.0, 20.0, 25.0 and 30.0% (w/w). These were gently stirred (200 rpm) for 30 min and stored overnight at 4 °C to facilitate hydration. The following morning, dispersions were stirred once more for 15 min at ambient temperature to further induce homogeneity in the system. Values of pH were adjusted as for agarose to vary between 4.0 and 8.0.

Binary mixtures of agarose and whey protein were made by preparing initially solutions of the individual components, as outlined in the preceding paragraphs. Appropriate amounts of these stock preparations were combined at 45 °C, a temperature at which both components remain stable in solution. In detail, 20 g of each stock solution comprising 2.0% agarose or 30.0% whey protein on a weight-per-weight basis were mixed in an 1:1 ratio to produce a mixture (40 g) of 1.0% agarose with 15.0% whey protein. Addition of NaOH or HCl adjusted the pH of mixtures within the working range.

2.3. Methods

Small-deformation dynamic-oscillation measurements in shear were made using a controlled strain rheometer (AR-G2 from TA Instruments, New Castle, DE, USA) with a 40 mm diameter parallel-plate geometry and 1 mm gap. A thin layer of silicon fluid (50 mPa s) was used to cover the exposed edge of the sample to prevent loss of moisture in the course of experimentation. This type of mechanical analysis determines the elastic (storage modulus, G') and viscous (loss modulus, G'') components of the network, complex viscosity (η^*) and a measure of the 'phase lag' δ ($\tan\delta = G''/G'$) of the material.

Experimental sequence for single agarose samples consists of cooling runs from 40 to 5 °C at rate of 1 °C/min, isothermal step at this temperature for 30 min and a frequency of 1 rad/s, followed by a frequency sweep from 0.1 to 100 rad/s at a strain of 0.1%. Whey-protein rheology involved a temperature sweep from 45 to 80 °C at 1 °C/min, frequency sweep from 0.1 to 100 rad/s at the end of the heating run, cooling from 80 to 5 °C a the same scan rate, isothermal step at the end of the cooling run for 30 min and a frequency of 1 rad/s, followed by a frequency sweep from 0.1 to 100 rad/s at a strain of 1.0%. Binary mixtures were loaded onto the rheometer at 45 °C, driven to 80 °C and then to 5 °C to reproduce the experimental protocol for single whey-protein preparations. In a second routine, binary mixtures were loaded onto the rheometer at 45 °C and driven to 5 °C to reproduce the experimental protocol for single agarose preparations, which allows whey protein to remain in the native conformation.

Differential scanning calorimetry was performed using MDSC Q2000 (TA Instruments, New Castle, DE, USA). The instrument interfaced a refrigerated cooling unit to achieve temperatures down to 0 °C and a nitrogen purge cell with a flow rate of 25 mL/min. Heat flow was calibrated using a traceable indium standard and the heat capacity response using a sapphire standard. Typically, about 12 mg of agarose, whey protein and the mixtures thereof would be analyzed. Temperature amplitude of modulation was ±0.53 °C while the period of modulation was 40 s. Hermetically sealed aluminum pans were used, with the reference being an empty pan. Single systems or binary mixtures were heated from 45 to 90 °C, cooled to 0 °C and then heated once more to 90 °C thus recording various first-order thermodynamic transitions at a scan rate of 1 °C/min to match the rheological routine.

Environmental scanning electron microscopy was used to provide tangible evidence of changes in network morphology and topology of binary mixtures as a function of thermal treatment and polymer composition (FEI Quanta 200 ESEM, Hillsboro, Oregon, USA). Gel cubes of about 10 mm^3 in size were prepared either by heating followed by cooling thus triggering first the thermal transition of whey protein followed by agarose gelation, or by cooling only. The latter routine structures the polysaccharide but leaves the whey protein unaffected as liquid inclusions in the binary mixture. Observing the microstructure of these high moisture-content gels requires exposure to a gaseous secondary electron detector (GSED) at an accelerating voltage of 20 kV and pressure of 5.75 Torr.

3. Results and discussion

3.1. Network formation in single gels of agarose polysaccharide and whey protein isolate

Much has been said about the structural and thermal properties of gels made by agarose. Perhaps the best-known aspect of the subject is the formation of cohesive gels even at concentrations as low as 0.1% seen in small-deformation rheometry and the very brittle nature of those gels under large-deformation compression testing (Aymard et al., 2001). The purpose of the present exercise is to provide a series of data for the follow up on phase behaviour of agarose/whey protein mixtures.

Fig. 1a illustrates the dynamic oscillatory properties of agarose preparations with controlled cooling at 1 °C/min. Agarose solutions are indistinguishable from water at high temperatures but upon cooling structure formation ensues, which is recorded as a sharp trace at temperature below 35 °C. It has previously been suggested that the long and flexible polysaccharide chains in solution participate in the formation of rigid double helices in gels (Normand, Lootens, Amici, Plucknett, & Aymard, 2000). This is followed by aggregation, which builds up a three-dimensional structure of considerable opacity and thermal hysteresis in melting and setting temperatures (also observed in this work but not shown presently).

Following a brief sample equilibration and a three-decade frequency sweep at the end of the cooling run, values of the solid-like component of the network were collected and plotted as a function of polymer concentration and adjusted pH in Fig. 1b. There is a continuous reinforcement in gel rigidity with increasing agarose concentration from 1.0 to 2.0% in preparations, which was also seen in the cooling profiles from 35 to 5 °C in Fig. 1a. This work has also varied the pH value from 4.0 to 8.0 in intervals of 0.5, which did not affect the ability of the polymer to form a three dimensional structure but impacted somewhat on the extent of molecular interactions in the gel. Calibration curves of that nature are a necessary part of the algorithm that will model phase behaviour in agarose/whey protein mixtures discussed in the following sections.

It is well known in the literature that besides increasing temperature and ionic strength with added counterions, changes in pH around the isoelectric point ($pI \sim 4.6$) profoundly affect the structural properties of a whey protein gel. This investigation aims to take advantage of changing viscoelasticity in the protein network as a function of pH variation (from 4.0 to 8.0) in subsequent mixtures with agarose. Fig. 2 a reproduces examples of heating profiles for whey protein systems that were scanned to 80 °C at a rate of 1 °C/min. Temperatures above 60 °C induce unfolding (denaturation) and subsequent association of the unfolded molecules into permanent structures within the experimental constraints, which

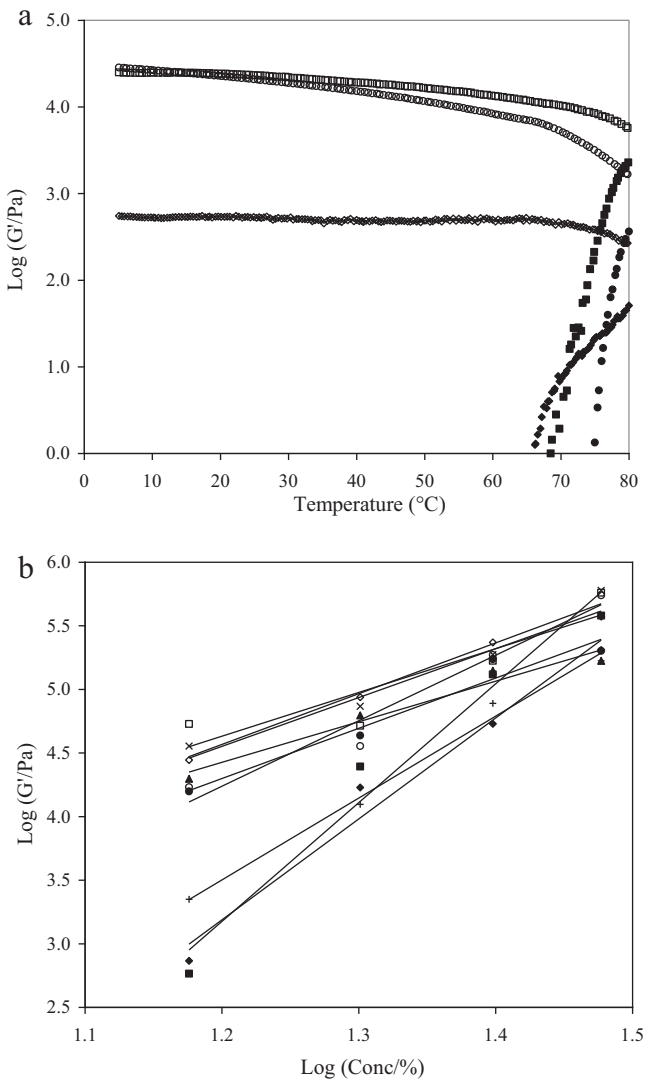

Fig. 2. (a) G' variation for 15.0% whey protein at pH 4.5 (heating, ♦; cooling, ◊), pH 5.0 (heating, ■; cooling, □) and pH 7.0 (heating, ●; cooling, ○) at a scan rate of 1 °C/min, and (b) calibration curves of G' at 5 °C as a function of whey protein concentration at pH values of 4.0 (♦), 4.5 (■), 5.0 (▲), 5.5 (+), 6.0 (○), 6.5 (●), 7.0 (◊), 7.5 (X) and 8.0 (□).

is recorded as a sharp increase in the values of storage modulus. These are opaque, aggregated structures due to high levels of the material used (15.0%), as compared to transparent, fine stranded gels at low protein concentrations or at pH away from the isoelectric point (Chantrapornchai & McClements, 2002).

Subsequent cooling records a monotonic increase in the values of G' in Fig. 2a without further change in moduli with time at the end of the experimental run (5 °C). Cooling of the material reinforces hydrogen bonding in addition to other secondary forces involved in structure formation at the denaturation temperature, i.e. ionic interactions, thiol-disulfide exchange, and non-specific hydrophobic forces (Alting, Hamer, de Kruif, Paques, & Visschers, 2003). As for agarose, calibration curves for subsequent modeling of the structural properties in binary mixtures were put together in Fig. 2b by plotting storage modulus values obtained at 5 °C against whey protein concentration (15.0–30.0%). Clearly, changes in pH values from 4.0 to 5.0, i.e. around the isoelectric point, have a pronounced effect on gel rigidity, which increases at least two orders of magnitude, e.g. from about $10^{2.7}$ Pa to 10^5 Pa at pH 4.0 in Fig. 2b. By contrast, the effect of pH on the agarose gel is less pronounced being

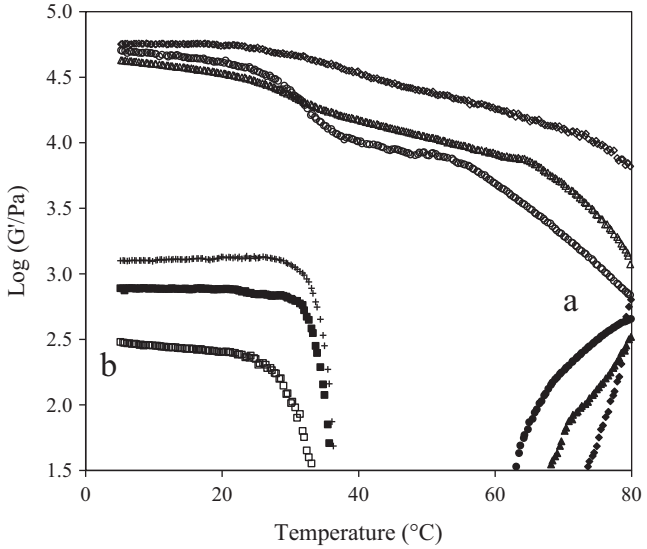

Fig. 3. (a) Heating (closed symbols) and cooling (open symbols) profiles of G' for mixtures of 1.0% agarose with 15.0% whey protein at pH 5.0 (♦, □), pH 7.0 (▲, □) and pH 8.0 (●, ○) at a scan rate 1 °C/min, and (b) cooling profiles of G' for mixtures of 1.0% agarose with 15.0% whey protein at pH 4.0 (□), pH 7.5 (■), and pH 8.0 (+) at the same scan rate.

confined to a maximum of half-a-decade in modulus values across the concentration range of the polysaccharide in Fig. 1b.

3.2. Thermomechanical and microscopy observations on the structural characteristics of agarose/whey protein mixtures

Experimental observations reported in the preceding section for single systems will be used to provide a guideline of the phase behaviour in aqueous binary mixtures. Following the analysis of single-component gels, a series of binary mixtures was prepared at 45 °C keeping the concentration of agarose and whey protein constant at 1.0% and 15.0%, respectively, and changing the pH within the range of 4.0–8.0. Two distinct experimental routines were then implemented; the first involved controlled heating of the composite system to 80 °C followed by cooling to 5 °C, with the second being a cooling run from 45 °C to 5 °C.

Fig. 3 illustrates the temperature-dependence of structure formation for selected pH values of our mixture. Heating results in protein denaturation and augmentation of the storage modulus whereas the agarose molecules remain in the disordered conformation. The gelling transition appears earlier and earlier with increasing pH, i.e. as the system moves away from its isoelectric point, an outcome that argues the importance of open globular structures and ionic interactions in the presence of counterions from the batch for incipient gelation. (*Note*: The early onset of network formation observed for whey protein at pH 4.5 and 5.0 in Fig. 2a should be due to vestigial precipitation near the isoelectric point, as opposed to the observations at higher pH 7.0 and 8.0 in Fig. 3, where precipitation in the binary mixture is further prevented by the presence of the protein-particle suspending agarose phase.) The experimental routine was then reversed reaching at a controlled scan rate a temperature range where agarose can undergo a coil-to-helix transition leading to gelation. Thus the liquid inclusions of the polysaccharide transform into gelled domains in the presence of the protein network, and this is monitored as a rise in G' values along the cooling run at temperatures below 35 °C.

The experimental procedure was concluded by cooling separate blends from 45 °C to 5 °C at the standard scan rate, and these experimental observations are also reproduced in Fig. 3. Structure formation is recorded as a sharp conformational transition, which

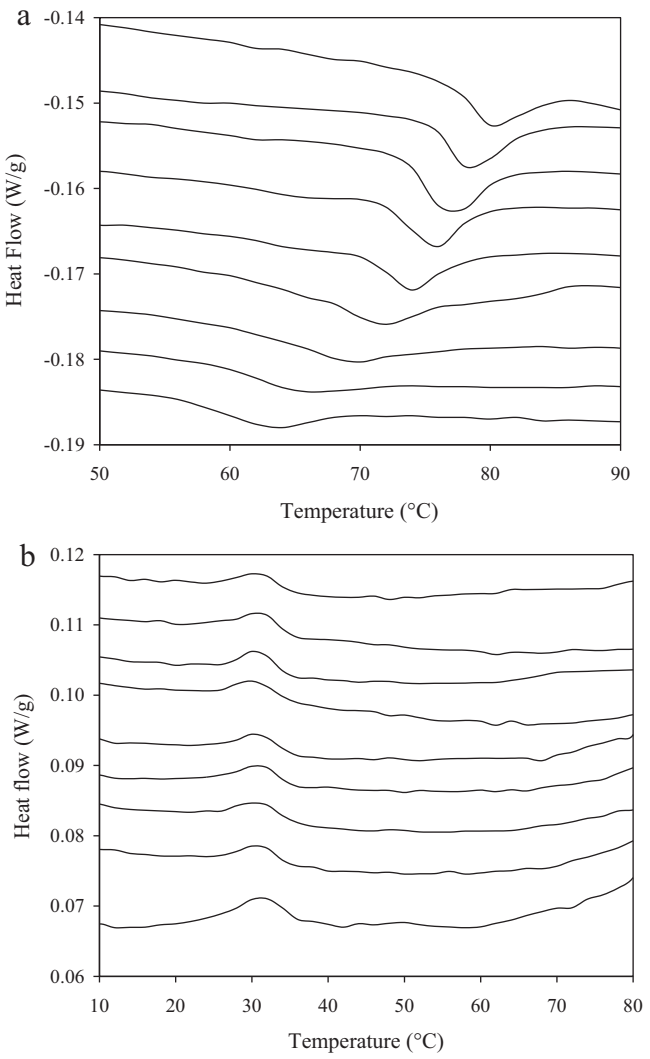

Fig. 4. (a) DSC endotherms for the heating profiles of 1.0% agarose with 15.0% whey protein mixture at pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 shown in the graph from top to bottom, and (b) DSC exotherms for the cooling profiles of 1.0% agarose with 15.0% whey protein mixtures at pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 shown in the graph from top to bottom (scan rate in both cases is 1 °C/min).

is characteristic of cooperative enthalpic associations in polysaccharide networks (Esquenet, Terech, Boue, & Buhler, 2004). The internal rearrangements and structure of the gels seem to settle within the lower range of the experimental temperatures, as indicated by the modulus traces that asymptotically approach constant values. Overall, structure formation indicates a dominant agarose network supported by similar cooling profiles of single agarose preparations in Fig. 1a. Shear-modulus variation at the end of the experimental routine is also consistent with data from the calibration curves as a function of adjusted pH in Fig. 1b. In the absence of heat treatment whey protein remains in the native non-gelling conformation but is capable of holding water in its low-viscosity phase.

Besides mechanical spectroscopy, experimental evidence from differential scanning calorimetry (DSC) can also provide a firm footing on the phase behaviour in aqueous preparations of binary mixtures (Mousia, Farhat, Blachot, & Mitchell, 2000). In doing so, fixed amounts of agarose and whey protein were blended at 45 °C, loaded at this temperature and heated to 90 °C at 1 °C/min, in accordance with the rheological routine, with Fig. 4a illustrating typical endothermic peaks as a function of reduced acidity for these mixtures (top to bottom spectra). A relatively sharp peak with a maximum heat flow temperature (T_{max}) of 79 °C denotes the cooperative conformational transition of the protein molecules upon heating at pH 4.0. By comparison, the process of thermal denaturation becomes increasingly gradual at higher values of pH (endothermic peaks become broader), as the protein exhibits a net negative charge. In addition, heat flow maxima culminate at lower temperatures, e.g. at 64 °C at pH 8.0, an outcome that is consistent with the early onset of gelation with increasing pH in heat treated samples of Fig. 3.

Subsequent cooling of the mixtures keeping the same scan rate produces well formed exothermic peaks throughout the experimental pH range (Fig. 4b). Maximum heat flow temperature for all samples remains unaffected by pH and culminates at about 32 °C. This is the cooperative process of coil-to-helix transition in agarose networks being congruent to the onset temperature of gelation recorded in cooled only samples in Fig. 3. Identical thermal events in terms of temperature sequence and overall peak form for each transition were recorded for the denaturation or gelation of single whey protein or agarose preparations (results not shown here), which argues that both components form gel networks in the mixture as in the individual preparations. Results also suggest that there are no specific interactions between agarose and whey protein (i.e. no formation of heterotypic junctions), which could distort the peaks of the individual gels and generate a new thermal event in the DSC spectrum (Nitta, Kim, & Nishinari, 2003).

Finally, images from environmental scanning electron microscopy were taken to provide tangible evidence of changes in network morphology and phase topology in agarose/whey protein mixtures as a function of external stimuli. Work focused primarily on thermally treated preparations, since it is known in the literature that cooling experiments yield dendritic polymer networks of agarose gels (Fujii et al., 2000), which support liquid inclusions of undenatured whey protein (we also observed this phase morphology in the cooled mixture but micrographs are not shown here). Fig. 5a illustrates the globular structures of a whey protein network at pH 4.0 having been heated to 80 °C. Structural knots in the acidic environment are rather fine, in the order of 5 μm, as compared to the protein spheroids at pH 7.0 (in the order of 20 or 30 μm) that appear to form a partially fused network (Fig. 5b). Cooling of the denatured protein structure in the presence of agarose stimulates further gelation hence yielding two component networks with contrasting refractive indices in Fig. 5c. At acidic pH, optically dense protein particles appear to form a continuous phase, which is interrupted by elongated polysaccharide chains. Phase separated patterns between a smooth polysaccharide phase and coarse protein spheroids are also evident in the mixture sampled at neutral pH (Fig. 5d).

3.3. Theoretical modeling of the phase behaviour in agarose/whey protein mixtures

Qualitative analysis in the preceding sections provided insights into network characteristics and the phase behaviour of our binary mixtures in relation to thermal treatment and change in pH. Once it is established that agarose and whey protein create a phase separated organization in the mixture, and the nature of phase continuity is reasonably understood from the experimental part of this work, the next step is to relate the physical properties of the components and their individual domains to the overall behaviour of the composite gel. Such explorations can be made by applying the so-called "blending laws" originated in synthetic polymer research (Gilsenan, Richardson, & Morris, 2003). The approach found considerable utility in the quantitative description of the structural properties of biopolymer composite gels by relating mechanical

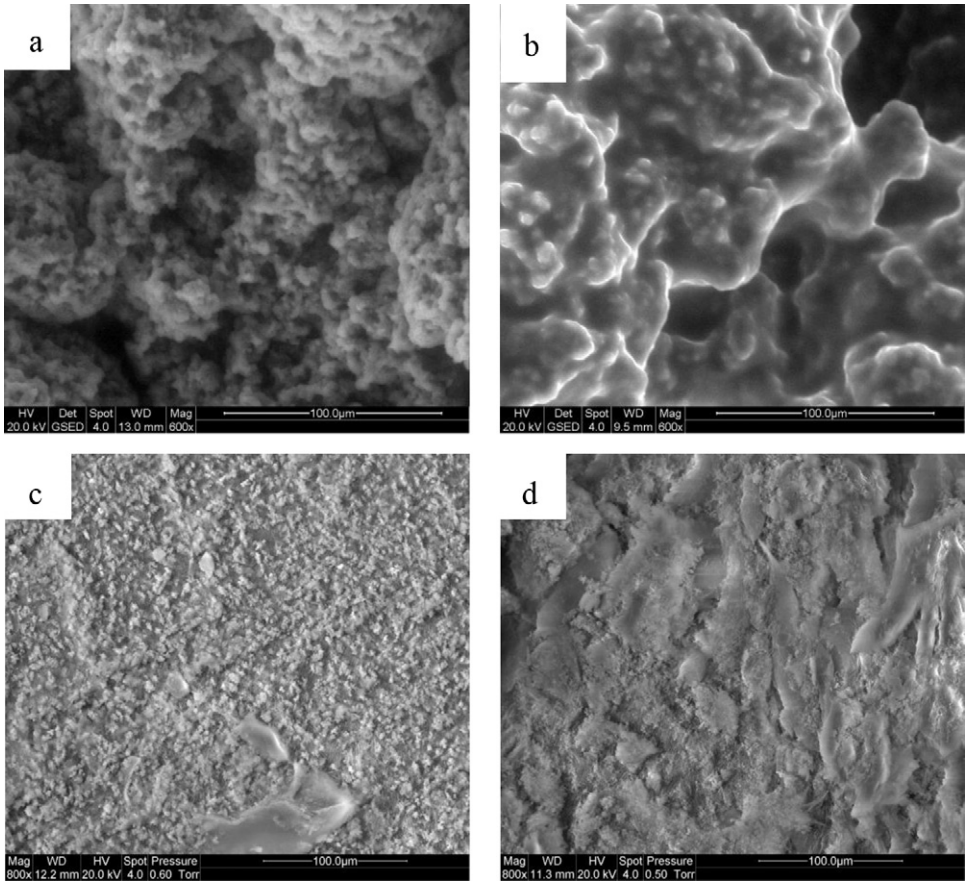

Fig. 5. ESEM images of (a) 15.0% whey protein at pH 4.0, (b) 15.0% whey protein at pH 7.0, (c) 1.0% agarose with 15.0% whey protein at pH 4.0 and (d) 1.0% agarose with 15.0% protein at pH 7.0, with all samples being thermally treated (heating followed by cooling); magnification is 100 μm.

properties of the two components to the overall mechanical behaviour of the composite gel so that aspects of phase behaviour, e.g. the solvent partition between polymeric phases, can be predicted. Such an achievement facilitates informed manipulation, for example, of the textural attributes of firmness and plasticity thus contributing desirable consistency in a variety of industrial formulations with superior structural functionality (Zoulias, Oreopoulou, & Tzia, 2002).

This work reproduces the mathematical expression of the blending laws that relates the overall storage modulus in shear of the composite agarose/whey protein gel to those of the two polymeric phases:

$$G'_c = \phi_X G'_x + \phi_Y G'_y \quad (1)$$

and

$$G'_c = \left(\frac{\phi_X}{G'_x} + \frac{\phi_Y}{G'_y} \right)^{-1} \quad (2)$$

where G'_c, G'_x and G'_y are the storage moduli in shear of the composite, *X*-phase polymer and *Y*-phase polymer, respectively, with ϕ_X and ϕ_Y ($\phi_X + \phi_Y = 1$) being the phase volumes of the two polymeric components *X* and *Y*.

Fig. 6 reproduces an example at pH of 4.5 of the computerized output designed to check the applicability of blending laws to the mixed systems that have been taken through the full thermal treatment, i.e. loading at 45 °C, heating to 80 °C and then cooling to 5 °C. Following this treatment, the two components form gelled networks, and an expedient way to predict the distribution of solvent between the two phases is to calculate the values of storage modulus for all possible distributions in order to find which one matches the experimental value of the mixture (Morris, 2009). In the present illustration, the solvent content of the whey-protein phase (S_{wp}) is plotted on the *x* axis. At very low values of S_{wp}, where most of the water is with the agarose phase, the protein is extremely

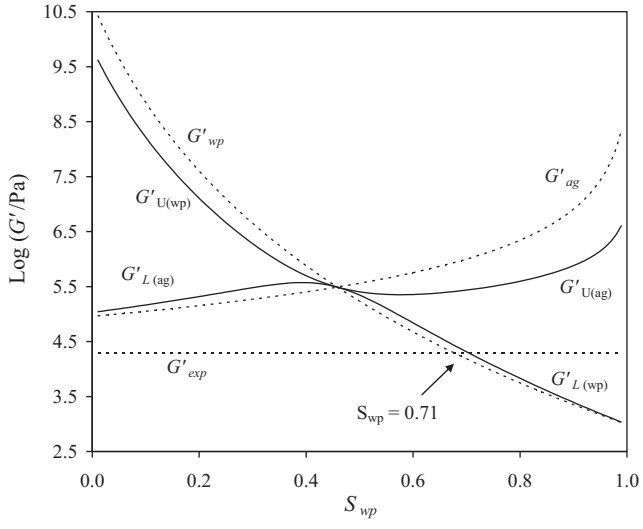

Fig. 6. Computerized modeling of the phase topology of 1.0% agarose with 15.0% whey protein mixture, which was exposed to heating and cooling, at pH 4.5 using the isostrain and isostress blending laws. Storage modulus values of agarose (G'_{ag}) and whey protein (G'_{wp}) are represented by dashed lines while the upper ($G'_{U(ag)}$; $G'_{U(wp)}$) and lower ($G'_{L(ag)}$; $G'_{L(wp)}$) bounds are illustrated as solid lines. Experimental composite modulus (G'_{exp}) taken at 5 °C is also shown to intersect the calculated lower bound at a specific value of S_{wp}.

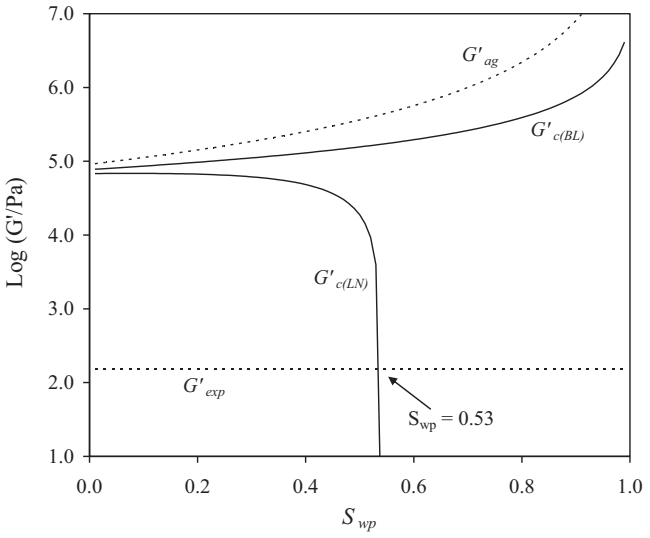

Fig. 7. Computerized modeling of the phase topology of 1.0% agarose plus 15.0% whey protein, which was exposed to a cooling routine only, at pH 4.5 using the Lewis–Nielsen and isostrain blending laws. Storage modulus values of agarose (G'_{ag}) are represented by a dashed line while the calculated composite moduli according to Lewis–Nielsen ($G'_{c(LN)}$) and blending ($G'_{c(BL)}$) laws are illustrated as solid lines. Experimental composite modulus (G'_{exp}) taken at 5 °C is also shown to intersect the Lewis–Nielsen predictions at a specific value of S_{wp}.

concentrated hence $G'_{wp} \gg G'_{ag}$. Conversely, at very high values of S_{wp}, $G'_{wp} \ll G'_{ag}$.

At one critical value of solvent partition, the moduli of the two phases cross over and under these conditions it becomes apparent from Eqs. (1) and (2) that the isostrain and isostress blending laws also yield this common value ($G'_{wp} = G'_{ag} = G'_U = G'_L$). Up to this point, the upper bound value corresponds to a whey protein continuous system, and the lower bound value to agarose continuous. At higher values of solvent partition, $G'_{U(ag)}$ relates to an agarose continuous phase and $G'_{L(wp)}$ to whey protein continuous. As a result, whey protein continuous curves run from the top left to the bottom right, whereas agarose continuous curves extend from the bottom left to the top right of the graph. Experimental moduli of the composite recorded at 5 °C appear to intersect a lower bound arrangement in blending-law modeling yielding an S_{wp} value of 0.71. This outcome is consistent with whey protein being denatured upon heating to create the continuous phase in the mixture and agarose forming hard filler particles upon subsequent cooling.

Thermal denaturation triggers drastic changes in the viscoelasticity of whey protein whose phase behaviour in mixture with agarose was examined using the blending laws. To further ascertain the effect of conformational changes on solvent partition between polymeric phases, agarose/whey protein mixtures were control cooled from 45 °C to 5 °C to alter the rigidity of the polysaccharide phase only. Using these experimental settings, quantitative analysis of the mechanical properties for the binary system of this investigation was carried out and the example of pH 4.5 is depicted in Fig. 7. Clearly, the basic blending laws cannot follow the change in structural properties once the discontinuous phase is in the form of liquid whey protein droplets, with the experimental value of the composite system falling well below their predictions. Our data argue that the modulus of the solid-like phase of agarose divided by the modulus of the liquid-like phase of whey protein ($G'_{wp} \ll 1.0$ Pa at 5 °C) yields values on the order of thousands.

This modulus ratio is roughly the correct indicator for synthetic polyblends and block polymers whose viscoelastic characteristics can be rationalized with the theoretical advance achieved by Lewis and Nielsen. The analytical expression of this framework of thought is given as follows (Petersson & Oksman, 2006; Shrinivas, Kasapis, & Tongdang, 2009):

$$\frac{G'_1}{G'_c} = \frac{(1 + A_i B_i \phi_2)}{(1 - B_i \psi \phi_2)} \tag{3}$$

$$B_i = \frac{[(G'_1/G'_2) - 1]}{[(G'_1/G'_2) + A_i]} \tag{4}$$

$$\psi = 1 + \left[\frac{(1 - \phi_m)}{\phi_m^2}\right] \phi_2 \tag{5}$$

where in Eqs. (3)–(5), G'_c, G'_1 and G'_2 are the shear moduli of the composite, continuous rigid phase of agarose and soft filler of whey protein, and ϕ_2 is the phase volume of the said filler. The framework takes into account the shape of the filler *via* an inverted Einstein coefficient ($A_i = 1/A$), which is very sensitive to the morphology of the composite. A further level of refinement was achieved by considering the concept of maximum packing fraction (ϕ_m) of the filler phase.

Fig. 7 shows the standard of agreement obtained using Eqs. (3)–(5) for the experimental shear modulus of the cooled only agarose/whey protein mixture. For the purpose of modeling, the Einstein coefficient was held constant at the theoretical value for dispersed spheres, $A = 1.5$ (Nielsen, 1974), hence the only adjustable parameter of the fit is the maximum packing fraction of the dispersed phase. The latter is a rather difficult parameter to obtain experimentally but, for each pH value of the aqueous mixture, experimental moduli of the composite at 5 °C intersect convincingly the part of the Lewis–Nielsen prediction that falls dramatically with S_{wp} at an ϕ_m value of 0.601. This estimate corresponds to loose packing of spheroidal inclusions (Torquato, Truskett, & Debenedetti, 2000), which is the expected outcome for the reduction of interfacial tension between two phase-separated polymeric domains. Solvent content for whey protein in its native conformation in Fig. 7 ($S_{wp} = 0.53$) is considerably lower than the denatured counterpart in Fig. 6.

The composition of Figs. 6 and 7 is quite detailed by reproducing storage-modulus predictions of the agarose/whey protein gel for all possible distributions of solvent in the mixture. To further demonstrate the distribution of solvent between the two polymeric phases and allow for meaningful comparisons in our systems where the pH values have been adjusted systematically, we utilized the "solvent avidity" factor, P (Clark, Richardson, Ross-Murphy, & Stubbs, 1983):

$$P = \frac{(S_{wp}/C_{wp})}{(S_{ag}/C_{ag})} \tag{6}$$

where C_{ag} and C_{wp} are the nominal (original) concentrations of the two macromolecules in preparations. Clearly, values of P-factor contrast the level of solvent found within phases per unit of the initial concentration of the corresponding polymer.

In Fig. 8, the outcome of composite bounds analysis, experimental storage moduli and solvent partition between phases shown in Figs. 6 and 7 has been reproduced by plotting against the P-factor derived from Eq. (6). Clearly, considerable changes are predicted in the distribution of solvent in the system as a function of thermal treatment and pH change to create three families of points. There is no systematic variation in P estimates, within the group of data for the cooling routine, remaining well below 0.10. It appears that the liquid-like whey protein phase allows diffusion of water molecules in the gelled agarose phase, which holds as the continuous matrix ten times more solvent than the protein per unit concentration. Reversing the experimental routine to heating followed by cooling creates a composite gel with whey protein as the supporting matrix that changes the pattern of water partition between the constituent phases. At acidic pH, constant values for the distribution of solvent in the system are obtained ($P = 0.25 \pm 0.10$), with agarose

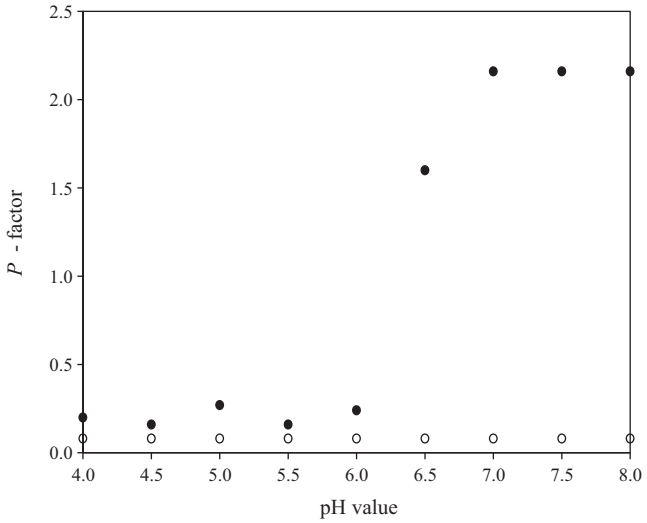

Fig. 8. Data for the relative solvent partition (*P*-factor) plotted against adjusted pH for the mixed systems of 1.0% agarose plus 15.0% whey protein, which were subjected to both heating and cooling (●) or a cooling only treatment (○).

remaining the "hydrophilic network". At alkaline pH, however, the *P*-factor increases sharply reaching values of about 2.20 in favor of the continuous whey-protein phase.

4. Concluding remarks

The present investigation is part of an effort to better understand segregative phase separation in aqueous biopolymer mixtures. Results demonstrate that the combination of complementary physical techniques such as mechanical spectroscopy, differential scanning calorimetry and electron microscopy can characterize the micromolecular organization of binary systems as a function of thermal treatment and change in pH. In terms of solvent partition between two polymeric phases, a helpful picture of the importance of conformational changes with experimental temperature or acidity in the phase behaviour of a mixed system is given. Rheological data were modeled using theoretical frameworks adapted from the synthetic polymer research.

Variation in the values of the *P*-factor for agarose/whey protein samples at a fixed polymer concentration is distinct from previous estimates reported in the literature for composite gels, e.g. gelatin/hydrolyzed starch, gelatin/low methoxy pectin and sodium caseinate/β-glucan. The latter group of binary systems exhibited isostrain or isostress phase behaviour according to the blending-law Eqs. (1) and (2) and classic phase inversion from one continuous matrix to another with increasing concentration of the second polymeric component (Gilsenan et al., 2003; Kasapis, Morris, Norton, & Clark, 1993; Kontogiorgos, Ritzoulis, Biliaderis, & Kasapis, 2006).

It was found that phase inversion in binary mixtures, due to increasing concentration of the second (*Y*) component, resulted in two distinct clusters of *P* predictions, one for the *X* – continuous composite and the other for the *Y* – continuous composite following phase inversion. The lack of systematic variation in these predictions with polymer concentration before or after phase inversion and the magnitude of *P* values in each cluster of points, which always favors the continuous-phase forming polymer, reflects fundamental differences in the interplay between the kinetics of ordering/gelation and microscopic phase separation in those materials.

In addition, designing experimental conditions that encourage formation of a bicontinuous mixture, where both polymers form a continuous network, allows increasing concentrations of a given component to exhibit favorable relative affinity for solvent in the system. This type of behaviour has been reported, for example, in gelatin/agarose gels where the presence of two continuous phases allowed the slower-gelling component (gelatin) to exhibit enhanced water holding capacity leading to a monotonic change in *P* values with increasing concentrations of the protein in the system (Shrinivas et al., 2009). This outcome for bicontinuous mixtures is distinct from the central finding of a single value of the *P*-factor observed in the distribution of solvent between the continuous matrix of polymer *X* and the discontinuous inclusions of polymer *Y* mentioned for several composite gels in the preceding paragraph (Gilsenan et al., 2003; Kasapis et al., 1993; Kontogiorgos et al., 2006).

This work complements research findings from the literature in presenting an unexplored aspect of segregative phase separation by adjusting the pH values in mixtures of the two conformationally dissimilar macromolecules of agarose and whey protein. Modeling of the rearrangement of water molecules between the two constituents has demonstrated that the *P* values are profoundly affected by pH in the aqueous system (Fig. 8). Thus, the proportion of solvent associated with the protein phase increases rapidly at slightly acidic and alkaline pH where the protein exhibits a higher degree of unfolding than below the isoelectric point (see also micrographs in Fig. 5). This is due to ionization of partially buried carboxyl, phenolic and sulfhydryl groups that cause certain unraveling of the polypeptide chains as they attempt to expose themselves to the aqueous environment (Monahan, German, & Kinsella, 1995).

Water diffuses in the anisotropic medium of the two polymers seeking osmotic equilibrium, but the decline in the amount of solvent kept in the agarose phase with increasing pH argues for mixed gels that are kinetically trapped in the local medium. Variation in solvent-partition data from this work, and earlier reports in the literature, suggest that *P* values derived under a particular thermal regime, kinetic rate of gelation, polymer composition of mixtures, applied shear rate, and pH variation in preparations should not be related to the Flory–Huggins interaction parameter (χ). This describes the energy of interaction between disordered polymeric segments and solvent in terms of segment–segment and solvent–segment pair interactions (Csaki, Nagy, & Csempesz, 2005), hence the molecular forces that govern their phase diagram at thermodynamic equilibrium in solution. It appears, however, that aggregation followed by gelation in whey protein or agarose phases arrest the composite system at intermediate conditions away from thermodynamic equilibrium.

Results of this work contribute to the school of thought that classic phase separation in solution leading to thermodynamic equilibrium between two polymer phases is not carried over to the gel state. In other words, there is no time-temperature superposition principle able to sustain the state of thermodynamic equilibrium in solution, as epitomized by the phase diagram, in a composite gel. Further, it appears that polymer-entrapping gelation is minimized and extensive phase separation is allowed to develop in the gel state by slowing down the rate of cooling or heating of a binary solution (Zhang, Jiang, & Zhu, 2006), (e.g. at 1 °C/min, as for this investigation). Extensive phase separation shifts the binodal curve of the phase diagram for solutions in the vicinity of the polymer-concentration axes in the gel state. This outcome allows successful utilization of the blending laws (Eqs. (1) and (2)) in the elucidation of the phase behaviour of composite gels (presently for the agarose/whey protein system), since the blending-law requirement of the two macromolecules being largely confined to their respective phases is now met.

Finally, insights gained from this work should be applicable to a wide range of binary biopolymer gels that include globular protein as a constituent. As far as we are aware, this is the first

attempt to model phase behaviour and solvent distribution in composite gels of polysaccharide and globular protein, as a function of pH, by adapting theoretical modeling from the synthetic polymer research. It was hypothesized that the distinct dependence of agarose and whey protein networks on pH should be reflected in the phase behaviour of their mixed gels. It is gratifying then to confirm this in the remarkable variation in solvent distribution between the two polymeric phases as a function of pH in Fig. 8. The universality of the pH effect on phase behaviour should be evaluated in composite gels of gelling polysaccharides (e.g. κ-carrageenan, deacylated gellan, and low methoxy pectin) and globular proteins (e.g. bovine serum albumin, ovalbumin, and molecular fractions of soy protein) with industrial interest. Informed manipulation of structural properties and textural consistency in these mixtures as a function of pH will assist in bridging the gap that has emerged between the recent advances in fundamental knowledge and the direct application to industrial formulations with a growing need for scientific input.

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