Associative and Segregative Phase Separations of Gelatin/κ-Carrageenan Aqueous Mixtures

ARTICLE *in* LANGMUIR · NOVEMBER 2006

Impact Factor: 4.46 · DOI: 10.1021/la061865e · Source: PubMed

Associative and Segregative Phase Separations of Gelatin/ κ-Carrageenan Aqueous Mixtures

Yapeng Fang,* Liangbin Li,† Chiharu Inoue, Leif Lundin, and Ingrid Appelqvist

Unilever Food and Health Research Institute, Olivier van Noortlaan 120, 3133AT Vlaardingen, The Netherlands

Received June 28, 2006. In Final Form: September 3, 2006

The effects of ionic strength, temperature, and pH on the phase separation behavior of type B pigskin gelatin/ sodium-type κ-carrageenan aqueous mixtures were investigated. Depending on the different combinations of temperature and sodium chloride (NaCl) concentration, the mixtures showed compatible, associative, and segregative phase separation behaviors. Additionally, a coexistence of associative and segregative (associative-*co*-segregative) phase separations was expected at low temperature and low NaCl concentration. These different phase separation events were observed using confocal scanning laser microscopy. Moreover, it was found that the segregative phase separation when alone is induced by the ordering of κ-carrageenan chains, while that in the coexistence region is induced by the ordering of gelatin chains. pH had a significant effect on the associative phase separation, resulting in morphologies changing from compatible solution to liquid coacervate and further to solid precipitate with decreasing pH. These were attributed to the dramatic changes of the charge density of amphoteric gelatin during the pH decrease.

Introduction

Aqueous biopolymer mixtures are in most cases characterized by a thermodynamic incompatibility that leads to a macroscopic phase separation.[1] According to Piculell et al.,[2] the phase separation of two biopolymers in a common solvent may be divided into two main categories: associative and segregative phase separation. In an associative phase separation, both biopolymers are enriched in one of the separating phases with the other phase containing mostly solvent. This type of phase separation is often obtained for oppositely charged biopolymer mixtures. In a segregative phase separation, two polymers are separated into two different phases. This is the case mainly for two nonionic biopolymers, two similarly charged polyelectrolytes, or a polyelectrolyte plus a nonionic biopolymer. Great interest has been shown in biopolymer phase separation because of its use in many industrial applications such as food and cosmetic microstructural designs,[3] microencapsulation,[4,5] and protein separation and purification.[6] In particular, associative-type phase separation is thought to have implications in many biological processes such as vesicle formation.[7]

A survey of abundant literature clearly shows that temperature, pH, ionic strength, charge density, chain conformation, etc. are important factors controlling the phase separation behaviors of biopolymer mixtures, particularly of charged biopolymer mixtures.[1,2,4,7,8] At a certain combination of these experimental factors, a biopolymer aqueous mixture usually exhibits only one of the two types of phase separation or a compatible behavior. By changing these experimental factors, a transition between associative and segregative phase separation can be generated continuously (via a region of borderline phase separation) or discontinuously (via a region of complete miscibility).[2,8] Contrary to this general rule, an interesting recent study on the mixtures of fish gelatin/κ-carrageenan by Haug et al.[9] demonstrated the possibility of the coexistence of both associative and segregative phase separation, although the associative phase separation is most likely to be kinetically trapped. Up to now, there has been no systematic study on how the combination of temperature, pH, and ionic strength controls the mutual transition and coexistence of associative and segregative phase separation.

Gelatin is a well-known protein extracted from mammalian or fish tissues. It has numerous applications in the food and photographic industries.[10,11] κ-Carrageenan is a negatively charged polysaccharide extracted from red seaweed[12] which has a linear sulfated backbone of alternating α-1,4- and β-1,3-linked galactose residues. κ-Carrageenan is widely used as a thickener, gelling agent, and stabilizer in the food industry. The aim of this work is to map out the phase separation behaviors of the aqueous mixtures of pigskin gelatin and κ-carrageenan at different combinations of temperature and NaCl concentration and examine the effects of pH. We also put emphasis on the relationship between phase separations and the temperature-induced conformational transitions of gelatin and carrageenan.

Materials and Methods

Materials. Gelatin, type B, from pigskin, was purchased from GELITA EUROPE (batch no. 618757). It has a weight-average molecular mass of 120 kDa as determined by an ALV/CGS-3 static light scattering instrument (ALV GmbH, Germany) at 50 °C and an isoelectric point of 4.9 as determined by a Nano-ZS ZetaSizer (Malvern Instruments, U.K.). Carrageenan (Genugel X-0909), with

* To whom correspondence should be addressed. E-mail: Yapeng.Fang@Unilever.com.

† Present address: National Synchrotron Radiation Laboratory, University of Science and Technology of China, Hefei, China.

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10.1021/la061865e CCC: $33.50
Published on Web 10/10/2006

a κ-fraction of 0.93, was obtained from Kelco (batch no. Bx728000). The weight-average molecular mass was determined to be 550 kDa at 50 °C. It contains the following ions: Na, 5.140%; K, 0.180%; Ca, 0.010%; Mg, 0.010% (w/w). Sodium chloride, sodium hydroxide, and hydrogen chloride (1 mol/L) used in this work are all of analytical grade.

Sample Preparation. Gelatin aqueous solutions were prepared by dissolving gelatin in Millipore water at 60 °C for 1 h. Note that the dissolution temperature was never allowed to rise above 60 °C to avoid the hydrolysis of gelatin chains at high temperatures. κ-Carrageenan aqueous solution was made by dispersing carrageenan powder into hot Millipore water at 95 °C under magnetic stirring for 1 h. The gelatin and κ-carrageenan solutions were mixed at 60 °C in proper proportions to get the desired concentrations of both polymers (%, w/w). Concentrated HCl and NaOH (1 mol/L) were used to adjust the pH. Different ionic strengths were obtained by adding a calculated amount of NaCl, taking into consideration the intrinsic ionic strength brought in by carrageenan.

Turbidity Measurements. All turbidity measurements were carried out on an UVIKON XL UV/vis spectrophotometer (SECOMAM, France) that was equipped with temperature-controlling cell holders driven by a Peltier device or a refrigerated circulator. Samples were placed in a quartz cell with an optical path length of 10 mm. Turbidity was recorded at a wavelength of 500 nm and was calculated as follows:

$$\tau = (-1/L)\ln(I/I_0)$$

where L is the optical path length (cm), I_0 the incident light intensity, and I the transmitted light intensity.

Differential Scanning Calorimetry (DSC). DSC measurements were made on a high-sensitivity microcalorimeter, DSC III (Setaram, France). About 0.8 g of sample was placed in the sample cell of the DSC instrument, and an equal amount of distilled water was used as the reference. DSC measurements were run from 50 to 5 °C at a cooling rate of 1 °C/min.

Confocal Scanning Laser Microscopy (CSLM). The microstructures of gelatin/κ-carrageenan mixtures were observed under a BioRad MRC 600 confocal scanning laser microscope equipped with an Ar/Kr mixed gas laser emitting at 488 and 568 nm. The CSLM instrument was connected to a Zeiss inverted microscope. About 5 mL of sample was put in a home-built temperature-controllable jacketed vessel, the bottom of which was made of a very thin glass slice (1 mm) that facilitates CSLM observations. The temperature was controlled within ±0.1 °C by a refrigerated water bath. Gelatin was labeled with a small drop of 0.01% fluorescein isothiocyanate (FITC) before being mixed with carrageenan solutions. The use of such a small amount of FITC guarantees a complete reaction with gelatin and no free FITC present. Gelatin-rich domains thus appear as bright regions in the obtained CSLM images.

ζ Potential Measurements. The ζ potential measurements of gelatin and κ-carrageenan in aqueous solutions at different pHs were performed on a Zetasizer Nano-ZS apparatus (Malvern Instruments, U.K.) that was combined with a pH autotitrator. The sample temperature was maintained at 25 °C by a heating coil. A disposable polystyrene cell with two electrodes was used.

Results and Discussion

State Diagram of Mixtures. The type B pigskin gelatin used in this work has an isoelectric point (pI) of 4.9. κ-Carrageenan is a negatively charged polysaccharide. The mixing of gelatin and carrageenan aqueous solutions, at pHs even above the pI, where gelatin has a negative overall charge, can lead to an associative phase separation, as the uneven distribution of cationic and anionic groups along the gelatin chain produces some positive patches that electrostatically attract carrageenan.[13]

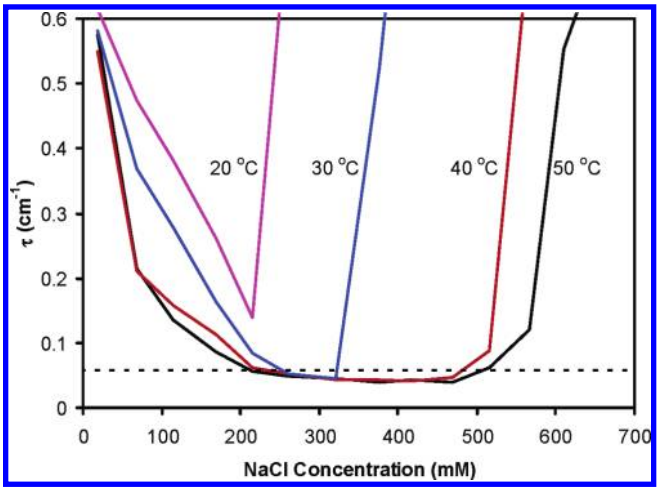

Figure 1. Titrations of turbidity at 500 nm as a function of the NaCl concentration for 0.75% gelatin/0.75% κ-carrageenan aqueous mixtures at pH 7 and different temperatures (indicated beside the curves). The dashed line provides a guideline for distinguishing compatible behavior from phase separation.

Figure 1 shows the change of turbidity of 0.75% gelatin/0.75% κ-carrageenan mixtures as a function of NaCl concentration measured at pH 7 and different temperatures. Take the turbidity curve at 50 °C for example. The turbidity first decreases with increasing NaCl concentration, which is typical of associative phase separation, since the degree of phase separation is reduced by the addition of salt. The addition of monovalent salts is supposed to suppress electrostatic attractions and hence associative phase separation.[14] When the NaCl concentration is beyond a certain value, the turbidity disappears almost completely and levels off as the NaCl concentration continues to increase. This means that the associative phase separation vanishes and the mixture enters a compatible region. The intriguing point is that, with further increasing NaCl concentration, the turbidity re-emerges and increases dramatically. This probably indicates that a second phase separation of gelatin/κ-carrageenan comes into play in the region.[15] As will be demonstrated later, this phase separation is of the segregative type and is induced by the ordering of carrageenan chains, which results in an asymmetry in the polymer−solvent interactions.

Since the compatible mixtures always have intrinsic turbidity, we nominally set a turbidity value (see the dashed line in Figure 1) to distinguish one-phase (compatible) systems from two-phase systems. CSLM observations testified that the choosing of this value was reasonable. Then, at each temperature, two critical NaCl concentrations, corresponding respectively to the transition from associative phase separation to the compatible region and that from the compatible region to the segregative phase separation region, can be worked out from the two intersections of the turbidity curve with the dashed line (the turbidity curve at 20 °C needs to be extrapolated at both sides of the valley to cross over the dashed line). Figure 2 plots the critical NaCl concentrations with temperature, which serves as a state diagram for the gelatin/κ-carrageenan mixture at pH 7. The diagram encompasses the regions (A) compatible, (B) segregative phase separation, and (C) associative phase separation and also predicts a region (D) where associative and segregative phase separations coexist. Clearly from the figure, the segregative phase separation boundaries depend strongly on temperature, while the associative phase boundaries show little temperature dependence. This can

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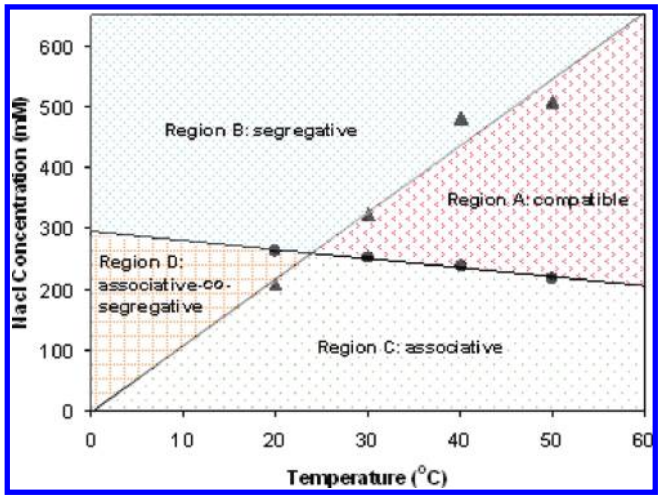

Figure 2. Derived state diagram based on Figure 1 for 0.75% gelatin/0.75% κ-carrageenan aqueous mixtures at pH 7. The diagram is comprised of four regions: compatible, segregative, associative, and associative-*co*-segregative regions. The solid circles are associative phase separation boundaries, and the triangles are segregative phase separation boundaries; the straight lines are drawn to guide the eye.

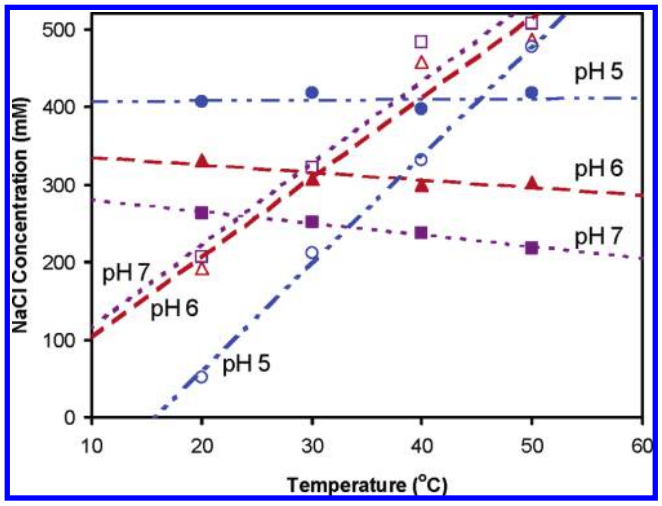

Figure 3. Effect of pH on the associative phase separation boundaries (solid symbols) and segregative phase separation boundaries (open symbols) of 0.75% gelatin/0.75% κ-carrageenan aqueous mixtures: pH 5 (●, ○), pH 6 (▲, △), pH 7 (■, □).

be interpreted by the fact that the associative phase separation is predominated by electrostatic interactions that are less temperature-dependent,[16] whereas the segregative phase separation is driven by the Flory−Huggins interactions of polymer−polymer and polymer−solvent that are much more temperature-dependent.[17,18] It should be emphasized that the state diagram is rather qualitative than quantitative. However, the direct CSLM observations into each of the regions can justify the state diagram, as will be discussed later.

The overall charge carried on amphoteric gelatin chains is significantly influenced by pH, and therefore, it is expected that pH will be an important parameter modifying the state diagram of gelatin/κ-carrageenan aqueous mixtures. Figure 3 shows how the associative phase boundaries and segregative phase boundaries

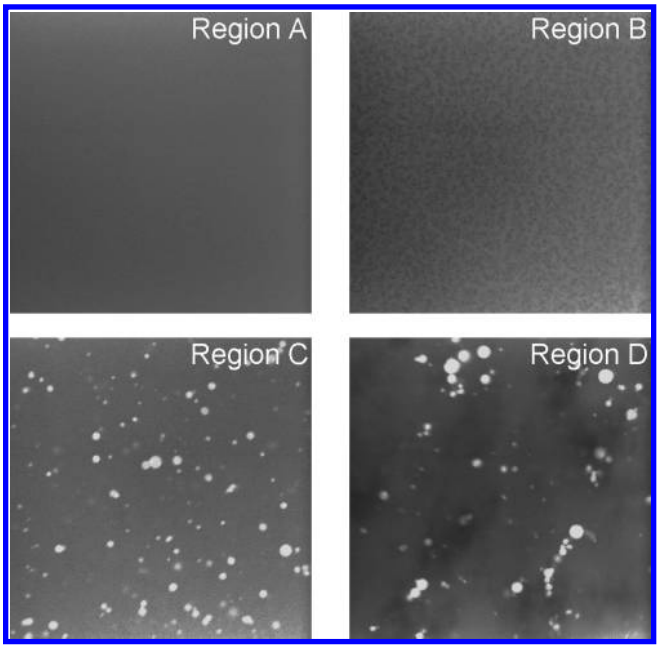

Figure 4. Examples of CSLM images taken at different regions of the state diagram (see Figure 2) of 0.75% gelatin/0.75% κ-carrageenan mixtures at pH 7: region A ([NaCl] = 300 mM, T = 50 °C), region B ([NaCl] = 300 mM, T = 20 °C), region C ([NaCl] = 50 mM, T = 50 °C), region D ([NaCl] = 50 mM, T = 5 °C). All the images have a size of 262 × 262 μm.

shift with changing pH. The decrease in pH effectively moves the associative phase separation boundaries to higher NaCl concentrations. This is due to the fact that decreasing pH makes gelatin chains more positively charged and hence strengthens the attraction between gelatin and κ-carrageenan, so more NaCl is therefore required to screen completely. On the other hand, the decrease in pH slightly shifts the segregative phase separation boundaries to lower NaCl concentrations. It is generally accepted that increasing ionic strength and decreasing pH have the equivalent effect of moving segregative phase separation boundaries to lower polymer concentrations.[19] Therefore, when other parameters are fixed (i.e., polymer concentrations, temperature), lowering the pH will make segregative phase separation start to occur at lower salt concentrations. The combinational effects of pH on associative/segregative phase boundaries lead to the broadening of the associative-*co*-segregative region (region D in Figure 2) when pH is lowered.

Evidence for the State Diagram from CSLM. To check the state diagrams obtained by turbidity measurements, CSLM was used to probe directly the phase separations and microstructures at the different regions of the state diagrams. Figure 4 shows the recorded CSLM images for 0.75% gelatin/0.75% κ-carrageenan mixtures at pH 7, located respectively at regions, A, B, C, and D of the state diagram shown in Figure 2. The phase structures obtained in the four regions are quite different. There are no discrete features in the region A image, indicating a homogeneous and compatible mixture. The region B image exhibits a low-contrast bicontinuous structure, which complies with the nature of segregative phase separation that forms gelatin-rich and carrageenan-rich phases. The region C image shows high-contrast emulsion-like structures, which is typical of associative phase separation (coacervation).[7,9] Both gelatin and κ-carrageenan are concentrated in the dispersed droplets. As for the region D image,

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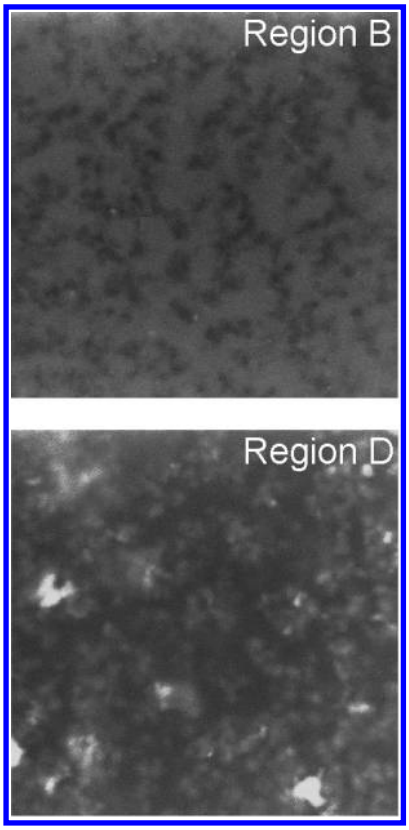

Figure 5. CSLM images taken at regions B and D of the state diagram (see Figure 3) of 0.75% gelatin/0.75% κ-carrageenan mixtures at pH 5: region B ([NaCl] = 450 mM, T = 40 °C), region D ([NaCl] = 200 mM, T = 20 °C). All the images have a size of 66 × 66 μm.

it retains the emulsion-like structure of region C, superimposed with a phase-separated background. This is in line with the prediction that associative and segregative phase separation coexist in this region.

The CSLM observations of 0.75% gelatin/0.75% κ-carrageenan mixtures at pH 5 once again can confirm the presence of four different phase regions in the state diagram. Particularly, they gave more visual evidence for segregative phase separation and associative-*co*-segregative phase separation (see Figure 5). The image taken at region B clearly shows a bicontinuous structure that is developed via the segregative phase separation of gelatin and κ-carrageenan. The region D image shows three phases; white spots are distributed in a cloudy background. The white spots result from the associative phase separation of gelatin/κ-carrageenan (that is, coacervate), and the cloudy background arises from a second segregative phase separation of the two polymers. This again justifies the prediction of the coexistence of associative/segregative phase separations in gelatin/κ-carrageenan mixtures.

The above results support the state diagrams obtained from turbidity measurements and also show the different microstructures associated with the different regions of the state diagrams. Freezing these structures by gelling one or both of the components, gelatin or κ-carrageenan, has the potential to design materials with significantly different properties, e.g., viscoelastic properties. Studies on the rheological properties of these systems are ongoing and will be reported elsewhere.

Mechanisms of Phase Separation. It is known that the associative phase separation in gelatin/κ-carrageenan mixtures is driven by the electrostatic interactions between the two polymers.[15] However, the mechanisms underlying the segregative phase separation remain ambiguous. To elucidate these, it is required to precisely map out the starting temperatures of phase separation at each ionic strength and the ordering temperatures of both gelatin and κ-carrageenan, instead of the rather schematic and qualitative diagrams as shown in Figures 2 and 3. We used the onset of turbidity increase upon cooling as a measure of the starting temperature of segregative phase separation and compared it with the onset ordering temperatures (coil−helix transition) of gelatin and κ-carrageenan obtained by DSC. Figure 6 shows the results obtained for the mixtures of 0.75% gelatin/0.75% κ-carrageenan when cooled at a rate of 1 °C/min.

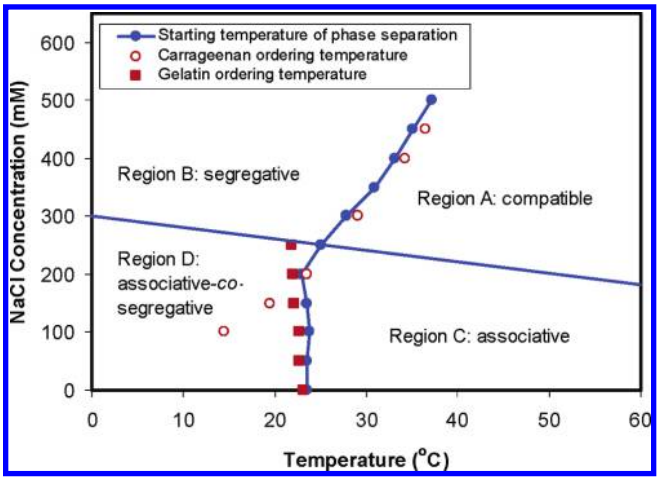

Figure 6. Relationship between the starting temperature of segregative phase separation and the ordering temperatures of gelatin and κ-carrageenan obtained for 0.75% gelatin/0.75% κ-carrageenan mixtures at pH 7. The starting temperature of segregative phase separation was obtained on the basis of turbidity measurements at a cooling rate of 1 °C/min, and the ordering temperatures of gelatin and κ-carrageenan were obtained from DSC measurements at the same cooling rate. The associative phase separation boundary (the nearly horizontal line) was taken from Figure 2, and its part in region D was based on uncertain extrapolation.

Clearly, the reconstruction brings about a slightly different state diagram from that shown in Figure 2; the segregative phase boundary in region D is almost perpendicular to the temperature axis rather than a sloping line as we expected. The start of segregative phase separation in region B is closely coupled with the onset ordering of κ-carrageenan, while that in region D coincides with the onset ordering of gelatin. This indicates that the segregative phase separation in region B is driven by the conformational ordering of κ-carrageenan, while that in region D is driven by the conformational ordering of gelatin. Indeed, the studies on gelatin/maltodextrin mixtures showed that the ordering of gelatin chains could induce the system to phase separate upon cooling.[18] This kind of conformational-ordering-induced segregative phase separation was believed to arise from the asymmetric polymer−solvent interactions after one component is ordered.[2,18] The mechanism behind the segregative phase separation of gelatin/κ-carrageenan seems to be decided by the component that has a higher ordering temperature upon cooling. The switching from κ-carrageenan-ordering-induced phase separation to gelatin-ordering-induced phase separation is simply due to the fact that the ordering temperature of κ-carrageenan is higher than that of gelatin in region B while it is lower in region D (please compare open circles and solid squares in Figure 6).

It is anti-intuitive that associative/segregative phase separations could coexist in region D, since thermodynamically the two phenomena are incompatible. However, this could be explained from the aspects of kinetics: when gelatin and κ-carrageenan are

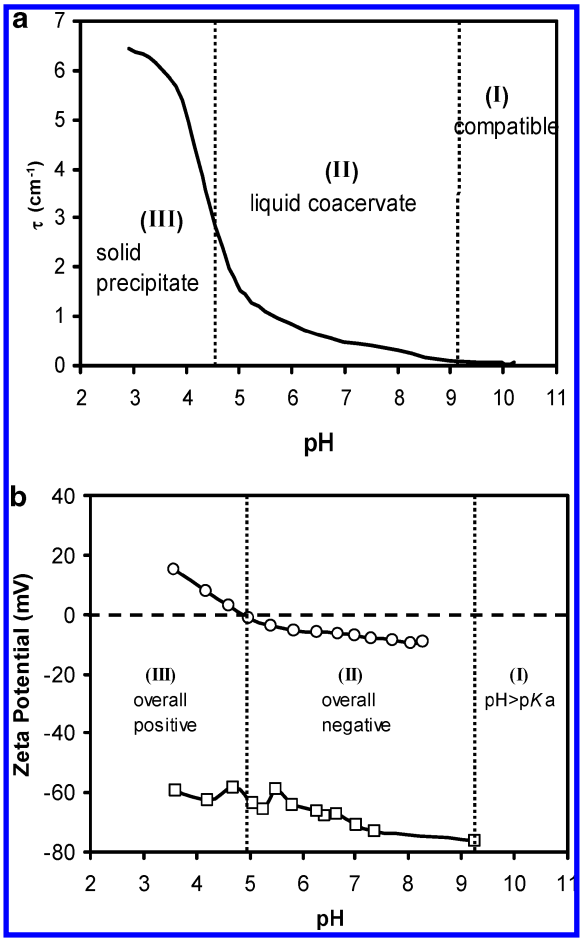

Figure 7. (a) Titration of turbidity as a function of pH at 50 °C for 0.75% gelatin/0.75% κ-carrageenan mixtures at [NaCl] = 25 mM. (b) Corresponding titrations of the ζ potential for gelatin (○) and κ-carrageenan (□).

mixed at higher temperatures and with low salt concentration, the associative phase separation first occurs, which forms two phases with one phase rich in both polymers and the other depleted. At a certain temperature upon cooling, the ordering of gelatin starts and kinetically traps or freezes the polymer-rich phase via the gelation of gelatin; at the same time, it induces a second segregative phase separation in the polymer-depleted phase where gelation does not occur because of the low gelatin concentration caused by depletion. Therefore, the coexistence region D is thermodynamically in a nonequilibrium state and kinetically entrapped by gelation.

Morphological Changes Induced by pH. It has been shown above that pH can markedly change the state diagrams of gelatin/κ-carrageenan mixtures, mainly via exerting effects on associative phase separation boundaries (see Figure 3). This section details how pH affects the behaviors of associative phase separation and the resulting morphologies. Figure 7a shows the titration of turbidity as a function of pH at 50 °C for 0.75% gelatin/0.75% κ-carrageenan mixtures at [NaCl] = 25 mM. With decreasing pH, the turbidity increases at pH $\approx$ 9 and has a second sharp increase at pH $\approx$ 5, which marks three pH regions: I, II, and III. For the purpose of comparison, the ζ potential titrations of gelatin and carrageenan at the same experimental conditions are shown in Figure 7b. Region I corresponds to a compatible region where the electrostatic attraction between gelatin and κ-carrageenan is very weak because most of the cationic groups of gelatin are deprotonated (pH > pK_a).[15] The CSLM observations in this region show no features (see Figure 8, top); gelatin and κ-carrageenan exist as individual chains or a soluble complex.[1]

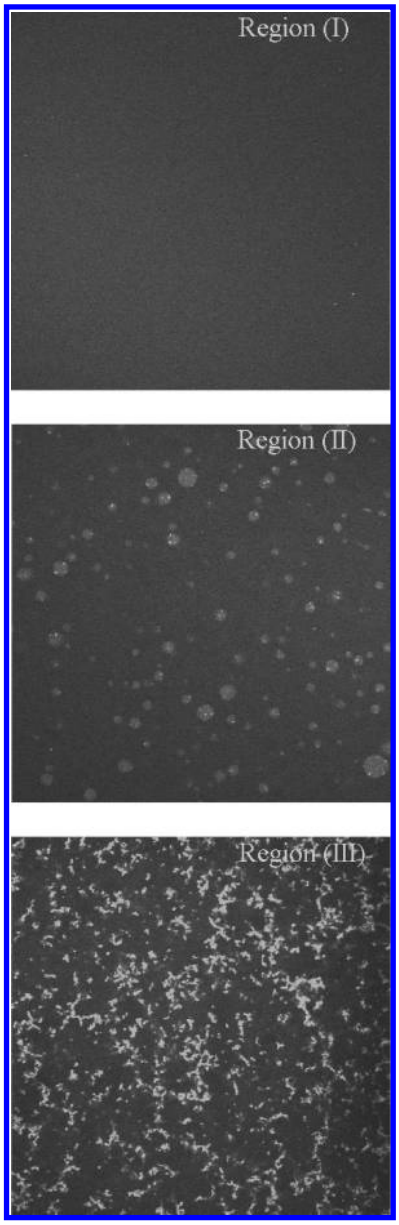

Figure 8. CSLM images taken at different pH regions for 0.75% gelatin/0.75% κ-carrageenan mixtures at 50 °C and [NaCl] = 25 mM: region I (pH 10), region II (pH 7), region III (pH 4). All the images have a size of 262 × 262 μm.

In region II, gelatin bears more and more cationic groups as the pH decreases. Although gelatin is overall negatively charged, the electrostatic attraction between gelatin chains and κ-carrageenan chains is already strong enough to lead to associative phase separation. The mixtures in this region exhibit an emulsion-like phase-separating structure (Figure 8, middle), and the droplets can grow with time by coalescence. The region is referred to as the liquid coacervate region. When pH is lowered from region II to region III, the gelatin chains start to become overall positively charged (Figure 7b). This leads to a sharp increase in turbidity that corresponds to the formation of fiberlike structures as observed by CSLM (Figure 8, bottom). The fiberlike structure is relatively stable and has no tendency to grow with time. This has been labeled as the solid precipitate region.

The transitions from compatible to liquid coacervate and further to solid precipitate regions are influenced by ionic strength. Figure 9 plots pH_l and pH_s as a function of NaCl concentration. pH_l is the pH at which turbidity shows the first increase and the mixtures enter the liquid coacervate region. pH_s is the pH where the

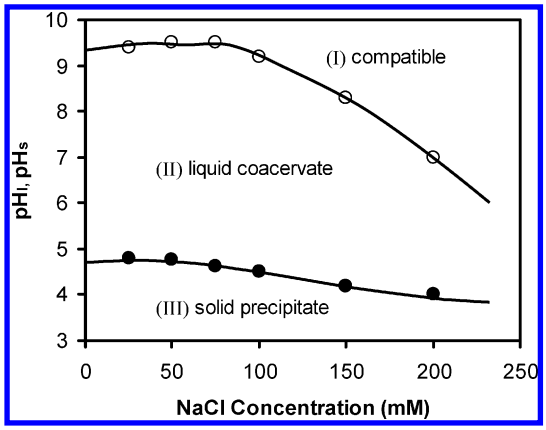

Figure 9. pH_l (○) and pH_s (●) against NaCl concentration for the mixture of 0.75% gelatin/0.75% κ-carrageenan at 50 °C.

differential of turbidity against pH has a negative maximum, characterizing the transition between liquid coacervate and solid precipitate. Below a NaCl concentration of 75 mM, pH_l and pH_s are almost independent of the NaCl concentration; actually pH_l shows a slight increase with increasing NaCl concentration. Thus, the addition of a small amount of salt seems to enhance the phase separation. Burgess has mentioned that associative phase separation could be reduced due to an unfavorable extended shape of the polymer chains arising from intramolecular repulsion at low ionic strength.[20] Weinbreck added another explanation that the presence of a small amount of salt could play a role in charge compensation for the polymer−polymer complex incapable of reaching electroneutrality and hence allow an effective phase separation.[14] On the other hand, when the NaCl concentration is above 100 mM, both pH_l and pH_s decrease with increasing salt concentration. This is simply due to the screening of intermolecular attraction between gelatin and κ-carrageenan in the excess of salt.

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Conclusion

The mixing of gelatin with κ-carrageenan usually leads to an associative phase separation that results from the electrostatic attraction between the two polymers. However, by tuning the salt concentration and temperature, the associative phase separation can be completely screened, and the system can be directed into the compatible region, the segregative phase separation region, and even a region where associative and segregative phase separations coexist. The segregative phase separations are induced by the ordering of either gelatin or carrageenan, depending on which polymer first starts to order upon cooling under different salt conditions. The associative-*co*-segregative phase separation region is believed to be in a kinetically trapped state rather than thermodynamically stable. The change in pH moves the boundaries of the state diagram of gelatin/κ-carrageenan mixtures and also significantly modifies the morphology of structures formed by associative phase separation.

The state diagram (e.g., Figure 2), together with the morphological effects of pH (Figure 9), serves as a guideline in designing different microstructures in gelatin/κ-carrageenan systems and therefore produces an important approach for both controlling and creating material behavioral properties, e.g., viscoelasticity.

Acknowledgment. We thank J. C. G. Blonk and G. van Dalen at Advanced Measurements and Imaging, UFHRI, Vlaardingen, for their help with the CSLM measurements and M. H. Bulter and R. Goodband at the Unilever Research Colworth House for their help with the DSC and turbidity measurements. The helpful discussions with Drs. Stephan Schumn, Rob Vreeker, Rob Groot, Robert Farr, Tim Foster, and Eddie Pelan are much appreciated.

LA061865E