Dielectric Analysis for In-Situ Monitoring of Gelatin Renaturation and Crosslinking

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ABSTRACT: This article probes the use of dielectric analysis (DEA) to monitor the physical changes that occur during gelatin renaturation and crosslinking. An interdigitated dielectric sensor was used to monitor ionic mobility in gelatin. In spite of the large concentration of water and ions in the gelatin model systems, a range of frequencies was found where DEA met all the requirements for ion viscosity measurements necessary to monitor aging gelatin. DEA was successful at detecting gelatin renaturation with a remarkably high signal-to-noise ratio. DEA was also capable of detecting crosslinking in the presence of formaldehyde, but only in the special case of fish gelatin. Unlike acid bone gelatin, which is semicrystalline at room temperature, fish gelatin remains fluid. Thus, fish gelatin provides a model system where crosslinking can be monitored in the absence of interference by renaturation processes. The dielectric response was also found to be sensitive to water transport between acid bone gelatin and a simulated pharmaceutical fill made of a PEG 600-water solution. In the case of acid bone gelatin, the kinetics of renaturation as detected from DEA closely matched those measured by monitoring the evolution of the melting point and of the enthalpy of melting as measured by differential scanning calorimetry (DSC). With dynamic mechanical analysis (DMA), the rise in the loss modulus with renaturation also closely tracked the increase in ionic viscosity. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 2765–2775, 2006

Key words: crosslinking; crystallization; dielectric analysis; gelatin; renaturation; sensors

INTRODUCTION

In pharmaceutical applications, gelatin capsules can be used to hold liquid, semisolid, or solid fills, whether in the form of soft elastic or hard capsules. This technology is particularly useful for the development of dosage forms for insoluble compounds and highly potent compounds and for early development through commercial dosage forms. Since gelatin capsules are made from a partially re-crystallized collagen protein of animal origin, they tend to be dynamic systems prone to physical and chemical interactions with the fill and with the surrounding environment.

In the case of soft elastic capsules, these interactions begin as soon as the capsule, made of a freshly extruded gelatin formulation, is sealed and in contact with the fill solution. From the initial moments of capsule shell manufacturing, complex chemical and physical changes occur and may continue over very long time scales. These long-term changes include gelatin renaturation, mass transport between the fill and the gelatin, loss of moisture to the surrounding atmosphere, and gelatin crosslinking due to the presence of aldehyde impurities in the fill. The realization that a thorough understanding of the kinetics of these long-term changes is critical to formulating stable gelatin dosage forms provided the motivation for this research.

Monitoring long-term changes using complex analytical tools can be tedious and expensive. This analytical challenge led us to explore the use of an *in situ* sensor in conjunction with automated data acquisition suitable for long-term stability experiments. In this study, we evaluated the suitability of dielectric analysis (DEA) for monitoring: 1) renaturation, 2) fill-gel interactions, and 3) crosslinking. This was accomplished by using simple gelatin model systems and comparing the dielectric response of maturing gelatin with that from more established techniques, such as differential scanning calorimetry (DSC), dynamic mechanical analysis (DMA), and isothermal calorimetry. DSC and DMA were selected in light of the fact that they have provided good insight into the process of gelatin renaturation and aging in earlier studies performed by other workers.[1–9] Isothermal microcalorimetry was selected for its sensitivity to chemical reactions between gelatin and formaldehyde.

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In DEA, a sinusoidal voltage is applied between electrodes placed in intimate contact with the sample, and the resulting sinusoidal current is measured.[10] The changes in amplitude and phase of the response are then used to calculate the dielectric constants. These constants provide insight on the mobility of ions and dipoles in the material. The loss component of permittivity, ε'', of a dielectric material may be expressed as:[11]

$$\varepsilon'' = \varepsilon''_{rd} + \frac{\sigma}{\omega\varepsilon_0} = \varepsilon''_{rd} + \frac{\alpha}{\eta\omega\varepsilon_0} \quad (1)$$

where:

ε''_{rd} is the permittivity component of dipole relaxation
σ is the bulk ionic conductivity
ε_0 is the permittivity of vacuum
ω is the frequency of the electric field
η is the bulk ionic viscosity
α is a material constant.

At low frequencies, ionic mobility dominates the response. Under these conditions, the dipole contribution of eq. (1) vanishes and ε'' becomes inversely proportional to both ionic viscosity and frequency. In the context of this study, ion viscosity is a measure of protein mobility in the gelatin. It is expected to be sensitive to changes in molecular mobility associated with structural changes, such as renaturation (i.e., crystallization) or chemically induced crosslinking.

To test the utility of DEA in gelatin systems, two types of gelatin were chosen: acid bone gelatin and fish gelatin. Acid bone gelatin is obtained by acid hydrolysis of collagen derived from animal by-products. A peculiarity of bovine gelatin is its capacity to form a specific triple-stranded helical structure not observed in hot solutions. The helical structure is stabilized by the proline and hydroxyproline amino acids. The formation of crystalline helical structures below the gelation point is called "renaturation" in the rest of this article. In contrast, fish gelatin has a lower proline and hydroxyproline content and, therefore, its gelation temperature is lowered below room temperature. This gelatin, therefore, allowed investigation of chemical crosslinking without the interference of renaturation. The term "crosslinking" will refer to the formation of chemical crosslinks in the presence of aldehydes. From a rubber elasticity standpoint, renaturation can be viewed as a thermoreversible crosslinking. For the sake of clarity, however, this terminology will be avoided whenever possible.

EXPERIMENTAL

Materials

Dry powder of 195 Bloom Acid Bone NF Gelatin (Kind and Knox, Sioux City, Iowa) was dissolved in deionized water held at 65°C in a water bath. The same procedure was followed with dry fish gelatin powder (Norland Products Inc, Cranbury, NJ) except that the bath temperature was 50°C. After mixing and complete dissolution, the two solutions were allowed to de-aerate overnight (at 50°C for the acid bone gelatin and at laboratory temperature for the fish gelatin). When not used, the preparations were stored at 5°C to prevent microbial degradation. The concentration of both solutions was 50% (w/w).

PEG 600 (Carbowax Sentry Polyethylene Glycol 600 NF, FCC grade, The Dow Chemical Company, Midland, MI) was used to prepare aqueous solutions simulating hydrophilic soft gel capsule fills. Details of the preparation of the aqueous based solution are provided in the next section.

Water to prepare gelatin solutions or PEG 600 solutions was obtained from two sources: 1) Milli-Q 4 bowl water system, Millipore Corp, Boston, MA, and 2) Milli-Q plus purification pack QPAK2, Millipore Corp, Boston, MA. Ion conductivity was below 18$M\Omega$-cm in both cases. For crosslinking studies, formaldehyde (37 w/w % solution in water, histological grade, Aldrich Inc, St Louis, MO) was used.

Procedures

Dielectric analysis (DEA)

Dielectric measurements were carried out on films of gelatin cast over interdigitated fringe field electrodes (1 in. rear exit Monotrode TMS, Netzsch Instrument Inc, Burlington, MA). Spacing between electrodes was 0.5 mm.[12] In the case of the fringe field dielectric sensing, the depth of material probed is roughly of the order of the spacing between electrodes. For this reason, film thickness was set at a minimum of 1.3 mm at the time of casting so that it would remain greater than or equal to electrode spacing after the shrinkage associated with gelation and drying. Film thickness was controlled with a casting knife (6 in. wide, 0–250 mils clearance, BYK-Gardner, River Park, IL).

A multichannel dielectrometer (DEA 230/10, Netzsch Instrument Inc., Burlington, MA) was used for data acquisition and analysis. Data acquisition was performed on two channels simultaneously, using software (Eumetric, version 8, Netzsch Instrument Inc., Burlington, MA) originally designed for monitoring the cure of molded composites with thermoset resin matrices.[10,13] Typical material constants entered in the acquisition software were: loss factor cutoff: 1; permittivity cutoff: 10^5; relaxed permittivity: 10; boundary layer radius cutoff: 25; and minimum permittivity: 10^{-6}. The electrode polarization correction option was turned off in all cases (all the permittivity values were relative to vacuum and are, therefore, dimensionless). High conductivity settings were used

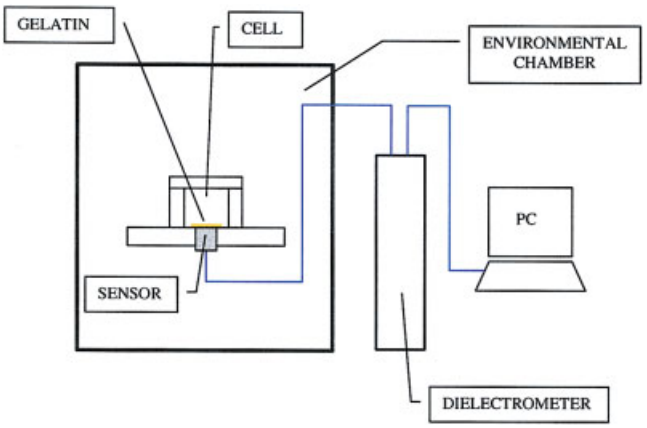

Figure 1 Schematic of the experimental set-up for DEA (top), with a view of the open environmental chamber (bottom). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in both the dielectrometer hardware and acquisition software.

The tool mount sensor was surface-mounted at the center of a stainless steel base plate. Once a gelatin film was cast over the sensor, a cylindrical stainless steel cell would be bolted to the base plate. The base plate could then be sealed with a top cover (also featuring a port for rapid fluid injection). Two cells of identical design were fabricated for this study, each with the same model of fringe field sensor.

For temperature and humidity control, the cells were placed inside an environmental chamber (SB1 160, Weiss Environmental Technology Inc, Menomonee Falls, WI) with cables from the back side of the sensors running through a sealed port hole on the side of the chamber, to the dielectrometer placed on the lab bench. The dielectrometer was interfaced with a bench top computer as depicted in Figure 1.

Several types of experiments were performed in the dielectric cells. In the case of the acid bone gelatin, cast films of gelatin were either exposed directly to the controlled temperature and humidity of the chamber (open cell) or placed in direct contact with the PEG-water solution (closed cell). In the open cell configuration, the cylindrical wall was removed for maximum exposure of the gelatin film to the convective air currents, and the humidity chamber was held at 30°C and 98% relative humidity (RH). In the case of the closed cell configuration, 40 g of 50% (w/w) PEG 600-water solution was poured over the gelatin film (weighing approximately 1.4 mg) and the cell was sealed. For crosslinking experiments, the PEG 600-water solution contained amounts of formaldehyde varying from 20 mg to 80 mg approximately. In all cases, temperature was maintained at 30°C by the environmental chamber.

The 50% (w/w) ratio of PEG 600-to-water was selected to achieve an equivalent RH of 98%, that is, such that water activity would be the same as in the open cell configuration. This ratio was determined by measuring the solubility isotherm of water vapor in PEG 600, using a dynamic moisture sorption gravimetric analyzer (see the method description below). The resulting plot was log-linear, and 98% RH corresponded to a water weight gain of 100%, that is, a 1 : 1 ratio of PEG 600-to-water.

In the case of fish gelatin, 4 g of the gelatin solution was poured over the sensor in a closed cell configuration, and the cell was kept inside the environmental chamber held at 30°C. To induce crosslinking of the fish gelatin, a 6.1% (w/w) formaldehyde solution was added to the 4 g of fish gelatin. A control without formaldehyde was run simultaneously in a second cell.

Differential scanning microcalorimetry (DSC)

The gelatin samples used for DSC were prepared by the same technique as used for DEA. The gelatin solutions were cast onto glass slides and the thickness controlled using a casting knife. The gelatin films were equilibrated in a sealed jar of PEG 600 solution and held in an oven at 30°C for approximately 30 h. Gelatin samples were removed from their conditioning environment and placed in a previously tared aluminum pan that was then hermetically sealed. The sample and pan were weighed on an analytical microbalance (Supermicro S4, Sartorius Corp., Bohemia, NY). Samples (weight range approximately 6–9 mg) and reference pans were placed in a DSC Q100 (TA Instruments) for calorimetric studies. The gelatin was equilibrated at 0°C and then heated above the crystalline melting point to 80°C at a rate of 20°C/min. The melting point and the enthalpy of melting were calculated with the TA Instruments Universal Analysis program. Cyclic tests were also performed beginning with heating to 80°C at a rate of 20°C/min to remove any thermal history of the sample. The first cycle involved cooling to 0°C at 20°C/min and then heating back up to 80°C at 20°C/min for an initial run with no renaturation. The sample was then cooled to 30°C at 20°C/min and held for a given period of renaturation, equilibrated to 0°C, and heated to 80°C at 20°C/min. This process was repeated with increasing values of hold time at 30°C, and involved heating to 80°C to observe the change in the melting point (T_m) and enthalpy of melting over a range of renaturation times. The graphical method used to define the melting point and the enthalpy of melting of acid bone gelatin is shown in Figure 2. Peak endotherm temperature was selected to define the melting point.

Dynamic mechanical analysis (DMA)

Solid-state rheology measurements were made using a custom designed apparatus in the Micromechanics

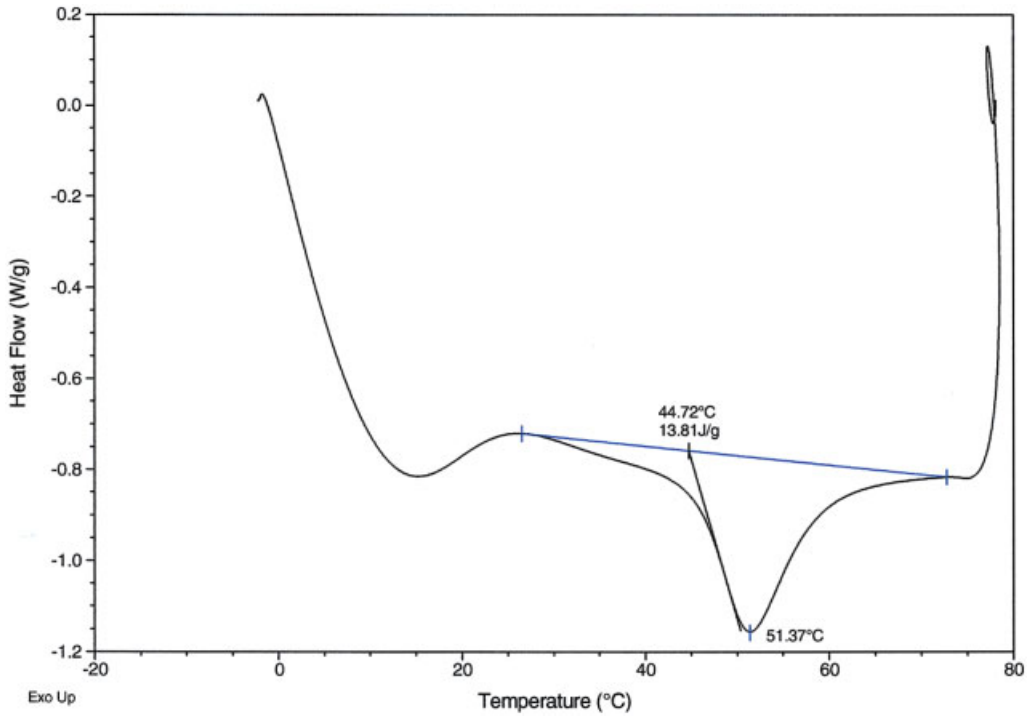

Figure 2 Graphical method used to determine the melting point and the enthalpy of melting of partially renaturated acid bone gelatin. The melting point was defined as the peak endotherm temperature. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Lab of Professor Kenneth Shull at Northwestern University (Evanston, IL). Gelatin samples were cast onto glass cover slips and placed under a PEG-water solution with an equivalent water activity of 98% RH. The samples under solution were held at 30°C with a stage heater (Physitemp, Clifton, NJ). A flat cylindrical punch was brought into contact with the gelatin layer using a piezoelectric stepping motor (Burleigh, Fischers, NY). Upon reaching an arbitrary load, a sinusoidal oscillatory strain was applied perpendicular to the sample. Load and displacement data were collected and analyzed with National Instruments LabVIEW software. The storage and loss modulii of the samples were calculated from measurements of the phase lag between the applied strain and the resulting stress. All measurements reported herein were conducted at a frequency of 0.02 Hz. For more details about the fixture and principles of operation, the reader is referred to an article by Shull and coworkers.[14]

Isothermal calorimetry

Gelatin samples were weighed into 3 mL glass ampoules using a Sartorius analytical balance. An electronic pipette was used for volumetric addition of formaldehyde (model *EDP2*, Rainin Instrument Co., Inc., Woburn, MA). The formaldehyde was pipetted into HPLC insert vials placed inside the 3 mL glass ampoule. Ampoules were crimped and loaded into the calorimeter immediately after their preparation. Power measurements were made at 30°C using a high sensitivity isothermal calorimeter (Thermal Activity Monitor, TAM model 2277, Thermometric AB, Jarfalla, Sweden). Digitam software (version 4.1, Thermometric AB, Jarfalla, Sweden) was used for data collection and analysis. The samples were equilibrated for 30 min prior to lowering them to the measuring position in the TAM. The power output was collected every 10 s over several days. In all experiments, the reference cells contained dry talc to balance the heat capacities.

Dynamic moisture sorption gravimetric analysis

The water sorption isotherm of water in PEG 600 was studied using a moisture balance (SGA-100 Symmetric Vapor Sorption Analyzer, VTI Corp., Hialeah, FL) fitted with a water bath circulation system (Julabo F25, Julabo Labortechnik GmbH, Steelbach, Germany). Data was collected using the VTI Isotherm (version 1.32) software supplied with the instrument. The isotherm was run at 30°C, by placing samples on a DSC pan inside the instrument. The drying temperature was 30°C. The RH steps were 5, 10, 20, 40, 50, 60, 70, 80, 90, and 95%. The equilibration criterion was 0.01% (w/w) in 180 min.

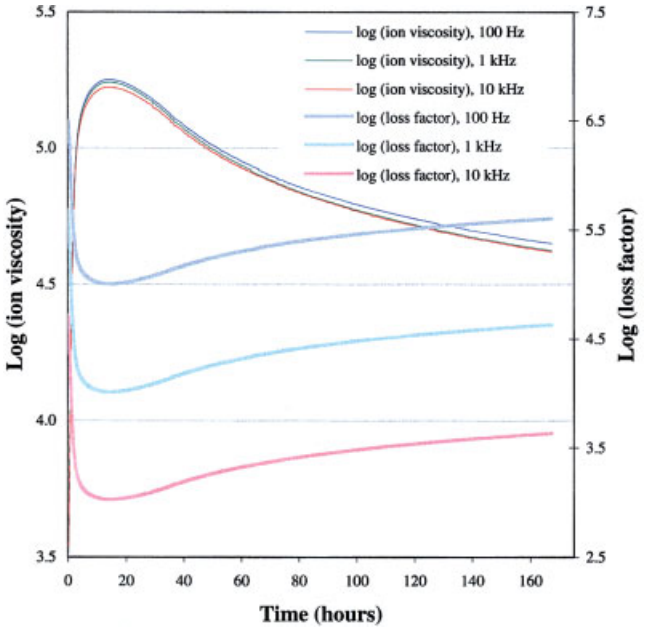

Figure 3 Frequency dependence of the dielectric response. Special case of gelatin undergoing renaturation in PEG 600-water at 30°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

Renaturation studies

The range of frequencies used for this study was selected to ensure that the dielectric response was dominated by ionic mobility. This is illustrated in Figure 3 where log(ε″) and log(ion viscosity) are plotted as a function of time in the case of a gelatin film undergoing renaturation in 50% (w/w) PEG 600-water. Three excitation frequencies were used: 100 Hz, 1 kHz, and 10 kHz. Note that the ionic viscosities obtained at these three frequencies were virtually identical. This frequency independence was maintained over a range of ionic mobility spanning nearly 2 orders of magnitude. At higher frequencies, dipole polarization occurred (not shown); while at lower frequencies, electrode polarization occurred due to the high concentration of water in the gelatin sample (not shown). The optimum frequency range was usually from 1 kHz to 10 kHz, as exemplified by the excellent overlap of the ion viscosity curves for these two frequencies (Fig. 3). Once frequency independence was verified, only one frequency was selected when reporting ionic viscosity, usually 10 kHz.

Figure 4 shows the dielectric responses of gelatin undergoing renaturation in 50% (w/w) PEG 600-water held at 30°C (98% equivalent RH) in one case, and in humid air held at 30°C/98% RH in the other case. To achieve this, two samples were cast from the same batch of gelatin mass onto the base plates of two separate cells. One cell was used in the open configuration (humid air), while the other was used in the closed configuration (PEG 600-water). Data from the closed cell was less noisy than that from the open cell because small variations in temperature and humidity associated with the control feedback loop cycles were dampened by the thick stainless steel walls.

With gelatin in direct contact with the PEG 600 solution, ion viscosity increased rapidly during the initial 10 h of renaturation. This was followed by a slow asymptotic relaxation towards a plateau that was reached much earlier by the sample held in humid air. Eventually, the two curves converged toward the same equilibrium, since water activity in the PEG solution was the same as in the humid air.

Figure 5 shows an overlay of DSC curves obtained by the cyclic method. In this case, a gelatin film was equilibrated in a sealed jar of PEG solution held in an oven at 30°C for approximately 30 h. Each DSC scan from left to right corresponds to longer isothermal renaturation times at 30°C. As renaturation time increases, note that the melting point shifts to higher temperatures and that the melting endotherms grow more pronounced. The first scan was made after 15 min of renaturation, while the last one was obtained near the end of the renaturation time.

A comparison of the DEA and DSC data show that the two techniques detect physical changes on the same time scale. Figure 6 shows a comparison of the enthalpy of melting measured from the cyclic DSC method with the dielectric response from gelatin renaturation in the PEG solution held at 30°C (closed cell). Figure 7 shows the melting point from the same

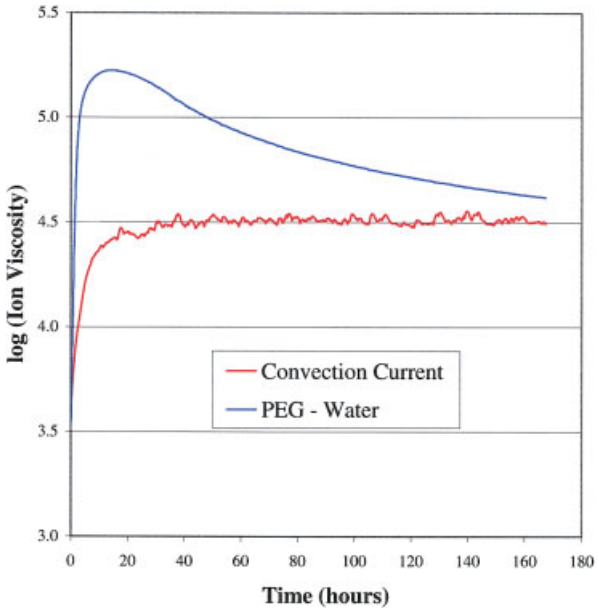

Figure 4 Comparison of gelatin renaturation in humid air held at 98% RH and in PEG-water (98% ERH), both at 30°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

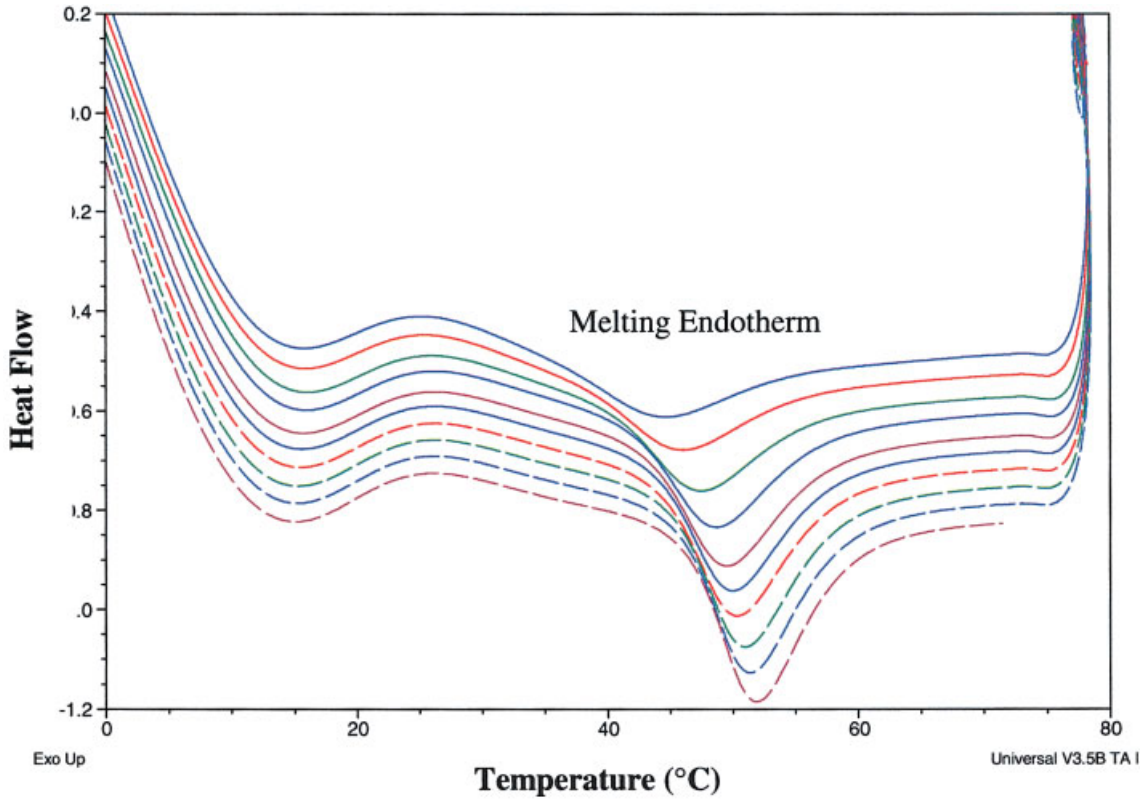

Figure 5 Example of DSC endotherm evolution as gelatin undergoes renaturation at 30°C. The melting point shifts to higher temperatures, and the heat of melting increases. The top scan was performed after 15 minutes of renaturation. For clarity, curves are shifted down as renaturation time increases. The bottom scan was performed after 15 h of renaturation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DSC experiment, instead of the enthalpy of melting. The comparison between DEA and DSC is not completely rigorous since the cyclic DSC scans were performed on equilibrated gelatin with a fixed amount of moisture, whereas the DEA was performed on gelatin renaturating while losing moisture to the PEG solution. Nonetheless, this was the first indication that with a well-designed set-up, DEA is capable of monitoring the renaturation process of gelatin. It should be noted that the limited software memory available for the cyclic DSC measurements did not allow us to explore longer renaturation times in this particular experiment. This is unfortunate, because cyclic DSC, by using the same sample repeatedly in a sealed pan yields inherently less noisy data than measuring independently prepared samples obtained at different equilibration time points. The cyclic DSC experiment allows monitoring of a closed system (from a mass standpoint) throughout the entire experiment and eliminates the experimental errors associated with weighing, moisture evaporation before pan sealing, and the use of a new pan for every time point.

To obtain renaturation data over 30 h, multiple samples were prepared and measured by DSC. As discussed above, this leads to increased scatter of the data as readily seen by examining Figures 8 and 9. For these experiments, the gelatin samples were exposed to humid air rather than a PEG solution, and the gelatin films used for DSC and DEA were made at the same time and under identical conditions. While DEA recorded renaturation *in situ*, to perform DSC scans, samples had to be taken out of the environmental chamber at various time intervals. In spite of this limitation, we were able to confirm that the time scale of renaturation as detected by DSC is the same as that of the ion viscosity signal from DEA. This time, the match between the two time scales was better than in the experiment with the PEG solution, most likely because gelatin sample histories were identical for both DSC and DEA. Additionally, we were able to explore longer renaturation times and to demonstrate that both techniques show a plateau after renaturation times exceeding 40 h.

Another method of choice to monitor gelatin renaturation is rheometry of dilute solutions of gelatin.[5–9] For this reason, an adaptation of this method was also introduced in this study to compare with the DEA data. A DMA apparatus customized to probe the surface of a film of concentrated gelatin (50 w/w %) was used. Such characterization would not have been possible with classical rheometry, due to the higher degree of stiffness of our sample in the gel state. As

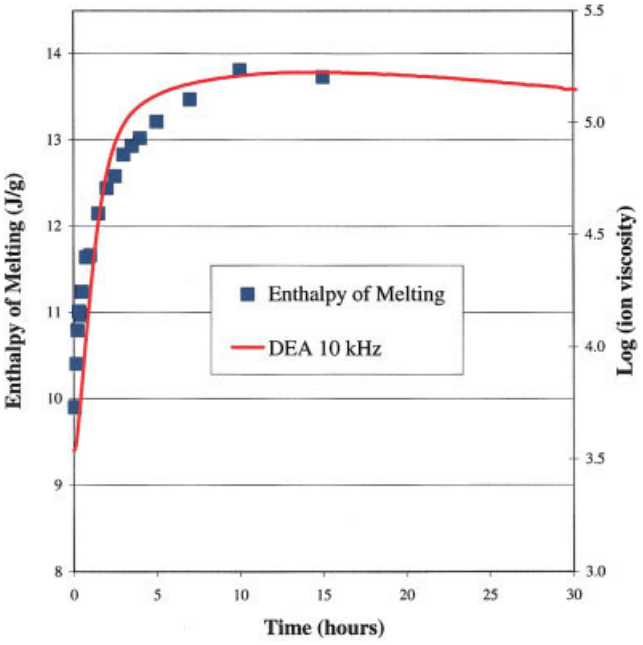

Figure 6 Comparison of the enthalpy of melting (DSC) and the ion viscosity (DEA) in PEG 600-water held at 30°C, as gelatin undergoes renaturation over 30 hours. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

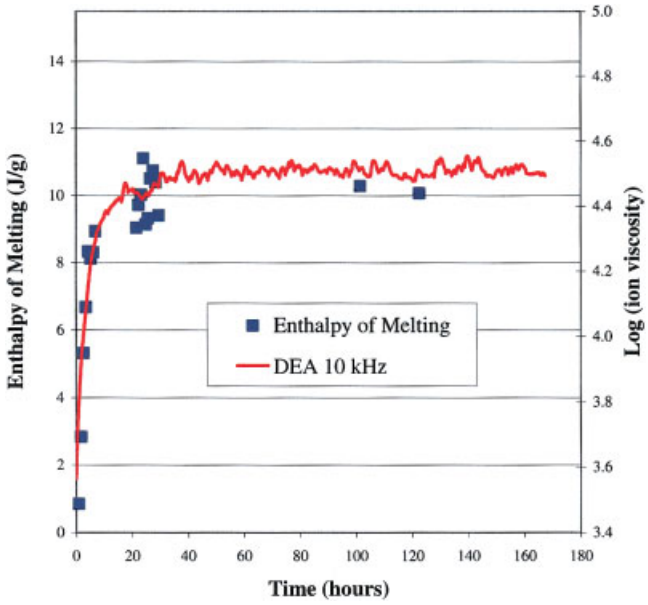

Figure 8 Comparison of the enthalpy of melting (DSC) and the ion viscosity (DEA) in humid air held at 30°C/98% RH, as gelatin undergoes renaturation over 180 hours. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

explained earlier, the gelatin film was immersed in PEG 600-water held at 30°C. In Figure 10, we superimposed loss modulus versus time and storage modulus versus time with the dielectric response of gelatin undergoing renaturation under identical conditions. The kinetics of renaturation as detected by the loss modulus (G″) and ion viscosity were noticeably slower

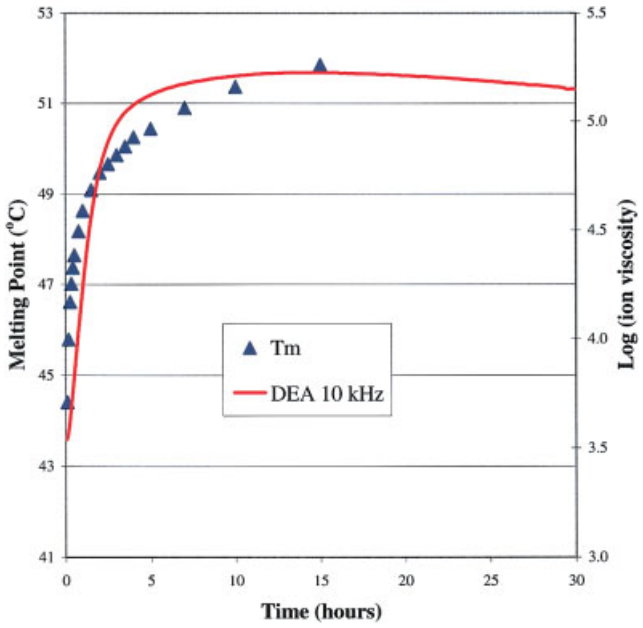

Figure 7 Comparison of the melting point (DSC) and the ion viscosity (DEA) in PEG 600-water held at 30°C, as gelatin undergoes renaturation over 30 hours. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

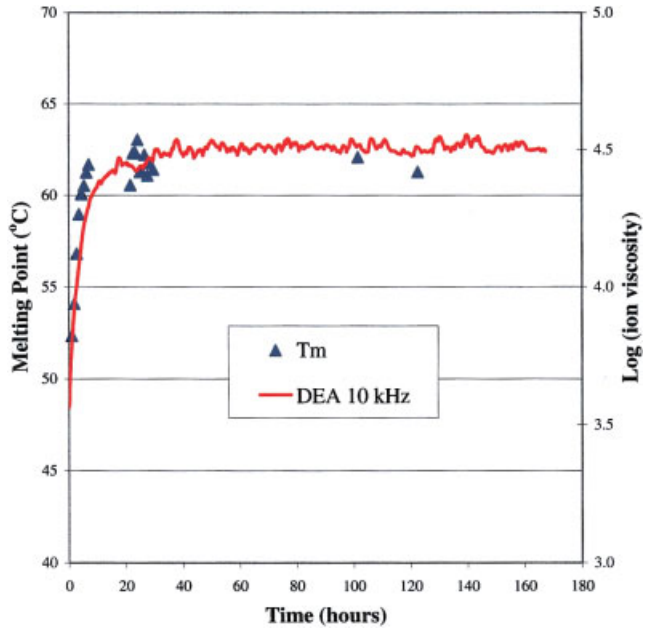

Figure 9 Comparison of the melting point (DSC) and the ion viscosity (DEA) in humid air held at 30°C/98% RH, as gelatin undergoes renaturation over 180 hours. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

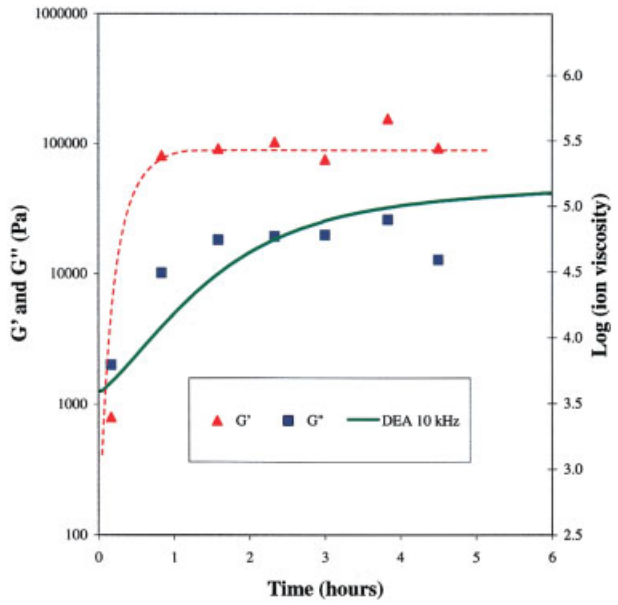

Figure 10 Comparison of the loss and storage modulii (DMA, 0.02Hz) and the ion viscosity (DEA) as gelatin undergoes the initial stage of renaturation in PEG 600-water held at 30°C. The dashed red line was added as a visual aid to help track G′. By contrast, the continuous green line is made of a raw experimental data points from DEA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

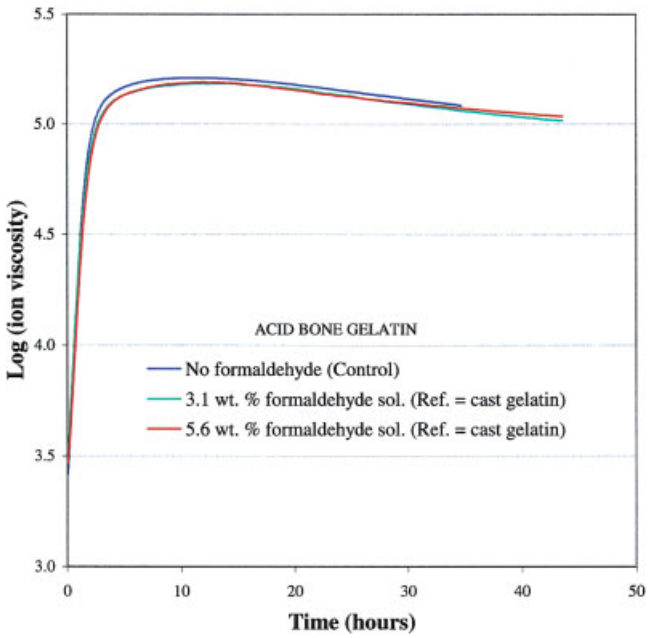

Figure 11 Effect of formaldehyde on the DEA response of acid bone gelatin undergoing renaturation in contact with PEG 600-water held at 30°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

than those detected by the storage modulus G′, with G′ reaching a plateau earlier than G″ and ion viscosity.

Crosslinking studies

Acid bone gelatin renaturation and potential crosslinking were monitored using a closed dielectric cell, with a gelatin film cast over the sensor and immersed in PEG-water solutions contaminated with varying amounts of formaldehyde. Temperature was held at 30°C. This configuration simulated the renaturation of gelatin soft gel capsules in contact with a hydrophilic fill containing aldehyde impurities. A control without formaldehyde was also run. The results are summarized in Figure 11. These data indicate that formaldehyde addition, even at high concentrations, had no detectable effect on the dielectric response. Despite the lack of DEA response, dissolution tests in de-ionized water indicated that the gelatin films exposed to formaldehyde had indeed crosslinked while the control had not. (After formaldehyde exposure, the gelatin capsule samples were recovered and immersed in 20 g of de-ionized water held at 37°C for one day. The state of the samples was recorded based on visual observations.)

The crosslinking kinetics of the same acid bone gelatin film exposed to formaldehyde vapor were also studied by isothermal calorimetry. Unlike the control, gelatin exposed to formaldehyde exhibited a pronounced exotherm, as shown in Figure 12. The reaction was still proceeding at the time the experiment was interrupted, that is, 40 h. The first 30 min of the

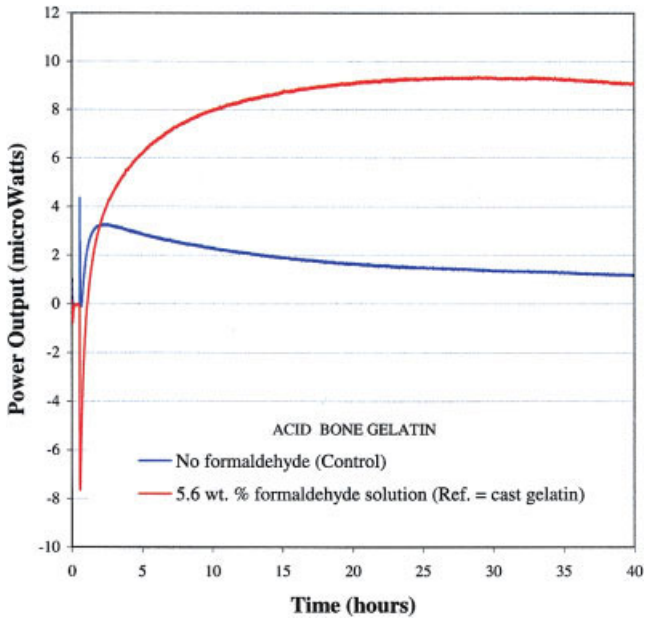

Figure 12 Effect of formaldehyde on the TAM response of acid bone gelatin immersed in PEG 600-water held at 30°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

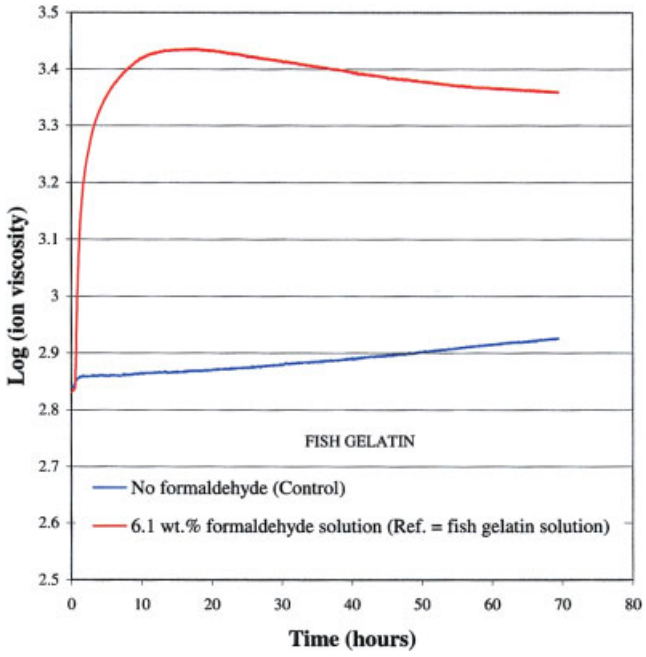

Figure 13 Effect of formaldehyde on the DEA response (100 kHz) of a fish gelatin solution held at 30°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

plot recorded the thermal equilibration phase. The disturbances observed at 30 min corresponded to the lowering of the sample holders into the measuring position.

The lack of detectable effect of chemical crosslinking on the dielectric response of acid bone gelatin undergoing renaturation (Fig. 11) led us to perform crosslinking experiments with fish gelatin held at 30°C. By remaining in the sol phase when cooled down to 30°C, the fish gelatin solution allowed us to perform crosslinking experiments without interference from renaturation. Such an experiment is illustrated in Figure 13. By contrast to the DEA experiment with acid bone gelatin, a clear dielectric response was detected as a result of formaldehyde addition (0.7 order of magnitude increase in ion viscosity). When the dielectric cells were open after 70 h, the fish gelatin solution exposed to formaldehyde had become an elastomeric solid, while the control remained a liquid. The 6.1% (w/w) formaldehyde solution added to the fish gelatin solution was close to the maximum amount (5.6 w/w %) of formaldehyde added to acid bone gelatin as shown in Figure 11. This means that it is unlikely that 6.1% (w/w) formaldehyde added to acid bone gelatin would have altered the dielectric response.

DISCUSSION

DEA was expected to monitor the kinetics of gelatin recrystallization and crosslinking by tracking induced changes in ionic mobility in the amorphous domains. Thus, the goal of this study was not so much to expand scientific knowledge on gelatin as it was to validate the suitability and practicality of using DEA for monitoring phenomena that are already well known in the field of gelatin physics and chemistry. Nonetheless, the data were interpreted in light of known literature results whenever possible.

Two types of gelatin were used: Type A acid bone gelatin from bovine source and gelatin from fish skin. Fish gelatin differs from bovine gelatin in that it has a lower content of proline and hydroxyproline residues. This structural difference sharply depresses the melting point, causing aqueous solutions of fish gelatin to remain liquid at room temperature. By contrast, hot solutions of bovine gelatin undergo a sol-gel transition when cooled to room temperature.

A 1 : 1 ratio of gelatin to water was used when preparing solutions of both types of gelatin. In the case of the acid bone gelatin, 50% (w/w) water is a relatively high moisture content compared to what is used in typical soft gel capsule formulations (8 to 12 w/w %). This aqueous system was selected to maintain a high degree of mobility without using other types of plasticizers, such as glycerin or sorbitol. This simple model system gave us a material capable of undergoing renaturation or crosslinking in a short time scale for accelerated testing, while preserving structural simplicity to facilitate the elucidation of structure-property relationships. It should be noted that all experiments with gelatin were conducted at 30°C. This temperature was selected because it is below the lowest melting temperature of acid bone gelatin (see Fig. 2 for an illustration of the melting range of a renaturated sample), yet high enough to achieve good temperature control with the highest possible molecular mobility in the gel state.

Evaluation of experimental techniques

That DEA would be successful at monitoring gelatin renaturation was not obvious at the onset of this work. Scientists familiar with the field are aware that electrode polarization[15] can lead to erroneous readings in materials containing high water and high salt concentrations. Approximate levels of the main ionic impurities in our gelatin (after deionization by the supplier) were as follows: chloride and phosphate (300 ppm), sulfate (4900 ppm), iron (2 ppm), calcium (1 ppm), and sodium (500 ppm).[16] The success of DEA in this application (i.e., the mitigation of electrode polarization effects) is attributed to the deliberate use of interdigitated electrodes with a relatively large interelectrode spacing compared to most sensors available on the market. Our work has revealed that DEA does have limitations for monitoring gelatin renaturation. Since the dielectric response is sensitive to moisture content

in the gelatin, ion viscosity is also sensitive to mass transport between gelatin and the surrounding media, either air or a liquid fill. This is clearly illustrated in Figure 4, which shows that the equilibration with a PEG 600 solution follows a different path than in an environment of humidified air of equal relative humidity. The reasons for this are explored below. In the future, great care should be taken to separate renaturation effects from moisture transport effects when using DEA.

In future gelatin studies spanning longer time scales (i.e., months or years), the dielectric analyzer would not need to be connected to the test cells continuously. Once the relatively large initial changes in dielectric response have taken place, the analyzer could be utilized for other applications and could be reconnected to the cells for periodic acquisition updates. The resulting data files could then be appended and plotted with any spreadsheet software.

The results of the crosslinking experiments indicate that DEA has limited applicability to the study of gelatin crosslinking and crosslinking inhibition. Such studies are possible with DEA only as long as a noncrystallizing model system, such as fish gelatin, is used. This result was very surprising at first, because the authors of this study expected crosslinking to significantly affect the amorphous regions of renaturated gelatin. In fact, most articles on crosslinking do not discuss the spatial distribution of crosslinks. Rather, they tend to focus on the chemistry of crosslinking[17–20] or use techniques that are not capable of distinguishing physical crosslinks from chemical crosslinks.[21,22]

Cyclic DSC allowed us to monitor gelatin renaturation in an entirely automated fashion. To our knowledge, this technique has not been used before to monitor gelatin. The quality of the data was very good. Unfortunately, the number of programmable cycles was relatively small and did not allow us to explore very long time scales. Consequently, memory upgrades should be considered for future work.

Solid state rheology in the form of a customized DMA apparatus probing the surface of a gelatin film immersed in simulated hydrophilic fills is also a novelty that deserves attention as a potential tool for soft gelatin capsule engineering. Although our results are preliminary, they do support the concept that the evolution of gelatin mechanical properties can be monitored from the very onset of gelation, and this under environmental conditions that are nearly identical to those experienced by newly manufactured soft gelatin capsules.

Lastly, isothermal calorimetry provided unique insight into the kinetics of gelatin crosslinking. In the case of acid bone gelatin, isothermal calorimetry was the only technique available to monitor crosslinking in real time. The signal-to-noise ratio was excellent in all cases.

Structure-property relationships

When the inability of DEA to detect crosslinking in acid bone gelatin undergoing renaturation is contrasted with the positive result for fish gelatin, an interesting interpretation emerges: crosslinking in acid bone gelatin may be confined to the crystalline regions of gelatin only. Not only would such a hypothesis makes sense physically (since the crystalline phase is by far denser than the amorphous phase), but it would also be entirely consistent with the absence of change in ionic mobility during renaturation. This is because free ions from gelatin salt impurities are expected to have the greatest mobility in the amorphous domains of the gelatin.

An earlier study of gelatin renaturation-crosslinking interactions by Watanabe and colleagues[23] does provide evidence that crosslinking occurs preferentially around the reformed collagen segments as long as the amount of crosslinks is relatively low. Watanabe's work was performed by using fluorescence anisotropy and fluorescence decay combined with circular dichroism. In our study, crosslink density may have been low due to the high moisture content of our model formulation. This means that one should be careful not to generalize our findings to gelatin formulations with a lower moisture content or exposed to higher amounts of formaldehyde.

Another fundamental question is the mechanism governing the drop in ion mobility during the renaturation of bovine gelatin. To explain the precipitous increase in ion viscosity during renaturation, it is helpful to keep in mind that the amount of water bound in the helical structure of gelatin has been estimated to be between 35 and 70% (w/w).[24] It follows that the crystallization process, by incorporating large amounts of water via hydrogen bonds, may give rise to a drop in water concentration in the amorphous phase of the gelatin. Such a redistribution of water between the amorphous and crystalline domains would inevitably result in reduced protein segmental mobility (and ionic mobility), since water is a potent plasticizer of amorphous gelatin.

The above deplasticization model is consistent with the data reported in Figure 4, where we compared the dielectric response of acid bone gelatin exposed to 30°C/98% RH to that of the same gelatin exposed to 30°C in PEG 600-water with an equivalent RH of 98%. During the initial stage of renaturation of the gelatin immersed in PEG 600-water, water was extracted rapidly into the hydrophilic PEG solution, thereby causing a faster loss of plasticity of the amorphous region than in the case of gelatin exposed to air. Eventually, the recrystallization process drew the water back from the PEG solution into the gelatin until thermodynamic equilibrium was reached. When equilibrium was approached, the dielectric responses from the two sam-

ples converged. This result suggests that a very hydrophilic fill, by extracting water rapidly before and after gelation, affects the kinetics of early renaturation.

Additional structure-property relationship information was provided by DMA. The fact that the storage modulus reached a plateau earlier than the loss modulus or ion viscosity suggested that the final crosslink density is reached relatively early during the renaturation process. According to rubber elasticity theory, G′ is proportional to the network node density.[25] In the case of renaturating gelatin, the network nodes are physical in nature and thermally reversible. They correspond to the crystalline domains of the hydrogel. By contrast, G″ and ion viscosity are measures of viscous dissipation during cyclic excitation (mechanical and electrical, respectively). G″ and ion viscosity are expected to increase as renaturation progresses. According to this view, the slower increase in loss modulus and ion viscosity is primarily related to the growth of existing crystalline regions, rather than to the nucleation and growth of new ones.

CONCLUSIONS

Comparison with DSC data shows that DEA at low frequencies is a promising technique for monitoring gelatin renaturation *in situ*. Compared to other techniques commonly used to characterize gelatin renaturation, such as DMA and DSC, sample preparation is much less labor intensive and the scatter of data tends to be reduced. In addition, data acquisition is completely automated and can be programmed to span thousands of hours, unattended, if necessary. The use of closed or open cells interchangeably enabled us to study renaturation of gelatin in contact with humid air or in contact with liquids whose composition was similar to those of soft gelatin capsule fills. This closely modeled gelatin physicochemical changes on the outer and inner surface of freshly made soft gelatin shell, respectively.

The crosslinking of bovine gelatin went undetected by DEA. This confined our crosslinking studies with DEA to fish gelatin whose gelation point is below room temperature. This surprising result, in combination with limited data from DMA, provided unexpected insight on the crosslinking mechanism at the molecular level. Finally, this work led us to investigate other experimental techniques for gelatin characterization. Although more labor intensive than DEA, programmed cyclic DSC and solid-state rheology on film samples should definitely be considered along with DEA for future gelatin renaturation studies.

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