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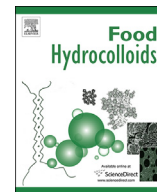
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Gelatin nanofibers: Analysis of triple helix dissociation temperature and cold-water-solubility



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ABSTRACT

Gelatin nanofibrous structures, characterized by high specific surface area and high porosity, have been widely researched for biomedical and food applications. The present paper researches the potential of electrospinning to produce a nanofibrous cold-gelling (or instant) gelatin product. Our results show that gelatin nanofibers are cold-water-soluble due to their high surface-to-volume ratio, facilitating easy water penetration and dissolution, and this for several gelatin types. Additionally, fast gelation after dissolution in cold water indicates that the electrospinning process does not significantly reduce the gelatin molecular weight, nor compromise triple helix formation. These conclusions were supported by thorough investigation of the internal gelatin structure, using a new approach based on modulated temperature scanning calorimetry. Oscillation rheology revealed that the nanofiber-based gels have moduli comparable to powder-based gels. Gelatin nanofibers can thus be used as instant gelatin product, without the drawbacks of traditional amorphous instant gelatins such as sensitivity to moisture, low wettability and low modulus of the cold gel. Using the approach reported here, every electrospinnable, but non-cold-water-soluble gelatin can be transformed into a cold-water-soluble variant, regardless of the type or modification. Electrospinning can thus offer enormous flexibility in materials selection, enabling the production of cold gels loaded with temperature-sensitive components, UV-cross-linkable cold gels, etc.

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1. Introduction

Gelatin is a biopolymer that is widely used, mainly in biomedical, photographic and food industries, due to its low cost and multifunctionality (Schrieber & Gareis, 2007). It is obtained through chemical hydrolytic degradation of collagen from animal bones, connective tissue or skin. Collagen consists of long chains of linked amino acids, with glycine, proline and hydroxyproline most abundantly present. The latter two are responsible for the

formation of a unique structure; they stabilize triple helix formation through interchain hydrogen bonds (Schrieber & Gareis, 2007). When collagen is hydrolyzed to form gelatin, either by acidic treatment (resulting in type A gelatin), alkaline treatment (resulting in type B gelatin) or enzymatic treatment, these helices can be destroyed in hot water, *i.e.* they are denatured. Upon cooling the resulting gelatin, however, some chains reassociate into triple helix structures. This triple helix formation in gelatin is thermo-reversible and characterized by a transition temperature and enthalpy. The temperature at which the gelatin triple helices dissociate upon heating, T_d , is dependent on the molecular weight, the amino acid composition, the amount of plasticizers present such as moisture, etc. (Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011; Rahman, Al-Saidi, Guizani, & Abdullah, 2010;

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Roussanova et al., 2014; Schrieber & Gareis, 2007). In literature, this dissociation temperature is sometimes also referred to as the denaturation temperature, even though no further degradation of the gelatin polypeptide chains is associated with the transition (Bigi, Panzavolta, & Rubini, 2004; Y. Z. Zhang, Venugopal, Huang, Lim, & Ramakrishna, 2006).

Above T_d , gelatin is soluble in water. Upon cooling, the coil-to-helix transition gives rise to intermolecular microcrystalline junction zones acting as physical cross-links, resulting in a hydrogel (Roussanova et al., 2014; Sobral & Habitate, 2001; Van Vlierberghe, Schacht, & Dubruel, 2011). In this case, T_d is also commonly referred to as gelling/melting temperature, pointing to the sol–gel transition. In gelatin hydrogels, T_d is generally lower than the human body temperature, which makes these materials suitable for “melt-in-mouth” food products, pharmaceutical delivery systems, etc. (Karim & Bhat, 2008; Roussanova et al., 2014).

The production of gelatin hydrogels is generally only possible by cooling from elevated temperatures, above T_d . In food industry, however, cold-water-soluble gelatin (or instant gelatin) has been developed for its use in easy-to-prepare gelatin-based products without the need of preheating. Traditional instant gelatins are currently widely used in ready-to-use cake mixes, whipped cream powders, etc. (Baines & Seal, 2012; Phillips & Williams, 2009, 2011). Additionally, for some applications, dissolution and subsequent gelation at room temperature could offer some major benefits, making the incorporation of temperature-sensitive components into gelatin hydrogels possible (Müller, 1989). This could open up unprecedented possibilities in food technology, as recently demonstrated by Pintado et al. who used instant gelatin for the formulation of emulsion gels containing non-meat fats as healthy meat alternatives (Pintado, Ruiz-Capillas, Jiménez-Colmenero, Carmona, & Herrero, 2015). Several strategies have been used in developing cold-water-soluble gelatins. Gelatin hydrolysate, for instance, is cold-water-soluble due to biochemical enzymatic degradation resulting in a low molecular weight product. Hydrolysates, however, are non-gelling (zero-Bloom) and thus not suitable for hydrogel formation (Phillips & Williams, 2009; Schrieber & Gareis, 2007). Cold water fish gelatin is characterized by low T_d due to lower proline and hydroxyproline content, and are thus often cold-water-soluble. The lower imino acid content, however, also adversely affects gel modulus (Gilsenan & Ross-Murphy, 2000; Karim & Bhat, 2008).

True cold-gelling gelatin, or instant gelatin, is obtained only when processed into the amorphous form, either by drum drying or dry-blending using carbohydrates, acids or urea (Leshik, Swallow, Leusner, & DiGiovacchino, 1985; Schrieber & Gareis, 2007). Dry-blending inherently suffers from large amounts of additives, often more than four times the amount of gelatin. Drum drying produces pure instant gelatin, but it has several drawbacks; ⁽¹⁾ small amounts of moisture can induce helix formation, having a pronounced negative effect on cold-water-solubility (Leshik et al., 1985), ⁽²⁾ low wettability during dissolution can lead to lump formation (Schrieber & Gareis, 2007), and ⁽³⁾ the ‘cold gels’ have a lower gel modulus (Schrieber & Gareis, 2007).

This paper presents a new strategy for the production of cold-water-soluble gelatin through electrospinning of gelatin nanofibers. In the electrospinning process, polymer solutions are processed into nonwovens consisting of sub-micron fibers. These nanofibrous membranes are characterized by high porosity and large specific surface area, typically around 90% and 20 m².g⁻¹ respectively (Wendorff, Agarwal, & Greiner, 2012). Gelatin nanofibers have been studied extensively for biomedical applications (Agarwal, Wendorff, & Greiner, 2008; Goh, Shakir, & Hussain, 2013; Kai, Jin, Prabhakaran, & Ramakrishna, 2013; Schiffman & Schauer, 2008; Sridhar et al., 2015) and, to a lesser extent, for the food

industry (Anu Bhushani & Anandharamakrishnan, 2014; Ghorani & Tucker, 2015; Kriegel, Arrechi, Kit, McClements, & Weiss, 2008; Nieuwland et al., 2013; Okutan, Terzi, & Altay, 2014). In food science and technology, nanofibers have already shown large potential to be used for the encapsulation of temperature-sensitive components, bioactive food compound delivery, active food packaging, edible coatings, clarification of beverages, etc. as recently reviewed by Anu Bhushani et al. and Scampicchio et al. (Anu Bhushani & Anandharamakrishnan, 2014; Hernandez-Sanchez & Gutierrez-lopez, 2015). For many of these applications, water-stability of the nanofibers at slightly elevated temperatures is a prerequisite, and a lot of effort has been invested in the development of cross-linking strategies to ensure water-stability of the nanofiber morphology (Jalaja, Kumar, Dey, Kundu, & James, 2014; Panzavolta et al., 2011; Y. Z. Zhang et al., 2006). The potential of uncross-linked gelatin nanofibers to be used as instant gelatin has not yet been investigated.

The present paper reports on the electrospinning of gelatin using acid-based solvent systems and the potential of this processing technique to produce cold-water-soluble gelatin products. The internal structure of gelatin nanofibers is investigated using thermal analysis. Within this, a new approach is reported for the analysis of the glass transition and triple helix dissociation temperatures, based on modulated temperature differential scanning calorimetry. Subsequently, the solubility of gelatin nanofibers in cold water is investigated. Additionally, mechanical properties of nanofiber-based hydrogels are characterized using oscillation rheology. Production of cold-water-soluble nanofibers through electrospinning, proposedly by increasing the surface-to-volume ratio of the gelatin product, is very promising in the search for true instant gelatins. Using this approach, every electrospinnable, but non-cold-water-soluble gelatin could be transformed into a cold-water-soluble variant without losing its gelling ability.

2. Experimental

2.1. Materials

Commercial gelatin isolated from pigskin by the acidic process (type A, 300 Bloom), and gelatin isolated from bovine bones by the alkaline process (type B, 260 Bloom), were kindly supplied by Rousselot, Ghent, Belgium. Both types were used as-received for the electrospinning process.

Solvents used for electrospinning were purchased from Sigma–Aldrich and used as received; acetic acid (99.8 v%) and formic acid (98–100 v%).

Cold-water-solubility was tested using demineralized water at room temperature, namely 19 °C.

Temperature calibrations of DSC were performed using indium (LGC).

2.2. Modification of type B gelatin

Type B gelatin was modified with methacrylic anhydride (1 eq.) and subsequently purified according to the method described by Van Den Bulcke et al. (Van Den Bulcke et al., 2000). 56.7% of the ε-amino groups were modified to methacrylamide side groups, as determined by ¹H NMR spectroscopy. This modification results in a UV-cross-linkable gelatin, which was also tested as starting material for the electrospinning process.

2.3. Electrospinning

2.3.1. The principle of electrospinning

In the electrospinning process, an electric field is applied

between a collector and a nozzle through which a polymer solution is flowing. When the electrostatic forces overcome the surface tension of the liquid, a jet is pulled towards the collector in a spiraling motion, facilitating solvent evaporation and the fiber drawing process (Pisignano, 2013). The nanofibers are randomly deposited onto the collector, resulting in a nonwoven membrane. The obtained nanofibrous product contains no to or a very low amount of residual solvent, which can easily be removed by a drying step prior to final end-use if demanded by the envisioned application (Nam, Huang, Agarwal, & Lannutti, 2007; Xie, Li, & Xia, 2008).

2.3.2. Characterization of the electrospinning solutions

The electrospinning solutions were characterized prior to use by their viscosity and conductivity. Viscosity was measured using a Brookfield viscometer LVDV-II (average error on measurements was 8%) and conductivity was determined by a CDM210 conductivity meter of Radiometer Analytical (average error on measurements was 11%). Additionally, the presence of triple helices in the electrospinning solutions was studied using polarimetry. When triple helices are formed in a solution, the optical activity of the solution is affected, measurable by a change in optical rotation by polarimetry (Djabourov, Leblond, & Papon, 1988; Gandhi, Yan, & Kim, 2014). Optical rotation of 13 wt% gelatin dissolved in the acid-based solvent systems was analyzed using a PerkinElmer polarimeter 341 (path length of 1 cm, mercury lamp with 436 nm filter). The gelatin powder was allowed to swell for 30 min at room temperature, after which the solution was heated to 45 °C in a water bath and measured in cooling for a temperature range of 45–10 °C (measurement every 5 °C).

2.3.3. Parameters of the electrospinning process

Acid-based solvent systems were chosen for electrospinning of all three gelatin types, namely acetic acid/water (AA/water) and acetic acid/formic acid (AA/FA). At certain ratios, these solvent systems allow for the dissolution of gelatin at room temperature without any triple helix formation in the electrospinning solution (as evidenced by optical rotation measurements reported in the [Electronic Supporting Information](#)), thereby guaranteeing a stable electrospinning process without clogging of the needle or the need for a complex heated setup. Our process optimization showed that gelatin is electrospinnable at concentrations between 13 and 15 wt % for AA/water solvent ratios between 90/10 and 50/50 or AA/FA solvent ratios between 90/10 and 0/100. Process stability was superior for 90/10 AA/water or 70/30 AA/FA. The gelatin electrospinning solutions were thus prepared by the dissolution of the gelatin powder as received (13 wt%) in 90/10 AA/water or 70/30 AA/FA. All data reported in the present paper were obtained using the unmodified type A gelatin, unless specifically stated otherwise. Further details on the optimization of the electrospinning process can be found in the [Electronic Supporting Information \(ESI\)](#).

The nanofibrous structures were produced on a multi-nozzle electrospinning setup suitable for production of large samples, with 18 gauge needles (inner diameter 0.838 mm), a tip-to-collector distance of 15 cm, a flow rate of 1 ml h⁻¹ and a voltage between 15 and 20 kV. All parameters (gelatin concentration, solvent ratio, tip-to-collector distance, flow rate and voltage) were adapted to allow for stable electrospinning and thus the production of uniform bead-free nanofibers. All electrospinning trials were performed at a relative humidity of 35 ± 5% and a temperature of 21 ± 3 °C.

2.4. Microscopy

The original gelatin powders were examined by optical

microscopy using an Olympus BX51 equipped with an Olympus UC30 camera. The electrospun samples were examined by scanning electron microscopy (FEI Quanta 200 F) at an accelerating voltage of 20 kV. Nanofiber sample preparation was done using a sputter coater (Emitech SC7620, coating with Au). The particle diameters of the powder and the nanofiber diameters were measured using ImageJ version 1.48. The average particle/fiber diameters and their standard deviations are based on 50 measurements per sample. Based on the average diameters, the surface-to-volume ratio of the powder can be estimated as 6/d [m⁻¹] and the surface-to-volume ratio of the nanofibers can be estimated as 4/d [m⁻¹]. The latter was recently verified by in-depth 3D image analysis of electrospun samples, showing that this approximation is in very close agreement with the experimental results (Choong, Yi, & Rutledge, 2015).

2.5. Thermal analysis

Modulated temperature differential scanning calorimetry (MTDSC) measurements were performed using a TA Instruments Q2000 equipped with a refrigerated cooling system and using nitrogen as purge gas (50 ml min⁻¹). The instrument was calibrated using Tzero™ technology, including a temperature calibration with indium. Samples were enclosed in the dedicated hermetic Tzero aluminum crucibles. Measurements were performed using a scan rate of 2.5 K min⁻¹ and a temperature modulation of ±0.5 K every 60 s. All thermal transitions were analyzed in heating. Glass transition temperature was determined as the temperature at the inflection point of the reversing heat flow trace at the baseline step. Dissociation temperature was determined as the temperature at the maximum in the non-reversing heat flow trace.

Sample preparation was done in one of two ways. ⁽¹⁾ Solid materials were measured by conditioning 3.00 ± 0.30 mg gelatin powder or nanofibers using a climate chamber (Weiss WK 340/40) for 24 h before closing the hermetic crucible. After DSC measurement, the moisture content was determined by drying at 60 °C under a purge flow of dry air until equilibrium was reached (mass change < 0.002% for 60 min) using a dynamic vapor sorption analyzer (DVS: TA Instruments, Q5000SA). The obtained values are reported in the [ESI](#). ⁽²⁾ Hydrogels were prepared by introducing gelatin powder or nanofibers in demineralized water at room temperature and heating at 60 °C for 15 min while stirring, after 30 min of swelling. 'Cold gels' were prepared without the heating step and directly enclosed in the hermetic crucibles after swelling/cold-gelling. The hot solutions were pipetted into the hermetic crucible and closed immediately for measurement of the 'hot gels', resulting in samples of 25 ± 5 mg containing 5 or 10 wt% gelatin.

The average error for dissociation enthalpy measurements of powder-based hydrogels using the first heating cycle was 9%.

2.6. Oscillation rheology

Mechanical properties of the hydrogels, *i.e.* storage modulus (G'), loss modulus (G'') and yield point, were measured using a Physica MCR 350 (Anton Paar) with plate–plate geometry. Shear strain amplitude sweeps were performed at 23 °C, using a shear gap of 1.5 mm and a constant frequency of 1 Hz. The hydrogels were prepared using the approach described for thermal analysis. The 'hot gels' were stored at 7 °C after cooling to room temperature. The 'cold gels' were kept at room temperature or stored at 7 °C after the swelling/cold-gelling step. The yield point was determined as the crossover point where $G' = G''$, as explained in literature (Mezger, 2011). The moduli were compared at strains within the linear viscoelastic region (0.2–1%).

3. Results and discussion

3.1. Electrospinning of cold-water-soluble gelatin nanofibers

Gelatin nanofibers have already been electrospun using several solvent systems. As for most polymers, toxic organic solvents including 2,2,2-trifluoroethanol (TFE) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were originally most commonly applied (Huang, Zhang, Ramakrishna, & Lim, 2004; Li et al., 2005; Y. Zhang, Ouyang, Chwee, Ramakrishna, & Huang, 2005; Y. Z. Zhang et al., 2006). More recently, also acidic solvent systems have been reported, most of which are acetic acid-based with in some cases small amounts of a secondary solvent such as water, ethyl acetate, 2,2,2-trifluoroethanol, dimethyl sulfoxide, ethylene glycol, formamide, etc. (Choktaweesap, Arayanarakul, Aht-ong, Meechaisue, & Supaphol, 2007; Jalaja et al., 2014; Ki et al., 2005; Panzavolta et al., 2011; Rujitanaroj, Pimpha, & Supaphol, 2008; Song, Kim, & Kim, 2008; Songchotikunpan, Tattiyakul, & Supaphol, 2008) Also the acetic acid-based solvent mixtures with lower toxicity such as aqueous acetic acid allow for the production of bead-free uniform nanofibrous nonwovens without the necessity of heating, making the production of nanofibers possible under milder and more sustainable conditions (Prat, Hayler, & Wells, 2014). Acid-based solvent systems with lower toxicity will therefore be used in the present paper. More specifically, an acetic acid/water (AA/water) and an acetic acid/formic acid (AA/FA) solvent system were selected. While AA/FA was not yet reported in literature, it offers a larger flexibility in terms of mixing with other (synthetic) polymers, making tailoring of nanofiber properties for specific applications possible. Solvent ratios allowing for stable and reproducible electrospinning of bead-free gelatin nanofibers were selected, namely 70/30 AA/FA and 90/10 AA/water. Polarimetric measurements showed that by using these solvent ratios, there is no change in optical activity of the polymer solutions upon cooling from elevated temperatures to room temperature (see ESI). Since the formation of triple helices upon cooling from the sol state is accompanied by a change in optical rotation, this indicated that there are no triple helices present when gelatin is dissolved in these solvent systems at room temperature, making stable electrospinning without heating possible while also avoiding potential gelation problems.

Since acid is known to degrade gelatin, the stability of the electrospinning solutions was investigated through viscosity measurements. Our results show that there is only a minor

decrease in solution viscosity over the course of 48 h, and this for both solvent systems (Fig. 1). This implies that also the decrease in molecular weight of the gelatin in the electrospinning solutions is limited. In the electrospinning process, electrospinnability and fiber diameter can be significantly influenced by a decrease in molecular weight (Andrady, 2008). In agreement with viscosity data, the electrospinning trials at different moments for over 48 h indicated similar electrospinning behavior and fiber morphology (Fig. 2). There is thus no significant influence of the possible gelatin degradation on the electrospinning process.

Both the unmodified gelatins (type A and type B) and the UV-cross-linkable gelatin (methacrylamide-modified type B) were electrospinnable using the acid-based solvent systems (see ESI). All electrospun samples were readily soluble in cold demineralized water (19 °C), regardless of the gelatin type/modification, the used solvent system, the dwell time in the electrospinning solution or the storage time of the nanofibers at room temperature and room humidity (22 ± 7 °C, $45 \pm 20\%$ RH). The powder used for the preparation of these gelatin nanofibers was non-cold-water-soluble (300 Bloom), indicating that the electrospinning process induces cold-water-solubility. Additionally, in contrast to drum dried amorphous gelatins sensitive to moisture-induced triple helix formation with loss of cold-water-solubility (Leshik et al., 1985; Schrieber & Gareis, 2007), the gelatin nanofibers remain cold-water-soluble when exposed to moisture. This suggests that the solubility of the nanofibers in cold water is not purely due to an amorphous gelatin structure. Analysis of the triple helix dissociation could thus offer some insight into the phenomenon, as shown by the case study of the type A gelatin powder in the following section.

3.2. Triple helix dissociation in gelatin nanofibers

Differential scanning calorimetry (DSC) is a popular technique for the analysis of triple helix dissociation, since the helix-to-coil transition is a measurable endothermic event (Dai, Chen, & Liu, 2006). In order to properly assess the internal gelatin structure, i.e. the glass transition T_g of the amorphous phase and the dissociation temperature T_d of the triple helices within the nanofibers or the original powder, a thorough DSC study was performed. In conventional DSC, however, glass transition and dissociation are difficult to distinguish. A modulated temperature technique (MTDSC) on the other hand, enables the separation of complex and overlapping effects by superimposing a sinusoidal wave on the linear heating ramp. The resulting signal is called the total heat flow, which can subsequently be broken down into a non-reversing and a reversing component by a deconvolution procedure (De Meuter, Rahier, & Van Mele, 1999; Reading & Hourston, 2006). Changes in specific heat are always visible in the reversing signal, whereas time-dependent processes such as triple helix dissociation end up in the non-reversing signal. MTDSC thus allows for convenient analysis of both T_g and T_d in the same experiment as illustrated in Fig. 3, without the need for quenching.

Since T_d is expected to decrease with decreasing molecular weight (Schrieber & Gareis, 2007), a comparison between gelatin nanofibers and the original gelatin powder can support the viscosity measurements from the previous section, showing that gelatin degradation in the acid electrospinning solution is limited. This comparison, however, should take into account the dependence of T_d on the moisture content. Indeed, with increasing water content, both T_g and T_d decrease significantly due to the plasticizing effect (Rahman et al., 2010). Both conditioned powders/nanofibers and hydrogels prepared by dissolution at 60 °C were studied, representing low and high moisture levels respectively. In order to obtain reproducible T_d -measurements reflecting the gelatin

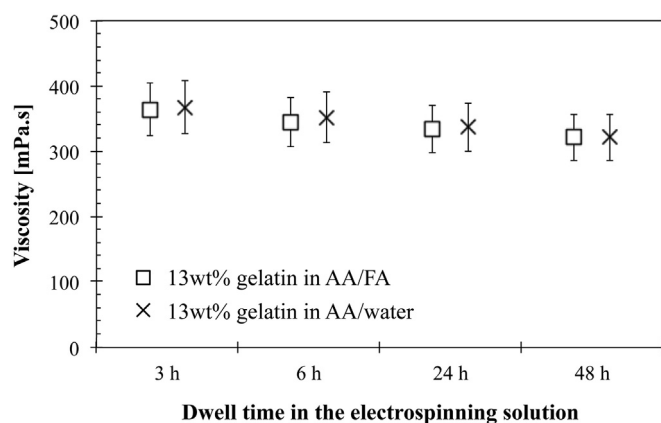


Fig. 1. Viscosity measurements of the electrospinning solutions with increasing dwell time show only a minor decrease over 48 h after introduction of gelatin into the acid-based solvent systems.

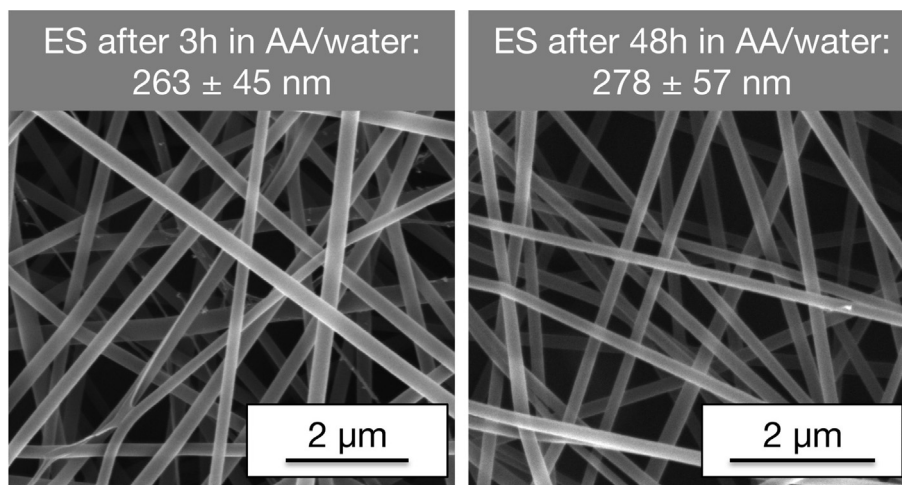


Fig. 2. SEM-images of gelatin nanofibers electrospun at 3 h and 48 h after introduction of 13wt% gelatin in 90/10 AA/water. No significant difference in process stability or fiber diameter was measured with increasing dwell time. Similar results were obtained for the AA/FA solvent system (see ESI).

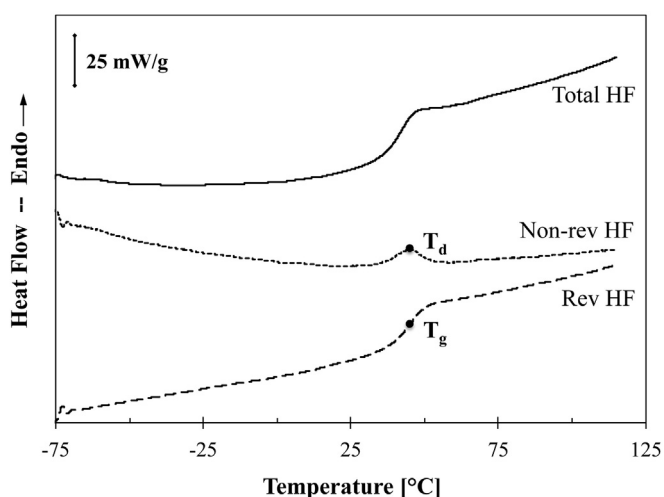


Fig. 3. Thermal properties of gelatin powder conditioned at 65% RH, measured using MTDSC. Overlapping glass transition and triple helix dissociation can be separated by analyzing the reversing and non-reversing heat flow signals respectively.

molecular weight, a first heating cycle in MTDSC is necessary. As illustrated in Fig. 4, T_d and ΔH_d are well reproducible after the first heating, which homogenizes the moisture content within the sample enclosed in the hermetic crucibles and erases thermal history. Therefore, the second heating cycle of the MTDSC measurements is most representative when analyzing the possible difference in molecular weight between the original gelatin powder and the nanofibers through thermal analysis.

Completely dry gelatin is a rigid, brittle, amorphous solid with a T_g above 100 °C (Lukasik & Ludescher, 2006). With increasing moisture content, T_g decreases and triple helix formation is possible. According to our measurements, T_g decreases from about 94 °C to about 10 °C with moisture concentration increasing from 7 to 23 wt%, and this for all samples. In hydrogels, the T_g is no longer measurable. Triple helix dissociation, on the other hand, is visible for all water concentrations, as expected. A clear decrease in T_d with increasing moisture content was recorded for conditioned gelatin samples with low moisture levels (Fig. 5). As previously reported in literature (D'Cruzand & Bell, 2005), our results indicate that a plateau is reached at high water concentrations, resulting in stable

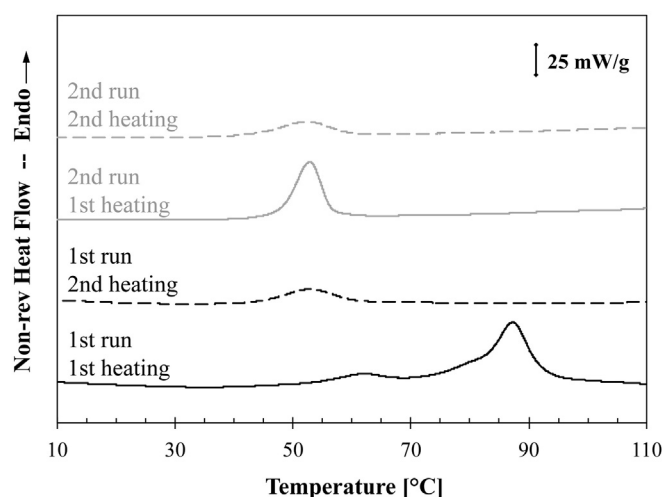


Fig. 4. Thermal properties of gelatin powder conditioned at 65% RH, measured using MTDSC. The second run (grey) was performed 10 days after the first run (black) while the sample was stored at room temperature. The first heating of each run (full lines) measures the sample as-conditioned, whereas the second heating of each run (dashed lines) measures the sample after cooling at 2.5 K min⁻¹.

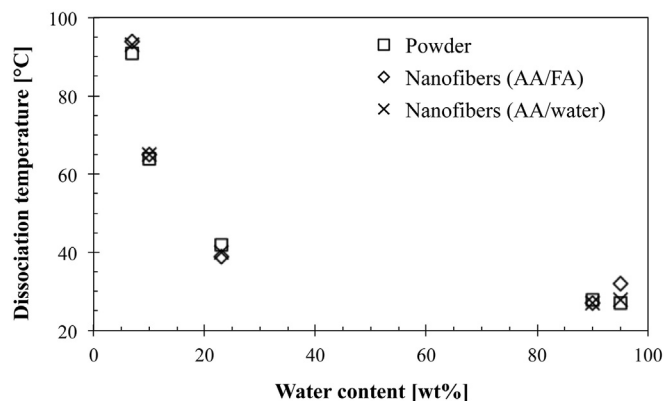


Fig. 5. Fast decrease of the dissociation temperature of gelatin with increasing water content, measured using MTDSC and based on the second heating cycle. No significant differences between powder and nanofibers were recorded.

T_d -values for gelatin hydrogels. More importantly, there were no significant differences in T_g or T_d between the original gelatin powder and the electrospun nanofibers. This not only confirms that there is no significant gelatin degradation, but also indicates that the electrospinning process does not alter the thermal stability of the amorphous phase or the gelatin triple helices. Gelatin selection for the electrospinning process can thus be done as a function of the application, without the need to account for a difference in thermal stability.

Traditional techniques for the production of instant gelatins are based on special drying methods to obtain an amorphous gelatin structure. Water can easily penetrate amorphous gelatin without the need for applying heat to cleave physical inter- and intramolecular bonds, making them cold-water-soluble (Schrieber & Gareis, 2007). The electrospinning process too, can significantly influence the crystalline structure of polymers (Dersch, Liu, Schaper, Greiner, & Wendorff, 2003). During fiber formation, the jet is substantially elongated, with strain rates as high as 10^4 s^{-1} , and the solvent is evaporated within milliseconds. Extensive stretching can cause significant orientation of polymer chains leading to more stable crystal phases (Steyaert, Delplancke, Van Assche, Rahier, & De Clerck, 2013). Fast solidification, on the other hand, generally leads to underdeveloped crystal structures and can thus also prevent triple helix formation. The gelatin nanofibers reported here, however, are not completely amorphous. Indeed, MTDSC analysis shows a clear helix-to-coil transition for all nanofibers, with dissociation enthalpies typically around 4 J g^{-1} after storage for several months at room temperature and room humidity ($22 \pm 7 \text{ }^\circ\text{C}$, $45 \pm 20\% \text{ RH}$). Although these ΔH_d -values are about 1/3 compared to the value obtained for the original powder, electrospinning does not lead to completely amorphous gelatin. This thus cannot be the reason for the cold-water-solubility of gelatin nanofibers.

Nanofibrous nonwovens are known for their large surface-to-volume ratio and high porosity, leading to improved wettability and large fiber-liquid contact area (De Schoenmaker, van der Schueren, De Vrieze, Westbroek, & de Clerck, 2011; Macagnano, Zampetti, & Kny, 2015). Water can thus easily penetrate gelatin nanofibrous nonwovens, possibly explaining the cold-water-solubility even though the structure is not completely amorphous. Consequently, gelatin nanofibers remain cold-water-soluble even after long storage time at room humidity (for over 6 months without climate control), since their diameters remain unchanged. This in contrast to amorphous instant gelatins, which are sensitive to moisture-induced triple helix formation. Additionally, the high specific surface area contributes to fast gelation at room temperature, without the wettability problems often encountered using amorphous gelatin. Indeed, within 10 min, a white hydrogel with structural integrity is formed (Fig. 6). The white color, which remains even after heating above $80 \text{ }^\circ\text{C}$, indicates that a small portion of the gelatin within the nanofibers is irreversibly cross-linked during the electrospinning process. These small insoluble parts of the fibers cause some light diffraction, turning the gel opaque. Since gelatin films, solution cast using the same solvent system as employed for electrospinning, result in clear hydrogels, this is probably due to the high degree of stretching of the polymer jet resulting in a mechanically induced chemical reaction. How this affects the thermal and mechanical properties of the resulting hydrogels, is studied in the next section, using type A gelatin nanofibers electrospun out of the AA/water solvent system.

3.3. Nanofiber-based gelatin hydrogels

Gelatin nanofibers are cold-water-soluble up to concentrations of 5 wt% and show good gelling ability at room temperature. This is

in contrast to the original gelatin powder, which shows no dissolution and only a small degree of swelling (Fig. 6), probably due to the remarkable difference in specific surface area. Indeed, the gelatin nanofibrous samples (average fiber diameter of about 270 nm) are characterized by a specific surface area about 1250 times larger than the original gelatin powder (average particle size of about 0.5 μm). The nanofiber-based 'cold gels' were compared to their 'hot' counterparts; powder- or nanofiber-based and prepared by dissolution at $60 \text{ }^\circ\text{C}$.

MTDSC analysis shows that the amount of triple helices in the 'cold gels' is slightly lower than the amount in the 'hot gels', as can be derived from a lower ΔH_d -value reported in Fig. 7. It is possible that upon dissolution at room temperature, the water has not yet completely penetrated into the core of all the nanofibers, lowering the amount of possible triple helix formation. Although heating to $60 \text{ }^\circ\text{C}$ does not render the white nanofiber-based 'cold gels' clear, the amount of triple helix formation does reach the same level as for the powder-based 'hot gels'. Consequently, the insoluble fraction of the nanofibers does not influence triple helix formation in nanofiber-based hydrogels.

Although thermal analysis indicates that the nanofiber-based 'cold gel' has a lower amount of triple helices, this does not result in a hydrogel with lower gel moduli, as demonstrated in Fig. 8. On the contrary, the 'cold gel' is characterized by slightly higher storage and loss moduli (G' and G'' respectively). This is probably due to a fiber-reinforcing effect. As recently demonstrated by Tonsomboon et al., introduction of gelatin nanofibers into alginate hydrogels significantly improves the mechanical properties compared to a neat hydrogel (Tonsomboon & Oyen, 2013). Our MTDSC results indicated that the gelatin nanofibers are not yet completely hydrated when dissolved at room temperature, allowing for some residual fiber-reinforcement.

Oscillation rheology shows that nanofiber-based 'hot gels' have mechanical properties similar to powder-based 'hot gels' (Fig. 8). Heating to $60 \text{ }^\circ\text{C}$, thus allows for complete water penetration without any significant fiber reinforcement remaining, which is in agreement with MTDSC results. Although these nanofiber-based 'hot gels' remain opaque, the insoluble fraction does not influence the mechanical properties of the hydrogels. Additionally, similar G' -values between powder- and nanofiber-based 'hot gels' point to comparable molecular weight values. Indeed, G' is known to decrease with decreasing molar mass (Eysturskard, Haug, Ulset, & Draget, 2009; Schrieber & Gareis, 2007). Rheology measurements thus again confirm our previous conclusion; there is no significant gelatin degradation during the electrospinning process.

In order to properly characterize the potential of cold-water-soluble gelatin nanofibers to be used as instant gelatin, the setting time of nanofiber-based hydrogels prepared at room temperature was studied using oscillation rheology. After dissolution at room temperature, the 'cold gels' were either kept at room temperature ($22 \pm 1 \text{ }^\circ\text{C}$) or stored in the fridge ($7 \text{ }^\circ\text{C}$). At room temperature, the 'cold gel' only reaches its final gel modulus after 24 h (Fig. 9a). However, even after 30 min the gel already exhibits good structural integrity. When put in cooled storage, the final gel moduli are already reached after 30 min (Fig. 9b). Indeed, a storage modulus of about 1.3 MPa was already recorded after 30 min in cooled storage, which is comparable to the storage modulus reaches after 24 h at room temperature (1.4 MPa) or the one recorded for 48 h in cooled storage (1.5 MPa, Fig. 8). Gelatin nanofibers are thus not only cold-water-soluble, but they show fast gelling after dissolution in cold water, especially when cooled.

4. Conclusions

In summary, a new concept for the production of a cold-gelling

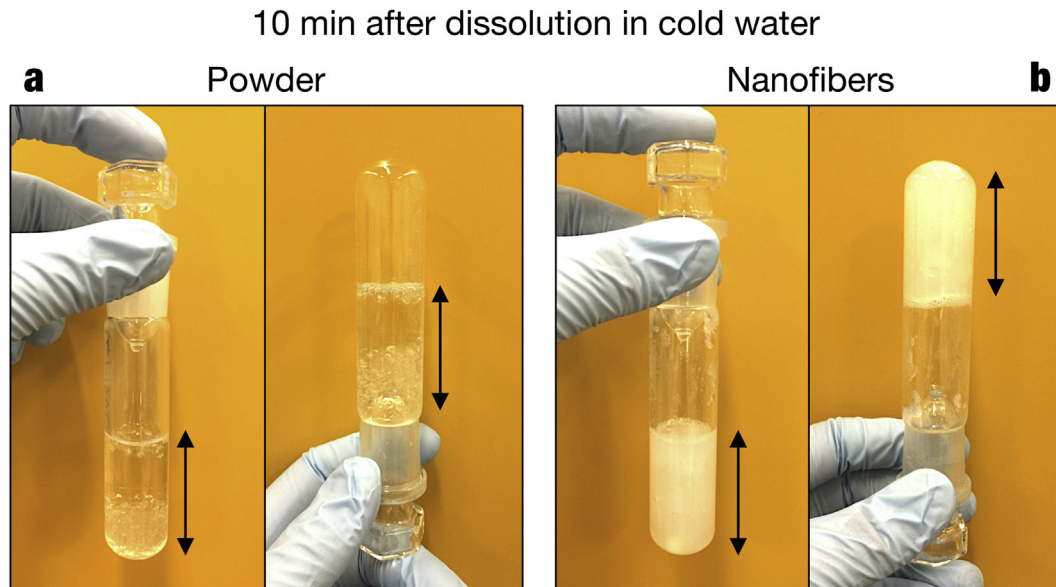


Fig. 6. Inverse tube test showing that gelatin powder does not readily dissolve in cold demineralized water (a), whereas gelatin nanofibers instantly form a white gel with structural integrity (b). Pictures were taken 10 min after introduction of 5 wt% gelatin into water at room temperature (19 °C).

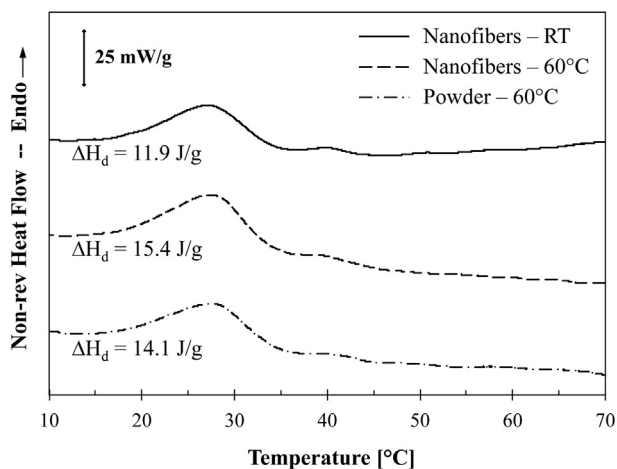


Fig. 7. Thermal properties of hydrogels prepared by dissolution of gelatin nanofibers/powder at room temperature/60 °C, measured using MTDSC and based on the first heating cycle. No significant differences in dissociation temperature were recorded. The nanofiber-based 'cold hydrogel' shows a lower dissociation enthalpy compared to the 'hot gels'.

(or instant) gelatin product was demonstrated by electrospinning of gelatin nanofibers using acid-based solvent systems. The nanofibers are characterized by high surface-to-volume ratio, high porosity and good wettability, facilitating their cold-water-solubility. Indeed, all electrospun samples were readily soluble in cold demineralized water, regardless of the gelatin type/modification, the used solvent system, the dwell time in the electrospinning solution or the storage time of the nanofibers at room temperature and room humidity. Viscosity measurements, thermal analysis and oscillation rheology all indicated that there is no significant degradation of the polypeptide chains, while nanofiber morphology indicates that the specific surface area of the nanofibrous samples is about 1250 times higher than the original gelatin powder. Additionally, thermal analysis showed that the cold-water-soluble gelatin nanofibers are not completely amorphous. Despite the latter property, a fast gelation process occurs upon dissolution

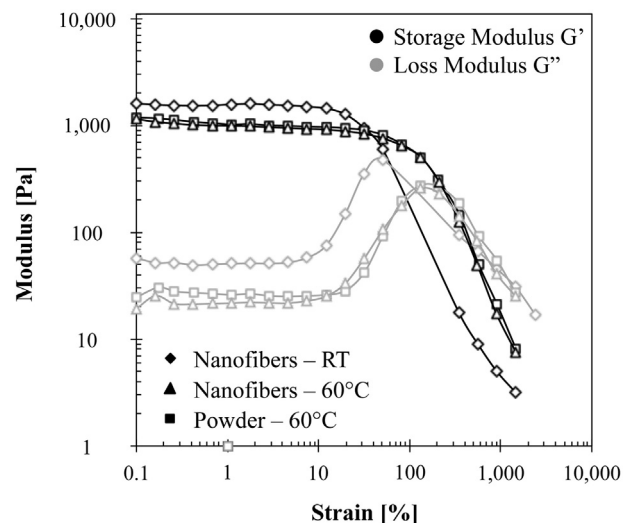


Fig. 8. Mechanical properties of hydrogels prepared by dissolution of gelatin nanofibers/powder at room temperature/60 °C and subsequent storage at 7 °C for 2 days, measured using oscillation rheology. The moduli of the nanofiber-based 'cold gel' are slightly higher. Properties of the nanofiber-based 'hot gel' are identical to the powder-based 'hot gel'.

in cold water, resulting in hydrogels with gel moduli comparable to powder-based gels. Gelatin nanofibers can thus be used as instant gelatin product, without the drawbacks of traditional amorphous gelatins; ⁽¹⁾ moisture-induced triple helix formation does not compromise cold-water-solubility, ⁽²⁾ the nanofibrous structure has high wettability, ensuring easy and fast gelation and ⁽³⁾ the gel modulus of the 'cold gels' is not adversely affected by the process. Using the approach reported here, every electrospinnable, but non-cold-water-soluble gelatin can be transformed into a cold-water-soluble variant, regardless of the type or modification. Electrospinning can thus offer enormous flexibility in materials selection, enabling the production of cold gels loaded with temperature-sensitive components, UV-cross-linkable cold gels, etc.

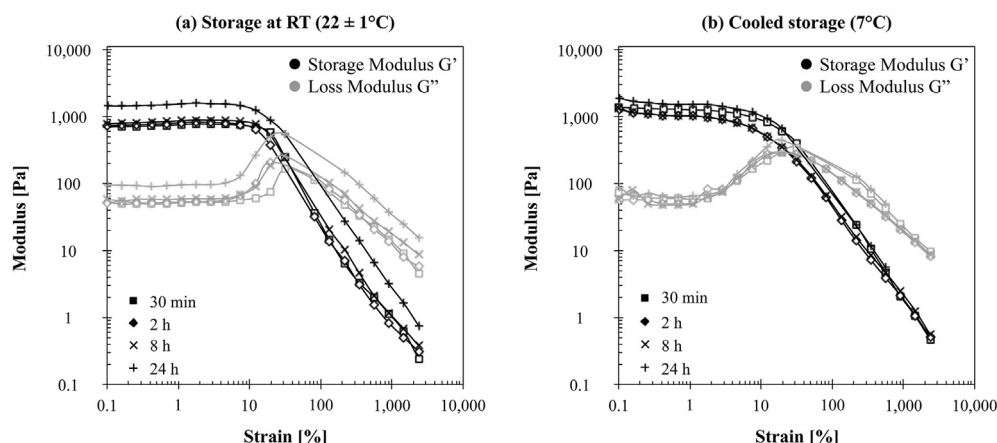


Fig. 9. Mechanical properties of hydrogels prepared by dissolution of gelatin nanofibers at room temperature and subsequent storage at room temperature/7 °C, measured using oscillation rheology. When stored at room temperature, the cold nanofiber-based gel only reaches its maximal moduli after 24 h (a), whereas cooled storage reduces this setting time to only 30 min (b).

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Symbols and abbreviations

G'	Storage modulus
G''	Loss modulus
AA	Acetic acid
DVS	Dynamic vapor sorption
ES	Electrospinning, electrospun
ESI	Electronic supporting information
FA	Formic acid
MTDSC	Modulated temperature differential scanning calorimetry
NF	Nanofiber
RH	Relative humidity
RT	Room temperature

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.foodhyd.2016.01.016>.

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