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Influence of Drying Procedures on Network Formation and Properties of Hydrogels from Functionalized Gelatin

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Summary: Side chain functionalization of gelatin with tyrosine-derived moieties, desaminotyrosine (DAT) or desaminotyrosyl tyrosine (DATT), has been reported to lead to physical networks stabilized by aromatic interactions and hydrogen bonds, while the inherent ability of gelatin chains to organize in helices is suppressed. Here, the treatment of DAT and DATT gelatin films at defined temperatures (drying at 5 °C, freeze-drying, and freeze-thawing) were explored for the potential to additionally stabilize the hydrogels by increasing the content of helical domains as additional physical netpoints. The influence of the drying procedures on the hydrogel properties such as network morphology and mechanical properties were analyzed by WAXS, swelling, and rheological measurements. The triple helix content had a stabilizing effect on gelatin-based hydrogels at temperatures below the helix-to-coil transition. However, this effect was less pronounced at physiological conditions above the transition temperature, resulting in rapid dissolution of the physical gelatin networks.

Keywords: gelatin; hydrogels; structure-property relations; supramolecular interaction; tyrosine

Introduction

Degradable polymers derived from the extracellular matrix are highly interesting as biomaterials for regenerative therapies, since they may promote material-cell interactions and potentially support the regeneration of damaged tissues when implanted as medical devices. Gelatin is a protein-based biopolymer, which is routinely used also in pharmaceutical applications such as oral capsules or absorbable haemostatic agents for parenteral application.[1]

As obtained by partial hydrolysis of collagen, gelatin contains a high amount of the repetitive tripeptides glycine, proline, and hydroxyproline, which give rise to the formation of left-handed single helices that further organize into triple helices stabilized by hydrogen bonds. In water, this supramolecular association is thermodynamically driven and leads to the formation of physical hydrogels with physicochemical properties that hardly can be tailored. Materials with improved performance, e.g., mechanical properties and stability under physiological conditions, can be achieved by chemical modifications of gelatin chains involving the formation of defined netpoints by covalent crosslinking,[2,3] physical crosslinking,[4,5] or application as composite hydrogels.[6] Physically crosslinked hydrogels are a particularly interesting approach for biomedical applications such as local drug releasing depots, since they may benefit from the reversible network formation. That can be realized with previously functionalized gelatin without the use of crosslinking agents, which might partially remain entrapped in the hydrogels as unreacted

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and potentially toxic substances. Additionally, the self-assembly of gelatin-based hydrogels upon cooling below the sol-to-gel transition temperature could enable quantitative loading of bioactive molecules or cells without exposure of the payload to crosslinking reagents.

Previously, physical gelatin networks were developed by functionalization of gelatin with the tyrosine-derived moieties desaminotyrosine (DAT) and desaminotyrosyl tyrosine (DATT). By introducing the sterically demanding phenolic groups, this functionalization suppresses triple helix formation, while introducing additional physical stabilization by π-π interactions and hydrogen bonds.[4,5] In these materials, it could be demonstrated that already a low degree of DAT/DATT functionalization, as given by the low amount of functionalizable lysine and hydroxylysine side chains (3 mol%) in gelatin,[7] can influence the macroscopic behavior of the resulting physical hydrogels significantly. Furthermore, by functionalization, increased incorporation of substances could be achieved due to additional stabilizing bonds, as shown for hydroxyapatite nanoparticles.[6]

However, for their use in biomedical applications, a shortcoming of DAT/DATT-functionalized gelatin without triple helices may be the limited capacity of the network to preserve its structure in the presence of an excess of water. Specifically, massive water uptake of the network may disturb the network integrity and result in rapid disintegration of the hydrogel. Previously, the main focus of gelatin functionalization with DAT/DATT was to suppress the formation of triple helices[5] in order to achieve hydrogels with controllable mechanical properties. Triggering triple helix formation in DAT(T) functionalized gelatin may be a concept to benefit from the inherent stabilizing forces of gelatin and create additional physical netpoints in the material (Figure 1). A first indication on the effect of the drying procedure on network properties can be seen from the enhanced triple helix formation by network drying below the sol-gel transition temperature, which resulted in different mechanical strength of the hydrogels at room temperature.[5]

In this study, various drying procedures at temperatures below the sol-gel transition should be studied to broadly explore potential stabilizing effects that might be effective both at room and body temperature. It was hypothesized that a controlled dehydration of gelatin chains may enhance

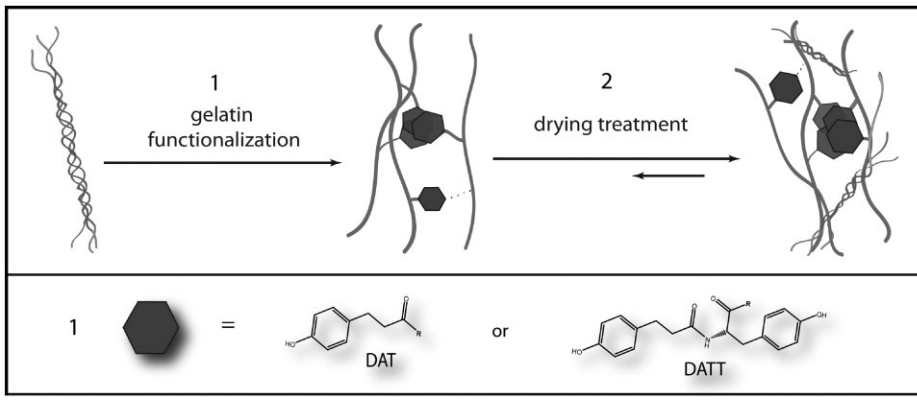

Figure 1.
Scheme of the study concept. Functionalization of gelatin with aromatic moieties results in diminished formation of triple helices and enhanced π-π interactions and hydrogen bonds.[5] In this study, a number of controlled drying procedures below the sol-gel transition temperature were explored to enhance hydrogel stability by inducing additional physical netpoints, such as triple helices, hydrophobic interactions, or hydrogen bonds.

the formation of triple helices or other physical interactions between gelatin chains. Slow evaporation of water from the hydrogels at 5 °C served as a standard drying procedure comparable to the conditions used in the previous study.[5] Additionally, different methodologies for controlled dehydration and in some cases repetitive dehydration/hydration cycles were applied, finally followed by freeze-drying to obtain a water-free, long-term storable material. Furthermore, the morphology of gelatin, DAT-, and DATT-functionalized gelatin (DAT-Gel and DATT-Gel, respectively) was assessed and discussed along with the macroscopic network properties such as swelling and rheological properties.

Materials and Methods

Materials

Gelatin (type A, porcine, Bloom 200), 3-(4-hydroxyphenyl) propionic acid (DAT), *N*-hydroxysuccinimide (NHS), *N,N*-diisopropylethylamine (DIPEA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and 2,4,6-trinitrobenzensulfonic acid (TNBS) were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany). Dimethyl sulfoxide (DMSO) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). O-*t*-Butyl-L-tyrosine, dichlormethane (DCM) and *N*-metyl pyrrolidon (NMP) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). All reagents and solvents were of analytical grade and used without further purification.

Gelatin Functionalization

Gelatin was functionalized with DAT and DATT by amide formation at lysine and hydroxylysine residues using EDC/NHS in DMSO as previously described.[4,5] DATT was synthesized in a two-step procedure as previously reported. The degree of functionalization (*d.f.*) with aromatic moieties was determined by a 2,4,6-trinitrobenzenesulfonic acid (TNBS) colorimetric assay ($n = 3$), which quantifies the free primary amino groups as described in literature.[8] For DAT- and DATT-Gel, *d.f.* of 91 mol% and 93 mol%, respectively, were calculated.

Drying of Gelatin Films

Films of gelatin, DAT-, and DATT-Gel were prepared by casting 5 wt.% aqueous solutions (dissolved at 45 °C) into polystyrene Petri dishes. The solutions were allowed to gel for 1 h at room temperature and then subjected to different drying procedures:

A. Slow drying at 5.0 ± 0.2 °C (24% r.h.) in a climate chamber (KBT Binder, Tuttlingen, Germany) until constant weight was achieved, which was obtained after 2 days of drying time, abbreviated as SE (slow evaporation).
B. Shock freezing by immersing in liquid nitrogen (−197 °C) with subsequent freeze drying, abbreviated as FF (fast freezing).
C. Slow rate freezing in an ethanol/ice bath (70 wt.% ethanol, −13 °C) with subsequent freeze drying, abbreviated as SF (slow freezing).
D. Freeze-thawing in either 3 or 10 cycles prior to freeze-drying (FT-3 and FT-10, respectively). Each freeze-thaw cycles involves freezing at −20 °C for 2 h, followed by thawing at room temperature for 2 h. Freeze-drying was performed at a Christ Alpha 2–4 LSC Lyophilizer (Matrin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) under a vacuum of 0.08 mbar.

Analysis of Triple Helices

Dry samples were analyzed by Wide-Angle X-ray Scattering (WAXS) using a X-ray diffraction system D8 Discover with a two-dimensional detector from Bruker AXS (Karlsruhe, Germany). The X-ray generator was operated at a voltage of 40 kV and a current of 40 mA. A copper anode and a graphite monochromator produced Cu Kα radiation with a wavelength of 0.154 nm. WAXS images were collected

from gelatin films (exposure times of 120 s per pattern) in transmission geometry with a collimator-opening of 0.8 mm at a sample-to-detector distance of 15 cm. Two-dimensional scattering patterns were integrated for analysis into one-dimensional curves of I vs 2θ. The relative content of triple helices (X_{TH}) was calculated by relating the intergrated areas of the triple helix peak to the integrated area of the amorphous peak as X_{TH} = (Area triple helix/Area amorphous) · 100 (%).

Swelling Studies

The swelling of films dried under different conditions was investigated in water at 10 °C for 48 hours in a climate chamber (KBT, Binder, Tuttlingen, Germany) (n = 6). The degree of swelling Q was calculated by Equ. 1

$$Q = 1 + \rho_g \cdot \left(\frac{m_{sw}}{m_d \cdot \rho_{H_2O}} - \frac{1}{\rho_{H_2O}} \right), \qquad (1)$$

where m_{sw} is the mass of the sample in the swollen state, m_d is the mass of the dry extracted sample, and ρ_{H_2O} (1 g · cm^{-1}) and ρ_g are the specific densities of water and gelatin, respectively. ρ_g was determined using an Ultrapycnometer 1000 (Quantachrome GmbH & Co. KG, Germany) with gelatin powders.

Rheological Characterization

The dynamic rheological analysis were performed on a stress-controlled rheometer (Haake MARS 2, Thermo Fisher, Karlsruhe, Germany) equipped with a plate-plate geometry of 20 mm and a Peltier temperature controlled system (temperature deviation 0.1 °C). A solvent trap was used to prevent solvent evaporation. The hydrogels were swollen to the equilibrium state for 24 hours in water at 10 °C and then measured under constant stress of 1 Pa and oscillatory frequency of 1 Hz in the linear viscoelastic region of the hydrogels (determined in frequency and amplitude sweeps). The thermomechanical behavior of the hydrogels was analyzed by measuring changes in the storage and loss moduli in the temperature range of 5 °C to 55 °C with a heating rate of 1 °C · min^{-1}. The gel-to-sol transition temperature was determined by differentiating the curve of the storage moduli G' (corresponds to its inflection point). The method-based statistical error for the rheologic determination of $T_{gel\text{-}sol}$ was 1–2 °C.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was applied for morphology analysis of cross-sections of the dried hydrogels. Before analysis, samples were attached on a sample holder and coated with a conductive iridium layer with thickness of 4 nm under vacuum (using sputter coater Q150T ES from Quorum Technologies, UK). SEM micrographs were taken at 200–400x magnification, operating at acceleration voltage of 5 kV using an Everhard-Thornley detector (Gemini Supra 40 VP, Zeiss, Jena, Germany).

Results and Discussion

The systematic investigation of DAT- and DATT-Gel in comparison to unfunctionalized gelatin should help to get a mechanistic understanding of morphological changes and to evaluate the capacity of the applied drying procedures to enhance intermolecular interactions in physical hydrogels.

It is believed that the formation of helices in gelatin is a kinetically controlled process.[5] If water will be withdrawn from the system at a relatively low rate as yielded here by drying at 5 °C (SE), the polymer chains are given extensive time for reorientation and interaction with each other. They can form ordered structures, which should be associated with the highest helix content. Freezing, on the other side, is a much faster process, possibly leading to a lower triple helix content. Since freezing of water in gelatin hydrogels leads to the removal of stabilizing hydrogen bonds to gelatin, it may be speculated that helices can organize depending on the cooling rate. As a consequence, physical gelatin hydrogels subjected to slower freezing, as in an

ethanol/ice bath (SF), may exhibit higher content of triple helices compared to hydrogels frozen by immersion in liquid nitrogen (FF). The final removal of water, except for SE, was performed by freeze-drying.

Samples subjected to SE (Figure 2A) appeared non-porous when studied at a similar magnification compared to the freeze-dried hydrogels, which showed a porous microstructure (Figure 2B–F). The high porosity of the freeze-dried samples is due to fixation of the overall volume of the sample in the frozen state, which is preserved during subsequent water sublimation. For SE, rearrangements take place during slow volume loss by water evaporation in the semisolid state of the samples. The crystallization pattern of water for those samples finally subjected to freeze-drying is strongly depending on the cooling rate, which therefore is supposed to predefine the porous morphology of the resulting dried materials. The ice nucleation rate is high in FF conditions, so small regular crystals apparently served as templates for the numerous small pores in these samples (Figure 2B). Freezing the samples at slower rates, as in SF, could be associated with a slower, possibly directional ice crystal growth[9] and formation of larger ordered ice crystals as indicated by large pores in Figure 2C.

As it is well established for other water soluble polymers that exhibit physical interactions, repeated dehydration and rehydration as in freeze-thawing cycles can trigger physical interactions in hydrogels. This technique is used to create, e.g., polyvinyl alcohol hydrogels through formation of crystallites in repeated freeze-thaw cycles.[10,11] Accordingly, repeated freezing of water should result in removal of stabilizing hydrogen bonds between gelatin chains, thus giving rise to self-arrangement of helical structures. Therefore, 10 cycles of freeze-thawing (FT-10) should lead to a higher degree of order in gelatin hydrogels compared to samples subjected to 3 freeze-thaw cycles (FT-3).

The X-ray scattering spectra of Gel, DAT-, and DATT-Gel revealed the morphology of the dried hydrogels. Three characteristic elements can be distinguished in the WAXS spectra: i) a broad halo at a scattering angle 2θ of about

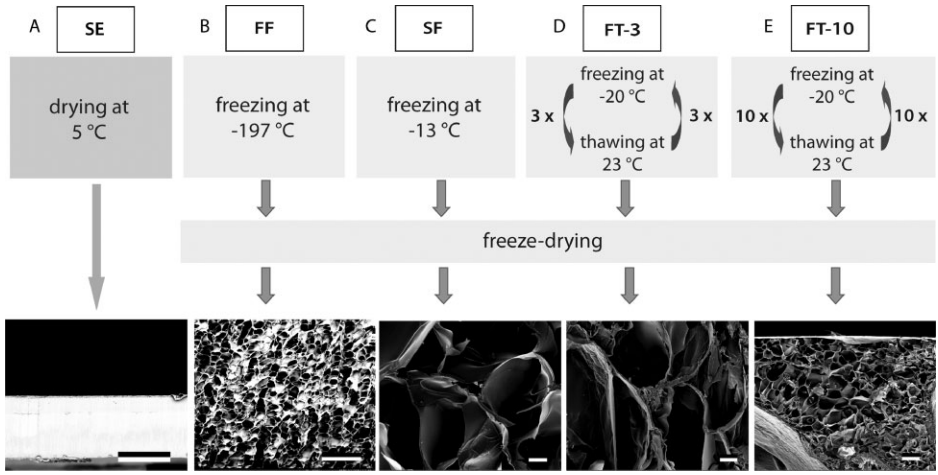

Figure 2.
Hydrogel drying protocols, sample abbreviations, and SEM micrographs (scalebars 50 μm) of resulting DATT gelatin samples: (A) SE, slow drying at 5 °C; (B) FF is immersed in liquid nitrogen and (C) SF is frozen at a slow cooling rate (EtOH/ice bath) prior to freeze-drying; (D-E) FT denotes freeze-thaw treatments (3 or 10 FT cycles) prior to freeze-drying. Gel and DAT Gel samples demonstrate similar porous structures as seen by SEM (data not shown).

21° corresponding to the amorphous region with predominantly randomly coiled proteins, ii) a peak at $2\theta = 7.5°$ representing the triple helix (TH) structures, and iii) a peak at $2\theta = 32°$ corresponding to the single helix scattering (Figure 3). The WAXS spectra and the calculated relative amounts of triple helices (X_{TH}) (Table 1) followed the trend, which has been proposed based on the expected advantageous effects of slow dehydration or repeated freezing.

In particular, the WAXS analysis illustrated that gelatin functionalization with DAT and DATT led to a decreased content of single and triple helices. For all hydrogels, drying at 5 °C best supported the association of triple helices, while rapid freezing with subsequent freeze drying (FF) resulted predominantly in amorphous samples. Slower freezing (SF) triggered the triple helix formation. Furthermore, DAT(T)-Gel hydrogels show a substantial increase of X_{TH} after 10 freeze-thaw cycles due to the repeated freezing of water along with phase separation of the gelatin phase, probably supporting interchain arrangement. This effect appears not very strong when applying three freeze-thaw cycles only (FT-3). Overall, the influence of the drying procedure on the triple helical morphology of functionalized and non-functionalized gelatin appears limited, which stresses the challenge to predictably control the triple helix association. These effects are not so strongly pronounced for non-functionalized gelatin, indicating the possibility to affect triple helix assembly on the one side by insertion of aromatic functional groups as defined physical netpoints and on the other side by drying procedures.

In the next step, the effect of different sample morphologies on their behaviour in an aqueous environment should be analysed. The degree of swelling (Q) of gelatin hydrogels is controlled by network properties defined by triple helices and additional physical interactions (e.g., π-π interactions, hydrogen bonds) or entanglements. In order to include contributions of all these interactions, a series of swelling studies with dried Gel, DAT-, and DATT-Gel films was performed at 10 °C, i.e. below the gel-to-sol transition temperature where triple helices are stable. It should be considered that pores are relevant for water entering the matrix during rehydration. Therefore, for the porous samples (FF, SF, FT), the equilibrium swelling state was reached much faster (after 8 h) in comparison to SE hydrogels that were equilibrated after 24 h (data not shown). In all cases, rheological experiments were performed after 24 h of equilibrium swelling.

Hydrogels from non-functionalized Gel demonstrated no remarkable alteration of Q induced by the applied drying procedures (Figure 4). For DAT-Gel, higher swelling of SE samples was observed compared to Gel. This tendency is supported by the lower X_{TH} after insertion of one aromatic functional group, i.e., a lower number of physical netpoints provided by triple helices. Apparently, DAT moieties sterically

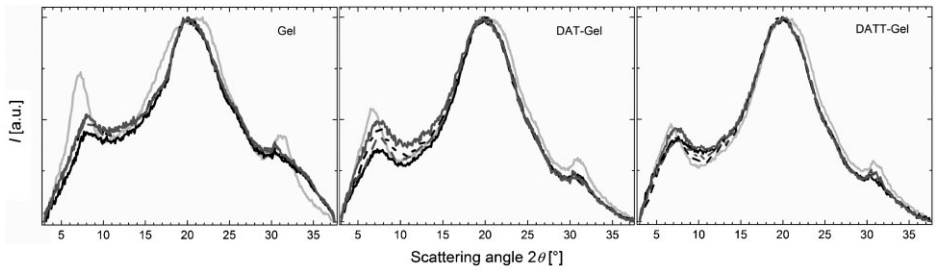

Figure 3.
Scattering spectra of dried gelatin, DAT- and DATT-gelatin hydrogel films after SE (light grey line), FF (black line), SF (black dashed line), FT-3 (grey dashed line) and FD-10 (grey line).

Table 1.
Summary of triple helix content X_{TH} of dried physical hydrogels from functionalized DAT and DATT-Gel, calculated from WAXS spectra, as well as the gel-to-sol transition temperature $T_{gel\text{-}sol}$ determined by the inflection point of the G' curve in rheological experiments.

	Gel		DAT-Gel		DATT-Gel	
	X_{TH} [%]	$T_{gel\text{-}sol}$ [°C]	X_{TH} [%]	$T_{gel\text{-}sol}$ [°C]	X_{TH} [%]	$T_{gel\text{-}sol}$ [°C]
SE	9.1	28	5.8	27	6.6	22
FF	3.9	30	4.0	22	3.9	24
SF	5.5	27	7.8	25	5.2	24
FT-3	4.7	29	4.1	24	3.4	23
FT-10	5.5	30	6.1	21	5.0	25

Compared to previous results,[5] the experimental procedure was slightly altered and lower values of $T_{gel\text{-}sol}$ were obtained. In particular, lower heating rates 1 °C · min^{-1} (2.6 °C · min^{-1} in reference[5]) for more precisely detecting the onset of thermal transitions were applied here. Additionally, due to the the high fluctuation in the G'' curves in the relevant temperature range, $T_{gel\text{-}sol}$ was calulated from the inflection point of the G' (crossover point of G' and G'' used in[5]).

suppressed helix trimerization but at the same time did not provide a comparative binding strength by other newly introduced physical netpoints. The strong reduction of Q upon faster drying procedures (FF, SF, FT) indicates successful formation of additional interactions in DAT-Gel by the different freezing conditions, which, however, showed limited systematic correlation with the content of triple helices.

Interestingly, the introduced DATT moieties in DATT-Gel not only decreased X_{TH}, but could also provide strong physical interaction that resulted in low swelling at SE conditions. A considerable reduction of Q was also observed for freeze-dried DATT Gel when compared to Gel samples. The drying procedures FF and FT-3 appeared to promote the strongest reduction of swelling at 10 °C, i.e., a physical

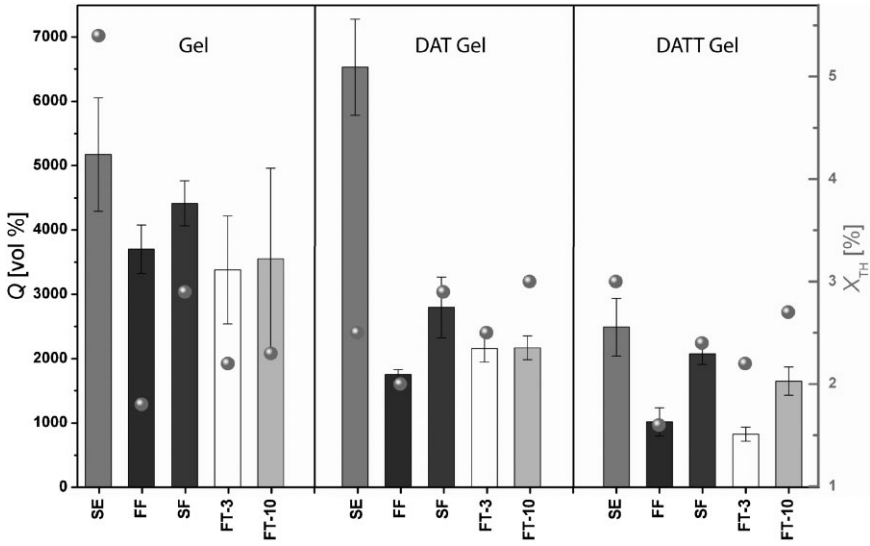

Figure 4.
Illustration of limited dependence of equilibrium swelling Q of gelatin (Gel), DAT and DATT gelatin (DAT and DATT Gel) hydrogels after 24 h in water at 10 °C (bars, scale on the left y-axis, n = 6) on the triple helix content, X_{TH} (data points as circles, scale on the right y-axis, data reprinted from Table 1). The drying treatments are abbreviated as follows: SE – drying at 5 °C, FF – fast freezing; SF – slow freezing; FT – freeze-thaw treatments (3 or 10 cycles); FF, SF, and FT were followed by freeze-drying.

network structure with increased crosslink density. A similar behaviour was observed also for DAT-Gel for these specific drying procedures, which seems surprising since FF samples contain the lowest X_{TH}. So, it could be concluded that contributions of other non-covalent binding forces such as hydrophobic interactions, which become more pronounced upon complete removal of water, contributed to a reduce Q in freeze-dried samples. Based on this analysis of hydrogel swelling in comparison to X_{TH}, it may be concluded that the triple helices are not in all cases the dominating interaction determining the hydrogel properties. This corresponds to earlier findings.[5]

The analysis of the thermomechanical properties of the hydrogels, swollen to the equilibrium state in water at 10 °C, was carried out by oscillatory rheology (Figure 5). The samples demonstrated to be soft gels with storage moduli (G') in the kPa range, which are in the linear regime at low temperatures below $T_{gel\text{-}sol}$. The interpretation and comparison of the G' values of the different gelatin based hydrogels should be carefully conducted, since G' also reflects the mechanical properties resulting from the ultrastructure of samples such as porosity and pore (size) distribution in case of porous samples, which may overlay effects of the organization on the molecular level. However, rheology is a valuable method to study the triple helix dissociation of physical gelatin hydrogels, expressed as $T_{gel\text{-}sol}$.

In non-functionalized gelatin samples, $T_{gel\text{-}sol}$ was higher compared to functionalized hydrogels, which can be assigned to the steric hindrance of triple helix formation by aromatic groups. Additionally, it should be considered that the helix length, which also contributes to the capacity of physical netpoints to withstand mechanical deformation, may be reduced in functionalized Gel. DATT-Gel networks demonstrate the lowest $T_{gel\text{-}sol}$ due to the bulky functional groups with two phenolic moieties. Generally, the specific effects of different drying procedures on $T_{gel\text{-}sol}$ of each material were limited. Above the $T_{gel\text{-}sol}$, similar moduli were observed for all materials in the sol state, suggesting that other physical netpoints in DAT(T) gelatin have insignificant impact on the rheological properties, at least after helix melting. This is in accordance with model systems of polyethylene glycol functionalized with tyrosine dipeptide derivatives, where associative interactions were reported only in the temperature range of 10 to 30 °C.[12] Below the $T_{gel\text{-}sol}$,

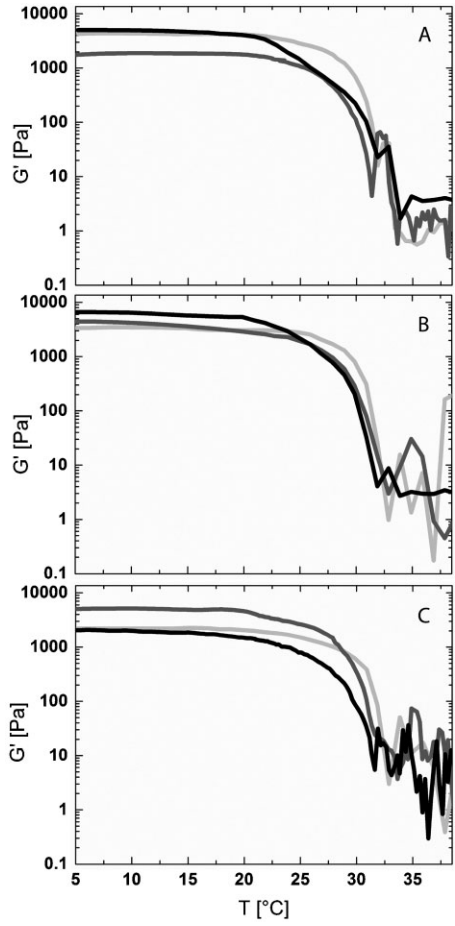

Figure 5.
Exemplary rheogramms (storage moduli G' in relation to temperature) of rehydrated hydrogels from gelatin (light grey), DAT- (dark grey), and DATT-Gel (black), subjected to A) slow evaporation, B) freeze-drying with fast freezing and C) 10 freeze-thaw cycles, at equilibrium swollen state. All samples were analyzed in the linear viscoelastic region ($\tau = 1$ Pa, $f = 1$ Hz) with a heating rate of $1\,°C \cdot min^{-1}$.

stronger physical interaction would have been expected for DATT compared to DAT. However, this did not translate into higher G' of DATT-functionalized gelatin, indicating that effects mediated by triple helices may have superimposed effects mediated by DAT(T) moieties.

Finally, it was examined whether the observed effects of drying procedures on network properties in the swollen state are effective also at temperatures above $T_{gel\text{-}sol}$. When incubating dried samples at 37 °C, which is above $T_{gel\text{-}sol}$ of all materials, no major differences between the functionalized and non-functionalized samples and drying procedures could be detected. Generally, the associative networks rapidly disintegrated (mass loss > 90 wt.% within 24 hours) when exposed to 37 °C with an excess of water regardless of the drying treatment and type of gelatin.

Conclusion

Previous reports on the effects of gelatin functionalization with aromatic moieties on the morphology of gelatin-based materials could be confirmed in this study, in which drying protocols were systematically varied and applied below the sol-gel transition temperature. It was identified that triple helices are not the main interactions governing stabilization in DAT(T)-Gel hydrogels, since X_{TH} did not systematically correlate in all cases with Q determined at 10 °C. Freeze-drying and freeze-thaw cycles are possibly suitable to trigger hydrophobic interactions in DAT(T)-Gel and lead to a considerably decreased swelling. The investigated drying treatments have a minor influence on the gel-to-sol transition temperature. The incubation of samples at 37 °C in water resulted in an almost complete disintegration of all physical hydrogels within 24 hours. A prolonged stabilization of the DAT and DATT gelatin physical hydrogels also at physiological conditions can possibly be achieved by introducing moieties that support the formation of stronger physical interactions, such as charge-transfer complexes or more hydrophobic moieties.[12]

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