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Research paper

Effects of processing conditions on the rheological behavior of collagen dispersions

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Abstract

Biomedical collagen preparations are mainly based on liquid aqueous preparations either used directly as injectables or transferred for example into solid implants or porous devices. In all cases the viscosity of the dispersion or solution has to be controlled or adjusted by pH, temperature, collagen concentration or crosslinking. We tested these effects on the rheological and structural properties of collagen fiber dispersions focusing on oscillatory rheometry. With increasing difference between pH and pI viscosity increased and went through a maximum with less rigidity of the fibers. The dispersions acted like a predominantly permanently linked network. This character changed to only partially linked at low collagen concentration due to isolation of the individual fibers. Up to 40°C, temperature is a tool suitable for adjusting viscosity without changes in the network structure. At 50°C, fourier transform – infrared spectroscopy (FT–IR) spectroscopy indicated the transition of the helical into random coil structure. Renaturation of the helices was found upon cooling but atomic force microscopy (AFM) indicated severe temperature induced damages of the fibers. Crosslinking with glutaraldehyde (GTA) leads to an increase in viscosity and the effect on the network structure depends on the processing conditions. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Collagen; Fiber; Rheology; Atomic force microscopy; Fourier transform - infrared spectroscopy; Complex viscosity; Loss modulus; Storage modulus; Crosslinking

1. Introduction

The current medical applications of collagen as a biodegradable material range from hemostats, sealants, implant coatings, artificial skin, bone graft substitutes to corneal shields and injectables for plastic surgery. Besides good biocompatibility and well-documented structural, chemical and physical properties [1–3], the main advantages of collagen are its biological activity and cellular attachment with their positive outcome on tissue regeneration or integration [4]. In most cases, collagen processing involves liquid aqueous preparations. These can on the one hand be used directly, e.g. as injectables for tissue augmentation [5,6] or as drug carriers for local application, e.g. for tumor treatment [7] or tissue regeneration [8]. On the other hand, solutions or dispersions may be transferred into solid implants, e.g. as sponges for tissue regeneration [9] or antibiotic wound dressings [10] as well as dense matrices for drug delivery [11]. In all cases the properties of the dispersion have to be adjusted and controlled for example with respect to viscosity to adjust intrudability [12] or pumping into molds for further processing by pH or temperature adjustment [13]. Viscosity at the same time secondarily affects characteristics such as pore size of porous sponges which is important for cell penetration [14].

We investigated the influence of pH, temperature, collagen concentration and chemical crosslinking on the rheological behavior of insoluble collagen fiber dispersions with different isoelectrical point using oscillating rheometry. Additionally, we studied the influence of temperature on the fibril structure of the collagen material with atomic force microscopy (AFM) and fourier transform - infrared spectroscopy (FT-IR) spectroscopy in order to identify temperature induced changes in the aqueous collagen preparations.

2. Materials and methods

2.1. Materials

Insoluble fibrous bovine tendon collagen c1 (pI ∽7.0) was provided by Innocoll GmbH, Saal, Germany. Insoluble

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fibrous bovine tendon collagen c2 (pI ∽5.1) was obtained from Genetics Institute Inc., Andover, MA, USA.

2.2. Preparation of dispersions

The lyophilized raw material was pre-swollen for 1 h in water with pH adjusted to 4.5 for c1 and 7.4 for c2 with acetic acid or sodium hydroxide if not stated otherwise. Subsequently, approximatley 50 ml dispersion were homogenized for 10 min in 1 min intervals with an Ultraturrax® 10 G tool (IKA, Staufen, Germany) at approx. 5000 rev./min (T controlled to <25°C by external cooling with a water/ice mixture). For crosslinking glutaraldehyde (GTA) was added to a 1% dispersion at GTA to collagenous amino groups ratios (GTA/-NH_2) of 0.04 and 0.25. After 24 h at 4–6°C, glycine was added (moles GTA:glycine 1:2) to remove residual aldehyde. All systems were centrifuged at 300 × g for 1 min to remove entrapped air-bubbles.

2.3. Rheometry

Oscillatory rheological experiments were performed on a Rheometrics RFR 7800 (Rheometrics, Bensheim, D) using a 0.02° cone/plate system (50 mm diameter) or parallel plate geometry (50 or 20 mm diameter). At first, the linear viscoelastic region was determined with a strain sweep at constant frequency ω of 0.1 rad/s. All subsequent frequency sweeps measuring storage modulus G', loss modulus G'' and complex viscosity η^* were performed at constant deformation in the linear viscoelastic region. The yield value of collagen dispersions was determined using classical continuous shear rheometry on a CS-10 Rheometer (Bohlin, Pforzheim, Germany) using a 4° cone/plate system (40 mm diameter) or a parallel plate geometry (40 mm diameter).

2.4. Fourier transform - infrared spectroscopy

FT-IR spectra were obtained on a Magna-IR 550 (Nicolet, Madison, WI, USA) using a temperature controlled thin film CaF_2 cell yielding a sample film of approx. 5.6 μm thickness [15].

2.5. Atomic force microscopy

One millilitre of a 0.1% collagen dispersion was transferred onto a glass slide and carefully washed with water adjusted to pH 4.5 for c1 and 7.4 for c2 with acetic acid or sodium hydroxide. The remaining collagen dispersion was dried for 24 h under a laminar air flow bench at room temperature. Images were taken with a Nanoscope 3 (Digital Instruments, Mannheim, Germany) using Nanoprobe® SPM tips. For temperature treatment, samples were heated to the designated temperature for 1 or 24 h and cooled to 25°C before further processing.

3. Results and discussion

All dispersions showed a shear-thinning flow behavior as η^* decreased almost linearly with rising frequency indicating structural viscosity. Using oscillatory rheometry exposing the samples to a sinusoidally fluctuating strain, information about the molecular structure of the gel network can be obtained. The storage modulus G' describes the elasticity of the material whereas the loss modulus G'' (= viscosity $\eta \cdot$ frequency ϖ) reflects the dissipated energy as a characteristic of the viscous properties. Within the pH spectrum tested for c1 (Fig. 1a), the lowest complex viscosity at pH 2.5 corresponded to electrostatic repelling forces between the protein chains with complete protonation. With rising pH, η^* went through a maximum at pH 3.5 as the attractive forces between the collagen fibers increased. However, with the pH approaching the isoelectrical point, the viscosity decreased again, aggregates were formed and water separated from the fiber bundles which led to inhomogeneity of the dispersion at pH 5.5. Slopes of the viscosity curves (∽ − 0.85) between −0.5 and −1 indicated that the aqueous collagen systems behave similar to a polymer network with predominantly permanent links [16]. As shown in Fig. 1a the type of network structure did not depend on pH. The small slopes and almost parallel pattern of the storage and loss modulus plotted versus frequency as well demonstrated the characteristics of a mostly permanent gel network for collagen preparations (Fig. 1b, G'' not shown for reasons of clarity). The minimal slope of G' of 0.08 at pH 3.5 indicated more flexible chains as compared to the rigid fibers at either pH 2.5 when the repelling forces are stronger (slope of 0.12) or at pH 4.5 and 5.5 when the structures aggregate (slope 0.13 and 0.12) [13]. Corresponding to the viscoelastic analysis of the systems, the yield value showed a maximum at pH 3.5 (Table 1).

For c2 the lowest viscosity resulted at pH 2.5 and 6.2 and the values increased with higher pH (Fig. 1c). This behavior could again be explained by stronger hydration and more stiff collagen structures with increasing net charge of the molecules rather than a decrease in interactive forces between fibers. By their higher slopes and by approaching each other at higher frequency, G' and G'' indicated more reversible physical entanglement at pH values close to the isoelectrical point (pH 2.5 and 6.2), whereas less dependence on frequency and a parallel pattern pointed to stronger fixation in a network at higher pH (Fig. 1d). Thus, the pH dependency in combination with the acidic pI of c2 corresponded to the results obtained for c1 with a neutral pI and careful pH variation is suitable for viscosity adjustment.

The slopes of η^*, G' and G'' were similar for 0.56%, 1% and 2.5% c1 dispersions at pH 4.5 (Fig. 2a,b) indicating the same, mostly permanent network structure of the samples. A substantial decrease in η^* by several orders of magnitude, an increase in the slope of η^* to −0.66 and an increase in the slope of G' and G'' was observed as the concentration was reduced to 0.1%. At the same time the yield point was

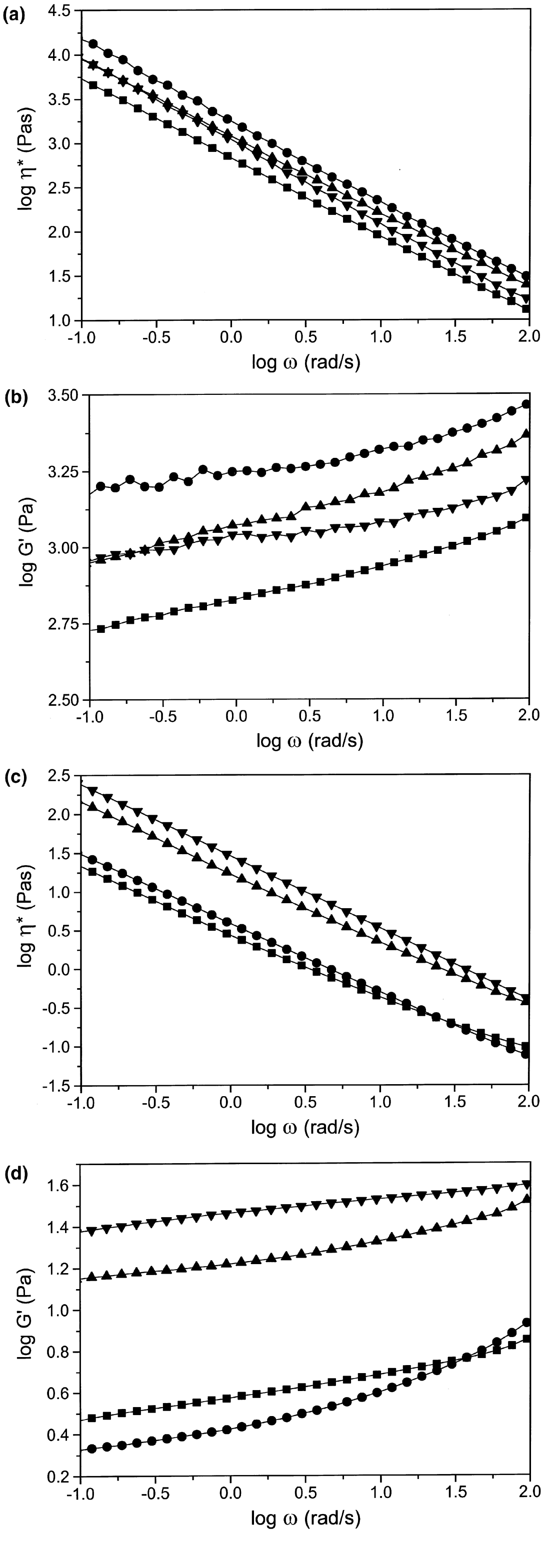

Fig. 1. Oscillatory rheometry: (a) the complex viscosity of c1 dispersions at pH 2.5 (■), 3.5 (●), 4.5 (▲) and 5.5 (▼) (1% at 25°C); (b) the storage modulus of c1 dispersions at pH 2.5 (■), 3.5 (●), 4.5 (▲) and 5.5 (▼) (1% at 25°C); (c) the complex viscosity of c2 dispersions at pH 2.5 (■), 6.2 (●), 7.4 (▲) and 11.0 (▼) (1% at 25°C); (d) the storage modulus of c2 dispersions at pH 2.5 (■), 6.2 (●), 7.4 (▲) and 11.0 (▼) (1% at 25°C).

Table 1

Yield values of c1 dispersions at different concentration, pH and temperature

Concentration (%)	pH	Temperature (°C)	Yield value (Pa)
1	2.5	25	36
1	3.5	25	241
1	4.5	25	39
1	5.5	25	40
0.1	4.5	25	< 0.05
0.56	4.5	25	18
2.5	4.5	25	330
1	4.5	30	19
1	4.5	40	12
1	4.5	50	2.8

markedly decreased from 330 Pa for a 2.5% c1 preparation as compared to less than 0.05 Pa at 0.1% (Table 1). This indicated an over proportional loss of interactions due to isolation of the collagen fibers resulting in a network which is only partially permanently linked [16].

With rising temperature, the viscosity of a 1% c1 dispersion pH 4.5 decreased and a stronger fiber flexibility could be detected as η*, G′ and G″ showed a drop in the slope in

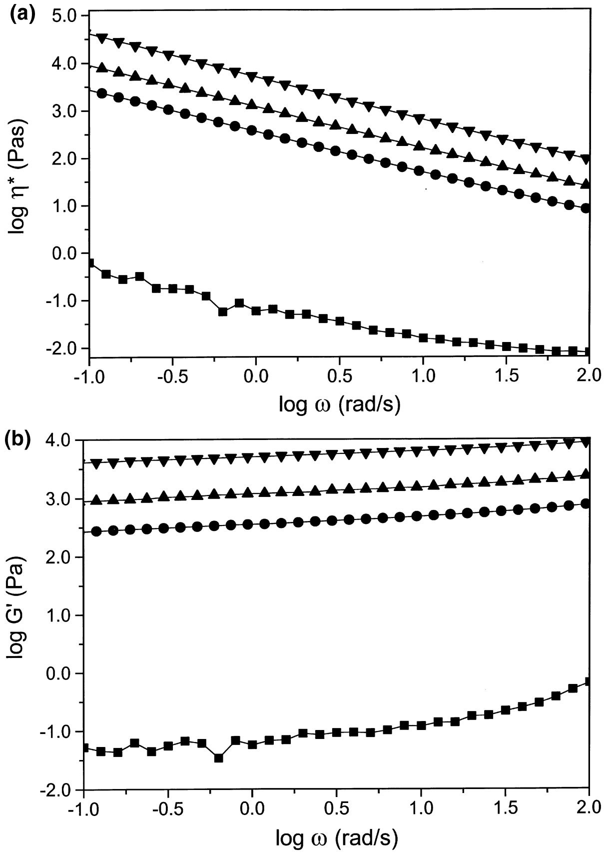

Fig. 2. Oscillatory rheometry: (a) the complex viscosity of c1 dispersions at 0.1% (■), 0.56% (●), 1% (▲) and 2.5% (▼) (pH 4.5 at 25°C); (b) the storage modulus of c1 dispersions at 0.1% (■), 0.56% (●), 1% (▲) and 2.5% (▼) (pH 4.5 at 25°C).

the frequency spectra at 40 and 50°C in comparison with the patterns obtained at 25 and 30°C (Fig. 3a,b). The yield values demonstrated a similar decrease with the most pronounced change between 40 and 50°C (Table 1). As differential scanning calorimetry had indicated, melting of c1 fiber dispersion occurs with a maximum at approximately 43°C [17]. Denaturation of collagen fibrils in solution represents an irreversible process [18]. However, for fibers, this transition is broader and more complex due to molecular inhomogeneity with respect to natural crosslinking and structural inhomogeneity on the fibril packaging level [19,20]. The extent of renaturation of collagen fibers depends on the initial crosslinking of the material [21]. Covalent intramolecular cross-linkages provide essential interchain contact, hence increasing the nucleation rate. Cooling the c1 dispersion previously heated to 50 down to 25°C did result in an increase in η* and a network structure which appeared to be similar to the initial 25°C sample but the initial viscosity was not reached. Consequently, careful warming of aqueous collagen fiber preparations below 40°C could provide a tool to adjust dispersion viscosity for casting and filling.

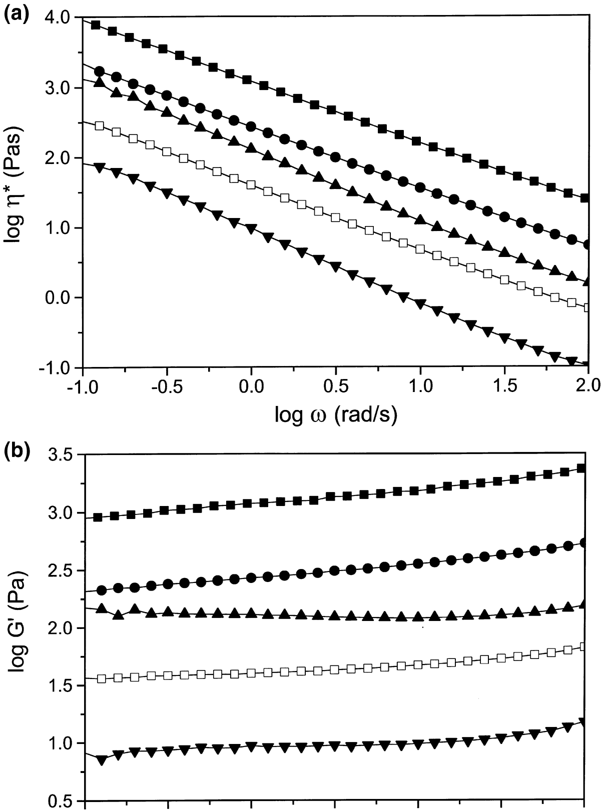

Fig. 3. Oscillatory rheometry: (a) the complex viscosity of c1 dispersions at 25°C (■), 30°C (●), 40°C (▲), 50°C (▼) and 25°C after heating to 50°C and cooling (□) (1% and pH 4.5); (b) the storage modulus of c1 dispersions at 25°C (■), 30°C (●), 40°C (▲), 50°C (▼) and 25°C after heating to 50°C and cooling (□) (1% and pH 4.5).

In order to further elucidate the collagen fiber denaturational transition with temperature, FT-IR studies were performed. The spectrum of the collagen dispersions demonstrated the characteristic pattern reflecting the amide I band at 1650 cm^{-1}, the amide II band at 1560 cm^{-1}, and the amide III band at 1244 cm^{-1}, resulting from N-H-stretching, C=O-

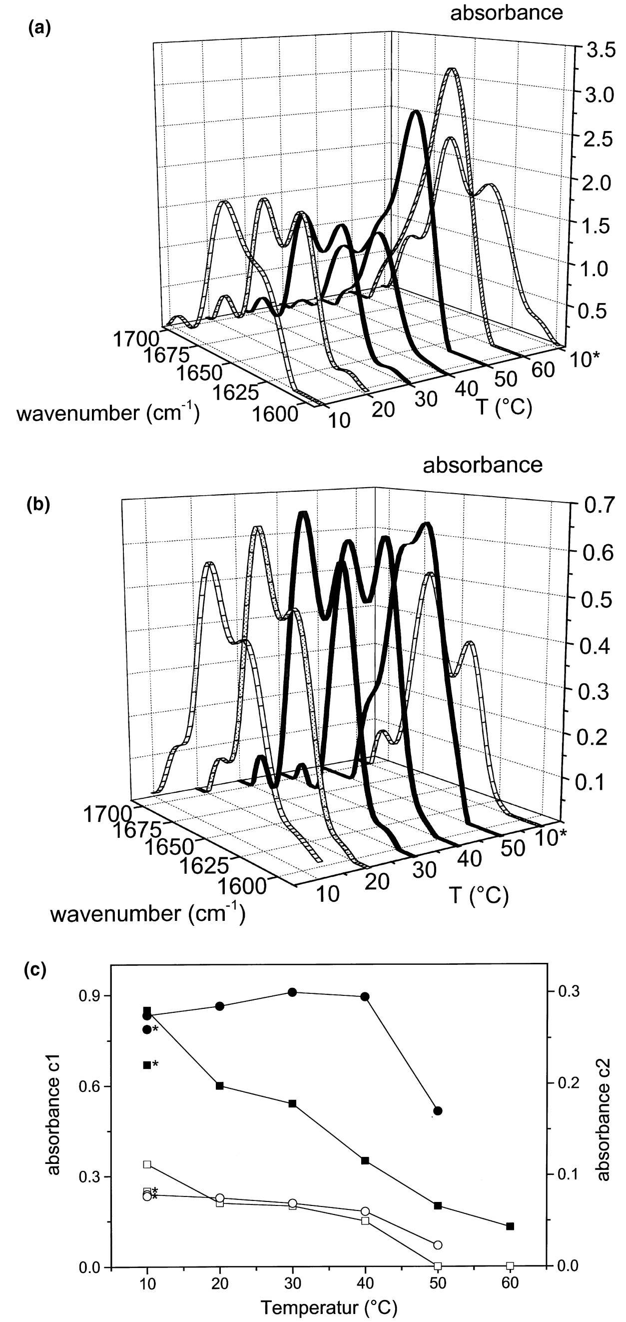

Fig. 4. (a) FT-IR spectra (deconvoluted) of 0.1% c1 dispersion at 10, 20, 30, 40, 50 and 60°C and at 10°C after heating to 60°C and cooling (10*); (b) FT-IR spectra (deconvoluted) of 0.1% c2 dispersion at 10, 20, 30, 40 and 50°C and at 10°C after heating to 50°C and cooling (10*); (c) absorbance of amide II (■) and amide III band (□) for 0.1% c1 dispersion and amide II (●) and amide III band (○) for 0.1% c2 dispersions at 1560 and 1244 cm^{-1} resp. as a function of temperature (*after heating and cooling).

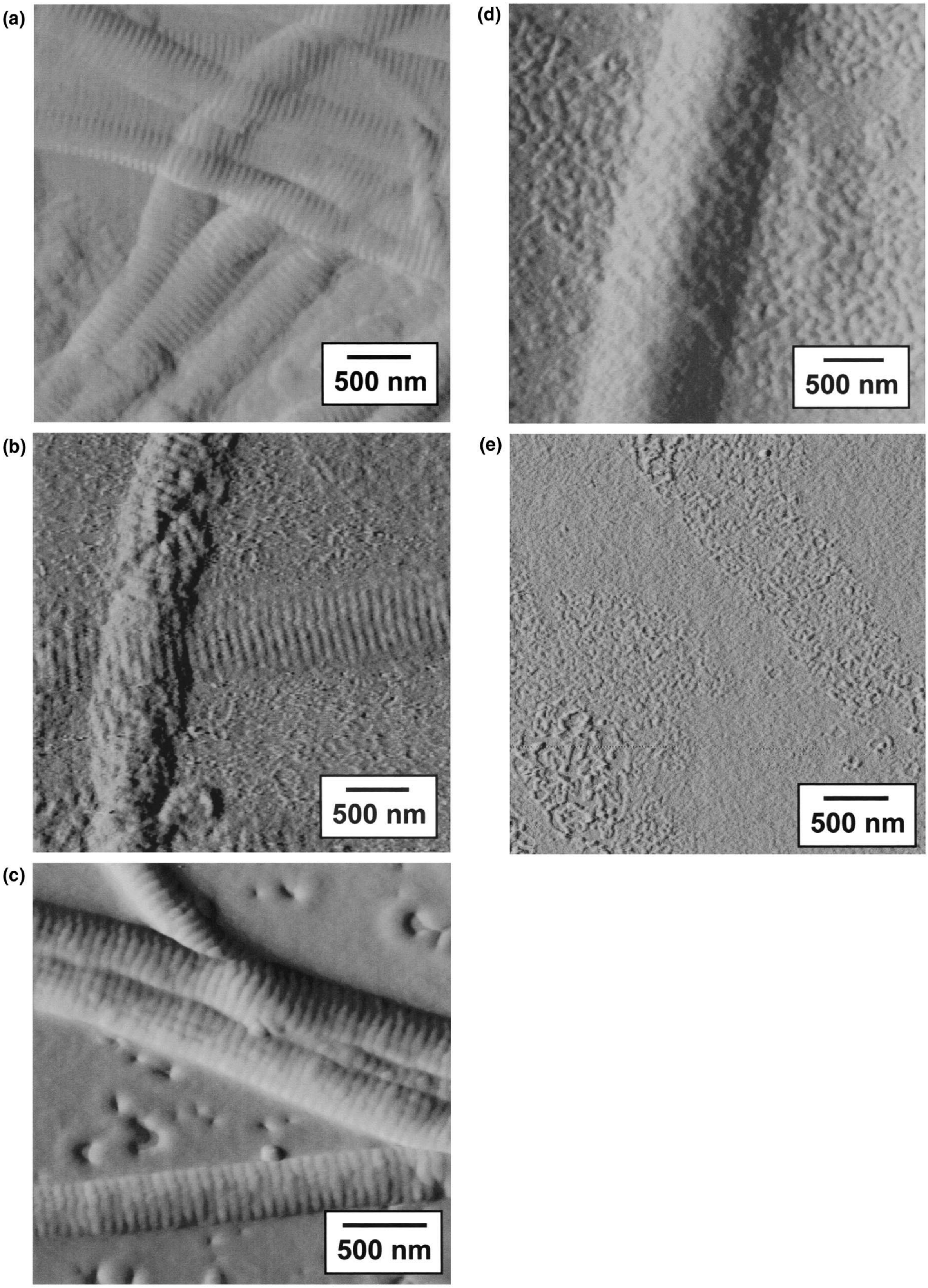

Fig. 5. AFM: (a) c1 at 25°C; (b) c2 at 25°C; (c) c1 at 25°C after heating to 40°C for 24 h and cooling; (d) c1 at 25°C after heating to 60°C for 1 h and cooling; (e) c2 at 25°C after heating to 60°C for 1 h and cooling.

stretching and N-H-bending vibrations [22]. Fig. 4a shows the amide I band region of a c1 dispersion pH 4.5 at temperatures between 10 and 60°C. Heating resulted in a shift between two peaks at 1660, and 1636 cm^{-1}, respectively. The move to lower wave numbers correlated with the transition from helical to random coil structure [23]. Between 10 and 20°C, raising the temperature has been shown to cause collagen fibers to rigidify through enhanced hydrophobic attractive forces [13] and may explain the difference in the peak ratios at 10 and 20°C. From 20 to 40°C there is only a slight shift and the most characteristic change occurs with the peak at 1660 cm^{-1}, disappearing almost completely upon heating to 50°C whereas rheometry had indicated a structural change already at 40°C. For c2 the same shift in the amide I band was detected with rising temperature (Fig. 4b). However, the transition is a more continuous process for c2 as compared to c1 fibers. Additional information can be obtained from the amide II and III bands whose intensity decreases with rising disorder from a helical to a random coil structure. Again for c1 a more pronounced transition between 40 and 50°C occurred whereas for c2 both peaks decreased almost continuously between 10 and 50°C (Fig. 4c). Upon cooling the temperature treated collagen dispersions to 10°C the amide I band of both c1 and c2 dispersion showed a similar ratio of the peaks at 1660 and 1636 cm^{-1}, as measured for the initial sample at 10°C (Fig. 4a,b). In addition, the amide II and III bands increased again to levels similar to the initial spectra measured at 10°C (Fig. 4c). Thus, the FT-IR investigations suggest reversible formation of helical arrangements. This renaturation process is related to the fiber characteristic of the material [21].

AFM images provided insight into the structural changes of the insoluble collagen dispersions with heating. The initial fibers of 0.05 to 2.5 μm diameter and 5 to 250 μm length show the characteristic periodic pattern of 67 nm staggering reflecting the lateral displacement spacing of the collagen molecules with c2 fibers being of more irregular shape and less pronounced periodicity than c1 fibers (Fig. 5a,b). Processing of the raw material not only induced chemical changes in c2 such as the conversion of the amides glutamine and asparagine to the corresponding acid form leading to the decrease in pI but also damaged three-dimensional arrangement of the triple helices. Heating to 30 and 40°C did not affect the microscopic appearance (Fig. 5c). At 60°C c1 fibers assumed an irregular shape without a hint of periodicity (Fig. 5d). The c2 fibers disintegrated resulting in small fragments (Fig. 5e) which still present helical structures as indicated by FTIR. AFM demonstrated that the structural change of the fibers by melting was not reversible, whereas FT-IR indicated helical arrangement of the breakdown products. Heating does not only result in a reversible conversion of the helical structures in the fibers but also breaking of crosslinks and the protein backbone. However, at temperatures below 40°C heating may be used to reduce the viscosity of collagen fiber dispersions for processing at least for short periods. The results indicated less resistance of c2 to thermal stress than c1.

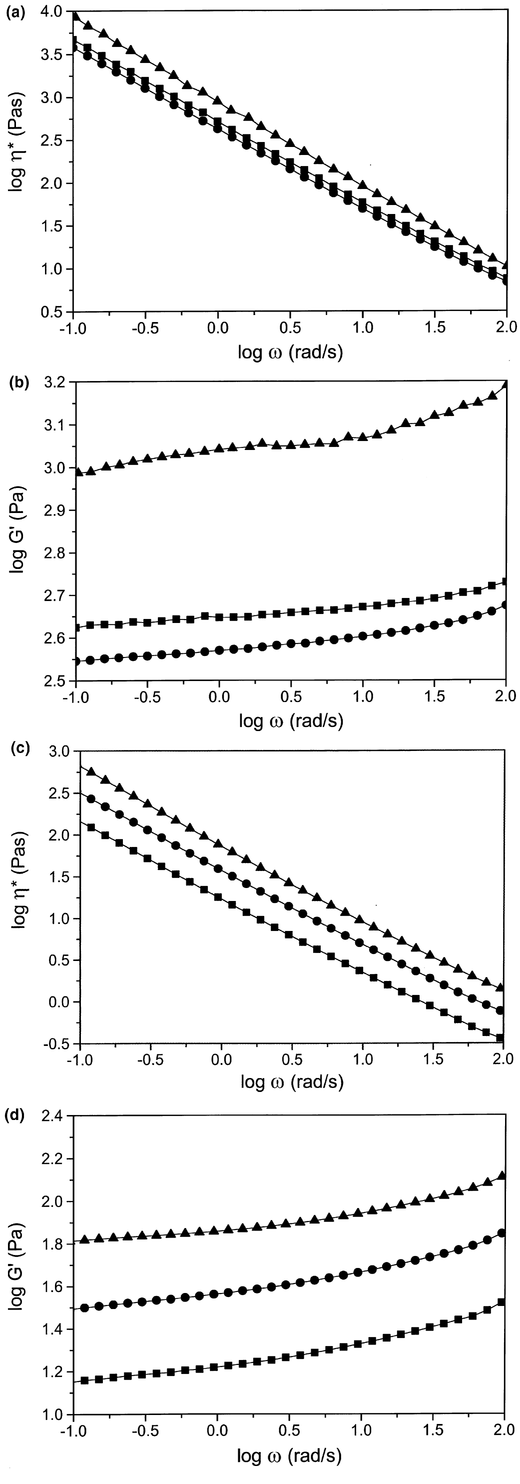

Fig. 6. Oscillatory rheometry: (a) the complex viscosity of c1 dispersions uncrosslinked (■), 0.04 GTA/NH_2 (●) and 0.25 GTA/NH_2 (▲) (1% and pH 4.5); (b) the storage modulus of c1 dispersions uncrosslinked (■), 0.04 GTA/NH_2 (●) and 0.25 GTA/NH_2 (▲) (1% and pH 4.5); (c) the complex viscosity of c2 dispersions uncrosslinked (■), 0.04 GTA/NH_2 (●) and 0.25 GTA/NH_2 (▲) (1% and pH 7.4); (d) the storage modulus of c2 dispersions uncrosslinked (■), 0.04 GTA/NH_2 (●) and 0.25 GTA/NH_2 (▲) (1% and pH 7.4).

Exogenous crosslinking of collagen is the preferred method to increase the in vivo persistence of collagenous biomaterials. Crosslinked collagen has been shown to be more resistant to deformation and flow than non-crosslinked material [12]. Crosslinking c1 dispersions at pH 4.5 with GTA did not result in a change in viscosity and network structure at a GTA/-NH_2 ratio of 0.04 (Fig. 6a,b). At a 0.25 ratio the viscosity increased and, in addition, the slope of G' increased with GTA treatment and the G'/G'' ratio increased from 5.4 for untreated systems to 10 for 0.25% GTA/-NH_2 indicating more rigid fiber structures. This effect is directly related to protein fiber fixation by crosslinking as well as reduced swelling and separation of water. The increase in viscosity is more pronounced for c2 dispersions (Fig. 6c,d) because crosslinking is performed at neutral pH for the material of acidic pI whereas c1 otherwise precipitates at pH 7.4. The reaction rate between collagen and GTA reaches its optimum between pH 7 and 8, and requires a free, non-protonated ε-amino group of lysine for the first addition to form an imine [2]. However, the slope of G' remains constant indicating similar network structure independent of GTA treatment and different mechanistic in the crosslinking processes. Thus, the effect of crosslinking during collagen processing very much depends on the conditions and, in addition, may be variable and difficult to control [24] strongly affecting rheological properties of collagen fiber dispersions.

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