Appl. Phys. A (2005)
DOI: 10.1007/s00339-005-3431-x

Applied Physics A
Materials Science & Processing

S. RAMMENSEE[1]
D. HUEMMERICH[2]
K.D. HERMANSON[1]
T. SCHEIBEL[2,✉]
A.R. BAUSCH[1,✉]

Rheological characterization of hydrogels formed by recombinantly produced spider silk

[1] Department of Physics, E22-Biophysics, Technische Universität München, 85747 Garching, Germany
[2] Department of Organic Chemistry & Biochemistry, Technische Universität München, 85747 Garching, Germany

Received: 18 June 2005/Accepted: 3 October 2005

ABSTRACT Many fibrous proteins such as spider silks exhibit impressive mechanical properties and are highly biocompatible leading to many potential biomaterial applications. For applications such as tissue engineering, polymer hydrogels have been proposed as an effective means of producing porous but stable scaffolds. Here, nanofiber-based hydrogels were produced from engineered and recombinantly produced spider silk proteins. The silk nanofibers are stable semi-flexible polymers which assemble into hydrogel networks. We studied the hydrogel rheology and determined the concentration dependence of the elastic modulus. AFM images indicate that the nanofibers might assemble into branch-like structures, which would also be consistent with the measured rheological behavior. Since the developed spider silk hydrogels are stable over weeks and show a high elastic modulus at low volume fractions, they are well suited for a broad variety of applications.

PACS 82.35.Pq; 83.80.Kn

1 Introduction

Silks are protein polymers that are naturally spun into fibers and threads by many animals such as silkworms and spiders. The proteins from which silk is made are usually produced by epithelial cells within specialized glands. After synthesis, the proteins are secreted into the lumen where they are stored prior to fiber spinning. The unique mechanical properties of the spun fibers make them especially interesting for many different applications [1]. However, there are additional approaches to build new materials based on these proteins such as the assembly of hydrogels.

Hydrogels are polymer networks formed from polymers that absorb water to a significant extent. Their porous structure and elastic properties make them useful for applications such as tissue engineering, drug delivery systems and functional coatings. Hydrogels can be formed by proteins, peptides, or other biopolymers such as alginates, or chitosan [2–9]. It has been shown previously that solutions prepared from dissolved silkworm silk also form hydrogels in vitro [10–13].

✉ Fax: +49-89-289-12469; E-mail: thomas.scheibel@fiberlab.de, abausch@ph.tum.de

The mechanical property of a hydrogel will depend on the material properties of its individual constituents. Many biopolymers, such as DNA, collagen, actin and neurofilaments, are semiflexible polymers, which demonstrate mechanical properties distinctly different from ordinary flexible polymers, such as polystyrene and polymethyl methacrylate. Networks of the semi flexible biopolymers are of technological interest because they demonstrate a high elastic moduli at low polymer volume fractions [14]. These unique mechanical properties are a result of the persistence length of the semiflexible polymers. Despite the natural abundance of such polymers there are almost no synthetic semiflexible polymers known.

Here, we show that spider silk hydrogels, like other networks of biological polymers, have viscoelastic properties which can be partly attributed to their semiflexible nature. However, structural evidence from AFM pictures also indicates that, unlike most other biopolymer networks, the silk nanofibers may form branch-like structures. As a result of the branching that occurs in the hydrogel, the storage moduli of both the chemically crosslinked and chemically noncrosslinked entangled networks show similar concentration dependencies.

2 Experimental

Dragline silk protein ADF-4 [15] from the garden spider Araneus diadematus has been used as a template for the synthetic silk construct C_{16} [16, 17]. The repetitive part of ADF-4 is generally composed of a single conserved repeat unit displaying only slight variations. These variations were combined to from one consensus module termed C (GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP), which was multimerized to obtain the repetitive protein C_{16}. The resulting C_{16} protein has a molecular mass of 48 kDa [16].

The C_{16} silk gene was expressed in the E. coli strain BLR [DE3] (Novagen). Cells were grown at 37 °C in LB medium to an $OD_{600} = 0.5$. Before induction with 1 mM IPTG (Isopropyl-ß-D-thiogalactoside), cells were shifted to 25 °C. Cells were harvested after 3–4 hours of induction.

C_{16} protein was purified and protein identity and purity was assessed as described by Huemmerich et al. [16]. Pellets of C_{16} were washed with 8 M urea and dissolved in 6 M

guanidinium thiocyanate (GdmSCN) before dialysis against 10 mM NH_4HCO_3. Precipitates formed during dialysis were removed by sedimentation at 50 000 × g for 30 min, and the remaining soluble silk proteins were lyophilized. Prior to analysis lyophilized protein was dissolved in 6 M GdmSCN followed by dialysis against 5 mM potassium phosphate pH 8.0. Aggregates were removed by sedimentation at 125 000 × g for 30 min. Protein concentrations were determined photometrically in a 1 cm path length cuvette at 276 nm using a previously determined extinction coefficient [16].

After the addition of 10% (v/v) methanol (final concentration), C_{16} self-assembled into nanofibers at concentrations between 5 and 30 mg/ml (Fig. 1). Depending on the protein concentration used, the nanofibers formed a hydrogel fiber network within a few days to one week. C_{16} hydrogels could easily be disrupted by agitation or shearing. Crosslinked hydrogels were made by applying ammonium peroxodisulfate (APS), and Tris(2,2′-bipyridyl)dichlororuthenium(II) (Rubpy) to the surface of the hydrogel. The amount of applied reactants was calculated to yield a final concentration of 10 mM APS and 100 μM Rubpy after allowing these compounds to enter the gel by diffusion over night. Subsequently, the hydrogels were exposed to visible light from a tungsten lamp for 1 min [19] and remaining liquid was removed from the gel surface.

For atomic force microscopy (AFM) analysis nanofibers of hydrogels were suspended in water to yield a final concentration of 80 μg/ml and incubated for 3 min on freshly cleaved mica. Samples were rinsed with water four times and air-dried prior to contact mode imaging using a Multimode SPM (Veeco, USA).

Dynamic rheological measurements of the crosslinked and non-crosslinked hydrogels were performed using a Physica MCR 301 with 25 mm parallel plate-plate geometry. The gap between the upper plate and the sample dish was set by first moving the upper plate approximately 2 mm above the surface of the sample. The upper plate was lowered very slowly (5 μm/s), while monitoring the normal force and was stopped at a limit normal force of 0.1 N. After the upper plate was stopped, the normal force was allowed to equilibrate to a constant value. In order to find a suitable stress to probe the material's linear viscoelastic properties at small strains but over a wide range of frequencies, the sample was sheared at 0.5 Hz and 1% strain in strain-controlled mode. The stress that was required in this test was then also used in the frequency dependent measurements, which were performed in stress-controlled mode. Finally, a constant deformation rate (5%/sec) was applied to the sample in order to study the behavior at large strains. Rheological measurements were conducted on samples with protein concentrations ranging from 5 to 30 mg/ml.

3 Results and Discussion

AFM images of dried hydrogels formed by the recombinant spider silk protein C_{16} indicate that they are formed by nanofibers with diameters of approximately 3 nm and lengths less than 1 μm (Fig. 1). Similar nanofibers have previously been observed to form from synthetic spider silk analogues [20], from diluted natural spider silk dope [21] or within the spin duct of a spider [22]. The nanofibers appear to be semiflexible with a persistence length on the same order of magnitude as their length. Many of the nanofibers also seem to assemble into branched structures. From the AFM images it could not be determined, if the branch-like structures are physical branches in each polymer fiber or are a result of nanofiber bundling. The macroscopic hydrogels are viscous and transparent (Fig. 2).

Similar to most concentrated polymer networks, the hydrogel made from recombinant C_{16} spider silk protein demonstrates viscoelastic behavior. When a linearly increasing strain is applied to the viscoelastic C_{16} silk networks, the stress changes proportionally to the applied strain. Figure 3 shows the stress/strain behavior of the crosslinked and non-

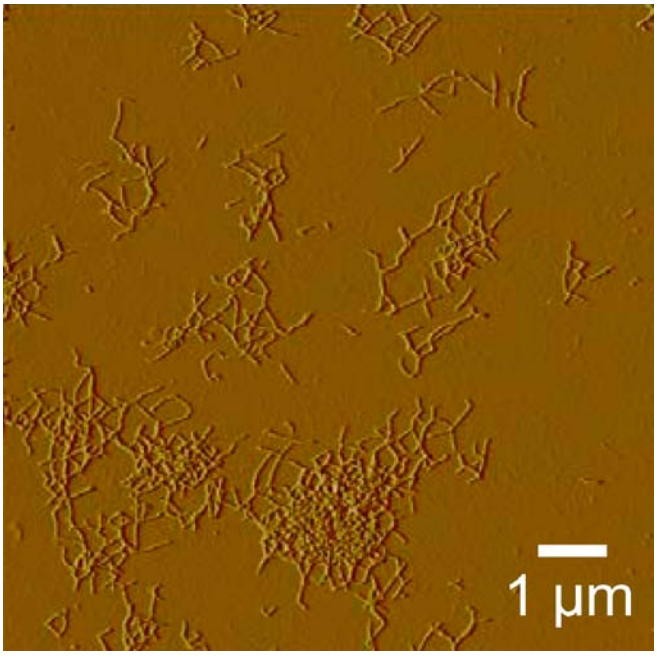

FIGURE 1 AFM deflection image of dried non-crosslinked C_{16} nanofibers. Some of the fibers appear to be interconnected or branched, although the fibers were not intentionally crosslinked

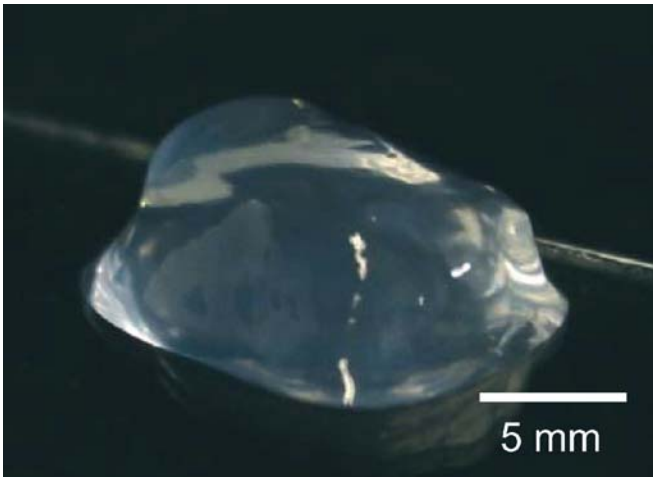

FIGURE 2 A hydrogel prepared from C_{16} protein. No flow of the hydrogel is observed

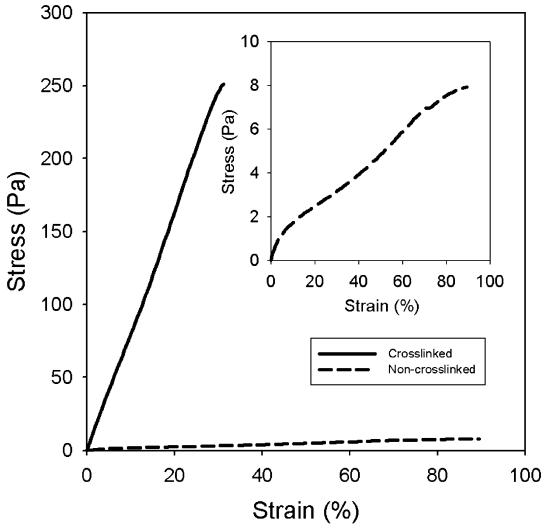

FIGURE 3 The stress/strain behavior of the crosslinked and non-crosslinked hydrogels at a concentration of 10 mg/ml. The crosslinked hydrogel demonstrates a higher stress at a given deformation than the non-crosslinked hydrogel. The crosslinked hydrogel flows after a 30% deformation is applied. *Inset*: stress/strain for the non-crosslinked network in larger scaling

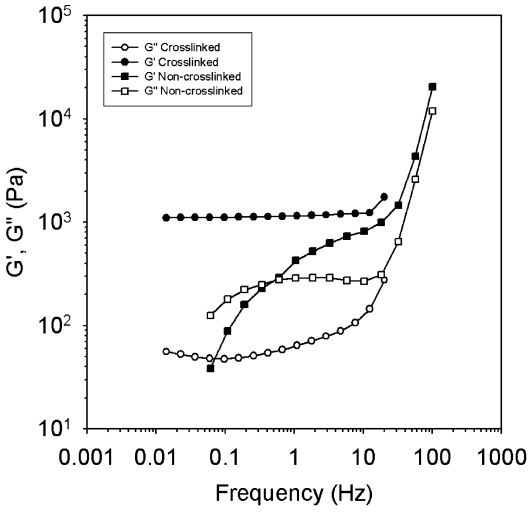

FIGURE 4 Frequency dependence of the storage modulus (G' – *filled symbols*) and loss modulus (G'' – *open symbols*) for both the crosslinked (*circles*) and non-crosslinked (*squares*) fiber networks at a concentration of 20 mg/ml. The storage modulus of the crosslinked sample is larger than the loss modulus and constant between a frequency of 0.1 and 10 Hz. The non-crosslinked sample shows a greater dependency on frequency, with a crossover frequency of 0.5 Hz and a plateau between 0.5 and 10 Hz

crosslinked hydrogels at a concentration of 10 mg/ml. The non-crosslinked C_{16} silk hydrogel has an initial shear modulus of 38 Pa. As strain is increased, the non-crosslinked hydrogel shows a lower stress response, and after a strain of 10% the response is relatively linear (Fig. 3 Inset). Once a strain greater than 90% is applied, the non-crosslinked hydrogel ruptures and flows. Unlike the non-crosslinked fiber network, the crosslinked network shows a linear stress response over all strains until it ruptures, has a much higher shear modulus of 820 Pa, and ruptures at a lower strain of 30%.

Dynamic viscoelastic measurements of the non-crosslinked fiber network, at a polymer concentration of 20 mg/ml, reveal that the storage modulus (G') and the loss modulus (G'') are dependent on the oscillation frequency (ω) (Fig. 4). The network demonstrates viscous behavior at low frequencies and elastic behavior at moderate frequencies, with a crossover at 0.49 Hz. The observed behavior of the hydrogel is similar to that expected for an entangled polymer network.

The non-crosslinked C_{16} silk hydrogel also displays much different dynamic viscoelastic behavior than that of the chemically crosslinked hydrogel (Fig. 4). Unlike the behavior of the non-crosslinked silk hydrogel, the crosslinked silk hydrogel demonstrates elastic behavior at all frequencies tested, and no crossover frequency is observed. The storage modulus of the crosslinked silk hydrogel is nearly constant at all frequencies, except at the highest frequencies tested. The crosslinked C_{16} silk hydrogel also demonstrates a higher storage and lower loss modulus than the non-crosslinked silk hydrogel.

As would be expected, the storage modulus of the crosslinked hydrogel is higher than that of the non-crosslinked network for all concentrations tested (Fig. 5). However, unexpectedly the storage moduli of both crosslinked and non-crosslinked networks increase with concentration c and have a $G' \sim c^2$ dependence. In the case of crosslinked linear semiflexible biopolymer networks, where the persistence length is

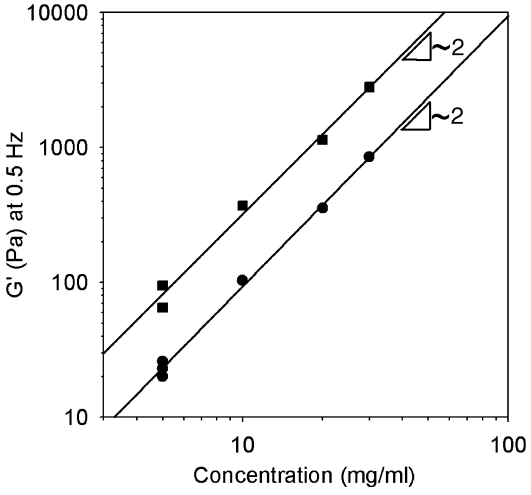

FIGURE 5 Concentration dependence of the storage modulus at a frequency of 0.5 Hz for both the crosslinked (■) and the non-crosslinked (●) hydrogels. Both networks have storage moduli that are proportional to the concentration squared $G' \sim c^2$. The same concentration dependency is found for frequencies between 0.5 and 2 Hz (not shown)

larger than the mesh size, the storage moduli of the polymer networks are expected to have a dependence of $c^{11/5}$, which is close to that which is observed for the crosslinked C_{16} silk hydrogel [14]. In the case of linear semiflexible biopolymer networks that are entangled but not crosslinked, the storage moduli are expected to have a lower concentration dependence of $c^{7/5}$ [23]. Such a dependency has been shown to be valid for other biopolymers such as F-actin [23, 24], but does not describe the dependency of the non-crosslinked silk C_{16} hydrogel. This discrepancy could be explained, if the branch-like structures observed in the AFM images are real physical branches in the polymer network. These physical interactions between the nanofibers could extend over many different nanofibers and thus result in microclusters. The mi-

croclusters would be interconnected and percolate the whole hydrogel, giving rise to the observed elastic behavior. The storage modulus of such a branched semiflexible polymer network or microcluster gel would be dominated by the thermal fluctuations of the single nanofibers. It is, therefore, expected that a branched semiflexible polymer network would demonstrate a similar concentration dependency to that which has been observed for linear semiflexible polymer networks such as actin.

For gelatin networks crosslinked by transglutaminase, Young's moduli between 15 and 120 kPa have been observed, depending on the purity and concentration (18%–26%) of gelatin used. The crosslinked silk hydrogel studied here has a Young's modulus of 8.4 kPa at 3% w/w assuming a Poisson's ratio of 0.5 [25]. It has also been found that hydrogels, formed by β-hairpin peptides assembled into physically crosslinked fibers, show a pure elastic behavior with a storage modulus of about 4 kPa at 20 mg/ml [8]. This value compares very well with the modulus obtained here of 1 kPa for the crosslinked silk hydrogels at 20 mg/ml. It has also been reported that the moduli of the β-hairpin fibers scaled in accordance to the accepted theoretical model for crosslinked semiflexible polymer networks.

In conclusion, the storage modulus scaling behavior of the non-crosslinked hydrogels studied here can not be explained within the framework of the most widely accepted models for linear semiflexible polymer networks. The branched nanofiber assemblies observed by AFM could explain the deviations from the current semiflexible polymer network models. In order to model such network structures further studies and theoretical concepts must be developed. Most importantly, since the spider silk hydrogels are stable over weeks and have a high elastic modulus already at low volume fractions, they are well suited for many different applications.

ACKNOWLEDGEMENTS The work was supported by SFB563 B14 (AB) and A9 (TS). KDH gratefully acknowledges supported by the Alexander von Humboldt Foundation. Support by Fonds der chemischen Industrie is also gratefully acknowledged.

REFERENCES

1 J.M. Gosline, P.A. Guerette, C.S. Ortlepp, K.N. Savage, J. Exp. Biol. **202**, 3295 (1999)
2 D.P. Knight, L. Nash, X.W. Hu, J. Haffegee, M.W. Ho, J. Biomed. Mater. Res. **41**, 185 (1998)
3 K.Y. Lee, D.J. Mooney, Chem. Rev. **101**, 1869 (2001)
4 B. Jeong, S.W. Kim, Y.H. Bae, Adv. Drug Delivery Rev. **54**, 37 (2002)
5 A.S. Hoffman, Adv. Drug Delivery Rev. **54**, 3 (2002)
6 H. Shin, S. Jo, A.G. Mikos, Biomaterials **24**, 4353 (2003)
7 J.L. Drury, D.J. Mooney, Biomaterials **24**, 4337 (2003)
8 B. Ozbas, K. Rajagopal, J.P. Schneider, D.J. Pochan, Phys. Rev. Lett. **93**, 268 106 (2004)
9 H. Yokoi, T. Kinoshita, S.G. Zhang, Proc. Natl. Acad. Sci. U.S.A. **102**, 8414 (2005)
10 Z.H. Ayub, M. Arai, K. Hirabayashi, Biosci. Biotech. Biochem. **57**, 1910 (1993)
11 T. Hanawa, A. Watanabe, T. Tsuchiya, R. Ikoma, M. Hidaka, M. Sugihara, Chem. & Pharmaceutical Bull. **43**, 284 (1995)
12 G.D. Kang, J.H. Nahm, J.S. Park, J.Y. Moon, C.S. Cho, J.H. Yeo, Macromol. Rapid Commun. **21**, 788 (2000)
13 U.J. Kim, J.Y. Park, C.M. Li, H.J. Jin, R. Valluzzi, D.L. Kaplan, Biomacromolecules **5**, 786 (2004)
14 F.C. Mackintosh, J. Kas, P.A. Janmey, Phys. Rev. Lett. **75**, 4425 (1995)
15 P.A. Guerette, D.G. Ginzinger, B.H.F. Weber, J.M. Gosline, Science **272**, 112 (1996)
16 D. Huemmerich, C.W. Helsen, S. Quedzuweit, J. Oschmann, R. Rudolph, T. Scheibel, Biochemistry **43**, 13 604 (2004)
17 T. Scheibel, Microbial Cell Factories **3**, 14 (2004)
18 D. Huemmerich, T. Scheibel, F. Vollrath, S. Cohen, U. Gat, S. Ittah, Current Biol. **14**, 2070 (2004)
19 D.A. Fancy, T. Kodadek, Proc. Natl. Acad. Sci. U.S.A. **96**, 6020 (1999)
20 E. Oroudjev, J. Soares, S. Arcdiacono, J.B. Thompson, S.A. Fossey, H.G. Hansma, Proc. Natl. Acad. Sci. U.S.A. **99** Suppl. 2, 6460 (2002)
21 X. Chen, D.P. Knight, F. Vollrath, Biomacromolecules **3**, 644 (2002)
22 J.M. Kenney, D. Knight, M.J. Wise, F. Vollrath, Eur. J. Biochem. **269**, 4159 (2002)
23 B. Hinner, M. Tempel, E. Sackmann, K. Kroy, E. Frey, Phys. Rev. Lett. **81**, 2614 (1998)
24 M.L. Gardel, M.T. Valentine, J.C. Crocker, A.R. Bausch, D.A. Weitz, Phys. Rev. Lett. **91**, 158 302 (2003)
25 M.K. McDermott, T.H. Chen, C.M. Williams, K.M. Markley, G.F. Payne, Biomacromolecules **5**, 1270 (2004)