Article

Non-Linear Behaviour Of Gelatin Networks Reveals A Hierarchical Structure

Zhi Yang, Yacine Hemar, loic hilliou, Elliot P. Gilbert, Duncan
James McGillivray, Martin A. K. Williams, and Sahraoui Chaieb

Biomacromolecules, **Just Accepted Manuscript** • DOI: 10.1021/acs.biomac.5b01538 • Publication Date (Web): 14 Dec 2015

Downloaded from <http://pubs.acs.org> on December 20, 2015

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

ACS Publications

Biomacromolecules is published by the American Chemical Society, 1155 Sixteenth Street N.W., Washington, DC 20036
Published by American Chemical Society. Copyright © American Chemical Society.
However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Non-Linear Behaviour Of Gelatin Networks Reveals A Hierarchical Structure

Zhi Yang^a, Yacine Hemar^{a,f}, Loic Hilliou^c, Elliot P. Gilbert^d, Duncan J. McGillivray^{a,g}, Martin
A.K. Williams^{f,g}, and Sahraoui Chaieb^{b*}

^a School of Chemical Sciences, The University of Auckland, Private bag 92019, Auckland 1142,
New Zealand

^b Division of Physical Sciences and Engineering, King Abdullah University of Sciences and
Technology (KAUST), Thuwal 23955-6900, Kingdom of Saudi Arabia

^c Institute for Polymers and Composites/I3N, University of Minho, Campus de Azurém, 4800-
058 Guimarães, Portugal.

^d Bragg Institute, Australian Nuclear Science and Technology Organisation, Locked Bag 2001,
Kirrawee DC, NSW 2232, Australia.

^e Institute of Fundamental Sciences, Massey University, Palmerston North 4442, New Zealand.

^f The Riddet Institute, Palmerston North 4442, New Zealand.

^g The MacDiarmid Institute, Palmerston North 4442, New Zealand

1
2
3 19 KEYWORDS: Gelatin, strain hardening, large deformation rheology, chemical cross-linking
4
5
6
7 20
8
9

10 21 ABSTRACT: We investigate the strain hardening behaviour of various gelatin networks -
11
12 22 namely physical gelatin gel, chemically-crosslinked gelatin gel, and a hybrid gel made of a
13
14 23 combination of the former two - under large shear deformations using the *pre-stress, strain*
15
16 24 *ramp, and large amplitude oscillation shear protocols*. Further, the internal structures of physical
17
18 25 gelatin gel and chemically-crosslinked gelatin gels were characterized by small angle neutron
19
20 26 scattering (SANS) to enable their internal structures to be correlated with their nonlinear
21
22 27 rheology. The Kratky plots of SANS data demonstrate the presence of small cross-linked
23
24 28 aggregates within the chemically-crosslinked network whereas, in the physical gelatin gels, a
25
26 29 relatively homogeneous structure is observed. Through model fitting to the scattering data, we
27
28 30 were able to obtain structural parameters, such as correlation length (ξ), cross-sectional polymer
29
30 31 chain radius (R_c) and the fractal dimension (d_f) of the gel networks. The fractal
31
32 32 dimension d_f obtained from the SANS data of the physical and chemically crosslinked gels is
33
34 33 1.31 and 1.53, respectively. These values are in excellent agreement with the ones obtained from
35
36 34 a generalized non-linear elastic theory that has been used to fit the stress-strain curves. The
37
38 35 chemical crosslinking that generates coils and aggregates hinders the free stretching of the triple
39
40 36 helix bundles in the physical gels.
41
42
43
44
45
46
47
48
49
50 37
51
52
53
54 38
55
56
57 39
58
59
60

Introduction:

Gelatin, which forms thermo-reversible gels, is a protein that is obtained by breaking the triple-helix structure of collagen into single-strand molecules¹. It is widely used as a gelling ingredient in food, cosmetic, and pharmaceutical products to provide elasticity, viscosity, and structural stability. In recent years, gelatin has been used in many emerging applications especially in the biomedical area for, e.g., encapsulation, tissue scaffolds, microspheres, and matrices for implants².

Physical gels of gelatin are formed when a temperature decrease transforms random coils into partially-renatured ordered triple helices³. For mammalian gelatin, if the temperature is increased again, to around that at body temperature, the triple helix conformation returns to a coil state once more, and thus the gel reversibly melts into a solution. Physical gelatin networks are mainly held together by hydrogen bonded junction zones⁴. Due to the thermal reversibility, physical gelatin gels are not stable at physiological temperature and above, which limits their applications in tissue engineering or drug delivery where gels are required to be stable for a certain period of time before dissolving. To overcome this drawback, and to stabilize the gelatin gels, chemical or enzymatic crosslinking is desirable. A variety of cross-linking agents have been utilized such as transglutaminase⁵ and glutaraldehyde⁶, as well as tannic acid⁷, caffeic acid⁸, bisvinyl sulfonemethyl⁹, genipin¹⁰, and carbodiimides¹¹. Several groups have also successfully prepared gelatin gels with a combination of physical and chemical crosslinking agents^{9, 12}. In this study, we choose to investigate glutaraldehyde as a cross linker because it is relatively inexpensive, easily available, and is an efficient gelatin cross-linker. Glutaraldehyde has been widely used during the past 50 years to immobilise and stabilise proteins through covalent intermolecular cross-links. It reacts mainly with the protein's amino groups, in lysine side-chains

and at the N-terminus, although minor involvement of other residues (arginine, histidine, tyrosine and cysteine) has been reported¹³.

The linear and non-linear elasticity are among the most important properties for both applications and a fundamental understanding of these gel networks under deformation. One specific non-linear behaviour of gelatin gels is their tendency to strain harden at large deformations, meaning that when they are deformed the observed stress increases faster than the strain¹⁴. Understanding the stress responses of these differently cross-linked gelatin networks under large deformations is extremely important for their food, biomedical, and tissue engineering applications. For example, the texture and aroma release during the chewing and masticating of gelatin-based foods are related to their mechanical properties under large deformation. Furthermore, gelatin gels used in artificial tendons and ligaments would be subjected to large stretch during real life body locomotion.

Unfortunately, most studies to date have focused on the linear rheology or small deformation rheology of various cross-linked and physical gelatin gels both experimentally and theoretically^{6, 15}. Of the few studies undertaken of large deformation or non-linear rheology of gelatin networks, most of them have focused only on non-cross-linked physical gelatin networks^{14, 16}.

The objective of this study is to correlate the large deformation rheological properties of various gelatin gel networks, namely gelatin physical gel, chemical gel, and mixed-crosslink gels to their internal network structures. The different gelatin gel networks can be achieved by setting the temperature below or above the gelation temperature and in the presence or absence of chemical cross-linkers.

1
2
3 85 **Materials and Methods:**
4
5
6 86
7
8 87 **Materials**
9
10 88 Porcine gelatin powder (bloom value 300, Sigma Aldrich USA) and glutaraldehyde solution
11
12 89 (Sigma Aldrich USA) were used without further purification.
13
14
15 90
16
17 91 **Methods**
18
19
20 92 *Crosslinking of various gelatin networks*
21
22 93 The protocols used to prepare the different gelatin networks are:
23
24 94 Physical gels
25
26
27 95 Gelatin powder was dissolved in Milli-Q water under mild stirring at 50°C for 1h until fully
28
29 96 solubilized to form a 3.0wt% homogenous solution. The sample, loaded onto the rheometer plate
30
31 97 preheated at 50°C, was annealed for 5 min and then the temperature decreased from 50°C to
32
33 98 20°C over 6 minutes (5°C/min) to initiate network formation. The physical gelatin network was
34
35 99 allowed to form at 20°C for 5h.
36
37
38
39 100
40
41 101 Chemically-crosslinked gels
42
43 102 Glutaraldehyde was added to 3.0wt% gelatin solution to achieve 0.2wt% glutaraldehyde in
44
45 103 gelatin solution at 35°C, vortex mixed, and loaded onto the rheometer plate that was preheated to
46
47 104 35°C. This gelatin chemical gel cross-linked by glutaraldehyde was allowed to form at 35°C for
48
49 105 5h.
50
51
52
53 106
54
55 107 Chemically and physically crosslinked gels
56
57
58
59
60

1
2
3 108 First, chemical networks were made following the above protocol. After that, the temperature of
4
5 109 the rheometer plate was cooled down from 35°C to 20°C (5°C/min) to allow for physical
6
7
8 110 networks (triple helix) to form at 20°C for an additional 5h.
9
10
11 111

12 112 *Rheology*

13 113 All rheological measurements were performed at least twice on duplicate samples.
14
15
16
17
18 114 Dynamic rheological measurement

19
20 115 Rheological measurements were carried out in an MCR 302 (Anton Paar GmbH, Graz, Austria)
21
22 116 stress-controlled rheometer fitted with a stainless steel plate and plate geometry (diameter: 50
23
24 117 mm) setting to a gap of 0.50 mm. Sunflower oil was placed around the exterior to minimize
25
26
27 118 water evaporation during measurements. During gelation, time sweep measurements were
28
29 119 performed at a constant frequency of 1Hz with a constant applied strain of 1% to monitor the
30
31
32 120 gelation kinetics until the storage modulus reached a plateau. At the end of the time sweep, the
33
34 121 frequency-sweep measurement was carried out at a constant strain of 1% for frequencies ranging
35
36 122 from 10^{-2} Hz to 10 Hz. Finally, the strain-sweep measurements were performed at a constant
37
38
39 123 frequency of 1 Hz for strains ranging from 10^{-1} % to 10^4 %. In these dynamic measurements the
40
41 124 elastic modulus G' , and the viscous modulus G'' were obtained.
42
43
44 125

45 126 Large deformation rheology measurements: Pre-stress protocol

46
47 127 To better quantify the non-linear behavior of the various gelatin gels, a differential measurement
48
49 128 was utilized. A low amplitude oscillatory stress $\delta\sigma$ was superposed to a constant applied pre-
50
51
52 129 stress σ_0 , and the differential elastic modulus, $K'(\sigma_0) = [\delta\sigma/\delta\gamma]_{\sigma_0}$ was determined as a function
53
54
55 130 of σ_0 at 1 Hz. The first applied constant stress was 1 Pa in amplitude. Subsequent pre-stresses
56
57
58
59
60

1
2
3 131 were, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000,
4
5 132 2200, and 2400 Pa until the network broke down. At each interval of applied constant stress,
6
7 133 small deformation oscillations (1 Pa) were conducted at frequencies ranging from 10^{-1} Hz to 100
8
9 134 Hz for 5 minutes. Finally, the differential elastic modulus at 1 Hz versus the applied constant
10
11 135 stress were obtained.
12
13
14

15 136

17 137 Large deformation rheology measurements: *Strain ramp* (constant shear) protocol

19
20 138 Another protocol that can be used to study the nonlinear rheology of biopolymer networks is to
21
22 139 use constant shear or a strain ramp, in which the strain is the control variable, which is increased
23
24 140 linearly in time, while the stress is measured. The advantage of this method is that the
25
26 141 nonlinearity, such as strain-hardening is observed directly, otherwise the stress-strain curve in
27
28 142 simple shear is a straight line for materials that do not show any strain-hardening¹⁴. This method
29
30 143 has been used to study the nonlinear rheology of gelatin¹⁴, and cross-linked actin networks¹⁷.
31
32
33

34 144

36 145 The stress-strain curve is obtained by applying a constant shear with shear rates ranging between
37
38 146 0.01 and 0.1 s^{-1} to various gelatin gels. Further, the pre-stress protocol above was modified
39
40 147 slightly to obtain the stress-strain curve in order to compare it with the one obtained from a
41
42 148 constant shear protocol. To achieve this, the small oscillation superposed stress described above
43
44 149 was removed; and constant stresses (2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, 1000,
45
46 150 1200, 1400, 1600, 1800, 2000, 2200, and 2400 Pa) were applied for 5 min and the resulting
47
48 151 strains were measured..
49
50

52 152

55 153 *Small angle neutron scattering (SANS)*

SANS experiments were performed on the 40 m Quokka instrument at the OPAL reactor at ANSTO, Sydney, Australia¹⁸. Three instrument configurations were used to yield a q range from 0.001 to 0.35 Å⁻¹ where q is the magnitude of the scattering vector defined as $q = \frac{4\pi}{\lambda} \sin \theta$ and 2θ is the scattering angle. These configurations were: (i) source-to-sample distance (SSD) = 20.1 m, sample-to-detector distance (SDD) = 20.2 m, using a wavelength of 8.1 Å with MgF₂ focussing optics and source aperture diameter 10 mm; and (ii) SSD = 11.9 m, SDD = 12.2 m and (iii) SSD = 7.9 m, SDD = 1.5 m and using a wavelength of 5.0 Å with source aperture diameter of 50 mm. A 10% wavelength resolution was used throughout and with sample aperture diameter of 10 mm.

To form the physical gels, the pure 3% w/w gelatin solution in D₂O was transferred into a quartz rheo-SANS cell at around 50 °C and cooled below the gelation temperature to 20 °C using a Julabo water bath to control the sample temperature. To ensure a relative steady state condition, the samples were left to gel at 20 °C for 5h before the SANS measurements. Chemically cross-linked gelatin gels were made by adding adequate amount of glutaraldehyde to obtain a final concentrations of 0.2wt % in 3wt% gelatin solutions in D₂O. The solution was prepared at 35°C, vortex mixed and placed into the rheometer cell preheated at 35°C. The sample was allowed to be cross-linked for 5h to form stable networks. SANS data analysis and model fitting was conducted using SasView software (www.sasview.org).

Results and Discussions:

Dynamic rheological properties of various gelatin gels

Three kinds of gelatin gels were formed in the rheometer and their gelation kinetics were monitored using time sweep measurements at a constant frequency of 1 Hz. The elastic moduli G' as a function of time are reported in Fig.1. After 5h for both physical and chemically cross linked gels and 10h for the chemical-physical hybrid gels, the gels have not reached equilibrium and demonstrate a continuously increasing elastic moduli over time. It has been suggested before that the network continues to develop following the elongation and rearrangement of the polymer chain, without a real equilibrium for physical and hybrid gels^{4, 19}. However, at longer time, i.e. 5h, the gelation kinetic is slower. Consequently, rheology experiments were performed after 5h for physical and chemically crosslinked gelatin gel and 10h for the hybrid gel. The effect of the gelation time on the strain hardening of gelatin gel is out of scope of in the current study.

For the physical gelatin gels, the gelation process occurs when the gelatin samples are cooled below their gelation temperature ($\sim 29^\circ\text{C}$)²⁰, in this study, to 20°C . To form purely chemically-crosslinked networks, glutaraldehyde was added to the gelatin at 35°C . Glutaraldehyde crosslinks gelatin through a nucleophilic addition-type reaction between its aldehyde group and the ε -amino groups of lysine in the gelatin molecule²¹. Mohtar et al.²² studied hoki skin gelatin cross-linked by glutaraldehyde and made similar observations on the increase of G' values that were proposed to be due to an increase in gel rigidity during the gelation time. Gels containing chemical and physical crosslinks (hybrids) were prepared in two steps. First, gelatin was cross-linked by glutaraldehyde at 35°C for 5h, where the formation of triple helices is prevented. G' was measured for gelatin physical gel at 35°C for 5h; no change of G' was observed suggesting the absence of helix formation and physical gel formation.

In the second step, the temperature was decreased below the gelation temperature at 20 °C to form triple helix physical networks. Therefore, this chemically cross-linked and physical gelatin networks are consecutive networks, i.e. the physical network is grown on top of a chemical one. The contribution to the linear elastic modulus of the chemical-physical gel (G'_{cp}) is both from chemical network (G'_c) and physical network formation (G'_p). G'_c is the elastic modulus reached after 5h of covalent cross-linking at 35 °C, and G'_p is the contribution arising entirely from triple helices formation after decreasing the temperature from 35 °C to 20 °C. The elastic contribution of these two networks is suggested to being purely additive ^{12b}. To test this hypothesis, a simple melting experiment for the chemical-physical gelatin gel was carried out (insert to Fig.1). The G' of chemical-physical gelatin gel after melting at 35 °C for 20 min is close to that of the pure chemical network formed in the first 5h. Consequently G'_p may be approximately obtained by subtracting the contribution of the chemical network G'_c from the total elastic modulus G'_{cp} . Specifically, the contribution from chemical networks was G'_c (110 Pa) while that from physical network was G'_p (60 Pa) resulting in a total gel elasticity G'_{cp} of 170 Pa. The linear rheological properties of these hybrid gels were dominated by chemical crosslinks.

As shown in Fig.1, the plateau value of G' for the hybrid gel is lower than that of the purely physical gelatin gel, suggesting the preformed chemically cross-linking has a detrimental influence on the formation of the helical network in the consequent physical gel formation. Similar observations were made previously on gelatin cross-linked by transglutaminase ¹². In the hybrid networks, the gelatin chains are preconnected by covalent bonds at several points along

their length; and this loss of flexibility limits the conformational changes necessary for the formation of the triple-helix junctions, possibly decreasing the length of the helices.

Figure.1. Elastic modulus G' as a function of gelation time. Symbols are: physical gelatin gel (■); hybrid gelatin gel (◀); chemical gelatin gel (●). The black line, blue dashed line and magenta line indicates the temperature profile of gelation of physical gelatin gel, chemical gelatin gel, and chemical-physical gelatin gel. For physical gelatin gel and hybrid gelatin gel, the measurement was conducted at 20°C. For chemical gelatin gel, the measurement was conducted at 35°C. Inset: chemical-physical gelatin gel measured at 35 °C after 600 minutes measurements to demonstrate the additive effect of physical and chemical crosslinkings.

The dynamic, viscoelastic behavior of the various gelatin gel networks was investigated by means of frequency sweeps. These measurements were obtained by applying a constant strain of 1%, well within the linear viscoelastic region. G' and G'' values as a function of frequency for different gelatin gels are shown in Fig.2. In all cases, the G' values were greater than the G'' values by a factor of approximately 100. The elastic modulus G' is nearly independent of the frequency in the range between 0.01 and 10 Hz. These findings suggest that all the gelatin samples formed strong gel networks demonstrating solid-like behavior²³.

Figure.2. Elastic modulus G' (solid symbols) and loss modulus G'' (open symbols) as a function of frequency. Symbols are: physical gelatin gel (■, □); hybrid gelatin gel (◀, ▶); chemical gelatin gel (●, ○). For physical gelatin gel and hybrid gelatin gel, the measurement was conducted at 20°C. For chemical gelatin gel, the measurement was conducted at 35°C.

246

247 The results of the strain sweep measurement for various gelatin gels are shown in Fig.3.

248 Qualitatively, all the gelatin gels exhibited similar trends of G' and G'' as a function of applied

249 strain. The strain sweep curve can be divided into three regions. First, up to a critical strain, both

250 G' and G'' remained independent of the applied strain. In this strain region, only reversible

251 deformations occur also known as the linear viscoelastic region (LVR). In the second region,

252 when the applied strain is further increased, both G' and G'' demonstrated an overshoot with

253 strain. It is noteworthy that the overshoots in G' and G'' do not occur at the same strain. Before

254 overshooting, G'' goes through a minimum, which is not observed for G' . This overshoot of G'

255 and G'' with strain depicts a typical strain-hardening behaviour for gelatin²⁴. In the third region,

256 at a high applied strain, both G' and G'' begin to decrease, suggesting that the gelatin gel

257 networks are starting to break. In this region, materials show more liquid behaviour than solid

258 behaviour which might relate to the flow of the fractured samples. To investigate if there is slip

259 upon shear and break up, strain sweep measurements were performed for gelatin physical gel

260 using plate-plate geometry with different gaps. As shown in Fig. S.1., the break-up (crossover of

261 G' and G'') shows up at similar strain, therefore the slip effect can be ruled out²⁵.

Figure 3. Elastic modulus G' (solid symbols) and loss modulus G'' (open symbols) as a function of strain. Symbols are: physical gelatin gel (■, □); hybrid gelatin gel (◀, ▶); chemical gelatin gel (●, ○). For physical gelatin gel and hybrid gelatin gel, the measurement was conducted at 20°C. For chemical gelatin gel, the measurement was conducted at 35°C.

Large deformation rheology: Pre-stress protocol

From the strain sweep measurements, it can be seen that all the gelatin gels demonstrate strain-hardening behaviour. To better characterize the nonlinear rheological properties of gelatin gels, a pre-stress protocol was used. In this approach, a constant level of stress was imposed and then a low-amplitude oscillatory deformation was superimposed to obtain the so-called differential modulus as a function of stress at a fixed frequency, $K'(\sigma)$, of the material in its stressed or strained state²⁶.

The behaviour of K' as a function of the applied constant stress, is shown in Fig. 4. When the elastic modulus is stress-independent, the differential modulus is the same as the elastic modulus $K'(\sigma_0) = G_0$. However, above the critical stress, K' increases until the network breaks. It is clearly seen that K' of different gelatin networks follow different power-law scalings with σ_0 . For the physical gelatin gel, in the strain hardening region, $K'(\sigma_0) \sim \sigma_0^{1.26 \pm 0.15}$ while for chemical gelatin gel, $K'(\sigma_0) \sim \sigma_0^{0.69 \pm 0.07}$. For the hybrid gelatin gel K' exhibits two power-laws. In the lower stress region, the power law exponent is similar to that of the chemical gelatin gel. In the high stress region, the power law exponent is similar to that of the physical gelatin gel. This finding indicates that the physical network within the hybrid gel dominates its strain hardening behaviour in the high stress region, whereas the chemical crosslinks play a more important role in the low stress region; although the power law fitting is merely indicative the power law dependence of K' with applied stress is higher for gelatin physical gel than chemical gel, as clearly seen in Fig.4.

The nonlinear response of all gelatin gels appears to deviate from an affine entropic elasticity model, which predicts an increase of $K'(\sigma_0) \sim \sigma_0^{1.5}$. This entropic nonlinear elasticity model assumes an affine deformation within the sheared samples and that the network elasticity originates from the resistance of individual network elements to stretching described by a worm-like chain model²⁶. However, in affine models, interactions between polymers are ignored so that polymers deform independently without affecting their neighbours. Such a deformation can only occur under ideal conditions²⁶. The affine (homogenous) deformation is not a reasonable assumption for physical and chemical crosslinked gelatin gels. First, it is suggested that structural non homogeneities can play a major role in the degree of non-affinity in polymer gels²⁶. The non-homogeneities are suggested to be present in chemically crosslinked gelatin gels

through our SANS studies (see below). Heterogeneities are also reflected by the fact that the chemical crosslinked gel showed a higher degree of deviation from the affine model compared to that of physical gel ($K'(\sigma_0) \sim \sigma_0^{1.5}$). Second, some researchers have adopted the non-affine nature of the network to predict the strain hardening in a collagen systems²⁷. This implies that the non-affine network may also provide a possible contribution to the strain hardening of gelatin gel²⁸. Third, as a hydrogel, the gelatin gel contains a large amount of water. Furthermore, the gelatin gel contains a “sol fraction”, *i.e.*, the dissolved gelatin molecules that have not participated in the infinite gel network. These viscous factors contribute to the nonlinear viscoelastic behaviour of gelatin gels, which cannot be described in the pure affine elastic models²⁸⁻²⁹. In the view of the above considerations, the worm-like chain affine model is too simplistic and ideal for explaining the strain hardening of various gelatin gels.

Figure.4. The differential elastic modulus, K' , as a function of applied constant shear stress, σ_0 , for physical gelatin gel (■); hybrid gelatin gel (◀); chemical gelatin gel (●). The solid black line at the right indicates $\sigma_0^{3/2}$. For physical gelatin gel and hybrid gelatin gel, the measurement was conducted at 20°C. For chemical gelatin gel, the measurement was conducted at 35°C.

Large deformation rheology: Strain ramp (constant shear study)

The comparison of data obtained with strain ramp, pre-stress protocols and large amplitude oscillation shear (LAOS) is presented in Fig. 5. The pre-stress method measures the nonlinear mechanical response at a specific frequency, while the strain ramp probes the system at a specific rate, and thus probes the response over a range of frequencies³⁰. LAOS is performed by subjecting a material to a large sinusoidal deformation and measuring the resulting mechanical response as a function of time²⁴. For the gelatin chemically-crosslinked and hybrid gels, the pre-stress and strain ramp protocols agree well over a decade of shear rates, as shown in Fig. 5 (B)

and (C), respectively. By contrast, for the physical gel, the strain rates with 0.1s^{-1} and 0.01s^{-1} show a deviation from the pre-stress protocol. The excellent agreement between pre-stress protocol and constant shear protocol performed on chemically cross-linked gels was also observed in F-actin cross-linked with permanent biotin-NeutrAvidin³⁰.

To better understand the nonlinear rheology of these gelatin gels, the BST equation with the scaling model was employed to fit stress-strain curves. In the BST equation³¹, the nonlinear stress response σ under shear deformation with strain γ is:

$$\sigma = \frac{2G}{n_{\text{BST}}} \frac{\lambda^{n_{\text{BST}}} - \lambda^{-n_{\text{BST}}}}{\lambda + \lambda^{-1}} \quad (1)$$

Where

$$\lambda = \frac{1}{2}\gamma + \sqrt{1 + \frac{1}{4}\gamma^2} \quad (2)$$

G is the linear elasticity modulus whereas n_{BST} is the nonlinearity parameter. It is noted that for $n_{\text{BST}} = 2$, eq. (1) reduces to the ideal rubber elasticity case $\sigma = G\gamma$.

To understand the nonlinearity parameter n_{BST} through a molecular interpretation of gelatin networks, a scaling model (based on the fractal structure of the polymers), a FENE model (based on the finite extensibility of the polymers), and a rod and coil model (based on the biochemical microstructure of gelatin) was developed¹⁴. It was found that the scaling model, with only one adjustable parameter, the fractal dimension d_f , could better describe the stress-strain curves in a quantitative way. The BST-scaling model was therefore employed to interpret the results. The relationship between the nonlinearity parameter n_{BST} and the fractal dimension d_f is as follows,

$$n_{\text{BST}} \approx \frac{d_f}{d_f - 1} \tag{3}$$

The summary of the linear elasticity modulus G , nonlinear elasticity parameter n_{BST} , and the fractal dimension d_f of various gelatin networks obtained using both pre-stress protocol and strain ramp protocol are listed in Table 1.

It is clearly seen that the value of n_{BST} obtained for the physical gels is higher than that of the chemically-crosslinked gels suggesting a higher degree of strain hardening behaviour observed in the physical gels. This behaviour was also observed in the larger exponent of the power-law scaling of the differential elastic modulus with applied constant stress in the physical gels than that of the chemically-crosslinked gels using the *pre-stress* protocol (Fig. 4). However, the value of n_{BST} for the hybrid gels is similar to that of the chemically-crosslinked gels. In the *pre-stress* protocol, two power law scaling regions for the hybrid gels and only one power-law scaling for chemically-crosslinked gel were observed. This may be because the single fitting parameter, n_{BST} , cannot fully describe the strain hardening behaviour of the hybrid gels that contain a hierarchy of structures each with its own mechanical and structural behaviour. The fractal dimension d_f of the physical gel obtained from different large deformations rheological approaches ranges from 1.38 to 1.40. For the chemically-crosslinked and hybrid gels, the fractal dimension d_f ranges from 1.48-1.49 and 1.45-1.47, respectively. Further the differences of onset of nonlinearity can be observed for all three gelatin gels using different large deformation protocols.

Both the difference of n_{BST} and the onset of nonlinearity obtained using different protocols could be attributed to the creep effect of these gelatin gels, since different protocols probe the strain

hardening behaviour at different time scales. As discussed before, the free dangling gelatin chains (“sol fraction”) and chains entanglement could cause creep and provide a viscous contribution to the strain hardening behaviour.

As can be seen in Fig. 5, all three kinds of gelatin gels exhibit different breaking strain using different large deformation protocols (for *Pre-stress* and LAOS protocol, the last data point corresponding to break-up), while the strain rate effect was insignificant on the stiffness (linear modulus) properties. These results are in agreement with other studies on the fracture properties of gelatin gel³². In this paper the strain hardening behaviour of various gelatin gel under large shear deformation is the principal focus. The BST model above was used to discuss the stress-strain curves and is based on fractal structure concepts that do not address fracture behaviour. The fracture (breaking) of various gelatin gels are governed by different mechanisms and depend on their structures¹⁶. The limits before network breaking do not lie in the same order in the three gels; in addition, the fracture mechanism for different physical or chemical cross linked gel are different. As can be seen in Fig.5, the scatter of the breaking strain using different large deformation protocols is more obvious in physical gelatin gel than that of chemically crosslinked gelatin gel. This could be due to the difference of fracture mechanism between physical and chemically crosslinked gels. For gels in which the polymer chains are cross-linked covalently (chemically crosslinked gelatin gel), fracture involves the breaking of covalent bonds in the cross-links or in a polymer chain. The fracture parameters are (nearly) independent of strain rate. This might be attributed to the fact that energy dissipation during deformation is small or virtually absent, owing to the very small permeability of the gels. The low ultimate strength of the chemical gel in figure 5B is probably due to the presence of micro-cracks or defects caused by the inhomogeneties that are minimal in physical gels³³.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

386

387
388
389
390
391
392

393

394

In terms of gelatin physical gel, large deformation of the gel may lead to the ‘unzipping’ of these junctions (triple helix). It has been suggested that for gelatin physical gel, both fracture stress and strain depend on the strain rate^{16, 34}. Unzipping of the bonds takes a finite time and this may itself cause the fracture strain or stress to become deformation-rate dependent. Also, fracture stress and strain depend on the stochastic nature of the structure and on the fracture force of the bonds. The latter parameters will also vary in a stochastic manner, resulting in a large scatter of the results³³.

Figure.5. Comparison of experimental stress-strain curves from Pre-stress protocol (black symbols), constant shear protocol with shear rate 0.1s^{-1} (blue symbols), 0.01s^{-1} (green symbols), and large amplitude oscillation shear (LAOS) (grey symbols) for A: gelatin physical gel, B: gelatin chemical gel, C: gelatin chemical-physical gel. Lines are fits to the BST equation. For physical gelatin gel and hybrid gelatin gel, the measurement was conducted at 20°C . For chemical gelatin gel, the measurement was conducted at 35°C .

	G (kPa)	n_{BST}	d_f
Physical gel (a)	0.50 ± 0.02	3.50 ± 0.055	1.40 ± 0.05
physical gel (b)	0.50 ± 0.02	3.65 ± 0.04	1.38 ± 0.04
physical gel (c)	0.50 ± 0.02	3.65 ± 0.04	1.38 ± 0.04
physical gel (d)	0.50 ± 0.02	3.50 ± 0.04	1.40 ± 0.04
Chemical gel (a)	0.11 ± 0.01	3.10 ± 0.05	1.48 ± 0.05
Chemical gel (b)	0.11 ± 0.01	3.05 ± 0.05	1.49 ± 0.05
Chemical gel (c)	0.11 ± 0.01	3.05 ± 0.04	1.49 ± 0.04
Chemical gel (d)	0.11 ± 0.01	3.10 ± 0.02	1.48 ± 0.02
Chemical-physical gel (a)	0.17 ± 0.04	3.18 ± 0.04	1.46 ± 0.04
Chemical-physical gel (b)	0.17 ± 0.03	3.20 ± 0.05	1.45 ± 0.05
Chemical-physical gel (c)	0.17 ± 0.05	3.15 ± 0.04	1.47 ± 0.04
Chemical-physical gel (d)	0.17 ± 0.03	3.15 ± 0.05	1.47 ± 0.05

Table.1 Summary of BST equation fitting parameters for gelatin physical gel, chemical gel, and chemical-physical gel. (a): Pre-stress protocol, (b): constant shear with shear rate 0.1s^{-1} , (c): constant shear with shear rate 0.01s^{-1} , (d) large amplitude oscillation shear (LAOS).

Small angle neutron scattering (SANS)

To investigate the structural differences between various gelatin gels, SANS experiments were conducted on the physical and chemically-crosslinked gelatin gels. SANS is a useful probe covering a large size range from the nanometers to fractions of a micrometer and covering the sizes of individual polymer chains and clusters³⁵.

1
2
3 412 Firstly, a Kratky plot is employed to investigate the structure differences between physical and
4
5 413 chemically-crosslinked gelatin gels (Fig.S.2). The shape of the Kratky plot yields information on
6
7 414 the conformation of the scattering unit ³⁶. An onset of a peak at low q is observed in the Kratky
8
9 415 plots of chemically-crosslinked gelatin gels and suggests the presence of frozen non-
10
11 416 homogeneities in the gel network ³⁷. The extent of heterogeneity of a polymer gel depends on the
12
13 417 polymerization mechanism and reaction conditions ³⁸. These non-homogeneities in the
14
15 418 chemically-crosslinked gelatin gel network could be due to the presence of cross-linking
16
17 419 aggregates. It is suggested that in the chemically-crosslinked gelatin gels, network construction
18
19 420 proceeds in a random and heterogeneous manner, with cross-links being formed in localized
20
21 421 regions, forming aggregates, which then come together ^{12a}. Such spatially heterogeneous
22
23 422 structures, with dense clusters linked by sparse networks, are also observed in other
24
25 423 glutaraldehyde cross-linked protein systems using small angle X-ray scattering ¹³. For the
26
27 424 physical gelatin gels, the Kratky plot indicates the relative homogeneous network in the studied q
28
29 425 range. In this gel the single-strand to triple-helix transitions occurs throughout the solution
30
31 426 thereby preserving a homogeneous network ^{12a,39}. However, it is worthy to note that the presence
32
33 427 of large non-homogeneities was also observed in the physical gels network in the ultra-low q
34
35 428 regime using USANS ⁴⁰.
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

430 Under large shear deformation, relatively large-scale structure clusters might be expected to
431 reorganize causing non-affine deformations. For the physical gels, both the rod-like structure of
432 triple helix bundles and flexible coils are believed to be present within the gel networks. When
433 the gels are deformed, the junction zones of the triple helix bundles may experience
434 deformations from compression and bending in addition to simple stretching⁴¹. For chemically-

crosslinked gelatin gels, small cross-linked aggregates and random coils within its heterogeneous networks are expected to experience various degrees of stretching and reorganization under large shear deformation. The assumption of an entropic affine deformation model is therefore likely too simplistic to describe the strain hardening of both the physical and chemically-crosslinked gelatin gels networks. It can be seen that the nonlinear response of these two gels deviates from an affine entropic stretching elasticity model, which predicts $K'(\sigma_0) \sim \sigma_0^{1.5}$ (Fig.4)⁴²; although for the physical and hybrid gels, an asymptotic 1.5 power-law scaling is observed at large stress prior to breaking. This may arise from the gel structural units undergoing significant stretching in an affine way prior to breaking thus making the entropic nonlinearity model in this regime a reasonable description of the system⁴³. A similar behaviour has been found with pectin gels; while strain-stiffening experiments reveal power law behaviour with exponents of around unity, their behaviour can be mapped onto the generic model asymptotically⁴⁴.

More insight into the nano-scale structure of these networks may be obtained by fitting the SANS data with an empirical model³⁶. One approach is to use the correlation length model³⁵ to interpret the SANS spectra of gelatin gels^{12a} and other hydrogel systems^{36, 45} over the whole q range. Another approach is to employ the fractal dimension (power-law) model and Guinier model to individually fit the SANS spectra of gelatin gel over specific q ranges^{40, 46}. In the following, we apply both approaches and describe our results in comparison to existing literature.

The scattering spectra for the gelatin physical and chemical gels were successfully fitted with a correlation length model as shown in Fig.6. The scattering intensity $I(q)$ is calculated as:

$$I(q) = \frac{A}{q^n} + \frac{C}{1 + (q\xi_L)^m} + \text{Bkg} \quad (4)$$

Where $I(q)$ is the scattering intensity, q is the scattering vector, and Bkg is the incoherent background scattering. The first term describes the power law scattering from large clusters (exponent= n) in the low q regime and the second term is a Lorentzian function that describes scattering from polymer chains (exponent= m) in the high q regime. This second term characterizes the polymer/solvent interactions. The parameter ξ_L is a correlation length for the polymer chains³⁵ and, in the case of a gel network, gives an indication of the gel mesh size.

Figure.6. Small-angle neutron scattering patterns of gelatin physical gel ($\square$) and chemical gel ($\circ$) in D_2O . The red solid line represents the fit of the correlation length model. The colored square box identifies the specific region for individual model fitting. See text for more information.

	n	ξ_L (Å)	m
Physical gel	2.46±0.04	69.2±2.4	1.82±0.01
Chemical gel	2.69±0.07	83.49±0.10	2.28±0.07

Table.2 Results from correlation length model fit to SANS patterns of gelatin physical and chemical gel

Parameters obtained from fits to the SANS spectra of the physical and chemically-crosslinked gelatin gels are summarised in Table 2. In the low q range, both gels may be described as mass fractal as indicated by power law exponent, n , of 2.46 ± 0.04 , and 2.69 ± 0.07 , respectively. The value of n between 2.4-2.6 could reflect weakly segregated network (2.5), randomly branched Gaussian chains (2.28) or indicate mass fractals (<3.0), amongst others⁵⁴. The differences in exponent, n , in the low q range that characterise the gel large-scale structure suggest different morphology of the networks⁴⁷.

In the mid- q regime, the Lorentzian exponent, m , extracted from the fitting can be employed to track differences in the local network morphology (1-10 nm) on the length scale of the polymer chains. The Lorentz exponent, m , may be related to the chain thermodynamics. The m value for physical gel and chemically crosslinked gel are 1.82 ± 0.01 and 2.28 ± 0.07 , respectively. The better the solvent, the more expanded a polymer coil is and the lower is the corresponding m . For linear polymer coils $m=1.67$ or 2 in a good solvent or a theta solvent, respectively. Branching usually increases m with respect to that of its linear counterpart. Randomly branched Gaussian

polymer chains are characterized by $m=2$ in a good solvent or $m=2.28$ in a theta solvent⁴⁸. For chemically cross-linked gel, $m=2.28$ indicating the presence of branched Gaussian chains in a theta solvent. For gelatin physical gel, $m=1.82$ suggesting presence of swollen linear chain in between good solvent and theta solvent. A fully swollen chain could suggest the presence of a “sol fraction”, i.e., the dissolved gelatin molecules that have not participated in the infinite gel network. These viscous factors contribute to the nonlinear viscoelastic behaviour of gelatin physical gels, which cannot be described with the pure affine elastic models, as discussed before^{29a 29b 28}. Such creep effects could partly explain the large scatter of breaking strain (see Figure 5) and the different n_{BST} values obtained from different large deformation protocols (over different time scales). Again, the presence of Gaussian branching chain could indicate the presence of heterogeneous structures in the chemically crosslinked gelatin gel due to random chemical crosslinking and with strain hardening originating from the response of different hierarchical units upon large deformation.

The Lorentzian correlation length for the physical and chemically-crosslinked gels are 69.2 ± 2.4 and 83.49 ± 0.10 Å, respectively. In physical gels, the correlation length can be understood as an average mesh size of the network⁴⁹. For chemically-crosslinked gels however, that correlation length does not reflect a ‘mesh size’ but rather the size of growing cross-linked aggregates^{12a}.

Fractal structures are objects that demonstrate a self-similar structure over a range of length scales⁵⁰. Gels can be treated as fractal structures and it is possible to quantify their self-similar structure through a geometrical parameter referred to as the fractal dimension d_f ⁵¹. Mass fractals exhibit a scattering intensity, $I(q)$, that obeys a power law equation given by⁵².

$$I(q) = \frac{A}{q^{d_f}} + B \quad (7)$$

Here B is the background scattering. The scattering profile of SANS data in the intermediate q -regime, $q=0.01$ to $0.08\text{\AA}^{-1}$ exhibited a power law dependence shown in Fig.9. We chose to examine the power law region exhibited in that q -regime based on a previous SANS study of physical gels⁵³. The fractal dimensions d_f of physical and chemically-crosslinked gels were around 1.31 ± 0.02 and 1.53 ± 0.03 , respectively. Notice that the ranger over which the power law is well presented is larger for chemical gels possibly because of the existence of inhomogeneities due to crosslinking. For polymer chains, a slope $n=1.67$ is typical for fully swollen coils and a slope $n=1$ is for rod-like structure⁵⁴. For the physical gel, an exponent $n=1.35$ indicates a semi-flexible extended structure of gelatin triple helix bundles⁴⁰. Furthermore, the $d_f=1.31\pm0.02$ deduced from this SANS study of physical gels is comparable with the d_f values (1.38-1.40) measured from model-fitting the stress-strain curve (Table.1). (The discrepancy might be due to the narrow data-fitting range and the large error that ensues.) For chemically-crosslinked gels, a power exponent $d_f=1.53\pm0.03$ suggests the existence of less rod-like, more swollen coils and small cross-linked aggregates within its network. This swelling is probably due to the local osmotic pressure that builds up between the inhomogeneities and the rest of the gel. As expected, the chemically-crosslinked gelatin gel is made only of random polymer coils and small aggregates with an absence of triple helix bundles due to gel formation above the transition temperature of the random coil to triple helix^{12a}. There is a good agreement between the d_f obtained from SANS model fitting ($d_f=1.53$) and from fitting the stress-strain curve for chemical gel ($d_f=1.48$ -1.49). The nonlinearity in rheology of the physical and chemically-crosslinked gelatin gels can be partly understood based on their fractal structures. The transition or the

529 crossover in the spectrum around $0.01 \text{ \AA}^{-1}$ occurs earlier in the q -values for chemically
530 crosslinked gels because of the osmotic pressure between the aggregates and the random coils.

531
532 The asymptotic region in the large q -domain ($q > 0.14 \text{ \AA}^{-1}$) is expected to reveal the local rigidity
533 whereby the chain cross-section makes a finite contribution to the measured structure factor^{51a}.
534 According to the Kratky-Porod equation, in the far q domain, one has

$$I(q) = I_0 \exp\left(-\frac{q^2 R_c^2}{2}\right) \quad (8)$$

535 where R_c is the chain cross-sectional radius. Fig.S.3 presents the Guinier plot in the high q -region
536 that enables the determination of R_c . For the gelatin physical gels and chemical gels, R_c values of
537 0.33nm and 0.34nm are obtained respectively. This compares well with other study where
538 $R_c = 0.35\text{nm}$ ^{46b}. The deviation of incoherent background signal deviate from a flat shape and
539 decreases with q is probably geometric such as the curvature of the detector or the angular
540 dependence of the transmission⁵⁵.

541 The SANS results reveal that the presence of structural differences between physical gelatin gel
542 and chemically crosslinked gelatin gel at various length scales. These structural differences are
543 proposed to contribute to the differences of their large deformation rheological properties.

544

545 Conclusions

The strain hardening behaviour of three gelatin gels: a physical gel, a chemically-crosslinked gel, and a hybrid network containing both physical and chemical crosslinks, have been studied by three large-shear deformation protocols, namely pre-stress, strain ramp, and large amplitude oscillation shear (LAOS). When using the pre-stress protocol, the different gelatin gels exhibit a different power law scaling of the differential elastic modulus towards applied constant stress. Specifically, for the physical gelatin gel, in the strain hardening region, $K'(\sigma_0) \sim \sigma_0^{1.26 \pm 0.15}$ while for chemically-crosslinked gelatin gel, $K'(\sigma_0) \sim \sigma_0^{0.69 \pm 0.07}$. For the hybrid network, there are two power law regions. In the small stress region, the power law exponent is similar to that of the chemically-crosslinked gelatin gel whereas in the large stress region, the power law exponent is similar to that of the physical gelatin gel. The results from the pre-stress, strain ramp, and the LAOS agree well in the case of the chemically-crosslinked and the hybrid gel but not as well for physical gels. Further, the BST-scaling model was employed to fit the stress-strain curves of the various gelatin gels; the nonlinearity parameter n_{BST} obtained from the physical gel (3.50-3.65) was found to be higher than that of the chemically-crosslinked gel (3.05-3.10) or the hybrid gel (3.15-3.20) indicating a higher degree of strain hardening in the physical gelatin gel at the strain investigated. The fractal dimension d_f obtained from model fitting is 1.38-1.40, 1.48-1.49 and 1.45-1.47, for the three gels above respectively.

Small angle neutron scattering revealed that the physical and chemically-crosslinked gels exhibit hierarchical structures. The Kratky plots of SANS data suggest that a relatively homogeneous network is formed in the physical gels, whereas in the chemically-crosslinked gels, there are some small cross-linked aggregates. As a result, higher deviation from a power law behaviour with exponent smaller than 1.5 in the strain hardening of the chemically-crosslinked gel

compared to that in physical gelatin gel comes as no surprise since non-affine deformation occurs in such heterogeneous structure. As discussed before, non-homogeneities can play a major role in the degree of non-affinity in polymer gels. Such non-homogeneities could hinder the free stretching of gelatin chains.

Through fitting the correlation length model to the SANS data, correlation lengths were obtained for these gels to be 69.2 ± 2.4 and 83.49 ± 0.10 Å, respectively. To further extract the structural parameters from the SANS, individual fits were performed on the power-law regime, and high- q Guinier regime. The cross-sectional radii of the gelatin chains for the physically-crosslinked and chemically-crosslinked gels were found to be 0.33nm and 0.34nm, respectively. The fractal dimensions d_f obtained from the power law fitting in the q -range ~ 0.01 to $\sim 0.06 \text{Å}^{-1}$ were 1.31 ± 0.02 and 1.53 ± 0.03 for these gels respectively, is comparable with the values of d_f obtained from fitting the stress-strain curves.

In summary, large deformation (strain hardening) and fracture behaviour of gelatin gels is related to their structure in a much more complicated and subtle way than small deformation behaviour. For all three gelatin gels, the differences in strain hardening and fracture behaviour can be observed when using different large deformation protocols, although the difference of strain hardening behaviour is much smaller. Using the BST model, the difference of strain hardening between gelatin physical gel and chemically crosslinked gel can be linked to their fractal structures differences measured by SANS. However, due to their hierarchical structure, other factors including dangling chains (viscous contribution), creeping effect, the elasticity difference between Gaussian chain and non-Gaussian chain (swollen chain), unzipping of triple helix

junction zones, structural reorganization under shear, and the presence of non-homogeneities (non-affine deformation) may also need to be considered to understand their strain hardening behaviour. For example, it is expected that the presence of dangling chain, unzipping of triple helix junction zones, and creep could play a more important role in determining the large deformation properties of gelatin physical gel as evidenced by the large scatter of breaking strain when probed at different time scales. However, in the case of chemically crosslinked gelatin gel, the non-affine deformation may become a more crucial factor in determining its large deformation properties due to presence of non-homogeneities. This is reflected by its larger deviation from the power law model (smaller than exponent 1.5) as suggested for polymers under affine deformation.

Associated content

Supporting Information

The supporting information is available free of charge on the ACS Publications website at DOI:
Strain sweep of gelatine physical gel using plate-plate geometry with different gap, Kratky plots for SANS patterns of gelatine physical gel and chemical gel, Guinier plot for SANS pattern of gelatine physical gel and chemical gel in high q regime.

Corresponding Author

Sahraoui chaieb (sahraoui.chaieb@kaust.edu.sa)

ACKNOWLEDGMENT

We acknowledge the support of the ANSTO, Australia, in providing the small angle neutron scattering research facilities (QUOKKA) used in this work. We thank Ferdi Franceschini from ANSTO, Australia for technical assistance. SC and ZY thank KAUST for financial support. LH

thanks the Portuguese Foundation for Science and Technology for financial support through project UID/CTM/50025/2013.

REFERENCES

1. Kuijpers, A. J.; Engbers, G. H. M.; Feijen, J.; De Smedt, S. C.; Meyvis, T. K. L.; Demeester, J.; Krijgsveld, J.; Zaat, S. A. J.; Dankert, J., Characterization of the Network Structure of Carbodiimide Cross-Linked Gelatin Gels. *Macromolecules* **1999**, *32* (10), 3325-3333.
2. (a) Fan, H.; Dash, A. K., Effect of cross-linking on the in vitro release kinetics of doxorubicin from gelatin implants. *Int. J. Pharm.* **2001**, *213* (1), 103-116; (b) Kang, H.-W.; Tabata, Y.; Ikada, Y., Fabrication of porous gelatin scaffolds for tissue engineering. *Biomaterials* **1999**, *20* (14), 1339-1344.
3. (a) Guo, L.; Colby, R. H.; Lusignan, C. P.; Whitesides, T. H., Kinetics of Triple Helix Formation in Semidilute Gelatin Solutions. *Macromolecules* **2003**, *36* (26), 9999-10008; (b) Guo, L.; Colby, R. H.; Lusignan, C. P.; Howe, A. M., Physical gelation of gelatin studied with rheo-optics. *Macromolecules* **2003**, *36* (26), 10009-10020.
4. Djabourov, M., Architecture of gelatin gels. *Contemp. Phys.* **1988**, *29* (3), 273-297.
5. (a) Babin, H.; Dickinson, E., Influence of transglutaminase treatment on the thermoreversible gelation of gelatin. *Food Hydrocolloids* **2001**, *15* (3), 271-276; (b) Mohtar, N. F.; Perera, C. O.; Quek, S.-Y.; Hemar, Y., Optimization of gelatin gel preparation from New Zealand hoki (*Macrurus novaezelandiae*) skins and the effect of transglutaminase enzyme on the gel properties. *Food Hydrocolloids* **2013**, *31* (2), 204-209.
6. Chiou, B.-S.; Avena-Bustillos, R. J.; Shey, J.; Yee, E.; Bechtel, P. J.; Imam, S. H.; Glenn, G. M.; Orts, W. J., Rheological and mechanical properties of cross-linked fish gelatins. *Polymer* **2006**, *47* (18), 6379-6386.
7. Zhang, X.; Do, M. D.; Casey, P.; Sulistio, A.; Qiao, G. G.; Lundin, L.; Lillford, P.; Kosaraju, S., Chemical cross-linking gelatin with natural phenolic compounds as studied by high-resolution NMR spectroscopy. *Biomacromolecules* **2010**, *11* (4), 1125-1132.
8. Kosaraju, S. L.; Puvanenthiran, A.; Lillford, P., Naturally crosslinked gelatin gels with modified material properties. *Food Res. Int.* **2010**, *43* (10), 2385-2389.
9. Hellio-Serughetti, D.; Djabourov, M., Gelatin hydrogels cross-linked with bis (vinylsulfonyl) methane (BVSM): 1. The chemical networks. *Langmuir* **2006**, *22* (20), 8509-8515.
10. (a) Kirchmayer, D. M.; Watson, C. A.; Ranson, M., Gelapin, a degradable genipin cross-linked gelatin hydrogel. *RSC Advances* **2013**, *3* (4), 1073-1081; (b) Bigi, A.; Cojazzi, G.;

- 649 Panzavolta, S.; Roveri, N.; Rubini, K., Stabilization of gelatin films by crosslinking with genipin.
650 *Biomaterials* **2002**, *23* (24), 4827-4832.
- 651 11. Liang, H. C.; Chang, W. H.; Liang, H. F.; Lee, M. H.; Sung, H. W., Crosslinking
652 structures of gelatin hydrogels crosslinked with genipin or a water-soluble carbodiimide. *J. Appl.*
653 *Polym. Sci.* **2004**, *91* (6), 4017-4026.
- 654 12. (a) Bode, F.; da Silva, M. A.; Smith, P.; Lorenz, C. D.; McCullen, S.; Stevens, M. M.;
655 Dreiss, C. A., Hybrid gelation processes in enzymatically gelled gelatin: impact on
656 nanostructure, macroscopic properties and cellular response. *Soft Matter* **2013**, *9* (29), 6986-
657 6999; (b) Bode, F.; da Silva, M. A.; Drake, A. F.; Ross-Murphy, S. B.; Dreiss, C. A.,
658 Enzymatically cross-linked tilapia gelatin hydrogels: physical, chemical, and hybrid networks.
659 *Biomacromolecules* **2011**, *12* (10), 3741-3752.
- 660 13. Kaieda, S.; Plivelic, T. S.; Halle, B., Structure and kinetics of chemically cross-linked
661 protein gels from small-angle X-ray scattering. *Phys. Chem. Chem. Phys.* **2014**, *16* (9), 4002-
662 4011.
- 663 14. Groot, R. D.; Bot, A.; Agterof, W. G., Molecular theory of strain hardening of a polymer
664 gel: application to gelatin. *J. Chem. Phys.* **1996**, *104* (22), 9202-9219.
- 665 15. Joly-Duhamel, C.; Hellio, D.; Ajdari, A.; Djabourov, M., All gelatin networks: 2. The
666 master curve for elasticity. *Langmuir* **2002**, *18* (19), 7158-7166.
- 667 16. Groot, R. D.; Bot, A.; Agterof, W. G., Molecular theory of the yield behavior of a
668 polymer gel: application to gelatin. *J. Chem. Phys.* **1996**, *104* (22), 9220-9233.
- 669 17. (a) Jensen, Mikkel H.; Morris, Eliza J.; Gallant, Cynthia M.; Morgan, Kathleen G.;
670 Weitz, David A.; Moore, Jeffrey R., Mechanism of Calponin Stabilization of Cross-Linked Actin
671 Networks. *Biophys. J.* **2014**, *106* (4), 793-800; (b) Lieleg, O.; Claessens, M. M.; Bausch, A. R.,
672 Structure and dynamics of cross-linked actin networks. *Soft Matter* **2010**, *6* (2), 218-225.
- 673 18. Gilbert, E. P.; Schulz, J. C.; Noakes, T. J., 'Quokka'—the small-angle neutron scattering
674 instrument at OPAL. *Phys. B.* **2006**, *385*, 1180-1182.
- 675 19. Joly-Duhamel, C.; Hellio, D.; Djabourov, M., All gelatin networks: 1. Biodiversity and
676 physical chemistry. *Langmuir* **2002**, *18* (19), 7208-7217.
- 677 20. Ronsin, O.; Caroli, C.; Baumberger, T., Interplay between Shear Loading and Structural
678 Aging in a Physical Gelatin Gel. *Phys. Rev. Lett.* **2009**, *103* (13), 138302.
- 679 21. Farris, S.; Song, J.; Huang, Q., Alternative Reaction Mechanism for the Cross-Linking of
680 Gelatin with Glutaraldehyde. *J. Agric. Food Chem.* **2010**, *58* (2), 998-1003.
- 681 22. Mohtar, N. F.; Perera, C. O.; Hemar, Y., Chemical modification of New Zealand hoki
682 (*Macrurus novaezelandiae*) skin gelatin and its properties. *Food Chem.* **2014**, *155* (0), 64-73.

23. Moura, M. J.; Figueiredo, M. M.; Gil, M. H., Rheological Study of Genipin Cross-Linked Chitosan Hydrogels. *Biomacromolecules* **2007**, *8* (12), 3823-3829.
24. Hyun, K.; Wilhelm, M.; Klein, C. O.; Cho, K. S.; Nam, J. G.; Ahn, K. H.; Lee, S. J.; Ewoldt, R. H.; McKinley, G. H., A review of nonlinear oscillatory shear tests: Analysis and application of large amplitude oscillatory shear (LAOS). *Prog. Polym. Sci.* **2011**, *36* (12), 1697-1753.
25. Yoshimura, A.; Prud'homme, R. K., Wall slip corrections for Couette and parallel disk viscometers. *J. Rheol.* **1988**, *32* (1), 53-67.
26. Wen, Q.; Basu, A.; Janmey, P. A.; Yodh, A. G., Non-affine deformations in polymer hydrogels. *Soft matter* **2012**, *8* (31), 8039-8049.
27. (a) Motte, S.; Kaufman, L. J., Strain stiffening in collagen I networks. *Biopolymers* **2013**, *99* (1), 35-46; (b) Vader, D.; Kabla, A.; Weitz, D.; Mahadevan, L., Strain-induced alignment in collagen gels. *PLoS One* **2009**, *4* (6), e5902.
28. Sun, W.-x.; Huang, L.-z.; Yang, Y.-r.; Liu, X.-x., Large amplitude oscillatory shear studies on the strain-stiffening behavior of gelatin gels. *Chin. J. Polym. Sci.* **2015**, *33* (1), 70-83.
29. (a) Haut, R. C.; Little, R. W., A constitutive equation for collagen fibers. *J. Biomech.* **1972**, *5* (5), 423-430; (b) Pioletti, D. P.; Rakotomanana, L.; Benvenuti, J.-F.; Leyvraz, P.-F., Viscoelastic constitutive law in large deformations: application to human knee ligaments and tendons. *J. Biomech.* **1998**, *31* (8), 753-757.
30. Broedersz, C. P.; Kasza, K. E.; Jawerth, L. M.; Münster, S.; Weitz, D. A.; MacKintosh, F. C., Measurement of nonlinear rheology of cross-linked biopolymer gels. *Soft Matter* **2010**, *6* (17), 4120-4127.
31. Blatz, P.; Sharda, S. C.; Tschoegl, N., Strain energy function for rubberlike materials based on a generalized measure of strain. *Trans. Soc. Rheol. (1957-1977)* **1974**, *18* (1), 145-161.
32. Forte, A. E.; D'Amico, F.; Charalambides, M. N.; Dini, D.; Williams, J. G., Modelling and experimental characterisation of the rate dependent fracture properties of gelatin gels. *Food Hydrocolloids* **2015**, *46*, 180-190.
33. Vliet, T., Large deformation and fracture behaviour of gels. *Faraday Discuss.* **1995**, *101*, 359-370.
34. (a) Ross-Murphy, S. B.; Morris, V. J.; Morris, E. R. In *Molecular viscoelasticity of xanthan polysaccharide*, *Faraday Symp. Chem. Soc.* Royal Society of Chemistry: 1983; pp 115-129; (b) McEvoy, H.; Ross-Murphy, S.; Clark, A., Large deformation and ultimate properties of biopolymer gels: 1. Single biopolymer component systems. *Polymer* **1985**, *26* (10), 1483-1492.
35. Hammouda, B.; Ho, D. L.; Kline, S., Insight into clustering in poly (ethylene oxide) solutions. *Macromolecules* **2004**, *37* (18), 6932-6937.

36. Saffer, E. M.; Lackey, M. A.; Griffin, D. M.; Kishore, S.; Tew, G. N.; Bhatia, S. R., SANS study of highly resilient poly (ethylene glycol) hydrogels. *Soft matter* **2014**, *10* (12), 1905-1916.
37. Karino, T.; Shibayama, M.; Okumura, Y.; Ito, K., SANS study on pulley effect of slide-ring gel. *Phys. B* **2006**, *385*, 807-809.
38. Di Lorenzo, F.; Seiffert, S., Nanostructural heterogeneity in polymer networks and gels. *Polym. Chem.* **2015**, *6*, 5515-5528.
39. Bastide, J.; Leibler, L., Large-scale heterogeneities in randomly cross-linked networks. *Macromolecules* **1988**, *21* (8), 2647-2649.
40. Helminger, M.; Wu, B.; Kollmann, T.; Benke, D.; Schwahn, D.; Pipich, V.; Faivre, D.; Zahn, D.; Cölfen, H., Synthesis and Characterization of Gelatin-Based Magnetic Hydrogels. *Adv. Funct. Mater.* **2014**, *24* (21), 3187-3196.
41. (a) Zhang, J.; Daubert, C. R.; Allen Foegeding, E., A proposed strain-hardening mechanism for alginate gels. *J. Food Eng.* **2007**, *80* (1), 157-165; (b) Onck, P.; Koeman, T.; Van Dillen, T.; Van der Giessen, E., Alternative explanation of stiffening in cross-linked semiflexible networks. *Phys. Rev. Lett.* **2005**, *95* (17), 178102.
42. Gardel, M.; Shin, J.; MacKintosh, F.; Mahadevan, L.; Matsudaira, P.; Weitz, D., Elastic behavior of cross-linked and bundled actin networks. *Science* **2004**, *304* (5675), 1301-1305.
43. Piechocka, I. K.; Bacabac, R. G.; Potters, M.; MacKintosh, F. C.; Koenderink, G. H., Structural hierarchy governs fibrin gel mechanics. *Biophys. J.* **2010**, *98* (10), 2281-2289.
44. Carrillo, J. M. Y.; MacKintosh, F. C.; Dobrynin, A. V., Nonlinear Elasticity: From Single Chain to Networks and Gels. *Macromolecules* **2013**, *46* (9), 3679-3692.
45. (a) Hule, R. A.; Nagarkar, R. P.; Hammouda, B.; Schneider, J. P.; Pochan, D. J., Dependence of Self-Assembled Peptide Hydrogel Network Structure on Local Fibril Nanostructure. *Macromolecules* **2009**, *42* (18), 7137-7145; (b) Hule, R. A.; Nagarkar, R. P.; Altunbas, A.; Ramay, H. R.; Branco, M. C.; Schneider, J. P.; Pochan, D. J., Correlations between structure, material properties and bioproperties in self-assembled [small beta]-hairpin peptide hydrogels. *Faraday Discuss.* **2008**, *139* (0), 251-264.
46. (a) Pezron, I.; Djabourov, M.; Leblond, J., Conformation of gelatin chains in aqueous solutions: 1. A light and small-angle neutron scattering study. *Polymer* **1991**, *32* (17), 3201-3210; (b) Mohanty, B.; Aswal, V. K.; Kohlbrecher, J.; Bohidar, H. B., Length scale hierarchy in sol, gel, and coacervate phases of gelatin. *J. Polym. Sci., Part B: Polym. Phys.* **2006**, *44* (12), 1653-1667.
47. Hule, R. A.; Nagarkar, R. P.; Altunbas, A.; Ramay, H. R.; Branco, M. C.; Schneider, J. P.; Pochan, D. J., Correlations between structure, material properties and bioproperties in self-assembled β -hairpin peptide hydrogels. *Faraday Discuss.* **2008**, *139*, 251-264.

48. (a) Gelade, E. T.; Goderis, B.; de Koster, C. G.; Meijerink, N.; van Benthem, R. A.; Fokkens, R.; Nibbering, N. M.; Mortensen, K., Molecular structure characterization of hyperbranched polyesteramides. *Macromolecules* **2001**, *34* (11), 3552-3558; (b) da Silva, M. A.; Bode, F.; Grillo, I.; Dreiss, C. c. A., Exploring the Kinetics of Gelation and Final Architecture of Enzymatically Cross-Linked Chitosan/Gelatin Gels. *Biomacromolecules* **2015**, *16* (4), 1401-1409.
49. Daoud, M.; Cotton, J. P.; Farnoux, B.; Jannink, G.; Sarma, G.; Benoit, H.; Duplessix, C.; Picot, C.; de Gennes, P. G., Solutions of Flexible Polymers. Neutron Experiments and Interpretation. *Macromolecules* **1975**, *8* (6), 804-818.
50. Schmidt, P. W., Small-angle scattering studies of disordered, porous and fractal systems. *J. Appl. Crystallogr.* **1991**, *24* (5), 414-435.
51. (a) Santinath Singh, S.; Aswal, V.; Bohidar, H., Internal structures of agar-gelatin co-hydrogels by light scattering, small-angle neutron scattering and rheology. *Eur. Phys. J. E: Soft matter Biol. Phys.* **2011**, *34* (6), 1-9; (b) Chaieb, S., Elasto-plasticity in wrinkled polymerized lipid membranes. *Sci. Rep.* **2014**, *4*, 1-5.
52. Manley, S.; Cipelletti, L.; Trappe, V.; Bailey, A. E.; Christianson, R. J.; Gasser, U.; Prasad, V.; Segre, P. N.; Doherty, M. P.; Sankaran, S.; Jankovsky, A. L.; Shiley, B.; Bowen, J.; Eggers, J.; Kurta, C.; Lorik, T.; Weitz, D. A., Limits to Gelation in Colloidal Aggregation. *Phys. Rev. Lett.* **2004**, *93* (10), 108302.
53. I. Pezron, T. H., M. Djabourov, and J. Leblond, In *Physical Networks*, Ross-Murphy, S. B., Ed. Elsevier, London: 1990; p 231.
54. Hammouda, B. *Probing nanoscale structures-The SANS toolbox*; **2010**.
55. Brûlet, A., Lairez, D., Lapp, A., & Cotton, J. P. Improvement of data treatment in small-angle neutron scattering. *J. Appl. Crystallogr.* **2007**, *40* (1), 165-177.

For TOC use only

TOC Figure

Small-angle neutron scattering patterns of gelatin physical gel ($\square$) and chemical gel ($\circ$) in D_2O . The data are fit using models (solid lines) that describe the hierarchical structures of gelatin at different length scales. The insets are schematic representations of the structural features characterized by the fitting.

794

795