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Dry vs. wet: Properties and performance of collagen films. Part II. Cyclic and time-dependent behaviours

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A B S T R A C T

Collagen constitutes one-third of human-body proteins, providing mechanical strength and structural stability. Films of collagen are widely used in tissue engineering as scaffolds for wound healing and corneal implants, among other applications, presupposing the investigation of their mechanical properties and performance under various loading and environmental conditions. Part I of this research (Bose et al., 2020) demonstrated a drastic change in the mechanical response of collagen films under *in-aqua* conditions when compared to dry specimens. It was also observed that collagen films exhibited a strain-rate-dependent hardening behaviour with a strain-rate-sensitivity exponent ranging from 0.02 to 0.2. In Part II, the cyclic and time-dependent behaviours of collagen films were analysed under different loading and environmental conditions. Strain ratchetting was observed for collagen subjected to cyclic loading under various stress levels and environmental (in-air and *in-aqua*) conditions, while the *in-aqua* samples demonstrated an increase in the stiffness (50% in the first cycle), which may be referred to as *cyclic stiffening*. In contrast, the dry samples showed a drop in the modulus after the first cycle, without any subsequent changes. Additionally, time-dependent viscoelastic properties were analysed, using dynamic mechanical analysis as well as creep and stress-relaxation techniques. Tan δ values for dry samples ranged from 0.05 to 0.075, while for hydrated ones it varied from 0.12 to 0.24. Collagen films exhibited primary and secondary creep stages, while the initial stress-relaxation was fast followed by a monotonous decay. The stress-strain-time data obtained from experiments were fitted in Prony series to estimate the relaxation moduli and times.

Keywords:

Collagen
Environmental conditions
Unloading modulus
Tan δ
Creep
Stress relaxation
Prony series

1. Introduction

Collagen is one of the most important proteins present in human body, contributing to the mechanical strength and structural integrity of connective tissues (Fratzl, 2008; Kadler et al., 2007; Buehler, 2006a,b). It has a complex hierarchical building blocks (see details in Part I of this study) beginning with tropocollagen molecules (1.5 nm diameter), assembled in collagen fibrils (100–500 nm diameter). The collagen fibrils, in turn, bundle up to form collagen fibres (few micrometres in diameter) and tissues (millimetres to centimetres) (Gautieri et al., 2011). This hierarchical order defines the complex viscoelastic behaviour of collagenous materials. Importantly, collagen films have significant applications in tissue regeneration such as corneal and wound healing (Ber et al., 2005; Sang et al., 2017; Li et al., 2019; Liu et al., 2012, 2014), substrates for sustained release of hormones (Maeda et al., 2001), dural substitutes (Collins et al., 1991), flexible electronics (Moreno et al.,

2015; Ghosh and Mandal, 2017) etc. thanks to their biocompatibility, biodegradability, flexibility and mechanical strength (Lee and Mooney, 2001; Fratzl, 2008). These potential biomedical applications and complex hierarchical structure mean that collagen-based materials are often exposed to various inelastic and time-dependent deformations under different environmental conditions.

The previous experiments has established that repeated cyclic stretching had a profound influence on mechanical properties of collagen-rich tissues such as skin (Kang and Wu, 2011; Remache et al., 2018; Pissarenko et al., 2019), tendons (Su et al., 2008), bones (Abdel-Wahab et al., 2011) etc. Collagenous tissues usually depict a complex visco-elastic behaviour: an increase in the accumulation of residual strain can lead to damage of the skin tissues (Kang and Wu, 2011; Remache et al., 2018) or increase in energy dissipation (Pissarenko et al., 2019). Some studies suggested that collagenous materials exhibited an increase in the stiffness following cyclic loadings (Su et al.,

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Table 1

Different environmental conditions used in cyclic and DMA test.

Specimen name	Testing environment	Specimen conditioning
Col _D	Ambient (dry)	–
Col _A	<i>In-aqua</i>	Pre-hydrated (10 min, water)
Col _H	Ambient	Pre-hydrated (10 min, water)

Table 2 Comparison of tan δ values of collagen from literature and current study.

Specimen Type	Dry State	Wet State	Reference
Corneal collagen	0.032–0.06		Hatami-Marbini and Rahimi (2015)
Corneal collagen (cross-linked)	0.028–0.048		Hatami-Marbini and Rahimi (2015)
Porcine stratum corneum	0.1	0.25	Yuan and Verma (2006)
Bovine cortical bone	0.035–0.1		Abdel-Wahab et al. (2011)
Femora of mouse	0.01–0.04	0.12–0.16	Pathak et al. (2011)
This study	0.05–0.075	0.12–0.24	

2008; Susilo et al., 2016), whereas, in collagen fibrils, the stiffness was nearly constant throughout the entire mechanical cycling (Liu et al., 2018). Thus, it would be interesting to investigate the mechanical response of pure collagen films under different loading (low-, intermediate- and high-stress regimes) and environmental conditions.

To analyse the time-dependent viscoelastic behaviour of collagen-based materials dynamic mechanical analysis (DMA) was used for tissues such as chordae (Wilcox et al., 2014), porcine and bovine cornea (Yuan and Verma, 2006; Hatami-Marbini and Rahimi, 2015), bones (Abdel-Wahab et al., 2011; Pathak et al., 2011), chicken collagen films (Oechsle et al., 2017) etc. Chordal tissues exhibited time-dependent storage modulus but a time-independent loss modulus (Wilcox et al., 2014). Further studies (Hatami-Marbini and Rahimi, 2015; Oechsle et al., 2017) suggested that the storage and loss moduli may be affected by treatment with various crosslinkers or incorporation of other protein sources/salts. Moreover, hydration can influence the damping coefficient, causing a significant change in storage and loss moduli, as observed in wet collagenous corneal tissues (Yuan and Verma, 2006) and bones (Pathak et al., 2011), leading to a drastic change in tan delta (Table 2). Hence, estimating the tan-delta values of pure collagen film in the dry and wet/hydrated state is important.

Another approach to analyse time-dependent properties of collagen is by means of creep and stress-relaxation tests. Previous studies on collagen-rich tissues such as tendons (Shepherd et al., 2014; Ganghoffer et al., 2016), skin (Pissarenko et al., 2019; Kang and Wu, 2011; Remache et al., 2018; Payne et al., 2015), bones (Abdel-Wahab et al., 2011; Novitskaya et al., 2014), heart valves (Anssari-Benam et al., 2011) etc. demonstrated viscoelastic responses. Moreover, collagen at molecular (Gautieri et al., 2012) and fibrillar (Shen et al., 2011; Gautieri et al., 2013) levels also exhibited a complex viscoelastic behaviour, attributed to various sliding mechanisms between molecules and fibrils/fibres (Gautieri et al., 2012). The creep behaviour of collagenous materials was generally characterised by primary and secondary creep (Bertassoni et al., 2016; Shen et al., 2011; Shepherd et al., 2014; Abdel-Wahab et al., 2011; Novitskaya et al., 2014) while the stress-relaxation was found to depend on the tissue hierarchy and other ground substances (Shen et al., 2011). From these experimental data, time-dependent properties are generally not extracted directly; instead, these data are usually fitted into viscoelastic models (e.g. the quasi-linear viscoelasticity (QLV) model, Maxwell-Weichert model or Prony series) to obtain the corresponding moduli and characteristic times. Despite several studies of the viscoelastic properties of collagenous tissues, there is limited work related to the time-dependent response of pure collagen films and determination of their associated moduli and relaxation times.

Part I of this work (Bose et al., 2020) demonstrates the importance of

in-aqua testing conditions, mimicking the *in-vivo* environment for potential biomedical applications, which drastically changes the mechanical response of collagen. It was found that dry collagen had elasto-plastic behaviour with strain-hardening, while *in-aqua*-tested collagen demonstrated a hyperelastic behaviour with a significant – nearly 2.5-fold – increase in its strain at failure (as compared to dry specimens), compensated by a reduction in its mechanical strength (by more than 90%). Still, studies related to collagen-based biological tissues such as ligaments, skins, tendons and bones demonstrated their more complex inelastic responses (Kang and Wu, 2011; Remache et al., 2018; Pissarenko et al., 2019; Su et al., 2008; Susilo et al., 2016) and time-dependent behaviour (Shepherd et al., 2014; Abdel-Wahab et al., 2011; Pissarenko et al., 2019; Remache et al., 2018). Hence, Part II of this work focuses on the inelastic and time-dependent response of collagen film in dry and hydrated or *in-aqua* conditions. It is divided into three parts: (a) evaluation of inelastic deformation and energy dissipation of collagen (uniaxial tensile cyclic tests dry and *in-aqua*); (b) assessment of frequency-dependent elastic and viscous responses of dry and hydrated collagen films; (c) investigation of the creep and stress-relaxation response of collagen under different stress and strain conditions, respectively, under ambient conditions. The normalised creep values (obtained from experiments) were used to estimate the Prony series coefficients in Abaqus Simulia.

2. Materials and methods

Collagen solutions (8 mg/ml) were prepared from bovine Achilles tendons (obtained from Sigma-Aldrich) using 0.05 M acetic acid (purchased from Sigma-Aldrich) and stored at 2–6 °C for 48 h. Next, the solutions were stirred for 4 h at 1500 rpm, maintaining a temperature of 2–6 °C. The solutions were then subjected to centrifugation for 180 s at 2500 rpm to remove the undissolved collagen, and then, degassed for 3 h to remove microbubbles. Lastly, the solution was poured in a polystyrene (PS) petri-dish and left in a fume hood for a couple of days to allow solvent evaporation. Fourier Transform Infrared Spectroscopy (FTIR) was used to identify the main functional groups of collagen before and after film formation (see Fig. 2, Bose et al., 2020 for details).

All mechanical testing (except DMA) were carried out using the Instron 5944 with a Bio-Puls Bath system (Instron 3130-100 Bio-Puls Bath, Instron, USA). The Bio-Puls system generated an environment suitable for *in-vivo* analysis of biomaterials. Dynamic mechanical analysis (DMA) was carried out with a Mettler Toledo DMA machine, using its tension mode. In this paper, collagen films tested at ambient conditions without being soaked in water are denoted as Col_D samples, while specimens tested *in-aqua* are referred to as Col_A samples (Table 1). In case of DMA, hydrated (Col_H) samples were tested as the machine could not operate in a water bath. It is worth mentioning that no significant difference in the mechanical properties were observed between films tested *in-aqua* at room temperature (RT) and at physiological temperature (37 °C) in Part I. Thus, only RT was considered in this paper.

2.1. Elasto-plastic behaviour

The elasto-plastic behaviour of collagen films was investigated under different cyclic loading regimes and environmental conditions. Specimens ($n = 5$) with dimensions of 45.00 mm $\times$ 5.00 mm $\times$ 0.05 mm (same as of tensile samples in Part I) were subjected to loading-unloading cycles under two different environmental conditions (Table 1): (a) at ambient temperature and humidity (Col_D samples); (b) inside a bio-bath containing water (Col_A samples). The Col_A collagen samples were pre-hydrated (in water) for 10 min before the loading and the increase in the thickness (approximately by 0.08 mm) was noted. Two cyclic loading regimes were employed for Col_D films were subjected: (i) incrementally increased load amplitude (nine cycles: starting at 5% of ultimate tensile strength (UTS) loading to 80% of UTS, with an increment of 10% of UTS loading from the second cycle; the UTS was 75

Fig. 1. (a) Stress-strain curve for incremental cyclic loading of in-air (Col_D) specimens (inset showing first three cycles). Effect of number of cycles on unloading modulus (b), total plastic strain (c), change in plastic strain (d) and dissipated energy (e).

MPa from Part I of Col_D samples); (ii) constant amplitude (40 cycles at 5%, 20% and 30% of UTS, representing low, intermediate and high load levels, respectively). The samples neither failed after 9 cycles in the first regime loading nor after 40 cycles after second regime. Col_A films were subjected to constant-amplitude cyclic loading for 20 cycles at 5%, 20%

and 30% of UTS (the level of UTS was 2.74 MPa for Col_A , see Part I), representing the low, intermediate and high stress levels. It is worth mentioning that none of the Col_A samples failed during these tests, and the stabilization in energy dissipation was obtained within 20 cycles. All the tests were carried out at 0.001 s^{-1} strain rate (see Part I for test

Fig. 2. (a) Stress-strain curves for monotonic cyclic loading for low, intermediate and high stress levels (5%, 20% and 30% of UTS, respectively). Effect of number of cycles on unloading modulus (b), total plastic strain (c) and change in plastic strain (d) for Col_D specimens.

results on the effect of strain rate on collagen behaviour).

et al., 2017), hydrogels (Kocen et al., 2017) etc.

2.2. Dynamic mechanical analysis

The viscoelastic behaviour of collagen was quantified using DMA. This technique separates the viscous response from the elastic one upon application of sinusoidal oscillation, usually in a range of frequencies from which the material's time-dependent behaviour was quantified.

A frequency sweep in DMA was performed at RT on collagen films ($n=3$) with dimensions 9.50 mm × 4.00 mm × 0.05 mm at constant strain. The force offset and amplitude were selected after conducting force sweeps for each sample to ensure accurate measurement of the modulus. The system was calibrated with a reference silicon bar sample with known material properties prior to the experiment. The tests were performed with Col_D and Col_H samples (Table 1). Hydrated specimens were used because the system does not operate in a water bath. It is worth mentioning that the dimensional changes (9.50 mm × 5.50 mm × 0.08 mm) for hydrated samples (pre-hydrated for 10 min) were used for data extraction. The frequency sweep was performed in the range of 0.1 Hz–10 Hz, which corresponds to the physiological frequency of normal human activities such as walking, running (Sweeney et al., 1965). This frequency range was earlier used to analyse DMA response of biomaterials such as chicken collagen (Oechsle

2.3. Creep and relaxation behaviours

Creep and relaxation behaviours of the studied collagen films were tested at various stress and strain levels. Specimens ($n=3$) with dimensions 45.00 mm × 5.00 mm × 0.05 mm (same as tensile samples in Part I) were subjected to both creep and stress-relaxation tests. For the former test, collagen films were subjected to 40%, 50% and 60% of UTS obtained from the tensile test; they correspond to 30 MPa, 37.5 MPa and 45 MPa, respectively. To estimate the creep exponent (η) using the Norton's creep law, medium stress levels was needed (Abdel-Wahab et al., 2011). The stress was ramped to the desired values and was kept constant for 5400 s. The corresponding deformation was recorded as displacement-time curves, used later to calculate the creep strain.

For stress relaxation, the extensions were ramped to deformation corresponding to tension stresses of 40, 50 and 60% of UTS loading; they were 1.4 mm, 1.92 mm and 2.52 mm, respectively. After this, the extension was constant for 5400 s, while the decrease in the force amplitude was recorded over time. The force-time values were used to generate corresponding stress-relaxation curves.

The Abaqus commercial software (Abaqus Simulia 6.13) was used to characterise the viscoelastic parameters (moduli and characteristic

times) of collagen by the relaxation modulus using the Prony series. The creep strain obtained from the experimental results was converted into the creep compliance using the following expression:

$$j_s(t) = \frac{\varepsilon(t)}{\sigma_c}, \quad (1)$$

where $\varepsilon(t)$ and σ_c are the strain at each time interval and the constant stress applied for the whole process, respectively. Normalised values of this compliances were used as an input for Abaqus software to obtain the corresponding dimensionless relaxation modulus and Prony-series parameters. The nominal stress-strain values (tensile data) were employed to describe the elastic behaviour of collagen, while normalised creep compliance was used in Abaqus as creep test data. The last value of normalised creep compliance was used as long-term modulus. Abaqus was then used to calculate the normalised dimensionless relaxation modulus ($e_R(t)$) and Prony-series parameters using the following relations:

$$e_R(t) = \frac{E_R(t)}{E_0}, \quad (2)$$

where $E_R(t)$ is the relaxation modulus at time t and E_0 is the instantaneous modulus, defined as:

$$E_R(t) = \frac{\sigma(t)}{\varepsilon}, \quad (3)$$

$$E_0 = E_R(0). \quad (4)$$

The dimensionless relaxation expression has the limits of $e_R(0) = 1$ and $e_R(\infty) = \frac{E_\infty}{E_0}$ and is defined by the Prony series expansion in the following way:

$$e_R(t) = 1 - \sum_{i=1}^N e_i (1 - e^{-t/\tau_i}), \quad (5)$$

where e_i and τ_i are the material constants and $i = 1, \dots, N$.

All the experimental results were statistically analysed using at least five specimens (for cyclic tests) and at least three specimens (for DMA, creep and stress-relaxation tests; this number was considered sufficient due to high reproducibility of results, with $R^2 > 0.95$) and is reported as mean $\pm$ standard deviation (S.D.).

3. Results and discussions

3.1. Elasto-plastic behaviour

3.1.1. Incremental cyclic loading (in air)

The tested collagen films (Col_D) demonstrated non-linear elastic-plastic behaviour for all the stress levels (nine cycles) in incremental cyclic loading. Typical stress-strain curves obtained for loading-unloading cycles are depicted in Fig. 1a, which revealed an increasing plastic deformation throughout the entire process. The inset (Fig. 1a) shows the presence of plastic deformation in collagen even at the lowest levels of stresses (first three cycles). The unloading modulus was calculated for each cycle to exclude the effect of plasticity; and an increase in the average modulus from the 1st to 9th cycle of 32% (from 1373 ± 118.5 MPa to 1812 ± 94.5 MPa) was observed (Fig. 1b), demonstrating a hardening behaviour. The unloading modulus was much higher than the $E_{\max}$ and E_{II} (1172 ± 193.3 MPa and 273.5 ± 33.1 MPa, respectively, obtained in Part I) for monotonous tension. This further denoted the effect of plastic behaviour through the entire deformation phase.

The plastic strain ε_p and change in plastic strain $\Delta\varepsilon_p$ were extracted from the cyclic curves and plotted with corresponding error bars in Fig. 1c and d, respectively. The latter parameter was calculated using the following relation:

$$\Delta\varepsilon_p(\%) = (\varepsilon_{n+1} - \varepsilon_n) \times 100, \quad (6)$$

where ε_n is the strain at n^{th} cycle.

The growing plastic deformation (as obtained from ε_p and $\Delta\varepsilon_p$) was also observed in other collagenous tissues such as bone (Dong et al., 2013) and mouse skin (Muñoz et al., 2008), subjected to progressive loading cycles. A strong effect of the progressive cyclic loading regime on accumulation of plastic deformation is apparent from Fig. 1 (c): initially very slow, the process accelerates, becoming a nearly linear one. This results in a typical sigmoidal curve for plastic strain increment (Fig. 1d), effectively reaching a plateau at the end of the test.

A hysteresis loss or dissipated energy was also assessed for the collagen film under progressive loading (Fig. 1e). It is the amount of energy dissipated during each loading-unloading cycle of deformation and obtained by integrating the area between loading-unloading curves. At lower stress levels (for first two cycles) the energy loss was rather very small at 0.037 MJ/m^3 (1st cycle) and 0.384 MJ/m^3 (2nd cycle), but increased to 248 MJ/m^3 for the last (9th) cycle signifying a more viscous behaviour at higher stress levels.

3.1.2. Constant-amplitude cyclic test

3.1.2.1. In-air cyclic test. Specimens of the collagen film (Col_D) were subjected to forty cycles with constant levels of load-amplitude 5%, 20% and 30% of UTS, corresponding to low- (3.75 MPa), intermediate- (15 MPa) and high-stress (22.5 MPa) regimes, respectively (Fig. 2a). The non-linear plastic deformation was observed across all the stress regimes. The maximum increase in deformation occurred in the first cycles for all the stress levels; it decreased gradually with continuing mechanical cycling. The unloading modulus (Fig. 2b) for the low stress level did not exhibit any specific trend, so it is insensitive to mechanical cycling. However, for intermediate and high stress levels, this modulus decreased (by 12% for the 5th cycle for the high-stress regime) after the first cycle, becoming later insensitive to increase in mechanical cycling. The almost constant modulus during this process suggests that the dissipation process was not accompanied by damage accumulation or stress softening (Liu et al., 2018). Still for all the loading conditions, the unloading modulus exceeded $E_{\max}$ (see Part I), a further demonstration of stiffening as a result of plastic deformation.

The levels of plastic strain and its incremental change $\Delta\varepsilon_p$ were calculated for different stress levels (Fig. 2c and d, respectively). The accumulation of plastic strain increased with the growing number of cycles (Fig. 2c). This behaviour can be referred to as *ratchetting*, commonly found in metals (Kang and Liu, 2008; Kang, 2008). Kang and Wu (2011) and Gao et al. (2015) also observed the ratchetting behaviour in collagen-rich tissues such as porcine skin and articular cartilage, respectively, during cyclic loadings. The ratchetting strain in collagen films exhibited their visco-elastic and plastic properties (Kang and Wu, 2011). The evolution of incremental plastic strain in cyclic loading (Fig. 2d) can be divided into two characteristic stages: Stage I- initial large plastic deformation in collagen due to recruitment or sliding mechanism of the fibrils; Stage II- plastic deformation reached the saturation, with predominantly viscoelastic response of the collagen material, within each cycle.

Apparently, accumulation of plastic strain with increase in the stress levels (Fig. 2c) was also observed in other collagen-based biological tissues (Kang and Wu, 2011). However, after 25 mechanical cycles, the values of the strain increments for all the stress levels were very close, confirming gradual stabilization of plastic deformation and damage.

Based on these observations, two distinct deformation mechanisms in the cyclically loaded collagen film may be highlighted: (i) the elastic response attributed to load-bearing capacity of collagen fibrils and fibres, found throughout the entire deformation stages; (ii) the irreversible plastic and damage responses linked to the initial intra-fibril/fibre sliding and matrix debonding, presented only during the initial loading.

Fig. 3. (a) Stress-strain curves for monotonic cyclic loading for low, intermediate and high stress levels (5%, 20% and 30% of UTS, respectively). Effect of number of cycles unloading modulus (b) and residual strain (c) for Col_A specimens.

3.1.2.2. In-aqua cyclic test. Pre-hydrated specimens (in water for 10 min) of the collagen film (Col_A) were loaded *in-aqua* with constant-amplitude cycles (20 cycles at 5%, 20% and 30% of UTS, corresponding to low-, intermediate- and high-stress regimes, respectively) in order to investigate the effect of water on elastic-plastic behaviour of collagen. None of the tested samples failed within 20 cycles of mechanical loading in all stress regimes. For *in-aqua* tests, the unloading was performed down to 0.5 MPa to avoid buckling of the samples as a result of excessive deformation in water. The collagen exhibited completely different non-linear response *in-aqua* (compared to that of Col_D) (Fig. 3a). It behaved more like a hyperelastic material. Still, the maximum plastic deformation occurred in the first cycle. Its level increased with the increase in the stress levels, showing some signs of saturation. The unloading modulus exhibited a stiffening behaviour with the increase in the number of cycles (Fig. 3b) (see also Susilo et al., 2016; Su et al., 2008), in contrast to the findings observed in Col_D (Fig. 2b), with nearly constant modulus. An increase of more than 50% in the modulus was observed for all level of stress tests: for low- 5.7 MPa–10.5 MPa; for intermediate- 18 MPa–28 MPa; for high- 22 MPa–38 MPa. This stiffening behaviour (referred to as *cyclic stiffening*) of collagen *in-aqua* conditions can be attributed to the reorientation or realignment of collagen fibrils along the loading direction (Susilo et al., 2016). The elastic moduli (E_I and E_{III}), obtained for the characteristic stages in quasi-static tests (in Part I of this paper), are also presented in Fig. 3b, demonstrating the effect of this stiffening.

The level of plastic strain caused by *in-aqua* mechanical cycling was more than an order of magnitude higher than that in the *in-air* tests. It also exhibited a ratcheting behaviour (Fig. 3c), similar in earlier reported for collagenous tissues such as porcine skin (Kang and Wu, 2011) and articular cartilage (Gao et al., 2015). It was suggested that water could act as plasticizing agent in collagenous material to allow

large-scale deformation for *in-aqua* conditions (Yuan and Verma, 2006).

3.1.2.3. Comparison between *in-air* and *in-aqua* tests

3.1.2.3.1. Hysteresis loss. The levels of hysteresis loss for a constant amplitude of cycling Col_D and Col_A were compared for each cycle and all stress regimes (Fig. 4). In all three loading regimes, the maximum energy dissipation was always observed in the first cycle for both Col_D and Col_A specimens, following gradual decrease as the number of cycles increased.

In the low stress regime (Fig. 4a), the energy dissipation dropped significantly after the first cycle for Col_A (87% from the 1st to the 2nd cycle), while this drop was less significant for Col_D specimens (27%). Moreover, the energy dissipation was higher for Col_A compared to Col_D throughout most of the process. The opposite was observed for both intermediate (Fig. 4b) and high (Fig. 4c) stress levels: the hysteresis loss for Col_D specimens was higher throughout.

These findings suggested that behaviour of Col_D was less viscous at low stress regimes; this changed at intermediate and high stresses. For both environments and all three stress levels a gradual stabilization of energy dissipated per cycle was achieved at the end of the tests.

3.1.2.3.2. Unloading modulus. The unloading modulus was calculated for each of the recovery curves extracted from the cyclic stress-strain graphs and both the normalised and absolute unloading modulus were plotted as function of a number of cycles (Fig. 5). The normalised unloading modulus exhibited contrasting trends: decreasing for Col_D and increasing for Col_A (Fig. 5a, c, e). Apparently, Col_D specimens were nearly insensitive to mechanical cycling at low (Fig. 5a) stress level, while Col_A specimens demonstrated cyclic stiffening. In case of intermediate (Fig. 5c) and high (Fig. 5e) stresses, the unloading modulus of Col_D specimen was higher for the first cycle, becoming

Fig. 4. Energy dissipation (or hysteresis loss) for low (a), intermediate (b) and high (c) levels of stress, corresponding to 5%, 20% and 30% UTS, respectively, for different environments (Col_D – in air and Col_A – in aqua).

effectively insensitive to subsequent mechanical cycling. This provides strong evidence that collagen did not exhibit damage accumulation or softening after the first cycling (Liu et al., 2018). However, for Col_A, cyclic stiffening was observed for both intermediate and high stress levels (Fig. 5c and e, respectively). For all the levels of stresses, the unloading modulus of Col_D was about two-orders of magnitude higher if compared to Col_A (Fig. 5b, d, f).

3.1.2.3.3. Residual strain. Residual strain represents the permanent deformation occurred for each cycle after the unloading process; its magnitudes were extracted for both Col_D and Col_A specimens for three used stress level. For comparability, these magnitudes were normalised with the level of strain attained after the first cycle (Fig. 6).

For all the stress levels and in both the environmental conditions, collagen depicted the ratchetting strain, previously reported in collagen-rich tissues such as porcine skin (Kang and Wu, 2011) and articular cartilage (Gao et al., 2015). At low stresses, the Col_A specimen exhibited significantly quicker strain accumulation than Col_D specimen (Fig. 6a). This difference reduced in the case of intermediate stress (Fig. 6b) effectively vanishing at high stress (Fig. 6c).

The upcoming section deals with the inelastic deformation of the collagen in-air and *in-aqua* conditions. Still, its long-term viscous time-dependent behaviour should also be assessed. Thus, in the following section, two approaches are used to determine the time-dependent response of the studied collagen film: (i) Dynamic Mechanical Analysis; (ii) creep and stress-relaxation tests.

3.2. Dynamic mechanical analysis: frequency sweep measurements

Rheological properties of polymers can be estimated in both solid samples and in melts. In this study, three different viscoelastic properties of the collagen in dry and hydrated/wet states were measured: storage modulus (E'), loss modulus (E'') and $\tan \delta$ (δ). Average curves with corresponding error bars depicting E' , E'' and $\tan \delta$ are plotted in Fig. 7 for the collagen film in dry and hydrated/wet state.

The storage modulus (E') represents the material's ability to store energy elastically. It was observed that it increased with the increase in the frequency for both Col_D and Col_H (wet) specimens (Fig. 7a), similar to other reported collagenous tissues such as mitral valve (Wilcox et al., 2014), stratum corneum of porcine (Yuan and Verma, 2006), bovine cornea (Hatami-Marbini and Rahimi, 2015) etc. The increase in the level of E' may be attributed to shorter time intervals for the collagen fibrils to relax with increase in frequency. The magnitude of E' measured for Col_D and Col_H differed drastically, by three order of magnitude; such a considerable change in the quasi-static modulus was also observed in the tensile tests of Col_D ($E_{\max} = 1172 \pm 193.3$ MPa and $E_{II} = 273.5 \pm 33.1$ MPa) and Col_H ($E_I = 2.88 \pm 0.74$ MPa and $E_{III} = 13.7 \pm 1.72$ MPa) collagen films (see Part I). Similar difference in the storage modulus was reported for wet stratum corneum as compared of dry ones (Yuan and Verma, 2006) and explained by disruption of interfibrillar bonds.

The loss modulus (E'') is linked to the viscous response and the ability of a material to dissipate energy. E'' obtained for Col_D and Col_H differed by two orders of magnitude (Fig. 7b). Moreover, the trend observed in Col_H was very different from that in Col_D. E'' decreased for

Fig. 5. Normalised (a, c and e) and absolute (b, d and f) unloading modulus for low (a and b) intermediate (c and d) and high (e and f) stress levels of collagen films subjected to in-air (Col_D) and in-aqua (Col_A) conditions.

the latter with the increase in the frequency, denoting a diminished viscous response due to restricted movement of the polymeric chains, which decreased interfacial dissipation (Abdo et al., 2019; Rath et al., 2016). So, Col_D collagen films behaved in a similar fashion to that of reinforced polymeric materials (Abdo et al., 2019; Venugopal et al., 2016; Rath et al., 2016). However, the hydrated collagen film depicted a

response more predominant in soft materials such as hydrogels (Yang et al., 2012a; Ibrahim et al., 2011; Kocen et al., 2017) and collagenous tissues (Hatami-Marbini and Rahimi, 2015; Oechsle et al., 2017). With the increase in frequency, it exhibited an initial decrease in E'' due to lower relaxation time of collagen fibrils. Whereas, the subsequent increase in E'' with the increase in frequency may be attributed to the

Fig. 6. Effect of cyclic loading on normalised residual strain for low (a) intermediate (b) and high (c) stress levels for collagen films subjected to in-air (Col_D) and in-aqua (Col_A) conditions.

presence of water molecules, acting as plasticizer disrupting the interfibrillar bonds (Yuan and Verma, 2006).

Tan delta (δ) or loss tangent, is the ratio of the loss modulus to the storage one; it characterises the damping capability of the material and its ability to dissipate energy. It is also referred to as *damping coefficient* and describes the time shift between an applied cyclic force and transmitted resultant force.

Tan δ values greater than 1 represent prominent liquid-like behaviour (viscous behaviour) while values less than 1 denote solid-like behaviour (elastic behaviour) (de Vries et al., 2017). The obtained values of tan δ for Col_D and Col_H were significantly lower than 1 (Fig. 7c), suggesting a solid-like behaviour of the collagen film. The hydrated specimens showed greater tan δ values as compared to dry ones throughout the used frequency range (Fig. 7c). Its magnitude for Col_D collagen film varied between 0.05 and 0.075, with a decreasing trend, revealing a slightly lower contribution of viscosity at higher frequencies. A striking difference was observed in Col_H where the tan δ varied in the range of 0.12–0.24, with an initial decrease followed by an increase.

Higher tan δ values represent more viscous behaviour; thus, the presence of water molecules in the Col_H samples affected the interfibrillar bonds, leading to a ductile response. Col_D samples exhibited higher elastic properties and behaved more like solid polymeric materials (Rath et al., 2016; Abdel-Wahab et al., 2011). On the contrary, the Col_H films depicted more viscous behaviour with tan δ values comparable to those of other wet collagenous materials like wet stratum corneum (Yuan and Verma, 2006) and wet bone (Pathak et al., 2011). The

obtained tan δ values were well within the respective ranges - values 0.028–0.1 and 0.12–0.25 - reported for other dry and wet collagenous materials such as cornea, bone and femora (Table 2), confirming the results obtained in this study.

An Ashby chart (Ashby, 2011) was used to compare mechanical properties (damping coefficient and tensile strength) of non-metallic engineering materials with those for pure collagen films in dry and wet conditions obtained in this study (Fig. 8). Apparently, the properties of dry collagen were in the range previously reported for collagen (yellow domain in Fig. 8) in CES Granta (Edupack, 2019), within the subset of biological materials. However, the wet collagen exhibited the mechanical behaviour outside the domain for collagen but rather close to that of soft biological materials, elastomers and foams. Hence, it would be preferable to characterise the mechanical response of collagen in conditions which wholly or partially mimic *in-vivo* environment.

3.3. Creep and stress-relaxation behaviour

Viscosity of collagen should also affect its long-term performance. To assess it, creep and relaxation tests were performed. In the former, collagen specimens were subjected to three different stress levels of 30 MPa, 37.5 MPa and 45 MPa (corresponding to 40%, 50% and 60% UTS, respectively) for 5400 s. An averaged creep response with respective data scatter was obtained at different loading conditions and presented in Fig. 9a. It was observed that in the initial few seconds the stress ramped to the desired amplitude, after which the deformation continued at a constant stress level. All the creep curves (Fig. 9a) have a similar

Fig. 7. Effect of frequency on storage modulus (a), loss modulus (b) and tan delta (c) for dry (Col_D) and hydrated (Col_H) collagen films.

Fig. 8. Ashby chart for loss coefficient plotted against tensile strength. The highlighted red and blue domains denote the mechanical properties of collagen tested in dry and wet conditions in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

trend, with increasing strain levels for higher stresses applied. The initial rapid strain growth (till about 3000 s) was followed by a much slower secondary creep for all the stress levels. A nearly constant magnitude of calculated strain rate was used to determine the end of the primary creep

stage. The presence of primary and secondary creep regimes was previously reported in collagenous materials such as dentin (Bertassoni et al., 2016), collagen fibrils (Shen et al., 2011), tendons (Shepherd et al., 2014) and bones (Abdel-Wahab et al., 2011; Novitskaya et al.,

Fig. 9. (a) Creep curves for collagen at various stress levels (30 MPa, 37.5 MPa, 45 MPa corresponding to 40%, 50% and 60% UTS), (b) isochronous curves for creep behaviour. Stress-relaxation (c) and normalised stress-relaxation (d) curves for different initial deformations - 1.4 mm, 1.92 mm and 2.52 mm (corresponding to 40%, 50% and 60% UTS).

Fig. 10. Normalised creep compliance curves obtained from experiments for 40%, 50% and 60% UTS (dotted lines) and fitted curve from Abaqus results with single set of Prony-series parameters (blue line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2014).

As well known, creep curves can be also used to estimate whether the material's viscoelastic behaviour exhibits linear scaling (Wineman and Rajagopal, 2000): in such a case, creep function J is proportional to the stress - $j(\sigma, t) = \sigma j(t)$. One way to study this is to consider the isochronous curves. Based on these curves (Fig. 9b), apparently, the studied collagen film depicted an almost linear viscoelastic response up to 60% of UTS.

A creep exponent (η) was estimated using the Norton's creep law for the secondary creep rate (for medium stress level) as follows:

$$\dot{\epsilon}_s = A(T)\sigma^\eta, \quad (7)$$

where $\dot{\epsilon}_s$ is the secondary creep-rate, $A(T)$ is the material parameter depending on the temperature T , and σ is the applied stress. The average values of parameters $A = 1.2 \times 10^{-13} \text{MPa}^{-\eta} \text{s}^{-1}$ and $\eta = 4.85$ were obtained by determining the intersect and slope of the graph plotted for the secondary creep rate against the applied stress in the double logarithmic scale.

The non-linear stress-relaxation response with time for three different levels of initial deformation - 1.40 mm, 1.92 mm and 2.52 mm, corresponding to 40%, 50% and 60% UTS, at 36 MPa, 46 MPa and 56 MPa, respectively - was obtained (Fig. 9c).

In all the cases, the stress relaxation followed the same trend: a fast

Table 3
Material constants obtained form Prony-series for collagenous materials.

Specimen Type	ϵ_1	ϵ_2	τ_1 (s)	τ_2 (s)	References
Fibrils	–	–	1.8 ± 0.2	63 ± 23	Yang et al. (2012b)
Porcine skin	0.34	0.32	52.6	2606	Remache et al. (2018)
Bones	0.118	0.0351	7.70	1061.7	Abdel-Wahab et al. (2011)
This study	0.31	0.13	64.8	1196	

initial relaxation transformed into a monotonous decrease in the stress with increasing time. At 1000 s, a drop in the stress of approximately 39% (from 36 MPa to 22 MPa), 37% (from 46 MPa to 29 MPa) and 32% (from 56 MPa to 38 MPa) occurred for 1.40 mm, 1.92 mm and 2.52 mm deformation, respectively. However, the subsequent decline in the stress from 1000 s to 5400 s was relatively low - 13.6% (from 22 MPa to 19 MPa), 10.3% (from 29 MPa to 26 MPa) and 8% (from 38 MPa to 35 MPa), respectively. Moreover, from normalised curves (Fig. 9d), it was also observed that relaxation was faster when the collagen was strained at lower deformation. Similar response was observed in collagen fibrils (Shen et al., 2011) with a quicker relaxation time. Generally, the relaxation time depended on the scale, varying from nanoseconds in collagen molecules (Buehler, 2006a,b; Gautieri et al., 2009) to some 10 s in collagen fibrils (Shen et al., 2011) to some 1000 s in tissues (Remache

et al., 2018; Abdel-Wahab et al., 2011), including collagen films in this study, which confirm that collagen hierarchy plays a significant role in stress relaxation. The viscoelastic response of a single fibril can be attributed to molecular rearrangement between collagen and water molecules (Shen et al., 2011), while at higher levels of the hierarchy, such as tendons and ligaments, this may be a result of the sliding mechanism of collagen fibres or fibrils (Screen, 2008) or uncrimping of the collagen fibres (Komatsu et al., 2007). Thus, it might be proposed that in collagen film two mechanisms - recruitment (unwinding and straightening) of fibres/fibrils and fibrils/fibres sliding - contributed to its stress-relaxation response.

3.3.1. Parameter estimation of Prony series

The normalised creep compliance obtained from experiments, for all

Fig. 11. Comparison between normalised relaxation modulus obtained with Abaqus and experimental data for various initial deformations: (a) 1.40 mm (corresponding to 40% of UTS); (b) 1.92 mm (corresponding to 50% of UTS); (c) 2.52 mm (corresponding to 60% of UTS).

the employed loading conditions (40%, 50% and 60% UTS), was fitted to extract an averaged normalised creep compliance curve (Fig. 10), to assess a single set of Prony-series constants (Table 3). The rapid decline in the contribution of subsequent terms in the Prony series allowed to limit analysis to the first two terms.

The relaxation moduli and times obtained for different collagenous materials vary significantly (Table 3). At microscopic level, the short relaxation time of fibrils is of the same order of magnitude as that of collagen embedded in hierarchical tissue (i.e. macroscopic level). This relaxation time was validated with both Maxwell-Weichert model (Shen et al., 2011) and estimation of Prony series parameter (Yang et al., 2012b). In contrast, the relaxation times of complex tissues such as skin and bone were higher (greater than 1000 s, Table 3). The long-term relaxation time obtained in this study was rather close to that of bones (Abdel-Wahab et al., 2011), while the short-term relaxation differed by an order of magnitude, as bone also contain hydroxyapatite. Alternatively, the short-term relaxation of skin (Remache et al., 2018) is close to the results of this study, while its long relaxation time varies due to the presence of ground substances such as proteoglycans (Shen et al., 2011). Also, the first modulus of skin is in close agreement to that obtained in this study.

The dimensionless normalised relaxation modulus calculated with Abaqus was compared with experimental data for initial stress levels corresponding to 40%, 50% and 60% of UTS. (Fig. 11). High levels of the coefficient of determination ($R^2 > 0.99$) were found for all the loading conditions, validating the determined Prony-series coefficient. So, in this study, the Prony-series coefficients of pure collagen at higher levels of hierarchy were determined; they can be used in simulations of viscoelastic properties of collagenous materials.

4. Conclusions

Although previous studies demonstrated the inelastic and time-dependent behaviour of collagenous tissues, there was a lack in the investigation of elasto-plastic and viscoelastic properties of pure collagen. This part of the work analysed the inelastic deformation and time-dependent behaviour of collagen under environmental conditions suitable for *in-vivo* biomedical applications. From the above experimental results, the main conclusions are:

- Cyclic loading with an increasing load amplitude resulted in an increase in the hardening behaviour of the collagen.
- For constant-amplitude cyclic loading, *in-aqua* samples demonstrated cyclic stiffening, with the growth of the modulus by about 50% at the end of 20th cycle in all the loading regimes, which may be attributed to the fibre orientation in the loading direction. On the contrary, in dry specimens, mechanical cycling resulted in a drop of the modulus - by 12% - (for high regime loading) after the 1st cycle; thereafter, the modulus was insensitive to the increase in the mechanical cycling.
- Ratchetting strain accumulation was observed for both dry and *in-aqua* cycling for all stress levels, as a result of plastic deformation.
- DMA tests provided in $\tan \delta$ values of 0.05–0.075 for dry collagen films, approximately three times lower than those for hydrated collagen (0.12–0.24), demonstrating the effect of hydration that affected the interfibrillar bonds, leading to ductile regime as earlier reported for wet stratum corneum (Yuan and Verma, 2006).
- The time-dependent (creep) behaviour exhibited a rapid increase in the creep strain (primary creep) followed by a rather slow secondary regime. The stress relaxation followed a typical exponential decay behaviour: a fast initial relaxation was observed up to 1000 s, leading to a slow reduction of stress.
- The experimental strain-time data obtained in tensile creep were used to extract the Prony series coefficients (relaxation moduli and times).

CRediT authorship contribution statement

Shirsha Bose: Investigation, Data curation, Writing - original draft.
Simin Li: Conceptualization, Methodology, Writing - original draft.
Elisa Mele: Conceptualization, Methodology, Writing - original draft.
Vadim V. Silberschmidt: Conceptualization, Methodology, Writing - original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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