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Electrospun collagen variability characterized by tensile testing

Ján Kužma[1,2] [0000-0002-2537-4119] Lukáš Horný[1][0000-0003-2667-249X] Tomáš Suchý[1,2][0000-0002-2656-4280] Monika Šupová[2][0000-0002-0902-9059] Zbyněk Sucharda[2][0000-0001-9068-3957]

[1] Faculty of Mechanical Engineering, Czech Technical University in Prague, Technická 4, Prague, 160 00, Czech Republic
[2] Institute of Rock Structure and Mechanics, Academy of Sciences of the Czech Republic, V Holešovičkách 41, Prague 8, 182 09, Czech Republic
kuzma@irsm.cas.cz

Abstract. The objective of this study was to verify repeatability of the preparation process of the collagen nanofibrous layers. The layers were fabricated by means of electrospinning. Constant conditions were held within the entire production process. It means that all samples were fabricated from collagen solution with the same chemical composition. Electrospinning conditions were also adjusted to have the same parameters, i.e. temperature, relative humidity, voltage and distance between electrodes. Finally, cross-linking procedure was also the same for all samples involved in the study. Elasticity of the material was assessed by means of uniaxial tensile test in a fully hydrated state of collagen samples. Internal structure of collagenous layers was studied by means of scanning electron microscopy. Although all technological conditions were held constant, mathematical modelling of the elastic behaviour revealed differences between samples. The results suggest that there is a certain variability of mechanical properties of electrospun hydrated collagen that is difficult to eliminate. By this fact, however, collagen is, to some extent, similar to biological tissue.

Keywords: Collagen, Constitutive model, Electrospinning, Hyperelasticity, Microstructure

1 Introduction

Collagen is one of the most occurring proteins. It makes up to 30% of all proteins in human body [1-6]. Collagen exists in the form of fibres which are formed by fibrils of chained monomers referred to as tropocollagen [5]. Tropocollagen molecule consists of triple-helix built from alpha chains of amino acids. Depending on a specific repeating arrangement of amino acids, at least 29 types of collagen are distinguished [3].

It is generally accepted that collagen provides structural strength and stability to tissues and organs [6, 7]. It has excellent biocompatibility, biodegradability and weak antigenicity [4]. It is considered as an excellent biomaterial and is used in various medical applications [4]. Collagen isolate may lose some properties due to hierarchical changes during the isolation and purification process [2]. Electrospinning is used to imitate collagen's natural fibrillary structure which should restore its physical properties [3].

During the last several years, electrospun collagen has been used in many studies and experiments in research fields covering, for example, regenerative medicine, tissue engineering, and drug delivery systems [8-12]. In applications at which the electrospun material will serve as a load-bearing element, the consistency of the mechanical properties may be crucial. One can name bone implants, where electrospun collagen enhances the rate of osseointegration [13, 14], or vascular prostheses, that may be based on collagenous matrix [15], as examples.

Mechanical properties of collagen materials can be also linked to their drug delivery capability. Since drug releasing mechanism is mostly based on a surface area which relies on the size and diameter of a structural fibre, it is clear that mechanical properties, naturally conditioned by an internal architecture, and drug delivery capability are mutually correlated. From this point of view, studies focused on reproducibility of the electrospinning fabrication of collagenous materials are an ordinary part of the materials science and development of reliable production technologies.

2 Methods

2.1 Material fabrication

Collagen nanofibrous materials were prepared based on 8 wt% collagen (VUP Medical, Czech Republic) phosphate buffer/ethanol solution to which was added polyethylene oxide as an auxiliary polymer (PEO, Mn 900,000, Sigma-Aldrich, Germany) in an amount of 8 wt% to the weight of collagen.

Electrospun materials were fabricated using a high voltage of 45 kV and the feeding rate was 80 $\mu l \cdot min^{-1}$. The temperature was kept at 26 °C, needle to collector distance was set to 20 cm and the relative humidity was kept at 23–26% (4SPIN, Contipro, Czech Republic). The electrospinning process was enhanced by combination with electroblowing. The flow rate of the preheated air (25 °C) was set at 10 $l \cdot min^{-1}$. All the electrospun materials were collected on a static collector (22 × 29 cm).

The stability of all the collagen layers was enhanced by means of cross-linking with a 95% ethanol solution containing EDC and NHS at a weight ratio of 4:1; the EDC and NHS (Sigma Aldrich, Germany) were used as received. Following a reaction period of 24 h at 37 °C. Finally, all the layers were washed in 0.1 M Na_2HPO_4 (2 × 45 min) and subsequently rinsed with deionised water (30 min). Layers were frozen at -15 °C for 5 h and lyophilised (BenchTop 4KZL, VirTis, U.S.A.).

Five groups of layers were fabricated in the above-mentioned procedure. They were obtained even from a single collagen batch.

2.2 SEM image analysis

The inner structure of specimens was characterized by means of scanning electron microscopy (SEM; Quanta 450 Microscope, FEI, U.S.A.) in high vacuum mode. The specimens were cut by a surgical knife prior to SEM analysis. The resulting cross-sections were coated with a thin layer of gold in an ion sputter (Emitech K550X, Quorum Technologies, UK).

The diameter of fibres was measured by means of ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://image j.nih.gov/ij/,1997–2015). The manual mode of the ImageJ analyser was used for the measurement of the average diameter of the fibres. At least 10 fibres were assessed at each of six SEM micrographs (mag. 10 000× n = 3, and 25 000x n = 3) for each layer type.

2.3 Mechanical testing

Tensile tests were conducted using a Zwick/Roell multipurpose testing machine equipped with a built-in video extensometer. By using contrasting marks on the surface of the samples, the video extensometer automatically determined the reference length and elongation of the samples. Tensile experiments were conducted at a constant clamp velocity of 0.1 mm·s^{-1}. The loading force was measured by the U9B (±25 N, HBM, Germany) force transducer.

Mechanical properties were evaluated by means of the uniaxial tensile tests of rectangular strips of the layers which were hydrated in physiological solution (the average width and length of the samples were approximately 10 mm and 40 mm, respectively).

During the test procedure, 5 preloading cycles were carried out as preconditioning. The sixth loading sequence was used in the subsequent mathematical modelling. To this end, the engineering strain defined as the ratio of the elongation of the sample to its reference length, and nominal stress defined as the ratio of applied force to the reference cross-section of the sample were determined from experimental records.

2.4 Model

To model fully nonlinear mechanical behaviour of hydrated collagen layers, hyperelastic material description was adopted. Hyperelastic material is characterised by the strain energy density function W. Fung-Demiray exponential model [16], frequently used in soft tissue biomechanics, was employed. Its particular form is expressed in the equation (1).

$$W = \frac{\mu}{2\alpha}\left(e^{\alpha(I_1-3)} - 1\right) \tag{1}$$

Here, μ is stress-like material parameter corresponding to infinitesimal shear modulus and α is dimensionless parameter modulating strain-stiffening response. I_1 is the

first principal invariant of the right Cauchy-Green strain tensor **C** which is obtained from $\mathbf{C} = \mathbf{F}^T\mathbf{F}$. **F** is the deformation gradient tensor.

Kinematics of the uniaxial tensile test was assumed to be described by (2). Here, $\boldsymbol{X}$ is a position vector of a material particle in the reference configuration and $\boldsymbol{x}$ is its position vector in the deformed state. In such a case, the deformation gradient **F**, that is defined as $\mathbf{F} = \partial\boldsymbol{x}/\partial\boldsymbol{X}$, has the form $\mathbf{F} = diag[\lambda_1,\lambda_2,\lambda_3]$ and $I_1 = \lambda_1^2 + \lambda_2^2 + \lambda_3^2$.

$$x_1 = \lambda_1 X_1 \qquad x_2 = \lambda_2 X_2 \qquad x_3 = \lambda_3 X_3 \tag{2}$$

In hyperelastic materials, the strain energy density function W serves as a potential function for stress. In case of the nominal (the first Piola-Kirchhoff) stress tensor **P**, this is expressed in the equation (3) which plays a role of the constitutive equation.

$$\mathbf{P} = \frac{\partial W}{\partial \mathbf{F}} - p\mathbf{F}^{-T} \tag{3}$$

In the equation (3), the assumption of incompressible behaviour of collagen was adopted. Which means that p is Lagrangean multiplier enforcing incompressibility constraint and has to be determined from a force boundary condition. Combining (1), (2), (3), incompressibility condition $det(\mathbf{F}) = 1$, and isotropy of the material, final expression for the nominal stress sustained by the sample in uniaxial tension applied in the direction 1 is obtained. It is expressed in (4). The relation between λ_1, used in (4), and engineering strain ε_{11}, measured in the experiment, is given by $\lambda_1 = 1 + \varepsilon_{11}$.

$$P_1 = \mu\left(\lambda_1 - \frac{1}{\lambda_1^2}\right)e^{\alpha\left(\lambda_1^2 + \frac{2}{\lambda_1} - 3\right)} \tag{4}$$

Employing least square method, parameters μ and α characterising every tested sample were determined. Least square optimization was carried out in multipurpose computer algebra system Maple (Maplesoft, Canda).

2.5 Statistical evaluation

Statistical analysis was conducted in Maple (Maplesoft, Canada). The coefficient of determination R^2 was used to evaluate a quality of the regression model (4). As usual, R^2 was considered to be equal to 1 minus ratio of the residual sum of squares to the total sum of squares. The resulting values of R^2 were displayed in a boxplot. Graphical methods were adopted to show whether differences in the mechanical behaviour of groups of samples exist. First, clusters of the material parameters μ and α in 2D phase space were created. Second, a convex hull for each group was added to the figure. Finally, diameters of collagen fibres obtained in SEM were compared by means of Kruskal-Wallis test to estimate if differences in the internal structure of collagenous layers exist. Dunn's test was used in post hoc analysis to show which groups differ. Significance level 0.05 was considered within the entire study.

3 Results and Discussion

A total number of 40 uniaxial tensile tests were conducted. Unfortunately, several samples did not survive the complete testing procedure and were eliminated from the subsequent analysis. Thus, final number of samples in each group was as follows: $n_1 = 7$, $n_2 = 8$, $n_3 = 8$, $n_4 = 5$, and $n_5 = 4$. Index i denotes the group number and $n = \Sigma n_i = 32$.

Figure 1 depicts resulting stress-strain responses and their models described by (4). The boxplot in the lower-right panel of Figure 1 summarizes coefficients of determination obtained in the least square minimization. Considering that none of R^2 is lesser than 0.97, it can be concluded that (4) is a suitable model for a description of hydrated collagen mechanical response.

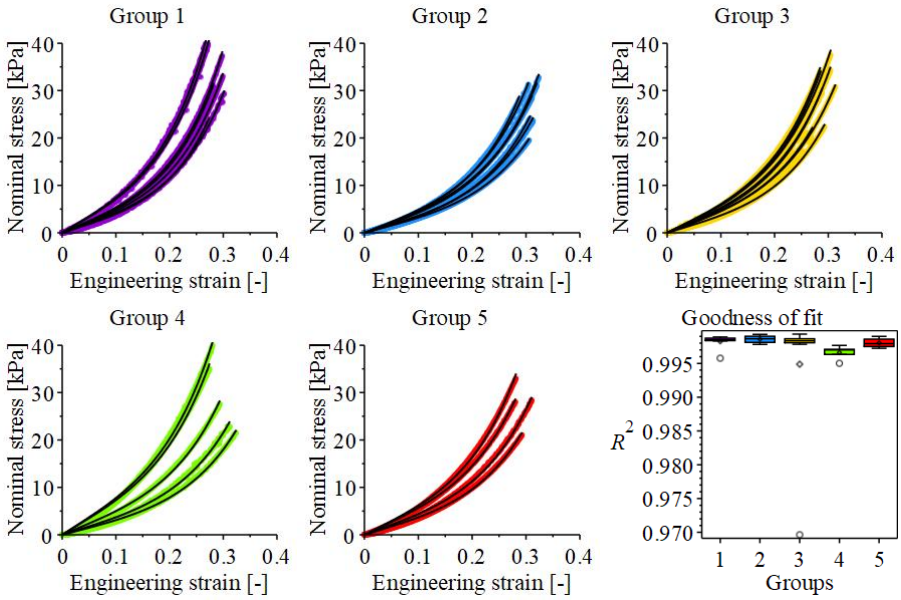

Fig. 1. Nominal stress-engineering strain relationships obtained from the experiment and fitted by the model (4). Bottom-right panel shows the boxplot of resulting coefficients determination.

Table 1. Expected values of the material parameters and their variability. IQR denotes interquartile range and SD denotes sample standard deviation.

Group	α [–]	IQR	μ [kPa]	SD
1	5.27	5.17-5.34	16.97	4.30
2	4.34	4.14-4.50	17.34	4.03
3	4.57	4.44-4.69	16.49	3.66
4	4.18	4.05-4.34	16.42	4.09
5	4.32	4.22-4.46	16.54	4.71

Mean values (arithmetic mean in case of μ and median in case of α) of the material parameters estimated in the nonlinear regression analysis are presented in Table 1.

Figure 2 in its left panel depicts all experimental data. Individual groups are distinguished by the same colours as in Figure 1. Since individual stress-strain curves mutually overlap, a hypothetical conclusion could be drawn that differences in the mechanical behaviour of the groups would be negligible. However, the right panel in the same figure, where convex hulls of material parameters are depicted, suggests that previous conclusion is false. At least Group 1, depicted with violet colour, seems to be different from others because it has an empty intersection with all other groups. Another conclusion, which can be drawn from the simplified cluster analysis, is that the parameter μ (horizontal axis) seems to fall into approximately the same range in all groups. Thus, in the model (4), it is only α which reflects differences between material groups.

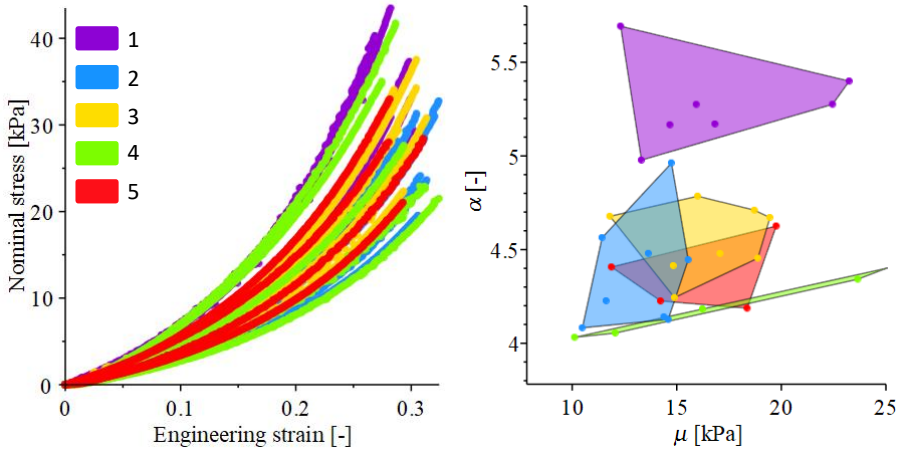

Fig. 2. Nominal stress-engineering strain relationships obtained from the experiment (left panel), two-dimensional graph of material parameters and convex hulls (right panel).

The panels of Figure 3 show microphotographs obtained via SEM microscopy. Fibrous structure of collagen electrospun is clearly visible. By means of image analysis, 60 measurements of fibre diameters were conducted in every group. Resulting data are compared in the boxplot presented in Figure 3. The same colour scheme as in Figure 1 and 2 is used again. The results clearly suggest that Group 1 is different from others (*p-value* < 0.001).

To be able to identify which groups mutually differ, Dunn's post hoc test was employed. Its results showed that Group 1 differs from all other groups (*p-value* < 0.001 in all cases). Also Group 2 differs from 3 and 4 (*p-value* = 0.01 and 0.001, respectively). Remaining pairs of groups did not show significant differences in fibre diameters.

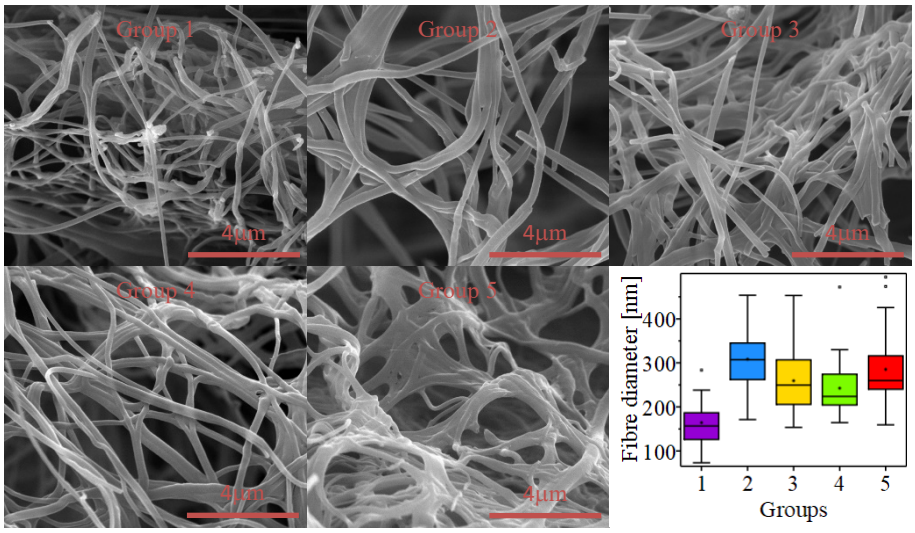

Fig. 3. Representative SEM microphotographs (mag. 25 000x). The boxplot of fibre diameters obtained in the image analysis.

Results suggest that, although constant conditions are kept during electrospinning, and the same collagenous solution is used, the final materials may differ in their mechanical properties and structure. Therefore, some extent of variability in mechanical properties of electrospun collagen should be taken into account in bioengineering applications.

Conflict of Interest

The authors declare that they have no conflict of interest.

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