Acta Biomaterialia 6 (2010) 3687–3693

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Structure–property relationships of a biopolymer network: The eggshell membrane

Fernando G. Torres *, Omar P. Troncoso, Franco Piaggio, Alfredo Hijar

Department of Mechanical Engineering, Catholic University of Peru, Lima 32, Peru

ARTICLE INFO

Article history:
Received 18 September 2009
Received in revised form 5 March 2010
Accepted 8 March 2010
Available online 21 March 2010

Keywords:
Eggshell membrane
Biopolymer
Polymer network
Mooney–Rivlin

ABSTRACT

The eggshell membrane (ESM) is a biopolymer network that may have potential applications in biomedicine, but it also may reveal important details regarding the behaviour of biopolymer networks. In this paper, we have studied the mechanical and morphological properties of the ESM in order to reveal important structure–property relationships. Light optical microscopy and atomic force microscopy were used to assess the morphology of the ESM. The mechanical properties of membranes and individual fibres were studied by means of tensile tests and nanoindentation tests, respectively. The mechanical behaviour of ESM networks in different environmental conditions showed a non-linear and a linear regime. As for elastomers and other biopolymer systems, the non-linear regime was modelled by the Mooney–Rivlin relation. The Young's modulus in the linear regime of the network was related to the Young's modulus of the individual fibres using Gibson and Ashby analysis for cellular solids. The results of morphological characterization were used to relate the properties of individual fibres to the properties of the whole networks. This enabled us to predict the macroscopical properties of the network based on the properties of the individual fibres. It was found that the ESM networks behaved as both Mooney–Rivlin and Hookean materials in different environmental conditions. This study helps elucidate the properties of the biopolymer networks found in nature and describes important mechanical properties for the use of the ESM as a biomaterial.

1. Introduction

The eggshell membrane (ESM) is a tissue found between the calcified eggshell and the albumen of eggs. This structure is a thin, highly collagenized fibrous membrane comprising inner (in contact with the albumen) and outer layers. It is mainly formed by types I, V and X collagen, making up 88–96% of its dry weight. The presence of other proteins, such as osteopontin, sialoprotein and keratin, has also been reported [1,2].

ESM is an industrial waste natural product that can be readily obtained in the food industry. Different studies have tried to use it in the recovery of gold from waste water [3,4]. Some characteristics of the ESM, such as the interconnected porous structure and the ability to transport nutrients to the developing embryo, suggest that it could be used as a biomaterial.

The biologically active ESM is essential for the formation of the egg, retaining the albumen and preventing the penetration of bacteria [5]. Due to these properties, both outer and inner ESM have been used in the past as a membrane for guided bone regeneration [6] and as a biological dressing to promote infection-free healing of wounds [7,8].

Although there are reports on the use of ESM as a biomaterial, not much information is known about its physical and structural properties relevant to this type of application, such as the pore and mechanical characteristics of the membrane. In fact, the ESM can be considered as a coherent network of fibrous proteins in which the behaviour of the structure is determined by both the individual fibres and the nature of the interaction between them. The physical properties of such a network must be known in order to control and improve the use of ESM for biomedical applications.

As in other tissue structures, fibrous biopolymers are the structural elements in ESM. Collagen is a biopolymer that is present in many biological systems. It forms several hard and soft tissues in animals, and is the main load-carrying element in blood containers, skin, tendons, cornea, etc. [9]. The collagen molecule has a triple-helical conformation and aggregates to form fibrils whose bundles form fibres.

The study of biopolymers based systems using a materials science approach can be useful in understanding the mechanical behaviour of such biological structures. Previous reports from this laboratory have assessed the mechanical properties of different systems formed by biopolymer networks, such as bacterial cellulose networks [10], spider silk [11] and collagen-based systems [12,13]. The study of scales from the Amazonian fish *Arapaima gigas*, has shown that this system behaves as a nanocomposite laminate structure of collagen and hydroxyapatite [12]. The tests

* Corresponding author.
E-mail address: fgtorres@pucp.edu.pe (F.G. Torres).

1742-7061/$ - see front matter © 2010 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2010.03.014

carried out on the biopolymer fibres from the byssus of the mussel *Aulacomya ater* show that such collagen-based fibre behaves like a Mooney–Rivlin material [13].

The aim of this paper was to study the morphological and mechanical characteristics of the outer ESM of hen eggs using a materials science approach. In order to assess the geometry of the membrane, morphological characterization was carried out with optical and atomic force microscopy. Atomic force microscopy nanoindentation tests were performed to estimate local mechanical properties. Finally, the mechanical behaviour of ESM in different media was studied using tensile tests at different conditions.

2. Materials and methods

2.1. Materials

ESM was obtained from commercial breeding lines of *Gallus gallus*. The outer membranes were carefully removed using clamps and washed with distilled water. The membranes were then stored in either water or albumen in order to avoid dehydration. Samples were cut out of the membrane in both latitudinal and hemispherical directions. The specimens were cut while immersed in water or albumen using a cutting tool specifically designed for this task.

2.2. Characterization techniques

2.2.1. Tensile tests

A MARK-10 bench top tensile testing machine equipped with a 10 N ± 0.5% load-cell was used. Rectangular samples (15 mm × 3.5 mm) were used for the measurements. The thicknesses of the membranes (around 100 μm) were obtained from digital micrographs, using a BRUNEL BMZ trinocular stereomicroscope equipped with a graticule in conjunction with image analysis software (ImageJ, National Institutes of Health, USA). The deformation of the sample was assumed to be equal to the separation of the crossheads. Three speeds were used: 10, 20 and 30 mm min^{-1}. Six samples were tested per condition.

Tests were made in three different states: dry, immersed in albumen and immersed in distilled water. For the tests performed with hydrated samples (immersed in water or albumen), a transparent polypropylene container was fitted to the tensile testing machine. The height of the container was enough as to allow the specimen clamps and the sample to be fully immersed in the fluid (water or albumen) during the test (see Fig. 1).

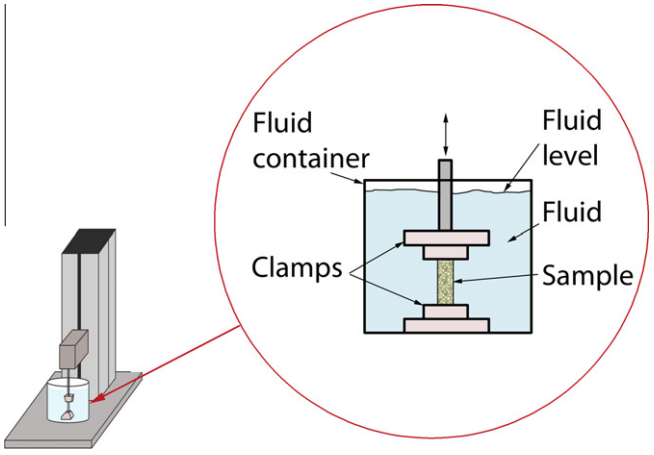

Fig. 1. Experimental rig used for tensile tests in hydrated samples.

Specimens for dry tests were dried in air for 24 h. The data were analysed using statistical tests including analysis of variance (ANOVA) and the Student–Newman–Keuls method for pairwise comparisons. The null hypothesis was rejected at a significance level of 0.05.

2.2.2. Morphology

The morphology of the hydrated samples was assessed with a Brunel inverted light optical microscopy and a Brunel BMZ trinocular stereomicroscope. The dried samples' morphology was assessed with an RJ Lee scanning electron microscope, using voltages in the range 10–20 kV. Specimens were mounted onto stubs and then gold coated as described in a previous report from this laboratory [14]. Further studies on dried samples were performed with a Nanosurf Easyscan 2 atomic force microscope (AFM) in the dynamic mode. A cantilever with a nominal spring constant of 42 N m^{-1}, a resonance frequency of 179 kHz and a tip radius of less than 10 nm was used.

2.2.3. Porosity estimation

The porosity of the ESM network was estimated by image analysis according to the following expression [15]:

$$P = 1 - \frac{n}{N} \tag{1}$$

where n stands for the number of pixels in the fibres and N is the total number of pixels in the micrograph.

2.2.4. Nanoindentation

The same AFM was used in the static mode for nanoindentation. Nanoindentation tests were performed to assess the mechanical properties of individual fibres (see Fig. 5b) avoiding void spots. The samples immersed in water were blotted dry and mounted in the AFM. Nanoindentation was carried out immediately after the sample was mounted in order to minimize the effect of dehydration of both the ESM fibres and the ESM.

In this test, the cantilever of the AFM is brought towards the sample while its deflection is recorded as a function of its vertical position. The measurements were repeated five times in each indentation point. The average values were used to plot the curve of the cantilever deflection against the height of the sample.

If we consider an infinitely stiff tip and a soft flat sample, the Hertz model can be used to predict the relation between indentation and loading force [16–19]. The Young's Modulus can be estimated by following Eq. (2):

$$z - z_0 = d - d_0 + \sqrt{\frac{k}{(\pi/2)[E/(1-\upsilon^2)]\tan\alpha}}\sqrt{d - d_0} \tag{2}$$

where E is the Young's modulus, υ is the Poisson ratio (taken as 0.5, corresponding to an incompressible material), k is the spring constant of the cantilever (0.28 N m^{-1}), α is the opening angle of the cone (50°), z is the height of the sample and d is the deflection of the cantilever. The 0 subscript accounts for the offset values.

3. Results and discussion

3.1. Morphology

As we have noted in the Introduction, ESM is a biopolymer network structure. Fig. 2a shows a hydrated sample in which the network is formed by several fibres arranged without a specific direction. Image analysis was used to make an estimation of the network properties. The fibre diameter was 1.95 ± 1.28 μm on average (Fig. 2b) and the mesh size was 3.96 ± 3.70 μm (Fig. 2c).

Fig. 3a depicts the ESM membrane morphology seen in SEM. The vacuum used for the SEM examination changes the morphol-

ID	Title	Pages
2164	Structure–property relationships of a biopolymer network: The eggshell membrane	7