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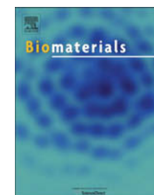

Microscale mechanical properties of single elastic fibers: The role of fibrillin–microfibrils

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ABSTRACT

Micromechanical properties of single elastic fibers and fibrillin–microfibrils, isolated from equine *ligamentum nuchae* using chemical and enzymatic methods, were determined with atomic force microscopy (AFM). Young's moduli of single elastic fibers immersed in water, devoid of or containing fibrillin–microfibrils, were determined using bending tests. Bending freely suspended elastic fibers on a micro-channeled substrate by a tip-less AFM cantilever generated a force versus displacement curve from which Young's moduli were calculated. For single elastic fibers, Young's moduli in the range of 0.3–1.5 MPa were determined, values not significantly affected by the presence of fibrillin–microfibrils. To further understand the role of fibrillin–microfibrils in vertebrate elastic fibers, layers of fibrillin–microfibrils were subjected to nano-indentation tests. From the slope of the force versus indentation curves, Young's moduli ranging between 0.56 and 0.74 MPa were calculated. The results suggest that fibrillin–microfibrils are not essential for the mechanical properties of single vertebrate elastic fibers.
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1. Introduction

Elastic fibers are essential structures which endow resilience to the extracellular matrix (ECM) by a passive, entropy-driven mechanism allowing stretching and recoil [1]. In vertebrates, the elasticity of several tissues and organs such as blood vessels, skin, lung, muscle, ligaments and cartilage [2–4] is provided by elastic fibers. Using macro-mechanical testing, the elasticity of elastic fiber-rich tissues has been a subject of study for years. Young's modulus of elastic fiber-rich tissue samples of purified dog or sheep aorta was determined to be in the range of 0.13–0.65 MPa [5]. Young's moduli between 0.1 and 0.8 MPa were calculated for tissue samples of purified pig aorta enriched with elastic fibers [6]. Young's modulus of single elastic fibers isolated from bovine *ligamentum nuchae* was, for the first time, determined by Aaron et al. [7] using a microtest apparatus attached to a polarizing microscope and was in the range of 0.4–1.2 MPa. The vertebrate elastic fibers contain at least two morphological components: amorphous elastin, which accounts for 90% of the elastic fibers, and microfibrils, which are 10–12 nm in diameter [1,8]. Fibrillin-1 is the major component of the microfibrils [3], although other, less abundant, molecules like microfibril-associated glycoproteins (MAGPs) [9–11], emilins [12–14], and latent transforming growth factor-β binding proteins (LTBPs) [15–17] have been identified.

Fibrillin–microfibrils are considered necessary for the assembly of a functional elastic fiber. During elastic fiber formation, fibrillin–microfibrils appear first and serve as a scaffold for the deposition of tropo-elastin [8]. Invertebrates lack elastin, and the essential elastic recoil of their tissue can only be provided by fibrillin–microfibrils, suggesting that fibrillin–microfibrils are elastic [18–20]. Young's modulus of the invertebrate microfibrillar network is in the range of 0.2–1.1 MPa [19–21]. The question arises as to whether vertebrate fibrillin–microfibrils also have similar mechanical properties and play a role in the mechanical properties of vertebrate organs and elastic fibers. Work on mammalian ciliary zonular filaments, a structure solely composed of fibrillin–microfibrils, gave insight into the mechanical properties of vertebrate fibrillin–microfibrils. X-ray diffraction and biomechanical testing of zonular filaments indicated that fibrillin–microfibrils have Young's moduli in the range of 0.19–1.88 MPa, similar in magnitude to invertebrates fibrillin–microfibrils and elastic fibers [22–24]. However, using

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molecular combing, Young's moduli of single fibrillin–microfibrils from zonular filaments was estimated to be 78–96 MPa, which are two orders of magnitude higher than the modulus determined from tissue samples [25]. From these data, the authors suggested that fibrillin–microfibrils play a role in reinforcing the vertebrate elastic fibers. Recent work by Lillie et al. also indicated that removal of fibrillin–microfibrils from the elastic fibers of porcine aorta results in a slightly decreased Young's modulus at low strain and a slightly increased modulus at high strain [6]. To date, there is debate on how fibrillin–microfibrils contribute to the mechanical properties of elastic fibers and in what order of magnitude Young's modulus of fibrillin–microfibrils ranges.

Since elastin is one of the most abundant proteins in human tissues, elastic fibers are regarded as highly suitable scaffolding material for tissue engineering applications. For the use of elastic fibers as a biomaterial, purity of the material is an important issue. Contamination with proteins may lead to unwanted immunological reactions. Efforts have been made to use purified intact elastic fibers as scaffolds in tissue engineering [26–29]. From the work of Daamen et al., it is suggested that purified intact elastic fibers devoid of fibrillin–microfibrils are preferred for scaffold purposes [29]. The mechanical properties of the purified elastic fibers, devoid of or containing fibrillin–microfibrils, should also be determined for an adequate use of these fibers in tissue engineering.

This study reports on the biomechanical properties of single purified elastic fibers. Highly purified elastic fibers, devoid of or containing fibrillin–microfibrils, were successfully extracted from equine *ligamentum nuchae* and measured with an atomic force microscope (AFM). Micromechanical bending tests, similarly as previously described [30], were for the first time performed on single elastic fibers to determine Young's moduli of the fibers. Young's modulus of fibrillin–microfibrils was determined using AFM-based nano-indentation. The contribution of fibrillin–microfibrils to the mechanical properties of the vertebrate single elastic fibers was evaluated using the combined results from bending and nano-indentation experiments.

2. Materials and methods

All experimental procedures were performed at room temperature, unless stated otherwise.

2.1. Isolation of elastic fibers

Elastic fibers, devoid of or containing fibrillin–microfibrils, were isolated from equine *ligamentum nuchae* using a modification of previously published work of Daamen et al. [31,32]. *Ligamentum nuchae* was pulverized under liquid nitrogen conditions using a pulverisette with a 1 mm sieve (Fritsch pulverisette 19, Idar-Oberstein, Germany). The pulverized tissue was subjected three times to an overnight extraction with 10 vol. (10 ml/g) of 1 M NaCl containing 0.02% (w/v) NaN_3 at 4 °C. After each extraction step, insoluble material was recovered by centrifugation at 5000 × *g* at 4 °C for 20 min. After the last extraction step, the pellet was washed with demineralised water and resuspended in 10 vol. of ethanol. After 90 min, the suspension was filtered through a paper funnel. This procedure was repeated with 10 vol. of chloroform/methanol (2:1) for 90 min, 10 vol. of acetone for 30 min, and 10 vol. of ether for 30 min. The resulting residue was dried in a desiccator. The dried material was extracted with 15 vol. of 97% formic acid with 1% (w/v) cyanogen bromide for 24 h under non-oxidizing conditions. After extraction, the suspension was diluted with 45 vol. of demineralised water and centrifuged at 5000 × *g* at 4 °C for 15 min. The pellet was washed with demineralised water until a pH of 6–7 was reached. After washing, the pellet was resuspended in 10 vol. of demineralised water and this suspension was divided in two parts. One half of the suspension was used to isolate elastic fibers devoid of fibrillin–microfibrils (Method I) and the other half was used to isolate elastic fibers containing fibrillin–microfibrils (Method II).

2.1.1. Method I

After centrifugation, the pellet was resuspended in 5 vol. of 0.5 M Tris–HCl pH 6.8 containing 4 M urea, 1 M β-mercaptoethanol, and 0.02% (w/v) NaN_3 and incubated overnight. This extraction step was repeated three times and after each step insoluble material was recovered by centrifugation at 5000 × *g* at 4 °C for 20 min. The resulting pellet was washed for 6 times with 5 vol. of demineralised water. After centrifugation, the pellet was resuspended in 5 vol. 0.1 M NH_4HCO_3, pH 8.2 containing 0.02% (w/v) NaN_3 and 10,000 U trypsin (T-4665, Sigma, St. Louis, USA) and incubated for 4 h at 37 °C. The suspension was centrifuged and the pellet was washed with demineralised water, followed by 3 overnight extractions with 5 vol. 1 M NaCl containing 0.02% (w/v) NaN_3. The resulting pellet was washed with demineralised water and the end product was stored at −80 °C.

2.1.2. Method II

After centrifugation, the pellet was resuspended in 5 vol. 0.2 M Tris–HCl pH 7.4 containing 0.05 M $CaCl_2$, 0.02% (w/v) NaN_3, and 500 U collagenase type VII (Sigma) and incubated for 4 h at 37 °C. The suspension was centrifuged and the pellet was washed with demineralised water, followed by 3 overnight extractions with 5 vol. 1 M NaCl containing 0.02% (w/v) NaN_3. The resulting pellet was washed with demineralised water and the end product was stored at −80 °C.

2.2. Purity assessment

2.2.1. Gel electrophoresis

Elastic fiber preparations were analyzed under reducing conditions (5% β-mercaptoethanol) on a 10% (w/v) polyacrylamide gel. Proteins were visualized by silver staining using a 0.1% (w/v) $AgNO_3$ solution.

2.2.2. Transmission Electron Microscopy (TEM)

Elastic fiber preparations were embedded in 1.5% (w/v) agarose, fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.4, post fixed with 1% (w/v) osmium tetroxide, dehydrated in ascending series of ethanol, and embedded in epon 812. Ultrathin sections (60 nm) were picked up on formvar-coated grids, post-stained with lead citrate and uranyl acetate, and subsequently imaged using electron microscopy (JEOL 1010, Tokyo, Japan).

2.2.3. Scanning Electron Microscopy (SEM)

Lyophilized elastic fiber preparations were mounted on stubs and sputtered with an ultrathin layer of gold in a polaron E5100 SEM coating system. Specimens were studied with an SEM apparatus (JEOL JSM-6310, Tokyo, Japan) operating at 15 kV.

2.2.4. Immune Fluorescence Assay (IFA)

Elastic fiber preparations were suspended in demineralised water and frozen in liquid nitrogen. 4 μm cryosections were cut and incubated in 1% (w/v) bovine serum albumin (BSA, fraction V, Sigma) in PBS to block aspecific binding sites. Sections were incubated overnight at 4 °C with rabbit anti-bovine type I collagen (1:50, Chemicon, Temecula, USA), rabbit anti-human fibrillin-1 (1:500, a kind gift of Dr. Dieter Reinhardt [33], and mouse anti-bovine elastin (1:500, clone BA-4, Sigma) diluted in PBS containing 1% (w/v) BSA. After washing with PBS, bound antibody was detected with 1:100 diluted goat anti-mouse IgG Alexa 488 or goat anti-rabbit IgG Alexa 488 (Molecular Probes, Eugene, USA) in PBS containing 1% (w/v) BSA for 90 min. Sections were washed in PBS and mounted in mowiol (4-88, Calbiochem, La Jolla, USA).

2.3. Micromechanical bending tests of elastic fibers using AFM

Quartz glass substrates with parallel micro-channels were prepared by reactive ion etching using an RIE Elektrotech system (Elektrotech Twin PF 340, UK). The width and depth of the channels were determined by AFM (home-built instrument) and SEM (LEO Gemini 1550 FEG-SEM, Oberkochen, Germany) measurements.

Diluted suspensions of elastic fiber preparations, devoid of or containing fibrillin–microfibrils, were prepared by adding 15 mg of preparation I or II to 20 ml demineralised water. Glass substrates were incubated in the diluted suspension for 10 min, and dried overnight.

A home-built AFM combined with an optical microscope was used for micromechanical bending tests. Bending experiments were performed using modified triangular silicon nitride cantilevers (coated sharp microlevers MSCT-AUHW, type F, spring constant $k = 0.5$ N/m, Veeco, Cambridge, UK). The tip of the AFM cantilever was removed using a focused ion beam (FIB) (FEI, Nova Nanolab 600 dual beam machine, Eindhoven, the Netherlands), which facilitated the positioning of the cantilever above the fiber, because the width of the cantilever was slightly wider than the fiber diameter. The spring constant of each tip-less cantilever was calibrated by pushing on a pre-calibrated cantilever as described elsewhere [34]. Before starting the measurement, the glass substrate containing the elastic fibers was immersed in 1 ml demineralised water and left to equilibrate for 15 min. Micromechanical bending tests were performed by bending the elastic fiber at the middle point of the channel using an AFM cantilever. Deflections versus piezo displacement curves were directly obtained from the micromechanical bending tests. From the results, forces versus displacement curves were derived to estimate Young's modulus of single elastic fibers. Local indentation of elastic fibers during bending was estimated by indenting the same fiber located on the glass surface.

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