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Research paper

Micromechanical analysis of native and cross-linked collagen type I fibrils supports the existence of microfibrils

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ABSTRACT

The mechanical properties of individual collagen fibrils of approximately 200 nm in diameter were determined using a slightly adapted AFM system. Single collagen fibrils immersed in PBS buffer were attached between an AFM cantilever and a glass surface to perform tensile tests at different strain rates and stress relaxation measurements. The stress–strain behavior of collagen fibrils immersed in PBS buffer comprises a toe region up to a stress of 5 MPa, followed by the heel and linear region at higher stresses. Hysteresis and strain-rate dependent stress–strain behavior of collagen fibrils were observed, which suggest that single collagen fibrils have viscoelastic properties. The stress relaxation process of individual collagen fibrils could be best fitted using a two-term Prony series. Furthermore, the influence of different cross-linking agents on the mechanical properties of single collagen fibrils was investigated. Based on these results, we propose that sliding of microfibrils with respect to each other plays a role in the viscoelastic behavior of collagen fibrils in addition to the sliding of collagen molecules with respect to each other. Our finding provides a better insight into the relationship between the structure and mechanical properties of collagen and the micro-mechanical behavior of tissues.

1. Introduction

The mechanical properties of tissues like tendons, ligaments, and bone are directly related to the arrangement of their constituent components. Collagen type I is the most abundant protein in the human body, and is the principal, tensile stress-bearing component, crucial for the strength and stability of a wide range of tissues. In this fibrillar type collagen the collagen triple helices, also called collagen molecules, are assembled in fibrils and cross-linked via the amino acids lysine and hydroxyl lysine present in their telopeptide regions (Silver et al., 2003; Ottani et al., 2002). The fibrils in turn are assembled in fibers, which depending on the type of tissue are assembled in fascicles like in tendon. The existence of other sub-structures in collagen fibrils has been debated for years. The *D*-periodic five-stranded microfibril, an assembly of five triple helices as a sub-structure, was first proposed by Smith (1968). Some recent studies suggest the presence of these microfibrils in fibrils. A longitudinal

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microfibrillar structure with a width of 4–8 nm was visualized in both hydrated (Raspanti et al., 2001; Habelitz et al., 2002) and dehydrated (Baselt et al., 1993) collagen type I fibrils using tapping mode AFM imaging. Three-dimensional image reconstructions of 36 nm-diameter corneal collagen fibrils also showed a 4 nm repeat in a transverse section, which was ascribed to the microfibrillar structure (Holmes et al., 2001). Using X-ray diffraction culminating in an electron density map, Orgel et al. (2006) suggested the presence of right-handed supertwisted microfibrillar structures in collagen fibrils.

As this hierarchical organization of collagen was revealed, many studies were initiated in an attempt to understand the relation between this structure and the mechanical properties as measured for tissue (Silver et al., 2003; Ottani et al., 2002; Puxkandl et al., 2002; Hulmes, 2002; Mosler et al., 1985; Sasaki and Odajima, 1996; Wang, 2006; Dowling and Dart, 2005; Magnusson et al., 2003; Silver et al., 2000). In this respect, it is of utmost importance to obtain the mechanical behavior of collagen fibrils, and to determine its contribution to the overall mechanical behavior of tissues. Stress–strain curves of collagen fibers and those of collagen-based tissues (such as tendon) in a hydrated state reveal a typical low stress, or 'toe' region, at low strains (<2%), followed by a 'heel' region where the slope slowly increases, and then followed by a linear region at higher strains (>3%) (Silver et al., 2003). Experimentally determined elastic moduli are typically between 0.1 and 1 GPa for these fibers and tissues (Butler et al., 1986; An et al., 2004; Pins and Silver, 1995). Applying tensile testing combined with synchrotron radiation diffraction on tissue samples, Mosler and co-workers (Mosler et al., 1985; Folkhard et al., 1987a,b) suggested that stretching of the collagen molecules and their sliding with respect to each other are the two major mechanisms to account for the elongation of collagen fibrils. This also forms the basis for the basis for simulation work done by Buehler (2006, 2008). In recent years, the development of micro- and nanomanipulation techniques like the atomic force microscope (AFM), offers a novel and direct means to measure the mechanical properties of materials on a micro- and nanometer scale (Smith et al., 2006; Kis et al., 2002; Guzmán et al., 2006; Oliver and Pharr, 2004), for example in the case of stretching individual DNA and protein molecules (Fisher et al., 1999; Carrion-Vazquez et al., 2000). With respect to collagen-based materials, studies have been initiated on the mechanical properties of individual collagen fibrils (10–500 nm in diameter) (Thompson et al., 2001; Graham et al., 2004; van der Rijt et al., 2006; Eppell et al., 2006; Strasser et al., 2007; Wenger et al., 2007; Svennson et al., 2010; Svensson et al., 2010). Most of the reported tests are limited to low strain ranges of typically a few percent, which provide very little information on the possible mechanism of the mechanical behavior of collagen fibrils. Eppel and coworkers have developed a specific MEMS device for tensile testing individual collagen molecules, that allows testing up to strains of 100% (Shen et al., 2008). Tests up to these strains are sufficient to break the fibrils, and thus determine the stress and strain at break. Although the presence of viscoelasticity of individual collagen fibrils was expected and has been observed by many researchers already, it had not been discussed and/or quantified in detail. Only recently, Svensson et al. studied this viscous behavior in more detail (Svensson et al., 2010). By stretching individual human patellar tendon fibrils at different strain rates and comparing the stress–strain curves with one recorded using a step-wise stress relaxation test (zero strain rate), the authors have separated the elastic and viscous component. Shen et al. using a microelectromechanical systems platform, performed in vitro coupled creep and stress relaxation tests on collagen fibrils isolated from the sea cucumber dermis (Shen et al., 2011). The time dependent behavior fitted well assuming a relaxation process described with two time constants.

In our recent research, we have measured the stress–strain behavior and time dependent mechanical properties of single native and crosslinked collagen fibrils to get more information on the viscoelastic behavior of the fibrils. Tensile tests at different strain rates and stress relaxation measurements of collagen fibrils have been performed using a home-built AFM system which has been adapted to allow stretching of collagen fibrils (initial length between 40 and 100 μm) up to 400 μm. The micro-tensile tests and stress relaxation measurements of collagen fibrils were performed on native fibrils and fibrils either cross-linked with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) or glutaraldehyde. Cross-linking with EDC results in "zero-length" cross-links, which in the case of collagen will lead to additional inter- and intra-molecular cross-links within the collagen fibrils (Olde Damink et al., 1996; Everaerts et al., 2007). Cross-linking with glutaraldehyde, however, introduces cross-links which can be at least 1.3 nm in length, leading to not only intra- and inter-molecular cross-links but also cross-links between microfibrils (Olde Damink et al., 1995). The differences obtained in the viscoelastic properties of these two differently cross-linked collagen fibrils, does not only provide additional evidence for the existence of microfibrils in the collagen fibril, but also indicates that the microfibrillar structure contributes significantly to the viscoelastic behavior of collagen fibrils.

2. Materials and methods

2.1. *Sample preparation for micro-tensile tests*

The general procedure for the isolation of (non)cross-linked collagen fibrils was described previously (Yang et al., 2008, 2007). In brief, a suspension of collagen fibrils was prepared from bovine Achilles tendon collagen type I (Sigma-Aldrich, Steinheim, Germany) by homogenization and filtration. Analysis of this suspension showed a high concentration of collagen fibrils. FTIR and DSC analysis indicated that these fibrils were not denatured in the isolation process (Yang et al., 2008). We therefore use the term native collagen fibrils. This suspension was further diluted to obtain a suitable suspension (~1 mg/ml) for depositing individual fibrils on a substrate, that are not crossing one another.

Furthermore, suspensions containing chemically cross-linked fibrils were prepared. Cross-linking of the fibrils was performed in the diluted suspensions (to avoid cross-linking