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Research Paper

Nanomechanical assessment of human and murine collagen fibrils via atomic force microscopy cantilever-based nanoindentation

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ABSTRACT

The nanomechanical assessment of collagen fibrils via atomic force microscopy (AFM) is of increasing interest within the biomedical research community. In contrast to conventional nanoindentation there exists no common standard for conducting experiments and analysis of data. Currently used analysis approaches vary between studies and validation of quantitative results is usually not performed, which makes comparison of data from different studies difficult. Also there are no recommendations with regards to the maximum indentation depth that should not be exceeded to avoid substrate effects. Here we present a methodology and analysis approach for AFM cantilever-based nanoindentation experiments that allows efficient use of captured data and relying on a reference sample for determination of tip shape. Further we show experimental evidence that maximum indentation depth on collagen fibrils should be lower than 10–15% of the height of the fibril to avoid substrate effects and we show comparisons between our and other approaches used in previous works. While our analysis approach yields similar values for indentation modulus compared to the Oliver–Pharr method we found that Hertzian analysis yielded significantly lower values. Applying our approach we successfully and efficiently indented collagen fibrils from human bronchi, which were about 30 nm in size, considerably smaller compared to collagen fibrils obtained from murine tail-tendon. In addition, derived mechanical parameters of collagen fibrils are in agreement with data previously published. To establish a quantitative validation we compared indentation

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results from conventional and AFM cantilever-based nanoindentation on polymeric samples with known mechanical properties. Importantly we can show that our approach yields similar results when compared to conventional nanoindentation on polymer samples. Introducing an approach that is reliable, efficient and taking into account the AFM tip shape, we anticipate that the present work may act as a guideline for conducting AFM cantilever-based nanoindentation of collagen fibrils. This may aid understanding of collagen-related diseases such as asthma, lung fibrosis or bone disease with potential alterations of collagen fibril mechanics.

1. Introduction

Collagens are the most abundant proteins in the human body. They are prevalent in organs, such as bones, skin and the respiratory system. One key characteristic of collagen-based tissues is their hierarchical architecture (Ottani et al., 2002; Fratzl and Weinkamer, 2007; Vashishth, 2007; Bechtle et al., 2010; Gautieri et al., 2011). At the lowest hierarchical level collagen consists of triple helical molecules ~300 nm in length and ~1.5 nm in diameter (Myllyharju and Kivirikko, 2004). These collagen molecules then self-assemble into larger organized cylindrical structures with diameters ranging from 30 nm to about 500 nm, i.e., the collagen fibrils (Kadler et al., 1996; Holmes et al., 2001). At the tissue level, collagen fibrils form fibres in tendons and ligaments (Silver et al., 2003), mineralized fibrils and fibres in bones (Weiner and Wagner, 1998) and highly oriented lamellae in the cornea (Komai and Ushiki, 1991). These structures progressively form larger structural elements resulting in tissues and organs with different biomechanical functionalities.

From the variety of collagen-based tissues, it seems evident that structure and function are highly correlated. Changes in structure at any hierarchical level of collagen-based tissues can occur in both physiological and pathological conditions (Myllyharju and Kivirikko, 2001). In such cases, the decline of tissue functionality can manifest itself as changes of the mechanical properties of the tissue at the macroscopic level. Prominent examples are osteogenesis imperfecta (Byers et al., 1991; Hulmes et al., 1995), diabetes mellitus (Paul and Bailey, 1996), chronic asthma (Holgate et al., 2010), aging and osteoporosis (Saito and Marumo, 2010) which have been associated with structural and biochemical changes at the collagen fibril level. To understand the origin of tissue functionality decline due to physiological or pathological changes, assessing the mechanical properties at all levels of tissue hierarchy down to the individual collagen fibril level is of high interest.

Several research groups have investigated the mechanical properties at the level of individual collagen fibrils by employing microelectromechanical systems (MEMS) (Eppell et al., 2006; Shen et al., 2008), in situ mechanical testing of tendon with small angle X-ray diffraction (SAXS) (Gupta et al., 2004) and testing individual collagen fibrils with atomic force microscopy (AFM, illustration of AFM cantilever-based nanoindentation shown in Fig. 1) (Heim et al., 2006; van der Rijt et al., 2006; Wenger et al., 2007; Yang et al., 2007, 2008; Grant et al., 2008, 2009, 2012; Svensson et al., 2010, 2013 Hang and Barber, 2011). Reported values for elastic modulus of collagen fibrils vary from 1.25 MPa (Grant et al., 2008) to 25 GPa (Yang et al., 2007). Table 1 summarizes the elastic modulus of collagen under different loading scenarios and environmental conditions (hydrated and dry). The variability of elastic modulus is to the largest part attributed to the hydration state of the fibrils, nevertheless also in dry state a large variability of reported elastic moduli (~1 to 25 GPa, cf. Table 1) is present, which may be attributed to differences in collagen origin, loading scenario, hydrated state and analysis method. AFM cantilever-based nanoindentation has recently gained in popularity, as a technique to mechanically assess collagen fibrils (Heim et al., 2006; Wenger et al., 2007; Grant et al., 2008, 2009, 2012), and importantly this technique has also shown great potential for translations in clinical diagnosis (Stolz et al., 2009; Plodinec et al., 2012).

Yet, despite the fact that AFM cantilever-based nanoindentation has gained popularity, there exists no standardized methodology for carrying out experiments and analysing data. The main limitation in nanoindentation experiments is the maximum indentation depth that can be achieved. The indentation depth is limited by the thickness of the sample and according to Bueckle's rule, which is generally used in conventional nanoindentation, the maximum indentation depth should be kept less or equal to 10% of the sample thickness (Bueckle et al., 1973) to avoid the influence of the underlying stiff substrate. Another limitation is the determination of the contact area i.e. knowledge of the AFM tip geometry. In most experiments conducted, the AFM tip shape is not directly determined for each tip but is most often assumed to be constant for all the AFM tips of the same type, with exactly the geometrical shape provided by the manufacturer. To improve the quality of the results a method of determining the AFM tip shape should be developed. Additionally, and in contrast to conventional nanoindentation, there is currently not a well-established analysis approach. Currently, different studies perform various analysis approaches, which makes cross-comparison of results difficult.

Here we propose a methodology and analysis approach for AFM cantilever-based nanoindentation experiments overcoming some important issues. In brief, we present results with regards to Bueckle's rule, compare the different analysis approaches (Hertzian and Oliver–Pharr analyses) most commonly used and present a protocol to determine the projected area function of the AFM tip using a reference sample. This study is aimed to be a reference for researchers wanting