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In situ mechanical and molecular investigations of collagen/apatite biomimetic composites combining Raman spectroscopy and stress-strain analysis

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ABSTRACT

We report the design, fabrication and application of a novel micro-electromechanical device coupled to a confocal Raman microscope that enables *in situ* molecular investigations of micro-fibers under uniaxial tensile load. This device allows for the mechanical study of micro-fibers with diameters in the range between 10 and 100 μm and lengths of several hundred micrometers. By exerting forces in the mN range, the device enables an important force range to be accessed between that of atomic force microscopy and macroscopic stress-strain measurement devices. The load is varied using a stiffness-calibrated glass micro-needle driven by a piezo-translator during simultaneous Raman microscopy imaging. The method enables experiments probing the molecular response of micro-fibers to external stress. This set-up was applied to biomimetic non-mineralized and mineralized collagen micro-fibers revealing that above 30% mineralization the proline-related Raman band shows a pronounced response to stress, which is not observed in non-mineralized collagen. This molecular response coincides with a strong increase in the Young's modulus from 0.5 to 6 GPa for 0% and 70% mineralized collagen, respectively. Our results are consistent with a progressive interlocking of the collagen triple-helices by apatite nanocrystals as the degree of mineralization increases.

Statement of Significance

Collagen and apatite are the main constituents regulating the mechanical properties of bone. Hence, an improved understanding of the impact of mineralization on these properties is of large interest for the scientific community. This paper presents systematic studies of synthetic collagen microfibers with increasing apatite content and their response to tensile stress by using a novel self-made electromechanical device combined with a Raman spectrometer for molecular level studies. The impact of apatite on the mechanical and molecular response of collagen is evaluated giving important insights into the interaction between the mineral and organic phases. Therefore our findings expand the fundamental understanding of the mechanics of the apatite/collagen system relevant for the design of bio-composites with similar bio-mimicking properties for e.g. bone regrowth in medical applications.

1. Introduction

Understanding the link between the mechanical and the molecular properties of micro-fibrillar materials is of great importance for the design of hard-soft matter composites such as collagen/apatite micro-fibers for biomedical applications, e.g. as growth templates for bone formation [1,2]. This is crucial for the controlled realization of composites with desired mechanical properties and optimized fracture toughness and Young's modulus.

In this context combining Raman microscopy with *in situ* stress-strain measurements is a powerful means to investigate the molecular response of materials to external tensile stress. Currently available commercial devices only allow for the study of bulk

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materials (dimensions of several hundred micrometers and above), which – as in the case of bone or mineralized tendon – have a complex three-dimensional microstructure where individual constituents at various hierarchical levels can contribute to the bulk mechanical properties [2–5]. This limitation prevents a direct interpretation of the obtained data since stress relief occurs along convoluted pathways due to the three-dimensional nature of the sample. Alternatively, atomic force microscopy (AFM) can be applied to study micro-fibers on the nanometer scale [6]. However, AFM on its own does not provide information on the molecular response to stress. These restrictions motivate our approach to design a dedicated stage for Raman-microscopy enabling the investigation of micro-fibers with bespoke glass micro-needles to apply calibrated forces in the mN range required for material systems such as mineralized collagen. This is motivated by the fact that the Young's modulus of collagen is of the order of hundreds of MPa and hence micro-fibers with diameters of 100 μm and below require external forces of the order of mN to obtain observable extension upon loading. Our research is driven by the interest in novel collagen/apatite composites as potential templates for implant overgrowth by bone [7] motivating our focus on collagen-based micro-fibers.

2. Collagen micro-fibers and Raman microscopy

Collagen is the most abundant fibrous protein found in the human body and other vertebrates [8]. It is the main building block of connective tissues such as skin and tendon as well as bone and teeth and consists of amino acid sequences arranged in a characteristic triplet (Gly–Pro–HyP), where glycine (Gly), proline (Pro) and hydroxyl-proline (HyP) constitute almost 30% of the polypeptide chain [8,9]. Type I collagen, as studied in our work, is assembled in a hierarchical fashion by a twisted left-handed helix, three of which can assemble into a right-handed superhelix forming the collagen molecule known as tropo-collagen. Single tropo-collagen molecules have a length of typically 300 nm and a diameter of 1.5 nm. The triple helices assemble in a staggered manner to form collagen fibrils with diameters of approximately 100 nm [10–12]. These fibrils tend to form extended micro-fibers on which our work concentrates. Hard apatite (Ap) deposited in conjunction with the soft collagen is a key component of bone, which in turn is a three-dimensional hierarchical bio-composite giving rise to the remarkable combination of fracture toughness and hardness in bone. Comprehending the correlation between mechanical properties and molecular structure requires a deeper understanding of the way in which this complex material responds to external forces and how it accommodates mechanical stress. Consequently, a wide range of techniques has been employed in the past decades to investigate the mechanical behavior of various biomaterials with particular focus on the tensile properties of fibrous materials with characteristic diameters ranging from the mm to the nm scale using different devices for mechanical testing.

Raman spectroscopy is a powerful tool for the study of the molecular structure of materials [13]. It is particularly useful for the investigation of water-containing bio-composites, since it does not suffer from the associated strong absorption observed in other types of spectroscopy, e.g. in FTIR [13]. Furthermore, it does not require large amounts of material or specific labelling of the sample. This is particularly important when combined with *in situ* stress-strain measurements as presented in this work. Collagen is an extensively studied biomaterial regarding its mechanical properties and various types of commercial or custom-built devices have been used for mechanical measurements [14,15]. Eppel et al. [16] studied the mechanical properties of single collagen fibrils under tensile stress using a custom-built micro-electromechanical device that allowed the use of immunofluorescence microscopy to image the fibrils. This is useful when measuring strain values and structural changes on the microscale, while nano-indentation was applied on single fibrils using an AFM tip [17]. Furthermore, AFM was used to exert tensile stress on collagen fibrils extracted from red deer antler with different levels of mineral content ranging from 30% to 60% for investigation of their mechanical response [6]. Other commercially available devices combined with Raman spectroscopy were also employed for *in situ* molecular investigations of the mechanical properties of pig-tail tendon [18] and human skin [19].

To the best of our knowledge, there has not been a systematic *in situ* Raman spectroscopy investigation of collagen micro-fibers as a function of calibrated mechanical loading for different degrees of mineralization. Studying micro-fibers rather than bulk specimens is important since hierarchical bio-composites such as bone or tendon will anisotropically respond to stress. Hence, the strain distribution strongly depends on the internal structure of the sample. This renders a decomposition of the different contributions to the stress-response (e.g. molecular stretching/bending and relaxation via alignment) difficult, if not impossible. Microfibrous samples can be uniaxially stretched reducing the possible pathways for stress-relaxation to molecular stretching and bending as well as inter-fibrillar glide. Our technique enables mechanical manipulation of micro-fibers on a length-scale between that accessible using AFM (maximum extensions <10 μm) and standard macroscale mechanical testing tools. Hence, this technique combined with *in situ* Raman spectroscopy can provide key insight into the correlation between the molecular structure of micro-fibers and their mechanical properties.

2.1. Experimental details

Our custom-built electromechanical device was designed to be compatible with a commercial Raman microscope for *in situ* studies. The unique characteristics of this device can be summarized as follows: i) it is possible to conduct Raman spectroscopy on a micro-fiber while simultaneously applying tensile load for *in situ* molecular investigations of structural changes accompanying mechanical deformation, ii) the load can be repeatedly applied and removed by displacing the calibrated micro-needle with a piezo-translator (PZT) to follow elastic or plastic deformation, iii) bespoke micro-needles with different fine tip lengths and/or diameters can be produced to provide spring constants of an appropriate magnitude for applying mechanical stress to microfibers. This device combined with Raman microscopy allows for the study of the molecular response of both mineralized and non-mineralized collagen under tensile stress, and for the investigation of how increases in collagen mineralization affect its mechanical properties. Fig. 1a and b show a schematic and a photographic representation of the setup integrated into a stainless steel case for mechanical stability and environmental control. The central elements of the micromechanical manipulator are two glass micro-needles with different stiffness values – a rigid needle is fixed to one end of the micro-fiber while a flexible needle attached to the opposite end of the micro-fiber acts as the calibrated force transducer. The flexible needle is mounted to a PZT and is calibrated gravimetrically using a set of defined weights. The flexible micro-needle can be uniaxially displaced by the PZT over a range of up to 450 μm, resulting in a calibrated force being applied reversibly to the micro-fiber. The static micro-needle attached at the opposite end of the micro-fiber must not bend during the micromanipulation experiment. This can be achieved by using a static micro-needle with approximately half the length and twice the diameter of the flexible micro-needle. Furthermore,