in situ Nanomechanical Investigations of Bone

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BY

FEI HANG

SCHOOL OF ENGINEERING AND MATERIALS SCIENCE
QUEEN MARY, UNIVERSITY OF LONDON
MILE END ROAD, LONDON, E1 4NS
Declaration

I declare that the work performed is entirely by myself during the course of my PhD studies at the Queen Mary, University of London and has not been submitted for a degree at this or any other University.

Fei Hang
Abstract

Mineralized collagen fibrils (MCFs) are the fundamental building blocks that contribute to the extraordinary mechanical behaviour of bone. Despite its importance in defining bone mechanics, especially the high resistance to fracture recorded in bone tissue, MCFs have yet to be mechanically tested and, thus, MCF contributions to the global mechanical properties of bone is unclear. In this thesis, a complete strategy for performing direct mechanical testing on nanosized fibrous samples including MCFs from bone using a novel in situ atomic force microscope (AFM) – scanning electron microscope (SEM) combination was established. This technique was used to mechanically test MCFs from antler bone tissue for the first time and resultant stress-strain behaviour was recorded to highlight the inhomogeneous response of fibrils, which is associated with fibrillar compositional heterogeneity. Mechanical properties of MCFs and bone tissue were found to be controlled by biomineralization process using additional tensile testing of MCFs and bulk samples from mouse limb bones at different ages.
Abstract

Extrafibrillar mineralization was found to have effects on the Young’s modulus of bone tissue rather than fibrils, indicating the importance of fibrillar interfaces in controlling overall mechanical behaviour of bone tissue. Interfaces between fibrils in bone were examined by carrying out single fibril pullout tests. A weak but reformable interface, dominated by ionic bonds between fibrils, was recorded and the sacrificial bond reforming activity at the interface was found to be dependent on pullout strain rate. Finally, considerations of bone as a fibrous composite was used to evaluate nanomechanical testing data, with approximately 50 % of the bone fracture energy accounted for in failure of fibril interfaces.
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List of Constants

\[ Z \] bone beam deflection
\[ M \] bending moment
\[ L \] length of the limb
\[ E \] Young’s modulus
\[ I \] second moment of inertia
\[ \rho \] density
\[ A \] cross-sectional area
\[ v \] velocity of sound in bone
\[ \nu \] Poisson’s ratio
\[ G_c \] critical energy release rate or fracture energy
\[ K_c \] critical stress intensity factor or fracture toughness
\[ F \] tensile force
\[ k \] cantilever spring constant
\[ d \] cantilever deflection
\[ r \] nanofibre radius
\[ L \] original fibre length
\[ \Delta L \] elongation of the fibre
\[ X \] retraction distance of the cantilever
\[ I_0 \] collected laser intensity
\[ I_1 \text{ and } I_2 \] reflected laser intensity
\[ \lambda \] wavelength of the laser
List of Constants

$I_m$ geometric moment of inertia
a frequency of the cosine function
b scaling constant
c decay rate of the laser intensity
$I_0'$ normalized laser intensity
$x'$ cantilever displacement
$\varepsilon$ strain
$\sigma$ stress
$D_l$ fibril diameter
$l_e$ fibril embedded length
$V_a$ activation volume
$H$ activation energy
$W$ work done to pullout the fibril
$H_{100\%}$ and $H_{0\%}$ activation energies for the boundary conditions of complete bond reforming (100 %) and complete bond failure (0 %)
$\alpha$ sacrificial bond reforming efficiency
$F_p$ maximum tensile force applied to the fibre before pullout
$U_f$, $U_d$ and $U_b$, strain energy stored in fibre free length region, debonded region and bonded region respectively
$G_t$ fibre interfacial fracture energy
$E_f$ and $E_m$ Young’s modulus of fibre and matrix
$A_f$ and $A_m$ Cross-sectional area of fibre and matrix
$\nu_m$ Poisson ratio of matrix
$W_f$ work of friction at the debonded interface
$a$ crack length
$f$ MCF volume fraction
$l_p$ pullout length of the fibrils
$G_c$ fracture energy from fibril fracture and interface failure
Chapter 1

Introduction

1.1 Background

Many biological materials incorporate biominerals within a polymeric framework in order to achieve a specific mechanical function. Bone is a prevalent example of a mineralized biological material with defined mechanical functions including transmitting the force of muscular contraction from one part of the body to another during movement, to protect vital organs and bone marrow as well as to enable the skeleton to maintain the shape of the body.

The material composition of bone is comprehensively understood, with soft materials predominantly of polymeric collagen and non-collagenous proteins acting as a framework for the formation of a harder mineral phase of plate-shaped crystals of calcium carbonated apatite. The organization of these elements of bone is complex but essentially based around nanofibres of collagen.
containing the mineral phase. Although bone exists in many shapes and different anatomy locations with various internal structures, all bone consists of a certain constituent called mineralized collagen fibrils (MCFs) which can be considered as the building blocks of bone [1-5]. MCFs can be both clearly identifiable using high resolution microscopy and used to form higher length scale structures in bone as shown in Figure 1.1.

Figure 1.1 Scanning electron micrographs showing the fracture surface of equine bone (A) and bovine bone (B) at comparable magnifications. Separated mineralized collagen fibrils are clearly identified in each sample as hair-like structures protruding from the bone surfaces.

The origin of the mechanical properties of bone is currently unclear, although numerous reports have indicated a strong relationship between bone hierarchical structure, especially the constituents of bone across different length
Chapter 1. Introduction

scales, with overall mechanical behaviour [1, 2, 4-9]. Of fundamental interest is the toughness of bone, especially as bone material is considerably tougher than many other natural materials despite the relatively poor mechanical properties of constituents [3, 10-15]. However, fundamental understanding of the nanoscale mechanisms operating in bone that provide this toughness still remain poorly understood [16]. The composition of bone is expected to define overall mechanical performance and over 80 % by volume of bone is occupied by MCFs [4]. Therefore, understanding how these fibrils organize and interact with each other in bone and, more importantly, how they behave mechanically is crucial in studying mechanics of whole bone and its toughening mechanism.

1.2 Current Studies on Mechanics of Bone

Common equipment employed to assess the structural properties of bone are a scanning electron microscope (SEM) and an atomic force microscope (AFM), used particularly for their high resolution imaging capability [15, 17-22]. AFM was initially introduced as a powerful image tool with atomic resolution [22-24] but was later used for mechanical testing including nanoindentation and direct mechanical testing on bone material. For example, many previous investigations examine the mechanical properties of bone at the nanoscale using AFM indentation techniques [25-38]. However, indentation testing is limited by the complex stress analysis required to interpret experimental data, so can be
considered as an indirect mechanical testing method. The indentation technique is also suitable for measuring surface mechanics rather than fibrous samples and is therefore not eligible for MCF mechanical testing. Only a limited number of studies [20, 39] have used AFM for direct mechanical testing of collagen fibrils, as shown in Figure 1.2, but no test considers mineralized fibrils from bone tissue.

Figure 1.2 Atomic force microscopy can be used to deform (but not fail) individual collagen fibrils from bovine tendon [20]. (A) An AFM probe with glue at its apex is used to pick up a collagen fibril from a Teflon coated surface with the one end of the fibril fixed in a drop of glue on the surface. (B) Schematic diagram indicating the AFM configuration, after fibril pick-up, used for mechanical testing.

Figure 1.2 highlights how isolated collagen fibrils from bovine tendon were mechanical tested using AFM. The stress-strain behaviour of collagen fibrils obtained in this test was used to define fibril elastic properties but the fibril
fracture properties including strength and ultimate strain were not measured due to the limited strain applied to the fibril by the experimental method. Additional research was carried out on mechanically testing collagen fibrils from sea cucumber using a micro electro mechanical (MEMS) device [40]. The individual collagen fibrils were tensile tested to failure with failure behaviour recorded. However, such a test is suitable for the selection of fibrils with relatively large diameters (usually up to microns) and is therefore not suitable for testing MCFs from bone tissue.

Figure 1.3 Individual collagen fibrils from sea cucumber tensile tested using (A) a MEMS device in ambient/humid environment. (B) Individual collagen fibrils were isolated from the bulk and tensile tested between the grips of the MEMS device [40].
These two examples of direct tensile tests on collagen fibrils using AFM and MEMS are of importance to soft tissue mechanics because it was first time the mechanical behaviour of collagen fibrils was recorded using reliable techniques. Critically, separation of the collagen fibrils from the parent material is required in both experiments. Unmineralized collagen fibrils from tendon and sea cucumber are usually packed in parallel arrays and can be easily separated by high frequency vibration ultrasound and chemical agents as used in these studies. However, mineralized collagen fibrils in bone are attached together with extrafibrillar matrix and mineral that become damaged when exposed to chemical or ultrasound techniques [19, 32, 39, 41-47]. A novel testing methodology is therefore desired for mechanically testing MCFs without using previous preparation techniques for unmineralized collagen fibrils, thus avoiding potential MCF damage, to allow understanding of MCF mechanical behaviour and their influence on whole bone mechanics.

1.3 Project Aims and Thesis Outline

1.3.1 Project Aims

This thesis details nanomechanical testing of MCF fibrous structures in bone materials and evaluates how the MCF unit contributes to the overall fracture behaviour of bone. In particular, little is known about how the MCFs
mechanically contribute to bone’s surprising toughness, especially as mechanical properties of bone constituents are considered to be relatively poor [2, 7, 9, 12, 15, 16, 36, 48-50]. The overall objective of this thesis is to address this question based on experimentally studying the mechanical properties of bone tissue at small scales appropriate to the MCFs in bone.

The main objectives of this project are therefore:

1. To develop a novel mechanical testing technique suitable for measuring individual nanofibres. Such a technique requires a suitable sample preparation method to separate individual MCFs from bone while avoiding induced damage from the preparation. The testing technique and associated sample preparation will form a universal mechanical testing strategy for MCFs.

2. Apply this new technique to bone materials and measure the mechanical properties of the individual MCFs.

3. Test the flexibility of the nanomechanical testing by studying MCFs from different ages of bone to obtain information on aging effects on the mechanical properties of MCFs.
Chapter 1. Introduction

4. Evaluate the interfacial properties of the interphase region between MCFs in bone tissue in order to understand the mechanical coupling between MCF assemblies.

5. Examine the nanoscale fibril related fracture behaviour and its contribution to the global fracture properties of bone.

1.3.2 Thesis Outline

In this study, a novel *in situ* nanomechanical testing methodology was developed for investigating nanofibrous samples including MCFs of bone. Individual MCFs from antler bone and mouse bones at different development stages have been successfully tensile tested. Furthermore, the interface between MCFs and surrounding non-collagenous proteins (NCPs) has also been mechanically evaluated by pulling out single collagen fibrils from the bone matrix. An analytical composite model was used to describe the overall fracture behaviour of bone material at the fibrillar level.

The second chapter of the thesis reviews existing bone literature, with emphasis on bone structural formation and theories developed from experimental results describing bone’s mechanical properties at different length scales. This literature
review will justify the importance of the constituent mechanical properties on overall bone mechanical behaviour.

In Chapter 3, detailed sample preparation methods for isolating individual mineralized collagen fibrils from bone are described. A novel in situ mechanical test method using a custom built atomic force microscope (AFM) combined with a scanning electron microscope (SEM) dual beam system is reported for performing tensile testing individual collagen fibrils from antler bone.

The fourth chapter details measurements of the mechanical properties of individual mineralized collagen fibrils from antler bone using the novel testing setup described in Chapter 3. The response of the mineralized collagen fibrils are examined in terms of their role in defining the toughness of antler through testing collagen fibrils from the surface of fractured antler specimens. Unprecedented insight into fracture behaviour of antler at the nanoscale is determined with novel fracture processes found to be intimately linked to inhomogeneous deformation at the fibrillar level.

The fifth chapter extends the mechanical testing of Chapter 4 to tensile testing of MCFs from different ages of healthy mouse bone. Mechanical properties of MCFs from different developmental stages of bone, where compositional changes in
whole bone are widely reported, are compared to bulk bone mechanical behaviour with implications of MCF mechanics on different ages of bone examined.

Chapter 6 uses in situ nanomechanical testing for pullout of MCFs from bone material in order to evaluate the interfacial properties between MCFs and NCPs. Interfacial behaviour in composite materials is critical in defining both deformation and failure, and the evaluations of interfacial failure in bone at nanometre length scales are expected to be instructive in defining whole bone mechanics.

Chapter 7 combines the mechanical properties of MCFs and their interfaces to describe, using analytical composite theories, the contribution of nanoscale mechanical behaviour towards whole bone mechanical performance. Finally, the eighth chapter summarizes the main contributions of thesis. Additional studies beyond the thesis and future work are also examined.
Chapter 2

Literature Review

2.1 Introduction

This chapter reviews the existing literature on bone structure and resultant mechanical behaviour, with emphasis on the prevailing results and theories regarding bone formation, hierarchy structure and composition of bone. The literature review is particularly important for justifying the experimental work of exploring mechanical properties of bone at the nanoscale.

2.2 Bone Formation and Secondary Bone

Bone is a composite material consisting of various different components, as described in Section 2.3 below, organized across different length scales to form whole bone incorporated within a skeletal structure. Bone material is distinct from other synthetic materials because of its ability to change and be in a
constantly changing structural state. While bones can be found from many different locations within a skeletal structure, long bones such as femur have been perhaps the most extensively investigated and provide the standard models used to describe bone structure. Before discussing the composition and structure of bone, one first needs to understand how the bone tissue is constructed hierarchically across different length scales during the bone formation process.

There are two main stages of the bone formation: primary and secondary ossification (known synonymously as osteogenesis) [12, 51, 52] which mainly involves three cellular activities i) the chondrocytes which produces cartilage for ossification in early stage ii) the osteoblasts which secrets collagen and form bone matrix that then differentiate into osteocytes embedded in bone tissue and iii) the osteoclasts which dissolve and absorb old bone tissue for remodelling. An example of growth process of long bone was demonstrated in Figure 2.1.

Figure 2.1 Schematic diagram showing the stages of endochondral ossification [53].
Bone formation is initiated by mesenchymal condensation where intercellular substances aggregate and condensed to form a cartilage model of the bone to be formed [54, 55]. The cartilage model grows in length and thickness by the continued cell division of chondrocytes, which is accompanied by further secretion of extracellular matrix to form cartilage for ossification [51, 56].

Following the appearance of cartilage model, a primary centre of ossification is formed by cartilage matrix mineralization, osteoclast activities and vascularization. The formation of bone then occurs at the epiphysial cartilage in primary ossification centre, which consists of ground substance (containing chondroitin-4-sulphate, chondroitin-6-sulphatc, and keratosulphate [57]) and loosely packed collagen fibrils (having diameter of 10-20 nm) [52, 58]. The matrix vesicles embedded in the epiphysial cartilage deliver mineral ions to the mineralization front [59-63] as shown in Figure 2.2.

A “woven” bone microstructure is formed during this rapid and unorganized mineralization in which the mineral component does not form in close association with the collagen and no regulated structures are formed. The collagen fibrils in this process are found to be too narrow for the mineral to deposit within them [58]. Instead, an extrafibrillar mineralization can be observed during this initial bone formation process, which indicates that the collagen does not play an appreciable role in directing the mineralization. The bone material produced during this process is called primary bone, which is usually found to exist shortly in the early development stage. The primary bone
can be also found in later development stages when skeleton is healing from trauma. During healing of bone fracture, the woven bone forms after collagen is secreted by osteoblasts. The mineralization process is similar to endochondral ossification. Mineral ions are delivered from matrix vesicles in sounding extracellular matrix to the newly formed collagen fibrils.

![Figure 2.2 Optical microscope image showing ossification from epiphyseal cartilage to mineralized collagen/woven bone [64].](image)

Primary bone is produced quickly to define the shape of whole bone that provides initial mechanical support, which is crucial in the early stage of bone development and in the recovery of bone fracture [53]. However, the collagen
fibrils constituents in primary bone contain a relatively low mineral content and are not organized in an efficient way to provide effective mechanical properties. The relatively poor mechanical properties of primary bone therefore require changes in this bone material in order to provide more effective mechanical function. Thus, secondary ossification occurs in order to provide constituent ordering and improved mechanical behaviour in bone at specific centres as shown in Figure 2.1.

The initiation of secondary ossification occurs at each end of long bone where the mesenchyme and blood vessels are developed in a similar process to primary ossification. The cartilage between the primary and secondary ossification centres is called the epiphyseal plate, and it continues to form new cartilage that is replaced by bone, a process that results in an increase in length of the bone as shown in Figure 2.1. Growth continues until the cartilage in the plate is complete replaced by bone and the bone growth stops. Meanwhile, secondary ossification proceeds by the existing primary bone tissue becoming dissolved by osteoclasts and absorbed into the extracellular matrix, which is then used by osteoblast to form a more organized and optimal structure. The changes in the primary bone material structure, referred to as bone remodelling, form bone material called secondary bone [65]. The shape of whole bone is therefore defined by the activities of osteoclasts and osteoblasts.

The collagen fibrils formed in secondary ossification by osteoblasts have a much larger diameter (70-100 nm) compared to those produced in primary woven
bone by chondrocytes [52]. Sufficient internal space is available in collagen fibrils from secondary bone to allow mineralization within fibrils, defined as intrafibrillar mineralization. The structure of collagen fibrils in primary and secondary bone is therefore different due to the mineralization process. While collagen fibrils in primary bone contain extrafibrillar mineral between the collagen fibrils, mineralization of fibrils in secondary bone occurs initially in the intrafibrillar spaces before extending to extrafibrillar mineralization.

2.3 Hierarchy Structure of Bone

During the secondary bone formation, the primary woven bone tissue is remodelled into a more optimal and organised structure [65]. The collagen fibrils produced in secondary ossification are highly organized and assembled into close packed and parallel lamellar structures used to form larger structures and eventually a complex hierarchy. Thus, discussions on the structural hierarchy of bone typically refer to the complex architectures found in secondary bone [66]. In a seminal review of bone structure provided by Weiner et al. [1], the whole structure of bone has been considered as seven levels of hierarchy (Figure 2.3) starting with nano-sized platelets of hydroxyapatite (HA) that are oriented and aligned within self-assembled collagen fibrils. These fibrils are layered in parallel arrangement referred to as lamellae which are arranged concentrically around blood vessels to form osteons. Finally, the osteons are either packed densely into compact bone or comprise a trabecular network of microporous bone, referred
to as spongy or cancellous bone. Skeletal whole bone therefore contains both compact and cancellous bone tissue.

Figure 2.3. The hierarchical levels of structure found in secondary osteonal bone, as demonstrated by Weiner and Wagner [1].
2.3.1 Level 1: Major Components of Bone

Level 1 of the seven level hierarchy of bone describes the major components of bone materials: the dahllite (carbonated apatite) crystals, type I collagen fibrils, and water. The carbonated apatite \((\text{Ca}_5(\text{PO}_4,\text{CO}_3)\text{OH})\) is the only inorganic component found in mature bone [1] and form crystallites with a flattened plate-like shape, with the average length and width of \(50 \times 25\) nm (Figure 2.3; Level 1) [51, 67] which are believed to be the smallest biologically formed crystals known [51].

The apatite crystal platelets are extremely thin with a remarkably uniform thickness of only few nanometres. Transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS) studies show that the thickness of the apatite crystals varies from 1.5 nm for mineralized tendon up to about 4.0 nm for some mature bone types [68, 69]. Due to the small size of the apatite crystals found in bone, the surface atomic structure as well as the mechanical properties of single crystals has not been experimentally measured using reliable methods. Atomic force microscopy (AFM) studies of synthetic apatite show that the surface is highly ordered and matches the structure of bulk equivalents [70]. Interestingly, bulk dahllite forms symmetrical hexagonal crystal structures reflecting unit cell symmetry whereas dahllite in bone has a unique plate shape.
Chapter 2. Literature Review

The organic constituent of bone consists of type I collagen used to construct collagen fibrils but also other diverse non-collagenous proteins (NCPs) [71]. In bone formation, NCPs are found to exist near the mineralization front between collagen fibrils and are also highly charged from an abundance of carboxylate groups from aspartic and glutamic acid residues, as well as phosphate from phosphoserine [51, 56, 72-74]. Although in low concentration, the NCPs are believed to perform an important function in the mineralization process of bone [51, 52, 75].

2.3.2 Level 2: Mineralized Collagen Fibrils (MCFs)

Type I collagen accounts for 90% of the organic/protein components by volume within bone and is therefore expected to be important in defining mechanical properties of the bulk bone material. Type I collagen is formed as a fibrous structure with the fibrils in bone having diameter of 80–100 nm as measured by TEM (Figure 2.3; Level 1). The lengths of individual MCFs remain unknown as the collagen fibrils tend to merge with neighbouring fibrils [76, 77]. Level 2 describes the mineralized collagen fibril (MCF) building block, which is defined by Weiner and Wagner [1, 2] as the smallest discrete basic unit used to construct bone materials (Figure 2.3; Level 2). The MCFs are therefore critical in defining the mechanical properties of bone, which is also the objective of this thesis.

The basic collagen fibril in bone is secreted by osteoblasts in the growth of bone. As shown in Figure 2.4, every individual collagen molecule is made up of three polypeptide chains about 1000 amino acids long [1] wound together in a triple
helix. A triple-helical molecule is thus cylindrically shaped, with an average diameter of about 1.5 nm, and lengths of 300 nm [51, 78, 79].

Collagen fibrils are formed from the self-assembled tropocollagen molecules. In a single collagen fibril, tropocollagen molecules are parallel to long axis of fibril, but their ends are separated by holes of about 35 nm as shown in Figure 2.5 (A) and neighbouring triple-helical molecules are staggered by 67 nm [80]. This quarter-stagger arrangement [31] shown in Figure 2.5 produces a characteristic and repeating gap region between the tropocollagen molecules that is occupied by water molecules. The mineralization process of bone replaces the water in gap regions within the collagen fibrils with plate-shaped mineral crystals [30] as shown in Figure 2.5. The formation of the mineral within the collagen fibrils is directed by the internal gap regions produced from the quarter-stagger arrangement [80, 81]. Single mineralized collagen fibrils can be therefore considered as a ceramic phase reinforced polymer system.
Figure 2.5 (A) 3-D organization of apatite minerals inside a single collagen fibril. Collagen molecules (represented by red rods) aggregate into a quarter-staggered pattern described by Hodge and Petruska [82]. Calcium phosphate (blue plates) nucleates in the gaps between the collagen molecules. (B) TEM image of mineralized collagen fibrils from mineralized turkey tendon showing the characteristic quarter-stagger pattern [83].

The mineral formed within the collagen fibril is typically of relative small dimensions and is not normally expected to be thermodynamically stable if existing outside of the organic matrix of the collagen fibril [44, 84-86]. The distribution of mineral components at different sites within the collagen network has been studied by Martin et al. [87] for canine whole bone and indicated 58% of mineral is intrafibrillar, 14% extrafibrillar, and 28% found at the gap regions.
between the ends of the tropocollagen molecules.

### 2.3.3 Level 3 Fibril Array

The third structural hierarchy level in bone denotes the fibril array incorporating packing of mineralized collagen fibrils. In most of the bone materials, MCFs are close packed into bundles or arrays along the long direction of their lengths [88] (Figure 2.3; Level 3). These fibril bundles are mostly composed of MCFs that sometimes merge with neighbouring fibril bundles [76]. Detailed information on how the MCFs are organized within fibril arrays still remains poorly understood and is controversial. Generally, two different fibril structural organizations are proposed in the literature [2, 89] as shown in Figure 2.6. Literature proposes that the two different arrays are formed in different bones to optimise the stress transfer in bones under different loading conditions [89-91].

![Figure 2.6 Schematic illustration of MCFs containing mineral plates (not drawn to scale, MCFs are shown in cylinders with black mineral crystals embedded) showing (A) an arrangement of mineralized collagen fibrils aligned both with](image)

52
respect to crystal layers and fibril axes (orthotropic symmetry). (B) Arrangement of mineralized collagen fibrils with only the fibril axes aligned (transversal isotropy) [1].

2.3.4 Level 4: Fibril Array Pattern

At level 4, the fibril array organizational patterns vary to produce a diverse range of structural types in bone tissues. As shown in Figure 2.7, organization of fibril arrays into a higher structure are generally divided into four categories: arrays of parallel fibrils, woven fibre structure, plywood-like structures and radial fibril arrays in different bone tissues [2, 49, 91].

In mature secondary lamellar bone, the plywood-like structure of Figure 2.7 (C) is the most common structure used to describe the organization of MCFs. Weiner et al made a detailed study of collagen organization in the lamellar bone from mouse limb bones [91, 92] and observed parallel layers of fibrils, each with a successive tilted angle of around 30° from layer to layer. A model was therefore proposed in which a lamellar unit is composed of five such sub-layers as shown in Figure 2.7 (C). Because only five and not six sub-layers oriented progressively every ± 30°, the lamellar unit structure is not symmetrical [93]. Variations in the thicknesses of the sub-layers occur for bones from different species, rather than the tilted angle between layers.
Figure 2.7 Electron micrographs and schematics highlighting the four most common fibril array pattern organizations in bone. SEM micrographs of fractured surfaces and schematic illustrations (not drawn to scale) of the basic organizational motifs indicate: (A) Array of parallel fibrils of mineralized turkey
tendon (scale: 0.1 mm) and schematic illustration showing the localized orthotropic symmetry of a fibril bundle. (B) Woven fibre structure of the outer layer of a 19 week old human fetus femur [94].

The schematic illustration shows fibril bundles with varying fibril diameters arranged in random orientations. (C) Plywood-like structure presenting fibril organization in lamellar bone from the fracture surface of a baboon tibia, with prominent fourth (large arrowhead) and fifth (small arrowhead) sub-layers [91, 95]. The corresponding schematic illustration shows the five sub-layer model described in [91] with sub-layers one (right hand side), two, and three arbitrarily composed of one fibril layer each, whereas sub-layers four and five are composed of four fibril layers each. Note that the fibrils in each layer are rotated relative to their neighbours (depicted by the change in direction of the ellipsoid cross-section), following the rotated plywood model [89]. (D) Radial fibril arrays from human dentin fractured roughly parallel to the pulp cavity surface. The tubules (holes) are surrounded by collagen fibrils that are approximately in one plane. The schematic illustration of the fibril bundles highlights the arranged in a plane perpendicular to the tubule long axis with no obvious preferred orientation within the plane [1, 2].

### 2.3.5 Level 5: Osteonal/Haversian System

At level 5, the bundles of mineralized collagen fibrils are organized into a higher length scale cylindrical structure unit known as the osteon as shown in Figure 2.3. Osteons are formed during remodelling of primary bone into secondary bone and
are initiated by osteoclasts removing primary bone material in a dissolution process as they migrate through the material. Such dissolution forms channels that are subsequently filled by osteoblasts. The osteoblasts excrete the more regularly organized layers of lamellar bone within the channel until only a narrow channel in the middle of the osteon remains to allow movement of blood vessels within the secondary bone [48] (Figure 2.3; Level 5). Other smaller capillary-like features (canaliculi) are also built into the structures and connect trapped osteoblasts, referred to as osteocytes. These osteonal structures are also called Haversian systems named after John Havers who discovered the lamellae structure in 1691 [96]. Primary bone is sometimes formed of osteons (primary osteon) but the slower formation of osteons relative to woven bone typically requires remodelling of woven bone to give secondary osteons, the most common structures found in mature secondary bones and described in the bone formation section 2.2 in this thesis.

2.3.6 Level 6: Architecture Level – Compact and Cancellous Bone

The level 6 architectural level identifies structural features over length scales of hundreds of microns and essentially describes the distinct regions of compact and cancellous bone. Osteons are formed into dense, solid bone tissue called compact bone or lighter, porous bone tissue called spongy bone or cancellous bone, which both can be found in secondary lamellar bones as shown in Figure 2.8.
Figure 2.8 (A) Micro-anatomy structure of a human femur head with compact and cancellous bone demonstrated. (B) Schematic diagram showing the structure of cortical bone [97].

2.3.7 Level 7: Whole Bone

At the highest scale level, whole bone represents the overall shape of the bone. In most of mammal animal’s skeletal system, bones exist in different shapes and sizes but are classified into three common types: long bone, flat bone, irregular bone and short bone as shown in Figure 2.9.

The long bones have a greater length comparing to width and usually exist in arms and legs to support motion. Flat bones are flattened and usually have a protective function such as the bones of the skull and sternum, which protect the internal organs from external impact. Short bones have relatively equal length and width and usually exist at joints such as carpal bones to facilitate motion.
Irregular bones possess little symmetry such as sphenoid bone and vertebrae and typically function as connective parts in the skeletal system.

Figure 2.9 Schematic diagram showing a range of different bone shapes found in the human skeleton [98].

The spatial organization in whole bone is defined by the microstructural units from the architectural level 6. These two levels are far larger than the mineralized collagen fibrils studied in this report and therefore are not discussed in detail. There is a considerable body of literature studying bone at large scales over decades such as the comprehensive reviews provided by Currey [99] and Thompson [55].
To conclude, all these 7 levels of bone are composed of structural differences in one magnitude between the subsequent levels, bridging from the ultrastructural component level to the whole bone [27]. In order to expedite a complete understanding of bone material, individual structural components and features at different levels contribute to the overall mechanical behaviour of bone. By examining bone at different hierarchical levels, a comparison between structure and material properties between levels can be established. However, each structural hierarchy level depends on the structural features of the levels below to provide function and structural support. Thus, the lowest discrete unit of MCFs must be critical in defining higher order mechanical behaviour and the overall mechanical response of bone, as will be discussed in the next section.

2.4 Bone’s Structure and Mechanical Properties

The earliest study on mechanical properties of bone can be perhaps traced back to the beginning of anatomy and orthopaedic surgery. However, initial studies highlighted the lack of interaction between biologists and mechanics researchers. In a symposium “The Mechanical Properties of Biological Materials”, Vincent and Currey noted: “...it was clear that the biologists who work on the mechanical properties of materials need much more to call upon the materials scientists for guidance on what is known already about the phenomena they are studying; on the other hand it was equally clear that the materials scientists had little idea of the
richness of biological diversity and of the pervasive ability of natural selection to provide the optimum solutions to very complex problems.”[36] Since the initiation of the symposium in 1980, a vast number of studies on mechanical behaviour of bone have been published and significant understanding achieved. This understanding was driven not by deep theoretical insights, but by the development of multidisciplinary subjects and the advances in materials testing techniques.

The mechanical behaviour of bone at macro scales is highly affected by the mechanical properties of its constituents [15]. However, the material properties of bone structure are strongly dependent on the scale of observation. Recent experimental and theoretical studies have provided new insight into the mechanics of bone, most notably through the use of advanced instrumentation that has permitted the examination of bone properties at ever-decreasing length scales, e.g. transmission electron [56, 100] and X-ray microscopy [69, 101], atomic force microscopy [17, 20, 102], and Raman spectroscopy [103, 104], as well as bottom-up multiscale simulation modelling [41, 105, 106].

In this section, the previous studies on mechanical properties of bone materials at different length levels using various methods are reviewed. As discussed in Section 2.3, bone is a composite material that exists on several hierarchical levels.
In the discussion of the structure of bone, hierarchy was reviewed from lowest length scale to whole bone, which roughly coincides with the bone development process i.e. bone is produced at small lengths scales initially but organization builds up larger length scale features. In this section, the review of studies on mechanical properties of bone starts from the highest level: the whole bone, because organizing the structural constitutes in bone tissue at different length scales is adapted due to the overall requirement of the mechanical functions of whole bone.

2.4.1 Level 7: Whole bone

The whole bone level is the largest length scale level of bone and represents the summation of the structural and material properties of all length scales of bone. At this whole bone level, the bone or skeletal system provides the mechanical functions of structural support and protection to internal organs as well as aiding motions. The structural geometry and organization of bone are adapted or ‘coincident’ with bone’s mechanical functions. Interaction between whole bone and other constituents of the body may include tendons, ligaments, muscles and other bones.

The shapes of whole bone are various according to the bone’s mechanical functions. While it is almost impossible to examine the physiological loading
conditions for all types of bones with different shapes, identifying the complete loading conditions for even a single type of bone with a certain shape is still difficult. This section will mainly discuss the mechanics of the most common bone we can find in skeletal system, which is long bone. The long bones are relatively simple in shape and classical engineering principles have been applied to understand the loading on such particular bone types.

Long bones provide common features for other bone types including a hollow internal structure with a relatively thick wall defining the whole bone’s external surface. Long bone is specifically expanded at each of its ends, with a cancellous bone structure found within these ends. The hollow structure of long bone is derived from mechanical function and the so called ‘minimum mass’ requirement that exists widely in nature [12]. In minimum mass analysis, the aim is to build a structure to perform a certain mechanical function with minimum mass.

A simple example is a limb bone loaded as a beam as shown in Figure 2.10. The limb bone acts primarily to exert loads from the environment and therefore needs to be stiff to withstand large bending moments. The distortion caused by bending on the bone must not exceed a limit. In evolution theory, natural selection will favour animals that function with the highest efficiency. This efficiency is when the function can be performed by such a structure produced
with the minimized cost to animals in terms of metabolic energy consumed and the time spent. Therefore, a mechanically optimized hollow structure was developed in most of the bone structures such as long bone.

![A mechanical analysis can be presented to highlight how the structure of bones is optimized towards a mechanical function. For the limb bone shown in Figure 2.10, the maximum deflection can be calculated as:

\[
Z = \frac{ML^2}{8EI}
\]

Equation 2.1

where \(Z\) is the maximum deflection of bone, \(M\) the bending moment applied, \(L\) is the length of the limb, \(E\) is the Young's modulus of the bone and \(I\) is the second moment of inertia. 
In Equation 2.1, the geometry and composition of the bone provide fixed values of $L$ and $E$ respectively. An external load is applied to the limb generates a bending moment $M$. To make sure the deflection of bone $Z$ does not exceed the criteria value to cause failure, a larger second moment of area $I$ is preferred. The parameter $I$ is typically defined for a beam with a round cross section in pure bending, as shown in Figure 2.11, as:

$$I = \sum y^2 \delta_{area}$$  \hspace{1cm} \text{Equation 2.2}

It is noticed that a larger $I$ can be achieved by increasing $y$, indicating that the simplest way of controlling deflection of beam under bending is to increase the beam cross section. However this will cause a corresponding increase in the total mass of the beam, which would therefore require more metabolic energy to produce the limb and is not preferable.

By assuming the beam density is $\rho$ with a cross area of $A$, the ratio of the mass to second moment of area is:

$$\frac{\rho LA}{I} = \frac{\rho L \sum \delta_{area}}{\sum y^2 \delta_{area}}$$  \hspace{1cm} \text{Equation 2.3}

Equation 2.3 suggests that a minimization of the beam mass and maximization of
the second moment of area of the beam for a fixed $I$, $L$ and $\rho$ can be achieved if the ratio $\frac{\sum \delta_{\text{area}}}{\sum y^2 \delta_{\text{area}}}$ is minimized. This condition is achievable if the beam mass is distributed as far as possible from the neutral axis.

![Diagram (A)](image1)

![Diagram (B)](image2)

![Diagram (C)](image3)

Figure 2.11 (A) A beam of original length $L$ under an external loading condition will (B) bend, causing a beam length change above and below the neutral plane. (C) Cross section of the beam showing the second moment of area will be different if loaded at different location on the cross section. $\delta_{\text{area}}$ is the unit area at a distance $y$ from the neutral plane.
To minimize the mass required to limit the deflection, bone uses a small cross section area and this area should be as far from the central neutral plane as possible. A hollow structure is therefore formed in bone materials that are expected to resist bending where the mass is far from the centre of mass. Thus the shape of whole bones can be insightful in illustrating the optimization of bone material occurring for mechanical function.

2.4.2 Level 6: Architecture Level

a. Compact Bone

As the compact bone carries most of the mechanical loading form environment, many researches on mechanical properties of bone focus on the compact bone tissue. Several mechanical properties of compact bone have been studied including elastic properties (mainly Young's modulus), strength (in different loading directions), fracture behaviour (including mechanisms and fracture toughness), creep rupture, and fatigue.

Bone is a type of anisotropic composite due to the orientation of various components in structure. Different loading conditions in mechanical testing have a considerable effect on the measured mechanical properties of bone. The highly organized structure of bone has been discussed in Section 2.3 and highlights the
orientation of constituents at different hierarchy levels in bone. A central feature of compact bone is the cylindrical shape of the osteons parallel to the bone long direction. The osteons containing calcium phosphate crystals are stiffer and stronger than the extracellular matrix existing between osteons [3, 99]. Hence if load is applied from different directions as shown in Figure 2.12, the stress will be distributed in osteons and interface regions in different ways.

When load is applied along the longitudinal axis, the stiffer osteons carry the load, resulting in bone exhibiting a maximum Young’s modulus and strength in this loading direction. Conversely, applying load on transversal direction will make osteon interface carry load directly which leads to a lower modulus and strength.

Figure 2.12 Loading bone in different directions. Brown cylinders represent osteons while the interface region is shown in yellow (not to scale).
In addition, the mechanical behaviour of bone, and indeed other biological materials, is affected by the testing environment. Therefore, the loading direction (such as longitudinal, circumferential and radial directions to the long axis of long bone as shown in Figure 2.13), the humidity of the specimen and the strain rate will influence the mechanical properties of bone and care must be taken in testing under physiological conditions. The testing condition will be carefully examined in the following discussion on different mechanical properties of bone.

![Figure 2.13 Schematic diagram showing three loading directions in mechanical test of bone material.](image)

**Elastic properties**

The elastic properties of bone material are somewhat ambiguous due to its inherent viscoelastic behaviour. However, two common methods are used to measure the Young's modulus of bone. The first is direct loading of a bone sample
to produce a stress-strain plot as shown in Figure 2.14. The apparent Young's modulus of the bone sample is found by taking the tangential value to the recorded data in the linear region of the stress strain curve at low strain values.

Figure 2.14 A typical stress-strain curve of cortical bone recorded in tensile test on human bone along longitudinal axis. The black line shows the linear response at small strain, with the slope defining the bone's Young's modulus. Yield stress and failure strain of the bone were also recorded as shown [107].

The second method of measuring elastic behaviour is through recording the velocity of a sound wave (usually using ultrasonic sound wave) in bone [12]. The sound velocity $v = \sqrt{\frac{E}{\rho}}$, where $E$ is the Young's modulus of bone and $\rho$ is the density of the media (which is bone in this case). However, the simple calculation
of Young’s modulus from recording the velocity of sound is only valid for isotropic materials, with the more complex structural anisotropy found in bone [12] making such calculations difficult.

Thus, direct mechanical testing of bone samples is a relatively straight-forward method with several advantages. In particular, the Young’s modulus of bone can be easily tested under different environmental situations. Both compact and cancellous bone can be tested without considering their structural characteristics i.e. consideration of bone as a continuum. Calculation of the Young’s modulus from velocity of sound measurements is indirect but can provide the information on derivation of all the stiffness coefficients and applied to specimens with complexe shapes or even in vivo [11, 108, 109]. A previous study compared elastic properties measured using ultrasonic and mechanical testing using applied loading methods on bovine bone specimens [110]. Results indicated ultrasonic testing values correlated with indirect mechanical testing of bovine bone selected from different anatomy locations. However, ultrasonic testing showed significantly higher elastic property values than the direct mechanical testing.

A considerable amount of data has been recorded for the mechanical properties of bone. Table 2.1 gives the values of Young’s moduli from different specimens of
human, canine and bovine bones by direct mechanical testing and the ultrasonic methods [109, 111]. In later research on bone specimens from bovine femur long bone, the mechanical properties of specimens were found to be higher along the long axis of the bone, which was believed to be associated with the bone function as a whole [110]. The Young’s modulus measured along the bone’s long axis is about 1.6 to 2.4 times higher than the modulus measured in the transverse direction. The variation in Young’s modulus with orientation has been validated by numerous researchers [11, 112-114].

Table 2.1 Young’s moduli of human and bovine bone specimens (in GPa). Reproduced from data in [12, 109, 111]. Subscripts 1, 2 and 3 represent the radial, circumferential and longitudinal directions relative to the long axis of the bone.

<table>
<thead>
<tr>
<th></th>
<th>Asbman et al.[111]</th>
<th>Reilly and Burstein [109]</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canine Human</td>
<td>Human Haaversian Fibrolamellar</td>
<td></td>
</tr>
<tr>
<td>Ultrasound</td>
<td>Tension Compression</td>
<td>Tension Compression Tension</td>
<td></td>
</tr>
<tr>
<td>$E_1$</td>
<td>12.8 12.0</td>
<td>12.8 11.7</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>3.0(25) 1.01(5)</td>
<td>1.6(5) 1.8(8)</td>
<td>0.17(25)</td>
</tr>
<tr>
<td>$E_2$</td>
<td>15.6 13.4</td>
<td>12.8 11.7</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>3.0(25) 1.01(5)</td>
<td>1.6(5) 1.8(8)</td>
<td>0.17(25)</td>
</tr>
<tr>
<td>$E_3$</td>
<td>20.1 20.0</td>
<td>17.7 18.2</td>
<td>23.1</td>
</tr>
<tr>
<td></td>
<td>3.6(38) 0.85(4)</td>
<td>3.2(3) 4.6(5)</td>
<td>5.4(6)</td>
</tr>
<tr>
<td>$G_{12}$</td>
<td>4.7 4.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$G_{13}$</td>
<td>5.7 5.6</td>
<td>3.3</td>
<td>3.6</td>
</tr>
</tbody>
</table>
As bone has some degree of viscoelasticity from its polymeric component collagen, the strain rate can influence the elastic properties but the effect is relatively small as the Young’s modulus is mainly affected by the presence of the hard mineral phase [11, 79, 115, 116]. Research comparing bone mechanically tested in tension under different strain rates highlighted a relatively poor dependence on Young’s modulus [108, 115, 116]. Specifically, a thousand times increase in loading strain rate caused a 40% increase the Young’s modulus of bone [115]. However, the strain rate used in these previous investigations is much higher that the loading frequency on bone in physiological condition. Ultrasonic testing essentially provides deformation of bone at loading rates equal to the velocity of sound. Therefore, bone testing using the relatively high loading rates of ultrasonic methods will give higher elastic modulus values recorded in
Table 2.1 compared to direct mechanical testing of bone under typically quasi-static loading rates.

**Strength**

The strength of bone is typically measured using direct mechanical testing. The stress-strain plots shown in Figure 2.14 used to define the elastic modulus can give the maximum load required to fail the bone material. As with elastic properties, bone strength is influenced by the direction of the testing relative to bone structure and loading rate. The strength of bone material recorded in the literature is summarized in Table 2.2. The results in Table 2.2 indicate significant differences in bone strength depending on the orientation of the test i.e. longitudinal, circumferential and radial as shown in Figure 2.13. In particular, the tensile strength and failure strain of bone material along the longitudinal orientation is significantly higher than in the circumferential direction. These observations correlate with mechanical testing on baboon bones indicating four times higher strength (289 MPa vs. 71 MPa) in the longitude direction compared to the circumferential [113]. Baboon bone also exhibited a 3 fold and 16 fold increase in failure strain and work to fracture in the longitudinal direction loading when compared to the transverse direction [61].
Table 2.2 Strength of human and bovine bones (in MPa) summarizing results found in previous studies [11, 12, 109, 112, 115].

<table>
<thead>
<tr>
<th></th>
<th>Tension Strength</th>
<th>Yield Stress</th>
<th>Ultimate Strain %</th>
<th>Compression Strength</th>
<th>Ultimate Strength</th>
<th>Ultimate Strain</th>
<th>Shear Strength</th>
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<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haversian</td>
<td>133</td>
<td>15.6(21)</td>
<td>0.031</td>
<td>205</td>
<td>1.9</td>
<td>0.3(20)</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>7.1(21)</td>
<td>0.7</td>
<td>131</td>
<td>1.3</td>
<td>0.3(20)</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.0</td>
<td>0.7</td>
<td>272</td>
<td>1.6</td>
<td>0.15(3)</td>
<td>7(12)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>157</td>
<td>3.3</td>
<td>171</td>
<td>10.0</td>
<td>0.49(6)</td>
<td>7(12)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>0.2(6)</td>
<td>190</td>
<td>7.2</td>
<td>0.13(31)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>0.13(31)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Bovine   |                  |              |                   |                      |                  |                |                |
| Fibrolamellar | 54          | 10.7(20)     | 0.14(20)          | 272                  | 1.6              | 0.15(3)        |                |
|          | 55                   | 4.7(6)      | 0.7               | 171                  | 1.6              | 0.15(3)        | 70             |
|          | 167                  | 5.9(6)      | 0.7               | 171                  | 1.6              | 0.15(3)        |                |
|          | 39                   | 68(6)       | 0.7               | 171                  | 1.6              | 0.15(3)        |                |
|          | 30                   | 3.2(6)      | 0.7               | 171                  | 1.6              | 0.15(3)        |                |

Table 2.2 Strength of human and bovine bones (in MPa) summarizing results found in previous studies [11, 12, 109, 112, 115].
Increases in tensile strength and failure strain along the longitudinal axis have been recorded in a number of additional studies [87, 109, 112]. The energy required to fracture the bone sample in the longitudinal direction was also shown to be 16 times higher than transverse direction, which coincided with physiological loading conditions of bone as most of the bones carry loading along the longitudinal axis [16, 117, 118]. For example, limb bone discussed in Section 2.4.1 has a main function of bearing a bending moment applied either in tension or compression along the longitudinal axis. Strain dependent strength additionally exists, with a higher tensile strength and higher strain rate recorded in some studies [10, 108, 117-119].

**Fracture Mechanics and Toughening Mechanisms**

The fracture behaviour of bone and the resultant toughness requires consideration of fracture mechanics. The first concept of fracture mechanics was developed in 1920 by Griffith [120] to describe how a crack would propagate in a material under external loading. The description of crack propagation in the material used energy considerations to determine if the stored strain energy could be released to propagate the crack by the creation of new surface area. Load was applied to a sample containing an artificial pre-crack until failure. A crack will extend in a solid material when the energy released from the material
as the crack grows exceeds the energy required to propagate the crack. The critical strain energy rate describes the energy dissipated during fracture per unit of newly created fracture surface area. The crack will grow when the stored energy release rate $G$ is greater than or equal to a critical value $G_c$ which is an inherent mechanical property of material. The critical energy release rate $G_c$ is also called fracture energy and will be used in this thesis. Tough materials that are characterized by a resistance to crack propagation have large $G_c$ values. The shape of the crack has provoked further analysis to consider crack geometry and dimensions, and how these parameters influence crack propagation within the material using stress analysis. Specifically, a crack will propagate when the stress at the crack tip reaches a critical value that overcomes the cohesive strength of the atoms just ahead of the crack. This critical value is also an inherent property of materials, which is defined as the critical stress intensity factor $K_c$. For materials with linear elastic behaviour, the critical stress intensity factor is related to the critical strain energy release rate by:

$$G_c = \frac{K_c^2 (1 - \nu^2)}{E} \quad \text{Equation 2.3}$$

where $\nu$ is Poisson's ratio and $E$ is Young's modulus. The application of fracture mechanics to solid materials has been extensively established but is less developed for bone due to the structural complexity where cracks may propagate.
at different length scales. The applicability of fracture mechanics to bone is therefore limited due to the linear elastic behaviour assumed by fracture mechanics that is not applicable in the rate-dependent viscoelastic behaviour of bone [16].

The fracture behaviour of bone can be characterized by $G_c$ or $K_c$ and these values are often calculated experimentally. However, a more direct method for calculating parameters to describe the failure of bone uses a ‘fracture toughness’ evaluation. The method for determining fracture toughness involves deformation of bone in order to record a load-displacement curve until failure. Deformation of bone is typically achieved using a three-point bending method as shown in Figure 2.15 (A). A notch is introduced at the mid-point of the bone and a crack propagates during loading. The area under the load-displacement curve indicates how much work is consumed in breaking the specimen into two as shown in Figure 2.15 (B). The energy required to break the specimen is considerably larger in bone that most other single phase materials [15], which can be explained mechanistically in terms of the nature of the crack path. A central feature of this bone fracture behaviour is that weak interphase regions between constituents at different levels provide multiple preferred paths for cracking, as opposed to a single crack pathway for many homogeneous materials. Interphase regions exist between the constituents in bone that have specific orientations, and provide the
basis for the marked anisotropy of the fracture properties of bone (bone is easier
to split and to break)\[6, 15, 16, 118\] and for the fact that the toughness is actually
lower in shear than in tension [14-16].

Figure 2.15 Three-point bending for toughness measurements. (A) Schematic
showing the three point bending test for measuring toughness. (B) A typical
load-displacement curve obtained from testing on mouse tibia bone in the
longitudinal direction [121].

The toughening of bone materials can be explained by both deformation or shielding mechanisms as reviewed by Launey et al. [15]. The strength and plasticity of bone are highly affected by the mechanical properties of its constituents which are strongly dependent on the scale of examination. Many studies have indicated the toughening mechanisms of bone are active predominantly at submicrometer levels [2, 9, 16, 122-126]. However, a smaller number of studies have suggested crack propagation at larger scales absorb the most energy [13]. While it is difficult to accept that small scale constituents such as MCFs do not contribute to crack propagation at any length scale, a model describing the relative contributions of the different length scales to toughness has yet to be provided.

Previous work has indicated that osteons are the main structural feature that appears to control toughening at large length scales of the order of several hundred micrometres [104]. The osteons in bone fracture provide a source of toughening that arises during crack growth rather than during crack initiation. Hence, osteons are not inherently tough but control the propagation of cracks in bone, known as shield toughening [115, 127]. As shown in Figure 2.16, the existing osteons in bone provide a longer crack propagation pathway, which
requires an increase in the overall work done to fracture of whole bone.

Figure 2.16 Anisotropy in the fracture behaviour of bone. (A) and (B) show the crack propagate along osteon interfaces and through osteons to create longer crack paths in three point bending tests on bone at the longitudinal direction (defined as the osteon parallel to specimen long axis). (A) Scanning electron micrograph showing crack tip and (B) schematically shows the crack growth. (C) and (D) demonstrate the crack developing in bone in the three point bending test on bone in the transversal direction (osteon vertical to specimen long axis). In the schematic diagrams (B) and (D), the osteons are represented in brown while the interface regions are in yellow.
In compact bone, the crack path of least resistance is invariably along the brittle hypermineralized interfaces called cement lines [14, 126, 128] between osteons and bone matrix. The cement lines are the preferential sites for major microcracks to form. When a crack propagates, the microcracks form near the crack tip area as shown in Figure 2.17 (A) where highest stresses are distributed. These microcracks have a typical spacing in the tens to hundreds of micrometres and are aligned primarily along the long axis of the bone, an orientation that directly results in the strong toughness anisotropy in bone. As these microcracks are mostly limited in the cement lines spaces, they are also called ‘monostrained microcracking’ [12, 115, 127]. Some recent simulations [9, 126] have clearly shown that, although microcracking is important intrinsically for toughness, its direct contribution to toughness is minimal and has been estimated to be less than 10 % [9, 126]. However, the importance of microcracking in toughening mechanisms is that it is essential for developing other significant toughening mechanisms at micron length scales and above [6, 129], including crack bridging and crack deflection [128, 130-133], which are the most potent toughening mechanisms in bone at large length scales. Microcracking may also important in providing signalling for bone remodelling which occurs in bone multicellular units (BMUs) including both osteoclasts and osteoblasts [134].
Fracture mechanics descriptions of crack growth highlight the competition between the direction of maximum mechanical driving force and the path of weakest structural resistance [14, 135]. High toughness is usually developed when these two paths exist orthogonally. For example, in three point bending testing on longitudinal oriented bone samples, the crack driving force is vertical to the preferable crack path (starting from microcracks in cement lines) which is along the osteons generating high toughness as seen in Figure 2.16 (B). In contrast, in the transversal direction, preferred mechanical and microstructural crack paths are nominally in the same direction as shown in Figure 2.16 (D). The orientation of the osteons can lead to significant (macroscopic) deflection of cracks that are attempting to propagate in the longitudinal oriented sample, which makes the longitudinal orientation much tougher than the transversal direction. Recent fracture mechanics measurements show that after only 500 μm of cracking, the fracture toughness, specifically the driving force for crack propagation, is more than five times higher in the longitudinal direction than in the transversal direction [128]. This toughening mechanism is named crack deflection [15] as shown in Figure 2.17 (B).
Figure 2.17 (A) to (D) Different toughening mechanisms in compact bone at the architectural level. (E) Example of an R-curve [136] (Fracture toughness vs. crack length) for an equine femur bone specimen.
The crack path and toughening mechanisms in the transversal oriented sample are quite different to the longitudinal direction. Preferable crack propagation paths are now parallel to the driving force which leads to the microcracks formed in cement lines in front of the crack tip and have the same direction as the crack path. The aggregation of these microcracks leads to the formation of the ‘secondary’ cracks or ligaments in the uncracked region ahead of crack tip as shown in Figure 2.17 (C). These secondary cracks act to bridge the crack and carry load which would otherwise be used to further crack propagation [9, 104, 126, 128, 137]. This mechanism called uncracked ligament bridging results in toughening but is a less significant mechanism than crack deflection [15, 128].

Another crack bridging mechanism can be found in tests on both longitudinal and transversal bone samples at smaller scale. Collagen fibrils bridging in many of the microcracks formed during in fracture affected area in bone [Figure 2.17 (D)] could help to stop crack propagation [133]. Researches showed that fibril bridging does not contribute significantly to the overall toughness (~1 MPa.m^{1/2}) for a single propagating crack [126, 128]. However, it is still important to carry out further study for its cumulative, integrated effect throughout the numerous microcracks that are generated in bone, which could lead to a novel toughening mechanism. The effect of collagen fibrils on bone toughness has been ignored in these previous studies due to the difficulty of examining samples at low
dimension but the influence of collagen fibril structural features on bone toughness has been highlighted as important in some simulation works [138]. Specifically, numerous fibrils pulled out from the bulk bone material around microcracks will create a relatively large new surface. The energy absorbed during the creation of this new surface area could contribute dramatically to the toughening mechanisms of bone. In addition, fracture between extrafibrillar mineral crystals and MCFs in the plastic deformation of bone initiates the formation of microcracks [139], which is the basic phenomenon involved in toughening mechanisms including crack deflection and uncracked-ligament bridging.

The multiple length-scale toughening acting in cortical bone leads to a characteristic resistance-curve (R-curve) behaviour of bone material where fracture toughness increases with crack extension [13, 136, 140], which is shown in Figure 2.17 (D). It is believed that R-curve behaviour should be sensitive to regional microstructural differences in bone [136]. With crack propagation, there is an increasing number of crack deflection and microcracks appearing in a neighbouring space away from the crack tip to enhance the fracture resistance. Further, crack propagation resistance in longitudinal samples is more sensitive to the regional structural differences comparing to transversal samples [127].
b. Cancellous bone

Mechanical properties of cancellous bone

The lighter, porous tissue in bones, called cancellous or spongy bone, also plays an important role in whole bone’s mechanical functions. The mechanical properties of cancellous bone are more complex than cortical bone as its mechanical properties are defined by both the mechanical properties of the cancellous bone material (which is the solid bone making up the trabeculae) and its porous structure. To isolate the material properties of cancellous bone from its structural effect, single trabeculae have been machined away from the cancellous bone matrix and mechanically tested using different testing methods. Table 2.3 summarizes a range of methods used for measuring mechanical properties of cancellous bone material including buckling testing, direct mechanical testing, nanoindentation, ultrasonic testing and computational simulation. In bulking tests, a single trabeculum was applied with compression from both ends and the load required for buckling the trabeculae was recorded to evaluate the Young’s modulus by Euler is buckling formula [141, 142].
Table 2.3. Recorded Young’s moduli of cancellous bone material taken from Currey [12].

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Bone type</th>
<th>Method</th>
<th>Young’s Modulus (GPa)</th>
<th>Wet/Dry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runkle and Pugh</td>
<td>1975</td>
<td>Human femur</td>
<td>Buckling</td>
<td>8.7</td>
<td>Dry</td>
</tr>
<tr>
<td>Townsend et al.</td>
<td>1975</td>
<td>Human femur</td>
<td>Buckling</td>
<td>14.1</td>
<td>Dry</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.4</td>
<td>wet</td>
</tr>
<tr>
<td>Ashman and Rho</td>
<td>1988</td>
<td>Bovine femur</td>
<td>Ultrasonic</td>
<td>10.9</td>
<td>wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human femur</td>
<td></td>
<td>12.7</td>
<td>wet</td>
</tr>
<tr>
<td>Choi et al.</td>
<td>1990</td>
<td>Human tibia</td>
<td>3-point bending</td>
<td>4.6</td>
<td>Wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortical</td>
<td>3-point bending</td>
<td>4.4</td>
<td>wet</td>
</tr>
<tr>
<td>Kuhn et al.</td>
<td>1989</td>
<td>Human ilium</td>
<td>3-point bending</td>
<td>3.7</td>
<td>Wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortical</td>
<td></td>
<td>4.8</td>
<td>Wet</td>
</tr>
<tr>
<td>Mente and Lewis</td>
<td>1989</td>
<td>Human femur</td>
<td>Cantilever + FEA</td>
<td>6.2</td>
<td>Wet</td>
</tr>
<tr>
<td>Ryan and Williams</td>
<td>1989</td>
<td>Bovine femur</td>
<td>Tension</td>
<td>0.8</td>
<td>Drying</td>
</tr>
<tr>
<td>Jensen et al.</td>
<td>1990</td>
<td>Human vertebra</td>
<td>Structural analysis</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Rho et al.</td>
<td>1993</td>
<td>Human tibia</td>
<td>Tension</td>
<td>10.4</td>
<td>Dry</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortical</td>
<td></td>
<td>18.6</td>
<td>Dry</td>
</tr>
<tr>
<td>Rho et al.</td>
<td>1993</td>
<td>Human tibia</td>
<td>Ultrasound</td>
<td>14.8</td>
<td>Wet</td>
</tr>
<tr>
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<td></td>
<td>20.7</td>
<td>Wet</td>
</tr>
<tr>
<td>van Rietbergen et al.</td>
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<td>3-D FEA</td>
<td>6</td>
<td>Wet</td>
</tr>
<tr>
<td>Turner et al.</td>
<td>1999</td>
<td>Human femur</td>
<td>Ultrasound</td>
<td>17.5</td>
<td>Wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortical</td>
<td></td>
<td>17.7</td>
<td>Wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human femur</td>
<td>Nanoindentation</td>
<td>18.1</td>
<td>Wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortical</td>
<td></td>
<td>20.0</td>
<td>Wet</td>
</tr>
<tr>
<td>Zysset et al.</td>
<td>1999</td>
<td>Human femur</td>
<td>Nanoindentation</td>
<td>11.4</td>
<td>Wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortical</td>
<td></td>
<td>16.7</td>
<td>Wet</td>
</tr>
<tr>
<td>van Lenthe et al.</td>
<td>2001</td>
<td>Bovine femur</td>
<td>FEA &amp; ultrasound</td>
<td>4.5</td>
<td>Wet</td>
</tr>
</tbody>
</table>

Young’s modulus of trabeculae listed in Table 2.3 is noted as being slightly lower than the values of compact bone in Table 2.1. In a following compression study between the individual trabeculae and cortical bone specimens with the same geometry and dimensions, it was found that the cancellous bone material has a
lower Young’s modulus comparing to compact bone [25, 26]. The nanoindentation tests on both individual trabeculae and cortical bone also suggest a similar variation [25, 26] which was further confirmed by ultrasound investigations on cancellous bone and was explained by the less organized structure in cancellous bone material [26].

**Mechanical function of cancellous bone and mechanically mediated bone adaptation**

Large volumes of whole bone are occupied by the cancellous structure to minimize mass [1, 3]. Two features can be easily observed from bone’s anatomy structure shown in Figure 2.8 (A). Firstly, cortical and cancellous bone can be clearly distinguished as two distinct structures. Secondly, trabeculae are arranged following a certain pattern in an angle associated with the direction of applied loads. This organization of trabeculae indicates bone possesses a degree of structural adaptation. Early theories first discussed in 1867 [143] presented drawings of trabecular arrangements in human’s proximal femur that were identical to the principal stress lines of a crane as shown in Figure 2.18. Wolff notably developed theory based on similar observations and defined the classical ‘Wolff’s law’ [143]:
“Every change in the form and the function of a bone or of their function alone is followed by certain definite changes in their internal architecture and equally definite secondary alterations in their external confirmation, in accordance with mathematical laws”

Figure 2.18 (A) Culmann’s schematic diagram showing principal stress line in an engineering crane. The crane was under a distributed vertical load from the top. (B) Wolff’s depiction of trabecular arrangement in proximal femur bone. Cited from Wolff [143].

Further bone adaptation theories provided a summary of the common mechanisms used to optimize bone when under external loading including:
• Trabeculae are oriented along axes of principal stress.
• The highest bone density can be found in areas of highest shear stress.
• Bone tissue adapts to external mechanical stimulation in an optimal manner.
• Bone cells respond to local force and adapt bone tissue correspondingly.

The theories of bone adaptation have been developed dramatically afterwards especially with the help of numerical simulations [144-148]. At present, the current state of bone adaption theories can be summarized as:

• The structural adaptation of bone is stimulated by mechanical strain [147].
• The development stage of bone is critical in defining how bone tissue responds to external load [147].
• The tolerance of mature bone to mechanical strain is reduced when compared to more immature bone and mature bone operates within a limited range of strain [147, 149].
• Bone structure developed due to mechanical adaptation show attributes of efficient or optimized structure [147, 148].
• Bone cells (mainly osteoblast and osteoclast) can directly respond to
applied mechanical strain [146, 148].

From discussion above, we can see how cancellous bone adapts to load and forms its unique architecture with trabeculae oriented along the principle stresses transferring applied loads effectively. An additional important mechanical function of cancellous bone is the ability to absorb energy from impact [1, 3]. Figure 2.19 shows the stress-strain curve of cancellous bone and indicates a characteristic shape optimized for energy absorption [150].

![Stress-strain curve of cancellous bone](image)

Figure 2.19 Stress-strain curve recorded in a cancellous bone compression test from Currey [12] based on data derived from Fyhire and Schaffler [150].
In the stress-strain curve of cancellous bone under compression testing, a clear linear response can be observed at the start of the compression test which is due to the elastic loading of bone material. Applied compressive strains further causes a drop in the stress until a plateau stress region is achieved as shown in Figure 2.19. The initial stress drop with strain is due to buckling of trabeculae. The ‘stepping’ indicates increasing numbers of trabeculae involved in the buckling. Finally, all trabeculae fail in bucking and the bone becomes compact to prevent from further collapse. These trabeculae buckling processes occur over a relatively large strain range to ensure that the work done is maximized during compressive testing of cancellous bone [12, 150].

2.4.3 Level 5-3: From Tissue to Fibrils

Several levels exist in bone’s structural hierarchy that are below the architecture level (macro level) and above fibrillar level (nano level). These include Haversian systems, lamellar structure and fibril arrays as discussed in Section 2.3. Although unique features existing at these levels, such as cement line or lacunae sites, are believed to have different mechanical properties comparing to bone tissue, most of the structural components at these levels have similar mechanical properties and compositional proportions comparable bone tissue at the architectural level [18, 37, 38].
2.4.4 Level 2: Mineralized Collagen Fibrils

The origin of the mechanical properties of bone is contentious, with structural organization over different length scales and constituent properties all expected to contribute [1, 2, 7, 49]. To isolate these constituents and their potentially synergistic effects is crucial in understanding the mechanism for bone deformation and fracture. MCFs are considered as the basic building blocks of bone as they are the smallest structural units containing all major materials in bone and all MCFs from different sources are expected to be the same in composition and material properties [2, 7, 49]. The collagen fibrils act as an organic framework in bone and determine, at least to some extent, the mechanical behaviour of bone. Bone is structurally a nanofibres composite of collagen fibrils acting as a template for the mineralization of carbonated apatite crystals. These mineral crystals exist both within the collagen fibril at the gap region and also between fibrils.

As the basic building blocks of bone, MCFs take more than 80% of the total volume of bone making it the most important constituent in defining mechanical properties of bone. Some studies suggest the spatial organization of collagen fibrils at higher levels determines the materials properties of bone at macro level. However, the mechanical properties of these structural constituents at higher levels are essentially defined by the collagen fibrils [2, 7, 49]. In addition, in bone
fracture, the MCFs bridging in cracks provides resistance to crack propagation [133] which contributes the high toughness of bone. Further study showed the fracture of extrafibrillar mineral crystals surrounding fibrils or delamination at the crystal/MCFs interfaces has been suggested as the cause of microcracks [139] which is the essential phenomenon of most of the potent toughening mechanisms.

Measurements and modelling of the mechanical properties of collagen and bone have been performed at molecular, micron, and macro scales. However, the scale around hundreds of nanometres, corresponding to the diameter of one fibril, is relatively unexplored. Despite the fact that we have a relatively complete understanding of structural information of MCFs in bone, their mechanical properties and the influence on overall bone mechanics, such as toughness in bone, has yet to be determined.

Several methods have been used to evaluate the mechanical behaviour of mineralized collagen fibrils in bone. With the development of material testing instrumentation, there are currently more techniques can be used to provide capabilities on low scale mechanical testing such as MEMS and AFM. However, the direct testing on isolated single mineralized collagen fibrils from bone tissue has yet to be obtainable using current methodology [20, 40, 105, 151]. In this
thesis, individual mineralized collagen fibrils and its system from bone are tested using a combination of atomic force microscopy (AFM) to manipulate and mechanically test individual mineralized collagen fibrils while scanning electron microscopy (SEM) provides high resolution in situ imaging.

Previous work has examined the mechanical properties of unmineralized CFs from a number of sources directly using AFM [20, 40]. AFM is often preferred to other techniques as both high-resolution imaging and mechanical deformation can be provided using a single AFM probe. First attempts on direct mechanical testing of in vitro-assembled human type I collagen fibrils were carried out by Graham and co-workers [39] using an AFM probe to pull an individual collagen fibril from a connecting substrate. However, the relatively low collagen fibril Young's modulus (32 MPa) suggested sliding of the collagen fibril from the AFM probe during testing, which was overcome using glue at the fibril-AFM probe junction to give modulus values approaching 0.8 GPa [20].

Further research was carried out on the mechanical properties of unmineralized individual CFs from sea cucumber using a microelectromechanical (MEMS) device [40]. The selection from this source was beneficial as the fibril diameters are typically relatively large, allowing manipulation of fibrils under optical microscopy for nanomechanical tests.
Figure 2.20 Schematic of direct mechanical testing on mineralized collagen fibrils. (A) Force spectroscopy experiment on collagen fibrils from Graham’s work. The fibrils are adsorbed between a clean glass surface and an AFM cantilever. The movement of the cantilever caused the resultant strain of collagen fibril and recorded by laser optical sensor [39]. (B) A similar test improved by van der Rijt et al using a drop of glue on the glass surface [20]. Both of the tests recorded relatively low modulus and did not measure the fracture properties of collagen fibrils.
Both AFM and MEMS devices are limited as individual fibrils are required to be first removed from the parent material sample, typically using chemical treatments that will dissolve hydroxyapatite in mineralized fibrillar assemblies. Other methods have overcome this limitation by using combinations of synchrotron x-ray diffraction together with tensile testing [152-154] and nanoindentation [31] to deform bulk bone materials and derive collagen fibril and mineral mechanics. While powerful, these techniques average the mechanical properties of the constituents over the volume of interest, typically many tens of cubic microns in the case of x-ray diffraction. Therefore, the mechanical properties of mineralized collagen fibril constituents as well as their influence on bone deformation and fracture are still to be ascertained.

2.4.5 Sub-structural Components of Bone

a, Calcium Apatite Mineral

The natural calcium appetite crystals from bone have yet to be isolated and tested due to the small size especially the extremely small thickness [68, 69]. Studies on artificial synthetic hydroxyapatite have shown that the Young’s modulus of synthetic powdered carbonated apatite was 109 GPa, whereas a larger value of 114 GPa can be observed for a large single crystal of hydroxyapatite [3]. The reason why dahllite in bone has a unique plate shape,
which differs from symmetrical hexagonal crystal structure of bulk dahllite, is still unknown.

b, Mechanical Properties of Collagen

The mechanical testing on bulk collagen materials such as tendon and cartilage that predominantly contain type I collagen has been previously carried out. The recorded materials properties on collagen itself (usually assembled in vitro or extracted from tendon and cartilage) generally show relatively large variations. The Young’s modulus values recorded on hydrated type I collagen from tendon ranged between 400 MPa and 1000 MPa, while failure strength having values from 50 to 100 MPa and the ultimate strain ranged from 4 to 20% [42, 46, 155-158]. The water content and arrangement of fibrils were believed to be crucial in determining the overall mechanical behaviour of the collagen material.

At the molecular level, plastic deformation of collagen involves the stretching of molecules and unwinding of tropocollagen molecules due to the entropic first and then the breaking of H bonds between molecules. The intermolecular sliding provides large plastic strain (up to 50% in physiological conditions) within collagen without catastrophic failure [105, 159-161].
c, Interaction Between Mineral Crystals and Collagen Molecules Within MCFs

Because the mineral phase has an elastic modulus that is more than an order of magnitude higher than that of collagen, the presence of the hydroxyapatite phase is critical to the stiffness of bone. Previous researches show a continuous increase in Young’s modulus with mineralization of collagen fibrils, ranging up to a factor of three for high mineral content [12]. Computational molecular modelling and experimental X-ray analysis suggest that under tension, slippage between mineral crystals and tropocollagen molecules is initiated and following by a continuous sliding between tropocollagen molecules as well as between mineral particles and tropocollagen molecules. This sliding at the molecular level enables a large regime of dissipative deformation as soon as yield point is reached which effectively increases the toughness [138] (Figure 2.21). Stress in fibrils can be maintained after slip started because of additional resistance to slip at the interface between collagen and mineral particles, which results in a dramatic increase in energy dissipation.
Figure 2.21 Computational simulation results of the stress-strain response of a mineralized fibril versus an unmineralized collagen fibril, demonstrating the significant effect of the presence of mineral crystals in collagen fibril on its mechanical behaviour [138].

In addition to providing a plastic deformation mechanism, this molecular behaviour of collagen and mineral phases under deformation within MCFs also represents a fibrillar toughening mechanism, which increases the energy dissipation compared to unmineralized collagen fibrils [105, 138, 151]. The existence of the nanoscale mineral crystals in MCFs increases the fibril Young’s modulus and fracture toughness. From the same computational simulation, the Young’s modulus, yield strain, and fracture strength in tensile tests of MCFs were found to be 6.2 GPa, 6.7%, and 0.6 GPa, compared to corresponding mechanical properties of 4.6 GPa, 5%, and 0.3 GPa from testing collagen fibrils without a
mineral phase [105, 138, 151].

**d, Non-Collagenous Proteins (NCPs)**

The final constituent of bone, which is often ignored in bone structure, are the two hundred or more so-called non-collagenous proteins (NCPs) [71] generally comprising less than 10% of the total protein content. Currently, there is little data on the mechanical behaviour or composition of NCPs but MCFs are known to be “glued” together by a thin layer (1–2 nm thick) of NCPs [162, 163]. When bone is externally loaded in tension, the applied load is resolved into tensile deformation of the MCFs and shear deformation in NCP regions [125]. Single molecule spectroscopy of fractured bone surfaces has confirmed that the NCP layer between fibrils is relatively weak but ductile and deforms by the successive breaking of a series of sacrificial bonds [164, 165]. The separation of individual MCFs during plastic deformation of bone is resisted by this NCPs layer via sacrificial bonds. The force required to break these sacrificial bonds has been estimated to be about 10%-50% of the force required to break the backbone of the protein chains of NCPs [162]. The NCPs may be also partially mineralized [166], which will increase its shear stiffness and reduce its deformability. Previous work has indicated that the interface between the NCPs and MCFs is formed of sacrificial bonds that break during plastic deformation of bone but
reform, which leads to a toughening mechanism whereby the matrix (NPCs) / fibre (MCFs) interface is disrupted beyond the yield point, and the matrix moves past the fibres forming to reform the matrix/fibre bonds continuously [162, 167, 168].

2.5 Conclusions

To conclude, bone is a hierarchical composite material composed primarily of assemblies of collagenous protein molecules, water, and mineral nanoparticles made of carbonated hydroxyapatite. The resultant bone structure is extremely tough, lightweight and adaptive to the physiological loading conditions experienced by the bone material. Methods of mechanically testing bone material at each hierarchical structural level and the correlated mechanical behaviour as well as bone toughening mechanisms were reviewed but a general lack of information at fibrillar length scales and their resultant contribution to overall bone mechanics was highlighted. Mineralized collagen fibrils are particularly crucial in defining overall mechanical behaviour of bone as they are the basic building blocks of bone. Specifically, the fibrillar network in bone plays an important role in the formation of microcracks and other toughening mechanisms in plastic deformation of bone.
Chapter 3

Methodology

3.1 Materials Preparation

Cortical bone samples were extracted from red deer and mouse for mechanical testing of their fibrillar system at the nanoscale. Antler bone was chosen due to its extraordinary toughness and absence of extrafibrillar mineralization. The mouse limb bone was chosen because of its rapid growth of a skeletal system and associated changes in the mineralization. Due to the considerable size variations found in whole antler bone and mouse limb bone, a range of sample preparation strategies were required. In this section, the preparation methods for antler and mouse bone samples used in this study are reported in detail.
3.1.1 Antler Bone Sample Preparation

A diversity of collagen fibril assemblies exist in bone materials with the antlers of deer notable as one of the toughest natural materials [49, 103, 129, 169]. Importantly, many of these apatite minerals are intrafibrillar in antler and provide a model composite fibre system of collagen reinforced with mineral, as opposed to many other bones where the mineral is also found in extrafibrillar spaces [103, 129, 154, 169, 170]. The prevalence of mineral within the collagen fibrillar framework in antler makes this material an ideal source of model mineralized collagen fibrils. Specifically, most of the mineral in antler is found within the fibril, resulting in a simple composite fibre of mineral reinforcing collagen molecules. Antler is notable as a bone material that exists outside the body and the cortical bone tissue in antler is known to have low water content varying from $13.2 \pm 1.2\%$ to $22.2 \pm 4.8\%$ depending on different times of the year [171]. This relatively dry state of the antler is importantly potentially compatible with the environment of an electron microscope chamber.

Samples were extracted from the main beam from an antler (after removal of velvet) of a mature red deer (Cervus elaphus). Because recent studies have shown that the selection of tissue region within the antler beam affects the composition and mechanical properties [154, 171], the samples were selected from the same compact cortical shell near the antler-pedicle junction. Small
beams of antler with dimensions of 3×20×0.2 mm were cut from the bulk material by using a water-cooling rotating diamond saw (Struers Accutom-5, Figure 3.1).

Figure 3.1 Struers Accutom-5 water-cooled rotating diamond saw.

The long direction of antler samples was oriented parallel to the antler main beam direction, which is also the principal osteonal axis as shown in Figure 3.2. The beams were transferred into Hank’s buffered solution and left overnight. This procedure allows full sample rehydration and mitigates mineral loss that may occur in distilled water or physiological saline. Samples were fractured perpendicular to the long axis to expose a number of individual fibrils at the fracture surface. Water on the surface of sample was removed by filter paper to avoid interference with SEM imaging.
Figure 3.2 (A) Optical image of an antler cross section, highlighting the compact cortical shell and the trabecular core. (B) Schematic representation of the sample: S shows the sample location and the orientation within the cortical bone C of an antler section; the trabecular region is indicated by T; (C) Optical microscopy image of a polished and HCl etched cortical bone cross section surface, showing
the typical tissue composition used for the experiments. (D) and (E) scanning electron micrographs on fractured antler samples at different magnifications showing exposed MCFs.

### 3.1.2 Mouse Bone Sample Preparation

Due to its relatively small size, the mouse limb bones were used directly as specimens without further cutting as for the antler. Fresh femur and radius bones from wild type 4 week and 10 week old mice were extracted as shown in Figure 3.3 (A). Limb bones were kept wet in Hank’s buffered solution during preparation and stored at -200 °C in wrapped gauze soaked in Hank’s buffered solution for up to 1 week until mechanical testing. Mouse bone samples were allowed to defrost at room temperature for 24 hours prior to mechanical testing. Soft tissue was removed from the bone surface using a blade following the defrosting stage and the fresh bone sample was kept in 70 % ethanol for 4 hours for fixation. Limb bone samples were then transferred to Hank’s buffered solution and left overnight to allow full sample rehydration and mitigate mineral loss that may occur in distilled water or physiological saline. All the processes above have been approved to have minimum influence on mechanical properties of bone [1, 7, 12, 16, 48, 49, 56]. Femur samples were used for direct tensile testing as it is easier to perform due to its larger size while radius samples were fractured perpendicular to the osteon long axis to expose the individual MCFs at
Chapter 3. Methodology

the fracture surface. Water on the surface of sample was removed by filter paper to avoid interference with SEM imaging. The fractured mouse radius bone was then transferred to the SEM chamber for MCF tensile testing as shown in the SEM image in Figure 3.3.

(A)

(B)

Figure 3.3 (A) Schematic skeletal system of a mouse indicating the location of femur and radius. (B) Fresh mouse radius bone from a wild type 10 week old male mouse with muscle and connective tissue covering the bone surface. (C)
Scanning electron micrograph showing individual mineralized collagen fibrils at the bone fracture surface.

### 3.2 in situ Nanomechanical Testing

As proposed in Chapter 1, a mechanical testing method, having ability in measuring nanofibres in their native state, is required. The nanomechanical testing setup used for this thesis is developed by integrating an atomic force microscope (AFM) for force measurements with a scanning electron microscope (SEM) to provide imaging capabilities. In this chapter, relatively simple samples of electrospun polyvinyl alcohol (PVA) and nylon-6 nanofibres were manipulated and tensile tested using the AFM-SEM setup in order to validate the method to evaluate the feasibility of this method. The complete stress-strain behaviour and failure of individual electrospun nanofibres was recorded and a diversity of mechanical properties observed, highlighting how this technique is able to elucidate mechanical behaviour due to structural composition at nanometre length scales.

#### 3.2.1 Introduction

Advances in nanotechnology and the growth in synthetic nanomaterial manufacture have required the development of specialized material mechanical
characterization methods at low dimensions. Nanofibres are one particular form of nanomaterial that show promise as a structural material in applications predominantly where the nanofibre acts as reinforcement in a composite [172-174]. Synthetic nanofibres typically exhibit superior mechanical properties along the fibre's principal axis, especially in polymeric materials incorporating uniaxial orientation of molecular chains [173, 175], and low defect density [176, 177] comparing to bulk material equivalents. Investigating the mechanical properties of nanofibres is therefore of critical importance in understanding the inherent nanofibre behaviour but is often challenging due to the relatively small length scales considered. Bone can also be considered as a nanofibrous composite consisting of MCFs with additional complex structural hierarchy that provides optimized mechanical properties. The effectiveness of biological composite materials in a number of mechanical applications, most notably for toughness, has motivated researchers to both understand structure-property relationships in biology and develop bio-mimetic materials incorporating such biological design features [178-181]. Current challenges exist in relating the nanoscale, and typically fibrous, components found in structural biological materials to their overall mechanical properties. Interestingly, nanofibrous components are common to most biological materials, from MCFs in this thesis that provide the organic framework in bone [17] to cellulose fibrils in wood [182]. Measuring the mechanical properties in both synthetic and biological
nanofibres is therefore relevant in determining the overall mechanical properties of structural materials incorporating nanofibre components. Critically, difficulties exist in testing constituents that have dimensions approaching the nanoscale, especially when testing nanofibres beyond elastic limits to failure.

Mechanical testing of an individual nanofibrous sample is expected to be the most direct method for examining mechanical behaviour but three practical challenges exist in this approach: i) observation of the nanofibre, ii) nanomanipulation of the nanofibre so that a mechanical test can be achieved and iii) accurate recording of the applied force and deformation during mechanical testing [30]. Conventional universal materials testing machines are typically able to test a number of materials that are observable optically and can be manipulated by hand, with the range of forces recorded during mechanical testing reflecting the sample dimensions in the millimetre range and larger. However, the relatively small size of nanofibres cannot be observed optically and requires greater manipulation precision and force resolution than provided by conventional methodologies. Simple approaches based on the earlier instrumented indentation [183] or more recent nanoindentation techniques [184] have been previously used to predict the tensile strength of materials, including one-dimensional nanomaterials, based on applied load and contact radius of the indenter tip with the sample [185]. However, the testing configuration is typically
difficult to control, with considerable assumptions in the material behaviour used to calculate mechanical quantities such as elastic modulus and yield strength [186-188].

During the last decade, atomic force microscopy has been frequently employed as a high-resolution force measurement tool coupled with its ability to image surfaces with nanometre resolution. AFM force spectroscopy has sufficient force resolution for investigating mechanical properties of surfaces [189-193], molecular and nanofibrous samples [164, 177, 194], bimolecular interactions [22, 195, 196] as well as single polymer and protein mechanics [24, 197-199]. However, in small scale fibre mechanical testing studies, manipulation of the fibrous samples through to mechanical testing requires direct observation of the sample to ensure correct alignment of the fibre along the loading direction and verification of sample failure.

The current state-of-the-art in direct tensile testing of nanofibres typically uses dedicated nanomanipulators to move an individual nanofibre to the end of an AFM probe while observing this manipulation using high resolution scanning electron microscopy, and was pioneered from investigations into carbon nanotube mechanics [164, 172, 200]. Gripping of the nanofibre to the AFM probe is achieved either by electron beam-induced deposition (EBID) [200] at the
nanofibre-AFM probe junction or manipulating the AFM probe into epoxy glue [164] prior to the nanofibre attachment to the probe apex. The free end of the nanofibre attached to the AFM probe is typically adhered to an immovable surface, thus providing gripping at both of the nanofibre ends. Direct tensile testing of the nanofibre is achieved by further manipulation of the AFM probe. The force applied to the sample causes the AFM cantilever beam to deflect during the test, with this deflection observed using high resolution microscopy. Thus, cantilever deflection can be defined as force if the spring constant of the cantilever is known. Similar tensile test procedures have been carried out on electrospun nanofibres including polyethylene oxide (PEO) [201] and nylon-6,6 [202] using optical microscopy to observe the manipulation and testing due to the fibre diameters being above the optical limits of light [30]. Finally, microelectromechanical systems (MEMS) have been used to measure the mechanical properties of fibrous materials but require significant manipulation accuracy to position the sample between gripping elements using optical microscopy or SEM to observe such manipulation [175, 203-205]. Typically, the force sensing system of piezoelectric ceramic devices used in MEMS is less accurate than the optical system used to measure force in the AFM.

Recent work has tensile tested electrospun polymer nanofibres with diameters less than 100nm using AFM probes and SEM imaging [206]. Firstly, the individual
electrospun nanofibre bridging across trenches in a substrate were hooked to an AFM probe. Tensile testing was performed by translating the AFM probe away from the fibre length to form a fibre ‘v’ shape during the deformation process. As with other tensile testing techniques, the force applied to the nanofibre was calculated from observing the AFM cantilever bending under the SEM while fibre deformation was also recorded by the SEM. However, tensile testing of these individual electrospun nanofibres requires both separation of nanofibres from one another and bridging of the nanofibre across substrate trenches. This preparation is often difficult to achieve in biological samples where nanofibre separation only occurs in aggressive chemical environments that damage samples [157, 207]. Tensile tests of such samples have to be carried out in their as-received or as-fabricated state. Critically, the inherent measurement of force applied to nanofibres from AFM cantilever observations is also less accurate than the AFM force spectroscopy where dedicated optical sensors are used to continually record cantilever deflections.

This section describes a novel in situ mechanical test method applied to a range of different nanofibrous specimens by incorporating ‘true’ AFM force spectroscopy with the imaging capabilities of a high resolution SEM. This ‘true’ AFM force spectroscopy uses an optical sensor to directly measure cantilever deflection as is used in stand-alone AFM and does not rely on imaging to measure
cantilever deflections with a correspondingly lower accuracy, as has been used previously [1, 25, 34-40]. Subtle variations in nanofibre mechanical performance are therefore expected to be accurately determined. Additional manipulation is provided by a focused ion beam (FIB) housed within the chamber of a dual beam configuration that allows sample sectioning following attachment to the AFM probe. Nanofibre gripping and manipulation are achieved by using accurate AFM piezopositioners while tensile testing experiments continually record cantilever deflection at high force resolution using AFM force spectroscopy. This configuration allows accurate stress-strain curves to be recorded during nanofibre tensile testing to failure. For this study, the application of the AFM-SEM system is explored by testing two synthetic materials of electrospun polyvinyl alcohol (PVA) and polyamide (nylon-6) nanofibres.

3.2.2 AFM-SEM Setup and **in situ** Tensile Test

Electrospun polymer fibre samples of PVA and nylon-6 were provided by Nanoforce Ltd. UK. Mechanical testing of individual nanofibres was carried out in the chamber of the SEM incorporating an AFM system. A custom built AFM system (attocube systems AG, Ger) with sample stage positioned 90 degrees to a conventional AFM system was used to allow access of the electron beam of the SEM (Quanta 3D ESEM, FEI Company, EU/USA) to the sample as shown in Figure 3.4. The AFM head consists of a cantilever on the cantilever plate attached to a
piezoscanner, with the principal cantilever axis in the same axis as the electron beam. Manipulation using the AFM setup is achieved using movement of the sample stage or the AFM probe. Sample stage movement is enabled using xyz piezopositioner situated underneath the sample stage plate as shown in Figure 3.4. The piezopositioners allow coarse movement with a maximum translation distance of 5 mm. Fine manipulation is provided by movement of the AFM probe attached to the cantilever using the piezoscanner connected to the AFM head. This piezoscanner provides a maximum travel distance of 40 μm with sub-nanometre resolution. The force detection scheme is based on an all fibre low-coherence optical interferometer system situated behind the AFM cantilever. The AFM in this study is compact enough to be installed within a SEM.

Samples were attached to the AFM sample stage as shown in Figure 3.4 by carbon cement and positioned such that the direction of the nanofibres within the sample was oriented in the horizontal plane and towards the AFM probe. Vacuum compatible glue (Poxipol, Arg) was placed at the side of the sample on the sample stage and the chamber taken to vacuum. Once under vacuum, the SEM was used to image the manipulation of the sample and AFM probe. Manipulation was carried out by moving the sample towards the AFM probe using the piezopositioners behind the sample stage. The glue at the side of the sample stage was first moved into contact with the AFM probe and then separated.
Removal of the AFM probe from the glue deposited a small amount of glue at the apex of the AFM probe. The piezopositioners were then used to move the fibrous sample towards the AFM probe so that a nanofibre protruding from the sample contacted the glue at the AFM probe apex, as demonstrated in Figure 3.5 (A).

Figure 3.4 (A) Schematic diagram showing the *in situ* configuration for tensile testing of a nanofibrous sample using combined AFM-SEM. The insert shows
fixing of the nanofibre with glue between the substrate and AFM cantilever. (B) Optical photograph showing AFM fitted on SEM sample stage when door of SEM chamber is open. Dashed rectangle indicates location of the AFM. Secondary Electron axis (SE) and Focused Ion Beam axis are also labelled in the photo. (C) Higher magnification optical side view image of the AFM on the SEM sample stage.

The contact of the free length of the nanofibre to the glue at the apex of the AFM probe was achieved consistently within a 3-5 minute timeframe from fixing the glue within the SEM chamber. The maximum timeframe for attachment of the nanofibre to the glue on the AFM probe was approximately 10 minutes, after which the glue viscosity was too high to allow penetration of the nanofibre. Manipulation and attachment of the nanofibre to the AFM probe was observed using the electron beam of the SEM at a long sample working distance (15 mm), low accelerating voltage (2 kV) and low electron beam current (40 pA) to ensure that no electron beam damage occurs, as has been shown for soft polymer systems [208]. In addition, previous work has indicated that the effect of vacuum on hydrated samples using the SEM imaging conditions above is minimal if specimens are exposed to vacuum for relatively short times of the order of a few tens of minutes [17, 208, 209]. After contacting with the nanofibre, the glue was allowed to cure for 10 minutes to ensure that one end of the nanofibre is securely
attached to the AFM probe.

Figure 3.5 Scanning electron micrographs showing a typical tensile test of electrospun nanofibres (PVA) using *in situ* AFM-SEM. (A) A single PVA nanofibre was attached to an AFM probe using epoxy glue at the end of probe. (B) Sectioning of the PVA nanofibre from the fibrous mat provides isolation at the end of AFM probe. (C) Insertion of the free PVA nanofibre end into glue provides the standard nanotensile testing configuration. (D) PVA nanofibre tensile testing
carried out after solidification of the glue was achieved by translation of the AFM probe away from the glue surface until the nanofibre failed at its mid-length. The deformation of epoxy glue at the anchor point with fibre and was carefully examined by pixel analysis and excluded from original tensile test result. The maximum deformation of epoxy glue is relatively small (0.33 \( \mu \text{m} \)) compared to the length of the tested fibre recorded before and after testing (10.2 \( \mu \text{m} \) and 12.1 \( \mu \text{m} \) respectively).

A relatively high energy focused ion beam (FIB) integrated within the SEM chamber, as well as electron beam with high accelerating voltage and beam current, were used to section the polymer nanofibres to achieve a desired isolated nanofibre length of 10-20 \( \mu \text{m} \) fixed to the AFM probe. FIB was used to section the nylon-6 samples while PVA nanofibres were sectioned by focusing the electron beam of the SEM at high magnification. The FIB accelerating voltage and current used for sectioning was 30 kV and 1.5 nA respectively and the electron beam was used at around \( 10^5 \times \) magnification with an accelerating voltage of 30 kV and beam current of 0.2 \( \mu \text{A} \). Fresh glue (Poxipol, Arg.) was subsequently introduced into the chamber and the free end of the nanofibre pushed into the semi-liquid glue, as shown in Figure 3.5 (C). The glue was allowed to cure after 10 minutes to ensure that the second nanofibre end was securely fixed.
Fixed electrospun polymer samples were tensile tested using the force spectroscopy of the AFM. Mechanical testing of all nanofibres along their principal fibre axis in the SEM plane of view was achieved by first observing the nanofibre by the SEM. The AFM probe was then moved by a small amount of the order of tens of nanometres above and below the SEM plane of view and the forced acting on the nanofibre as recorded by the AFM examined. A small movement above or below this plane will cause the AFM cantilever to bend towards the glue, resulting in a recorded force, when the nanofibre in the SEM plane of view. Misaligned nanofibres not lying in the SEM plane of view cause asymmetric AFM cantilever bending during both movement above and below the SEM plane of view. Hence, only manipulated nanofibres with their length in the SEM plane of view are tensile tested.

Force spectroscopy was achieved by translation of the AFM probe away from the sample stage. This translation caused a bending of the AFM cantilever corresponding to the applied force acting on the attached individual nanofibre until rupture failure occurred. All nanofibres tensile tested failed in the middle of their free length, away from the holding glue and the substrate. The force applied to each nanofibre sample was calculated from the deflection of cantilever recorded using the optical fibre setup behind the cantilever. Cantilever deflection was converted to force using the thermal noise method [23].
The calculation of the stress in the nanofibre specimens require the accurate determination of their diameter. The measurement of nanofibre diameters was achieved by recording a series of nanofibre images along the nanofibre length using the SEM prior to mechanical testing. The SEM parameters used for manipulation were selected for nanofibre diameter observation using fast electron beam scanning speeds (~ 60 Hz) and rapid switching between relatively low to brief high magnification capture of the nanofibre image and back to low magnification. This methodology was applied to ensure no melting or deformation of the nanofibre occurred due to minimal exposure of the nanofibre to the electron beam.

After nanofibre failure, images around the nanofibre failure point were selected from the original series of SEM images taken prior to mechanical testing and a total of approximately 6 images per nanofibre sample underwent pixel analysis using ImageJ (NIH, USA) to determine an average nanofibre diameter. We note that previous studies on mechanical properties of nanofibres from various groups have also relied on measuring nanofibre diameter using the SEM [5, 6, 11, 13, 25, 34, 40].

The force applied on the specimen is detected by an all fibre low-coherence optical interferometer system situated behind the AFM cantilever as shown in
Figure 3.6. A non-linear sine curve of laser signal intensity vs. retract distance as shown in Figure 3.7 (A) is produced with cantilever bending during mechanical testing. The original data was recorded in the experiment and converted to a stress-strain plot as shown in Figure 3.7 (B). The testing technique is different to many previous AFM setups [25, 26] due to the non-linear data analysis applied to the interferometer employed in this system.

Figure 3.6 Schematic diagram of the laser interferometer used in the AFM illustrating the interference signal measured. Approximately 4 % of the laser light is reflected at the glass-air interface with an intensity of $I_1$ while ~96 % of the light is transmitted and partially reflected at the AFM cantilever with an intensity of $I_2$. The intensity $I_2$ depends on the reflectivity of the AFM cantilever.
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3.2.3 Data Analysis Principle

The fibrous samples were tensile tested by translation of the AFM probe away from the sample stage as described above. The tensile stress applied to the nanofibre can be calculated using Hooke’s Law:

\[ \sigma = \frac{F}{\pi r^2} = \frac{k d}{\pi r^2} \]  
Equation 3.1

where \( F \) is the tensile force acting on the nanofibre, \( k \) is the spring constant of the cantilever, \( d \) is the cantilever deflection and \( r \) is the nanofibre radius.

When the cantilever is translated away from the sample stage, the retraction distance of the piezoscanner X behind the cantilever is recorded by AFM. This translation causes cantilever bending and deformation of the fibrous sample. The retraction distance \( X \) is therefore equal to the sum of the cantilever bending \( d \) and the sample deformation \( \Delta L \). Hence, the nanofibre strain \( \varepsilon \) can be calculated using:

\[ \varepsilon = \frac{\Delta L}{L} = \frac{X - d}{L} \]  
Equation 3.2

where \( \Delta L \) is the elongation of the nanofibre which excludes the glue deformation as shown in Figure 3.5 (D), \( L \) is the original fibre length taken from SEM images of fibrous samples at the start of tensile testing and \( X \) is the retraction distance of the cantilever.
Equations 3.1 and 3.2 indicate that the determination of cantilever bending is required to measure both the stress and strain behaviour of the nanofibre during tensile testing. A laser interferometer optical fibre situated behind the AFM cantilever was used to accurately determine the degree of bending during the tensile test as shown in Figure 3.6. The interference of a laser beam reflected from the back of the cantilever to the end surface of a fibre optic interact constructively or destructively, defined as reflected laser intensity, as the optical fibre-cantilever distance changes. A typical sinusoidal reflected laser intensity-distance plot for nanofibre tensile testing is shown in Figure 3.7 (A).

Figure 3.7 Force spectroscopy plot from tensile testing of an individual nanofibre. (A) Original sinusoidal laser intensity-distance curve recorded in force spectroscopy. The Y-axis is reflected laser intensity while the X-axis indicates the piezoscanner retraction distance. Failure of the nanofibre is observed at a
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retraction distance of 15 μm. (B) Resultant stress-strain curve converted from
the sinusoidal curve based on Equation 3.4

Figure 3.7 (A) clearly indicates a sinusoidal variation in the collected laser
intensity as the cantilever bends due to piezoscanner translation during the
tensile test. The collected laser intensity $I_0$ is defined by the intensity of incident
lights $I_1$ and $I_2$ as well as the optical path length difference between the two,
defined as $2(D+d)$ as shown in Figure 3.6, thus:

$$I_0 = I_1 + I_2 + 2\sqrt{I_1 I_2} \cos \left( \frac{2\pi}{\lambda} \times 2(D + d) \right)$$  
Equation 3.3

where $I_0$ is the reflected laser intensity, $I_1$ and $I_2$ are the reflected laser intensities,
$D$ is the initial cantilever-fibre optic distance and $D+d$ is the cantilever-fibre optic
distance as the tensile test proceeds. $\lambda$ is the wavelength of the laser (1330 nm)
used in the laser interferometer setup. The cantilever deflection $d$ is therefore
calculated from:

$$d = \arccos \frac{I_0 - I_1 - I_2 \frac{\lambda}{4\pi} - D}{2\sqrt{I_1 I_2}}$$  
Equation 3.4

Equation 3.4 can therefore be applied to the collected laser light intensity data to
determine the cantilever bending. The force sensitivity of every test was
evaluated by defining the resolution of data collection in the sinusoidal
intensity-displacement curve. The laser intensity changing between two adjacent data points is converted to the minimum displacement recorded in the test by Equation 3.4. The force resolution can be then calculated from the minimum displacement of the cantilever recorded. For tensile testing of electrospun PVA fibres, 618 data points were collected in total with the smallest force recorded as \((5.4 \pm 0.8) \times 10^{-4} \, \mu\text{N}\). Tensile testing of electrospun nylon-6 fibres recorded 410 data points and the force resolution was \((1.65 \pm 0.3) \times 10^{-3} \, \mu\text{N}\). 768 data points were collected for the tensile test and the minimum force recorded was \((1.74 \pm 0.5) \times 10^{-3} \, \mu\text{N}\). The accurate determination of laser intensity \(I_1\) and \(I_2\) is crucial in force sensing but is usually difficult in practice due to the following challenges below.

**a. Intensity Drop**

In Figure 3.7 (A), a noticeable drop of laser intensity occurred as the cantilever deflected in the original data plot. This intensity reduction is mainly caused by the tilt of the cantilever along the long axis, which is schematically demonstrated in Figure 3.8. Bending of the cantilever as force is applied to the AFM probe will cause a deviation \(x\) of the reflected laser onto the fibre optic and a corresponding deviation angle \(\theta\) as shown in the Figure 3.8. Reflected laser light is therefore deflected away from the core of fibre optics. The displacement of the reflected
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spot can be calculated using (in a linear approximation):

\[
\frac{y}{D + d} \approx \sin \theta \quad \text{Equation 3.5}
\]

\( \theta \) is given as:

\[
|\theta| = \frac{FL^2}{2EI_m} \quad \text{Equation 3.6}
\]

where \( I_m \) is the geometrical moment of inertia; \( E \) is Young's modulus of material of the cantilever. The deflection of the cantilever \( d \) can be calculated using this equation:

\[
d = \frac{FL^3}{3EI_m} \quad \text{Equation 3.7}
\]

Figure 3.8 Schematic diagrams showing the deviation of laser light reflecting off a deflecting AFM cantilever.

Under external force applied to the AFM probe, the deflection of the AFM
cantilever leads to a shift of reflected laser beam away from core of fibre optics. The laser intensity recorded by optical sensor is therefore reduced with the deflection of cantilever, which causes the drop in the reflected laser light intensity. The deviation of laser spot on fibre optics is $y$ shown in Figure 3.8 where:

$$y \approx (D + d) \sin \theta$$  
Equation 3.8

Hence, if we assume a 450 µm long cantilever, with a spring constant of 0.2 N.m$^{-1}$ that displaces by 30 µm, which is the typical value in experiment, and a cantilever positioned 40 µm away from the fibre optics initially, a minimum laser spot displacement $y$ of 3 µm will be produced. The intensity drop in the dataset can be fitted with a Lorentzian function such as:

$$I_0 = \frac{(1 + \cos 2ax) \times b}{2[1 + (x/c)^2]} + \text{background}$$  
Equation 3.9

where $a$ gives the frequency of the cosine function, $b$ is a scaling constant, $c$ determines the decay rate of the laser intensity and the background offsets laser intensity from zero. An example is shown in the following Figure 3.9
Figure 3.9 A typical Lorentzian fitting on original data obtained in a tensile testing experiment on fibre sample. The black line is the original data plot. Red line is the Lorentzian fitting.

The schematic in Figure 3.10 shows how the AFM cantilever bends in different ways and the effect on the resultant data recorded under different experiment modes.
Figure 3.10 Schematic diagram of cantilever in different working modes. (A) Z-Spectroscopy is the usual force spectroscopy tool used for indentation and compression test. The cantilever bends up by applying compressive load. (B) Dither-Spectroscopy is the maintenance mode in which the cantilever is driven by dither piezo mounted behind the cantilever, which only changes the distance between cantilever and fibre optics. The cantilever is not loaded and therefore does not bend. (C) In nanofibre experiments, the cantilever is under tensile load and bends down.
b. Laser Beam Position

The laser intensity recorded by the fibre optic during AFM cantilever bending is determined not only by the magnitude of the cantilever bending but also by the laser spot location on cantilever, which is shown in Figure 3.11. If the laser spot is located at a distance $L_1$ away from the cantilever root, the bending $d_1$ recorded by the laser intensity will be much smaller than the real cantilever deflection $d$ as shown in the Figure 3.11 below.

Figure 3.11 Schematic diagrams showing different relative positions of the fibre optics along the AFM cantilever (positions $d_1$ and $d_0$). Orange rectangles represent different locations of fibre optics after installing AFM cantilever. When considering a constant cantilever bending, the distance between cantilever and fibre optics at each of these positions change due to different laser positions. The
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insert shows the bottom view from the AFM probe with the circular in orange representing the fibre optics.

From Equation 3.7, \( d_1 \) is given as:

\[
\frac{d_1}{d_0} = \frac{L_1^3}{L^3} \rightarrow d_1 = \left(\frac{L_1}{L}\right)^3 \times d_0 \quad \text{Equation 3.10}
\]

The laser intensity recorded at the \( d_1 \) position in the experiment is:

\[
I_0 = I_1 + I_2 + 2\sqrt{I_1 I_2} \cos \left\{ \frac{4\pi}{\lambda} \left[ D + \left(\frac{L_1}{L}\right)^3 \times d_0 \right] \right\} \quad \text{Equation 3.11}
\]

Therefore, the location of the laser spot has a third power effect on the laser intensity recorded in data plotting. The period of the cosine function above is defined by the relative location of the laser spot on the AFM cantilever. When a new AFM cantilever is installed, the relative laser position will typically change. The accurate determination of the laser position on the AFM cantilever is relatively difficult in practice as an invisible laser is used in the optical system.

3.2.4 Calibration and Data Analysis

To solve the problems of the non-linear change in the reflected laser light
collected by the optical fibre during AFM cantilever deflection, a calibration approach is applied. A reference dataset is recorded after nanofibre tensile testing by first firmly attaching an AFM probe to a solid substrate of silicon fixed to the AFM sample stage using epoxy glue within the SEM chamber, in a similar method to the nanofibre attachment. The AFM head was translated away from the substrate, which caused the cantilever to bend away from the solid substrate. Cantilever deflection in this case is exactly equal to the travelling distance of the AFM head which is defined as cantilever displacement.

To eliminate the drop of laser intensity, the data obtained from calibration and experiment are both normalized into $I_0 \in (-1, +1)$ and $I'_0 \in (-1, +1)$ which are shown in Figure 3.12 (B) and (D). We noticed that the wavelength in the experimental data plot is ‘expanded’ compared to the calibration data plot. The expanded wavelength can be described as the displacement value in experimental data that is equal to cantilever deflection plus sample elongation. Meanwhile, the cantilever displacement recorded in calibration is equal to the cantilever deflection.
Figure 3.12 An example of a laser intensity versus cantilever displacement plot obtained from tensile testing on an individual electrospun PVA fibre and subsequent calibration. (A) Part of the original laser intensity with cantilever displacement plot recorded during tensile test. (B) The same dataset after normalization. (C) Part of the original laser intensity with cantilever displacement plot of calibration. (D) Calibration data plot after normalization.

The relation between laser intensity and cantilever displacement after normalization in calibration can be defined as:

$$I'_0 = f(x')$$  \hspace{1cm} \text{Equation 3.12}$$

where $I'_0$ is the normalized laser intensity and $x'$ is the cantilever displacement.

Similarly, the laser intensity and cantilever displacement relation in experiment
data after normalization is:

\[ I_0 = f(x) \quad \text{Equation 3.13} \]

where \( I_0 \) is the normalized laser intensity and \( x \) is the cantilever displacement. To calculate sample stress and strain, the cantilever deflection needs to be accurately determined which can be derived from the laser intensity recorded. Equation 3.11 showed that the laser intensity is determined by the relative position of the laser spot on the AFM cantilever, which is difficult to measure as an invisible laser used. However, once the AFM probe is installed, the laser spot remains at the same position on the cantilever and the relation between laser intensity and cantilever deflection is constant. Therefore, the cantilever deflection at the red spot \((x, I_0)\) in the experiment is the same as the cantilever bending at the green spot \((x', I_0')\) in calibration shown in Figure 3.12 (B) and (D) as the laser intensity at these two points have the same value and phase angle. Meanwhile, at the green spot in the calibration, the cantilever deflection \(d\) is exactly same to the cantilever displacement \(x'\). Therefore, the sample strain at the red spot \((x, I)\) in the experiment can be calculated using:

\[ \varepsilon = \frac{x - d}{L} = \frac{x - x'}{L} \quad \text{(when } I_0 = I_0') \quad \text{Equation 3.14} \]
where $L$ is the original length of sample. The tensile load applied on sample is calculated as the cantilever spring constant $k$ times cantilever deflection. So the stress of sample at red point is calculated:

$$\sigma = \frac{kd}{\pi r^2} = \frac{kx'}{\pi r^2} \quad \text{(when } I_0 = I_0')$$  \hspace{1cm} \text{Equation 3.15}

For example, at the red data point in Figure 3.12 (A) plot: $x = 2.37 \, \mu m$, $I_0=6.38 \, V$. After normalization: $x=2.37 \, \mu m$, $I_0=1 \, V$. The corresponding data point in normalized calibration plot is the green point Figure 3.12 (D) where the $x= 1.25 \, \mu m$, $I_0'=1 \, V$. If the fibre length $L=10 \, \mu m$, radius $r= 40 \, nm$, spring constant of cantilever $k=0.2 \, N.m^{-1}$, the stress and strain of tested fibre at that point can be calculated as:

$$\epsilon = \frac{x - x'}{L} = 11.2 \% \quad \text{Equation 3.16}$$

$$\sigma = \frac{kx'}{\pi r^2} = 49.8 \, MPa \quad \text{Equation 3.17}$$

### 3.2.5 Accuracy and Errors

As discussed in section 3.2.3, different laser positions on the AFM cantilever affects the outputting cantilever deflection signal and influences the accuracy of the force measurement. Therefore, in this section, the accuracy and errors of the
force measurements in the AFM system are discussed by considering the laser located at the back of AFM cantilever as shown at position \( d_0 \) in Figure 3.11.

a. Accuracy

As defined in Equation 3.15, the force applied to the sample is calculated from the cantilever deflection in the test, which is equal to the cantilever displacement recorded in calibration as discussed in Equation 3.12 and Equation 3.13. Therefore, the force resolution of the AFM is defined as the product of the smallest recorded displacement of the cantilever and the spring constant of the cantilever. The Attocube AFM system used in this study has a smallest displacement of 0.36 nm at 300 K and 0.23 nm at 4 K for z axis piezo movement [210]. Mechanical tests were carried out at room temperature, which approximates to 300 K and therefore provides a cantilever deflection resolution of 0.36 nm. The AFM cantilever spring constants (K) used in nanofibre mechanical testing in this thesis are typically ranged between 0.01 Nm\(^{-1}\) to 0.2 Nm\(^{-1}\). Therefore, the smallest force that is measured by the AFM is equal to the product of the cantilever deflection resolution (0.36 nm) and K = 0.01 Nm\(^{-1}\), to give a minimum force of 3.6 pN. The nanomechanical tests produce the recorded forces by movement of the piezo positioner in order to provide displacement of the AFM cantilever. In this thesis, 2048 data points were sampled during each
tensile test during a 20 μm z axis piezo displacement. The accuracy of the smallest detectable piezo displacement is therefore 9.8 nm, which corresponds to a force resolution of around 100 pN when using a cantilever with a spring constant of 0.01 Nm⁻¹. The forces and displacements used in the AFM setup are considerably smaller than micro electro mechanical systems (MEMS). In particular, MEMS devices measure forces in the range of tens of nano-Newton up to hundreds of micro-Newton[211-214].

Finally, the SEM used in this study has a spatial resolution of 1.0 nm in secondary electron imaging mode at 30 kV, 2.0 nm at 2 to 3 kV and 3.0 nm at 1 kV under high vacuum operation mode[215].

b. Errors

The mechanical oscillation of the AFM cantilever is the major factor in the force measurement error and is dominated by the vibration of the SEM sample stage. In addition, the interference of electronic components in the AFM controller unit can also affect the stability of the signal output. All these effects will lead to a resultant “oscillating” signal as shown by the recorded variation in the laser intensity, in volts, over time as recorded in Figure 3.13. The average signal is shown in Figure 3.13 as being, on average, 5.455 V with a range from 5.45 V to
5.46 V. As shown in Figure 3.12 (A), the largest slope in the first period of the sine curve is recorded to be 0.52 μm/V at the point with Y value of round 5.4 V. The intensity-displacement relation is approximately linear near the largest slope point where the cantilever displacement can be calculated from the slope directly. Therefore, the 0.01 V noise in laser intensity signal corresponds to a displacement of approximately 0.01 V × 0.52 μm/V = 5.2 nm. Hence, the general error of all the interferences on the force measurement was estimated to be about 0.1 nN from the noise level of the original signal in a 1 kHz bandwidth using a 0.02 Nm-1 cantilever as shown in Figure 3.13. AFM cantilever with lower spring constants of 0.01 Nm-1 have smaller measured force noise ranged between 0.05 to 0.08 nN. As the mechanical forces recorded using tensile testing of nanofibres range from 314 to 1413 nN, a force error of 0.1 nN represents a maximum 0.03 % error in the recorded forces.

![Figure 3.13 Plot of laser intensity reflected from the AFM cantilever against time](image-url)
Chapter 3. Methodology

for the AFM integrated within SEM (1 kHz data sampling bandwidth, cantilever spring constant is 0.02Nm-1).

The accurate determination of the stress-strain behaviour when mechanically testing samples requires the reliable measurement of the sample’s dimension, especially the cross section area. As shown in Equation 3.12, the radius of the nano fibrous samples has a square dependence on their stress level. High magnification secondary electron images of sample were taken from different view angles in prior of testing to verify a circular cross-section. The radius of the nanofibre was carefully measured from SEM images by using pixel analysis software (ImageJ, NIH, USA). However, the samples tested in the experiment are made up of non-conductive polymeric materials. Hence, charging effects due to electrons from the SEM beam residing on the nanofibre surface influences the SEM image quality and causes distortion of the resultant image. The evaluation of the charging effect was carried out by measuring the radius of nylon-6 samples with and without gold sputter coating. The nanofibre radius was calculated by averaging 6 measured radius values at different random positions along the fibre length. The measured nanofibre radius for 20 individual non-coated nylon-6 nanofibres showed a larger standard deviation of 14 % compared to 8 % for nylon-6 nanofibres with a gold-coating, indicating a 6% error caused by the charging effect alone (non-coated sample are measured in images taken under 3
kV and gold-coated sample are imaged under 30 kV). The error in values of stress arising from inaccuracies in measuring the nanofibre radius from SEM images are calculated to be around 12% which is significantly higher than the measured force noise level of the AFM cantilever. Therefore, errors in values of stress are dominated by the fibrous sample radius measurement using SEM.

3.2.6 Results of Tensile Test on Polymeric Nanofibres and Discussion

The reliability and accuracy of the presented novel in situ mechanical testing setup was evaluated by performing measurements on synthetic nanomaterials. Electrospun PVA, nylon-6 nanofibres were tensile tested to failure with the resultant stress-strain curves for individual nanofibres shown in Figure 3.14. The stress-strain behaviour of all of the nanofibres are different, indicating that the AFM-SEM technique used in this work is able to elucidate mechanical behaviour due to varying structural compositions. A summary of the nanofibre mechanical properties is listed in Table 3.1. The deformation of the electrospun nanofibres is initially linear but with increasing non-linearity indicating viscoelastic behaviour for both PVA and nylon-6 at increasing tensile strain. PVA and nylon-6 stress-strain curves indicate considerable linear behaviour compared with typical polymer materials, which exhibit considerable plastic deformation. The origin of this stress-strain behaviour in electrospun fibres is unknown but may
be due to the difficulty in plastic deformation at reduced length scales which has been previously suggested [175, 185, 204, 206, 208].

Table 3.1 Mechanical properties of PVA and nylon-6 nanofibres measured by in situ AFM-SEM and their bulk equivalents. $E$ is the Young’s modulus measured for each sample in their linear region up to 4% strain. $\sigma_{\text{UTS}}$ is the ultimate tensile strength and $\epsilon_{\text{UTS}}$ is the ultimate tensile strain of the sample. Comparable film and bulk properties were measured from PVA films or taken from literature for nylon films (*see [206]).

<table>
<thead>
<tr>
<th>Materials</th>
<th>Status</th>
<th>Diameter (nm)</th>
<th>$E$ (GPa)</th>
<th>$\sigma_{\text{UTS}}$ (Mpa)</th>
<th>$\epsilon_{\text{UTS}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA</td>
<td>fibre</td>
<td>130.6±57.2</td>
<td>0.47 ±0.27</td>
<td>62.2±18.5</td>
<td>21.3±4.7</td>
</tr>
<tr>
<td></td>
<td>film</td>
<td>0.20±0.06</td>
<td>31.8</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td>nylon-6</td>
<td>fibre</td>
<td>113.0±16.3</td>
<td>1.32±1.52</td>
<td>78.1±6.0</td>
<td>31.5±22.2</td>
</tr>
<tr>
<td></td>
<td>film*</td>
<td>N/A*</td>
<td>47±0.5</td>
<td>168±14.8</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.14 Tensile stress-strain curves for different fibrous samples. (A) 5 PVA nanofibres exhibit a linear stress-strain relationship before 10 % strain followed by non-linear behaviour indicating ductile yield in one of the samples. (B) One of the five nylon-6 nanofibres (N3) shows double yield point behaviour in the stress-strain plot, suggesting crystal structure changes during deformation, while other nanofibres exhibit clear yield behaviour.

The Young’s modulus for PVA nanofibres is $0.47 \pm 0.27$ GPa. The PVA films, prepared by solution casting from the same polymer solution used for electrospinning and measured using conventional tensile testing (Instron, UK) in air, gave a Young’s modulus of $0.20 \pm 0.06$ GPa. The PVA nanofibres therefore have mechanical properties that are slightly higher than the bulk isotropic PVA films, as compared in Table 3.1. The high Young’s modulus of PVA nanofibres indicates potential structural anisotropy along the tested direction [30, 175, 201, 205, 206, 216]. We note that the PVA nanofibres can swell under the influence of
water, with dry fibres giving Young’s modulus values of around 3 GPa [216]. As the nanofibres in this work are far below such a value, we conclude that water is still bound within the PVA nanofibres despite conducting the tests in the vacuum chamber of an SEM.

The ultimate tensile strength of nylon-6 nanofibres obtained in our experiment is comparable to the results recorded in a previous indirect tensile test on electrospun nylon-6 nanofibres with similar diameters using a hooking method [206]. Nylon-6 cast films showed a tensile strength of $47 \pm 0.5$ MPa with a strain to failure of $168 \pm 14.8$ % [206]. The nanofibres tested in this work exhibit lower ductility but higher strength compared with mechanical properties for film or bulk materials reported in other studies [201, 204, 206], possibly due to the electrospinning processing method. Interestingly, the electrospun nylon-6 nanofibre exhibits a two stage stress-strain plot in test N 3 as shown in Figure 3.14, which can be related to the double yield point behaviour of nylon-6 detailed in previous works and observed in other materials [217-221].

The mechanical deformation of polymer fibres at reduced length scales has been previously studied extensively. For example, an increase in the elastic modulus of electrospun fibres with smaller fibre diameter has been reported and confirmed to be an effect associated with an increase in fibre crystallinity [222, 223] and
supramolecular confinement [224, 225]. The molecular organization within electrospun fibres has been shown to be size dependent with a result in reduced plasticity and corresponding ductility [223]. Indeed, plasticity effects at reduced length scales have been observed in thin films, with a number of reviews on the topic [226].

3.3 Conclusions

A novel in situ tensile testing method was developed to be used in this thesis for measuring the mechanical stress-strain behaviour of nanofibrous materials in tension to failure. This method has a capability of testing nanofibres in their as-received or as-fabricated state and is therefore applicable to measure the mechanical properties of biological nanofibres like the mineralized collagen fibrils in bone. To demonstrate the feasibility of the in situ AFM-SEM method, the stress-strain behaviour of nanofibrous samples of electrospun PVA and nylon-6 were measured. Electrospun PVA and nylon-6 nanofibres exhibited an increased tensile strength and elastic modulus when compared to bulk equivalents. The mechanical properties of PVA nanofibres were shown to be similar to hydrated samples, indicated that the vacuum of the SEM does not dehydrate such samples within the testing timeframes used in this work and therefore indicate that this AFM-SEM technique can fulfil the requirements of this study.
Chapter 4

Nanomechanical Properties of Individual Mineralized Collagen Fibrils from Bone Tissue

4.1 Introduction

Section 2.3.2 and 2.4.4 in Chapter 2 defined how mineralized collagen fibrils (MCFs) are distinct building blocks for bone material and perform an important mechanical function. A diversity of MCF assemblies exist in bone materials with the antlers of deer [49, 103, 169] notable as one of the toughest natural materials. Importantly, many mineral crystals are found to exist within the intrafibrillar region in antler as opposed to many other bones where the mineral is also found in extrafibrillar spaces [103, 154, 169, 170]. The mechanical properties of these MCFs and their influence on the overall bone mechanics, such as toughness in antler, has yet to be determined.
In this chapter, the *in situ* nanomechanical testing method of combined AFM-SEM defined in Chapter 3 is used to manipulate and measure the mechanical properties of individual MCFs from antler. The recorded stress-strain response of individual MCFs under tension shows an initial linear deformation region for all fibrils followed by inhomogeneous deformation above a critical strain. This inhomogeneous deformation is indicative of fibrils exhibiting either yield or strain hardening and suggests possible mineral compositional changes within each fibril. A phenomenological model is used to describe the fibril nanomechanical behaviour.

### 4.2 Materials and Methods

#### 4.2.1 *in situ* Nanomechanical Testing

Antler samples for *in situ* nanomechanical testing were prepared as reported in Section 3.1.1. Cortical bone extracted from antler main beam was cut into small beams with long axis parallel to osteons and rehydrated in prior of experiment. Nanomechanical testing of individual MCFs was performed using a combined AFM-SEM as described in Chapter 3. A schematic of the combined AFM-SEM setup is shown in Figure 4.1 (B) and highlights how the AFM probe is perpendicular to the fracture plane of the antler and along the principal axis of the exposed collagen fibrils.
Figure 4.1 (A) Scanning electron micrograph showing a typical testing configuration for tensile testing of MCFs. The image shows a large number of exposed collagen fibrils observed at the fracture surface of antler bone. An individual collagen fibril protruding from the fracture surface is attached to the glue at the end of the AFM probe. Translation of the AFM probe away from the fibril causes tensile deformation of the fibril until failure occurs which is shown in the inserted image. (B) Schematic diagram showing the combined SEM-AFM setup.

Attachment of an individual collagen fibril was carried out according to the methodology defined in Section 3.2 in the previous chapter. However, whereas validation of the technique was performed on synthetic nanofibres that are manufactured in an isolated form, MCFs are bound together in bone and require adaption of the fibre mechanical testing technique. Clamping of an individual collagen fibril to the end of the AFM probe was achieved by first translating the end of the AFM probe into a droplet of glue (Poxipol, Arg) contained within the
SEM chamber as described on synthetic nanofibre manipulation in Chapter 3. Removal of the AFM probe from the glue deposited a small amount of glue at the apex of the AFM probe. The AFM probe was subsequently moved towards the free end of the exposed collagen fibril until contact between the fibril and the glue at the AFM probe apex was achieved as shown in Figure 4.1 (A). This contact was achieved consistently within a 3-5 minute timeframe from the fixing of glue within the SEM chamber. Manipulation and attachment of the collagen fibril to the AFM probe was observed using the electron beam of the SEM at long working distance (15 mm) and low accelerating voltage (2 kV) to ensure that no electron beam damage occurs, as has been shown for soft polymer systems [227]. Solidification of the glue occurred approximately 10 minutes after contacting with the fibril free end. Thus, the ends of the individual MCF was fixed both to the apex of the AFM probe and the bone surface. This gripping of the individual MCF is somewhat different to the validation testing on synthetic nanofibres in Chapter 3, where fixing of the nanofibre using glue was used for both of the fibre ends. We note that the manipulation of collagen fibrils requires SEM imaging as opposed to AFM imaging. AFM imaging is suitable for examining bone specimens where the collagen fibrils are in the plane of the fracture surface [19, 228] but is unable to image surfaces where the collagen fibrils are perpendicular to the fracture surface due to the instability of this surface to imaging using the AFM probe.
Fibrils attached to the AFM probe were used for subsequent tensile testing by translation of the AFM probe away from the fracture surface. This translation caused a corresponding bending of the AFM cantilever until failure of the individual collagen fibril as shown in Figure 4.1(A) inset. The force applied to the collagen fibril was calculated from the spring constant of the AFM cantilever, found using the thermal noise method [23] and measured the cantilever deflection using an optical interferometer setup (Attocube Systems, Ger) situated behind the cantilever. The calculation of the stress in the collagen fibril requires the accurate determination of the fibril diameter. The diameter of MCFs was measured by pixel analysis in the SEM images captured before mechanical test using ImageJ (NIH, USA). The diameters of collagen fibrils mechanically tested in this work have a mean value of 92.4 ± 12 nm. All mechanical testing ensured that the collagen fibril axis is in the same plane as the SEM image, otherwise force applied to the fibril will cause fibril orientation as opposed to deformation. This configuration was achieved by moving the AFM probe attached to the collagen fibril above and below the SEM plane of view. A small force will be recorded during this out of plane movement if the fibril axis is aligned with the plane. Thus, only fibrils with their principal axis in the SEM view plane are tested. All fibrils tested failed in the middle of their free length, away from the holding glue and the bone surface.
4.2.2 Compositional Study Using EDS

X-ray energy dispersive spectroscopy (EDS) microanalysis within an SEM (Inspect SEM, FEI Company, EU/USA) was used to investigate the composition of antler samples used in experiments and verify if the regions containing the mechanically tested collagen fibrils are mineralized. Chemical composition (Ca/P) ratios have been previously used as a marker in EDS for the calculation of the mineral distribution in bone tissue [28, 229, 230] and is thus employed in our study. Twenty EDS spectra within an area of 100×100 µm² were collected at the tested fracture surface of antler in order to determine the calcium content.

4.2.3 Thermal Gravimetric Analysis

The effect of the SEM vacuum on the hydration of bone samples was examined using Thermo Gravimetric Analysis (TGA). TGA is typically used to record the weight of a sample as the sample temperature increases. For bone materials, TGA has been used to heat a sample and record the weight loss due to removal of water from the bone structure [102]. Therefore, TGA is a suitable technique to record the hydration state of bone before and after exposure to the SEM vacuum. Antler bone samples with dimensions 3×10×0.2 mm were cut from the main beam of same antler bone used for in situ mechanical testing. Bone samples were first hydrated by leaving in Hank’s buffered solution for 20 hours. The samples were removed from solution and excess water removed from the sample surface.
using filter paper. Samples were split into four groups, with each group containing 5-6 bone samples. The first group was transferred into the TGA furnace immediately and heated from 40 °C to 250 °C with a heating rate of 30 °C.min⁻¹ with 60 mL.min⁻¹ nitrogen flow. The other three groups were moved into the SEM chamber operating at a pressure of 3.4 × 10⁻³ Pa for 15, 30 and 45 minutes respectively, followed by analysis using the TGA.

4.3 Results

4.3.1 Mechanical Behaviour of Individual MCFs

The stress-strain behaviour of 6 individual collagen fibrils was measured using the AFM-SEM with the results shown in Figure 4.2. All fibrils show a linear stress-strain response during initial tensile loading up to strains of between 2 % to 3.7 %. This strain value at the limit of the linear response corroborates previous deformation of hydrated antler using x-ray studies [154] indicating that the collagen fibrils are also hydrated despite the vacuum environment of the SEM. The linear modulus in Region I of the collagen fibril stress-strain curve is highly reproducible with a value of 2.4 ± 0.4 GPa and is considerably larger than previous collagen moduli [20, 40], indicating that the fibrils have some degree of mineralization that improves their stiffness.
Figure 4.2 (A) The stress-strain plot for tensile testing of individual MCFs. The MCFs show a linear stress-strain regime with a linear elastic modulus of $2.4 \pm 0.4$ GPa. At higher strains above approximately 2-3 % strain, the MCFs exhibit either yield or an apparent increase in the tangential modulus. The insert shadow area shows the inhomogeneity of the strain response with increasing stress. (B) Failure strength verse ultimate strain recorded by stretching collagen fibrils until failure. The arrow shows increasing strength along with decreasing fracture strain, indicating a ‘brittle like’ change.

Further tensile deformation of a MCF caused an observed transition to a mechanically inhomogeneous Region II. Fibrils in Region II exhibit either yield behaviour or strain hardening including higher modulus and ultimate strength, but a decrease in the fibril ultimate strain to failure. No fibril failure was observed in the SEM image during the tensile testing until catastrophic failure. The last data point in the stress-strain behaviour of the MCFs from Figure 4.2
was recorded just before this catastrophic failure. The detailed mechanical properties of all six collagen fibrils are recorded in Table 4.1.

Table 4.1 Mechanical properties of six individual MCFs tensile tested to failure using combined AFM-SEM

<table>
<thead>
<tr>
<th>Test No.</th>
<th>Diameter (nm)</th>
<th>Modulus I (GPa)</th>
<th>Modulus II (GPa)</th>
<th>$\varepsilon_{UTS}$ (%)</th>
<th>$\sigma_{UTS}$ (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72.9</td>
<td>2.30</td>
<td>1.95</td>
<td>5.14</td>
<td>125.68</td>
</tr>
<tr>
<td>2</td>
<td>102.5</td>
<td>2.29</td>
<td>0.96</td>
<td>6.46</td>
<td>89.05</td>
</tr>
<tr>
<td>3</td>
<td>90.7</td>
<td>1.91</td>
<td>0.98</td>
<td>6.72</td>
<td>82.22</td>
</tr>
<tr>
<td>4</td>
<td>96.0</td>
<td>3.03</td>
<td>3.85</td>
<td>5.81</td>
<td>185.00</td>
</tr>
<tr>
<td>5</td>
<td>86.0</td>
<td>2.30</td>
<td>2.78</td>
<td>5.37</td>
<td>129.50</td>
</tr>
<tr>
<td>6</td>
<td>106.0</td>
<td>2.46</td>
<td>1.18</td>
<td>6.23</td>
<td>100.66</td>
</tr>
</tbody>
</table>

Mean ± SD  92.4±12.0  2.38±0.37  1.95±1.17  5.96±0.62  118.68±37.67

4.3.2 Compositional Analysis

The antler bone mineral content at the fracture surface was evaluated using EDS following previous works [28, 229, 230]. Preliminary SEM back scattered images shown in Figure 4.3 indicated regions of high and low mineralization at the fracture surface. The corresponding EDS analysis of elements present at the fracture surface, including O, Na, Mg, S, P and Ca, indicated that the calcium content varied considerably from 31.64 % to 61.34 %. This calcium content shows that the bone is not only mineralized but there is a large variation in the mineral content.
Figure 4.3 Scanning backscattered electron micrograph of the antler bone fracture surface. The light regions on the image indicate higher mineralization. The corresponding Ca/P ratio in the image varied from 1.48 to 3.12, indicating hydroxyapatite mineral is present \[28, 230\] and the content varies throughout the antler bone.

### 4.3.3 Water Content Change in Bone Samples Exposed to SEM Vacuum Studied by TGA

The amount of water in the bone samples calculated from TGA can be plotted against exposure to SEM vacuum time as shown in Figure 4.4 below.
Figure 4.4 Plot of water content in bone with time of SEM vacuum exposure. The water content in bone was measured from the weight loss recorded during TGA tests by heating bone samples up to 200 °C.

The bone samples directly taken from Hank’s buffered solution contain 15.64 ± 2.60 % of water while bone samples exposed to the SEM vacuum contained progressively less water, with bone samples exposed to vacuum for 45 minutes containing 8.79 ± 0.61 % of water. The water content in bone remained at 13.35 ± 1.52 % when exposed to vacuum for 15 minutes, which is the same (within error) as the water content in bone samples prior to SEM vacuum exposure and similar to the water content in fresh antler bone recorded in a previous study [171].
The manipulation and mechanical testing of the MCFs were accomplished within a time frame of 10-15 minutes after introducing to the SEM vacuum, indicating that the samples were still hydrated. This low water loss due to the short time of exposure in vacuum has also been observed in synthetic nanofibres swollen with water in Section 3.2. In addition, during the mineralization process of CFs, the water in the gap regions of fibril is replaced by mineral phase which makes MCFs less hydrated comparing to CFs [45]. We therefore conclude that our nanomechanical techniques tested bone in its hydrated state. Table 4.2. shows the average weight of bone samples with exposure to the SEM vacuum and the weight percentage of water contained in the sample calculated from the removal of water during TGA.

<table>
<thead>
<tr>
<th>Exposure time (Mins)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (μg)</td>
<td>4.15±1.01</td>
<td>3.24±0.45</td>
<td>3.72±0.70</td>
<td>4.13±0.91</td>
</tr>
<tr>
<td>Water (%)</td>
<td>15.64±2.60</td>
<td>13.35±1.52</td>
<td>9.84±0.94</td>
<td>8.79±0.61</td>
</tr>
</tbody>
</table>
4.4 Discussion

The deformation behaviour of individual collagen fibrils in tension described by Region I and II are indicative of structural or compositional variation within the fibrils themselves. A mechanistic description of the deformation of individual collagen fibrils can be made by comparing our experimental results in this work with molecular dynamics simulations [138]. These simulations show some similarity with our individual MCF tensile tests, with an initial linear response followed by heterogeneous deformation in the MCF stress-strain behaviour. The initial linear behaviour was mostly from elastic behaviour of the MCF constituents and the tensile modulus calculated from this Region I was dependent on the amount of mineral contained within the fibril. The presence of mineral phase increases the local yield regions in MCFs when tensile load is applied, which suggests the MCFs fail locally to ensure that the majority of the fibril length remains undamaged after exposure of the fibrils during sample preparation [138]. The linear stress-strain fibril response of the MCFs in Figure 4.2 after primary mechanical tests provided an experimental validation of this mechanism as testing of fibrils after yield will give a non-linear response.

The heterogeneous deformation zone, defined by Region II in this work, was shown in the molecular simulation to be also due to the amount of mineral in the
collagen fibrils [43, 138]. The mineralized collagen fibrils in the simulation work displayed a characteristic stress-strain curve that is similar to Figure 4.5 (B) here, indicating that deformation in Region II is due to intermolecular slippage and failure at the mineral-tropocollagen macromolecule interface. We note that other simulations show how intermolecular slippage in non-mineralized collagen fibrils are suppressed when crosslink density is increased [105]. The tropocollagen molecules in Buehler’s simulation work [138] do not vary the crosslink density between these tropocollagen molecules. However, variations in the mineral content within the MCFs may define the two observed stress-strain behaviours in Figure 4.5, with the amount of mineral analogous to the crosslinking behaviour in the simulations. A relatively large mineral content as shown in Figure 4.5 (A) may enhance the binding between tropocollagen molecules, causing molecular extensions at relatively high strains and an increase in the tangential modulus. A relatively low amount of mineral as shown in Figure 4.5 (B) conversely weakly binds the tropocollagen, with sliding between the tropocollagen and resultant fibril yield observed.
Figure 4.5 Tensile stress-strain curves for mineralized collagen fibrils showing two distinct mechanical behaviours. Tropocollagen uncoiling occurs initially in both types of fibrils within Region I. In (A) the fibril shows an enhanced elastic modulus in Region II due to the mineral increasing the stress transfer between tropocollagen macromolecules. In (B) the low mineral density within the fibrils allows sliding between tropocollagen molecules, resulting in a plastic deformation.

The proposed variation in mineral content causing the two observed MCF stress-strain behaviours can be evaluated from EDS investigations within the bone samples. The compositional EDS analysis in Section 4.3.2 shows a variation in mineral content throughout bone that suggests a corresponding variation in the collagen fibril mineral content. Table 4.1 supports this assumption that the heterogeneous fibril strain comes from the compositional variation with collagen fibrils with a higher tensile modulus in Region I, as defined in Figure 4.5 (A), also exhibiting an increased tensile modulus in Region II. The increased tensile
modulus in Region II relative to Region I cannot be simply due to mineral content and must be due to a stiffening of the fibril with strain. Classical work from tendon, essentially an aligned unmineralized CF array, shows how crimping of tropocollagen molecules is removed with increasing strain, resulting in an increased tensile modulus [105, 155, 231]. The increased tensile modulus in MCFs as shown in Figure 4.5 (A) may therefore be due to the removal of crimping in the MCF. However, molecular dynamics simulations do not show this behaviour as the model assumes an uncrimped, uniaxially aligned tropocollagen network [138]. The stress-strain curves recorded in MCFs tensile testing are similar to the simulated stress-strain behaviour for uncrimped MCFs. The mineral phase is acting as ‘crosslink’ between tropocollagen molecules to enhance the fibril tensile modulus.

While the nanomechanics of collagen fibrils are expected to be due to mineral content, there are some other alternative mechanisms could lead to the different mechanical response of MCFs in the region II of the stress-strain curves. In the first region of the stress strain curves, all the fibrils exhibit a similar response to the applied load, which could be explained by the distinct mechanics of the tropocollagen molecules and the apatite crystals within the fibrils. The tangential modulus of the MCFs in the first region is defined by the interaction of the tropocollagen and mineral phase which is indicative a possible similar degree of
mineralization. The various responses recorded in the region II could come from the different interactions between mineral crystals due to the different distribution of the minerals within collagen when tropocollagen molecules start to slip between each other under strain. Mineral crystals in strain-hardened fibrils are brought together and contact to each other due to the slippage of tropocollagen molecules and start to bear load to provide a higher resistance to strain while this interaction of minerals may be absent in yield fibrils. In addition, the change in tropocollagen residue sequences [41, 151, 160, 232], hydroxyapatite crystal shapes [233, 234] as well as their texture and orientation [233] are considered to have some degree of influence on the mechanical behaviour of individual MCFs but are not as significant as the mineral component.

The implications of nanomechanical heterogeneity have been more widely studied and previous work has illustrated how heterogeneous deformation in mineralized tissue aids energy dissipation [31, 122, 162]. The principal mechanical function of antler is energy absorption during impact and therefore promotion of heterogeneous deformation is favourable. Thus, our work highlights how heterogeneous deformation originates from the nanomechanical behaviour of the MCFs themselves.
4.5 Conclusions

In conclusion, the stress-strain behaviour of individual MCFs from antler bone was measured using combination AFM-SEM. An initial region of homogeneous fibrillar deformation was succeeded by inhomogeneous mechanical behaviour above applied strains of 2-3.7 %. A molecular mechanism is proposed to explain these different fibril mechanical responses to external load. The nanomechanical testing technique developed in this work may also be applicable to the measurement of bone at different mineralization and diseased states.
Chapter 5

Mechanical Properties of MCFs from Differing Ages of Bone

5.1 Introduction

The capacity to measure the mechanical properties of individual MCFs using novel experimental AFM-SEM techniques has been defined in the previous two chapters. This Chapter aims to address the ability of the technique to elucidate potential differences that may exist in MCFs. To further this aim, MCF fibrils from bone of different ages are considered due to bone being a biological material with capability of renewing and remodelling its structure over time defining its material properties [48, 51, 56]. The effect of this structural renewing and remodelling in bone on MCF mechanics can therefore be assessed. The mechanical properties of bone are generally known to change with age, with
bone mineral density (BMD) typically used to describe the susceptibility of different ages of bone to [235-237]. Considerable debate remains as to whether the fracture behaviour of bone can be reliably predicted by assessing bone density alone [238-243]. Recent clinical observations indicate a correlation in bone mineral density of healthy people and patients who suffer fractures due to age related diseases [238]. Moreover, experimental and simulation studies on changes in collagen materials with age also show the limit of considering bone mineral density alone in the mechanical study of aging bone [79, 104, 137, 156, 244]. An important aspect of bone tissue quality is the relative amounts and the material properties of its main constituents of collagen fibrils and apatite crystals. The influence of apatite/collagen ratio on mechanics of bulk bone material in aging has been widely studied [245-248], although the basic building block of MCFs from different ages has yet to be mechanically assessed. The evaluation of the mechanical properties of MCFs from different ages of bone may therefore be beneficial in potentially defining overall bone mechanical behaviour or providing further understanding on the influence of MCFs in the changing mechanics of whole bone. In this chapter, MCFs taken from the limb bones of 4 and 10 week old mice are mechanically tensile tested to failure using in situ AFM-SEM nanomechanical testing methods as described in Chapter 4. The mechanical behaviour of individual MCFs from different ages of mouse bone was compared to results from bulk samples. Thus, the effects of aging on bone nanomechanical
behaviour can be elucidated.

5.2 Materials and Methods

5.2.1 Animals

C57BL/6J wild type mice (4 and 10 weeks) were freshly obtained from the Medical Research Council (MRC, UK). Mice were kept in accordance with UK Home Office welfare guidelines and license restrictions.

5.2.2 Sample Preparation of Bone Tissue

The right and left femur from each animal were exercised and cleaned to remove adhering soft tissue from the femur limbs. As recorded in Section 3.1.2, mouse femur bones were kept wet in Hank’s buffered solution during preparation and stored frozenly. Bulk mechanical testing on whole mouse bone femur was performed by fixing the distal and proximal end lobes in dental cement (FiltekTM Supreme XT, 3M ESPE, USA) to grip the samples in a custom built testing device (M110.1DG, Physic Instrumente, UK).
Figure 5.1 Sample preparation for tensile testing. (A) Schematic of mouse skeleton showing the location of the femur (ellipse). (B) Optical image of an isolated femur bone. (C) Custom made micro milling setup used to remove bone leaving 0.1 mm thick of anterior quadrants. (D) Bony ends of Femur included in the dental cement (FiltekTM Supreme XT, 3M ESPE, USA) and milled mid diaphysis.

Embedded femurs were machined to form a necked region in the mid diaphysis.
using a custom-made micromilling machine. The milling machine consisted of two motorised linear stages (M110.1DG linear stages Physic Instrumente, UK), which are connected to the computer via 2 motor controllers. A high speed rotatory milling tool (Dremel 300, Dremel Inc, UK) with 0.8 mm diameter cutting tool was fixed and specimen was held in a Hank’s buffered solution fluid chamber connected to the linear stage as shown in Figure 5.1(C). Specimens were machined from the posterior quadrants leaving a 100 µm thick anterior quadrant. The width and the thickness of each specimen were measured after milling using a Basler A101f monochrome CCD camera (Basler Vision Technologies, Ger). High resolution (1024 × 768) optical images of the milled specimens were captured, transferred to computer and dimensions measured using ImageJ software (NIH, USA). The typical dimensions of the gauge regions were approximately 0.10 mm (thickness), 1.0 mm (width) and 4.0 mm (length). SEM was used to examine if bone samples were damaged during the machining protocol and damaged samples were discarded.

5.2.3 Tensile Testing of Bulk Bone Tissue

A customized tensile testing machine as shown in Figure 5.2 was used to deform the femur samples by applying bi-directional motion of two DC encoder stages (M110.1DG, Physic Instrumente, UK), which has a minimum incremental motion of 0.1 µm.
Figure 5.2 Schematic view of the micro tensile testing machine. The sample is immersed in Hank's buffered solution. Tensile load is applied along the schematically prepared femur long axis as shown in the inset.

The main features of this tensile tester are (i) bi directional tensile testing capability and (ii) sample testing in a fluid chamber (this provides physiological environment to the sample during the experiment). Load was measured with a 22 N model 31 tension/compression load cell (SLC31/00005, RDP electronics, UK) and sample holders were moved with linear stages. Due to an initial slack-range in the grips, the stress-strain curve shows an initial toe-in region, which was not considered during the data evaluation. A consistent strain rate of
0.02 \( \text{\%}.s^{-1} \) was applied on the bone sample by moving bi-directional stages in opposite direction at a velocity of 0.001 mm.s\(^{-1}\). 6 samples taken from 4 week old mouse femur bone and 4 samples from 10 week old were tensile tested up to applied strains of 3 \%. Resultant stress-strain plots were recorded.

### 5.2.4 in situ Nanomechanical Testing of MCFs

The fresh radial bones from 4 and 10 week old mouse bone were extracted and prepared as described in Chapter 3, Section 3.1.2. MCFs exposed at the fracture surface were tensile tested to failures by in situ AFM-SEM setup reported in Chapter 3, Section 3.2. To determine the stress-strain relation of samples, the diameters and lengths of the MCFs were accurately measured by pixel analysis using high magnification SEM secondary electron imaging taken before and after nanomechanical experiments using ImageJ (NIH, USA). Resultant stress-strain plots from tests on 6 MCFs from 4 week old mouse bone and 5 MCFs from 10 week old mouse bone were recorded.

### 5.3 Results

The mechanical stress-strain responses for tensile tested bulk bone samples are shown in Figure 5.3 (A). All bone samples show a relatively linear increase in stress with applied strain in these plots. The stress-strain behaviour for 10 week
old mouse bone showed a larger linear (Young's) modulus of approximately 4.5 GPa when compared to 1.2 GPa for 4 week old mouse bone as shown in Figure 5.3 (B).

Figure 5.3 Mechanical behaviour of femur samples from 4 and 10 week old mouse bone recorded during tensile testing showing (A) stress-strain plots of bone samples with linear regressions and (B) summary plot showing the Young's modulus calculated from stress-strain plot linear regressions for the 4 and 10 week old bone.

Stress-strain plots for individual MCFs from 4 and 10 week old mouse bone tensile tested by the AFM-SEM are shown in Figure 5.4.
Chapter 5. Mechanical Properties of MCFs from Different Ages of Bone

Figure 5.4 Stress-strain behaviour of individual mineralized collagen fibrils from mouse radius bone at 4 and 10 weeks respectively.

All fibrils show a linear stress-strain response during initial tensile loading up to strains of between 2 % to 4 %, followed by a more variable stress-strain behaviour beyond strains of 4 % as described for individual MCFs examined in antler bone. Young’s moduli are calculated from this initial linear region and are similar in both bone age groups, displaying values of $2.54 \pm 0.60$ GPa and $3.65 \pm 0.81$ GPa respectively. The calculated mechanical properties of the testing individual MCFs is shown in Table 5.1 below and indicates an additional similarity in the ultimate strength and strain in both age groups.
Table 5.1 The mechanical behaviour of MCFs tensile tested to failure.

<table>
<thead>
<tr>
<th>Age</th>
<th>Length (μm)</th>
<th>Diameter (nm)</th>
<th>Strength (MPa)</th>
<th>Ultimate Strain</th>
<th>$E$ (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
<td>1.25</td>
<td>92.0±9.3</td>
<td>138.43</td>
<td>0.052</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>2.55</td>
<td>118.8±9.7</td>
<td>89.05</td>
<td>0.065</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>1.67</td>
<td>103.8±10.2</td>
<td>82.22</td>
<td>0.067</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>1.71</td>
<td>110.4±12.5</td>
<td>185.25</td>
<td>0.054</td>
<td>2.83</td>
</tr>
<tr>
<td></td>
<td>1.27</td>
<td>93.1±10.5</td>
<td>129.50</td>
<td>0.054</td>
<td>3.41</td>
</tr>
<tr>
<td></td>
<td>1.92</td>
<td>109.5±8.7</td>
<td>100.10</td>
<td>0.062</td>
<td>2.35</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>1.73±0.44</td>
<td>104.6±9.6</td>
<td>120.76±35.27</td>
<td>0.059±0.006</td>
<td>2.54±0.60</td>
</tr>
<tr>
<td>10 weeks</td>
<td>1.02</td>
<td>97.2±9.1</td>
<td>113.73</td>
<td>0.070</td>
<td>4.12</td>
</tr>
<tr>
<td></td>
<td>2.08</td>
<td>118.7±6.3</td>
<td>131.89</td>
<td>0.078</td>
<td>2.36</td>
</tr>
<tr>
<td></td>
<td>2.45</td>
<td>105.2±14.6</td>
<td>154.10</td>
<td>0.068</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>1.57</td>
<td>89.3±8.6</td>
<td>188.94</td>
<td>0.055</td>
<td>4.81</td>
</tr>
<tr>
<td></td>
<td>1.48</td>
<td>108.1±7.5</td>
<td>204.66</td>
<td>0.041</td>
<td>3.44</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>1.72±0.50</td>
<td>103.7±10.0</td>
<td>158.66±34.03</td>
<td>0.062±0.013</td>
<td>3.65±0.81</td>
</tr>
</tbody>
</table>

5.4 Discussion

The mechanical behaviour of the bulk bone material and the MCFs can be compared in order to ascertain the influence of the nanoscale on larger scale bone mechanics. The linear stress-strain behaviour average over all of the collected experimental data for the individual MCF and bulk bone at different ages is plotted in Figure 5.5 below. The variation in Young’s modulus for bulk bone samples with age is clearly observed in Figure 5.5 (A). This variation is expected to due to the different composition of bone developing from 4 to 10 weeks. Critically, the increase in the Young’s modulus for bulk bone with
increasing aging is not observed for the average stress-strain behaviour of MCFs in Figure 5.5 (B). Within error, the stress-strain response of MCFs from 4 and 10 week old bone are comparable, indicating a weak or absent effect of increasing age. Our results therefore indicate that the mechanical properties of the MCF unit in bone do not define the mechanical properties of bone at larger length scales as represented by the bulk bone data.

Figure 5.5 Linear regressions of average stress-strain plots for bulk bone and MCFs samples from 4 week and 10 week old mouse bone. Error bars are standard deviations (see Table 5.1).

Previous studies have shown how mechanical properties of bone at different ages depend on the mineral content [51, 244, 249]. To obtain the information of mineralization of bulk bone samples at different ages, compositional analysis
using Quantitative Backscattered Scanning Electron microscopy (qBSE) was performed. The qBSE images were collected from the mid shaft of the cortical bone from 1 week to 16 weeks (left to right in Figure 5.6) for wild type mice. A significant increase in mineralization shown as increased brightness in Figure 5.6 is observable, especially for the bone aging from 1 to 10 weeks.

Figure 5.6 BSE microscopy images of mid shaft cross sections of limb bones from 1 week to 16 weeks for wild type mice.

The increase in the Young's modulus of bulk bone must therefore be related to the increase in the degree of mineralization present on the bone as shown in Figure 5.6 and observed in previous literature [1, 2, 51, 86, 132, 230, 244, 249, 250]. In particular, the stiffer mineral phase has been shown to influence the
elastic properties and fracture behaviour of bone when varying mineral percentage, orientation and morphology [1, 2, 15, 48, 56, 86, 132, 249] and has been indicated as being the dominant phase in defining bone mechanics [1, 2, 56, 249, 251]. However, this increase in the mineralization of whole bone with age does not show a corresponding increase in the Young’s modulus of individual MCFs from the mouse bone, despite the expected mineral content influence on fibril mechanics as discussed in Chapter 4. The slight increase in MCF Young’s modulus from 4 to 10 weeks in Figure 5.5 is much smaller than in bone tissue. In addition, a molecular dynamics simulation study on mechanistic description of the deformation of individual MCFs showed that the Young’s modulus of MCFs is dependent on the amount of mineral that exists within the fibrils [138] due to the mineral phase increasing tropocollagen crosslinking, which promotes load transfer within fibrils and leads to increased bone stiffness.

The discrepancy between increased mineralization increasing bulk bone Young’s modulus but not the MCF Young’s modulus can be evaluated further by considering the BSE microscopy images in Figure 5.6. Calcium concentration was used to represent Bone Mineral Density (BMD) and was calculated from the grey scale of each individual pixel in the BSE microscopy images of Figure 5.6, baselined against standard reference materials of carbon and aluminium, as carried out in previous literature [229]. Calcium concentration within each pixel
in the BSE microscopy image is determined from its grey scale comparing to reference materials. The ratio between the number of pixels with certain calcium concentration and total number of pixels in one image is defined as the frequency of appearance and used to evaluate the Bone Mineral Density Distribution (BMDD). The Full Width at Half Maximum (FWHM) of the BMDD is taken as the width at the half height of peaks in the diagram of frequency of appearance verse calcium concentration for examining the distribution in local variation of mineral content. A high FWHM represents a large variation in BMDD within bone tissue.

Figure 5.7 highlights how a significant reduction in the distribution of calcium as defined by the Ca FWHM values is observed from 1 to 4 weeks and stabilizes from 4 weeks to 16 weeks. Table 5.2 summarizing the data presented in the figure. In contrast, a dramatic increase in average calcium concentration was observed from 1 to 4 weeks and gradually stabilizes from 4 weeks to 16 weeks. These two observed phenomena are described by considering the mineralization process in bone. In primary ossification, the mineralization occurs mainly in the extrafibrillar regions as the narrow collagen fibrils formed have relatively limited space for mineral to deposit. In the later secondary ossification, the collagen fibrils have much larger diameters to allow mineral palettes to form within internal spaces. However, the initial location of mineral deposition i.e. within the fibrils or the intrafibrillar regions in secondary ossification is unclear [18, 83].
Figure 5.7 Quantitative analytical diagrams of BSE microscopy images taken from bone of different ages. (A) Frequency of Appearance against calcium concentration with age. (B) Histogram of FWHM of BMDD plotted with development which corresponds to the distribution in local variation of Calcium content and (C) Mean calcium concentration in form of weighted fraction (Ca
wt %) calculated from (A) was plotted as a function of development (1 week to 16 weeks).

Table 5.2 Quantitative analysis of qBSE images taken on bone at different development stages (1 to 16 weeks).

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Ca % Mean</th>
<th>Ca FWHM</th>
<th>Ca % Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.36</td>
<td>17.67</td>
<td>18.5</td>
</tr>
<tr>
<td>4</td>
<td>23.85</td>
<td>5.86</td>
<td>27.67</td>
</tr>
<tr>
<td>7</td>
<td>25.15</td>
<td>5.12</td>
<td>29.81</td>
</tr>
<tr>
<td>10</td>
<td>26.09</td>
<td>4.85</td>
<td>28.70</td>
</tr>
<tr>
<td>16</td>
<td>27.86</td>
<td>4.65</td>
<td>27.70</td>
</tr>
</tbody>
</table>

In ossification, the organic matrix acts as a template to induce inorganic crystal growth. The organic matrix therefore promotes heterogeneous nucleation of apatite and inhibits homogeneous nucleation [51, 83] by providing large contacting area between extracellular fluid containing mineral ions and collagen matrix. The formation of calcium apatite crystals occurs randomly in the mineralization front area [12, 45, 51, 55] so a random distribution of nucleation sites throughout bone tissue will occur, exhibiting a large local variation of calcium concentrations in early stage development of bone as indicated in the large Ca FWHM for 1 week old bone in Table 5.2. As the collagen fibril widths produced in secondary ossification are significantly larger than the extrafibrillar
spaces (~100 nm fibril diameters comparing to 1-2 nm spacing between fibrils), mineralization in secondary ossification occurs initially in the relatively large spaces found in the gap regions within collagen fibrils where water is replaced by hydroxyapatite (HA) crystals [12, 18, 33, 45, 50]. Once the gap regions are filled with mineral within a fibril, we propose that the HA mineral can only form between the collagen fibrils in the spacing as shown in Figure 5.8:

Figure 5.8 Schematic sketches showing three stages of biomineralization on collagen fibrils in mouse bone. Tropocollagen molecules are denoted as blue cylinders and the hydroxyapatite mineral crystals are red particles with random shapes. Negatively charged molecules (most of which are Non-Collagenous Proteins as discussed in Chapter 2) existing in the extrafibrillar space are shown in green and bonded via positive calcium ions. (A) Calcium and phosphate ions migrate to the gap regions (initially filled with water) within collagen fibrils and
initiate nucleation of HA crystals. (B) All the gap regions within collagen fibrils are completely filled with mineral crystals. (C) Mineral continues to grow within the extrafibrillar space and cover the fibril surfaces.

We can therefore assume that, in this study, the 1 week old mouse bone is at the first stage of mineralization. The mineral crystals form predominantly and randomly in the gaps regions found within the collagen fibrils. Relatively large variation in local bone mineral density distribution (BMDD) but low average calcium concentration is therefore observed. Beyond 1 week and up to 4 weeks the mineral continues to deposit within the gap regions of the fibril (shown in Figure 5.8 (B), resulting in an increase in the Ca concentration for 4 week old bone as shown in Table 5.2. Beyond week 4, the gap regions within the collagen fibrils are saturated with mineral and the process of mineralization slows as the mineral ions become more difficult to nucleate in the intrafibrillar spaces. This slowing of mineralization results in more stable BMDD and mineral concentration values beyond week 4. While mouse skeleton is considered as mature at 10 to 12 weeks [38, 68, 244], our results indicate that mouse bone becomes mature beyond 4 weeks. Thus, this phenomenological model indicates that the MCFs from 4 week old mouse bone are effectively fully mineralized within their gap regions. MCFs from 10 week old mouse bone will show no further increases in their mineralization, resulting in similar Young’s modulus
values when compared to MCFs tested from 4 week old mouse bone. However, the mineralization occurring between the MCFs is apparently responsible for increases in bulk bone Young’s modulus. Structural difference at the nanoscale between 4 and 10 week old bone occur due to the formation of extrafibrillar mineral. Consideration of the space between collagen fibrils filled with non-collagenous proteins (NCPs) may be the key to understanding the increased Young’s modulus of bone at 10 weeks. In particular, the extrafibrillar space contains mineral that provides more efficient linkage between fibrils and therefore improved the load transfer between fibrils leading to a higher Young’s modulus. Furthermore, the Young’s modulus recorded in 10 week MCFs from mouse radial bone is comparable to the value of bone tissue at same age (3.65 ± 1.8 GPa versus 4.7 ± 1.3 GPa) indicating an effective stress transfer between fibrils in bone. As the extrafibrillar space is relatively limited with only 1-2 nm in thickness [152, 162], it is reasonable to assume the mineral crystals on the fibril surfaces might contact mineral in adjacent fibrils to form a rigid network of apatite mineral [252]. The increased connectivity between fibrils promotes the stress transfer and may lead to a nanoscale stiffening mechanism.

On the other hand, the MCFs and bone tissue tested from antler bone showed two different Young’s modulus values but in same order as recorded in Chapter 4 (2.4 ± 0.4 GPa of MCFs versus 5 to 7 GPa for antler bone tissue [103, 170, 171]). The
difference between measured Young’s modulus of MCFs and bone tissue could arise from apatite mineral at the fibril surface connecting together leading to an enhanced network stiffness as discussed for mouse bone above. Indeed, mineral present at the surface of individual MCFs from antler may not contribute to the mechanical behaviour measured using AFM-SEM as poor stress transfer between the straining fibre and surface mineral may exist. This may indicate a potential underestimation in mechanical properties of isolated MCFs compared to arrays of MCFs found in bone where stress transfer can occur between all constituents. The measurement of the Young’s modulus may also give rise to errors between individual MCFs and bulk bone samples. Bone is a type of viscoelastic material having a nonlinear stress-train response. The Young’s modulus is measured as the tangent slope of the stress-strain curve at relatively small strain [12, 103, 109, 112]. However, in this thesis, the Young’s modulus of fibrils is calculated by linear regression of the data points on the stress-strain curve due to the limited data collected by AFM. These two measurement methods record different modulus values as shown in Figure 5.9. In fact, some fibrils exhibit a Young’s modulus higher than 8 GPa by choosing the first two points for linear regression. The Young’s modulus of mouse bone tissue and corresponding MCFs were measured using the same linear regression method and therefore have comparable values. However, other bone samples may differ considerably due to the assignment of this tangential slope in stress-strain curves.
Figure 5.9 Young's modulus measured by different methods. (A) Tangent slope of stress-strain curve at small strain is recorded as Young's modulus. (B) Young's modulus is measured as the linear regression of data points on a stress-strain curve.

5.5 Conclusions

To conclude, 4 week and 10 week old mouse limb bones were representatively chosen for evaluating mechanical properties of bone and MCFs at different ages. Conventional mechanical testing methods were used to measure the mechanical behaviour of bulk bone and stress-strain plots were recorded. Individual MCFs from 4 and 10 week old mouse bone were tensile tested to failure using AFM-SEM techniques demonstrated in Chapter 4. In addition, an increase in the degree of mineralization at increasing bone age was examined by qBSE.
Homogeneity of mineralization and the bone mineral density were found to be increasing with age. The Young’s modulus of both bulk bone and MCFs was found to increase with age, with the increased amount of mineral in the older bone responsible for this increase. However, the increase in the Young’s modulus for MCFs was significantly less than for bulk bone tensile testing. A mechanism was developed that considered the mineralization of collagen fibrils and suggested that the mineral content in MCFs from 4 and 10 week old bone is compositionally similar. The interphase region containing mineral between collagen fibrils is described as varying with bone age and is more critical in defining the mechanical properties of bulk bone. To study the extrafibrillar region further, the NCPs layer between MCFs is mechanically evaluated in the next chapter.
Chapter 6

Nanoscale Interfacial Behaviour in Bone

6.1 Introduction

The mechanical performance of bone has been evaluated throughout the thesis in terms of the collagen fibril units. As with any composite material described in Section 2.3.2 of Chapter 2, the mechanical properties of a composite are defined not only by fibres but also by the matrix and fibre-matrix interface. Bone is perhaps unique in being a composite material containing a high volume (> 80 %) of fibrous material [12], resulting in a relatively small phase between MCFs. Despite the relatively small volume fraction of the matrix material, the interface formed between MCFs and NCPs should influence overall bone behaviour from composite considerations. While interfaces in bone at constituent length scales is the subject of this Chapter, interfacial behaviour in a number of layered biological
structures, especially shell materials, have been previously evaluated [253-255].

The propensity for interfacial failure in biological layered composites and the large fracture area involved has led to the pursuit of novel biomimetic layered composite structures based on shell materials fabricated with high volume fractions of ceramic mineral phase within a polymeric matrix [256-258]. These biomimetic composites show unprecedented work of fracture values due to extensive ductility and fracture at nanometre length scales. Bone is notable as a composite structure that exhibits considerable toughness through using fibrous constituents of MCFs as opposed to the layered platelets. The origin of bone toughness is therefore contentious and potentially different to shell materials, with hierarchical deformation over a range of length scales [7, 259], microcracking mechanisms [128, 132], heterogeneous failure [31, 260] and distinctive load transfer between constituents [261] proposed as defining bone toughness. Many of the proposed failure mechanisms in bone depend, at least in some part, on the mechanical behaviour of the nanomaterial constituent properties found in bone. These bone constituents can be evaluated in composite terms as a high volume fraction of collagen nanofibres reinforced by mineral platelets of hydroxyapatite. The mineral is often found in the collagen fibrils themselves but can be extracellular. The critical material constituent not considered in previous Chapters but present in what should be considered as the interphase region, describing both the interface and the matrix phase between
MCFs, is the non-collagenous protein (NCP) region. The NCP region found within the relatively small spaces of 1-2nm between collagen fibrils is amorphous and includes a number of proteins, most notably osteopontin [75, 262] and proteoglycan tethers between collagen fibrils [231]. A number of studies have examined the mechanical properties of both unmineralized collagen fibrils [20, 40] from various sources and mineralized collagen fibrils from bone tissue [17, 260]. However, the NCP region between the collagen fibrils is expected to be critical in defining the toughness of whole bone but is often poorly understood.

Recent work has indicated deficiency of specific proteins in NCPs cause loss of bone strength, while the transfer of stresses between collagen fibrils is expected to be critically dependent on the mechanical behaviour of the NCP region [231, 263]. Indeed, classical mechanical analysis of composites of fibres bound together by a polymer matrix highlights the importance of the fibre-matrix interfacial adhesion on stress transfer. Composite theory has been extensively exploited in nanocomposite interfacial mechanics, such as efficient stress transfer at carbon nanotube-polymer interfaces [172, 264] or poor stress transfer in graphene-polymer interfaces [265], but is rarely utilized in understanding the effect of nanoscale interfaces between the collagen fibrils in bone. Interfacial mechanical considerations in bone have been made [125, 261] but lacks evaluation of collagen fibril-NCP interface mechanics.
The mechanical characteristics of the NCP interface region between collagen fibrils in bone therefore remains elusive despite its importance in bone and other fibrous material toughness. Direct evaluation of nanoscale interfaces has been achieved using advanced atomic force microscopy (AFM) techniques, utilized to manipulate and remove nanofibres partially embedded within a polymeric matrix material [172, 264]. These direct nanofibre pullout measurements give quantitative information on the mechanical behaviour of the interface between the nanofibre and matrix, allowing the evaluation of both strong and weak nanocomposite interfacial mechanics. This Chapter therefore exploits the direct mechanical testing ability of the AFM-SEM technique to evaluate the interfacial properties at collagen fibril-NCP interfaces in bone using a pullout configuration to provide understanding on the role of nanoscale interfaces in bone toughness.

6.2 Materials and Method

6.2.1 Material Preparation

Antler bone samples were prepared using methods described in Chapter 3 and 4. Briefly, samples were extracted from the main beam of antler from a mature red deer and the velvet removed. All samples were selected from the same compact cortical shell near the antler-pedicle junction. Small beams of antler with dimensions of 3×20×0.2 mm with the long axis parallel to principal osteonal
direction were cut from the bulk material using a water-cooling rotating diamond saw and stored in 70% ethanol solution. Before mechanical evaluations, samples were left in Hank’s buffered solution overnight to allow full sample rehydration that mitigates mineral loss, which may occur in distilled water or physiological saline. Water on the surface of the sample was removed by filter paper to avoid interference with SEM imaging. Hydrated antler bone samples were subsequently fractured perpendicular to their long axis to expose mineralized collagen fibrils and immediately transferred to the chamber of an SEM containing the AFM setup.

6.2.2 in situ AFM-SEM Fibril Pullout Experiment

Bundles of fibrils at the bone fracture edge were selected with an individual fibril protruding from the centre of the bundle as shown in Figure 6.1. Mechanical testing of the NCP was achieved following a pullout configuration, as has been achieved in synthetic fibrous nanocomposites [164, 172]. The AFM probe within the SEM chamber was first translated into a glue (Poxipol, Arg) droplet containing within the chamber, to allow pickup of glue at the apex of the AFM probe. The AFM probe was then translated towards the free end of the MCF protruding from a bundle as shown in Figure 6.1.
Figure 6.1 (A) Scanning electron micrographs showing an AFM probe containing glue at its apex attached to an individual MCF partially embedded in a fibril bundle at the fracture surface of antler bone. (B) The exposing fibril was pulled out by the AFM with images at high magnification used to measuring the fibril embedded length $l_e$.

The attachment of the free end of the exposed individual MCF to the AFM probe containing glue was performed within a 10 minute time window to ensure that the glue was still liquid during the MCF attachment. The AFM probe was subsequently moved away from the bone surface, which caused an increase in the tensile stress within the fibril and an equal but opposite shear stress within the NCP surrounding the MCF. Fibrils were observed to detach from the bone surface at a critically applied force, measured using the optical interferometer setup to record the deflection of the AFM cantilever in the AFM system as
reviewed in Section 3.2. These MCF pullout experiments were performed in the SEM vacuum chamber for less than 20 minutes to ensure that water loss from the bone was minimized. Previous mechanical testing on bone constituents in SEM described in Section 3.2 highlighted how bone at nanometre length scales retains the same mechanical properties as fully hydrated bone samples [260] within the testing time frame used here. We note that the selection of an individual MCF for pullout was not controlled as the length of the MCF embedded within the bone tissue was not known. Therefore, only around 2 in 10 individual MCFs selected for pullout actually became pulled out of the bone tissue, with the other tested MCFs fractured within the free length part.

### 6.3 Results and Discussion

Individual mineralized collagen fibrils were pulled out from rehydrated bone sample using \textit{in situ} AFM-SEM. The mechanical properties of the NCP interphase region around the MCFs is calculated by recording the force applied to the MCF by the AFM system. As shown in Figure 6.1, a force $F$ is applied at the free end of MCF with the effective force parallel to the protruding MCF long axis calculated accurately by knowing the off-axis translation angle $\theta$ (<30°). The force required to pull the MCF out of the bone surface $F_p = F \cos \theta$. The force applied on the
MCFs and the displacement of the AFM cantilever during the pullout was recorded and shown in Figure 6.2 for tests on five different collagen fibrils within the same bone region. The force applied to the MCF increased linearly with progression time of the experiment until a maximum force, $F_p$, was reached, which caused failure of the MCF-NCP interface and a rapid drop in the force $F$ exerted by the AFM until the MCF was separated from the bone sample. A linear increase of applied force $F$ with experimental progression time has been observed for other nanofibre pullout experiments [264, 266-269], indicating that the MCFs in this work pullout as opposed to fracture within the bulk bone material and subsequent pullout.

![Figure 6.2 Plot showing the force applied to the partially exposed MCF during pullout against progression time for the pullout experiment. The force increases](image-url)
linearly with progression time until a maximum force $F_p$ is reached, which causes failure of the interface and rapid separation of the MCF from the bulk bone sample.

The strength of the interface between the MCF and surrounding NCP is characterized by the interfacial shear strength ($\tau$) and is calculated from the maximum force applied to the exposed MCF to cause pullout from the surrounding NCP:

$$\tau_i = \frac{F_p}{\pi D_f l_e}$$  \hspace{1cm} \text{Equation 6.1}

where $D_f$ is the fibril diameter and $l_e$ is the length of fibril embedded within the bone. Equation 6.1 assumes the stress generated at the MCF-NCP interface during pullout is uniformly distributed along the fibril embedded length. The evaluation of the interfacial shear strength at the MCF-NCP interface thus requires accurate determination of fibril diameter $D_f$ and embedded length $l_e$. SEM was used to measure the diameter and length of the MCF before and after pullout testing. The diameter of the MCF fibrils, measured at 5 equidistant points along the MCF free length, were reasonably constant before and after pullout testing with an average $D_f = 115.6 \pm 33$ nm. The length of the MCF after pullout consists of the MCF free length before pullout and the embedded length $l_e$. Thus, subtraction of the MCF length before pullout from the MCF length after pullout
provides \( l_e \). SEM imaging of the MCF free lengths before and after pullout using pixel analysis (ImageJ, NIH, USA) gave a range of \( l_e \) values from 510 to 880 nm. The strain in the free length of the MCF during pullout testing is less than 1 % when considering a maximum applied force of 0.3 \( \mu \)N, the fibril diameter and the MCF Young’s modulus defined in Chapter 4, indicating negligible fibril strain contributions to the measured mechanical behaviour in this work.

The calculated MCF-NCP interfacial shear strength \( \tau \) is 0.65 \( \pm \) 0.15 MPa using Equation 6.1 and the results in Figure 6.2. This shear strength for the nanoscale interfaces found in bone is lower than values recorded from pullout of engineering fibres from conventional fibre reinforced polymer composites [172, 264, 266, 269]. However, engineering composites are often optimized for effective stress transfer between the reinforcing fibres and require relatively high \( \tau \) values up to approximately 50 MPa [269, 270]. The MCF fibres in this work therefore exhibit low interfacial shear strength, which is conducive for toughness as cracks propagating through bone will be deflected at the weak interfaces between the MCFs and surrounding NCP. The vast area available at these nanoscale interfaces in bone may be a considerable energy absorbing process.

A stress-based analysis as described above can be indicative of the mechanical behaviour of the MCF-NCP interface but provides little understanding of the
fundamental nature of the interface. However, energy based criteria has been used to understand molecular mechanisms present at interfaces in bone material [152]. Specifically, the work done $W$ to pullout the fibrils from the NCP is given as:

$$W = \int_0^{F_p} Fl_e = \frac{1}{2} F_p l_e$$  \hspace{1cm} \text{Equation 6.2}

Previous studies suggest that macroscopic plasticity of bone is mainly due to the plastic deformation occurring in extrafibrillar spaces as elastic deformation is retained within the fibrils [5, 153, 234]. Thus, substantial work is required for the fibril pullout process during breaking of bone, which contributes to the plastic deformation of the extrafibrillar NCPs from MCF sliding and separation. At molecular length scales, the NCP material consists of proteins including osteopontin [262], proteoglycans [271, 272] and fetuin A [273] that are anchored to the mineral sites on the MCFs and form an ionic network. The ionic bonds existing in the thin layer of extrafibrillar NCPs bridge between negatively charged protein molecules and divalent calcium ions, providing a molecular ‘glue’ to bind the MCFs together. The work done to pullout the MCF from surrounding NCP therefore causes failure of these sacrificial ion bonds in the NCP glue. However, a number of works have shown how the interfacial region can reform [152, 167, 168, 274], indicating that the work of pullout may have to fail sacrificial ionic bonds multiple times in the NCP before complete MCF separation from the bone bulk.
We therefore propose a model MCF-NCP system having partially contacting protein molecules between MCFs as shown in Figure 6.3. Most of the mineral content is found within the MCFs in antler bone, with little mineral found in the NCP region. Mineral will therefore be present on the MCFs surface mainly at the gap region of the MCF, as defined in the Hodge-Petruska scheme [82] and the staggered arranged mineral arrangement [43], and will cover approximately 60% of the MCF surface. The negatively charged protein molecules are therefore bound to the mineral regions of the MCF as proposed by Salih et al. [275] as well as Hartmann and Fratzl [168] as shown in Figure 6.3.

Figure 6.3 Schematic of the sacrificial ionic bonding system in NCPs region with pink indicating MCFs, the blue region defining the extrafibrillar NCPs glue layer and red denoting mineral crystals. Each short black dash line in NCPs layer represents an activation volume. (A) NCPs are shown anchored to the mineral
sites of the MCF. (B) The negatively charged NCP molecules are bonded together by positively charged divalent calcium ions. (C) MCF pullout causes shear in the NCP layer where ionic bonds are assumed to reversibly form process of MCFs pullout. Thus, during plastic deformation of the interface glue region, ionic bonds fail as negatively charged NCPs molecules are separated but reform when the NCP molecules contact neighbouring NCP molecules during the pullout process. The NCP bonding will reform and break continually with the fibril pullout movement, resulting in a large number of bonds breaking relative to a non-reforming pullout process as show in (D).

Under shear force applied to the MCF-NCP interface during pullout testing, the negative charged protein molecules connected via calcium ions will slide to separate and the ionic bonds will break and potentially reform the network in the extrafibrillar region. To study this reforming behaviour of sacrificial bonds, two extreme conditions can be assumed: i) failure and continued reforming of all bonds from the NCP in contact with the separating MCF during the pullout as indicated in Figure 6.3 (C) or ii) the complete failure of bonds at the MCF-NCP interface during pullout. In i) the work done will be used to break a larger number of bonds due to reforming events than condition ii). The activation energy $H$ is associated with the work done to break bonds over a unit volume and has been previously recorded as approximately 1 eV for a 1 nm$^3$ volume of bone.
material in which one or several sacrificial ionic bonds exist [152, 162, 167, 168].

Figure 6.3 (C) shows the condition where bonds existing at the MCF-NCP interface reform during the interface failure. Each short dashed line represents a unit activation volume defined as ‘unit’ in which a number of sacrificial bonds exist. At position $X_0$, bonds between the MCF and NCP will break but not reform as the MCF is pulling out of the NCP. However, at position $X_n$, the unit will break and reform by $n$ times where $n$ is the number of negatively charged molecules existing along the long axis of the MCF. The whole extrafibrillar space can be considered as a hollow cylinder with thickness of 1 nm as reported previously [152, 168, 276] and the cylinder can be divided into $n$ ‘rings’ along its long axis. Each ring contains $\pi D / \sqrt[3]{V_a}$ units of activation volume. At the cross section shown in Figure 6.3 (C), the units within the intrafibrillar space at one side of the MCF will break and reform $n(1+n)/2$ times during fibril pullout. Therefore, over the whole interface area, the total number of units breaking and reforming will be:

$$N_{100\%} = \frac{\pi D}{\sqrt[3]{V_a}} \times \frac{n(1+n)}{2} \approx \frac{\pi D}{\sqrt[3]{V_a}} \times \frac{n^2}{2}$$  \hspace{1cm} \text{Equation 6.3}$$

where the $\sqrt[3]{V_a}$ represents the unit length of a side in a single cubic activation volume which is equal to 1nm. $V_a = 1\text{nm}^3$, $\sqrt[3]{V_a} = 1\text{nm}^2$.
Conversely, the total number of units broken if no reforming occurs \((N_{0\%})\) as shown in Figure 6.3 (D) can be stated as:

\[
N_{0\%} = \frac{\pi D}{\sqrt{V_a}} \times n
\]  
Equation 6.4

In the Hodge-Petruska model of MCFs [82] with staggered arranged mineral arrangement [43], approximately 60% of the MCF surface is covered with mineral and attached with negatively charged protein chains. Therefore \(n\) can be estimated using

\[
n = \frac{0.6l_e}{\sqrt{V_a}}
\]  
Equation 6.5

The activation energy \(H = W/N\) and provides boundary values of 0.011±0.0025 eV and 2.21±0.40 eV for bond reforming interfaces and complete interfacial failure respectively. The calculated activation values lower than previous literature value of 1 eV, and indicate complete bond reforming underestimates the activation energy whereas no interfacial bond reforming overestimates this activation energy. Thus, Equations 6.3 and 6.4 define boundary conditions, indicating the interfacial bonding between the MCF and NCP will only reform partially.
To explore partial interface reforming, MCF pullout testing using the different pullout velocities were compared to the work done \( W \) during pullout as shown in Table 6.1. The calculated activation energies for the boundary conditions of complete bond reforming \( (H_{100\%}) \) and complete bond failure \( (H_{0\%}) \) are also shown in Table 6.1. The reforming of an individual sacrificial bond is a probability event controlled by the molecular kinetics of NCPs protein chains. However, over the whole interfacial space, the efficiency of the activation volume units reforming has a statistical certainty in a single fibril pullout event due to a large number of sacrificial bonds involved. Based on Equation 6.3, 6.4, and 6.5, the total number of activation volume reforming can be calculated as:

\[
N = N_{0\%} + N_{100\%} = \frac{\pi D_l (0.6\alpha l_e)^2}{2V_a} + \frac{\pi D_l 0.6 l_e (1 - \alpha)}{\sqrt[3]{V_a}} \quad \text{Equation 6.6}
\]

The reforming efficiency \( \alpha \) is defined as the proportion of activation volume units required to reform in order to provide a literature activation energy \( H=1 \text{ eV} \) in the fibril pullout and can be calculated as:

\[
\alpha = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \quad \text{Equation 6.7}
\]

where: 
\[
a = \frac{0.36\pi D_l l_e^2}{2V_a}, \quad b = -\frac{\pi D_l 0.6 l_e}{\sqrt[3]{V_a}}, \quad c = -\frac{\pi D_l 0.6 l_e}{\sqrt[3]{V_a}} - \frac{W}{H}; \quad H = 1\text{ev}.
\]
By assuming all the sacrificial bonds within an activation volume unit share an equal chance of breaking and reforming, the probability of a single bond reforming $P$ in the fibril pullout is the same as the activation volume reforming efficiency $\alpha$ and listed in Table 6.1.

Table 6.1. Data showing the geometry, work done and calculated interfacial behaviour in MCF pullout tests.

<table>
<thead>
<tr>
<th>$L_e$ (nm)</th>
<th>Diameter (nm)</th>
<th>Work ($\times 10^{-14}$J)</th>
<th>$\tau$ (MPa)</th>
<th>$H_{100%}$ (eV)</th>
<th>$H_{0%}$ (eV)</th>
<th>Reforming efficiency (%)</th>
<th>Pullout velocity ($\mu$m s$^{-1}$)</th>
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<tr>
<td>700</td>
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<td>0.011</td>
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<td>3.95</td>
</tr>
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<td>540</td>
<td>81</td>
<td>2.93</td>
<td>0.79</td>
<td>0.014</td>
<td>2.22</td>
<td>9.00</td>
<td>2.09</td>
</tr>
<tr>
<td>650±</td>
<td>115.6±</td>
<td>5.36±</td>
<td>0.65±</td>
<td>0.011±</td>
<td>2.21±</td>
<td>8.02±</td>
<td>2.55±</td>
</tr>
<tr>
<td>150</td>
<td>33</td>
<td>2.30</td>
<td>0.15</td>
<td>0.0026</td>
<td>0.40</td>
<td>1.32</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Figure 6.4 shows the bond reforming efficiency plotted against the pullout velocity. In particular, the bond reforming efficiency is expected to decrease as the pullout velocity is increased due to the reduction in time available for ionic bonds to reform at the MCF-NCP interface. Figure 6.4 supports this assumption by showing a relatively rapid drop in bond reforming efficiency as the pullout velocity increases fivefold from 0.5 to 2.5 $\mu$m.s$^{-1}$. The reforming efficiency of
sacrificial ionic bonds in extrafibrillar space is highly dependent on the calcium ions density within the NCP regions. The speed of calcium ions migration to new bond reforming sites, which is decided by the permeability of NCP matrix, is critical on the reforming process. More rapid pullout testing would therefore suggest insufficient time for the calcium ions to transport sufficiently to bond reforming sites, which leads to a lower reforming efficiency.

Figure 6.4 Plot of the sacrificial ionic bond reforming efficiency against individual MCF pullout velocity speed for separation of MCFs from the surrounding NCP region. The dashed line shows the trend of the reforming efficiency increasing with decreasing pullout speed.

The calculation of activation energy for pullout of individual MCFs from a
surrounding NCP binding ‘matrix’ based on AFM pullout experiments is instructive in defining a partial capability for bonding reforming in bone at the nanoscale. In addition, the molecular mechanism for bond reforming is expected to be rate dependent and is supported by experimental pullout data. We also note that the MCF pullout was achieved in less than 0.4 seconds. This pullout time corresponds to an average testing strain rate of $4.27 \pm 1.23 \text{s}^{-1}$, which is higher than the strain rate ($\sim 1.59 \text{s}^{-1}$) used in testing to mimic the real loading condition of antler bone in daily use and under sudden impact in combat [116]. Therefore, under physiological loading conditions, antler bone will be expected to exhibit a higher reforming efficiency than in this work.

6.4 Conclusions

In this chapter, individual MCFs were pulled out from surrounding NCP matrix regions. Stress based analysis was used to show that the MCF-NCP interface is weak, with energy based analysis suggesting an ability of the MCF-NCP interface to reform during mechanical testing. The promotion of interfacial failure at the nanoscale with ability to reform this interface is noted as being beyond current synthetic nanocomposite design. Specifically, difficulties lie in producing composites with dispersed, high volume fractions of nanofibres that provide
significant fracture surface after interfacial failure and the ability of interfacial bonding to reform during the fracture process. The number of ionic bonds failing at the fibril interface is therefore being maximized by reforming in order to increase the overall work done to break the bone material.
Chapter 7

Mineralized Collagen Fibril Contributions to the Fracture Behaviour of Bone

7.1 Introduction

The nanoscale mechanical properties in bone, specifically the mechanical behaviour of MCFs and their associated interfaces, have been elucidated in previous Chapters but the relationship between nanoscale behaviour and whole bone behaviour is unclear. This Chapter attempts to make predictions on the contribution of the nanoscale to overall bone mechanics using simple fracture considerations and composite models. The use of analytical composite models is established in engineering materials and is therefore appropriate for the MCF-type fibre reinforced structures of bone. The fracture toughness of bone material is of particular importance and is related to the experimental fibril
mechanics obtained in this work. In particular, the mechanical properties of MCFs from antler bone have been evaluated in terms of their deformation and failure behaviour in Chapter 4 and the mechanical properties of their interfaces in Chapter 6. Therefore, the evaluation on the contribution of nanoscale fibril fracture and pullout to the overall fracture resistance of antler bone is attempted here.

7.1.1 Pullout Theory: Energy Balance Model

Observation of bone fracture surfaces in Chapters 4-6 indicates a propensity for the MCFs to pullout from the bone matrix material. Indeed, the relatively weak interfacial shear strength reported in the previous Chapter would support the promotion of failure at the MCF-NCP interface. Descriptions of the contribution of an individual interface on overall composite behaviour rely on consideration of the fibre pulling out from the composite material. In fibre pullout testing, a gradually increasing tensile force is applied to the fibre partially embedded within the matrix until a critical force causes complete failure of the fibre-matrix interface, resulting in the fibre being pulled out from the composite, with a force-displacement curve typically recorded in this process. By applying a suitable load transfer model, the interfacial shear strength, interfacial friction coefficient and interface crack propagation behaviour can be measured. The interfacial shear strength is given by using a simple force balance of:
\[ \tau_i = \frac{F_p}{2 \pi r l_e} \]  

Equation 7.1

where \( F_p \) is the maximum tensile force applied on fibre before pullout, \( r \) is the fibre radius and \( l_e \) is embedded length of the fibre. This theory is straightforward but ignores the effects of matrix elasticity, uneven distribution of tensile load in the interfacial space, embedded length and Poisson’s ratio. More complete descriptions of the fibre pullout process proposed by Chua and Piggott [277] incorporate the following: i) lateral pressure applied on the fibre from matrix; ii) interfacial friction coefficient; iii) interfacial fracture energy; iv) fibre embedded length and free length.

The latest energy-based interface model proposed by Jiang and Penn [278] is preferable in recent studies [264] as consideration of all factors listed above are made based in the energy balance analysis. In this model, the experiment system consists of three parts: i) fibre free length, ii) debonded region of fibre and iii) bonded region between the fibre and matrix. The strain energy stored in these regions as well as work of friction in fibre sliding out of the matrix are considered in an energy balance analysis to derive the pullout load. Specifically, a crack propagates at the interface between the fibre and matrix when the energy released from the system provides sufficient energy to create new surfaces for crack propagation in the bonded region of the composite and work to overcome
friction in the debonded region is:

\[ \frac{\partial U_f}{\partial a} + \frac{\partial U_d}{\partial a} + \frac{\partial U_b}{\partial a} = 2\pi r G_f + \frac{\partial W_f}{\partial a} \]

Equation 7.2

where \( U_f, U_d \) and \( U_b \) are the strain energies stored in the fibre free length region, debonded region and bonded region respectively. \( G_f \) is the fibre interfacial fracture energy and \( W_f \) is the work of friction at the debonded interface. The crack length is described by \( a \) so that no crack initiates when \( a = 0 \).

By assuming negligible friction between the debonded fibre and matrix, the interfacial fracture energy \( G_f \) can be calculated from the maximum pullout force \( F_p \):

\[ F_p = \left( \frac{n'}{r} \right) l_o \sqrt{\frac{E_f A_f 2\pi r G_f}{(2 + \beta)}} \]

Equation 7.3

where:

\[ n' = \sqrt{\frac{E_m}{E_f(1 + \nu_m) \ln R/r}} \]

Equation 7.4

and

\[ \beta = \sqrt{\frac{E_f A_f}{E_m A_m}} \]

Equation 7.5

where \( E_f \) and \( E_m \), are the Young’s modulus of the fibre and matrix respectively. \( A_f \) and \( A_m \) are the cross-section area of the fibre and matrix, \( \nu_m \) is Poisson’s ratio of
the matrix and $R$ is the distance orthogonal from the fibre surface that is perturbed when pulling the fibre from the matrix.

The analysis above can be applied to the pullout data using *in situ* AFM-SEM as reported in Chapter 6. The nanoscale interfacial fracture energy in fibrillar systems of bone can be determined by using Equation 7.3, as has been carried out in numerous fibrous composite systems [1]. One condition of applying Equation 7.3 is that the interfacial friction between the fibre and matrix should be negligible. When two contacting objectives are in relative movement, the molecular interaction between the surfaces is generally described as a friction force. The ‘friction’ in collagen fibril pullout from bone is essentially the break and reforming of sacrificial ionic bonds existing within the extrafibrillar space. As discussed in Chapter 6, the number of sacrificial bonds reforming in particular pullout tests is less than 10% of the total bonds under high pullout speed. The friction raised from sacrificial bonds in the pullout is relatively low and is ignored in this study. It is therefore expected to be reasonable to extend the concepts and model above to MCFs pullout experiments.

When using Equation 7.3 for fibril pullout analysis, the matrix needs to be clearly defined as $n$ and $\alpha$ are highly dependent on the matrix properties. In this study, the matrix may be defined as the antler bone tissue surrounding the tested fibril. As shown in Figure 7.1 (A), when the fibril is pulled out from the bone matrix, the
neighbouring MCFs are also affected by the shear stress transferred via the extracellular NCPs layer. Alternatively, the matrix could be defined as the NCPs that exist between fibrils. Only the fibril with load applied directly with surrounding NCPs layer is affected by shear stress as shown in Figure 7.1 (B). However, this assumption indicates that the NCPs layer does not transfer load between MCFs, which is contradictory to observations (fibril interfacial shear strength recorded as 0.6 MPa) in Chapter 6.

Figure 7.1 Schematic diagrams showing stress distribution in the pullout fibril and surrounding bone tissue. MCFs are represented by orange cylinders with blue extracellular NCP regions existing between the MCFs. (A) Adjacent MCFs are deformed by the shear load transferred from NCPs whereas (B) Surrounding bone tissue is not affected if assume NCP do not transfer load which is contradictory to the phenomenon observed in Chapter 6.
7.2 Results

If we assume that a relatively large amount of matrix as bone tissue surrounding is deformed during a pullout event, \( \alpha \) in Equation 7.3 can be neglected as the matrix area \( A_m \) is much larger than the fibril area \( A_f \). Equation 7.3 can be therefore rearranged as:

\[
\frac{F_P}{l_e} = \left( \frac{K' n'}{r} \right) \sqrt{G_f} \tag{Equation 7.6}
\]

Where \( K' \) is a constant defined by the geometry and Young’s modulus of MCFs:

\[
K' = \sqrt{E_f A_f \pi r} \tag{Equation 7.7}
\]

Equation 7.6 can be solved by first plotting the variation in the maximum pullout force \( F_P \) against the embedded fibril length \( l_e \) using the data collected in Chapter 6. The maximum pullout force \( F_P \) plotted against the fibril embedded length is shown in Figure 7.2 with a linear regression used to give the ratio \( \delta F/\delta l_e \).
Figure 7.2 Maximum pullout force required for pulling out of individual MCFs from bone matrix plotted against fibril embedded length. Red dash line shows the linear regression.

The fibril interfacial fracture energy $G_f$ can be calculated using Equation 7.6 from the linear regression in Figure 7.2. In addition, average MCF radii of $57.8 \pm 16.5$ nm were tested in pullout, the Poisson's ratio of antler bone matrix $\nu_m$ is recorded as 0.3-0.32 [103, 116, 170, 171, 279] and the Young's modulus of wet compact bone tissue from red deer antler is recorded to be ranged between 5.1 to 7.8 GPa [103, 116, 170, 171] (indeed, our samples of antler bone are the same source as Currey's 2009 work [171] with Young's modulus of 7.2 GPa). Finally, the constant $n$ contains the stress transfer parameter $R/r$ which cannot be determined in this fibril pullout test. Therefore, the calculated fibril interfacial
fracture energy $G_f$ is plotted against $R/r$ as shown in Figure 7.3. Typically, $R/r$ values vary from 2 - 3 for weak interfacial adhesion up to 9 for strong adhesion [280, 281]. Hence, the calculated $G_f$ from Equation 7.6 is plotted against stress transfer parameter $R/r$ as shown in Figure 7.3 and ranged from 2.17 to 6.87 $\text{J.m}^{-2}$.

Figure 7.3 Calculated interfacial fracture energy from pullout of individual MCFs from bone matrix is plotted with different stress transfer parameters $R/r$. Error bars indicate the standard deviation of the interfacial fracture energy values calculated from five individual fibril pullout tests.

The calculation of interfacial fracture energy shown in Figure 7.3 requires interpretation of $R/r$ values. The results of fibril pullout experiments in Chapter 6 indicate relatively low interfacial shear strength values, at least relative to
engineering composites, which suggest that small $R/r$ values operate in bone. However, $R/r$ values form a complex relationship with the elastic properties of composite constituents as shown in Equation 7.4. Additional complications in determining $G_f$ from $R/r$ values arise from the elastic response assumed in the analysis and the relatively small amount of matrix existing between the MCFs that can be deformed.

$G_f$ describes the fracture energy release in unit area of MCF-NCP interface fractured. Figure 7.4 show a crack propagating perpendicular to the osteon orientation (which is also the principle orientation of MCFs). Such crack propagation will cause failure of individual MCFs, followed by pullout to leave exposure of fibril ends and pullout voids at the fracture surface.

Figure 7.4 Schematic showing the propagation of a crack perpendicular to the osteon orientation in bone leading to fibril fracture and pullout. MCFs are
represented by orange cylinders with blue extrafibrillar NCP regions existing between the MCFs.

The energy required for crack propagating at the fracture surface combines the work done to break MCFs and the fibre interfacial fracture energy $G_t$. Considering MCFs are closely packed in bone and share interfaces with neighbouring fibrils, the total fracture energy required to fail the interfaces between MCFs can be calculated as:

$$\Delta U_i = \frac{f l_p G_t}{r} \quad \text{Equation 7.8}$$

where $f$ is the MCF volume fraction, taken as 82% [12], $l_p$ is pullout length of the fibrils exposed at the fracture surface, $G_t$ has a value ranging from 2.17 to 6.87 J.m$^{-2}$ as calculated from energy based analysis in Figure 7.3. The total fracture of bone therefore consists of the total interfacial fracture energy given in Equation 7.14 but also a contribution from the failure of MCFs. The total energy required to fracture MFCs during propagation of a crack in bone can be calculated from the area under stress-strain curves recorded in Chapter 4, thus:

$$\Delta U_f = f \sigma \epsilon l_p \quad \text{Equation 7.9}$$

where $\sigma$ is the maximum MCF failure stress and $\epsilon$ is the maximum MCF failure
strain, taken from average values in Chapter 4. Hence, the total fracture energy $G_c$ released when considering MCF pullout and fibril failure is the summation of Equations 7.8 and 7.9 to give:

$$G_c = f l_p G_f + f \sigma \varepsilon l_p$$  \hspace{1cm} \text{Equation 7.10}

The fibril pullout length $l_p$ can be measured by pixel analysis on scanning electron microscopy images. However, fibrils might be oriented in an angle to the SEM image plane and direct measurement on SEM image therefore might not give real length of fibrils. To measure the true fibril length and eliminate the error from various fibril orientations, SEM images on fibrils were taken from different angles as shown in Figure 7.5. The real fibril length is given by the trigonometry calculation as:

$$l_p = \frac{l_{30}^2 - \cos^2 30 l_0^2}{\sqrt{1 - \cos^2 30}}$$  \hspace{1cm} \text{Equation 7.11}

where $l_0$ and $l_{30}$ are the fibril pullout lengths measured when observing the fracture surface at $0^\circ$ and $30^\circ$ degrees respectively in the SEM as shown in Figure 7.5.
Figure 7.5 Scanning electron micrographs of fracture surfaces in antler bone from different viewing angles. (A) Image taken with sample stage at 0 degree tilt. (B) Image taken when sample stage was tilted by 30 degrees.

106 fibrils were randomly chosen with their lengths measured over the whole fracture surface. The distribution of fibril pullout length was plotted in Figure 7.6. An average fibril pullout length of \(2.8 \pm 1.7 \, \mu\text{m}\) with an average fibril radius of \(51.3 \pm 6.3 \, \text{nm}\) was recorded. The total fracture energy directly associated from fibril pullout and failure mechanisms at a bone fracture surface calculated from Equation 7.10 gives a range of values from 139.8 to 334.7 J.m\(^{-2}\), in which the fibril fracture energy contributes less than 2 J.m\(^{-2}\).
Figure 7.6 Distribution of fibril pullout length at antler bone fracture surfaces.

7.3 Discussion

The fracture energy of nanoscale interfaces between MCFs in bone calculated using energy based analysis is much lower than values for synthetic engineering composites reinforced with fibres chemically modified to promote strong interfacial adhesion, suggesting that the molecular interaction between MCFs is weaker than covalent bonds in the chemically modified interface in artificial composites. For example, a previous study [282] gave a range of interfacial fracture energy values for glass fibres pulled from a variety of polymers such as vinyl ester (13–34 J.m⁻²), polyamide 6 (24–93 J.m⁻²) and polyamide 6.6 (52–61 J.m⁻²) [264]. On the other hand, the fracture energy of the MCF interface
calculated here is comparable to ionic bonded interfaces in ceramics [283-285],
which confirmed previous simulation studies indicating how ionic bonds are
dominant at fibrillar interfaces in bone [152, 167, 168, 274].

The relatively low interfacial fracture energy calculated in this Chapter
importantly needs to be placed in context with the fracture of whole bone. As
discussed in Section 2.4.2, the multiple length-scale toughening acting on cortical
bone leads to a unique rising resistance-curve (R-curve) behaviour for bone
fracture [6, 136, 137, 140]. The fracture toughness of bone material increases
with crack extension in bone. Vashisht et al. [286, 287] have reported rising
R-curve behaviour in antlers of red deer and demonstrated that the superior
toughness of antler bone is due to its enhanced ability to form microcracks
during deformation and fracture. The fracture work recorded previously in
R-curve measurements on antler bone using in situ synchrotron X-ray method
has shown low initiation fracture energy of 5.71 to 17.93 J.m$^{-2}$ with crack length
of 0.02 mm that increases to almost 1 kJ.m$^{-2}$ when crack length reaches 1 mm in
all loading directions [288]. Other studies have shown a much higher fracture
energy from 30 kJ.m$^{-2}$ [170, 171] to 60 kJ.m$^{-2}$ [15, 288] recorded when cracks
propagate in the transverse direction relative to the long axis of bone.

The difference the fracture energy of bone, which is of the order of 10 kJ m$^{-2}$, and
the initiation of cracking is approximately 3 orders of magnitude. This difference is significant as initiation of cracking does not involve the large amount of plastic deformation and microcracking occurring in bone beyond crack initiation. Indeed, the energy based analyses used above ignore frictional behaviour associated with pullout, which could contribute significantly to the overall fracture energy of bone. Therefore, our calculated fracture energy results should be compared to crack initiation behaviour only. Considering the fibril interfacial fracture energy $G_i$ calculated varies from 2.17 to 6.87 J.m$^{-2}$, the nanoscale fibril interfaces contribute roughly 50 % of the initiation fracture energy in antler bone. It is therefore reasonable to assume that when cracking initiates in bone, the major mechanism is the separation of fibrils around a crack. The interfacial fracture is dominant in fracture initiation in antler bone at this stage.

When a crack extends to up to 1 mm, the fracture energy is recorded to be approximately 1 kJ.m$^{-2}$ in all loading directions [288]. The total fracture energy directly associated from fibril pullout and failure mechanisms at a bone fracture surface calculated from Equation 7.10 gives a range of values from 139.8 to 334.7 J.m$^{-2}$ in which fibril interfaces contribute more than 90 %. Our result indicates the nanoscale contributions of MCFs, especially the fibril interfaces, to the fracture process at this stage is significant with up to ~33 % of the total energy required to fail bone by assuming only simple flat surfaces are produced in
fracture as shown in Figure 7.4 (B). The real contributions of MCFs could be higher as i) crack propagates in deflected and twisted paths generate larger fracture areas as demonstrated in Figure 7.7 (A), ii) there might be more cracks existing away from fracture sites which leads to an underestimation on number of interfacial failure events used for calculated fracture energies.

Crack propagation in bone material beyond crack lengths of 1 mm cause large variability in the fracture energies of bone. For cracks propagating transverse to the osteon direction, the fracture energy of antler bone can reach values of up to 31 kJ.m\(^{-2}\) [116, 171] to 60 kJ.m\(^{-2}\) [15, 288]. This fracture energy is considerably higher than for longitudinal splitting of bone as considerable delocalized failure of bone occurs. Thus the total number of MCF fibril events contributing to the total fracture energy of bone in transversal direction cannot be simply made by summation of the individual events at the fracture as described using Equation 7.10. Instead, the total number of events within the fracture volume, which extends considerably beyond the apparent bone fracture surfaces observed, must be considered but is difficult to know accurately. Similarly, splitting antler bone along it longitudinal direction only requires a fracture energy of 6 to 10 kJ.m\(^{-2}\) [15, 116, 171, 288] due to failure being restricted more towards the apparent fracture surfaces of bone as opposed to the larger failure zones occurring in transverse cracking. However, the total number of failure events in both longitudinal
splitting and transverse cracking are significantly larger than assumed at the propagating cracks as assumed in Equation 7.10. Figure 7.7 highlights the differences in the damage zone for longitudinal and transverse crack growth. In particular longitudinal crack propagation in Figure 7.7 (A) shows a smaller damage zone than transverse cracking in (Figure 7.7 (B), which was confirmed by X-ray tomography studies [288]. Transverse cracks therefore produce a larger failure region than actual fracture surface, increases the fracture energy considerably when compared to longitudinal cracking which greatly enhances the fracture resistance of antler bone. Therefore, both transverse and longitudinal failure in bone represents the failure mechanism away from the failure described using Equation 7.10. Smaller cracks below 1 mm must therefore reflect Equation 7.10 most closely.

In addition, weak interfaces between fibrils enable the occurring of nanocracks away from fracture site due to the heterogeneous mechanical behaviour of fibrils as recorded in Chapter 4. In the stress affected region ahead of a crack tip, the strain mismatch between fibrils with different Young's moduli leads to the failure of interfaces and separation of fibrils. The nanocracks formed away from a crack tip will accumulate, resulting in an increased stress away from the crack tip and eventually formation of microcracks as shown in Figure 7.7 (B). The appearance of microcracking absorbs additional energy and promotes other toughening
mechanisms as discussed in Section 2.4.2.

Figure 7.7 (A) and (B) Scanning electron micrographs of crack growth path at a fracture tip in antler bone [288]. Transverse crack propagates against osteon orientation in (A) with red arrows show the crack ‘twisting’ during crack growth. (B) Cross section image of osteons showing how ‘micro cracking’ occurs away from a crack tip. (C) and (D) Synchrotron X-ray computed micro-tomography images of cracks occurring in both the longitudinal and transverse orientations of antler bone [288] with cracks shown in purple. Central canals in osteons (hollow volume in osteons) and vascular channels are represented in brown. Both longitudinal and transverse directions exhibit significant cracking. However,
cracking in the transverse orientation (D) shows more failure volume due to crack deflection and microcracking generated in the neighbouring space near the principal crack tip [288].

7.4 Conclusions

In this chapter, energy based analyses using results from pullout testing of MCFs was carried out. A fibril interfacial fracture energy ranging from 2.87 to 6.87 J.m$^{-2}$ was calculated and was found to be comparable to ionic bonded interface values, which is coincident with previous simulation result that fibrillar interface is dominated by ion bonds. Around 50% of the work done to initiate the crack was indicated to be due to the failure of the nanoscale fibrillar interfaces which makes it a potent mechanism in crack initiation. The total energy required to both fracture MCFs, using data from Chapter 4, and the calculated interfacial fracture energies were used to develop total fracture energy for bone based on the MCF contribution. Around 33% of the energy required to crack bone with relatively small crack lengths was needed to fail MCFs, although the fibril strength is a minor contribution, and their associated interfaces. This total contribution from MCFs assumed the failure was restricted to the surface, which is considerably different to larger transverse and longitudinal splitting fracture behaviour where
crack lengths are above 1 mm. Both longitudinal and transversal fracture of bone have failure regions that are significantly larger than the apparent fracture surface of bone although longitudinal cracking creates smaller fracture regions when comparing to transverse fracture.
Chapter 8

Conclusions and Future Work

8.1 Summary of the Thesis

In the first chapter, the need for research in the field of MCF mechanics and its influence on overall mechanical behaviour of bone tissue was established. Objectives of the project along with thesis outline were defined. Chapter 2 reviewed existing literature for bone material with emphasis on: i) the bone formation process (ossification) and biomineralization, ii) compositional information, iii) structural hierarchy, iv) bone mechanics at different length scales including current studies on collagen fibrils and MCFs as well as multiple length-scale toughening mechanisms. This review justified the importance of the constituent mechanical behaviour on the overall mechanical properties of bone.
Chapter 3 reported on the *in situ* nanomechanical testing methodology combining AFM with SEM for investigating nanofibrous samples including MCFs from bone in detail, including sample preparation processes and the nanomechanical testing setup as well as data collection and analysis. Samples were prepared without applying chemical treatments to retain the compositional and mechanical characteristics of MCFs, validated by the similarity of MCF mechanics to more incomplete fibril strain data in the literature and the apparent initial linear stress-strain response of MCFs at the bone fracture surfaces. The suitability of the AFM-SEM setup for mechanical testing of nanoscale fibrous samples was evaluated by performing tensile tests on synthetic electrospun PVA and nylon-6 polymer nanofibres. Stress-strain curves recorded on PVA and nylon-6 indicated that the AFM-SEM testing method is suitable for testing nanofibres. In addition, mechanical properties of PVA nanofibres measured after exposing to vacuum in the SEM chamber for a relatively short time are comparable to hydrated bulk PVA film, indicating that water is retained in the sample within the testing time frame used.

The AFM-SEM technique in Chapter 3 was applied to individual MCFs in Chapter 4 to show a two-stage stress-strain behaviour of individual MCFs from antler bone during tensile testing to failure. Fibrils exhibited a uniform linear stress-strain response in a first stage followed by inhomogeneous deformation
above MCF strains of 2-3.7 %. The inhomogeneous mechanical properties of MCFs were explained by the compositional heterogeneity at the nanoscale recorded using BSE microscopy. A phenomenological model was proposed to explain the origin of high fracture resistance of antler bone at the fibrillar level due to inhomogeneous fibril deformation.

As mineralization level in collagen fibrils was found to be crucial in defining MCF's mechanical behaviour in Chapter 4, MCFs isolated from different ages of mouse bone with different degrees of mineralization were tensile tested in Chapter 5 and compared to bulk mouse bone samples. Little variation in the Young’s modulus of MCFs from different ages of bone was observed whereas bulk bone samples showed a significantly stronger age dependent elastic response. These results suggest that MCFs from the ages of bone tested have a similar degree of mineralization despite the bulk bone samples having significantly larger Young's modulus variations. A fibrillar biomineralization process was proposed where mineral ions first migrate to the free spaces within fibrils and replace the water in the gap regions gradually. After all gap regions in fibrils are filled with mineral, apatite crystals continue to grow into extrafibrillar spaces beyond the fibril surface, leading to small increases in the overall bone mineralization as the extrafibrillar space is limited for mineral to deposit within. The appearance of extrafibrillar mineral does not change the Young's modulus of
the fibril itself but promotes stress transfer between fibrils and results in an improved stiffness of bulk bone. The importance of nanoscale interfaces between the fibrils in bone is therefore noted and studied further in next two chapters.

In Chapters 6 and 7, the interface between MCFs and surrounding NCPs in antler compact bone was mechanically evaluated by pulling individual MCFs out of the antler bone matrix. The shear strength of the MCF-NCP interface was calculated and the sacrificial bonds reforming activities at the interface examined with the reforming efficiency estimated in Chapter 6. The bond reforming activity was found to be dependent on the strain rate of pullout testing, controlled by NCP molecular kinetics. Thus, high pullout strain rates do not provide sufficient time for sacrificial bonds to reform whereas slower pullout rates provide a higher NCP reforming efficiency at the interface region. Critically, the interfacial shear strength was found to be relatively small, at least compared to interfaces in engineering composites, which indicated a propensity for bone to fail at the MCF-NCP interface. The potential for interfacial failure at the nanoscale was developed further in Chapter 7 in order to examine the contributions of failure at nanoscale interfaces on whole bone mechanics. The MCF interfacial fracture energy $G_f$ was defined as a key parameter and evaluated using energy based analysis approaches, showing values similar to the ionic bonded interfaces coincident with previous simulation studies. The calculated MCF interfacial
fracture energy was evaluated using analytical composite models and found to contribute significantly more than MCF failure to toughness, with an overall 50% contribution of interfacial failure to the total fracture energy when cracks initiate in antler bone. It is therefore reasonable to suggest that fibril mechanics at the nanoscale have a critical role in defining overall mechanical properties, especially the fracture behaviour of bulk bone material. The relative importance of MCF mechanics is in contrast to a number of previous models that indicated the origin of bone’s extraordinary toughness is built upon the multiple toughening mechanisms due to microstructural arrangements in bone. These toughening mechanics at micro and macroscopic scales must therefore be at least partially based on MCF contributions.

8.2 Future Work

The work presented in the thesis is pioneering in direct mechanical study on MCFs from bone tissues but present a number of future opportunities. Specifically, mechanical testing usually requires large number of samples tested but a larger statistical data set is difficult to achieve in this study due to the difficulty in nanoscale manipulation and performing nanofibrous tensile testing experiments. Although every experiment in this thesis was performed on at least
5 to 6 samples, which meets minimum statistics requirement, larger data sets would provide information on the material variation in bone.

The reforming efficiency of sacrificial bonds in extrafibrillar spaces during fibril pullout was found to be dependent on strain rates. Further fibril pullout tests using strain controlled testing devices to enable the pullout of fibrils at lower physiological loading speeds would be obviously desirable and is not currently available in the AFM setup used. In addition, studies on NCP molecular kinetics of straining and relaxation is needed for a better explanation on reforming efficiency. Molecular simulation studies linked to the experimental pullout data could be instrumental in revealing molecular kinetics behaviour of NCPs.

Finally, the fibril interfacial fracture was found to contribute significantly to the fracture of bone tissue when cracks initiate. However, the contribution of fibril interfacial failure to larger cracking events is much more difficult to evaluate as the number of interfacial failure events is difficult to evaluate as the failure zone extends significantly beyond the crack tip. For example, transverse cracking of bone samples show a significant diffuse cracking zone beyond the crack tip. Future work investigating the total amount of surface area created within the total failure zone in front of a propagating crack would be more instructive in determining the contribution of nanoscale interfacial failure on overall bone
toughness. One possible approach to examine this problem is to perform *in situ* fracture testing on bone and observe cracks with high resolution X-ray tomography to obtain a better approximation of the accumulation of nanoscale crack surfaces during propagation of millimetre crack lengths in bone material.

### 8.3 Major Findings of the Thesis

All the objectives proposed in first chapter were successfully accomplished and the main findings in this thesis are listed below:

- The novel *in situ* AFM-SEM nanomechanical testing method established in this thesis was proven to be suitable for nanofibrous samples.

- Dehydration of specimens by exposing to vacuum in the SEM chamber was found to be minimum within the time frame used in all the *in situ* experiments (usually 20 mins) including testing on MCFs from bone and PVA nanofibres indicating the applicability of this technique on hydrated samples.

- MCFs from antler bone have a two-stage stress-strain behaviour. A linear response with Young’s modulus of 2.4 ± 0.4 GPa was recorded before 2 - 3.7 %
strain of fibrils followed by an inhomogeneous deformation. An ultimate strength of $118.7 \pm 38.7$ MPa was recorded with failure strain of $6.0 \pm 0.6\%$.

- Fibrillar inhomogeneous mechanical behaviour is associated with its compositional heterogeneity. Intrafibrillar mineral crystals perform as crosslink points between tropocollagen molecules to enhance stiffness. Different mechanical properties of fibrils are expected to lead to nanoscale heterogeneous deformation in bone which promotes energy dissipation.

- The appearance of extrafibrillar mineral during mineralization process does not alter the mechanical properties of individual MCFs themselves but provides stronger connectivity between fibrils, which enhances the stiffness of bone tissue.

- Fibrillar interfacial shear strength was calculated to be $0.6 \pm 0.15$ MPa by pulling out MCFs from antler bone, indicating relatively weak bonding between fibrils. The reforming of sacrificial ionic bonds between fibrils was found to be dependent on the pullout speed.

- A fibrillar interfacial fracture energy $G_f$ ranged from $2.17$ to $6.87$ J.m$^{-2}$ was calculated and found to be similar to values recorded on ionic bond
dominated interfaces, which confirmed simulation results from the literature describing MCFs linked together by ionic bonds.

- The direct contribution from MCFs on antler bone fracture energy is about 50% when crack initiates but potentially reduces when cracks extend further.
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