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Hierarchical interconnections in the nano-composite material bone: Fibrillar cross-links resist fracture on several length scales

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Abstract

Bone is a complex and very important multi-constituent bio-composite. In this work, we focus on the arrangement of bone constituents from the nanoscopic to the microscopic scale, and investigate the influence of their arrangements on the fracture mechanisms of the whole composite. We find that bone, on the nanoscopic scale, consists of mineralized collagen fibrils held together by a non-fibrillar organic matrix, which results in a primary failure mode of delamination between mineralized fibrils. In turn, these mineralized fibrils form one of three types of filaments that span microcracks in fractured bone samples, possibly resisting the propagation of these cracks.

Keywords: Bone; AFM; Mechanical properties; Non-collagenous proteins; Filaments; Collagen; Fibrils

1. Introduction

Bone is one of the most important and most complex biominerals. It has become the focus of intensive study in light of the rising average population age and associated bone diseases. In recent years, it has become increasingly clear that, in addition to the insights achieved in the biological sciences and medicine, information on the nanoscopic architecture of the bone is needed to understand and predict bone fracture, e.g. [1]. Bone consists mainly of mineralized collagen fibrils. The collagen fibrils, being the main organic component in bone, are reinforced with nanoscale hydroxyapatite particles [2–8]. This results in a mineral reinforced protein fibril of ≈50–100 nm diameter. These fibrils are the elementary building block for the large variety of bones in the body. To facilitate the function of the specific bone, they are arranged in several possible patterns [9].

In addition to its biomedical significance, bone has been used as a model for many artificial bio-ceramic composites [10,11]. In many of these artificial composites, a combination of a soft polymer matrix reinforced with stiff particles is used as an approximation of the interaction between collagen and hydroxyapatite. Such materials are based on the crystal–polymer interactions on the molecular and nanoscopic level. In this paper, we present additional strength-increasing mechanisms in bone that may add to the quality of artificial bio-mineral composites.

2. Materials and methods

Trabecular bone samples were cut from fresh bovine and human vertebrae. Samples were frozen and cut on a band saw into cubes approximately $4.5 \times 4.8 \times 4.0$ mm, where the shortest dimension was in the direction of the

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spinal column. The marrow was removed from the trabeculi using a pressurized stream of phosphate buffered saline (PBS) solution or water.

A buffer (40 mM CaCl, 110 mM NaCl, 10 mM HEPES, brought to pH 7.0 by addition of small amounts of 1.0 M NaOH) was used for storage.

2.1. Atomic force microscopy (AFM)

Single trabeculae were extracted from human and bovine bone cubes under a dissecting microscope and mounted on steel sample discs with 2-ton epoxy. The samples were then rinsed in de-ionized water. Remaining water was removed by placing the mounted samples in a centrifuge tube on top of a Kimwipe. The samples were centrifuged for a few seconds after which all samples were placed in a vacuum desiccator and evacuated to below 1 Torr. Samples were imaged in contact mode (Fig. 1A) or tapping mode (Fig. 1B) under nitrogen atmosphere.

2.2. Scanning electron microscopy (SEM)

Trabecular bone cubes were polished and cleaned with pressurized water to remove loose residues. The bone samples were compressed (under PBS solution) in a small, SEM-compatible vise that fits in the chamber of an FEI (XL 40 Sirion) scanning electron microscope. After compression, residual salts were removed by immersing the clamped sample in a Milli-Q (Millipore-purified) water-supplied flow-through system. The samples were then dried in a vacuum oven (10^{-3} Torr, 30 °C) and gold/palladium-coated by sputtering for SEM imaging.

Human trabecular bone was purchased from a tissue bank and prepared using the same procedure as used for the bovine. All the images chosen for this paper are representative examples of features observed several times in different samples of human as well as bovine bone.

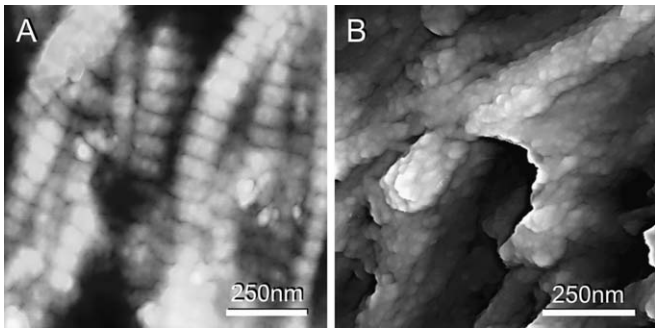

Fig. 1. High resolution images of the primary components of bone: (A) AFM image of the outer surface of human trabecular bone. The outer surface consists mainly of collagen fibrils showing the characteristic 67 nm D-banding. (B) AFM image of mineralized fibrils on a fracture surface of bovine trabecular bone. Here, in the interior of a trabecula, the collagen fibrils are mineralized with hydroxyapatite particles to form the primary building block of bone.

2.3. Environmental SEM

Bone samples were extracted from bovine vertebrae in the same way as for the conventional SEM images. Samples were then cut into 1 mm thick slices and sanded with 1200 grit sandpaper. The sample was placed in a custom made holder in which it could be loaded while mounted on the stage of an environmental scanning electron microscope (ESEM, Philips XL30). The samples were kept in storage buffer and the uncoated samples were loaded in the ESEM while being moist. In the ESEM, excess water was evaporated through the vacuum system. The sample was cooled to 3 °C in an environment of close to 100% humidity. The preloaded sample was imaged to find a suitable crack with bridging fibrils. To further strain the bone, the chamber of the ESEM was vented and the sample was rewetted. The strain was increased by tightening a clamping screw in the custom sample holder. After blotting the sides of the sample with a Kimwipe and evaporation of the excess water in the vacuum chamber of the ESEM, the crack was located again and imaged.

2.4. Nanojet

Nanojet (Nanonozzle Plasma JET Microfabrication Tool) is a new tool for nanoscale localized chemical etching by gaseous species (free radicals) [12,13]. Radicals are created from a mixture of two gases (SF_6 and O_2) within a cavity, powered by a microwave generator, operating at 100 W and 2.45 GHz (Electro-medical supplies). The radicals are transported through a capillary, which is tapered to form a nanonozzle. The electrically neutral radicals are forced in the direction of the substrate by a pressure gradient along the tube. In contrast to kinetic etch techniques such as focus ion beam (FIB) which operate with high-energy ions (5–100 keV), the chemically active radicals in Nanojet have thermal energy and therefore do not mechanically damage the bone surface. Chemical etching of the substrate takes place at the surface only, leading to high selectivity of the etching with respect to different materials or their densities in the composite.

Due to the directionality of the molecular beam emerging from the high aspect ratio nanonozzle, a localized etching can be performed. By scanning the substrate under the nozzle, a pattern can be generated with nearly the same resolution as the nozzle diameter.

We etched the sample for 1 h with a nozzle opening of 300 nm, which was positioned 100 nm above the sample. The sample was imaged by SEM after the first etch to observe the changes on the bone surface. To find the same place on the bone surface after each treatment and to see the evolution of the surface morphology, the surface was mechanically marked, which made it easily detectable during the SEM imaging. The formed topological details were also used to navigate and find the same surface area.

The etch procedure was repeated three times, where after each etch, the same spot was imaged. For SEM imag-