Video Article

Fragility Assessment of Bovine Cortical Bone Using Scratch Tests

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Abstract

Bone is a complex hierarchical material with five distinct levels of organization. Factors like aging and diseases like osteoporosis increase the fragility of bone, making it fracture-prone. Owing to the large socio-economic impact of bone fracture in our society, there is a need for novel ways to assess the mechanical performance of each hierarchical level of bone. Although stiffness and strength can be probed at all scales – nano-, micro-, meso-, and macroscopic – fracture assessment has so far been confined to macroscopic testing. This limitation restricts our understanding of bone fracture and constrains the scope of laboratory and clinical studies. In this research, we investigate the fracture resistance of bone from the microscopic to the mesoscopic length scales using micro scratch tests combined with nonlinear fracture mechanics. The tests are performed in the short longitudinal orientation on bovine cortical bone specimens. A meticulous experimental protocol is developed and a large number (102) of tests are conducted to assess the fracture toughness of cortical bone specimens while accounting for the heterogeneity associated with bone microstructure.

Video Link

The video component of this article can be found at https://www.jove.com/video/56488/

Introduction

In this study, we measure the fracture toughness of bovine compact bone from the mesoscale (osteons) to the microscale (lamellar level) using a novel micro scratch technique[1,2,3,4,5]. Fracture processes including crack initiation and crack propagation in bone are directly influenced by length scales owing to the different structural constituents and organization at different levels of hierarchy. Therefore, assessing bone fracture at smaller length scales is essential to yielding a fundamental understanding of bone fragility. On the one hand, conventional tests such as three-point bending, compact tension, and flexure tests are commonly conducted on bovine femur and tibia for fracture characterization at the macroscopic scale[6,7,8]. On the other hand, to measure the fracture toughness at the microscopic scale, Vicker's indentation fracture was proposed[9]. Micro indentation was performed using the Vicker's indenter to generate radial cracks. Furthermore, the Oliver Pharr nanoindentation fracture toughness method was performed using a sharp cube corner indenter[10].

In the above nanoindentation based fracture toughness studies, the lengths of the cracks thus generated were measured by the observer and a semi-empirical model was used to calculate the fracture toughness. However, these methods are irreproducible, subjective, and the results are highly dependent on the observer's skill due to the need to measure the crack lengths using optical microscopy or scanning electron microscopy. Moreover, scratch tests were conducted at the nano-scale, but the underlying mathematical model is not physics-based as it does not account for the reduction in strength due to cracks and defects[11]. Thus, a gap of knowledge exists: a method for fracture assessment at the microscopic level based on a physics-based mechanistic model. This gap of knowledge motivated the application of micro scratch tests to compact bone by focusing first on porcine specimens[5]. The study has now been further extended to understand bovine cortical bone.

Two different orientations of the specimens are possible: longitudinal transverse and short longitudinal. Longitudinal transverse corresponds to fracture properties perpendicular to the longitudinal axis of the femur. Whereas, short longitudinal corresponds to the fracture properties along the longitudinal axis of femur[5]. In this study, we apply scratch testing to bovine cortical bones to characterize the bone's fracture resistance in the short longitudinal direction.

Protocol

NOTE: The protocol described here, follows the animal care guidelines of the Illinois Institutional Animal Care and Use Committee.

1. Specimen Procurement

1. Collect freshly harvested bovine femurs from a United States Department of Agriculture (USDA)-certified slaughterhouse and transport them in plastic air tight bags in a cooler.
   NOTE: For the study conducted here, femurs were collected from animals that were 24 - 30 months old, corn-fed, and weighed about 1,000 - 1,100 pounds.
2. Freeze the femurs at -20 °C until the start of the specimen preparation procedure. This temperature keeps the femurs fresh[12,13,14].

2. Cutting, Cleaning, and Embedding the Specimens

1. Thaw the frozen femurs in a container with water for about 2 h at room temperature.
2. Cut multiple discs about 10 - 15 mm thick from the mid-diaphysis region using a table top diamond band saw to produce specimens with uniform cross-sectional area of the cortical bone.
3. Use a dissection kit to remove any soft tissue or flesh attached to the cortical bone.
4. Cut the cross sections of the femurs obtained in step 2.2 using a diamond-wafering blade on a low speed saw under wet conditions along the longitudinal axis of the bone to obtain multiple roughly cuboidal sections.
   NOTE: Here, only specimen preparation and scratch tests performed on the short – longitudinal specimens are discussed. However, except for the direction of cutting, the preparation procedure remains the same for the transverse orientation.
5. Clean the specimens in a solution prepared using 1.5% anionic cleaner and 5% bleach for a duration of 20 min in an ultrasonic cleaner.
6. **Embed the cortical bone specimens in acrylic resin (herein polymethyl methacrylate (PMMA)) for ease of handling and stability.**
   1. To embed the specimens, first coat the walls of the mold with a release agent. Then mix the acrylic resin and hardener in a beaker, as per instructions given by the PMMA manufacturer.
   2. Place one of the cut cortical bone specimens into each mold with the surface to be scratched facing downwards. Pour the acrylic resin mix into these prepared specimen holders. Let the specimens cure for a duration of up to 4 - 5 h.

7. Cut the embedded specimens into 5-mm thick discs, exposing the surface to be scratched, using the low speed saw and mount the specimens on to metal (aluminum) discs of diameter 34 mm and height 5 mm using cyanoacrylate adhesive.
8. Wrap the specimens in a gauge soaked in Hanks Balanced Saline Solution (HBSS) and refrigerate at 4 °C until further use[15,16].

3. Grinding and Polishing Protocols

NOTE: A pre-requisite to high-precision testing at small-length scales is a smooth and levelled surface of specimens. Previous polishing protocols[13,17] result in a large surface roughness, leading to substantial inaccuracy in measurement. The challenge lies in achieving low average surface roughness, less than 100 nm, over a large area 3 x 8 mm^2 surface.

1. Grind the bovine cortical bone specimens at room temperature using 400 grit and 600 grit Silicon Carbide papers for 1 min and 5 min, respectively. Maintain the grinder-polisher at base speeds of 100 rpm and 150 rpm, respectively.
2. Machine grind the bovine cortical bone specimens at room temperature on the 800 and 1,200 grit papers for a duration of 15 min for each step. Maintain the grinder-polisher at a base speed of 150 rpm, head speed of 60 rpm, and operating load of 1 lb.
3. Polish the specimens using 3 µm, 1 µm, and 0.25 µm diamond suspension solutions in the same order on a hard, perforated, non-woven cloth for a duration of 90 min each at room temperature. Maintain the operating load for each step at 1 lb with the base and head speeds of the polisher at 300 rpm and 60 rpm, respectively.
4. Polish the specimen using 0.05 µm alumina suspension solution on a soft, synthetic rayon cloth for a duration of 90 min at 1 lb with base and head speed of 100 rpm and 60 rpm, respectively, also at room temperature.
5. Put the specimens in a beaker with de-ionized water and put the beaker in an ultrasonic bath for 2 min in between each consecutive step of grinding and polishing to clean the residue and avoid cross contamination.
6. View the surface features using optical microscopy and SEM imaging.
   NOTE: As shown in **Figure 1**, osteons, Haversian canals, cement lines, interstitial regions, and lacunae were observed on the bovine cortical bone specimens. These imaging methods reveal the porous, heterogeneous, and anisotropic nature of cortical bone specimens. Additionally, advanced surface examination of the specimens was performed to assess the quality of the polished surface. A representative polished surface is shown in **Figure 2**.

4. Micro Scratch Test

NOTE: Micro scratch tests are performed on the polished bovine cortical bone specimens using a micro scratch tester (**Figure 3**). A diamond Rockwell indenter with a tip radius of 200 µm and apex angle of 120° is used for the study. The instrument allows the application of a linear progressive load up to 30 N. Furthermore, the instrument is equipped with high-accuracy sensors to measure the horizontal load, penetration depth, and acoustic emissions generated due to scratching. The instrument can capture the panoramas of scratch grooves.

1. Prior to the testing of cortical bone specimens, calibrate the Rockwell indenter tip using polycarbonate as reference material[3].
2. Place the cortical bone specimen on the stage and choose the site of scratch test using the optical microscope set up integrated to the micro scratch tester module.
3. Apply a linear progressive load with a start load of 30 mN and end load of 30 N. The loading rate should be set to 60 N/min and the scratch length to 3 mm.
4. Perform series of scratch tests on the short longitudinal (**Figure 3b**) bovine cortical bone specimens as illustrated in **Figure 3**.
5. Wet the specimen surface with HBSS after a set of every three to four scratch tests to keep them hydrated.
6. Analyze the scratch test data based on non-linear fracture mechanics modelling[2].

Representative Results

Atomic force microscopy was used to measure the roughness of the polished surface. As a rule of thumb, the specimen qualifies as a well-polished one if the surface roughness is an order of magnitude smaller than the surface features of interest. In this case, the measured surface roughness of 60 nm over a 40 µm x 40 µm area clearly falls within this criterion.

Figure 4 shows the force versus penetration depth graphs of representative scratch tests performed on the short longitudinal bovine cortical bone specimen. While the vertical force is the prescribed incremental load, the horizontal force is the measured resistance experienced by the probe. **Figure 5** shows the scanning electron microscopy images of the fractured short longitudinal bovine cortical bone surface. This image shows chipping and flaking of the surface and occurrence of intrinsic toughening mechanisms such as micro cracking, crack deflection, and crack bridging. The micro scratch test data is analyzed using MATLAB scripts based on non-linear fracture mechanics modelling[2]. Prior to the occurrence of the fracture process, there would be plastic dissipation[18]. As the penetration depth increases, fracture processes are activated.

Based on microscopic observation, we consider a single crack propagating as shown in **Figure 3b**. We build a nonlinear fracture mechanics model[1,2] to predict the scaling of the scratch force. A homogeneous transverse isotropic microstructure is considered for the cortical bone at the tissue level. **Figure 6** shows the force scaling of the fracture toughness of the short longitudinal cortical bone specimens. A ductile-to-brittle transition is introduced by varying the penetration depth. In the brittle and fracture-driven regime, the scratch force F_T is proportional to the quantity $\sqrt{2pA}$, where $2pA$ is the probe shape function[1,2,3,4,5]. Therefore, the fracture toughness, $K_C = F_T/\sqrt{2pA}$[1,2,3,4,5] converges toward a constant. Furthermore, a K_c value which corresponds to a brittle fracture is reported on the force scaling plot for a single test as shown in **Figure 6**. 102 micro scratch tests were conducted on the short longitudinal bovine cortical bone specimens as shown in **Figure 7**. Outlier tests correspond to the specimens which were tested after one week of preparation and storage in the saline solution. Storing the specimen for a very long duration altered the surface due to precipitate formation from the saline solution leading to different fracture toughness values. The overall fracture toughness value obtained is 4.05±0.63 MPa$\sqrt{m}$. The literature reported fracture toughness values in the range of 2.5 to 5.5 MPa$\sqrt{m}$[6,8]. These results show that the fracture toughness values reported from the micro scratch tests are in accordance with literature.

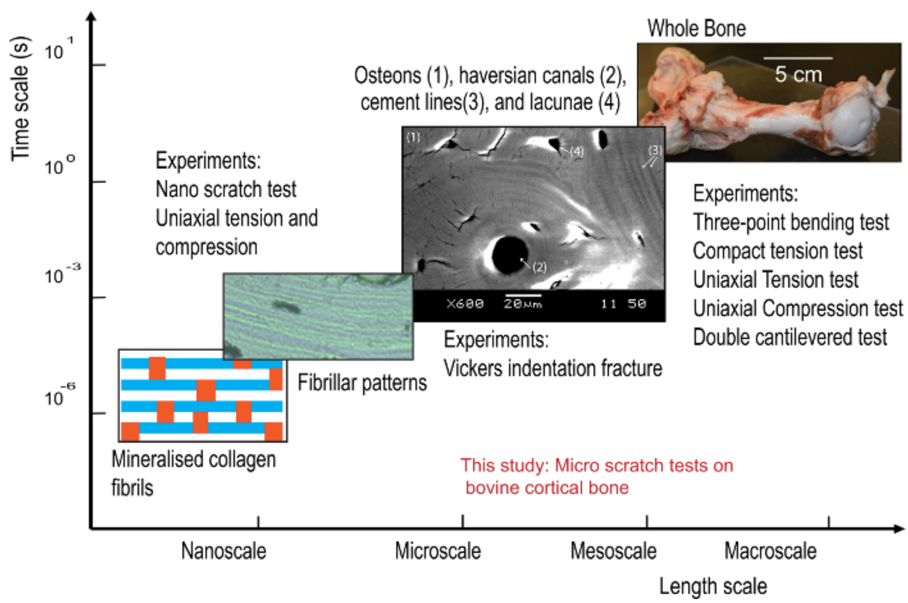

Figure 1: A graph showing the different hierarchical levels of bone specimens and the experimental investigations conducted at each level. The horizontal axis corresponds to the length scale ranging from macroscale to nanoscale and the vertical axis corresponds to time scale at which the experiments corresponding to each level are conducted. (Image Credit: Kavya Mendu). Please click here to view a larger version of this figure.

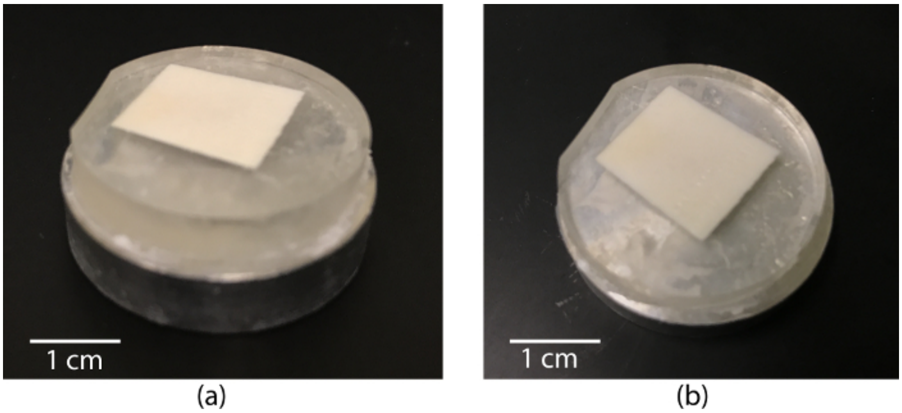

Figure 2: Digital photographs of (a) aluminum discs used as a base for the specimens and (B) well-polished short longitudinal bone specimen. Please click here to view a larger version of this figure.

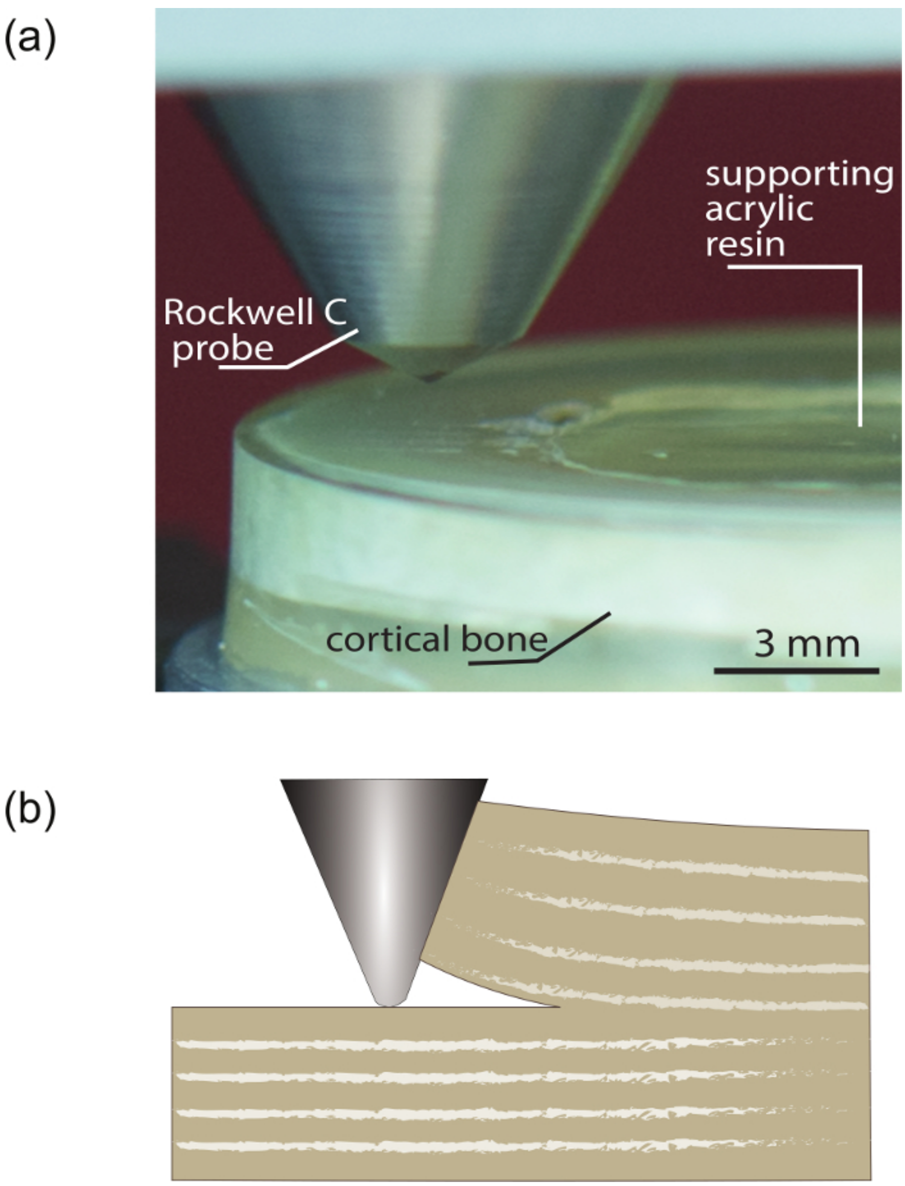

Figure 3: Micro scratch test. Digital photograph of the micro scratch test on the bovine cortical bone specimen (**A**). A Rockwell probe having an apex angle of 120° probing the cortical bone specimen embedded in Polymethyl Methacrylate. **(B)** Schematic of a scratch probe ploughing the bone material showing the advent of a mixed mode of fracture in a short longitudinal specimen. (Credits: Ange-Therese Akono, Amrita Kataruka, and Kavya Mendu). Please click here to view a larger version of this figure.

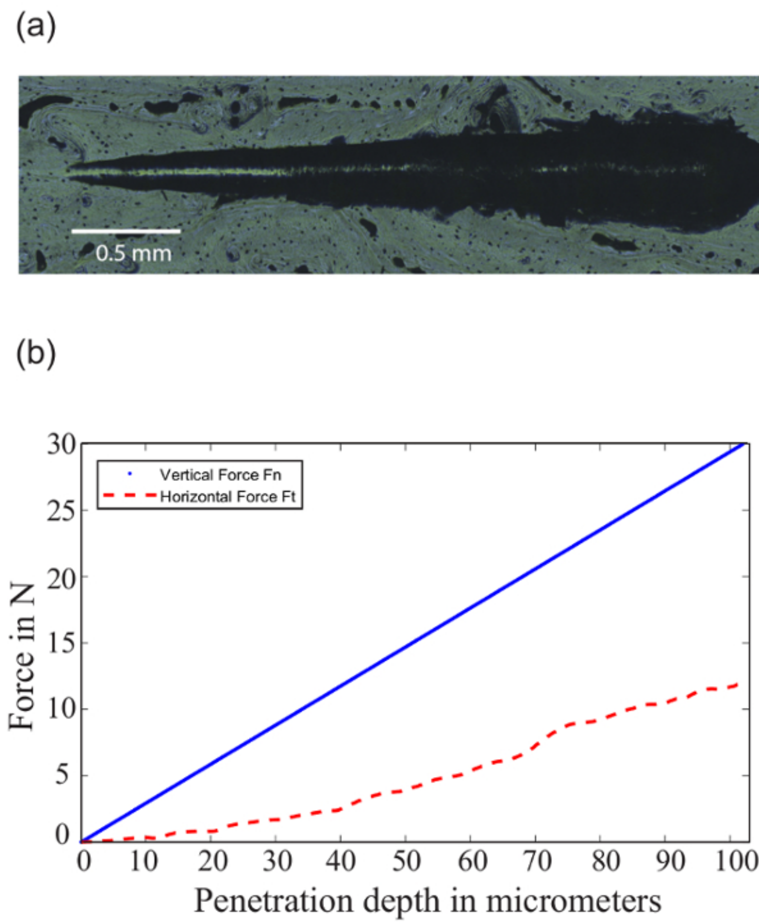

Figure 4: Scratch groove. Optical microscopy image of the panorama of the scratch groove (**A**). **(B)** Corresponding plot of the force versus depth along the length of the scratch groove. Horizontal force corresponds to the resistive frictional force detected by the sensors attached to the micro scratch tester stage and the vertical force corresponds to the progressive linear force applied onto the cortical bone specimen. Please click here to view a larger version of this figure.

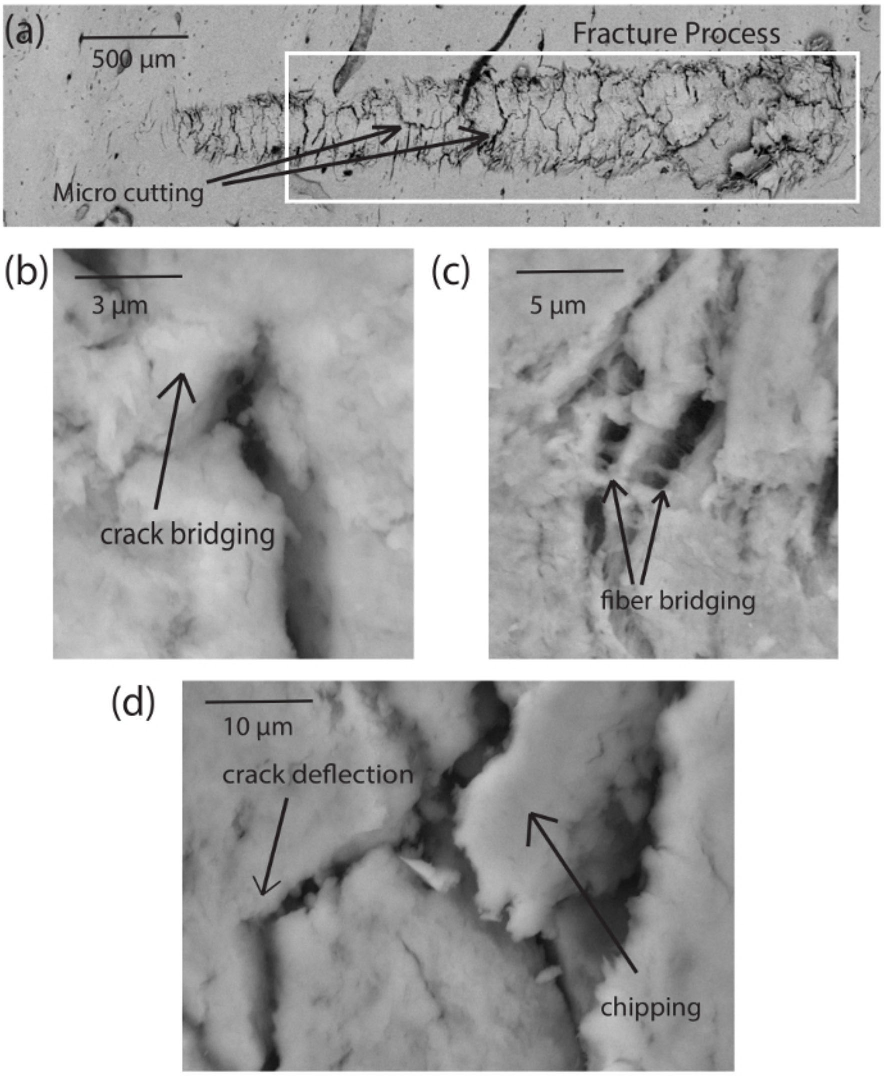

Figure 5: Scanning electron microscopy (SEM) images. SEM images of the scratch groove showing micro mechanisms such as crack deflection, crack bridging, fiber bridging, and chipping at different magnification levels **(A)** 40X **(B)** 10,000X **(C)** 2,400X **(D)** 5,000X. Captured using the low vacuum Scanning Electron Microscope at the Frederick Seitz Material Science Laboratory and Beckman Institute, University of Illinois at Urbana Champaign. Please click here to view a larger version of this figure.

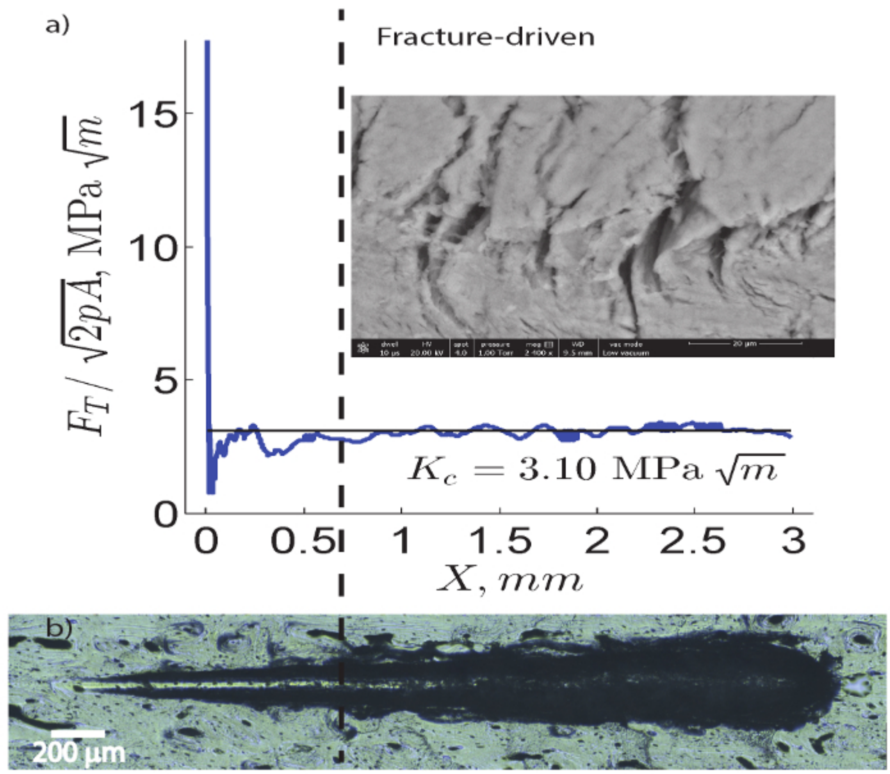

Figure 6: Scratch force and micro scratch image. (A) Scaling of the scratch force along the length of the scratch shows the convergence of fracture toughness. F_T is the horizontal force and $2pA$ is the probe shape function that depends on the geometry and penetration depth. **(B)** Panoramic optical microscopy image of a micro scratch on bovine bone in the short longitudinal direction. Please click here to view a larger version of this figure.

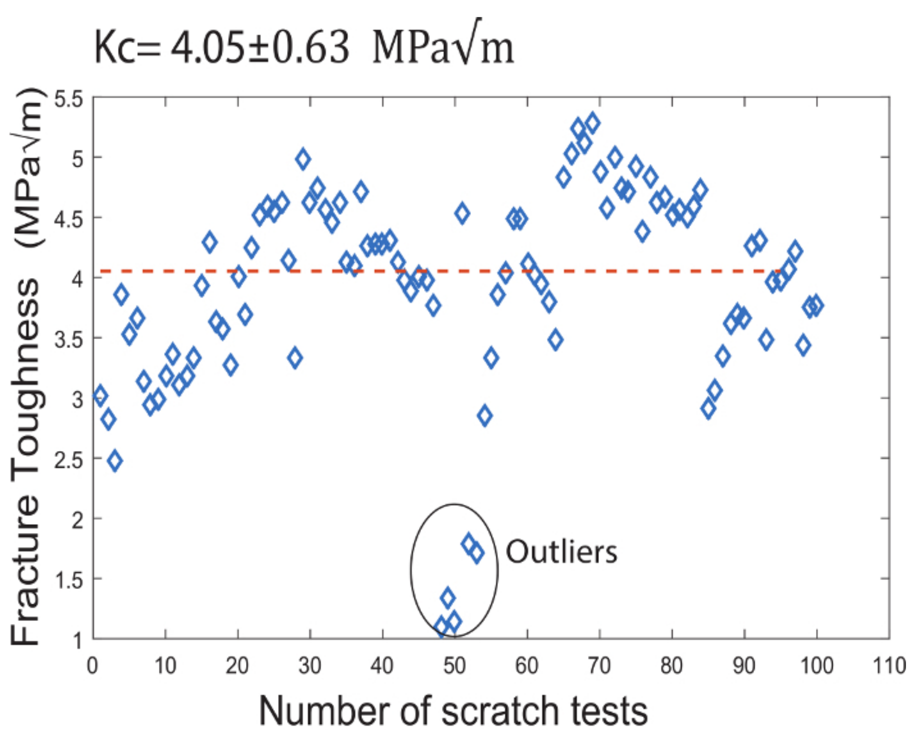

Figure 7: Fracture toughness. Plot showing the fracture toughness values of the 102 micro scratch tests conducted on the short longitudinal bovine cortical bone specimens. Please click here to view a larger version of this figure.

Discussion

Micro scratch tests induce a mixed-mode fracture[3]. Furthermore, in the short longitudinal bovine cortical bone specimens, fracture processes are activated as the probe digs deeper. For a 3-mm long scratch, the prismatic volume probed is around 3,600 µm long, 600 µm wide, and 480 µm deep. This large volume helped in predicting a homogenized response. A non-linear fracture mechanics model enabled us to extract the fracture resistance based on the *J*-integral calculation[1,2,4].

Bovine cortical bone specimens provide a larger area for testing when compared to the porcine specimens which were used for the earlier publication[5]. However, there is a corresponding difference in the size of microstructure features from porcine to bovine cortical bone specimens. This led to the development of a new polishing protocol for the bovine specimens. Furthermore, during the development of the method, it was observed that the prepared bovine cortical bone specimens need to be tested within one week after preparation. This is to avoid residue formation on the bovine specimens due to saline solution, which might drastically affect the test results.

In addition, the tests conducted on the short longitudinal bovine cortical bone specimens had controlled environmental conditions and standardized specimen preparation protocols. This led to a reduction in the variability of the test results from the previously reported 23% for short longitudinal porcine cortical bone specimens[5] to 15% for the short longitudinal bovine cortical bone specimens in this study. However, in **Figure 7**, outlier test results can be attributed to various reasons like duration of storage in saline water or location of the scratch itself. Nevertheless, given that bone is heterogeneous at the meso- and microscopic length-scales, a certain amount of variability is expected.

Scanning electron microscopy shows the incidence of fracture processes during these scratch tests. Toughening mechanisms such as micro cracking at the meso scale, crack deflection, and crack bridging at the microscale and fiber bridging at the sub-micron scale were observed (see **Figure 5**). This is in accordance with the toughening mechanisms reported earlier in the literature[19]. Thus, micro scratch tests determine the fracture properties of bovine cortical bone specimens from the meso scale to micro scale.

The method that we propose here requires a small number of specimens and enables testing of the specimens at smaller length scales. For instance, ductile to brittle transition is introduced at the macroscopic scale by working with specimens of different sizes while having a constant aspect ratio. According to the size effect fracture assessment technique, at least 5 different sized specimens are required to estimate a fracture toughness value[20,21]. Thus, to estimate 102 fracture toughness values, macroscopic testing needs around 510 specimens which involves a lot of time and resources. Thus, this method we propose estimates the fracture toughness at a faster rate and is more economical. Furthermore, understanding the fracture characteristics at different hierarchical levels enables us to comprehend the mechanics of bone more efficiently. In addition, testing is efficient, reproducible, and can easily be carried out under a wide range of environmental controls. For instance, testing specimens submerged in a saline solution in an environmental chamber may be carried out to simulate *in vitro* conditions. In addition, the method will also be applied to test bone fracture toughness in the longitudinal transverse direction to capture anisotropy in bone. Thus, our method is a novel means for the fracture assessment of biological tissues.

Disclosures

The authors have nothing to disclose.

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