1993

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A SIMPLE FREEZE FRACTURE TECHNIQUE FOR
SCANNING ELECTRON MICROSCOPY OF COLLAGENOUS BIOMATERIALS

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Abstract

A simple freeze fracture technique for the ultra-structural analysis of fragile collagenous biomaterials is presented. Following standard methods of preparation and dehydration, fragile biomaterials are fractured with a sharp blade attached to a modified slide hammer. The sliding mass imparts a sudden impact load on the frozen surface of the material, creating a distortion free cleavage plane across the material. More traditional methods including bending and cutting with scissors introduce artifacts indirectly associated with stress concentration produced by bending, and sample compression at the edges with cutting. The impact loading of the sample during fracture results in preservation of edge detail, showing clear structural features up to the surface of the collagenous biomaterial.

Key Words: Freeze fracture, scanning electron microscopy, biomaterials, collagen, sample preparation technique.

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Introduction

Prior studies [3] employing scanning electron microscopy (SEM) of fragile collagenous biomaterials observed ultrastructural details of the material prepared by a simple freeze fracture technique. That study compared cut and snapped reconstituted collagen fibers. The fracture induced by cutting resulted in compression artifacts on the surface of the biomaterial at the fracture site. The snapping technique was observed to preserve edge detail not found in the cut samples. However, the bending or snapping technique results in material failure along stress concentrators, or risers, possibly due to oriented fibrillar substructure in the reconstituted collagen fiber. Therefore, the fracture face produced by snapping may preferentially show structural imperfections, rather than the average cross-sectional morphology. The technique presented here is a modification of these methods, and utilizes a specially prepared slide hammer which produces fracture via the application of a sharp impact load, resulting in fracture faces with clean structural detail of the interior as well as surface edges.

Materials and Methods

Hammer design

A slide hammer commonly used in automobile body repair, was modified by the addition of an aluminum chuck to hold a utility knife razor blade, which served as the cutting edge of the fracture system. Figure 1 shows the general design characteristics of the slide hammer assembly. The system consists of a cast iron cylindrical mass (0.5 kg), which is free to slide on a steel central rod. The end opposite the handle was threaded to accommodate the addition of an aluminum chuck designed to hold a utility knife razor blade, and a cylindrical striking surface. The knife blade is held in place by a simple screw clamp mechanism, built into the chuck. This design ensures that the blade is mounted on the axis of the steel rod and the cylindrical mass. In principle, the blade mounted in the chuck is held over the sample, and the mass allowed to fall, impacting the striking surface, and propagating the impact load

through the chuck to the knife and the material.

Sample preparation and freeze fracture

In the example presented here, collagen fibers isolated from rat tail tendons (RTT) of Sprague Dawley rats, were air dried, bundled in groups of 5-6 fibers approximately 1 cm long with sutures (9-0 nylon, Ethicon, Somerville, NJ) tied at each end. The sutures held the fibers together for easier processing, and clearly marked the unfractured ends of the sample, as fracture surfaces were created at the midpoint between the sutures. The RTT fibers were then dehydrated through an increasing series of ethanols (35-100%) just prior to freeze fracture. Freeze fracture was performed in a pool of liquid nitrogen contained in an insulated styrofoam container, large enough to accommodate an aluminum anvil (aluminum block dimensions 3.5 cm X 4.5 cm X 2.5 cm) and a specially prepared transport container. Approximately 4 liters of liquid nitrogen were added to the styrofoam box, containing the anvil and the transport container, which were allowed to equilibrate with the liquid nitrogen. The chuck-blade end of the slide hammer was then immersed in the liquid nitrogen, and allowed to equilibrate. The handle and mass were kept above the surface of the liquid nitrogen. Additional nitrogen was added to maintain the fluid level above the flat surface of the anvil. The dehydrated sample of rat tail tendon was then rapidly transferred from the 100% ethanol and immersed in the liquid nitrogen. The sample was placed on the aluminum anvil, and the blade of the slide hammer positioned above the sample, oriented for perpendicular and oblique fracture faces (Figure 2). Rather than actually touching the sample surface, the blade is held slightly above the sample, and the mass allowed to fall striking the chuck-blade and fracturing the sample in the plane of the blade. The fractured sample was then transferred in aluminum foil pouches, filled with liquid nitrogen and placed in the transport container. The container plus the sample were then placed in a Bell jar and allowed to return to room temperature under high vacuum (100 mTorr) overnight [3]. The RTT fibers at room temperature were then mounted on aluminum stubs with a conducting silver paste (Ted Pella, Inc., Redding, CA), with the fibers supported upright by glass fragments from a pasteur pipette [3], thus exposing the fractured surfaces. The mounted RTT fibers were sputter coated with a thin (40 nm) coating of gold-palladium, and viewed on an Amray 1400 SEM operated at an accelerating voltage of 20 kV.

Results

Figures 3 and 4 show low and high magnification views of the characteristic fracture face produced in isolated rat tail tendon fibers using this method. An oblique fracture face (Figure 3) was produced by angling the plane of the blade approximately 60° with respect to the fiber axis. The micrograph shows clean structural detail within the interior of the fractured fiber and no evidence of compression in the plane of the fracture. The fracture edge viewed at higher magnification shows

Figure 1. Photograph of slide hammer system. The slide hammer consists of a cylindrical mass (M) of 0.5 kg with a hole through the long axis. A steel rod (R) inserted through the hole, serves as a guide for the mass, and is threaded at one end to hold an aluminum chuck (C). Via a simple screw clamping mechanism (SC), a utility knife razor blade (K) is attached to the slide hammer with the plane of the knife blade parallel to the axis of the steel rod. White bar on ruler indicates 6 cm.
Figure 2. Schematic representation of freeze fracture technique. The frozen sample is placed on the surface of the aluminum anvil, which is submerged in liquid nitrogen. The blade of the liquid nitrogen cooled chuck is then positioned just slightly above the sample in the preferred orientation with respect to the sample, and the mass allowed to fall striking the chuck and propagating the impact load through the chuck and blade, thus fracturing the sample in the plane of the blade.

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preservation of structural detail from the surface into the interior of the collagen fiber (Figure 4).

Discussion

The results of this analysis indicate that the modified slide hammer presented in this study may be a useful and simple technique for the freeze fracture of fragile collagenous materials. Robards and Sleytr [2] review a variety of freeze fracture techniques which involve the application of either tensile, compressive, bending, or shearing forces to the sample. The unique impact loading generated by the sliding mass produces distortion-free fracture faces, and sharply defined edges with clear structural detail at the transition from the surface to the interior along the edge of the fracture face. In addition, the slide hammer fracture device can be created from inexpensive and readily available materials with a minimum of machining.

This technique has been successfully used for the freeze fracture of a composite tendon ligament prosthesis of polylactic acid and collagen fibers [1], as well as reconstituted collagen fibers (Pins, unpublished data). In each of these different materials, the fracture face appeared with comparable preservation of edge detail noted in the samples of rat tail tendon in the micrographs shown above.

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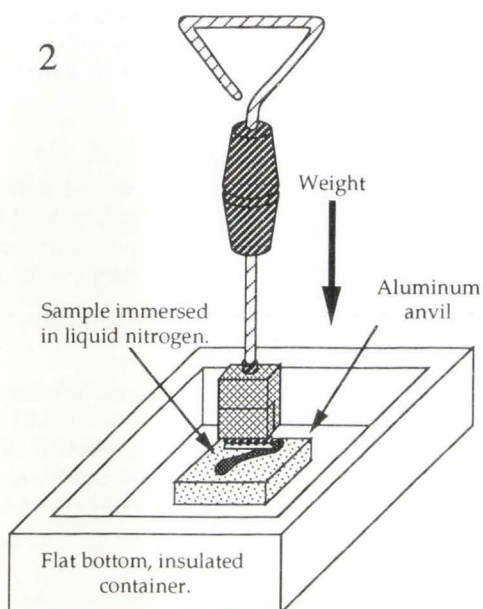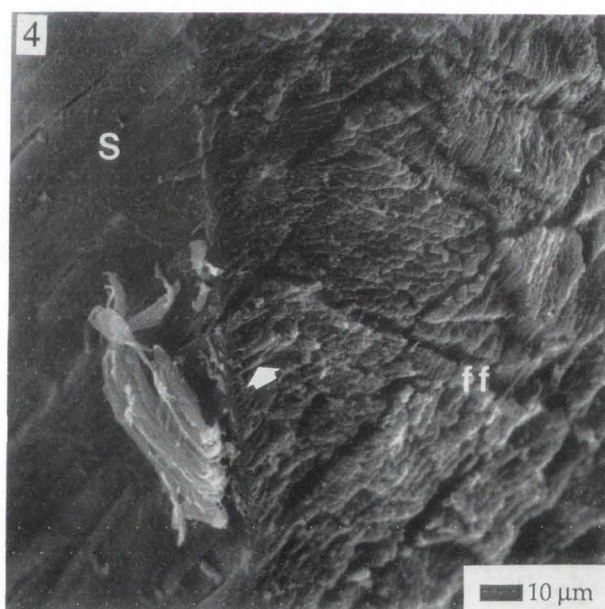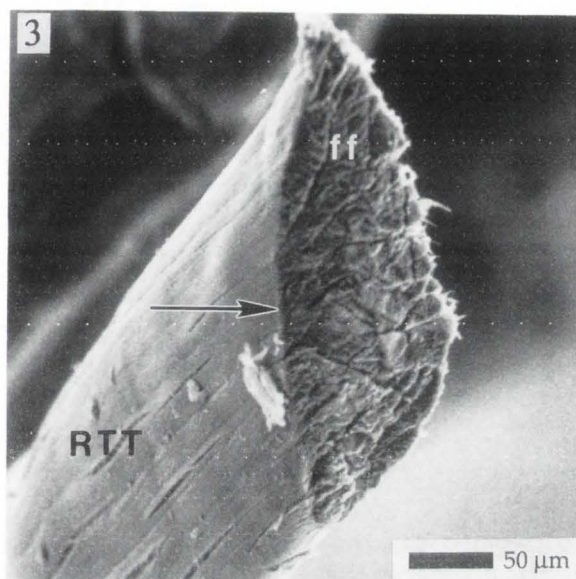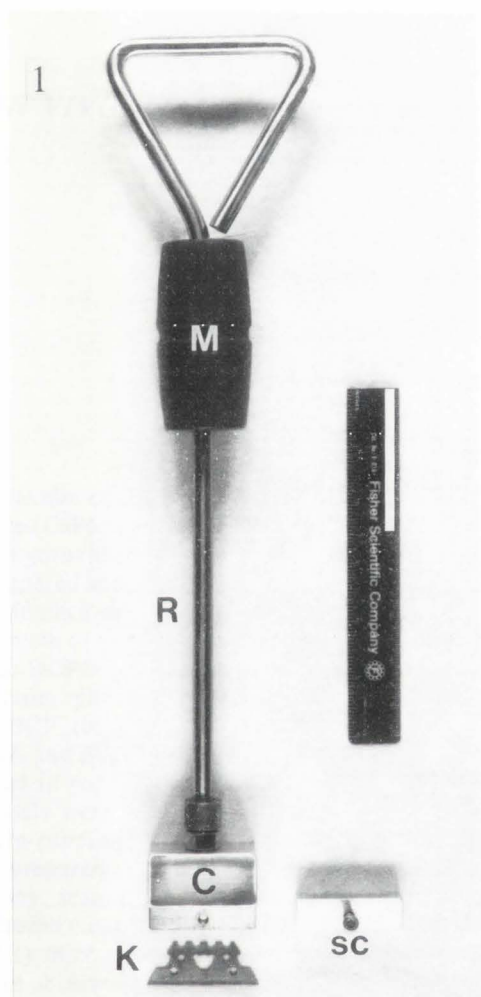
Figures 3 and 4. Scanning electron micrographs of slide hammer fractured rat tail tendon fiber (RTT). **Figure 3** shows the fracture face produced in the RTT collagen fiber at an angle of approximately 60° with respect to the fiber axis. Arrow indicates fracture direction, and the fracture face (ff) shows a lack of compression induced distortion in sample. **Figure 4** shows a high magnification view of the leading edge of the fracture face shown in Figure 3. The arrow marks an individual collagen fibril just below the surface of fractured fiber, clearly showing maintenance of substructural detail at the fracture face-surface transition region. ff: fracture face; S: surface of the RTT fiber.