Manipulation of Collagen Fibers for Mechanical Characterization

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Introduction

Collagen is the most abundant protein in the mammalian anatomy, manifesting itself in a wide variety of forms, functions, and types. In biological systems, collagen fibers provide the major mechanical support for cell attachment, thus developing the shape and form of tissues[1]. Collagen is used as scaffolding material in tissue engineering, but must first be characterized for mechanical strength. Traditional methods for fiber characterization are not suitable for sub-micron size collagen. The Zyvex L100 Manipulator System is the instrument of choice for characterizing fibers on these length scales. This application note presents a brief description of collagen, its applications, and describes how the Zyvex L100 can be used to manipulate microscopic collagen fibers for mechanical characterization.

Collagen proteins are comprised of polypeptide chains (α-chains) that form a unique triple-helical structure that is 300 nanometers long and 1.5 nanometers in diameter. Each a-chain has a repeating amino acid structure of Gly-Xaa-Yaa, where *Gly* is glycine and *Xaa-Yaa* can be any amino acid pairing, though most often are proline and hydroxyproline[1,2]. There are more than twenty disparate collagen types that exist in animal tissue, five of which are known to form fibers: Types I, II, III, V, and XI. These types tend to self-assemble into periodic, cross-striated fibers, which can reach centimeters in length and tens of microns in diameter. Type I collagen is the predominant fiber-forming collagen type and is found in bones, skin, teeth, and tendons. Type II collagen, considered the second most abundant, is found in cartilaginous tissue, developing cornea, and vitreous humor[1].

Collagen in Tissue Engineering

Type I collagen is regenerated in response to tissue injury and as a result of fibrotic disease[1]. However, many processes do not have efficient regenerative capabilities, if any at all. For example, the anterior cruciate ligament (ACL) has little innate ability to heal itself. Current medical treatments for damaged or lost tissue include transplantation, reconstruction, drug therapy, prosthesis, and medical devices. Tissue engineering has been investigated as an alternative to these treatments. Tissue engineering is a multidisciplinary field with goals of replicating the functions of living tissues both *in vivo* and *in vitro*. Appropriate scaffolding material is needed to provide a basis of support and encourage cell regeneration. Because collagen proteins are a major structural element in so many of the body's tissues and organs, collagen fibers are a logical choice for scaffolds[3].

Collagen Fiber Characterization Overview

The mechanical and material properties of any implanted biological substance must be ascertained before deemed as an acceptable therapy. The scaffold should be biocompatible, biodegradable, suitable for cell attachment, and have a 3-dimensional, porous structure. The scaffold must also have mechanical properties that duplicate the tissue being replaced[4].

The implanted scaffold must promote the development of mechanically stable structural support, thus fulfilling its inherent role as a scaffolding element. For this reason, mechanical properties of the collagen fibers must be characterized before they can be considered as a scaffolding material. Upon application of a load, the collagen fiber must return to its original length (within some small percent error) to be considered an effective load bearing support structure. The mechanical characterization involves tensile and compression testing[3].

Though elastic, most collagen exists in a crystalline-polymer form that is susceptible to molecular defects upon loading and compression. The ability to gauge the molecular response of collagen fibers during mechanical testing is invaluable for understanding the mechanisms and processes involved in protein assembly and fiber formation. This information will affect the methods by which scaffolds are synthesized.

Limitations of Current Characterization Techniques

Current techniques for measuring mechanical properties of collagen involve using custom built[3] or proprietary tensile loading systems[5,6]. These instruments clamp onto the fibril and apply a tension. The established methods for characterizing collagen scaffolds are ineffective for structures with lengths below one centimeter and diameters below ten microns. Collagen fibers are made up of a hierarchy of structures, starting with amino acids that form proteins that are 300 nanometers in length and 1.5 nanometers in diameter. The proteins further assemble into fibrils of varying lengths and diameters. The fibrils form fibers that can reach centimeters in length and hundreds of microns in diameter[7]. One major limitation of the instruments mentioned above is that they can only accommodate macroscale fibers and fibrils, and are not applicable to fibers on length scales that require optical microscopes.

For any test of fibers that are on these length scales, handling the fibers is the biggest barrier to success. Zyvex Corporation's L100 Manipulator System is the solution to the problems limiting the science of collagen fiber characterization **(Figure 1)**. The L100 can be used under an optical microscope to grasp, pull, and twist hydrolyzed and dry collagen fibers for mechanical characterization.

In addition to limitations in dexterity, current techniques cannot replicate fatigue-inducing conditions experienced by ligaments *in vivo*, such as muscles in a state of exercise. A collagen fiber experiences continuous loading and relaxation as ligaments support flexing and relaxing of muscles during constant periods of activity such as running or swimming. Additionally, the elastic limits for bending are relevant when attempting to replicate ligaments that support bending of the extremities. Pronation and supination cause ligaments to feel a tensile and torsional stress, which tensile loading equipment cannot replicate. The Zyvex L100 can be used to facilitate mechanical characterization of collagen fibers by applying both tensile and torsional stresses.

There is also no established technique for integrating atomic force microscopy into the mechanical characterization apparatus. Atomic Force Microscopy (AFM) has been used to image collagen fibers[8], and can be used to learn about changes in their molecular structure upon loading and compression. A means of integrating an AFM with the L100 is being investigated for imaging fibers under stress.

Most collagen macromolecules and fibers are highly crystalline polymers that exhibit large relaxation and retardation times, attributing to their function as the stress-bearing extra-cellular matrix of biological tissue. The major collagen classes (I and II) are often found as co-polymers with the remaining fibril-forming types. For example, the Type I–III collagen pairing is found in arterial walls[1]. Because collagen fibers are polymeric, their mechanical strength can be gauged using standard polymer fiber characterization techniques.

The L100 as a Characterization Tool

The Zyvex L100 is a piezo-motor actuated manipulation tool with sub-micron resolution for use with either scanning electron or optical microscopes. **Figure 1** shows the L100 System head. The modular head unit consists of two four-degrees-of-freedom (DOF) positioners and one three-DOF positioner. **Figure 2** is a schematic describing the nomenclature for the positioning directions. The axial direction (or Y-axis) is defined as being motion towards the center of the sample stage. All actuators have 100 nanometer precision, which is more than sufficient to manipulate collagen fibers.

The L100 is interfaced via the Zyvex Optical Docking Station*, which consists of an optical stereomicroscope, X-Y-translation stage, connector harness, and ring light. All of these components are mounted onto an optical breadboard for easy adjustment. The stereomicroscope can be connected to a charge coupled device (CCD) camera for signal acquisition.

The L100 System achieves its manipulation capabilities with quick-disconnect tools that attach to the end of the positioners. Zyvex's NanoEffector™ tools includes static probes such as wires, and more dynamic tools, such as microelectromechanical systems (MEMS) based, thermally actuated grippers.

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* Zyvex Corporation offers a wide range of accessories for the Nanomanipulator suite of instruments, including the Optical Docking Station and NanoEffector™ tools. For more information, contact Zyvex at 877.998.3999 ext. 271 or email sales@zyvex.com.

The Loop Test

The loop test is an alternative to tensile tests and is particularly applicable to short fibers. Monofilaments for composite materials have been tested for mechanical strength using the loop test[9]. In this test, a fiber is contorted to form a loop. The L100 can apply a torsional stress to a collagen fiber, thereby creating a loop. The radius of curvature of the loop is diminished by pulling the fiber in opposite directions, and the bending strength can be measured at the point just before the fiber breaks. When used in the Zyvex Optical Docking Station, real-time image capturing can be performed for analysis. Loop test theory and applications are discussed in subsequent sections of this note.

Fatigue Testing

The L100 can be used to mimic tensile fatigue testing on collagen fibers. For sub-micron scale collagen fibers, the L100 can be used to position the collagen fiber onto a Zyvex MEMS-based oscillator with a preload. The MEMS oscillator can replicate continuous stress/relax states of the fiber, simulating muscle exercise.

Handling collagen fibers

There are two key steps to follow when preparing a collagen sample for manipulation with the L100. The first step is rehydration, where the moisture in the dried collagen fiber is replenished. The second is binding the collagen fiber to a probe. This can be done using cyanoacrylate-ester based glue. These steps are discussed below.

Rehydration

Collagen will lose water over time, and must be rehydrated in order to maintain its intrinsic elasticity. Hydrating collagen fibers can be done with phosphate-buffered saline (PBS) in a spray or soak, or with an aqueous bath. The soak is performed before any manipulation work. The collagen is placed in a bath or in soaked gauze for an hour[5]. The PBS spray is administered during manipulation to maintain hydration, and should be done every 60 seconds[3]. This will ensure the collagen maintains it elasticity during the test.
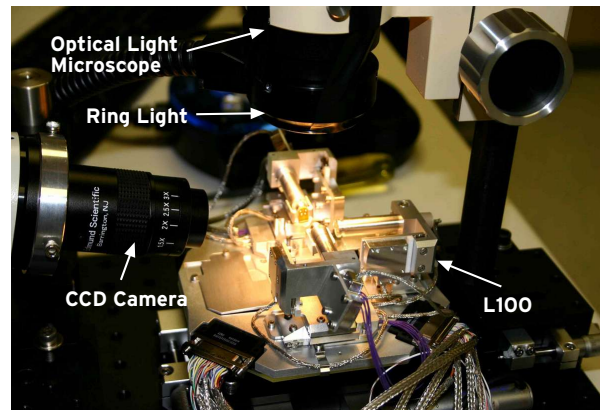

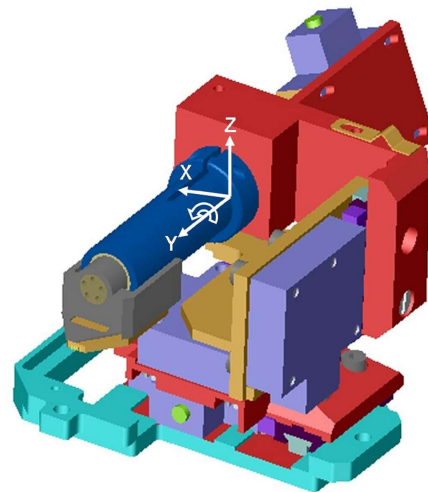**Figure 1** The L100 Assembly System in the Optical Docking Station.

Figure 2 A schematic of a four-degree-of-freedom positioner, highlighting the axial nomenclature.

Cyanoacrylate-ester Adhesive

The L100 uses MEMS grippers to strip collagen fibers from the fibril sample. Thus, the fiber must be strongly bound to the sample holder. After rehydrating the fiber, dip the probe in a cyanoacrylate-ester based glue, and place the fiber onto the probe with a pair of tweezers. The glue must be dry before attempting any manipulation. **Figure 3** shows a 250 micron diameter tungsten probe (the sample holder) with a 10 micron diameter collagen fiber (rat tail tendon) affixed to it using a cyanoacrylate-ester based glue.

Manipulation

The first task in any manipulation experiment is to set-up the L100. To perform such experiments as the loop test, place the probe with the collagen fiber into the three-degree-of-freedom (DOF) positioner. This positioner should be located between two four-DOF positioners. These positioners have MEMS gripper NanoEffectors and are initially positioned at the extreme ends of their axial rotation. The grippers should be "powered-closed" so that that the applied force can be varied. As a powered-closed gripper encloses a fiber, it can squeeze the fiber beyond the contact point by increasing the actuation voltage.

The L100 is used with the Optical Docking Station when performing collagen manipulation tasks. The L100 fits into the X-Y-translation stage with its standard mounting ring. The cables are connected with the adapter assembly. Center the L100 with the X-Y-translation stage and bring the collagen fiber into the focal plane of the optical microscope.

When the collagen fiber is in focus, begin bringing a MEMS gripper into contact with it. **Figure 4a** shows a powered-closed metal-MEMS gripper approaching a collagen fiber. The gap between the flexure arms of the gripper is on the order of 6 microns. The gripper is loaded onto a four-DOF positioner. **Figure 4b** shows the gripper closed onto a collagen fiber. Grasp the fiber with the grippers and back the positioner away from the fiber until a smaller fibril is free, as shown in **Figure 5**. Grasp the free end of the fiber with the second MEMS gripper. A tension is then applied to the fiber by moving the positioners away from each other **(Figure 6)**. To form a loop in the fiber, a torsional stress is applied by bringing the axial rotators to their opposite extrema. Moving positioners towards each other relieves the applied torsion, which causes the fiber to twist. Continue to position the NanoEffectors until the loop is created. **Figure 7** shows the fiber formed into a loop.
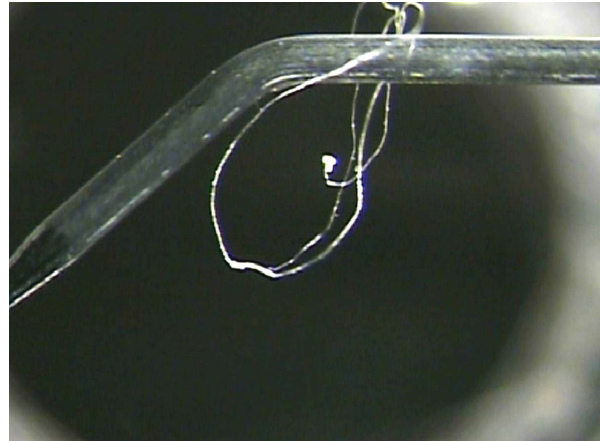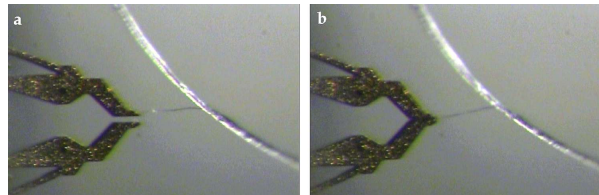

Figure 3 The probe with a collagen fiber glued to it.

Figure 4a-b A MEMS gripper grasping onto a collagen fiber. The gripper opening is 6 microns.

Figure 5 A MEMS gripper holding a free collagen fiber.

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Zyvex has published an application note on operating MEMS grippers with the nanomanipulator, which is available at www.zyvex.com[10].

Fiber Characterization

The L100 can manipulate sub-micron size collagen fibers for mechanical characterization. Traditional tensile tests are ineffective at calculating the strength of small fibers. The loop test is a more effective method for characterizing small fibers, and the L100 is perfect for manipulating them.

Loop Test Theory

Because collagen fibers are polymeric, they can be characterized using polymer fiber characterization techniques. One such technique is called the loop test. This test measures the bending strength of fibers by measuring the smallest radius of curvature a fiber will allow before breaking. The relationships between the physiometric parameters of the fiber and the extrapolated information regarding the compliance are described below.

Figure 8 shows a schematic of a looped fiber. The fiber has a thickness (diameter) D and is formed into a loop with radius of curvature ρ. The fiber loop is pulled in the $\pm\hat{y}$ directions forcing compression and tension about point **A**, the point on the bend where breaking will occur[9]. The values for critical compressive strain, ε_{cr}, and skin strain, ε_{sk}, are determined by the following equations:

$$\varepsilon_{sk} = \frac{D\rho}{2} \quad \textbf{(Eq. 1)}^{[9]},$$

$$\varepsilon_{cr} = \frac{D}{2\rho} \quad \textbf{(Eq. 2)}^{[11]}.$$

The radius of curvature can be measured by analyzing data collected through the optical stereo-microscope. The loop test requires adroit manipulation of fibers, especially at sub-micron length scales. The Zyvex L100 is designed for manipulating structures on these length scales, and can easily apply the necessary stress on collagen fibers for mechanical characterization.

Figure 6 The collagen fiber pulled taut.

Figure 7 The collagen fiber formed into a loop with the L100.

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Conclusions

Collagen is a major structural support element in mammalian organs and tissues. Thus, it is being used as a scaffolding material in tissue engineering, a field with an emphasis in tissue and organ regeneration. In order for collagen to be considered as a candidate for tissue engineering scaffolds, the mechanical properties of the scaffold must be determined. The mechanical properties of formed collagen fibers are assembly dependent, so the molecular response of the fibrils under load must also be ascertained in order for tissue engineers to grow site specific collagen *in vitro* and *in vivo* that meet the requirements for bioimplantation. The L100 Manipulation System can perform the tasks necessary to fully characterize the fibers and scaffolds grown and used by engineers for tissue engineering. Integrating Atomic Force Microscopy into the L100 platform is being investigated as a tool for molecular analysis. The ability to manipulate sub-micron size fibers, coupled with Zyvex NanoEffector™ tools and accessories makes the L100 the premier instrument for collagen fiber characterization.

Figure 8 A schematic of the fiber loop. The top image is the loop; The bottom image is a close up showing the D parameter (fiber diameter)[9].

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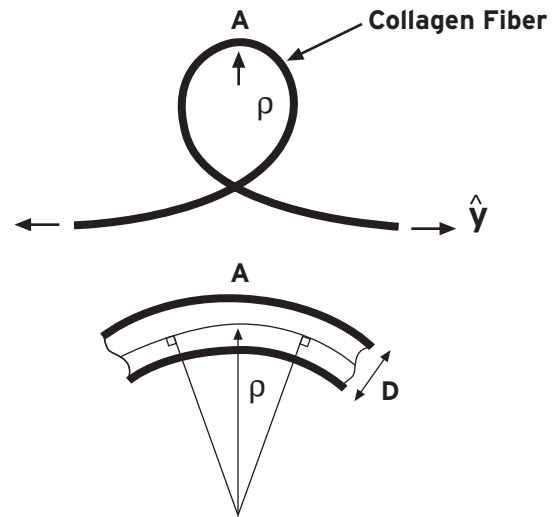11. S. Fidan, *et al., Comp. Sci. and Tech.* 49 (1993): 291–297.