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A Method for Determination of Stiffness of Collagen Molecules

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This study developed a micromechanical test method for determination of stiffness of collagen molecules. The novelty includes the adoption of an optical tweezer system for loading a single collagen molecule and the development of a binding technique for gripping the collagen molecule termini for the micromechanical test. This methodology will potentially help us to understand the mechanical properties and functions of collagen ultrastructure.

Connective tissues such as ligaments and tendons are the major structures for withstanding mechanical forces and maintaining joint stability. Collagen is the predominant protein in these connective tissues. The deviation of collagen from its normal molecular structure causes various disorders, such as excessive joint laxity, poor ligament healing, abnormal fracture healing, tendon adhesions, enthesitis, soft tissue degeneration, inherited diseases, collagen-related skin diseases, or abnormal wound healing (1-3). These observations suggest that collagen is the major source of mechanical strength in connective tissues. Although extensive biochemical and structural studies have been performed to understand collagen and its pathological conditions, the mechanical properties and functions of collagen have rarely been studied beyond gross anatomical structures. The major impediment to more detailed mechanical analysis has been the paucity of experimental methods to test individual collagen fibers or individual collagen molecules.

Deviating from the traditional biomechanical method for gross mechanical tests of soft tissues, this study presents a technique that can be used for measurement of stiffness of a single collagen molecule. The novelty of the method includes: 1) adopting a state-of-the-art instrument, an optical tweezer system (Laser Tweezers 980/1000 Module, Cell Robotics, Inc., Albuquerque, NM) (4,5), for measurement of the stiffness of a single collagen molecule, and 2) developing a binding technique for gripping the collagen molecule termini for this micromechanical test.

MATERIALS AND METHODS

In this study, single collagen molecules were stretched *in vitro* by using the optical tweezer system under light microscopy as illustrated in Fig.1, and the details are described in the following paragraphs. In brief, a solution containing collagen molecules, small polystyrene (PS) beads and large PS beads was prepared and placed onto a coverglass. Both small and large beads were precoated with heparin for the molecule binding. The concentration of molecules and beads was carefully adjusted to create a high incidence of a single molecule attachment onto a small bead at one terminus and the coverglass through a large bead at the other terminus, or, in short, a bead-molecule-coverglass linkage. The coverglass was mounted onto the X-Y stage. The invisible molecule was stretched through the visible beads. After the optical tweezer system was on, any suspended or linked small beads could be trapped into the laser trap by slowly moving the X-Y stage. If the small bead was linked with a molecule whose other terminus was attached on a large bead (Fig.1), the optical tweezers would experience a force F_m from the molecule attachment against the trapping force F_{trap} which is equal to the F_m at equilibrium. The trapping force was precalibrated as a function of the distance from the small bead center to the laser trap center. The deformation of the molecule was measured as the displacement of the small bead center from the large bead center. The stiffness of the molecule was then calculated.

The following is the description of the methodology, including 1) preparing the collagen molecules for the stiffness testing and 2) measuring the collagen molecule stiffness.

Preparing the Bead-Molecule-Coverglass Linkage

Molecule preparation. The collagen molecule samples were prepared from type I procollagen (6). Procollagen was used because this molecule not form intermolecular cross-links. The samples would, therefore, have homogeneous single molecules for the tests. The detailed isolation of procollagen was given by Olsen et al. (7). Briefly, leg tendons were dissected from 17-day-old chick embryos, digested with trypsin and collagenase, and then tendon fibroblasts were pelleted. Tendons from twelve dozen embryos yielded about 2×10^9 cells. Cells were placed in suspension culture for 6 hours. Type I procollagen was isolated from the culture medium of the suspension cultures by ammonium sulfate precipitation; a yield of about 1.5 mg procollagen was obtained from 2×10^9 cells. Procollagen purity was confirmed by SDS-PAGE analysis, and protein identification was confirmed by amino acid analysis.

Formation of the bead-molecule-coverglass linkage. The method used for formation of the bead-molecule-coverglass linkage was based on quantitative study of heparin binding sites on the collagen molecules (6,8), which showed that both the N-terminus and C-terminus of collagen are the binding sites for heparin. In this study, two steps were taken to formulate the bead-molecule-coverglass linkage. At first, both the PS large and small beads were coated with heparin, the method

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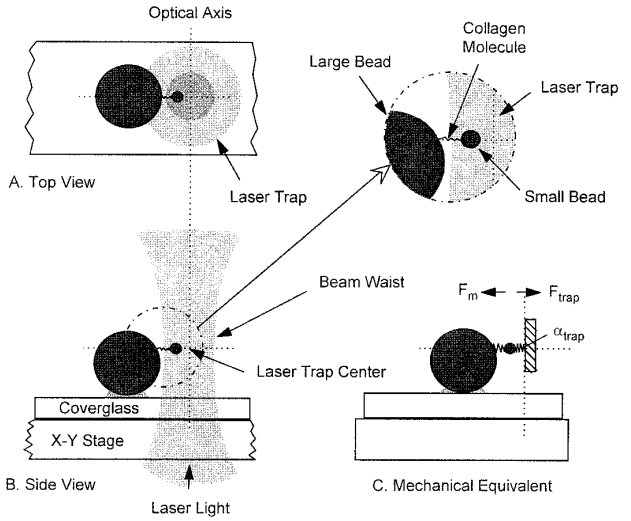

FIG. 1. An illustration of the essential features of the experiment. (A) The top view and (B) the side view of the configuration during elongation. A collagen molecule is attached onto the coverglass through a large bead (3.15 μm in diameter) at one terminus, and onto a small bead (0.49 μm in diameter) at the other terminus. The coverglass is fixed to an X-Y stage. The molecule is then stretched by moving the X-Y stage while the small bead is confined within the laser trap. (C) The mechanical equivalent of the experimental geometry. F_{trap} is the trapping force, and F_m is the force exerted by the molecule on the bead. At equilibrium, these two forces are balanced.

provided by Pierce (Rockford, IL). In principle, the reactions involve the hydrazone linkage between the amino-functionalized beads and the terminal aldehyde group of heparin. This is further stabilized to a single bond by adding sodium cyanoborohydride. Amino-functionalized PS beads of two sizes were purchased (Bangs Laboratories, Inc., Carmel, IN), the small beads (0.49 μm in diameter) to be manipulated by the optical tweezers and the large beads (3.15 μm in diameter) to be anchored onto the coverglass (Fig.1).

The coupling procedure was completed as follows: One and a half grams of heparin (sodium salt) (Sigma Chemical Company, St. Louis, MO) were dissolved in 20 ml of 0.2 M sodium carbonate. A total surface area of up to 2,000 mm^2 of amino-functionalized PS beads was then added to the heparin solution. In a fume hood, 160 mg of sodium cyanoborohydride was added and mixed to dissolution. This was allowed to react for at least 72 hours at room temperature, with gentle mixing. Upon completion of the reaction, unreacted amine groups on the PS beads were blocked by the addition of 0.5 gm of acetaldehyde. The heparin-derivatized beads were stored at 4°C in the presence of a preservative (0.05% sodium azide) until needed.

The next step was the solution preparation for coupling collagen termini to the beads and coverglass. The goal was to have a high incidence of a single molecule attachment onto a small bead at one terminus and the coverglass through a large bead at the other terminus. The methodology was mixing about equal numbers of the beads with molecules in the solution, which was similar to those used in the previous studies for other molecules (9,10). The numbers of beads and molecules were estimated before coupling. The bead number was calculated by dividing the total solid contents by a single bead volume, and the beads were examined by optical microscopy. The molecular number was estimated by dividing the total molecular weight in the solution by a single molecular weight (molar mass/Avogadro's number). The pH value of the buffer was 6.7 in order to achieve high affinity tethering of collagen/heparin coupling. This value was based on an unpublished data from J.D. San Antonio: The pH dependency of heparin binding to collagen was tested by affinity coelectrophoresis using the method described previously (11). High affinity interactions were found at pH 5-7.

The coupling procedure started by placing the large bead solution onto the coverglass (Fig.2A). The large beads were anchored onto the coverglass through non-covalent bonds after the solvent evaporated. The collagen molecules were first mixed with the small beads for 5 minutes (Fig.2B), and then mixed with the large beads on the coverglass for 15 minutes at room temperature (Fig.2C). The reason for mixing the collagen molecules with the small beads before the large beads was to increase the chance for the molecules to bind onto the small beads, since the surface area ratio between the two bead sizes is 41:1. The solution had a slightly higher ratio of (both large and small) beads to collagen molecules (1.1 : 1) to create more opportunity for the single molecule binding. Finally, the reaction was stopped by washing out unreacted beads and molecules using the same buffer.

Measuring the Collagen Molecule Stiffness

Force calibration. Before the measurement of collagen stiffness, the force applied to the molecule had to be calibrated. At equilibrium, the force applied to the molecules is equal to the laser trapping force applied to the bead (Fig.1). It was demonstrated that the trapping is mechanically equivalent to a spring (12), and the trapping force F_{trap} is equal to the trapping stiffness α_{trap} multiplied by the displacement of the bead center from the laser trap center y, i.e.,

$$F_{trap} = \alpha_{trap} y.$$

Several methods have been proposed for calibration and mutual comparison of the trapping stiffness (13-15). In this study, calibration was not performed because the system used did not have enough resolution. Instead, the calibration results from a previous study (10) were adopted because the beads in both studies had identical size. The α_{trap} used was 0.5 pN/nm for applied 1000 mW laser power.

Micromechanical tests of collagen molecules. The bead-molecule-coverglass linkage shown in Fig.1 provides the essential mechanical

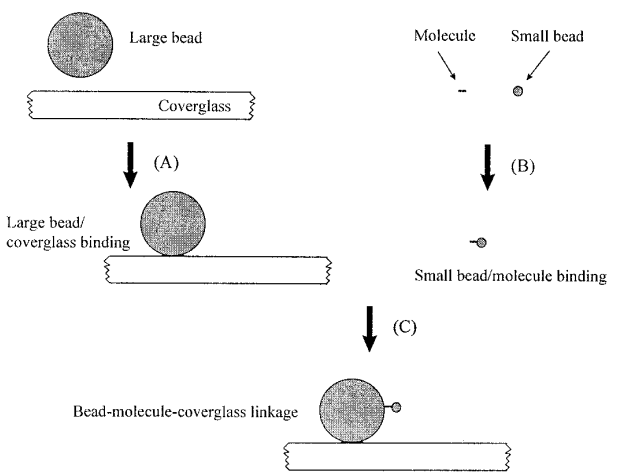

FIG. 2. The coupling procedure for formation of the bead-molecule-coverglass linkage. (A) The large beads were anchored onto the coverglass through non-covalent bonds after the solvent evaporated. (B) The collagen molecules were mixed with the small beads for 5 minutes. (C) The mixture of the molecules and small beads was then placed onto the coverglass for 15 minutes for attaching the large beads. Efforts were made to increase the incidence for formation of the single molecule attachment in the bead-molecule-coverglass linkage by having a slightly higher ratio of (both large and small) beads to collagen molecules (1.1 : 1) in the mixed solution. Finally, the reaction was stopped by washing out unreacted beads and molecules using the same buffer.

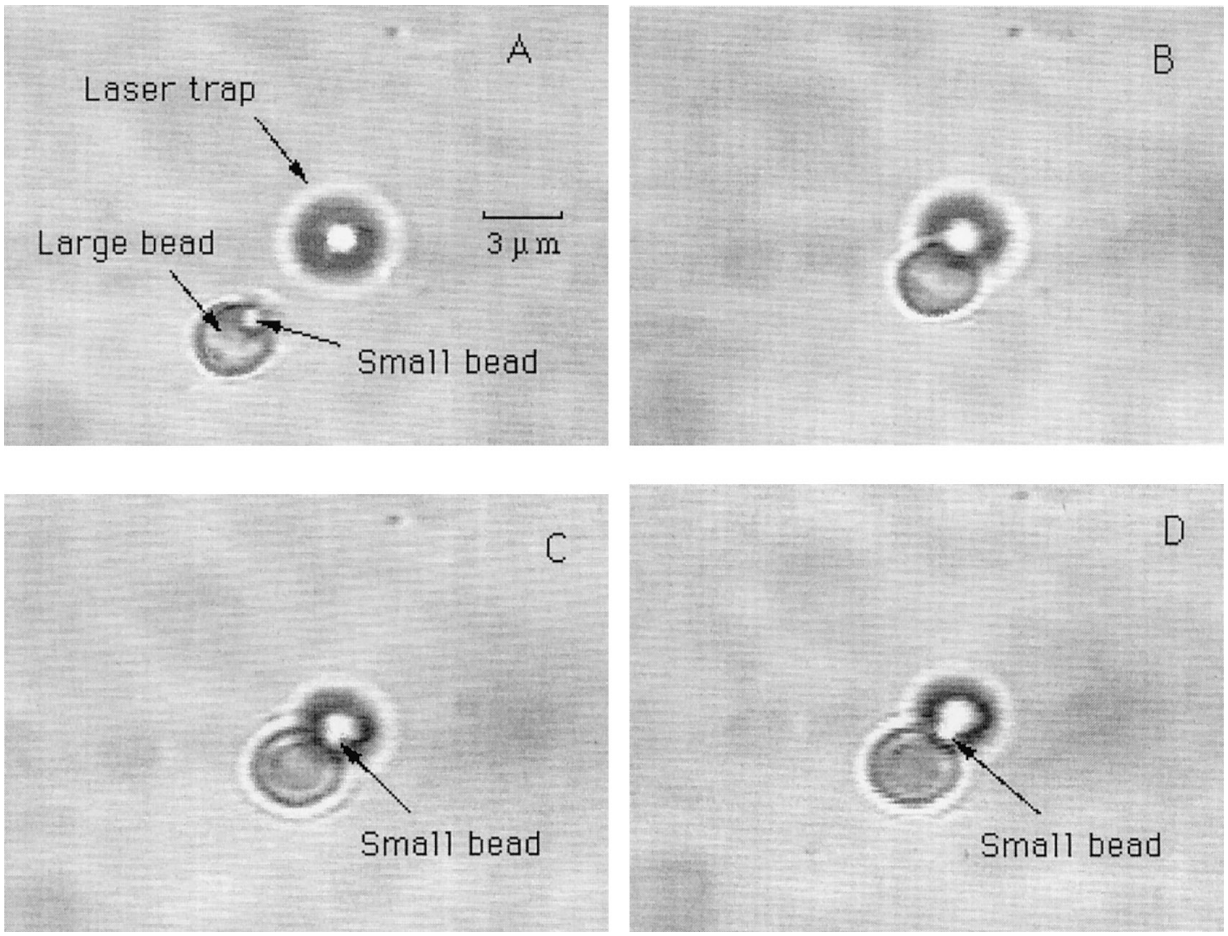

FIG. 3. The images of stretching a typical collagen molecule through the bead-molecule-coverglass linkage by the optical tweezers (top view). (A) An instant before the linkage moved into the laser trap. (B) The initial unloaded position when the small bead was trapped in the trap center. (C) The molecule was stretched by moving the X-Y stage with the small bead confined within the trap. (D) The molecule was further stretched. The schematic of this test is shown in Fig.1A.

structure for collagen stiffness testing. Typical linkages were identified under light microscopy by slowly moving the X-Y stage. A small bead was caught by the optical tweezers and confined within the trap. Initially, the bead was adjusted to the trap center so that no force was on the molecule. By moving the X-Y stage to different positions, the collagen molecule was stretched through the bead, and the distances between the laser trap center and the small bead center and between the small bead center and the large bead center were measured, respectively, throughout the course of elongation.

Determination of collagen molecule stiffness. From the micromechanical tests of the collagen molecules, the relationship between the applied force on the molecule F_m and the molecule deformation d (or the load-deformation curve) was determined. The molecules were considered as a spring-like structure, the stiffness was, therefore, the slope of the load-deformation curve.

RESULTS

The microscopic images of a typical bead-molecule-coverglass linkage were shown at four subsequent positions (Fig.3). The optical tweezers generated a laser trap to manipulate the small bead in order to stretch the molecule. The large bead was fixed onto the coverglass. The small bead had a diameter of 0.49 μm but appeared larger on the images because it was slightly out of focus. Fig.3A shows the linkage before moving into the trap. With the laser trap applied to the small bead, the bead was positioned at the trap center as the initial unloaded position (Fig.3B). This frame was selected once the minimum distance between the laser trap center and large bead center ($d0$) was observed.

Next, the collagen molecule was stretched to two subsequent positions by moving the X-Y stage with the small bead confined within the trap (Figs.3C&D), and the distances between the trap center and the small bead center (y) and between the small bead center and the large bead center ($d1$) were each roughly measured in turn. The deformation of the molecule was considered to be the distance difference d between $d1$ and $d0$. The force applied on the molecule F_m was equal to the trapping force F_{trap} defined

as $F_{trap}=\alpha_{trap}y$. A total of five sample trials were performed. The average stiffness was 0.34±0.11 pN/nm.

DISCUSSION

This study introduced a method for measurement of the stiffness of collagen molecules, a parameter that was impossible to determine previously. Although it is preliminary, the result reveals at least two important factors: 1) The bead-molecule-coverglass linkage through heparin binding is suitable for the stiffness measurement. 3) The method could potentially be used for determining the micromechanical properties and functions of various types of collagen and other extracellular molecules.

Although the results are encouraging, limitations exist in the current system that can be resolved in future studies. The major limitation is the resolution of the optical tweezer system. The microscope used in the optical tweezer system from Cell Robotics, Inc. is a conventional light microscope and only has a resolution of 0.2 μm. Such a resolution means that the deformation measurement errors could be as high as 69% of the collagen length (290 nm). In order to determine the deformation of collagen molecules, the optical tweezer system has to be enhanced to quantitatively measure nanometer displacemetns by optical trapping interferometry (10,12), video-based centroid tracking (16) or a high-resolution photodiode position detector (17). In addition, for the same reason, the X-Y stage (0.2 μm resolution) also has to be upgraded to a piezo one with a picometer precision (10).

With these modifications, a number of unanswered questions from this study can be addressed. First, the deformation of the molecule can measured accurately. According to a previous study (10) which measured force and velocity of single kinesin molecules using the optical tweezer system with interferometry, the displacement measurement errors were estimated to be less than ±5% over the range of 0 $\sim$ 200 nm. Second, force calibration can be carried out. Different methods for calibration of force applied on the molecules proposed previously (13-15) can be tested and validated under the same conditions of solution and beads as in our collagen tests. Third, such a accuracy also allows the determination of the initial unstretched position of the molecule before applying the load. It is reasonable to assume that the molecule starts to be loaded once the displacement and force are beyond the noise level. Also, the possibility of multi-molecule linkage can be examined. Theoretically, it is possible that there is more than one molecule formed in some bead-molecule-coverglass linkages. This nanometer detection would allow easy stiffness differentiation between single molecule and oligometric molecule attachments (e.g., a single molecule is roughly twice as soft as two molecules in parallel). Finally, the bead motions may not be strictly parallel to the molecular axis. This problem will be solved by stretching the molecule through the small bead at different trap center heights (varying the bead location along the Z-axis). Theoretically, stretching the molecule parallel to the X-Y stage will not require a vertical force component. By comparing the force-deformation curves at different trap center heights, the one with the minimum vertical force for a given horizontal deformation will be selected.

In summary, this study developed a micromechanical test method for determination of stiffness of collagen molecules. The novelty includes the adoption of an optical tweezer system for loading a single collage molecule, and the development of a binding technique for gripping the collagen molecule termini for this micromechanical test. Although the finding was preliminary, this study enabled us—for the first time—to measure the mechanical properties of a collagen molecule. This methodology will potentially help us to understand the mechanical properties and functions of collagen ultrastructure.

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