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Using the Micropipette Aspiration Technique to Measure Type I Collagen Single Molecule's Elasticity

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Abstract

Type I collagen is the most abundant collagen protein in human skin. Its biomechanical properties have long been a subject of investigation. However, measurements of its elasticity using different methods have often yielded conflicting results. While Atomic Force Microscopes and Optical Tweezers have often been used extensively to probe the stiffness and elasticity of single collagen molecules, the Micropipette Aspiration Technique has not yet been applied to type I collagen. This paper reviews the strengths and limitations of AFM and optical tweezers compared to MAT, and positions MAT as a promising approach for measuring the elasticity of collagen monomers. A persistence length in the range of approximately 10-20nm is anticipated to be obtained using the Marko-Siggia model. Additionally, the experiments expected to explore how variations in temperature and pH affect molecular mechanics. Although MAT has its own limitations, it offers the potential to provide new insights that could advance understanding of collagen biomechanics.

Keywords: Micropipette Aspiration Technique, Type I collagen, Collagen's elasticity measuring, Atomic force microscopes, Optical tweezers

1. Introduction

1.1 Type I Collagen

Proteins are large, complex molecules that play a critical role in human skin. Collagen is the most abundant protein in the human skin, being 30% of total protein. In addition, type 1 collagen is a fibrillar type collagen, and it is the most abundant collagen in the human body. Type I collagen is the major fibrillar protein of the extracellular matrix (ECM). Although recent studies show that type I collagen can regulate cells' adhesion, migration, and signal transduction behavior, it has the primary function of providing a structural scaffold to maintain tissue integrity (Jones et al., 2010). As shown in Fig. 1, type I collagen usually appears in triple helix form, where two a1 chains and one a2 chain coil around each other. Until today, there have been at least 25 types of collagens identified, and they are all named as type I, type II, type III collagens etc. There are similarities between fibrillar collagens type I, II, III, V, and XI: they all consist of 338 consecutive Gly-X-Y tripeptides, where X represents frequently proline and Y represents frequently hydroxyproline. Typically, questions around how to measure the flexibility of collagens have always been a hot topic. Even though there have been numbers of hydrodynamic methods and electron microscopy being used to explore the answers to the questions, these precise methods somehow always reach conflicting conclusions (Sun et al., 2002).

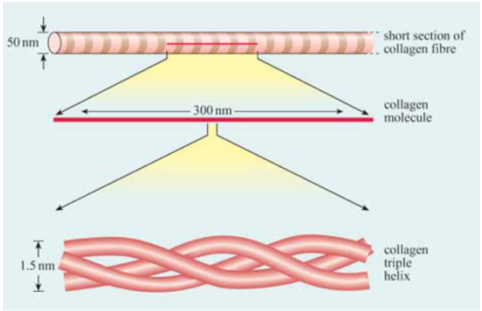

Figure 1. Type I Collagen Structure (Type 1 Collagen Structure, n.d.).

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1.2 Previous Methods

Since the 1980s, various mechanical measurement tools have been developed, with atomic force microscope (AFM) emerging as a widely used, essential technique for probing nanoscale forces (Kim, 2021). Introduced by Binnig et al. (1985) as a combination of scanning tunneling microscopy and stylus profilometer, AFM allows measurement of forces at specific, spatially defined regions. It has been used to study the topography and mechanical properties of collagen fibrils and single type I collagen molecules under near-physiological conditions, enabling insights not attainable with techniques such as X-ray crystallography (Bozec and Horton, 2005; Strasser et al., 2007). However, AFM has several inherent limitations when handling molecules with complex structure, which can restrict its applicability for studies on type I collagen (Khattignavong et al., 2023). Type I collagen's triple helical structure presents a mechanical and structural complexity that can be difficult to probe accurately with AFM. In addition, AFM provides limited control over the direction of molecular stretching and is highly sensitive to environmental factors such as humidity, temperature, and buffer conditions. Sample preparation steps, including drying, may also induce distortion. Furthermore, the cantilever's limited force range may prevent detection of subtle molecular transitions.

In contrast, Micropipette Aspiration Technique is well-suited for complex structured molecules, provided their size is compatible with the micropipette. MAT allows molecules to be manipulated in solution, preserving near-native conditions, and offers greater control over applied forces and stretching direction. These advantages make MAT a more reliable and physiologically relevant method for measuring the elasticity of type I collagen compared to AFM.

Optical Tweezer, which also emerged in the 1980s, uses a highly focused laser beam to exert attractive or repulsive forces on small particles. They have been widely applied in mechanobiological studies such as mechanotransduction, cell micro-rheology, membrane mechanics, and cell-cell interaction (Kim, 2021). However, like AFM, optical tweezers also have their inherent limitations. Their force is limited to a range of approximately 0.1-100 pN (Neuman and Nagy, 2008), which may be insufficient to fully stretch type I collagen or capture all of its mechanical transition. Potentially underestimating its elasticity. Additionally, the requirement to attach collagen molecules chemically to the beads may distort the conformation of the molecules, or introduce artifacts that affect the measured mechanical properties.

MAT, in contrast, can apply a larger and more precisely controlled range of forces, allowing full stretching of collagen and detection of subtle mechanical transitions. Furthermore, as long as the molecules can fit into the micropipette, MAT can manipulate fibrous, elongated, or branched molecules without the need for chemical attachments. These advantages make MAT a more suitable technique for measuring the elasticity of type i collagen compared to optical Tweezers.

2. Theoretical Background

2.1 Methods to Isolate Molecules

Since the experiment focuses on individual type I collagen molecules, it is necessary to isolate single molecules on the substrate. One common approach is the dilution method performed by Bozec and Horton (2005), in which a highly diluted collagen solution is applied to a hydrophilic substrate. This ensures sparse absorption of molecules and increases the likelihood that only one molecule is present for imaging or mechanical manipulation. Additional strategies, such as repeated single-molecule force spectroscopy measurements, can further enhance single-molecule isolation by probing sparsely distributed collagen monomers, reducing the chance of multiple molecules being pulled simultaneously.

2.2 Micropipette Aspiration Technique

The Micropipette Aspiration Technique (MAT) was invented by Heinrich Schnitger in 1957. A fine hollow needle is used by the experimentalists to separate a portion of a cell with vacuum pressure, and the geometric changes of the cell shape are measured in order to determine the cell's elastic property. The Micropipette Aspiration Technique was invented to be capable of measuring a large force range from approximately 10 pN to a few hundreds nanoNewton.

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This large range of force is the Micropipette Aspiration Technique's advantage compared to many other techniques, including AFM (Baghini et al., 2013). With all these advantages, the Micropipette Aspiration Technique has long been used as a technique to assess cell elastic properties (Wu et al., 2020). In fact, the Micropipette Aspiration Technique has become the most widely used method to measure cell elasticity with its few equipment requirements (Liu et al., 2019). The Micropipette Aspiration Technique has been used to measure the elasticity of breast cancer, both stem-like and non-stem-like, by aspirating cancer cells one-by-one into small glass micropipettes (Mohammadalipour et al., 2014).

The Micropipette Aspiration Technique was designed to test biomacromolecules or large structural molecules, typically over several hundred nanometers, and flexible molecules. For example, the Micropipette Aspiration Technique is used to study soft cells like neutrophils and red cells (Hochmuth, 2000). The Micropipette Aspiration Technique is often applied on DNA, liposomes, polymer chains, and long fibrillar proteins, collagen, testing. The length of a type I collagen molecule is approximately 300 nm and has the width of about 1.5 nm (Henriksen and Karsdal, 2016). Due to its great length, a type I collagen molecule is a macromolecule, and the Micropipette Aspiration Technique is applicable to do tests on bigger molecules with its 500 nm tip. Based on other previous studies, type I collagen molecules' flexible regions are indicated by their viscoelastic measurements, microscopic observations, and amino acid sequence. As a flexible macromolecule, type I collagen molecules perfectly suit the Micropipette Aspiration Technique's requirement for testing elasticity (Freeman et al., 2005).

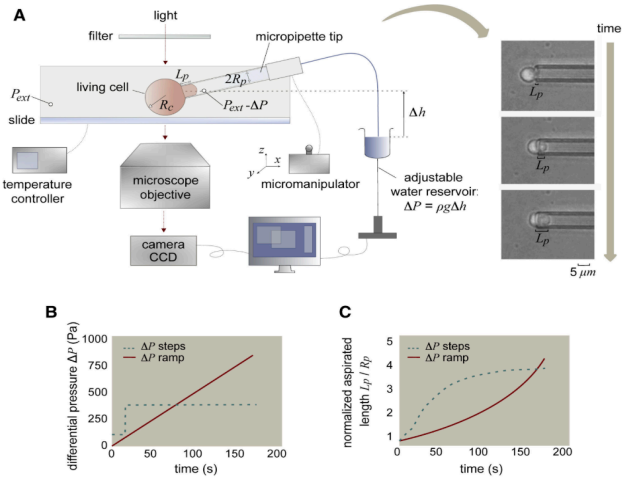

Figure 2. Scheme of the Micropipette Aspiration Technique Experiment. (A) Experimental setup used to simultaneously adjust the differential pressure ΔP, the temperature, and the position of the micropipette. (B) Graph shows two types of curves when different Δapplied over time. (C) Plot of a aspirated length as a function of time for the two types of experiment (González-Bermúdez, 2019).

During the experiment, an adjustable water reservoir would be attached to the micropipette and apply a suction pressure of ΔP to the micropipetten as shown in Fig. 2. The equation to calculate the suction pressure is $\Delta P = pgh$, which is the height difference between the top of the water reservoir and the tip of the micropipette (h) times the weight of water (pg). If there is a flow, the following equation would be taken into account: $\Delta P = pgh\ (1 - U/U_f)$. U represents the actual velocity of the aspirated material and U_f represents the velocity of the material if flowing freely (González-Bermúdez et al., 2019).

3. Proposed Experiment

3.1 Testing Elasticity

When testing the elasticity of type I collagen, the study would first isolate the molecule. To achieve this, it would create a high dilution of the collagen stock solution: specifically, at a ratio of 1:1000 using phosphate-buffered saline (PBS). This dilution level can ensure low surface coverage, thereby significantly increasing the likelihood that only one single collagen molecule would be adsorbed onto the coverslip in a spatially separated manner. This low-density is essential for avoiding molecular overlap. Then, the experiment would put 100μL of the diluted solution by micropipette on a hydrophilic glass coverslip or a freshly cleaved mica substrate, both of which offer an ultra-flat surface ideal for molecular manipulation. Glass Coverslip offers a smooth, slightly negatively charged, hydrophilic surface with moderate adhesion. In contrast, freshly cleaved mica substrate provides an atomically flat, strongly hydrophilic, negatively charged surface that allows stable adsorption without significant molecular distortion. In this particular experiment, a freshly cleaved mica substrate is preferred because its ultra-flat surface enhances the likelihood of isolating a single, spatially separated collagen molecule. And may also minimize surface-induced

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distortion and preserve the molecule's native elasticity. The sample would then be placed in a humidity-controlled incubation chamber to prevent evaporation and maintain consistent environmental conditions. A few drops of water may be dropped beside the sample to observe if evaporation happens. A 5 - 10 minute incubation period would allow a small number of collagen molecules to adhere to the coverslip through nonspecific surface interaction while excess molecules remain in the diluted solution. Next, after a few molecules attached to the coverslip, the unbound or loosely associated molecules would be rinsed out on the sample with PBS. The sample would then subsequently dried under a stream of dry nitrogen gas, using a mild, low-pressure flow to minimize mechanical stress and ensure that the collagen molecules maintain their native conformation prior to micropipette aspiration, a step feasible because type I collagen molecules are generally highly resistant to distortion due to their strong mechanical properties and resistance to proteolytic degradation.

After that, the collagen-coated substrate would be placed on the stage of the cell suspension chamber. A micropipette would be mounted onto a micromanipulator for precise positioning against the collagen. The micropipette tip would have an inner diameter of around 300 to 500 nm. The inner diameter is chosen to correspond with the diameter of the type I collagen molecule (around 1.5 nm in width and 300 nm in length), ensuring the compatibility between the pipette geometry and molecular size. Although there seems to be a size mismatch between the width of the molecule and the inner diameter of the micropipette, as long as the molecule's width is smaller than the micropipette, there would be no geometric blockage or clogging that may affect the result of the experiment. The setup of the experiment would be similar to Fig. 3. To apply negative pressure, a water reservoir would be connected to the opposite end of the micropipette with the water column creating a controlled suction force. This setup enabled the micropipette to aspirate the collagen molecule on the end into the micropipette. As suction pressure gradually increases, the molecule would experience a stretching force. The goal of the experiment is to gradually pull the molecule gently into the micropipette without breaking it. During the experiment, environment temperature would be controlled in regular room temperature, which is around 20 to 22 °C or 68 to 72 °F. There is not a hard requirement for the temperature, but it needs to remain constant throughout the whole experiment since it is an important control group that would be needed in the later calculation section.

3.2 Collecting Data

Throughout the experiment, a pressure gauge would be used to measure the suction pressure in precise units while the deformation of the molecule during aspiration would be visualized and recorded by the optical microscope along with a digital camera. The captured video footage would provide a detailed record of the molecule's elongation, or geometric change, in response to the applied force. Since the length of the type I collagen molecule is around 300 nm, the picture of the stretched molecule would be compared to the picture of the stretched molecule and the original molecule to calculate the length of the molecule after stretching. This visual data would then be fit into the Worm-Like Chain model to calculate the elastic parameter. The temperature of the room would be also recorded by a thermometer.

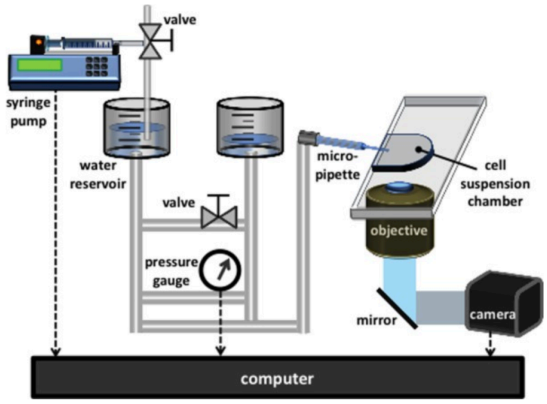

Figure 3. Example Set up of the experiment (Lee and Liu, 2014).

Worm-like chain and glassy WLC models describe semiflexible biopolymer networks whose relaxation spectra broaden exponentially when polymers interact adhesively (Kroy and Glaser, 2007). Later, Kurniawan et al. extended this concept suggested previously into a 3D simulation of a realistic, cross-linked WLC network. She applied this model to investigate how the network responds nonlinearly to small and large strains by analyzing the influence of its structural parameter (Mohammadkhah and Klinge, 2023). The Worm-Like Chain model can help calculate a molecule's elasticity by providing the mathematical relationship between the force applied to a polymer and its resulting extension. The WLC equation that can be used to determine the elastic stiffness of the molecule is

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$$F = \frac{k_b T}{l_p}\left[\frac{1}{4(1-\frac{x}{L_0}+\frac{F}{K})^2} - \frac{1}{4} + \frac{x}{L_0} - \frac{F}{K}\right]$$

This is the Marko-Siggia form of the equation that was suggested in 1995. In this equation, F represents the applied stretching force in Newtons, x represents the end-to-end extension of the molecule in meters, L_0 represents the contour length, l_p represents the persistence length, which is the bending stiffness, k_b represents the Boltzmann constant 1.38×10^{-23} J/K, and T represents Temperature in Kelvin. In the proposed experiment, F may be obtained from the pressure gauge; x from the video footage; and T from the thermometer. This equation may be used to find l_p, which is the elastic stiffness of the molecule (Marko and Siggia, 1995).

3.3 Elasticity Differences Under Different pH Conditions

Different pH may affect type I collagen's function. Shtrauchler et al. (2024) did experiments examining how different pH influences the structure, properties, and function of type I collagen (Bronner-Shtrauchler et al., 2024). As Bronner-Shtrauchler et al. (2024) described, when collagen's pH is at 7 and 7.8, it possesses a higher storage modulus and a lower permeability compared to ph is 6.5 and 7.4. In previous studies, Greenfin horse-faced filefish skin collagen (GHSC), which was identified as a type I collagen, has been investigated to study the effect of pH on its structure. As a result, a higher pH will cause GHSC's morphology to change from a well-textured stack to a loose silk-like structure. GHSC's viscoelasticity also changed: GHSC solution changes from a dominant viscous to elastic behavior when pH increases, and its function also changes to be used in injectable biomaterials (Wu et al., 2024).

Since that different pH would change the structure of type I collagen, the study may use the MAT to test the stiffness of the single type I collagen molecule under different pH conditions. To test this, it simply needed to add another step before starting the experiment: change the pH of the collagen. In markets, the commercial type I collagen is usually dissolved in 0.01 N HCl with a pH at around 2-3. NaOH can be gently added to raise the pH of the collagens. In addition to the original, acidic solution, a neutralized version and a basic version of the type I collagen may be created. It is recommended to perform all steps on ice or at 4 °C to prevent premature gelation or neutralization. After changing the pH of the solution, the experiment may repeat the previous proposed steps to test the elasticity difference among the three solutions. As a prediction, since in previous studies Wu et al. (2024) had proven that the higher the pH, the looser the structure, this may predict that as the type I collagen becomes more basic, it would be more stiff and have higher elasticity.

3.4 Elasticity Differences Under Different Temperatures

More than just changing the pH condition, it's also possible to change the temperature conditions and observe if different temperatures would create elasticity differences on type I collagen monomers. Previous research indicated that under human regular body temperature, the preferred conformation of collagen is a random coil rather than helix. This means that type I collagen, which is in a triple helix form, is intrinsically unstable rather than stable in human temperature (Leikina et al., 2002). With such background information, the scientists may test type I collagen single molecule's elasticity under different temperatures to determine under which temperature would type I collagen be the most stable.

In order to perform this task, the solution temperature would be changed when the experiment starts. This would require a temperature-controlled stage or chamber that can gradually change the temperature of the low concentrated type I collagen solutions to different versions: cold storage (2 to 8 °C), room temperature (around 20 to 22 °C), and heated stage (around 70 to 80 °C). The three specific ranges of temperatures were selected to represent distinct experimental conditions. The 2 to 8 °C range corresponds to standard refrigerated storage conditions, which makes it an appropriate reference for cold storage. Similarly, the 20 to 22 °C range is a widely accepted definition of room temperature and was chosen to represent the experimental conditions. Finally, a range of 70 to 80 °C has been selected for the heated stage based on the findings of Gevorkian et al. (2009), who reported that the Young's modulus of type I collagen exhibits a positive correlation with the temperature between 45 to 80 °C. Moreover,with additional evidence of complex intermolecular structural transitions occurring specifically between 70 to 80 °C (Gevorkian et al., 2009).

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Furthermore, as denaturation of type I collagen is reported to occur only above 120 °C, the use of 70 to 80 °C is considered experimentally feasible. After setting the sample to different temperatures, the experiment would then repeat the previous steps on these collagens and collect the elasticity data that would later be compared.

4. Expected Result

We expect that using the Micropipette Aspiration Technique on individual type I collagen monomers will produce measurable force-extension curves that align with theoretical predictions derived from the worm-like chain model. After fitting the collected data using the Marko-Siggia formula, it can be anticipated to obtain a persistence length in the range of approximately 10-20 nm, which would be in consistency with the values reported in the previous studies, such as the work performed by Chang and Buehler (2014).

Under the limitations of unintended multi-molecule aspiration events, pipette drift, and molecular instability during suction, it is to be expected to observe a certain degree of data variability. However, the experiment is planned to address this by performing several hundreds to several thousands aspiration experiments to obtain a sufficiently large dataset. By analyzing this dataset statistically, researchers would be able to filter out outlier data points that deviate significantly from the majority. Through this approach, the majority of usable data is expected to cluster around a consistent and reproducible mean contour length and force-extension profile, reflecting the typical behavior of single collagen monomers.

Furthermore, it is predicted that the mechanical behavior of type I collagen monomers would vary depending on the pH of the surrounding environment. Specifically, the collagen molecule will be expected to exhibit a more stable and consistent elastic property under a neutral pH (around pH 7), which closely mimics physiological conditions. When the collagen is exposed to more acidic or more basic pH levels, the experiment anticipated observing deviations in both persistence length and force-extension behavior, possible due to change in molecular structure, conformation, or partial unfolding of the collagen triple helix.

Likewise, the elasticity of the collagen monomer is also predicted to vary as the temperature changes. Accordingly, the elasticity of the collagen would be anticipated to increase as the temperature increases. This is based on the previous study performed by Gevorkian et al. (2009) that proved type I collagen's structure would first stabilize and then increase when temperature increases between 45 to 80 °C. However, if the sample temperature reaches 120 °C, it would cause heat-denaturation, which all these effects would then disappear (Gevorkian et al., 2009).

5. Discussion

Several practical hurdles must be carefully addressed before Micropipette Aspiration Technique can yield reliable single-molecule data on type I collagen. One of the primary challenges is ensuring that only a single molecule is being pulled and measured during each aspiration event. While the widely used dilution method —dispersing collagen at extremely low concentrations — significantly increases the probability of isolating a single molecule, it cannot guarantee that only one molecule is involved in every measurement. Even under optimized conditions, there remains a non-negligible possibility that two or more molecules may be aspirated simultaneously.

Because there is no perfect physical method to prevent multi-molecule aspiration entirely, a robust safeguard relies on statistical validation and careful post-experiment data filtering. A large ensemble of force-extension measurements, typically compromising hundreds to thousands individual pulling events, are collected. Outliers — data points that deviate substantially from the main distribution — are then identified and excluded. For example, if one measurement under the same applied pressure shows an unusually large elongation compared to the majority of the dataset, it is likely that multiple molecules were involved, and that data point would be filtered out to preserve the integrity of single-molecule analysis.

In addition to isolating single molecules, another challenge involves stabilizing the type I collagen molecule during the aspiration process. Molecular stability during aspiration is critical. A collagen molecule must remain tethered by only one end while the free end is drawn into the micropipette. When using the MAT, it is important that type I collagen maintain a stable position so it can be aspirated by the micropipette without the need of additional

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forces. If the molecule is very unstable when being aspirated, it would increase the possibility of failing the test every time, and increase the possibility of aspirating two molecules at the same time since molecules may overlap each other if being unstable and move around.

6. Conclusion

Elasticity testing of type I collagen has long remained a persistent and unresolved challenge within the field of molecular biomechanics. Researchers have explored a variety of experimental approaches, including well-established techniques such as Atomic Force Microscope (AFM) and Optical Tweezers, in an effort to quantify the elasticity properties of collagen molecules at the single-molecule level. Yet, these efforts so often lead to conflicting results with each of these methods' limitations.

Despite this, applying the Micropipette Aspiration Technique to measure the elasticity of type I collagen has been an area where, surprisingly, no one has ever tried. This is noteworthy because both type I collagen's size and flexibility indicates that it is a collagen particularly well-suited for analysis using the Micropipette Aspiration Technique.

Atomic Force Microscope, Optical Tweezer, and the Micropipette Aspiration Technique are all widely recognized as powerful and versatile tools used in the study of molecular mechanics. Each technique has gained prominence within the scientific community due to its distinctive advantages and specialized application compared to other techniques. Given this, it is proposed that applying the Micropipette Aspiration Technique to type I collagen monomer may offer new insights into its mechanical behavior — insights that have not been accessible through existing techniques alone. Even though using the Micropipette Aspiration Technique may also cause other challenges, for instance, hard to stabilize molecules during aspiration, the advantages brought by this technique would only cover such minimal challenges. By exploring this method on this collagen, there may be findings uncovered that contribute meaningfully to people's understanding of collagen mechanics.

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