Determination of Local Elastic Modulus of Soft Biomaterial Samples Using
AFM Force Mapping

By

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Force Mapping

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Abstract

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Conventional methods of mechanical testing cannot measure properties of soft materials at the nanoscale. In fields such as tissue engineering, it is important to distinguish the bulk elastic modulus from the surface elastic modulus and to characterize the spatial distribution of material with non-uniform stiffness. One of the important new methods of testing is the force mapping using the atomic force microscope. The existing force mapping approaches often suffer from omitting important effects that might result in artifacts. The most important effects include taking into account sample’s adhesion and viscoelasticity and considering more realistic probe shapes. In this work, we have applied a method that take into consideration probe shape and sample adhesion and developed elastic modulus mapping. This inclusive approach can be applied to samples with adhesion that exhibit indentation large enough that requires indenter shape models more sophisticated than the traditional paraboloid shape model. Sample viscoelasticity can be revealed by comparing parameters obtained in opposite scanning directions.

The application developed methodology is illustrated with the study of composite biomaterial scaffolds that are used to support the differentiating cells. Samples are composed of four groups: uncrosslinked electrochemically aligned collagen (ELAC),

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uncrosslinked Bioglass incorporated ELAC (BG-ELAC), genipin crosslinked-ELAC and crosslinked electrochemically compacted collagen (ECC, unaligned). The force mapping on BG-ELAC sample did not exhibit any area with high elastic modulus (measured modulus was around 0.6 MPa), indicating that there is no Bioglass particle protrusion observed at the BG-ELAC surface. Elastic modulus of ELAC molecules appears softer (~0.1 MPa) for samples without Bioglass added. Adding genipin crosslinker to collagen threads made the ELAC and ECC samples stiffer even more than uncrosslinked BG-ELAC with characteristic values of elastic modulus approximately 0.97 MPa and 1.28 MPa for crosslinked-ELAC and crosslinked ECC respectively. More extensive studies are necessary to fully investigate effect of crosslinking ratio and adding Bioglass at different concentrations on the elastic modulus values of collagen samples. Methodology reported here is a suitable tool for such studies and can be applied to other soft and potentially heterogeneous biomaterial samples.
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Symbols

$H$  Hardness of material

$P_{\text{max}}$  Maximum load

$A$  Projected area

$\nu, \sigma$  Poisson ratio

$E$  Elastic (Young’s modulus)

$E^*$  Reduced Young’s modulus

$S$  Stiffness of material

$S_{\text{max}}$  The initial contact stiffness

$P$  Applied load

$h$  The deformation of sample

$R$  Radius of curvature

$a$  Radius of contact area

$h_f$  The final depth of the residual hardness impression

$h_s$  The drift of the surface at perimeter of contact

$h_c$  Vertical displacement during the contact

$h_{\text{max}}$  Total displacement at any time during loading

$V_1$  Photodiode voltage

$\Delta Z_p$  Vertical displacement of piezo actuator

$Z_p$  The position of the base of the cantilever

$U_1$  The potential energy of the cantilever spring

$U_2$  The potential energy pf tip-sample interaction

$U_t$  Minimum potential at the equilibrium position of the tip

$U_s$  Potential energy due to the sample deformation
$U_c$ The energy resulting from the bending of the cantilever

$U_{cs}$ Tip-sample potential caused by surface forces

$d$ The cantilever deflection

$k_c$ The spring constant of the cantilever

$k_s$ Sample stiffness

$k_{interaction}$ Spring constant of tip-sample interaction

$D$ Tip-sample separation

$\delta$ The indentation depth

$L$ The length of the cantilever

$w$ Cantilever width

$\theta$ Cantilever thickness

$C^*$ A constant related to the selection of tip-sample contact point

$\alpha$ The semivertical angle of the tip
Abbreviations

LH$_2$ Liquid hydrogen
CNTs Carbone nanotubes
ECM Extracellular matrix
SEM Scanning electron microscopy
TEM transmission electron microscopy
AFM Atomic force microscope
ROC Radius of curvature
vdW van der Waals
PEO polyethyleneoxide
LRW London Resin White (plastic)
JKR Johnson, Kendall and Roberts Theory
DMT Derjaguin, Muller and Toporov Theory
ELAC Electrochemically aligned collagen
BG-ELAC Bioglass incorporated ELAC
ECC Electrochemically compacted collagen
Std Standard deviation of values
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Chapter 1 Introduction

1.1 Why are Mechanical Properties Measured?

The use of different kinds of materials depends on their mechanical properties, from hard structural materials to soft biomaterials. Knowing the mechanical properties of materials can facilitate their improvement [1]. For example, the cross linking process is sometimes recommended in plastics for improving their resistance to mechanical and chemical influences [2]. Here are several examples of processes where knowing the mechanical properties of polymeric materials is essential for successful development. Because of the rapid development of plastics and its derivatives, the plastic recycling process has become one of the largest global concerns over the last several decades. To prevent pollution and to recover both energy and material, mechanical properties of plastic materials have been manipulated to produce easily recycled plastics. To achieve this goal, investigation of the properties that are responsible for plastic degradation, e.g. the ability to granulate, and hence to reuse the reground materials have definitely been successful [3]. In the space field, a tank made from thin polymer is used to store liquid hydrogen LH$_2$, which is used to protect the astronauts from the high level of cosmic radiation at cryogenic temperatures. Investigation of mechanical properties such as permeability and Young's modulus of the film has been performed at cryogenic temperatures to monitor the stiffness of the LH$_2$ container and hence to preserve the LH$_2$ level [4]. In membrane manufacturing, mechanical properties for membranes used for water filtration, methanol fuel cells, coating and other important applications are undoubtedly worthwhile. Membranes should exhibit a proper strength that keeps their integrity during the achievement of their role even in
critical environments such as high pressure or temperatures [5]. Lastly, it was recently demonstrated that stiffness of substrates affects development and locomotion of cells [6, 7]. Therefore, knowing the local elastic modulus is important for the development of approaches aiming at controlling cell behavior.

1.2 Mechanical Properties at Nanoscale

Development of the nanocomposite industry has provided new functional materials. Mechanical performance can be controlled by adjusting the mechanical properties of material in nanoscale [8], even better than those of micro-particle-filled composites [9]. For example, the interfacial strength between the fiber and matrix in nanocomposite polymeric material is responsible for the general behavior of this material [8]. Carbon nanotubes (CNTs) have the highest strength and stiffness and the lowest density among all known materials [10]. Mixing polymeric material with CNTs creates smart (damage resistant with high surface to volume ratio) polymer matrix nanocomposites and improves the material mechanics [11]. CNTs are also widely used in polymer reinforcement and coating surfaces that require a robust mechanical performance such as in gears, drive shafts, body panels in airplanes [12,13], and instrumentation sensors such as probes in atomic force microscopy [14].

Decreasing sizes of materials to nanoscopic scale has become desirable to achieve durability and strength [15]. Over the last several years, understanding and probing nanomechanical properties have become of significant interest. In the biological field, the investigation of nanomechanical properties can lead to identifying biofunctions and
diagnosing many health problems [16]. For example, decreased rigidity is a marker for cancer cells, in addition to other diseases such as Alzheimer's dementia, and vascular and kidney diseases that occur due to losing the elasticity of cells [17], while stiffening of red blood cells in malaria is correlated with the deaths due to this disease [18]. Therefore, changes in mechanical properties of cells may affect disease progression.

One important recent application of nanoscience technology is in the field of tissue engineering, where substrates called scaffolds are tailored to support growing cells. The scaffolds are designed to mimic the in vivo chemical, topographical, and mechanical properties of the organ [19]. To build a strong tissue, scaffolds first should be mechanically strong to be easily handled. Second, the scaffold and tissue site in which the former is to be implanted have to be mechanically consistent with each other. Third, scaffolds should promote cell adhesion, which also strongly depends on consistency of the mechanical properties between cells and scaffolds that should be similar to that between cells and the extracellular matrix, ECM [20-30].

Development of materials with new and improved functions by manipulating nanoscale characteristics requires quantification of the mechanical properties of materials in that scale. This requires knowledge of important parameters, methods and theories that have been used to determine nanomechanical properties of materials.

1.3 Parameters for Studying Mechanical / Nanomechanical Behavior of Materials

One of the first quantities that has been determined as a mechanical characteristic of material is the hardness, $H$, which is a measure of material resistance to indentation, or
how much the material will deform under a load [31]. When a hard ball is pressed with a
fixed normal load into a smooth hard surface and equilibrium has been reached, the
indenter and hence the load are removed and the diameter of the permanent impression is
measured. Then, the hardness of the smooth surface is the ratio of the maximum load (at
the equilibrium $P_{\text{max}}$) to the projected area, $A$, of the indentation [32]

$$H = \frac{P_{\text{max}}}{A} \quad (1.1).$$

One more mechanical property of materials is the Poisson’s ratio, $\nu$, which
expresses how much the material extends orthogonally to the direction of applied force. It
is the ratio of the transverse / orthogonal strain to the strain along the direction of elongation
[33]. The Poisson’s ratio for soft materials approximately equals 0.5 [9].

The elasticity (elastic modulus) of material, $E$, is the ratio of stress to strain, where
stress is the deformation force per contact area, and the strain is the amount of the
deformation relative to the unperturbed state [34]. The elastic modulus is the material's
resistance to being elongated or compressed elastically. It is a constant value which can be
extracted from the linear portion of a stress-strain diagram. Figure 1.1 illustrates the stress
/ strain relationship for different kinds of materials. The deformation is elastic as long as
the relation is linear.
Figure 1.1 shows the stress-strain curve and illustrates different kinds of deformation. The slope of the linear region is the elastic (or Young’s) modulus [http://www.totalmateria.com/]

The stiffness, \( S \), of material is an amount of force, \( P \), that is required to deform the material structure [35]

\[
S = \frac{P}{h}
\]  

(1.2)

where \( h \) is the deformation of the sample caused by the force along the same degree of freedom.

1.4 Mechanical Properties Measurements

One of the first analytical approaches to study mechanical properties of materials was by Boussinesq in 1885 [36]. He first used potential theory to compute stresses and displacements for a homogenous-elastic body loaded vertically by a rigid-axisymmetric
indenters [36]. Then, the same procedure was used to derive solutions for other common indenter geometries such as conical and cylindrical [37, 38]. In 1896, Hertz recognized that the elastic contact plays a key role in mechanical properties determination [31] since the first response to interactions of two objects is elastic deformation [39]. He considered two elastic bodies with different radii of curvature and different elastic constants in contact at a point. Figure 1.2, [39] illustrates Hertz’s model for the elastic response when $R_1 = \infty$ for a flat surface and $R_2 = R$ for a spherical indenter [39].

![Diagram of Hertz model](image)

Figure 1.2 shows Hertz model for studying elastic contact between two surfaces with different radii and elastic constant [39].

By applying the load $P$, the bodies deform and create a contact area with radius of contact, $a$, which increases with the penetration depth, $h$, according to the equation:

$$a = \sqrt{Rh} \quad (1.3)$$
For elastic sample, removal of the load decreases the contact area back to the initial point contact. For an applied load, \( P \), and the indenter radius \( R \), the reduced elastic modulus \( E^* \) can be obtained from the contact area radius – load dependency:

\[
E^* = \frac{3PR}{4a^3} \tag{1.4}
\]

where

\[
\frac{1}{E^*} = \frac{(1 - v_1^2)}{E_1} + \frac{(1 - v_2^2)}{E_2} \tag{1.5}
\]

\( E_1 \) and \( v_1 \) are the elastic modulus and the Poisson ratio of the flat surface, and \( E_2 \) and \( v_2 \) are the elastic modulus and the Poisson ratio for the indenter. Unlike Boussinesq's model, Hertz's model can be used to analyze the effects of non-rigid indenters, since it can deal with a wide range of indenter elastic constants [31]. Hertz's approach is a basis for many experimental and theoretical works in contact mechanics [40]. However, Hertz's model can be used only in the case of a very low surface energy (surface adhesion), and the indenter load should be much higher than surface forces so the latter can be neglected. Meanwhile, the load should be limited to prevent wear and plastic deformation [41, 42].

Most materials exhibit a mixture of elastic and plastic behavior, and the indentation process might exhibit inelastic deformation. Moreover, application of the Hertzian model is limited to few simple shapes of indenters. Consequently, this approach has been replaced by elastic-half-space analysis, which was introduced by Sneddon [43]. He developed the Hertzian theory further by using general indenters of axisymmetric shape that can also be
described as a solid of revolution of a smooth function (e.g., a flat ended cylindrical punch, a parabolic of revolution, and a cone, and other shapes) to indent elastic half-space.

In the early 1970s, as an extension of Sneddon’s work, Bulychev, Alkhin, and co-workers [44-48] were interested in measuring the elastic modulus from load-displacement indentation. They used a microhardness testing machine to obtain load-displacement curves, Figure 1.3 [35], and determined the elastic modulus by using the equation:

\[
S = \frac{dP}{dh} = \frac{2}{\sqrt{\pi}} E_r \sqrt{A} \tag{1.6}
\]

Figure 1.3 shows the load-displacement indentation curve for typical materials with an elastic-half-space characteristic. \(h_f\) represents the final depth of the residual hardness impression [31].

where \(S\) is the stiffness of the upper linear portion (the elastic behavior) of the unloading data, \(E_r\) is the reduced modulus, and \(A\) is the projected area of elastic contact, which is the optically measured area of hardness impression. Therefore, the modulus of material can
be determined by measuring the initial unloading stiffness from the slope of the unloading curve and the contact area [31]. This equation was originally established for a conical indenter in elastic contact theory, though Bulychev et al. obtained reasonable results with this equation using spherical and cylindrical indenters, and expected the same applicability for other indenter geometries with square and triangular cross sections. Parr, Oliver and Brotzen also emphasized that the equation is suitable for any indenters with a body of revolution of smooth function (spherical and cylindrical) [49]. In turn, King in 1987 confirmed by the finite element calculation that this equation can be applied also on indenters of square and triangular cross sections, which are not bodies of revolution of a smooth function [50].

Due to the fact that thin film industries and hence mechanical investigations on a very small scale have become one of the foremost concerns during 1980's, instruments of submicron indentations, which are called traditional indenters, have been developed [51-54]. However, imaging a hardness impression for a very small indentation as a procedure to obtain the contact area was very difficult and time consuming. Focusing on this problem, Oliver, Hutching and Pethica proposed a simple method to determine the contact area in terms of the indenter area function; it is a cross-section area of the indenter as a function of the distance from its tip (contact depth) [55,56]. At the peak load, the material deforms and takes the shape of the indenter at some depth, which can be obtained from the load-displacement data in Figure. 1.3. There are two obvious depths that can easily be extrapolated from the load-displacement curve, the maximum displacement at the
maximum load, $h_{\text{max}}$, and the residual depth after final unloading, $h_f$. Then, the projected area of contact can be estimated from the shape function [56].

In 1986, Doerner and Nix provided an approach, which was considered as the most comprehensive method until 1992, interpreting the load-displacement data acquired from deep-sensing indentation instruments to determine the elastic modulus of thin films. According to this approach, the elastic indentation can be subtracted from the total displacement to calculate the hardness of material, and the elastic modulus is extracted from the initial unloading stages (unloading stiffness) [57]. By extrapolating the depth at zero load, the area of contact can also be calculated using the Oliver, Hutching, and Pethica approach [55, 56] to find the resolution. The authors observed in some materials that the elastic behavior of the initial unloading stages is similar to that of a flat cylindrical punch. In other words, during this stage the area of contact remains unchanged and hence its unloading portion is linear. Therefore, the slope of the linear portion $dP/dh$ (unloading stiffness) can be determined. However, the flat-punch approximation is not completely suitable for real material behavior. It was found that a large number of materials do not have any linear part in their unloading curve. In 1992 [31], the indentation load-displacement data for fused silica, soda-lime glass, a single crystal of aluminum, tungsten, quartz, and sapphire were obtained using Berkovich (a three sided pyramid) indenters, and the nonlinearity in unloading curves for each material was observed. Dynamic measurements of contact stiffness proved that the reason for this curvature reflects changes in the contact area continuously as the indenter is withdrawn [31].
To analyze these complex data, modeling Berkovich indenters, a method of taking into account the curvature in unloading curves and determining the depth in the absence of linear portions have been reported by Oliver and Pharr [31]. Based on the Sneddon theory, which had derived analytical solutions for several indenter geometries, conical and paraboloid of revolution shapes were selected to compare with elastic unloading of Berkovich indenters. Like Berkovich indenter, the conical indenter has a cross section area that is a function of $h^2$. Additionally, the conical indenter tip is similar to that of the Berkovich indenter; they have similar geometries at a small indentation depth. The paraboloid of revolution was chosen as a realistic indenter (the real indenters are rounded at the apex because it is impossible for indenter to be perfectly sharp). Previous methods had attempted to manipulate the unloading curvature, in which a straight line of the upper portion of the unloading curve was taken to measure the slope (the stiffness). However, the stiffness value depends on how much of the data points are in this line. Further, the stiffness obtained from the first and final unloading are very different because of the creep in the first curve. Attempts have been made to minimize the creep by holding the indenter for a period of time before the final unloading, yet a problem related to the data point-line fitting still exists.

It was found that the stiffness obtained from the power law, Sneddon's expression, for the first unloading data is only a little greater than that for the final curve. This indicates that the creep is not observed and hence the power law is recommended to be used for analyzing data. Parameters used in this analysis are shown in Figure 1.4 [31], which is a scheme of a cross section of indentation.
Figure 1.4 shows a scheme of the across section through the indentation of a typical sample in the case of elastic half space [31].

In this figure, \( h_{\text{max}} \) is the total displacement at any time during loading, \( h_c \) is the vertical displacement during the contact, \( h_s \) is the drift of the surface at the perimeter of contact, and \( h_f \) is the final depth of the residual hardness impression after the indenter is fully withdrawn and the surface has elastically recovered. Another scheme that illustrates the key parameters is given in Figure 1.5 [31]. Based on Sneddon's model, the loading-unloading data were analyzed in the elastic response region. Because of the nonlinearity, the initial contact stiffness, \( S_{\text{max}} \), was measured only at the peak load, \( P_{\text{max}} \),

\[
S_{\text{max}} = \frac{P_{\text{max}}}{h_{\text{max}}} \quad (1.7)
\]

Then, the elastic modulus can be determined by

\[
E_t = \frac{S_{\text{max}}\sqrt{\pi}}{2\sqrt{A}} \quad (1.8)
\]
Figure 1.5 illustrates the key parameters in Fig. 1.4 for an elastic half space material [31].

To obtain the contact area, $A$, the indenter geometry and hence its area function, $f(h)$, should be known. Then, the projected area can be calculated at the peak load by

$$A = f(h_c) \quad \text{(1.9)}$$

where

$$h_c = h_{\text{max}} - h_s \quad \text{(1.10)}$$

The $h_{\text{max}}$ can be experimentally measured at the peak load. According to Sneddon's expression for the shape of the surface outside the contact area [44], $h_s$ for the conical indenter is given by:

$$h_s = \frac{(\pi - 2) (h - h_f)}{\pi} \quad \text{(1.11)}$$
where \((h - h_i)\) represents only the elastic displacement. The Sneddon's expression of the stiffness, \(S\), over this distance for the conical indenter is:

\[
S = \frac{2 P}{(h - h_f)} \quad (1.12)
\]

Therefore: for the projected area at the peak load \(P_{\text{max}}\)

\[
h_s = \varepsilon \frac{P_{\text{max}}}{S_{\text{max}}} \quad (1.13)
\]

where \(\varepsilon\) equals to \(2(\pi - 2) / \pi \approx 0.72, 0.75\) or 1 for a conical indenter, a paraboloid of revolution indenter or a flat punch indenter, respectively. Hence, \(h_s\) for the flat punch equals \(P_{\text{max}} / S_{\text{max}}\), then \(h_c\) is given by:

\[
h_c = h_{\text{max}} - \frac{P_{\text{max}}}{S_{\text{max}}} \quad (1.14)
\]

which is the same as that in the Doerner and Nix expression for a flat punch. For a conical indenter:

\[
h_c = h_{\text{max}} - 0.72 \frac{P_{\text{max}}}{S_{\text{max}}} \quad (1.15)
\]

and for a paraboloid of revolution:

\[
h_c = h_{\text{max}} - 0.75 \frac{P_{\text{max}}}{S_{\text{max}}} \quad (1.16)
\]
Using $\epsilon = 0.72$ or 0.75 depends on which of the indenter geometries are the best for describing the experimental data. Depending on the power law exponent, $m$, values that fit in the unloading curve, the unloading was best described by a paraboloid of revolution [31]. Although the difference with the conical $\epsilon$ is insignificant, there are other reasons for selecting paraboloid geometry to represent the Berkovich indenter in this experiment. The pressure distribution around the tip of the paraboloid is more realistic. Second, it is relatively difficult to deal with a conical indenter because any plastic deformation on the apex leads to complete loss of important characteristics, especially the elastic properties [31].

**1.5 Nanoindentation Techniques**

Traditional indentations for measuring mechanical properties have additional indirect steps; they are restricted by imaging the surface after the experiment in order to determine the area of hardness impression (the plastically deformed area) [58]. Scanning electron microscopy (SEM) or transmission electron microscopy (TEM) is required after microindentation experiments. Further, imaging the shallow indenters by these techniques is often unclear and leads to inaccurate measurements. For these reasons, traditional indentation measurements have become inconvenient and time consuming. After development of nanoscale science, traditional indentation measurements became more tedious and difficult for acquiring reliable results since microindenters do not allow for the investigation of nanomechanical properties. In the last 20 years, it was realized that there is a deep relationship between nanoscale interactions and the macroscale behavior of materials [9]. This implication has been used to improve and develop materials in the
engineering and biological fields [9]. For example, dispersing nanoparticles uniformly in polymer matrices improves their mechanical properties such as modulus, hardness, and even damage resistance.

Nanoindentation technique has been developed and widely used to determine elastic modulus, hardness, and other mechanical properties at nanoscale, e. g. in studying properties of thin-films [59]. Its applications have dramatically grown during the past 20 years in medical and microbiological fields to diagnose and monitor the mechanical properties of cells that are linked to disease progression [60]. In the nanoindentation approach, an indenter with a known tip geometry and nanoscale radius is loaded with gradually increasing tiny forces to penetrate the sample by a small amount of depth, and the mechanical properties can be estimated using the indentation force-displacement data [60, 58]. The advantage of this technique over traditional indentations is the ability to directly determine mechanical properties by analyses of indentation load-displacement data alone, without the need for imaging the hardness impression [61]. It was found that using nanoindenters with soft materials results in the “skin effect”, which is the overestimation of modulus at small indentation depth [62]. This occurs because of the existence of viscoelastic behavior or sample creep (especially when a sharp probe is used), which leads to an error in the surface detection and hence to the use of an incorrect projection contact area in the calculation of the modulus. In addition to the nonlinearity of the stress-strain relationship, the skin effect can also originate from analyzing the data by using a model that does not take surface adhesion into the account [62]. Indentations by nanoindenters can only be analyzed by the Oliver and Pharr method, in which a small
indentation is made by an arbitrary sharp indenter (e.g. Berkovich indenter), and does not take surface adhesion into account [63].

1.5.1 Nanoindentation by Atomic Force Microscope, AFM

A number of investigations of nanomechanical properties for soft materials and thin layers, as in biomaterial, biological, and microbiological studies, by non-destructive and in situ measurements have dramatically increased during the last decades [64-70]. In the diagnostic field, measuring nanomechanical properties of cells is becoming a useful tool to monitor disease progression [71].

At present, the direct local probing of mechanical properties of hard, soft, thin layer, and complex materials non-destructively and in situ with nanoscale resolution has become a reality after the development of the atomic force microscope, AFM [72]. It is considered to be the most accurate technique for quantitative measurements of mechanical and dynamic properties at nanoscale [17]. A small tip diameter (radius of curvature down to <10 nm) makes the AFM instrument able to apply small loads (<1 nN) locally and for relatively hard samples produces very small deformation (0.02 nm) [17]. Consequently, a very high spatial image resolution can be obtained by AFM [17] and local topographic information can be gained. Spatial resolution in topographic imaging is related to the mechanical properties. AFM is a versatile tool that can be used for variety of materials whether conducting or insulating, and under variety of conditions (vacuum, air or liquid medium) [58, 60, 71, 72].
The general principle of AFM technique is fairly simple as illustrated in Figure 1.6. The sample mounted on a piezo-ceramic electric scanner is contacted by a sharp tip fixed at the end of a flexible cantilever that is held in the probe holder. During indentation measurements, the tip first goes down to contact with the sample and then indents into the surface until a previously specified value of compressive force is applied to the sample [67]. This part of the motion cycle is called “approach”. Then the probe reverses its motion and brings the probe to the initial position. This part of the motion is called “withdraw”. Once the tip senses the surface forces, the flexible cantilever bends, and its deflection is measured with high precision by the optical lever method. It is based on focusing a laser beam on the top of the probe, so the cantilever deflection causes the laser reflection to a new position on a segmented photodiode as shown in Figure 1.6.

Figure 1.6 shows the principle scheme of the atomic force microscope.
Thus the deflection changes the photodiode voltage, $V_t$, which is monitored as a function of the vertical displacement of the piezo actuator, $\Delta Z_p$ as shown as deflection vs. displacement graph in Figure 1.7 [67]. During the probe approach the deflection voltage remains constant (in the absence of long-range forces) until the tip jumps to contact with the surface under influence of short-range (nanometer scale) surface attractive forces [67]. This results in a rapid decrease in deflection voltage (region B-C in Figure 1.7). A further displacement of the piezo actuator increases the repulsive forces that deflect cantilever in the opposite direction, causing an increase in the $V_t$ signal (region C-D in Figure 1.7). When the repulsion reaches the maximum, the unloading process starts, and the cantilever deflection will gradually decrease until the tip separates from the surface. In many cases, during the unloading process the tip adheres to the surface, leading to an increase in the attractive forces (negative value) until the force reaches maximum tensile value (point E in Figure 1.7). Then the tip separates from the sample (region E-F in Figure 1.7).

![Deflection-displacement curve](image)

Figure 1.7 shows deflection-displacement curve involving different types of tip-sample interactions [67]

### 1.5.2 Forces during the Tip-Sample Contact
In AFM measurements surface forces play a major role in forming nanometer scale contacts between the two surfaces [58]. Therefore, to achieve nondestructive characterization with high spatial resolutions, the AFM operation should be within the limited range of attractive and repulsive forces. Two major forces during tip-sample interactions are the repulsive force and the attractive van der Waals (vdW) force [73, 74]. The repulsive force results from the overlap of electrons between the tip and sample atoms; therefore, it is a short range (~ angstroms) and localized force. The long-range vdW force (typical range 5-15 nm) originates from induced dipoles (polar molecules attract nonpolar molecules by changing the arrangement of their electrons and thus inducing the dipole moment) and/or dispersion force interactions and, unlike the repulsive force, it is not very localized and can include hundreds of the nearest atoms [75]. This force is responsible for the nanoscale jump to contact and for the same pair of materials varies depending on surface roughness. Therefore, especially for biological and soft samples, to keep the sample undamaged and to minimize the error in measurements of elastic parameters, the vdW force should be controlled. This can be achieved for example by performing experiments in a liquid medium. It was found that the AFM measurements have higher signal to noise ratio in water than in a vacuum or air because of the significant reduction of vdW force [76, 77]. This substantial decrease in vdW force is mainly attributed to increasing the uniformity of the polarizable system created during the tip-sample contact in a liquid medium [75].

There is another type of attractive force when performing AFM measurements in ambient air at considerable humidity that is called the capillary force. It results from
condensation of water at the place of tip-sample contact, thus creating a liquid meniscus bridging the tip and sample surfaces [78]. It might lead to excessive attraction and hence, inaccurate measurements. One convenient way to eliminate capillary force is to perform the experiment in a liquid medium [79].

The tip-sample interaction forces acting between the tip and the surface during the approach-withdraw cycle are illustrated in Figure 1.8. The cantilever, which is considered as a Hookean spring, has a potential energy of $U_1 = \frac{1}{2} k_c d^2$, where $k_c$ is the spring constant and $d$ is the deflection of the cantilever. The tip-sample interaction has a potential energy $U_2$ that includes the sum of energies of the attractive and repulsive forces. The second derivative of this potential with respect to the tip-sample separation $D$ is the spring constant of interaction $k_{\text{interaction}} = -\frac{dU_2}{dD}$. This spring constant is positive for repulsive forces and negative for attractive forces. The equilibrium position of the tip corresponds to the minimum of total potential, $U_t = U_1 + U_2$. If $k_c < -k_{\text{interaction}}$, the cantilever will jump in contact with the sample surface, and if $k_c > -k_{\text{interaction}}$, the cantilever will jump out of contact with sample surface [39]. For elastic deformation, the total potential of the system can be described by:

\[
U_t = U_{cs}(D) + U_c(Z_c) + U_s(\delta) 
\tag{1.17}
\]

\[
= U_{cs}(D) + \frac{1}{2} k_c Z_c^2 + \frac{1}{2} k_s \delta^2 
\tag{1.18}
\]
where $U_{cs}$ represents the tip-sample interaction potential caused by surface forces, $U_c$ is the energy resulting from the bending of the cantilever, $U_s$ is the potential energy due to the sample deformation, $k_s$ is the sample stiffness, which is a resistance to the sample deformation, $\delta$ is the indentation depth, and $D$ is the actual tip-sample separation $D = Z_p - (Z_c + \delta)$ where $Z_p$ is the position of the base of the cantilever (piezo position). At the contact point, $D$ equals 0 and $Z_p = Z_c + \delta$. At equilibrium, maximum indentation, we have $k_s \delta = k_c Z_c$ [71].

Figure 1.8 represents the forces between tip and sample surface as a function of tip-sample distance [https://www.researchgate.net/].

1.6 Measurements of Nanomechanical Properties by AFM

The first investigation of mechanical properties using AFM was by Burnham and Cotton in 1989 [80]. They used the force mode of AFM for three different materials. Pure
elastic (elastomer), pure plastic (gold), and a material in between (graphite) were indented by a tungsten tip with radius $R = 100$-$200$ nm and attached to cantilever with $k_c = 50 \pm 10$ N/m. If such a cantilever is bent by 20 nm, then the applied load can be as large as 1000 nN. The characteristic force curves of the three materials are shown in Figure 1.9 [80]. Sneddon's solution for a flat-ended cylindrical indenter [43] was used to calculate the elastic modulus values, which were comparable with what had been obtained by other methods. This work started application of AFM as a method to directly determine the mechanical properties of a wide range of materials.

Figure 1.9 displays the force curves of three different materials: a) elastomer, b) purely plastic (gold) and c) the most common elastic half-space material (graphite) [44].

Later, different AFM methods were developed to measure mechanical properties of materials. These methods are bending/tensile test [81-85], dynamic AFM [86-90], as well as nanoindentation methods [91-96].
1.6.1 Bending / Tensile Testing

In a bending / tensile test, the tensile load is applied on the fibrous specimen and the resulting elongation is measured. The sample is mounted on a microscope stage equipped with a camera to determine the deformation and geometrical dimensions of the sample [85]. The load acting on the sample is determined by a piezo resistive AFM cantilever whose tip is placed at the free end of the specimen. Then, the elastic modulus of the sample can be determined using standard beam theory [84] assuming a uniform rectangular cross section cantilever:

\[
E = \frac{4L^3F}{w\theta^3}\delta
\]  

(1.19)

where \( L \) is the length of the cantilever, \( w \) is the cantilever width, \( \theta \) is the cantilever thickness, and \( F/\delta \) is the slope of load-displacement curve. This method is suitable for in situ applications; however, it has significant drawbacks [84]. First, while the force measurements are carried out by the piezo resistive AFM cantilever, the displacement cannot be measured by AFM because of the hysteresis in the piezoelectric AFM cantilever; hence, an additional device such as SEM is needed to measure the displacement accurately [84]. Even with additional accessories, accurate and reproducible results are limited to measurements only in a vacuum state [84] and for only stretched materials such as polyethyleneoxide (PEO) nanofiber as illustrated in Figure 1.10 [85].

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1.6.2 Applications of Dynamic AFM to Characterize Mechanical Properties

Dynamic atomic force microscope techniques have been developed to obtain high resolution topographic images and compositional information about sample surfaces in gentle and non-destructive ways [86]. This method is especially suitable for soft samples that can be easily damaged by high adhesion, friction or high electrostatic forces, or for materials that cannot be tightly fixed on their substrates. Dynamic AFM methods include the amplitude modulation AFM (tapping mode) and the non-contact mode.

1.6.2.1 Force Modulation-AFM, FM-AFM

The dynamic mode technique called force-modulation, AFM, allows the imaging of areas on hard surfaces. In this technique the sample height is modulated when measuring a topographic image and a map of surface elasticities is created (force modulation image) by observing the modulation of cantilever amplitude at the frequency of sample modulation.
[97]. After keeping the tip-sample force constant by the feedback loop, a small motion (~1-10 nm amplitude) $\Delta Z_m$, is introduced to the sample surface in $Z$ direction. This leads to an additional cantilever deflection, $d$, and hence a variation in the tip-sample force. The upper and lower positions of the cantilever are measured to calculate the cantilever deflection $d$. Then, the quantity $d / \Delta Z_m$ is used to build the force modulation image. The amount of variation of the average force depends on the dynamic response represented in the viscoelastic properties of the sample. This response is characterized by the sample spring constant, $k_s$, as follows:

$$ k_s = \frac{\Delta F}{\Delta Z_m - d} $$

where $(\Delta Z_m - d)$ is the sample deformation by the acting force $\Delta F$. In the absence of adhesion forces (no energy dissipation) we have:

$$ k_c d = k_s (\Delta Z_m - d) $$

$$ \frac{\Delta Z_m}{d} = \frac{k_c}{k_s} + 1 $$

If $k_c / k_s << 1$, the sample is much stiffer to be deformed and the contrast based on surface elasticities cannot be obtained. Therefore, stiff cantilevers (depending on the elastic modulus of the sample, $k_c > 1$ N /m for polymeric samples and up to ~1000 N/m for hard samples) are necessary for acquiring the force modulation images. During force modulation imaging the soft areas deform more than hard areas on the sample surface (the
cantilever deflection $d$ is less over the soft area). According to the Hertz model (with no adhesion force), the force $\Delta F$ acting on a surface of modulus $E_s$, and required to deform it by pressing a spherical indenter of radius $R$ to the depth $\delta$ is expressed as:

$$\Delta F = (E^2_s R \delta^3)^{1/2}$$

(1.23)

The first application of force modulation model was by P. Maivald et al. in 1991 [97], in which a contrast resulting from variations in local surface elasticity and topographic images were obtained for carbon fibers and epoxy composite, $E$ ranges from 3.5 GPa to $2.3 \times 10^2$ GPa. Figure 1.11 [97] displays these images, for which a stiff cantilever ($k_c = 3000$ N/m) was fabricated with a diamond tip of radius ($R = 10 \, \mu$m) to apply a repulsive force of $10^{-3}$ N in air.

Figure 1.11 shows force-modulation imaging of carbon fiber and epoxy composite. Panels in the figure show a) topographic image, b) stiffness in the force modulation image. Here bright spots correspond to stiff areas. Image width is 32 µm. The figure reproduced with permission from IOP science [97].
As illustrated in the topographic image in Figure 1.11, carbon fibers show up higher than the surrounding epoxy. The force modulation image exhibits the variation in local surface elasticity and also reveals this structure across the cross section of fibers. Features in the force modulation image generally do not match perfectly with features in the topographic image confirming that each image has its own information source [97]. This technique can be used to characterize nanoscale heterogeneity of mechanical properties and surface defects. However, application of this technique is limited to relatively hard samples [98]. On soft samples and samples with adhesion, force-modulation imaging might produce excessive lateral forces that will damage samples. Moreover, on sticky samples, the adhesive force adds to the average compressive force. Consequently this might produce false contrast in the image. Force modulation measurements were previously carried out on tissue embedded in London Resin White, LRW (a hydrophilic acrylic resin used for embedding biological samples) [99], which have modulus values almost equal 0.5 GPa. The image of elasticity was damaged during measurements as shown in Figure 1.12 [99].
Figure 1.12 illustrates topography (A) and elasticity (B) of etched LRW embedded sample. Panel B shows the difficulties of measuring elastic differences in the components [98].

1.6.2.2 AFM Phase Imaging

Tapping mode imaging is performed by exciting cantilever oscillation at or near its resonance frequency. Then the tip at the end of the oscillating cantilever interacts with the sample surface, and the local force gradient shifts the resonance frequency of the cantilever. This in turn changes the amplitude of the cantilever oscillation. Imaging is performed by scanning across the sample surface and using a feedback loop to maintain the amplitude of the cantilever oscillation. It is noticed that the phase of the oscillating cantilever depends on the mechanical and adhesive properties of the sample. More specifically, the phase shift for the cantilever excited at its resonance frequency is proportional to the energy dissipated during each contact of the tip with the sample. Phase shift can be measured along with the topographic image. Contrast in the phase image indicates the variation in elastic and/or
adhesive properties of the sample surface [100]. Therefore, phase imaging cannot be used to obtain quantitative information about the elastic modulus.

1.7 Current Applications of AFM Nanoindentation on Soft Materials and Associated Challenges

The ability to continuously and accurately measure stiffness at various indentation depths [101] nominates Nanoindentation methods to be used in determination of nanomechanical properties of a wide variety of materials [102]. However, the relatively large indentation forces (several μN) applied in this technique restricts it from being used with soft materials, on which the nonlinear stress-strain relationship likely occurs. For example, the strong indentation of biological cells can cause a disruption of cellular components [103]. Furthermore, the skin effect, which is the overestimation of modulus at small indentation depth and hence exceeding the elastic limit, occurs with soft samples measured by nanoindenters, in which the data are analyzed by the Oliver-Pharr model [104]. Another downside in nanoindenters is the need of additional optical device to complete the measurements. This external procedure causes time consuming and decreases the accuracy of measurements, which became depend on the resolution of this device [101].

The recent applications on soft materials, such as tissue engineering and biological fields, concern with investigation of nanomechanical properties and submicro features on the outermost surface. Further, imaging and concurrently determining nanomechanical characterization under physiological conditions became very important for either monitoring cell progression or improving biomaterials [101]. These multiple purposes, in
addition to the versatility of using different models to analyze data, demand the using of AFM nanoindentation for applying forces of a few piconewton and achieve very small deformation (~ 0.1 nm) [101], and to simultaneously image the indentation site under desirable conditions [105]. Recently, AFM nanoindentation has been used to study the skin effect of different polymers using probes with different radii (22, 810 and 1030 nm) and analyzing the data by different models (Oliver-Pharr, JKR, Hertz and DMT) [104]. This report stated that the skin effect disappeared by using the large radius probes and analyzing the data by either JKR or DMT, which take the adhesion into account. When using the dull probes, the results started to be close to the bulk moduli at indentations 2-3 nm, which considered as the smallest depth for soft samples to reach the bulk modulus. However, in the case of sharp tip the bulk moduli could not be reached until the indentation was 90 nm. Consequently, the skin effect originates from either using a sharp tip that can break the stress-strain linearity, or using improper model such as Oliver-Pharr to extract modulus values [104]. In biological field, AFM nanoindentation was applied to distinguish between healthy and disease cells and also monitor the disease progression [106]. Intensive studies have been made by AFM nanoindentation on normal, benign and tumor cells [107, 108]. It was found that the flexibility of tumor cell diffusion can be attributed to its lower stiffness with respect to normal cell [108, 106]. Because of the AFM ability to characterize micro and nanoscale feature sizes, AFM nanoindentation has widely applied to study microorganisms and viruses. Mechanical heterogeneity of bacterial surface and its biofilm have been examined [109]. Determination of local stiffness on viral surfaces demonstrated that their elasticity related to the virus inactivation reaction [110]. Additionally, this approach has been used to determine elastic modulus of hydrogels and thin layers of soft
materials [68, 111, 104, 112, 113], and some of the results were agree with macroscopically measured values [68, 121]. More developed AFM nanoindentation method has been performed using force-volume mode [114], which also called force mapping, in which a matrix of force curves are collected across the sample surface. It has been widely used with soft samples as a solution to avoid the lateral forces that result from contact-imaging [115]. During force mapping, the tip is completely separated from the surface before indenting the other point. Some of its recent applications are to produce maps of elasticity and topographic images for human cells at different stages of disease progression.

Nonetheless, challenges for AFM nanoindentation to achieve accurate measurements on soft samples still exist. These difficulties are related to either sample nature such as viscoelasticity and adhesion phenomena, or experimental setup represented in probe geometry and size, the cantilever stiffness, identification of probe-sample contact point [104], and using improper model to extract the Young's modulus of materials [101].

1.7.1 Effects of Viscoelasticity in Force-Indentation Measurements

Viscoelastic materials have important applications as adhesives, coatings, biosensors and lubricants [111]. Therefore, determining the viscoelastic behavior accurately for these materials has recently a considerable concern. Attempts have been made to access the viscoelastic property of soft materials; one of them is the study the dependence of tip-sample adhesion on loading speed and also on temperature, but these procedures have lack of quantitative information regards the viscoelastic characterization [116]. To investigate the interfacial viscoelasticity of materials, microparticles were attached to a tipless AFM to study the adhesion with mica surface [117, 118] and then
analyzing the data by different theories to explore the adhesion contributions [117, 120, 121]. Recently, Cappella et al proposed an approach to determine the viscoelasticity by studying the variation of elastic modulus with changing both time and temperature of the experiment [122]. Yet, these attempts used indirect ways to investigate the viscoelastic behavior. It used the frequency of voltage instead of piezo displacement rate, which directly related to the complete travel of the piezo that can reflect the irreversible sample deformation [122, 123]. Another suggestion to provide information about viscoelasticity was the strain rate. Using a conical probe in AFM nanoindentation to explore the viscoelasticity in term of the strain rate was arguable procedure. Although the self-similarity of the conical probe as an axisymmetric shape, the stress field from this tip is highly complicated because the surface points located in the tip domain are not experiencing the same strain during the indentation process [111]. For this reason, indenter rate was introduced instead of strain rate to characterize viscoelastic behavior and its repeatability. In this work [111], the variations of Young's modulus with both temperature and the indenter rate changes were identified, which reflected the viscoelastic behavior of poly propylene glycol material. Figure 1.13 shows the dependence of evaluated Young's modulus on both temperature and indenter rates.
Figure 1.13 shows the dependence of elastic modulus of polyethylene glycol on indenter rate and temperature. The elastic modulus changes significantly at high indenter rate and low temperature [111].

The plots illustrate the high dependence of modulus on the indenter rate at low temperature. It was found that the viscoelastic property is reproducible as long as the basic AFM steps have achieved [111].

1.7.2 Cantilever and Tip Characteristics

Even though the sharp tip with ROC~ 10 nm provides high resolution and can sense the supported cellular membranes [124], the tip of large ROC is recommended for soft and/or thin [74] samples to minimize plastic deformation and reduce damaging [125, 126]. The large tip radius exerts a slight pressure and contact gently with soft samples. However, in many cases the dull probes likely provided incorrect values of elastic modulus if the sample roughness would not be taken into account [101]. Spherical tips have ROC similar or even larger than the roughness of biological surfaces. This leads to the incorrect estimation of
the tip-sample contact point. Therefore, a relatively sharp probe with ROC smaller than sample roughness should be considered [127]. The Geometrical changes in the tip shape have significant effects on elastic modulus values. Using spherical probe with (ROC ~ 2) μm causes a dramatic decrease in modulus compared to that using pyramidal probe (ROC ~ 50 nm) [128]. On the other hand, using probes with the same ROC but different spring constants can affect Young's moduli. The spring constant is a marker to what extent the cantilever can deflect, and by this deflection the mechanical properties of samples will be detected [101]. To achieve an accurate detection for surface elasticity, the cantilever stiffness should be close to that of sample. Indentation of soft materials by stiff cantilever can exceed the elastic limit and damage the sample. Soft cantilever will be damaged if it scans a much stiffer surface.

### 1.7.3 Effects of Indentation depth

There are two main effects related to the penetration depth and lead to obvious errors in elastic modulus values. First, the indentation that similar or less than sample roughness affects the true tip-sample contact area. In other words, very small indentation in biological surfaces leads to miss contact with many points beneath the tip curvature and hence large error in elastic modulus calculations [101]. To solve this problem, the penetration depth must be much larger than sample roughness [129]. The rule of thumb can be applied in this case, which stated that the roughness / indentation depth ratio should be $\leq \frac{1}{10}$ [101].

The second reason is represented in the substrate effect, which is the overestimation of Young's modulus [101], and can be seen in the case of compliant material with small
thickness [130, 131]. This effect appears at the indentation site that either pile-up when the tip indents the soft thin film deposited on a hard surface, or sink-in when the indentation occurs at a hard material fixed on soft substrate [132]. To eliminate the substrate effect, the indentation depth must be less than 10% of the entire sample thickness [31, 133]. Based on Oliver and Pharr's, models have been developed to improve the accuracy of modulus. Hay and Crawford [134] were enabled to eliminate the substrate effect and accurately determine elastic modulus through an indentation depth up to 25% from the sample thickness. However, it was noted that in AFM indentation measurements substrate effects might be expected when the radius of contact area is similar to the sample thickness, even though sample indentation might still be small in comparison to the sample thickness [135].

Therefore estimation of geometry of tip-sample contact is necessary in order to understand whether the substrate effect can be avoided in particular experiment. Overall, the main steps to deal with soft thin materials in AFM nanoindentation technique are to examine the sample roughness and its total thickness, then perform an indentation ten times more than roughness scale. For thin samples using sharp probe and low indentation force might be necessary to avoid systematic error associated with hard substrate. For analyzing data of thin films, using infinite thickness models may cause large errors in elastic modulus values [135, 136, 137]. In the latter reference, Akhremitchev and Walker used Dhaliwal and Rau model [138] to calculate elastic modulus for finite sample thickness and indicate that the errors result from models of linear elasticity can be an order of magnitude.

1.7.4 Effects of Model Selection
Models that do not take into account the obvious phenomena of materials cannot provide accurate values of modulus. Even though Hertzian and Oliver-Pharr models are considered as conventional methods for many materials, challenges occur when applying these methods on compliant and hydrated biomaterials [113]. For example, the Oliver Pharr model can only deal with the elastic-plastic response of the sample without taking the adhesion into account [139], resulting in large errors in modulus values of adhesive biological samples. Other viscoelastic models have been proposed to calculate the storage modulus of viscoelastic materials under a very low load to achieve only Hertzian interactions [140], but the very small indentation can increase the error in modulus values. Applying load much higher than adhesion forces or performing the experiments under water can also minimize the adhesion and facilitate using the viscoelastic models. However, using high load or water might damage the sample or change its physical properties, respectively. Therefore, a comprehensive model that compromise between all significant phenomena is required to diminish the error ratio. In fact, it is still complicated to develop a model that can deal with both viscoelastic and adhesion properties of material [141]. This can be attributed to the dependence of both adhesion and viscoelastic properties on frequency.

The obstacle to use Hertz model with adhesive samples is that the adhesion property of biological samples enlarges the contact area between tip and sample surface, which conflicts with Hertzian concept that requires a very small contact area to ignore the adhesion. The JKR model can be applied to model the indentation by a large spherical probe that creates a large contact area, and then the adhesion can be taken into account.
However, the JKR model has some limitations since it cannot be valid for a wide range of materials such as viscoelastic samples, and works only with a perfectly spherical tip [113]. To develop this model and make it more versatile, a model which based on the adhesive interaction between AFM tips and samples, called two-point method, has been proposed in previous work [142]. Many problems related to soft thin films and large adhesive materials have been overcome by modeling the induced adhesive tip-sample interactions. In this method, only a passive indentation is induced by tip-sample adhesion, minimizing the interference from the substrate compared to what is found using the indentation method [142]. Furthermore, it does not require locating the tip-sample contact point, which is a challenge in adhesive soft samples, since the adhesive interactions are represented by fitting the whole retraction force curves. In this report, the two point method was tested on a series of poly (dimethyl siloxane) polymer samples with different degrees of crosslinking, and the results were consistent with macroscopic data [143]. Because of the high accuracy of this model [144, 140], it was later applied in several researches. One of them was for calculating the elastic modulus of poly (vinyl acetate), PVAc, films in both glassy and rubbery states. First, elastic modulus values were calculated from measured force curves by using Hertz equation. Then, the modulus values were used to get the JKR theoretical curves, which exhibited a good agreement with the experimental data in the case of glassy samples, indicating the validation of two-point method for calculating the elastic modulus of glassy polymers. On the other hand, a little deviation between the experimental data of rubbery samples and the theoretical curves can be observed. This inconsistency might be attributed to the viscoelastic interaction between the probe and sample, indicating that the two-point method works only with elastic manner [140, 142]
1.7.5 Problems with Contact Point Identification

One of the most common problems during nanoindentation of soft materials is misidentification of contact point, Guo and Akhremitchev [145] stated that almost 10% of systemic errors are related to incorrect selection of contact point. Missing the contact point can result from either the high level of noise in the instrument or the high adhesion on the sample surface, which considerably exists in biological materials [144]. Attempts have been made to correctly identify the tip-sample contact point on soft and adhesive samples. Jaasma et al [146] extracted the contact point from the linear relationship of derivative of cantilever deflection to its base position. Extrapolation data to zero value yields the point at contact or separation position. However, this method requires data to be in the elastic limit (very small indentation). On the other hand, at this position the interactive forces are significant and cause high level of noise that can affect the accuracy of derivation. To manipulate this situation, measurements might be performed at sufficiently large indentation to minimize the level of noise [146]. Yet, this leads definitely to damage the soft biological surfaces. Another suggestion has been introduced by Guo and Akhremitchev [145], their idea start from the fact that accurately determination of Young’s modulus from Hertizian expression (equation 1.24) is limited with a correct value of the indentation depth, $\delta$.

$$F = \frac{4ER^2}{3(1-\nu^2)}\delta^3$$  \hspace{1cm} (1.24)

Where $F$ is the load, $R$ is the probe’s radius of curvature, $\delta$ is the indentation, $\nu$ is the Poisson ratio of the elastic solid, $E$ is the Young’s modulus. Yet, it is difficult to obtain
the accurate value of $\delta$ because of the hardly determine the tip-sample contact point. Therefore, Guo and Akhremitchev wrote the Hertzian equation in a way by which the Young’s modulus can be calculated from the dependence of applied force on the tip-sample distance, $D$, regardless their contact point. They states that the relation between $D$ and $\delta$ can be written as:

$$\delta = C - D$$ \hspace{1cm} (1.25)

Therefore, the Hertzian equation can be expressed in terms of equation (1.25) as follows:

$$F^{2/3} = C^* - \left[ \frac{4\pi R Z}{3(1-\sigma^2)} \right]^{2/3}$$ \hspace{1cm} (1.26)

$C^*$ is another constant that reflects the selection of tip-sample contact point.

From this linear equation, the post contact region points can be plotted and then the Young’s modulus can be extracted from the slope of the line. Noticeable, a systematic error of only $\sim 10\%$ might occur, which results from either the probe radius of curvature or the estimated Poisson’s ratio. This uncertainty is found to be less than that of cantilever spring constant [145]. Yet, this approach can be used only in elastic limit where the indentation is non-interactive [144].

1.8 The Aim of Research

Methods of measuring elastic properties of materials described above suffer from different limitations that prevent comprehensive characterization of mechanical properties of soft samples. Specifically, techniques capable of mapping mechanical and adhesive properties often provide only a relative map of elastic properties (e.g. force modulation and
phase imaging described above). Techniques capable of extracting the absolute value of
the local modulus (force-indentation measurements) often report only statistical values of
the elastic modulus without providing information regarding the spatial distribution of
moduli values [147-150]. More advanced studies that generate maps of elastic moduli of
soft samples do not consider the effects of adhesion and viscoelasticity [151,152]. It is the
goal of the current study to develop and test methodology that is capable of measuring
mechanical and adhesive properties of soft materials and to simultaneously report
heterogeneity and spatial distribution (mapping) of the measured parameters, including the
effects of depth sensing, adhesion, and viscoelasticity.

The developed approach is used to characterize the elastic properties of collagen-
Bioglass samples that are important substrates in tissue engineering efforts [153]. Selection
of these samples for testing measurement methodology is rationalized below.

The rational design of artificial scaffolds for tissue engineering requires inclusive
studies of the extracellular matrixes (ECMs) to successfully mimic their characteristics. 
ECMs are natural scaffolds that provide structural support to cells and surrounding tissues
[152]. Figure 1.14 shows an image of the natural ECM of bone tissue.
Figure 1.14 represents a cross section showing osteons. A unit of cells, an osteocyte, has a 5-20 \( \mu m \) diameter, is 7 \( \mu m \) deep, and contains 40-60 cells [https://www.78stepshealth.us/].

ECMs mainly contain bioactive molecules such as proteins, glycosaminoglycans and glycoproteins. Quantities and local distribution of the ECM components determine the mechanical properties, shape, and function of the whole organ. For example, collagen is the most common component in bone and skin tissues [154]. Therefore, collagen can be the main component for designing an artificial scaffold in bone engineering. However, collagen concentration must be controlled since it can affect both cell-cell adhesion and scaffold porosity, which has basic roles in cell-scaffold adhesion. Moreover, an increase in collagen concentration results in high crosslinking of ECM, which leads to a limitation in the supply of gases and nutrients to the cells and release of a hypoxia-related signaling process [155]. Scaffold stiffness should also be controlled, since it is responsible for mechanical strength during and after implantation [156]. Meanwhile, ECM stiffness values are different from one tissue to another. The stiffness values of osteogenic, myogenic, and neuronal tissues are 34 kPa, 11 kPa, and 0.1 kPa, respectively [156]. Therefore, the artificial scaffold should have a stiffness value compatible with that kind of tissue.
Moreover, cells interacting with scaffolds interact with the superficial layer of the biomaterial, while the mechanical testing of scaffold materials typically measures the total modulus. Thus, it is important to compare the elastic properties at the surface with the bulk values. In addition, the spatial heterogeneity of mechanical properties on a biologically-relevant length scale should be characterized to provide additional information for scaffold design.

Experimental testing will be performed on collagen samples loaded with different amounts of Bioglass, to mimic the ECM of bone tissue. Figure 1.14 shows a cartoon of an artificial scaffold as expected at microscale.

Figure 1.15. displays a cartoon of an artificial scaffold with cells attaching between collagen fibrils in microscale

Elastic properties of collagen samples will be mapped using the AFM force mapping technique. The variation of the elastic modulus with depth will be sensed by analyzing portions of the force curves corresponding to different penetration depths
(different levels of maximum applied force). The spatial heterogeneity of the modulus will be characterized by using different scan sizes, corresponding to the pixel-to-pixel distances of 20, 95, and 470 nm. This will allow the separation of systematic variations in the elastic modulus with characteristic dimensions from random point-to-point variations.

In order to perform depth sensing, relatively sharp AFM probes will be used. Therefore the tip of the probe might indent the sample to a depth noticeably exceeding the radius of curvature of the probe. However, standard methods of extracting elastic properties in indentation measurements utilize very simple probe shape models (e.g. conical or paraboloidal tip models). The tip of the AFM probe is usually somewhat rounded at the apex and then transitions to a conical shape. Consequently, if a simple probe shape model is used in data analysis, then the effects of the probe shape might be perceived as the depth dependence of the elastic modulus. To test and correct for this possible artifact, the hyperboloidal tip shape model will be used because it has both of these features: rounded tip shape that transitions into cone [137]. Data analysis will explicitly take into account sample’s adhesion to avoid artifacts related to testing mechanical properties for sticky samples [142].
1.9 References


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Chapter 2. Experimental Section

2.1 Samples and AFM setup

In this study, four different collagen-based biomaterial samples were prepared: 1) uncrosslinked electrochemically aligned collagen (ELAC), 2) uncrosslinked Bioglass incorporated ELAC (BG-ELAC), 3) crosslinked-ELAC and 4) crosslinked electrochemically compacted collagen (ECC; unaligned). ELAC and BG-ELAC were synthesized based on previously published protocol [1]. Briefly, acid soluble monomeric collagen solution (Purecol, 3.1 mg/mL, Advanced Biomatrix, CA) was first dialyzed against water for 24 hrs. Bioglass particles (GLO160P/-20; ~ 20 µm particle size; MO-SCI Corporation, MO) were suspended at 200 mg / ml in water. Then, the Bioglass particles were mixed with dialyzed collagen at the desired ratio, 30% Bioglass : 70% collagen (w / w). This mixture was then applied between two stainless steel wire electrodes ( electrode spacing: 1.8 mm), and electric field was applied (3 V, 30 min). Electric field triggers the formation of a pH gradient that in turn imparts a positive charge to the collagen molecules close to the anode and negative charge to the collagen molecules close to the cathode. Because of their repulsion with the same charge on the electrodes, the collagen molecules self-assemble along the isoelectric point. During this process, the Bioglass particles become entrapped within the collagen framework to form a highly aligned and densely packed Bioglass incorporated ELAC thread (BG-ELAC) [1]. Collagen only threads (ELAC) were made by the same way but without adding Bioglass particles. For the third group of samples, ELAC threads (without Bioglass) were crosslinked via incubation in 0.625% genipin solution (in 90% ethanol) for four hours at 37 ⁰C [2]. The
last group of samples were the crosslinked unaligned ECC collagen matrices which were prepared in a similar manner but by replacing the stainless steel wire electrodes with planar graphite electrodes [3].

2.2 Experimental Design

For AFM measurements, the samples were fixed on clean clipped corner plain microscope slides [Thermo Fisher Scientific ESCO]. The thread diameter of ELAC samples ranged from 250 µm to 300 µm, but appeared wider when deposited on the substrate. The ECC unaligned samples are flat with a width of almost 5 mm. The cover glasses were fixed with epoxy glue on plain glass microscope slides. Figure 2.1 shows a photograph for a filament of the ELAC sample after fixing on the slide.

All AFM measurements were performed on fresh samples using a Molecular Force Probe 3D AFM (MFP-3D, Asylum Research, Santa Barbara, CA). Samples were placed on the AFM scanner, and two separate positions of the xy scanner were identified such that AFM probe can be moved to these positions for force indentation measurements. Before the AFM measurements, the samples were re-hydrated in a phosphate buffer saline (PBS) pH ~ 7, to mimic the physiological environment. Then, the AFM head was placed properly on the scanner and the measurements started from one of the identified positions. The scanning was performed at two different spots for each sample (two samples for the uncrosslinked and two for crosslinked collagen) and three different scan sizes to investigate the large scale and short scale heterogeneity, respectively.
Figure 2.1 shows photograph of 1 cm long filament of ELAC sample deposited on a microscopic glass slide. After deposition on glass apparent width is 1 mm.

All AFM measurements were performed using silicon nitride probes model NP-S20 (Veeco, Santa Barbara, CA). To quantify force acting on the probe the cantilever spring constant was measured by the thermal noise method [4], and the typical value was approximately 100 pN / nm. Because of the heterogeneity of the Si$_3$N$_4$ cantilever material, it might be suspected that the spring constant of cantilevers might vary significantly [5]. Therefore the spring constant was measured for each cantilever used in the measurements.

The manufacturer specifies radius of curvature of the AFM tip as approximately 20 nm. If the tip radius is very sharp it might damage the soft surface; if it is very dull, no high spatial resolution can be obtained. The tip of AFM probe used in the elastic modulus measurements was imaged using scanning electron microscope (SEM, Philips XL30). Figure 2.2 shows the SEM image of the typical tip used in this research. The resolution of this image is not sufficient to resolve features on a scale of 10 nm, but the image allows to see that the probe has a sharp tip with a radius of curvature < 50 nm. This dimension indicates that the probe was not significantly damaged during the AFM measurements.
The AFM measurements followed standard measurement procedure. After fixing the cantilever in the probe holder and then in the AFM head, the laser beam was focused on the back of cantilever close to the tip position to give the maximum reflection signal, and the deflection was adjusted to zero. Then, the deflection sensitivity was calibrated on the glass surface, and afterwards the spring constant was also calibrated using the built-in thermal motion analysis procedure.

The measurements were carried out at three different spots that were previously selected. One spot was selected on the glass area that was not covered with biomaterial. This spot was used for measuring the optical lever sensitivity in PBS (typical values of inverse sensitivity range from 27 nm / V to 31 nm / V) and to image the bare glass surface for comparison. Figure 2.3 shows the glass surface image after cleaning by deionized water and drying with a flow of N₂.
Figure 2.3 shows images for clean-dry glass surface before fixing the sample. The A panel shows deflection image and the B panel shows the topographic image. The topographic image is flat because this scale is typical for collagen samples.

The other two spots were on the collagen sample with a distance of ~ 2 mm in between. Two spots are selected to investigate the large scale heterogeneity of elastic properties. At each spot, first, a contact mode image of a square area of 20 × 20 \( \mu \text{m}^2 \) was obtained to ensure the tip is scanning over the sample (not substrate) surface. Figure 2.4 shows typical image on ELAC sample in contact mode at a relatively low set point of 0.5 V. Low setpoint is selected to maintain the integrity of the sample surface.

Optical lever sensitivity was calibrated after each scan to perform accurate indentation measurements [5].
Figure 2.4 shows the typical deflection (A) and height (B) image of ELAC sample with scan size of 20 μm × 20 μm obtained before force mapping.

### 2.3 Force Mapping

Force curves were collected by moving the AFM probe towards and away from the sample surface. Motion towards the sample surface was reversed once the deflection of the cantilever reached the pre-determined trigger value. After each measurement, the position of the sample was changed to a new location. Measurements continued until a map of 32 × 32 force plots were collected [6]. The resulting force volume is a two-dimensional grid across a specific area of sample surface, which can be used to extract a map of the mechanical, adhesive, and viscoelastic properties over this area. During the force mapping, the relative trigger was selected at 25 nm, meaning that the maximum compressive force will be limited to $25 \cdot k_c \approx 2.5$ nN for all force curves. The force curve before the trigger is reached is called the approach force curve and after the trigger the
retracting curve starts. Collected data are stored on the computer and then analyzed using custom software written for Matlab.

At each spot, the force mapping is performed over three different scan sizes, 640 nm × 640 nm, 3 μm × 3 μm, and 15 μm × 15 μm, corresponding to 20 nm, 95 nm, and 470 nm distances between two nearby force plots. The rationale for mapping small scan sizes is to investigate the small scale heterogeneity of elastic modulus and to maintain the optical lever sensitivity, since it might vary for large scan sizes.

2.4 Data Analysis

The AFM instrument measures cantilever deflection voltage and sample displacement. To extract information about the local mechanical properties of materials these data should be converted to force and indentation values. The experimentally measured force vs. indentation dependence is compared with predictions of theoretical models. In this section instrument calibration and several theoretical models are considered.

2.4.1 Calibration of cantilever sensitivity and force constant

The AFM instrument measures cantilever deflection voltage and sample displacement. To extract information about the local mechanical properties of materials these data should be converted to force and indentation values. To obtain force and indentation values, the cantilever deflection sensitivity should be calibrated.

When the laser beam reflects off the cantilever, it goes to the split photodiode and produced the deflection voltage. When the end of the cantilever moves the deflection
voltage changes as illustrated in Figure 2.5. The sensitivity tells us how much the voltage changes with a cantilever motion (the slope of $\Delta V$ vs. $\Delta Z$), where $\Delta Z$ is the change in piezo position, the deflection [7].

Calibration of optical lever sensitivity was carried out by changing the z piezo position until the tip reached a hard surface (the deflection occurs) and change in the deflection voltage was recorded. Figure 2.6 shows the relationship between changes in deflection voltage and displacement, and the calibration of the optical lever sensitivity.

![Diagram of Optical Lever Sensitivity](http://www.nanophys.kth.se/)

Figure 2.5 shows the dependence of voltage value on the cantilever motion. The repulsive force bends the cantilever up and shifts the laser, and hence the voltage, to the positive value (Figure adapted from [http://www.nanophys.kth.se/](http://www.nanophys.kth.se/))

Optical lever sensitivity is measured on a hard surface:

$$\text{OLS (optical lever sensitivity)} = \frac{\Delta V}{d} \quad (2.1)$$
where $\Delta V$ is the change in the deflection voltage and $d$ is the cantilever deflection.

Figure 2.6 shows a deflection vs. $z$ displacement curve on a hard surface.

The measured value is used to convert the deflection voltage to the deflection distance at other positions on the sample:

$$d = \frac{\Delta V}{\text{OLS}} = \Delta V \times \text{Inv. OLS} \quad (2.2)$$

where Inv.OLS is the inverse optical lever sensitivity. Displacement of the base of cantilever is the sum of the cantilever deflection $d$ and the indentation $\delta$.

$$Z = d + \delta \quad (2.3)$$

Then

$$\Delta Z = \Delta V \times \text{Inv. OLS} + \delta \quad (2.4)$$
Therefore, the cantilever sensitivity should be calibrated on surface that is much harder
than the samples (such as mica or a glass slide) to ensure that $\delta$ is close to zero in equation
(2.4) so that for calibration

$$\text{Inv. OLS} = \frac{\Delta Z}{\Delta V} \quad (2.5)$$

The value of Inv.OLS should be relatively low (less than 100 nm / V); higher values
lead to a weak sensitivity, meaning that a large cantilever deflection is needed to achieve a
little change in voltage. Consequently, low sensitivity results in considerable contribution
of electronics noise to the measured signal, degrading performance of the instrument. Also,
low sensitivity might indicate that the AFM probe got damaged by handling or during
experiments. When the cantilever deflection is calibrated, the sample indentation can be

calculated as.

$$\delta = Z - \Delta V \times \text{Inv. OLS} \quad (2.6)$$

The applied force can be calculated as follows:

$$F = k_c \times d \quad (2.7)$$

where $d$ is the cantilever deflection. Selecting cantilever with particular spring constant
requires taking into account several considerations. The most sensitive measurements can
be obtained when the cantilever spring constant is comparable to that for the sample.
However, the deflection voltage of a very soft cantilever in presence of adhesive forces
might exceed the voltage range of the photodiode. On the other hand, deflection of too
stiff cantilever will have significant contribution from the system electronic noise [4]. In
measurements spring constant of cantilevers was calibrated using the built-in thermal noise method [4].

During the experiments, the Inv.OLS values in PBS were between 24 nm / V and 31 nm / V. The spring constant determined by the thermal noise method for a long wide cantilever ranges from 98 pN / nm to 125 pN / nm, so the maximum applied forces calculated by Eq. (2.6) range from 2.4 nN to 3.8 nN. After calibration, the force-displacement curve can be obtained as shown in Figure 2.7.

![Figure 2.7](image.png)

Figure 2.7 shows a typical force-displacement curve

### 2.4.2 Indentation Models

Indentation values were obtained as described in eq. 2.6. However, it should be noted that the displacement Z does not have zero corresponding to the sample surface, but is determined up to some constant. Therefore the extracted indentation will also have some offset. This issue in detail is discussed in section 1.7.5. This problem can be particularly
significant for soft samples [8]. Therefore, initially indentation is determined with an offset (as shown in Figure 2.8) and the position of the contact point is used as an adjustable parameter during data reduction.

![Figure 2.8](image)

Figure 2.8 shows a typical force-indentation curve

For example, the force-indentation dependence without adhesion for paraboloidal probe is fitted using the model [8]:

\[
F = \frac{4}{3} \frac{E}{1-\sigma^2} \sqrt{R \delta^3}
\]

(2.7)

where \(\delta\) is the indentation determined with offset as in Figure 2.8 with an added offset (\(\delta = \delta_{\text{exp}} + \delta_{\text{off}}\)), \(F\) is the applied force, \(R\) is the probe’s radius of curvature, \(\sigma\) is the Poisson ratio of the sample and \(E\) is the elastic (Young’s) modulus of the sample. Then \(\delta_{\text{off}}\) is used as a fitting parameter. The Poisson ratio cannot be obtained separately from the elastic modulus in indentation measurements. Therefore another fitting parameter in this model is the modified elastic modulus \(E^*\)
\[ E^* = \frac{E}{1 - \sigma^2} \]  \hspace{1cm} (2.8)

As can be seen from SEM image of the AFM probe, the initial curved apex then transitions into somewhat conical shape. Therefore, if indentation is large then paraboloidal shape model given by eq. 2.8 might be inaccurate. Therefore probe shape that contains both the rounded apex and conical body can be considered. One of such shapes is the hyperboloidal shape [8] that is shown in Figure 2.9.

![Diagram of Paraboloidal and Hyperboloidal Shapes](image)

Figure 2.9 shows comparison of paraboloidal and hyperboloidal shapes that have the same radius of curvature \( R \). In the figure \( \alpha \) is the semi-vertical angle of hyperboloidal probe.

For a hyperboloid probe profile force-indentation dependence cannot be derived as a closed-form expression. However, this dependence can be calculated using the radius of contact area as a parameter [8]:

\[ F = \frac{Ea^3}{(1 - \sigma^2)R} \left[ \xi^2 + \frac{\xi}{2} (1 - \xi^2) \left\{ \frac{\pi}{2} + \arctan \left( \frac{1}{2\xi} - \frac{\xi}{2} \right) \right\} \right] \]  \hspace{1cm} (2.9)
\[ \delta = \frac{a^2}{2R} \zeta \left[ \frac{\pi}{2} + \arctan\left(\frac{1}{2\zeta} - \frac{\zeta}{2}\right) \right] \]  
(2.10)

where

\[ \zeta = \frac{R \cot (\alpha)}{a} \]  
(2.11)

Here \( a \) is the radius of contact area, \( \alpha \) is the semi-vertical angle of the tip and other parameters as described for the paraboloidal probe model eq. 3.7.

The modified elastic modulus values obtained from data analysis with the above models that do not include adhesion are used as an initial guess to fit the data with models that consider adhesion [6]. In fitting models with adhesion one more fitting parameter is used: the energy of adhesion, \( \gamma \). Here for both probe shape model the equations use the radius of contact area, \( a \), that connects force and indentation. For the paraboloidal probe the JKR theory (after Johnson, Kendall and Roberts [9]) gives force and indentation as:

\[ F = \frac{4a^3E^*}{3R} - \sqrt{8\pi a^3E^*\gamma} \]  
(2.12)

\[ \delta = \frac{a^2}{R} - \sqrt{\frac{2\pi a\gamma}{E^*}} \]  
(2.13)

The JKR model takes into account elastic deformation of the sample surface, but it ignores the long range attractive forces. Samples used in this study are soft and elastic deformation is significant. Moreover measurements do not detect long range attractive forces. Therefore it is reasonable to apply this model to analyze indentation data on
samples studied here. Similar model that takes adhesion into account can be derived for the hyperboloidal probe model [6]:

\[
F = \frac{E^* A}{R} \left( \frac{a^2 - A^2}{4} \left( 2 \arcsin \left[ \frac{a^2 - A^2}{a^2 + A^2} \right] + \pi \right) + a A \right) - \sqrt{8\pi E^* a^3 \gamma} \tag{2.14}
\]

\[
\delta = \frac{a A}{4R} \left( 2 \arcsin \left[ \frac{a^2 - A^2}{a^2 + A^2} \right] + \pi \right) - \frac{2\pi a \gamma}{E^*} \tag{2.15}
\]

Here \( a \) is radius of contact area, \( A = R \cot(\alpha) \), and other parameters as described above.
2.5 References


Chapter 3. Results

AFM images for all samples, ELAC, 30% BG-ELAC, crosslinked ELAC and crosslinked unaligned collagen have been collected before and after force mapping to confirm the integrity of measured surface after force mapping. Figure 3.1 shows the height (right panel) and deflection (left panel) images for the typical sample of each type collected after performing the force mapping measurements; scan size in all four images was 15 µm × 15 µm. If there was any damage during the mapping, squares of 640 nm and 3 µm would be visible in the middle of images below. Absence of such squares indicates no damage to the sample surface using the selected range of forces during indentation measurements.
Figure 3.1A-D show deflection (left) and height (right) images at 15 µm scan size. Panels A-D show images of typical ELAC, 30% BG-ELAC, crosslinked-ELAC, and crosslinked unaligned collagen samples, respectively.

Approach and withdraw force plots have been acquired for each point in the map (32 × 32 = 1024 force plot). The force-indentation data were analyzed using custom software written for Matlab. Figures 3.3-3.6 show typical approach and withdraw force curves for each sample. Force curves were analyzed using the least squares fit of the JKR model eq. 2.12 and 2.13 to the data. For each force curve analysis provides elastic modulus ($E^*$, see eq. 2.8), position of the surface and for curves exhibiting adhesion, energy of adhesion parameter as well.
Figure 3.2 displays fitting typical approach (A) and withdraw (B) force plots of ELAC sample using paraboloidal probe shape. Data analyzed to maximum compressive force of $F_{\text{max}} = 1$ nN.

Figure 3.3 shows fitting typical approach (A) and withdraw (B) force plots of BG-ELAC sample using paraboloidal probe shape. Data analyzed to maximum compressive force of $F_{\text{max}} = 1$ nN.
Figure 3.4 displays fitting typical approach (A) and withdraw (B) force plots of crosslinked-ELAC sample using paraboloidal probe shape. Data analyzed at $F_{\text{max}} = 1$ nN.

Figure 3.5 shows fitting typical approach (A) and withdraw (B) force plots of crosslinked unaligned collagen sample using paraboloidal probe shape. Data analyzed at $F_{\text{max}} = 1$ nN.
Analysis of all force plots (1024 analyzed for each map) provides maps of elastic modulus ($E^*$) for each sample at two spots and three different scan sizes to characterize local elastic modulus and investigate the large range and short range heterogeneity. As an example, Figure 3.7 illustrates the heterogeneity in maps of elasticity over three scan sizes: 640 nm, 3 µm and 15 µm for crosslinked-ELAC sample. Separate maps are shown for approach and withdraw scan direction. In analysis range of compressive forces was limited to 1 nN maximum force; the JKR paraboloidal probe shape model was used.
Figure 3.6 shows the maps of elasticity of crosslinked-ELAC at the three different scan sizes, and approach and withdraw scan directions using 1 nN maximum force and paraboloidal probe shape model. Numbers on axes indicate pixel position. Blue pixels show places were collected force plots could not be analyzed.

The average of elastic modulus values for each map with the corresponding standard deviation were calculated using Matlab program. Tables 3.1 to 3.14 display these values for the four different samples.

Table 3.1. Elastic modulus values and their corresponding standard deviations (Std) of sample 1, 1st spot map (1024 force curves) of ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.
### Table 3.2. Elastic modulus values and their corresponding standard deviations (Std) of sample 1, 2nd spot map (1024 force curves) of ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^*$, MPa</th>
<th>$F_{\text{max}}$ 1 nN</th>
<th>$F_{\text{max}}$ 2 nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach Std</td>
<td>0.104</td>
<td>0.151</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.135</td>
<td>0.221</td>
<td>0.148</td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.098</td>
<td>0.124</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.111</td>
<td>0.149</td>
<td>0.054</td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.091</td>
<td>0.117</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.116</td>
<td>0.145</td>
<td>0.082</td>
</tr>
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</table>

### Table 3.3. Elastic modulus values and their corresponding standard deviations (Std) of sample 2, 1st spot map (1024 force curves) of ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^*$, MPa</th>
<th>$F_{\text{max}}$ 1 nN</th>
<th>$F_{\text{max}}$ 2 nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach Std</td>
<td>0.139</td>
<td>0.146</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.129</td>
<td>0.140</td>
<td>0.006</td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.141</td>
<td>0.132</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.122</td>
<td>0.128</td>
<td>0.037</td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.114</td>
<td>0.116</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.111</td>
<td>0.114</td>
<td>0.032</td>
</tr>
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</table>
Table 3.4. Elastic modulus values and their corresponding standard deviations (Std) of sample 2, 2nd spot map (1024 force curves) of ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^*$, MPa</th>
<th>$F_{\text{max}}$ 1 nN</th>
<th>$F_{\text{max}}$ 2 nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach</td>
<td>0.090</td>
<td>0.075</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.006</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.094</td>
<td>0.081</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.006</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>3 um</td>
<td>Approach</td>
<td>0.103</td>
<td>0.087</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.008</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.103</td>
<td>0.095</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.008</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>15 um</td>
<td>Approach</td>
<td>0.118</td>
<td>0.109</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.011</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.113</td>
<td>0.132</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.013</td>
<td>0.037</td>
<td></td>
</tr>
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Table 3.5. The average of elastic modulus values and their corresponding standard deviations (Std) of all spots on ELAC samples using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^*$, MPa</th>
<th>$F_{\text{max}}$ 1 nN</th>
<th>$F_{\text{max}}$ 2 nN</th>
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</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach</td>
<td>0.111</td>
<td>0.172</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.034</td>
<td>0.038</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.166</td>
<td>0.253</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.054</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>3 um</td>
<td>Approach</td>
<td>0.105</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.045</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.122</td>
<td>0.148</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.038</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>15 um</td>
<td>Approach</td>
<td>0.122</td>
<td>0.142</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.038</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.114</td>
<td>0.161</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.076</td>
<td>0.065</td>
<td></td>
</tr>
</tbody>
</table>
**Table 3.6.** Elastic modulus values and their corresponding standard deviations (Std) of sample 1, 1st spot map (1024 force curves) of 30% BG-ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^*$, MPa</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$F_{\text{max}}= 1$ nN</td>
</tr>
<tr>
<td>640 nm</td>
<td>Approach</td>
<td>0.510</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.488</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.111</td>
</tr>
<tr>
<td>3 um</td>
<td>Approach</td>
<td>0.572</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.534</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.197</td>
</tr>
<tr>
<td>15 um</td>
<td>Approach</td>
<td>0.527</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.131</td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.499</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.130</td>
</tr>
</tbody>
</table>

**Table 3.7.** Elastic modulus values and their corresponding standard deviations (Std) of sample 1, 2nd spot map (1024 force curves) of 30% BG-ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^*$, MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$F_{\text{max}}= 1$ nN</td>
</tr>
<tr>
<td>640 nm</td>
<td>Approach</td>
<td>0.529</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.101</td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.463</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.107</td>
</tr>
<tr>
<td>3 um</td>
<td>Approach</td>
<td>0.570</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>Withdraw</td>
<td>0.525</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.132</td>
</tr>
<tr>
<td>15 um</td>
<td>Approach</td>
<td>0.605</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.199</td>
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<tr>
<td></td>
<td>Withdraw</td>
<td>0.590</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.169</td>
</tr>
</tbody>
</table>
Table 3.8. Elastic modulus values and their corresponding standard deviations (Std) of sample 2, 1st spot map (1024 force curves) of 30% BG-ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^\ast$, MPa</th>
<th>$F_{\text{max}}$ = 1 nN</th>
<th>$F_{\text{max}}$ = 2 nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach Std</td>
<td>0.578</td>
<td>0.709</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.058</td>
<td>0.068</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.719</td>
<td>0.852</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.106</td>
<td>0.106</td>
<td></td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.542</td>
<td>0.761</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.154</td>
<td>0.210</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.804</td>
<td>1.020</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.296</td>
<td>0.340</td>
<td></td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.392</td>
<td>0.568</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.198</td>
<td>0.299</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.593</td>
<td>0.796</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.373</td>
<td>0.371</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.9. Elastic modulus values and their corresponding standard deviations (Std) of sample 2, 2nd spot map (1024 force curves) of 30% BG-ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, Scan direction</th>
<th>$E^\ast$, MPa</th>
<th>$F_{\text{max}}$ = 1 nN</th>
<th>$F_{\text{max}}$ = 2 nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach Std</td>
<td>0.777</td>
<td>0.756</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.164</td>
<td>0.137</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.704</td>
<td>0.771</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.136</td>
<td>0.123</td>
<td></td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.826</td>
<td>0.792</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.225</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.731</td>
<td>0.786</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.187</td>
<td>0.171</td>
<td></td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.778</td>
<td>0.780</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.205</td>
<td>0.173</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.770</td>
<td>0.782</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.155</td>
<td>0.162</td>
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</table>
Table 3.10. The average of elastic modulus values and their corresponding standard deviations (Std) of all spots on 30% BG-ELAC samples using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
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<th>( E^* ), MPa</th>
<th>( F_{\text{max}} = 1 ) nN</th>
<th>( F_{\text{max}} = 2 ) nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach Std</td>
<td>0.600</td>
<td>0.646</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.109</td>
<td>0.098</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.594</td>
<td>0.700</td>
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</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.115</td>
<td>0.107</td>
<td></td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.628</td>
<td>0.689</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.182</td>
<td>0.175</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.649</td>
<td>0.762</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.203</td>
<td>0.213</td>
<td></td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.575</td>
<td>0.640</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.183</td>
<td>0.189</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.613</td>
<td>0.715</td>
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</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.206</td>
<td>0.231</td>
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</tr>
</tbody>
</table>

Table 3.11. Elastic modulus values and their corresponding standard deviations (Std) of map (1024 force curves) of crosslinked-ELAC sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, scan direction</th>
<th>( E^* ), MPa</th>
<th>( F_{\text{max}} = 1 ) nN</th>
<th>( F_{\text{max}} = 2 ) nN</th>
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</thead>
<tbody>
<tr>
<td>640 nm</td>
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<td>0.609</td>
<td>0.715</td>
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</tr>
<tr>
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<td>Std</td>
<td>0.133</td>
<td>0.126</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>0.580</td>
<td>0.769</td>
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</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.119</td>
<td>0.145</td>
<td></td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.881</td>
<td>1.490</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.520</td>
<td>1.210</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>1.340</td>
<td>2.240</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>1.060</td>
<td>1.940</td>
<td></td>
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<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.924</td>
<td>1.670</td>
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</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.470</td>
<td>1.190</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>1.480</td>
<td>2.450</td>
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<td></td>
<td>Std</td>
<td>0.962</td>
<td>1.860</td>
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</table>
Table 3.12. Elastic modulus values and their corresponding standard deviations (Std) of 1st spot on crosslinked unaligned collagen sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, scan direction</th>
<th>$E^*$, MPa</th>
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<td>$F_{\text{max}} = 1 \text{ nN}$</td>
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<td>Approach Std</td>
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<tr>
<td></td>
<td></td>
<td>0.226</td>
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<td>Withdraw Std</td>
<td>2.470</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.628</td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.732</td>
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<tr>
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<td>Withdraw Std</td>
<td>1.790</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.587</td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.570</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.316</td>
</tr>
<tr>
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<td>Withdraw Std</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.730</td>
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</table>

Table 3.13. Elastic modulus values and their corresponding standard deviations (Std) of 2nd spot on crosslinked unaligned collagen sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
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<th>$E^*$, MPa</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>$F_{\text{max}} = 1 \text{ nN}$</td>
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<td>Approach Std</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.196</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>2.120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.650</td>
</tr>
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<td>3 um</td>
<td>Approach Std</td>
<td>0.842</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.241</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>1.530</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.538</td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.772</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.253</td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>1.320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.466</td>
</tr>
</tbody>
</table>
Table 3.14 Elastic modulus values and their corresponding standard deviations (Std) of all spots on crosslinked unaligned collagen sample using maximum compressive forces of 1 nN and 2 nN, extracted by fitting with JKR model.

<table>
<thead>
<tr>
<th>Scan sizes</th>
<th>Std, scan direction</th>
<th>$E^*$, MPa</th>
<th>$F_{max}=1$ nN</th>
<th>$F_{max}=2$ nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>640 nm</td>
<td>Approach Std</td>
<td>0.981</td>
<td>1.575</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.211</td>
<td>0.290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>2.295</td>
<td>3.040</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.639</td>
<td>0.616</td>
<td></td>
</tr>
<tr>
<td>3 um</td>
<td>Approach Std</td>
<td>0.787</td>
<td>1.290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.214</td>
<td>0.290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>1.660</td>
<td>2.435</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.562</td>
<td>0.517</td>
<td></td>
</tr>
<tr>
<td>15 um</td>
<td>Approach Std</td>
<td>0.671</td>
<td>1.014</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.285</td>
<td>0.396</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Withdraw Std</td>
<td>1.300</td>
<td>1.930</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.600</td>
<td>0.698</td>
<td></td>
</tr>
</tbody>
</table>

Results shown in these tables indicate that the samples are relatively soft with modulus $E^*$ in are range from approximately 0.1 MPa to approximately 3 MPa. It should be noted that to obtain values of corresponding elastic modulus, results for modulus $E^*$ should be multiplied by factor $(1 - \sigma^2)$. The Poisson ratio $\sigma$ ranges from 0.5 to 0, so the factor ranges from 0.75 to 1. This represents the inherent uncertainty in extracting elastic modulus using this method. This uncertainty can be reduced if reasonable guess about Poisson ratio can be made.
Chapter 4. Discussion

4.1 Elastic modulus measurements

Previous section shows parameters obtained by fitting the JKR model to data collected on four different types of samples. Results show that for ELAC, BG-ELAC, crosslinked-ELAC and crosslinked-ECC samples the average extracted moduli obtained from approach curves using 1 nN maximum compressive force are 0.12 MPa, 0.61 MPa, 0.97 MPa and 1.28 MPa with 0.05 MPa, 0.17 MPa, 0.54 MPa and 0.41 MPa standard deviation, respectively. The same order was found at $F_{\text{max}} = 2$ nN. Figure 4.1 shows these average data with their standard deviations shown as error bars. In previous work, it was found that Bioglass incorporated within ELAC threads improves the elastic modulus significantly [1]. The higher standard deviation value of BG-ELAC than for ELAC sample indicates less uniformity of the former. Crosslinked samples have higher Young’s modulus than uncrosslinked collagen threads. The same observation has been reported in previous work [2], which stated that the modulus value of soft materials decrease with decreasing the crosslink ratio [2]. Kishore and coworkers determined the ultimate tensile stress (UTS) for ECC material with and without treatment with genipin crosslinker [3]. They observed a significant increase in UTS by adding genipin to the ECC matrices [3]. The crosslinked-ECC unaligned exhibits higher modulus than crosslinked aligned, crosslinked-ELAC sample. The higher std value of elastic modulus for crosslinked-ELAC than that of crosslinked ECC unaligned sample might be attributed to the presence of regions with significantly different values of the modulus as shown in Figure 3.6. No such regions are
observed for unaligned sample. Figure 4.2 shows the force maps of one spot of crosslinked ECC unaligned sample at the three different scan sizes.

Figure 4.1 displays the average values of Young’s modulus of ELAC, BG-ELAC, crosslinked-ELAC and crosslinked-ECC collagen threads at A) $F_{\text{max}} = 1$ nN, and B) $F_{\text{max}} = 2$ nN. Standard deviations are shown as error bars.
Figure 4.2 shows the force maps of one spot of crosslinked ECC unaligned sample at the three different scan sizes.

Elastic modulus values measured here can be compared with elastic modulus values of collagen gels, in which collagen molecules are organized in hydrogels with a lot of empty space between individual molecules, and with the elastic modulus is in the sub-kPa
Therefore, the densification-alignment and unalignment processes of the investigated samples very significantly increases modulus in comparison with hydrogels. The measured values are close to the elastic modulus of stiffer biological materials like cartilage and meniscus (100 kPa – 1 MPa range) [5, 6]. However, this value is significantly (about two orders of magnitude) lower than the elastic modulus of protein materials in crystalline state [7].

The elastic modulus of similarly prepared BG-ELAC samples was reported earlier in paper by Kishore and co-workers [1]. Results for the bulk modulus obtained using the dynamic mechanical analyzer were approximately 10 times higher than values reported here. It might be suggested that in part the observed difference results from uncertainty in the radius of curvature of the AFM probe. However, the nominal value of the tip radius was specified with 5 nm uncertainty. This means that if the extreme values of 15 nm and 25 nm were used, they would provide modulus values different by ~13% from the value obtained using the expected radius. The measured elastic modulus is proportional to $R^{-\frac{1}{2}}$ (see eq. 2.7). Therefore the radius should be approximately 100 times lower to reconcile the observed difference, making the probe unrealistically sharp with 0.2 nm radius of curvature. Therefore other explanation for observed difference is needed. This difference in modulus values can be attributed to the testing direction. The modulus value of anisotropic material depends on the direction of the applied force. In the previous work [1], the applied force of the tensile test used to determine the elastic modulus is parallel to collagen alignments, whereas the force applied by AFM is perpendicular to the alignments. Furthermore, the observed difference can be explained by noting that AFM tests elastic
modulus of the near-surface layer. Therefore it can be suggested that the surface of samples is considerably softer than the middle part of material. From the typical force-indentation curves shown in Figure 3.2 – 3.6, it can be seen that the soft surface layer extends to at least several tens of nanometers into the sample surface because after some relatively short distance (about 50 nm – 150 nm) the fit curves start to deviate noticeably from the data. It appears that samples are stiffer beyond the surface layer. For ELAC sample the elastic half-space model fits the indentation data reasonably well up to the depth of approximately 150 nm (Figure 3.2) and for BG-ELAC sample the range of good fit is approximately 50 nm (Figure 3.4). For crosslinked-ELAC, the reasonable fitting is at depth up to approximately 35 nm while that for crosslinked-ECC (unaligned) at ~ 25 nm. The consistence of these depths coincides with that order in Figure 4.1. By applying a specific force the tip travels largest distance into the softest, and the shortest into the stiffest material. The upturn of the force-indentation data from the fit lines generated using the JKR model can be observed for all samples.

4.2 Viscoelasticity

As with most biological and soft materials \[8, 9, 10\], our samples exhibit viscoelastic behavior that can be seen as hysteresis between the approach and the withdraw force curves when the probe applies compressive force to the sample (hysteresis at tensile forces is caused by adhesion) \[9, 11, 12\]. Generally such hysteresis in the region of compressive forces can be attributed to either plastic or viscoelastic behavior \[13\]. After the sample is deformed plastically, the deformation persists after removal of the force. However, the viscoelastic behavior is a time dependent deformation that occurs after the force is
applied/removed to/from the sample [14]. Therefore, if force curves collected on the same spot continue to exhibit hysteresis then observed hysteresis is of viscoelastic nature. For all samples tested here the continuously reproduced hysteresis indicates the viscoelastic behavior. Figures 3.3 – 3.6 show this hysteresis as a difference in slope and offset between approach and withdraw curves for ELAC, BG-ELAC, crosslinked-ELAC and crosslinked-ECC, unaligned, samples in the region of compressive (positive) forces.

The model that is used here to analyze indentation data does not explicitly include viscoelastic effects. The observed viscoelasticity can be quantified as differences in the elastic modulus obtained for the approach and withdraw force curves. Such difference would mainly manifest itself as difference in the slope of force-indentation curves near the point of reversal of probe’s motion. Therefore higher-force regions of force curves should be considered to compare viscoelastic behavior across the samples. This type of hysteresis is typical for viscoelastic polymeric materials [15] and biomaterials [16]. It can be noticed that the approach and withdraw force curves shown in Figures 3.3 – 3.6 have similar slopes for low compressive forces (below ~1 nN). Consequently effects of viscoelasticity can be quantified as horizontal offset between approach and withdraw curves. Such offset is zero for purely elastic samples. Figures 4.3 and 4.4 show histograms of approach-withdraw offsets obtained for different samples at different scan sizes, as indicated in corresponding panels.

Figure 4.3 compares force curve offsets at different scan sizes and Figure 4.4 compares values obtained at different spots on the sample surface. Offsets seem to be similar at different scan sizes with BG-ELAC samples exhibiting lower offset than the
other two samples. Large offset observed on ELAC sample might be expected because this sample is not crosslinked. Somewhat surprising is the low offsets measured for BG-ELAC sample observed at different scan sizes and at different spots.

Figure 4.3 displays the approach-withdraw offset for ELAC, BG-ELAC and crosslinked-ECC, unaligned samples at $F_{\text{max}} = 1$ nN using paraboloidal tip model. The numbers inside the chart are the positions of histograms ± one standard deviation error in this position.
Figure 4.4 shows the approach-withdraw offset for the different spots using data collected with 15 μm scan size.

This reveals that the Bioglass particles affect the viscoelastic behavior of collagen threads: It appears that samples with added Bioglass exhibit more elastic-like behavior. For ELAC sample, it can be seen that the offset distribution dramatically changed
throughout the short scale of the scan size (from 58.7 – 8.6 to 6.4 + 1.7 from 3 μm to 15 μm, respectively).

For crosslinked-ECC, the viscoelasticity is larger than for BG-ELAC sample and this is consistent at different scan sizes and spots. In the case of crosslinked-ELAC a continuous change in viscoelastic offset was observed throughout changing the scan scales. Figure 4.5 shows the slow increase of the mean value of approach-withdraw offset for crosslinked-ELAC sample over the three different scan sizes, 640 nm, 3 μm and 15 μm. This regularity might be attributed to the fact that this sample consists of regions with relatively low and high elastic modulus, as seen in Figure 3.8. Only “low” modulus region was tested at scan size of 640 nm, and this region corresponds to lower viscoelastic offset that is shown on the left panel of Figure 4.5. As higher modulus areas appear in the image, so the more pronounced is the right hand side shoulder in the histograms. These observations are consistent with earlier notion that crosslinking increases elastic modulus but also increases viscoelastic offset between approach-withdraw curves.
Figure 4.5 displays the slow increase of viscoelasticity mean value of crosslinked-ELAC with increasing the scan sizes as higher modulus areas are included in testing.

In the comparison of mean values of offset for the four types of sample at 15 μm scan size, the offset distribution is overlapped within the two spot ranges of ELAC and BG-ELAC samples, revealing the similar manner in viscoelasticity at the given scan size. On the other hand, crosslinked materials show clearly increase in the offset most probable values from each other and the first two samples.

4.3 Heterogeneity of Surface

The topographic map of ELAC sample in Figure 2.4 shows variations that could be associated with local organization of collagen threads. The corresponding map and histogram of elastic modulus are shown in Figure 4.6. It can be observed that the distribution of elastic modulus is not wide with clear peak around the most probable value. As discussed in the previous sub-section, the elastic modulus extracted from the withdraw force curves is shifted towards higher values. Distance between force plots collected in this map is approximately 0.5 μm, and considerable point-to-point variation in the elastic
modus observed in the modulus maps here indicate that the characteristic scale for variation in the elastic modulus for this sample is less than 0.5 µm. The force maps collected at 640 nm scan size shown in Figure 4.7 exhibit considerably smoother variation in the modulus with characteristic scale of modulus variation of 100 nm. This scale is similar in size with the size of the tip-sample contact area estimated as $2(R \cdot \delta)^{1/2}$ which reaches value of ~110 nm at maximum indentation. Figures 4.6, and 4.7 show the maps and histograms for both scan directions, approach and withdraw. The withdraw maps show slightly higher variation in distribution of modulus and thus higher standard deviations. Higher width of distribution of the withdraw curves can be attributed to the higher uncertainty in the modulus extracted by simultaneously taking into account the adhesion of samples.

![Elastic modulus maps](image)

Figure 4.6 shows force maps and their corresponding histograms of ELAC sample at 15 µm scan size
Unlike the ELAC sample, which does not have a significant increase of heterogeneity with increasing scan size, the standard deviation of elastic modulus for BG-ELAC is considerably higher and it increases with increasing the scan size, as shown in Figure 4.8. This is expected for composite materials since the larger scan size can include more and more features with various mechanical properties. This consistence has been shown in previous report [18], in which the samples appears to be strongly heterogeneous with increasing the scan size.

Figure 4.7 shows elastic modulus maps and corresponding histograms measured on ELAC sample at 640 nm scan size.
Figure 4.8 shows elastic modulus maps and their corresponding histograms of BG-ELAC at different scan sizes: a) 640 nm, b) 3 µm, and c) 15 µm.

Elastic map with scan size 640 nm for BG-ELAC shows noticeably sharper variation of features than for ELAC sample. This can be attributed to the considerably lower size of the contact area; here characteristic size $2(R \cdot \delta)^{1/2}$ is approximately 60 nm. Crosslinked-ECC sample, which has a resolution of approximately 45 nm shows features with more sharp dimensions than BG-ELACs’. Figure 4.9 characterizes some features of the four types of sample at the smallest scan size, 640 nm, in which the point to point distance equals 20 nm. The small features reflect the high modulus of surface, on which the tip-
sample contact area is smaller than that on the lower modulus materials. Even though crosslinked-ELAC has a characteristic size even less than BG-ELACs’ (approximately 53 nm), it exhibits sudden variations in force the map, indicating that the feature dimensions are smaller than 53 nm.

Figure 4.9 displays force maps of ELAC and BG-ELAC, crosslinked-ELAC and crosslinked-ECC, unaligned samples at 640 nm scan size. The higher modulus samples show sharper features.

### 4.4 Depth Sensing and Tip Shape Artifacts

Tables 3.5, 3.10, 3.11 and 3.14 display the elastic modulus values of samples, ELAC, BG-ELAC, crosslinked-ELAC and crosslinked-ECC, unaligned samples at maximum forces of 1 nN and 2 nN, using a JKR model for paraboloidal tip shape [18]. For all samples there are differences in elastic modulus values determined at different level of compressive forces. By using the t-test these differences are significant although their elastic maps exhibit unobvious differences. In a previous study [19], there was a slight difference in modulus values between the core and shell of the collagen fibril, and both showed similar morphological structures [20, 15]. However, findings for individual collagen fibrils cannot be directly applied here because of difference in preparation
conditions and modification procedures of these materials. Figure 4.10 displays 15 µm × 15 µm elastic modulus maps for ELAC, BG-ELAC, crosslinked-ELAC and crosslinked-ECC, unaligned samples obtained using a paraboloidal tip model at different levels of maximum (depth) applied force. First, The BG-ELAC maps show essentially no different topographic features indicating absence of objects with different elastic moduli at depth up to ~250 nm.

\[ F_{\text{max}} = 1 \, \text{nN} \quad \text{ELAC} \quad F_{\text{max}} = 2 \, \text{nN} \]
### BG-ELAC

$F_{\max} = 1 \text{ nN}$

<table>
<thead>
<tr>
<th>Elastic modulus [MPa]</th>
<th>Mean 0.392 Std 0.198</th>
</tr>
</thead>
</table>

$F_{\max} = 2 \text{ nN}$

<table>
<thead>
<tr>
<th>Elastic modulus [MPa]</th>
<th>Mean 0.568 Std 0.299</th>
</tr>
</thead>
</table>

### Crosslinked-ELAC

$F_{\max} = 1 \text{ nN}$

<table>
<thead>
<tr>
<th>Elastic modulus [MPa]</th>
<th>Mean 0.924 Std 0.470</th>
</tr>
</thead>
</table>

$F_{\max} = 2 \text{ nN}$

<table>
<thead>
<tr>
<th>Elastic modulus [MPa]</th>
<th>Mean 1.670 Std 1.190</th>
</tr>
</thead>
</table>

Figure 4.10 Force maps and corresponding histograms of ELAC, BG-ELAC, crosslinked-ELAC and crosslinked-ECC, unaligned samples using paraboloidal tip at different indentation depths.

Data in the previously mentioned tables and histograms indicate that the samples appear stiffer and with higher variation (higher std) at higher indentation force. The higher standard deviation with increasing indentation depths might reflect higher heterogeneity in direction perpendicular to sample’s surface within the sample. Increase in the elastic modulus with increase of the applied force has observed when measuring elasticity of living cells [21]. However, for soft sample such as corneal membrane, the indentation profiles indicated considerable uniformity of material with depth [20]. The increase of modulus values with the indentation depth in part can be attributed to the tip shape artifact and not to actual increase of elastic modulus with increase of depth sensing. The AFM tip
does not have ideal shape of paraboloid of revolution, but rather shape with rounded apex that becomes a cone, similar to shape of hyperboloid [22]. To avoid this tip shape artifact in the depth sensing, studies the hyperboloid probe with adhesion model [18] was applied. As an example, the results of crosslinked-ELAC samples are shown in Table 4.1.

Table 4.1. Elastic modulus values (MPa) and their standard deviations (Std) for one spot on crosslinked-ELAC at ($F_{\text{max}} = 1 \text{ nN}$ and $2 \text{ nN}$) using paraboloidal and hyperboloidal models.

<table>
<thead>
<tr>
<th>Scan sizes And directions</th>
<th>E, MPa, Standard deviation</th>
<th>$F_{\text{max}}, \text{JKR}$</th>
<th>$F_{\text{max}}, \text{Hyperboloid}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 nN</td>
<td>2 nN</td>
</tr>
<tr>
<td>Approach 640 nm withdraw</td>
<td>Mean</td>
<td>0.609</td>
<td>0.715</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.133</td>
<td>0.126</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.580</td>
<td>0.769</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.119</td>
<td>0.145</td>
</tr>
<tr>
<td>Approach 3 μm withdraw</td>
<td>Mean</td>
<td>0.881</td>
<td>1.490</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.520</td>
<td>1.210</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>1.340</td>
<td>2.240</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>1.060</td>
<td>1.940</td>
</tr>
<tr>
<td>Approach 15 μm withdraw</td>
<td>Mean</td>
<td>0.924</td>
<td>1.670</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.470</td>
<td>1.190</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>1.480</td>
<td>2.450</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>0.962</td>
<td>1.860</td>
</tr>
</tbody>
</table>

To achieve the same indentation by both paraboloid and hyperboloid probe shapes with the same radius of curvature, the higher force should be applied with the hyperboloidal probe because the former shape displaces more material as illustrated in Figure 4.11.
Figure 4.11 compares shapes of the paraboloidal (gray) and hyperboloidal (blue) probes and indicates expected indentation depths at two different force levels, as indicated in the figure.

However, the results in Table 4.1 show that using hyperboloid probe model decreases the modulus values obtained at different indentation forces, indicating that the paraboloidal tip underestimated the modulus values. This artifact has increased with increasing the applied force. By using t-test, there are insignificant differences between paraboloid and hyperboloid modulus values in the case of $F_{\text{max}} = 1$ nN, whereas the differences became significant at $F_{\text{max}} = 2$ nN. Figures 4.12 a and b show the increase of modulus difference between paraboloidal and hyperboloidal model at $F_{\text{max}} = 2$ nN over the given scan sizes on crosslinked-ELAC sample.
Figure 4.12 in panels a and b show the difference in $E$ values by using paraboloid and hyperboloid probes over different scan sizes for crosslinked-ELAC sample at $F_{\text{max}} = 1 \text{ nN}$ and 2 nN, respectively.

The same effect was also found in ELAC material. The reason for increasing the tip artifact with increasing the indentation depth can be attributed to the considerable
change in the tip shape at larger indentation corresponding to $F_{\text{max}} = 2 \text{ nN}$ as illustrated in Figure 4.11.

4.5 Conclusion

A comprehensive approach was developed to determine and characterize surface distribution of elastic modulus for soft biomaterials at nanoscale under physiological conditions. The developed method takes into account sample adhesion and can be used for probes with geometry that deviates from simple paraboloid of revolution geometry. The hyperboloidal probe shape is added into the analysis in order to test for probe shape artifacts. Elastic modulus values obtained for modified collagen threads are in the range of hundreds of kilopascals. The characteristic dimensions of surface features visible in elastic maps are consistent with estimated size of the tip-sample contact area. Even though the visual differences between modulus maps at different maximum forces are unobservable, difference of mechanical properties measured at the surface layer and at the deeper probe penetration are significant as confirmed by the t-test.

No Bioglass protrusions at the surface were detected for BG-ELAC sample, however elastic modulus of surface collagen molecules appear stiffer than for ELAC-sample. It appears that Bioglass affects packing order of the surface collagen molecules even though it is not present at the top sample layer. Crosslinked materials showed higher elastic modulus than BG-ELAC threads, however crosslinking also increases viscoelastic features observable in indentation experiments. Further more extensive studies are necessary to determine the concentration dependence of the Bioglass, or crosslinker, affecting the elastic modulus of the surface of BG-ELAC threads. This can be extended
with comprehensive multi-disciplinary study of effects of crosslinking ratio or adding Bioglass on cell response [1, 3]. The rationale for using this method is its ability to separate the elastic modulus in microscale dimensions and of superficial layer that is sensed by cells from the elastic modulus of the bulk of substrate.

The developed technique allows the detection of viscoelasticity of the surface layer of the material. This opens an opportunity to determine to what degree viscoelastic properties (in contrast to the purely elastic) of the surface layer affect cell differentiation and growth. However, to fully explore this idea the data analysis should be extended to take viscoelastic properties into account explicitly. To start, it can be performed by adopting viscoelastic models that do not take adhesion into account [21-23], and then utilize much more complex models that includes both adhesion and viscoelasticity [24-26].
4.6 References


