

MULTI-SCALE MEASUREMENT OF MECHANICAL PROPERTIES OF SOFT SAMPLES WITH ATOMIC FORCE MICROSCOPY

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INTRODUCTION

The atomic force microscope (AFM) has rapidly become one of the most widely used methods for studying mechanical properties of living cells [1]. Atomic force microscopy involves laser tracking of the deflection of a small (200 to 300- μm long) cantilever probe as its tip (~ 50 nm radius of curvature) scans, indents, or otherwise interacts with the sample. In particular, nano-indentation with AFM (Fig. 1) is well suited for cell mechanics applications due to its high sensitivity (sub-nanoNewton), high spatial resolution (sub-micron), and the ability to be used for real-time measurements in a physiologic aqueous cell culture environment.

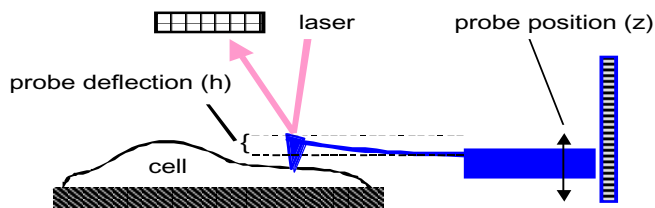


Figure 1: Schematic of the AFM indentation experiment.

Since the earliest AFM investigations of cell mechanics [2], the prevalent method of analyzing AFM indentation data has been application of the so-called "Hertz model" of contact between a cone and a semi-infinite elastic half space (this problem was actually solved by Love [3]). Based on such studies, biological cells are typically found to have an elastic modulus ranging from 1 to 100 kPa [1]. However, the classical analysis assumes infinitesimal sample deformation, whereas for soft biological samples such as cells the local deformations near the sharp probe always fall into the finite strain regime [4]. In addition, the classical analysis assumes the sample is well characterized as a homogeneous continuum. In contrast, the microstructure of cells and many other biological materials consists of a heterogeneous distribution of filamentous proteins, often with dimensions comparable in scale to the size of the standard AFM probe

tip. This permits correlation of apparent local mechanical properties with underlying structures [5]. However, it is unclear how these local properties may relate to the macroscopic bulk properties of the sample. In some cases, it may be these bulk properties that are most important for modeling and understanding how a cell interacts with, and responds to, changes in the extracellular mechanical environment [6].

We have recently published a new analysis method whereby accurate compensation for the AFM tip geometry allows computation of an apparent elastic modulus as a function of indentation depth that can reveal nonlinearity and heterogeneity of material properties from standard AFM indentation tests [4]. The objective of this study is to validate this method using agarose hydrogels, which are comparable in stiffness to living cells but are structurally and mechanically much simpler. In particular, we test the ability to compensate for indenters of different size and geometry, and compare the microscopic mechanical properties with those obtained from standard macroscopic tests, thus spanning 5 orders of magnitude in scale.

MATERIALS AND METHODS

Test Materials

Agarose is a neutrally charged, thermoreversible hydrogel. 3% wt/vol agarose (Type VII, Sigma-Aldrich) was prepared with 0.01 M phosphate buffered saline (PBS) and gelled into 60 mm petri dishes at a thickness of ~ 2 mm. A 1.5% wt/vol agarose solution was also prepared by 1:1 dilution with PBS. Two petri dishes for each agarose concentration were prepared, and were stored at room temperature fully hydrated in PBS and sealed with parafilm until testing.

Microscopic Material Testing

AFM indentation was conducted with samples submerged in PBS on a Bioscope AFM (Digital Instruments) mounted on the stage of an inverted light microscope (IX-70, Olympus). V-shaped silicon nitride cantilever probes (Digital Instruments) having a nominal spring constant of $\kappa = 0.06$ N/m were mounted on the AFM. Two types of AFM probes that differed in their tip geometry were used (Fig. 2): the standard silicon nitride pyramid tip, and custom probes with a 10- μm

polystyrene microsphere tip (NovaScan Technologies). The mounted probes were submerged in PBS for at least 20 min prior to testing to allow for thermal equilibration at room temperature. For each agarose sample, an array of 9 indentations covering a 20x20 μm square region of the sample was performed at an indentation rate of 1 Hz and a z-range of 3 μm , with the force curve sampled at 512 points.

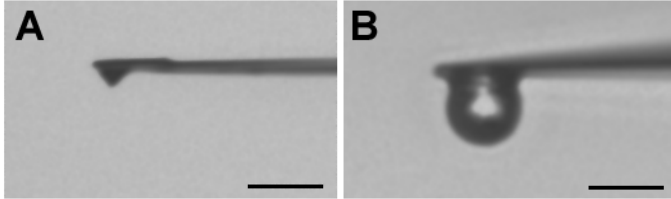


Figure 2: AFM tip geometries. (A) Standard SiN₄ pyramid. (B) 10 μm polystyrene sphere. Scale bar, 10 μm .

AFM indentation data (deflection, h , vs probe position, z) were analyzed using custom software to identify the contact point and convert the data to indentation force ($F = \kappa \times h$) vs indentation depth ($D = z - h$). As previously described [4], the following generalized contact equation is applied to each individual force-depth data point, i , to calculate an apparent pointwise modulus, E_{pp}^i , representing the material properties of the sample:

$$E_{pp}^i = \frac{F^i}{2\pi \bullet \phi(D^i)} \quad (1)$$

The analytic function $\phi(D)$ is determined by the geometry of the AFM probe: the pyramidal tip was modeled as an equivalent blunt cone with a 50-nm tip radius and a semi-angle $\theta = 40^\circ$, and the polystyrene bead was modeled as a sphere with radius $R = 5 \mu\text{m}$.

Bulk Material Testing

Following the AFM indentation tests, three cylindrical agarose disks were cored with a 4.76-mm diameter tissue trephine from the central region of each petri dish. Bulk material testing of agarose disks was performed in unconfined compression using a custom-designed computer-controlled testing apparatus [7]. Fully bathed in PBS, each sample was first equilibrated in creep to a tare load of $\sim 0.02 \text{ N}$, equivalent to an applied stress of $\sim 1 \text{ kPa}$, and from this offset, stress relaxation tests were performed with a ramp speed of $1 \mu\text{m/s}$ until reaching 10% strain. Young's modulus was calculated from the ratio of the equilibrium stress to the applied strain.

Statistical Analysis

The effect of measurement technique on elastic modulus was tested using ANOVA, with Sheffe's S post hoc analysis. Statistical significance was accepted for $p < 0.05$. Results are presented as mean $\pm$ SD.

RESULTS

Values of E_{pp} were essentially independent of indentation depth (i.e. linear elastic), so data are compared at $D = 100 \text{ nm}$ for convenience (Fig. 3). There were no significant differences between the average elastic modulus for the 3% agarose gel obtained with the AFM pyramid ($26.3 \pm 4.2 \text{ kPa}$), the AFM microsphere ($28.1 \pm 6.7 \text{ kPa}$) and bulk unconfined compression ($24.3 \pm 0.6 \text{ kPa}$). However, the modulus of the 1.5% agarose gel obtained with the AFM pyramid ($9.8 \pm 1.5 \text{ kPa}$) was significantly different ($p = 0.0001$) from the values obtained by the other two techniques ($3.7 \pm 1.8 \text{ kPa}$ and $3.4 \pm 2.4 \text{ kPa}$, respectively).

DISCUSSION

The findings suggest that when the AFM indenter geometry is properly taken into account, our analysis yields microscopic material

properties that are consistent with bulk properties of agarose gels. The greatest discrepancy occurred for the 1.5% gel indented with the pyramidal tip. The average pore diameter of such samples is approximately 100 to 300 nm [8]. In comparison, the contact diameter between sample and probe at $D = 100 \text{ nm}$ is approximately 150 nm for an equivalent blunt cone [9]. Therefore, the homogeneous continuum assumption may not have been applicable for this case. The improved agreement with the AFM pyramid for the 3% gel may have reflected a smaller pore size with the increased gel concentration [8]. The contact diameter for the sphere is approximately 1400 nm at $D = 100 \text{ nm}$, substantially greater than the agarose pore size at either concentration.

Whereas the bulk properties were determined at equilibrium, the microscopic properties were measured at an indentation rate of 1 Hz. Note that the characteristic time constant (T) for a biphasic material is proportional to $D^2(Ek)^{-1}$ where D is taken to be the indentation depth, E is the Young's modulus and k is the hydraulic permeability of agarose [10]. For $E = 25 \text{ kPa}$, $k = 1 \times 10^{-13} \text{ m}^4/\text{Ns}$ [11] and $D = 500 \text{ nm}$, we get $T = 0.0001 \text{ sec}$, i.e. the characteristic frequency is 10,000 Hz. From this analysis it is apparent that although the AFM tests were performed at 1 Hz, because of the scale at which the tests were performed (sub-micron indentation), this frequency is well below the characteristic frequency of the flow dependent viscoelastic response of agarose. Thus, the material properties acquired in this manner can be considered quasi-static equilibrium properties, and are appropriate for comparison with those obtained by bulk unconfined compression.

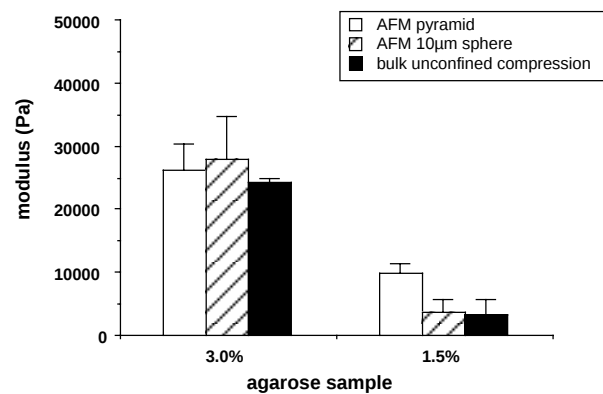


Figure 3: Elastic modulus for 3.0% and 1.5% agarose samples measured by AFM indentation and unconfined compression.

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