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Toward the realization of reproducible Atomic Force Microscopy measurements of elastic modulus in biological samples

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ABSTRACT

The validation of the AFM method for elastic modulus E measurement in soft materials ($E < 5$ MPa) is still missing. The interest of measurements in materials with $E < 5$ MPa is mainly biological, including soft tissues and single cells. For the diagnosis of malignant human tumors, a change in cell elasticity, within tissues, has recently been recognized as a marker of metastatic potential. To measure a cell elasticity difference, reproducible E measurements in biological samples are needed. In this work a robust method for a metrological validation of E measurements in the range 500–5000 kPa was developed, based on the realization of thick E standard samples and on the study of the interactions between the measurement process and the sample at micro- and nano-scale. E measurement reproducibility limit of 4% has been reached. This allows designing a very sensitive and reproducible measurement of E in biological samples representing thus a powerful diagnostic tool for cancer detection.

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1. Introduction

Atomic Force Microscopy (AFM) allows high-resolution imaging of biological samples and the characterization of mechanical properties of very soft and non-homogeneous materials, such biological samples, by detecting repulsive and attractive cell surface forces (Cross et al., 2007; Kuznetsova et al., 2007). Young's modulus or elastic modulus (E) is a measure of materials stiffness; it can be measured by AFM (Kuznetsova et al., 2007; Darling et al., 2007; Costa, 2004) and gives information on biological sample (e.g. single cell within a tissue) elasticity.

The validation of the AFM method for E measurement in materials with $E < 5$ MPa is still missing (Carrillo et al., 2005). In the low range, the E measurement by AFM is influenced by the interaction between the measurement system and the material of which E is measured. Therefore, a metrological characterization of the system interaction needs to be determined. The interest of E measurements in materials with $E < 5$ MPa is mainly biological: soft tissues and single cells or cell cultures exhibit E in this range (Wenger et al., 2007).

Recently, a change in cell E has been recognized as a marker of disease such as cancer (Cross et al., 2007, 2008; Guo et al., 2012). Changes in the extracellular matrix and cytoskeleton structure have been found translating into cell elasticity changes (Bhadriraju and Hansen, 2002). In 2007, Cross et al. found a difference in E between living human metastatic cancer cells and the corresponding benign cells; they measured by AFM that malignant cells are 70% softer than benign cells. Current and traditional analyses for cancer cell detection (such as cytomorphological and immunohistochemical analyses) (Lekka et al., 2012a, 2012b) are qualitative morphological analysis; they relies on cytoskeleton remodeling leading to cell shape changes. However traditional methods for malignant cells diagnosis have a limitation; frequent morphological overlap between tumor and normal cell types occurs (Cross et al., 2007). Cross et al. also demonstrated that AFM measurements of E well-correlate with traditional methods of cancer cell detection. Therefore, AFM mechanical analysis offers the powerful tool to quantitatively distinguish malignant cells from normal cells for cancer detection. To measure a cell elasticity difference, reproducible elasticity measurements of the biological sample are needed and the target reproducibility must be lower than the expected cell elasticity difference (70%). As a consequence the measurement method, AFM Force Spectroscopy, must be validated for reproducibility.

Investigation for cancer detection can involve single cells (Lekka et al., 2012a; Li et al., 2008) and tissues (Lekka et al., 2012b) coming from biopsies. Consequently, investigations should cover measures at macro-, micro- and nanoscale, respectively for analyzing the extended E in a tissue, the specific E of a single cell and also E of

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defined cells substructures at nanoscale. It has been shown (Lekka et al., 2012a) that E measured at single cell level and tissue level (nano-, micro- and macro- levels) can be different, and the combination of the two AFM measurements offers a precious set of information about cancer detection. To perform reproducible E measurement on different biological samples (tissues, single cell, cells substructures) the AFM method must be validated in different scale ranges. In addition, high indentation speeds must be tested in order to perform measurements in time limits compatible with cellular processes of living cells such as cell mobility (lifespan: seconds) and cell division, apoptosis (lifespan: minutes).

With this work a robust method for a metrological validation of E measurements in the range 500–5000 kPa was developed, based on the realization of thick samples showing a homogeneous E value on macro-, micro- and nanoscale, and on the study of the interactions between the measurement process and the sample. Sylgard 184 was chosen as modeling material for soft tissues, as also described in our previous work (Demichelis et al., 2013; Demichelis et al., 2014). Sylgard samples in biological elastic range of 50–5000 kPa were prepared. Indentations with the AFM sensor were performed to characterize surface homogeneity and viscoelastic behavior of samples. Its use as multiscale standard was also investigated. Operative measurement settings were obtained for the realization of reproducible elasticity measurements on biological materials.

Results obtained in this work will allow designing a very sensitive and reproducible measurement of E in biological samples aimed in measuring elasticity differences below 5%.

2. Experimental

2.1. Sylgard as E standard in the range 500–5000 kPa

The validation of the AFM *Force Spectroscopy* method on soft materials requires E standards. The standard must have an E defined in all its volume, must present homogeneity properties and stability over time. Procedures for preparation of standards must be defined; they can invalidate the employ of the standard since influence the sample homogeneity in all directions, both xy plane and z direction.

Polydimethylsiloxane PDMS is a viscoelastic polymer of cross-linked chains that can be prepared curing short PDMS chains with hydrogenated-PDMS chains. The chemical curing reaction (hydrogen addition to the vinyl ends of PDMS chains, catalyzed by Pt and heat) causes the internal re-arranging of the random-distributed PDMS chains that expose to the surface idrofobic $-CH_3$ groups. This material, commercialized as Sylgard 184, consists of the base agent (short PDMS chains) and the curing agent (hydrogenated-PDMS chains) that must be blending each other. Sylgard can be a good candidate as standard in this context since presents a tunable E varying the base/curing ratio, allows to realize very low E materials (in the range 500–5000 kPa), presents a very homogeneous surface at a microscopic level (Demichelis et al.,2014) and let to constructs mechanically stable samples.

2.2. Principal influence quantities affecting the interaction between the measurement process and sample

The AFM Force Spectroscopy method allows obtaining an experimental force–distance curve when indenting a sample, the shape of the force–distance curve reflects the sequence of sample layers with possible different elasticity. E values strongly fluctuate at very low indentation depths (nearly the contact point, i.e., the sample surface). E reaches a plateau by increasing the indentation depth and finally increases when the substrate stiffness is sensed (JPK application note, 2014).

The elasticity measured in each layer depends on indentation speed because of the viscoelastic behavior of the sample (McCrum et al., 2003). When the characteristic time of indentation is smaller than the sample relaxation time (high indentation speed), the outcome is a higher resistance of the sample because interfacing with the PDMS viscous behavior, it results in an apparently higher E. Vice versa, when the indentation time is longer than the sample relaxation time (low indentation speed), the sample has the possibility to move away from the indenting probe diffuse from the bulk to the sample surface. The outcome is, thus, a lower resistance that results in an apparently lower E. It follows that the indentation speed plays an important role.

The contact mechanics model employed will affect the measured interaction between system and sample. The Hertz contact model was chosen for simplicity of calculation, since E value was not concern of this work, just E reproducibility was investigated in function of nominal E values.

Another influence quantity affecting the interaction is the indenter. It is defined by the cantilever elastic constant, the tip radius and shape, and the photodiode sensitivity when hanged to the AFM instrument. In this work indenter was chosen based on previous measures (Demichelis, 2013; Demichelis et al., 2014), its choice is not object of this paper.

3. Materials and methods

3.1. Preparation of Sylgard samples

Fresh Sylgard 184 (Dow Corning) rectangular samples, 0.5 cm height, were realized in a grid plastic stamp, with nominal base/curing ratio of 15, 25 and 55 by weight. Stirring time of 2 min and curing time of 24 h were set. The stamp was put on the AFM stage and each compartment was filled with deionized water, for AFM measurements in liquid. The employed storing method consisted of storing the samples at room temperature, without water on the surface, covering them with a plastic cup, washing the sample surfaces with ultrapure water prior to perform AFM measurements.

3.2. AFM measurement setup

Force measurements of Sylgard samples were performed in liquid (deionized water) to avoid the jump-to-contact effect (Demichelis et al., 2014). AFM Force Spectroscopy measurements were realized with a JPK Nanowizard II instrument preparing suitable nanoindenters. The nanoindenter for the E measurement at microscale level was realized gluing a SiO_2 sphere (GmbH microparticles, nominal diameter 7.75 μm) on the top of a Silicon tipless cantilever. A bio-compatible adhesive (Dymax OP-29 optical glue) and a rigid tipless cantilever were employed (Nanosensors TL-NCH, nominal elastic constant k 40 N/m, no coating). To perform E measurement at nanoscale level, a commercial rigid Silicon Nitride cantilever with a pyramidal tip was chosen (AppNano ACTA, nominal elastic constant k 40 N/m, face angle of the quadratic pyramid 31°, radius of the edge tip less than 10 nm, Al coating).

1 V was set as approaching parameter of indenter (corresponding to a cantilever deflection setpoint of 25 nm when approached to the sample), default feedback parameters for the approach are employed (i-Gain 150 kHz, p-Gain 0.0048). 0.4 V was set as final relative setpoint of cantilever during the Force Spectroscopy measurements (corresponding to a maximum load of 400 nN for the employed cantilever at the end of the extend process); the maximum experimental z length measurement was set equal to 5 μm.

3.3. E calculation

Young's modulus E [Pa] of Sylgard sample was calculated from the classical Hertzian model for a spherical indenter (Eq. 1) (Ladjal et al., 2009), when using the cantilever with the glued sphere, and for a four-sided pyramidal indenter (Eq. 2) (Lin et al., 2007), when using the cantilever with the pyramidal tip as follows:

$$F = \frac{4E}{3 \times (1-\nu^2)} \times R^{1/2} \times \delta^{3/2} \quad (1)$$

$$F = \frac{E}{(1-\nu^2)} \times \frac{\tan(\alpha)}{\sqrt{2}} \times \delta^2 \quad (2)$$

in which F [N] is the force that the indenter develops against the sample, ν is the Poisson ratio (in this case 0.5), R [m] is the radius of the spherical indenter, α [°] is the face angle of the tip, δ [m] is the indentation depth.

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