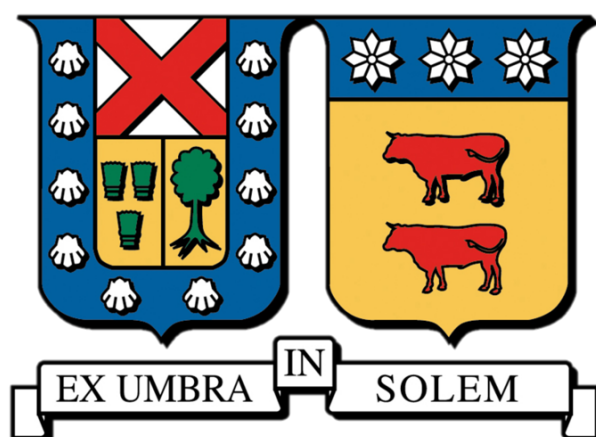


Nanomechanical Properties of Biopolymers

Thesis submitted as required to obtain the degree of
Master of Science in Physics.

Physics Department,
Federico Santa María Technical University,
Valparaíso, Chile, 2024.



Presented by Diego Jaramillo-Pinto.

ADVISOR : Dr. Tomas P. Corrales
CO-ADVISOR : Dr. Yusser Olguín
COMMITTEE : Dr. Patricio Häberle
Dr. Francisco Melo

“Vuelve a disfrutar del viaje, el cielo va a esperar que el ciclo acabes. Estado material es parte del sueño universal para encontrarte. El río va a tocar todas las puertas para encontrar un camino al mar.”

Héctor Mella Á.



*This thesis is dedicated to my grandmother Elsa.
Rest in peace with your beloved god, corazón de escarcha.*

Acknowledgments

Firstly, I would like to thank my parents: I have never felt that anything could possibly go wrong with my life, since I have you watching my steps. Never judging, always supporting. That's the best gift of life. My dear partner, Simona: we have evolved together during these years, understanding ourselves and life as a whole. Your company has been crucial not only for this writing process, but for everything. Thanks for being you, and for letting us share our lives. Don't ever forget to just live in the present and share your happiness with the world. Thanks to my advisor, Prof. Dr. Tomas P. Corrales, for his guidance during this process. I just can't think of a better advisor. You always supported me in all my ideas, no matter how far-fetched they seemed, and you even helped/pushed me to have two international internships (in Europe and California, where I'm going to start a Ph.D. in a few weeks) in less than a year! The lab environment is the result of your good guidance. Let's keep doing things with passion. Talking about the lab: Nicole, you're not even in the lab anymore, but I will always be grateful for your help. Dragica, you saved my life in Germany! Thanks!! Benja, Seba, Chava, Pacha, Cony, your company in the group is a treasure. I hope to keep in contact with you, and collaborate forever! SPMKidZ: Martín and José, I passed on my knowledge to you before leaving the lab, and you even had a contribution to this thesis (Fig.4.11 a),c),e))! I don't know if you will keep on this, but I'm sure that you will accomplish whatever you want. Keep moving from the heart. Don't do drugs. Thanks for letting me be your "mentor" for a few months.

Thanks to my co-advisor Prof. Dr. Yusser Olguín. Your collaborative guidance in terms of the thesis topics, added to the role that Prof. Dr. Filipe Natalio played in this work, made it an enjoyable and multidisciplinary one. Thanks to Dr. Michael Kappl, from the Max Planck Institute for Polymer Research, who not only received me on his group in Mainz, Germany, for some weeks, but also gave me the opportunity to have hours-long talks with him in the lab that made my AFM-understanding grow a lot. On the same page, I want to acknowledge Prof. Dr. Roberto Andresen (My new Ph.D. advisor!), who also received me within his group at University of California, Merced, during a couple of weeks, which greatly opened my science-understanding. Let's keep collabs!

This work was funded by ANID through project Fondecyt Regular 1211901 "Multiscale Mechanical Properties of Hygroscopic Nanofibers", Fondecyt Iniciación 11201056 "Modelling and optimization of a microbioreactor useful in peripheral nerve tissue engineering" and "Millennium Nucleus in NanoBio-Physics" project NNBP NCN2021.021. I also want to acknowledge my university, for giving me financial support by "Programa de Incentivos a la Iniciación Científica" (PIIC-2023-2), and their master's stipend scholarship.

Abstract

In recent times, biopolymer materials have been studied due to their wide range of applications and sustainability. Among these, biopolymer-based hydrogels are fundamental in medical research, such as neural tissue engineering. Cellulose-based materials with tailored properties can be obtained by combining the growth of cotton in tissue culture with molecularly-designed glucose building-block feeding. This project aims to study the nanoscale effects of biological incorporation of fluorine-based molecules on cotton and characterize the nanomechanical properties of various biopolymer-based hydrogels for neural tissue growth using environmental Atomic Force Microscopy (e-AFM).

We present a protocol for synthesizing cotton fibers using enzymatic digestion to yield nanofibrils. Both digested and non-digested cotton fibers were measured using e-AFM, including imaging and nanoindentation (for non-digested fibers) under different relative humidities. Collagen-, PEGDA-, GelMA-, and Alginate-based hydrogels prepared for neural tissue engineering were measured under liquid conditions.

Results indicate that both control and modified cotton macrofibers have micro- to nano-structures arranged along their elongation axis, with noticeable heterogeneities in their local mechanics, presumably due to their internal arrangement. In modified samples, the fluorine atom may also contribute to these differences. Control cotton nanofibers swell at specific spots when exposed to higher relative humidity, suggesting a semicrystalline structure. Modified cellulose nanofibers exhibit heterogeneous swelling, with almost periodic changes in stiffness and height, likely due to the fluorine atom's interference with their wettability.

Biopolymer-based hydrogels showed rugosity (RMS) values around 2 nm, except for Collagen, which had an RMS of around 15 nm, potentially due to UV light exposure. The Young's moduli of the gels ranged between 0.53 and 4.1 MPa.

Resumen

En la actualidad, los materiales basados en biopolímeros han sido ampliamente estudiados debido a su rango de aplicaciones y sostenibilidad. Entre estos materiales, los hidrogeles basados en biopolímeros son fundamentales para la investigación médica, como la ingeniería de tejido neural. Además, combinando el crecimiento de algodón en cultivo de tejidos con alimentación de bloques de glucosa diseñados molecularmente, fibras de algodón con propiedades adaptadas pueden ser obtenidas. Este proyecto tiene como objetivo estudiar los efectos a nanoescala de la incorporación biológica de moléculas basadas en flúor en el algodón y caracterizar las propiedades nanomecánicas de varios hidrogeles basados en biopolímeros para el crecimiento de tejido neural utilizando Microscopía de Fuerza Atómica ambiental (e-AFM).

Presentamos un protocolo para sintetizar fibras de algodón utilizando digestión enzimática basada en celulasa para obtener nanofibras. Tanto las fibras de algodón digeridas como las no digeridas fueron medidas utilizando e-AFM, incluyendo imágenes y nanoindentación (para fibras no digeridas) bajo diferentes humedades relativas. Hidrogeles basados en colágeno, PEGDA, GelMA y alginato preparados para la ingeniería de tejido neural fueron medidos en condiciones líquidas.

Los resultados indican que tanto las macrofibras de algodón de control como las modificadas tienen micro- a nanoestructuras dispuestas a lo largo de su eje de elongación, con heterogeneidades notables en su mecánica local, presumiblemente debido a su ordenamiento interno. En las muestras modificadas, el átomo de flúor también puede contribuir a estas diferencias. Las macrofibras modificadas muestran ciertos cúmulos entre estructuras más pequeñas. Las nanofibras de algodón control se hinchan en puntos específicos cuando se exponen a una mayor humedad relativa, sugiriendo una estructura semicristalina. Las nanofibras de celulosa modificadas exhiben una hinchazón heterogénea, con cambios casi periódicos en la rigidez y altura, probablemente debido a la interferencia del átomo de flúor con su humectabilidad.

Los hidrogeles basados en biopolímeros mostraron valores de rugosidad (RMS) alrededor de 2 nm, excepto el colágeno, que tuvo un RMS de alrededor de 15 nm, potencialmente debido a la exposición a luz UV. Los módulos de Young de los geles oscilaron entre 0.53 y 4.1 MPa.

Contents

Acknowledgments	iii
Abstract	v
1 Introduction	1
1.1 Cellulose	4
1.2 Hydrogels	9
2 Measurement Fundamentals	13
2.1 Atomic Force Microscopy Fundamentals	13
2.1.1 AFM Imaging	15
2.1.2 Phase Imaging Mode	17
2.1.3 AFM Force Spectroscopy	17
2.2 Environmentally controlled AFM (e-AFM)	18
2.2.1 Humidity controlled AFM	19
2.2.2 Liquid environment AFM (L-AFM)	20
2.3 Hertz Model of solids in contact	21
3 Experimental Setups and Protocol	23
3.1 Sample Preparation	23
3.1.1 Cotton Macrofibers	23
3.1.2 Nanocellulose Synthesis	23
3.1.3 Hydrogels	24
3.2 Microscopy Setup	24
4 Results and Discussion	27
4.1 Cellulose Project	27
4.1.1 Macrofibers	27
4.1.2 CNF Morphology	31
4.1.3 Humidity controlled experiments	31
4.1.4 Comparison with standard acid hydrolysis protocol	36
4.2 Nanomechanics of hydrogels	38
5 Conclusions and Outlook	41
Bibliography	43

Chapter 1

Introduction

It is of general knowledge that petroleum has been used as a vital resource for humanity since centuries, with medical and fueling applications, for example. It was not until the First Industrial Revolution that this resource started to have a great impact in the worldwide economy, and that it started to be completely exploited as a non-renewable natural resource, for its uses not only in energy but also in the creation of materials. In terms of materials derived from petroleum, we can easily think in plastics, synthetic rubber, nylon, lubricants, among others. One of the biggest problems with the petroleum industry is that contamination occurs quite often as a result of its uses, leading to significant ecological impacts [1]. In response to the effects of the un-ending demand of fossil-fuels as petroleum, a new generation of sustainable materials have been studied in recent times, such as bio-based materials, whose building blocks comes from living matter [2].

The present work can be placed in the field of physics of materials, and is particularly focused on **biopolymers**, which are *naturally occurring* polymers produced by living organisms. Biopolymers are materials that have been extensively studied throughout the years [3], since they offer a solution to achieve an environmentally-friendly alternative and help reducing waste derived from plastics [4] for many industries. The application of biopolymers range from textile industry and regenerative medicine to food industry. An example of the latter is the work of Bezjak et al. [5], where they made an edible scaffold with parallel microchannels based on marine biopolymers, showing that these microchannels allowed the formation of muscle-like structures (Fig. 1.1). Another example of application in muscle-tissue engineering is the work of Taborda et al. [6], where they used the electrospinning technique [7] to create a scaffold with aligned nanofibers from a blend of three different biopolymers (Fig. 1.2), which presented a high rate of cell growth.

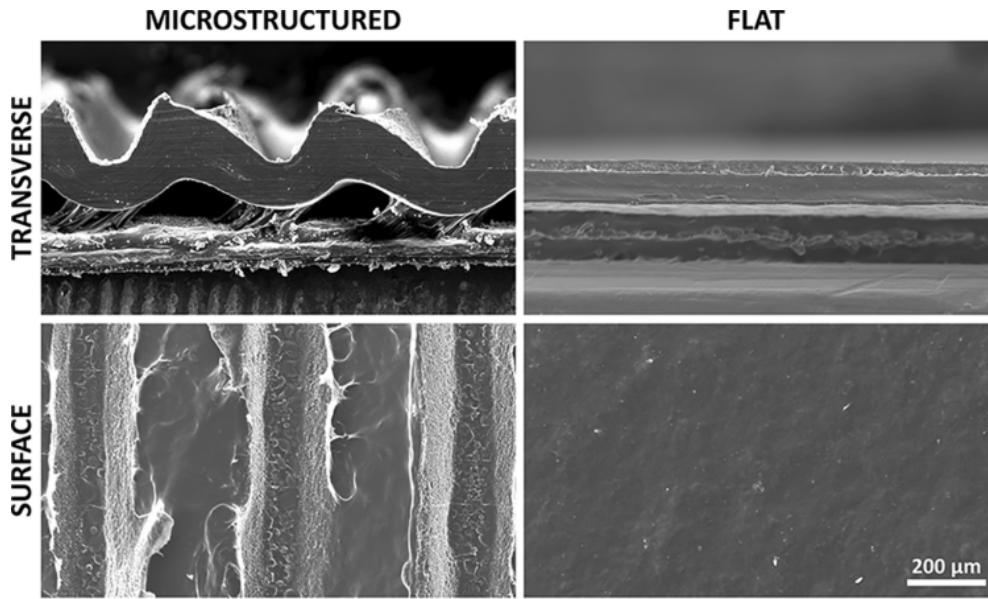


Figure 1.1: SEM images of the scaffolds created in the work of Bezjak et al. [5]. In the left column, one can see the periodic microstructures created on the scaffold, from a transverse and top view. Right column shows the counterpart, scaffolds without the microstructure-process.

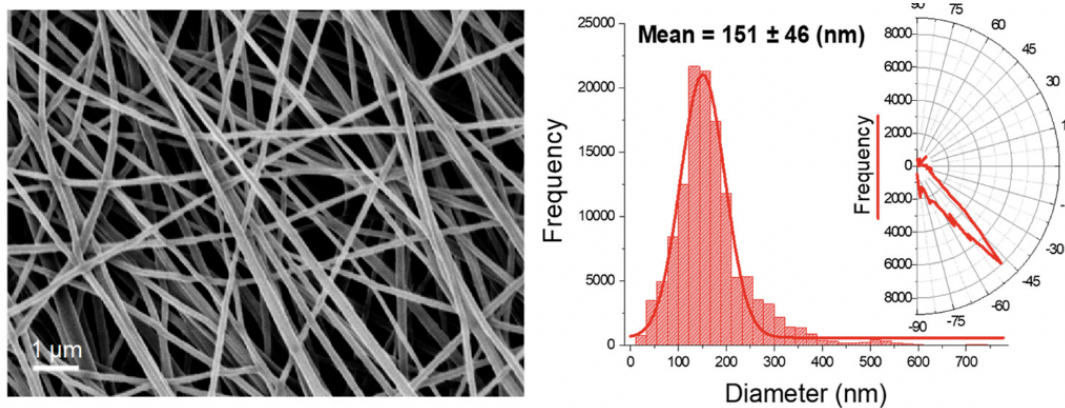


Figure 1.2: Left image shows a SEM micrograph of an electrospun nanofiber scaffold, made out of a biopolymer blend (Salmon gelatin, Chitosan, PVA). On the right, we can see a diameter histogram and that the sample has a relative mean fiber orientation at -48° . Adapted from Taborda et al. [6].

In terms of structure, a **Polymer** (or macromolecule) is a set of N molecular units linked together by covalent bonds, forming a chain. The molecular units (or monomers) could correspond to different structures, but what defines a polymer is that modifying some quantity $n \ll N$ of monomers, does not affect its properties. Also, N is denoted as the degree of polymerization. One of the simplest polymers that we can synthesize is the polyethylene (PE), which as its name says, is a chain composed of N ethylene chemical units. Polymers in general have a similar structure to that of PE (Fig. 1.3), usually being based on a Carbon back-bone, and replacing the Hydrogen atoms. It is important to know how a chain could be distributed in space to study it, since crystalline or amorphous forms can be presented.

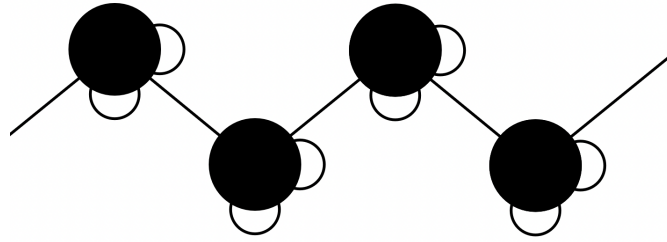


Figure 1.3: Polyethylene representation. Black and white spheres represent Carbon and Hydrogen respectively. Its chemical unit is CH_2 .

In order to understand polymers, various models, that will not be discussed here, are developed. Many of them are covered in Strobl [8]. Another thing that is worth to notice in this work, is that a polymer array can be either hydrophilic or hydrophobic (like or dislike water respectively), meaning that their response to environmental relative humidity will be determined by their internal capability to absorb water. It has been proved, for example, that collagen nanofibers present heterogeneous swelling [9], i.e. depending on the zone of the fiber, it interacts differently with water. It is visible, also, on Atomic Force Microscopy (AFM) phase images, which refer to different energy dissipation).

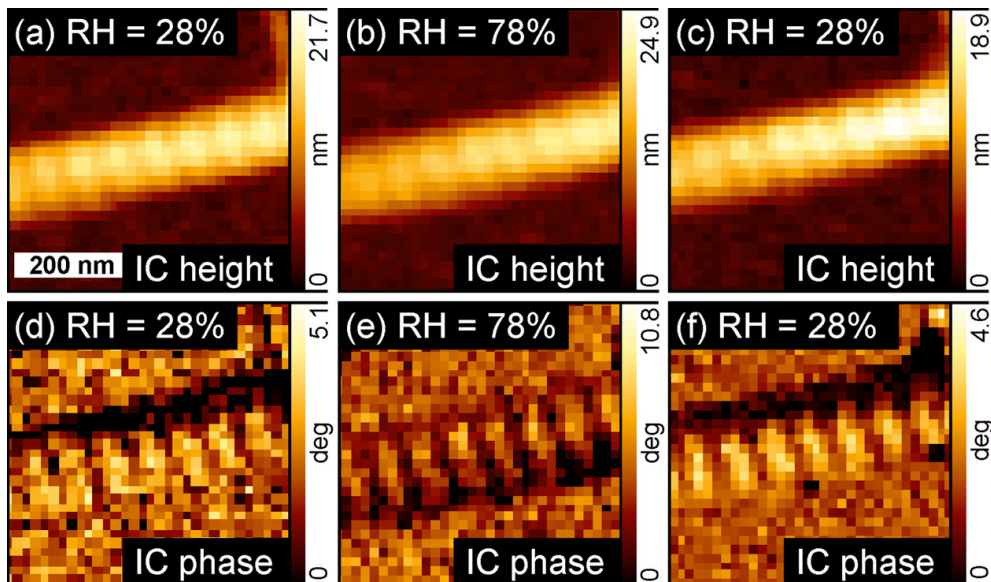


Figure 1.4: IC-mode AFM images of a collagen fibril before swelling [height (a), phase (d)], after 20 h of swelling [height (b), phase (e)], and after drying [height (c), phase (f)]. Image taken from Spitzner et al. [9]

Swelling of structures is influenced by the material's Degree of Crystallinity (DoC) [10]. Nanoscale swelling has also been studied in the work of Menacho et al. [11], where they studied both experimentally and theoretically the fiber-water interaction of a biopolymer with a high DoC (PVA), at different relative humidity levels (See Fig. 1.5).

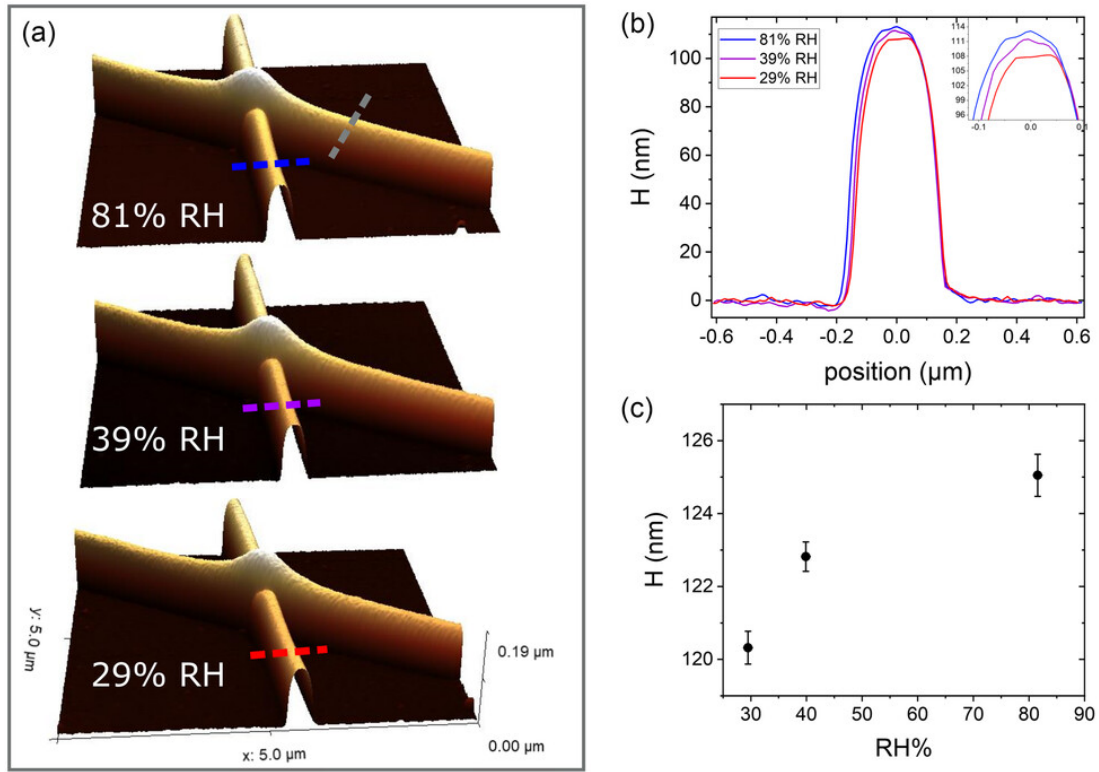


Figure 1.5: (a) 3D Topographies of two overlapping PVA fibers at different relative humidities (RH). (b) Cross-section of the bottom nanofiber for different humidity values. (c) Height of the nanofiber, obtained over three successive images of the same region, averaged from both bottom and right-side cross sections as a function of humidity. Taken from Menacho et al. [11].

1.1 Cellulose

One of the biopolymers that will be studied in this work is Cellulose. Cellulose is quite an interesting biopolymer, since it is considered semi-crystalline [12], and nowadays has many applications. For example, in the textile industry, cotton (cellulose-based material) is considered as a promising basis for wearable smart textiles (this concept will be discussed later, around Fig. 1.10) [13]. Vast research efforts have been placed on **nanocellulose**, using different methods to synthesize it, while working with different sources. In the work of Chakraborty et al. [14], a general analysis on characterization techniques was made, making clear that AFM is a strong tool to understand the properties of nanocellulose. They also present a schematic of the plant-based cellulose structure (Fig. 1.6). This work concludes that AFM is able to provide details of surfaces than can be complementary to Scanning Electron Microscopy (SEM) and yield mechanical/chemical properties of the sample that validate some results obtained with Infrared Spectroscopy (IR).

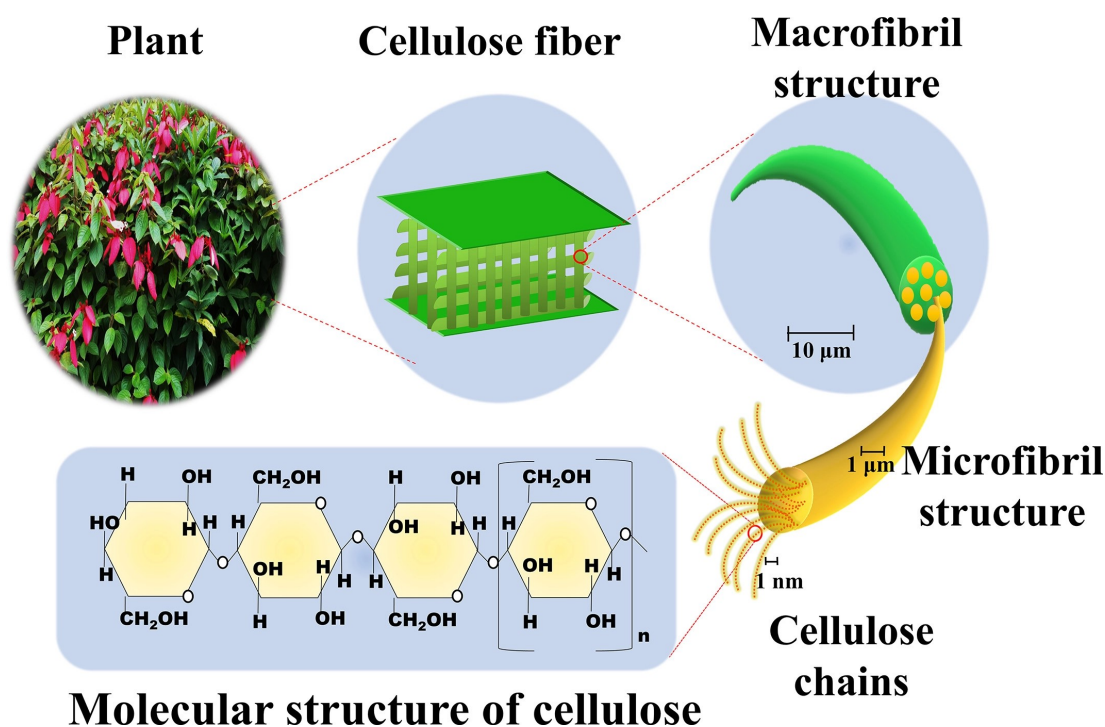


Figure 1.6: Plant-based Cellulose schematic structure. (Image taken from Chakraborty et al. [14]).

There have been successful methods to synthesize nanocellulose from different sources. For example, in the work of Fahma et al. [15], cellulose nanofibers (CNF) were obtained from **oil palm empty-fruit-bunches** by sulfuric acid hydrolysis (See Fig. 1.7). In that experiment, the average thickness of CNFs was within 1-3.5[nm], and their DoC rounded 53.83% and 58.78% depending on the time of exposition to the sulfuric acid. CNFs had cylindrical form. A fundamental point of this work is that they evidenced that in the hydrolysis process, the acid generally has troubles penetrating the crystalline regions, so the **amorphous region of cellulose is attacked first**. This can be seen with X-Ray Diffraction (XRD), noting that when the cellulose was exposed to the sulfuric acid, it first increased its DoC, while as the time of exposition to sulfuric acid passed, it decreased. Another interesting work is that from Li et al. [16], where CNF synthesized via hydrolysis using sulfuric acid are shown, but this time they come from **cotton**. The fibers had cylindrical form (rod like) and a smooth surface, with average lengths varying from 90.5[nm] to 664.9[nm], and widths varying from 7[nm] to 19.2[nm] (See Fig. 1.8). In this work, information regarding the crystallinity of nanocellulose from different sources was reviewed, showing variations from 54% to 87.1%.

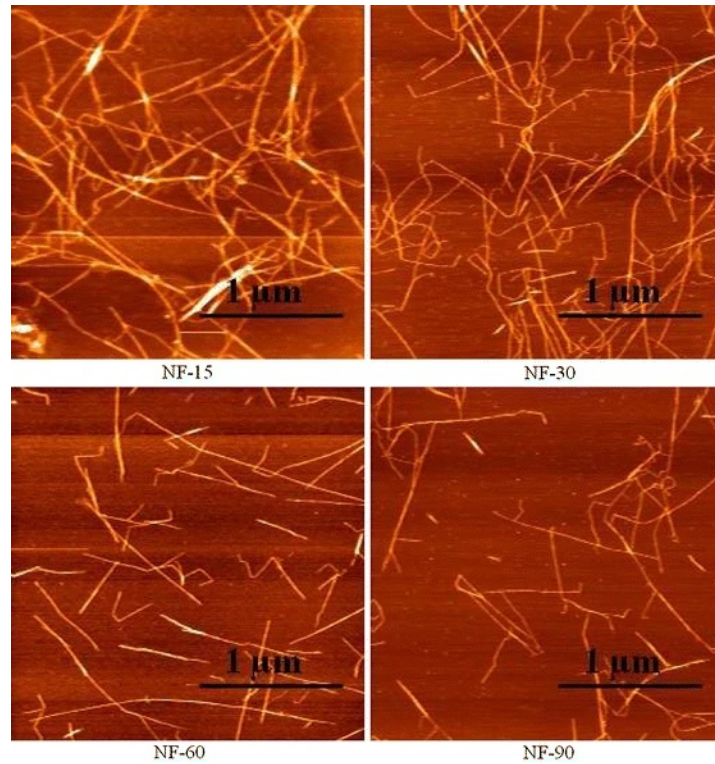


Figure 1.7: AFM images of CNF obtained from oil palm empty-fruit-bunches. The number after the dash on the code of the images refers to the time exposed to sulfuric acid, in minutes. (Image taken from Fahma et al. [15]).

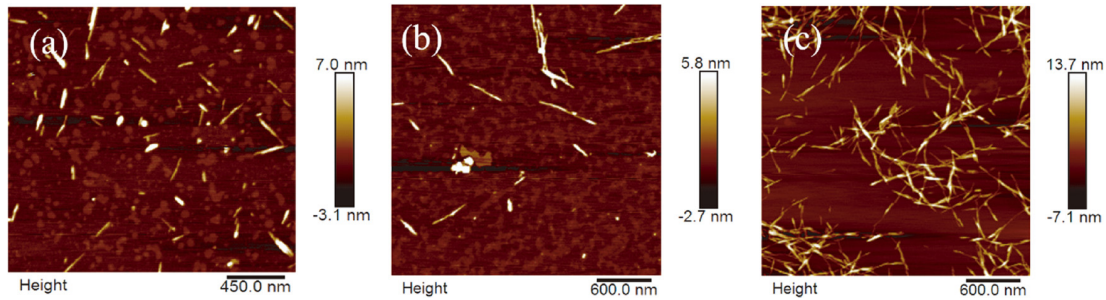


Figure 1.8: AFM images of cotton-based nanocellulose digested by sulfuric acid. Fibers with different times of exposition to sulfuric acid are shown (decreasing from left to right). Their lengths and width decrease with more time of exposition. Image taken from Li et al. [16].

The work of Ottesen and Syverud [10] concluded that there is a correlation between DoC and swelling. Higher DoC implies lower swelling (See Fig. 1.9). That evidence should imply that nanocellulose from different sources (with different DoC) would have different swelling properties. In this work, they did not find any evidence for distinct crystalline and amorphous domains in natural cellulose. They only reported a general swelling of the structures.

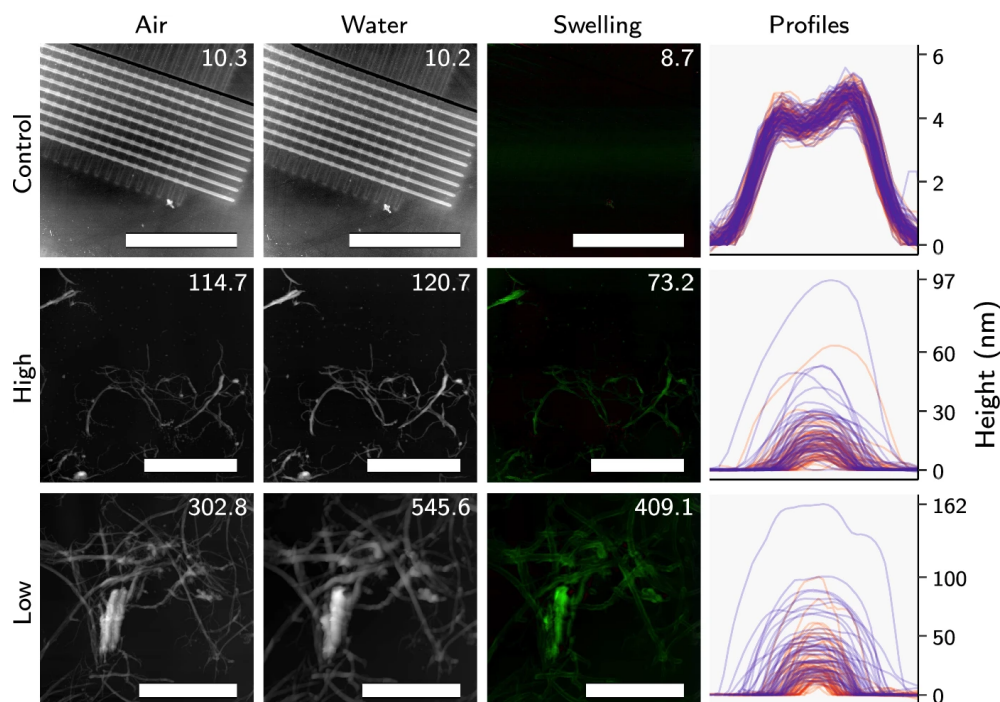


Figure 1.9: AFM micrographs of CNF with high DoC and low DoC in air or water as indicated. In the swelling column, the change in height. Scale bar is 5[μm]. Top right corner of the images indicates the max height registered. (Image taken from Ottesen and Syverud [10]).

In 2017, **biological** fabrication of cellulose fibers with **tailored properties** was achieved by Natalio et al. [13], where cellulose with magnetic and fluorescent properties were obtained. This was an important advancement towards improving the characteristics of cotton, thinking of it as a basis of smart textiles (adaptive materials that integrate multi-functionalities to dressing clothes [17], Fig. 1.10). Fig. 1.11 shows a scheme of the method used by Natalio et al. [13]. In summary, they combined *In vitro* cotton culture with molecularly designed glucose derivatives, obtaining unnatural properties from cotton fibers, such as magnetism and fluorescence. In plants, cellulose fibers are composed of β -glucopyranose units linked by the β -1,4-glycan bonds extruded as nanofibrils to the extracellular space of the plant ovule, where they self-assemble into larger micro to centimeter size structures through H-bonds. Natalio et al. [13] conclude that biological systems combined with appropriate molecular designs can be exploited in order to obtain functional composite materials, implementing the concept of **material-farming**. Thinking in the smart textiles industry, the idea of modifying cotton in order to control its wetting properties arises. This could not only lead to generate non-electronic cotton based smart textiles with hydrophobic responses to water, but also, to understand on a more fundamental level how the CNF extrusion process works and how it can be interfered by the biological incorporation of fluorine atoms.

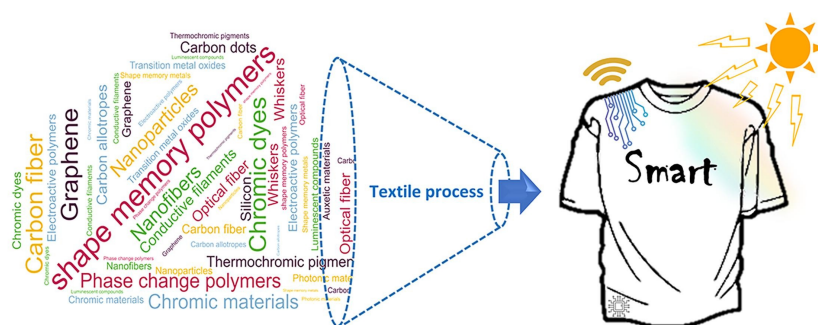


Figure 1.10: Representation of the use of different advanced materials in the preparation of smart textile products. (Image taken from [18]).

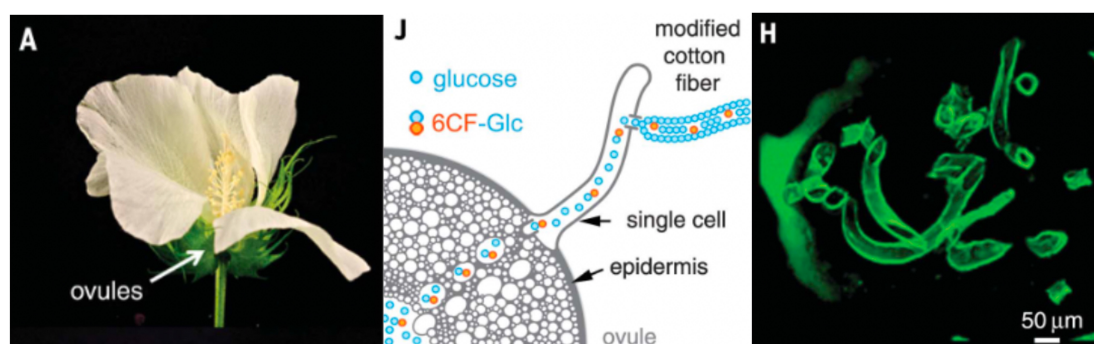


Figure 1.11: Schematic of the biological incorporation of fluorescent tagged glucose into cotton fibers. (Adapted from Natalio et al. [13]).

The idea for this project is to use this method to incorporate the glucose-based molecule 3-deoxy-3-fluoro-d-glucopyranose (3-dfg) in fertilized cotton ovules, and then detect its effect at the nanoscale. 3dfg is a molecule that, in simple words, is a glucopyranose (as the ones that composes cellulose, see Fig. 1.6) with its -OH group from Carbon-3 replaced by a Fluorine atom.

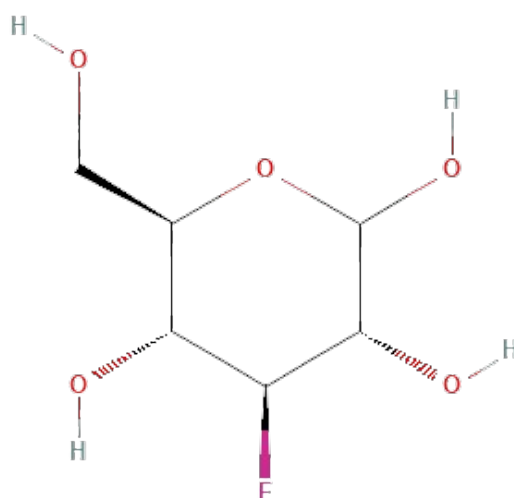


Figure 1.12: 3-deoxy-3-fluoro-D-glucopyranose molecule representation [19].

1.2 Hydrogels

The second biopolymer-based structures that we will study in this project are hydrogels, focusing on nerve tissue engineering. Hydrogels are created when *hydrophilic* polymeric chains are crosslinked (Fig. 1.13), typically forming 3D structures. Biopolymer-based Hydrogels have a wide range of applications, considering that when hydrated, they are in general soft materials with a considerable amount of water content. This can be used to simulate many biological structures and has lately attracted research groups [20].

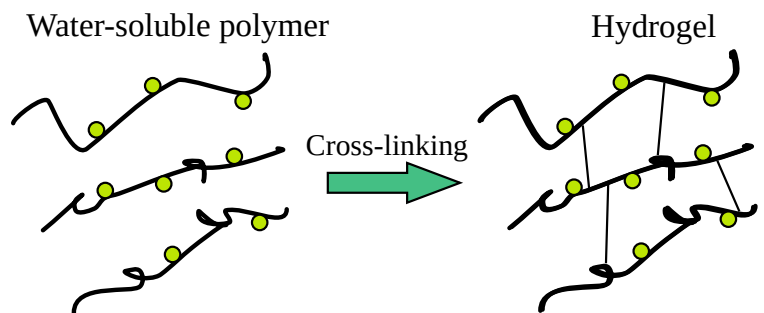


Figure 1.13: Representation of a hydrogel structure.

In terms of biomedical applications, for example, in tissue engineering, hydrogels are used to emulate the extracellular matrix (ECM) as scaffolds. Collagen is the most used biopolymer in tissue engineering, since it is the main component of the mammalian tissues' ECM [21]. The type I collagen, the most common for this applications, is a triple-helical protein of periodic polypeptide chains. Collagen fibrils self-assemble into bundles that are crosslinked to obtain the hydrogels [22]. An AFM image of a collagen gel is presented in Fig. 1.14.

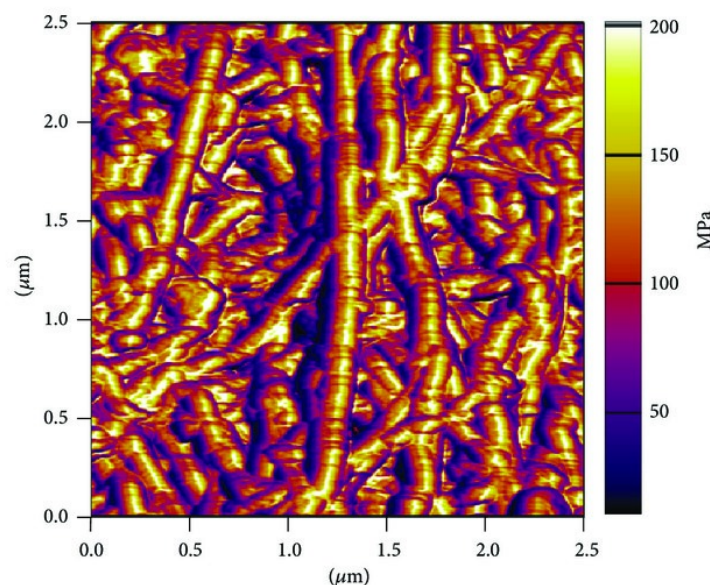


Figure 1.14: AM-FM image of a collagen gel. Image from Stylianou et al. [23]

There is also research that has shown the benefits of mixing traditional biopolymer-based hydrogels with novel materials, such as Carbon NanoTubes (CNT). For example, the work of Ye et al. [24] showed that mixing CNT with PEG (Polyethylene Glycol) improved considerably the cell viability compared to the pure PEG hydrogel, when studying PC12 cells. The cell adhesion in general of pure PEG hydrogels is minimal, but it was not the case for the CNT-PEG gels. The authors correlate this with the low rate of protein adsorption presented by the pure PEG hydrogel (c-PEG-0 in Fig. 1.15).

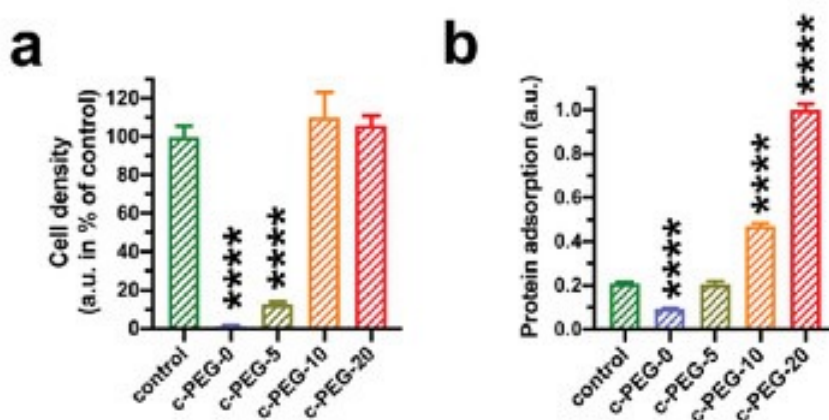


Figure 1.15: a) Cell density of PC12 cells after incubation on PLL-coated glass substrates (control) and different CNT-PEG composite samples. c-PEG-0,5,10,20 refers to different CNT concentrations, where 0 is pure PEG. b) Normalized ECM protein adsorption of control and different CNT-PEG composite samples. Adapted from Ye et al. [24]

In the particular case of peripheral nerve tissue engineering, hydrogels made from compounds such as Gelatin Methacrylate (GelMA), Polyethylene Glycol Diacrylate (PEGDA), Collagen, and Alginate have been shown to be good candidates to serve as biomimetic ECM scaffolds [25, 26, 27, 28, 29]. This is mainly due to their tunable mechanical properties, compared to other types of hydrogels. The Peripheral Nervous System (PNS) serves as the extensive network of nerves that branch out from the Central Nervous System (CNS) and connect with the rest of the body. It is composed by nerves, ganglia, and sensory receptors distributed throughout the body, facilitating communication between the CNS and various tissues, organs, and limbs. Nerves are bundles of nerve fibers (axons) and their associated connective tissues extend from the CNS to different parts of the body. They carry signals to and from the CNS, allowing communication with muscles, glands, and sensory receptors. Ganglia are clusters of nerve cell bodies and support cells located outside the CNS. They serve as relay points for incoming and outgoing nerve signals. Sensory Receptors detect various stimuli such as touch, temperature, pain, pressure, and chemical changes throughout the body. Examples include photoreceptors in the eyes, mechanoreceptors in the skin, and chemoreceptors in the taste buds and olfactory epithelium. Having this in mind, and understanding that most signals are carried out by electrical pulses, we can understand that the environmental electrical activity could affect the development of the neural system. In the

same work of Ye et al. [24], they remark the importance of electrical activity for neurons to keep the homeostasis. In fact, the work of Ma et al. [26] shows how changes in homeostasis could lead to neuropsychiatric disorders. However, not only electrical signals play a role in neuronal development. The work of Koser et al. [30] showed the importance of mechanosensing in the axon growth in developing brain. They showed, for example, that axons Retinal Ganglion Cells (RGC) that were exposed to the same chemical environment but to substrates with different stiffness grew different. In fact, after 24 hours, axons seeded on stiffer substrate were considerably longer than their counterpart (Fig. 1.16).

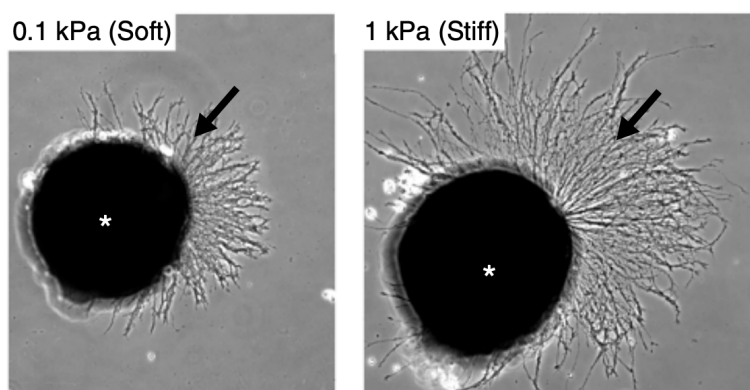


Figure 1.16: Cultures of Xenopus eye primordia (asterisks) on (a) soft (0.1 kPa) and (b) stiff (1 kPa) substrates. Arrows indicate axons. Adapted from Koser et al. [30].

Along with the stiffness of the surface, it is also important to characterize its roughness [31]. Roughness affects protein adsorption [32], and thus, cell-adhesion, which is mediated by binding proteins. Depending on the cell type and culture conditions, there can be threshold roughnesses for the cells to grow [33, 34]. So, to understand the neuronal behavior in the different processes (for example, when regenerating tissue from an injury), we would need to study not only the chemical-electrical signals, but also how the environmental mechanics affect them. This makes imperative to have mechanical characterizations of the substrates used for brain-cells culture, for example, which motivates the second part of this work [35].

In summary, this thesis aims to study the nanoscale effects of biological incorporation of fluorine-based molecules on cotton, and also to characterize the nanomechanical properties of different types of biopolymer-based hydrogels for neural tissue growth. This will be done through different experiments using Atomic Force Microscopy under different conditions, including humidity-controlled and liquid-environment experiments. In chapter 2, AFM fundamentals will be introduced, as well as basic deformation theory concepts and the Hertz Model for solids in contact. Chapter 3 will focus on experimental setups and protocols, while in chapters 4 and 5 the results and conclusions will be presented.

Chapter 2

Measurement Fundamentals

In this chapter, fundamentals of AFM will be introduced, considering the environmental conditions and modes of operation. Then, the Hertz Model of contact mechanics will be presented.

2.1 Atomic Force Microscopy Fundamentals

Atomic Force Microscopy (AFM) is a type of Scanning Probe Microscopy (SPM), invented in the 1980's, after the Scanning Tunneling Microscopy (STM) [36] and Scanning Near-Field Optical Microscopy (SNOM) [37]. AFM uses a probe (tip) to get high-resolved details of a surface through the tip-sample interaction. AFM has its own branches, such as Magnetic Force Microscopy [38] (a magnetized tip scans a magnetic sample) and Kelvin Probe Force Microscopy [39] (measures contact potential differences). An important difference between AFM and its predecessors is that it allows imaging topography of **conducting and isolating surfaces**, with atomic resolution [40]. Atomic resolution with AFM has been proven to be obtainable in vacuum, and even at room temperature [41].

The basic components of AFM (see Fig. 2.1) are a Laser, Tip, Cantilever, Piezo actuators and Photodiode (PD). The tip is the component that will interact with the sample, and is attached to a cantilever, generally made of silicon. The cantilever is used to measure the interaction between the tip and the surface, through the signal that the PD receives from the laser, which is focused on the backside of the lever. The laser points the cantilever, and is deflected and caught by the PD, which typically has 4 quadrants (photosensitive regions). The PD generates a current proportional to the photons caught in every quadrant. The Piezo actuators are piezo electric crystals that move the cantilever across the surface, and control the tip-sample distance.

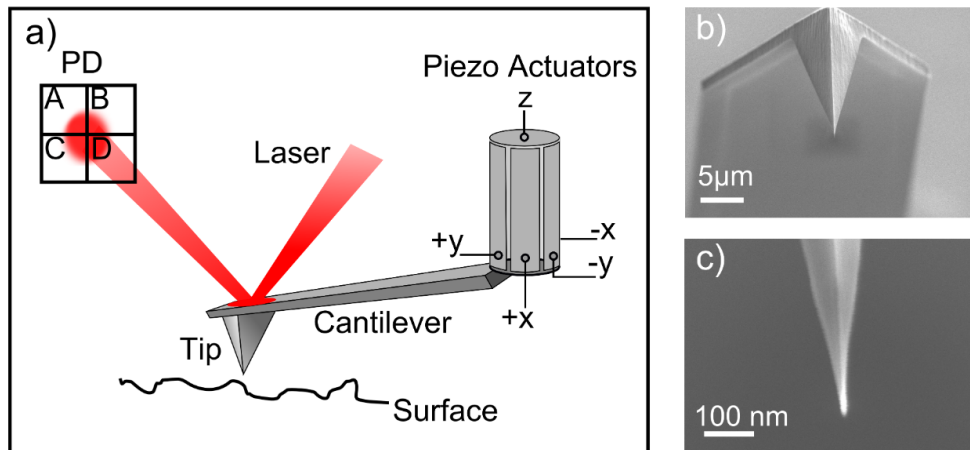


Figure 2.1: a) Basic AFM components representation. b) SEM image of a commercial cantilever with its tip. c) Zoom of a cantilever tip. (Image taken from Corrales [42]).

AFM topographical images are generated considering the tip-sample interaction, which is a mix of short and long range forces, that can be understood with a Lennard-Jones potential (see Fig. 2.2).

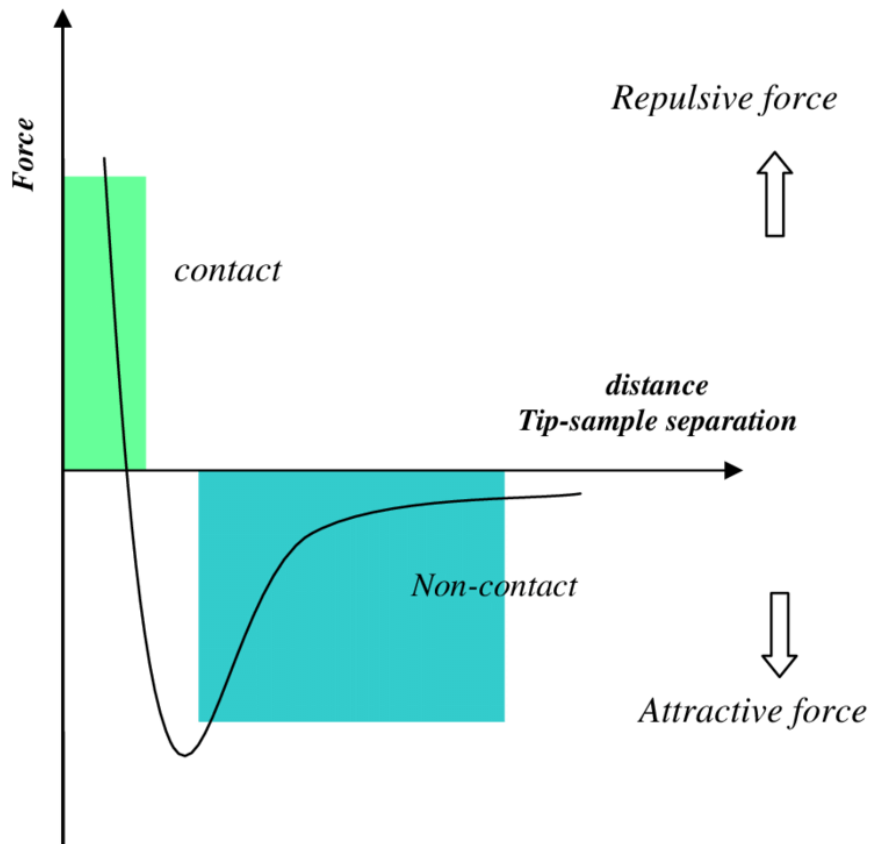


Figure 2.2: Representation of tip-sample interaction as Lennard-Jones potential, in terms of distance. The distances marked correspond to different AFM modes. (Image taken from El-Desawy [43]).

2.1.1 AFM Imaging

There are three principal modes for the AFM to operate creating topographical images:

- **Contact mode:** Cantilever-sample distance is in the repulsive region of Fig. 2.2. The probe scans keeping a constant tip-sample force, considering that the interaction will be repulsive, thus being able to do a 3D topographical map [44]. From this mode, the Force spectroscopy that will be presented later was developed.
- **Tapping mode:** The cantilever vibrates near its mechanical resonance frequency. The resonance frequency is obtained doing a frequency sweep, using an additional piezo-actuator located on the cantilever holder. Once approached to the sample, the tip will *tap* or briefly touch the surface, reducing the vibration amplitude [45]. This can be understood as the tip intermittently moving from the contact to the non-contact regime of Fig. 2.2. By taking the variation between the free-vibration amplitude and the vibrating amplitude that the tip has in every point as a setpoint, a topographical map of the surface can be obtained. This mode is also known as Amplitude Modulation (AM) mode.
- **Non-contact mode:** The cantilever vibrates near its resonance frequency. As the tip approaches close enough to interact with the sample, the force between them would be enough to produce a shift in the frequency from the resonance one. The change in frequency is related proportionally to the gradient of the interaction force. To see this, we start considering the frequency of an interacting cantilever and then doing the considerations shown in [42]:

$$f = \frac{1}{2\pi} \sqrt{\frac{k^*}{m}} \quad (2.1)$$

$$= f_0 + \frac{1}{4\pi m} \sqrt{\frac{m}{k_0}} k^* \quad (2.2)$$

$$\Rightarrow \frac{\Delta f}{f_0} = \frac{1}{2} \frac{k^*}{k_0} = -\frac{1}{2k_0} \frac{dF}{dz}. \quad (2.3)$$

f_0 is the non-interacting frequency of the lever, k^* the effective spring constant, and k_0 the lever's spring constant. By taking the shift in frequency as a setpoint, a topographical image is created. This mode is also known as Frequency Modulation (FM) mode.

The reason for using different modes is that they are optimized for different types of samples and objectives. Contact mode has the advantage, for example, that it can measure lateral forces on a sample, but at the same time the disadvantage that the same movement can damage the tip or the sample. In the work of Zhong et al. [46], they show differences between contact mode AFM and Tapping mode, in a polymer surface, concluding that tapping mode was better at imaging (see Fig. 2.3), as it generated images without damaging the tip nor the sample, while contact mode induced irreversible damage in the polymer.

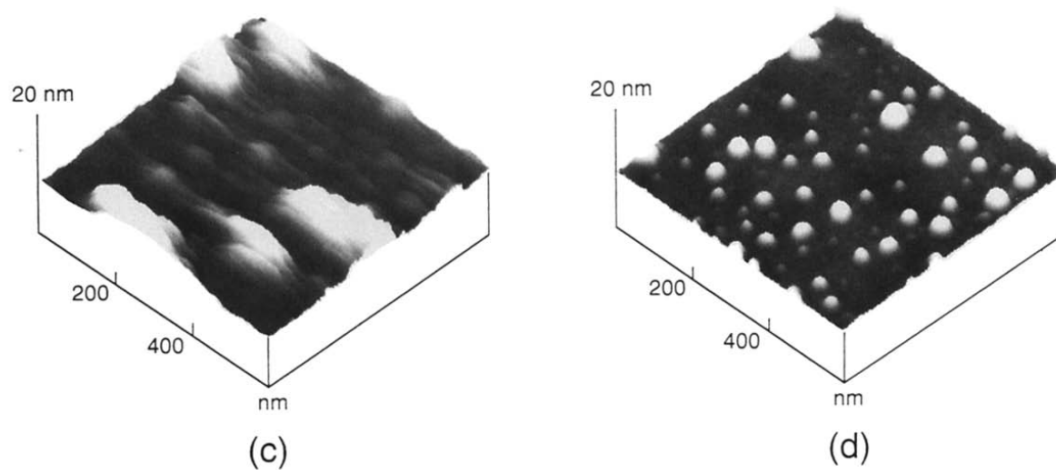


Figure 2.3: Comparison of AFM (c) Contact mode, (d) Tapping mode imaging on a Fractured polymer/silica fiber surface. (Image adapted from [46]).

If the sample's topographical features are very small compared to the tip radius, which is commonly in the order of nanometers, the image generated can be distorted, since the tip would be hitting the same feature for a longer period (See Fig. 2.4). This is a problem that can be solved generally decreasing the tip radius as much as it is needed, as well as adjusting the scanning velocity. Nevertheless, depending on the environment-tip interaction, this may not be enough (will be discussed more in Sec. 2.2). Another method to look into more details of the surface's properties using AFM is through the Phase Imaging mode, that will be presented next.

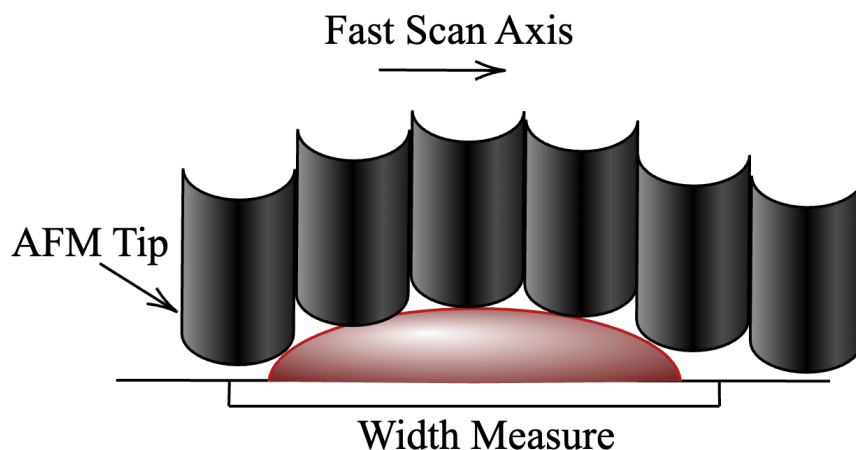


Figure 2.4: Graphical representation of how the dimensions of a sample measured with AFM can disturb the real topography if the tip radius is comparable to the sample scale.

2.1.2 Phase Imaging Mode

Based on the tapping mode, Phase Imaging goes beyond topography, and gives information about the variations of materials properties, such as adhesion, friction, and viscoelasticity [47]. Basically, the cantilever has an input with a certain frequency, which is shifted every time it interacts with a surface, due to the energy loss. This phase shift is recorded, and thus a phase contrast map is obtained.

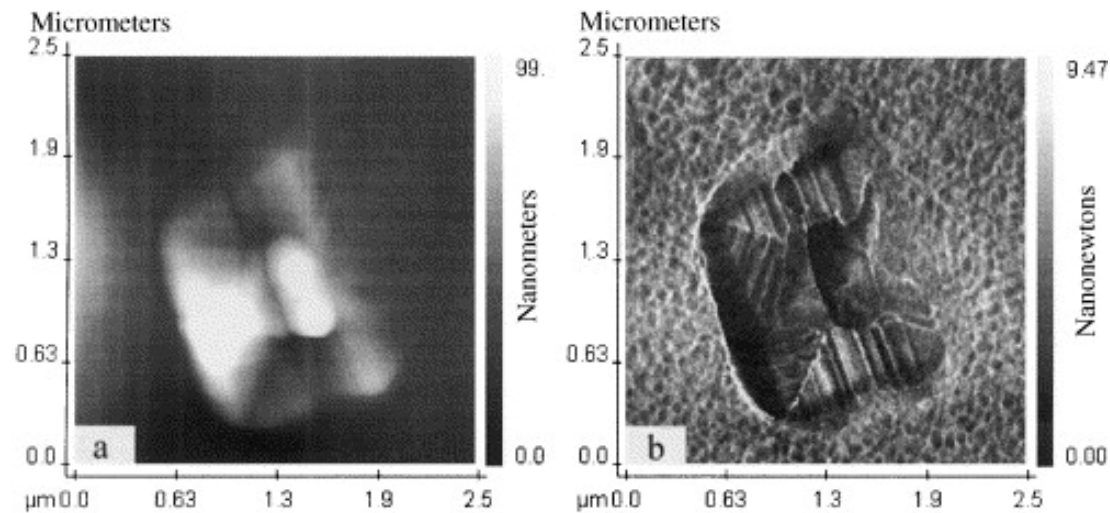


Figure 2.5: (a) Topographic AFM image of a ferroelectric PZT film showing a large PZT crystal. (b) Phase-contrast image of the same region as (a), showing the large crystal composed of a central crystallite, surrounded by four twinned crystals. The twinned crystallites contain black and white striations. The fine granular structure of the bottom PZT film can be also easily distinguished. Image taken from Pang et al. [48].

An example of the details that can be obtained using the phase imaging mode can be seen in the work of Pang et al. [48] (Fig. 2.5). In this example, we can see a big difference between phase and topography, when considering the nano-scale details. Nano- to micro-structures can be more easily spotted through phase imaging, thanks to the difference in the energy dissipation in every the tip-sample interaction. This difference can be due, for example, to the stiffness of the material. Of course, it will always be a mixture of different parameters affecting.

2.1.3 AFM Force Spectroscopy

Force Spectroscopy measurements consists on approaching the AFM tip onto a single point of the sample's surface in order to deform it, while the cantilever's vertical deflection is measured by the Photodiode [42]. The movement is made by the Z-piezo actuator. In the moment that tip and sample are in contact, their deformation is understood as the one of two Hookean bodies (if the sample is deformable). This assumption is made under the understanding that the deformation should be small enough compared to the sample's total dimensions, so it can be considered an elastic deformation and not pass to the

plastic regime. Then, the deformation of the sample (δ_s) can be obtained in terms of total deformation (δ_t) and the cantilever's deformation (δ_c). Knowing the cantilever's deformation and its spring constant (usually obtained from thermal noise method, as explained in the work of Hutter and Bechhoefer [49]) the deflection signal can be converted into applied force.

To measure a soft sample, a reference on a hard and undeformable sample is taken. This allows to calibrate the deflection sensitivity of the cantilever, i.e., how the deflection and the PD voltage are related. Once the reference is done, a Force graph in terms of tip-sample distance can be made. This is shown in Fig. 2.6, where approach-retract curves can be seen.

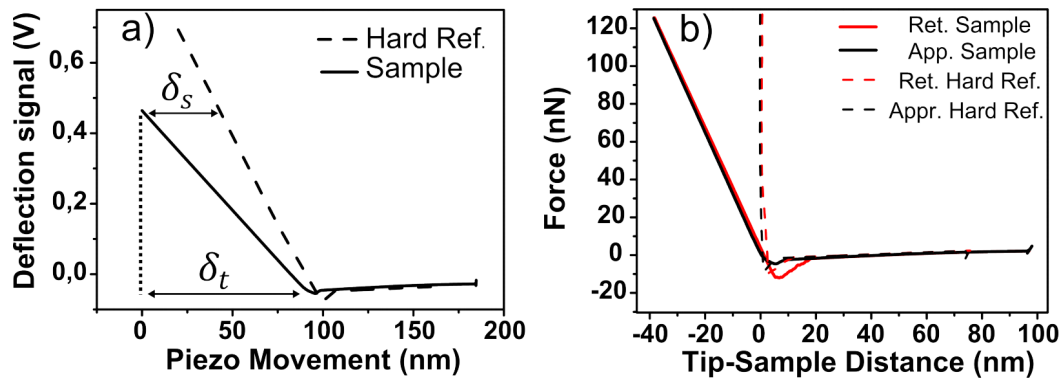


Figure 2.6: a) Reference curves, used to calibrate the microscope's sensitivity. Hard and soft samples are illustrated. b) Final converted Force curves in terms of tip sample distance. Image taken from Corrales [42].

Fig. 2.6 shows another interesting phenomena that usually occurs on deformable samples, the hysteresis. Hysteresis of the approach-retraction curve can be caused by multiple factors, such as capillarity of the sample (dependent on the surface tension), sliding the tip on the surface, and others. Having the force-distance curves obtained from a tip-sample interaction, we can identify the elastic regime of the curve, and use a force value to obtain the Young's modulus of the sample using Eq. 2.9 (explained in the following section).

2.2 Environmentally controlled AFM (e-AFM)

The traditional AFM technique operates in ambient conditions, where the sample is scanned in air or vacuum. However, many biological and soft materials require a liquid environment to maintain their functionality and integrity. Also, as we saw in the first chapter, the exposition to different relative humidity levels of the samples can give us insights on how it interacts with water at a molecular level. Then, controlling the environment of the AFM chamber is something of great interest for this project.

2.2.1 Humidity controlled AFM

Real e-AFM setup will be presented later, but here we are introducing the schematic idea of how the environmental chamber of the AFM would be (Fig. 2.7).

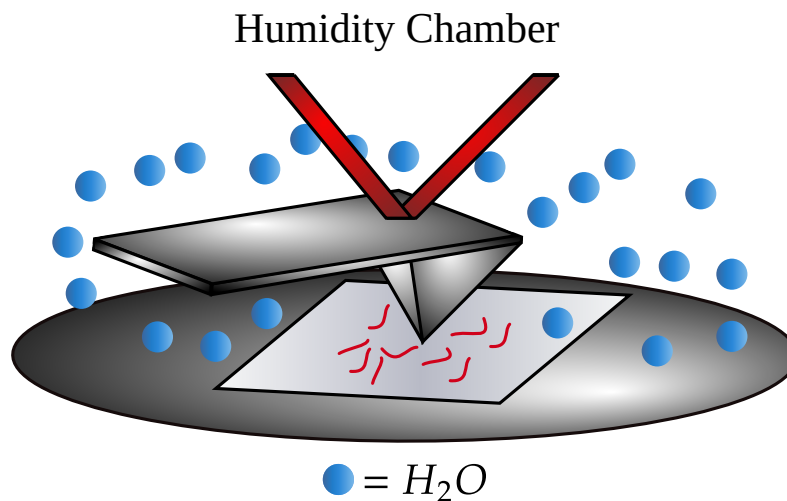


Figure 2.7: AFM humidity chamber schematic.

Something to take into consideration while doing any measurement that has to do with humidification, is that the tip-sample interaction could be hardly affected by the tip-water interaction too. In fact, it is common that the sharp tip could create some capillary-bridges (See Fig. 2.8) when interacting with the sample, enhancing effects similar to what was shown in Fig. 2.4. This can affect the interpretation of the sample's width, would not alter the measurement of the sample's height.

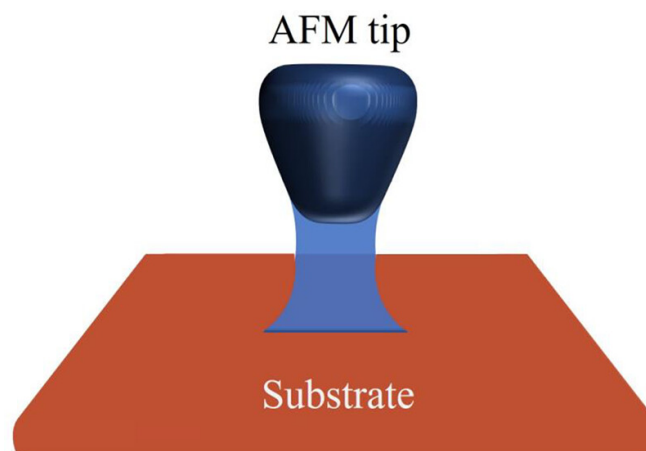


Figure 2.8: Representation of the capillary bridges than can be formed between tip and substrate when exposed to environmental humidity. Imagen taken from Payam [50].

2.2.2 Liquid environment AFM (L-AFM)

Liquid Atomic Force Microscopy (L-AFM) allows to study interactions, dynamics, and properties of samples immersed in liquids such as water, organic solvents, or biological buffers. If one wants, for example, to study the nanomechanical properties of hydrogels in a hydrated state using AFM, the experiment would have to be performed in a liquid environment. The configuration will rely on the sample to analyze, but the idea is to submerge both, the AFM cantilever and the sample in liquid. The main technical challenge of this method is the presence of liquid itself, which could introduce additional forces and interactions compared to the dry environment. In particular, the liquid environment can deeply affect how the photo-detector receives the laser signal (remember Fig. 2.1). This can be improved using cantilevers with a gold-coating on their back, for example. This technical problems do not imply by any term that resolution is lost using this method. In fact, as one can see in the review work of Sumaiya and Baykara [51], first atomic resolution out of vacuum measurements with AFM were obtained in liquid environment by Ohnesorge and Binnig [52]. The liquid AFM compared to the environmental one has advantages, as avoiding the formation of capillary bridges (Fig. 2.8), and also the friction forces are reduced¹.

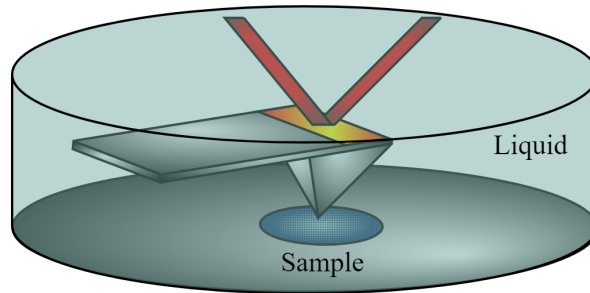


Figure 2.9: L-AFM setup representation. In this representation, a gold-covered back cantilever is presented.

¹Even with that, the group of Sumaiya et al. [53] in 2022 was able to achieve true atomic resolution imaging in air, using Conducting Atomic Force Microscopy, but that is out of the scope of this work.

2.3 Hertz Model of solids in contact

In this section, some important results, as well as the considerations taken by the Hertz Model will be briefly presented. The work of Hertz [54] considered two spheres in contact, as represented in Fig. 2.10. Note that Fig. 2.10b. accounts of a circular contact surface, with radius a .

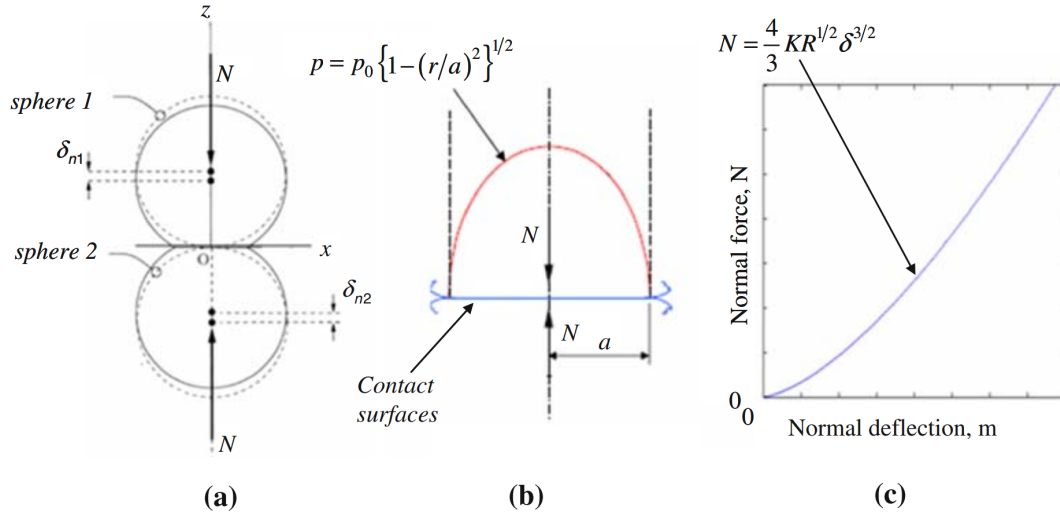


Figure 2.10: a) Action of the normal force (N), normal deflection (δ) and resulting contact surface. b) Contact pressure profile between the spheres. c) Typical normal force vs normal deflection curve. (Image taken from [55]).

Following Hertz (and, as a more contemporary approach, the analysis made in the work of Dintwa et al. [55]), the maximum pressure is given at the centre of the contact surface, $r = 0$, and is given by:

$$p_0 = \frac{3F}{2\pi a^2}, \quad (2.4)$$

where F refers to the applied force, that was named as N in Fig. 2.10. The term a can be written in terms of the effective stiffness (K) and the effective radius of curvature (R) as:

$$a = \left(\frac{3FR}{4K} \right)^{1/3}. \quad (2.5)$$

The definitions of R and K are the following:

$$\frac{1}{K} = \frac{1 - \nu_1^2}{E_1} + \frac{1 - \nu_2^2}{E_2}, \quad (2.6)$$

$$\frac{1}{R} = \frac{1}{R_1} + \frac{1}{R_2}, \quad (2.7)$$

where ν_i and R_i are the Poisson's ratios and radii of curvatures of both spheres. Finally, the normal deflection will be given by:

$$\delta = \left(\frac{9F^2}{16RK^2} \right)^{1/3}. \quad (2.8)$$

In case that one of the materials is much harder than the other one, having a Young's modulus of E , one of the terms of Eq. 2.6 would disappear, yielding:

$$\delta = \left(\frac{9F^2 E^2}{16R(1 - \nu^2)} \right)^{1/3}. \quad (2.9)$$

For this model to be theoretically developed, many assumptions were taken into account [55]: 1) materials of contacting bodies should be isotropic and homogeneous. 2) static loads applied. 3) dimensions of the bodies are much larger than contact radius. 4) materials are Hookean (linearly elastic). 5) no friction effects are considered. 6) small deformations. 7) static loads are applied. In reality, many of these assumptions cannot be applied. Nevertheless, the work of Dintwa et al. [55] showed that some of these conditions are not mandatory to be satisfied in order to get correct results using the model.

To apply this model, we need to know the Poisson's ratio (ν) of the material, which is defined explicitly as:

$$\nu = \frac{\text{Transverse Strain}}{\text{Longitudinal Strain}}, \quad (2.10)$$

and describes the coupling between axial and transverse deformations [56]. i.e., it describes how a material changes in shape when stretched or compressed. In general, for incompressible materials such as rubber, ν tends to have a value of 0.5. Even if we know this value tends to considerably deviate from that [56], during this work we will not experimentally determine the Poisson ratio of our samples and only consider it to be 0.5. The work of Smith et al. [56], for example, shows a facile method to quantify ν for gels, but it escapes from the scope of this work.

Chapter 3

Experimental Setups and Protocol

In this chapter, the sample-preparation protocols will be described, as well as the e-AFM setup. Additionally, tools and equipment used for the synthesis and AFM analysis will be presented. Macroscopic cotton samples and hydrogels were provided by our collaborators.

3.1 Sample Preparation

3.1.1 Cotton Macrofibers

For treated and control cotton samples, a small (yet visible) macroscopic fiber was isolated from bulk cotton using tweezers. Then, it was fixed to a glass coated with a fresh epoxy film, created through blade coating (See Fig. 3.1). The blade is embedded with an epoxy mix on its edge, and then used to create a thin film on the glass. A thin thread of cotton is placed over the epoxy film using tweezers, and left to dry at room temperature for 24 hours. After this, samples were measured using an environmental atomic force microscope (e-AFM).

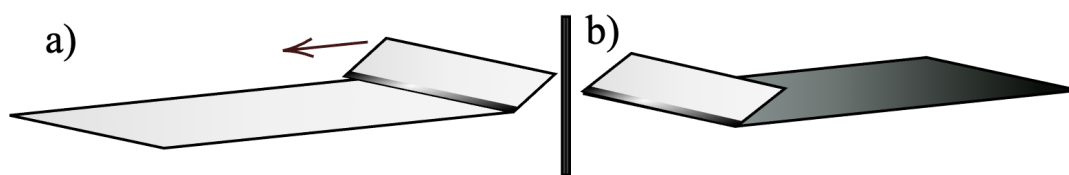


Figure 3.1: Schematic representation of the blade coating of glasses. a) Glass and blade before the coating. b) Glass and blade after the coating.

3.1.2 Nanocellulose Synthesis

Treated and control cotton samples weighing approximately 10 mg were enzymatically digested using *Trichoderma reesei* cellulase (*Trichoderma reesei* ATCC 26921, Sigma, Israel) (1.8mg/mL) in ultra pure water and kept at 25°C using a thermoblock for 16h . This process can be iterated to get more days of digestion. Then, $10\mu\text{L}$ of the resulting solution was diluted 1 : 500 in doubled distilled water and kept at 4°C until further measurements were performed.

Finally, 10 μL of the diluted solution was placed onto a silicon surface, previously cleaned with isopropanol, and placed in an incubator at 10°C for a drying period of 5h. Once dried, the samples were measured using an e-AFM. In this work, the control CNF was analyzed after 3 days of digestion, while the modified CNF was digested two days.

3.1.3 Hydrogels

¹ Collagen solution was prepared by dissolving 1.5 mL of collagen (Collagen I) in 30 mL of 20 mM acetic acid. A total of 2 mL of the solution was deposited in each well of the six-well plate, left to incubate for two hours, and then washed three times with PBS (Phosphate-Buffered Saline) pH 7.4. On the other hand, PEGDA hydrogel was prepared by mixing 1 g of PEGDA and 31 mg of Darocur 1173 (photoinitiator), dissolved in 10 mL of HEPES. A total of 1.5 mL of solution was deposited in each well of a six-well culture plate and exposed to UV radiation (0.4 joules), the wells were washed three times with PBS pH 7.4. Alginate hydrogels were prepared by ionic polymerization by mixing 800 μL of an alginate solution and 1 mL of 100 μM CaCl_2 . A total of 2 mL of the mixture is placed in each well of the six-well culture dish, allowed to rest for 30 min, and washed 3 times with PBS pH 7.4. The alginate solution is prepared by dissolving 0.5 g of alginate in 50 mL of culture medium (RPMI 10% HS, 5% FBS, 1% AB) for a final concentration of 0.01% w/v. Finally, the synthesis of methacrylated gelatin (GelMA) hydrogel was prepared from a modified protocol by reaction of gelatin with methacrylic anhydride. From a 10% (w/v) gelatin solution in PBS (pH 7.5) at 50°C, methacrylic anhydride was added slowly (0.2 mL/min) under vigorous stirring until a 20% v/v gelatin solution was formed. The resulting solution was diluted 1:2 in PBS and dialyzed for 3 days in Milli-Q water at 40°C. The product was freeze-dried to obtain a powder in varying amounts. Treatment of the culture surfaces was performed by dissolving 0.1 g of GelMA in 2 mL of PBS, plus 500 μL of culture medium and 100 μL of Irgacure (photoinitiator) 4:5:1, 1.5 mL of the mixture was deposited per well in six-well plates and irradiated with UV 0.2 joule. Further details are provided in [35].

3.2 Microscopy Setup

For all the Cellulose characterization, we used a NanoSurf Flex AFM C3000, mounted with a humidity chamber (HC). The HC is connected by plastic tubing that injects a flow of gas, which is a mixture of two entries. The first entry comes from a bottle with a mixture of water vapor, air and nitrogen (99.998% purity, from INDURA). Water vapor is obtained with a piezo electric pulverizer, used to create humid air. The second line is composed of pure nitrogen, which can be used to dry the environment. Also, there is an exit hose, that controls the pressure in the HC, and pushes out the original air. The range of humidities can sweep from around 2% and 80%. Additionally, a BME280 sensor, controlled by Arduino is on the floor of the HC. BME280 sensor

¹These hydrogels were prepared by our collaborators, and the details were provided by them. All this work was already published and can be found in Olguín et al. [35].

has been proven to be a good option to measure temperature, pressure and humidity of different environments [57], being a low cost option to consider in order to even monitoring the quality of air in cities like Santiago, Chile [58]. The cantilevers used for this part of the work were Supersharps **SSS-FMR** by NanoSensors (Watsonville, CA, USA). An Anti-static mat with a measured resistance of around $1.9[M\Omega]$ is used to mount the AFM probes, specially the SSS-FMR cantilever, since a small quantity of static electricity can damage the tip if discharged. Optical images of the epoxy-glued macrofibers were taken with a Zeiss Primotech (Jena, Germany).

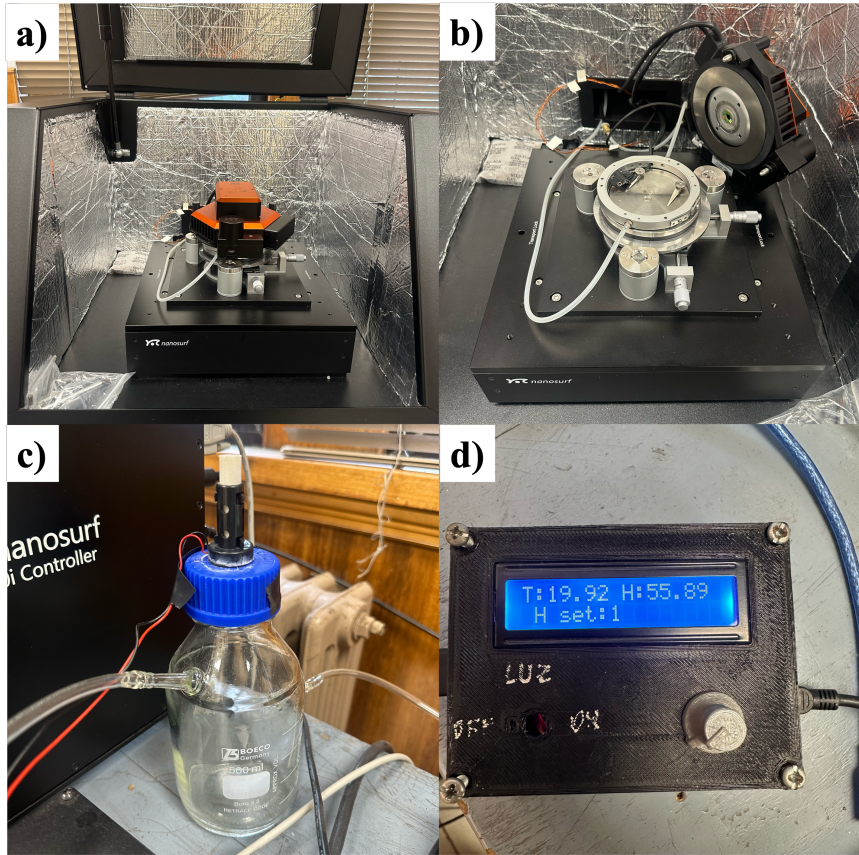


Figure 3.2: a) Flex AFM with mounted humidity chamber (hc) inside its insulating chamber. b) Top view of the AFM's hc containing a BME280 sensor. c) Bottle with a piezo-vibrator system for mixing humid air. d) Digital screen displaying real-time temperature and relative humidity from the sensor inside the hc.

For the Hydrogels' experiments, the collagen sample was measured using the Flex AFM, while alginate, PEGDA, and GelMA were measured with a JPK NanoWizard3. The AFM chips used for this research were qp-BioAC by NanoSensors (Watsonville, CA, USA). These AFM chips have three different cantilevers with different elastic constants and dimensions. Each cantilever has a tip radius of around 10 nm and a Uniqprobe coating that consists of a reflective gold layer. This coating is applied to the backside of the cantilever, which faces the detector. This coating helps reduce the bending of the AFM cantilever and its chemical stability when measuring in liquid environments. In particular, the AFM measurements were performed in a PBS solution.

Chapter 4

Results and Discussion

4.1 Cellulose Project

In this section, results on control and treated cellulose will be presented. Results include macrofibers and cellulose nanofibers.

4.1.1 Macrofibers

Both types of samples -control and modified- were fixed to a glass through the blade coating technique, as presented in Chapter 2. The obtained macrofibers are shown in Fig. 4.1.

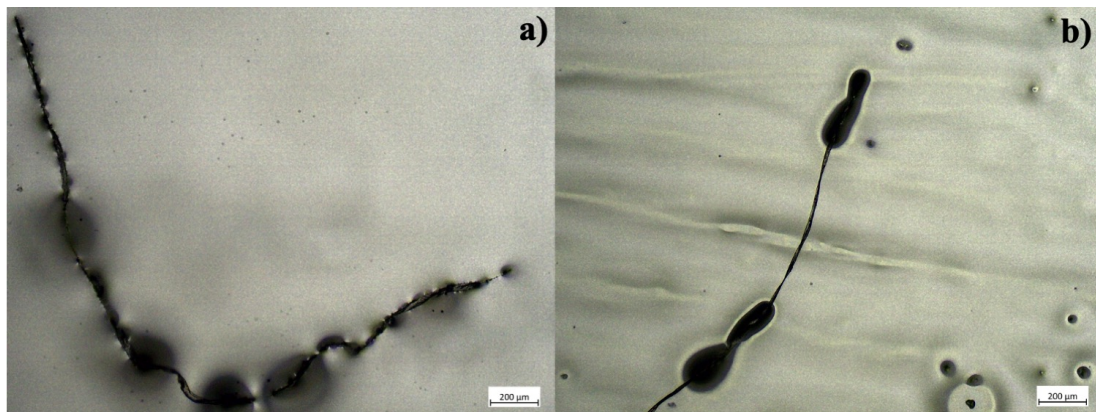


Figure 4.1: Epoxy-glued macrofibers on a glass surface. a) Control Fiber, b) 3-dfg Fiber. Scale bar is 200(μm).

Using an optical microscope, we identified zones where the fibers were not covered by epoxy, and analyzed them with AFM.

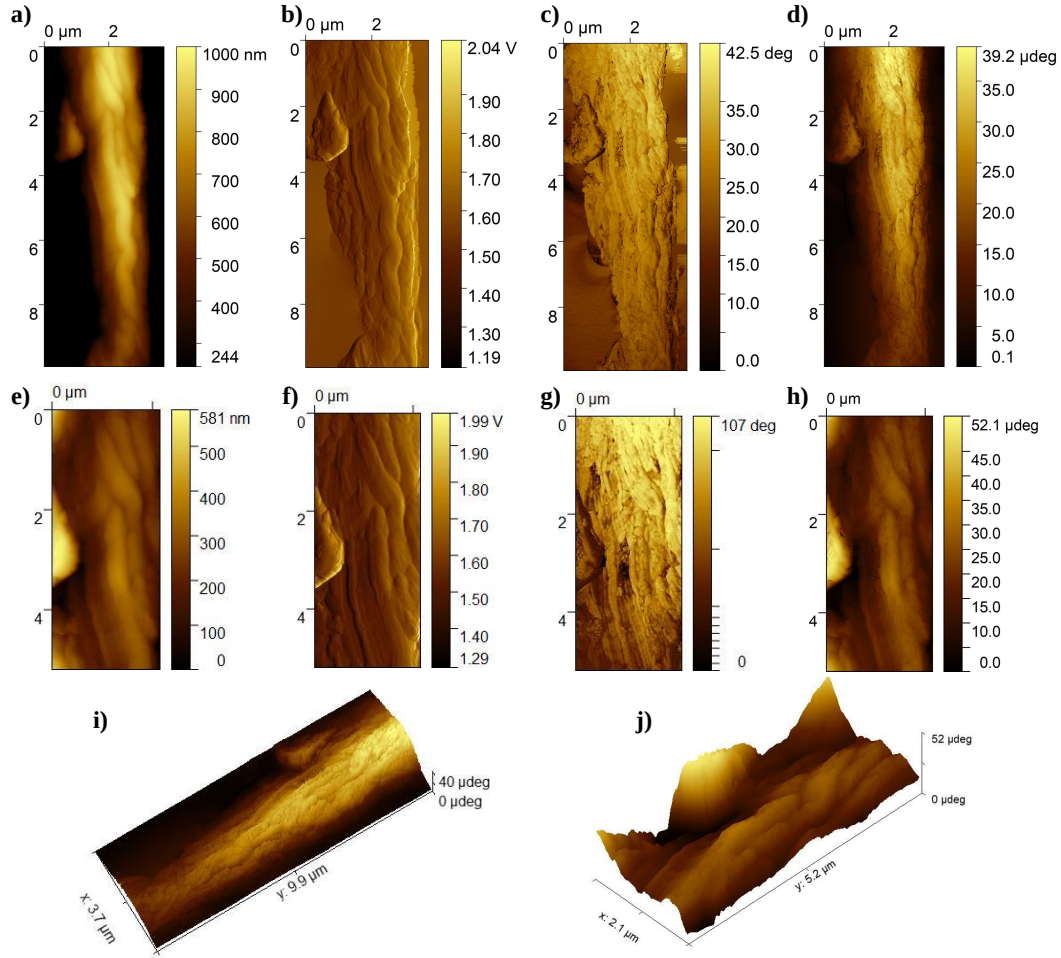


Figure 4.2: AFM images of the control macrofiber supported over a dry epoxy film. The middle row of images (e,f,g,h) correspond to a cropped spot of the first row (a,b,c,d). a), b), c), are Topography, Amplitude and Phase channels, respectively, as well as e), f), g). d) and h) correspond the convolution of topography and phase channels. i) and j) correspond to 3d representations of d) and h), respectively.

In Fig. 4.2 we can see AFM images of the macrofiber at one specific spot. One can see that there are micro- to nano-structures that compose the macrofiber, and that are aligned with its elongation axis. This agrees with what we expect, for example, from the structures presented in Fig. 1.6. Just by looking at the images, although, it is difficult to state if those structures are the actual cellulose nanofibers that we will be looking for in the next section. Phase images suggest that contrast arising from the tip-sample interaction is not completely homogeneous within the macrofiber - leading us to think that the arrangement of the macrofiber's components could be changing its local stiffness.

After looking for some spots within the fiber, we did force spectroscopy experiments on it (Fig 4.3). Maximum value of force applied was 150nN.

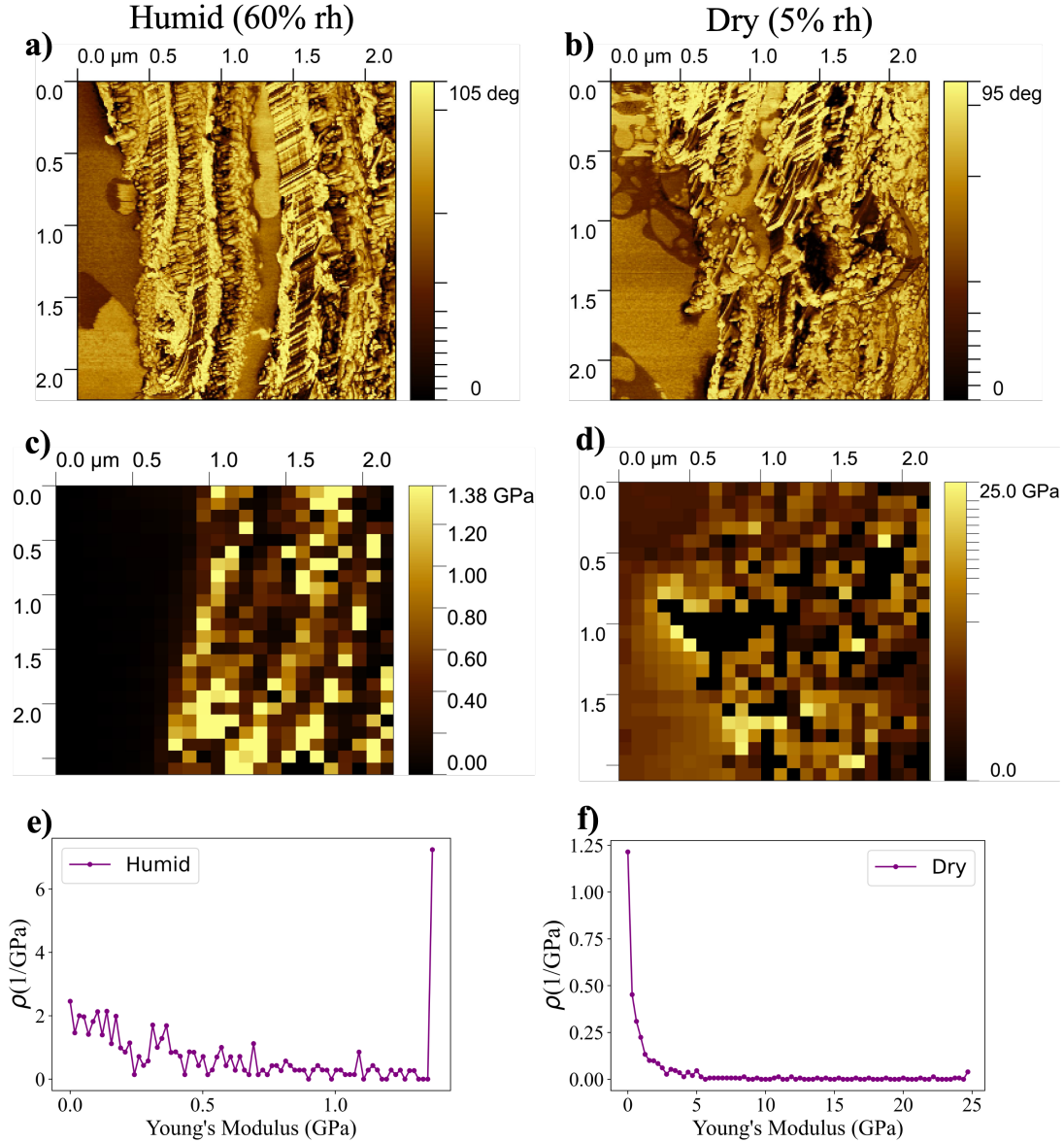


Figure 4.3: Humidity-controlled experiments on a control Macrofiber. Left column (a,c,e) represents a phase image, mechanical map and a Young's modulus distribution for a humid ($rh \approx 60\%$) state fiber (background filtered), respectively. Right column (b,d,f) are the analog for a dry state ($rh \approx 5\%$).

We first measured with a relative humidity close to 60%, and then, close to 5%. We can see from Fig. 4.3 a),c) and b),d), a clear contrast between the fiber and the epoxy background for both cases. Even with that, in terms of quantifying the stiffness of the sample, we can just confirm what we inferred from Fig. 4.2: the arrangement of macrofiber's components affect it's local stiffness. The Young's modulus distribution for both cases (humid and dry) is heterogeneous, not showing any bell-like distribution after masking and separating the fiber from the background. Having this in mind, we did not try the same experiment for modified macrofibers. In retrospective, we could have tried different methods

if we wanted to test the mechanical properties of the fiber. For example, using colloidal probe AFM [59] instead of a sharp tip, or just using a macroscopic tensile test as in the work of Kuperman et al. [60]. In terms of morphology, we can notice that left and right column seem completely different. We do not think that this is a morphological change due to humidity changes, but due to the nanoindentation experiment. It is possible that we used too much force against the humid fiber, which is expected to be softer than when dried, so we re-arranged its micro-components. We think that imaging in terms of relative humidity without applying nano-indentation could have given us more clues about the swelling of the macrostructures, maybe to spot some heterogeneities clearer, but we were not able to do so in this thesis. From Fig. 4.3 e) and f), even if it is difficult to confirm, Young's moduli distribution suggest that the dry ($E \approx 0 - 1.5 \text{ GPa}$) state is harder than the humid ($E \approx 0 - 25 \text{ GPa}$). AFM images were also obtained from modified 3dfg macrofibers (Fig. 4.4).

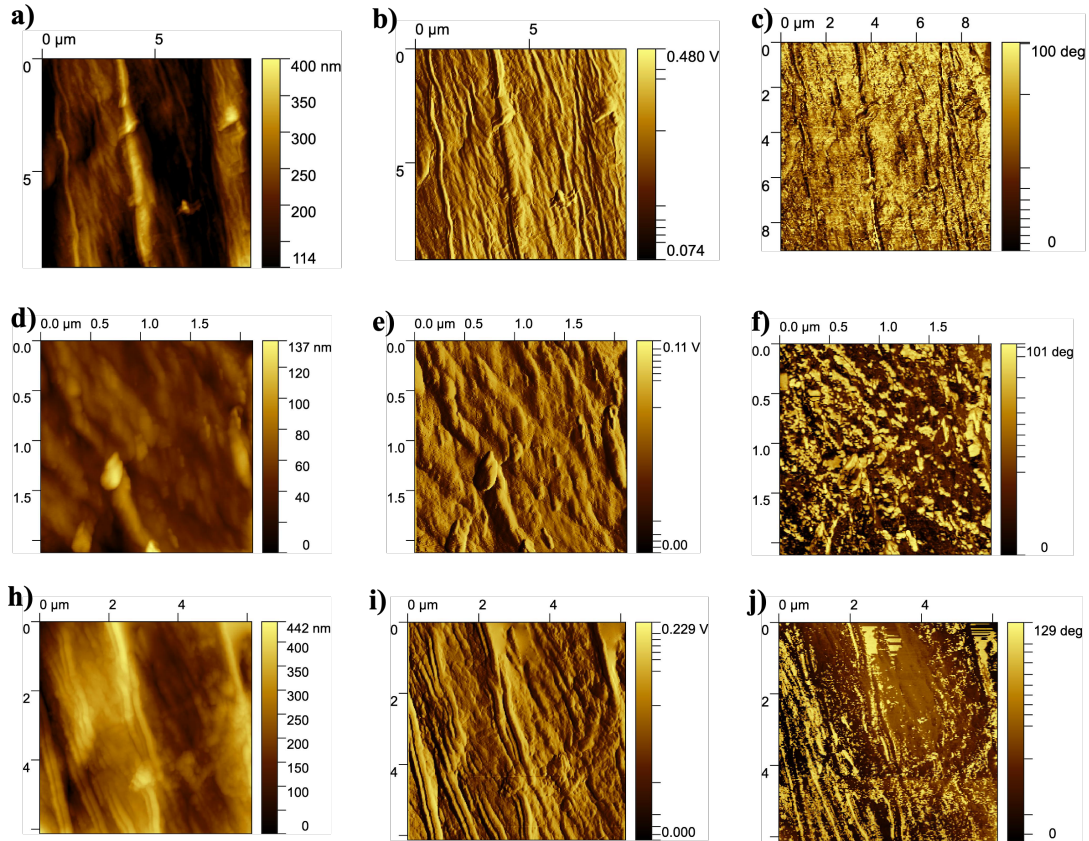


Figure 4.4: AFM Images of the modified macrofiber over three zones. (a,b,c), (d,e,f) and (h,i,j), correspond to Topography, Amplitude and Phase channels for “Zone 1”, “Zone 2” and “Zone 3”, respectively.

Fig. 4.4 shows that modified cotton macrofibers present some differences when compared to the control ones. In particular, there are some globular structures above the nano- to micro-structures observed. We think that this blobs can be created due to the interference of the fluorine atoms on their internal structures. In general, considering Fig.4.4d-j), modified macrofibers seem to be more heterogeneous than control ones, but they keep an orientation towards their elongation axis.

4.1.2 CNF Morphology

4.1.3 Humidity controlled experiments

First, we can see that control and modified macro-samples behave different when exposed to the same cellulase-water dilution (Fig. 4.5). This can be considered as an indicator that something different is happening with the digestion of control and modified samples. Control sample seems to dilute in the solution, while modified sample seems to absorb it.

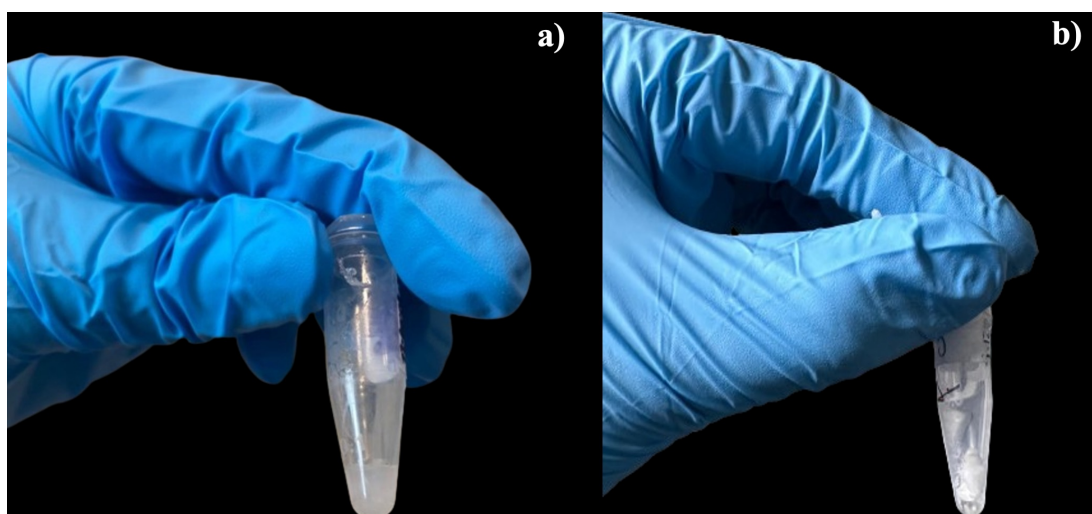


Figure 4.5: Photography of stock solution after 1 day of enzymatic digestion. a) Control, b) Modified samples.

Once digested, humidity-dependent AFM images were obtained in the control CNF. Fig. 4.6 a) shows a CNF at a dry state, and Fig. 4.6 b) at a humid state. At a first look, we can see that control CNF suffered swelling at specific points. In Fig. 4.6 b), white boxes indicate the swelling zones 1, 2 and 3.

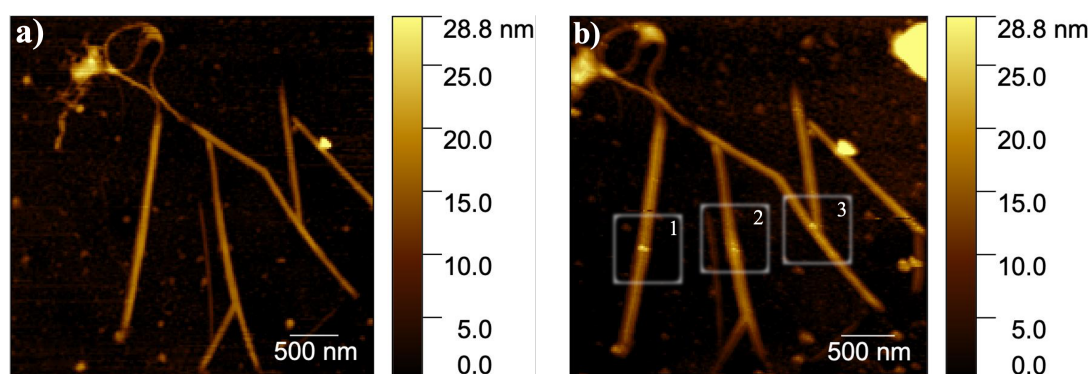


Figure 4.6: AFM topographical images of control enzymatically-digested CNF. Left Panel: Dry state ($< 10\%$ rh). Right panel: Humid state ($> 70\%$ rh). Zones 1,2,3 denote swelling points.

We will focus now on studying swelling zones 1 and 2 of Fig. 4.6 (white boxes 1 and 2).

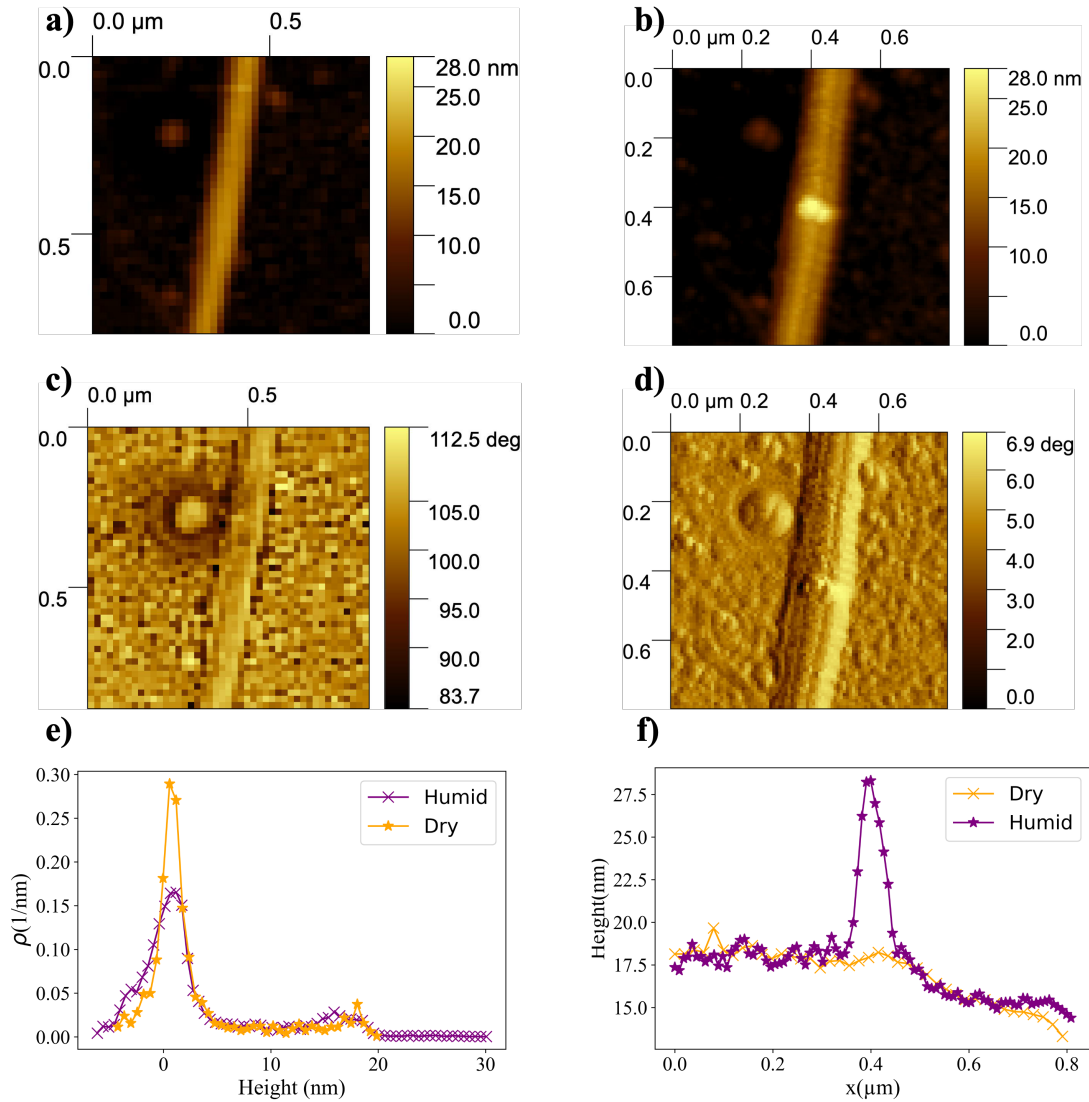


Figure 4.7: AFM Experiment on control CNF. a),c) are Topography and Phase contrast channels, respectively, at $rh < 10\%$. b),d) are the same with $rh > 70\%$. Both are a crop of Fig. 4.6 Zone 1. e) Height distribution of the zone in both states. f) Cross section height taken along the long axis of the fiber, following its elongation axis, in both dry and humid states.

From Fig.4.7 e),f), we can notice that, along the fiber, there is a change in height at a certain point, of around 10nm. The question here is if this increment of height is due to the fiber itself swelling at a certain point, or there are just water-nanodroplets that were adsorbed over the fiber surface at specific points. This can be clarified studying the phase images (Fig.4.7c),d)). If there were a water droplet on the fiber's surface, there would be a noticeable color contrast in the phase images, due to the difference in energy loss from tip-water and tip-cellulose interactions. The low contrast of the phase-shift in the humid state (Fig. 4.7 d)) makes us believe that the fiber itself is presenting swelling at specific points.

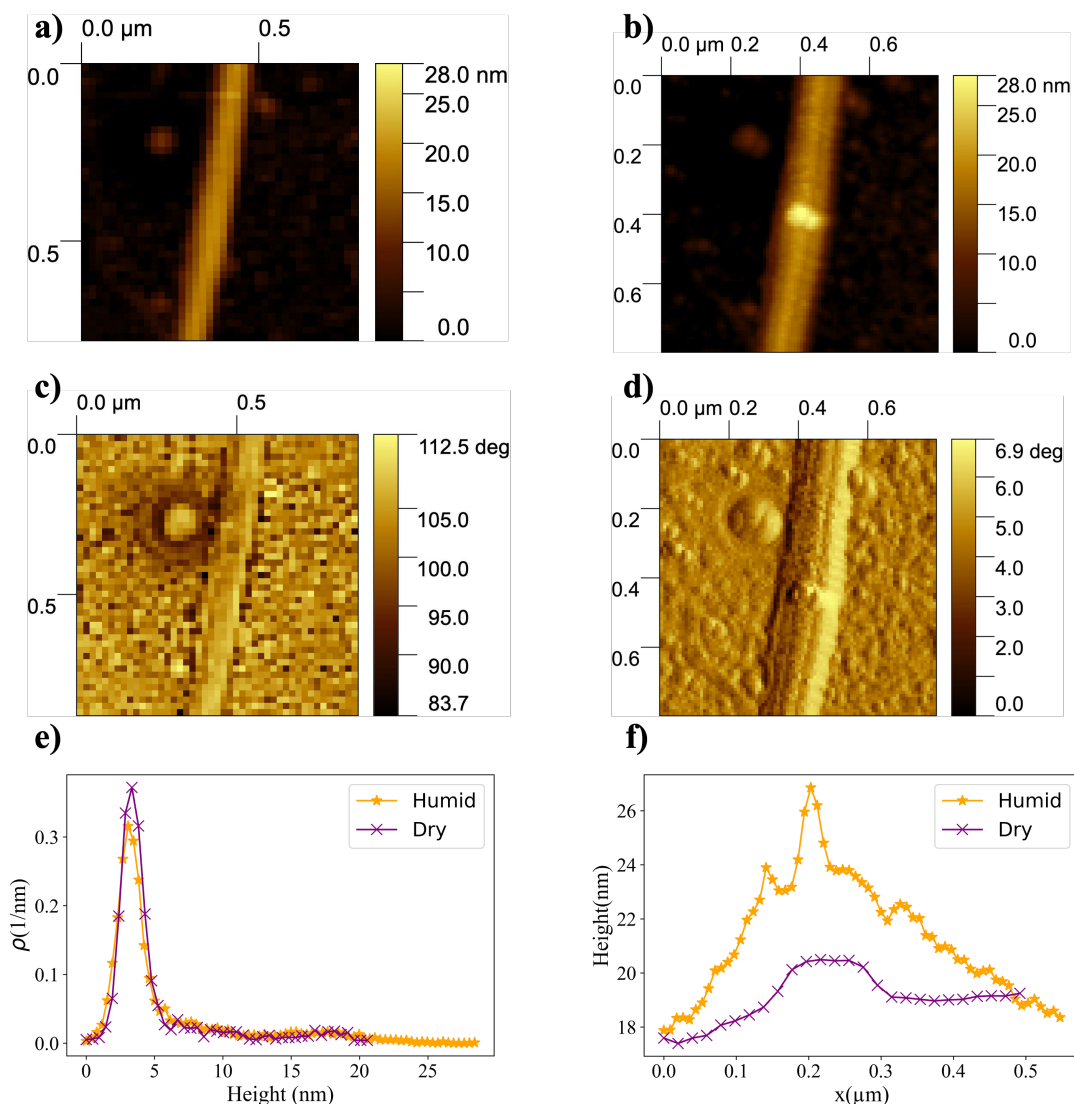


Figure 4.8: AFM Experiment on control CNF. a),c) are Topography and Phase contrast channels, respectively, at $rh < 10\%$. b),d) are the same with $rh > 70\%$. Both are a crop of Fig. 4.6, from what we will call Zone 2. e) Height distribution of the zone in both states. f) Cross section height of the fiber, following its elongation axis, of both states.

In Fig. 4.8, we can see the same behaviour as in Zone 1, but here, the contrast in the swelling point with respect to the rest of the fiber is even lower. This, again, makes us think that the fiber itself is swelling at that point, and not just adsorbing the water on its surface.

In the work of Ottesen and Syverud [10] (See Fig. 1.9), they showed that when exposed to water, the whole nanofibril-structure swelled, and they found no evidence for distinct crystalline and amorphous domains in natural cellulose. Here, we think that we found an important evidence of these distinct amorphous and crystalline domains, being the amorphous domains the ones that swell when exposed to water. In fact, in the same work, they showed that depending on the DoC of the structure, the swelling capability changed. We think that, through enzymatic digestion, we obtained CNF composed by crystalline

structures linked by amorphous ones. A fundamental difference between our work and the one of Ottesen and Syverud [10] is that we synthesized the CNF through cellulase digestion, while they used homogenization of cotton linters.

For the case of 3-dfg (modified) fibers, we can see a different behaviour when exposed to water (Fig. 4.9).

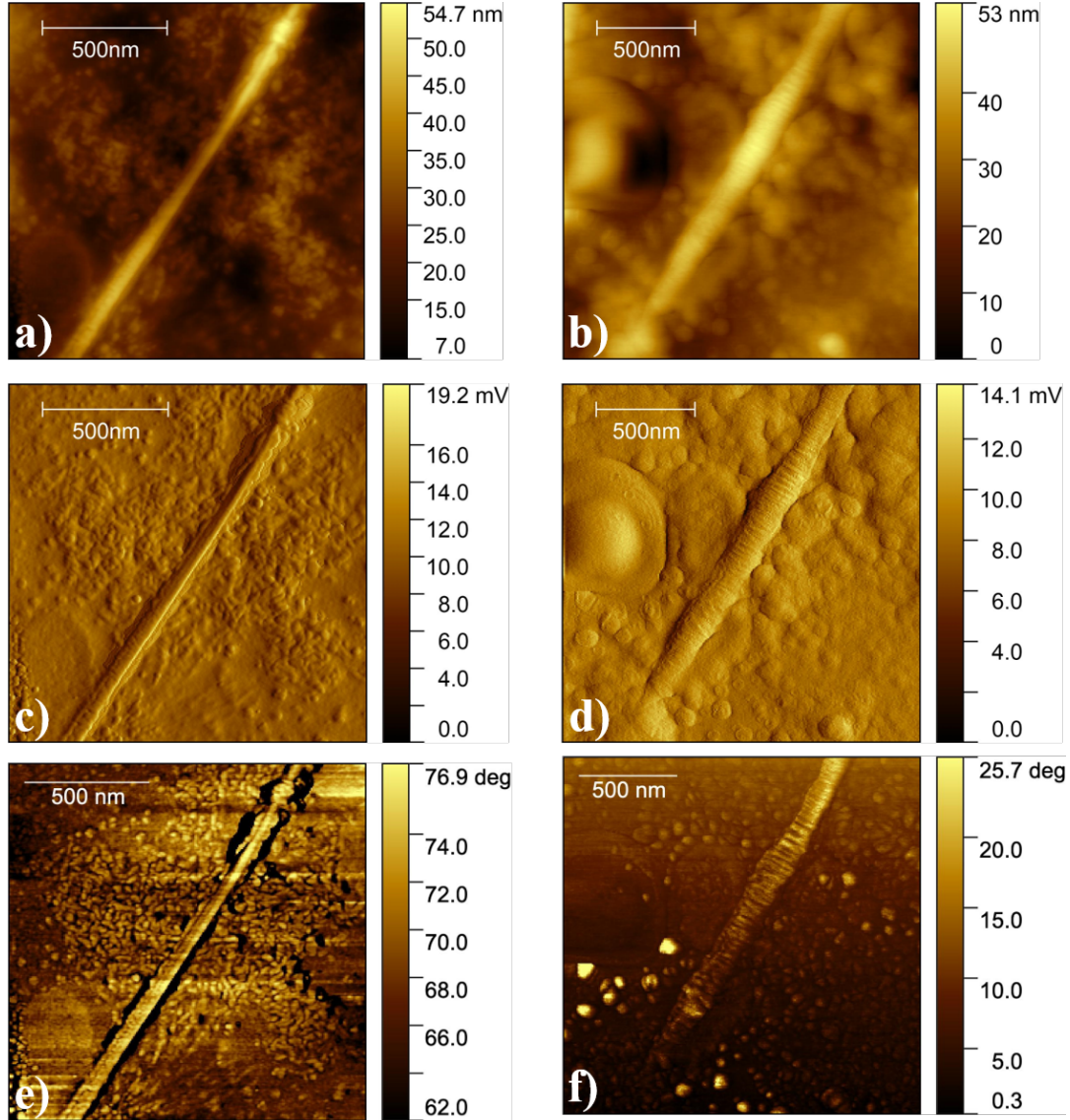


Figure 4.9: Humidity-controlled experiments on a 3dfg CNF. Left column panels (a,c,e) present Topography, Amplitud and Phase channels at a dry state ($rh < 10\%$), respectively. Right column panels (b,d,f) represent their counterpart at a humid state ($rh > 65\%$).

In this case, it seems like the whole structure presented swelling, including the background. It is difficult to quantify if the fiber itself grew in radius, since it seems to be surrounded by a little film of material, probably as a result of the enzymatic digestion. So, the change in height could be affected by the background. Also, as we discussed in Ch. 2, width measurements with AFM are not something to trust in, especially when working with humid environments. Having said that, what we can notice clearly, is that the images of the humid

state present rugosities-contrasts, that were not present in the dry state, or at least are enhanced by the humidity. If we take cross sections of the topography and phase channels, for example, we can see that this contrast appears in both channels (Fig. 4.10). This period can be quantified, taking for example the distance between ten of the peaks (Fig. 4.10 d)), yielding an average value of $37 \pm 8(nm)$.

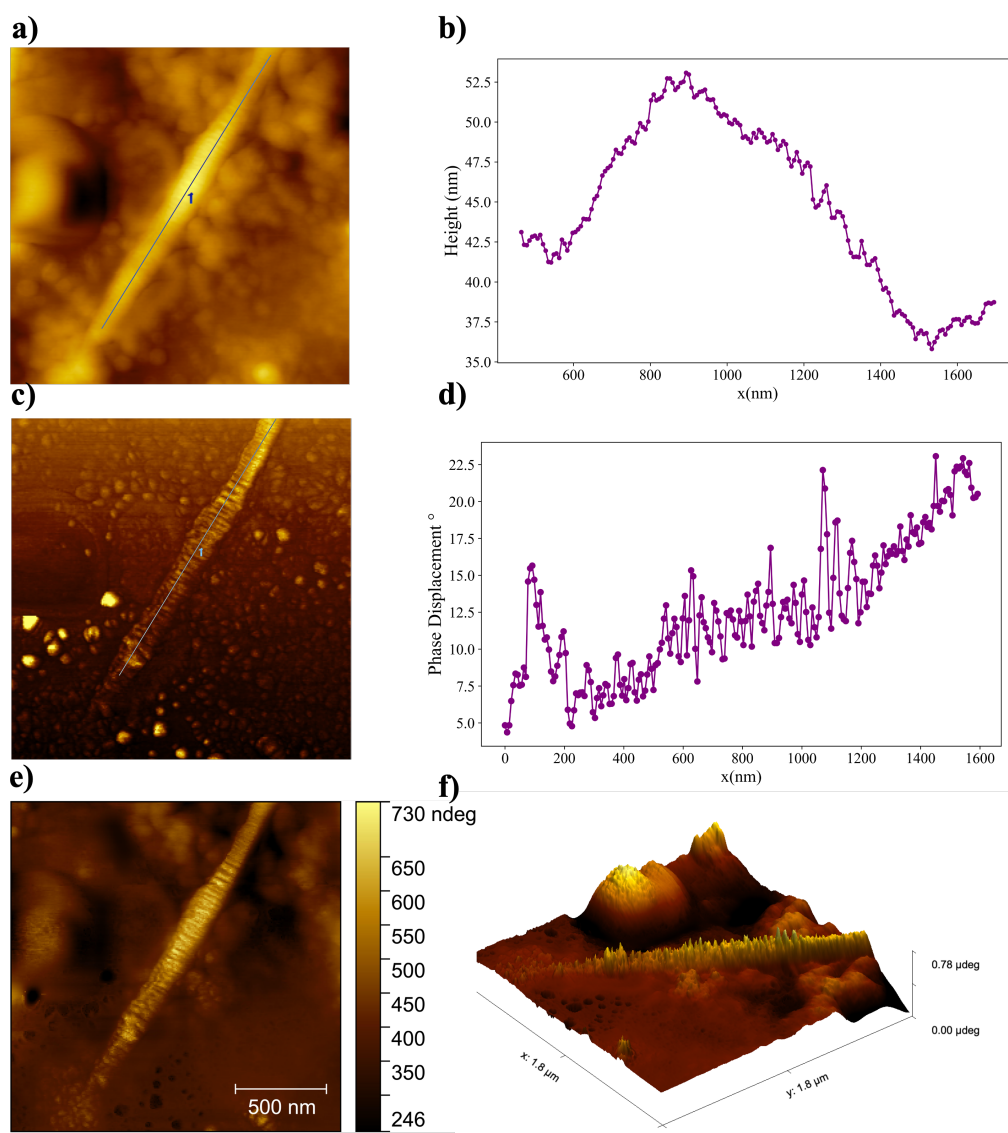


Figure 4.10: Same spot as Fig. 4.9. a),c) show cross sections on Topography and Phase channels, respectively. b),d) are the plot of the cross sections taken in a) and b), respectively. e) is a convolution between topography and phase channels. f) is the 3d representation of e).

This phenomenon has been reported before for fibrillar structures. For example, in Spitzner et al. [9], they spot the D-band pattern for Collagen through studying the swelling of collagen fibers (Fig. 1.4). We think that the periodicity we found could be related to different regimes of 3dfg modified fiber and unmodified cellulose, causing swelling heterogeneities. These swelling heterogeneities, both for natural and modified CNFs, have not been reported in literature.

4.1.4 Comparison with standard acid hydrolysis protocol

To compare our enzyme-based protocol with a standard sulfuric acid hydrolysis in natural fibers, our collaborators sent us a sample of sulfuric acid-digested CNFs over mica. They followed a protocol similar to the one described by Jiang and Hsieh [61], to treat the same natural cotton fibers.

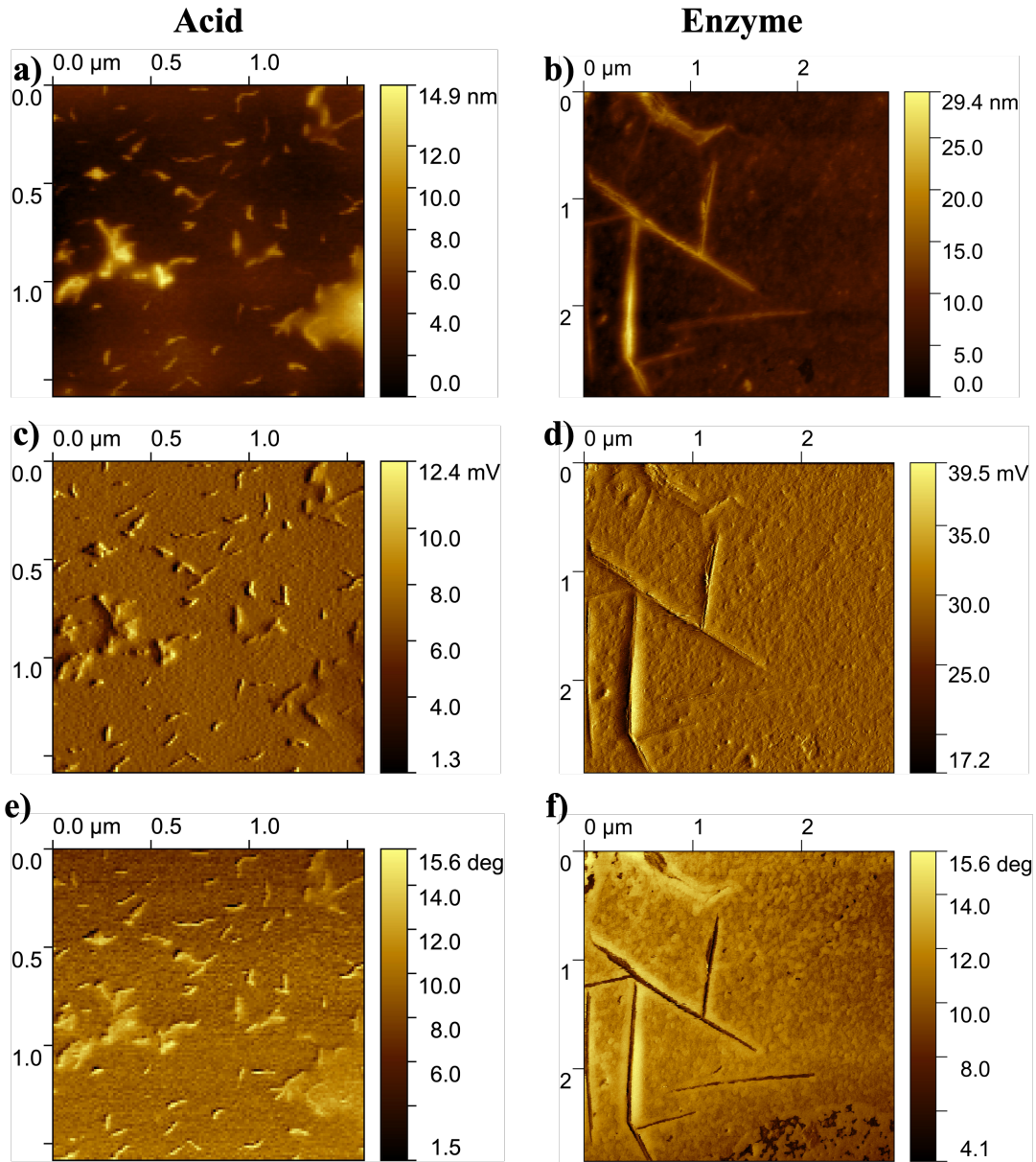


Figure 4.11: AFM images of CNFs. Left column pannels (a),c),e)) show topography, amplitude, and phase channels of sulfuric acid-digested CNFs. Right column pannels (b),d),f)) are their enzyme-digested counterpart.

From Fig. 4.11, we can notice that enzymatic digestion yields, in general, longer structures with higher height. Enzyme-digested CNFs lengths are in the order of $1\mu m$, in contrast to the acid-treated structures, which have lengths of around $200nm$. In terms of height, enzyme-digested structures range between $8 - 25nm$, and the acid-treated between $2 - 10nm$. From phase images (Fig.4.11 e),f)), we

can see a higher contrast between the fibers and the substrate in the enzyme-digested sample, but this can be due to the difference between substrates (mica for acid-treated, silicon for enzyme-digested).

Considering our results, we believe that our enzymatic digestion protocol leads to bigger structures -when compared to sulfuric acid treatment- that are composed by crystalline and amorphous regions. Probably, and as we discussed in Ch. 1, acid-treated cellulose is a more crystalline structure.

4.2 Nanomechanics of hydrogels

¹For the different hydrogels, we obtained topographical images and mechanical moduli histograms (Fig. 4.12).

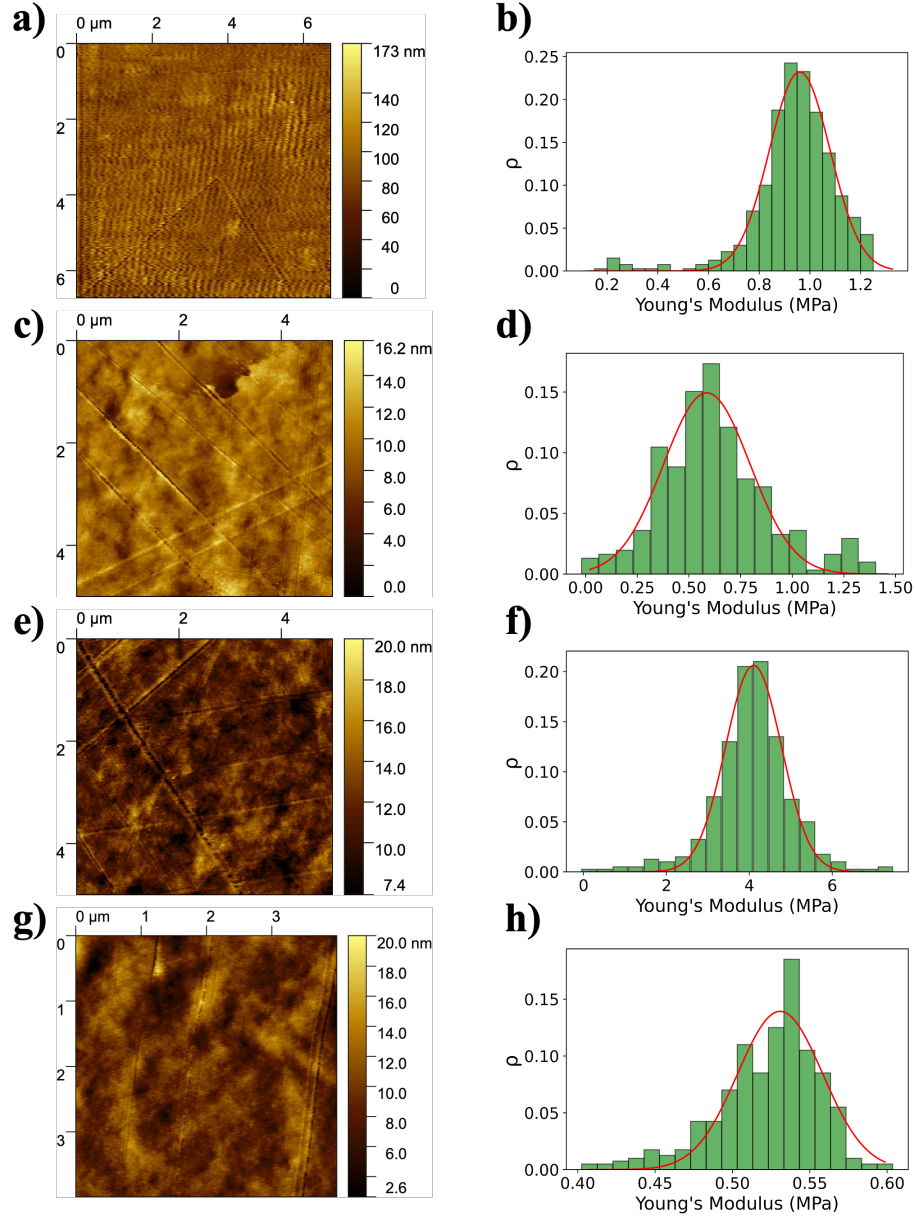


Figure 4.12: (a),c),e),g)). AFM topographical images of collagen, GelMA, PEGDA, and alginate, respectively. (b),d),f),h)). Young's modulus histograms of collagen, GelMA, PEGDA, and alginate, respectively.

¹All the results and discussions from this section have already been published by our group in Olguín et al. [35].

To obtain the mechanical moduli presented in Fig. 4.12 (right column), 400 force-distance curves were taken over a region of a few micrometers, in liquid environment of PBS. These curves were fitted using the hertz model (Eq. 2.9) and considering a Poisson's ratio $\nu = 0.5$ for every gel. For the case of Gelma, some curves were disregarded, and 306 were used to fit and obtain Fig. 4.12 f). The topographical parameters obtained from these measurements are presented in Table 4.1.

Table 4.1: AFM measurements of topographical parameters for hydrogels.

	Collagen	GelMA	PEGDA	Alginate
RMS(nm)	14.6	1.6	1.6	2.0
Young's Modulus (MPa)	0.96 ± 11	0.59 ± 0.21	4.10 ± 0.67	0.53 ± 0.03

Firstly, we can compare the Young's moduli of these hydrogels with literature, and see that they are within their expected values [62, 63]. For the case of rugosities (RMS), they were calculated with the software Gwyddion. RMS stands for "*Roughness Mean Square*". The roughness values for Collagen, GelMA and Alginate are within expected from literature [64, 65, 66]. For the case of PEGDA, measured RMS is considerably lower than what has been reported in literature [67, 68, 69]. In general, increasing the levels of crosslinking on the hydrogels increases the surface stiffness and reduces its roughness [70, 71]. In this work, the PEGDA hydrogel was crosslinked with a higher UV radiation power than in literature (i.e., higher level of crosslinking), so it makes sense to obtain higher RMS values. These hydrogels were successful when seeding PC12 neural cells on them [35].

Chapter 5

Conclusions and Outlook

This work explored the nanomechanical and morphological characterization of two different biopolymer systems: Hydrogels and Cellulose. For cellulose, we used environmental-atomic force microscopy (e-AFM) to detect the nanoscale effects of biological incorporation of a fluorine-based molecule (3-deoxy-3-fluoro-D-glucopyranose) in cotton, and studied the swelling properties of natural and modified cellulose at its macro- and nano-scale components. For the hydrogel systems, we characterized the surfaces of Collagen, Alginate, PEGDA and GelMA hydrogels in liquid environments (PBS). Sample preparation protocols were implemented, such as blade coating for cellulose macrofibers and cellulase-enzymatic digestion for CNF. A liquid-mode AFM protocol was also implemented to measure the hydrogels.

Both control and modified cotton macrofibers have micro- to nano-structures arranged along their elongation axis, with noticeable heterogeneities in their local mechanics, presumably due to their internal arrangement. In modified samples, the fluorine atom may also contribute to these differences. Modified macrofibers show globules between smaller fibrillar structures. Control cotton nanofibers swell at specific spots when exposed to higher relative humidity, suggesting a semicrystalline structure. We believe that our experiments evidence amorphous and crystalline domains within natural CNF, differing from earlier results [10], most likely due to the difference in the hydrolisis method, i.e., acid or enzymatic hydrolisis. Our results show that enzyme-digested structures seem to be bigger than acid-treated ones. Modified cellulose nanofibers exhibit heterogeneous swelling, with a D-like pattern that is seen in height and phase with a period of $37 \pm 8(nm)$, likely due to the fluorine atom's interference with the wettability of the nanofibers. We think that this period could be related to the modified fiber's repeat unit length (as D-spacing), but further research on it is necessary to confirm. Biopolymer-based hydrogels showed rugosity (RMS) values around 2 nm, except for Collagen, which had an RMS of around 15 nm, potentially due to UV light exposure. PEGDA hydrogel showed lower RMS when compared with literature, probably due to the different exposition to UV light used in this work too. The Young's moduli of the gels ranged from 0.53 to 4.1 MPa. Future research in this topics will focus on trying to correlate the effect of these mechanical properties of the gels with the effect of electrical stimulation on PC12 cell culture proliferation. By now, no correlation could be set [35].

Bibliography

- [1] Fowzia Ahmed and ANM Fakhruddin. A review on environmental contamination of petroleum hydrocarbons and its biodegradation. *International Journal of Environmental Sciences & Natural Resources*, 11(3):1–7, 2018.
- [2] Ashish George, MR Sanjay, Rapeeporn Srisuk, Jyotishkumar Parameswaranpillai, and Suchart Siengchin. A comprehensive review on chemical properties and applications of biopolymers and their composites. *International journal of biological macromolecules*, 154:329–338, 2020.
- [3] Kathleen Van de Velde and Paul Kiekens. Biopolymers: overview of several properties and consequences on their applications. *Polymer testing*, 21(4): 433–442, 2002.
- [4] María Flórez, Patricia Cazón, and Manuel Vázquez. Selected biopolymers' processing and their applications: A review. *Polymers*, 15(3):641, 2023.
- [5] Dragica Bezjak, Nicole Orellana, Jorge H. Valdés, Tomás Corrales, and Cristian A. Acevedo. Towards understanding the role of microstructured edible scaffolds for cultured meat production. *Food and Bioprocess Technology*, 07 2023.
- [6] María I Taborda, Karina N Catalan, Nicole Orellana, Dragica Bezjak, Javier Enrione, Cristian A Acevedo, and Tomas P Corrales. Micropatterned nanofiber scaffolds of salmon gelatin, chitosan, and poly (vinyl alcohol) for muscle tissue engineering. *ACS omega*, 8(50):47883–47896, 2023.
- [7] Wee E Teo and Seeram Ramakrishna. A review on electrospinning design and nanofibre assemblies. *Nanotechnology*, 17(14):R89, 2006.
- [8] Gert R Strobl. *The physics of polymers*, volume 2. Springer, 1997.
- [9] Eike-Christian Spitzner, Stephanie Röper, Mario Zerson, Anke Bernstein, and Robert Magerle. Nanoscale swelling heterogeneities in type i collagen fibrils. *ACS Nano*, 9(6):5683–5694, 2015. PMID: 25961780.
- [10] Vegar Ottesen and Kristin Syverud. Swelling of individual cellulose nanofibrils in water, role of crystallinity: an afm study. *Cellulose*, 2021.
- [11] Willy Menacho, Karina N. Catalan, Tomas P. Corrales, and Horacio V. Guzman. Quantitative dynamic afm hydration-adsorption design for hygroscopic and bio-compatible polymeric nanofibers. *Small Structures*, 2024.

- [12] S. Park, J.O. Baker, and M.E. et al. Himmel. Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnol Biofuels*, 3(10):0–10, May 2010. ISSN 2731-3654.
- [13] Filipe Natalio, Regina Fuchs, Sidney R. Cohen, Gregory Leitus, Gerhard Fritz-Popovski, Oskar Paris, Michael Kappl, and Hans-Juergen Butt. Biological fabrication of cellulose fibers with tailored properties. *Science*, 357(6356):1118–1122, September 2017. ISSN 0036-8075.
- [14] Ishita Chakraborty, Sintu Rongpipi, Indira Govindaraju, Sib Sankar Mal, Esther W Gomez, Enrique D Gomez, Ranjan Dutta Kalita, Yuthika Nath, and Nirmal Mazumder. An insight into microscopy and analytical techniques for morphological, structural, chemical, and thermal characterization of cellulose. *Microscopy Research and Technique*, 2022.
- [15] Farah Fahma, Shinichiro Iwamoto, Naruhito Hori, Tadahisa Iwata, and Akio Takemura. Isolation, preparation, and characterization of nanofibers from oil palm empty-fruit-bunch (opefb). *Cellulose*, 2010.
- [16] Ming Li, Beihai He, Yiyi Chen, and Lihong Zhao. Physicochemical properties of nanocellulose isolated from cotton stalk waste. *ACS Omega*, 6(39): 25162–25169, 2021.
- [17] Rebecca R. Ruckdashel, Dhanya Venkataraman, and Jay Hoon Park. Smart textiles: A toolkit to fashion the future. *Journal of Applied Physics*, 129(13): 130903, 2021.
- [18] Carlos Rafael Silva de Oliveira, Afonso Henrique da Silva Júnior, Ana Paula Serafini Immich, and Juliane Fiates. Use of advanced materials in smart textile manufacturing. *Materials Letters*, 316:132047, 2022. ISSN 0167-577X.
- [19] <https://pubchem.ncbi.nlm.nih.gov/compound/3-Fluoro-3-deoxy-D-glucopyranose>. Accessed: 2023-2-28.
- [20] Carla Huerta-López and Jorge Alegre-Cebollada. Protein hydrogels: The swiss army knife for enhanced mechanical and bioactive properties of biomaterials. *Nanomaterials*, 11(7):1656, 2021.
- [21] Kuen Yong Lee and David J. Mooney. Hydrogels for tissue engineering. *Chemical Reviews*, 101(7):1869–1880, 2001.
- [22] Elizabeth E. Antoine, Pavlos P. Vlachos, and Marissa Nichole Rylander. Review of collagen i hydrogels for bioengineered tissue microenvironments: Characterization of mechanics, structure, and transport. *Tissue Engineering Part B: Reviews*, 20(6):683–696, 2014.
- [23] Andreas Stylianou et al. Atomic force microscopy for collagen-based nanobiomaterials. *Journal of Nanomaterials*, 2017, 2017.
- [24] Lijun Ye, Haichao Ji, Jie Liu, Chien-Hua Tu, Michael Kappl, Kaloian Koynov, Johannes Vogt, and Hans-Jürgen Butt. Carbon nanotube–hydrogel

- composites facilitate neuronal differentiation while maintaining homeostasis of network activity. *Advanced Materials*, 33(41):2102981, 2021.
- [25] Lihua Luo, Yan He, Ling Jin, Yanni Zhang, Fernando P Guastaldi, Abdulkhaleg A Albashari, Fengting Hu, Xiaoyan Wang, Lei Wang, Jian Xiao, et al. Application of bioactive hydrogels combined with dental pulp stem cells for the repair of large gap peripheral nerve injuries. *Bioactive materials*, 6(3):638–654, 2021.
- [26] Fukai Ma, Zhifeng Xiao, Bing Chen, Xianglin Hou, Jianwu Dai, and Ruxiang Xu. Linear ordered collagen scaffolds loaded with collagen-binding basic fibroblast growth factor facilitate recovery of sciatic nerve injury in rats. *Tissue Engineering Part A*, 20(7-8):1253–1262, 2014.
- [27] Ying Bu, Hai-Xing Xu, Xin Li, Wen-Jin Xu, Yi-xia Yin, Hong-lian Dai, Xiao-bin Wang, Zhi-Jun Huang, and Pei-Hu Xu. A conductive sodium alginate and carboxymethyl chitosan hydrogel doped with polypyrrole for peripheral nerve regeneration. *RSC advances*, 8(20):10806–10817, 2018.
- [28] Rachel Chapla, Mera Alhaj Abed, and Jennifer West. Modulating functionalized poly (ethylene glycol) diacrylate hydrogel mechanical properties through competitive crosslinking mechanics for soft tissue applications. *Polymers*, 12(12):3000, 2020.
- [29] Kan Yue, Grissel Trujillo-de Santiago, Mario Moisés Alvarez, Ali Tamayol, Nasim Annabi, and Ali Khademhosseini. Synthesis, properties, and biomedical applications of gelatin methacryloyl (gelma) hydrogels. *Biomaterials*, 73:254–271, 2015.
- [30] David E Koser, Amelia J Thompson, Sarah K Foster, Asha Dwivedy, Eva K Pillai, Graham K Sheridan, Hanno Svoboda, Matheus Viana, Luciano da F Costa, Jochen Guck, et al. Mechanosensing is critical for axon growth in the developing brain. *Nature neuroscience*, 19(12):1592–1598, 2016.
- [31] Aftin M Ross, Zhongxiang Jiang, Martin Bastmeyer, and Joerg Lahann. Physical aspects of cell culture substrates: topography, roughness, and elasticity. *Small*, 8(3):336–355, 2012.
- [32] Ugo Covani, Luca Giacomelli, Adriano Krajewski, Antonio Ravaglioli, Lorenza Spotorno, Patrizia Loria, Saradindu Das, and Claudio Nicolini. Biomaterials for orthopedics: A roughness analysis by atomic force microscopy. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, 82(3):723–730, 2007.
- [33] B Majhy, P Priyadarshini, and AK Sen. Effect of surface energy and roughness on cell adhesion and growth—facile surface modification for enhanced cell culture. *RSC advances*, 11(25):15467–15476, 2021.

- [34] WA Loesberg, J Te Riet, FCMJM Van Delft, P Schön, CG Figdor, S Speller, JJWA Van Loon, XF Walboomers, and JA Jansen. The threshold at which substrate nanogroove dimensions may influence fibroblast alignment and adhesion. *Biomaterials*, 28(27):3944–3951, 2007.
- [35] Yusser Olguín, Mónica Selva, Diego Benavente, Nicole Orellana, Ivan Montenegro, Alejandro Madrid, Diego Jaramillo-Pinto, María Carolina Otero, Tomas P Corrales, and Cristian A Acevedo. Effect of electrical stimulation on pc12 cells cultured in different hydrogels: Basis for the development of biomaterials in peripheral nerve tissue engineering. *Pharmaceutics*, 15(12): 2760, 2023.
- [36] Alberto Lopez-Gasso. Scanning tunneling microscopy: The new eyes and hands of the scientists, 03 2018.
- [37] DW Pohl. Scanning near-field optical microscopy (snom). In *Advances in optical and electron microscopy*, volume 12, pages 243–312. Elsevier, 1991.
- [38] U. Hartmann. Magnetic force microscopy. *Annual Review of Materials Science*, 29(1):53–87, 1999.
- [39] Manuel Nonnenmacher, MP o’Boyle, and H Kumar Wickramasinghe. Kelvin probe force microscopy. *Applied physics letters*, 58(25):2921–2923, 1991.
- [40] Hans-Jürgen Butt, Brunero Cappella, and Michael Kappl. Force measurements with the atomic force microscope: Technique, interpretation and applications. *Surface science reports*, 59(1-6):1–152, 2005.
- [41] Franz J. Giessibl. Afm’s path to atomic resolution. *Materials Today*, 8(5): 32–41, 2005. ISSN 1369-7021.
- [42] Tomas P. Corrales. *Local dynamics and bending mechanics of mesostructured materials*. PhD thesis, Mainz, 2013.
- [43] Mohamed El-Desawy. Characterization and application of aromatic self-assembled monolayers. 2007.
- [44] Sylwia Babicz-Kiewlicz. *Analysis of Atomic Force Microscopy Surface Images by Means of Higher-harmonic Tip Vibrations Modes*. PhD thesis, 2015.
- [45] Chih-Wen Yang, Ing-Shouh Hwang, Yen Fu Chen, Chia Seng Chang, and Din Ping Tsai. Imaging of soft matter with tapping-mode atomic force microscopy and non-contact-mode atomic force microscopy. *Nanotechnology*, 18(8):084009, jan 2007.
- [46] Q. Zhong, D. Inniss, K. Kjoller, and V.B. Elings. Fractured polymer/silica fiber surface studied by tapping mode atomic force microscopy. *Surface Science Letters*, 290(1):L688–L692, 1993. ISSN 0167-2584.
- [47] Eriko Nagao and James A. Dvorak. Phase imaging by atomic force microscopy: Analysis of living homoiothermic vertebrate cells. *Biophysical Journal*, 76(6):3289–3297, 1999. ISSN 0006-3495.

- [48] Geoffrey KH Pang, KZ Baba-Kishi, and A Patel. Topographic and phase-contrast imaging in atomic force microscopy. *Ultramicroscopy*, 81(2):35–40, 2000.
- [49] Jeffrey Hutter and John Bechhoefer. Calibration of atomic-force microscope tips. *Review of Scientific Instruments*, 64:1868 – 1873, 08 1993.
- [50] Amir Farokh Payam. Modeling and analysis of the capillary force for interactions of different tip/substrate in afm based on the energy method. *ACS Measurement Science Au*, 3(3):194–199, 2023.
- [51] Saima A Sumaiya and Mehmet Z Baykara. Atomic-scale imaging and spectroscopy via scanning probe microscopy: An overview. *Journal of Vacuum Science & Technology B*, 41(6), 2023.
- [52] F. Ohnesorge and G. Binnig. True atomic resolution by atomic force microscopy through repulsive and attractive forces. *Science*, 260(5113):1451–1456, 1993.
- [53] Saima A Sumaiya, Jun Liu, and Mehmet Z Baykara. True atomic-resolution surface imaging and manipulation under ambient conditions via conductive atomic force microscopy. *ACS nano*, 16(12):20086–20093, 2022.
- [54] Heinrich Hertz. Ueber die berührung fester elastischer körper. 1882(92): 156–171, 1882.
- [55] Edward Dintwa, Engelbert Tijskens, and Herman Ramon. On the accuracy of the hertz model to describe the normal contact of soft elastic spheres. *Granular Matter*, 10:209–221, 2008.
- [56] Ariell Marie Smith, Dominique Gabriele Inocencio, Brandon Michael Pardi, Arvind Gopinath, and Roberto Carlos Andresen Eguiluz. Facile determination of the poisson’s ratio and young’s modulus of polyacrylamide gels and polydimethylsiloxane. *ACS Applied Polymer Materials*, 0(0):null, 0.
- [57] Gleb V. Shevchenko, Nikita A. Glubokov, Anton V. Yupashevsky, and Anna S. Kazmina. Air flow sensor based on environmental sensor bme280. In *2020 21st International Conference of Young Specialists on Micro/Nanotechnologies and Electron Devices (EDM)*, pages 432–435, 2020.
- [58] Matías Tagle, Francisca Rojas, Felipe Reyes, Yeanice Vásquez, Fredrik Hallgren, Jenny Lindén, Dimitar Kolev, Agot K Watne, and Pedro Oyola. Field performance of a low-cost sensor in the monitoring of particulate matter in santiago, chile. *Environmental Monitoring and Assessment*, 192(171), 2020.
- [59] Michael Kappl and Hans-Jürgen Butt. The colloidal probe technique and its application to adhesion force measurements. *Particle & Particle Systems Characterization: Measurement and Description of Particle Properties and Behavior in Powders and Other Disperse Systems*, 19(3):129–143, 2002.

- [60] Ofir Aharon Kuperman, Peterson de Andrade, XiaoMeng Sui, Raquel Maria, Ifat Kaplan-Ashiri, Qixiang Jiang, Tanguy Terlier, Jacob Judas Kain Kirkensgaard, Robert A Field, and Filipe Natalio. Harnessing precursor-directed biosynthesis with glucose derivatives to access cotton fibers with enhanced physical properties. *Cell Reports Physical Science*, 5(5), 2024.
- [61] Feng Jiang and You-Lo Hsieh. Cellulose nanocrystal isolation from tomato peels and assembled nanofibers. *Carbohydrate Polymers*, 122:60–68, 2015.
- [62] Molly E. Ogle, Gilad Doron, Matthew J. Levy, and Johnna S. Temenoff. Hydrogel culture surface stiffness modulates mesenchymal stromal cell secretome and alters senescence. *Tissue Engineering Part A*, 26(23-24):1259–1271, 2020.
- [63] Paul A Janmey, Daniel A Fletcher, and Cynthia A Reinhart-King. Stiffness sensing by cells. *Physiological reviews*, 100(2):695–724, 2020.
- [64] Stylianos Vasileios Kontomaris, Andreas Stylianou, and Anna Malamou. Atomic force microscopy nanoindentation method on collagen fibrils. *Materials*, 15(7):2477, 2022.
- [65] Yong Hou, Leixiao Yu, Wenyan Xie, Luis Cuellar Camacho, Man Zhang, Zhiqin Chu, Qiang Wei, and Rainer Haag. Surface roughness and substrate stiffness synergize to drive cellular mechanoresponse. *Nano letters*, 20(1): 748–757, 2019.
- [66] Marilyn C McNamara, Ryan J Pretzer, Reza Montazami, and Nicole N Hashemi. Shear at fluid-fluid interfaces affects the surface topologies of alginate microfibers. *Clean Technologies*, 1(1):265–272, 2019.
- [67] M Munz. Microstructure and roughness of photopolymerized poly (ethylene glycol) diacrylate hydrogel as measured by atomic force microscopy in amplitude and frequency modulation mode. *Applied surface science*, 279: 300–309, 2013.
- [68] Zhi-Peng A Wang, Chang-Lin Tian, and Ji-Shen Zheng. The recent developments and applications of the traceless-staudinger reaction in chemical biology study. *RSC advances*, 5(130):107192–107199, 2015.
- [69] Fahimeh Askari, Mojgan Zandi, Parvin Shokrolahi, Mitra Hashemi Tabatabaei, and Elnaz Hajirasoliha. Reduction in protein absorption on ophthalmic lenses by pegda bulk modification of silicone acrylate-based formulation. *Progress in biomaterials*, 8:169–183, 2019.
- [70] Jui-Yang Lai, Li-Jyuan Luo, and David Hui-Kang Ma. Effect of cross-linking density on the structures and properties of carbodiimide-treated gelatin matrices as limbal stem cell niches. *International journal of molecular sciences*, 19(11):3294, 2018.
- [71] Chloe N Grover, Jessica H Gwynne, Nicholas Pugh, Samir Hamaia, Richard W Farndale, Serena M Best, and Ruth E Cameron. Crosslinking and composition influence the surface properties, mechanical stiffness

and cell reactivity of collagen-based films. *Acta biomaterialia*, 8(8):3080–3090, 2012.

