

**OPTIMIZATION OF SAMPLE PREPARATION METHODS FOR THE
MECHANICAL CHARACTERIZATION OF TISSUE ARCHITECTURES**

**A DISSERTATION SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MATERIALS SCIENCE AND NANOTECHNOLOGY**

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August 2017

OPTIMIZATION OF SAMPLE PREPARATION METHODS FOR THE MECHANICAL CHARACTERIZATION OF TISSUE ARCHITECTURES

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August 2017

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ABSTRACT

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August 2017

There is now mounting evidence that mechanical signals are as crucial as genetic and biochemical feedback mechanisms for directing and organizing complex cellular behaviors, and material characterization tools are routinely being employed in biomedical research to investigate the physical aspects of cell-to-cell communication. Atomic force microscopy (AFM) is a surface characterization tool that is compatible with aqueous environments and has recently emerged as an important technique for the mechanical analysis of biomaterials such as proteins, nucleic acids, cells and tissues. However, owing to the natural heterogeneity of biological materials and the diversity of sample preparation methods, AFM results in the literature are characterized by large discrepancies between individual studies, which prevents the drawing of general conclusions from the existing research. While the effects of factors such as AFM probe morphology and fixation length on measurement quality have been described individually in the literature, previous studies typically focus on the analysis of bacterial and eukaryotic cells rather than tissues. Consequently, a detailed comparison of tissue preparation methods for AFM analysis is lacking.

The present thesis describes the mechanical characteristics of four murine tissues (heart, liver, spleen and kidney) following sample preparation using three commonly employed methods (paraffin-embedding, cryosectioning and agarose-embedding). Fixatives used in the paraffin-embedding process are observed to greatly increase the elastic modulus of tissues due to the irreversible cross-linking of the tissue extracellular matrix. Cryosectioning and agarose-embedding, in contrast, provide elasticity values that are more consistent with the live condition of the tissue, but suffer from high tip-sample adhesion that must be compensated through the use of high ($\sim 10 \mu\text{m}$) measurement distances and/or non-adhesive AFM probes. In addition, agarose-embedded sections are subject to stringent measurement conditions due to the lack of long-term storage options. While cryosectioning is found to be an ideal compromise between data quality and ease of measurement, perfusion with 4% PFA is observed to increase tissue elasticity even under cryosectioning, showing that perfusion is not a recommended step in tissue preparation for AFM.

Keywords: atomic force microscopy, sample preparation, fixation, Young's moduli

ÖZET

DOKU YAPILARININ MEKANİK İNCELENMESİ İÇİN ÖRNEK HAZIRLAMA YÖNTEMLERİNİN OPTİMİZASYONU

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Ağustos 2017

Günümüzde hücrelerin davranışlarının genetik ve biyokimyasal mekanizmalar kadar mekanik sinyallerle de kontrol edildiği hakkında detaylı bilimsel kanıt bulunmaktadır ve malzeme analiz yöntemleri biyomedikal alanda hücre-hücre iletişiminin fiziksel yönlerini araştırmak için sıklıkla kullanılmaktadır. Atomik kuvvet mikroskobu (atomic force microscope, AFM) sıvıda ölçüm alabilen bir yüzey karakterizasyon cihazıdır ve son yıllarda proteinler, nükleik asitler, hücreler ve dokular gibi biyomalzemelerin mekanik analizinde önemli bir yöntem olarak ortaya çıkmıştır. Ancak biyolojik malzemelerin doğal olarak heterojen olmaları ve örnek hazırlama yöntemlerinin çeşitliliği nedeniyle, literatürdeki çalışmalar ve araştırma grupları arasında AFM sonuçları büyük farklar göstermektedir. Bu durum ise yapılan çalışmalardan genel sonuçlar çıkarılmasını engellemektedir. Her ne kadar literatürde ölçüm frekansı ve fiksatif kullanım süresi gibi faktörlerin ölçüm kalitesine etkisi detaylandırılmış olsa da, önceki bu çalışmalar genellikle bakteri ve ökaryot hücreleri hakkında yapılmış olup, dokuların ölçümü konusu ise incelenmemiş bulunmaktadır. Dolayısıyla, AFM analizi için kullanılan doku hazırlama yöntemlerinin karşılaştırması, literatürde eksik olan bir konudur.

Bu çalışma dört fare dokusunun (kalp, karaciğer, dalak ve böbrek) üç sık kullanılan örnek hazırlama yönteminden (parafin blok, dondurarak kesme (cryosectioning) ve agaroz blok) sonraki mekanik özelliklerini araştırmaktadır. Parafin blok yönteminde kullanılan fiksatif kimyasalların, dokudaki hücrelerarası matris yapısını cross-link ederek elastik modül değerlerini kayda değer bir miktarda arttırdığı gözlemlenmiştir. Parafin blok yönteminin aksine, dondurarak kesme ve agaroz blok yöntemleri canlı dokuya daha yakın elastikiyet sonuçları vermekte, ancak AFM ucunun örneğe yapışması sebebiyle yüksek ölçüm uzaklıkları ($\sim 10 \mu\text{m}$) veya yapışmayan AFM uçlarının kullanılmasını gerektirmektedir. Ayrıca, agaroz blok örneklerinde uzun süre saklanama imkanı bulunmadığı için hızlı ölçüm yapılmalıdır. Dondurarak kesme data kalitesi ve ölçüm kolaylığı açısından ideal bir yöntem olarak görülmektedir, ancak %4 PFA ile perfüzyon bu yöntemde bile doku elastikiyetini artırdığı için AFM için perfüze doku kullanımını tavsiye edilmemektedir.

Keywords: atomik kuvvet mikroskobu, örnek hazırlama, fiksasyon, Young modülü

ACKNOWLEDGEMENTS

During these past six years I have toiled hard and often, as many will attest, though not always to further my thesis, as many will attest also. It has been an exceedingly long journey, and I could not have made it without the contributions of friends, family and colleagues who have helped me through every step of the way. It is here that I address those who have supported me through thick and thin.

My advisor, Dr. Ayşe Begüm Tekinay, has my endless gratitude for guiding me and my fellow graduate students with competence through these difficult years, and my co-advisor, Dr. Aykutlu Dâna, will always be appreciated for his keen mind and timely advice. I also owe much to Dr. Turgay Tekinay, who first welcomed me to UNAM and fostered many a long-lasting collaboration, Dr. Mustafa Özgür Güler, whose measured feedback has delivered us from countless critical mistakes, and Dr. Emine Deniz Tekin, from whom I have learned much in the way of molecular simulation.

I had the good fortune to share my research environment with many brilliant men and women of science. I would be a much more competent scientist, I think, if I had some of Hakan's capacity for independent thinking, Rashad's talent to get his ideas across, Büşra's careful and methodological way of working, Gözde's sharp eye and stable hand in surgery, Berna's ability as a judge of character, Yasin's coolness in precarious situations, Öncay and Ceren's willingness to tackle difficult research with little time and resources, Murat's devotion to his life choices, Mustafa's sense of justice, Gülistan's research ethic, Melis and Göksu's broad-minded understanding of people and the literature alike, Arif and Ruslan's peerless expertise in all things chemical, Melike's aptitude to do cutting-edge research while running an entire laboratory on

her own, Elif's skill at turning wide-ranging data into coherent manuscripts, Fatih and Nurcan's ability to perform a doctorate's worth of work in the duration of an MS, Nuray's commitment to do good science regardless of the required effort, Özge's flair for handling umpteen different research topics with gusto, Oğuz's cheerfulness in the face of lengthy and daunting work, Canelif's frankness, İdil's perseverance and good humor, and Ahmet Emin's way of living in the moment. I hope that they, too, will remember this humble biologist and his knack for words – my e-mail address is always open to those who would like a bit of proofreading or some insect trivia, though my mobile phone might not be so!

As for my circle of close friends; Tolga Tarkan Ölmez, Ahmet Emin Topal, Ebuzer Kalyoncu and Ömer Faruk Sarioğlu, thanks for your companionship for all these years. But don't think you'll get kind words like the folks above! I will never forget Tolga's conviction in upholding what he thinks is right (but is egregiously wrong), Ebuzer's crafty get-rich-quick schemes, Ahmet Emin's strange non-sequiturs and Ömer Faruk's indispensable role as a voice of reason in our unruly little group.

But it is my family that deserves the biggest thanks of all, and I would not have made it *anywhere* without the endless support of my mother Ayşe and my sister Jülide. You both know that I am no good at expressing sentimentality, but I dearly hope that you understand how much your encouragement has meant to me in difficult times.

Lastly, I would like to thank TÜBİTAK for providing me with financial support through the TÜBİTAK BİDEB 2211-C programme, which has made my doctoral studies possible.

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CHAPTER 1

Introduction

This chapter has been modified from the following publications:

Ozkan AD, Topal AE, Dana A, Guler MO, Tekinay AB (2016) Atomic force microscopy for the investigation of molecular and cellular behavior. *Micron* 89:60-76.

Ozkan AD, Topal AE, Dikecoglu FB, Guler MO, Dana A, Tekinay AB (2017) Atomic force microscopy probe modification methods and their applications in the imaging of biological materials. *Seminars in Cell and Developmental Biology* (accepted manuscript).

1.1. The atomic force microscope

1.1.1. Principle and relevance to biological sciences

At its simplest, atomic force microscopy (AFM) is a characterization method that measures the topology and material properties of surfaces by recording the deflection of a metallic probe (or “tip”) as it moves over the target surface. AFM can be operated under three principal modes: In contact mode, the tip is dragged directly over the surface and deflects away due to repulsive Coulombic interactions, while in non-contact mode it is held at a short (typically <100 nm) distance over the sample and oscillates at a frequency that depends on the attractive van der Waals forces acting upon it. In tapping or intermittent contact mode, the tip is kept oscillating above the sample, and the oscillation frequency changes as the tip approaches the surface at regular intervals¹. Contact and intermittent modes are particularly suitable for the probing of biological samples due to their applicability in liquid media²: Despite considerable losses in resolution, a liquid environment allows cellular imaging in a native environment and, more importantly, permits the direct investigation of mechanical changes on a live cell surface in response to an introduced stimulus³. Time-lapse elastographs taken in this fashion have been utilized for a diverse array of applications, including to visualize the formation of amyloid⁴ or collagen⁵ fibers under different environmental conditions, determine how membrane integrity is altered in the presence of antibiotics⁶, or record the production and dissolution of cytoskeletal elements during cell movement⁷. In addition, it is possible to utilize the AFM tip as a stimulus to elicit a response from the target cell, and the probe itself can be

functionalized with ligand molecules to determine the affinity of the cell membrane to a particular biological moiety.

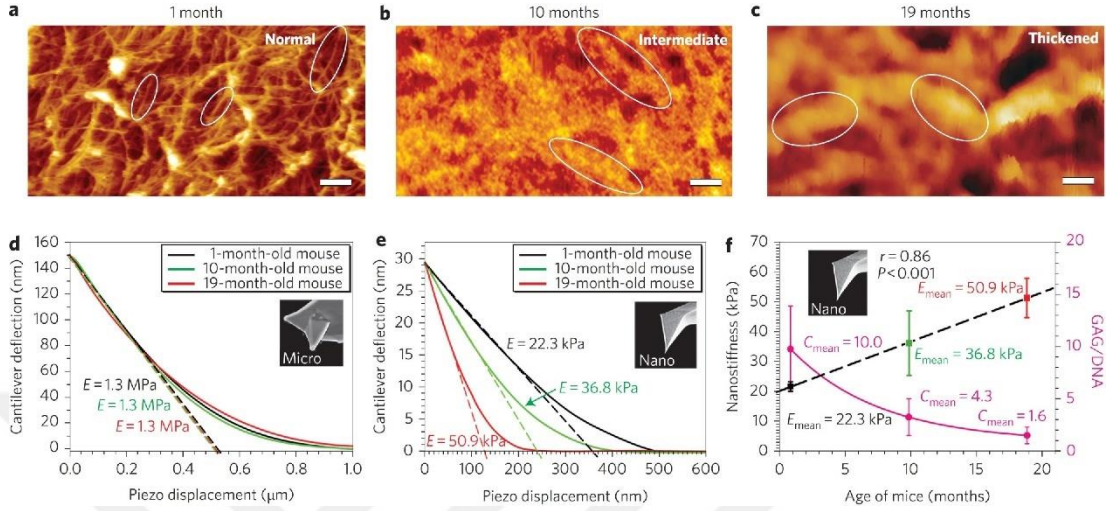


Figure 1.1. Comparison of micro- and nanoindentation for the identification of changes associated with aging in the cartilage of C57BL/6 mice. Microindentation results could detect no difference between 1, 10 and 19-months old individuals, while nanoindentation was able to determine that the cartilage elasticity of non-arthritis mice changes with age, demonstrating the importance of tip morphology for the acquisition of accurate AFM results. Replicated with permission from Stolz *et al.*⁸

Despite this flexibility, cells and tissues are exceptionally susceptible to measurement errors in AFM, as biological materials are far from ideal substrates for this technique. AFM was originally developed for the imaging of nano-to-microscale samples with low surface roughness, while eukaryotic cells on flat surfaces may measure in excess of $10 \mu\text{m}$ in height and $100 \mu\text{m}$ in diameter, which is beyond the vertical and horizontal ranges of many commercial systems. In addition, mammalian cells do not survive in the absence of certain environmental conditions, and while the necessary temperature

and CO₂ ranges can be maintained by specialized attachments for biological imaging, measurements must still be performed in a growth or differentiation medium that contains a wide range of proteins and growth factors. These biomolecules readily attach to available surfaces and create a corona that may interfere with measurement, while physical contact with the sample may also foul or abrade the probe during contact-mode imaging or force-displacement measurements, especially for complex tissue samples⁹. Furthermore, probe morphology and measurement conditions have been reported to significantly alter AFM results: Chiou *et al.*, for example, found that effective Young's moduli of NIH3T3 and 7-4 cells were two-folds higher when measured by sharp tips compared to flat or bead-attached cantilevers¹⁰, while Carl and Schillers similarly demonstrated that spherical probes produce significantly lower Young's modulus values compared to conventional, sharp-tipped AFM probes for the elasticity analysis of Chinese hamster ovary cells, despite exhibiting consistent results over a wide range of probe radii (0.5-26 μm)¹¹. Consequently, despite the ability of AFM to characterize material surfaces with excellent resolution and high repeatability, results associated with cells and tissues should always be considered in the context of their measurement conditions.

1.1.2. Analysis of tip-sample interactions

AFM is not limited to the surface characterization and can be used to determine the elastic modulus of a broad range of biological and non-biological samples. In these measurements, the AFM tip indents the sample until the repulsive forces acting against it exceed a pre-determined threshold, and the strength of these forces, when expressed in relation to the distance by which the tip is forced into the sample, allows the

calculation of the sample elastic modulus. In addition to indentation, forces associated with tip retraction are also measured by AFM, and adhesive interactions between the tip and the sample can be quantified by evaluating the difference between tip approach and retraction curves (which corresponds to the work performed by adhesive forces between the two materials). A variety of contact models have been used to describe the relationship between tip forces and the depth of indentation, taking into account factors such as surface roughness, tip morphology and force-induced deformation of the tip and the sample. In the context of AFM imaging, principal among these are the Hertz model, which assumes that both indenting and indented objects are uniform, smooth, non-deforming and linearly elastic¹²; the Sneddon model, which is a modification of the Hertz model for conical indenters; and the DMT and JKR models, which include additional force terms to account for tip-sample adhesion¹³. As the JKR model is more suitable for highly deformable surfaces, it is generally preferred for the AFM analysis of biological materials, although other models will also yield comparable elastic moduli under most conditions. It should also be noted that, in addition to model choice, parameters associated with each model (such as spring constants and tip radii) should be known for accurate fitting.

Other types of information can be derived from force-distance curves; for example, the sudden detachment of the sample (or a part thereof) manifests as a sudden “bump” in the retraction curve and can be used for the domain analysis of proteins. Accordingly, the unfolding of each domain of a complex protein is reflected as a single detachment event, and the comparative strength of each domain can be evaluated through the order in which they unfold¹⁴. Similarly, the adhesive forces between two

biologically affine molecules, such as a receptor and its ligand or an antigen and its corresponding antibody, can be quantified through force-distance curve analysis. Even entire cells can be used as probes to determine the affinity between two distinct cell types, and AFM is frequently employed in this capacity for the analysis of immune cell interactions – Zhang, Wojcikiewicz and Moy, for example, reported that Jurkat cells (T-lymphocytes) formed stronger adhesions to HUVECs with longer interaction times, and used antibody blocking to demonstrate that, following 0.25 seconds of binding, the adhesion molecules E-cadherin, ICAM-1 and VCAM-1 were responsible for 18%, 39% and 41% of the interaction between the two cell types¹⁵.

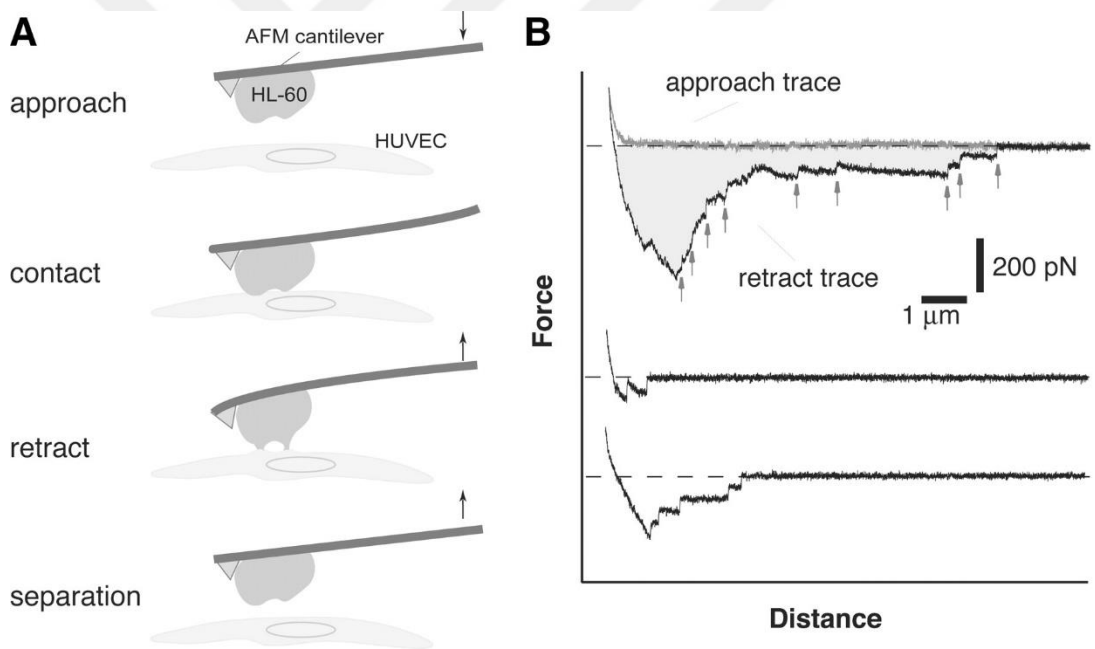


Figure 1.2. Approach and retraction curves showing the binding interaction between HL-60 cells and HUVECs. Top, middle and bottom curves show the strong interaction between the two cell types, loss of this interaction in the presence of an anti-VCAM-1 antibody, and relatively weak binding of HL-60 to BSA. Replicated with permission from Zhang *et al.*¹⁶

In another study, this group also demonstrated that ICAM-1 and VCAM-1 partially mediated the interactions between monocytic human promyelocytic leukemia cells (HL-60 cells) and HUVECs, while β_1 -integrins played a comparatively stronger role in attachment and $\alpha V\beta_3$ -integrins had no significant role in this process¹⁶. In addition, the leukocyte-endothelial cell association was inhibited through the disruption of VLA-4/VCAM-1 binding by the cRGD sequence, which is a well-known motif for cell attachment and presumably competes with adhesive cell membrane proteins for binding sites¹⁷. Cellular detachment was associated in both cases with multiple rupture events that correspond to the stretching and/or breaking of receptor-ligand bonds.

Adhesive interactions are also of fundamental importance for tumorigenesis and cancer metastasis, and Yu *et al.* have found that surface-coated ephrin-A1 (but not soluble ephrin-A1) stimulates collagen I binding in PC3 prostate cancer cells by enhancing the adhesive capacity of β_1 -integrins, which may be relevant to the metastasis of these cells into collagen I-rich bone tissue¹⁸. In addition to mammalian cell lines, the adhesion of bacterial and single-celled eukaryotic cells can also be quantified through cell-functionalized probes; for instance, a single *Staphylococcus aureus* bacterium can be used to obtain adhesion images by probing the interaction forces between the bacterium and skin corneocytes. Like cancer cells, cellular recognition and binding mechanisms are crucial for the initial invasion, immune detection and phagocytic destruction of bacterial and fungal pathogens, and Mostowy *et al.* have demonstrated the importance of septins for the cellular entry of *Listeria monocytogenes* by measuring the force of interaction between invasion proteins on *Listeria* and Met receptors on HeLa cells in the presence and absence of these

proteins¹⁹. In a similar vein, El-Kirat-Chatel and Dufrêne have shown that the yeast *Candida albicans* adheres strongly to the J774A.1 murine macrophage cell line (with interaction forces reaching ~3000 pN) through mannan/mannose receptor interactions in a time-dependent manner, and suggested that the phagocytosis of the yeast cell by the macrophage is responsible for the gradual increase in adhesive forces²⁰. It is also known that *C. albicans* can form biofilms alongside the bacterial pathogen *Staphylococcus aureus*, and this group also used mutant *Candida* strains to show that bacteria adhere preferentially to fungal hyphae but not the cells themselves, and that the Als protein family and O-mannosyl groups are crucial for the establishment of *Candida-Staphylococcus* interactions²¹.

1.2. Use of AFM in biomechanical characterization

1.2.1. Use of AFM in the characterization of prokaryotic cells and exudates

In addition to quantifying biomolecular interactions involving either purified proteins or whole cells, AFM has received considerable attention for its ability to directly measure the mechanical properties of cells, tissues, membranes, biofilms and other large-scale biological assemblies, which cannot easily be characterized in their native environment by other imaging and mechanical analysis tools. A representative selection of AFM studies on the mechanical characterization of bacteria, yeasts and cellular secretions is provided in Table 1.1. It is readily evident that several means of sample preparation are available for measurement, and that values such as tip-sample adhesion, F-d curve slopes and cellular spring constants can all be used to compare the

mechanical integrities of biological samples; consequently, only these studies detailing the full range of measurement conditions were included in the table.

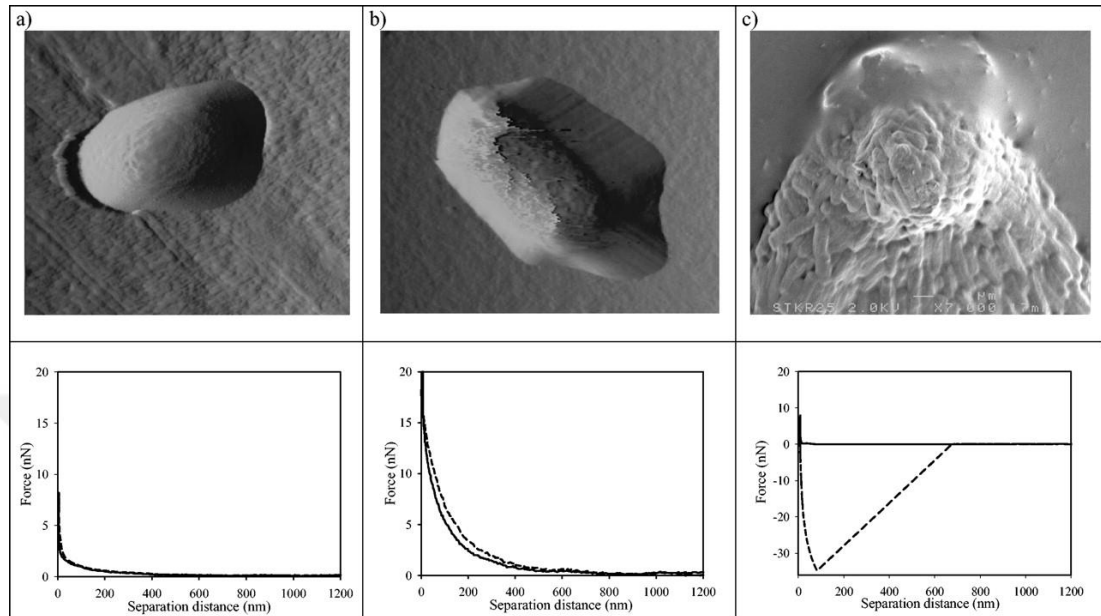


Figure 1.3. AFM topographs and associated force-distance curves of *Klebsiella terrigena* ATCC33527 cells following attachment to (a) Isopore polycarbonate membranes, (b) poly-L-lysine-coated glass slides and (c) AFM cantilevers, showing that fixation method greatly alters both the morphology and elastic moduli of bacterial cells. Replicated with permission from Vadillo-Rodríguez *et al.*²⁸

Both air and liquid imaging have been performed for mechanical investigations; however, biological materials may display large changes in elastic behavior depending on environmental humidity. As such, samples in air tend to have much larger Young's moduli compared to samples imaged in liquids (*e.g.* a 10-fold difference was observed in between the elastic moduli of air-dried and rehydrated murine sacculi from *Escherichia coli*²³), while fixation and cell immobilization can likewise have a major

impact on elasticity. As previously mentioned, it is usually preferable to compare results within studies rather than assuming a given stiffness value will apply under other experimental conditions, as AFM results of cells and tissues will by necessity reflect inherent differences in measurement techniques and sample preparation methods, as well as the natural variance in the material properties of bacterial cells and their secretions.

Table 1.1. Mechanical characterization of single-celled organisms by AFM.

Sample	Tip properties	Imaging conditions	Elastic properties	Reference
Sulfate-reducing bacteria	Silicon nitride, $k = 0.06 \text{ N/m}$ (nominal)	Contact mode, air, sample on mica	(Adhesion) -3.9 to -4.3 nN at cell surface, -5.1 to -5.9 nN at cell-substrate boundary, -6.5 to -6.8 nN at cell-cell boundary	24
Enterococcal <i>Escherichia coli</i> , wild-type and dispersin mutant	Silicon, $k = 2.8 \text{ N/m}$ (nominal)	Contact mode, liquid (distilled water) and air, sample on gelatin-treated mica	(F-d slope) 0.133 for wild-type strain on agar, 0.069 for wild-type strain in broth, 0.81 for dispersin mutant on agar, 0.78 for dispersin mutant in broth	25
<i>Bacillus subtilis</i> , <i>Micrococcus luteus</i> , <i>Pseudomonas putida</i> , two strains of <i>Escherichia coli</i>	Silicon nitride, $k = 0.32 \text{ N/m}$ (nominal)	Contact mode, liquid (HM buffer), sample on APTES coverslip	(Spring constant) varies between 0.16 ± 0.01 to 0.41 ± 0.01 , higher in Gram-positive cells	26
<i>Pseudomonas aeruginosa</i>	Silicon nitride and silicon nitride with silicon oxide tips, $k = 0.07 \pm 0.01$ (calibrated by the thermal method)	Contact mode, liquid (milliQ water), sample on poly-L-lysine-coated glass	(Spring constant) $0.044 \pm 0.002 \text{ N/m}$ for unfixed, $0.11 \pm 0.03 \text{ N/m}$ for glutaraldehyde-fixed cells; creep deformation behavior also investigated	27
<i>Klebsiella terrigena</i>	Silicon nitride, $k = 0.06 \text{ N/m}$ (nominal)	Contact mode, liquid (potassium phosphate buffer at pH 6.8), sample on polycarbonate membrane filter/poly-L-lysine-coated glass/immobilized on tip by glutaraldehyde fixation	(Adhesion) $-0.26 \pm 0.05 \text{ nN}$ for membrane filter, -0.5 ± 0.2 for poly-L-lysine, $-35 \pm 2 \text{ nN}$ for glutaraldehyde fixation, other adhesion parameters also measured	28

Two <i>Streptococcus salivarius</i> strains	Silicon nitride, $k = 0.03 \text{ N/m}$ (nominal)	Contact mode, liquid (deionized water or 0.1 M KCl solution), sample on polycarbonate membrane filter	(Adhesion and repulsion) Fibrillated strain shows a larger repulsion range, interpreted to reflect the layer of fibrils; retraction results in three adhesion forces potentially corresponding to three different lengths of fibrils observed by electron microscopy	29
<i>Desulfovibrio desulfuricans</i> , <i>Pseudomonas</i> sp. and an unidentified local marine isolate	Silicon nitride, $k = 0.12 \pm 0.02 \text{ N/m}$ (calibrated by the thermal method)	Contact mode, liquid (artificial seawater), sample coated on tip and brought into interaction with metals	(Adhesion) All three isolates adhere to aluminum better than mild steel, stainless steel 316 and copper; <i>Desulfovibrio</i> and <i>Pseudomonas</i> adhere better than the marine isolate.	30
<i>B. mycoides</i>	Silicon, $k = 0.064 \text{ N/m}$ and 0.4 N/m	Contact mode (constant height), liquid (0.145 M NaCl), sample coated on tip and brought into interaction with glass	(Adhesion) $7.4 \pm 3.7 \text{ nN}$ of adhesion to hydrophilic glass surface, $49.5 \pm 14.42 \text{ nN}$ of adhesion to hydrophobic-coated glass surface	31
Marine bacterial depositions	Silicon, $k = 45.7 \text{ N/m}$ (calibrated by the added mass method)	Tapping mode, air, sample deposited on fluoridated and non-fluoridated polyurethane	(Young's modulus) between 1.5 and 2.2 GPa	32
Bacterial depositions, suspected to be extracellular polymeric substances	Silicon nitride, k varies from 0.03 to 0.5 N/m (nominal)	Contact mode, liquid (MilliQ water), sample deposited on polystyrene	(Adhesion) Forces of $0.8 \pm 0.2 \text{ nN}$ observed over bare polystyrene, as opposed to $0.2 \pm 0.2 \text{ nN}$ after cell attachment	33
<i>P. aeruginosa</i> pili	Silicon nitride, $0.008 \pm 0.004 \text{ N/m}$	Contact mode, liquid (water), sample attached to poly-L-lysine-coated tips and brought into interaction with mica surface	(Adhesion) Rupture forces of 95 pN during retraction.	34
<i>E. coli</i> biofilms	Silicon nitride, $k = 0.07\text{-}0.4 \text{ N/m}$	Contact mode, air, sample deposited on glass	(Adhesion) Pull-off forces of 122.65 pN for cell-tip interaction and 51.79 pN for glass-tip interaction	35
Bacterial cellulose fibers	Silicon nitride $k = 1.03 \pm 0.05 \text{ N/m}$ (calibrated by the thermal method, nominal k of 0.5 N/m not used due to large discrepancy)	Contact mode, air, sample on silicon nitride-coated silicon grating	(Young's modulus) $78 \pm 17 \text{ GPa}$	36

<i>E. coli</i> and <i>E. coli</i> spheroplasts	Silicon nitride, $k = 0.1$ and 0.01 N/m (nominal, actual spring constants calibrated by the thermal method)	Contact mode, liquid (TBS2 buffer), sample on APTES/Glut mica	(Spring constant) 0.194 N/m for intact cells, 0.571 N/m for fixed spheroplasts	37
<i>Saccharomyces cerevisiae</i>	Silicon nitride, $k = 0.008 \pm 0.4$ N/m (calibrated by the thermal method)	Contact mode, liquid (milliQ water), sample on polycarbonate membrane filter	(Young's modulus) 6.1 ± 2.4 MPa on bud scar, 0.6 ± 0.4 MPa on surrounding cell wall	38
<i>Aspergillus nidulans</i>	Silicon nitride, $k = 0.47 \pm 0.06$ N/m (calibrated by the thermal method)	Contact mode, liquid (PBS), sample on poly-L-lysine-coated glass	(Spring constant and Young's modulus) 0.29 ± 0.02 N/m and 110 ± 10 MPa for wild-type hyphae in complete medium, decreases for mutant strain lacking a chitin synthesis gene, as well as in the presence of 0.6 M KCl.	39

1.2.1.1. Bacterial cell surfaces

Unlike many vertebrate cell lines, bacterial cells are not dependent on a highly specific set of environmental conditions to survive, and can tolerate extended AFM imaging sessions without detrimental effects^{40, 41}. Their ease of procurement, non-demanding growth conditions and the fact that many laboratory species either are, or serve as models for, common pathogens make bacteria popular targets for AFM imaging. Bacteria must be immobilized prior to imaging in liquid media, as their mobility otherwise makes it impossible to image. Even sessile bacteria can be laterally pushed by the AFM tip⁴². Immobilization can be performed by drying and rehydrating, electrostatic binding to a positively charged surface (*e.g.* gelatin or poly-L-lysine), entrapment in adhesive proteins, covalent binding to amino- or carboxyl-functionalized surfaces or by physical confinement in microwells or porous membranes^{42, 43}. It should be kept in mind that the immobilization method may modify the surface properties of the entrapped cells, *e.g.* by directly altering the bacterial

surface chemistry or triggering a defense mechanism against the environmental stresses associated with the immobilization technique. Due to the small sizes of bacteria, it is also possible to functionalize the AFM tips with bacterial cells, which can then be used to test the interaction between the bacterium and material surfaces. Ting *et al.*, for example, used such tips to show that the Gram-negative bacteria *Massilia timonae* and *Pseudomonas aeruginosa* adhere better to stainless steel surfaces than does the Gram-positive *Bacillus subtilis*⁴⁴.

AFM studies of bacteria frequently focus on the mechanisms by which certain molecules inhibit the growth of pathogens. Many antibiotics (*e.g.* beta-lactam antibiotics, polymyxins and glycopeptide antibiotics) act by inhibiting the synthesis of bacterial cell walls or membranes, and thereby alter the membrane integrity of the affected bacteria. Other antibiotic-mediated effects, such as membrane thinning⁴⁵ or pore formation⁴⁶, can also be observed by AFM, and the effects of less conventional antibiotics, such as plant extracts⁴⁷ and peptide sequences^{48, 49}, can also be assessed. The antimicrobial peptide PGLa has been demonstrated to lower the stiffness of *Escherichia coli* membranes and create micelle-like structures around cell membranes prior to their eventual rupture, while garlic extract was also associated with membrane disruption.

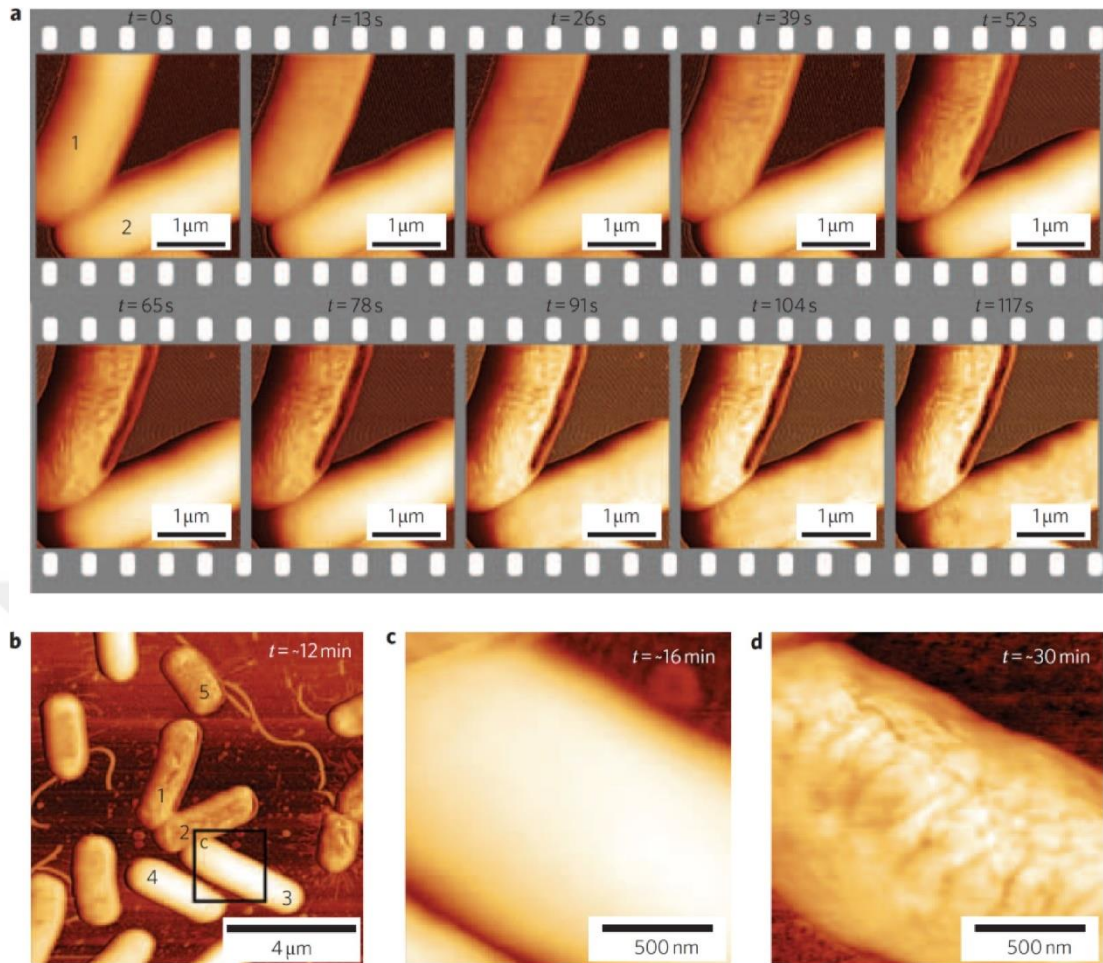


Figure 1.4. The effect of an antimicrobial peptide (CM15) on *E. coli* cell walls, as observed by high-speed AFM. (a) Disruptions begin to appear on cell surfaces as early as $t = 13$ s and increase in severity with time. (b, c) Although some bacteria resist the effects of the peptide, these individuals nevertheless react to prolonged treatment (d, at $t = \sim 30$ min). Replicated with permission from Fantner *et al.*⁵⁰

Chitosan and chitooligosaccharides were also tested for their antimicrobial effect on *E. coli* and *Staphylococcus aureus*, and the thick peptidoglycan wall of *S. aureus* was found to allow this bacterium to better retain its overall morphology, despite experiencing a significant decrease in cell rigidity⁵¹. While the effects of antibacterial

molecules may be apparent even under dry imaging, it is also possible to quantify the resulting changes in a time-dependent manner by characterizing the mechanical properties of bacterial cells in liquid before and after introducing the antibiotic in question into the medium⁶. This technique has the advantage of ensuring that the changes observed are fully due to the antibiotic, as opposed to a combination of it and the drying process, as bacterial cell walls may greatly alter their mechanical properties in response to relative humidity⁵².

A variety of subcellular elements can also be identified on membrane surfaces using AFM. Both bacterial and eukaryotic cells can be used in these efforts, and lipid bilayers can be substituted as simplified models of cell membranes^{53, 54}. The structure and function of membrane proteins^{53, 55, 56}, pore complexes⁵⁷, gap junctions⁵⁸, amyloid aggregates⁵⁹ and a variety of membrane-component lipids⁶⁰, as well as the mode of action of cholera toxin⁶¹, were investigated by atomic force microscopy. Of particular interest are the recent developments in AFM-based rapid, high-resolution imaging methods, which have granted substantial insight into the nature of small surface elements. Processes such as the self-assembly of bacterial S-layer proteins⁶², protein folding⁶³, misfolding⁶⁴ and crystallization⁶⁵ events, the motion of “walking” proteins⁶⁶, and drug-membrane interactions⁶⁷ have been the subject of real-time imaging studies.

1.2.1.2. Bacterial secretions, exudates and biofilms

It is well-known that cells in multicellular organisms enhance their survival and coordination through the production of an extracellular matrix (ECM); however, this property is not unique to higher eukaryotes. Bacteria also secrete proteins,

polysaccharides and quorum sensing molecules that relay information between conspecific cells and serve as a buffer against environmental stresses. In addition, the surface attachment of bacteria is also mediated by extracellular depositions, the adhesion capacities of which can be measured through AFM. Colanic acid production and surface lipopolysaccharide lengths, for example, were previously demonstrated to determine the strength of attachment of *E. coli* cells, as colanic acid-overproducing mutants were found to exhibit stronger attachment while shorter surface lipopolysaccharides were associated with a lack of adhesive capacity⁶⁸.

Bacterial biofilms are also of special interest with regards to AFM characterization. Biofilms are extracellular polysaccharides that are secreted to facilitate the attachment of bacteria (or other unicellular organisms) to a surface, and protect the adhering cells against hostile environmental factors such as antibiotics, detergents and heavy metals⁶⁹. Biofilms are undesirable elements in many settings, and suitable means to inhibit their formation is necessary to prevent potential health hazards in food, agricultural and medical industries. As such, the mechanical properties of biofilms, as well as the mechanisms by which antifouling molecules act against biofilm production, have been investigated by using AFM and other mechanical characterization techniques^{70, 71}. Corrosion damage and adhesive capacity of biofilms on a variety of metal surfaces have been detailed in the literature: Holden *et al.* report that unsaturated and liquid-grown biofilms of *Pseudomonas putida* respond differently to drying, suggesting that the biofilm composition is altered for optimal growth in dry and wet environments⁷². In another report, Tay *et al.* detail the effect of silver ions on *Staphylococcus epidermidis* biofilms and propose a mechanism through which silver

destabilizes the biofilm structure by binding to the electron donor groups provided by the biofilm components, thereby weakening the hydrogen bonds that hold the biofilm matrix together⁷³.

1.2.2. Use of AFM in the characterization of eukaryotic cells and tissues

A selection of AFM studies on the stiffness characterization of eukaryotic cells and tissues is provided in Table 1.2. AFM of mammalian cells and tissues is often undertaken for disease diagnosis or stem cell differentiation studies, as diseased tissues often display mechanical characteristics distinct from their healthy counterparts and stem cells are widely known to alter their differentiation pathways and mechanical characteristics depending on external stimuli. Consequently, AFM of cancer and stem cells can provide greater insight into the pathways required for events such as metastasis and lineage commitment. However, measurement of these samples is more difficult than single-cellular organisms or non-living biological materials such as biofilms and other secretions, as mammalian cells require a specific set of environmental conditions to survive and environmental stress can have a major impact on their mechanical properties. Nonetheless, cell culture conditions can be replicated to some extent within the liquid cell of an AFM, and a large number of successful investigations have been made regarding the mechanical character of living mammalian cells and tissues, either cultured *ex vivo* or collected immediately prior to imaging.

Table 1.2. Mechanical characterization of mammalian cells and tissues by AFM.

Sample	Tip properties	Imaging conditions	Elastic properties	Reference
NIH3T3 fibroblasts	Silicon nitride, $k = 0.018 \text{ N/m}$ (calibrated by thermal method)	Contact mode, liquid (DMEM containing D-glucose (1000 mg/L) and 10% fetal bovine serum, fresh, warmed Ringer's solution used as medium replenishment), sample on fibronectin-coated glass	(Young's modulus) 4 to 100 kPa over the cell surface, lower around the nucleus than in the periphery	74
Breast cancer lines (MCF-10A and MCF7)	Silicon nitride, $k = 0.01 \text{ N/m}$ (nominal) modified with a $4.5 \mu\text{m}$ diameter polystyrene bead	Contact mode, liquid (culture medium), sample on glass	(Young's modulus) 0.2 to 1.2 kPa range, malignant cell line (MCF7) 1.4 to 1.8 times softer than benign cell line (MCF-10A)	75
Osteoblasts, mesenchymal stem cells and osteosarcoma cells	Silicon nitride, $k \sim 20 \text{ N/m}$ (calibrated using thermal method)	Contact mode, liquid (25 mM HEPES), fixed cells on polystyrene, glass or collagen-coated glass	(Young's modulus) 0.7 ± 0.1 to $2.6 \pm 0.7 \text{ kPa}$ range, lower Young's modulus for MG63 osteosarcoma cells on collagen	76
Zonal articular chondrocytes	Spherical gold-coated borosilicate bead ($5 \mu\text{m}$ diameter), $k \sim 0.065 \text{ N/m}$ (calibrated by thermal method)	Contact mode, liquid (DMEM), sample on poly-L-lysine-coated glass	(Young's modulus) Instantaneous moduli at $0.55 \pm 0.23 \text{ kPa}$ for superficial, $0.29 \pm 0.14 \text{ kPa}$ for middle/deep cells; relaxed moduli at $0.31 \pm 0.15 \text{ kPa}$ for superficial, $0.17 \pm 0.09 \text{ kPa}$ for middle/deep cells; apparent viscosities at $1.15 \pm 0.66 \text{ kPa s}$ for superficial, $0.61 \pm 0.69 \text{ kPa s}$ for middle/deep cells	77
Cardiac muscle, skeletal muscle and endothelial cells	Silicon nitride, $k = 0.03$ to 0.05 N/m (calibrated using thermal method)	Contact mode, liquid (growth medium), sample on glass slide	(Young's modulus) Moduli of $100.3 \pm 10.7 \text{ kPa}$ for cardiac muscle, $24.7 \pm 3.5 \text{ kPa}$ for skeletal muscle and 1.4 ± 0.1 to $6.8 \pm 0.4 \text{ kPa}$ depending on the region tested for epithelial cells	78
Cardiac myocytes	Silicon nitride, $k = 0.06 \text{ N/m}$ (nominal)	Contact mode, liquid (culture medium), sample on laminin-coated petri dish	(Young's modulus) $35.1 \pm 0.7 \text{ kPa}$ for cardiomyocytes from 4-months old rats, $42.5 \pm 1.0 \text{ kPa}$ for cardiomyocytes from 30-months old rats.	79
LLC-PK1 and MDCK kidney epithelial cell lines	Silicon nitride, $k = 0.12 \text{ N/m}$ (nominal)	Contact mode, liquid (artificial urine)	(Young's modulus) $1.5 \pm 0.8 \text{ MPa}$ for LLC-PK1 cells, $5 \pm 1.5 \text{ MPa}$ for MDCK cells, oxalate treatment decreases Young's modulus to $1.2 \pm \text{MPa}$ for LLC-PK1 cells (other stiffness parameters also measured)	80
Neuronal growth cones	Silicon nitride, $k = 0.006 \text{ N/m}$ (nominal)	Contact and dynamic modes, liquid (L15/ASW medium), sample on poly-L-lysine-coated glass	(Young's modulus) 3-7 kPa for the C domain, 7-23 kPa for the T domain, 10-40 kPa for the P domain	81

Healthy and pathological erythrocytes	Silicon nitride, $k = 0.03$ (nominal)	Contact, liquid (PBS), sample on poly-L-lysine-coated glass and fixed by glutaraldehyde	(Young's modulus) Moduli of 26 ± 7 kPa for healthy erythrocytes, 43 ± 21 kPa for hereditary spherocytosis, 40 ± 24 kPa for thalassemia and 90 ± 20 kPa for G6PD deficiency samples	82
Liver endothelial cells	Silicon nitride, $k = 0.032$ N/m	Contact, liquid (serum-free endothelial cell medium), cells on collagen-coated petri dishes with and without glutaraldehyde fixation	(Young's modulus) Moduli of 2 kPa for living cells and over 100 kPa for fixed cells	83
Oral squamous cell carcinoma, normal and malignant lines	Silicon nitride tip, $k = 0.01$ to 0.1 N/m; APTES-modified silicon oxide sphere tip, $k \sim 0.5$ N/m (calibrated using Sader method)	Contact, air, sample prefixed with 2% PFA and fixed with 3.7% PFA	(Young's modulus) Median values of 6.75 MPa for "normal" and 4.36 MPa for metastatic cancer cells. Elasticity measurements taken using sphere-modified tips.	84
PC-3 prostate cancer cells	Silicon nitride tip, $k = 0.012$ N/m (calibrated using thermal method)	Contact, liquid (culture medium), samples treated with anticancer drugs or DMSO control for 24 h prior to analysis.	(Young's modulus) $c.$ 3 kPa for untreated cells, increased to $c.$ 6-12 kPa in a dose-dependent manner following drug treatment. Frequency-dependency of the elastic modulus was also tested and found to change significantly for Celebrex, BAY, Totamine, TPA and VPA treatment, but not for DSF, MK and Taxol. This effect is linked to the fact that the former drugs may alter crosslinking rates of cytoskeletal filaments, while the latter only change fiber length and thickness.	85
Normal and cancerous bladder epithelium cells	Silicon nitride tip, $k = 0.011$ to 0.018 (calibrated using thermal method)	Contact, liquid (culture medium), sample on glass	(Adhesion energy) average of 8.17×10^{-16} J for normal, 26.95×10^{-16} J for cancer cells (Young's modulus) average of 27.57 kPa for normal, 2.46 kPa for cancer cells	86
Porcine articular cartilage	Silicon nitride, $k = 0.06$ N/m (nominal) and borosilicate glass beads with $r = 2.5$ μm , $k = 0.06$ and 13 N/m (nominal)	Contact, liquid (PBS), tissues on poly-L-lysine-coated glass	(Dynamic elastic modulus) On the order of 2.6 MPa for borosilicate glass beads, about 100-fold lower for sharp tips	87
Mouse articular cartilage of arthritic Col9a1 ^{-/-} knockout mice	Silicon nitride, $k = 0.06$ N/m (nominal) and microspheres, $k = 10$ and 12 N/m	Contact, liquid (PBS), bulk tissues glued on a round Teflon disk	(Dynamic elastic modulus) 1.3 ± 0.4 MPa for microspheres (no change recorded between ages) and 22.3 ± 1.5 kPa to 50.9 ± 4.7 kPa for sharp tips (gradual increase with age)	88

Aortic intima of rats	Not listed, tip mounted on a custom platform for <i>in vivo</i> AFM imaging	Anaesthetized living animals	(Young's modulus) 0.4 – 0.5 MPa range for blood vessels without drug influence, raised to c. 1.0 MPa in the presence of nitroglycerin and decreased back to c. 0.3 MPa in the presence of norepinephrine	89
Anterior human corneal stroma	Phosphorus-doped silicon, $k = 25$ and 33 N/m (calibrated by an optical method, as described by Sader <i>et al.</i>)	Contact, liquid (15% dextran), bulk tissue placed on Teflon cell without attachment	(Young's modulus) Between 1.14 and 2.63 MPa, consistent across the indentation depths (between 1.0 and $2.7\ \mu\text{m}$)	90, 91
Human corneal basement membrane	Borosilicate glass, $k = 0.06$ N/m (nominal)	Contact, liquid (PBS), a 3×3 mm tissue piece dissected and glued onto a well on steel disk	(Young's modulus) 2 to 15 KPa, mean of 7.5 ± 4.2 kPa average for the anterior basement membrane; 20 to 80 KPa, 50 ± 17.8 kPa average for the Descemet's membrane	92
Monkey lenses	Gold-coated tip, $k = 0.01$ N/m (nominal), calibrated by the relationship between applied voltage and cantilever deflection	Contact, liquid (BSS), lenses dissected and placed on Teflon slide	(Young's modulus) 1.720 ± 0.88 kPa	93
Human bone	Various	Various	(Young's modulus) 16.6 ± 1.1 to 27.1 ± 1.7 for dry adult tibiae (lower for children), 13.4 ± 2.0 to 22.7 ± 3.1 for dry adult vertebrae (lower for wet samples), 16.58 ± 0.32 to 26.6 ± 2.1 for dry adult femoral midshaft (lower for wet samples and in the femoral neck), other bone measurements and tissue hardnesses also noted	94
Bovine ocular tendon fibers	Silicon nitride tip, $k = 0.02$ N/m (calibrated using thermal method)	Contact, air (sampling chamber kept at 100% humidity), sample glued on glass petri dish	(Young's modulus) 60 ± 2.69 MPa for lateral rectus, 59.69 ± 5.34 MPa for inferior rectus, 56.92 ± 1.91 MPa for medial rectus, 59.66 ± 2.64 MPa for superior rectus, 57.7 ± 1.36 MPa for inferior oblique and 59.15 ± 2.03 for superior oblique tendons. Differences between tendon elasticities are not statistically significant.	95
Breast tissue sections	Borosilicate glass tip, $k = 0.06$ N/m (nominal), individual tips calibrated using thermal method	Contact, liquid (PBS supplied with protease inhibitors and propidium iodide), samples sectioned by cryomicrotomy	(Young's modulus) c. 400 Pa in healthy and non-invasive tumor regions, 4-fold increase in average stiffness in invasive tumor front. Higher average stiffness in the invasive front is caused by highly stiff (>5 kPa) regions in this area. Aggressive breast cancer subtypes are also found to exhibit higher Young's moduli, quantified in terms of upper 10% stiffness.	96

Benign and aggressive prostate tumors	Silicon nitride tip, $k = 0.06$ N/m (nominal), individual tips calibrated using thermal method	Contact, liquid (physiological buffer), sample sliced by razor and glued on glass	(Young's modulus) 3.03 ± 0.64 kPa for benign, 1.72 ± 1.22 for cancerous tissues. The average Young's modulus for cancer samples with Gleason scores in the 2-7 range was 2.07 ± 1.30 , this value was 1.39 ± 0.48 for samples with Gleason scores in the 8-10 range. In addition, metastatic tumors had an average modulus of 1.06 ± 0.58 , while non-metastatic cancer tissue had an average elasticity value of 1.99 ± 1.24 . These values reflect the tissue microenvironment and contrast macro-scale elastography results, in which more aggressive cancers are stiffer.	97
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1.2.2.1. Cancer diagnosis and characterization

While medical advances have led to significant decreases in the incidences of many cancers, cancer still remains to be one of the most important diseases in recent history. It is well-established that cancer cells exhibit marked differences in stiffness and elasticity compared to their healthy counterparts⁹⁸; however, diagnostic applications of mechanical characterization methods are currently limited. Effective diagnostic techniques must ascertain the presence (or absence) of disease with high confidence and using minimal amounts of sample tissue and time, and despite the ability of mechanical measurements to effectively distinguish between healthy and tumor cells, it is unlikely that a biopsy at an early stage of disease would yield tumor cells in numbers necessary to perform diagnosis based purely on mechanical data. Nonetheless, once the presence of a tumor is confirmed using more conventional methods, AFM may serve as a valuable tool for its characterization: Laidler *et al.*, for example, report the possibility of utilizing AFM to determine whether a suspected breast or prostate tumor is malignant on the basis of its elastic properties⁹⁹. In addition, AFM cantilevers can be functionalized with antibodies for disease-specific markers and used in the detection of cancer and other disorders¹⁰⁰; however, this technique

effectively converts the AFM into a biosensor or sorting system instead of relying on its capacity for mechanical characterization.

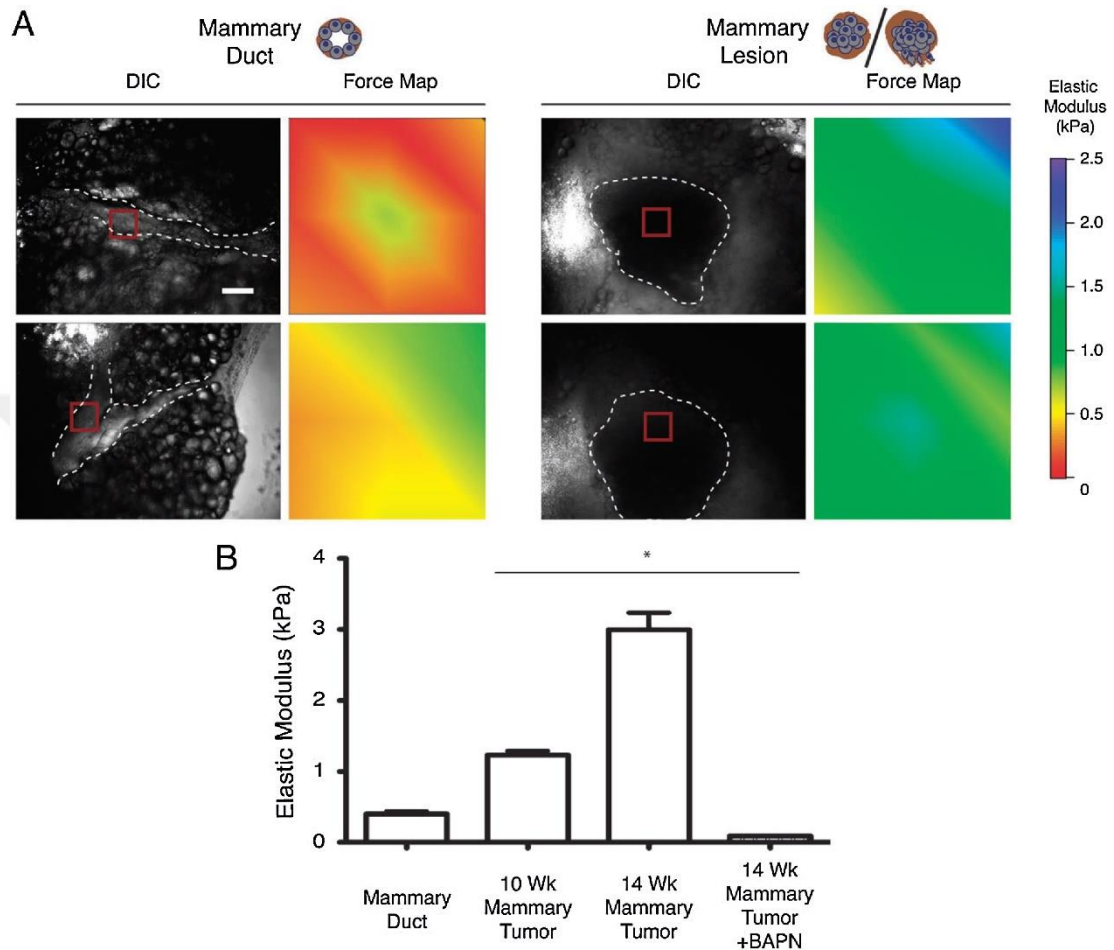


Figure 1.5. Changes associated with mammary tumor formation, as quantified by AFM. Normal mammary duct tissue is less stiff compared to tumor tissue (a), and the tissue elastic modulus increases with tumor age (b). The inhibition of collagen cross-linking by BAPN prevents the tumor-associated increase in stiffness, suggesting that ECM modifications are involved. Replicated with permission from Lopez *et al.*¹⁰¹

Greater utility lies in the characterization of tumor cells and their interactions by AFM, which may further the current understanding of cancer biology and allow the design

and evaluation of novel cancer drugs. Discrepancies between the elasticities of cancer and non-malignant cells, for example, have been recorded in human lung, breast, pancreas, fibroblast, prostate, adenocarcinoma and other cell lines^{102, 103}. These changes are suspected to increase the mobility of malignant cells during metastasis, and decreases in cell stiffness appear to be progressive, with more malignant cells expressing lower Young's moduli. Fuhrmann *et al.* reported that the dysplastic Barrett's esophagus cell lines are less rigid compared to metaplastic cells, which in turn are softer than their healthy counterparts¹⁰⁴. Other changes in cell morphology may also occur to facilitate metastasis, and these too can be quantified by AFM. Sokolov *et al.*, for example, have determined that cancerous human cervical epithelial cells display two brush layers, in contrast to the single-length molecular brush of non-malignant cervical epithelium, which may also assist in invasion attempts by these malignant cells¹⁰⁵. In addition, the tumor microenvironment also appears to contribute significantly to the alterations in tumor elasticity: Instead of the expected decrease in Young's moduli, Weaver *et al.* observed a time-dependent increase in tumor stiffness during mammary tumor development in PyMT mice, and this increase in rigidity was also reflected in epithelial cells taken from the tumor site. However, cells taken out of their native environment and grown *in vitro* had lower Young's moduli compared to their *in vivo* counterparts, and the inhibition of the ECM-crosslinking enzyme lysyl oxidase mitigated the gradual increase in the elastic modulus of mammary gland tumors¹⁰¹. As such, tissue culture samples and *in vivo* tumor cells may not necessarily agree in elastic properties, as the stiffness of the former depends on the cytoskeletal properties of the cells, while that of the latter is largely mediated by the tissue microenvironment.

Drug responses of cancer cells can also be quantified by AFM. Zhang *et al.* observed that HeLa, HepG2 and C6 cells experience dose-dependent morphological changes on their cell membranes following treatment with colchicine or cytarabine. Surface roughness increased and pores appeared on the cell membrane after drug administration and before MTT-quantifiable decreases in viability could be observed, suggesting that the surface alterations represent an early response to drug presence¹⁰⁶. Such differences may be monitored to evaluate the effectiveness of drug candidates. Changes associated with gene deletion or restoration can also be observed through AFM: Zhou *et al.* reported that the expression of BRMS1 (restoring breast cancer metastasis suppressor 1) induces increases in cell adhesion capacity, cellular spring constant and Young's modulus, which supports the idea that BRMS1 expression is associated with cytoskeletal rearrangements that are unfavorable for metastatic activity. Cell morphology and roughness were also altered following BRMS1 expression, possibly as a consequence of cytoskeletal modifications¹⁰⁷.

1.2.2.2. Diagnosis of other diseases

While cancer-associated changes in cell elasticity are particularly drastic, cancer is by no means the only condition to alter the mechanical properties of affected tissues. A variety of other conditions, including malaria, sickle cell anemia, hepatic fibrosis, cardiovascular disease, renal stiffness, muscular dystrophies and bone disorders, have also been associated with notable changes in the elastic properties of the affected tissues¹⁰⁸⁻¹¹². Mechanical diagnosis methods have been devised for some of such conditions, and AFM can be utilized to study the structural and mechanical properties of affected tissues; but it must be noted that, as with cancer, AFM-based diagnostic

methods for these diseases currently appear to be more suited towards supporting conventional diagnosis. However, the high-resolution imaging and mechanical probing capacity of AFM is ideal for the determination of the causes underlying the progression of the diseases in question.

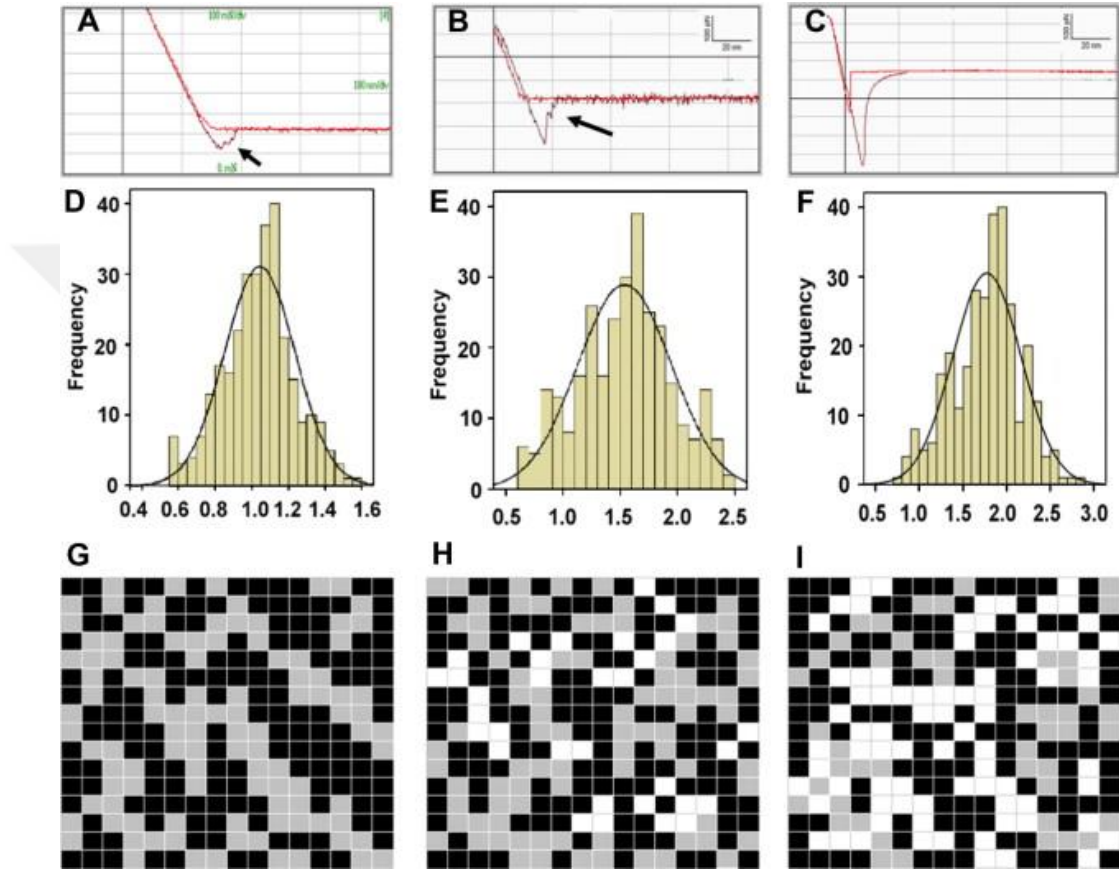


Figure 1.6. Young's modulus measurements of erythrocytes from young and healthy (YHP; a, d and g); old and healthy (OHP; b, e and h) and old and type-II diabetic (ODP; c, f and i) individuals. Erythrocytes exhibited higher stiffness in ODP individuals compared to YHP or OHP ($1.78 \pm 0.39 \times 10^5$ N/m² vs. $1.04 \pm 0.19 \times 10^5$ N/m² and $1.53 \pm 0.41 \times 10^5$ N/m²). In addition, OHP and ODP erythrocytes exhibited higher adhesion compared to YHP erythrocytes (420 ± 25 pN and 510 ± 63 pN v. 200 ± 38 pN, respectively). Replicated with permission from Jin *et al.*¹¹³

Several reports on AFM-based disease characterization exist in the literature. Szymoński *et al.*, for example, demonstrate that red blood cells belonging to patients with hereditary blood diseases generally bear a higher Young's modulus compared to healthy erythrocytes⁸². In particular, gluteraldehyde-fixed, poly-L-lysine-immobilized erythrocytes of patients with hereditary spherocytosis, thalassemia or G6PD deficiency were stiffer compared to normal cells, while patients with anisocytosis displayed two distinct peaks in their histogram of Young's moduli, corresponding to healthy and diseased populations of cells. In patients with hereditary spherocytosis, changes in cell morphology were also observed. Similarly, Vatner *et al.* have investigated the effects of aging on cardiac myocytes by AFM indentation, comparing 4 months- and 30 months-old rats in order to assess whether myocyte health influences the aging-associated diastolic dysfunction of the left ventricle¹¹⁰. Myocytes were found to significantly increase in stiffness with age, suggesting that the observed dysfunction can be linked at least in part to the malfunction of individual myocytes. The effect of aging on cellular Young's moduli was also noted by Sokolov *et al.*, who have shown that older human epithelial cells are more rigid than their younger counterparts, and have further demonstrated that an increase in the density of cytoskeletal elements is responsible for the age-related increase in stiffness^{114, 115}. The age-related progression of osteoarthritis was also quantified on the articular cartilage of normal and arthritic mice, and nanometer-scale tips (but not microbeads) were shown to be able to resolve the gradual changes in cartilage stiffness⁸⁸.

Another interesting frontier in AFM-based disease characterization is the investigation of malfunctioning proteins that are involved in the pathogenesis of neurodegenerative

disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis. These disorders severely decrease the quality of life, are exceptionally common among the elderly and have no definitive cures, which renders it crucial to gain further insight into the nature of their causative agents. As such, the formation of amyloid plaques has been investigated in detail using a variety of biological, chemical and material characterization methods, including several AFM-based studies. Lansbury *et al.*, for example, reported on the *in vitro* formation of metastable A β amyloid fibril precursors that may later develop into complete amyloid assemblies, and suggest that the halting of this mechanism may prevent the onset of Alzheimer's disease by retaining the precursor fibrils ("protofibrils") in their benign intermediate form⁴. In addition, Kowalewski and Holtzman demonstrated that the size, shape and production kinetics of A β aggregates were altered depending on the surface on which the aggregation occurs¹¹⁶ (*i.e.* particulate assemblies were generated on the hydrophilic mica surface, while β -sheets formed on the hydrophobic graphite), and suggested that the β -sheet forming behavior of A β on graphite may yield useful information on how protein folding occurs *in vivo*. The ability of A β peptides to form ion-channel like structures without interacting with other membrane components was also confirmed using AFM, with trimeric, tetrameric, pentameric and hexameric pore structures being identified in topographic images. Both D- and L-enantiomers of A β peptides were able to form these channels, suggesting that the pore formation mechanism is not stereospecific⁵⁹.

1.2.2.3. Stem cell differentiation

Fluctuations in mechanical properties do not necessarily suggest aging-related deterioration or a disease state, as healthy cells may also respond to environmental signals by altering their membrane integrity. This is most obviously observed in stem cells, as differentiation induces fundamental changes not only in cell morphology and expression patterns, but also in membrane content and stiffness¹¹⁷⁻¹¹⁹. Since the maintenance, recruitment and differentiation of stem cells are intimately linked to the mechanical properties of their immediate environment, it is feasible to use AFM measurements in order to determine the factors that drive the differentiation process in these cells. Such factors are relatively clear in some cases, such as the mesenchymal stem cell differentiation into myogenic, chondrogenic or osteogenic lineages, but the mechanical triggers behind the differentiation of other cells are less understood. In addition to stem cell differentiation, changes in the microenvironmental conditions of tissues can be assessed using AFM or other mechanical characterization methods, especially in situations involving the slow recovery of a damaged system, as in the cases of bone fracture healing. Other processes, such as the local remodeling and ECM secretion of cells in a tissue culture environment, or the capacity of biomimetic materials to imitate the mechanical environment of their tissue model, also fall within the purview of AFM.

The differences between stem and derived cells have also been investigated by AFM. Guilak *et al.* have confirmed that chondrocytes, osteoblasts and adipocytes, the primary differentiation products of mesenchymal stem cells, display different rigidities, and determined that undifferentiated mesenchymal stem cells are similar to

adipocytes in their mechanical characteristics¹²⁰. Schieker *et al.* reported that the two subcategories of human mesenchymal stem cells, flat cells and rapidly self-renewing cells, can also be differentiated on the basis of their morphological and adhesive characteristics⁷⁶, with the former type appearing topographically similar to human osteoblasts and displaying a high adhesion to the surface, while the latter exhibited characteristics similar to MG63 osteosarcoma cells and displayed a smoother topography. Even stem cell fate can be predicted by mechanical properties prior to the appearance of visible indicators. Gonzalez-Cruz and Darling reported that adipose-derived stem cells can be classified according to their differentiation potential to adipogenic, osteogenic or chondrogenic lineages on the basis of their mechanical behavior¹²¹. As could be expected; softer, larger and more pliant adipose-derived stem cells are more likely to differentiate into adipocytes, while smaller and more rigid stem cells are inclined towards osteogenic or chondrogenic differentiation. In addition, the effect of stem cells on an *in vivo* environment can be determined following their implantation: Mesenchymal stem cells injected into the site of a myocardial infarction have been recorded to decrease the rigidity of the myocardium, which was associated with reduced fibrosis and a better prognosis for the postinfarcted heart.

The importance of the ECM for the maintenance and differentiation of stem cells is both obvious and paramount^{122, 123}. It is therefore unsurprising that the mechanical properties of ECM elements are vital in providing the signals responsible for inducing stem cells to differentiate, or for retaining them in their quiescent state^{119, 124}. Great differences exist in the ECM rigidities of adult tissues, from 0.1 kPa in brain to >30 kPa in bone¹²⁵, and excessively soft or rigid tissues may result in suboptimal

differentiation (see *e.g.* Engler *et al.* for an account of aberrant myotube formation associated with unsatisfactory surface stiffness¹¹²). In addition to adult stem cells in mature tissues, there is also evidence that endo-, meso- and ectodermal progenitors are arranged into distinctive germ layers with the assistance of differences in tensile strength^{126, 127}: Cell-cortex tensions of endodermal progenitors in gastrulating zebrafish embryos are higher than that of the mesodermal progenitors, which in turn are larger than that of ectodermal progenitor cells¹²⁸.

1.2.2.4. Extracellular secretions and tissue microenvironments

AFM can be used to measure the native stiffness of tissue micro- and nanoenvironments, although other methods, such as microindentation, can also be used for the micromechanical investigation of larger areas of tissues. Bone, cartilage and the ocular tissues have been frequently investigated using AFM, and age- or disease-related effects have been found to be reflected in tissue stiffness. As with bacterial and eukaryotic cells, the method of sample preparation can greatly alter the elastic modulus of tissues, and sampling location likewise has a substantial effect on mechanical properties^{88, 94}. It must also be kept in mind that the micro- and nanoscale stiffnesses of a given tissue may differ greatly, *e.g.* the microstiffness of porcine articular cartilage was observed to be over 100-fold greater than its nanostiffness, and the age-related effects of osteoarthritis on the articular cartilage of mice could only be observed through nanoscale tips^{8, 87}. As such, care must be taken not to compare the results of cell- or tissue-based AFM studies to literature examples that use dissimilar conditions of measurement.

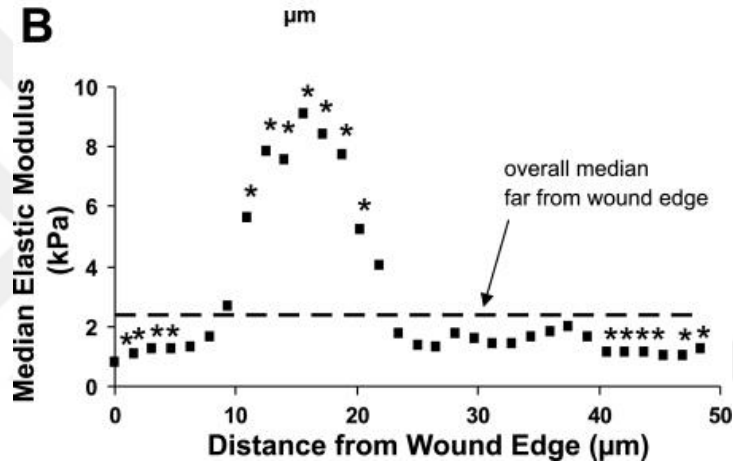
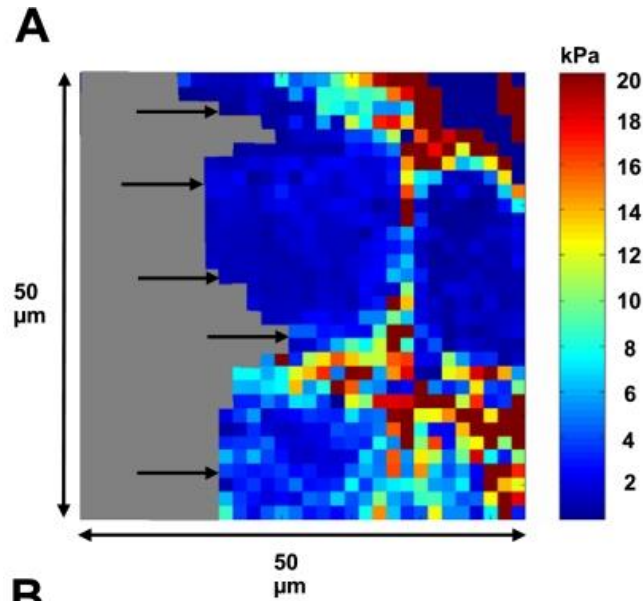


Figure 1.7. Elastic moduli of 16HBE human bronchial epithelial cells, as expressed in relation to the distance to a scratch wound in cell culture, show a transitory increase in stiffness at the wound edge. Replicated with permission from Wagh *et al.*¹²⁹

The extent of natural heterogeneity within a given cell population or tissue sample can also be established using AFM. An AFM apparatus adapted for use in living brain tissue, for example, has been used to demonstrate that the rat hippocampus is heterogeneous, and that hippocampal subregions are associated with different elastic moduli¹³⁰. The wound healing process has also been characterized using AFM, and the

leading edge of the wound was found to exhibit a spatially limited increase in stiffness to recruit fibroblasts to the wound site. This localized peak is created by cytoskeletal changes in the cells of the wound edge, and disappears if the expression of the actin fiber-regulating protein RhoA is disabled, resulting in a lack of fibroblastic recruitment to the site of injury¹²⁹. In addition to tissues, an individual cell can also be analyzed to identify distinct regions on its surface: Scheuring *et al.* have characterized the mechanisms used by red blood cells to contort during their passage through blood vessels, utilizing a method in which the function of known structural proteins are altered and the resultant changes on the cell surface are measured to map these proteins onto the mechanically heterogeneous regions present on the erythrocyte¹³¹. The addition of MgATP was utilized to elicit changes on the membrane elements on the extracellular (“outwards”) and cytoplasmic (“inwards”) sides of the erythrocyte, which behaved in two distinct ways and therefore allowed the detailed characterization of membrane structures and molecular compositions on both sides.

In addition to characterizing the structure of previously deposited matrix microenvironments, AFM can also determine how cellular exudates are secreted outside cells, especially with regards to the membrane budding and fusion events that occur during the secretion process. Research in this direction focuses mostly on cells involved in the secretion of hormones or digestive fluids, such as pancreatic acinar cells¹³² or growth hormone (GH)-secreting cells¹³³. Secretion in acinar cells was found to depend on large (500-2000 nm) apical “pits” dotted with 3-20 smaller (100-180 nm) “depressions” or “fusion pores”, the depths of which correlate with the extent of amylase secretion. Similar pits and depressions were also present in GH-secreting

cells, and the existence of GH within these structures was confirmed using gold-tagged GH antibodies, demonstrating that the depressions were secretory in nature. A combination of atomic force microscopy and confocal microscopy was also used to demonstrate that the secretory vesicle swelling process is regulated by a GTP-binding protein¹³⁴.

1.3. Importance of mechanical signaling in the context of biological research

1.3.1. Mechanotransduction pathways

Despite their reliance on mechanical signals for their survival and proliferation, cells are limited in their ability to directly sense physical stresses and must use proteins such as stretch receptors and mechanosensitive ion channels to integrate mechanical stimuli into biochemical pathways. The process of transferring mechanical information into metabolic and genetic elements is called *mechanotransduction* and involves the action of a broad spectrum of membrane, cytosol and ECM elements. In addition, mechanical sensing is associated with specific intermediate molecules and transcription factors that produce the required responses at the gene and protein levels by continuing the signaling cascade initiated by mechanoresponsive biomolecules. Different subsets of these signaling molecules are activated in response to different types of mechanical stimuli, allowing cells to react appropriately to a wide range of environmental conditions: For example, Shikata *et al.* demonstrated that focal adhesion kinase (FAK) is phosphorylated at different locations depending on whether cells are exposed to shear or cyclic stretching stress, allowing each stress type to produce specific changes

in focal adhesion point distribution and GTPase activation rates in human pulmonary artery endothelial cells^{135, 136}. Similarly, Papadopoulou *et al.* reported that cyclic stress was more effective than static stretching in activating osteogenesis-related pathways in primary human periodontal ligament fibroblasts¹³⁷, while Jacobs *et al.* have shown that calcium release in hFOB 1.19 human fetal osteoblasts is stronger in response to steady and pulsed flow compared to oscillating flow¹³⁸.

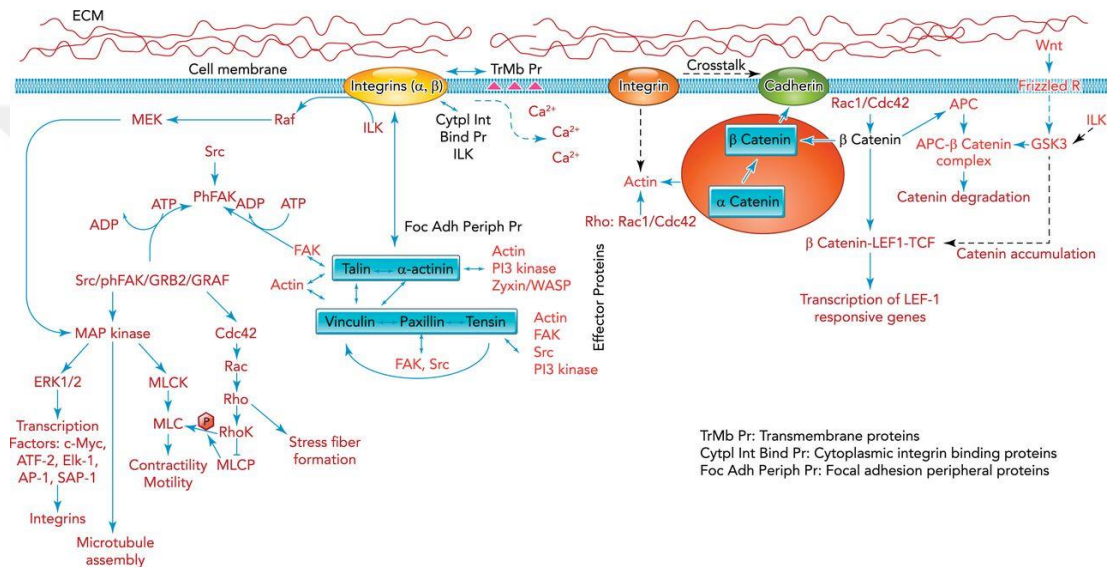


Figure 1.8. A general overview of mechanotransduction pathways and the associated signaling cascades. Replicated with permission from Suki *et al.*¹³⁹

These stress type-dependent alterations in cellular responses are also reflected at higher levels of biological organization, as Rosenfeld *et al.* observed that human umbilical vein endothelial cells (HUVECs) arranged into vessel-like tubules in gelatin-based 3D scaffolds under both cyclic and static stretching, but were aligned at a 30° – 60° angle from the direction of cyclic stress while showing no angular divergence from the stress axis under static stretching¹⁴⁰.

While the ability of cells to exhibit stimulus-specific responses to a variety of mechanical signals is well-recognized, the molecular pathways underlying these behaviors are characterized only to a lesser degree: As mechanosensory mechanisms are of particular importance to the biology of hard tissues such as cartilage and bone, which must continuously respond to external stresses to adequately support body musculature and internal organs, many currently known mechanical sensing pathways have first been described in these tissues. However, while mechanotransductive elements such as focal adhesion points, calcium-gated channels, matrix metalloproteinases, ECM proteoglycans and YAP/TAZ signaling components have long been appreciated to play an important role in the biology of tissues that are regularly exposed to significant external forces¹⁴¹⁻¹⁴⁴, recent discoveries have made it abundantly clear that mechanical signaling is much broader in scope and contributes significantly to the biological activity of every cell and tissue. Disparate and far-reaching processes such as nuclear organization¹⁴⁵, cellular differentiation¹⁴⁶ and embryonic development¹⁴⁷ have been demonstrated to depend on mechanical stimuli in addition to biochemical signals, suggesting that mechanical aspects of cell-cell and cell-ECM signaling are as important for the regulation of cellular behavior as well-established receptor/ligand interactions. A selection of such mechanical signaling pathways is provided below.

1.3.1.1. Role of membrane-associated elements

Cells do not exist independently of their external environment, but instead are integrated into it through membrane-bound proteins – accordingly, each individual cell is connected to other cells and the ECM through adhesion molecules that tether the cell to its associated niche and detect the mechanical changes that may occur in its surroundings. Fundamental for this interaction is the class of transmembrane proteins called integrins, which regulate ECM binding and are composed of α - and β -components that may assemble in 24 combinations in humans¹⁴⁸. Integrin molecules are initially synthesized in a “bent” conformation, in which both subunits are clustered together, and the activity of proteins such as fibronectin, actin, kindlin and talin are required to “unbend” them and enhance their ligand-binding (usually RGD-binding or LDV-binding) capacity^{149, 150}. These molecules also alter the membrane distribution of integrin and stimulate the formation of large protein assemblies called *focal adhesion points*, where mechanical stresses are converted into biochemical signals through force-mediated protein activation: Talin, for example, contains an amino acid motif that binds to the adhesion-strengthening protein vinculin, but is ordinarily concealed within the protein structure. External forces, however, can trigger the exposure of this binding site, promoting the recruitment of vinculin to the integrin-actin binding complex¹⁵¹. Similarly, myosin-II is able to transmit stretching forces to integrin $\alpha 5 \beta 1$ and induce a switch between “relaxed” and “tensioned” states in its interaction with fibronectin – the former only requires the presence of the RGD motif on fibronectin, while the latter also entails the participation of the adhesion synergy site, thus increasing the adhesive strength associated with $\alpha 5 \beta 1$ binding¹⁵². In addition,

focal adhesion kinase (FAK) is another integrin-binding element that is involved in the mechanotransduction of external forces through its phosphorylation at three tyrosine residues (Y396, Y576 and Y577), which is mediated by mTOR in response to pulling forces acting on integrin¹⁵³.

Cadherins are the other principal class of mechanotransductive membrane proteins, and where integrins are essential for cell-ECM binding, their role is to modulate cell-cell adhesion. As with integrins, the protein complexes formed by cadherins are linked to the actin cytoskeleton of the cell, and the distribution of the former is linked to the contractions produced by the latter¹⁵⁴. Instead of focal adhesions, however, the protein complex associated with cadherins is the *adherens junction*, which is formed with the participation of another class of proteins called *catenins*. In addition to serving as a structural element in adherens junctions, catenins also participate actively in mechanotransduction: Like talin, α -catenin contains a vinculin-binding domain that is exposed in response to stretching forces, which increases the strength of the adherens junction under mechanical stress¹⁵⁵. As such, vinculin is a crucial element in both focal adhesion points and adherens junctions, although the mechanism of its activation differs in cell-cell and cell-ECM connections: Cadherin-mediated signaling occurs through the phosphorylation of vinculin at the Y822 residue, which is performed by Abelson tyrosine kinase following its activation by cadherin in response to external forces, while integrin-mediated signaling does not depend on this pathway¹⁵⁶. Other proteins, such as FAK, are also known to participate in both cadherin and integrin complexes, allowing cells to effectively respond against mechanical stimuli by coordinating their cell-cell and cell-substrate interactions¹⁵⁷. In addition, it is worth

noting that cadherin-mediated mechanotransduction triggers the activation and membrane localization of AMP-activated protein kinase (AMPK), a metabolic regulator that allows cells to meet the massive energetic demands associated with the cytoskeletal rearrangements required to act in response to mechanical stress^{158, 159}.

1.3.1.2. Role of cytoplasmic, nuclear and ECM elements

While membrane-bound proteins are able to locally regulate parameters such as membrane elasticity in response to mechanical stress, long-term adaptation against external forces requires the activation of regulatory proteins at the gene level. As such, mechanosensitive proteins are typically supported by a set of cytosolic intermediate elements that establish a channel of communication between the cell membrane and the nucleus. Calcium, for example, is a crucial regulator of mechanotransduction in the cytoplasm, and stretch-activated calcium channels are responsible for modulating its intracellular concentration in response to external stress¹⁶⁰. Small metabolites such as cyclic AMP and proteins such as G-proteins, phospholipases and MAP/ERK pathway kinases are likewise linked to many metabolic processes, and their activity is at least partially regulated by mechanosensitive membrane proteins such as integrins and the aforementioned stretch-activated ion channels¹⁶¹⁻¹⁶⁴. In addition to membrane elements, the ECM can also generate a series of intermediate molecules to participate in cellular signaling in response to mechanical stress: Basic fibroblast growth factor (bFGF), for example, is generally bound to perlecan in the cartilaginous ECM, but stretching forces weaken this interaction and facilitate the release of this growth factor from the proteoglycan matrix. Following its release, bFGF can then interact with its membrane receptor to establish a signaling cascade with the cell interior¹⁶⁴. In a similar

manner, the cytoskeleton can react to mechanical changes either in tandem with the cell membrane, through its connections with focal adhesion complex proteins such as talin and vinculin¹⁶⁵, or independently by linker elements such as plectin, which directly connects intermediate filaments to the nuclear envelope¹⁶⁶.

The nucleus, too, is integrated within an extensive network of direct and indirect mechanoregulatory signals: YAP and TAZ, transcriptional regulators that alter their localization patterns following their activation, represent the best-known elements of nuclear mechanotransduction and play an important role in both organ formation and cancer development¹⁶⁷. Both of these proteins are associated with the establishment of size constraints during tissue growth and, if not regulated by upstream elements of the Hippo pathway, their activity in the nucleus promotes the excessive development of the tissue(s) in question. Their role in cancer is also linked to their principal function, as many aggressive neoplasms are characterized by the aberrant activation of YAP/TAZ or deactivation of YAP/TAZ inhibitors such as the kinases MST1/2 and LATS 1/2, resulting in unchecked cellular proliferation¹⁶⁸. As could be expected from its role in regulating organ size, the YAP/TAZ pathway responds strongly to mechanical cues in the environment, and especially to the stretch receptors of the cytoskeleton. Stresses associated with stiffer cytoplasmic and extracellular environments favor YAP/TAZ activation, while softer cells and substrates promote the deactivation of these regulatory elements¹⁶⁷. In addition to YAP/TAZ, other nuclear proteins are also known to be involved in mechanotransduction, including lamins and emerin, and chromatin itself has been speculated to respond directly to mechanical stress^{141, 166}.

1.3.2. Mechanical organization of cells and tissues

As documented in the previous sections, cells constantly perceive and act upon a mechanical Umwelt that constraints and regulates their behavior, and such rigid biomechanical feedback mechanisms would not have evolved without strong selective pressures for their development. The principal function of mechanotransduction pathways is to produce cooperative cellular responses against both internal and environmental factors: In addition to their ability to sense the biophysical aspects of their immediate surroundings, cells can alter the elastic moduli of their own cytoskeleton or ECM to direct certain events such as tissue development, which require the precise coordination of a broad range of cell types. The embryonic heart, for example, is formed from a tubular precursor that forms a loop to create the cardiac chambers, and this looping is mediated by a difference in elasticity between the two sides of the heart tube – the stiffer side is less prone to bending and therefore forms the inner curvature of the loop, while the softer side is more compliant and forms the outer curvature¹⁶⁹. Likewise, muscle-induced mechanical stimulation is necessary for skeletal development due to the crucial roles played by the YAP/TAZ pathway during this process, and mouse embryos lacking muscle development show skeletal aberrations resulting from a corresponding lack of mechanical stimuli¹⁷⁰. In addition, wing development in *Drosophila* has been linked to the impact of the mechanical stress acting on the cytoskeleton, with higher cytoskeletal tension being associated with more pronounced wing development through the inhibition of the LATS1-homolog Wts¹⁷¹. Even the earliest stages of embryonic organization have been linked to cellular coordination based on mechanical signals, as the endodermal, mesodermal and

ectodermal layers are reportedly separated based on the strength of the junctions between cells of each dermal layer¹⁴⁷.

In addition to organogenesis, mechanical signals have also been implicated in the coordinated action of cells during wound repair, as 16HBE human bronchial epithelial cells have been shown to increase their stiffness at the wound edge in a RhoA-dependent manner, and this effect was speculated to act as a stimulus for cellular recruitment to the site of injury¹²⁹. Scar formation is another aspect of wound healing that is closely linked to mechanotransduction mechanisms, and the FAK complex in particular has been implicated in the formation of fibroses in tissues such as lungs, heart, kidneys and liver¹⁷². Changes in elasticity can also be used as a metric for evaluating the effectiveness of novel treatment methods, as Berry *et al.* used elastic moduli to investigate the success of mesenchymal stem cell treatment for heart infarctions and found that MSC-treated hearts were intermediate between healthy cardiac tissue and untreated infarctions in terms of mechanical stiffness¹⁷³. Lastly, mechanical signals are crucial not only in the functioning of healthy tissue, but also the disruptions in tissue function associated with the tumor environment: As described in Section 1.2.2.1., cancer cells are generally recognized to be softer than their healthy counterparts and increase their ability to metastasize by lowering their elastic moduli, although this trend may not necessarily hold in tumor microenvironments. In addition, matrix-mediated feedback mechanisms may directly contribute to the development of cancer, as the overexpression of the receptor tyrosine kinase has been reported to render mammary epithelial cells both more invasive and more responsive to environmental stiffness¹⁷⁴.

1.4. The importance of comparative studies in the mechanical characterization of biomaterials

As demonstrated in this chapter, mechanical signals are fundamental in determining the behavior of cells across all levels of organization. However, despite the unmatched flexibility of atomic force microscopy, its use for the characterization of the mechanical Umwelt of cells has so far been limited. This effect is attributable to the fact that cells are incredibly variable substrates and AFM is highly sensitive to small changes in sample properties, which creates a “trash in, trash out” scenario where individual AFM results are highly reliable but cannot be generalized because they only hold for the precise set of environmental conditions under which each measurement has been made. Comprehensive studies that evaluate the effect of individual sample preparation and measurement techniques on AFM results would partially circumvent this problem by offering qualitative and quantitative metrics to compare results from different groups, but such research has been limited in the literature. The present thesis aims to address this deficit by providing a thorough investigation of tissue elastic moduli under a wide variety of sample fixation and measurement conditions.

CHAPTER 2

Optimization of sample preparation methods for the AFM analysis of tissues

2.1. MATERIALS & METHODS

2.1.1. Animal experiments

Tissues used in the present study were derived from animals used in control groups of unrelated experiments and for the isolation of primary cell cultures. Spleen, liver, kidney and heart tissues of adult male BALB/C mice (n = 3) were dissected and washed in PBS prior to embedding protocols, and used as the sample material for AFM studies. In addition, the aforementioned four tissue types have also been derived from a 11 day-old BALB/C mouse (n = 1), and spleen, lung, kidney and heart tissues were recovered from an adult male BALB/C mouse (n = 1) perfused with 4% PFA to serve as age and pre-fixation controls, respectively.

2.1.2. Paraffin-embedding and deparaffinization

Spleen, liver, kidney and heart tissues were fixed in 4% paraformaldehyde for 48 h, dehydrated in a series of 70%, 80%, 90% and 100% ethanol (EtOH) for two washes of 1 h each, and incubated overnight in 100% EtOH prior to embedding. Following this process, samples were infiltrated twice with 100% xylene and twice with a 1:1 mixture of xylene:paraffin for 1.5 h each, and embedded in paraffin blocks afterwards. 5 µm tissue sections were then taken from each sample using a Leica RM2245 semi-automated microtome. Tissue sections were subsequently deparaffinized and rehydrated by incubation in 100% xylene (twice), 1:1 mixture of xylene:EtOH, 100% EtOH (twice), 90% EtOH, 80% EtOH, 70% EtOH and 50% EtOH for 3 min per wash.

Samples were kept in PBS prior to AFM analysis; all samples were imaged within 6 h of their deparaffinization.

2.1.3. Cryosectioning

Spleen, liver, kidney and heart tissues were embedded in Sakura® Finetek Tissue-Tek® O.C.T. compound, frozen in liquid nitrogen, and stored at -80 °C prior to cryosectioning. 20 µm tissue sections were taken from tissue blocks using a Leica CM1850 cryostat operating at -30 °C, transferred to positively-charged slides, briefly air-dried to ensure the attachment of the sample to the slide, and washed in PBS prior to AFM measurements. All samples were measured within 6 h of their reconstitution in PBS.

2.1.4. Agarose-embedding and vibrotomy

Liver and kidney tissues were embedded in 4% low-melting point agarose at *ca.* 45-50 °C to minimize heat-induced damage to tissue integrity. Tissue blocks were incubated for 1 h at room temperature for agarose to solidify, and 150 µm tissue sections were subsequently taken in ice-cold PBS using a Leica VT1200 S automated vibratome. Sections were transferred to positively-charged slides and briefly air-dried prior to AFM analysis in PBS. All samples were imaged within 12 h of the dissection of the relevant organ.

2.1.5. Atomic force microscopy analyses

All AFM measurements were performed in PBS using octafluorocyclobutane (C_4F_8) coated cantilevers. Fluorotips were produced from standard SiNi cantilevers (SiNi-100, Budget Sensors) by coating with C_4F_8 under 150 W for 1 h in an inductively coupled plasma (ICP) device, according to a protocol modified from Topal *et al.*, and the longer cantilevers (200 μm length, 60 pN/nm nominal spring constant) were used for force-distance measurements using an Asylum Research MFP-3D AFM. Cantilever spring constants were calibrated using the thermal method prior to each series of measurements. For deparaffinized and cryosectioned samples, each indentation map was acquired over a 20 μm x 20 μm area at a resolution of 16 x 16 pixels, with three such areas being taken per section and three sections being mapped for each sample. Consequently, a total of 27 maps were taken for each tissue, with 9 maps representing each animal (with the exception of cryosections, which used a total of 18 maps). For agarose-embedded samples, mapping over wide areas was complicated by the roughness of the surface and the vertical limit of the AFM, and twelve 5 μm x 5 μm areas were instead mapped at a resolution of 8 x 8 pixels for each section. Accordingly, 108 maps were acquired for each tissue type, with 36 maps representing each animal. In either case, a total of 6912 (= 108 x 8 x 8 or 27 x 16 x 16) force-distance measurements were made for each sample type, at a maximum indentation depth of 10 μm and a deflection set point of 1 V (which was decreased to 0.5 for one set of cryosectioned maps to determine the effect of deflection set point on Young's modulus results).

2.1.6. Curve-fitting analyses

Retraction curves of F-d maps were analyzed using a contact point determination (CPD) algorithm developed by Chang *et al.* with minor modifications¹⁷⁵. Briefly, the CPD algorithm was modified to use the retraction curve rather than the approach curve, and to perform fitting on the entire curve from the contact point onwards rather than defining an additional limit before the end. Fits were performed with a Poisson's ratio of 0.33 and G threshold of 1 under a conical geometry, with spring constants matching those obtained using the thermal calibration method. In addition, as the algorithm tends to provide exceptionally low Young's moduli for incorrectly fit data, cutoff thresholds were used as follows: a minimum of 5 kPa for deparaffinized tissues, a minimum of 0.5 kPa for perfused-and-cryosectioned tissues, and a minimum of 0.005 kPa and maximum of 5 kPa for agarose-embedded tissues. In addition, Young's moduli above the highest values observed in spleen samples were generally discarded. Discarded curves never constituted a majority, with a minimum of 4842, 5972 and 4311 force-distance curves being used for the analysis of agarose-embedded, deparaffinized and cryosectioned samples, respectively.

2.1.7. Statistical analyses

All values are presented as mean $\pm$ standard error of mean, except where noted. Student's t-test and one-way analysis of variance (ANOVA) with Tukey's and Bonferroni's post-correction tests (applied as appropriate) were used to quantify the differences between each group, and p-values below 0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism v7.03.

2.2. RESULTS

2.2.1. AFM analysis of deparaffinized mouse tissues

An overview of elasticity calculation results associated with deparaffinized tissues is provided in Figure 2.1. It is readily apparent that the elastic moduli of individual tissues cannot be distinguished using deparaffinized samples, with only liver tissue giving a minor but statistically significant difference under this preparation method. This effect is not unexpected, as paraffin-embedding by necessity entails the use of fixants such as formalin and paraformaldehyde, which irreversibly crosslink the tissue matrix. Nevertheless, some unfixed tissue regions may remain, as all tissue histograms had a notable presence of sub-10 kPa components.

Elasticity comparison of deparaffinized tissues

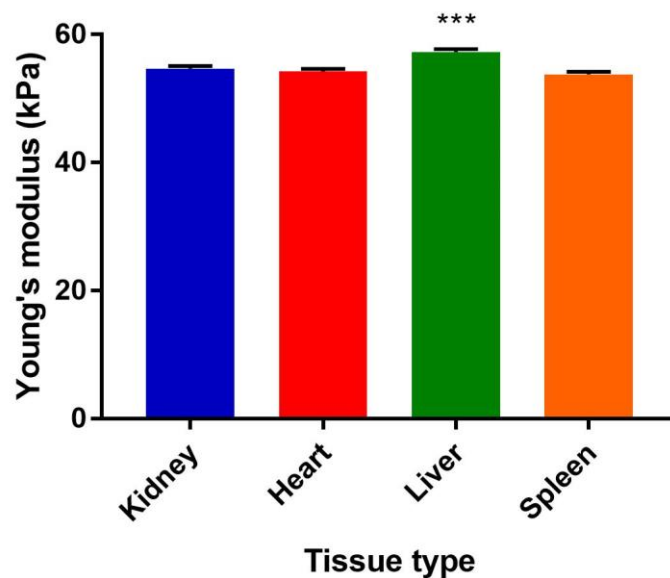


Figure 2.1. Comparison of Young's moduli associated with deparaffinized tissues.

Elastic moduli are in the 40 kPa-60 kPa region and show little variance.

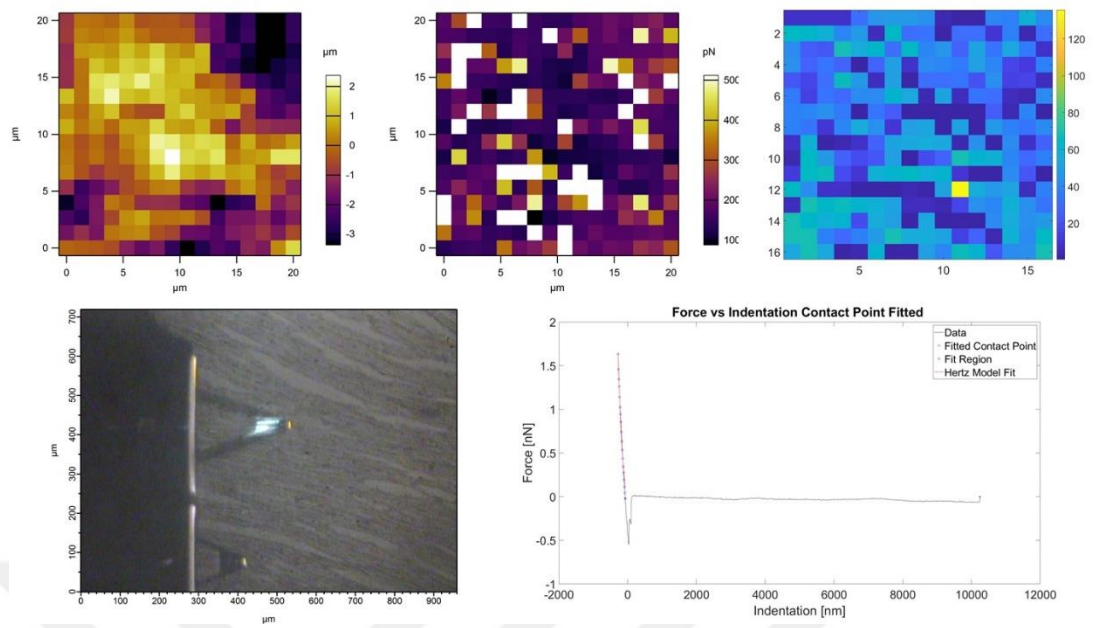


Figure 2.2. Representative height, adhesion, elastic modulus maps, appearance under light microscopy, and a sample force-distance curve for deparaffinized heart tissue.

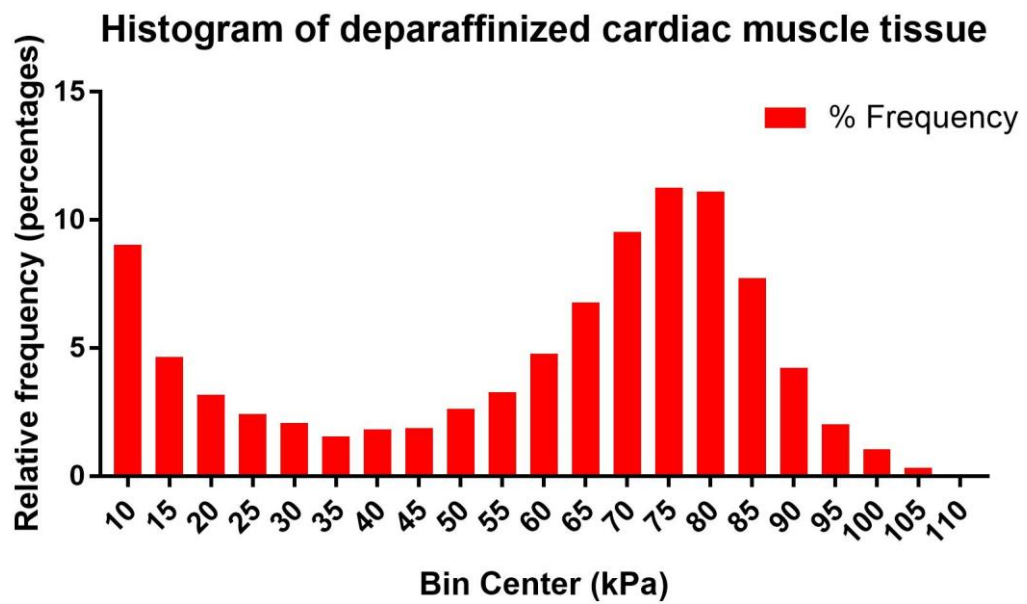


Figure 2.3. Young's modulus distribution associated with deparaffinized heart tissue. The small peak around 10 kPa is notable and potentially represents tissue remnants that were unaffected by the fixation process.

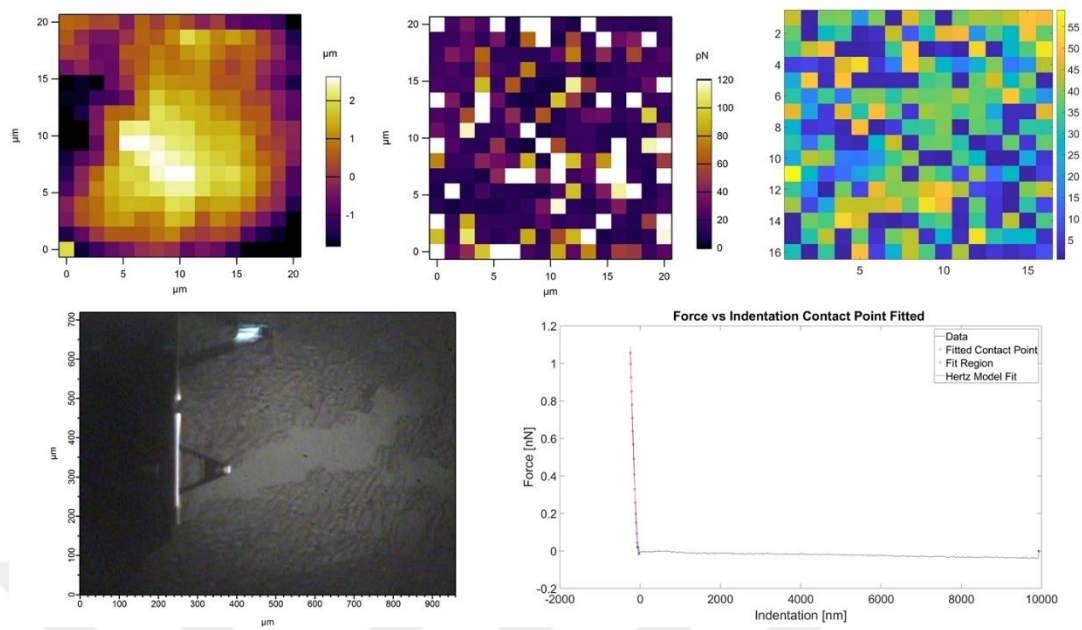


Figure 2.4. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for deparaffinized kidney tissue.

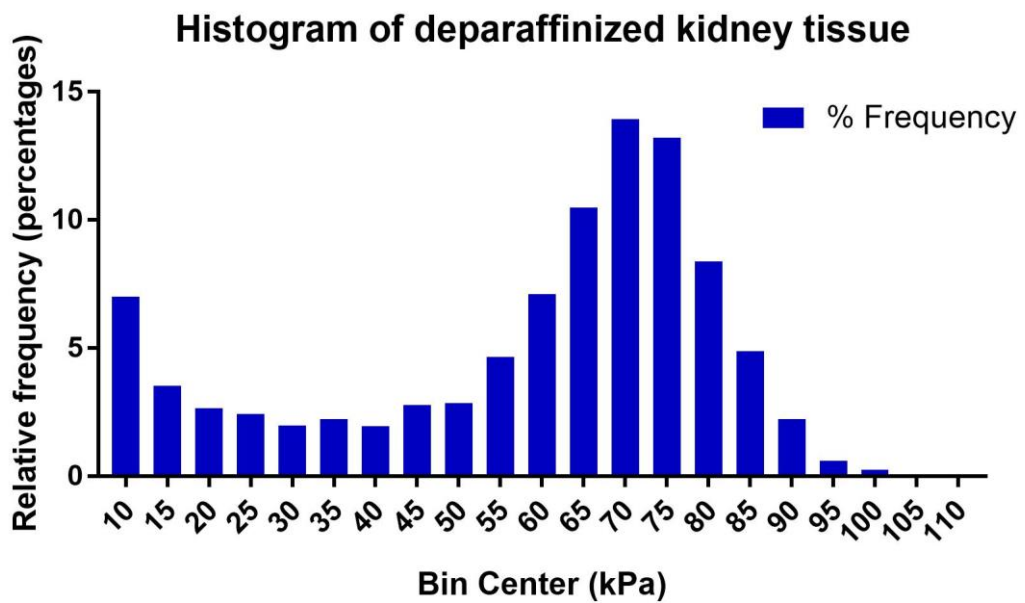


Figure 2.5. Young's modulus distribution of deparaffinized kidney tissue.

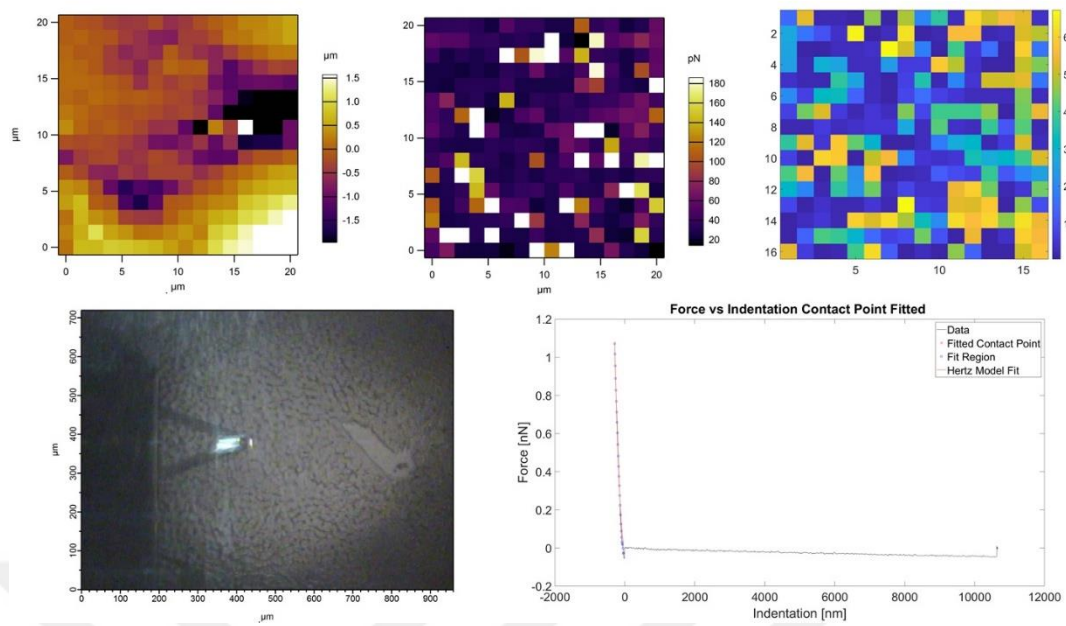


Figure 2.6. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for deparaffinized liver tissue.

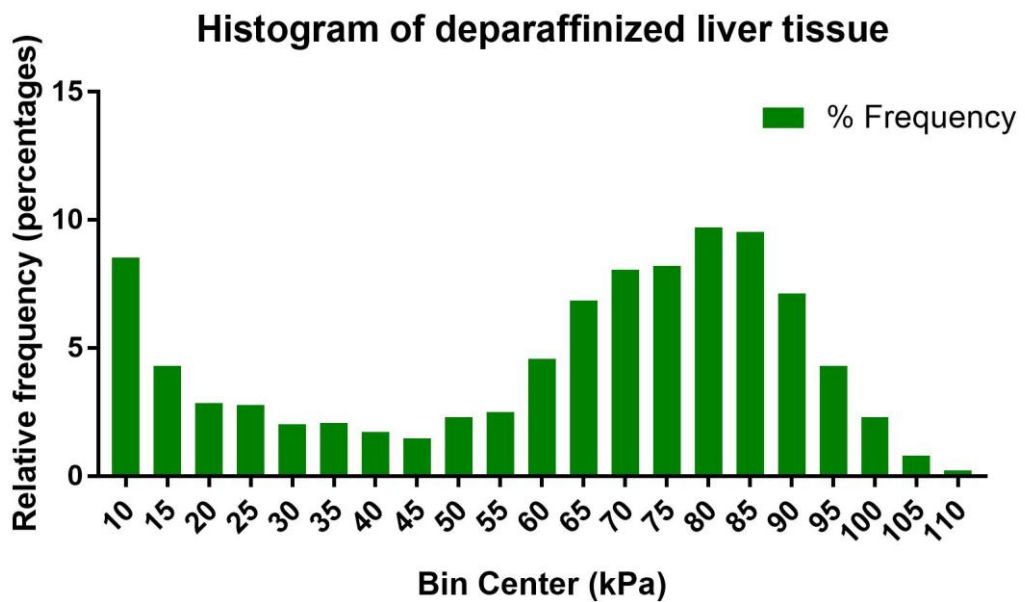


Figure 2.7. Young's modulus distribution associated with deparaffinized liver tissue.

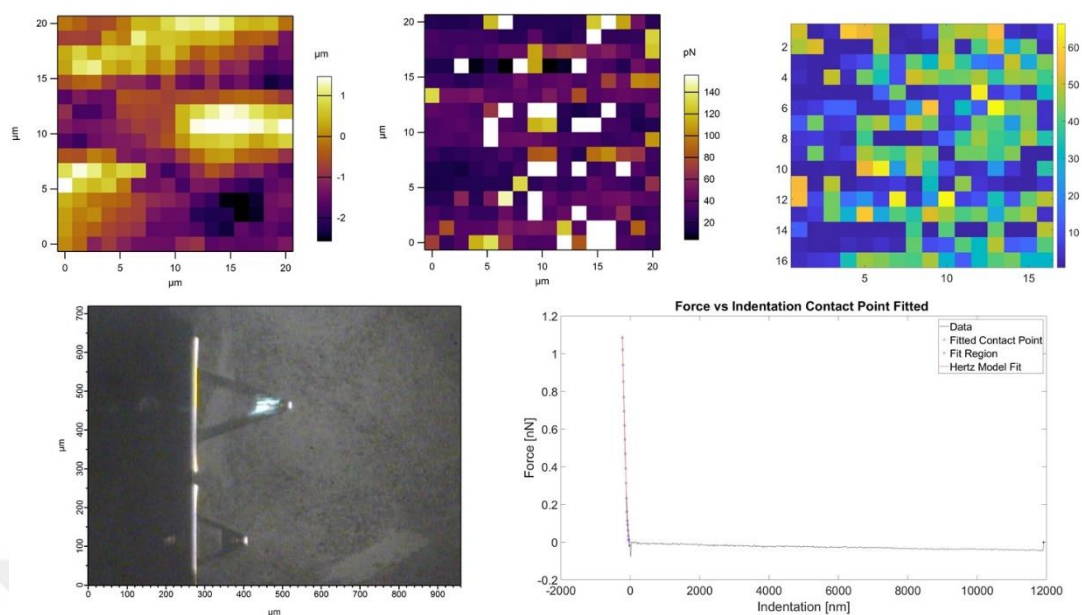


Figure 2.8. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for deparaffinized spleen tissue.

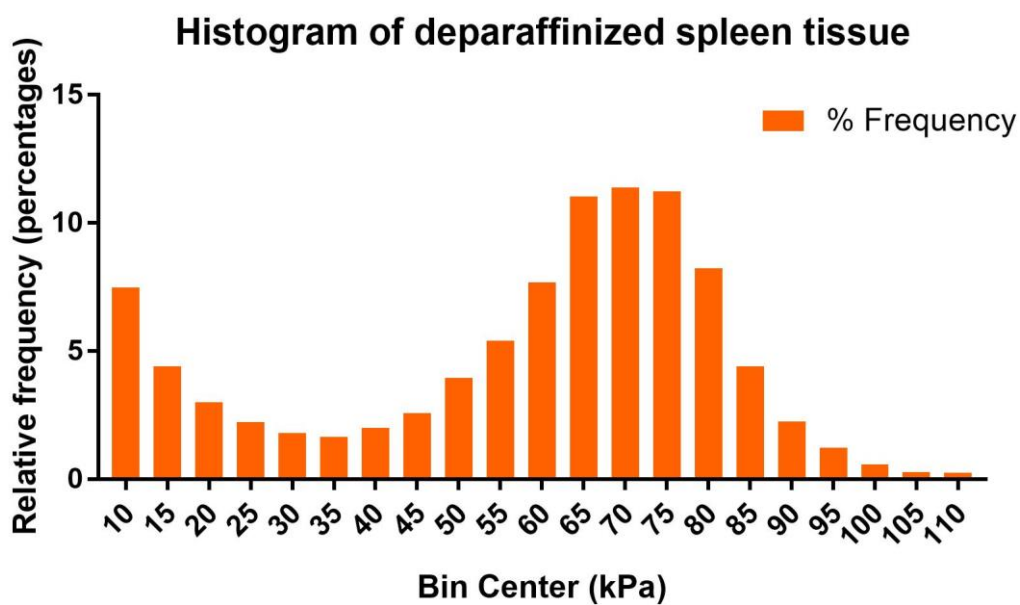


Figure 2.9. Young's modulus distribution associated with deparaffinized spleen tissue.

2.2.2. AFM analysis of cryosectioned mouse tissues

Unlike deparaffinized samples, significant differences are apparent between individual sample types for cryosectioning, which is a relatively non-invasive method and does not involve the use of strong fixatives. It is notable that heart tissue exhibits a considerably higher elastic modulus (4.46 ± 0.07 kPa) than the other sample types tested (1.36 ± 0.02 , 1.32 ± 0.02 and 1.62 ± 0.03 kPa for kidney, liver and spleen tissues, respectively), which is consistent with the requirement of heart tissue to constantly endure strong mechanical stresses (Figure 2.10). In contrast, there was no statistically significant differences between kidney and liver samples, and only small differences between them and spleen, as these tissues are not ordinarily exposed to external forces.

Elasticity comparison of cryosectioned tissues

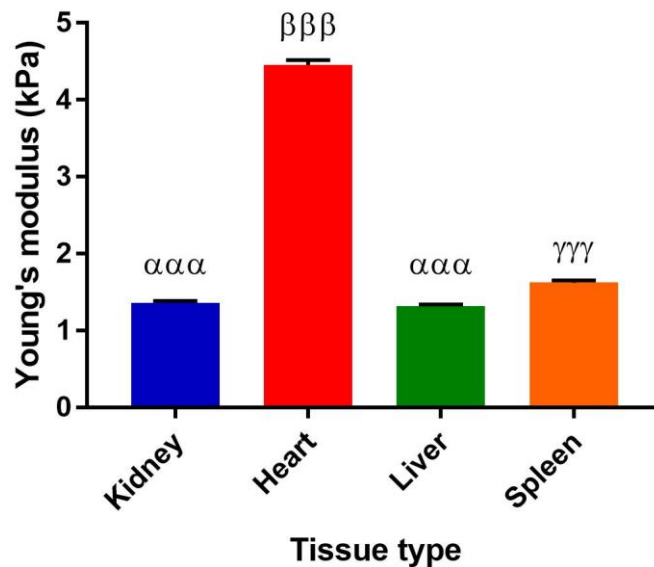


Figure 2.10. Comparison of Young's moduli associated with cryosectioned tissues. Elastic moduli are in the 1 kPa – 5 kPa range, with heart tissue exhibiting significantly higher stiffness.

Cryosectioning was also used to determine the effect of different sample types on calculated elastic moduli. In particular, one set of samples ($n = 9$ maps) was analyzed using a deflection threshold of 0.5 V, and elastic moduli associated with that set were found to be consistently lower than these obtained for a deflection threshold of 1 V. This effect is attributable to the fact that the tip indents the sample to a greater extent under the 1 V threshold, creating a steeper curve that is fit to a relatively higher elastic modulus. In contrast, the “truncated” curve associated with the 0.5 V threshold would be fit to lower E values. However, it is also worth noting that bending-induced fluctuations and drifts experienced by the cantilever can affect deflection values and introduce a factor of uncertainty to these deflection threshold-based measurements.

Cryosectioned tissues measured at 1 V and 0.5 V thresholds

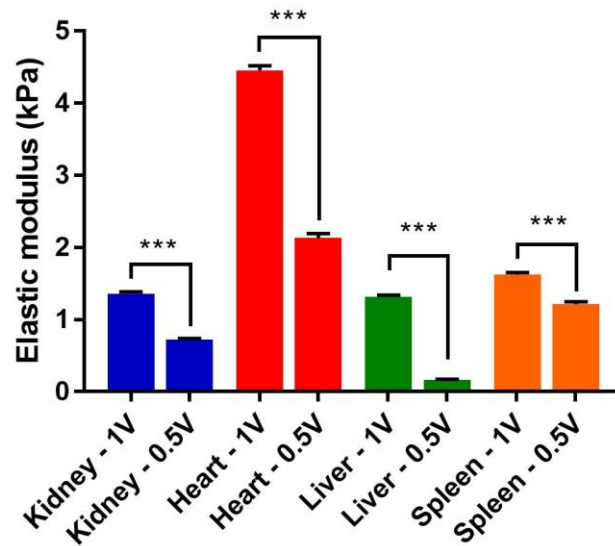


Figure 2.11. Comparison of Young’s moduli associated with cryosectioned tissues under different deflection thresholds. Tissues measured at 0.5 threshold consistently exhibit lower calculated Young’s moduli (0.72 ± 0.01 , 2.13 ± 0.06 , 0.17 ± 0.01 and 1.21 ± 0.03 for kidney, heart, liver and spleen tissues, respectively).

In a similar manner, elastic moduli associated with 11-day old mice were measured using cryosectioning as the sample preparation method, and every tissue of these mice were found to be softer than their adult counterparts ($E = 0.26 \pm 0.003$, 0.17 ± 0.002 , 0.14 ± 0.002 and 0.18 ± 0.003 kPa for kidney, heart, liver and spleen tissues). This observation is also in agreement with the literature, as tissues of fetuses and infants lack mature ECM deposition and crosslinking, which contribute strongly to the elastic modulus of tissues. Similar effects have also been recorded for aging and disease states, which are also characterized by altered secretion of ECM components^{79, 88}. While Figure 2.12. only compared P11 and adult samples on a per-tissue basis, it's also worth noting that all results between individual tissues for P11 samples are statistically significant. This result suggests that, unlike paraffinized samples, P11 tissues retained their unique mechanical characteristics under cryosectioning.

Cryosectioned tissues of adult and p11 mice

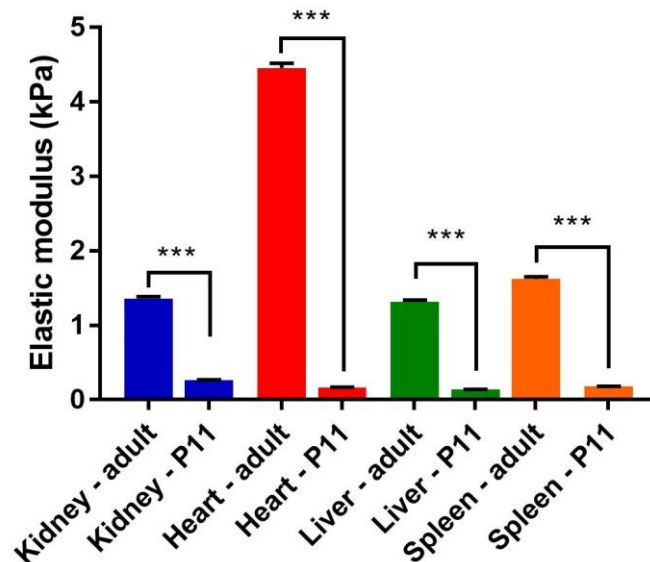


Figure 2.12. Comparison of Young's moduli associated with cryosectioned tissues of adult and newborn (day 11) mice.

Lastly, elastic moduli associated with perfused kidney, heart, lung and spleen tissues have been compared with their non-perfused counterparts and were found to be consistently higher ($E = 7.98 \pm 0.16$, 11.66 ± 0.21 , 7.35 ± 0.15 and 10.5 ± 0.16 for perfused kidney, heart, lung and spleen tissues), suggesting that even short periods of fixation are sufficient to change the Young's modulus of tissues (Figure 2.13). In addition, heart and lung tissues exhibited higher elastic moduli than other perfused and non-perfused tissues, which is likely because of their proximity to the fixative agent used in the perfusion process (which is administered directly to the heart). However, elastic moduli of perfused tissues were still much lower than paraffin-embedded tissues, suggesting that fixation length is still important in determining tissue stiffness.

Elasticity comparison of perfused and non-perfused tissues

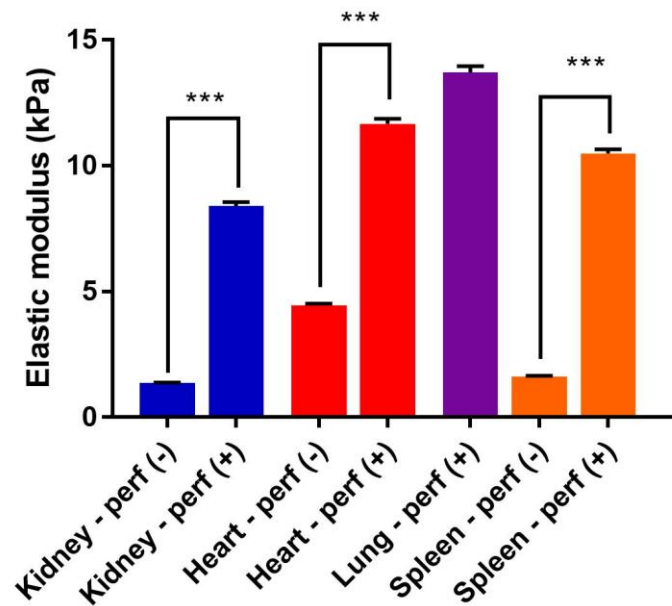


Figure 2.13. Comparison of Young's moduli associated with cryosectioned tissues with and without perfusion.

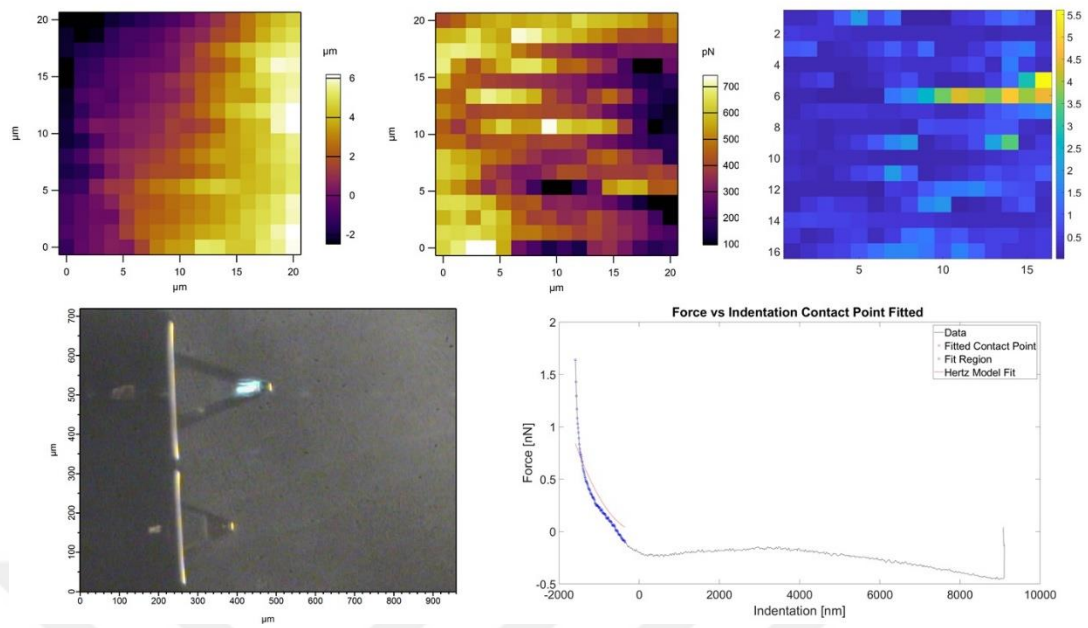


Figure 2.14. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for cryosectioned kidney tissue.

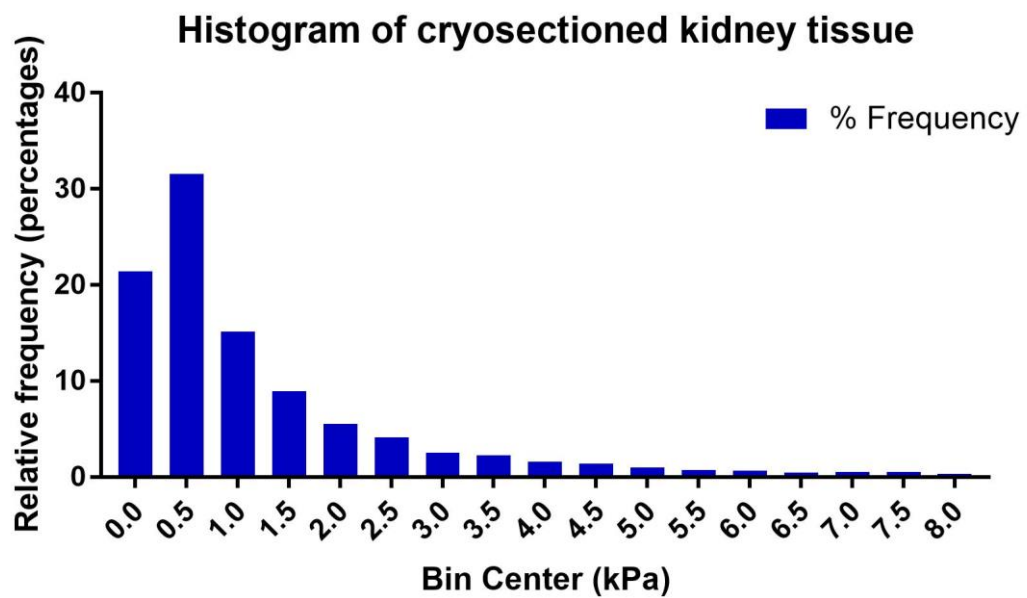


Figure 2.15. Young's modulus distribution associated with cryosectioned kidney tissue.

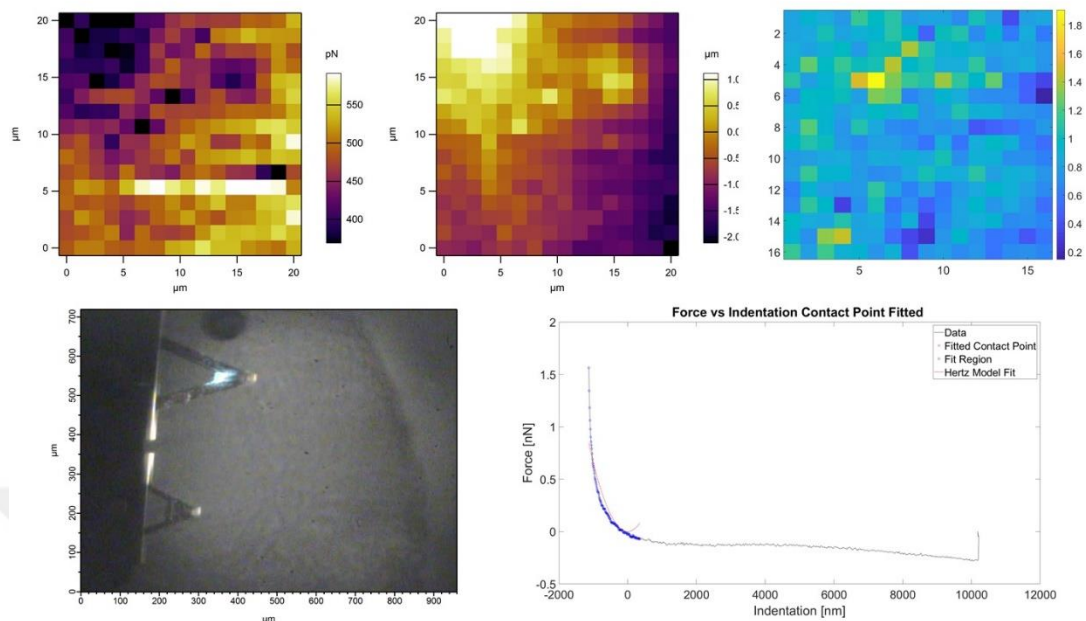


Figure 2.16. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for cryosectioned heart tissue.

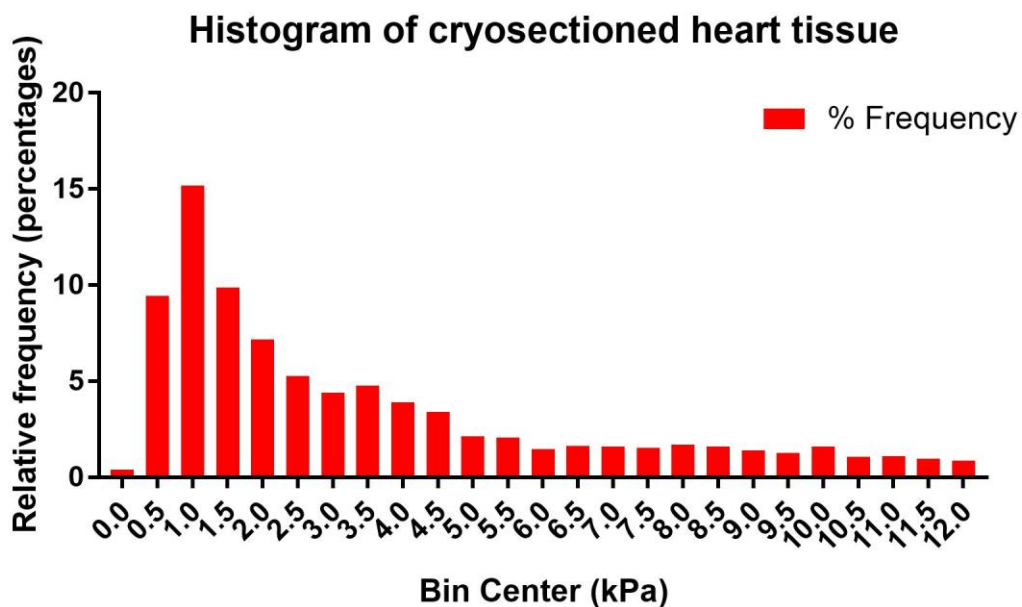


Figure 2.17. Young's modulus distribution associated with cryosectioned heart tissue.

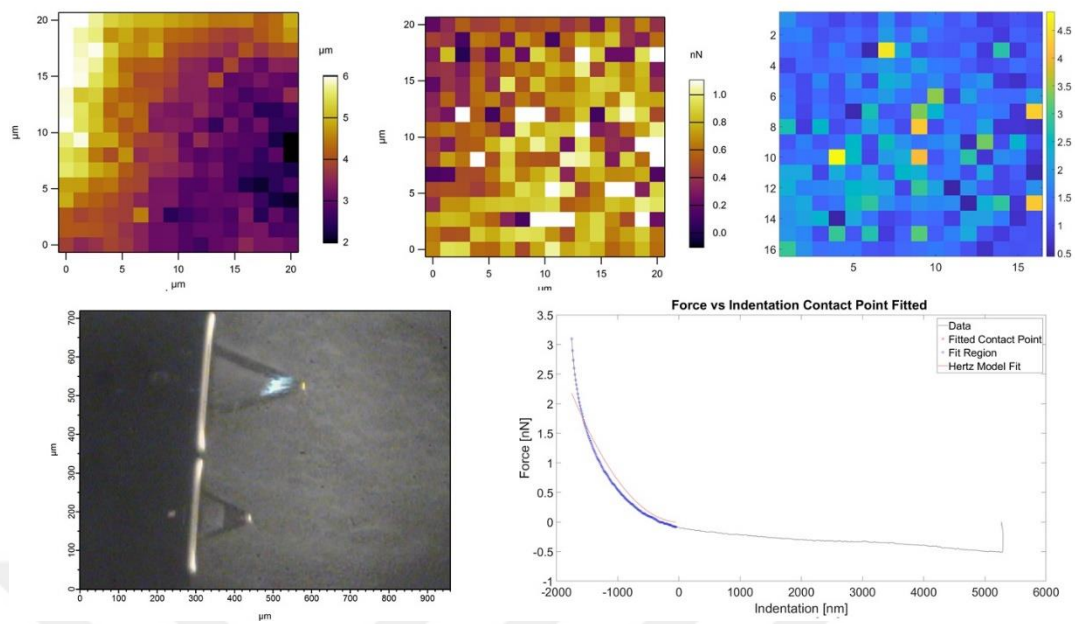


Figure 2.18. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for cryosectioned liver tissue.

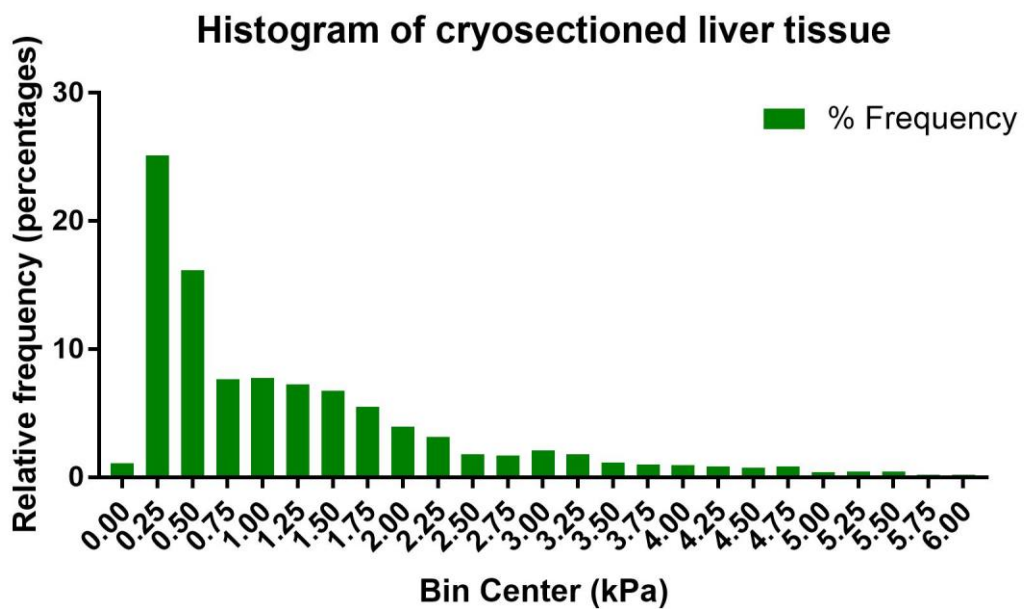


Figure 2.19. Young's modulus distribution associated with cryosectioned liver tissue.

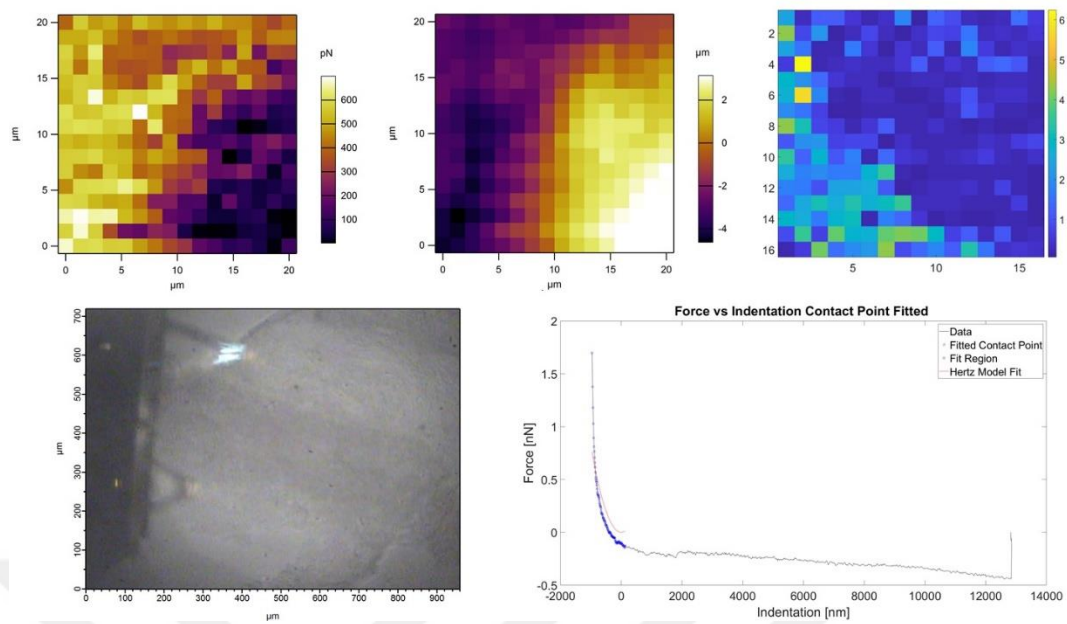


Figure 2.20. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for cryosectioned spleen tissue.

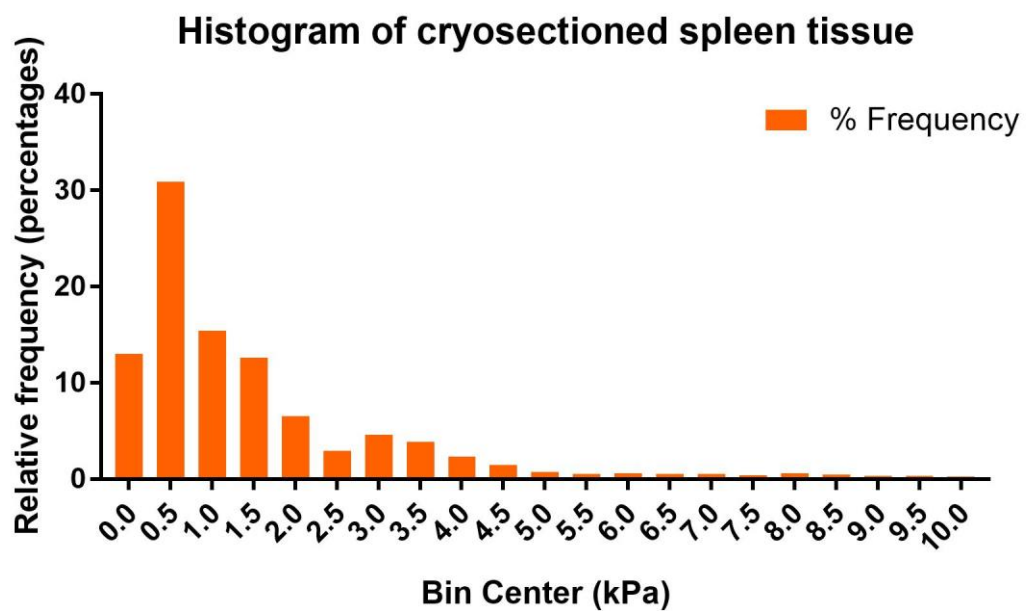


Figure 2.21. Young's modulus distribution associated with cryosectioned spleen tissue.

2.2.3. AFM analysis of agarose-embedded mouse tissues

Average Young's moduli associated with agarose-embedded kidney and liver tissues are provided in Figure 2.22. While heart and spleen could not be measured due to these tissues resisting the vibratome blade, agarose-embedding is the least invasive technique used in the present study and can be assumed to accurately represent the native elastic modulus of tissues. Kidney and liver results show that the elastic moduli of agarose-embedded tissues are even lower than cryosectioned samples, suggesting that the tissues may have been infiltrated by O.C.T. medium or affected by the freezing process during cryosectioning. However, differences between agarose-embedding and cryosectioning were not statistically significant for either tissue (see Figure 2.27).

Elasticity comparison of agarose-embedded tissues

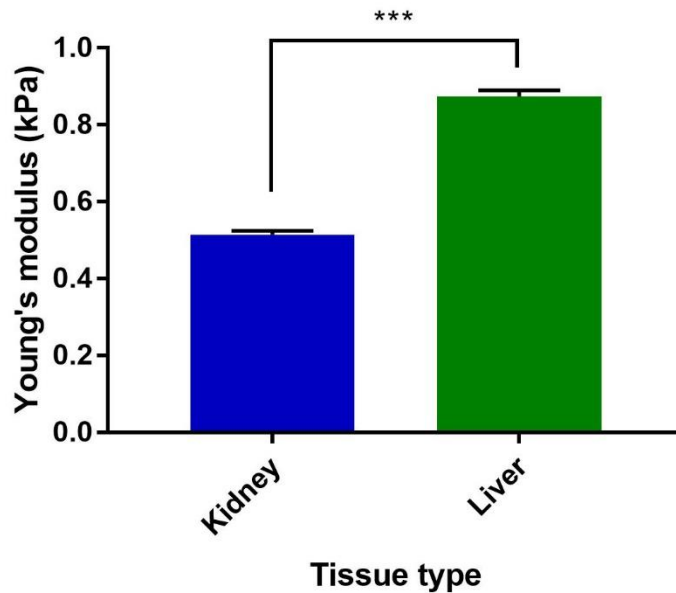


Figure 2.22. Comparison of Young's moduli associated with agarose-embedded tissues.

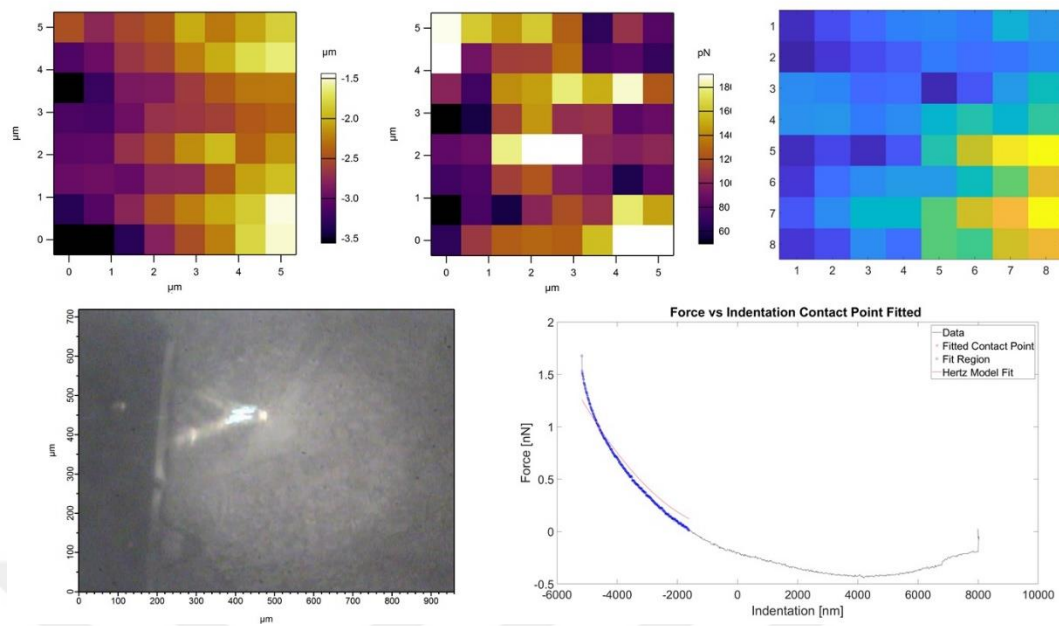


Figure 2.23. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for agarose-embedded kidney tissue.

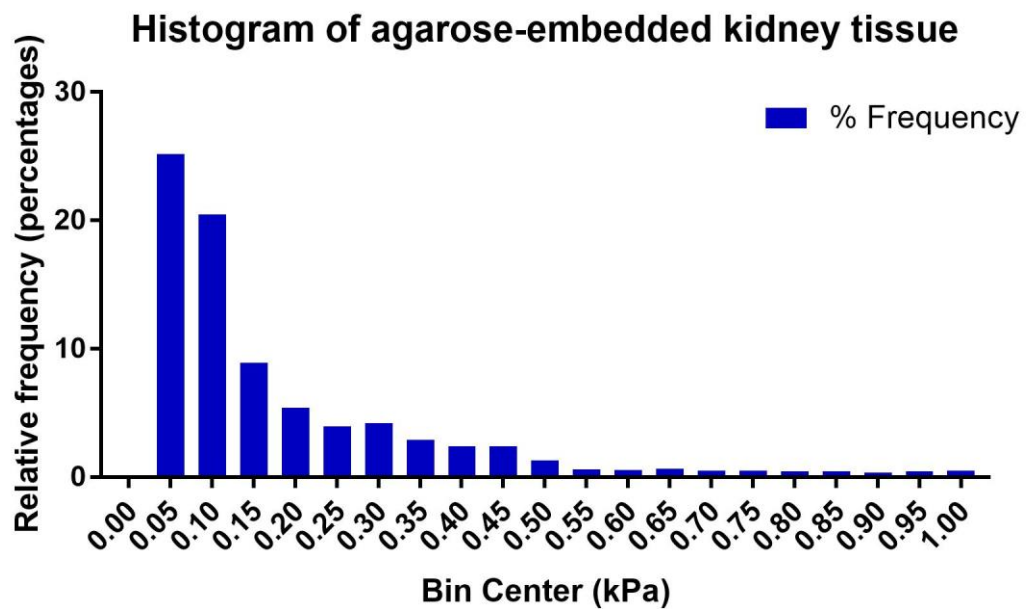


Figure 2.24. Young's modulus distribution associated with agarose-embedded kidney tissue.

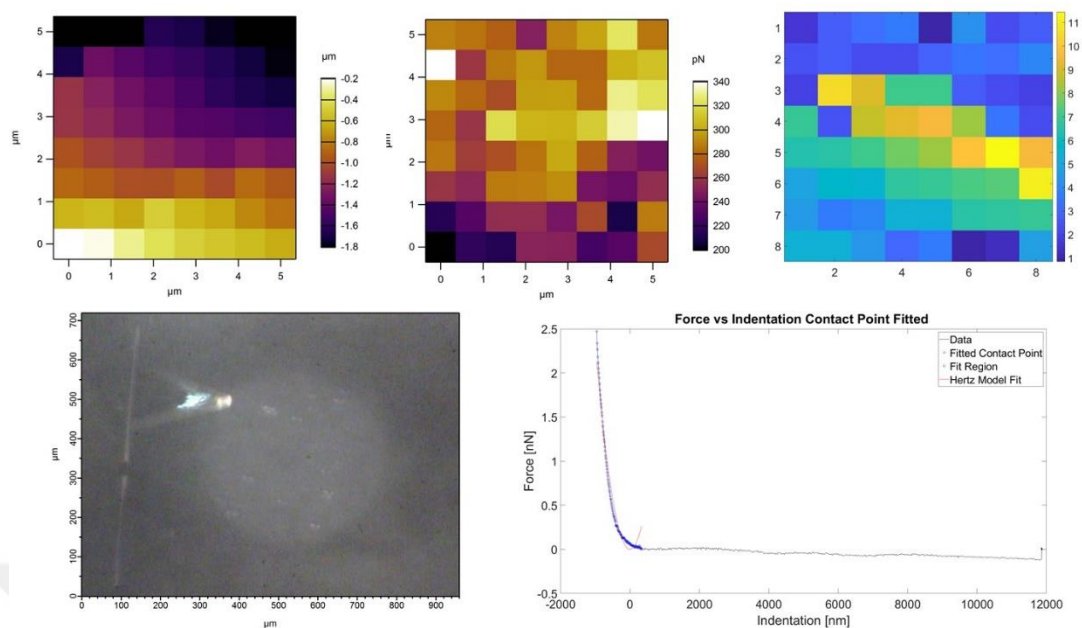


Figure 2.25. Representative height, adhesion, elastic modulus maps, general appearance under light microscopy, and a sample force-distance curve for agarose-embedded liver tissue.

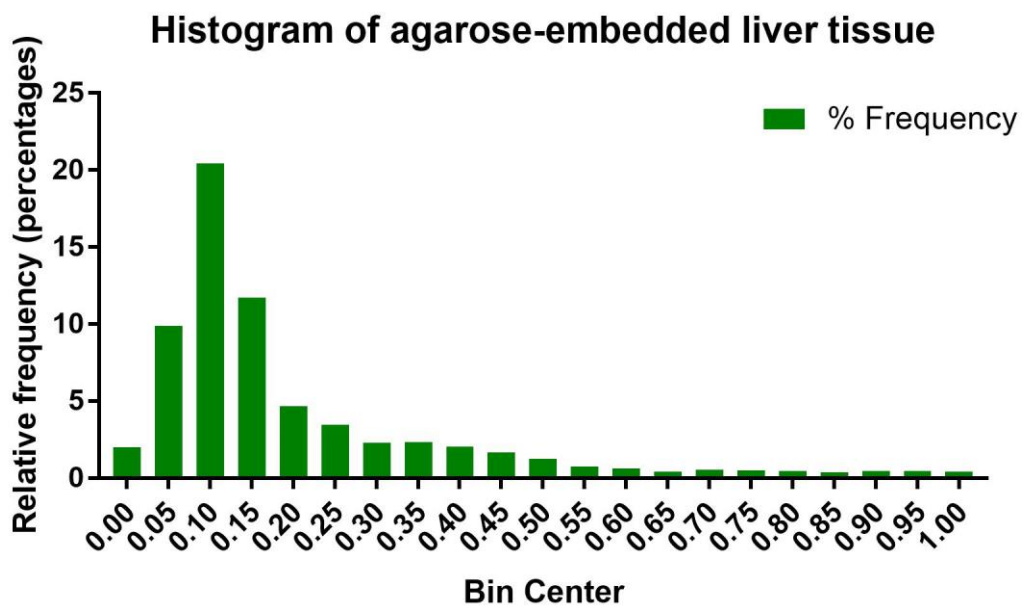


Figure 2.26. Young's modulus distribution associated with agarose-embedded liver tissue.

2.3. DISCUSSION

A broad overview of all samples and fixation methods tested in the present study is provided in Figure 2.27. It is readily apparent that deparaffinization greatly increases the elastic modulus of tissues, while there is no statistically significant difference between cryosectioning and agarose-embedding. The latter method is closely representative of the native elastic modulus of tissues, but also requires very stringent measurement conditions that make it impractical for routine application, as tissues prepared using this method are not suitable for storage of any kind and will quickly decay. Consequently, the ability of cryosectioning to yield elastic moduli similar to agarose-embedded samples while providing an easy means of storage at -80 °C or -20 °C is invaluable for the AFM analysis of biomaterials. However, AFM studies must also consider that the method of animal euthanization used for sample gathering can also alter the elastic modulus of tissues, as perfused samples were shown to exhibit a different stiffness profile compared to non-perfused tissues (Figure 2.13). Furthermore, the configuration of the AFM itself can also alter the results, as evidenced by the fact that lower deflection thresholds result in the calculation of lower elastic moduli in otherwise identical tissue samples (Figure 2.11). Therefore, the present study underlines the fact that every aspect of sample preparation and measurement can change AFM results of biological materials; consequently, it is imperative that all environmental, equipment-based and fixative processes are fully detailed in any AFM study on tissues and other biological samples. Unfortunately, existing reports often omit these details, which is a principal reason for the major and unresolvable discrepancies between AFM results in the literature.

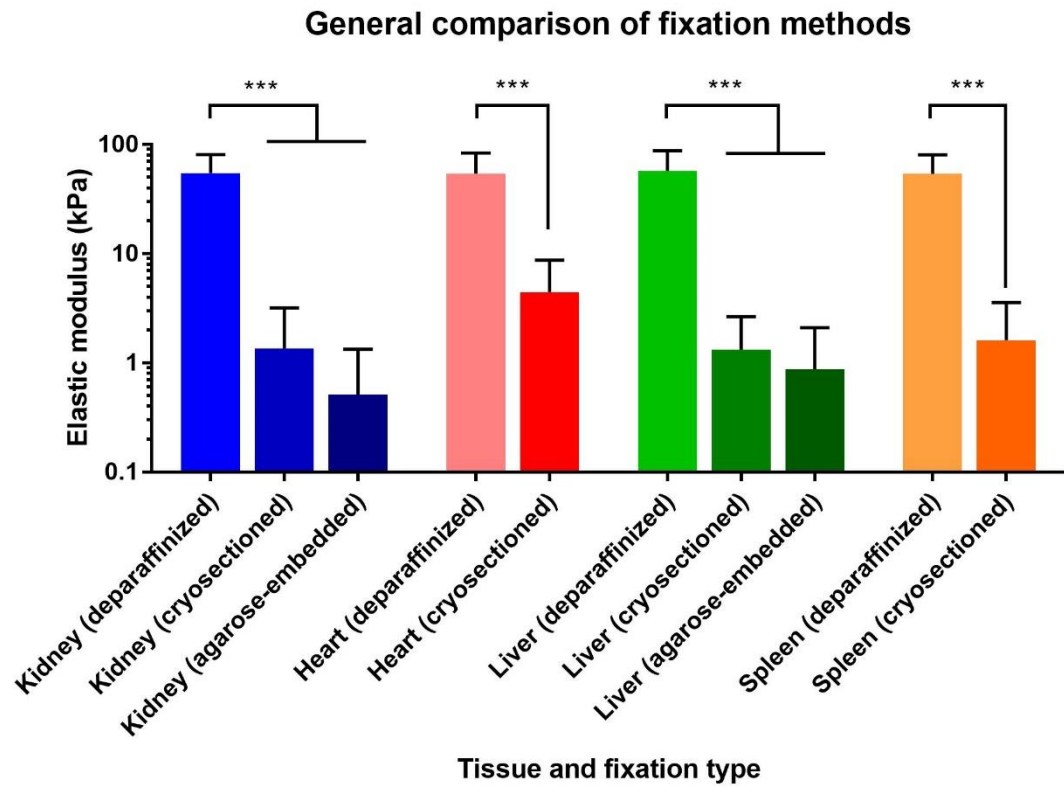


Figure 2.27. Comparison of Young's moduli associated with all tissues under all sample preparation methods tested. Error bars describe standard error rather than the standard error of mean to better highlight significances between each sample.

Nevertheless, elasticity studies of both human and murine tissues are common in the literature and employ both AFM and other mechanical characterization tools. Diagnosis of liver fibrosis, for example, is possible through transient elastography, a technique that uses ultrasound to determine the elastic modulus of intact liver tissue in a non-invasive manner. Even the grade of fibrosis can be assessed through this method, as fibrotic livers with higher METAVIR scores have been found to exhibit higher elastic moduli in one study (with 7.1 kPa, 9.5 kPa and 12.5 kPa identified as critical cut-off points for fibroses with METAVIR scores over F2, F3 and F4, respectively)¹⁷⁶.

Similarly, Wells *et al.* used rheometry analysis to show that the shear modulus of rat liver tissue is below 1 kPa in healthy animals but rises to the 3 kPa – 22 kPa range under fibrosis as a result of increased matrix deposition and cross-linking^{177, 178}. It is notable that, despite using different measurement methods, these results are broadly similar to the present study, which identifies the elastic modulus of cryosectioned mouse liver tissue as 1.32 ± 0.02 kPa and agarose-embedded liver tissue as 0.87 ± 0.02 kPa (Figures 2.10 and 2.22). Cardiac tissue, however, is generally reported to exhibit a higher elastic modulus than found in the present analysis, with Berry *et al.* reporting 18 ± 2 kPa from healthy ventricular myocardium¹⁷³ (as compared with 4.46 ± 0.07 kPa for heart tissue in Figure 2.10). However, that result was acquired from intact heart tissue without microtomy, while the value in the present study is derived from cryosectioned samples. In addition, results associated with heart tissue were higher than those observed for other, softer tissues such as liver, spleen and kidney, which is also consistent with the literature.

2.4. CONCLUSIONS

Elastic modulus measurements are known to vary considerably depending on the method used; for example, differences of several orders of magnitude are common between indentation and stretching experiments in a range of tissues¹⁷⁹. This study further demonstrates that even a single, well-established method can yield greatly different Young's moduli depending on the method of sample preparation. Deparaffinized tissues, despite their ease of preparation and storage, were found to be of little use for AFM measurements, producing similar elastic moduli due to the effect

of crosslinking. In contrast, agarose-embedded samples had the lowest Young's moduli and presumably represented the true elasticity of native tissue architectures to the greatest extent; however, their lack of long-term preservation greatly limited their practical use. Cryosectioned tissues serve as an intermediate between these two methods, allowing easy storage while providing elastic modulus values that are similar to agarose-embedded tissues and can be used to distinguish between individual tissue types. Nevertheless, measurement conditions such as deflection threshold are also observed to impart considerable changes on calculated Young's modulus values. Consequently, the present work suggests that AFM results in the literature should only be taken as valid with respect to their particular set of environmental and device-based variables, which should be reported in full detail for any AFM study to be considered reliable and repeatable.

CHAPTER 3

All-Atom Molecular Dynamics Simulation of the Dependence of Peptide Assembly and Thermal Stability on Counterion Identity

This chapter has been modified from the following publication:

Ozkan AD, Tekinay AB, Guler MO, Tekin ED (2016) Effects of temperature, pH and counterions on the stability of peptide amphiphile nanofiber structures. *RSC Advances* 6: 104201-104214.

3.1. INTRODUCTION

Peptide amphiphiles (PAs) are biomolecules that are commonly used in therapeutic applications due to their capacity for self-assembly, which is facilitated by non-covalent interactions between individual PA units and allows the formation of high-aspect ratio nanofibers. Hydrogel scaffolds formed by PA assemblies show great potential in tissue engineering and regenerative medicine¹⁸⁰⁻¹⁸² due to their biofunctionality, biocompatibility and biodegradability¹⁸³⁻¹⁸⁵. While the self-assembly mechanism is based on noncovalent interactions, it is also responsive to environmental factors such as temperature, pH and the presence of enzymes¹⁸⁶⁻¹⁹¹. The effect of temperature on the self-assembly mechanism of PA molecules has been studied extensively in the literature¹⁹²⁻¹⁹⁴. Miravet *et al.*, for example, reported that the palmitoyl-KTTKS sequence transitions from nanotapes to micelles at temperatures over 293 K, with the exact transition temperature changing with the concentration. Similarly, Hamley *et al.*¹⁹⁵ observed a reversible thermal transition in the self-assembly mechanism of the palmitoyl-KKFFVLK sequence, while Niece *et al.*¹⁹⁶ detailed the specifics of nanofiber formation over a wide range of pH values on both positively-charged and negatively-charged PA molecules. Accordingly, negatively-charged PAs were found to preferentially self-assemble at lower pH values, while positively-charged PAs assemble at higher pH, and the mixture of the two PA types is able to self-assemble at neutral pH. Toksoz *et al.*¹⁹⁷ also studied the self-assembly of positively, negatively and neutrally charged PAs under different environmental conditions, while Chen *et al.*¹⁹⁸ showed the importance of the amino acid sequence in assembly morphology using PAs containing repeating arginine/aspartic acid units.

Ghosh *et al.*¹⁹⁹ synthesized six different PA molecules that exist as either isolated PAs or spherical micelles at pH 7.4 and self-assemble into fibers at pH 6.6, which emulates the acidic extracellular environment of tumor tissue. Dehsorkhi *et al.*²⁰⁰ have studied the effects of pH change (at pH 2, 3, 4 and 7) on the self-assembly of palmitoyl-KTTKS, and observed that PA assemblies alter their morphology from tapes to twisted fibers, and again to tapes, and finally to spherical micelles under gradually decreasing pH values. Deng *et al.*²⁰¹ designed a PA molecule by covalently attaching dodecanoic acid to an A β fragment to study the pH-dependent conformational changes that occur in amyloid assemblies, and found that tape-like nanofibrils and nanoribbons are the main forms at pH 3 and 10, respectively. Guo *et al.*²⁰² showed that lauryl-GAGAGAGY molecules, the structural motif of which was derived from silk fibroin, exhibited pH-sensitive assembly: When the value of pH decreased from 11 to 8, the morphology of self-assembled nanostructures changed from nanofibers to nanoribbons. Lastly, Dagdas *et al.*²⁰³ comprehensively studied the mechanical properties of self-assembled lauryl-VVAGERGD molecules by altering the temperature and pH. Their PA nanofibers were formed by Na⁺-dependent self-assembly at pH 7, Ca⁺²-dependent self-assembly at pH 7 and H⁺-dependent self-assembly at pH 2. These assemblies generally displayed similar morphologies under SEM, TEM and AFM, as well as similar chemical properties under FT-IR and room-temperature circular dichroism (CD) spectroscopy, although a small difference was observed in CD between calcium- and hydrogen-dependent assembly conditions, and more marked differences were observed at elevated temperatures under oscillatory rheology.

Experimental results have shown that tuning the self-assembly of the PA molecules by changing the environmental variables such as temperature and pH greatly affects the morphology of the resulting aggregate nanostructures. Even though the role of environmental variables on the morphological properties of resulting nanostructures has been investigated experimentally, theoretical investigations in that context have so far been limited. In particular, while simulation studies have been performed to investigate the effect of temperature on self-assembly, the effect of pH is seldom considered due to the inherent limitations of standard explicit-solvent molecular dynamics (MD) methods, which involve constant-proton algorithms that cannot simulate lower or higher pH values. Nevertheless, Cote *et al.*²⁰⁴ used the multiscale MD simulations to investigate the pH-dependent self-assembly of palmitoyl-IAAAEEEE based fibers. Coarse-grained discontinuous MD was used for kinetic details, and all-atom, constant-pH molecular dynamics (CpHMD) simulations were used for thermodynamical features. In CpHMD simulations, alkyl tails were excluded and the initial structure was prepared as a tetramer by placing four PA molecules in a parallel β -sheet conformation with protonated Glu residues. After 100 ns of standard all-atom MD with fixed protonation states, the final structure was taken as an input for CpHMD simulations. To quantitatively predict pH-dependent conformational changes, the total number of backbone hydrogen bonds was calculated as a function of pH and the transition between random coil and the β -sheet structures was observed in the range of pH 6-7. Fu *et al.*^{205, 206} observed a large number of different nanostructures through the self-assembly of a model PA, palmitoyl-VVVAAEEEE, by changing the temperature (ranging from T=260 K to T=550 K) under a discontinuous MD simulation algorithm. In a previous study by this group²⁰⁶, the self-

assembly of this PA sequence was observed to depend on the temperature and electrostatic interactions between individual molecules. Specifically, nanofibers were formed at the temperature range of 376 K – 414 K (moderate region) with weak electrostatic strength, while spherical micelles were observed at the temperature range of 300 K – 338 K (low region) and again with weak electrostatic strength.

Recently²⁰⁷, MD simulations of multiple cylindrical PA nanofibers revealed the structural properties of the nanofibers and the role of molecular interactions on their stability. In particular, the number of the PA molecules on each layer was found to determine the overall stability of the nanofiber. 7-, 9- and 12-layered nanofibers were found to trigger the formation of spherical micellar assemblies, while 13-, 14-, 16- and 19-layered nanofibers supported the formation of nanofibers after 50 ns of simulation. Other nanofibers, consisting of 17, 18, 20 and 21 layers, were unable to support the formation of stable structures and disassembled within 1 ns of simulation time. Both hydrophobic (VVAG) and hydrophilic (ERGD) sections of the model PA were found to play key roles in the assembly process, with VVAG-VVAG, D-Na⁺ and E-R interactions serving to organize the aggregation of the peptide molecules. The 19-layered nanofiber, containing 12 PA molecules per layer²⁰⁸, was found to be the most stable configuration among the simulated structures, and the diameter of nanofiber (8.4 nm) was found to be close in agreement with experimental observations^{184, 209}. For these reasons, the 19-layered system was ultimately determined as the model of interest for future simulation studies.

In this work, we investigated the effects of temperature and charge neutralization with different ions (Na⁺, Ca⁺², Cl⁻) and pH (pH 7 and pH 2) on the stability of preassembled

cylindrical PA nanofibers. Lauryl-VVAGERGD was chosen as the model molecule. This sequence contains the bioactive RGD peptide motif, which has been shown to support cellular adhesion and proliferation *in vitro*, and of which thermal behavior was investigated experimentally²⁰³. Na⁺ and Ca⁺² mediated assemblies were modelled at pH 7, as is the assumed standard of united-atom MD, while the effects of pH on peptide aggregation behavior was investigated through the inclusion of additional H⁺ ions within the initial PA structure. As noted above, this path was taken because standard explicit solvent MD simulations are constant-proton algorithms and cannot directly simulate molecular assembly at different pH values (simulation details are provided in the Methods section). In addition, PA nanofiber behavior was not investigated under basic pH, as negatively-charged PA structures do not self-assemble under these conditions, which lack the requisite counterions.

3.2. MATERIALS & METHODS

3.2.1. Simulation details

Lauryl-VVAGERGD nanofibers were formed under three different formulations; PA molecules at pH 7 with Na⁺ ions (PA1); PA molecules at pH 7 with Ca⁺² ions (PA2); and PA molecules at pH 2 with Cl⁻ ions (PA3) (Figure 3.1). In PA1 and PA2, the total net charge is -2, with charged residues being Glu (E) = -1, Arg (R) = +1, Asp (D) = -2 (including the end-chain COO⁻ = -1). As a result, the excess of negative charge on the cylindrical nanofiber was neutralized by adding a sufficient number of Na⁺ ions to the PA1-based nanofiber and Ca⁺² to the PA2-based nanofiber. To represent pH 2 in PA3, Glu (E) and Asp (D) side chains were protonated with Glu (E) = 0, Arg (R) = +1 and

Asp (D) = 0, so that the total net charge of the PA3 molecule was +1. A sufficient number of Cl⁻ ions were added to the PA3-based nanofiber to neutralize the system. Each structure was contained in a rhombic dodecahedron box of SPC type water molecules²¹⁰, and energy minimization was performed on the solvated-electroneutral systems using a steepest-descent algorithm to get suitable starting conformations. Each system was equilibrated with NVT and NPT (100 *ps* for each) prior to a 70 *ns* MD production run with GROMACS 4.5.6²¹¹. GROMOS 53a6²¹² force field combined with Berger lipid parameters²¹³ was used to represent the PA molecules. Initial velocities were assigned from a Maxwell–Boltzmann distribution. To obtain the trajectories required for reconstructing the time evolution of PA nanofibers, Newton’s equation of motion was solved numerically using the leapfrog algorithm with a 2 *fs* integration time-step. The linear constraint solver (LINCS) algorithm²¹⁴ was applied to all hydrogen bonds. The Particle Mesh Ewald (PME) method²¹⁵ was used in calculating electrostatic interactions and a 1.0 *nm* cut-off was set for the calculation of the van der Waals interactions. Simulations were performed with periodic boundary conditions in all directions at constant temperature and pressure. Various static temperatures were simulated using a velocity rescaling thermostat²¹⁶ with two coupling groups (PA and non-PA groups) and with a coupling time constant of 0.1 *ps*. The pressure was maintained at 1 *bar* using an isotropic Parrinello–Rahman barostat²¹⁷ with a coupling time constant of 2.0 *ps*. Coordinates and energies were saved at every 10 *ps* for the trajectory analysis. Snapshots were obtained using visual molecular dynamics (VMD) software²¹⁸.

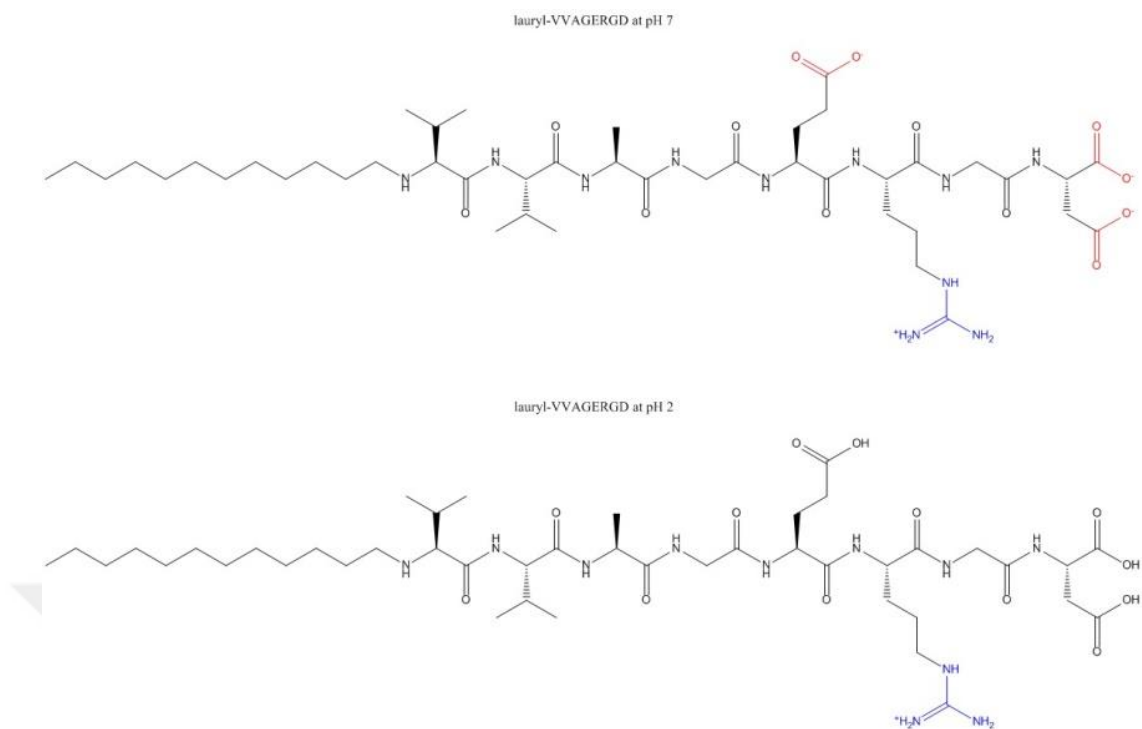


Figure 3.1. Chemical structures of lauryl-VVAGERGD at pH 7 and pH 2. Charged groups at each pH are shown in red (for negative charges) and blue (positive charges).

For all three formulations, MD simulations were carried out at room temperature (300 K) and higher temperatures (310 K (representing body temperature), 318 K, 338 K and 358 K) for 70 ns. Following the analysis of these simulations, two more simulations were carried out to better understand the temperature-dependence of the PA1-based nanofiber. First, the final configuration at the end of the 70 ns simulation at 338 K of the PA1-based cylindrical nanofiber was extracted to heat it up to 358 K. In addition, temperature was raised at every 20 ns steps in a second simulation, from 300 K to 358 K. In each case, self-assembly simulations were started from a configuration in which 228 PA molecules were used to construct cylindrical nanofiber structures as described previously by Tekin.^{207, 208}

3.3. RESULTS

The present work describes an extensive MD study of the temperature-dependent trajectory of PA-based cylindrical nanofibers prepared at pH 7 and pH 2 with different charge-neutralizing ions. Stability of cylindrical nanofibers under PA1, PA2 and PA3 configurations was analyzed at five different temperatures using the RMSD of the backbone atoms by comparing the energy-minimized state of the initial cylindrical nanofiber and the equilibrated system after 70 *ns* (Figure 3.2a-c). For all three fiber structures, RMSD values at 300 K and 318 K were below/around 2 nm and the structures maintained their cylindrical form, indicating their stability under these conditions. PA1 and PA3 nanofibers were also stable at the physiological temperature (310 K), with the exception of a minor, temporary increase in RMSD values (between 39.3 ns and 46.5 ns) for PA3 nanofibers, which was observed not to result in fiber disintegration. The PA2 nanofiber, however, was not stable, as indicated by the increase of its RMSD values over 2.0 nm after 45 ns and strong fluctuations from that point onwards to the end of the simulation. Despite this initial picture, however, unexpected results were observed for simulations associated with high temperatures, showing both temperature-dependent disassembly and eventual reassembly. As shown in Figure 3.2a, although the RMSD of the PA1-based cylindrical nanofiber at 338 K was greater than 2.0 nm after 27 ns, the structure retained its cylindrical form. However, the unstable PA nanofiber eventually started to lose its cylindrical form and split into two after 55 ns (Figure 3.3).

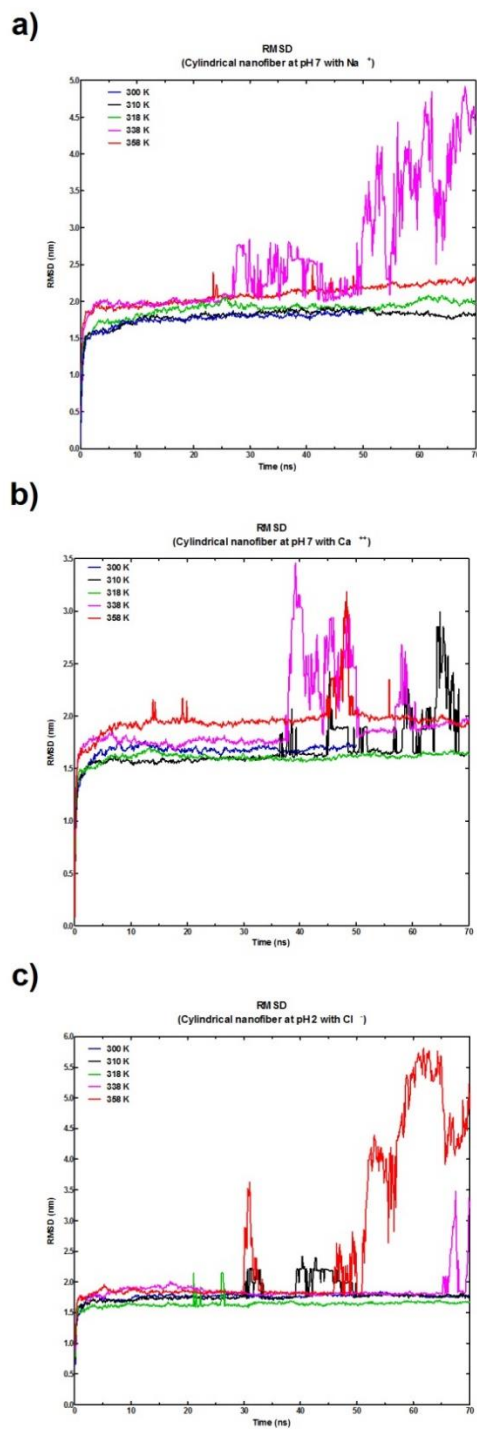


Figure 3.2. RMSD graphs of **a)** PA1, **b)** PA2, and **c)** PA3 nanofibers. RMSD values greater than 2 *nm* are assumed to represent nanofiber disintegration, which typically occurs at 338 *K* or 358 *K*.

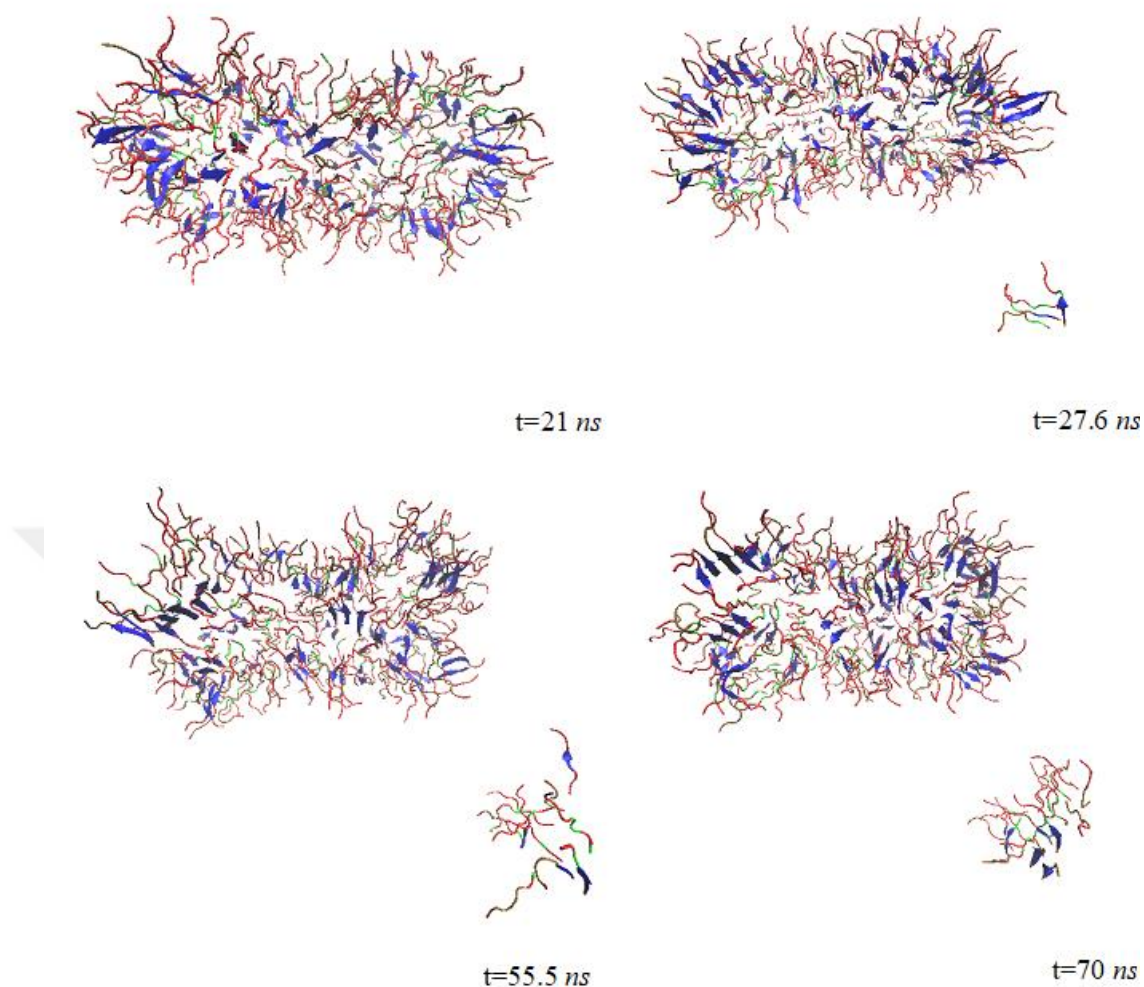


Figure 3.3. Snapshots of PA1-based nanofibers at 338 K. The nanofiber was observed to lose its cohesion after 55 ns, and the peptide structure eventually split into two.

As also shown in Figure 3.2a, the RMSD of the structure at 358 K was slightly above 2 nm after 22 ns and reached its peak values around this time frame. This temporarily increase was associated with the breakoff of a small PA fragment from the cylindrical nanofiber (see Figure 3.4). However, disintegration was not present at the higher temperature of 358 K, unlike the case observed at 338 K.

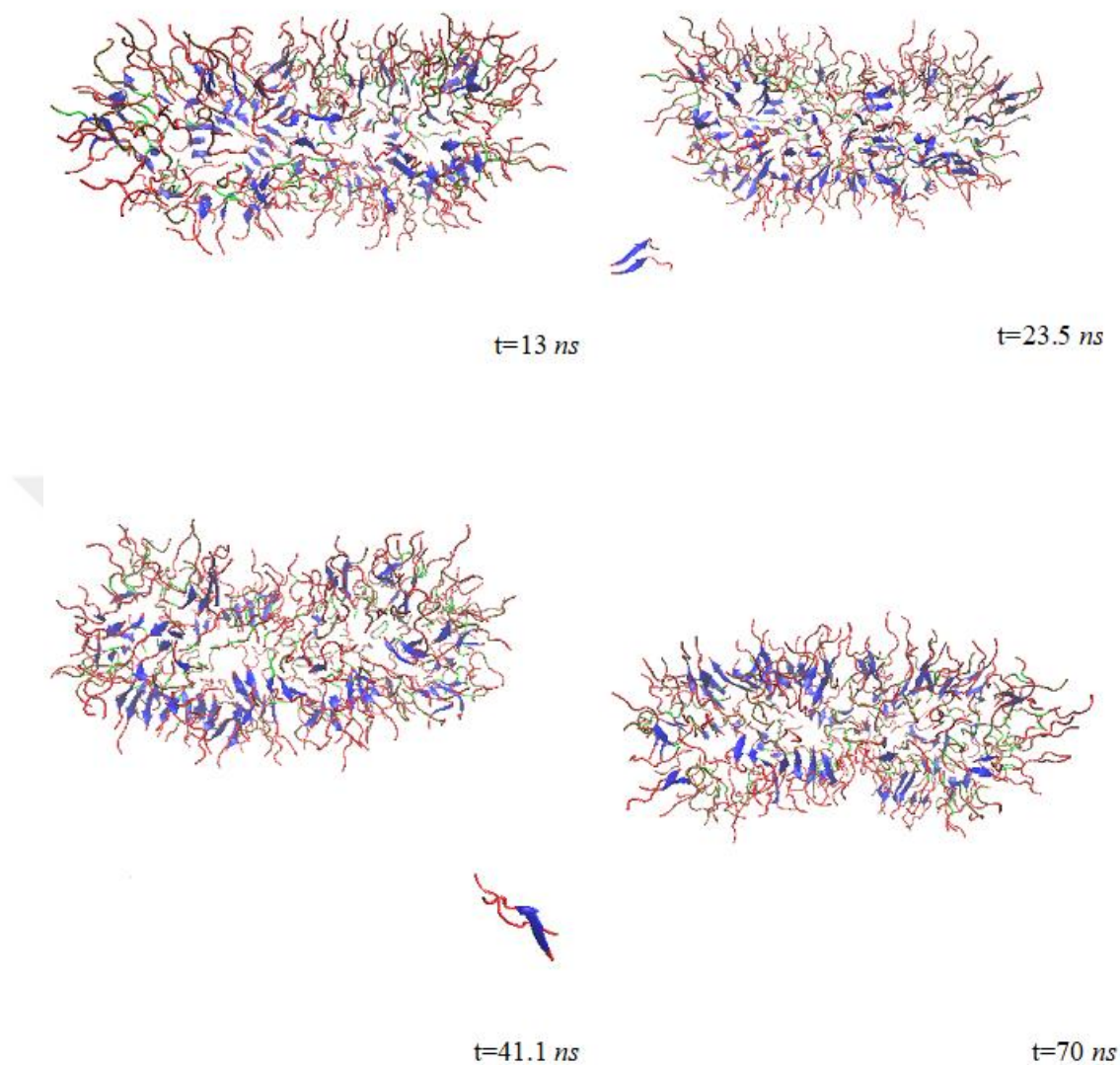


Figure 3.4. Snapshots of the PA1-based nanofiber at 358 K. Despite the departure of some PA molecules from the network, the system retained its overall structure.

Since disintegration of the cylindrical nanofiber could be expected as the temperature increases, two more simulations were performed to determine the reason behind the absence of nanofiber disassembly at 358 K. In the first simulation (simulation I), the configuration of the PA1-based cylindrical nanofiber after 70 ns of simulation at 338

K was extracted and heated to 358 K. In the second simulation (simulation II), temperature was raised in 20 ns intervals from 300 K to 358 K. In simulation I, the starting structure was in two pieces, as shown in Figures 3.5 and 3.6, with RMSD values around 4.4 nm. These pieces combined after 18.3 ns to reform the initial cylindrical PA assembly with an RMSD value of *ca.* 2 nm. After 34.2 ns, the RMSD increased again, but no disintegration was present and the fiber merely lost some of its structural integrity in a manner analogous to 338 K and 358 K results.

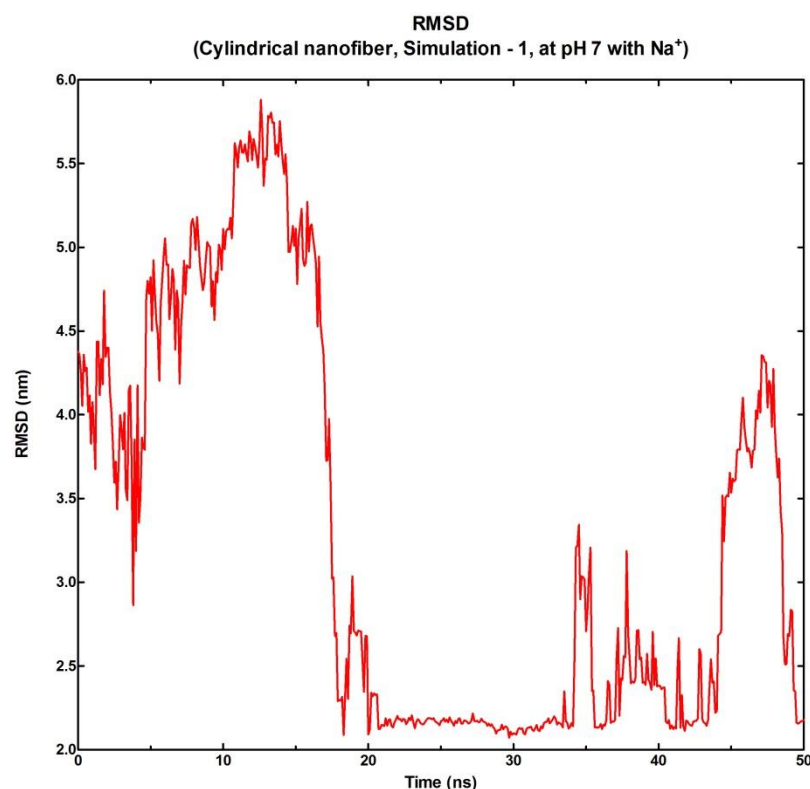


Figure 3.5. RMSD graph of simulation I for the PA1 nanofiber. The initial structure was taken from its 338 K configuration and heated to 358 K. The peptide system regained its structure in the 20 ns – 35 ns interval, but exhibited fluctuations in the nanofiber radius after this time period.

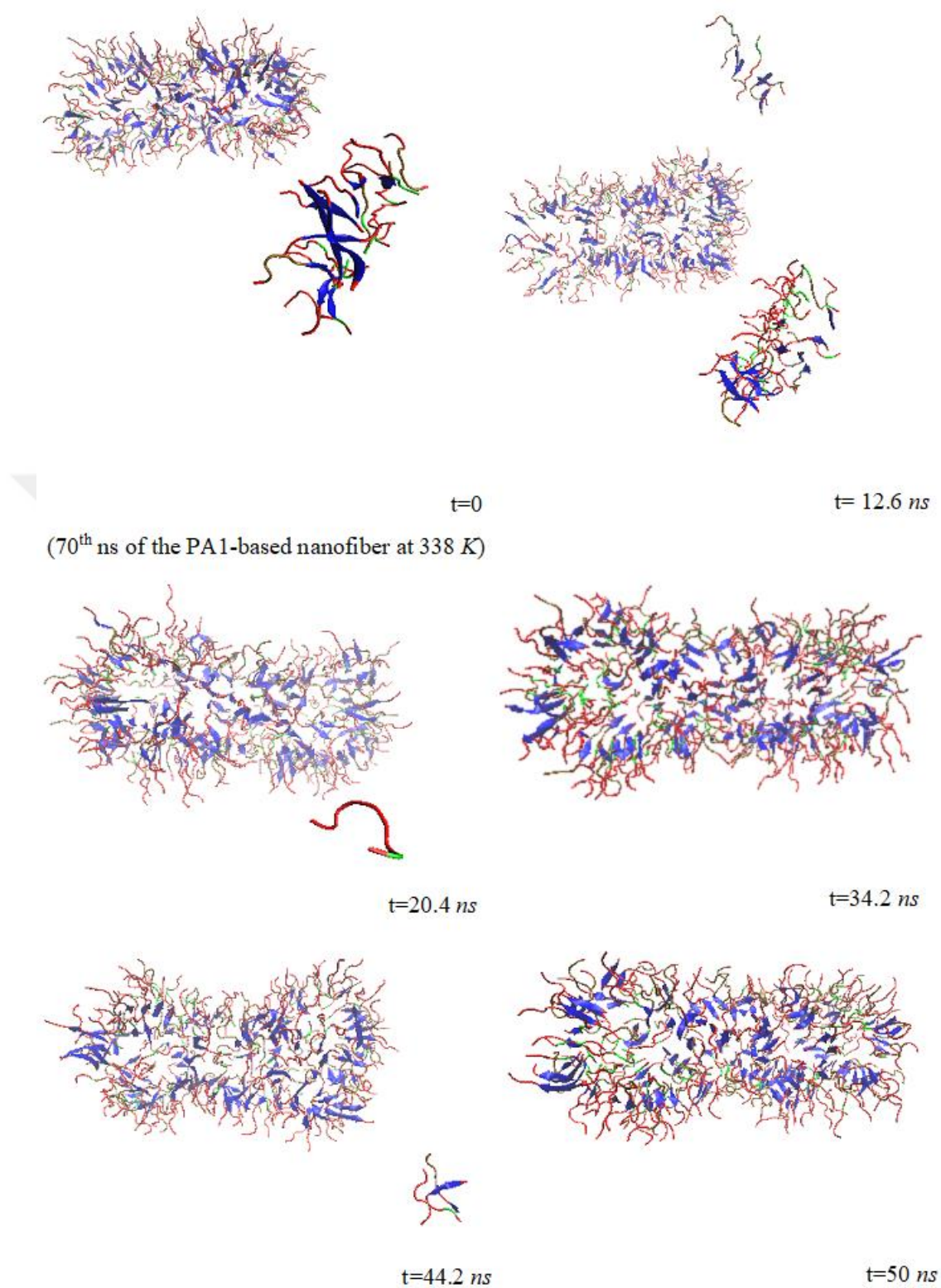


Figure 3.6. Snapshots of simulation I for the PA1 nanofiber. As with the RMSD results, the snapshots suggest that the system reassembled after 20 ns of heating.

In simulation II, the temperature of the system was increased from 300 K to 358 K gradually at 20 ns intervals. RMSD values (Figure 3.7) started fluctuating after 97 ns, and the structure began to crumble after 112 ns, which corresponds to 358 K (Figure 3.8). However, no disintegration was found to occur at 338 K, which is the temperature threshold at which directly-heated PA1 nanofibers start to disassemble. Since 20 ns of simulation at 338 K might not have been sufficient to observe disintegration, this part of the simulation was also extended to 70 ns. However, no fiber disassembly was present even after this extended simulation period, with RMSD values consistently staying under 2 nm (Figure 3.9).

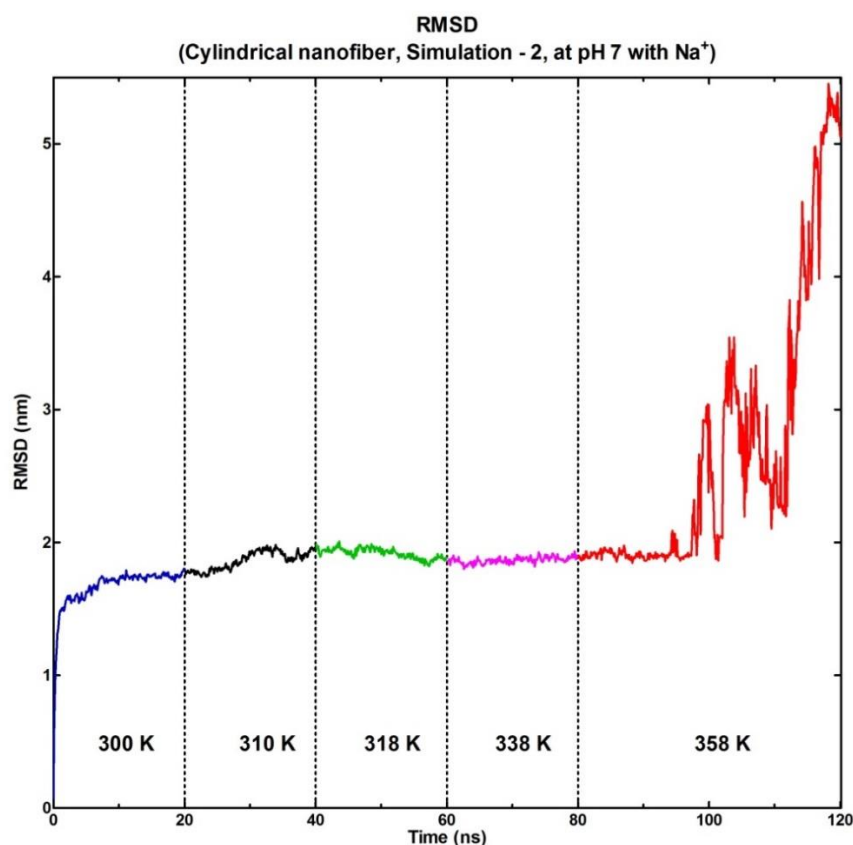


Figure 3.7. RMSD graph of PA1 nanofiber gradually heated from 300 K to 358 K (Simulation II). Disassembly of the nanofiber system was observed only at 358 K.

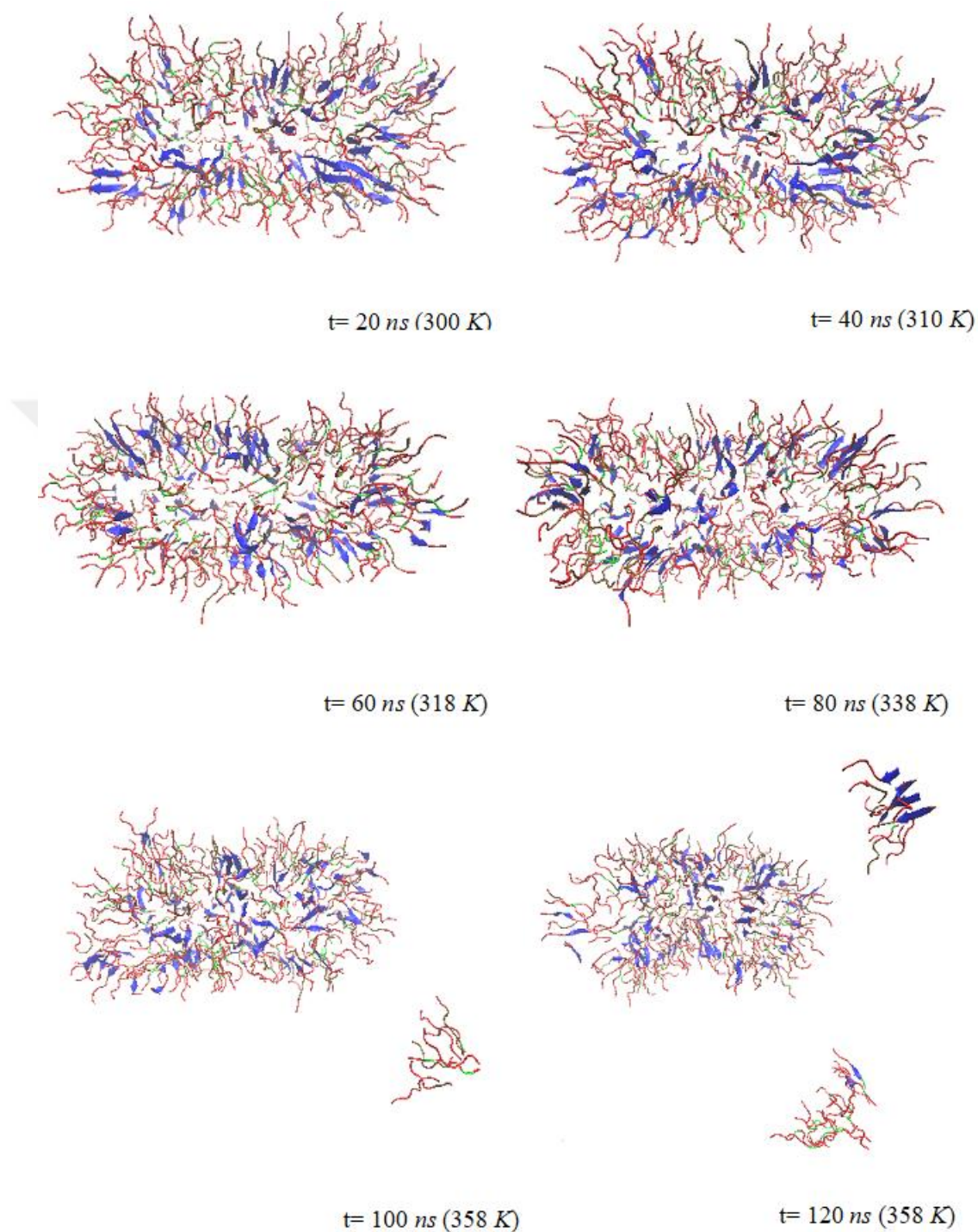


Figure 3.8. Snapshots of simulation II for the PA1 nanofiber. Fiber disassembly was observed to occur in the 100 ns – 120 ns interval, which corresponds to 358 K.

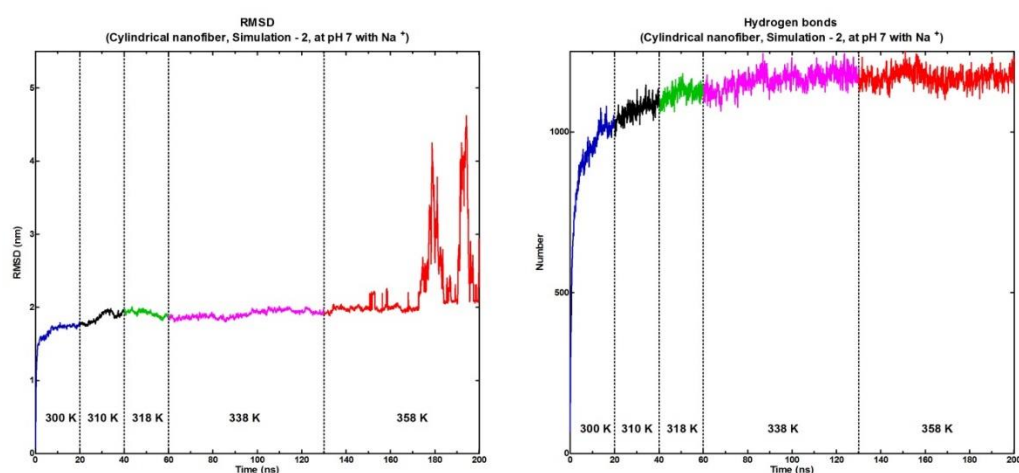


Figure 3.9. Extended simulation for the RMSD values and number of H-bonds associated with the gradual heating of the PA1 nanofiber (Simulation II).

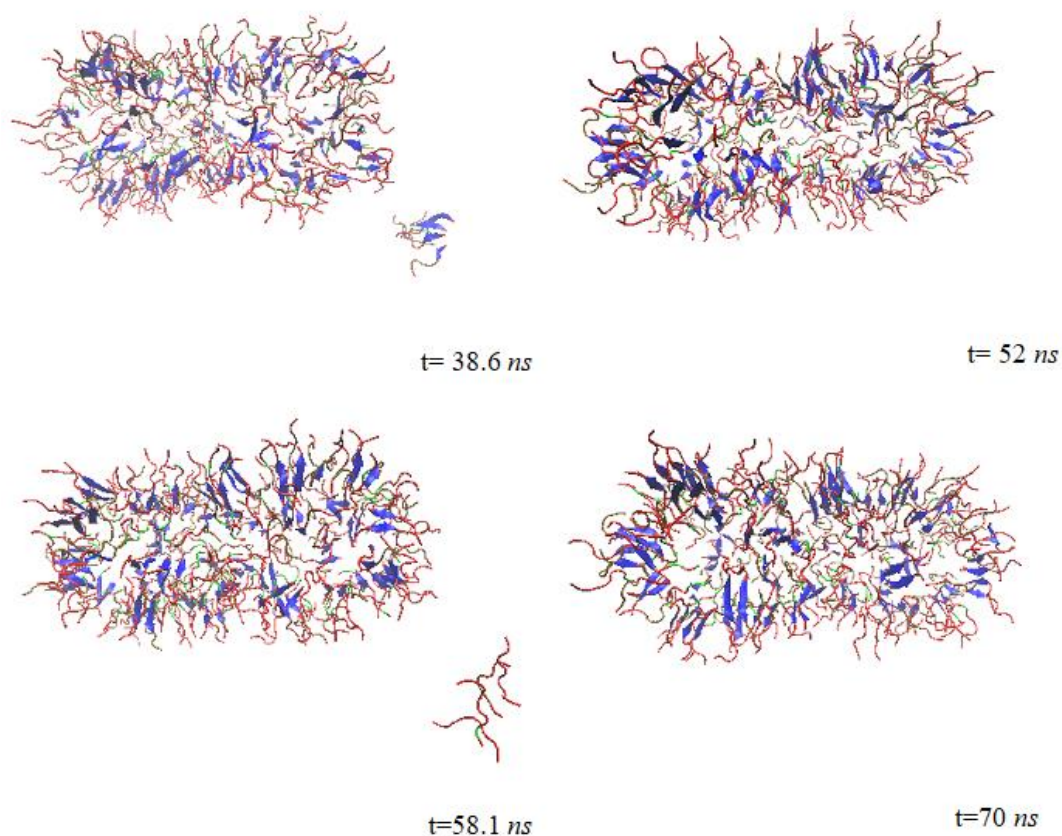


Figure 3.10. Snapshots of the PA2 nanofiber at 338 K. Although the nanofiber remained relatively intact, its radius and subunit configuration were unstable.

The RMSD value of the PA2-based nanofiber was above 2.5 nm after 38 ns at 338 K, and some of the PA molecules broke away from the nanofiber at this time period (Figure 3.2b and Figure 3.10). But between 51 and 57 ns, the structure took the form of a cylinder, disintegrated, and eventually re-assumed its cylindrical shape between 60.7 and 70 ns (RMSD < 2.0 nm). Likewise, some disassembly between 45.4 - 49 ns was observed for the PA2 configuration at 358 K, but the nanofiber was found to largely maintain its initial structure (Figure 3.2b and 3.11).

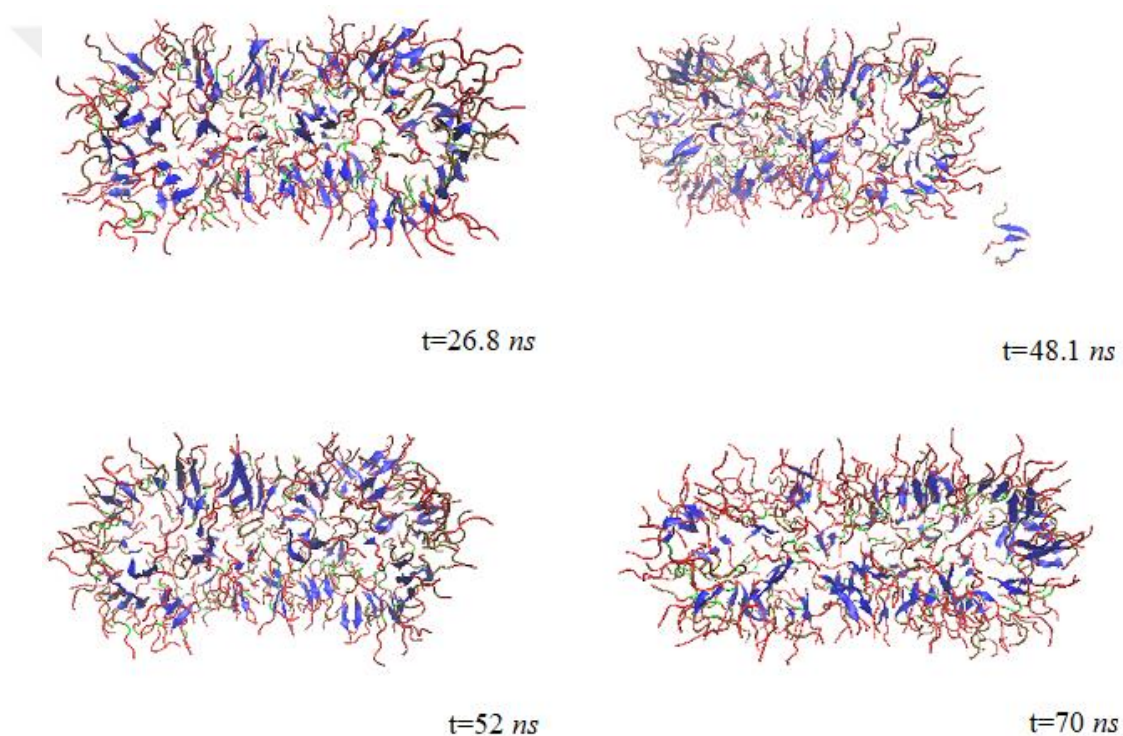


Figure 3.11. Snapshots of the PA2 nanofiber at 358 K. The structure was generally able to retain its structural integrity, although slight fluctuations existed around 48 ns.

Comparative analysis of simulation results associated with PA1 and PA2 configurations at 338 K and 358 K shows that the change in the RMSD at 338 K is greater than the change at 358 K for both structures. However, the PA2 nanofiber was

unstable and cycled between disintegrated and intact fiber structures, while fiber disassembly could not be reversed in the PA1 nanofiber. In addition, large fluctuations were observed in RMSD values for the PA2 nanofiber at 310 K, which is unexpected given the relatively low temperature used for the simulation. Consequently, Na^+ ions appear to be more effective as charge neutralizers than Ca^{+2} ions in mediating the stability of peptide nanostructures, and PA1 was therefore used in subsequent studies.

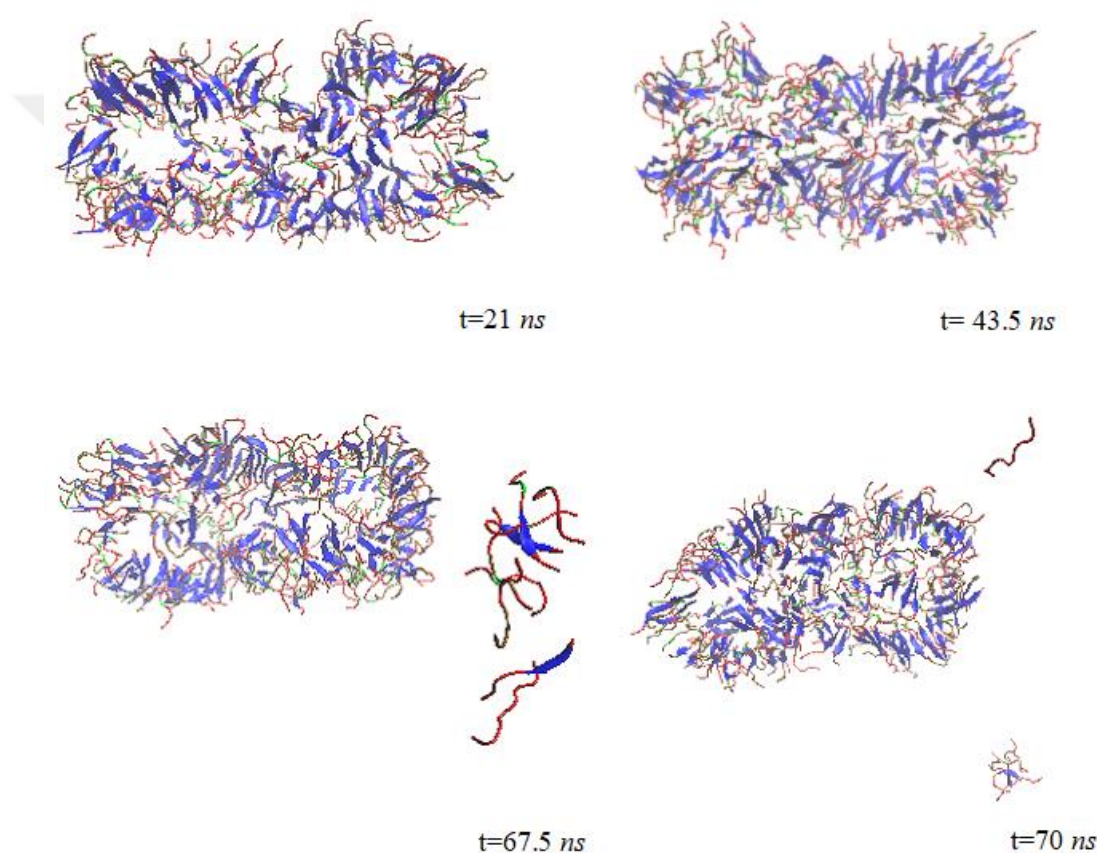


Figure 3.12. Snapshots of the PA3 nanofiber at 338 K. While the overall structure was generally stable, small peptide fragments were observed to leave the peptide structure towards the end of the simulation period, suggesting that fiber disassembly is beginning to occur at this temperature.

In the RMSD analysis of the PA3 configuration at 338 K (Figure 3.2c), some disintegration was observed towards the end of the simulation at 338 K (Figure 3.12), while the entire structure was broken into 3 sections and completely lost its cylindrical form at 358 K (Figure 3.13). This behavior forms a strong contrast with the disassembly kinetics of PA1, which disintegrates around 338 K, but keeps its initial form at 358 K.

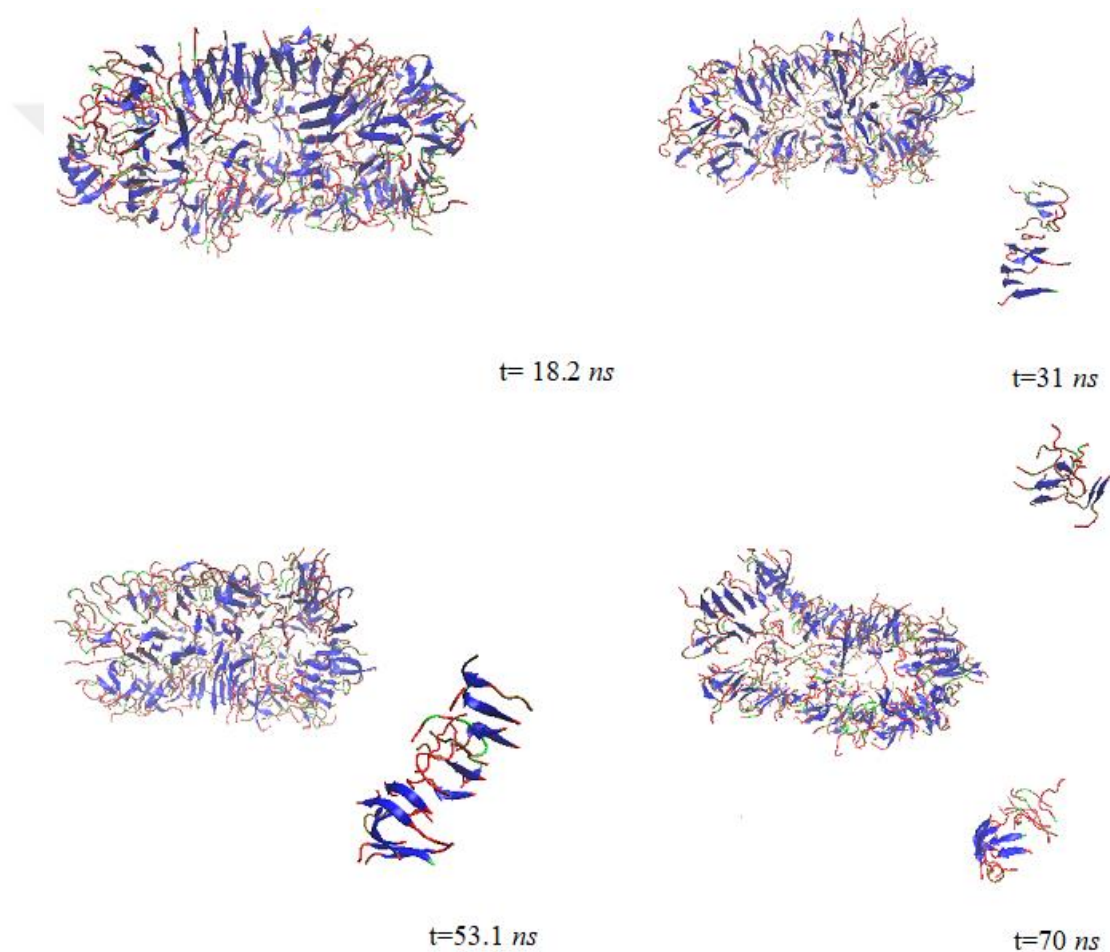


Figure 3.13. Snapshots of the PA3 nanofiber at 358 K. The nanofiber was much less stable compared to 338 K results, and the structure rapidly split into three sections at this temperature point.

The secondary structure content of each nanofiber as a function of time at different temperatures was determined by the Dictionary of Secondary Structure of Proteins (DSSP) program designed by Kabsch and Sander²¹⁹. At each temperature, all three PA-based fibers had random coils as their dominant secondary structure; at the ranges of 69%-65%; 63%-67% and 59%-53% for PA1, PA2 and PA3, respectively. β -sheets were the second most-adopted structures in all nanofiber configurations, ranging between 9%-11% for the PA1 nanofiber, 9%-11% for the PA2 nanofiber and 15%-21% for the PA3 nanofiber. Thus, the PA molecules prepared at low pH had more β -sheet (or less random coil) structures at each temperature, suggesting that acid-mediated self-assembly results in more organized structures. In addition, as seen in Table 3.1, β -sheet percentages decreased in the case of disintegration.

Table 3.1. Percentage of β -sheet and random coil conformations associated with PA systems under each temperature.

T (K)/PA	300 K	310 K	318 K	338 K	358 K
PA1					
β -sheet	9%	11%	11%	9% (disintegration)	10%
Coil	69%	66%	66%	66%	65%
PA2					
β -sheet	10%	9% (disintegration)	11%	10%	11%
Coil	67%	67%	65%	65%	63%
PA3					
β -sheet	15%	18%	18%	21%	19% (disintegration)
Coil	59%	57%	55%	53%	54%

Hydrogen bonds between the PA molecules were also analyzed by assuming the existence of the bond for O-H distances of 0.35 nm or below, and OHN angles of 30 degrees or less (Figure 3.14). There was a slightly greater number of H-bonds associated with the PA3 configuration compared to the PA1 nanofiber at each

temperature point. It is worth noting that a similar result was obtained from the DSSP analysis, as the PA3 nanofiber exhibited a greater percentage of β -sheets at each temperature (*cf.* Table 3.1). In addition, the number of H-bonds generally increased with increasing temperature.

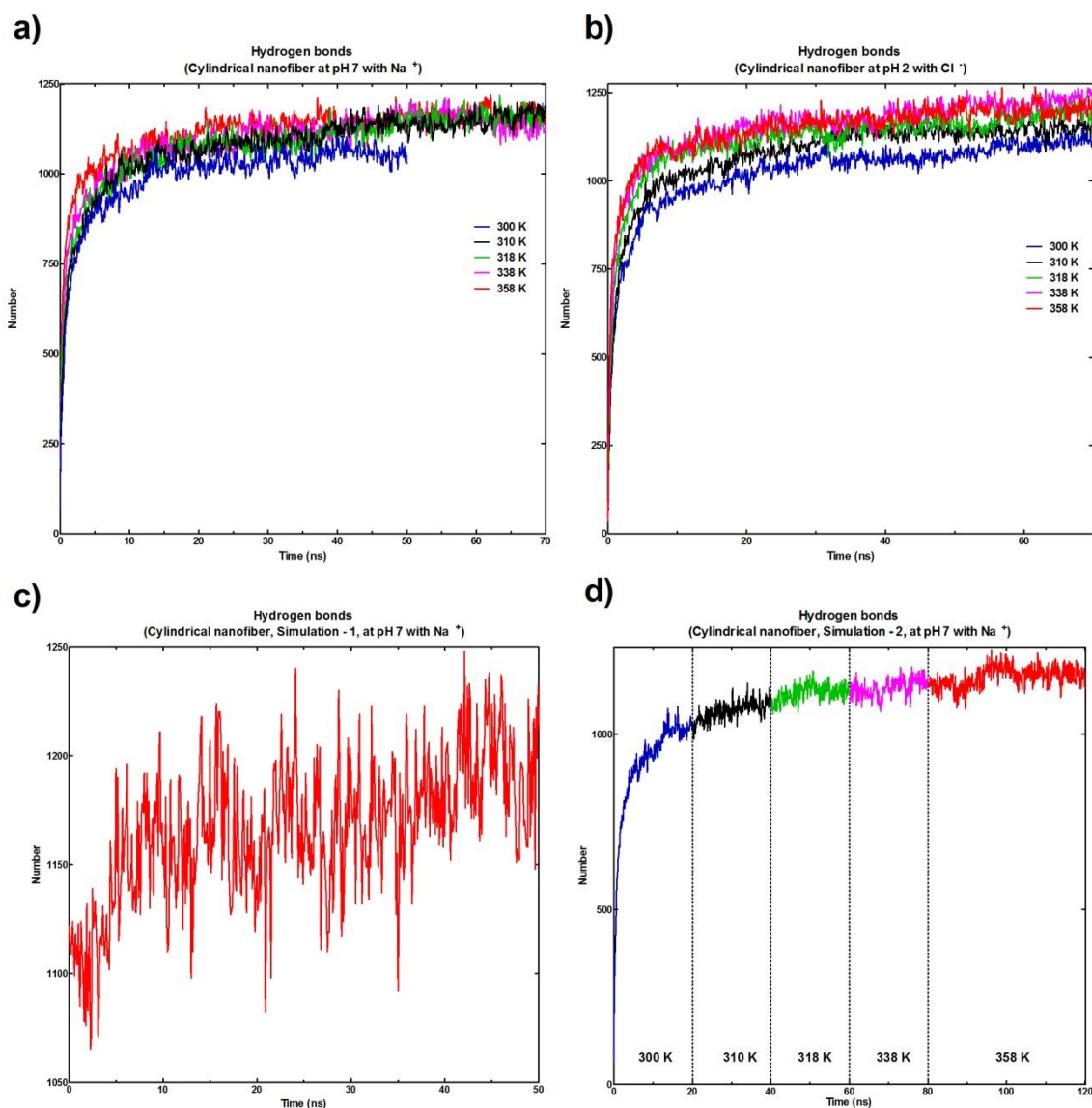


Figure 3.14. Number of H-bonds formed between PA molecules as a function of simulation time. H-bonds were observed to increase with temperature, but did not differ considerably between the PA systems tested.

3.4. DISCUSSION

RMSD analyses outlined in the present study suggest that temperature increase results in the loss of nanofiber structure, with the 338 K and 358 K simulations in particular demonstrating that higher temperatures compromise the compactness of the peptide backbone. The fact that the PA1 nanofibers in 338 K simulations exhibited a total disassembly in nanofiber structure also merits some note, since a similar effect was not observed in the 358 K results (see Figure 3.2a). Proteins and peptides often exhibit irreversible changes in their assembly characteristics following heating, which usually (but not necessarily) accompany the loss of biological functions and drive the structure to a more stable equilibrium following the disruption of electrostatic interactions²²⁰. Therefore, the structure at 338 K may possibly represent an "intermediate state" where the thermal energy provided is capable of disrupting the nanofiber structure but not sufficient for the nanofiber to reach another stable configuration at the time periods tested. Indeed, the disassembly observed at 338 K was not present in a simulation of peptide nanofibers that were gradually heated to 300 K, 310 K, 318 K, 338 K and 358 K for 20 ns each (simulation time was also extended by an additional 50 ns at 338 K to determine whether reassembly is possible under longer time scales, see Figure 3.7) suggesting that a slow transition into higher temperatures may increase the stability of the peptide backbone. As shown in Figure 3.5, the simulation was initiated from a disassembled configuration at 338 K, which was heated to 358 K. The increased heat presumably caused the nanofiber to reach a more stable state in a local energy minimum, increasing its resistance to further heat-mediated disassembly. The existence of intermediate states in peptide systems was previously shown

experimentally by Tantakitti et al.¹⁹⁴, who observed that competing interactions between repulsive and attractive forces can "lock" or "trap" supramolecular peptide nanosystems into thermodynamically unfavorable states, which are defined by local minima in the energy landscape of the system. In addition, movements between these minima have been shown to greatly alter the biological functions of a β -sheet forming peptide, with one state promoting cell survival and a second, metastable state leading to cell death. The transient formation of an intermediate structure was also observed in simulations by Yu and Schatz²²¹, who reported that the SLSLAAAGIKVAV sequence first assembles into a "pillar-like" form before assuming its final shape as a cylindrical nanofiber.

Secondary structure analyses suggest that the peptide nanofibers predominantly exhibit random coil and secondarily β -sheet structures. This result is partially in agreement with Dagdas *et al.* and underlines a characteristic feature of many peptide assemblies²⁰³. Valine and alanine residues in particular are β -sheet promoting amino acids, and the VVAG motif in the lauryl-VVAGERGD peptide is capable of mediating its self-assembly through β -sheet formation²²². Minimal changes were observed between Na^+ and Cl^- -mediated assembly; however, the Cl^- -mediated assembly (the PA3 nanofiber) was also more predisposed towards β -sheet formation compared to the Na^+ -mediated (the PA1 nanofiber) system, suggesting that pH change creates a stronger effect on assembly behavior than does the inclusion of cations. Curiously, secondary structure predictions were not strongly dependent on temperature, while Dagdas *et al.* observed that Ca^{+2} and Cl^- -mediated assemblies react differently to temperature increases: Ca^{+2} -crosslinked nanofibers were relatively resistant to thermal

stress up to 338 K, while HCl-treated nanofibers reacted immediately to temperature changes from 308 K onwards. The difference between these observations may result from the ability of small assemblies to still retain their secondary organization despite the disassembly of the nanofibrous peptide network, which would affect CD measurements (as CD spectra are sensitive to meso-scale aggregate effects) but not MD simulations²²³. The present work therefore suggests that the temperature-dependent changes in Dagdas *et al.*'s CD spectra are not from the interactions within individual nanofiber units, but possibly from the disassembly of nanofiber bundles, truncation of nanofiber length and other mesoscale structural effects that may contribute to CD signals. Differences between the behaviors of macro- and microscale assemblies were also proposed as the reason that Dagdas *et al.* observed relatively small changes in the oscillatory rheology of Ca⁺²-crosslinked nanofibers, while a large loss of β -sheet signatures was present in their CD spectra at identical temperatures. Our simulation experiments introduce an additional layer of complexity to this model, suggesting that, even though the core nanofiber was intact until higher (338 K – 358 K) temperatures, the overall nanofiber structure might be altered by temperature and the method through which the peptide hydrogel has been formed. In addition, small differences in the temperature responses of Cl⁻, Ca⁺²- and Na⁺-crosslinked gels may play more important roles in biological systems, into which peptide hydrogels are frequently implanted²²⁴⁻²²⁶.

While we only investigated the changes that occur in response to temperature and pH changes, it is known that the biocompatibility and cell-recruiting, drug-encapsulating, immune system-inducing and differentiation-promoting capacities of PAs depend

strongly on their structural properties^{182, 227, 228}. For example, Moyer *et al.*¹⁹⁰ have shown that the location and length of the alkyl tail can drastically alter the morphology of pH-responsive, self-assembled peptide amphiphile systems, and that cylindrical nanofibers had around 7-fold higher encapsulation efficiency for camptothecin compared to spherical assemblies. In contrast, Mumcuoglu *et al.*²²⁹ demonstrated that cell-penetrating peptide nanospheres were uptaken by the cells at greater concentrations compared to nanofibers, because peptide nanofibers bound strongly to the cellular membrane and could be internalized only to a lesser degree. In addition, two lysine-rich peptides were shown by Newcomb *et al.*²³⁰ to exhibit markedly different cytotoxic effects depending on their hydrogen bonding interactions, with the H-bond-rich β -sheet forming peptide showing minimal toxicity and the H-bond-poor sequence rapidly disrupting the integrity of both cell membranes and liposomes. As electrostatic interactions can greatly alter the macrostructure of the peptide system, it is probable that the gelation agent used for their self-assembly will play a role in determining their morphology and, consequently, effects in biological systems, which should be considered for the design of peptide systems for use in regenerative medicine.

3.5. CONCLUSION

The present study demonstrates that the stability of PA nanofibers under temperature changes depends heavily on the conditions used to precipitate the self-assembly process. In particular, the Cl^- -mediated assembly of the lauryl-VVAGERGD PA was reversed only at 358 K, while Na^+ -mediated assembly experienced a loss in structure

at 338 K but retained its organization at 358 K. While extended simulation times did not elicit any change in the organization of the PA1 nanofiber, the pattern of heating appeared to change the structural response of the self-assembled peptide amphiphile system: PA1 was observed to be stable at 358 K when heated directly to that temperature, but lost its morphology under a temperature ramp from 300 K to 358 K. In addition to the temperature and pH changes, counterion-based differences may also exist in factors such as mechanical elasticity, protease resistance and drug release capacity, which may be especially pronounced in the ion-rich environment experienced by PA gels used in *in vivo* therapeutic applications. As such, the considerations outlined herein should be taken into account for the design and modeling of future peptide systems.

CHAPTER 4

Conclusion and future perspectives

Methods in physics, such as fluorescence and electron microscopies, have been fundamental in advancing our knowledge in basic biology. In a similar manner, computational methods are now widely appreciated to be critical for the characterization of a broad range of biological phenomena, and especially for the coherent analysis of data generated by high-throughput screening and sequencing efforts. However, even these methods are hindered by the enormous complexity of biological environments, which are full of redundancies, counterintuitive systems and constant variance due to their evolutionary history. Consequently, method optimization is an underappreciated yet critical part of modern biology, and the first section of the present study explores its relevance to a highly popular mechanical characterization method in atomic force microscopy. The findings of this chapter, although intuitive in some ways (*e.g.* deparaffinized samples can easily be predicted to have higher Young's moduli due to the fixation process, while cryosectioned and agarose-embedded tissues are treated much more mildly), underlines the fact that even minor alterations can have major consequences in AFM measurements – the full range of measurement conditions are seldom reported in the literature, and this study makes a good case for this trend to change. In addition, it should be stressed that the present study does not test every possible variable – factors such as fixative concentration, fixation length, section thickness, measurement medium and temperature, AFM tip radius and morphology, indentation depth and contact models used in the analysis of force-distance curves may all significantly affect elasticity measurements using this technique. Consequently, follow-up studies involving a larger number of variables can be performed to better establish the effect of each external factor on the measurement of elastic moduli.

The second part of the present thesis details the use of all-atom molecular dynamics simulations for the characterization of peptide assembly behaviour, and like the first section, serves to underline the significant impact that environmental variables have on biomaterial properties. The temperature stability of the peptide nanofiber structures was found to depend on counterion identity, but contrary to what might be expected, calcium ions were found to induce lower stability compared to sodium ions. Calcium-based self-assembly involves the formation of crosslinks within and between individual peptide nanofibers, which requires the localization of calcium ions into specific locations to serve as bridges. This organization, although readily accomplished in nature, is difficult to replicate in simulation over short time periods, which is a potential reason for the observed inefficiency of calcium in mediating fiber stability. In addition, fiber structure was found to be lost at 338 K and re-established at 358 K under some initial configurations (Na^+ and Ca^{++} at pH 7), which is curious and warrants further study. One potential reason for this effect is that sudden heating to higher temperatures allows the fibers to bypass an energy barrier to reach a lower energy state, while the energy provided at lower temperatures is only sufficient to facilitate the disassembly of the fiber, which is consistent with literature reports that suggest certain peptide sequences are consolidated and become more stable after heating. As with the first part of this thesis, however, the configurations used in the second section represent only a small sampling of the peptide structures that exist in nature, and future studies focusing on the effects of counterions on the assembly of other morphologies (such as micelles and nanoribbons) would contribute greatly to our understanding of peptide assembly kinetics. The interaction of these peptide structures with proteins can also be simulated to better understand their *in vivo* behaviour.

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APPENDIX A

**Code developed for the construction of peptide
nanofibers**

The following PyMol script was used for constructing peptide nanofiber structures for various purposes. Note however that it was not employed for the simulations detailed in Chapter 3.

For two-PA nanofibers (accepts two PAs, called initialPA and initialPA2, variables bolded)

```
from pymol import cmd
from pymol import math
import numpy
import itertools

peptideperlayer = 10
numberoflayers = 8
planeangle = 60
distancebetweenlayers = [0,0,15]
withinlayerangle = 90
longaxis = 15
shortaxis = 10
helicityangle = 18
cmd.align("initialPA", "initialPAprime")
cmd.orient ("initialPA")
cmd.copy ("initialPA2","initialPA")
cmd.rotate ("[0,0,1]","180","initialPA2")
cmd.translate ("[-50,0,0]","initialPA2")
cmd.zoom ("initialPA initialPA2")
cmd.orient ("initialPA initialPA2")

a = range(numberoflayers)
s = range(peptideperlayer)
for x in a:
    for y in s:
        if (y % 2 == 0):
            cmd.copy("PA%s%s" %(y, x), "initialPA")
            cmd.orient ("initialPA initialPA2")
            anglecomponent =
numpy.radians(x*helicityangle+y*360//peptideperlayer)
            numbercomponent =
math.sqrt(longaxis*longaxis*math.sin(anglecomponent)*math.sin(anglecomponent)
+ shortaxis*shortaxis*math.cos(anglecomponent)*math.cos(anglecomponent))
            radius = longaxis*shortaxis//numbercomponent
            ellipsedistance = [radius,0,0]
            coneangle = numpy.radians(planeangle)
```

```

conecorrection = math.cos(coneangle)*radius
cmd.translate (ellipsedistance,"PA%s%s" %(y, x))
cmd.rotate ("x",withinlayerangle,"PA%s%s" %(y, x))
cmd.rotate("y",planeangle,"PA%s%s" %(y, x))
conecorrectionvector = [0,0,radius]
cmd.translate (conecorrectionvector,"PA%s%s" %(y, x))
cmd.orient ("initialPA initialPA2")
cmd.rotate ("z",y*360//peptideperlayer,"PA%s%s" %(y, x))
for _ in itertools.repeat(None, x):
    cmd.translate (distancebetweenlayers,"PA%s%s"
%(y,x))
    for _ in itertools.repeat(None, x):
        cmd.rotate ("z",helicityangle,"PA%s%s" %(y, x))
    else:
        cmd.copy("secondPA%s%s" %(y, x), "initialPAprime")
        cmd.orient ("initialPA initialPA2")
        anglecomponent =
numpy.radians(x*helicityangle+y*360//peptideperlayer)
        numbercomponent =
math.sqrt(longaxis*longaxis*math.sin(anglecomponent)*math.sin(anglecomponent)
+ shortaxis*shortaxis*math.cos(anglecomponent)*math.cos(anglecomponent))
        radius = longaxis*shortaxis//numbercomponent
        ellipsedistance = [radius,0,0]
        coneangle = numpy.radians(planeangle)
        conecorrection = math.cos(coneangle)*radius
        cmd.translate (ellipsedistance,"secondPA%s%s" %(y, x))
        cmd.rotate ("x",withinlayerangle,"secondPA%s%s" %(y, x))
        cmd.rotate("y",planeangle,"secondPA%s%s" %(y, x))
        conecorrectionvector = [0,0,radius]
        cmd.translate (conecorrectionvector,"secondPA%s%s" %(y, x))
        cmd.orient ("initialPA initialPA2")
        cmd.rotate ("z",y*360//peptideperlayer,"secondPA%s%s" %(y,
x))
        for _ in itertools.repeat(None, x):
            cmd.translate (distancebetweenlayers,"secondPA%s%s"
%(y,x))
            for _ in itertools.repeat(None, x):
                cmd.rotate ("z",helicityangle,"secondPA%s%s" %(y,
x))
cmd.delete ("initialPA initialPA2 initialPAprime")

```

For single-PA nanofibers (accepts one PA, called initialPA, variables bolded)

```

from pymol import cmd
from pymol import math
import numpy
import itertools
peptideperlayer = 24
numberoflayers = 5
planeangle = 60
distancebetweenlayers = [0,0,15]
withinlayerangle = 180
longaxis = 15
shortaxis = 10
helicityangle = 10
cmd.orient ("initialPA")
cmd.copy ("initialPA2","initialPA")
cmd.rotate ("[0,0,1]", "180", "initialPA2")
cmd.translate ("[-50,0,0]", "initialPA2")
cmd.zoom ("initialPA initialPA2")
cmd.orient ("initialPA initialPA2")

a = range(numberoflayers)
s = range(peptideperlayer)
for x in a:
    for y in s:
        cmd.copy("PA%s%s" %(y, x), "initialPA")
        cmd.orient ("initialPA initialPA2")
        anglecomponent =
numpy.radians(x*helicityangle+y*360//peptideperlayer)
        numbercomponent =
math.sqrt(longaxis*longaxis*math.sin(anglecomponent)*math.sin(anglecomponent)
+ shortaxis*shortaxis*math.cos(anglecomponent)*math.cos(anglecomponent))
        radius = longaxis*shortaxis//numbercomponent
        ellipsedistance = [radius,0,0]
        cmd.translate (ellipsedistance, "PA%s%s" %(y, x))
        cmd.rotate ("x", withinlayerangle, "PA%s%s" %(y, x))
        cmd.rotate("y", planeangle, "PA%s%s" %(y, x))
        cmd.orient ("initialPA initialPA2")
        cmd.rotate ("z", y*360//peptideperlayer, "PA%s%s" %(y, x))
        for _ in itertools.repeat(None, x):
            cmd.translate (distancebetweenlayers, "PA%s%s" %(y,x))
        for _ in itertools.repeat(None, x):
            cmd.rotate ("z", helicityangle, "PA%s%s" %(y, x))
cmd.delete ("initialPA initialPA2")

```