Determination of Parameters to Induce Self-Assembly and Alignment of Collagen on a Conductive Substrate

Undergraduate Honors Thesis
Presented in Partial Fulfillment of the Requirements for
Graduation with Distinction in the
Department of Mechanical Engineering at
The Ohio State University

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November 2019

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Abstract

Through an engineering perspective, bone is an interesting material whose composition and properties are influenced by external stimuli that maintains its mechanical integrity. To achieve this, bone has a hierarchical structure and heterogeneous compositions mostly consisting of type I collagen and inorganic minerals. Type I collagen is a fibrillar protein acting as a basic building block in bone tissue and as a response to external loads, is assembled with minerals in an orderly fashion forming the mineralized collagen matrix. This process, known as the mineralization process, controls the mechanical stiffness of bone during bone formation, remodeling, and healing. Although the mineralization of collagen structures is well-known, its role and mechanism of assembly during the bone mineralization process has not been fully understood yet. And as one of the hypotheses to explain its mechanisms, collagen is known to have mechanoelectric coupling properties, which is also not well understood. Thus, in order to understand the role of collagen during bone mineralization, it is necessary to fabricate the collagen matrix on a conductive substrate to test and confirm its mechanoelectric properties. In this study, we investigated the quality and quantity, through Atomic Force Microscopy (AFM), of the assembled collagen matrix on a conductive substrate with a variation of several conditions: collagen concentration, ion concentration, and pH level. In addition, we studied effect of an electric field on the assembly process. For the results, we obtained a well-assembled collagen matrix on the conductive substrate but found that the electric field on the substrate does not affect the alignment of assembled collagen matrix for the current experimental setup. Adjustment of the experiment is required.

Acknowledgements

I would like to thank my advisor, Dr. Hanna Cho – her willingness to take me into her research lab without any prior experience in research, her immersing me without any hesitation into the its exciting and difficult world, and with hopes of improving and refining my "research muscle", by pointing out any inadequacies to fix, exercise, strengthen, and remodel them. Being involved in her lab has introduced the idea of research and all its fascinations to me and started me in a path to which I can hopefully keep on walking. I personally stumbled a lot in starting research, went around in circles and disoriented, learning to walk in the territory I really knew nothing about. But her patience in guiding me in its workings and how to navigate it has been a big service and a source of encouragement to me.

Likewise, I would like to thank Jinha Kwon for being a pattern to how a researcher should be — resourceful and knowledgeable in research, and meticulous and systematic in experiments and documentation. His patience and availability made my entrance into research, what could have easily been a disaster, a smooth guidance also.

I would like to also thank the staff and faculty that have also advised me on different aspects of this research, specifically Dr. Castro, Chris Adams, and Ross in the Electronics Shop.

The final gratitude towards my friends and family, without whom I could not achieve all that I have so far.

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Chapter 1: Introduction

1.1 Collagen

Collagen is a protein which maintains the shape, strength, and integrity of various tissues in mammal bodies. The most abundant is Type I, consisting of osseous tissue responsible for the structure and strength in bones. Figure 1a shows the alignment of the bone microstructure in response to external tension and compression forces. According to the Wolff's law, bone has ability to remodel its internal architecture over time to be stronger to resist the mechanical loading that exerting on the bone (Wolff, J.J., 1996).

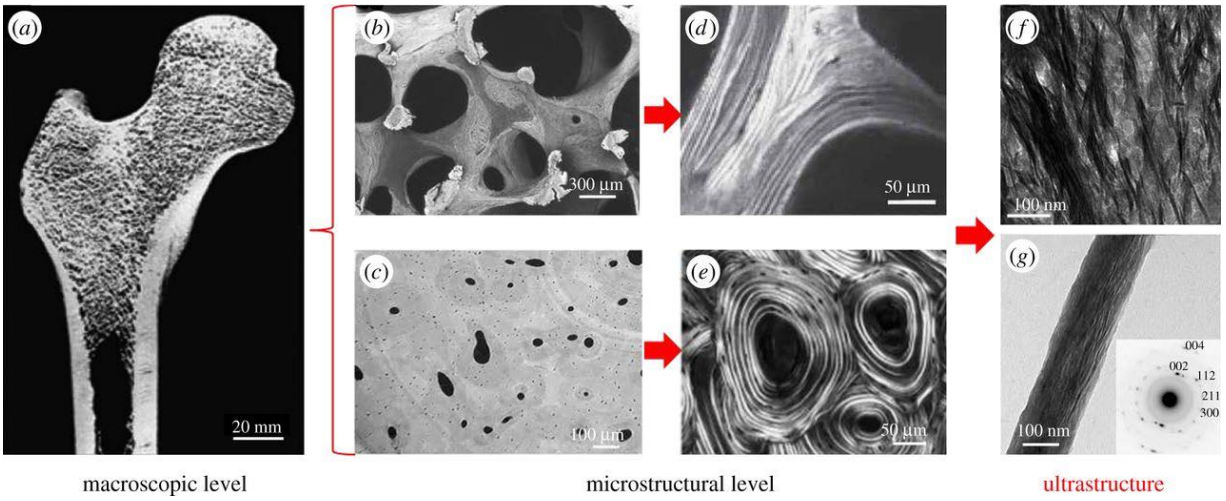


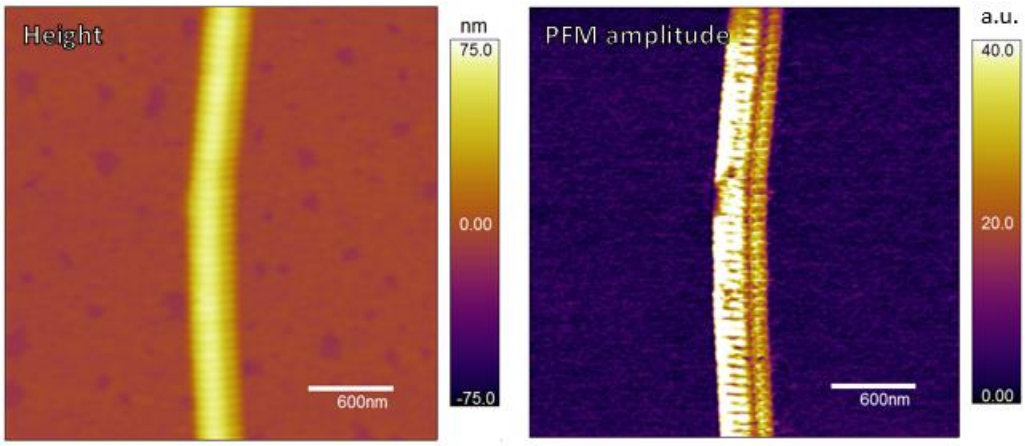
Figure 1: Bone at different hierarchical levels. Macroscopic level showing the femur bone. (a) A view inside the end of the femur bone demonstrates the bone microstructure alignment orthogonal to the applied force of the joints. (b) The trabecular (porous section) bone network. (c) The compact (dense section) bone. Both show the bone microstructure. (d) and (e) show the lamellar structure, composed of matrix of collagen fibers. Both show the bone ultrastructure. (f) and (g) show bundles of collagen and a single collagen fiber, which constitute the bone ultrastructure. (Georgiadis, M., Müller, R., & Schneider, P. 2016)

In Figures 1f-g, the alignment of the collagen fibers in the bone ultrastructure sets the pattern for the formation of the bone microstructure, aligning in such a way that gives the bone the ability to carry the mechanical loads and stresses applied by the joints (Hulmes, D.J.S., 2008). These collagen fibrils in bones can be considered a "smart material," demonstrating a response by aligning itself to withstand the applied mechanical forces (Martin, R.B., Burr, D.,

2015). Understanding this mechanism is extremely useful for the design of new generation materials as well as improving clinical treatments. However, the role of the collagen and the mechanism to control the inner structure of the bone has not been fully investigated yet.

1.2 Mechanoelectric coupling of collagen

In order to explain the bone adaptation mechanism to external loading and the changes in its structure, two main hypotheses have been proposed: mechanoelectric coupling of the collagen (i.e., piezoelectricity) and the streaming potential theory. Collagen can be considered as transducers exhibiting those hypothesized functions. After the 1960s, the streaming potential theory became widely accepted and generally considered as the primary mechanism. The reason for this is due to bone being immersed in fluids, such as bone marrow, and the resulting difficulty in determining the piezoelectric characteristics in wet conditions. However, while the streaming potential theory successfully described the electric charge surrounding the collagen, the mechanism of how the collagen matrix is assembled in an orderly manner was still not clearly determined. Meanwhile, it was found that collagen fibrils have piezoelectric tensors with different amplitudes depending on the loading direction. Figure 2 below shows the testing of the mechanoelectric properties of collagen fibrils using Piezoresponse Force Microscopy.



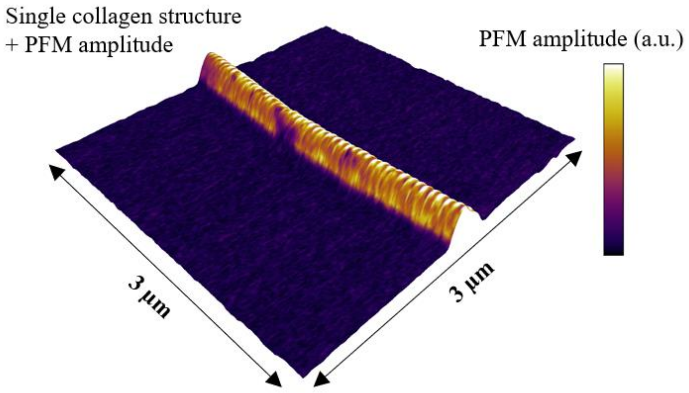


Figure 2: Testing of mechanoelectric coupling of collagen fibril with Piezoresponse Force Microscopy

In addition, since the pores of the bones are very small, whose diameters span only a few nanometers wide, the collagen fibers are exceptionally proximal to one another, allowing the consideration that the piezoelectricity of collagen can be dominant even in wet conditions. In order to investigate the relationship between external loading and the piezoelectricity of the collagen matrix, it is necessary to develop a test bed which comprise of a conductive substrate coated with a well-assembled collagen matrix.

1.3 Objective of the Research

In this study, we investigated the quality and quantity of assembled collagen matrix coated on a conductive substrate dependent on a variety of conditions. Here, a silicon substrate coated at the top surface by platinum was used because a silicon-based substrate can be used easily in MEMS fabrication techniques to build the future test bed. Moreover, while the ideal conditions of collagen concentration, the electrolyte and the ionic concentration, and pH level on the Mica substrate have been well established, there are currently no studies on the investigation of conditions which result in an assembled collagen matrix on a conductive substrate. In addition, with consideration that the collagen molecules have polar hands characterized by a N-

terminal (NH^{3+}) and C-terminal ($COOH^{-}$) at either ends which result in a polarization along the fibrillar direction, we hypothesized that an electric field applied on the conductive substrate would affect the alignment of the collagen matrix during the assembly process. Here, we examined the effect of electric field on the collagen assembly process.

1.4 Significance of Research

This is the first study to investigate the conditions affecting the assembly process of the collagen matrix on a conductive substrate. The result of this study provides knowledge about the behavior of the collagen fibrils during the assembly process on conductive substrate with a variation of different of conditions. In addition, it can be used for future study to investigate the role of the piezoelectricity of the collagen during the bone mineralization process. With a comprehensive understanding of its assembly behavior and electromechanical functionality, there is great potential in numerous biological applications and in the design of innovative materials.

1.5 Overview of Thesis

The thesis, containing 5 chapters, will be organized as follows. Chapter 2 will introduce a more thorough background of collagen characteristics and its self-assembly process. A detailed listing of the variant parameters stated above, and the basis for varying those parameters will also be covered in this chapter. The experimental methods applied for the preparation of the samples, along with a background on the imaging apparatus, Atomic Force Microscopy, will be detailed in chapter 3. The results and its analysis will be detailed in chapter 4. Finally, the summary and conclusion of this research, along with the key contributions of this thesis and future directions of study will be detailed in chapter 5.

Chapter 2: Background and Literature Review

2.1 Type I Collagen in Bones

Type 1 collagen is the most common type found in skin, tendon, bone, and various other parts of the body. A single collagen molecule is composed of polar molecules with three polypeptide chains in repeating helical pattern, resulting in a long, thin fibrillar form with length of 300 nanometers. These molecules then are arranged in a staggered manner, cross-linking with each other at their ends to form a bigger collagen fibrillar structure as shown in Figure 3. One strand of longitudinal-lined collagen molecules will have a periodic spacing of 67 nanometers between each molecule, which is shown as a characteristic of a collagen fibril called D-spacing. A topographical image of type I collagen fibril with its D-spacing obtained by Atomic Force Microscopy (AFM) is shown in Figure 4. The bone is an organ comprised of a hierarchically organized material constructed by a fiber-reinforced composite material. The collagen fibrils studied in this research mingle together with other fibrils forming a collagen matrix, and with increasing structures, forms a collagen fiber, then osteons in cortical bone or osseous tissue in trabecular bone with minerals. This is shown in the hierarchical structure of bone in Figure 5.

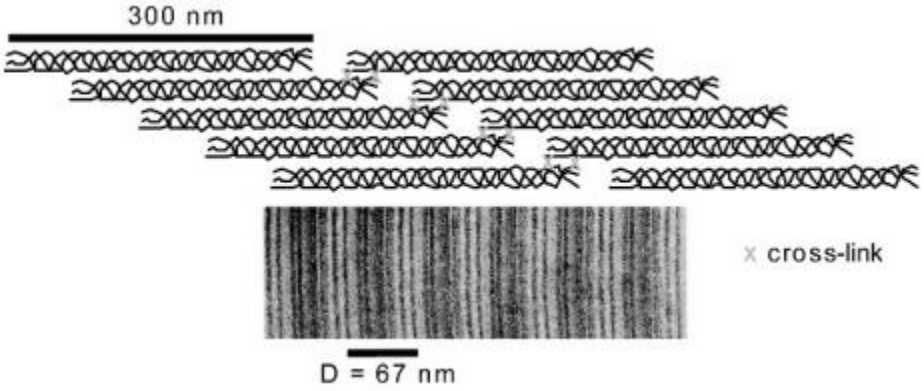


Figure 3: Molecular structure of a type I collagen fibril (Hulmes, 2008).

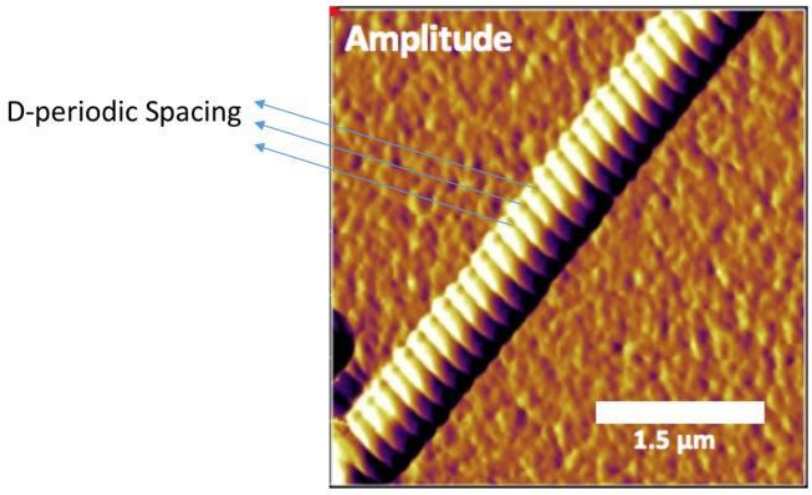


Figure 4: The topographical image of a single type I collagen fibril captured using AFM.

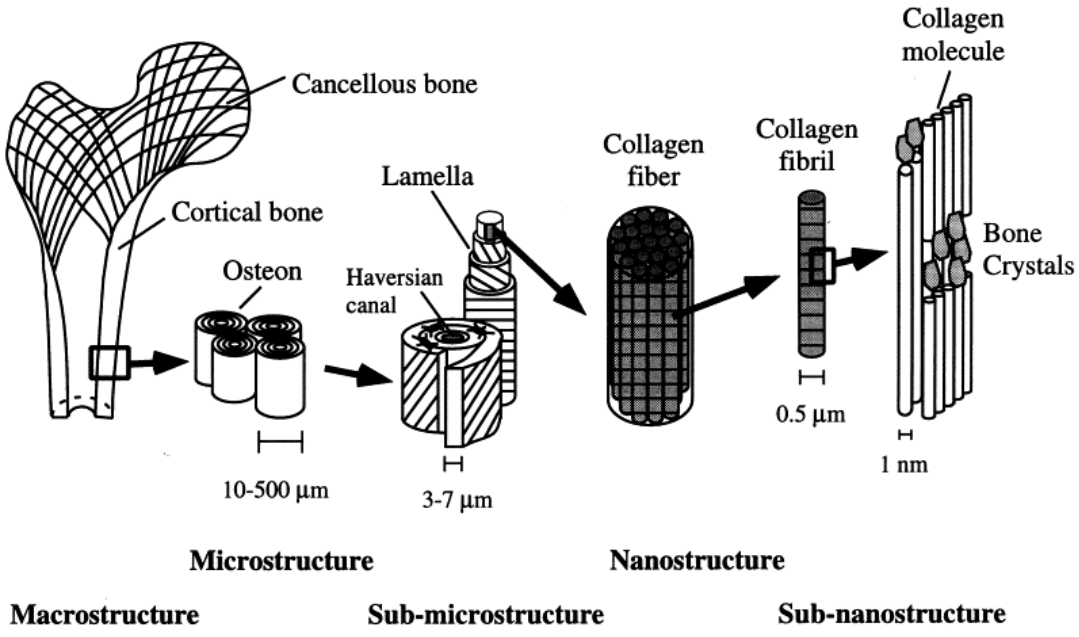


Figure 5: Hierarchical structure and components of bones (Rho, 1998).

The inner architecture of bone shows an anisotropic structure through which the design is optimized to resist mechanical loading applied onto the bone. This has its basis on Wolff's Law, which states that bones in healthy persons or animals adapts to the external loads by changing its

structure. The increase of loading results in the remodeling of the bones so that it becomes stronger to resist the loads. This is illustrated in Figure 6, which shows the anisotropy of a femur bone, patterned directionally to resist the loads applied.

In order to understand this advantageous feature of the bone, it is essential to investigate the composite material of bone in the sub-nanometer scale because of its hierarchy as shown in Figure 5. Bone is a heterogenous material consisting mainly type I collagen and crystalline carbonated apatite (i.e., hydroxyapatite). After the collagen molecules are secreted by bone forming cell (i.e., osteoblasts) to build a structural matrix, the matrix is strengthened by the subsequent mineral deposition. Consequently, the mineralization process is a key factor of modulating mechanical property of bone by controlling the structural and compositional heterogeneity of bone.

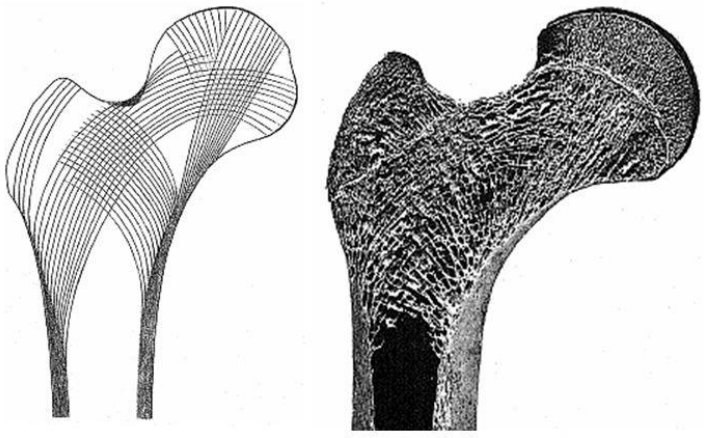


Figure 6: Cross-section of femur bone, illustrating directionality of bone formation.

2.2 Collagen Self-Assembly

During the collagen assembly process, the collagen molecules are assembled into collagen fibril, and the collagen fibrils develop the structural matrix.

This collagen fibrillogenesis is dependent on a few key parameters, including collagen concentration, pH, and the presence of ions and its concentration in the collagen solution. In this study, the effect of these factors is investigated on the conductive substrate which is based on silicon substrate coated by platinum.

2.2.1 Collagen Concentration

When performing experiments in vitro with collagen, the stock collagen solution is diluted to an ideal concentration suitable for the formation of fibrils. Fibrillogenesis is induced by the addition of collagen into fibril forming buffers. The role of collagen concentration greatly affects the overall structure of the resulting fibrils, along with the morphology and structures of the fibrils (F. Gobeaux, 2007). Therefore, in this study, collagen concentration is a parameter to find an ideal concentration for the conditions of this experiment. Figure 7 shows the results of the study, with varying collagen concentration of ranging from 40 to 300 mg/mL concentration.

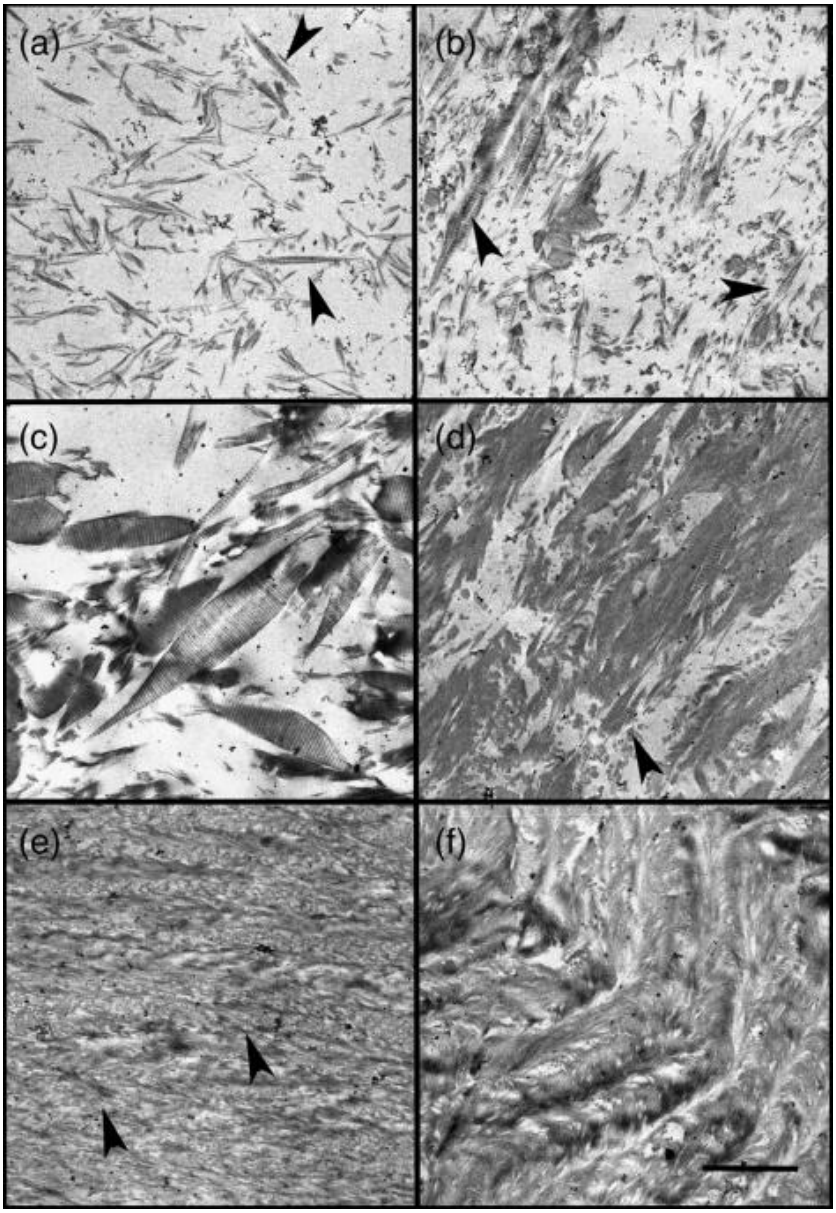


Figure 7: Effect of collagen concentration on fibril structure. (a) 40 mg/ml, (b) 75 mg/ml, (c) 100 mg/ml, (d) 200 mg/ml, (e) 300 mg/ml, (f) >> 300 mg/ml. (F. Gobeaux, 2007)

2.2.2 pH

pH is known to be essential in the electrostatic interactions between the collagen molecules during fibrillogenesis. The overall stability of the collagen fibrils as well as the diameter and the D-spacing of the fibrils is dependent on the pH level of the solution (Li, Y, 2009). In a study of fibrillogenesis in a range of pH, it was shown that fibrillar structures were

present in solution with pH ranging from 5.5 to 9.5. Under 5.5 pH, there was no formation of fibril structures, while higher pH, ranging from 9.7 to 10.3, it resulted in greater spacing between the collagen fibrils. After pH of 10.5, the fibrils did not attach onto the surface (Jiang, Hörber, 2004). Figure 8 show that from a pH range of 7.1 to 9.2, there were collagen fibrils present on the surface. The varying degrees of diameter with different pH is due to the electrostatic interactions between the collagen molecules. With a lower pH, there is more availability in the H^+ ions attaching to the collagen molecules, which causes more repulsion between the collagen molecules. The thinner but more spaced out collagen fibrils shown in the figure illustrates this phenomenon.

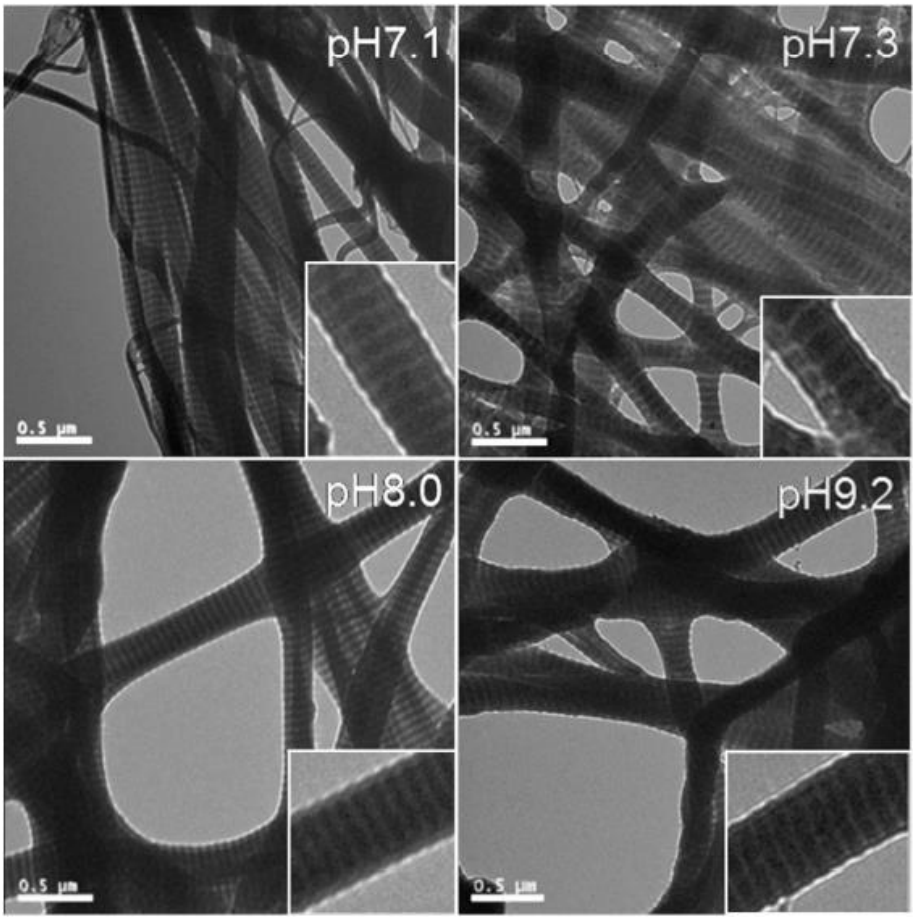


Figure 8: Effects of pH on collagen fibrillogenesis. (Li, Y, 2009)

2.2.3 Electrolytes and Ionic Concentration

The appearance and organization of the collagen fibrils is dependent on the presence of electrolytes. Variations of electrolytes and their concentrations on the effect of collagen fibrillogenesis were studied on Mica substrate, which is widely used for current studies in collagen fibrillogenesis. It was found that potassium ions gave the characteristic D-spacing of collagen fibrils, and demonstrated better fibrillogenesis than other kind of ions such as NaCl or $MgCl_2$as shown as Figure 9. The periodic D-spacing of the collagen fibrils was also studied by a variation of range from 50 to 300 mM concentration of the potassium ions on Mica substrate. The presence of potassium ions also inhibited more than one layering of the collagen fibrils on the surface (Jiang, F., Hörber, H., 2004).

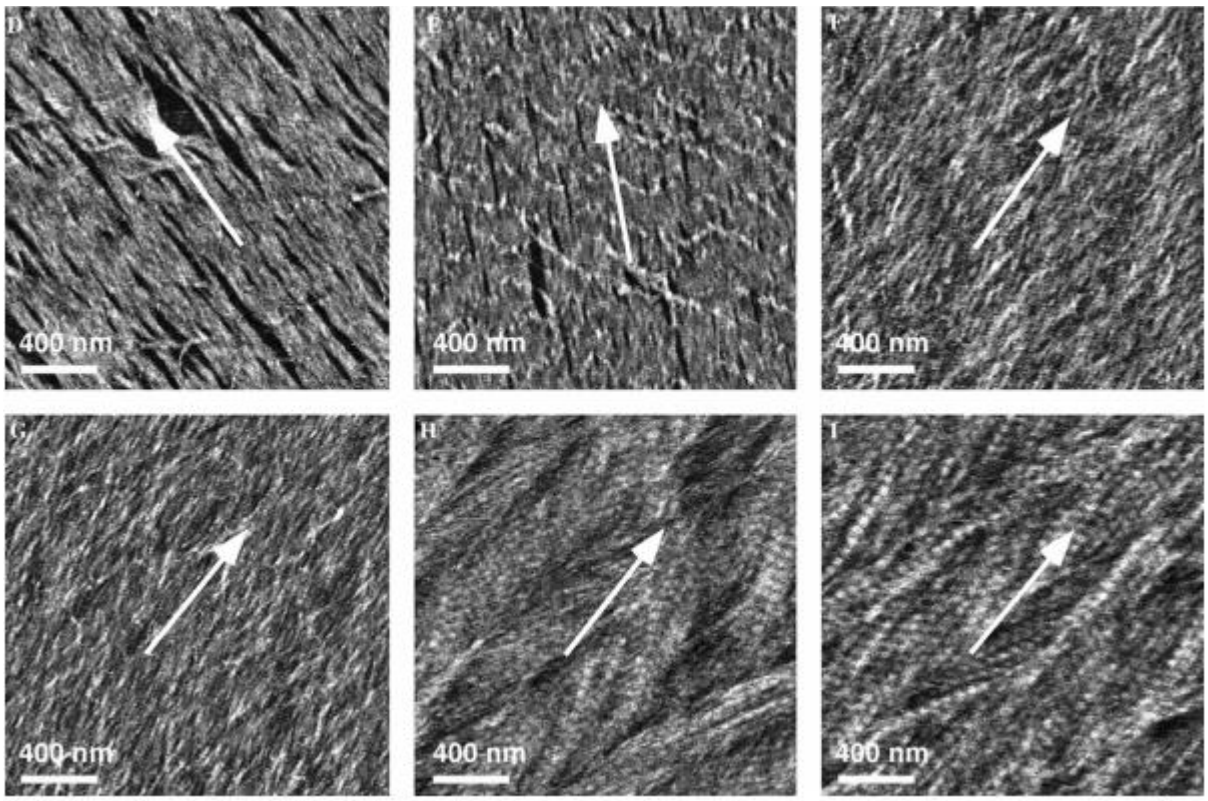


Figure 9: Self-assembly of collagen fibrils with varying electrolytes. (d) 200 mM NaCl, (e) 50 mM KCl, (f) 100 mM KCl, (g) 200 mM KCl, (h) 200 mM KCl, and (i) 5 mM MgCl2. (Jiang, F., Hörber, H., 2004)

2.3 Collagen Alignment

Collagen alignment in the assembly process is one of the key factors to understand the role of collagen matrix during the mineralization process. Although the feature of collagen alignment has been studied in many papers, there has not been any studies done conductive substrates. The studies of fibrillogenesis that is mentioned in the above sections were performed on a mica substrate, which has characteristics in the atomic structure conducive to the adsorption and alignment of collagen. For this study, in order to be able to characterize the piezoelectric properties of collagen fibers, there needs to be an alignment of collagen fibrils on a metallic substrate to convey electrical signals. A method explored in this study to have a well-aligned collagen matrix on a metallic substrate is through the utilization of the polarity in the collagen molecules with the application of an electrical field during fibrillogenesis.

2.4 Ideal Metal Surface for Collagen Coating

The preceding research to this current research was related to the determination of the ideal metal substrate most conducive to the collagen coating on its surface. The metal surfaces silicon, platinum, and gold were chosen as platforms for potential testing of the piezoelectricity of the collagen fibrils. The determination of the material was based upon the well-known features of biocompatibility and resistance to corrosion.

The ideal metal surface was determined by inducing self-assembly of collagen fibrils onto the surface and testing the conductivity of the metal. The resulting collagen assembly is shown in Figure 10. The result was that platinum was the best candidate for further testing of the piezoelectricity of collagen fibers (Deng, Z. 2018).

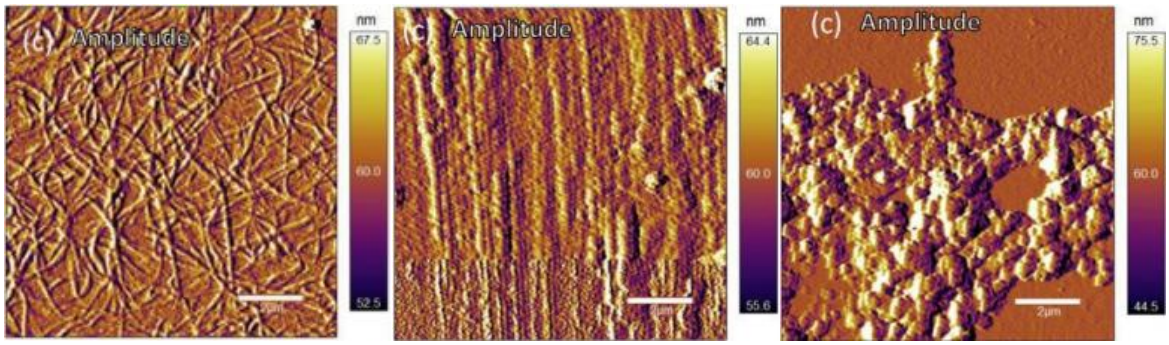


Figure 10: Collagen fibrillogenesis on silicon, platinum, and gold surfaces.

2.5 Atomic Force Microscopy

AFM is a high-resolution scanning probe microscopy. The major functions of the AFM are imaging, force measurement, and manipulation. In this study, the Asylum Research MFP-3D Infinity AFM, shown in Figure 11, was used to obtain topographic image of the collagen matrix.

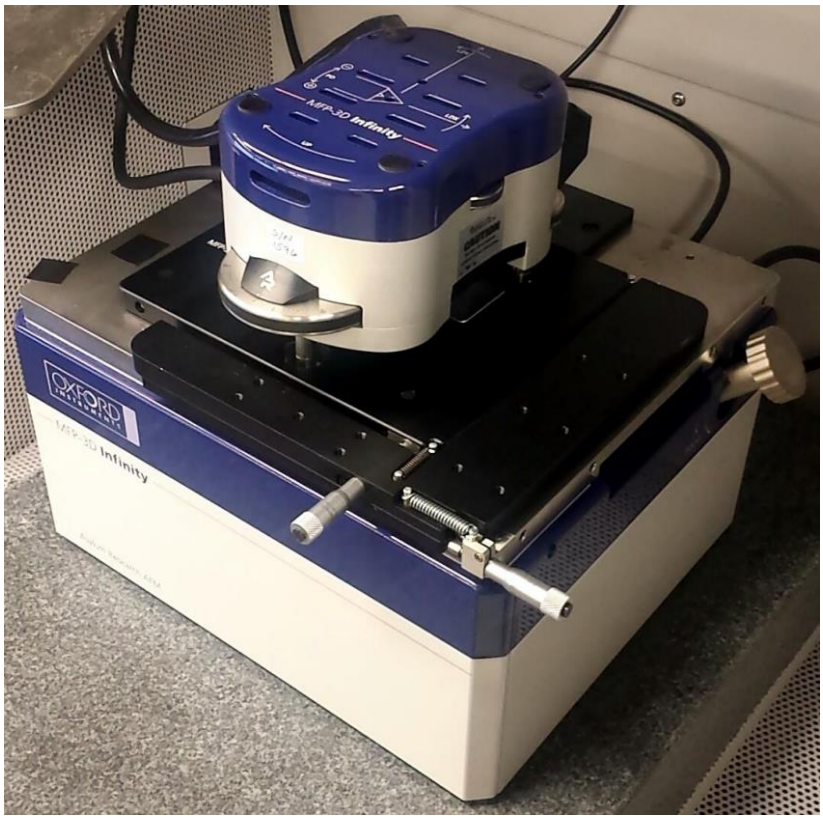


Figure 11: Asylum Research MFP-3D Infinity AFM.

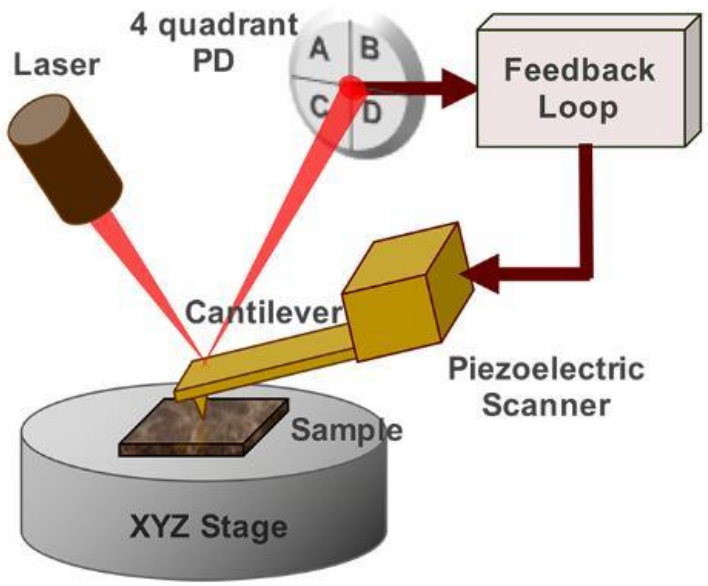


Figure 12: AFM operational components.

Basically, the AFM system utilizes cantilever beam which has sharp tip at the end as shown in Figure 12. By approaching the tip to the sample surface, the force between tip and sample is generated due to the contact. This generated force causes deflection of the cantilever which is captured by photodiode embedded in the system. By calculating the deflection of the cantilever, the height information of the sample is measured. Finally, by scanning the sample, three-dimensional topography of the sample is obtained.

Generally, there are two operational modes to obtain topographic image of a sample: contact mode and tapping mode. In contact mode, the cantilever tip is kept in contact onto the surface of the sample. By scanning the sample, the morphology of the sample is obtained. In the other hand, the cantilever tip is oscillated far from the sample, and the tip approaches to the sample. Eventually, the tip taps on the sample resulting in the amplitude change of the tip oscillating. By tracking the amplitude change, the morphology of the sample is obtained.

In this study, the tapping mode was used because it is more sensitive to morphological change of a sample, and the contact mode can cause mechanical damage to the sample.

Chapter 3: Experimental Methods

3.1 Design of Experimental Procedure

The experimental methods for this research was based on the literature reviews and the methods and procedures listed. Instead of the mica surface used for the previous studies, the methods were repeated for a platinum surface. A well-aligned collagen fibril arrangement on the platinum substrate would then be tested for piezoelectric properties.

The parameters varied in this experiment were the ones stated above: collagen concentration, pH, and ionic concentrations. In addition, different methods to have collagen on the metal substrate were considered, along with their respective incubation times. The variation of each parameter was done separately, independent from one other. The systematic approach to this experiment was to find the ideal condition of one parameter, and once it was found, the next parameter was varied to find the best conditions, added to the previous ideal conditions.

3.2 Sample Preparation

3.2.1 Metal Substrate Cleaning

To ensure that the collagen fibrillogenesis would not be disrupted by any foreign contaminants on the surface of formation, the substrate was cleaned. The substrates were submerged in acetone that was heated to 45 deg Celsius for 10 minutes to eliminate oils and organic residues on the surface. Then they were submerged again into methanol for 5 minutes. Afterwards, they were placed in the ultra-sonicator filled with deionized water and sonicated for 20 seconds. The substrates were then removed and placed on glass slides to dry for usage.

3.2.2 Preparation of Collagen Solutions

Type I collagen extracted from rat tail (Advanced Biomatrix, Inc. and Corning, Inc.) was used in this study. The collagen concentrations were 4.0 mg/mL (Advanced Biomatrix, Inc.) and 4.42 mg/mL (Corning, Inc.), both in 0.02 N acetic acid. A phosphate-buffered saline (PBS) was prepared with deionized water. For the addition of potassium electrolytes with a fixed concentration, the amount of KCl was calculated and added to the PBS solution. The collagen stock was added at the last step to prevent the start of fibrillogenesis until all aspects of the experiment was set up. After the addition of collagen stock into PBS + KCl solution, the solution was swirled to ensure the thorough dispersing of the collagen molecules inside the solution. The resulting PBS + KCl + collagen solution was applied directly to the metal substrate. The study utilized a submersion method, where the substrate was submerged in a dish filled with the collagen solution. The dish and the submerged substrate were placed then into an oven of 37 deg Celsius to begin fibrillogenesis onto the substrate.

3.2.3 Voltage Application

For application of voltage to the metal substrate, the substrate required an addition of wires to attach to the power source. Wires were attached to the metal on the corners of the substrate by CircuitWorks Conductive Silver Epoxy (TedPella). The substrate with the wires attached were cured in the oven at 70 deg Celsius for 1 hour. The length of the curing time was to ensure the epoxy completely solidified, and that maximum conductivity and adhesion was obtained between the interface of the wire and the platinum. Figure 13 shows the resulting substrates prepared for voltage application.

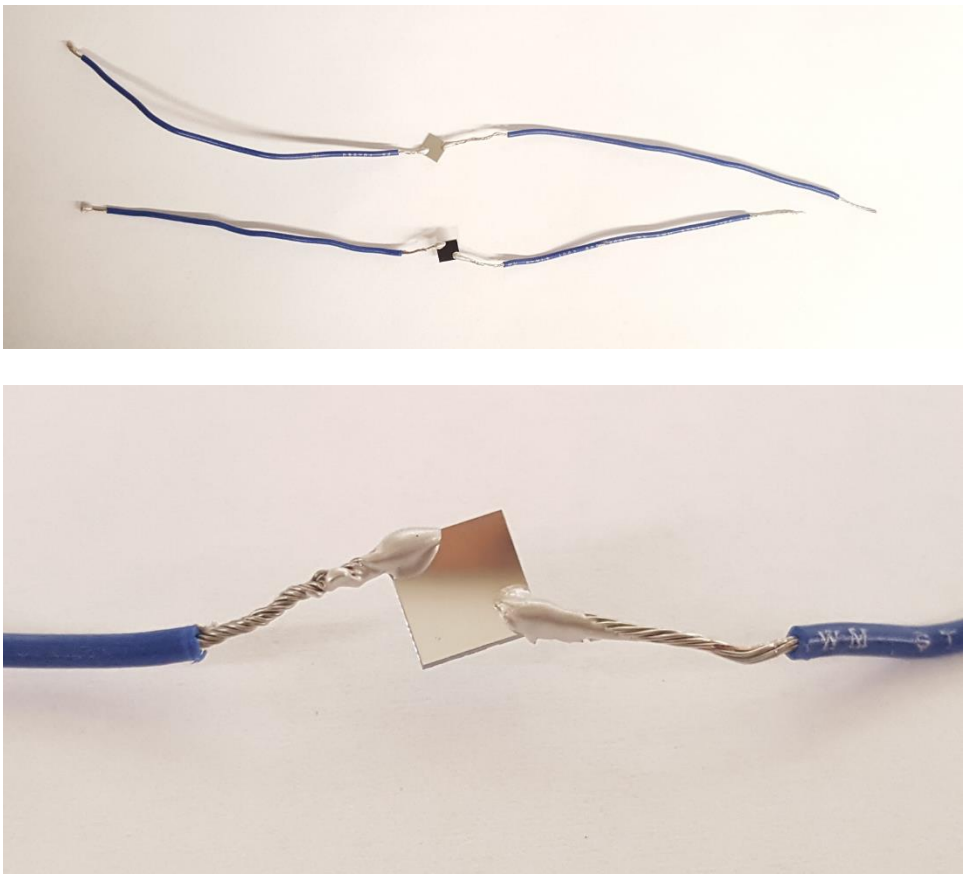
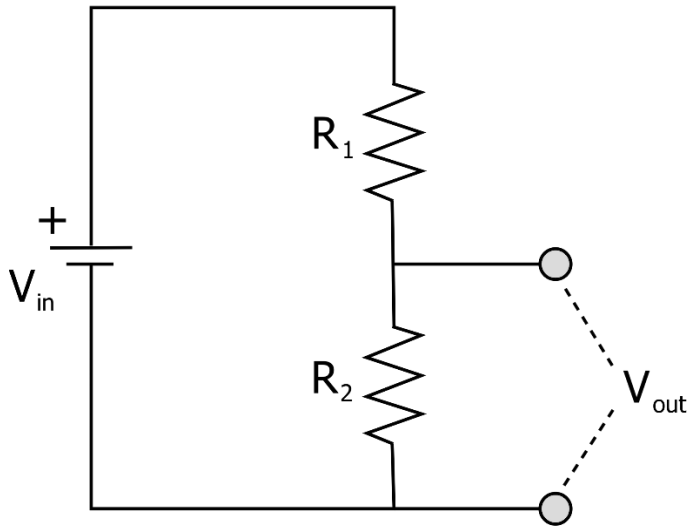


Figure 13: Platinum substrate with wires for electric field application

In order to apply a fixed amount of voltage to the metal substrate, a voltage divider circuit was set up, shown below.



A 3V DC power supply was used for the V_{in} terminal. The substrate was placed as V_{out} in the circuit and the measured resistance of the substrate was set as R_2, which measured a consistent value of around 10 Ω. The applied voltages for V_{out} were 0, 10, 100, 500, and 1000 mV to see a trend in the effect of voltage on collagen. The resistance value of R_1 was calculated with the equation shown:

$$R_1 = \frac{V_{out}}{V_{in} * R_2} - R_2$$

The substrate was placed in an oven at 37 deg Celsius. The power supply was turned on at the start of the experiment, before the collagen solution was placed on the substrate to ensure that there was a voltage across the substrate when the collagen comes in contact with the substrate.

3.3 AFM imaging

After the completion of the incubation time for collagen formation, the substrate was removed from the oven, disconnected from any circuits, and submerged in deionized water to remove any additional layers of collagen or excess minerals that have attached to the surface. After rinsing, the substrates were placed on glass slides to air dry in preparation for AFM imaging.

The AFM tapping mode with OMCL-AC240TS cantilever (OLYMPUS, Inc.) which has resonance frequency at around 70 kHz and 2 N/m nominal spring constant was utilized in the imaging of the samples. The samples were scanned at room temperature. The drive frequency was 85 kHz with a drive amplitude of 1.5 V. The setpoint voltage varied from 500 to 800 mV

and the integral gain varied from 5 to 15 V to get a better resolution of the images between the different scan sizes.

Chapter 4: Results and Discussion

4.1 Overview

In this study, different parameters were investigated to obtain the optimal conditions to have a well-aligned collagen matrix on a platinum substrate. Collagen concentration, pH level, and KCl concentration were varied to promote collagen self-assembly on the metal surface. An application of an electric field was varied to promote an alignment of the collagen fibrils during self-assembly. The variation of each parameters is detailed below along with the results and their interpretations.

4.2 Collagen Self-Assembly

4.2.1 Collagen Concentration

For the determination of ideal collagen concentration, the range of concentration was determined by many of the previous studies done on similar experiments. Two concentrations, 10 ug/mL and 32 ug/mL were tested on a Mica substrate, as a precedence for the values that were to be used for the metallic substrate. Figure 14 below show the resulting images from the respective collagen concentrations.

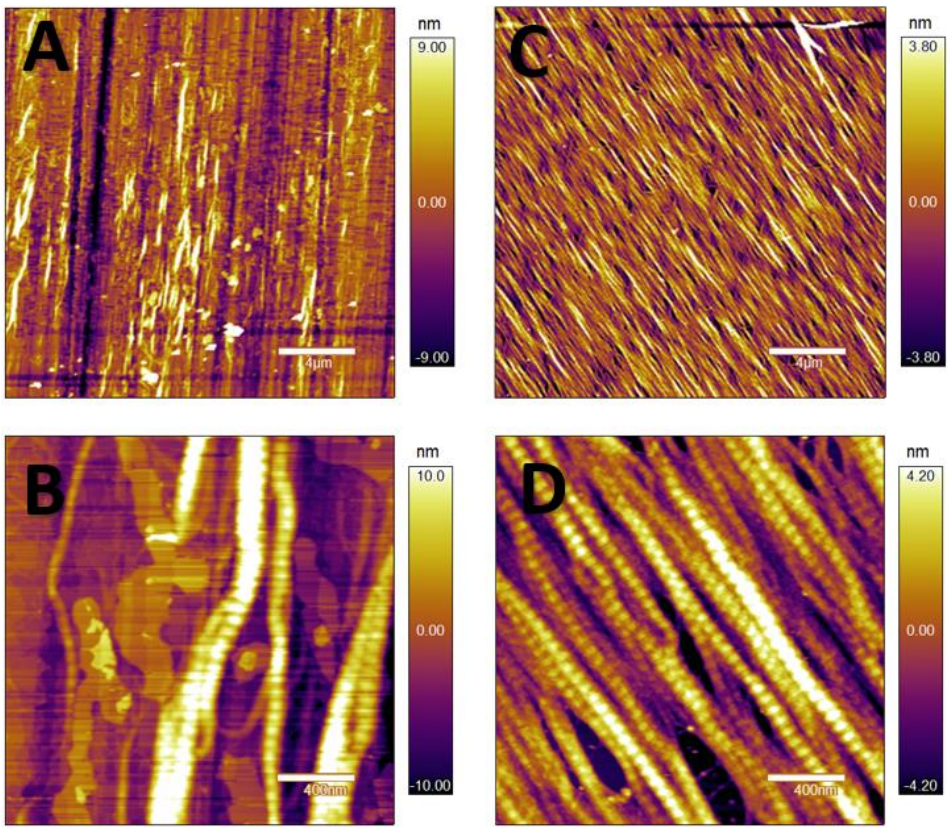


Figure 14: Scanned height for variation of collagen concentration. (a) and (b), 10 ug/mL. (c) and (d), 32 ug/mL.

Collagen concentration of 32 ug/mL was chosen as precedence for the experiments. The images from the AFM shows a well-coated surface of collagen fibrils for the respective collagen concentration. The closer scanned size (B, D) confirms that the fibril structures are collagen fibrils, as indicated by the characteristic D-spacing shown.

4.2.2 pH

The AFM images of self-assembled collagen on platinum substrates under the conditions of pH 5.0, 7.3, and 9.0 are shown in Figure 15 below. The results are consistent with the literature (Li, Y, 2009), as there is a slight increase in the diameter of the collagen fibrils with

increasing pH. The absence of collagen fibrils at pH 5.0 also confirm the literature (Li, Y, 2009) as they do not attach to the substrate with pH under 5.5.

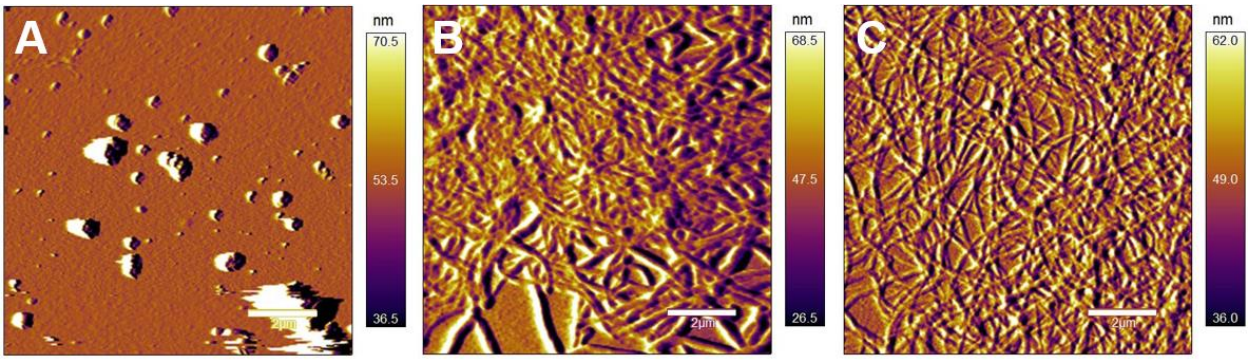


Figure 15: Scanned height for variation of pH. (a) pH 5.0, (b) pH 7.3, (c) pH 9.0.

4.2.3 Ionic Concentration

The ionic concentration was varied, ranging from 0 mM to 400 mM of potassium ions. Figure 16 and 17 show the resulting scanned images. The sample with no ionic concentration showed no fibrils on the surface. The closer scanned image shows some collagen molecules that ~~may~~ would have started to assemble. The sample with 50 mM potassium ions shows collagen fibrils. However, the closer scans do not show the D-spacing that collagen fibrils are to have. The ideal potassium concentration was shown to be between the ranges of 100 to 200 mM. The fibril structure is present, along with the characteristic D-spacing. The 300 mM potassium concentration showed no fibrils on the surface.

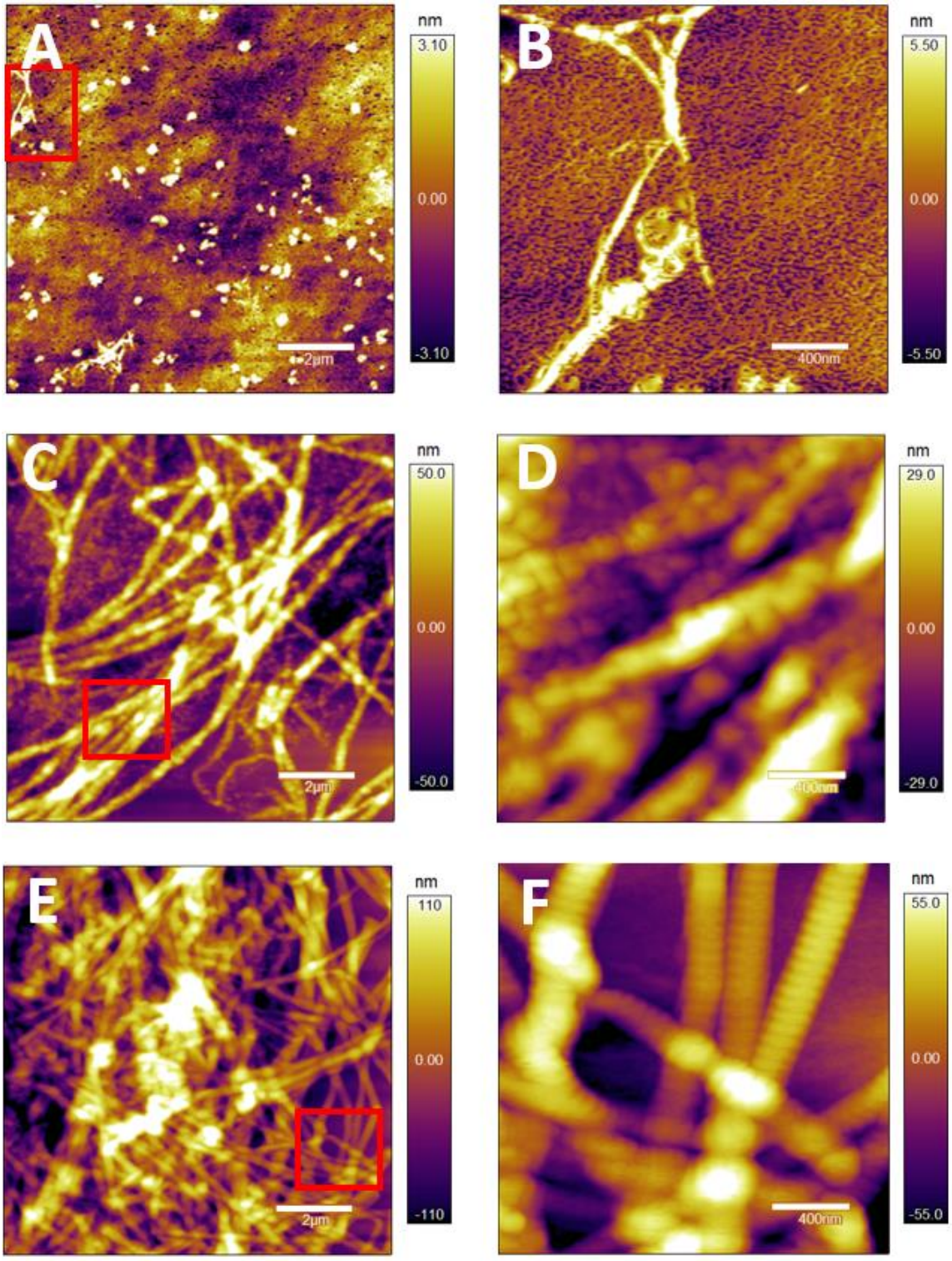


Figure 16: Scanned height for variation of KCl concentration. (a) and (b) 0 mM, (c) and (d) 50 mM, (e) and (f) 100 mM. Left column, scan size of 10 um to show overall spacing and coverage of fibrils. Right column, scan size of 2 um to show characteristic D-spacing of collagen fibril.

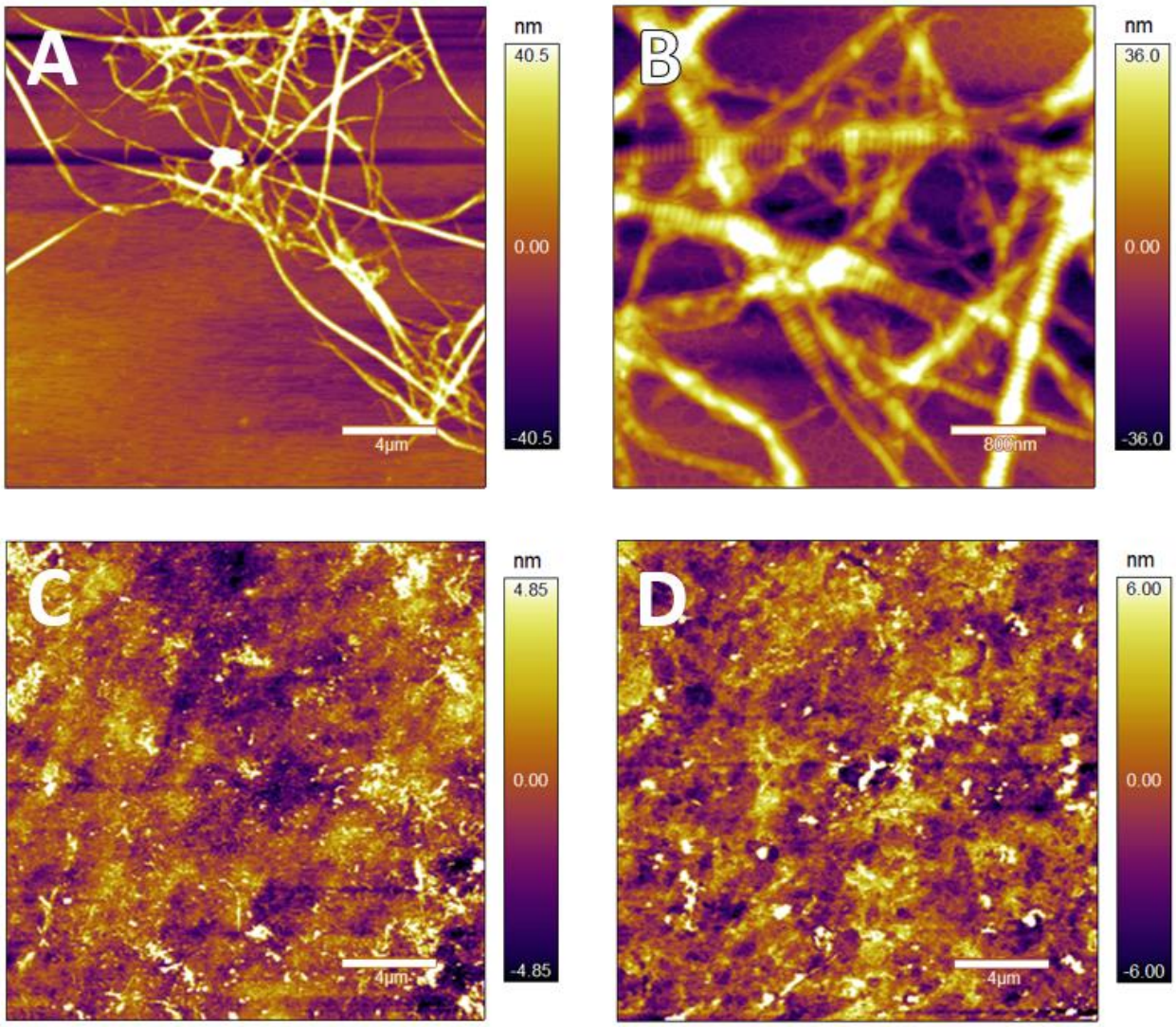


Figure 17: Scanned height for variation of KCl concentration. (a) and (b) 200 mM, (c) and (d) 300 mM. Left column, scan size of 10 um to show overall spacing and coverage of fibrils. Right column, scan size of 2 um to show characteristic D-spacing of collagen fibril.

4.3 Collagen Alignment

For collagen alignment, an electric field was induced onto the platinum surface. The voltages that were applied were 10 mV, 50 mV, 100 mV, 500 mV, and 1 V. The substrates were imaged using the Scanning Electron Microscope (SEM) to get an image of a larger area in order to check whether the collagen fibrils had aligned in a certain direction. Figure 18 show the

results for the different voltages applied. Unaligned collagen fibrils were present for all varying voltages.

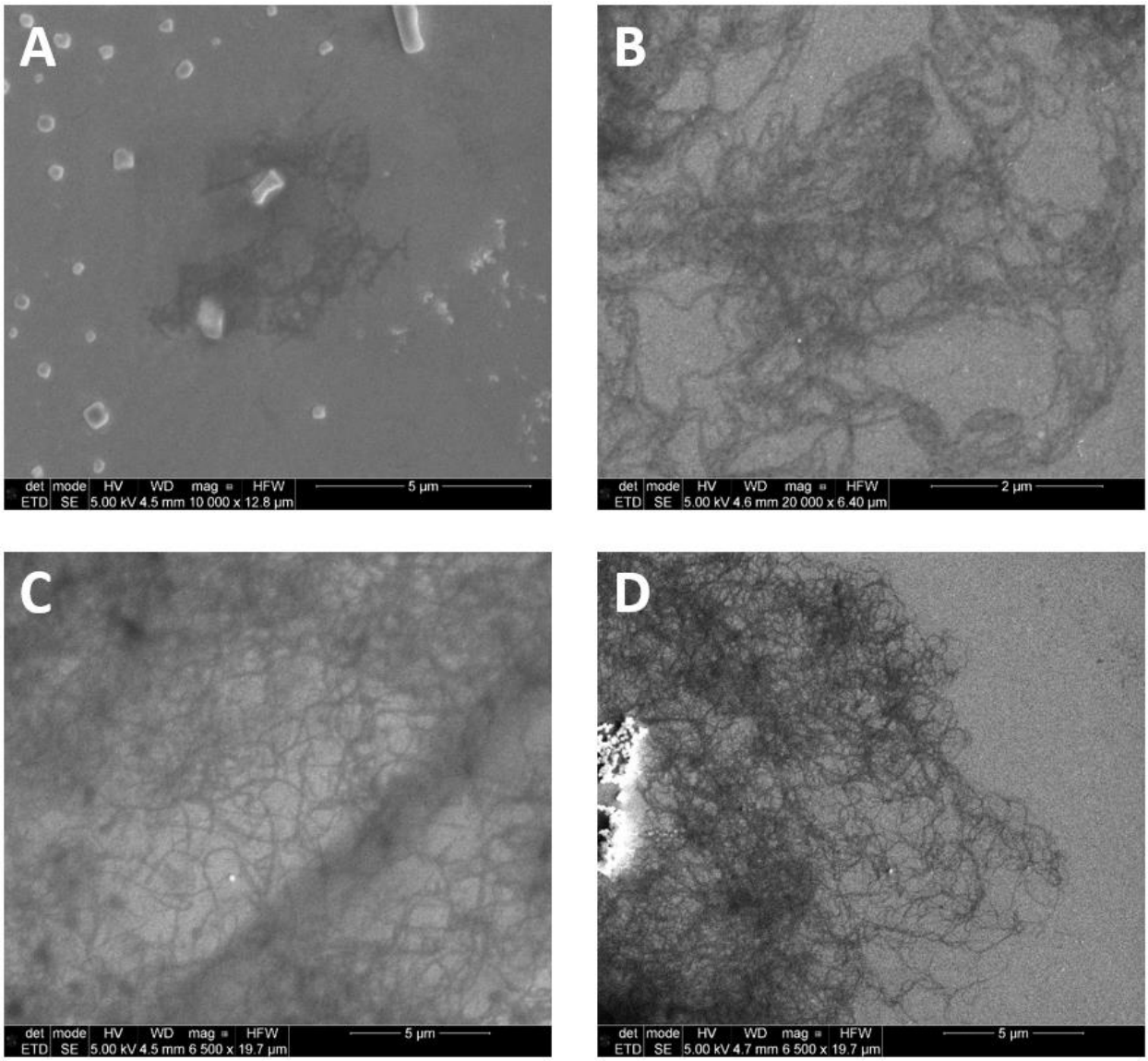


Figure 18: SEM images for variation of voltage application. (a) 0V, (b) 100 mV, (c) 500 mV, (d) 1 V. Scales are 5 um for a, c, d, and 2 um for b. Unaligned collagen fibrils present for every voltage application.

Figures 19 and 20 show a scanned image near the corners of the substrate. Figure 19 shows some blotches that are thought to be collagen fibers. There seems to be a general direction

in which these blotches seem to be aligned. Figure 20 shows the AFM images of some of those blotches. The scanned areas show some fibril-like structures. In confirming they are collagen fibrils, however, the closer scanned images do not show the characteristic D-spacing.

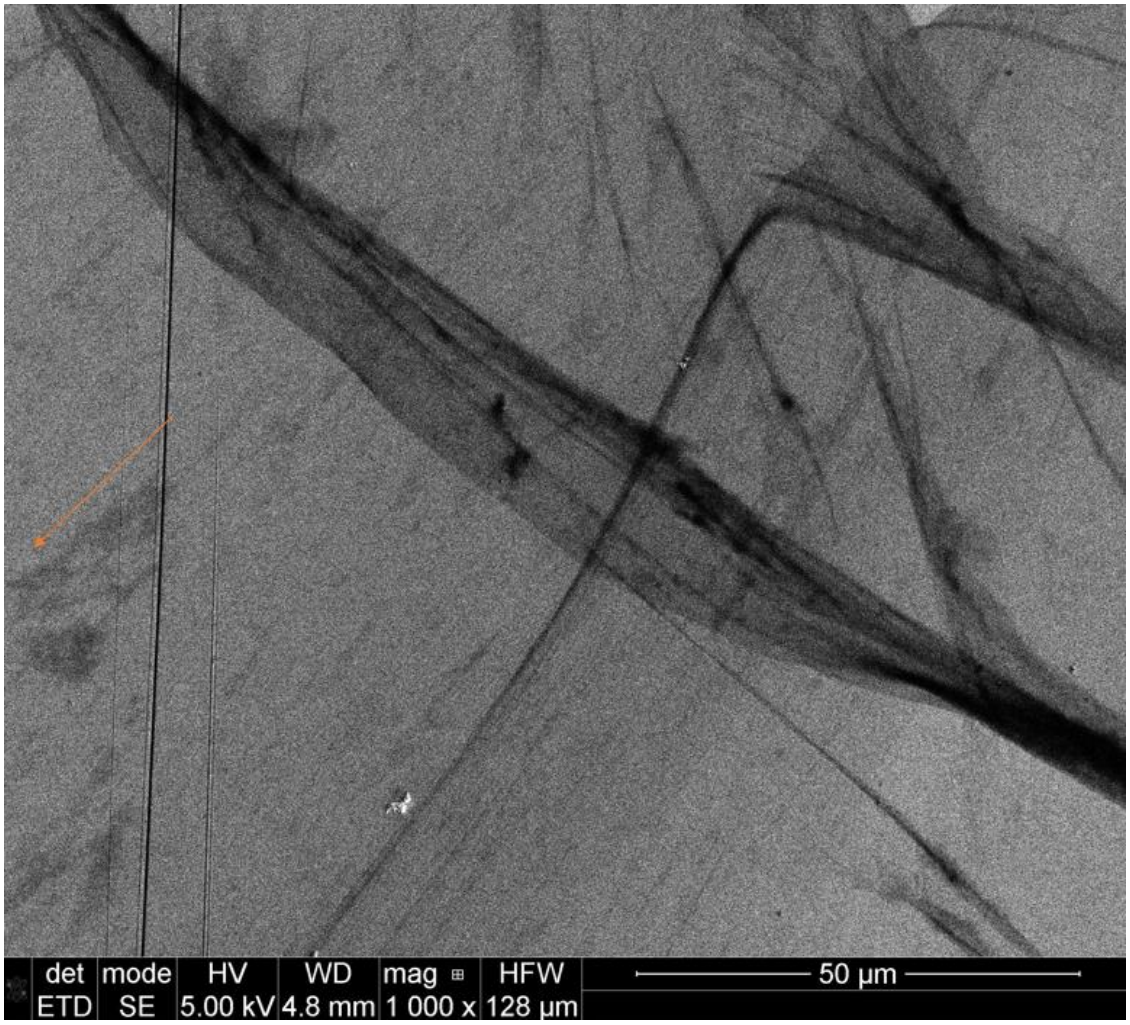


Figure 19: SEM image of 1V application. 50 um scanned size. Directional alignment of blotches thought to be clumps of collagen fibrils.

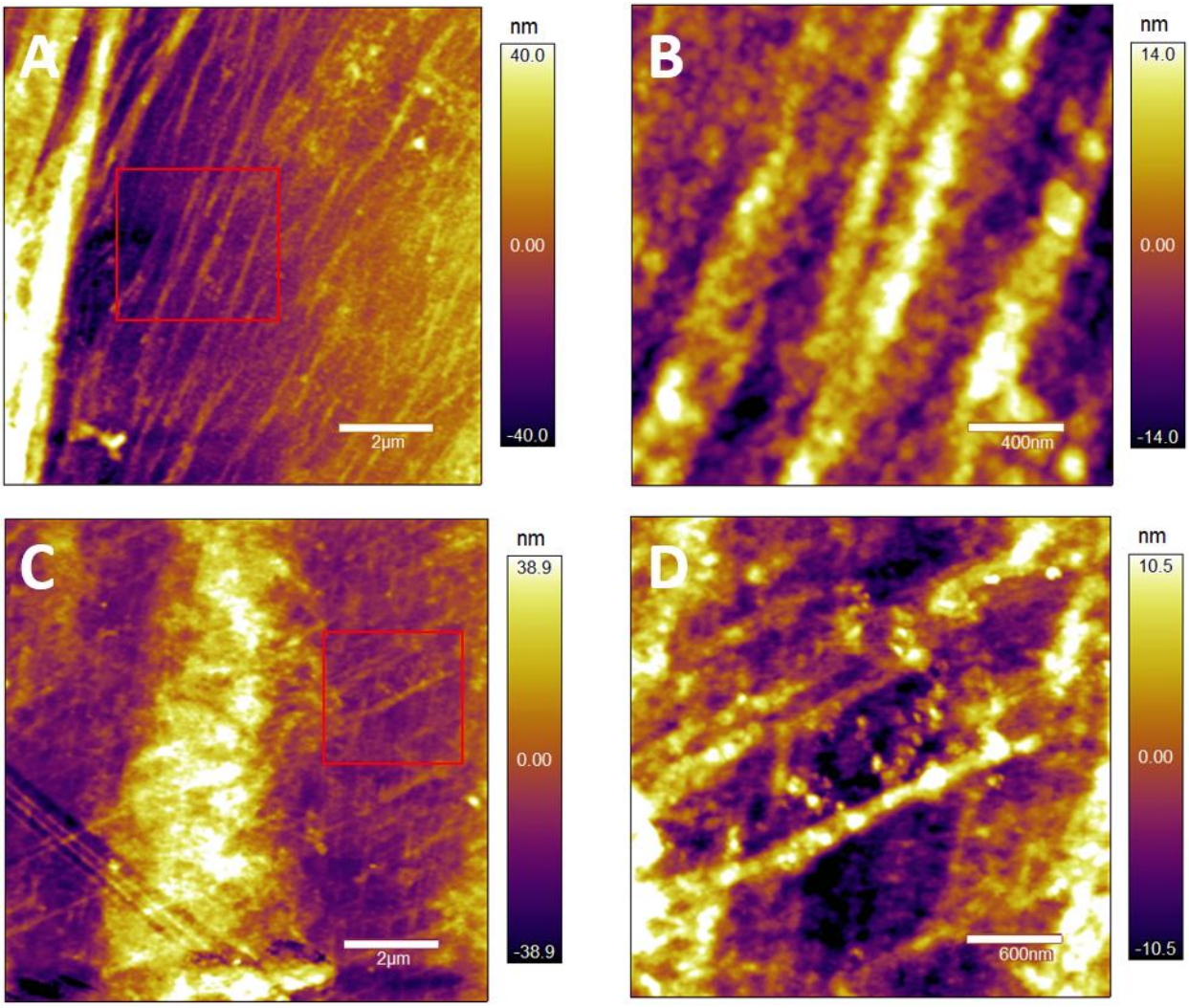


Figure 20: Scanned height for 1 Volt application. (a) and (b) on one location. (c) and (d) on another location.

Due to the polarity of the collagen molecules, it was hypothesized that an application of voltage onto the substrate would cause the alignment of the collagen molecules during its self-assembly. With an assumption that this holds true, the reason why it could not have aligned for this experiment is due to the substrate being submerged in an ionic solution. The electric field applied to the substrate may have not been uniform due to the sharp edges of the substrate and also the discharge of the electric field onto the ionic solution. One way to mitigate this to pipette

a droplet of the collagen solution onto the substrate instead of immersing the entire substrate into the solution.

Chapter 5: Conclusion

5.1 Contributions

Previous extensive studies of collagen were done on Mica substrate. There is a lack of studies on the self-assembly of collagen on a conductive substrate. The significance of this study was the determination of certain parameters, such as collagen concentration, pH, and ionic concentration that is conducive to the self-assembly of collagen on a conductive substrate. Parameters producing the self-assembly of collagen on a conductive substrate have been refined. Preliminary data with application of electric field causing collagen fibril alignment has been obtained. Further refinement of the experimental methods can be tested to affirm whether an electric field would cause collagen fibril alignment.

5.2 Future Work

The alignment of collagen can be further explored with refinement of the experimentation with voltage application on the substrate. Instead of submerging the substrate, the solution can be pipetted with droplets on the surface of the substrate to prevent extensive leaking of electric charge from the surface into the ionic solution. To mitigate for potential heating of the substrate, a better circuit with more current limiting components can be implemented.

As mentioned previously, the long-term goal of this study is to elucidate the mechanisms of bone mineralization. More specifically, this study is to explore one of the theories responsible

for this process—the piezoelectricity of the collagen matrix. A platinum substrate with well-coated and aligned collagen fibrils will comprise the test bed that will be utilized to test and affirm piezoelectric properties.

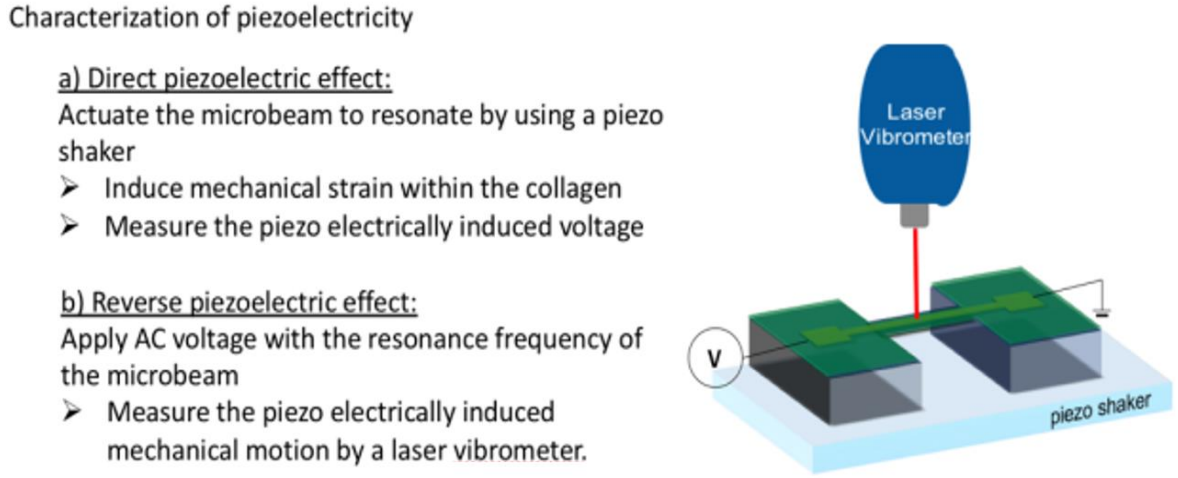


Figure 21: Schematic of future experiment

This testing will be conducted on a microbeam fabrication with a piezoelectric shaker to induce mechanical strain within the collagen film. The setup is shown in Figure 21. The electric potential generated by the piezoelectricity can be measured by the voltage across the electrodes on the microbeam. To characterize the reverse piezoelectric effect, a voltage will be applied through the electrodes onto the collagen film. The motion induced by its piezoelectricity will be measured by the laser vibrometer.

5.3 Summary

The self-assembly of collagen on a platinum-coated substrate was refined with the variation of parameters, such as collagen concentration, pH, and ionic concentrations. A method

of obtaining the alignment of collagen on the platinum surface was explored by the application of an electric field. Further refinement of alignment methods can be studied and applied into future experiments to study the role of collagen piezoelectricity in the mechanism of bone mineralization.

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