UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Físicas y Nanotecnología

TÍTULO: Collagen Remineralization Induced by an Electric Field

Trabajo de integración curricular presentado como requisito para la obtención
del título de Ingeniero en Nanotecnología

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Urcuquí, agosto 2019

Urcuquí, 22 de agosto de 2019

SECRETARÍA GENERAL
(Vicerrectorado Académico/Cancillería)
ESCUELA DE CIENCIAS FÍSICAS Y NANOTECNOLOGÍA
CARRERA DE NANOTECNOLOGÍA
ACTA DE DEFENSA No. UITEY-PHY-2019-00013-AD

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Acknowledgements

I would like to thank to my tutors Gema Gonzalez and Werner Bramer for the guidance through this project. Especially for the help and the teachings during its development. Thanks to my family who supports me, without them nothing of this would have been possible.

Resumen

El colágeno es la proteína más prevalente en todo el cuerpo humano, esta proteína tiene muchas propiedades importantes que sostienen las funciones biológicas. Como la principal proteína que compone el hueso, es importante comprender los mecanismos relevantes relacionados con la curación de fracturas. La curación de fracturas se ha abordado desde diferentes fenómenos, entre ellos se ha demostrado que los Electric Stimulus (ES) son efectivos, pero con un mecanismo de acción poco claro. Los mecanismos propuestos discutidos en la literatura son el resultado de experimentos *in vivo* e *in vitro* que son muy complejos, porque ambos involucran células y proteínas. Hasta donde sabemos, ninguna investigación en este campo ha abordado el mecanismo para la biomineralización del colágeno por ES sin los otros componentes biológicos. En este proyecto, estudiamos la biomineralización del colágeno con ES aplicado con Capacitive Coupling (CC) en Simulated body fluid (SBF). Determinamos si la polarización de la carga de colágeno es uno de los mecanismos por los cuales el estímulo eléctrico mejora la curación de fracturas. Nuestro trabajo muestra que los electrodos aplicados directamente sobre el colágeno mejoran su biomineralización, por lo tanto, la polarización del colágeno parece ser un mecanismo involucrado en ES utilizado para la curación de fracturas.

textbf Palabras clave: Colágeno, biomineralización, acoplamiento capacitivo, estimulación eléctrica, fluido corporal simulado.

Abstract

Collagen is the most prevalent protein in the whole human body, this protein has many important properties that sustain biological functions. As the main protein composing bone it is important to understand the relevant mechanism that are related to fracture healing. Fracture healing has been addressed from different phenomena among them the ES has been demonstrated to be effective, but with unclear action mechanism. The proposed mechanisms discussed in the literature is the result of *in vivo* experiments and *in vitro* experiments which are very complex, because they both involve cells and proteins. No investigations in this field, as far as we know, have addressed the mechanism to collagen biomineralization by ES without the other biological components. In this project, we study the biomineralization of collagen with ES applied with CC in SBF. We determine if collagen charge polarization is one of the mechanisms by which electric stimulus enhances fracture healing. Our work shows that electrodes directly applied on collagen enhances its biomineralization, therefore collagen polarization appears to be one mechanism involved in ES used for fracture healing.

Keywords: Collagen, biomineralization, capacitive coupling, electrical stimulation, simulated body fluid.

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Chapter 1

Introduction

Body healing constitutes a huge area of research and study. Analyzing human body response to stimulus is the key to achieve progress in wound healing. The stimulus of body self response is usually a less invasive way to treat wounds which is an advantage [1,2], because the self body response will not usually cause any allergic reaction or higher complications. In this category, Electric Stimulus (ES) has attracted attention and several studies have been developed in this field [2–4]. Most of these investigations focus on a way that ES enhances bone healing [1–8]. In this project we will study the effect of electrical stimulation on collagen response in order to accelerate the remineralization in a novel manner. We will use a different approach than most investigations have reported as far as we know, since the literature has addressed this problem either by using *in vivo* studies or *in vitro* studies with cells and proteins. In this project we use ES to induce collagen biomineralization in simulated body fluid, without the presence of cells.

Bones are structures composed mainly of three components: collagen, hydroxyapatite and H_2O [9,10]. In general bone composition is: 65% of inorganic compound (Hydroxyapatite), 25% organic matrix(Collagen) and 10% water [4,9–13]. Collagen is a protein formed by a repetitive pattern of three polypeptides chains formed by around 1000 aminoacids each it works as the main building block for many tissues in the body, like bones [9,10]. As mentioned before, collagen alone is not the only component in bones, there is also a mineral called hydroxyapatite. The general chemical formula of hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$ [12]. There are several possible replacements for anion (OH) and cation (Ca) [10,12,13]. The family of hydroxyapatite found in bones is also known as carbonated apatite, it is denoted as $(Ca_5(PO_4, CO_3)_3(OH))$ [9,10]. Collagen fibrils mineralized with hydroxyapatite and structural H_2O are the main components of bone [9,10,14]. Still bone structure is more complex than that, there are several levels of organization in the 3D macro structure of bone [9–11].

We can describe collagen from nanostructure to macrostrcutre starting from the polypeptide chain. The structure starts from the amino acid chain composed of ((Proline, Hydroxiproline)(Glycine)(Proline, Hydroxiproline))$_x$ then three polypeptide chains will join together to conform a specific triple helix structure (tropocollagen), the staggered arrangement of tropocollagen will conform fibrils [9,10,15]. This fibrils will make up fibers [9,10,15] and depending on the organization of the collagen fibers there are several types of bone tissue [9,11]. From fibers as mentioned above there are

further structures dependent of the 3D arrangement of them[11]. There are seven levels of hierarchical organization for bone[9]. This levels of organization are created thanks to biological mechanism inside the body, to reach the known macro structure of bone. We can apreciate the collagen hierarchical of a tendon in figure 1.1

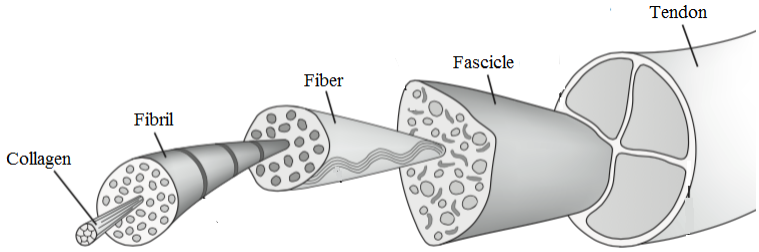

Figure 1.1: Hierarchical organization of collagen in tendon[16].

Collagen is a protein with crystalline structure[4,5], in a similar way tropocollagen staggered array has periodic structure[9–11,14]. This periodic structure is called fibrils and it has gaps every 64 nm, this gaps are filled by molecules like water and Hydroxyapatite[9,10]. Hydroxyapatite is also found in the inter matrix of fibrils which conform fibers[9,10]. As mentioned above the 3D conformation of this fibers is relevant to the final properties of the collagen[11].

Summarizing, we have seen that bone tissue is such a complex system and the principal components are collagen(25%) and hydroxyapatite(65%). In addition unmineralized collagen has an electrostatic feature, this might give bone signaling paths to start healing and recover its complete form[4]. This feature is piezoelectricity and causes that bone have a charge polarization resultant from mechanical loading[4,14].

Collagen is the protein constituent of bone tissues, research has been developed around collagen properties and interactions[4–6]. Collagen is a piezoelectric material[4,5]. A piezoelectric material is a material that undergoes charge polarization when it is been mechanical deformed and viceversa[4,14]. Since collagen is subjected to constant mechanical effort during activities (i.e walking) piezoelectric effect is working all the time. This piezoelectric behavior of collagen presumably is one of the mechanism that starts signaling pathways for the formation of apatite in collagen empty spaces (gaps and inter fibrils spaces)[4,5,11]. In consequence is relevant to study the charge polarization effect for mineralization of collagen.

Electric stimulation has been used by different authors in order to induce bone mineralization[3–6]. This experiments uses ES delivered through electrodes inside samples. This experiments (*in vivo* and in vitro) showed to cause an stimulus over samples mineralization.

From the literature we obtain the following approaches in ES performed fracture healing studies: Applied Direct Current (ADC), Capacitive Coupling (CC) and Inductive Coupling (IC). Everything related to *in vivo* and *in vitro* studies with cells, cellular mechanism and proteins using ES[2,3,5–8,17–20]. Jørgensen reported a test applying a constant electric field CC in an injured human and found an improvement in healing time by 30 percent[5]. Another experiment was carried out by Griffin, *et al.* in an *in vitro* experiment with osteoblast. These authors analyzed the osteoblast proliferation and Alkaline phosphatase activity they found that a degenerate sinusoidal electric wave in CC was the best stimulation for osteoblast and enzymatic activity[6]. Other experiments were also carried out in *in vivo*

systems reporting similar results establishing that ES is effective in the healing process[3,5–8,17–20]. However *in vivo* experiments and *in vitro* experiment are not able to determine the exact mechanisms by which the electric current stimulates the healing process[3]. This might be due to a high amount of variables to handle, this is especially true in the *in vivo* experiments. The same problem is present in *in vitro* experiments that work with osteoblasts and osteoclasts, which are very complex structures, thus complicating the analysis of the experiment.

According to the reviewed experiments on remineralization and bone healing, ES has had a certain degree of success. However, as far as we know all experiments using ES address the bone regeneration issue in a biological way (experiments related to cells and proteins)[1–8,17,18]. Therefore, the proposed experiment for this present work will be the study of the effect of ES on collagen in the presence of simulated body fluid. In order to understand the effect of ES on collagen biomineralization, we studied the direct and indirect application of an electric field over a collagen macro structure with CC configuration. To determine if electric field polarizing collagen and fluid ions is a process that enhances hydroxyapatite formation. This result might be indirectly related to a biological mechanism that undergoes in bone healing. This project did not use cells and proteins, thus reducing the complexity of the system. This gives a greater control over variables and makes the analysis more precise, therefore clarifying if there might be a physical mechanism in collagen due to ES that acts on bone mineralization.

There are several ES to be applied, direct current, periodic current and non-periodic current. ADC is a set up that applies ES directly inside bone placing two electrodes. CC is a set up that applies ES capacitor like not interacting with inner fluids. Lastly IC applies ES using electromagnetic induction. Direct current have shown to be useful, but it has been reported that presents secondary effects[5]. Unexpected secondary effects showed that direct current does not work properly with the biological mechanism of regeneration. In consequence a periodic current and a non-periodic current are considered in this project.

There are several experimental set ups possible as mentioned above. Investigations showed that electric field applied with CC and IC interacts with cellular mechanism (signaling pathways) and release proteins and stored Ca which help to synthesize more collagen[4–6]. For this project we will use CC. In ES we can choose various patterns and parameters. Intuitively as hydroxyapatite is composed of both anions and cations the ES experiment proposed will be a squared wave. Many experiments showed promising results on this set up on periodic ES[3,5]. However the most precise mechanism for a proper biomimetization could possibly be a non-periodic signal that mimics a random mechanical effort that bones are submitted in every day life. It has been reported that an experiment with degenerative sinusoidal wave (with non periodic pattern) has positive results[6]. Following that, in this work we are going to test a non-periodic signal generated with Chua's circuit. Chua's circuit provides a non-periodic signal useful to perform this task.

Consequently, because there is enough evidence that ES might be involved in biomineralization of collagen from all stated above. The project proposal is to study collagen remineralization in simulated body fluid medium with a specific set up with two ES signals periodic and non-periodic. We generated both electric signals and delivered them with capacitor like plates over collagen.

1.1 Objectives

The general objective of this work is to study the effect of ES on collagen hydroxyapatite mineralization.

1.1.1 Specific objectives and Activities

- Obtain collagen from bone demineralization.

  Obtain bones from rabbit legs.

  use EDTA solution to demineralize the collagen samples.

- Design a circuit to generate non-periodic signal.

  Design chua's circuit.

  Test the chua's signal and obtain circuit main parameters (Central frequency and Voltage Peaks).

- Design a system to apply the signal in CC mode on the samples in the presence of simulated body fluid.

  Construct flexible electrodes with adaptable shape.

  Construct a system to continuously deliver SBF to the system without touching electrodes to avoid electro-chemical reactions.

- Study the effect of periodic and non-periodic signals on the biomineralization of collagen

  Do a SEM analysis of the samples.

  Retrieve mineralization speed data related to weigth gain over time.

This research will first review similar work in order to determine the best parameters for action. Then we analyze the bone structure specially in relation to the complexity of collagen. Finally we review collagen piezoelectricity properties which play a major role in this project. In a similar way we will justify the parameters applied, the circuit design and the preparation of materials.

Chapter 2

Literature Review

This chapter will focus on describing the main aspects mentioned in introduction as well as summarizing, briefly, the characterization techniques used in this project.

2.1 Bone structure

Bone is a tissue composed by a protein called collagen. Collagen has several levels for description: tropocollagen, collagen fibrils and fibers. Thanks to several mechanisms of action the simple aminoacids chains will compose several hierarchical structures[9,15]. As we can observe in figure 1.1 collagen has several levels of organization[9,15].

Aminoacids are the most basic structures of collagen. The common aminoacid chain is composed of Proline, Glycine and Hidroxiproline and then organized in tropocollagen, fibrils and fibers[21,22]. The nanostructure organization levels are shown in Figure 2.1. It is imperative that Glycine is repeated every three aminoacids[21], so the collagen aminoacid chain formula will be $(Gly, X, Y)_a$. Where X and Y can be replaced for prolyne or hydroxiproline and this whole small chain repeated a times. This repeated aminoacid chains are joining other two chains and organize further in to a triple helix[9,11,15]. The triple helix structure has very wide conformation, this is due to the 3D conformation of the triple helix which is not planar and there are various possible angles between aminoacid planes[9,21]. This basic structure of collagen is called Tropocollagen which is about 280 nm in length and 1.5nm width[9,15]. Tropocollagen stacked in arrays form fibrils and these fibrils which are about 1 um join together creating collagen fibers of about 10um[15].

Going deeper in the tropocollagen arrangement we observe that this triple helix structure is packed together parallel to each other but shifted from each other by 64nm[9,11]. Also, at their ends they present a gap of about 35 nm[9,11,14]. This gap is really important because in the fibril composite structure it presents a channel thanks to this gap, and there water and minerals (hydroxyapatite) that conform part of the fibrils and further part of the fibers inner spaces[9,11,14].

Part of the versatility of collagen resides in the aminoacid chain capacity to accept certain amount of replacements[21,22], and the other part of the versatility resides in the components that are able to be placed in fibril gaps[9] and

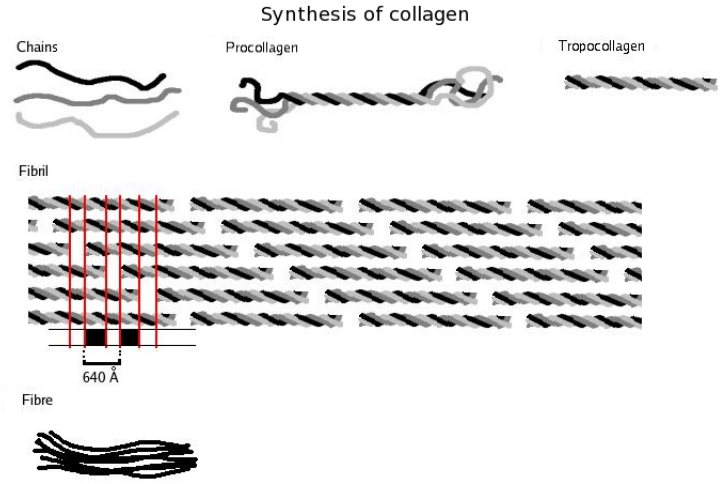

Figure 2.1: Collagen nanostructure organization from aminoacids to fibers[23].

fiber inner spaces[11]. There are also studies that show that minerals are placed in between collagen fibrils[10,11], thus making the 3D structure play an important role in bone properties[11]. This is where hydroxyapatite is an important factor, because as we have mentioned, different ions can replace apatite ions altering its stoichiometry[9,10,14]. In fact stoichiometric of hydroxyapatite is very hard to find in biological systems[10]. Thanks to this, collagen is able to exhibit different mechanical properties in different tissues. We can observe in Figure 2.1 the fibril spacing and the organization of collagen.

2.2 Piezoelectricity and electric stimulus

2.2.1 Piezoelectricity

The classical piezoelectric effect is the relation between mechanical strain and charge polarization on the material which produces a certain potential and viceversa[4,14]. In the most basic mathematic description of piezoelectric effect the strain component X_i is related to a component E_j of electric field by a power expansion that we can see in equation 1, where the subscripts refer to constants and components of a reference axis in the crystal[4]. In consequence, for effects like piezoelectricity, the direction in which strain is applied is important[4]. A material will exhibit the piezoelectric effect depending on its internal structure[4]. Materials that are centrosometric cannot exhibit the piezoelectric effect[4]. Applying a stress on a piezoelectric material will cause it center of gravity to move from its equilibrium position, thus generating an asymmetric charge distribution[4]. In the case of collagen stress applied shows the piezoelectric effect[4,14]. This happens thanks to either the displacement of bonds or the displacement of ions[4]. Furthermore experimental scientists have shown that rabbit bone longitudinal sections methaphysis, epiphysis and dyaphisis have different electric potentials in relation to each other[5]. This shows that a large and well organized

pattern of electric potentials are playing a role on bone tissue, ratificating the importance of this investigation.

$$X_i = C_{1ij}E_j + C_{2ij}E_j^2 + C_{3ij}E_j^3 + ... \quad (1)$$

2.2.2 Electric stimulus

Here we describe the role of Electric Stimulus (ES). Artificially inducing polarization on the fluid and collagen will help to demonstrate if this electric polarization is one of the mechanisms that induces collagen biomineralization, therefore helping in bone healing process. ES refers to the stimulus made by electric current over collagen. ES differentiates in to two important parts. The first one is an electric signal that can be direct current, periodic current or non-periodic current. The second one is the set up of electrodes that delivers the signal. This assemblies are the following Applied Direct Current (ADC),Capacitive Coupling (CC) and Inductive Coupling (IC).

There are several facts that are useful to consider on each one of previous set ups, resulting that the best for this work is capacitive coupling set up. Capacitive coupling is selected because it shows a good performance and it is mechanically suitable for the experiment. In order to choose the ES signal parameters and shape, a review of previous works is given below.

The ADC involves the direct application of a current through anode and cathode electrodes applied directly to the tissue[3,7,8]. There are various *in vitro* and *in vivo* studies of this procedure[3,6]. *In vitro* studies showed that ADC stimulates osteogenesys by electrochemical reaction at the cathode $O_2 + 2H_2O + 4e^-/rightarrow4OH$, this reaction seems to increase the pH and this environment enhances bone healing[3,17,18]. Another product of this electrochemical reaction seems to be hydrogen peroxide which enhances osteoclast differentiation and this enhances bone formation[17,18]. There are studies that performed around 10 *in vitro* studies and 35 clinical studies using the ADC set up with different levels of confidence. Studies showed some inconsistencies in clinical trials[3]. Therefore, ADC stimulation is not a suitable approach according to these results. Early works performed on ADC with direct current signal in bone healing issue studies[5]: Besset et al. (1994) implanted electrodes on dog Femurs and induced a small ADC of 10 to 100 *uA*. The results showed an extensive callus formation and a rapid osteogenesis was found on the cathode. In the anode there formed a brownish material over bone presumably some kind of denatured protein. Cieszynki (1963) performed an experiment on rabbits with one electrode plate placed on a fractured leg and other plate on the back using 250 to 4000 *uA*. The results showed that a positive potential on the back caused a subcutaneous tumor and in the leg increased fracture strength by 1.5 while negative potential did not show improved healing effect. A further study of Cieszynky of this effect on human bones did not show a consistent pattern. Minkin *et al.* (1968) showed a depressed growth rate in eight week old rabbits by an ADC stimulus of 70 *uA*. Therefore, it has been shown that there is a close relation between bone tissue and electric stimulus.

On the other hand CC common set up consists in locating two electrodes in different sites without direct contact to the bones[3,7,8]. This forms an electric field around the desired spots. Common frequencies used are around 20 Hz to 200 kHz and voltages around 1 to 10 V[3,8]. There are several studies on CC applications, four *in vitro* studies and eleven clinical studies[3]. The mechanisms related to CC appears to be the activation of calcium gates by voltage activated receptors of cells releasing calcium storage on the medium[3,19]. In general, CC appears to decrease the healing time of fractures[3].

Torben Jørgensen performed clinical studies with capacitive coupling on human patients[5].The investigation used 1 Hz on the plates simulating walking speed, also it delivered 20 to 100 uA direct current on the plates. The signal and current waveform applied on the electrodes can be observed in figure 2.2 and 2.3 (retrieved from their paper). It is similar to a charging discharging capacitor. The results obtained after treating 57 fractures were the following: the stability of fracture was obtained thirty percent faster in the electrically stimulated group, there is a greater incidence of skin infection on the treatment group and one patient developed osteitis when applied only positive signal.

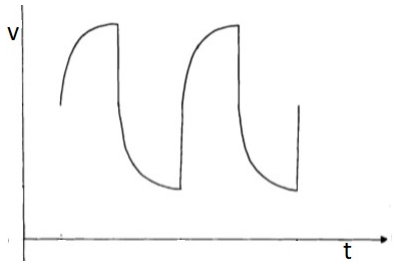

Figure 2.2: Voltage vs time signal applied in Torben Jørgensen study[5].

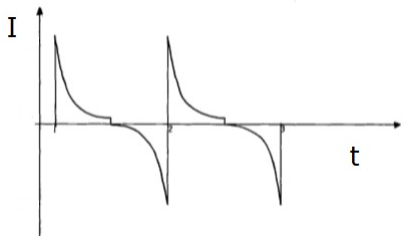

Figure 2.3: Current vs time signal applied in Torben Jørgensen study[5].

Michelle Griffin performed osteoblast-like cells stimulation with degenerate electrical waveform[6]. The degenerate wave form is shown in Figure 2.4. Using this signal they compared between two experimental *in vitro* configurations. The first experiment is the signal applied inside the fluid by placing two Ag-AgCl electrodes inserted in synthetic rubber-agar bridges. They applied 10 mV/mm electric field with 16 Hz. The last experiment was performed with CC configuration applying the same signal with 10 mV/mm electric field and 16 Hz. The results of this work showed that the degenerative signal with CC compared to no stimulated group have increased the cytotoxicisity and decreased proliferative effect but it had higher alkaline phosphatase activity and higher mineralization effects. The signal applied directly in the fluid appears to have lower cytotoxicity than CC but barely more cytotoxicity than control and less cell proliferation than the control, yet it exhibits the higher alkaline phosphatase activity and the highest minerals nodule formation. Summarizing the degenerative signal seems to increase the mineralization of these cells independently of the used set up. There are several works either *in vivo* or *in vitro* working on bone healing process using ES, yet the underlying mechanism is still unclear[3]. There are three main experimental set ups

for delivering ES the first one is ADC stimulus, the second one is CC and the last one is IC stimulus[3,7,8]. Yet all the works on this field work with ES on cells and proteins. In contrast this work will study collagen bioremineralization response to ES in simulated body fluid. Given that, the parameters and set ups are reviewed from the literature in order to choose the best parameters to be used in this project. Accordingly this investigation will use a similar signal provided by a Chua's circuit.

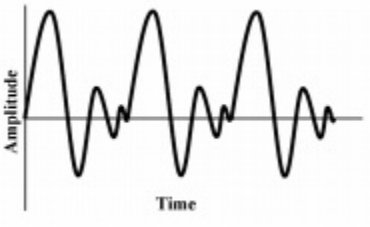

Figure 2.4: Degenerative sine wave applied to osteoblast like cells in Michelle Griffin experiment[6].

Inductive coupling has been performed by various researchers[3,7,8,20], twenty one *in vitro* studies and fifty nine clinical studies were accomplished[3]. These experiments were made placing coils on the skin over the fracture site[3]. The current flows through these coils generating an electromagnetic field as described by Faraday Law of induction. The electric fields created were in the range of 1 to 100 mV/cm depending on the fracture size[3,7,8]. Summarizing IC mechanism seems to enhance the calcium uptake on bone by activating cells pathway signaling[3,20]. Also, it enhances osteoblast cell proliferation[3,20].

There are more investigations performed on related topics to enhance cell proliferation using mechanical strain frequency[24,25]. These studies determine certain parameters that we can extrapolate to our work. This are useful bases to decide which frequency range we can use. Because they determine frequencies at which cells have optimal work. Rosenberg performed an experiment with mechanical strain over cell culture[24]. The experiment tested mechanical vibration in a range of frequency of 20 - 60 Hz, finding that the best frequency for cell proliferation was 20 Hz and the best frequency for metabolic activity was 60 Hz. Another experiment relating cycle number and frequency of uniaxial strain on osteoblast was done by Kaspar[25]. These experiments showed and optimal frequency for 1 Hz at a fixed cycle number. Despite the fact that mechanical strain frequency might not be transformed directly to electrical frequency on collagen these investigations provides a range of frequency parameters to be applied.

2.3 Circuit design

In this section we will review the circuit designed to generate the non-periodic signal. This circuit is called chua circuit. Chua circuit is a system with three degrees of freedom which uses capacitors, inductor and negative resistance (a resistance that provides energy instead of dissipating it) to generated a non periodic signal. The periodic signal is provided by a commercial wave generator equipment.

2.3.1 Chua circuit

Electronics have a wide range of application in circuits, analog devices allow to construct circuits with many different features according to our necessities, for instance, a full-wave positive triangular signal. Operational amplifier is one of the most used devices because it works with dc and ac current at the same time providing many features, depending on the set up we can have voltage gain with the positive feedback, full wave of any kind of signal, a certain gain dependent oon the device and more.

The first Chaotic system was proposed by Edward Lorentz. He provided an example of chaos using a set of differential equations describing an idealized model of the atmosphere[26,27]. The model was hard to be tested experimentally, so a group of physicist tried to develop an electrical circuit that mimics those equations. This circuit was very complicated and complex[26]. Leon Chua observed this circuit, and after a failed test of that circuit he wondered if there was a chance to build a much simpler circuit that exhibits chaotic behavior that is not governed exactly by Lorentz equations[26]. He was successful at this task and removing all the unnecessary components of the circuit, he provided the simple circuit that we know nowadays.

The differential equations ruling over chua circuit are presented below (2, 3, 4). One condition to fulfill chaotic behavior is to have three degrees of freedom on the system, that means we will need three scalar values at a moment to describe the state of the circuit[26,27]. For instance, using a capacitor can be seen as a current source dependent on the change in voltage[26,27] as we can see in equation 5 below. And the inductor can be treated as a voltage source that has a voltage dependent on the change in current[26,27] as we can see in equation 6. That's why chua circuit is constructed as described below, each energy storage component is adding a degree of freedom to the system. Also, it is important to have a non-linear component which for the chua circuit is the chua's diode.

$$C_1\dot{V}_1 = \frac{V_2 - V_1}{R} - f(V_1), \tag{2}$$

$$C_2\dot{V}_2 = \frac{V_1 - V_2}{R} + i, \tag{3}$$

$$L\dot{i} = -V_2 - ri, \tag{4}$$

$$C = \frac{Q}{V}, \tag{5}$$

$$V = L\frac{\delta I}{\delta t}. \tag{6}$$

In figure 2.5 we can observe the circuit diagram. Equation 2 is obtained from the close loop analysis of the right most circuit in the diagram and using kirchoff's law, where V_1 is the voltage in C_1, C_1 is the capacitance of capacitor 1, V_2 is the voltage in C_2, R is the central resistance value and $f(V_1)$ is a function that describes the current of chua's diode. Equation 3 comes from the second capacitor in a similar way using kirchoff's law, where i is inductor current. In this case we are replacing physical inductor with a simulated inductor called gyrator where the current i is obtained with equation 7, where V_P is the voltage at point P. Equation 4 is obtained from the leftmost part of the circuit, which will be the voltage generated at the inductor. The term r will be the parasitic resistance of the inductor.

As mentioned before, in our diagram, the inductor is replaced by a gyrator. The amount of inductance created by the gyrator can be calculated with equation 6 and r will be the resistance of the gyrator[28].

$$L = \frac{R7R9R10C}{R8} \tag{6}$$

$$i = \frac{V_P - V_2}{R_7} \tag{7}$$

Chua's diode is an active resistor on the circuit. The active resistor is a component that introduce energy into the

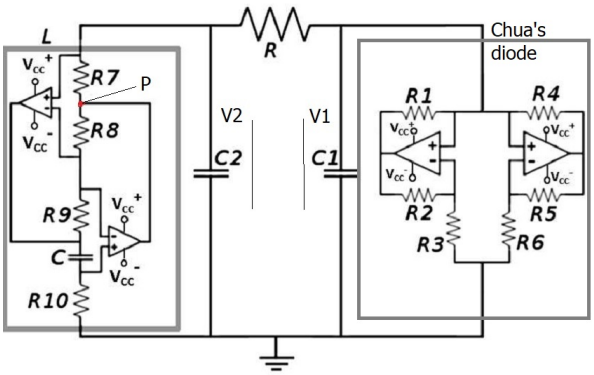

Figure 2.5: Chua circuit basic diagram[28]

circuit instead of dissipating energy. Yet, after surpassing the linear range operational amplifier, it will behave as a normal resistance[26]. Connecting two of them in parallel will prevent this linear behavior. We can observe the result in Figure 2.6: voltage vs current behavior of the chua's diode. Using an operational amplifier we built a Chua's diode. This device is an active resistor in the circuit and it will help us to obtain a non periodic signal. It was used for the first time to make Lorentz's equations in 1983 at the University of Waseda (Japan). After that, it has been well documented in a variety and complicated ways. It has been used in communication, simulated using software like Matlab or Multisims. The non periodic signal or chaotic signal is a good tool when we need to simulate a random behavior because it is non periodic and has really big changes for little variations on the initial parameters of the system[26]. A practical use of this non periodic signal is to simulate the random walk of a person which is a matter of interest when using electric current to reproduce polarization on collagen. Resulting in induction of biomineralization on collagen. The resulting Chua circuit signal is shown in Figure 2.7 and 2.8. Figure 2.8 shows the variation of V_1 voltage and V_2 voltage.

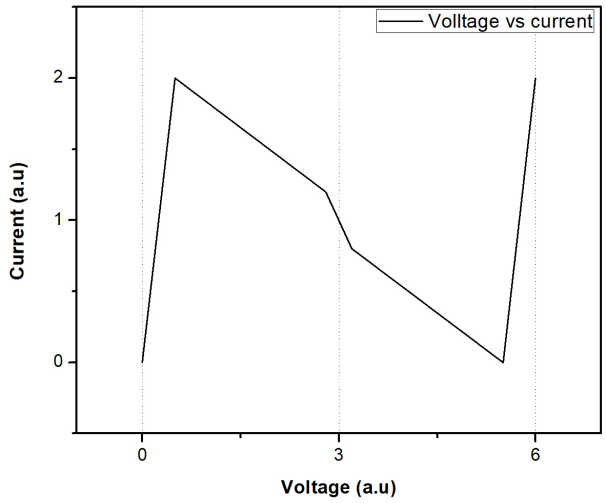

Figure 2.6: Chua's diode Voltage vs Current diagram

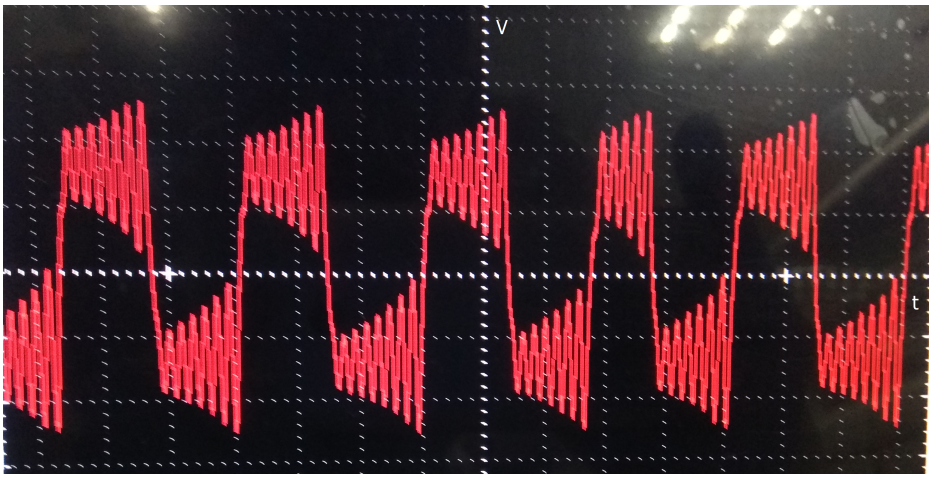

Figure 2.7: Chua's circuit signal measured on an oscilloscope.

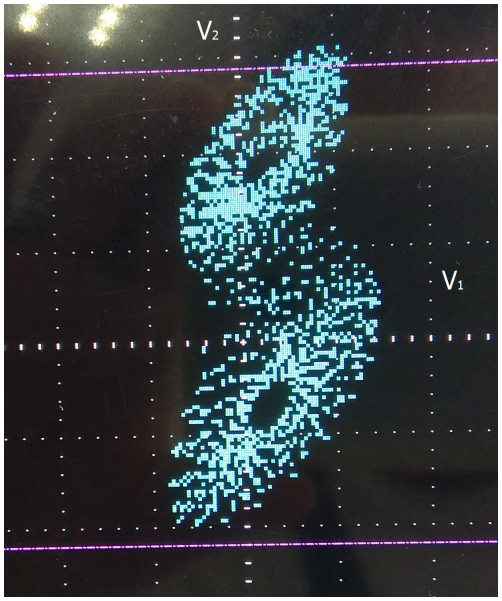

Figure 2.8: Chua's circuit signal measured on an oscilloscope between V_1 vs V_2.

2.4 Characterization techniques

2.4.1 Scanning electron microscopy

Scanning electron microscopy (SEM) is a useful technique in order to look at the surface structure of materials. This technique uses an electron beam generated by high voltage to "scan" sample surface. The working principle is that electrons directed from the source interact with the sample. This interaction produces : secondary electrons, backscatered electrons and photons. The photons are a product of the recombination of secondary electrons expelled from the sample in inner levels of energy. When an inner electron is expelled from the sample an electron should jump from higher level to fill that space, this recombination will produce characteristic photons for every atom. Secondary electrons and backscatered electrons will give surface images and qualitative composition of the sample, and an x-ray detector will detect characteristic photons produced by the sample. This last resource is called Electron dispersive spectroscopy (EDS). It is a helpful tool too obtain a quantitative analysis of the sample composition. Finally, a characteristic of backscatered electrons is that their energy is dependent on the nuclei size making imaging contrast related to the atomic number of the elements present in the sample.

2.4.2 X-ray nanotomography

X-ray nanotomography uses a source of x-rays and a x-ray detector. The working principle is the samples absorption of photons. Transmitted x-ray photons are detected and then related to the source photons. Doing this various times

rotating the sample will construct a complete 3D image of the sample.

Chapter 3

Experimental part

3.1 Experimental set up

This work has 3 major experimental parts, the first one is about bone demineralization in order to obtain the collagen fibers from the bone macroestructure. The second part will be the circuit design and parameters we have to follow in order to have controlled electric field applied over bone. The last part of the experiment will be the bone remineralization in Simulated body fluid (SBF). This last part will cover the system to deliver current into the bone complex along which the were circuits previously developed. The bones were obtained from rabbit. The following process will explain the protocol to treat the rabbit bone. This process includes cutting the raw meat, cleaning the bone surface very carefully, cutting bone section and then cleaning the bone marrow.

3.2 Bone demineralization

Rabbit was used to obtain bones. They were frozen as soon as they were obtained and all the processes were performed under cold conditions, close to ice packages $0^0 - 10^0 C$. The protocol followed was as follows:

(a) One rabbit leg was retrieved from a container full with ice, while the remaining legs were kept on the container.

(b) Meat surrounding bones were removed with a scalpel until bones only had fat and cartilages on it.

(c) With the scalpel the diaphysis of the bone was scraped off, with this most of the visible fat and meat was removed in that process.

(d) Clean the bare bone with distilled water, e) we performed a washing with $NaOH$ at concentration $1N$ in order to dissolve meat, proteins and fat from the surface of the bone.

(e) The bone was washed again with water in order to clean $NaOH$ from it.

(f) The last two washes were performed with Ethanol at 70% in order to remove all the remaining fat on the surface.

(g) Right after a distilled water wash.

This process was repeated for each rabbit leg, in total we have four front legs and four hind legs that give us a total of sixteen rabbit bones. Once all the process is complete bones were separated by kinds and then sealed in glass containers and put inside the freezer.

The next step was to cut the bone with an automatic serrated saw. Once bones were cut we had access to marrow. With a normal injector, a distilled water pressure was applied in order to withdraw most of the marrow. Subsequently repose in water for 5 minutes and for another 5 minutes in ethanol. After that, with another injector, ethanol was injected with pressure into the hollow of the bone in order to retrieve remaining fat and the rest of the marrow components. The last wash was performed with more water injected with pressure into the hollow and into the surface. The last step was to dry with absorbent paper and store in glass containers with an average 120 ml of Ethylenediaminetetraacetic acid (EDTA) $0.30M$ and save them on the fridge.

The preparation of EDTA 0.30 M followed is: Dissolve 124.39 g of EDTA over 1100 ml of distilled water. Then place 750 ml of water in the glass container and start stirring vigorously. After that we added EDTA powder slowly and gradually. Once all the powder is in the solution we added NaOH in order to regulate the pH. NaOH should be placed until the solution reached a pH of 8. The pH reached in the solution was 8.20. Once the solution turns transparent it is an indicator that we are getting close to PH and EDTA is correctly disolving in the solution. Stirring was performed for 4 hours more until no particles or suspensions could be seen in the recipient.

The amount of EDTA placed in excess on the storage glass was dependent on the volume of the bone, We have the following values: Tibias 130ml, Femur 105 ml, Humero 100 ml, radio and cubito 100ml. Samples demineralizated are shown in figure 3.4.

3.3 Circuit designs

The following schemes of the circuit have been developed in order to build the electrical part: The first is a voltage amplificator to measure the piezoelectricity observed in figure 3.1. Its amplification is 1000 times Vin. The second circuit is Chua's circuit. Fundamentally, a chaotic system requires that its components have the following characteristics[26–28]:

- One or more nonlinear elements.
- One or more locally active resistors
- Three or more elements of energy storage.

While there are many ways to build a standard Chua's circuit and many variations on the standard, for simplicity, we will focus here on the version made only from resistors, capacitors, and op-amps show in figure 2.5.

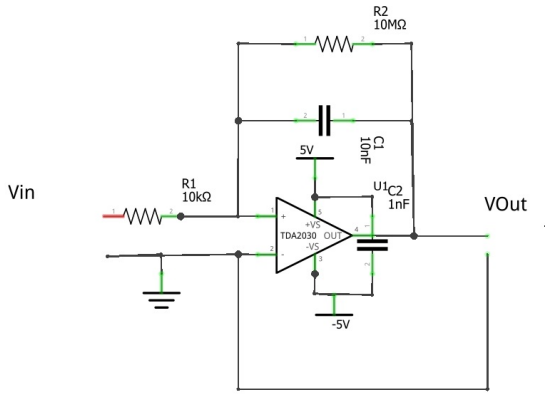

Figure 3.1: Circuit schematic of amplifier circuit[29].

Table 3.1: Data from oscilloscope display of Chua circuit

Wave Pulse	Segments	Value of segment	Base time	Period (ms)	Frequency (Hz)	Varianze
0.2	3	0.2	5	3	333	92
0.2	3	0.2	5	3	333	92
0.2	3.5	0.2	5	3.5	286	45
0.2	6.5	0.2	5	6.5	154	87
0.2	4.5	0.2	5	4.5	222	19
0.2	6	0.2	5	6	167	74
0.2	3.3	0.2	5	3.3	303	62
0.2	4.5	0.2	5	4.5	222	19
0.2	4.5	0.2	5	4.5	222	19
0.2	6.5	0.2	5	6.5	154	87
0.2	4	0.2	5	4	250	9
0.2	8	0.2	5	8	125	116
0.2	3.5	0.2	5	3.5	286	45
0.2	4.5	0.2	5	4.5	222	19
0.2	3	0.2	5	3	233	92
			Average	241	Varianze	58

A central frequency was measured averaging waves from Chua's circuit signal resulting in a central frequency of $241Hz$, we can observe oscilloscope data retrieved from display in table 3.1. Chua's circuit due to its nature is highly sensitive to loads and any perturbations. In order to have signal isolated from the base Chua's circuit we conect the signal to an operational amplifier in voltage follower mode. This makes our main signal isolated from any load. The

last step is a voltage divider to set the biggest peak of Chua's circuit to $1V$. The following materials and specific values are used: $R = 2.5k\Omega(pot)$, $R_1 = 220\Omega$, $R_2 = 220\Omega$, $R_3 = 2.2k\Omega$, $R_4 = 22.0k\Omega$, $R_5 = 22.0k\Omega$, $R_6 = 3.3k\Omega$, $R_7 = 100\Omega$, $R_9 = 1.0k\Omega$, $R_{10} = 2.5k\Omega(pot)$, $C = 100nF$, $C_1 = 10nF$, $C_2 = 100nF$, $L = 15mH$.

On the other hand, periodic signal was provided by a function generator with the following parameters: Signal square, Frequency 241 Hz, 1 V_{pp} and 0 V offset. We can observe both signals compared in the oscilloscope in figure 3.2

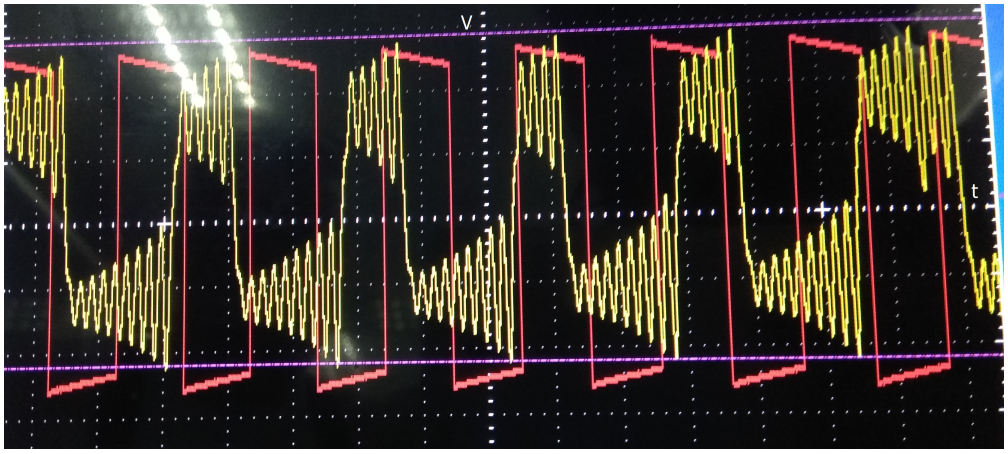

Figure 3.2: Chua signal vs periodic signal displayed in oscilloscope

3.4 Bone remineralization

3.4.1 Simulated body fluid preparation

The preparation of SBF 1.5 was performed as follow: The reagents were added in 400 ml of distilled water in the same order as listed in Table 3.2, after that, pH was adjusted with HCl 1 M to 7.0 and lastly volume was adjusted to 500 ml.

3.4.2 Experiment set up

For the remineralization experiments we proposed two experimental set ups. The first one consist of a dynamic system which places electrodes in the exterior part, surrounding collagen and delivers SBF fluid through the bone insides. The delivery system is a series of sterilized pipes with a flux of 0.018 $\frac{ml}{s}$, this will be called dynamic experiment. The second experiment is a static situation similar to the common CC set up. 5 ml of SBF are placed in a test tube and copper electrodes of 1 cm^2 are placed surrounding tubes connecting the signal, this will be called static experiment. We can observe the set up in figure 3.4 and schematic diagram in figure 3.3.

Table 3.2: Reagents for 0.5 L of SBF 1.5

Reagent	Weight (g)
$NaCl$	5.997
$NaHCO_3$	0.2625
KCl	0.168
K_2HPO_4	0.171
$MgCl_2.H_2O$	0.229
$CaCL_2H_2O$	0.2085
Na_2SO_4	0.0535
$C_4H_{11}O_3(tris)$	4.543

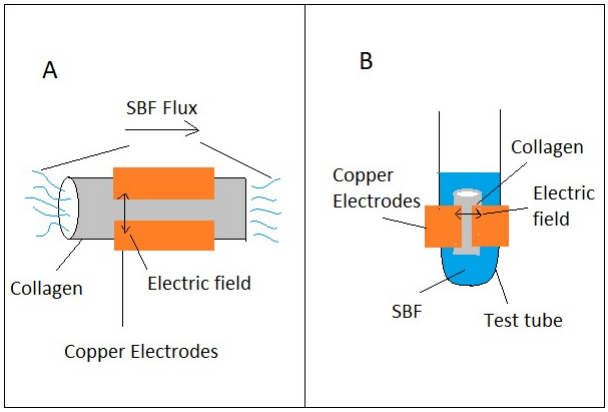

Figure 3.3: Experimental set up schematic, (A) dynamic experiment and (B) static experiment

The remineralization process will be monitored weighing bone mass in order to observe the amount of minerals retrieved by bones and then building the curve of weight over time we can get the speed of bone remineralization. Furthermore, scanning electron microscopy and Electron dispersive spectroscopy (EDS) elemental analysis studies will be done in order to obtain information about the quality of remineralization. Also, x-ray nanotomography is performed to see the change in the volume of the sample.

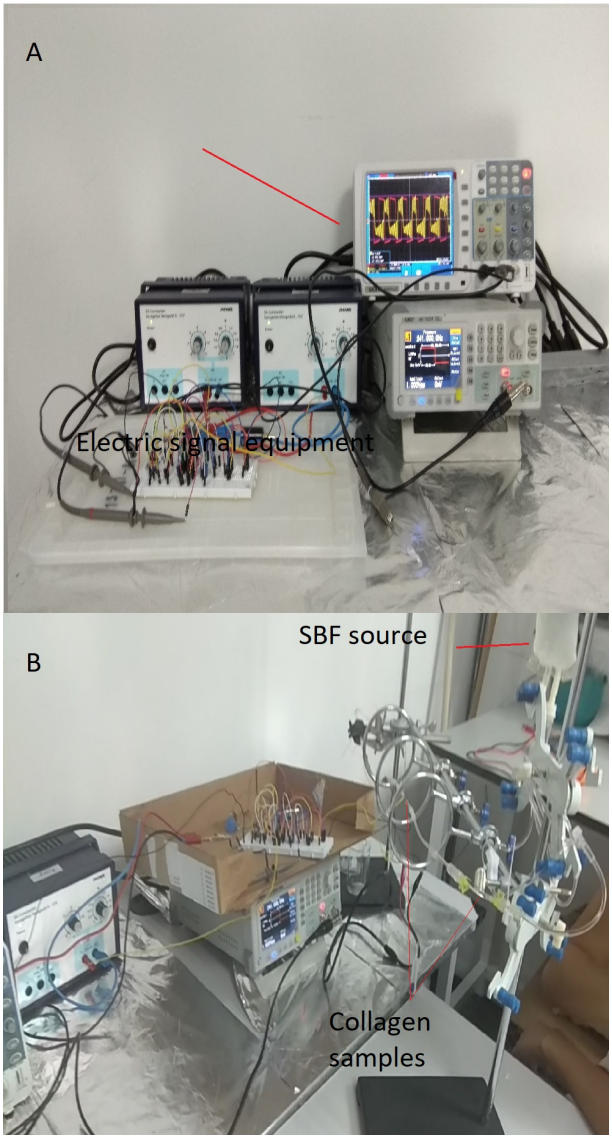

Figure 3.4: Experimental set up, (A) is the experiment ES equipment, (B) is experiment mechanical fluid system.

Chapter 4

Results and Discussion

4.1 Results

The results section will cover the main findings of the experiment. Figure 3.3 shows a scheme of the two types of experiments carried out, (A) is the dynamic experimental scheme, while (B) is the static experiment scheme. For both experiments dynamic and static the following signals were applied: periodic signal, Chua signal (chaotic signal) and no signal. Electrodes are placed as shown in figure 3.3 covering collagen for dynamic experiment and covering test tube for static experiment. The samples that were under each type of signal were weighted after different periods.

4.1.1 Dynamic experiment

Data obtained from the dynamic experiment are shown in table 4.1 and figure 4.1A. Individually normalized graph is presented in figure 4.1B to notice better the relative variation. Table 4.1 shows the weight variation of samples under the different treatments over different periods of time, under the dynamic experiment and figure in a similar way present the weight variations over time. The first part of the graph shows an abrupt drop, attributed to dehydration of

Table 4.1: Weight variation of samples for different signal treatments over time on dynamic experiments

Time (days)	Weight Periodic (mg)	Weight Chua (mg)	Weight Control (mg)
0-10	-176 ±1	-159 ±1	-234 ±1
10-18	16 ±1	8 ±1	23 ±1
18-22	40 ±1	22 ±1	-29 ±1

the external part of collagen sample that was exposed to air. Therefore, sample lost a lot of water and this explains the abrupt drop of the weight. Different types of water are found in collagen tissues: free and bonded water and structural water[9,10]. Free and bonded water act directly on the surface of the triple helix of collagen forming an interconnected network[9,10]. This is the water that is lost externally during this experiment. Structural water has the function to

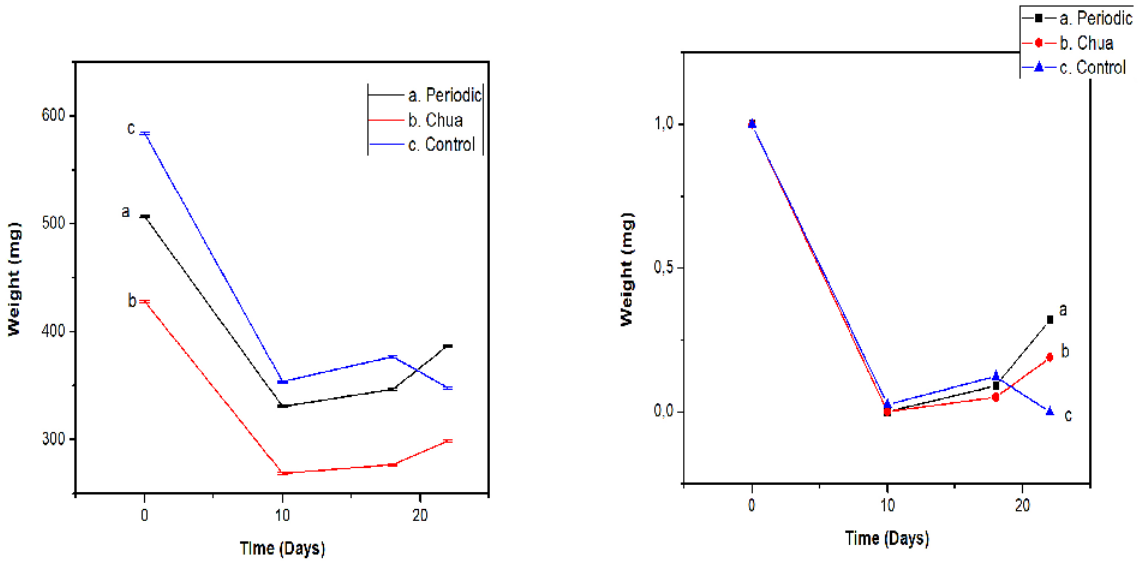

Figure 4.1: Collagen Weight vs Time of treatments: periodic, Chua and control for dynamic experiment, (A) Raw data, (B) Normalized data.

maintain the integrity of the collagen triple helix, acting as a bridge between the alpha chains strengthening the structure[9,10]. This water was maintained, since the integrity of the collagen was not affected. Furthermore, the interstitial part of the collagen sample was always in presence of SBF and this is the region that we are studying. The weight gain obtained without taking in account the first measure in $\frac{w\%}{w}$ in 12 days is: for Periodic treatment $16.92\%\frac{mg}{h}$, for Chua treatment $11.15\%\frac{mg}{h}$ and for control $-2\%\frac{mg}{h}$. These results show us that the periodic electric field influence over the sample increased the amount of minerals received from the solution.

SEM characterization of samples are presented below in figure 4.2, (a) periodic sample, (b) Chua sample and (c) control sample. EDS analysis is presented on table 4.2, 4.3 and 4.4 showing the atomic concentration percentage (At%) of the elements present. SEM results of backscatered electrons images show distribution of particle depositions on collagen. EDS shows the composition of the precipitates that we can observe in some samples, indicating Ca deposition on the surface which confirms mineralization. A distribution of calcium deposits on the surface of sample can be observed for all the different samples that were submitted to the different type of signals in the dynamic experiment. There is an important difference in the distribution of these Ca deposits depending on the applied signal. In the sample that was under a periodic signal, large agglomerates were observed. While in the sample that was under Chua signal a very fine uniform distribution of Calcium deposits was formed. Probably this distribution of Ca is more convenient since the hydroxyapatite crystals will grow from this in a more homogeneous and uniform way. The control sample shows very few depositions, indicating that Electric stimulus favors Calcium depositions. Figure 4.3 shows x-ray tomography of sample dynamic under Chua treatment where we can observe the

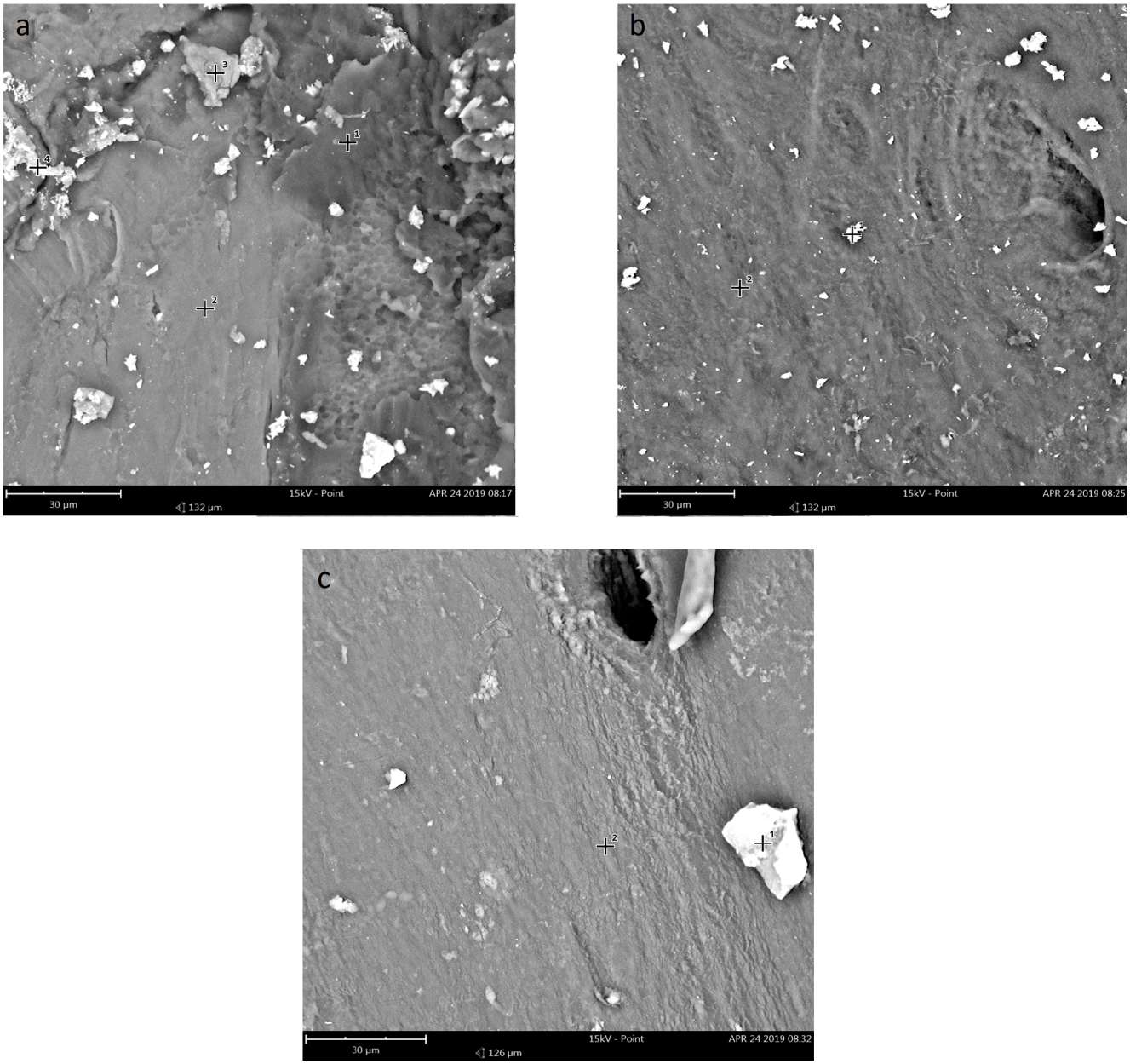

Figure 4.2: SEM image of dynamic experiment, (a) periodic sample, (b) Chua sample and (c) control sample

3D morphology of collagen samples. The other tomography 3D images from all the different samples, are presented in the appendix

Table 4.2: Analysis of different ions on the sample indicated in the image spot 1-4 of figure 4.2a, for sample undergoing periodic signal for 22 days on the dynamic set up

Spot 1			Spot 2			Spot 3			Spot 4		
Element	At%	Error	Element	At%	Error	Element	At%	Error	Element	At%	Error
C	83.6	1.0	C	50.1	1.1	C	72.7	1.1	C	38.5	1.5
O	16.4	0.1	O	22.9	0.3	O	26.6	0.1	O	36.5	0.0
			Cl	0.7	0.2	Cl	0.4	0.5	Ca	1.4	0.1
			N	26.0	1.3	Ca	0.3	0.9	N	22.8	2.4
			Na	0.3	1.2				Na	0.7	0.5
									Cl	0.2	0.8

Table 4.3: Analysis of different ions on the sample indicated in the image spot 1-2 of figure 4.2b, for sample undergoing chua signal for 22 days on the dynamic set up

Spot 1			Spot 2		
Element	At%	Error	Element	At%	Error
O	47.3	0.0	C	43.0	0.9
Ca	3.1	0.1	O	26.0	0.2
C	28.2	1.7	Cl	0.7	0.3
N	21.4	4.8	N	30.3	1.1

Table 4.4: Analysis of different ions on the sample indicated in the image spot 1-2 of figure 4.2c, for sample undergoing no signal for 22 days on the dynamic set up

Spot 1			Spot 2		
Element	At%	Error	Element	At%	Error
C	60.4	1.0	O	31.0	0.1
Ca	3.0	0.3	Cl	1.2	0.6
O	28.5	0.2			
Cl	0.3	1.0			

The combined tailoring effect of wettability in Simulated body fluid (SBF) and collagen polarity charge under the applied electric field enable binding of inorganic ions Ca^{2+}, Cl^{-}, Na^{+} on the surface, these are the nuclei that are initially formed and that will result in accelerated mineralization and formation of hydroxyapatite with time on the polarized surface. Placing a test charge in the center of the sample will give us a distance about 4 mm to the electrodes. The electric potential produced by periodic signal and non periodic signal are denoted as E_1 and E_2 respectively. This potential is dependent on time, yet ignoring the time dependence and fixing electric potential to the max constant peak of electric signal the electric field can be obtained from parallel plates approximation. Because

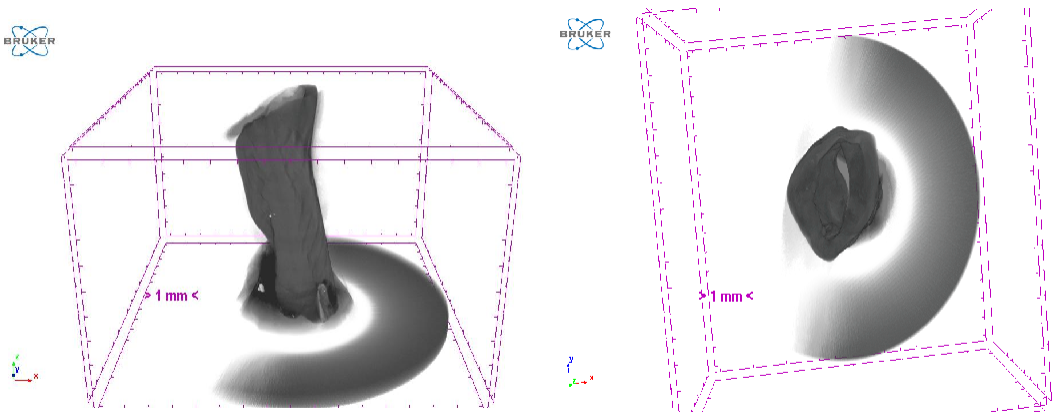

Figure 4.3: 3D x-ray morphology image of sample Chua of dynamic experiment

plates around test tubes and collagen samples make the form of a half cylinder we relate this half cylinder radius to the rectangle distance formed from unfolding this cylinder. Approximating distance of the plates by $r \sim d$. Electric field is equal to $E = -\vec{\nabla}V$, we can say that our voltage is constant overall. Thanks to this, the problem reduces to $E = -\frac{V_{max}}{d}$. This approximation is useful to compare E_1 with E_2 because radius are different on both experiments (static and dynamic) while dynamic experiment has a radius of formed cylinder equal to 4 *mm*, the radius from static experiment is 8 *mm*. So $E_1 = -\frac{V_{max}}{4}$.

4.1.2 Static experiment

For static experiments, we use two sizes of samples for each type of signal applied. Two replicas of each samples were tested. The aim was to see if the effect of bone collagen size will affect the mineralization process of the samples under the same electric field. The other important point to remember is that these samples were completely immersed in the SBF so they do not dehydrate, and on the contrary to the dynamic experiment the electrodes were located in the external part of the asay tube at an average distance between plates of 8 *mm*. So the SBF and the collagen were under the applied field but the collagen was in the center of the tube at a distance of 8*mm* from the plates. In the other hand we have the static experiment with six samples. We have two samples for each treatment. Description of samples is similar to the dynamic experiment. Samples described as A are small collagen sample from tibia and samples described as B are bigger samples from humerus. Data obtained from the Static experiment are shown in table 4.5 and figure4.4. Average normalized data is also presented in figure 4.5 in order to observe data. The weight gain obtained overall expressed in $\frac{w\%}{w}$ in 19 days is: for Periodic treatment in average 6.59%, for Chua

Table 4.5: Weight variation of samples for different treatments over time on static experiment

Time (days)	Periodic1(mg)	Periodic2(mg)	Chua1(mg)	Chua2(mg)	Control2(mg)	Control1(mg)
0-7	-2 ±1	-17 ±1	-5 ±1	-18 ±1	-11 ±1	3 ±1
7-15	16 ±1	36 ±1	32 ±1	25 ±1	34 ±1	8 ±1
15-19	-5 ±1	0 ±1	-4 ±1	5 ±1	-2 ±1	5 ±1

treatment average 8.00% and for control in average 8.45%. This experiment shows us that both treatments are not helping on the remineralization time. This might be attributed to the magnitude of the electric field on the collagen molecule being about half lower ($E_2 = -\frac{V_{max}}{8}$) than in dynamic experiments and also the effect of electric field on the ions of the SBF solutions will have an effect on their movement and migration to the collagen surface. Size does not seem to change the mineralization process, yet there is no big difference in size between samples A and B. The SEM images of the backscatered electrons are showing particle distribution of samples presented in figure 4.6 which shows (a) periodic sample, (b) chua sample and (c) control sample, and figure 4.7 which shows (a) periodic sample, (b) chua sample and (c) control sample. EDS tables with atomic concentration of particles are presented in tables 4.6 to 4.11. In figure 4.6a it can be observed a large proportion of Calcium deposits forming large agglomerates. This small sample compared with the larger sample from figure 4.7a which has more numerous deposition of precipitates but smaller size. This can be due to the smaller area under the same SBF volume. However under the Chua signal the opposite effect was observed and the deposition was higher in the larger sample from figure 4.7b, while in figure 4.6b the smaller sample we have smaller particles and some concentrated agglomerates.

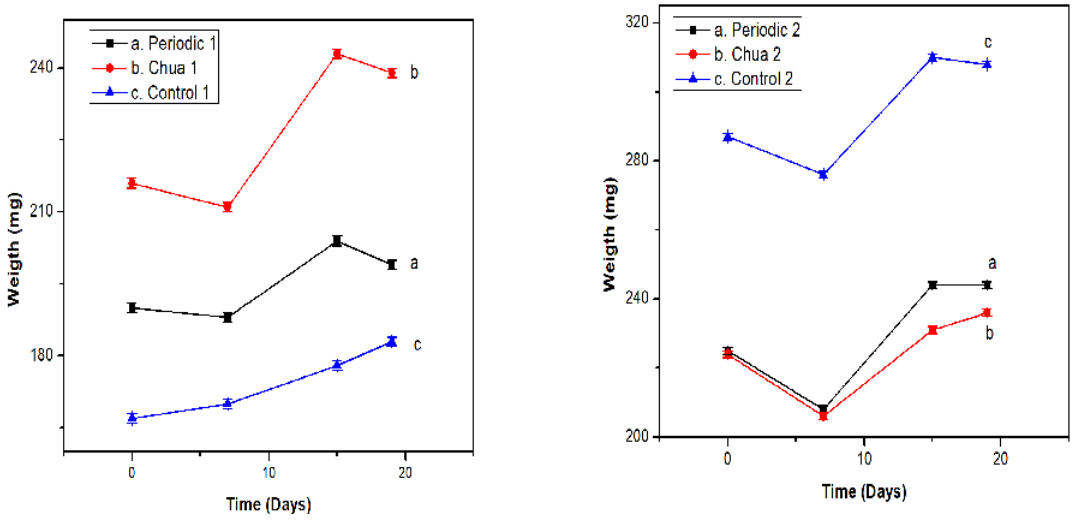

Figure 4.4: Time vs Collagen weight of treatments: periodic, Chua and control for static experiment. (A) Are small samples from tibia denominated small samples and (B) are big samples from humerus denominated large samples.

Table 4.6: Analysis of different ions on the sample indicated in the image spot 1-2 of figure 4.6a, for sample undergoing periodic signal for 19 days on the static set up

Spot 1			Spot 2		
Element	AC	Error	Element	AC	Error
Ca	6.5	0.0	C	48.1	0.7
O	51.0	0.1	Cl	0.8	0.2
C	22.2	1.7	O	17.5	0.2
N	20.1	4.8	N	19.5	2.4
Si	0.2	0.7	Ca	0.2	0.6
			Na	0.4	0.5

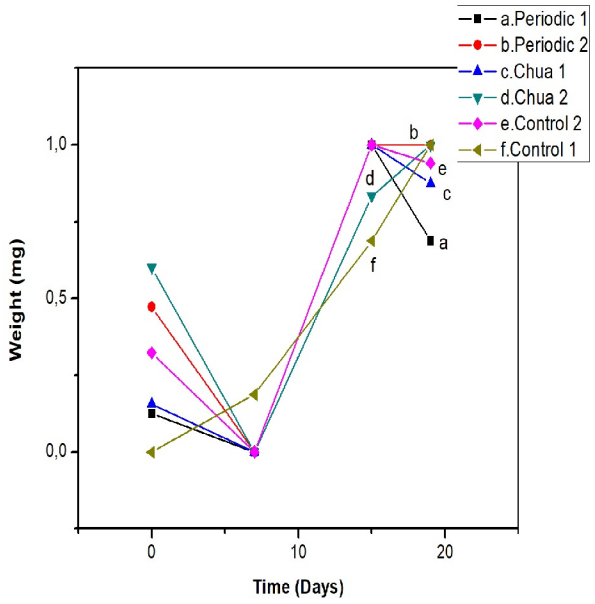

Figure 4.5: Normalized collagen weight vs time of samples: a. periodic average , b. chua average, c. control average for static experiment the average values were taken between small and large samples.

Table 4.7: Analysis of different ions on the sample indicated in the image spot 1-3 of figure 4.7a, for sample undergoing periodic signal for 19 days on the static set up

Spot 1			Spot 2			Spot 3		
Element	AC	Error	Element	AC	Error	Element	AC	Error
C	40.3	1.0	C	37.3	1.4	C	40.1	1.1
O	28.1	0.2	O	32.2	0.1	O	28.0	0.1
Cl	1.0	0.2	Si	0.9	0.2	Cl	1.2	0.2
N	29.3	0.9	Cl	0.8	0.2	N	29.2	1.1
Na	1.3	0.4	N	27.6	2.8	Na	1.4	0.7
			Na	1.3	0.4			

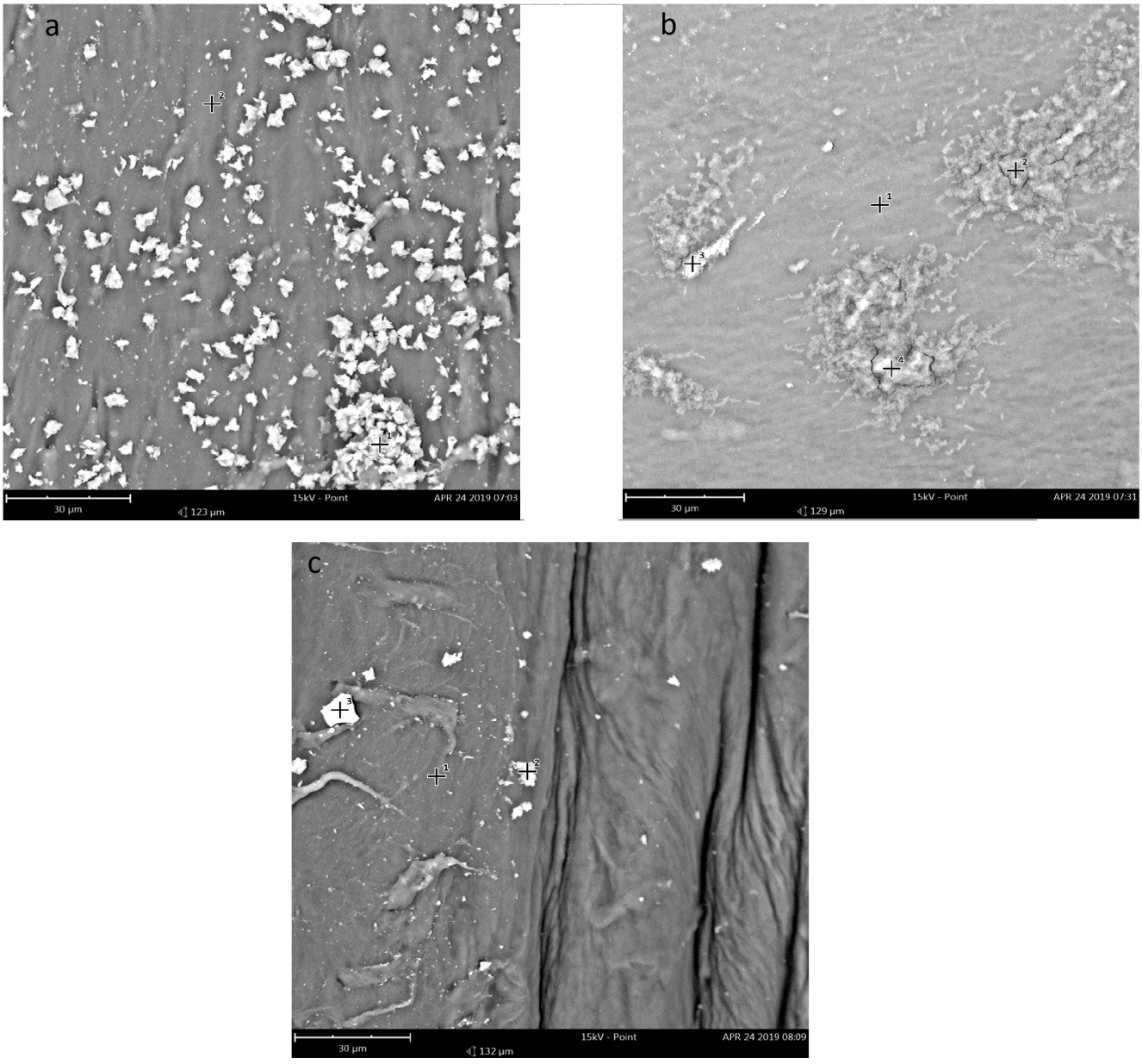

Figure 4.6: SEM image of small samples from tibia A, (a) periodic sample, (b) chua sample and (c) control sample.

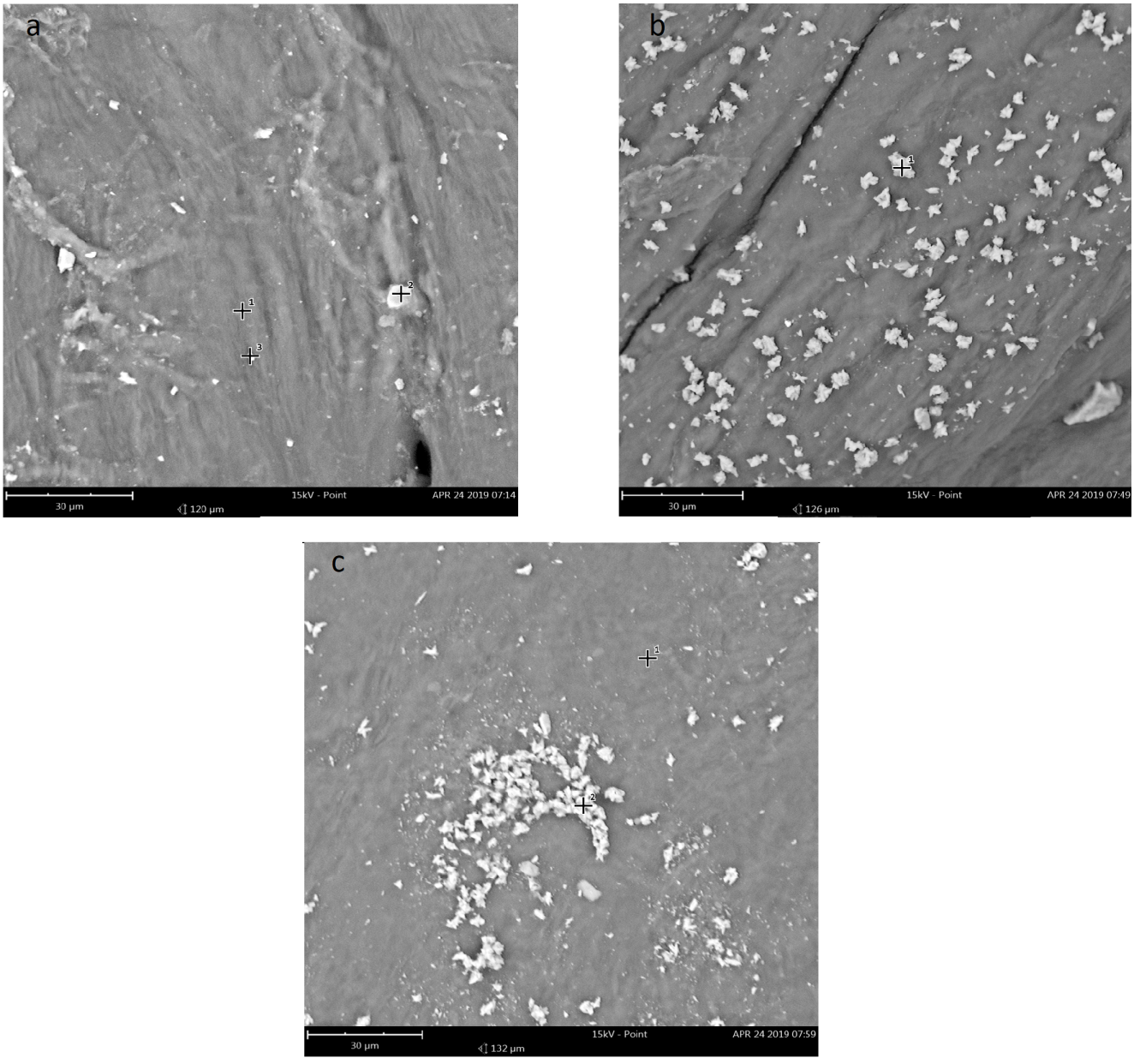

Figure 4.7: SEM image of larger samples from humerus B, (a) periodic sample, (b) chua sample and (c) control sample.

Table 4.8: Analysis of different ions on the sample indicated in the image spot 1-4 of figure 4.6b, for sample undergoing chua signal for 19 days on the static set up

Spot 1			Spot 2			Spot 3			Spot 4		
Element	AC	Error	Element	AC	Error	Element	AC	Error	Element	AC	Error
C	43.8	1.2	C	43.2	1.4	C	45.5	0.9	C	48.1	1.1
Cl	1.5	0.3	O	26.7	0.1	Cl	1.8	0.4	Cl	0.8	0.2
O	24.1	0.1	Cl	1.0	0.2	O	26.8	0.4	O	14.3	0.3
N	29.7	0.5	N	28.3	4.0	N	24.4	3.2	N	21.3	0.6
Na	0.8	0.9	Na	0.6	0.5	Na	1.1	1.3	P	0.2	1.3
S	0.2	0.8	P	0.2	1.1	S	0.2	1.2	S	0.2	1.5
									K	0.2	2.2
									Na	0.3	0.7

Table 4.9: Analysis of different ions on the sample indicated in the image spot 1 of figure 4.7b, for sample undergoing chua signal for 19 days on the static set up

Spot 1		
Element	AC	Error
Ca	43.8	1.2
O	1.5	0.3
N	24.1	0.1

Table 4.10: Analysis of different ions on the sample indicated in the image spot 1-3 of figure 4.6c, for sample undergoing periodic signal for 19 days on the static set up

Spot 1			Spot 2			Spot 3		
Element	AC	Error	Element	AC	Error	Element	AC	Error
C	41.4	1.2	C	38.0	1.0	C	47.9	1.1
O	26.9	0.2	O	27.3	0.1	O	16.1	0.1
Cl	0.7	0.3	N	33.8	0.8	Cl	0.4	0.3
N	30.5	1.2	Cl	0.4	0.3	N	23.2	0.6
Na	0.5	0.8	Na	0.5	0.5	Na	0.3	0.4

Table 4.11: Analysis of different ions on the sample indicated in the image spot 1-2 of figure 4.7c, for sample undergoing no signal for 19 days on the static set up

Spot 1			Spot 2		
Element	AC	Error	Element	AC	Error
C	45.9	1.1	O	43.1	0.0
O	25.4	0.2	Ca	2.7	0.1
Cl	0.7	0.3	C	30.4	1.3
N	27.4	1.7	N	23.6	3.8
Na	0.6	1.2	Cl	0.3	0.5
S	0.1	1.0			

Figure 4.8 shows x-ray tomography of sample dynamic under chua treatment where we can observe the 3D morphology of collagen samples. The other 3D tomography images from all the different samples, are presented in the appendix

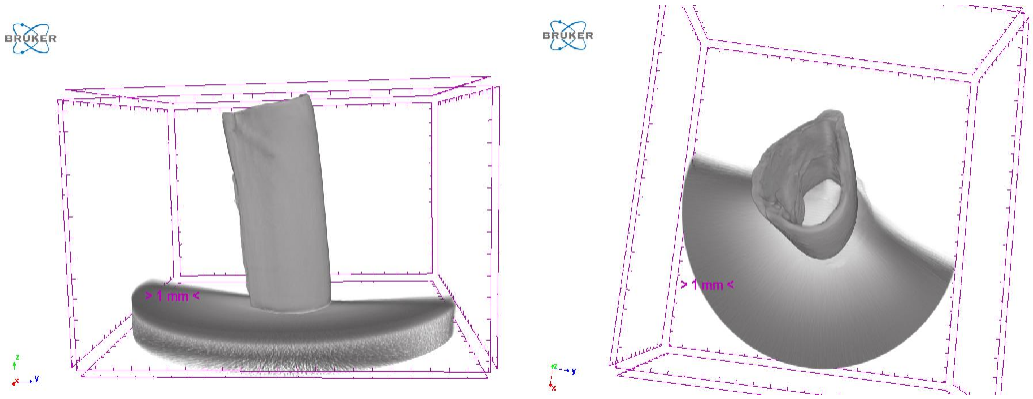

Figure 4.8: 3D x-ray morphology image of sample control2 of static experiment

4.2 Discussion

The results for the dynamic experiment show a positive influence of the treatment over the samples because there is a higher gain in weight. From SEM images we observe a tendency in samples under chua signal, the calcium deposits spread in a uniform way a cross the surface. On the other hand, periodic sample have bigger stacks of calcium agglomerates.

The results for the static experiment did not show enhancement on mineralization with any treatment, in contrast there is a negative effect. There are various ways to approach this result. Taking in consideration that both samples had the same parameters on demineralization, mineralization and electric stimulus, we have to address the discussion from the differences on the experimental set up. One reason of success of the dynamic experiment could be that even though the flux of SBF was very little, this interaction on the collagen sample generates mechanical vibrations. Rosenberg stated that fluid flux is a common set up for generating mechanical effort which might also affect collagen activity[24]. This mechanical effort as showed in other investigations[24,25], this can work on to the biomineralization process. However, the control sample also was submitted to flux. Therefore the difference on electrodes positioning of both set ups affect the electric stimulus. The electric field on the dynamic experiment is applied directly onto the surface of the collagen which makes the electric field very strong, in the case of static experiment the electric field is applied outside a glass test tube, which is a very indirect application reducing the effectiveness of electric field. This makes that collagen might not be polarized or very little polarized on static the experiment. There is also an electrostatic effect on ions in SBF that acts under the electric field. In the dynamic experiment the polarization source is right next to the collagen exterior making that the electrodes polarization acting directly on the collagen walls attracting ions to bone inner walls. On the other hand for static experiment the glass is in contact with electrodes and bone is in the middle of the tube, thus the polarization source is far away from collagen and presumably ions will be concentrating on the edge of test tube and not in the middle where collagen is placed.

Chapter 5

Conclusions & Outlook

5.1 Conclusions

Collagen structure was successfully demineralized with SBF, and experimental set up was designed and implemented with the tools that were constructed in order to deliver Electric Stimulus (ES) to collagen demineralized samples. Results showed that collagen polarization might be a mechanism by which ES enhances bone mineralization, therefore this will have an effect on fracture healing. Dynamic experiment were the most effective set up for mineralization, a marked difference in mineralization was obtained with respect to the static experiment set up. On the other hand chua signal was the most effective for collagen mineralization and this was observed specially in dynamic experiment, and chua signal showed a very good distribution of calcium particles on collagen surfaces. From these results we conclude that Electrical stimulation induces collagen biomineralization.

5.2 Recommendations

Project should be developed further adding relevant statistical analysis using more samples. Other aspects to consider is a comparison between other ES that were not considered in the present study, in order to find the most effective one.

Appendix A

Long Appendix 1 Heading

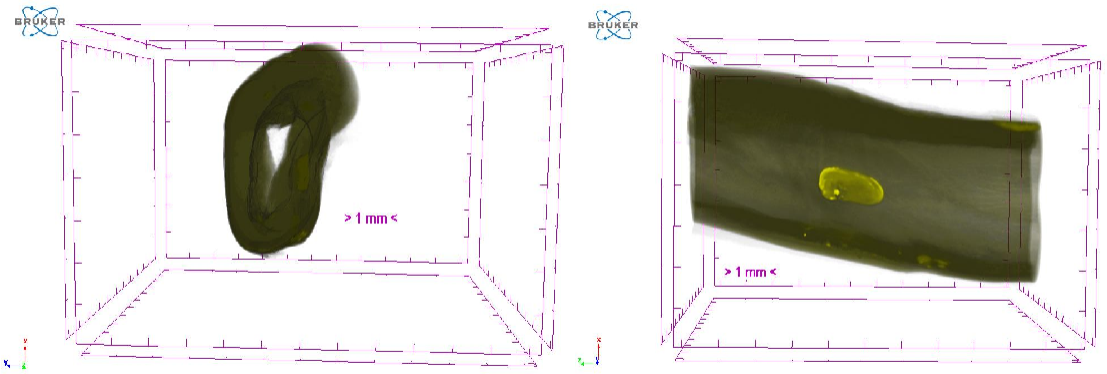

Figure A.1: 3D x-ray image of sample periodic of dynamic experiment

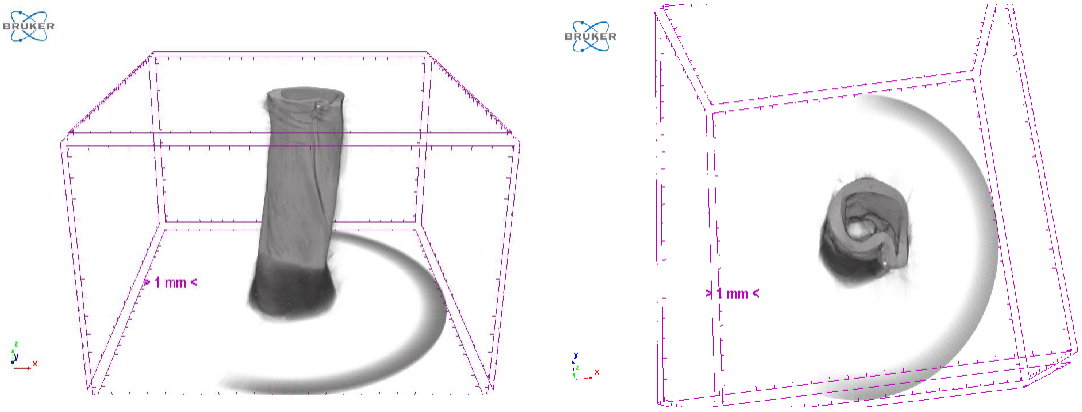

Figure A.2: 3D x-ray image of sample control of dynamic experiment

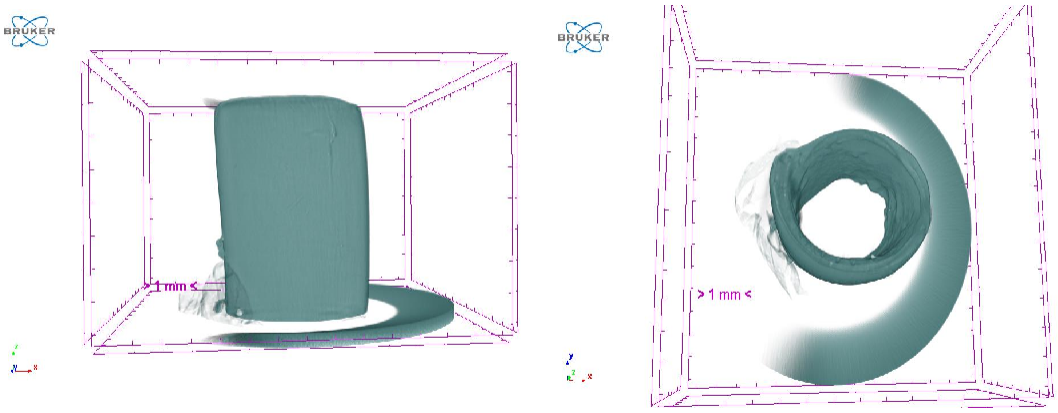

Figure A.3: 3D x-ray image of sample periodic1 of static experiment

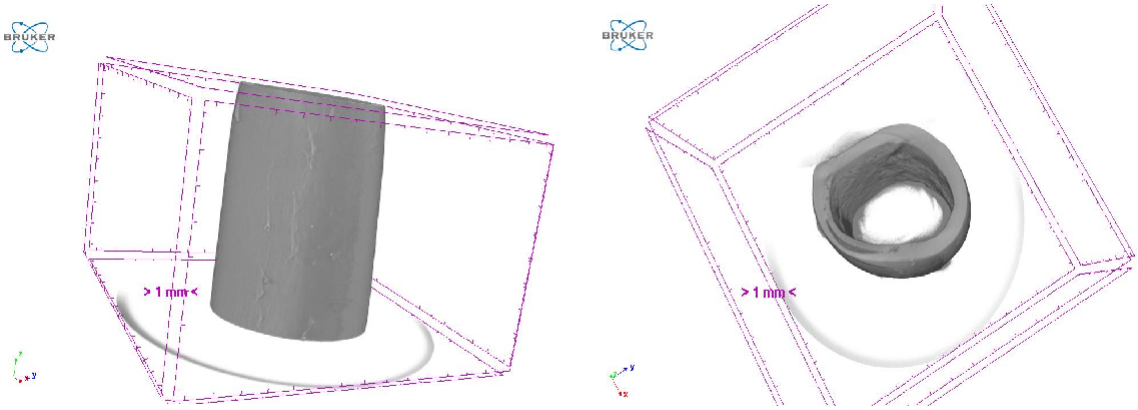

Figure A.4: 3D x-ray image of sample periodic2 of static experiment

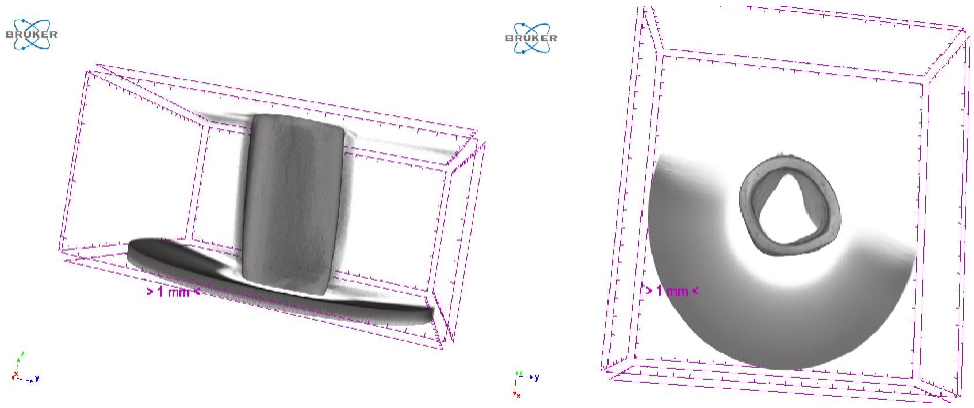

Figure A.5: 3D x-ray image of sample chua1 of static experiment

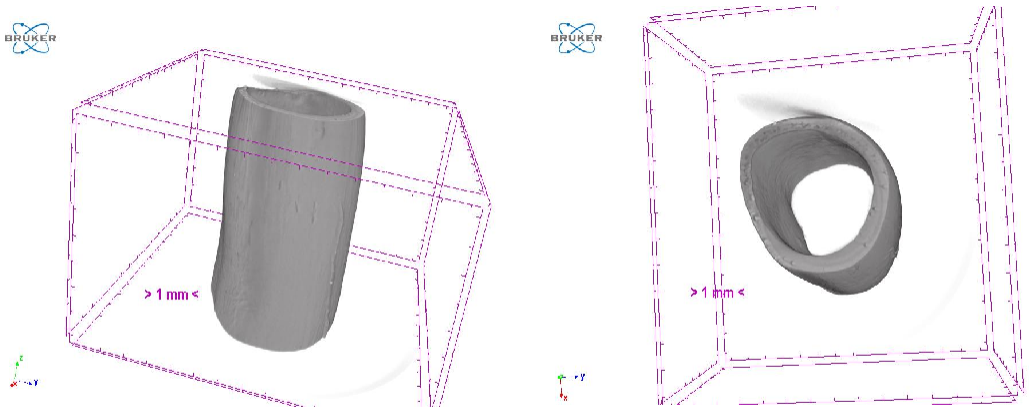

Figure A.6: 3D x-ray image of sample chua2 of static experiment

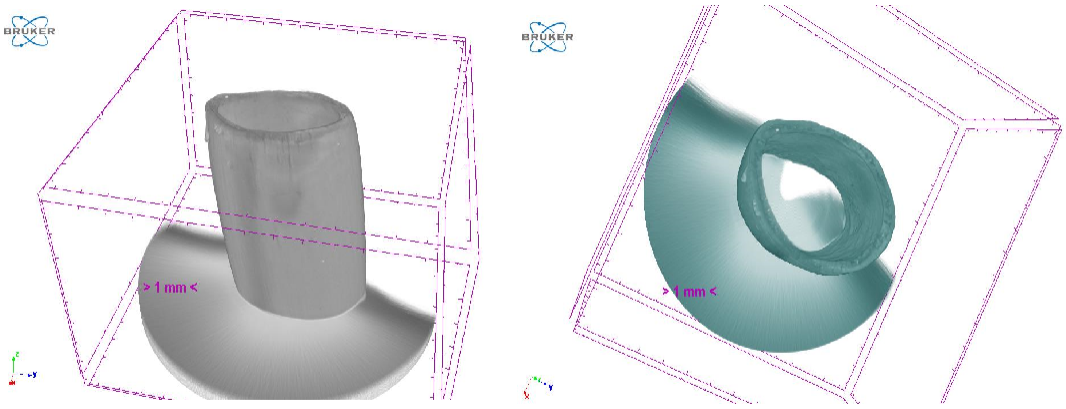

Figure A.7: 3D x-ray image of sample control1 of static experiment

Bibliography

[1] Victoria, G.; Petrisor, B.; Drew, B.; Dick, D. Bone stimulation for fracture healing: What's all the fuss? *Indian journal of orthopaedics* **2009**, *43*, 117.

[2] Zhao, M.; Penninger, J.; Isseroff, R. R. Electrical activation of wound-healing pathways. *Adv Skin Wound Care* **2010**, *1*, 567–573.

[3] Griffin, M.; Bayat, A. Electrical stimulation in bone healing: critical analysis by evaluating levels of evidence. *Eplasty* **2011**, *11*.

[4] Shamos, M. H.; Lavine, L. S. Physical bases for bioelectric effects in mineralized tissues. *Clinical Orthopaedics and Related Research®* **1964**, *35*, 177–188.

[5] Jørgensen, T. E. The effect of electric current on the healing time of crural fractures. *Acta Orthopaedica Scandinavica* **1972**, *43*, 421–437.

[6] Griffin, M.; Sebastian, A.; Colthurst, J.; Bayat, A. Enhancement of differentiation and mineralisation of osteoblast-like cells by degenerate electrical waveform in an in vitro electrical stimulation model compared to capacitive coupling. *PloS one* **2013**, *8*, e72978.

[7] Ryaby, J. T. Clinical effects of electromagnetic and electric fields on fracture healing. *Clinical Orthopaedics and Related Research®* **1998**, *355*, S205–S215.

[8] Aaron, R. K.; Ciombor, D. M.; Simon, B. J. Treatment of nonunions with electric and electromagnetic fields. *Clinical Orthopaedics and Related Research®* **2004**, *419*, 21–29.

[9] Weiner, S.; Wagner, H. D. The material bone: structure-mechanical function relations. *Annual review of materials science* **1998**, *28*, 271–298.

[10] Vallet-Regi, M.; González-Calbet, J. M. Calcium phosphates as substitution of bone tissues. *Progress in solid state chemistry* **2004**, *32*, 1–31.

[11] Chen, J.; Burger, C.; Krishnan, C. V.; Chu, B.; Hsiao, B. S.; Glimcher, M. J. In vitro mineralization of collagen in demineralized fish bone. *Macromolecular Chemistry and Physics* **2005**, *206*, 43–51.

[12] Legeros, R. Z. Apatites in biological systems. *Progress in crystal growth and characterization* **1981**, *4*, 1–45.

[13] Gruselle, M. Apatites: a new family of catalysts in organic synthesis. *Journal of Organometallic Chemistry* **2015**, *793*, 93–101.

[14] Fukada, E.; Yasuda, I. On the piezoelectric effect of bone. *Journal of the physical society of Japan* **1957**, *12*, 1158–1162.

[15] Buehler, M. J. Nature designs tough collagen: explaining the nanostructure of collagen fibrils. *Proceedings of the National Academy of Sciences* **2006**, *103*, 12285–12290.

[16] Blumpkin999, Tendon hierarchy and organization.png. 2017; `https://commons.wikimedia.org/wiki/File:Tendon_hierarchy_and_organization.png`.

[17] Baranowski, T. J.; Black, J. *Mechanistic approaches to interactions of electric and electromagnetic fields with living systems*; Springer, 1987; pp 399–416.

[18] Bodamyali, T.; Kanczler, J.; Simon, B.; Blake, D.; Stevens, C. Effect of faradic products on direct current-stimulated calvarial organ culture calcium levels. *Biochemical and biophysical research communications* **1999**, *264*, 657–661.

[19] Brighton, C. T.; Wang, W.; Seldes, R.; Zhang, G.; Pollack, S. R. Signal transduction in electrically stimulated bone cells. *JBJS* **2001**, *83*, 1514–1523.

[20] Bassett, C. Fundamental and practical aspects of therapeutic uses of pulsed electromagnetic fields (PEMFs). *Crit Rev Biomed Eng* **1989**, *17*, 451–529.

[21] Persikov, A. V.; Ramshaw, J. A.; Brodsky, B. Prediction of collagen stability from amino acid sequence. *Journal of Biological Chemistry* **2005**, *280*, 19343–19349.

[22] Eastoe, J. The amino acid composition of mammalian collagen and gelatin. *Biochemical Journal* **1955**, *61*, 589.

[23] Solitchka, Collagen biosynthesis (en).png. 2005; `https://commons.wikimedia.org/wiki/File:Collagen_biosynthesis_(en).png`.

[24] Rosenberg, N.; Levy, M.; Francis, M. Experimental model for stimulation of cultured human osteoblast-like cells by high frequency vibration. *Cytotechnology* **2002**, *39*, 125–130.

[25] Kaspar, D.; Seidl, W.; Neidlinger-Wilke, C.; Beck, A.; Claes, L.; Ignatius, A. Proliferation of human-derived osteoblast-like cells depends on the cycle number and frequency of uniaxial strain. *Journal of biomechanics* **2002**, *35*, 873–880.

[26] Lavröd, J. The Anatomy of the Chua circuit. **2014**,

[27] Siderskiy, V.; Kapila, V. Parameter matching using adaptive synchronization of two Chua's oscillators. *International Journal of Bifurcation and Chaos* **2014**, *24*, 1430032.

[28] Sirderskiy, V. How to build Chua's circuit. 2019; `http://www.chuacircuits.com/howtobuild1.php`.

[29] Fritzing free software.

Abbreviations

ADC Applied Direct Current 2, 3, 7, 9
At% atomic concentration percentage 22, 24

CC Capacitive Coupling xi, xiii, 2–4, 7–9

EDS Electron dispersive spectroscopy 13, 19
EDTA Ethylenediaminetetraacetic acid 16
ES Electric Stimulus xi, xiii, 1–4, 7–9, 35

IC Inductive Coupling 2, 3, 7, 9

SBF Simulated body fluid xi, xiii, 15, 24, 26
SEM Scanning electron microscopy 13