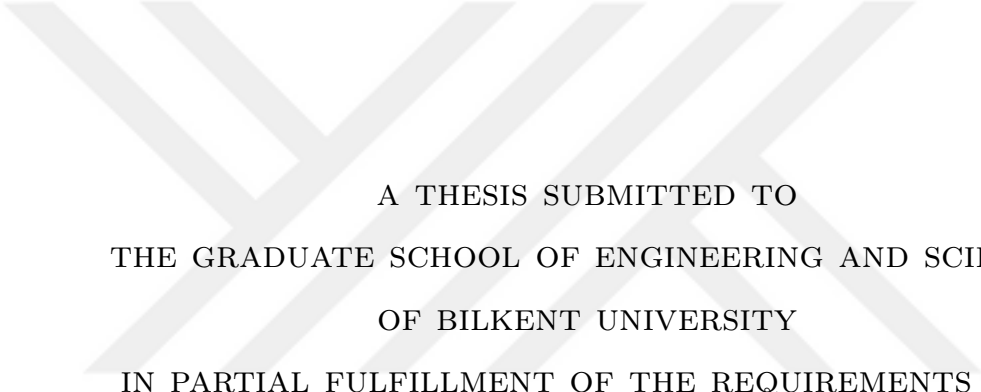


BIOINSPIRED ORGANIC-INORGANIC COMPOSITE MATERIALS



A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
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IN
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By
Egemen Deniz Eren
May 2016

Bioinspired Organic-Inorganic Composite Materials

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May 2016

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.



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ABSTRACT

BIOINSPIRED ORGANIC-INORGANIC COMPOSITE MATERIALS

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M.S. in Materials Science and Nanotechnology
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May 2016

Nature has been an inspiration and information source for scientists over centuries, for developing new materials. A great effort has been spent in order to understand biological materials. The biomineralization process is observed in the nature and it creates perfectly hierarchical structures, which give the living organisms extraordinary properties. It is also a fact that along with the nature; living creatures such as nacre and bacteria employ biomineralization in order to produce minerals for protection and navigation purposes. In addition, bone is a composite material which protects the internal organs and provides mechanical support and is a result of biomineralization process.

In this thesis, the biomineralization processes of living organisms and bone is mimicked by employing peptide amphiphile nanofibers as templates for inorganic materials production. Glutamic acid residue is used in order to mimic the negatively charged domains for proteins, which play crucial roles in biomineralization process in some organisms and bone. In order to mimic the structure of sea shell, which is composed of calcium carbonate, and bone, which consists of calcium phosphate, relevant mineral solutions were used. In conclusion, when organic and inorganic components are used together, they demonstrate superior mechanical properties, when compared to organic molecules alone.

Keywords: Peptide amphiphiles, self-assembly, biominerals, mineralized composite gels, template directed inorganic mineral synthesis, calcium phosphate, calcium carbonate, enhancement in mechanical properties.

ÖZET

BİYOESİNLENİLMİŞ ORGANİK-ANORGANİK KOMPOZİT MALZEMELER

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Malzeme Bilimi ve Nanoteknoloji, Yüksek Lisans
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Mayıs 2016

Doğa, bilim adamları için yüzyıllardır önemli bir ilham ve bilgi kaynağı olmakla beraber, yeni malzemelerin geliştirilmesi anlamında her zaman benzersiz bir kaynak olmuştur. Doğanın kendi başına elde edebildiği bu malzemeleri ve yapıları anlamak için büyük çabalar sarf edilmektedir. Doğada şahit olduğumuz biyomineralizasyon mekanizması, mükemmel bir düzende hiyerarşik (aşamalı) yapılar oluşturarak, canlılara üstün nitelikli özellikler kazandırır. Deniz canlıları ve bakteriler gibi canlılar biyomineralizasyon işlemini kullanarak kendilerini korumak, yönlerini bulmak gibi birçok amaç için çeşitli malzemeler ürettikleri bilinen bir gerçektir. Bu canlıların yanı sıra, insanlarda da bulunan ve ayakta durmak ve iç organlarımızı korumak gibi birçok görevi olan kemik de biyomineralizasyon mekanizmasının bir ürünüdür. Buradan yola çıkarak, bu tez çalışmasında peptit amfil molekülüleri kullanılarak doğada tanık olduğumuz biyomineralizasyon mekanizması taklit edilmiştir. Peptit tasarımında kullanılan glutamik asit amino asiti, doğada biyomineralizasyon işleminde rol alan protein yapısındaki eksi yüklü bölgeleri taklit etmek için kullanılmıştır. Bununla birlikte, deniz canlılarının kabuğunun yapısında bulunan kalsiyum karbonatı ve insan kemiğinin yapısında bulunan kalsiyum fosfatı taklit edebilmek için bunlara uygun olarak mineral başlangıç malzemeleri seçilmiştir. Sonuç olarak, organik ve anorganik yapılar karıştırıldıklarında ortaya çıkan kompozit malzemenin, sadece organik malzemeye kıyasla mekanik özelliklerinin daha iyi olduğu gösterilmiştir.

Anahtar sözcükler: Peptit amfiller, kendiliğinden bir araya gelme, biyomineral, mineralize olmuş kompozit gel, şablon yönelimli anorganik mineral sentezi, kalsiyum fosfat, kalsiyum karbonat, mekanik özelliklerde artış.

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Chapter 1

Introduction

1.1 Biomineralization

Biomineralization is a process during which organisms employ biomolecules and biological substances to create minerals in order to use them in various ways. The formation of bone is also a result of mineralization process. The phenomenon of biomineralization occurs in the presence of an organic and inorganic substance. While organic molecules are thought to regulate the formation of biominerals, inorganic substances give the structure several properties such as toughness, strength, and magnetically responsivity [1]. The proteins, which are responsible for formation of biominerals, such as osteonectin, collagen type I, bone sialoprotein and osteocalcin, employ negatively charged domains to obtain the hierarchically structured composite material [2,3,4]. Highly critical role of such proteins suggest that biomineralization process usually occurs in mild conditions in terms of temperature, pH and etc., in which the proteins must be functional [5]. Bone is a perfect example of hierarchically structured composite materials and comprises collagen molecules as organic substrate and nanoscale hydroxyapatite minerals as inorganic content [6,7]. While 70% of the bone is made from inorganic minerals, 30% is made of organic substances [8]. The composite structure and hierarchical nature of the bone is responsible for its superior mechanical properties such

as toughness and strength [9]. While, inorganic biomaterials give the composite structure the ability of pressure resistance and toughness; organic content, which is made of collagen molecules, is responsible for elasticity and resistance to stress. Composite structures can also be found in the shells of mollusks, in which polymorphs of calcium carbonate can be found as inorganic component and nacrein protein as organic substance [10,11]. The nacrein protein plays an important role in biomineralization and creation of the composite structure. Crystal phase of calcium carbonate can be controlled by one of the domains of this protein by binding calcium ions, while the other domain of nacrein protein generates a carbonate ion [10,11]. The shell of nacre can be classified as a composite/hybrid material which consists of such architecture, defined as "brick and mortar" arrangement, where aragonite crystals behave as bricks and protein fibers behave as mortar [12,13,14].

1.1.1 Calcium Phosphate

Calcium phosphates are the minerals which contain calcium ions together with orthophosphates (PO_4^{3-}) or metaphosphates (PO_3^-), and they can also form compounds containing $H_2PO_4^-$ and HPO_4^{2-} ions. In the structure of bone and teeth, both HPO_4^{2-} and PO_4^{3-} can be found, in addition, hydrated calcium phosphate contains OH^- ions, thus they belong to the basic apatitic calcium phosphate family [15]. Various calcium phosphates and their chemical formula can be seen in Table 1.1. Calcium phosphates can be found as minerals, and one of the most important type of calcium phosphate is apatite. They can be used in implant applications such as surface coating on metals or in composites. Table 1.2 describes the physical properties, lattice parameters and densities of various phases of calcium phosphates and apatites. When a compound has a general formula of $X_{10}(YO_4)_6Z_2$ and has a crystal structure of hexagonal system, space group, $P6_3/m$, then it can be described as apatite. The structure of apatite is highly tolerant to substitutions, therefore, X, Y and, Z can be replaced by various types of other ions [15,21]. Among all the apatite types, hydroxyapatite has drawn attention owing to its bioactivity, biocompatibility and similarity of its chemical

<i>Abbreviation</i>	<i>Ca/P Ratio</i>	<i>Composition</i>
HAp	10/6	Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$
FAp	10/6	Fluorapatite, $Ca_{10}(PO_4)_6F_2$
ClAp	10/6	Chloroapatite $Ca_{10}(PO_4)_6Cl_2$
MCPM	1/2	Monocalcium phosphate monohydrate, $Ca(H_2PO_4)_2H_2O$
MCPA	1/2	Monocalcium phosphate anhydrous, $Ca(H_2PO_4)_2$
TTCP	2	Tetracalcium phosphate, $Ca_4P_2O_9$
β -TCP	3/2	Tri-calcium phosphate
ACP	-	Amorphous calcium phosphate
α -TCP	3/2	α -tricalcium phosphate, α - $Ca_3(PO_4)_2$
OCP	8/6	Octacalcium phosphate, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$
DCPD	1	Dicalcium phosphate dehydrate, $CaHPO_4 \cdot 2H_2O$
DCPA	1	Dicalcium phosphate anhydrous $CaHPO_4$

Table 1.1: Abbreviations of various types of calcium phosphates [16]

<i>Abbreviation</i>	<i>Ca/P Ratio</i>	<i>Crystal Structure</i>
HAp	10/6	Hexagonal
FAp	10/6	Hexagonal
ClAp	10/6	Hexagonal
TTCP	2	Monoclinic
β -TCP	3/2	Rhombohedral
α -TCP	3/2	Monoclinic

Table 1.2: Table 1.2 Crystal structures of different phases of calcium phosphate and apatites [16–20].

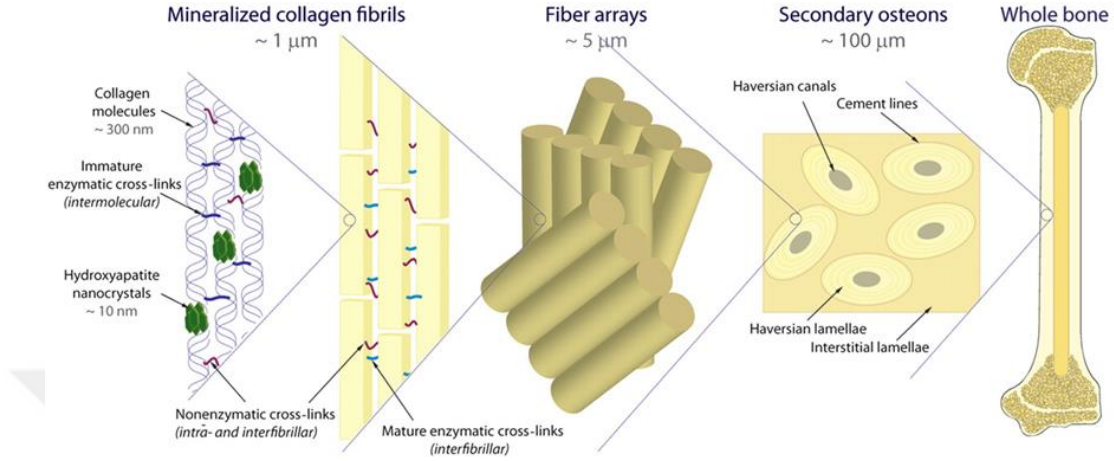


Figure 1.1: Structure of bone (Reproduced with permission from Reference [24] Copyright 2011 Proceedings of the National Academy of Sciences of the United States of America)

composition to bone. The stoichiometric hydroxyapatite has the general chemical formula of $Ca_{10}(PO_4)_6(OH)_2$ and it has the Ca/P ratio of 1.67 [15,21,22]. It is well known that hydroxyapatite minerals promote rapid growth of bone and bonding between surface of the implant material and bone tissue [23].

1.1.2 Calcium Carbonate

Calcium carbonate is a chemical compound with the chemical formula of $CaCO_3$. It can be found in various places on Earth and various types of living organisms. It can be found in the composition of rocks, in addition to in rivers and ocean in soluble form, and finally in the skeletons and shells of living organisms [25,26]. It is also the fundamental substance of shells of marine organisms, snails, pearls and eggshells [25,27]. One of the most interesting properties of calcium carbonate is its polymorphs. It nucleates in three anhydrous crystalline forms which are calcite, aragonite and vaterite [28]. While calcite is the most stable form of calcium carbonate, aragonite is less stable than calcite and vaterite is the least stable one. As in calcium phosphate, calcium carbonate can also coexist in the shell, of the living organism as composite structure. Nacre, for instance, has a hierarchical

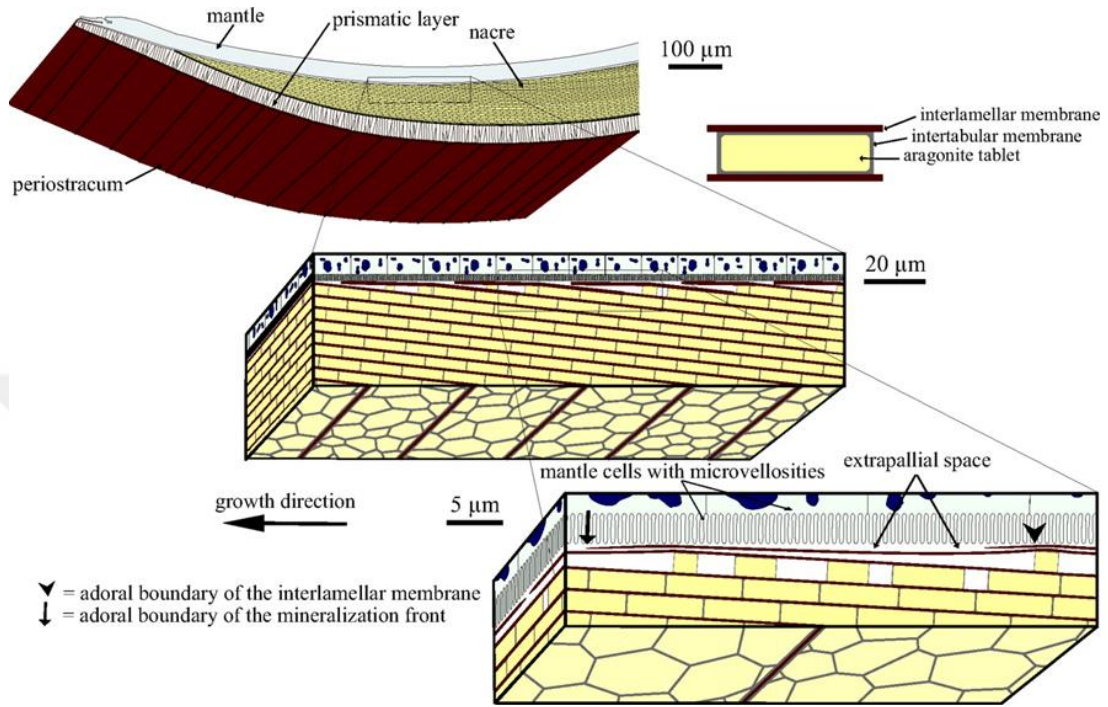


Figure 1.2: Structure of shell of the nacre (Reproduced with permission from Reference [30] Copyright 2009 Proceedings of the National Academy of Sciences of the United States of America)

structure of both organic and inorganic compounds; also, it can be classified as a composite material and is composed of 95 wt % aragonite and 5 wt % of proteins and polysaccharides [12,29]. Owing to its "brick and mortar" arrangement, shell of nacre exhibits much higher fracture toughness compared to mineral by itself [12,29].

1.2 Peptide Amphiphiles and Their Self-Assembly Mechanism

Peptide amphiphiles (PAs) are molecules, which are composed of two fundamental domains, hydrophilic peptide sequence and hydrophobic alkyl tail. The building blocks of peptide sequence are amino acids, which consist of an alpha

carbon which is in the center of the structure where an amino group, a carboxyl group, a hydrogen atom and a side chain R are attached to that alpha carbon [31]. While amphiphilic property is the main triggering element for the self-assembly of PA molecules, there are several effective factors which effect the self-assembly and stability of resulting assemblies such as electrostatic interactions between charged amino acids, hydrophobic interactions, hydrogen bonding and Π - Π stacking [32,33].

In order to obtain peptide amphiphile molecules, a peptide bond (amide bond) must be formed between two amino acids. This amide bond is a covalent bond which occurs between the carboxyl group of the first amino acid and amine group of another amino acid. By using different amino acids, various types of peptide sequences with different properties could be obtained [31]. Once self-assembly of peptide amphiphile molecules is triggered, they form β -sheet secondary structure which allows them to form 1D nanostructures through hydrogen bonding [33].

In the self-assembly mechanism of PA molecules, there are several factors that play critical role such as hydrogen bonding, Π - Π stacking, hydrophobic, van der Waals, and electrostatic interactions [31,33,34]. There are different amino acids which can contribute to the self-assembly of peptide amphiphiles by hydrophobic interactions and Π - Π stacking such as non-polar and aromatic amino acids. In addition, polar amino acids provide hydrogen bonding or electrostatic interactions due to their charged or uncharged characteristics [31,33,34]. Besides the individual amino acids, the peptide backbone also provides stability to the system by forming hydrogen bonds. When take into consideration individually, these interactions are weak compared to covalent interactions, however, once they come together and effect the structure, these interactions can stabilize the gel systems. Self-assembly of peptide amphiphile molecules is generally regulated by noncovalent interactions, and these interactions both drive and stabilize the secondary structure of peptides [35].

Self-assembly of peptide amphiphile molecules can be triggered by various external parameters such as pH, light, temperature and ionic strength [36]. When compared to all alternatives, adjustment of pH is the easiest approach to govern and regulate the self-assembly of peptide amphiphile molecules [37,38,39,40]. Differently from the adjustment of pH, the impact of metal ions and ionic strength

on the self-assembly mechanism of peptide amphiphile molecules were investigated by changing the cation type, which can be mixed with the peptide-based systems [41,42,43]. By altering the type of cation, rheological properties of peptide amphiphile nanofibers can be controlled precisely [42,43]. Along with the rheological properties, structural properties of peptide amphiphile nanofibers can be controlled by changing the cation; for instance, by adding the calcium ions, self-assembly of peptide molecules can be triggered. Furthermore, following the addition of calcium ions, mineral precursors can be added so that mineralized peptide nanofibers can be obtained [44].

1.2.1 Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) is a method introduced by Bruce Merrifield in order to synthesize a tetrapeptide in 1963 [45]. This method is advantageous due to its easy, inexpensive and rapid nature. In this method, the synthesis of peptide amphiphile molecule begins with the loading of protected amino acids on polymeric supports, namely resin. The fundamental logic behind the solid phase peptide synthesis is depends on the repetitive cycles of deprotection and coupling. C-terminus amino acid, which has protected N-terminus, is coupled with the free unprotected N-terminus amino acid which is on the resin. In order to get rid of the excess amount of reagents and improve the purity of the final product, the resin is washed with acetic anhydride after each coupling. At the end of the synthesis, in order to separate the peptide solution from the resin, trifluoroacetic acid (TFA) is employed.

1.2.2 Design of Peptide Amphiphiles

As can be seen from Figure 1.3, there are usually four regions of peptide amphiphile molecules which are a hydrophobic tail, a β -sheet forming peptide domain, charged amino acids region and finally a bioactive region [31,44]. The amphiphilic behavior of PA molecules is a result of the hydrophobic domain of

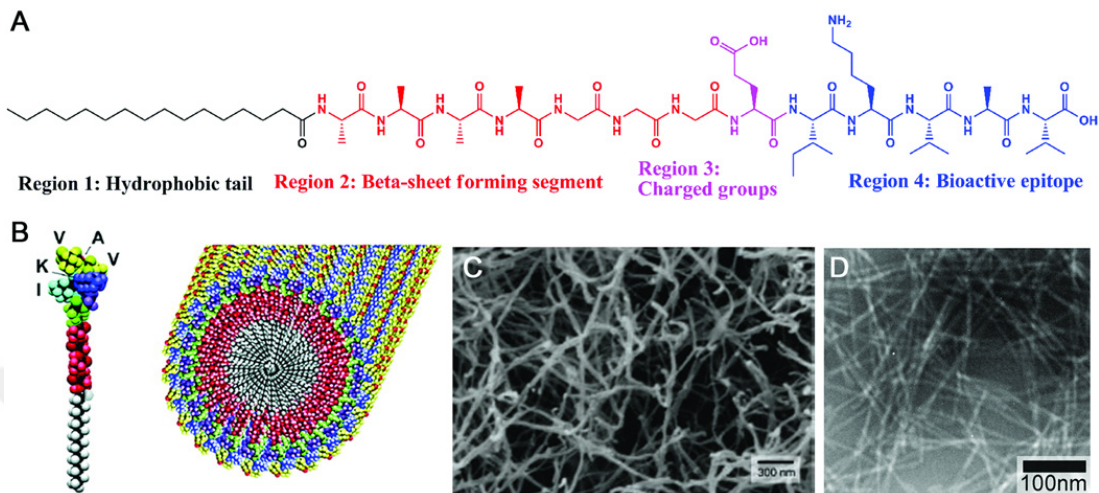


Figure 1.3: Structure of peptide amphiphile nanofibers (Reproduced with permission from Reference [31] Copyright 2008 John Wiley and Sons)

the sequence which can be lauryl groups [38]. The domain which is next to the hydrophobic region of the peptide amphiphile sequence is the domain that triggers the β -sheet formation. Thanks to that region, PA molecules can create β -sheet secondary structure which leads the formation of 1D network [31]. The third region of the PA molecule consists of charged amino acids which are highly critical in terms of solubility of the molecule. Moreover, this domain can be employed as the functional domain for a variety of purposes, without altering the nanofiber geometry [31]. The last region in PA design is the bioactive domain which is responsible for various features and can be used in biomedical applications such as; drug delivery, regenerative medicine and tissue engineering [23,44,46,47].

1.3 Applications of Peptide Amphiphiles

Smaller molecules can self-assemble into 3D structures by employing non-covalent interactions such as hydrogen bonding, hydrophobic, electrostatic, van der Waals, metal-ligand and Π - Π interactions [48]. The phenomenon of self-assembly can be found in nature and living cells ubiquitously. For instance, self-assembly of lipids is the fundamental of formation of cell membrane, formation of DNA double

helix, and folding of proteins. Therefore, both self-assembly process and nature can be an inspiration for scientists to develop new biodegradable, bioactive, and biocompatible materials [49]. Owing to their amphiphilic nature, peptide amphiphilic molecules can be found in various forms and shapes and can be used in various applications such as regenerative medicine, drug delivery, and template in synthesis of inorganic materials.

1.3.1 Regenerative Medicine

Self-assembled peptide amphiphiles can be suitable candidates to be used in tissue engineering and regenerative medicine as scaffolds and cell culture matrices considering their fibrous network. Owing to their excellent abilities to mimic the cellular environment in terms of providing chemical, biological and physical cues, they can be used as synthetic extracellular matrices (ECM). Several bioactive domains can be added to peptide amphiphile sequence in order to bind specifically to heparin, VEGF, TGF β -1, BMP-2 or integrin to obtain scaffolds for angiogenesis [50,51], bone [52] and articular cartilage regeneration [53]. Moreover, the self-assembled peptide amphiphile molecules demonstrated the capacity to promote neurite outgrowth [54].

1.3.2 Drug Delivery

In order to obtain specific targeting and efficient cellular internalization of drugs, self-assembled peptide nanofibers can be employed. To promote bioavailability and penetrate through the cell membrane, the hydrophobic domain of peptide amphiphile molecules plays a critical role. In addition the bioactive domain plays an important role in interaction with the cell [55]. Liposomes, which are functionalized by peptide amphiphiles can be used as drug delivery systems owing to their biodegradability, biocompatibility, and cell membrane mimicking properties [47,56]. Apart from liposomes, fibrous network of the self-assembled peptide

nanofibers can be employed as the drug carrier platform. For instance, oligonucleotide can be used as a drug to cure several diseases such as cancer [57], and delivery of oligonucleotide drug can be achieved by employing positively charged micellar and fibrillary peptide-based nanostructures [58,59].

1.3.3 Template Effect in Synthesis of Inorganic Materials

The directed formation and growth of the inorganic nanostructures can be sustained by the peptide amphiphile molecules where template effect plays an important and outstanding role. By employing these types of organic materials, the directed production of inorganic structures can be achieved in terms of size and morphology. Moreover; due to the ease of modification of the chemical structure of peptide amphiphile molecules, various types and shapes of inorganic structures can be fabricated [44–69]. Stupp group reported several works based on biomineralization of hydroxyapatite by employing self-assembled peptide nanofibers [44,63,70]. They were inspired by the phosphophorylated protein family which are present in dentin, composed of repetitive units of Asp-Ser(P)-Ser(P) and Ser(P)-Asp [71]. Therefore; they used a phosphoserine residue in the sequence of peptide design to mimic the structure of the native protein. This design leads the templated formation of hydroxyapatite crystals onto the surface of the peptide amphiphile nanofibers [44].

Zare et al. fabricated mineralized nanofibers by employing collagen fibrils as template and calcium phosphate as biomaterials [72]. Their strategy incorporates a feed solution which carries both polymeric molecules and inorganic cations. This feed solution interacts with a nanoporous membrane which is in an anion bearing solution and finally allows to the formation of mineralized nanofibers at the end of the pores.

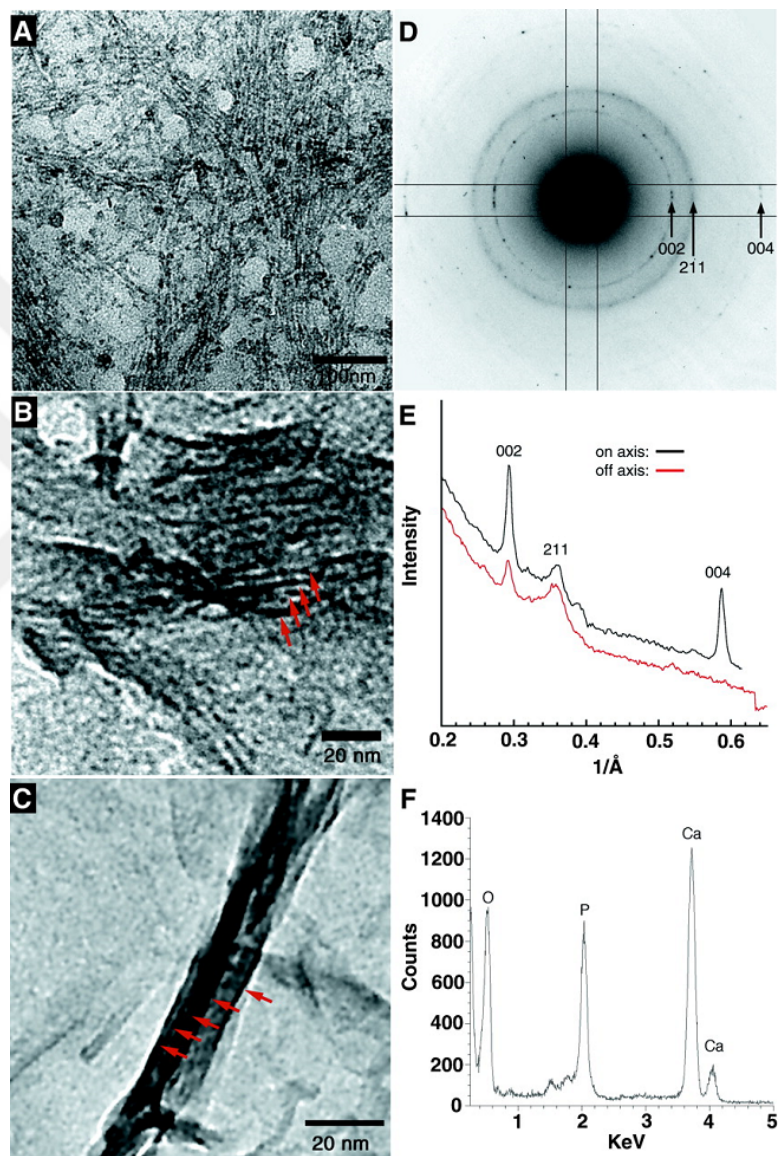


Figure 1.4: Mineralized peptide amphiphile nanofibers. A) TEM image of mineralized nanofibers which are incubated for 10 min in CaCl_2 and Na_2HPO_4 solution. B) Hydroxyapatite crystals can be seen after 20 min of waiting. C) Mature HA crystals cover the PA fibers. D) Electron diffraction pattern and E) plot of intensity versus inverse angstroms show the presence of hydroxyapatite crystals inside the peptide amphiphile fibers. F) EDS profile of mineralized nanofibers. (Reproduced with permission from Reference [44])

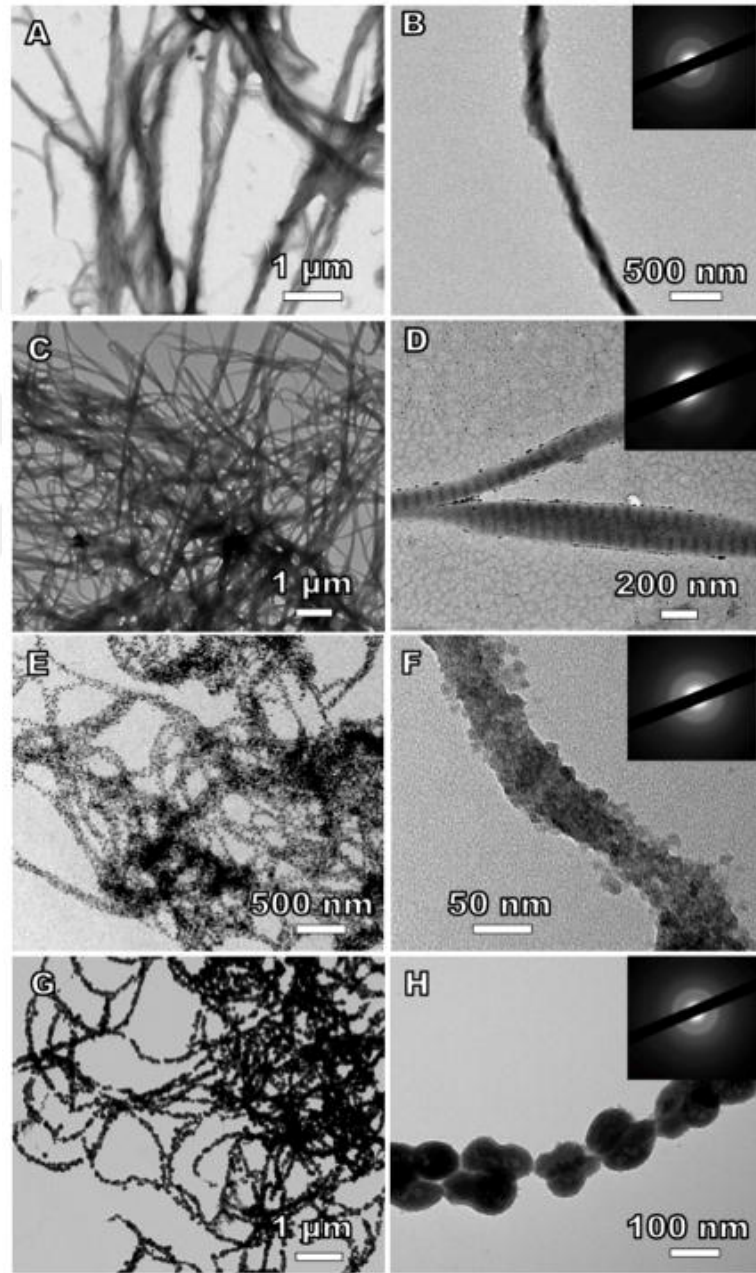


Figure 1.5: Collagen fibrils: A, B) unmineralized; C, D, E, F, G, H) mineralized in different molarities of CaCl_2 solutions (Reproduced with permission from Reference [72])

Chapter 2

Biom mineralization of Peptide Amphiphile Nanofibers

2.1 Introduction

The fabrication of biom mineralized peptide amphiphile nanofibers was done using a negatively charged peptide amphiphile design in order to mimic the negatively charge domains of proteins which play important role in biom mineralization in bone and shell of nacre. In order to trigger the formation of nanofibers, different approaches can be employed such as adjusting pH or introducing different cations into the system. In this study, Ca^{2+} ions were used to trigger the self-assembly of the peptide amphiphile molecules. Moreover, the Ca^{2+} ions that are attached to the glutamic acid residues of the peptide amphiphile molecules will act as nucleation sites for the growth of the inorganic minerals on the surface of the nanofibers. After the formation of self-assembled peptide nanofibers, proper mineral solutions were used to fabricate the desired mineralized composite material. Peptide amphiphile molecules were used as the organic components of the composite material and they behave as template in terms of the growth of the biom minerals. Calcium carbonate and calcium phosphate were used as the

inorganic components of composite material. Enhancement in mechanical properties can be determined by fabricating mineralized peptide nanofibers. Further investigation of cell studies were conducted to determine the biocompatibility of the mineralized composite material. Moreover, alkaline phosphatase activity was tested to determine the osteogenic differentiation of the Saos-2 cells by using biomineralized peptide amphiphile nanofibers.

2.2 Chemicals and Reagents

Chemicals and reagents which are used in synthesis of peptide amphiphile molecules, and in preparation of mineralized peptide amphiphile nanofibers can be seen below.

2.2.1 Chemical and Reagents to Synthesis Peptide Amphiphile Molecules

9-Fluorenylmethoxycarbonyl (Fmoc) protected amino acids, Fmoc-Glu(OtBu)-Wang resin and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from NovaBiochem and ABCR. Lauric acid, N,N-diisopropylethylamine (DIEA), piperidine, acetic Anhydride, and trifluoroacetic acid (TFA) were purchased from Merck. N,N-Dimethylformamide (DMF), dichloromethane (DCM), and diethyl ether were purchased from Sigma-Aldrich. Triisopropylsilane was purchased from Alfa Aesar. The chemicals were used as received, without any purification.

2.3 Characterization Methods

Several characterization methods were employed in order to determine the structural and chemical properties of mineralized peptide nanofibers. To determine the

structural and morphological properties of the mineralized and non-mineralized PA nanofibers, TEM, AFM, and SEM were employed. XRD was used to determine the type of the minerals which are first nucleate onto surface of the nanofibers, and alter the structure of the nanofibers. Later, TGA was employed in order to find the organic/inorganic ratio of the composite materials. Moreover, mechanical properties and self-healing properties of the mineralized peptide amphiphile nanofibers were characterized by rheological measurements. Finally, Live-Dead assay and alkaline phosphatase (ALP) activity assay were conducted to mineralized and non-mineralized materials.

2.3.1 Liquid Chromatography-Mass Spectroscopy (LC-MS)

1 mL of water was used to dissolve 1 mg of peptides. The solutions were then sonicated for 15 min. LC-MS measurements were taken with Agilent Technologies 6530 Accurate-Mass Q-TOF LC-MS with electrospray ionization (ESI) source equipped with reverse-phase analytical high performance liquid chromatography (HPLC). Agilent Zorbox Extend-C18 column was used with 0.1 % (v/v) ammonium hydroxide water (A) and 0.1% (v/v) ammonium hydroxide acetonitrile (B) as mobile phase. The flow of mobile phase was 0.65 mL/min with composition of 98% A 2% B at first 2 minutes. From 2 to 20 min, the flow of B increased until 100% B and turned back again to 2% again for the next five minutes. LC chromatogram was obtained at 220 nm.

2.3.2 Preparative High Performance Liquid Chromatography (Prep-HPLC)

An Agilent 1200 preparative reverse-phase HPLC system equipped with a Gemini 5 μ C18 110A column with size of 100x21.20 mm 5 μ for negatively charged sample was used to purify the peptides. 0.1 % (v/v) ammonium hydroxide–water (A) solution and 0.1% (v/v) ammonium hydroxide–acetonitrile (B) solution were used

for the mobile phases. 100 mg of peptides were dissolved in 10 mL of 0.1 % (v/v) ammonium hydroxide–water and injected into the system.

2.3.3 Critical Point Dryer (CPD)

Ethanol exchange was done prior to CPD to exchange the water content of the composite materials with ethanol. Because water was immiscible in CO₂, ethanol was used as the intermediate solvent to dehydrate the gels, later being infiltrated by CO₂. Ethanol itself was miscible in both water and CO₂. The hydrogel samples were dehydrated in gradually increasing concentrations of 20%, 40%, 60%, and 80% (v/v) ethanol solutions to avoid sample shrinkage for 20 min in each solution, and subsequently transferred to 100% ethanol for 3 hours of waiting period. The samples were dried at the critical point of carbon dioxide (31 °C and 1072 psi) using the Tousimis Autosamdri-815 B, Series C critical point dryer to obtain undamaged dry peptide network of the gel.

2.3.4 Transmission Electron Microscopy (TEM) and Scanning Transmission Electron Microscopy (STEM)

The samples were prepared and mineralized on carbon-coated TEM grid to preserve nanoscale properties of mineralized peptide nanofibers. This is highly critical, especially to avoid the artifacts during the TEM sample preparation. A drop of aqueous peptide amphiphile (0.05% w/v) was mounted on the holey grid. After that, 5 mM of CaCl₂ and 5 mM of (NH₄)₂HPO₄ solutions were dropped on the holey grid at 1 to 10 ratio respectively, and waited for several minutes to obtain calcium phosphate minerals. In addition, instead of adding (NH₄)₂HPO₄, Na₂CO₃ was added as the mineral source to obtain calcium carbonate minerals decorated on the surface of the peptide nanofibers. The samples were also analyzed by EDX which indicated the presence of calcium and phosphate. In order to determine the effect of peptide amphiphiles, control experiments were conducted in the absence of E₃-PA. To prepare control samples, ultra-pure water

was dropped onto carbon-coated TEM grid instead of E₃-PA, then CaCl₂ solution was added and (NH₄)₂HPO₄ or Na₂CO₃ was added subsequently to obtain the formation of calcium phosphate and calcium carbonate, respectively.

2.3.5 Atomic Force Microscopy (AFM)

Mineralized peptide nanofibers were also imaged by AFM in tapping mode after deposition onto a silicon wafer. First of all, E₃-PA (0.05% w/v) solution was dropped onto a silicon wafer, subsequently, 5 mM of calcium chloride solution was dropped to induce nanofiber formation of E₃-PA. Finally, 5 mM diammonium phosphate solution was added in order to provide calcium phosphate mineralized peptide nanofibers. To create calcium carbonate mineralized peptide nanofibers, sodium carbonate solution was added and kept at room temperature for drying. A commercial microscope (MFP3D, Asylum Research) was used in the experiments.

2.3.6 Scanning Electron Microscopy (SEM)

Morphology of mineralized and non-mineralized peptide nanofiber hydrogels were analyzed with FEI Quanta 200 FEG scanning electron microscope after the gels were critical point dried. Critical point drying is crucial in order to preserve the true structure of peptide amphiphiles. The gels formed for SEM analysis had a total volume of 60 μ L and were made by combining 20 μ L of E₃-PA solution with 20 μ L of CaCl₂ solution and 20 μ L of a mineral source for calcium carbonate minerals (NH₄)₂HPO₄ solution was used, and for calcium carbonate minerals Na₂CO₃ solution was used. After the mineralized peptide nanofiber hydrogels were formed, the water was slowly exchanged with a series of water-ethanol mixtures until the gel was in 100% ethanol. To protect the gel structure of the samples, the ethanol exchange was performed gradually prior to the critical point dryer process. No macroscopic changes in the gel appearance were observed during the ethanol exchange process. The critical point dried samples were then sputter coated with 6 nm of a gold-palladium alloy. Finally, FEI Quanta 200

FEG scanning electron microscope with an ETD detector was used to visualize of resulting networks. To determine the effect of the E₃-PA, control experiments were also conducted in the absence of peptide amphiphile molecules.

2.3.7 X-Ray Diffractometer (XRD)

A panalytical Xpert Pro MPD (Multi-Purpose X-Ray Diffractometer) was used in order to obtain XRD spectra of mineralized and non-mineralized peptide amphiphiles. In order to determine the presence and type of minerals, four different experiments were conducted. The gels formed for XRD analysis had a total volume of 560 μ L and they were mixed the ratio of 1:10:10 by following the mixing order of E₃-PA, CaCl₂, (NH₄)₂HPO₄ or Na₂CO₃, respectively. Lastly, the samples immersed into liquid nitrogen were lyophilized, and powdered samples were analyzed by XRD. To determine the differences between mineralized and non-mineralized peptide nanofibers, two control experiments were conducted. Similarly, E₃-PA (1% w/v) was dropped inside the Eppendorf tube. Afterwards, calcium chloride was used to obtain self-assembled peptide nanofiber and hydrogen chloride was used to adjust pH to 2 and obtain peptide nanofiber hydrogel.

2.3.8 Thermal Gravimetric Analysis (TGA)

A TA Instruments TGA Q500 was employed in order to determine the organic/inorganic content of the mineralized and non-mineralized peptide nanofibers. Several different experiments were conducted by using mineralized peptide nanofibers and non-mineralized peptide nanofibers as a control group. To prepare TGA samples, same procedure which was used in the preparation of XRD samples was followed. The temperature range used in the experiment was between 0 and 800 °C, and heating rate was 15 °C /min in N₂ environment.

2.3.9 Rheological Measurements

An Anton Paar Physica RM301 Rheometer was used in order to characterize the viscoelastic properties and effects of minerals regarding the mechanical properties of mineralized peptide amphiphiles. The self-assembly and mineralization of the peptide amphiphiles were induced on the plate of the rheometer. In order to determine the rheological differences between mineralized and non-mineralized peptides amphiphiles, four different experiments were employed. In mineralized peptide nanofiber hydrogels, 250 μ L of (1% w/v) E₃-PA was placed on the plate of the rheometer, and 25 μ L of CaCl₂ solution was added drop by drop in order to provide self-assembled peptide nanofibers. Finally, 25 μ L of (NH₄)₂HPO₄ solution was added to obtain calcium phosphate minerals. In order to obtain calcium carbonate minerals, the same procedure was followed, however, instead of using (NH₄)₂HPO₄ solution, Na₂CO₃ solution was used. Additionally, to determine the mechanical effects of the minerals, two control experiments were carried out where CaCl₂ or HCl was used as inducing agent for the nanofiber formation. All measurements were carried out at room temperature. Gelation kinetics of the mineralized and non-mineralized peptide nanofibers were characterized with time-dependent rheology. During the time-sweep test, strain, and angular frequency were held constant at 0.01% and 10 rad s⁻¹ respectively. To determine the linear viscoelastic range (LVR) of the gels, amplitude sweep test was conducted at a constant angular frequency of 10 rad s⁻¹ with logarithmically ramping the strain amplitude from 0.01 to 1000%. The self-healing (thixotropic) properties of mineralized peptide nanofiber hydrogels were also examined. The self-assembly and mineralization of the peptide amphiphiles were conducted on the plate of the rheometer to avoid disturbance of the inner structure of the materials. In order to identify the differences of self-healing properties between mineralized and non-mineralized peptide nanofiber hydrogels, four different experiments were conducted. In mineralized peptide nanofibers, 250 μ L of (1% w/v) E₃-PA was placed on the plate of the rheometer, and 25 μ L of CaCl₂ solution was added drop by drop in order to provide self-assembly of peptide amphiphile molecules. Finally, 25 μ L of (NH₄)₂HPO₄ solution was added to obtain calcium phosphate minerals, which were decorated on the surface of peptide nanofibers. In order to obtain calcium

carbonate minerals, Na_2CO_3 solution was used and the same procedure was followed instead of using $(\text{NH}_4)_2\text{HPO}_4$ solution. During thixotropic measurements, after the formation of mineralized peptide nanofibers, the sample was allowed to stay 15 min to have stable hydrogels and three steps were followed. The first step was load phase in which mineralized peptide nanofibers were distorted in LVR, 0.01% for 5 min. The second step was decomposition phase, where, the strain was increased to 1000% logarithmically for 1 min to break the chemical bonds and deform the mineralized peptide nanofibers. Finally, recovery phase was employed for 10 min by decreasing strain to 0.01%. All the measurements were carried out at room temperatures.

2.3.10 Cell Culture and Maintenance

Saos-2 cells (human osteosarcoma cell line, ATCCR HTB-85TM) were used in all cell culture experiments including viability and ALP activity assay. Cells were cultured in 75 cm² flasks at 37 °C in a humidified incubator and supplied with 5% CO². The Saos-2 cells were maintained in DMEM (low glucose) with L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell experiments were carried out after the cells reached 90% confluency. The culture medium was changed every 34 days. Cells were seeded under the same conditions used for their maintenance. For ALP activity assay, the seeded cell medium was replaced with osteogenic medium (DMEM with 10% FBS supplemented with 10 mM β -glycerophosphate, 50 $\mu\text{g mL}^{-1}$ ascorbic acid and 10 nM dexamethasone) after reaching confluency.

2.3.11 Live Dead Assay

The viability of Saos-2 cells incubated on mineralized peptide nanofibers and uncoated tissue culture plates (TCP) for 24 h was studied by Live-Dead Assay (Invitrogen). Briefly, cells were seeded on nanofibers and uncoated 96-well tissue culture plates at a density of 5×10^3 cells/well for 24 h. After incubation

under standard conditions, cells were stained with $2\mu\text{M}$ Calcein-AM and $4\mu\text{M}$ ethidium homodimer I (EthD-1) in PBS (1X) for 30 min at room temperature. After incubation, images were taken at three random points per well with a fluorescent microscope at 100x magnification. Viable cells were imaged under fluorescence microscope. All samples were studied in triplicate. Live and dead cells were counted by using Image J and number of live cells was calculated for each sample.

2.3.12 Alkaline Phosphatase (ALP) Activity Assay

The ALP activity of Saos-2 cells was analyzed by quantifying the degradation of p-nitrophenol due to endogenous ALP activity after 3 and 7 days of culture in osteogenic medium. Briefly, cells were seeded on PA nanofiber-coated and uncoated 48-well TCP surfaces at a density of 2×10^4 cells/well and the cell medium was replaced with osteogenic medium after cells reached 90% confluency. At predetermined time points, cells were rinsed with PBS and protein extraction was performed using M-PER protein extraction kit (Thermo) with 5% protease inhibitor solution at $150\mu\text{L}$ per well for 30 min on a shaker. After centrifugation at $14,000g$ for 10 min, supernatants containing the protein fraction were removed and BCA protein assay performed to quantify the amount of protein obtained from the cells as described in the manufacturers protocol. For ALP activity, $50\mu\text{L}$ of the protein sample was incubated with $150\mu\text{L}$ of p-nitrophenol phosphate substrate in 96-well plates for 30 min on a shaker. Serial dilutions of p-nitrophenol in 0.25 M NaOH solution were used as standards. Finally, the optical density of the samples was determined at 405 nm using a microplate reader. ALP results were normalized to the total amount of protein at each time point tested.

2.4 Peptide Synthesis

Fmoc solid phase peptide synthesis method was employed to synthesize Lauryl-Val-Val-Ala-Gly-Glu-Glu-Glu-OH ($\text{E}_3\text{-PA}$). For $\text{E}_3\text{-PA}$, Fmoc-Glu(OtBu)-Wang

resin (100-200 mesh) (Novabiochem) served as the solid support. Carboxylate group activation of 2-mole equivalents of amino acid was succeeded by 1.95-mole equivalents of N,N,N,N-Tetramethyl-O-(1H-benzotriazole-1-yl) uronium hexafluorophosphate (HBTU), and 3-mole equivalents of diisopropylethylamine (DIEA) for 1 mole equivalent of functional sites on the solid resin. Fmoc groups were removed at each coupling step with 20% piperidine/dimethylformamide (DMF) for 20 min. Amino acid coupling time was set to be 2 h at each cycle. Lauric acid served as the source of the hydrophobic domain and its coupling mechanism was similar to amino acid coupling. 10% acetic anhydride-DMF solution was used to block the unreacted amine groups after each coupling step. Cleavage of protecting groups and peptide molecules from the solid support was carried out by using trifluoroacetic acid (TFA) cleavage cocktail (95% TFA, 2.5% water, 2.5% triisopropyl silane) for 3 h. Excess TFA was removed by rotary evaporation. Synthesized E₃-PA was then precipitated in diethyl ether overnight. The precipitate was collected by centrifugation and dissolved in ultra-pure water. This solution was frozen at -80°C followed by lyophilization. The purity of the peptide amphiphile was assessed using Agilent 6530 quadrupole time of flight (Q-TOF) mass spectrometry with electrospray ionization (ESI) source equipped with reverse-phase analytical high performance liquid chromatography (HPLC). Synthesized E₃-PA was purified with a preparative HPLC system (Agilent 1200 series). E₃-PA was then freeze-dried and reconstituted in ultrapure water at pH 7.4 before use.

2.5 Preparation of Biomineralized Peptide Amphiphile Nanofibers

Stock solutions of calcium chloride (CaCl₂), diammonium phosphate (NH₄)₂HPO₄ and sodium carbonate (Na₂CO₃) were prepared in ultra-pure water. Synthesized E₃-PA was also dissolved in ultra-pure water at neutral pH, 1% (w/v). Initially, negatively charged E₃-PA was dropped onto silicon wafer and, positively charged CaCl₂ was added to E₃-PA to trigger self-assembly of peptide amphiphile with charge neutralization and obtain self-assembled fibrous network by employing

non-covalent interactions. To compare the effect of calcium and hydrogen ions, both of the ions were used separately to obtain self-assembled nanofibers. In order to obtain a thermodynamically stable gelation, they were allowed to stay for 30 min. After having a stable hydrogel, mineral precursors were dropped onto the silicon wafer separately to obtain mineralized peptide nanofiber hydrogels. The same procedure was employed to determine the effect of minerals on morphology by altering waiting time. The volumetric ratio of CaCl_2 and mineral precursors ($(\text{NH}_4)_2\text{HPO}_4$ and Na_2CO_3 to $\text{E}_3\text{-PA}$ within the mineralized hydrogels was determined as 1:1. In order to prepare the control samples, ultra-pure water (with the same volume of $\text{E}_3\text{-PA}$) was added to the calcium chloride and mineral precursors. First, ultra-pure water was dropped onto a silicon wafer, and calcium chloride solution was added. Finally, $(\text{NH}_4)_2\text{HPO}_4$ or Na_2CO_3 solution was added to obtain calcium phosphate and sodium carbonate minerals, respectively.

2.6 Results and Discussion

Biomaterialized composite materials were obtained by employing organic molecules as template. This organic structure was synthesized by using solid phase peptide synthesis (SPPS) method to obtain peptide amphiphile molecules. In this work, peptide amphiphile molecules comprise three domains which are hydrophobic alkyl tail, a sequence of amino acids that triggers the formation of β -sheet and charged residue. The first region of the peptide sequence is responsible for hydrophobic interactions; therefore, it gives the structure amphiphilicity. The second domain of the peptide sequence is responsible for triggering the formation of β -sheet. Finally, the last domain of the peptide sequence contains charged amino acids which play a crucial role in terms of the template effect of the peptide amphiphile molecules. Nanofiber formation triggered by Ca^{2+} plays a critical role in terms of the biomineralization process. Free Ca^{2+} ions which are decorated onto surface of the peptide amphiphile nanofibers later interact with the mineral source and promote the nucleation and growth of the minerals.

The negatively charged $\text{E}_3\text{-PA}$ was designed and synthesized to contain an alkyl

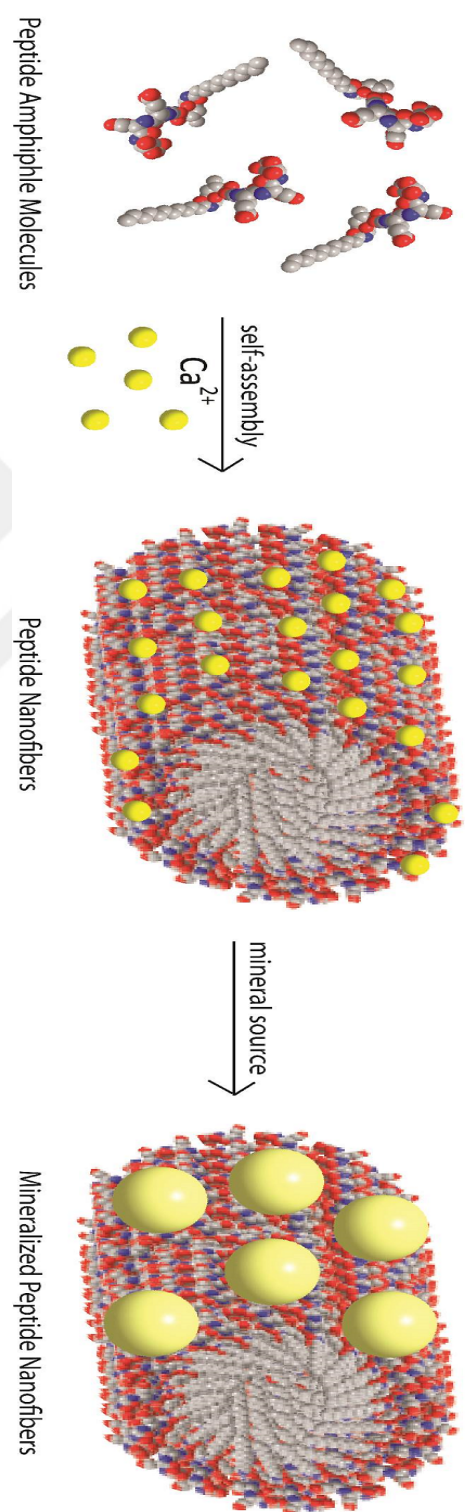


Figure 2.1: Preparation of Biomineralized Peptide Amphiphile Nanofibers

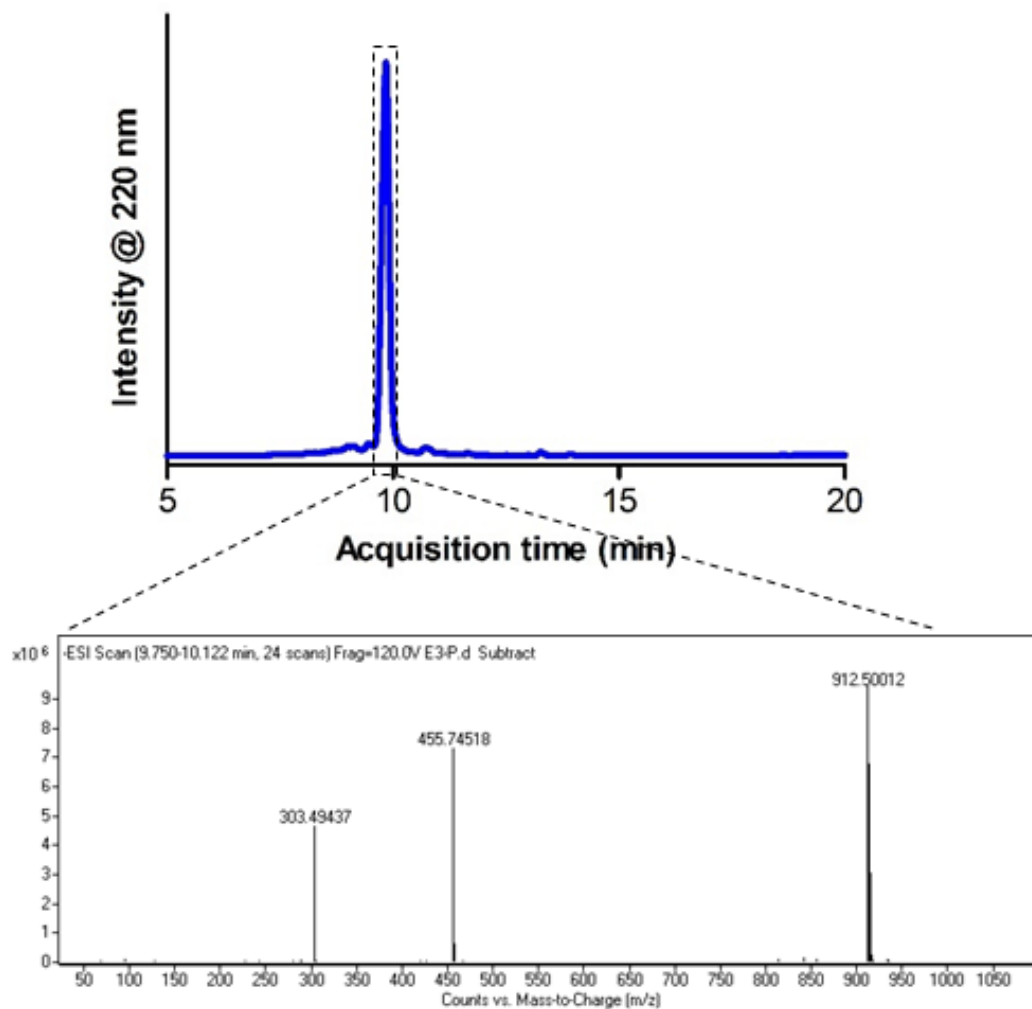


Figure 2.3: Characterization of E₃-PA by using LC-MS (a) liquid chromatogram of E₃-PA by the absorbance at 220 nm, (b) mass spectrum of E₃-PA. MS: (m/z) calculated 913.5008, [M-H]⁻ measured 912.50012, [(M-2H)/2]⁻² measured 455.74518. [(M-3H)/3]⁻³ measured 303.49437.

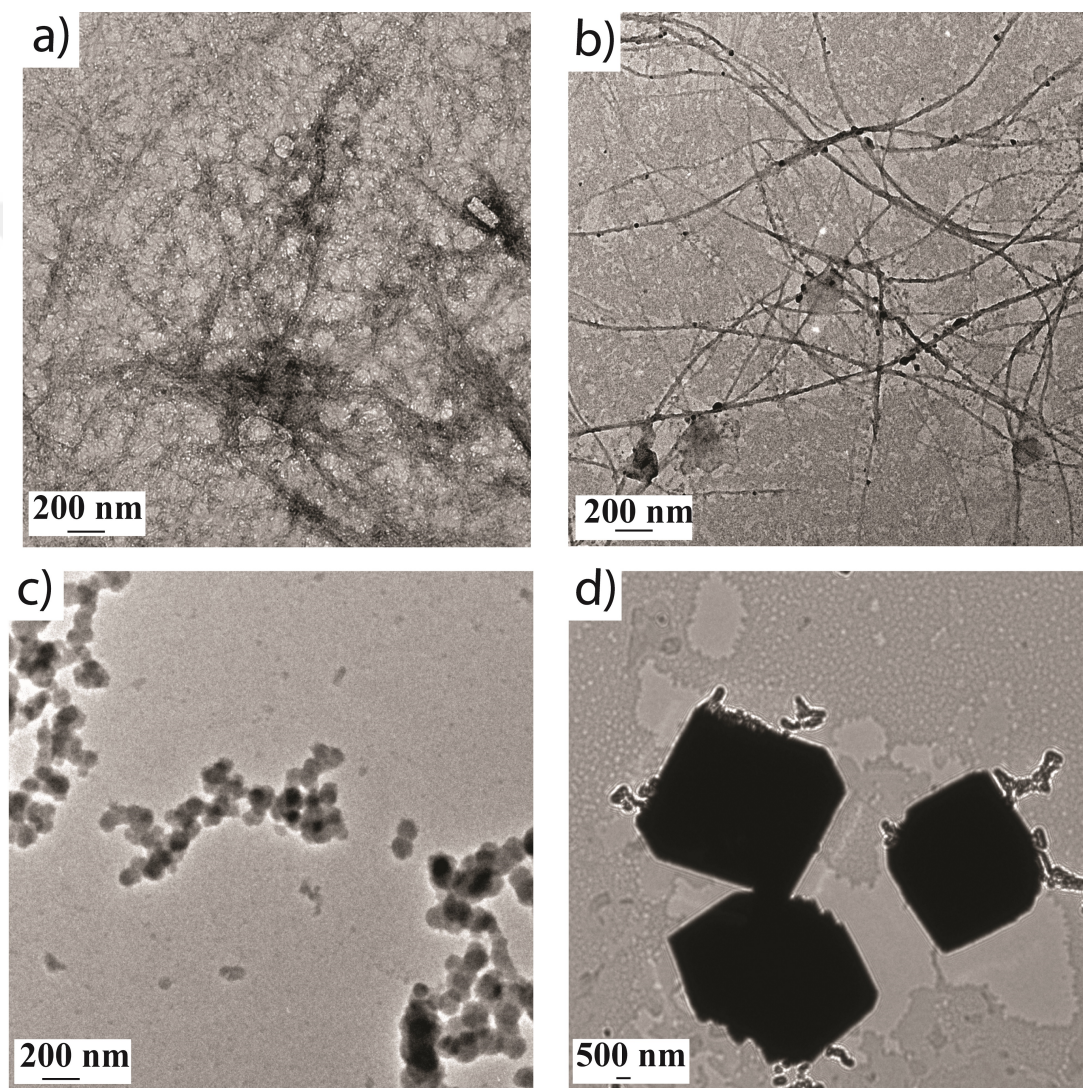


Figure 2.4: Mineralized peptide amphiphile nanofibers: (a) calcium phosphate mineralization after 30 min; (b) calcium carbonate mineralization after 30 min. Minerals without the presence of peptide amphiphile nanofibers: (c) amorphous calcium phosphate minerals after 30 min; (d) the most stable form of calcium carbonate (calcite minerals) after 30 min of mineralization.

minerals [70,73–75]. Also, after 90 min of waiting, morphology alteration in calcium carbonate mineralized peptide nanofibers can be observed (Figure 2.4). Similar to the other control experiments, 90 min of waiting changes nothing in the absence of E₃-PA. Template effect of peptide amphiphile molecules cannot be observed after 90 min of waiting (Figure 2.4).

Finally, nanofiber formation can be observed without the addition of mineral sources. Ca²⁺ ions induce the formation of nanofibers by binding to glutamic acid residues decorating the surface of the nanofibers (Figure 2.5)

AFM was also conducted to compare the differences of morphological properties between mineralized and non-mineralized peptide amphiphile nanofibers. In the case of mineralized peptide nanofibers, minerals on the surface of the nanofibers can be observed clearly (Figure 2.6); however, in the case of non-mineralized peptide nanofibers, there is no sign of mineral content (Figure 2.6).

In addition, scanning electron microscopy (SEM) characterization was conducted to determine the effect of minerals on the morphology of peptide amphiphile nanofibers and to understand the template effect of peptide amphiphile molecules. XRD results which will be discussed later; prove that after allowing the gels to mineralize for 24 h, calcium phosphate, and calcium carbonate mineralization took place in both gel systems. Template effect of E₃-PA can be seen from the SEM images (Figure 2.7). However, when E₃-PA was not used, random distribution of calcium phosphate and calcium carbonate minerals was obtained due to the absence of glutamic acid residues (Figure 2.8). The porous morphologies observed in mineralized peptide nanofibers after 24 h can be employed for tissue engineering applications since both calcium carbonate and calcium phosphate is biocompatible and pore sizes in mineralized peptide nanofibers can promote cell proliferation, adhesion, and differentiation [64,76,77].

To determine the type of the minerals which deposit and grow onto the surface of the peptide amphiphile nanofibers, X-ray diffractometer (XRD) was employed after gels were incubated for 24 h. The mineralized peptide amphiphile nanofibers were analyzed by XRD, which showed the characteristic peaks of hydroxyapatite

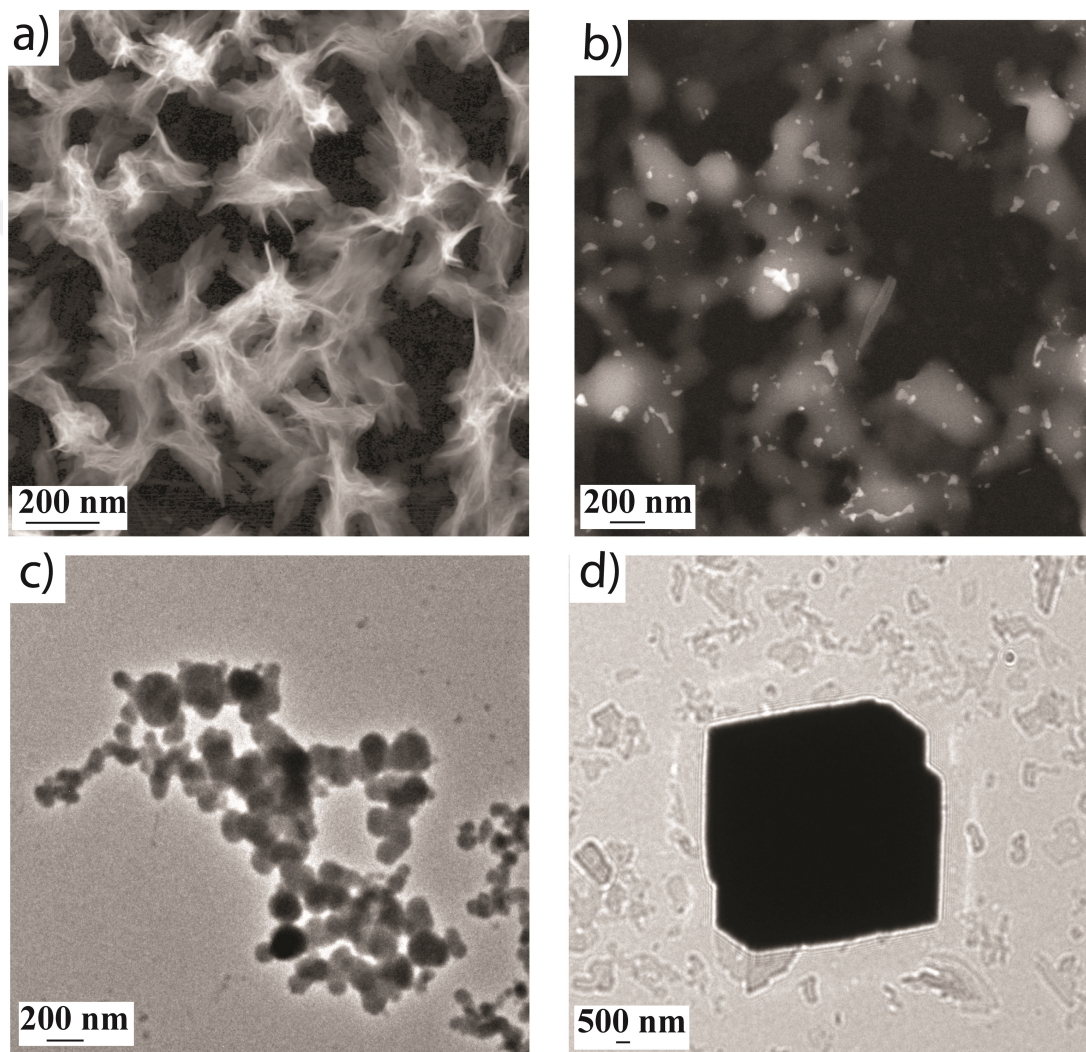


Figure 2.5: (a) Formation of plate-like hydroxyapatite crystals in presence of peptide amphiphile molecules after 90 min of mineralization, (b) mineralization of calcium carbonate in presence of peptide amphiphile molecules after 90 min, (c) amorphous calcium phosphate mineralization after 90 min in absence of peptide amphiphile molecules, (d) formation of calcite crystals after 90 min of mineralization.

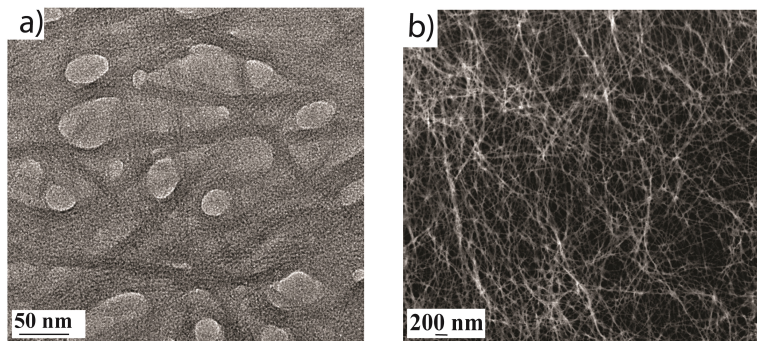


Figure 2.6: (a,b) Peptide amphiphile nanofibers without mineralization process.

and calcite [64,78–85] (Figure 2.9).

The diffraction peaks at (211), (300) and (004) match with the expected peaks of HA (Figure 2.10). In addition, diffraction peaks at (104), (110), (113) and (202) correspond to expected peaks of calcite (Figure 2.11).

Non-mineralized peptide amphiphile nanofibers were also characterized by XRD, and results indicate that there is no characteristic peak which corresponds the HA or calcite (Figure 2.12) (Figure 2.13) (Figure 2.14). The peaks which can be observed in all four gel systems belong to the inevitable content of NaCl salt even after five times of washing prior to preparation of XRD samples (Figure 2.14).

Moreover, TGA was employed to determine the weight loss and organic/inorganic ratio of mineralized peptide nanofibers under elevated temperatures. TGA of the HA / E₃-PA composite material demonstrates a mass loss of 7% up to around 200 °C which corresponds to adsorbed water content (Figure 2.15). In addition, there is a characteristic mass loss between 200 °C and 260 °C, which was attributed to the morphology change of the DCPD (dicalcium phosphate dihydrate) impurity phase to DCPA (dicalcium phosphate anhydrous). As all organic molecules, peptide amphiphile nanofibers are softer and weaker compared to biominerals; therefore, melting and denaturation of those molecules occur earlier compared to biominerals. From this point of view, the weight loss between 300 °C and 600 °C can be attributed to the loss of peptide amphiphile

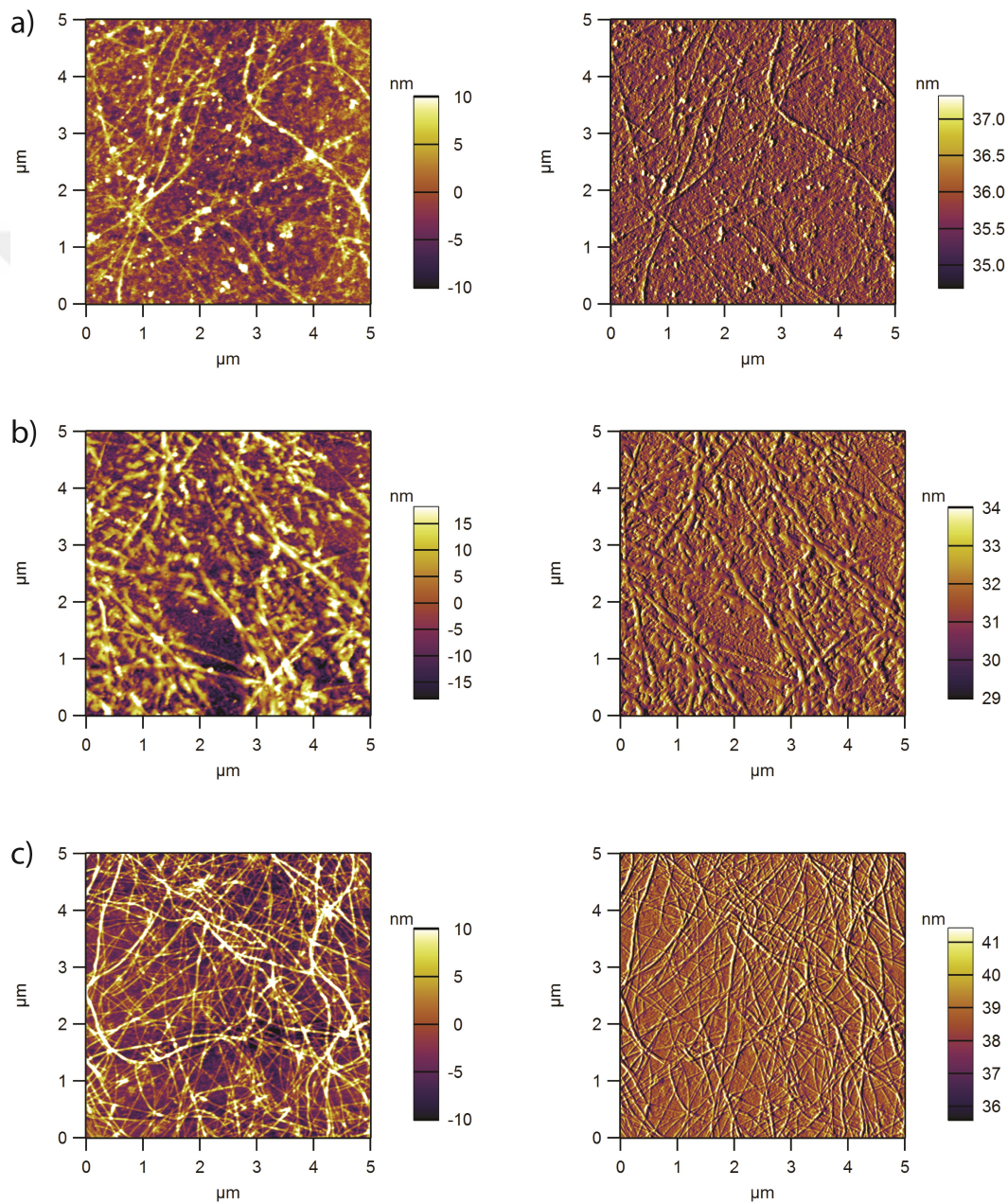


Figure 2.7: AFM images of (a) calcium phosphate, and (b) calcium carbonate mineralized peptide amphiphile nanofibers, (c) non-mineralized peptide amphiphile nanofibers.

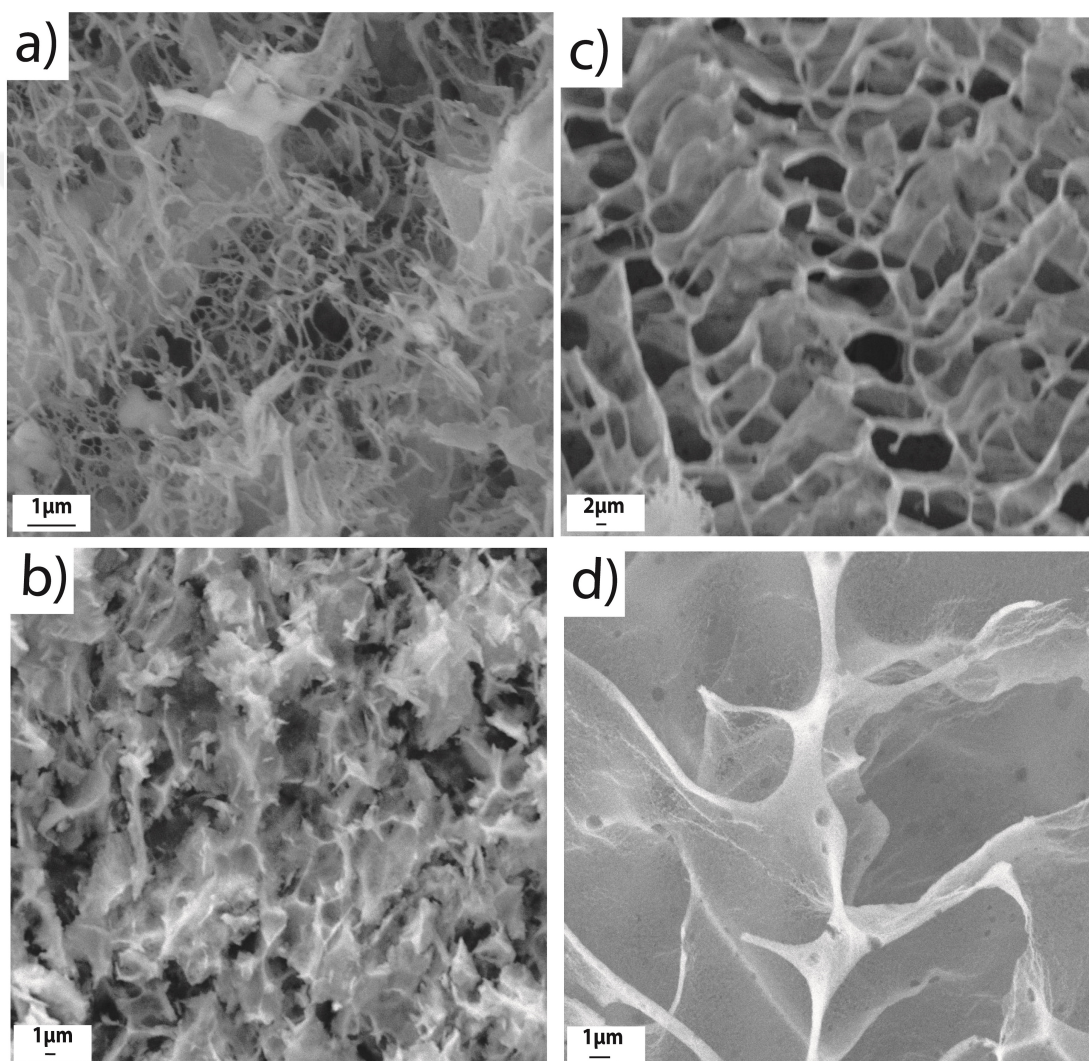


Figure 2.8: SEM images of (a, b) calcium phosphate mineralized peptide amphiphile nanofibers after 24 h of incubation; (c, d) calcium carbonate mineralized peptide amphiphile nanofibers after 24 h of incubation.

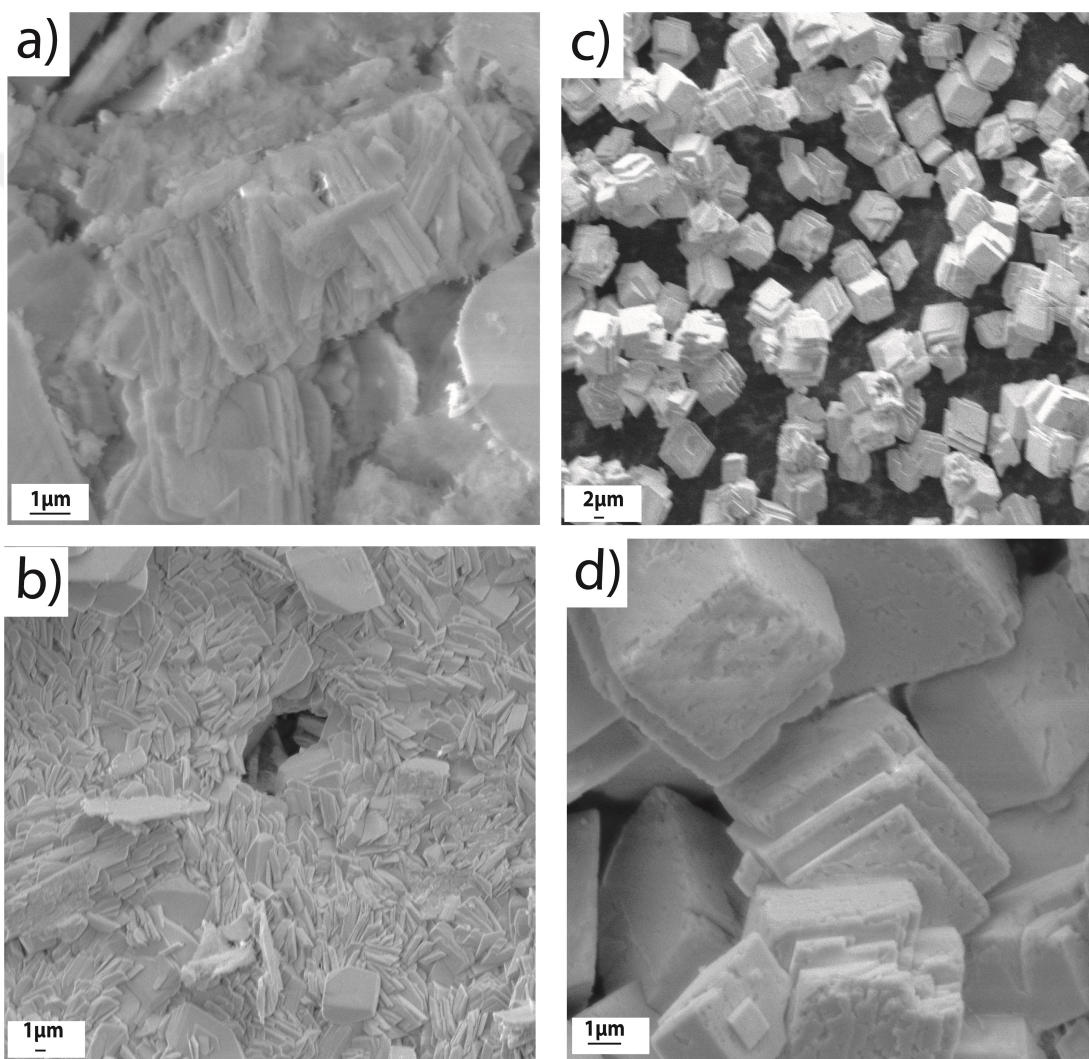


Figure 2.9: SEM images of (a, b) calcium phosphate mineralization in the absence of peptide amphiphile molecules; (c, d) calcium carbonate mineralization in the absence of peptide amphiphile molecules.

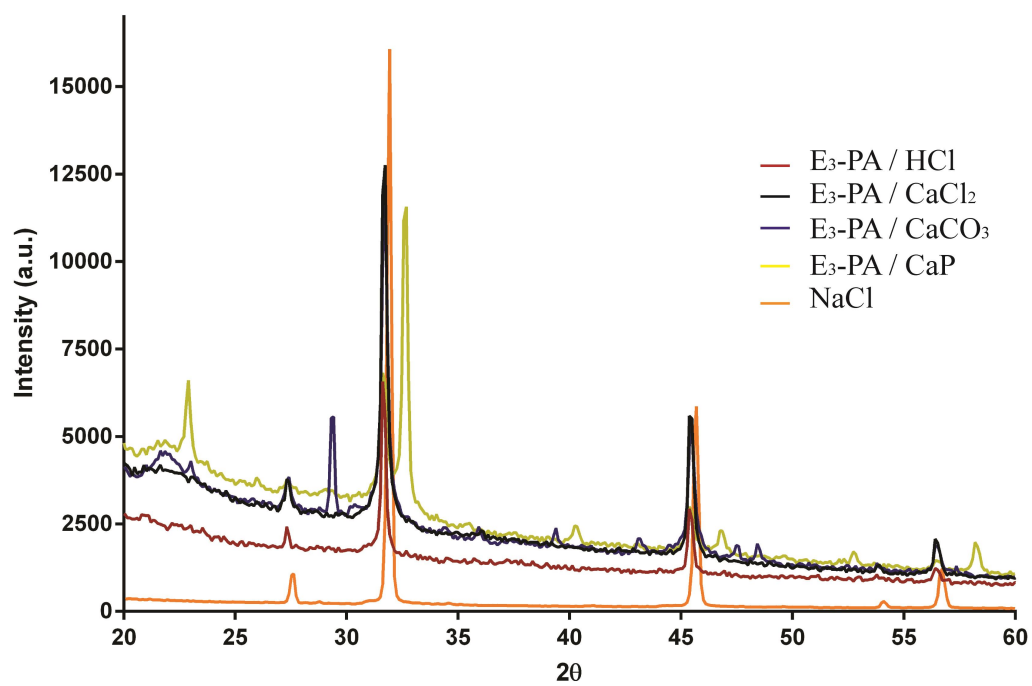


Figure 2.10: XRD patterns of mineralized, and non-mineralized peptide amphiphile nanofibers and NaCl powder.

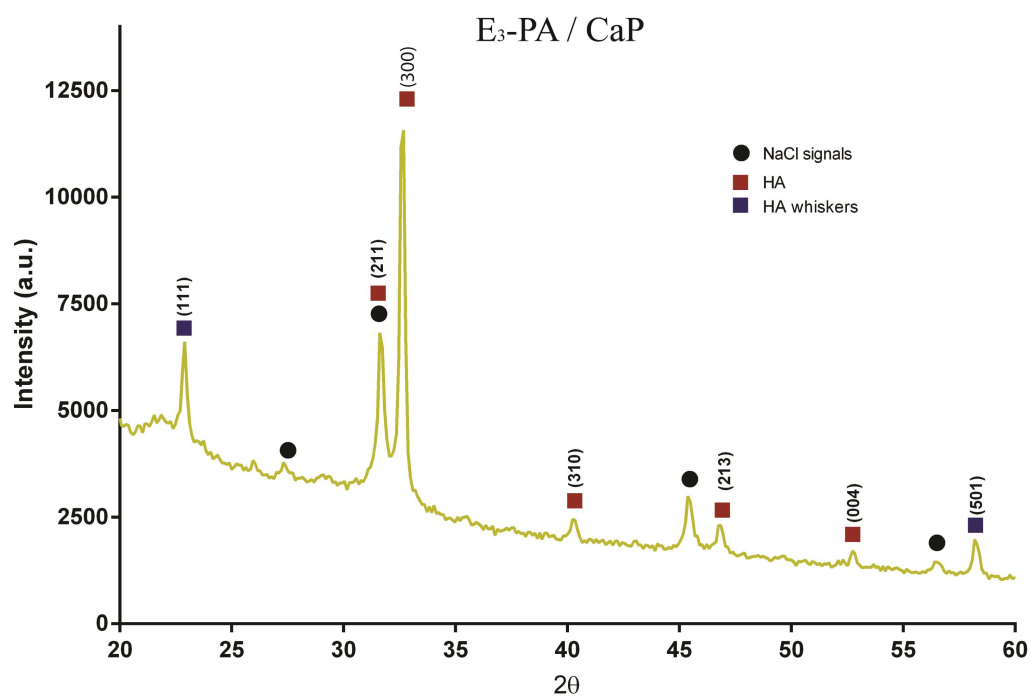


Figure 2.11: XRD patterns of calcium phosphate mineralized peptide amphiphile nanofibers.

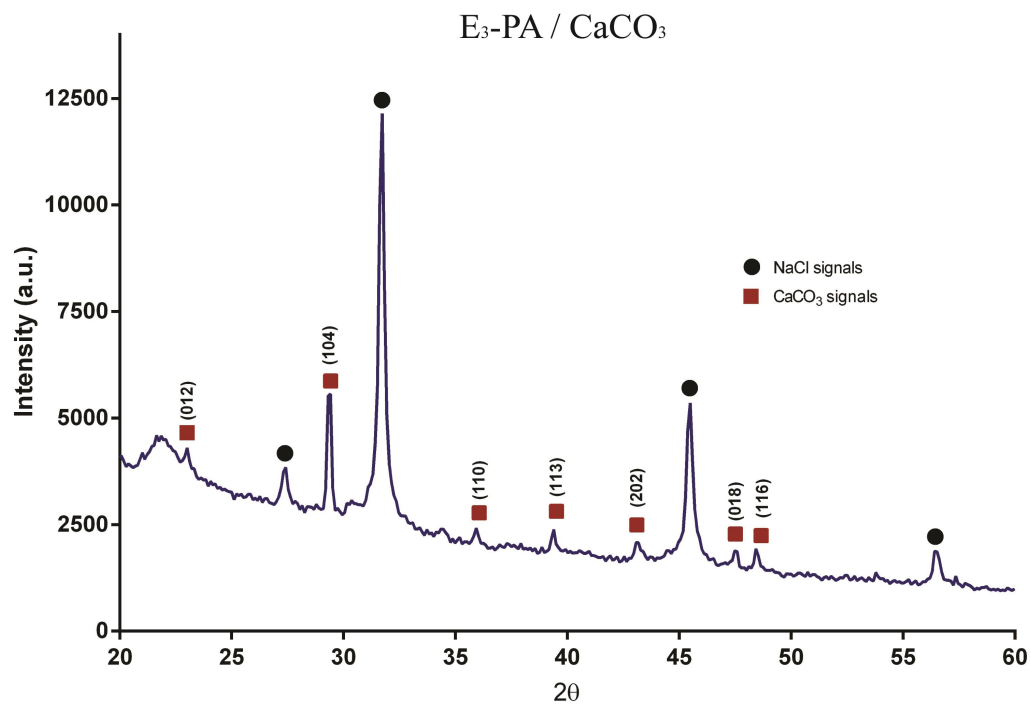


Figure 2.12: XRD patterns of calcium carbonate mineralized peptide amphiphile nanofibers.

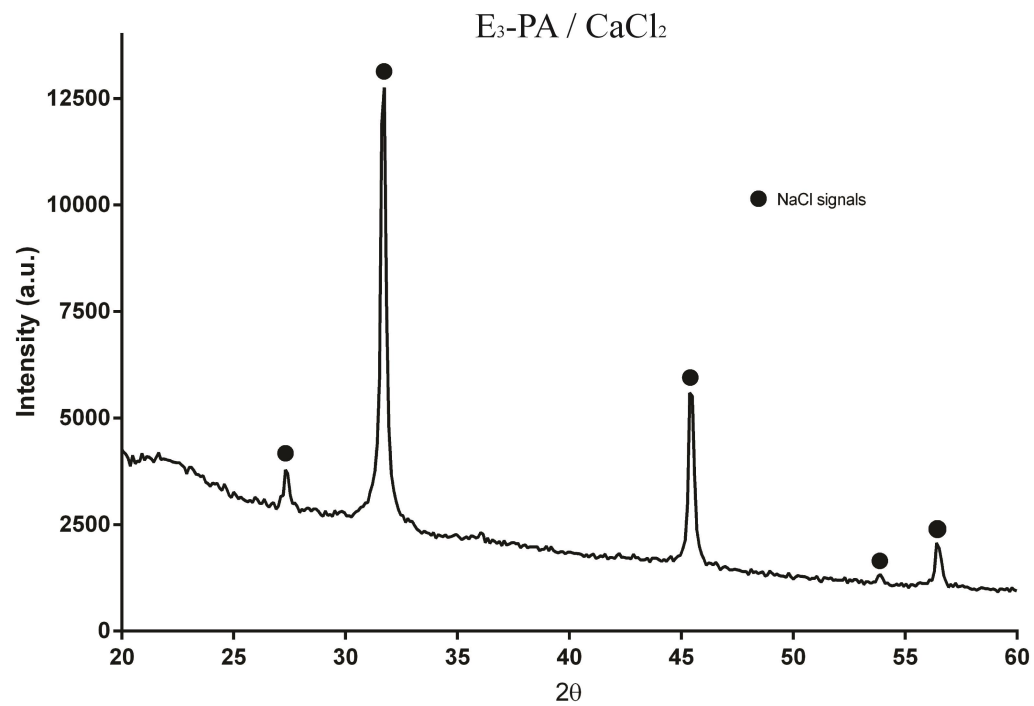


Figure 2.13: XRD patterns of non-mineralized peptide amphiphile nanofibers. The triggering mechanism of self-assembly is calcium ions.

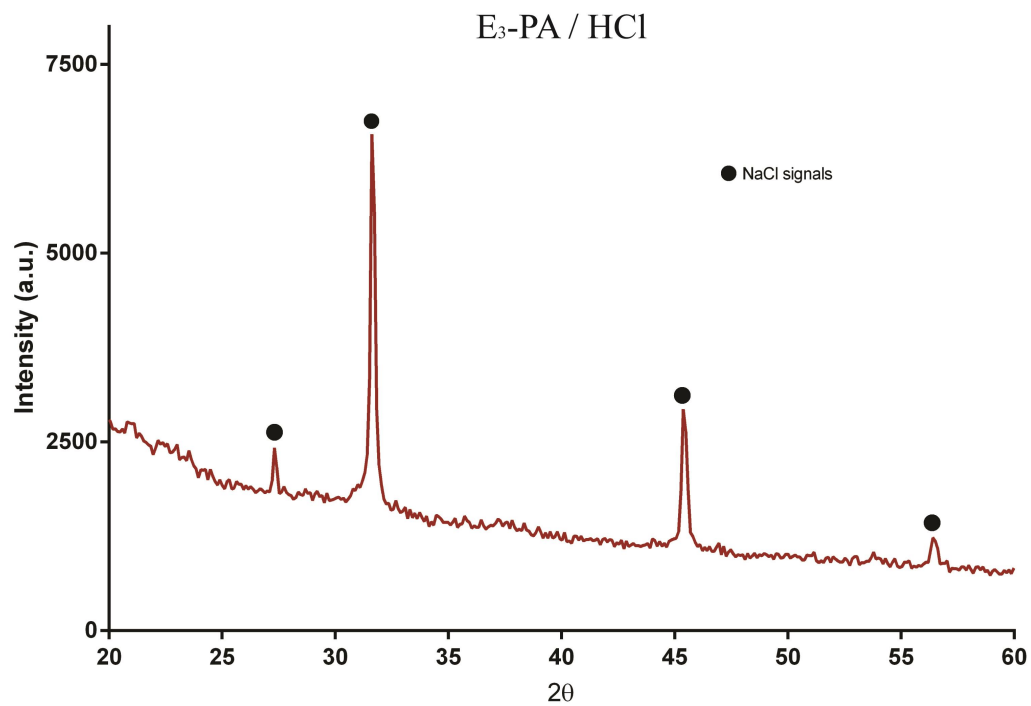


Figure 2.14: XRD patterns of non-mineralized peptide amphiphile nanofibers. The self-assembly mechanism is triggered by lowering the pH by using H^+ ions.

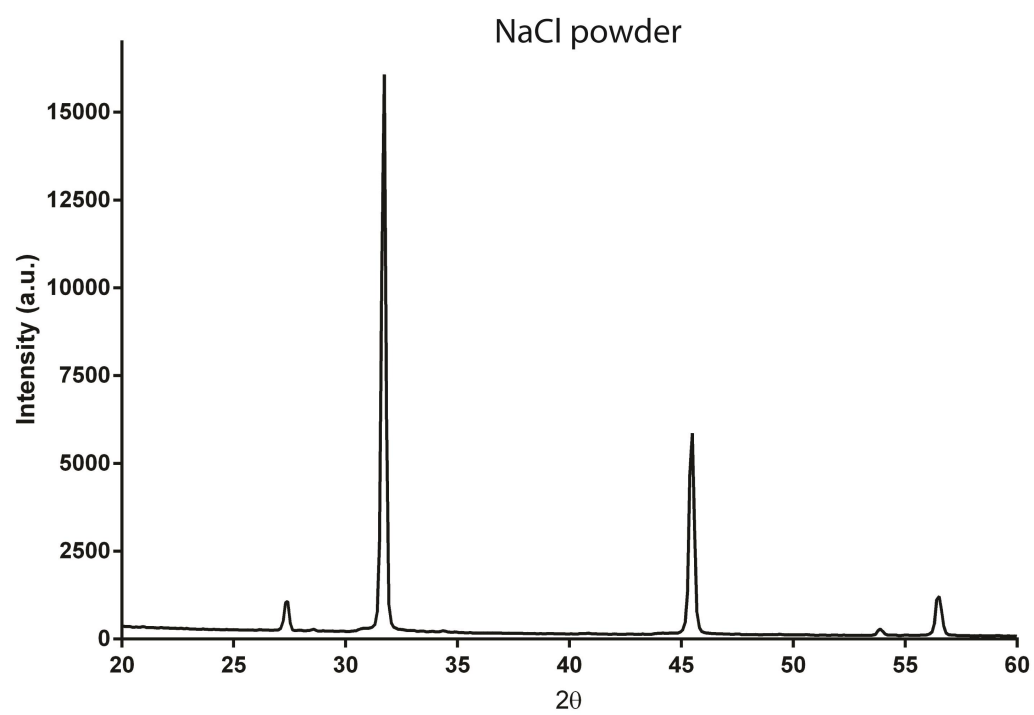


Figure 2.15: XRD patterns of NaCl salt.

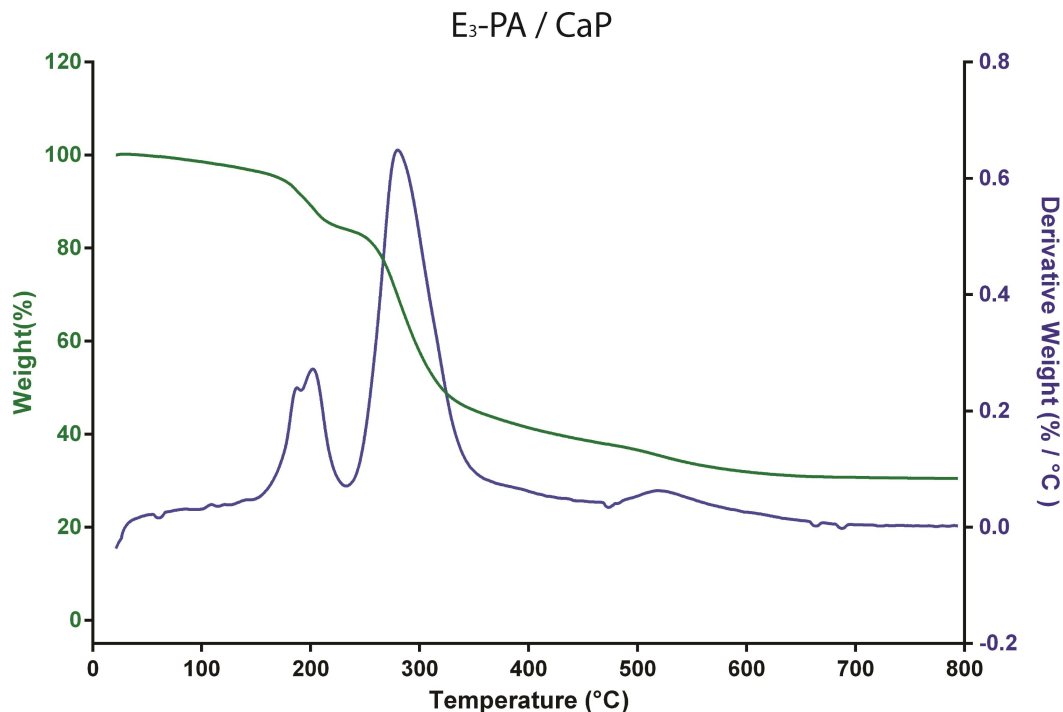


Figure 2.16: TGA of calcium phosphate mineralized peptide amphiphile nanofibers.

nanofibers. Finally, the observation of decomposition of HA minerals cannot be observed because higher temperatures are required to obtain the decomposition of HA [86,87,88].

Furthermore, CaCO_3 / E₃-PA composite material was characterized by TGA. The weight loss of 7.4% from 25 °C to 300 °C corresponds to the loss of water, which occurs on the surface of the mineralized peptide nanofibers and occurs in the crystal structure of calcium carbonate. Also, it can be observed that between 300 °C and 600 °C, decomposition of peptide amphiphile nanofibers occur. Finally, the mass loss between 600 °C and 800 °C can be attributed to the decomposition of CaCO_3 to CaO and CO_2 [89–94](Figure 2.16).

Non-mineralized peptide amphiphile nanofibers were also characterized by TGA. Initially, a mass loss of 7-8% can be observed between 20 °C to 250 °C, which corresponds to adsorbed water inside the structure. Above 250 °C, there

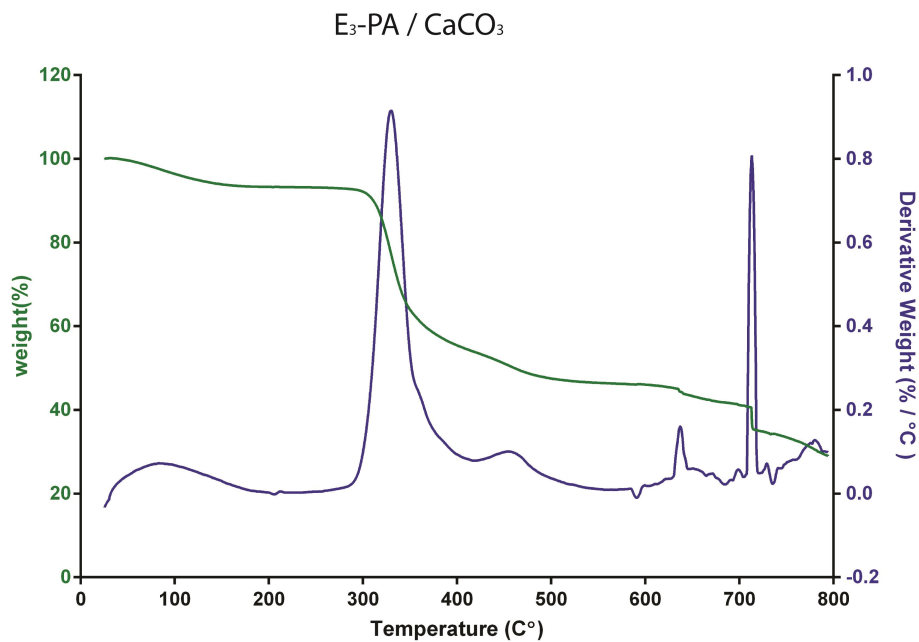


Figure 2.17: TGA of calcium carbonate mineralized peptide amphiphile nanofibers.

were no characteristic peaks to prove the presence of HA or calcite (Figure 2.17).

To determine the viscoelastic properties of mineralized and non-mineralized peptide amphiphile nanofibers, the rheological characterization of composite materials was investigated. By conducting rheological analyses, it is possible to measure storage modulus, G' , and loss modulus, G'' , of the materials. The rheological experiments prove that the mechanical stiffness of peptide amphiphile nanofibers improves by mineralization of nanofibers.

As known from nature; both bone and nacre are excellent examples of composite materials that are combinations of organic and inorganic components. That extraordinary association helps those natural materials to combine strength with toughness which makes them damage-tolerant. As our results indicate, while non-mineralized peptide amphiphile nanofibers demonstrate G' values ranging from 0.5 kPa to 2 kPa, G' values of mineralized peptide amphiphile nanofibers increase up to 9 kPa to 13 kPa. This situation could be explained by several factors; one of

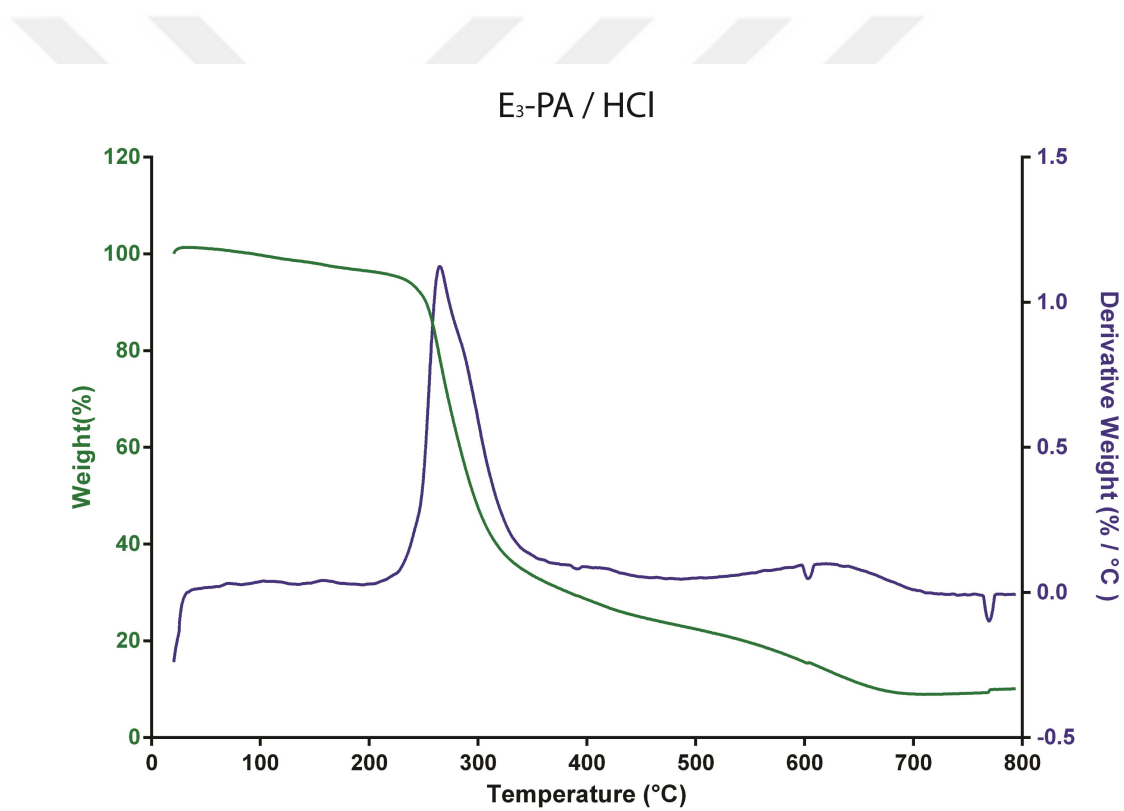


Figure 2.18: TGA of non-mineralized peptide amphiphile nanofibers.

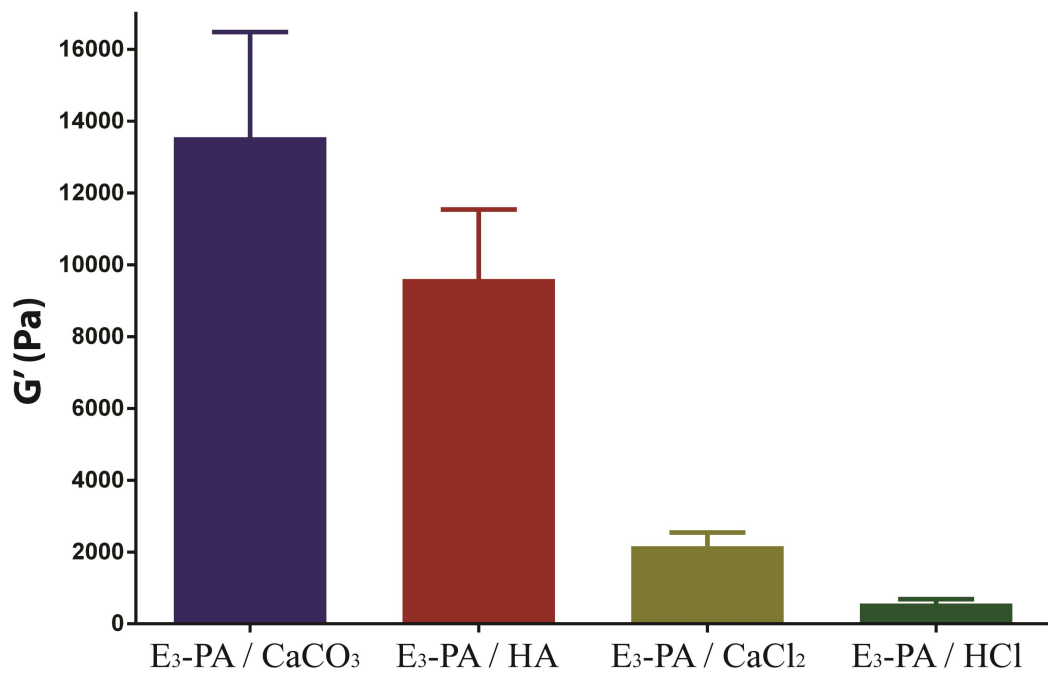


Figure 2.19: Equilibrium storage modulus of mineralized and non-mineralized peptide amphiphile nanofibers.

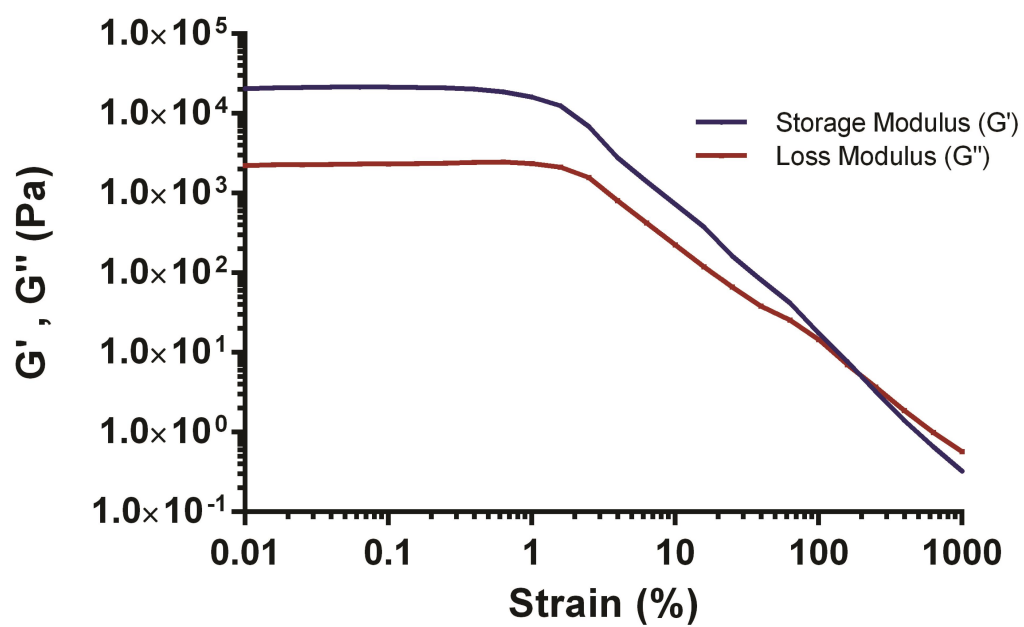


Figure 2.20: Amplitude sweep test of calcium phosphate mineralized peptide amphiphile nanofibers.

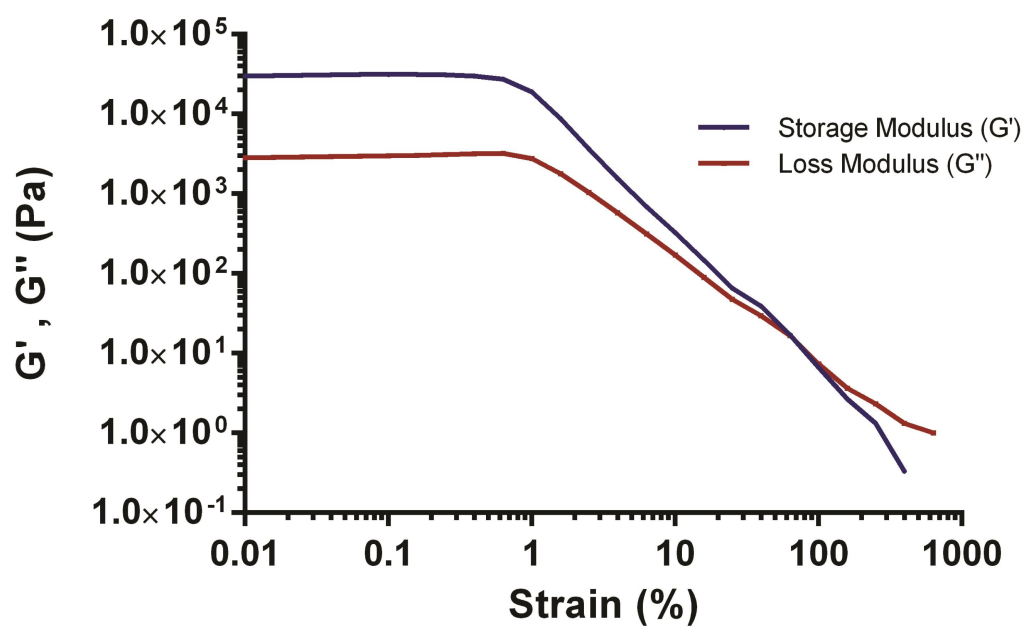


Figure 2.21: Amplitude sweep test of calcium carbonate mineralized peptide amphiphile nanofibers.

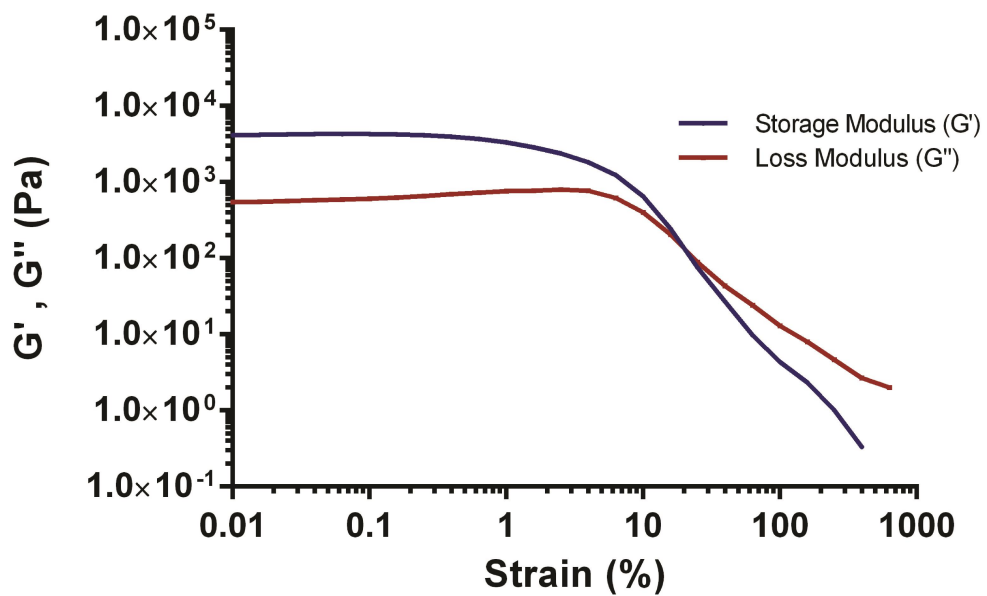


Figure 2.22: Amplitude sweep test of non-mineralized peptide amphiphile nanofibers. Self-assembly is triggered by calcium ions.

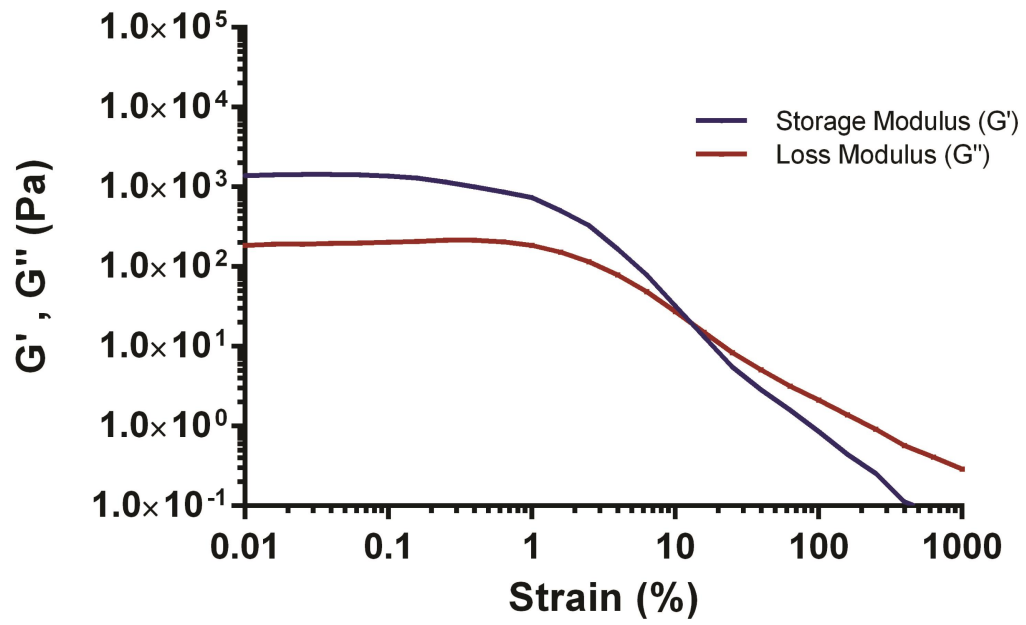


Figure 2.23: Amplitude sweep test of non-mineralized peptide amphiphile nanofibers. The self-assembly mechanism is triggered by lowering the pH by using H^+ ions.

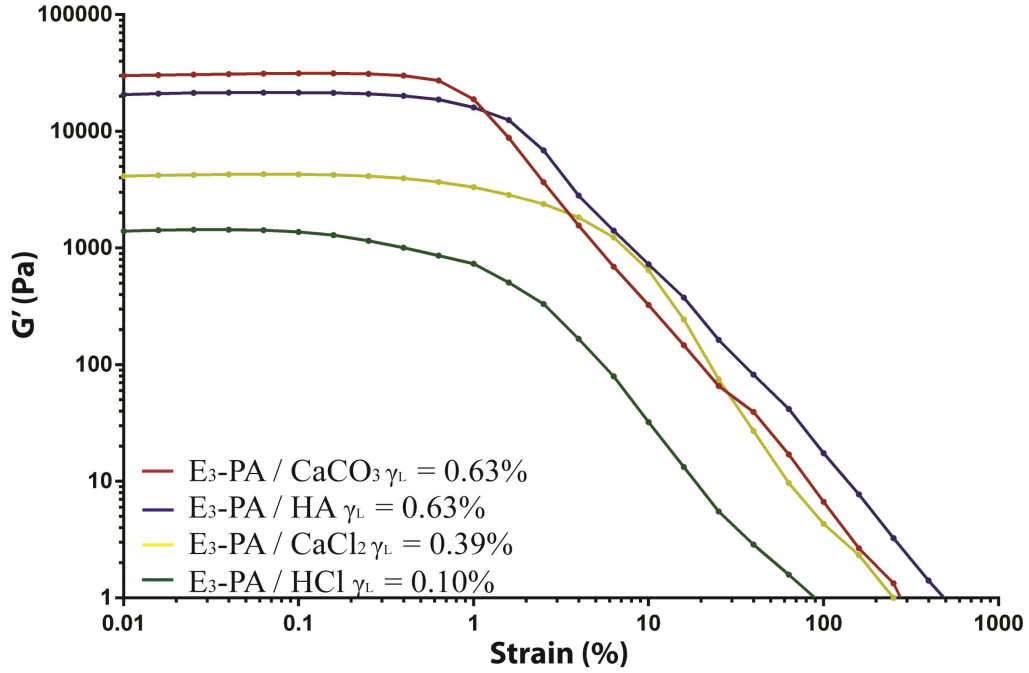


Figure 2.24: Limiting strain values of mineralized and non-mineralized peptide amphiphile nanofibers.

them is, by creating biominerals inside the polymeric structure, energy dissipating points are created. Therefore, in the case of any applied force to the structure, minerals absorb the energy and prevent the structure from falling apart. Another reason is the, energy dissipating points form plastic zones which cover the cracks and reduce the driving force for a crack to continue its path.

Finally, rheology characterization method was used to determine the limiting strain amplitude of mineralized and non-mineralized peptide amphiphile nanofibers. In a region which is called linear viscoelastic range (LVR), under elastic deformation, materials can preserve their elastic properties by keeping the storage modulus constant. Beyond the specific boundary of limiting strain range, called limiting strain amplitude (LSA), plastic deformation occurs and the modulus of the materials start decreasing under increasing strain values. The elasticity of the materials can be determined by measuring the length of the LVR, which can also be considered as a measure of stability of the materials. The results showed that LVR of mineralized peptide nanofibers were slightly higher

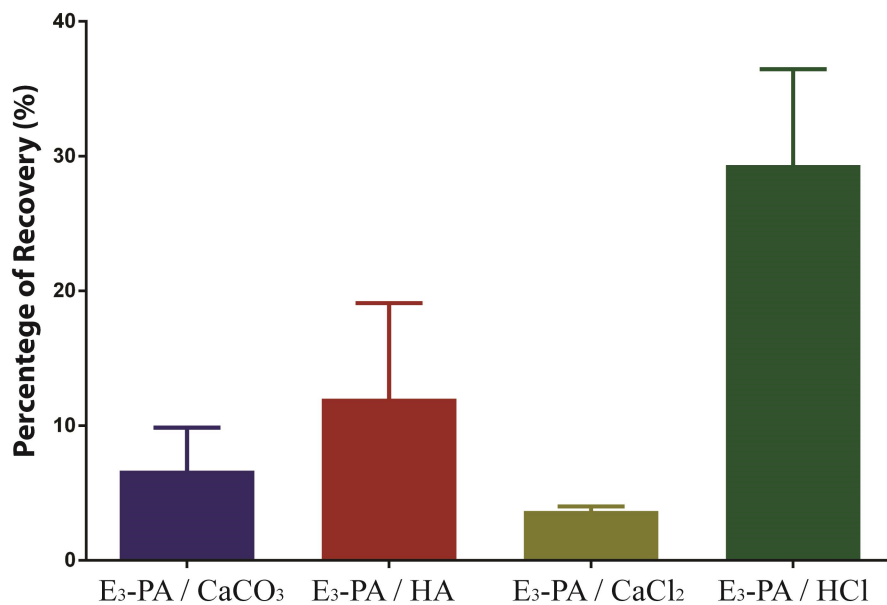


Figure 2.25: Self-healing properties (recovery rates) of mineralized and non-mineralized peptide amphiphile nanofibers.

compared to non-mineralized peptide nanofibers. Therefore, stability of these composite materials is higher compared to non-mineralized counterparts (Figure 2.23). Moreover, the self-healing ability of mineralized and non-mineralized peptide amphiphile nanofibers was determined by performing the thixotropic test. To carry out the thixotropic test, high shear load was applied to the samples; which breaks both covalent and non-covalent bonds along with the minerals. The recovery rates of mineralized and non-mineralized gels were also calculated. When peptide nanofiber formation is triggered by H^+ ions, the recovery rate of the gel is calculated to be almost 40%; however, when gelation is triggered with Ca^{2+} ions, recovery rate was observed to be 4% (Figure 2.24).

These results match with a study, where they demonstrated that, when gelation is triggered by $CaCl_2$ rather than HCl , calcium-mediated ionic bridges form stronger both intra and interfiber cross-links than hydrogen bonds [43]. Therefore, stronger calcium-mediated ionic bridges are much harder to recover by themselves

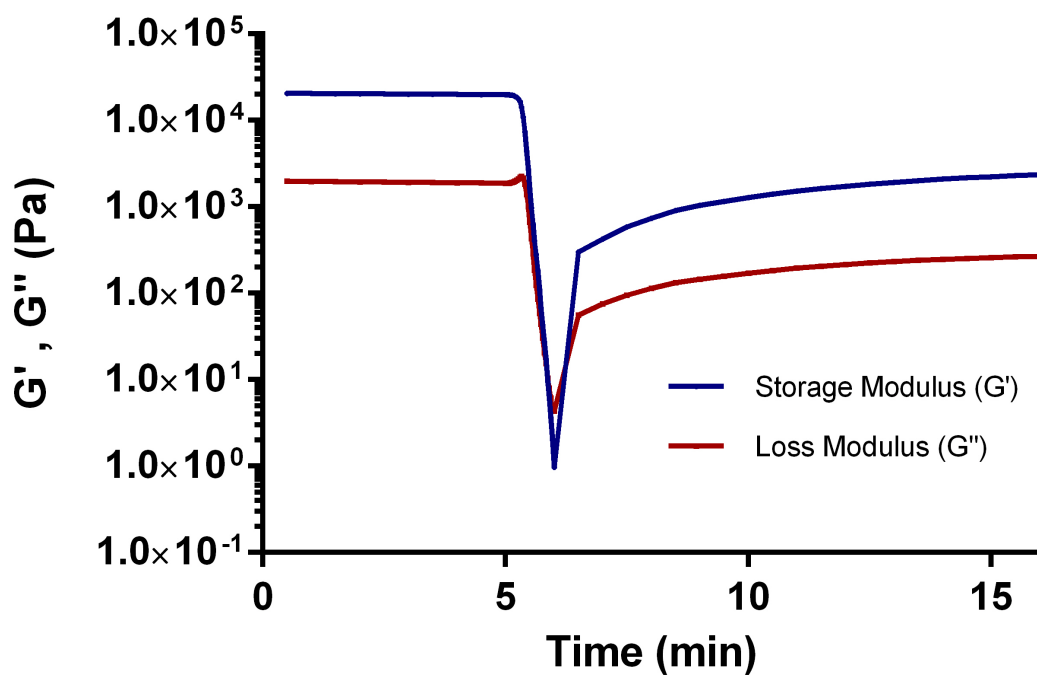


Figure 2.26: Thixotropic behaviour of calcium phosphate mineralized peptide amphiphile nanofiber gels.

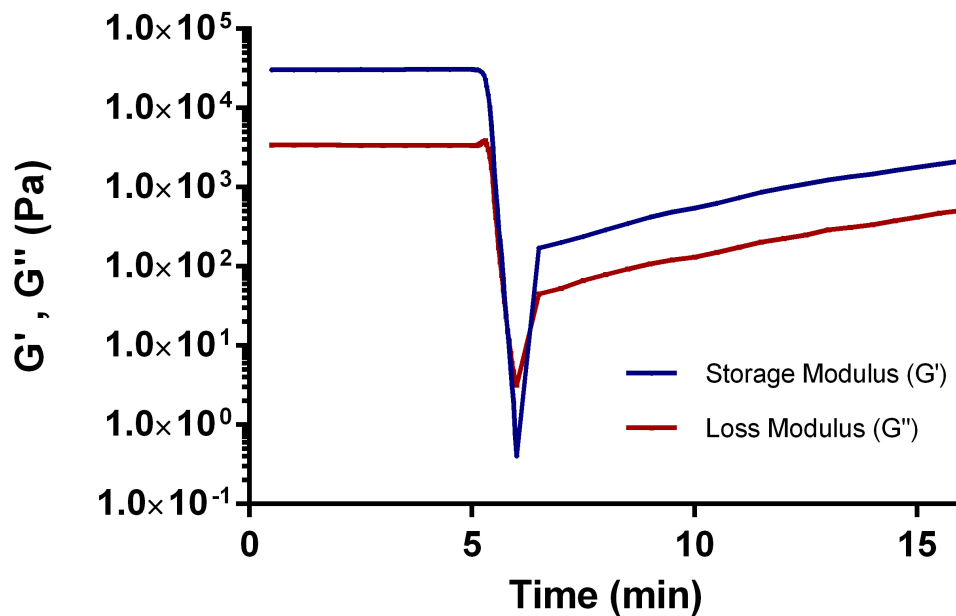


Figure 2.27: Recovery rate of calcium carbonate mineralized peptide amphiphile nanofiber gels.

compared to hydrogen bonds formed by protonated acids. Mineralized peptide amphiphile nanofibers were also characterized regarding their self-healing properties. Both calcium phosphate and calcium carbonate bearing composite gel systems demonstrate fewer recovery rates compared to HCl / E₃-PA gels. However, the recovery rate of composite gels is higher compared to CaCl₂ / E₃-PA gel system. There could be two reasons for that observation. The soluble ions inside the composite structure, or the behavior of organic content in composite gels. After high shear load was applied, all the covalent and non-covalent bonds and biominerals were deformed inside the structure. Once recovery phase was started, soluble ions inside the gel may again create supersaturation points and initiate the nucleation of minerals onto nanofibers which leads the increment in recovery rate. Another possible reason is the behavior of organic content, which acts as a viscoelastic glue inside the composite structure and prevents the deformation of biominerals under high shear loads.

In addition, after a careful examination it can be observed that the recovery rate of calcium phosphate mineralized peptide nanofibers is higher compared to

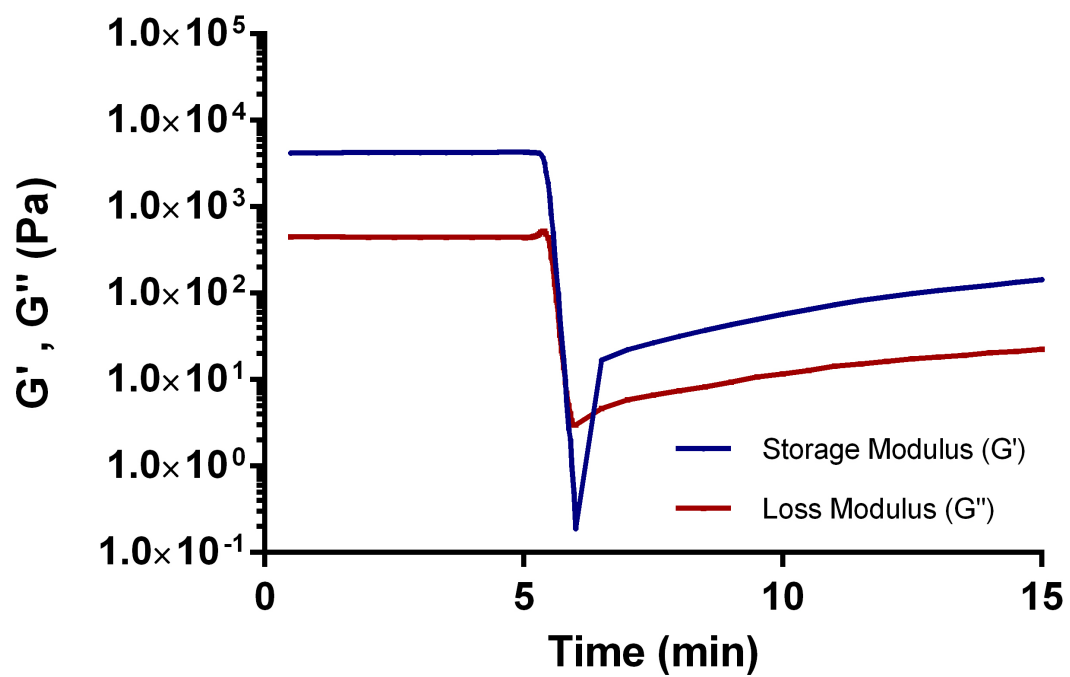


Figure 2.28: Recovery rate of non-mineralized peptide amphiphile nanofiber gels. Self-assembly is triggered by calcium ions.

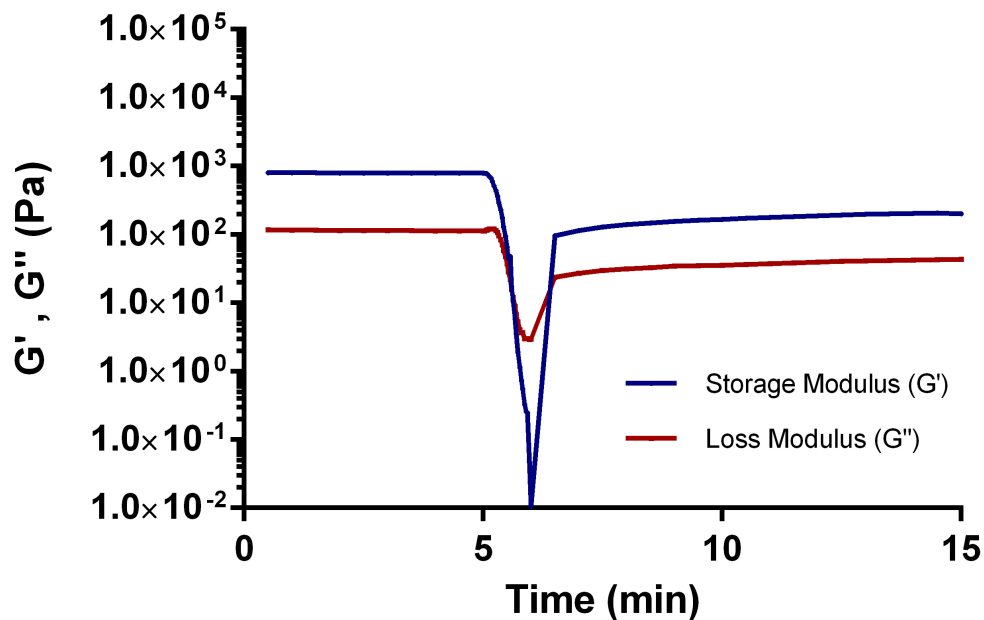


Figure 2.29: Recovery rate of non-mineralized peptide amphiphile nanofiber gels. Self-assembly is triggered by H^+ ions.

calcium carbonate mineralized peptide nanofibers. The TEM results show that, formation of calcium phosphate minerals are slower compared to formation of calcium carbonate minerals inside the organic content. It can also be proved by organic/inorganic ratio from the TGA results. Before the decomposition of the materials occurs the calcium carbonate amount is higher than the calcium phosphate amount inside the organic structure. Therefore, increasing mineral content decreases the self-recovery of the composite material.

Moreover, the effect of mineralized peptide nanofibers on the viability of Saos-2 cells was tested by calcein AM staining after 24 h of incubation. The Saos-2 cells were viable on all surfaces over the 24 h period. We did not observe any significant difference in the viability of cells on peptide nanofiber scaffolds and bare glass surface (Figure 2.29 and Figure 2.30). These results showed that peptide nanofibers provide a biocompatible environment.

Biom mineralization is the process by which hydroxyapatite is deposited in the

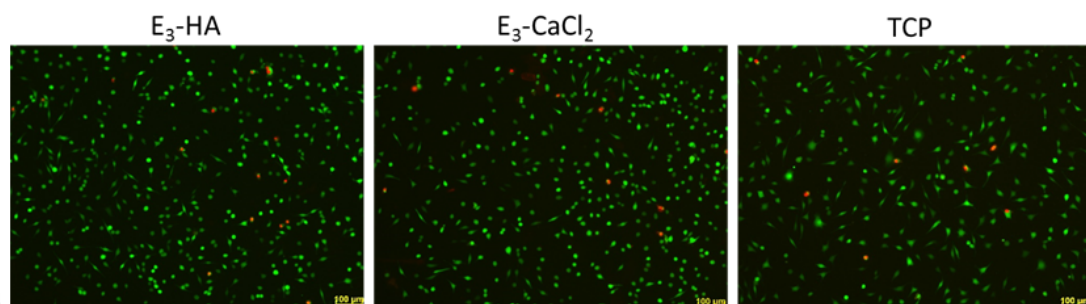


Figure 2.30: Viability of Saos-2 cells on mineralized and non-mineralized peptide nanofibers after 24 h incubation. Live cells–green. Dead cells–red.

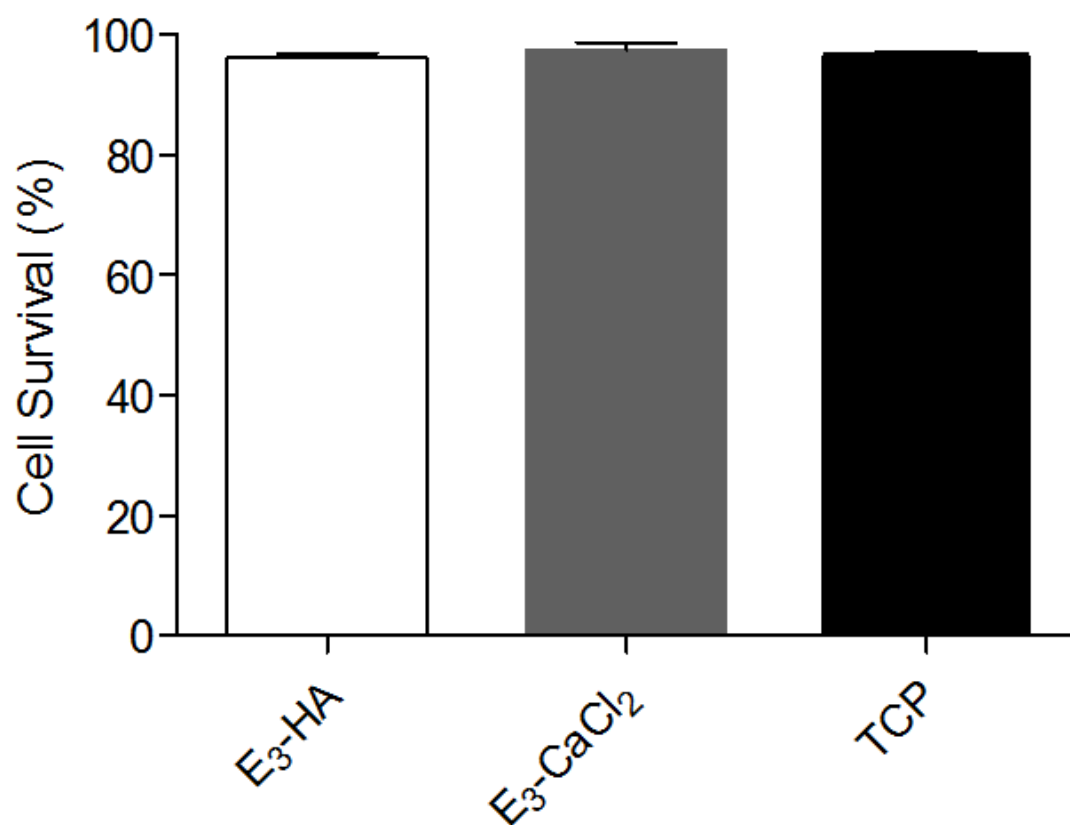


Figure 2.31: Viability of Saos-2 cells on mineralized and non-mineralized peptide nanofibers after 24 h of incubation.

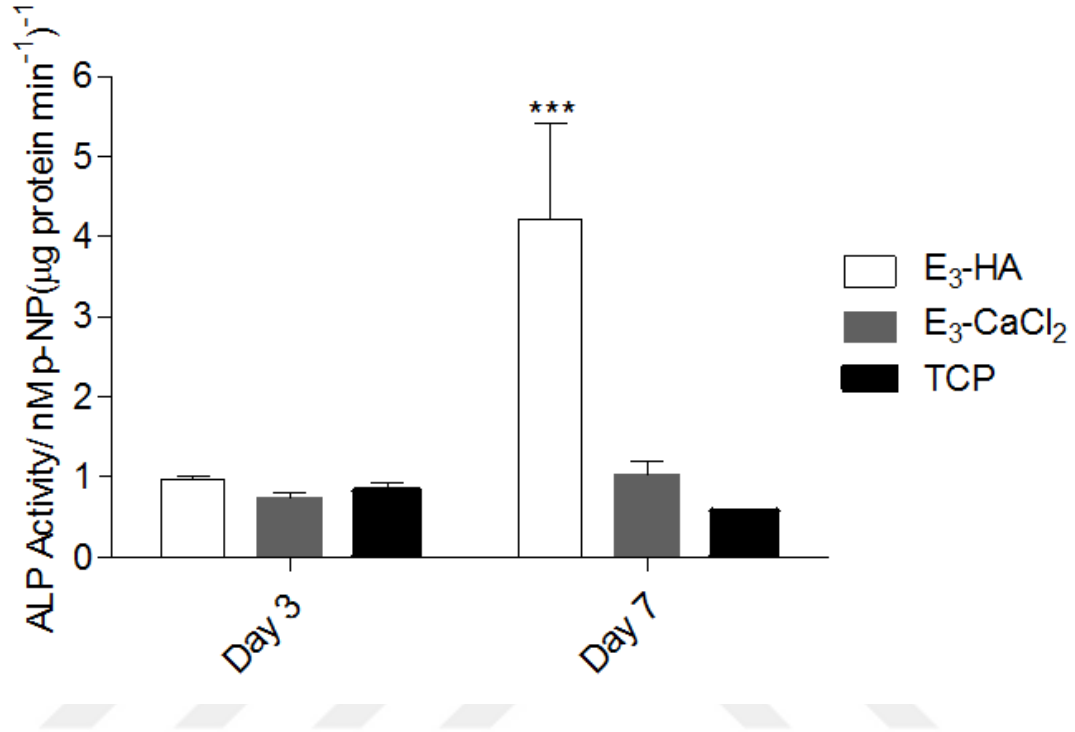


Figure 2.32: Effect of mineralized and non-mineralized peptide nanofibers on alkaline phosphatase activity of Saos-2 cells on days 3 and 7, *** $p < 0.001$.

extracellular matrix. The first step of mineralization in bone tissue is the formation of hydroxyapatite crystals within matrix vesicles that bud from the surface membrane of osteoblasts. This is followed by propagation of hydroxyapatite into the extracellular matrix and its deposition between collagen fibrils. Alkaline phosphatase (ALP) hydrolyzes pyrophosphate and provides inorganic phosphate to promote mineralization [95]. The early phase of in vitro osteogenic differentiation can be characterized by the expression of alkaline phosphatase (ALP) at mRNA and protein level [96]. Therefore, we tested the ALP activity of Saos-2 cells cultured on mineralized peptide nanofibers on days 3 and 7. Maximum ALP activity was observed on day 7 for calcium phosphate mineralized peptide nanofiber composite material.

Chapter 3

Conclusion

The calcium carbonate and calcium phosphate mineralized peptide nanofibers can be formed by employing peptide amphiphile molecules as template, and a self-assembly triggering solution. In our study, peptide amphiphile molecules were synthesized by using solid phase peptide synthesis (SPPS) method. The self-assembly of peptide amphiphile molecules was triggered by Ca^{2+} ions to obtain the formation of β -sheet secondary structure. In addition, the functional glutamic acid residues on the last region of peptide amphiphile molecules, which mimics the negatively charged feature of protein found in nature, provided nucleation sites for the formation of the inorganic material on the surface of the nanofibers.

The preparation of mineralized peptide amphiphile nanofibers was started by initiating the self-assembly of the peptide amphiphile molecules by dropping the CaCl_2 solution into the peptide solution. After triggering self-assembly, instant formation of gels was observed. Then, the solution which triggers the formation of minerals was dropped onto the gels. Depending on the desired mineral, diammonium phosphate or sodium carbonate can be used to obtain calcium phosphate or calcium carbonate, respectively. They were allowed to be mineralized from 30 minutes to 90 minutes to determine the effect of the minerals depending on the incubation time.

Several characterizations were performed in order to determine the properties of

mineralized and non-mineralized peptide amphiphile nanofibers, such as, LC-MS, SEM, TEM, XRD, TGA, AFM and rheology. In order to determine the molecular weight of the peptide amphiphile molecule, LC-MS was conducted. It was determined that the molecular weight of the E₃-PA was 912.50012 g/mol.

The transmission electron microscopy results show the nucleation and growth of the biominerals on the surface of the peptide amphiphile nanofibers. While, after 30 min of incubation, the minerals onto surface of the fibers can be observed, after 90 min of incubation, the specific structures belong to the calcium phosphate and calcium carbonate can be seen. In addition, the difference between structures in the absence and presence of the peptide amphiphile molecules can be seen. Overall, template effect of peptide amphiphile molecules in terms of the mineralization of the calcium carbonate and calcium phosphate can be observed. Moreover, by conducting atomic force microscopy, the difference between mineralized and non-mineralized peptide amphiphile nanofibers can be observed.

The scanning electron microscopy results of the composite materials showed the morphological differences between the materials which were fabricated in the presence and absence of the peptide amphiphiles. While, in the presence of the peptide amphiphile molecules, template effect of the organic content can be observed, in the absence of the peptide amphiphile molecules, the randomly distribution of the minerals occurred.

The X-ray diffractometer results for the mineralized peptide amphiphile samples demonstrated the peaks which correspond to the hydroxyapatite and calcite. On the other hand, analysis of the non-mineralized peptide amphiphile nanofiber samples demonstrated the sodium chloride salt content which was inevitable due to the processes of dissolving and triggering self-assembly. NaOH was used to dissolve the peptide amphiphile molecules and CaCl₂ or HCl was used to trigger the self-assembly of the peptide amphiphile nanofibers. Because of the Na⁺ and Cl⁻ ions which are present in the peptide solution, they form NaCl salt, which we could not get rid of even after 5 times of washing.

The TGA was conducted to determine the organic/inorganic content of the composite materials. According TGA results, calcium phosphate mineralized peptide nanofibers contain less minerals compared to calcium carbonate mineralized peptide amphiphile nanofibers. This is because the kinetics of the growth of the

minerals are different. TEM results of the samples which were incubated for 90 min in the absence of the peptide amphiphile molecules also prove this theory, because after 90 min, while calcium carbonate reaches its most stable form calcite, calcium phosphate still preserves its amorphous phase.

The rheological and thixotropic measurements demonstrated that mineralized peptide nanofibers were mechanically superior compared to non-mineralized counterparts and recovery rate of mineralized peptide nanofibers were considerably high.

Finally, *in vitro* studies were conducted to determine the biocompatibility and alkaline phosphatase activity of the mineralized composite material. The mineralized composite materials provided a biocompatible environment to the cells, and promoted osteogenic differentiation.

Since biominerals and peptide amphiphile molecules are both biocompatible, mineralized composite gel systems can be utilized in tissue engineering. For instance, ECM stiffness plays a crucial role in cell proliferation, adhesion and other functions of the cell. Therefore, applying mineralized peptide nanofibers to defected areas in bone or tooth could be suitable for rapid regeneration of damaged tissues. Also, by designing the sequence of peptide amphiphile molecule, various types of organic-inorganic composite materials could be synthesized. Since the composition of tooth and bone are different from each other, such peptides could be employed to produce desired composite material.

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