

**CATALYTIC AND BIOACTIVE NANOSTRUCTURES FOR
REGENERATIVE MEDICINE APPLICATIONS**

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By

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CATALYTIC AND BIOACTIVE NANOSTRUCTURES FOR REGENERATIVE
MEDICINE APPLICATIONS

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ABSTRACT

CATALYTIC AND BIOACTIVE NANOSTRUCTURES FOR REGENERATIVE MEDICINE APPLICATIONS

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PhD in Materials Science and Nanotechnology

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Peptide nanostructures provide a remarkable toolbox for designing nature inspired smart materials. Synthetic peptide nanomaterials can be tailored with chemical, physical and biological signals to be utilized in a wide range of applications in biomedicine. With the increasing demand for complex nanostructures with facile preparation methods, bioinspired smart nanomaterials have gained more importance for achieving multifunctional hybrid materials. In particular, biological or chemical cues can be integrated into supramolecular designs to generate bioactive materials.

This thesis describes nature inspired combinatorial methods for designing peptide nanostructures, which display catalytic and biologically functional moieties. These multifunctional peptide nanostructures were synthesized by using solid phase peptide synthesis. Designed peptide units were in accordance with the relevant biological function and they self-assemble to form nanofibrous networks mimicking the extracellular matrix. This combinatorial approach allows a wide range of applications including artificial catalysis, cell cultivation, biomineralization and live-cell labeling.

In this thesis, the self-assembled catalytic and bioactive peptide nanostructures were utilized in artificial enzyme studies, biomineralization and tissue regeneration. The results show that these new artificial enzymes display both catalytic and biological functions of their natural counterparts such as proteins.

In the first chapter, basic concepts of self-assembly, artificial catalysis approach, biomineralization and bioactive peptide nanostructures were explained. In the second chapter, multicomponent artificial catalyst model formed by self-assembly was investigated. Designed artificial peptide molecules were characterized structurally and catalytic capability of this *de novo* system was shown with both model and actual substrate. In the third chapter, ALP inspired catalytic, ion coordinating and biomineralizable peptide nanostructures were examined, bioactivity of this enzyme inspired materials was shown with multiple cell lines and using 2D and 3D cell culture methods. In the fourth chapter, enzyme responsive dentin sialophospho-protein like materials were exhibited instead artificial catalyst. Multi-responsive material induced biomineralization similar to dentin sialophospho-protein which controls mineralization during dentinogenesis. In the fifth part, peptide nanostructures were applied as bioorthogonal catalyst for live-cell tagging study, fluorophore tagged living cells by peptide catalyst was imaged by confocal microscopy. The last chapter covers novel materials inspired by unique nature of collagen and its bioregenerative capacity investigated with stem cells and preliminary cartilage induction was obtained.

Keywords: Peptide amphiphile (PA), self-assembly strategy, artificial catalysis, enzyme, supramolecular designs, biomedical applications

ÖZET

KATALİTİK VE BİYOAKTİF PEPTİT NANO YAPILARIN REJENERATİF TIP ALANINDA UYGULAMALARI

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Doğadan esinlenerek oluşturulan ve günümüz araştırmalarında önemli bir yer taşıyan akıllı malzemelerin geliştirilmelerinde peptit tabanlı yapılar dikkate değer bir yer tutmaktadır. Sentetik peptitlerin kimyasal ya da biyolojik fonksiyonel gruplarla kolay manipüle edilebilir olması, bu yapıları medikal ve endüstriyel alanlarda ilgi çekici kılmıştır. Günümüzün gelişen teknolojisi ile birlikte üretim ve kullanım açısından pratik malzemelere olan ihtiyaç gün geçtikçe artmaktadır. Artan ihtiyaçlar doğrultusunda çok fonksiyonlu hibrit yapılar ihtiyacı karşılamak için önemli bir kaynak olarak görülmeye başlanmıştır ve bu tarzdaki çok fonksiyonlu yapıların tasarımında doğadan ilham alarak tasarlanan peptit malzemelerin kullanımı yaygın hale gelmiştir. Doğadaki bir çok farklı kaynaktan esinlenerek ve aynı zamanda farklı özellikleri bir araya getirerek oluşturulan bu tasarımlar, çoklu fonksiyonları olan biyoaktif supramoleküler yapılar geliştirmek için kullanılmıştır.

Bu tezde farklı bir çok disiplini bir araya getirerek ve doğal malzemelerin doğadaki yapı ya da fonksiyonları da göz önünde bulundurularak hazırlanan, katalitik ve biyolojik işlevler yapacak şekilde dekore edilmiş peptit yapılardan bahsedilmektedir. Peptit yapıların sentezleri katı faz peptit sentez metodu ile yapılmıştır. Amaca göre tasarlanan peptit molekülleri kendinden toparlanabilmekte olup, öztoparlanım sadece supramoleküler düzenlenmeye değil aynı zamanda düzenlenmeye bağlı katalitik bölge formasyonunu ve yaklaşan moleküllerin etkileşimine bağlı aktivite artışını sağlamaktadır. Bu birden fazla yöntemi kullanarak oluşturulan metot ile yapay kataliz, biyomineralizasyon, hücre etkileşimi ve canlı hücre etiketlenmesi gibi bir çok alanda uygulama imkanı elde edilmiştir. Özetle bu tezin içeriğinde kendinden toplanımlı katalitik ve biyoaktif yapılar kullanılarak oluşturulan; yapay enzim, biyomineralizasyon ve rejenatif ilaç uygulamaları bulunmaktadır. Bu çalışmalar gelecekte spesifik amaçlara göre kompleks biyomalzemelerin tasarımlarının doğadaki sistemlere yaklaşması için ümit vermektedir.

Bu tezin ilk bölümünde tezin içeriğindeki çalışmalar için genel bir giriş verilmiş olup, temel kendinden toplanım mekanizması, yapay kataliz yöntemi, biyomineralizasyon ve biyoaktif peptitler anlatılmıştır. İkinci bölümde ilk çalışma olan birden çok bileşenin kendinden toplanımı ile oluşturulan yapay katalistten bahsedilmiştir. İçerikte bu yapıların karakterizasyonunun yanı sıra, model ve doğal sübstrat varlığındaki katalitik etkinliğide gösterilmiştir. Bir başka kataliz çalışması olan üçüncü bölümde, alkalın fosfatazdan esinlenerek geliştirdiğimiz katalitik, iyon koordine kapasitesine sahip ve biyomineralizasyonu indükleyen peptit yapılar anlatılmaktadır. Bu yapıların biyoaktivitesi, 2D ve 3D hücre kültürleme metodları ve farklı hücre tipleri kullanılarak gösterilmiştir. Dördüncü kısımda ise yapay enzim modellerinden farklı olarak enzimle

kontrol edilebilen ve dentin sialofosfo-proteinden esinlenerek geliştirilmiş peptit modellerinden bahsedilmektedir. Farklı indüktörler tarafından manipüle edilebilen peptitler dentinogenesis sırasında dentin sialofosfo-proteinin görevine benzer olarak biyomineralizasyonu desteklemiştir. Beşinci bölümde canlı hücreleri etiketlemek için geliştirilen biyortogonal katalist çalışması anlatılmıştır ve bu katalist ile etiketlenen canlı hücreler konfokal mikroskopi analizi ile görüntülenmiştir. Tezin son bölümünde ise yapısal olarak eşsiz olan kolajen moleküllerinden esinlenerek hazırlanmış olan yeni peptit moleküller ve bu moleküllerin kıkırdak rejenerasyonunda kullanılabileceğini gösteren öncül çalışmalar anlatılmıştır.

Anahtar Kelimeler: Peptit amfifiller, kendiliğinden bir araya gelme stratejisi yapay kataliz, enzimler, supramoleküler tasarım, biyomedikal uygulamalar.

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ABBREVIATIONS

ALP	: Alkaline phosphatase
β-gly	: β-glycerophosphate
BMP-2	: Bone morphogenetic protein-2
BSA	: Bovine serum albumin
CD	: Circular dichroism
Col-I	: Collagen type I
DCM	: Dichloromethane
DFT	: Density Functional Theory
DMF	: N, N-Dimethylformamide
DMEM	: Dulbecco's modified Eagle's medium
DMP1	: Dentin matrix protein -1
Dopa	: 3,4-Dihydroxy-L-phenylalanine
DSPP	: Dentin sialophosphoprotein
ECM	: The extracellular matrix
EDTA	: Ethylenediaminetetraacetic acid
EdU	: 5-ethynyl-2'-deoxyuridine
EDX	: Energy-dispersive x-ray spectrometry
ESI	: Electrospray ionization
FBS	: Fetal bovine serum
FTIR	: Fourier transform infrared spectrometry
GAG	: Glycosaminoglycan
HA	: Hydroxyapatite
HPLC	: High performance liquid chromatography

LC-MS	: Liquid chromatography-mass spectrometry
MBHA	: methylbenzhydramine
MCF-7	: Michigan Cancer Foundation-7 breast cancer cell line
Osp	: Osteopontin
PA	: Peptide amphiphile
PBS	: Phosphate-buffered saline
rMSC	: Rat mesenchymal stem cell
Runx2	: Runt related transcription factor-2
Q-TOF	: Quadrupole time of flight
SBF	: Simulated body fluid
SDS	: Sodium dodecyl sulfate
SEM (Microscopy)	: Scanning electron microscope
SEM (Statistics)	: Standard error of the mean
STEM	: Scanning transmission electron microscope
TIS	: Triisopropylsilane
TCP	: Tissue culture plate
TEM	: Transmission electron microscope
TFA	: Trifluoroacetic acid
XRD	: X-ray diffraction
XPS	: X-ray photoelectron spectrometry

CHAPTER 1

Introduction: Concepts in Peptide Based Catalysts and Biomaterials

1.1 General Introduction

The discovery of solid-phase peptide synthesis has facilitated the development of synthetic peptides for a wide range of applications[1]. Among synthetic biomaterial derivatives, peptide-based materials hold a significant place due to their unique structural and functional features. Hydrogel-forming peptide amphiphile (PA) molecules in particular offer new material designs for a broad range of applications, as a result of their easily tailorable structure, ability to be functionalized through a broad variety of chemical groups, interaction with other inorganic/organic materials (*e.g.* for mineralization or metal binding) and bioactivity. Synthetic peptide hydrogels can be formed by many structures as mono, di- and tripeptides, and may contain complex motifs such as α -helices, β -sheets, coiled-coils, β -hairpins and triple helices. These structures can be used to develop materials with advanced multifunctional properties through simple chemical modifications.

Peptides are typically prepared by the solid-phase peptide synthesis method (SPSS), which supports their high-yield chemical synthesis on a solid mesh and eliminates the necessity of elaborate purification steps. SPSS is a useful technique for natural peptide synthesis and has replaced difficult bacterial protein expression protocols. In addition, non-canonical or dextrorotatory amino acids can be included within peptide sequences

using this efficient chemical synthesis technique. Briefly, SPSS starts with small polymer beads that are tagged with functional linkers, on which peptide sequence can be built. The growing peptide remains covalently attached to the bead during the sequential amino acid linkage process, and acid treatment is used after the synthesis of the complete sequence to cleave the peptide from the solid bead. The immobilization of the peptide makes it easy to remove excess reagents and byproducts through filtration, and all reagents used can then be flushed away. Protection groups are also an important component of synthesis reaction, as these side groups are used to prevent side reactions or undesirable couplings during reaction. Only the coupling site is deprotected and is capable of linking with the next amino acid, and an exposed N-terminal amine is generally used for this process. These wash cycles and deprotection procedures are then repeated until the complete peptide is synthesized[2].

Self-assembly is another key point of our research, and is a molecular organizing process induced by local interactions among disordered components through interactions among precursor moieties. Self-assembling peptides can undergo spontaneous assembly and organize into ordered structures resembling folded proteins. These peptides are then applied as building blocks of a broad range of materials and devices; and self-assembling peptides in particular have found applications in areas varying from biomaterials to molecular electronics.

Peptide systems utilize molecular recognition processes to create hierarchical architectures from unordered precursors, emulating the natural process of protein assembly. Accordingly, the diversity and functionality of peptide systems are second only to the proteins they imitate, making peptide-based materials promising candidates for applications in various areas. This thesis focuses on these applications; focusing on

peptide-mediated catalysis, the use of peptide systems as biomaterials and combining catalytic and biological properties in one peptide system that can replicate the multifunctional nature of biological enzymes.

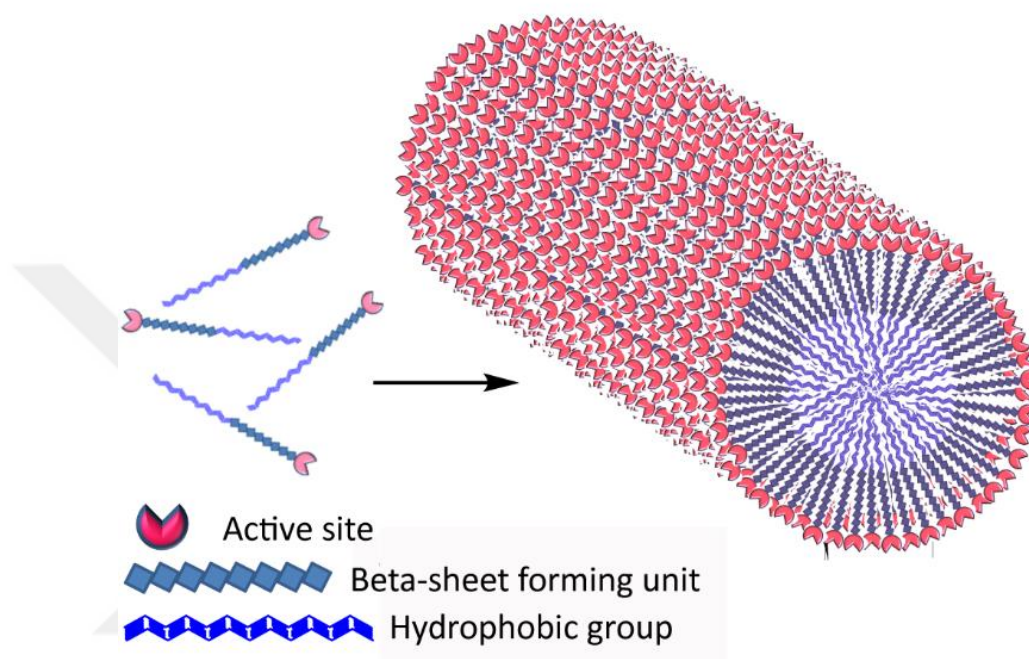


Figure 1.1 Illustration of the self-assembly process

1.2. Enzyme Kinetics

1.2.1. Introduction

Enzymes are the most complex macromolecules in living organisms due to their extraordinary stereochemical and mechanistic properties. Enzymes have various functions and contribute greatly to cellular, tissue and systemic homeostasis. The basic function of these dynamic macromolecules is to mediate the catalysis and transformation of other biomolecules. Recently, developments in nanotechnology have brought a new outlook for enzymes, which are now considered to be natural nanomachines capable of catalyzing complex chemical reactions. Indeed, enzymes are able to catalyze reactions at incredible turnover rates, which can reach around a million substrate molecules per second, while retaining high specificity [3]. Therefore, enzymes attracted great attention for nanocatalysis research and their complex action mechanisms have become a topic of great interest. In particular, artificial degradable small molecules provide as an alternative approach for developing artificial materials for catalysis studies.

Enzyme-inspired catalysis studies focus on developing simpler and more effective molecules by mimicking native catalysis mechanisms, usually for medical and industrial applications. Artificial enzyme studies have been primarily focused on polymers, synthetic organic molecules and inorganic reagents. However, these structures have several shortcomings such as toxic precursors (monomers, crosslinking agents etc.), volume loss after application and low biodegradability. Various types of supramolecular nanostructures and hydrogels have drawn great interest in catalysis

studies because of their ability to avoid these issues in addition to being simple to produce and exhibiting complete biodegradability and high biocompatibility.

The action of the catalytic triad is one of the most studied mechanisms in the context of enzymatic catalysis research. The catalytic center of triple action enzymes are mainly composed of a Ser-His-Asp motif [4]. In this mechanism, a separate function is performed by each component molecule – the serine serves as a nucleophile, the histidine is the general base/acid, while the aspartate orients histidine to generate enhanced catalytic action. Electrostatic interactions are not the only reason for efficient turnover mechanism, as the cyclic structure of action site is formed through hydrogen bonding and sustains the charge relay mechanism among residues [3]. Essential residue preferences and the stereochemical fit between the catalyst and substrate are other crucial requirements of enzyme catalysis and must be considered for the development of effective enzyme mimics.

Phosphatases are another important group of enzymes and serve to eliminate a phosphate group from a biomolecule by cleaving phosphoesters into phosphate ions. Phosphorylation/dephosphorylation reactions are one of the key regulators of biological mechanisms, as protein phosphorylation is a common posttranslational modification and serves a regulatory function in many proteins. Alkaline phosphatase (ALP) is one of the most common phosphatases in vertebrates, and functions in alkaline environments to dephosphorylate biomolecules such as proteins, nucleotides or small phosphorylated molecules like pyrophosphates and ATP [5]. One of the main functions of this enzyme is the regulation of bone mineralization, and it is secreted extensively by osteoblasts during the bone remodeling process [5].

The mechanisms and actions of these enzymes are inspiring for scientists. However, the elaborate expression and purification steps associated with protein isolation limits the practical applications of enzymes due to time- and cost-related concerns. The development of synthetic enzymes is a promising means of utilizing enzymes in a cost-effective manner in a wide range of application areas, including medicine, pharmaceutical industry, nanofabrication and energy [6-8].

Intrigued by these requirements, we explored peptide based artificial enzyme designs and their kinetics under physiological conditions in search for a stable, biocompatible and bioactive artificial enzyme. We also explored the bioactivity of this artificial enzyme with cell culture experiments to verify whether it could exhibit the bioactivity of its natural counterparts. As such, we sought to engineer an artificial system that can replicate not only the catalytic features of enzymes, but also the regulatory functions that are frequently undertaken by enzymes in biological systems.

1.2.2. The Catalytic Triad and its Catalytic Mechanism

Catalytic triads are found in proteases and are mainly responsible for their proteolytic activity. The key residues for this triple action unit are serine, histidine and aspartic acid. The imidazole ring on histidine serves as a general acid/base and activates the hydroxyl group on side residue of serine, which functions as the nucleophilic unit of catalytic action. There are also other types of triads: The active site of cysteine proteinases, for example, feature a triad of cysteine, histidine and asparagine, which uses cysteine as a nucleophile for the initial stages of catalysis. In contrast, there are also proteases that utilize activated water molecules to initiate catalytic action. The

significance of structural motif and side group interactions are evident by their ubiquitous presence in mediating enzymatic action.

The catalytic reaction mechanism of catalytic triads can be explained by two half reactions. The first part of the reaction is called acetylation, and the substrate is cleaved by the enzyme to form an intermediate structure at this step. The second part, called deacetylation, occurs after intermediate formation and is involved in the hydrolysis of the substrate to end the intermediate state through short term interactions and ultimately restore the catalytic site to its original state. In short, cleavage process occurs as follows: the substrate first binds to the enzyme and positions itself within the hydrophobic pocket of the active site, and is then cleaved after it reaches an ideal arrangement for the enzymatic reaction to occur. In serine based catalytic triads, the collapse of the tetrahedral intermediate displaces the serine, and the change in the conformation of the active site results in the compression of hydrogen bonding between histidine and aspartic acid.

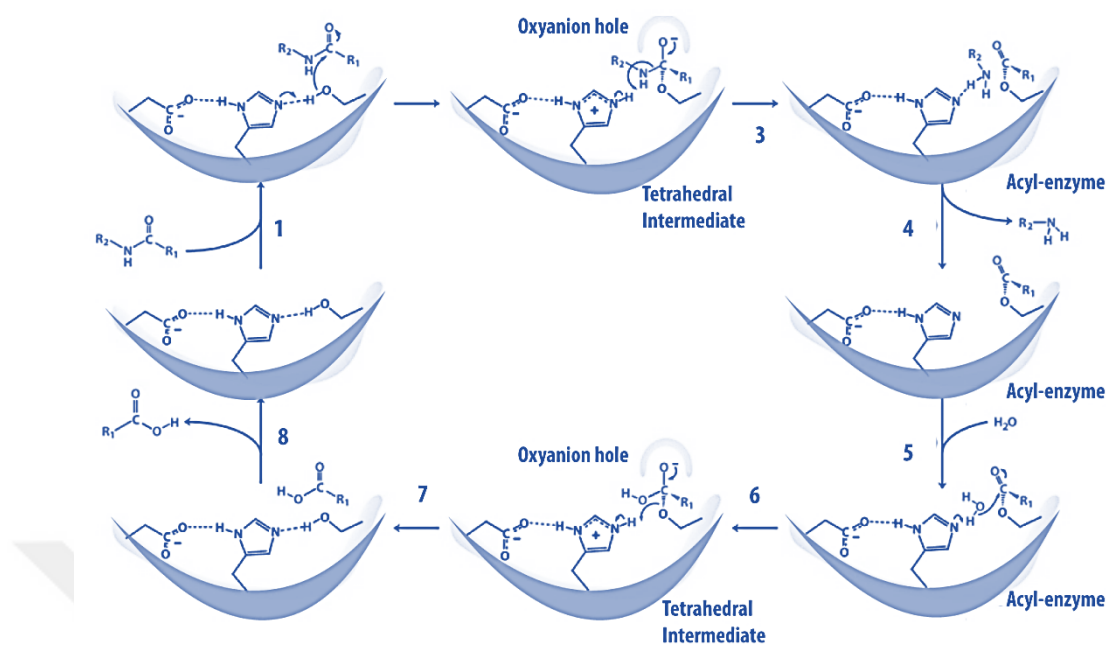


Figure 1.2 Reaction mechanism of catalytic triad catalysis.

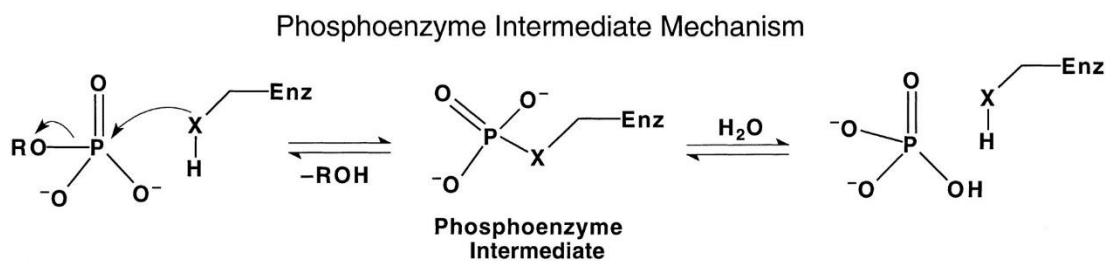
This effect leads to stronger interactions between the residues and creates a lower-barrier hydrogen bond, which increases pKa of histidine from 6.5 to >12, enhancing the general base character of histidine. This basic histidine is in an ideal position to effectively deprotonate the serine residue, and interacts with it to generate a highly nucleophilic alkoxide ion from the serine hydroxyl group. The alkoxide ion attacks the substrate and forms a tetrahedral acyl-enzyme/substrate complex, and the unstable negative charge on the carboxyl oxygen of the substrate is stabilized by the residues in the oxyanion hole. The unstable negative charge on the intermediate complex leads its collapse, leading to the formation of a new intermediate and subsequent breakdown of the substrate. Following this process, a strongly nucleophilic hydroxide ion is generated at the active site through interaction with an incoming water molecule, which is deprotonated by general base interaction. Lastly, the ester bond of the acylenzyme is cleaved by the hydroxide to generate a second tetrahedral intermediate ion, which reacts with oxygen in the catalytic pocket to restore its negative charge.

1.2.3. Phosphatase Mechanism

Phosphatase catalysis could be explained through two possible mechanisms. Since the mechanism of phosphoenzyme catalysis is still not fully understood and various types of phosphatases could have different mechanisms. These mechanisms involve catalysis through either direct transfer or an enzyme intermediate. Figure 1.3 details the two principal possibilities by which phosphatases may remove phosphoryl groups from the phosphoester units of biomolecules. ALP and tyrosine phosphatases represent two classes of phosphatases which use phosphoenzyme intermediate state during their catalytic action. Metal ion-assisted phosphatases -metallophosphatases- administer a

metal coordinated water group as a nucleophile and proceed to the direct transfer of the phosphoryl group. In Figure 1.3., the active nucleophile is represented with the symbol X, *e.g.* the hydroxyl unit of serine in serine phosphatases or the thiol unit of cysteine in tyrosine phosphatases.

The mechanism of ALP catalysis was previously investigated with various imidazole presenting organometallic structures; however, ALP possesses other functions and organic constructs having such functionalities have not been reported in the literature. The imidazole-containing histidine residue was found to be a fundamental moiety for the catalytic action of ALP. These residues incorporate Zn^{II} ions to aid in organophosphate coordination and facilitate the deprotonation of water, which is converted into an intermolecular nucleophile. This nucleophile subsequently attacks the ester bond of a phosphate monoester to initiate the mineralization process in biological systems [9]. Zn^{II} ions are also involved in substrate binding and cleavage, and the multivalency of the enzyme system is an important parameter for its hydrolytic activity [10-14].



Direct Transfer Mechanism (Metal Ion Assisted)

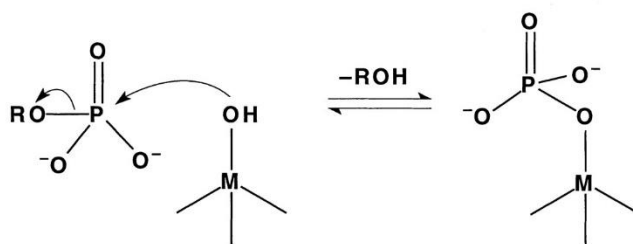


Figure 1.3 Reaction Mechanism for Phosphatases. Two possible mechanisms of phosphoenzymes.

1.2.4. Enzyme Kinetics

Reaction rates and kinetics are dependent on the type of reaction involved and especially to the types of interaction between molecules. For enzymatic reactions, rate changes as the reaction progresses. The catalytic reaction rate starts high and slowly reaches a plateau as time increases and the substrate concentration decreases. Additionally, intermediate products also affect the rate of reaction. As such, a series of rate laws have been described to correlate reaction rate with the concentrations of various components in the system.

1.2.4.1. Zero-Order Reaction

The rate of zero-order reaction is equal to the rate constant, as the rate is not affected by reactant concentration. The reaction stops immediately when the limiting reactant is completely exhausted.

The numerical representation of this type of reaction is as follows:



$$r = k$$

where k refers to the rate constant and has the unit of $\text{mole L}^{-1} \text{sec}^{-1}$.

1.2.4.2. First-Order Reaction

The rate of reaction is directly proportional to the concentration of one of the reactants in first order reactions.

The equation associated with this reaction is as follows



Differential Rate Law: $r = k [A]$

where k refers to the rate constant and has the unit of $\text{mole L}^{-1} \text{sec}^{-1}$.

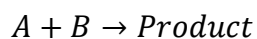
1.2.4.3. Second-Order Reaction

For a second-order reaction, the rate of reaction is directly proportional either to the square of the concentration of one of the reactants or to the product of two reactants.

The equation for this type of reaction is provided below:



or



Differential Rate Law: $r = k [A]^2 = k [A] [B]$

where k refers to the rate constant and has the unit of $\text{mole L}^{-1} \text{sec}^{-1}$.

1.2.4.4. Michaelis-Menten Mechanism

Substrate concentration-independent enzymatic behavior can be described by a simplified Michaelis-Menten model. This model involves the transition state of an enzyme E binding to a substrate S to form a complex ES, which transforms the substrate into a product P and frees the enzyme to catalyze other reactions.

The reaction can be described as:



where E refers to the enzyme, S is the substrate, and ES is an enzyme-substrate transition state.

In addition, its rate may be described as:

$$v = \frac{d[P]}{dt} = \frac{V_{max} [S]}{K_m + [S]}$$

where V_{max} stands for the maximum rate reached by the system at its maximum substrate concentrations, where the rate reaches a plateau and the reaction is saturated.

The Michaelis constant K_m represents the binding affinity between the enzyme and the substrate, and is the substrate concentration where the reaction rate is half of V_{max} .

The equilibrium constant of the reaction can be derived as follows:

$$K = \frac{k}{k^{-1}}$$

1.2.5. Catalytic Nanostructures

The discovery of enzymes, the unraveling of their catalytic mechanism and the mimicking of their properties have been attracting interest for a long time. This discipline has been substantially maintained due to the ever increasing knowledge on enzyme mechanisms and an expansion of synthetic skills. Increasing information on enzymes and synthetic techniques enables us to reproduce the enzyme's function, mimic a chemical reaction/chemical transformation (covalent bonds) or molecular recognition. Peptides have been utilized as promising artificial enzyme candidates due to presence of amino acids in the active sites of native enzymes. Ever since, either on scaffold or self-assembled peptides provides a basis for artificial enzyme investigations. An overview of the main types of peptide based enzyme mimicry is given in this article.

1.2.5.1 Catalysts

Enzymes are sophisticated proteins which maintain metabolic equilibrium in living creatures. From the digestion of nourishment to the replication of DNA, enzymes function in many vital activities as highly selective catalysts which accelerate both the rate specificity and catalytic rate of metabolic reactions. Active sites of enzymes, catalytic groups, often collaborate with specific cofactors or coenzymes for catalytic efficiency [15]. These catalytic proteins can be mainly classified as functional group enzymes or as metalloenzymes. Functional group enzymes enable catalytic reaction only via functional groups available in the side chains of the polypeptide backbone of

the enzyme. The metalloenzymes immobilize metal-ions with their functional groups for catalytic purposes [16].

Designing enzyme-like synthetic molecules, either by applying biochemical or physico-chemical construction of active sites of protein structure and function or with the aid of computational methods, is a promising area of research with the potential to tremendously impact medicine and industrial chemistry. Artificial enzymes also provide a powerful method for dissecting enzyme mechanisms of natural systems [9]. Enzymatic catalysis occurs through the binding of a substrate close to functional groups in the enzymes [17, 18]. As a result of this, it is possible to imitate catalysis mechanism by using functional groups of proteins in a small molecule which has ability to bind substrate. There are many reported models of enzymatic activity or enzyme active sites based on synthetic organic molecules such as macrocyclic compounds and molecular assemblies [19]. Novel strategies based on amino acids or peptides as characteristic molecular moieties have resulted in a remarkable expansion of the field of enzyme mimics or artificial enzymes. Similar to peptide chains that form active sites in enzymes, the self- assembled nanofibers of short peptides aim to preserve the essence of enzymes in a simpler system than proteins. [20-22].

This part will summarize studies on peptide assisted or peptide constructed as artificial enzymes. There are different types of structures will be explained from peptide modified macromolecules to self-assembled protein mimics.

1.2.5.2. Active Site Mimicking Peptides

Since the discovery of triadic mechanism of serine proteases the functional group enzyme mimicking has received a significant amount of attention to develop artificial enzymatic structures.

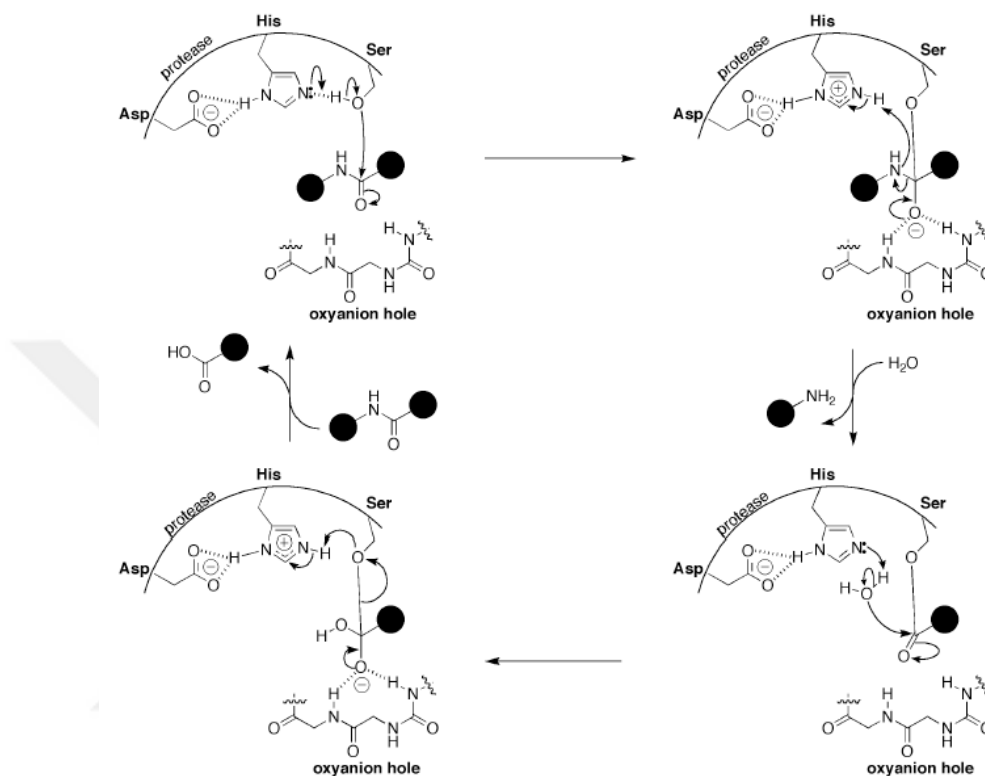


Figure 1.4 Catalytic Mechanism of Serine Proteases.

Depiction of steps of catalytic action of triads.

The cooperative interaction of functional groups of the catalytic triad can be delicately manipulated to construct peptide-based mimics of these enzymes. Scientists has been inspired by this three amino acid based catalytic mechanism and tried to design artificial systems to simulate catalytic activity.

The discovery of solid-phase peptide synthesis, [1, 2] facilitated preparation of synthetic peptide. Solid-phase split-mix libraries of peptides could be prepared and screened for catalytic properties. Since enzymes are polypeptides could be used as

synthetic mimics of enzymatic activity [1]. In addition, peptides are usually more available for catalytic property tuning compared to proteins, which provides ease and important research method for revealing enzymatic mechanisms.

Despite, the preliminary studies to construct peptidic serine hydrolase mimics, it was an promising approach, [23] showed low reproducibility and activity of these systems were considered as uncertain [24] Different methods, such as hydrolysis of activated esters instead of hydrolysis of amide bonds, were also described for this purpose (Figure 1.5). Peptides with secondary structures like α -helix (Figure 1.4, D) or β -sheet [25] (Figure 1.5, E) have also shown hydrolytic activity. Other example are cyclic peptides, the Asp-His-Ser catalytic triad residue attached onto the cyclic peptide [26] (Figure 1.5, B) or triad amino acids placed in the ring instead external attachment (Figure 1.5, D). However, they both showed poor hydrolytic activity due to lack of required catalytic space for hydrogen bonding.

Even though the catalytic activity of peptidic artificial enzyme design was inadequate, the facile side which these bio-mimetic systems that comprising a high density of functional groups could be synthesized and analyzed illustrated the usefulness and applicability of peptide-chemistry for the construction of enzyme mimics. Hydrolytic peptide dendrimer investigated by Reymond et al. is one of the [27] Catalytic groups containing peptide denrimers are developed as efficient and rapid method (Figure 1.5, G), revealing peptidic constructs with almost enzyme-like activity-profiles. Unfortunately, these dendrimers were only capable of hydrolysis of activated esters whereas hydrolysis of amide-bonds constructs remained elusive. Finally, self-assembled peptide constructs (Figure 1.5, F and G) have also been used as serine

hydrolase mimics [28]. Although these constructs had a poor activity, they represented a novel approach in the construction of peptidic hydrolysis catalysts.

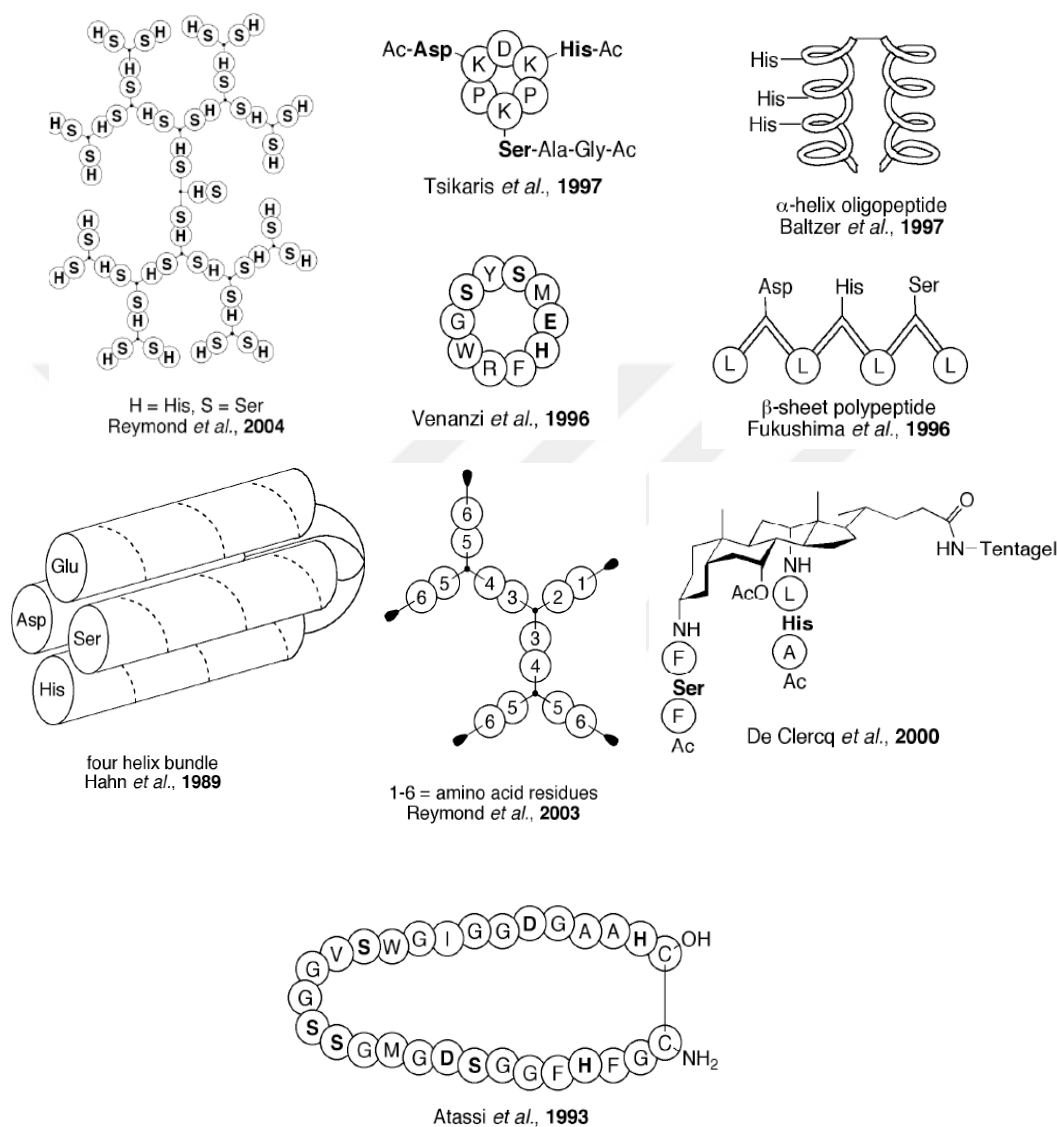


Figure 1.5 Examples of peptides and peptide scaffold as hydrolase mimics.

1.2.5.3. Peptidic constructs as mimics of metalloenzymes

Artificial metalloenzymes are mainly designed by using inorganic or organometallic approaches. In this section, peptide construct occupant enzyme mimic structures will be explained.

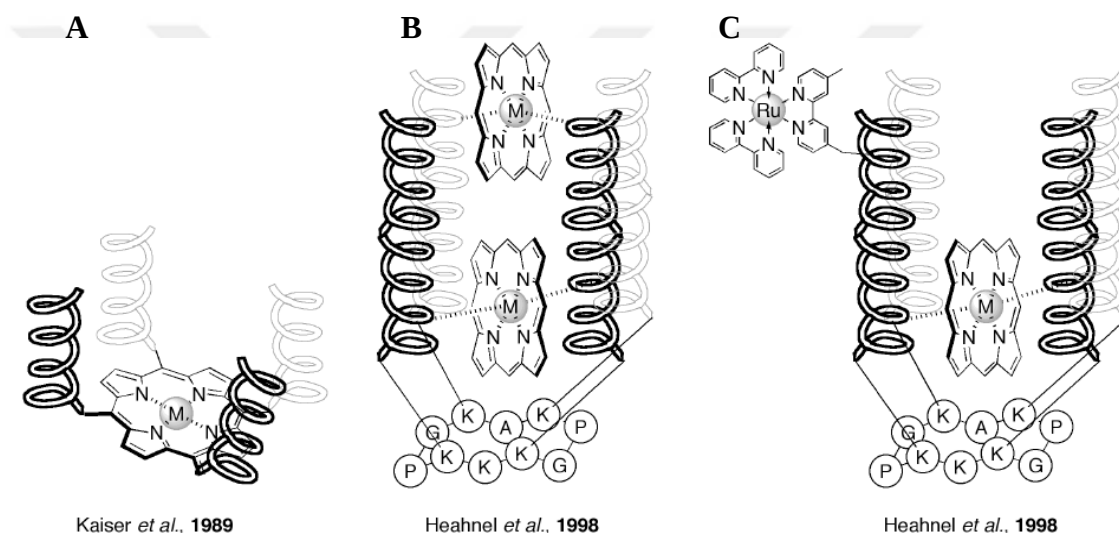


Figure 1.6 Typical Examples of Template –Assisted Synthetic Proteins

Enzyme mimicking peptide composites like scaffold peptides[29] provides tunable chemical properties of metal center and this is helpful for elucidation of the role of proteins in native metalloenzymes [30] Study of Kaiser et al. is a good example of hemeoproteins, which comprising helical peptides that were positioned onto porphyrin to catalytic site and hydrophobic core in a same construct.. (Figure 1.6, A) [31]. Modified approach was designed by Hähnel et al. which is utilizing helical and RAFT

(cyclo template) peptide conjugation as a water-soluble cytochrome b model protein (Figure 1.6, B). These kinds of combination based structures are practical in determining the importance of the amino acid sequence on the stability, activity, the coordination chemistry of the metal-ion and the functional properties of the model protein [32]. The alterability of such designs are also useful to develop combinatorial synthesis of model-proteins and cofactors like Cu(II), flavin or Ru-bipyridine (Figure 1.6, C) [33]. Furthermore, other metal binding related properties like redox potential or charge transfer and folding stability can be assessed. The combinatorial design proved to be very informative and has resulted in the construction of Cu(II)-binding model proteins in which the properties of the metal-center could be modified by subtle changes in the metal-center adjacent helices [29].

The characteristics of immobilized metal-ions of metalloenzymes are influenced by the surrounding protein tertiary structure which is a limiting factor for investigations of model-peptides. Using artificial combinatorial scaffolds might lead to a better understanding of the interaction between metal ion and peptide/protein or cofactors. In conclusion, enhanced enzyme mimics or artificial metalloenzyme might improve our understanding of enzymatic activity.

1.2.5.4. Scaffold-Assisted Peptidic Constructs

In the previous part, metalloenzyme mimicking designs was explained, the synthetic scaffold has important role for catalysis studies and these scaffolds will be explained [29]. The scaffold based approaches allowed scientists to replace the bulk of the

protein when investigating the interaction between metal-ion and protein mimic thoroughly.

Reported scaffolds in the literature can be classified as diversity oriented, pre-organization oriented, or combination of scaffolds. On the other hand, diversity oriented scaffolds provides potential for introduction of different peptides. Although pre-organization oriented scaffolds are not suitable for different peptide attachment, this method is capable of positioning engaged peptides. However, the combination of these two types is more prospering.

The scaffolds have only been merely investigated in terms of their application in solid-phase chemistry. Yet, there are some studies which have managed to achieve synthetic protein-like structures. The cyclotrimeratrylene (CTV) construct is a good example as a template for the construction of collagen mimics, synthetic receptors, and for the preparation of trivalent amino acid glycoconjugate [34] [35] [36]. Analogously, calix[4]arene scaffolds have been utilized for the preparation of cyclic-peptide holding constructs and enzyme mimics.

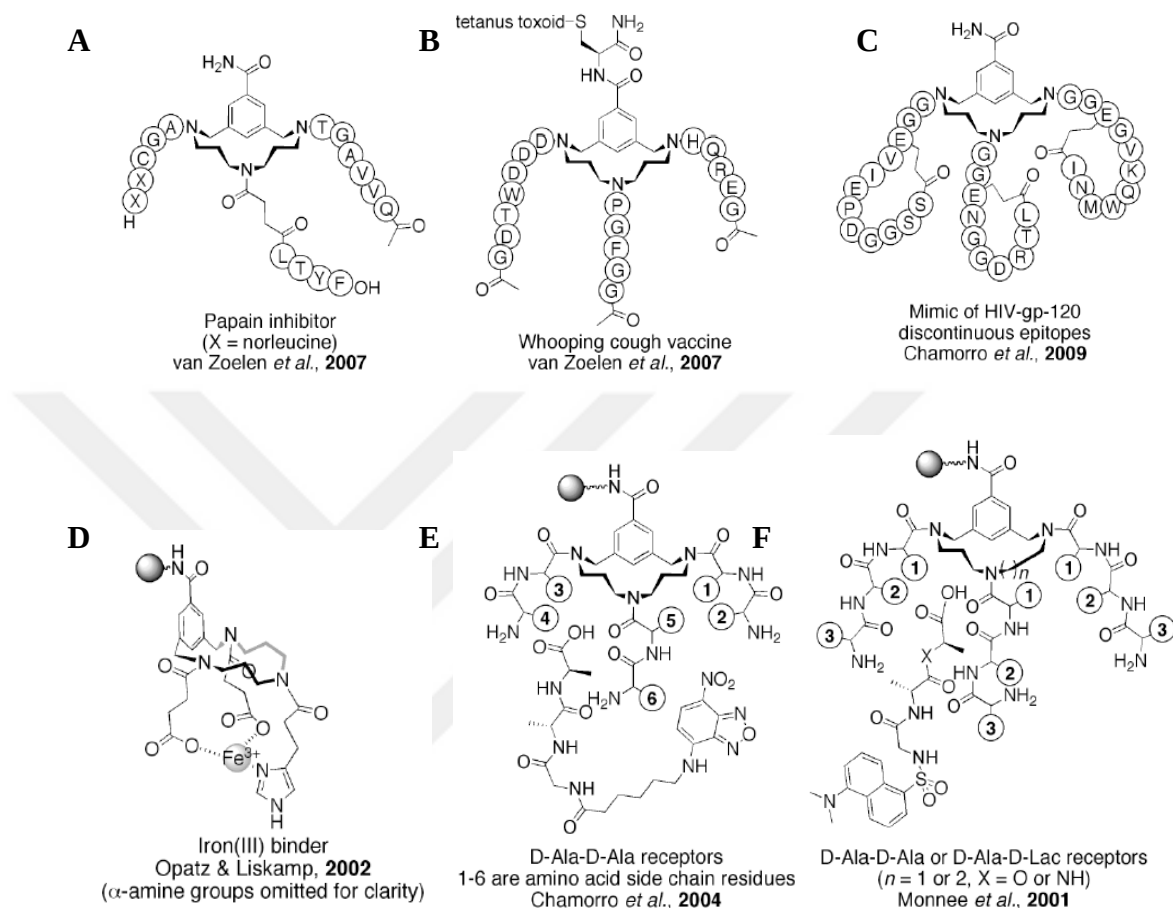


Figure 1.7 Overview of applications of TAC-based peptide constructs. Different possible applications of TAC peptides, a) papain inhibitor, b) cough vaccine, c) mimic of HIV, d) Iron(III) binder, e) D-Ala-D-Ala receptors, f) D-Ala-D-Ala or D-Ala-D-Lac receptors, summarized in this figure.

Another scaffold, which was developed by Madder et al., is based on construction of serine hydrolase mimics [37]. As an example of scaffold composite, the RAFT scaffold designed by Mutter et al. and the triazacyclophane (TAC)-scaffold [38] combination have been some of the most common applied scaffolds in peptide chemistry. Initially, the scaffold libraries of iron (III) (Figure 1.7, D) or fluorescently (Figure 1.7, F) labeled peptide binding tripodal peptidic receptors were designed. Subsequently, these constructs become the first examples of biologically relevant receptors which is composed of three different peptide chain integrated scaffolds. These constructs can also be designed as enzyme inhibitors. For example, a synthetic mimic of papain inhibitor (cystatin mimic) was simulated by using TAC-scaffold (Figure 1.7, A) [39]. Furthermore, the TAC-scaffold was used as a platform to generate a synthetic vaccine, which has developed protective antibodies against whooping cough (Figure 1.7, C) [40]. Moreover, the non-stop solid-phase synthesis of a protein mimic containing three peptide loops as a mimic of discontinuous epitopes was recently unclosed (Figure 1.7, F). These applications revealed the enormous potential of TAC-scaffold peptides in medicinal chemistry and chemical biology. Although the TAC-scaffolds have been used in many biologically relevant systems—ranging from the construction of artificial synthetic receptors for pathogenic organisms to synthetic vaccines – their applicability as scaffolds for the construction of peptide-based enzyme (active site) mimics was unexplored.

1.2.5.5. Self-assembled Peptidic Construct

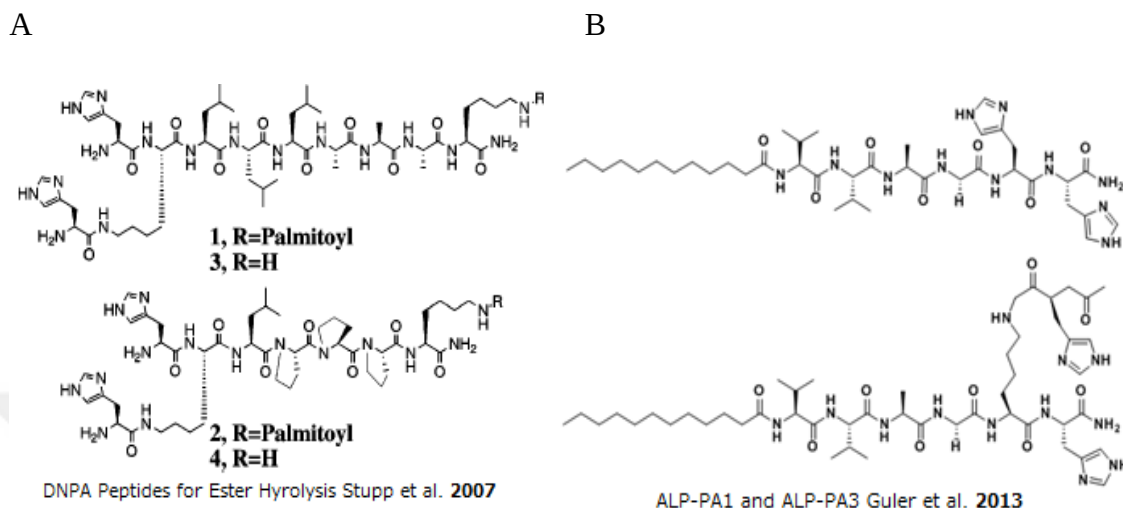


Figure 1.8 Examples of Self-Assembled Peptide Catalysts a) palmitoyl conjugated b) lauric acid conjugated self-assembled peptide catalyst.

Apart from previous studies, there is one more peptidic approach which has capacity for developing breakthrough catalytic mimics, which is self-assembled peptides. Self-assembled PA have attracted interest in the field of nanotechnology for its potential for application in many discipline. In particular, nanofibers can facilitate enzymatic reactions through high density of active sites displayed on the fiber surface. A number of studies have been conducted based on this method and one of this is a nanofiber ester catalyst was developed by Stupp et al. In this study, second rate ester catalysis obtained through β -sheet forming peptide fibers (DNPA) which has imidazole functionalized surfaces. (Figure 1.8, A) [41].

Another recent study is metalloenzyme inspired peptide structures. Metalloenzymes have different hydrolytic activity compared to functional group enzymes as explained

before. This kind of enzymes mainly uses metal ions for catalysis by immobilizing them with functional groups of peptides. In this study, ALP mimics was determined as model enzyme by mimicking its polynuclear zinc binding domain. The AP simulating constructs were tagged with two histidine group to immobilize the Zn^{II} ion to gain metalloenzymatic response. (Figure 1.8, A) This peptides mimicked ALP like activity in terms of both their catalytic and scaffolding features. However, self-assembled enzyme mimic studies are still rare and this subject needs more attention to develop facile and effective structures [42].

1.2.5.6. Perspective for Nanocatalysts

In previous sections, many different artificial enzyme mimics and several approaches for the construction of enzyme active sites were explained. The functional group enzyme mimicking peptides, metalloenzyme mimicking peptides, scaffolded enzyme mimics and self-assembled artificial catalyst were explained and exemplified shortly. Artificial enzyme studies are started and especially based on serine hydrolases, as shown in each part. Although there are many good studies and examples, peptide based artificial enzyme studies are still at early stage and require more effort to develop better mimics and constructs in catalysis for further purposes like drug design and medical applications. The problem about the development of enzyme mimics is highly specific arrangement of catalytic residues in natural enzymes. In order to mimic these well-organized structures, scientists need to further explore and understand active sites, and then apply or simulate these mechanisms with artificial site modified scaffolds. Artificial enzymes will be good spares of natural enzymes in considering time

consuming and expensive synthesis of proteins and immunogenic side effects of animal derivatives. As a result of this, harmless, facile, cheap and effective enzyme mimics in demand for future medical application.

1.3. Bioactive Materials and Biomineralization

The complex nature of cell-matrix interactions and the fundamental role of extracellular matrix (ECM) in the regulation of cellular behavior have been investigated to great detail, and recent advances in nanotechnology and materials science opened up new avenues in developing new materials for regenerative medicine. In this context, peptide nanofiber systems emerged as a promising tool and have commonly been used as modifiable synthetic scaffolds in regenerative medicine studies. Both structural and functional properties of peptide nanofiber systems can be engineered to resemble the ECM and present biomolecular recognition sites that can induce specific interactions with cell surface receptors. These controllable properties of peptide based nanostructures have been extensively used for tissue regeneration and for extracellular matrix studies to alter cellular responses and behaviors.

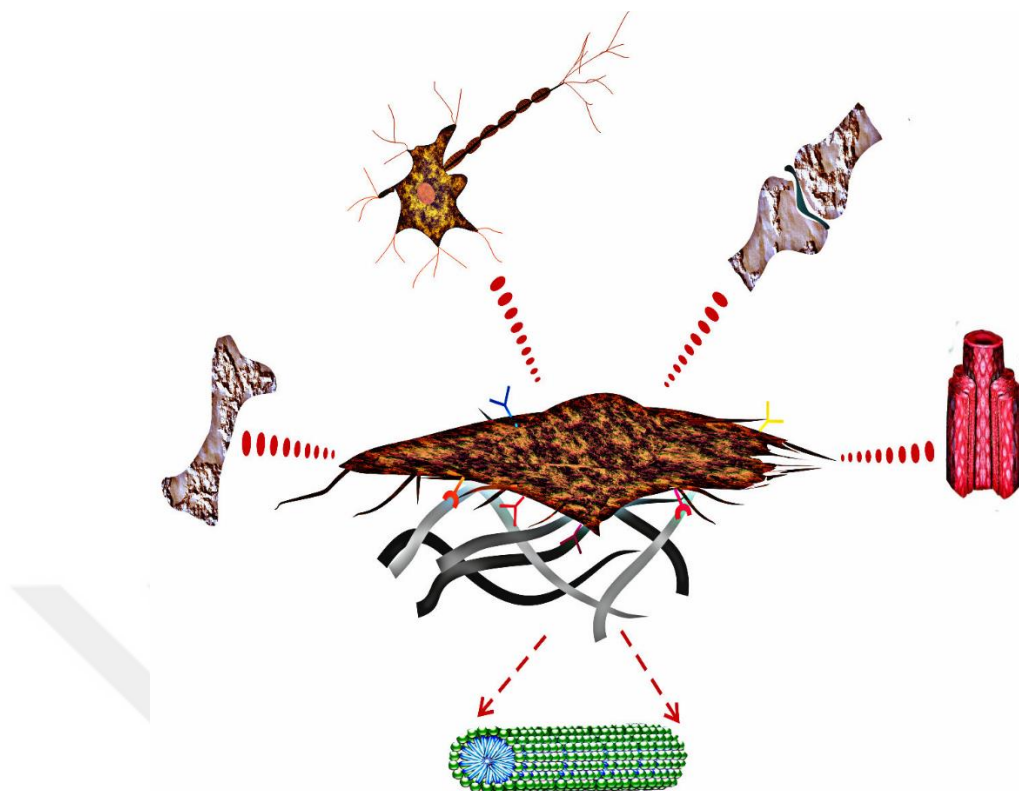


Figure 1.9 Regenerative applications of peptide nanomaterials.

Recent advances in biomaterial research has focused strongly on eliminating numerous issues associated with complex networks. Self-assembled hierarchical structures, produced by bioengineering and peptide synthesis methods, are especially promising for this context, as they are easy to design and nonetheless serve as useful models for the fibrous networks found in natural extracellular systems. They can therefore be used to gain a better understanding of the cell-matrix interactions that occur in nature, and offer new perspectives for the development of materials with wide-ranging application areas in regenerate medicine. In addition, peptide-based nanofiber structures can display the biochemical properties and the complex architecture of their natural counterparts, which makes them ideal for tissue regeneration applications due to

biodegradability, biocompatibility, quick assembly, water storage capacity and bioactivity [43].

The microenvironments provided by biomaterials should be compatible with cellular activities, and to ensure the normal function of cells; the physical, biochemical and mechanical properties of biomaterials should be designed to closely resemble these found under natural conditions. As such, a greater understanding of cell-biomaterial and cell-ECM interactions is key for developing functional materials that can effectively interact with and support cellular behavior, Peptide nanofibers decorated with receptor recognition sequences are frequently used as growth and differentiation-regulating microenvironment for regenerative purposes, and have seen considerable success in this capacity, having been used for the regeneration of tissue as disparate as cartilage, neurons and skin. However, the present thesis focuses on the use of peptide nanostructures for the regeneration of mineralizable tissues, as exemplified by bone and teeth.

Degradable small molecules, including PA, have recently emerged as an alternative approach for developing implantable materials for tissue regeneration. Bone regeneration studies have been primarily focused on polymers and synthetic proteins. However, these structures have several shortcomings like toxic precursor materials (monomers, crosslinking agents etc.), volume loss following application, and low biodegradability.

Various types of supramolecular nanostructures and hydrogels have attracted great interest in regenerative medicine because of their ease of production, complete biodegradability and biocompatibility. These inert nanostructures can be applied to deformed bone tissue as implantable or injectable materials under simple procedures

and with minimal invasiveness. Investigation of self-assembled peptide nanostructures is a growing field with great potential to generate new, facile and effective bone regenerative applications. Here, we describe some of the significant contributions to the field of bone regeneration with self-assembled peptide structures.

Bone formation is a well-orchestrated and complex biological process, and bone regeneration strategies should therefore meet a strict set of requirements to allow the proper remodeling of the deformed site. In general, bone tissue consists of both inorganic components (primarily a specific type of mineral called hydroxyapatite) and a wide variety of organic materials, most of which is type I collagen. The inorganic mineral phase constitutes 65% of the wet weight of the bone tissue, while the organic components and water contribute to the rest [44]. Organic extracellular components found in bone matrix can be classified as insoluble (*e.g.* collagen) and soluble (*e.g.* growth factors, transcription factors) factors, and play a critical role in bone organization and remodeling [45]. Organic components, and especially collagen, provide resistance to tension, while the inorganic mineral phase contributes to resistance against compression. When the bone is demineralized, the remaining organic phase provides flexibility and resistance to fracture; the removal of organic matrix consequently makes the bone rigid and brittle. As a composite structure consisting of organic and inorganic components, the hierarchical organization of the bone matrix not only serves as a structural and mechanical support to cells, but also provides biochemical cues that regulate cell and tissue functions.

During bone regeneration, osteoinduction and osteoconduction are governed by different factors (mechanical, biological, and chemical) that interplay each other and these components should be considered carefully while constructing materials for bone

regeneration. While a diverse set of materials have been utilized to build such scaffolds, inert and mechanically supportive metals and alloys have so far been used as permanent bone implants. These metallic implants and surface modification techniques lack osteoinductive properties, despite their success in osteoconductive features to accelerate the bone healing process. To improve cell attachment and to induce bone differentiation process, bioactive molecule (ECM proteins, growth factors) attachments to implant surfaces are critical to obtain adequate bone healing and controlled mineralization. Proteins and growth factors are large molecules bearing short peptide sequences which can trigger downstream processes, especially cell adhesion and differentiation among many other roles. Accordingly, peptide structures with short bioactive units draw significant attention for bone remodeling studies.

Table 1 Summary of bioactive sequences for bone regeneration

Peptide	Origin	Applications
RGDS	Found in ECM proteins, mostly in fibronectin and binds to integrin	Cell adhesion, attach cells to the bioactive surface for osteogenic activity
IKVAV	Laminin	Cell adhesion, spreading, migration
YIGSR	Laminin	Cell adhesion
DGEA	Collagen type I	Osteoblast specific binding via alpha2-beta1 integrin
GFOGER	Collagen IV	Collagen mimetic sequence
KRSR	Binds to transmembrane proteoglycans	Selectively increase osteoblast adhesion with bio-adhesive moiety functionalization
GAG-PA	Heparan sulphate mimicking peptide	Protein-based extracellular matrix components, glycosaminoglycans (GAGs) regulate bone formation.
E3-PA + DOPA-PA	Noncollagenous matrix proteins	Mineralization and osteogenic differentiation
RADA16	Originally designed as ionic self-complementary oligopeptides	
ALK	Osteogenic growth peptide	Osteogenic differentiation of osteoprogenitors
DGR	Osteopontin	
PRG	2-unit RGD motifs	

Most commonly used bioactive short peptides are cell-binding epitopes, including RGDS, IKVAV and YIGSR. The RGDS sequence in particular has frequently been

used to facilitate cell attachment. The RGD motif is found in fibronectin, osteopontin and sialoprotein, and fibrous materials that possess the RGD sequence can mimic the function of these properties [46, 47]. The adhesion of bone marrow derived stem cells, for example, was investigated on RGDS-containing peptide PA surfaces, and the activity of these scaffolds was compared with epitope-free peptides. Stem cells were encapsulated into PAs and the coassembled system was injected *in vivo*, and RGDS containing PA gels was found to promote cell viability substantially better than the epitope-free control group [48].

The primary component of bone ECM is collagen I fibers, and bioactive short sequences derived from this protein are ideal targets for the induction of bone tissue remodeling. DGEA, the best-studied collagen I epitope, is found in the $\alpha 1$ helix of collagen, and its osteoinductive characteristics were utilized intensively for bone regeneration studies. For example, DGEA peptide coated hydroxyapatite surfaces are known to enhance the differentiation of mesenchymal stem cell into an osteogenic fate [49]. However, bioactivity is not enough for adequate regeneration in some cases, and more than one component may be required to enhance activity. For example, another study using CGGDGEAG sequence reported a lack of adhesion by rat calvarial osteoblasts onto peptide surfaces [50]. To eliminate adhesion problem and improve the osteoinductive potential of DGEA-PA, RGDS-PA, and S-PA peptides were utilized in different combinations, and according to histochemical staining and PCR results, the RGDS-PA and DGEA-PA combination effectively upregulated osteogenic differentiation [51]. Another short, collagen-derived peptide sequence is GFOGER, which binds to the osteogenesis-regulating integrin $\alpha 2\beta 1$. Differentiation of osteoprogenitor cells into osteoblasts can be triggered by using this sequence, and

GFOGER coated polycaprolactone scaffolds were shown to support the remodeling of critical sized defects in rats, inducing significant regeneration in nonhealing femoral defects compared to peptide-free control groups [52].

In addition to cell attachment and proliferation, peptide sequences were also used as growth factor binding units. Binding units located on gel scaffolds can attract growth factors to the desired location and support the healing process. Bone morphogenetic protein-2 (BMP-2), a well-studied bone differentiation regulating factor that plays significant roles during osteogenesis, was targeted for this purpose. BMP receptor-binding peptides with osteopromotive domains, DWIVA and A4G3EDWIVA, were found to be adequate for promoting the bone regeneration process [53]. In the ECM, glycosaminoglycans (GAG) also interact with BMP-2, and sulfated GAG mimetic peptide-BMP interactions were used to promote osteoblast maturation and mineralization of osteogenic cells. The GAG mimicking ability of the peptide nanofibers and their interaction with BMP-2 promoted osteogenic activity and mineralization of osteoblastic cells [54].

A mussel inspired, DOPA-mediated surface adhesion strategy was also used in the literature to direct efficient osteoblast maturation on implant surfaces. For this purpose, E₃-PA/Dopa-PA and DGEA-PA/Dopa-PA combinations were used to induce osteoblast formation and subsequent bone-like mineralization. Hydroxyapatite boosting glutamic acid and DOPA residues on fiber surfaces induced significant osteogenic activity as a consequence of enhanced mineral deposition, since inorganic materials like hydroxyapatite can also induce osteoblast maturation during the bone formation process [55]. As such, it is clear that mineral inducing peptide scaffolds have important roles for the remodeling of bone.

Peptide based soft bone grafts can also serve as structural frameworks. These materials can be designed in order to have structural components that complement the effect of signaling epitopes, further enhancing their bioactivity. For example, micro-patterned, RGDS-modified scaffolds bearing 20-40 nm holes were found to upregulate osteogenic marker expression compared to non-patterned surfaces. In particular, whole patterned surfaces presented high density epitopes that were able to enclose cells and stimulate differentiation [56]. In another example, phosphoserine-bearing PA nanofibers were used to induce mineralization. This matrix presented a convenient template for the deposition of hydroxyapatite, the basic mineral component of bone, due to possessing a suitable structure for crystal packing [50]. As peptides also allow the design of nano-patterned surfaces, they can serve as versatile building blocks for generating templates that can induce inorganic material deposition.

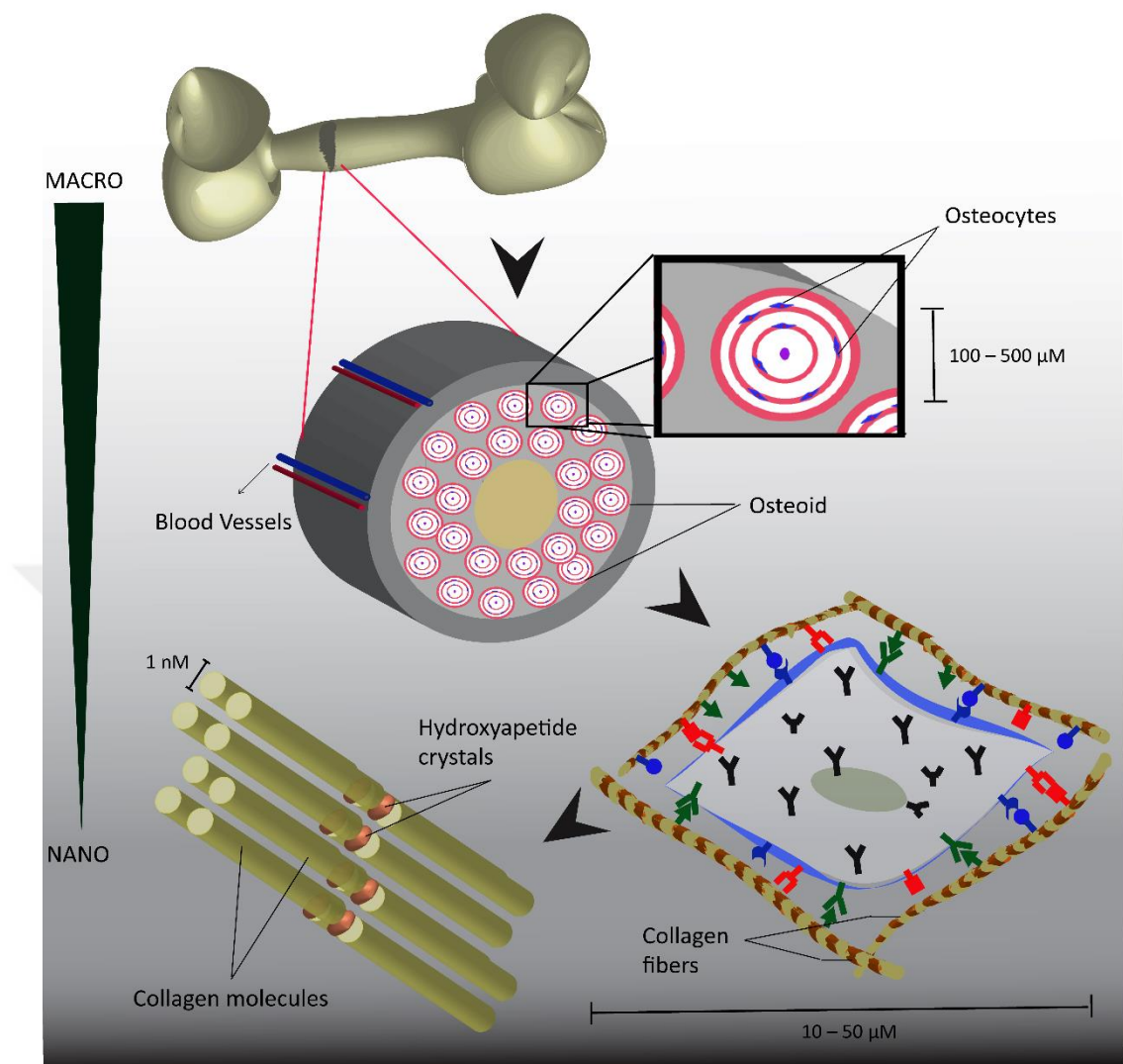


Figure 1.10 Structural and cellular components of bone structure. (Reproduced from “Therapeutic Nanomaterials” 2016, Wiley)

CHAPTER 2

Catalytic Supramolecular Self-Assembled Peptide

Nanostructures for Ester Hydrolysis

2.1. Objective

Essential amino acids in catalytic sites of native enzymes are important in nature inspired catalyst designs. Active sites of enzymes contain coordinated assemblies of multiple amino acids, and catalytic action is generated by the dynamic interactions among multiple residues. However, catalysis studies are limited with the complex and dynamic structure of the enzyme; and it is difficult to conclusively attribute a given function to a specific residue. Minimalistic approaches involving artificial catalytic sites are promising for the investigation of enzyme function in the absence of non-essential protein components, and self-assembling peptide nanostructures are especially advantageous in this context. Here, we describe design and characterization of an enzyme-mimetic catalytic system formed by the self-assembly of separate essential residues (Ser, His, Asp) displayed on PA molecules. The function of each residue and their combinations on the nanostructures in hydrolysis reaction was studied. The catalytic self-assembled nanostructures were used for efficient ester hydrolysis such as a model substrate (pNPA) and a natural substrate (acetylcholine) highlighting the key role of self-assembly for catalytic domain formation to test efficiency of *de novo* designed catalyst as a catalytic triad model.

2.2. Introduction

Enzyme-like synthetic systems designed by using the information derived from the identification of evolutionarily conserved domains or computational optimization are highly promising for the development of novel catalytic materials in medicine and in industrial chemistry [8]. The design of enzyme mimetic catalysts is complicated because of the three-dimensional conformations of the proteins. An enzyme is composed of several amino acid residues with optimal three dimensional arrangements. Functional cooperative residues can only be determined after testing large numbers of possible peptide sequences and heavy computation [7, 57]. Reductionist approaches are helpful in order to reduce the workload associated with the complicated nature of proteins.

Novel strategies based on small organic molecules, supramolecular structures, polymers and inorganic materials have resulted in remarkable expansion of the field of enzyme mimics or artificial enzyme [8, 58-63]. Although these strategies are effective in terms of catalytic efficiency, covalently constructed hydrolytic modules have still not advanced enough to design enzyme-like structures, since genuine enzymes fold into their active and dynamic conformation by self-assembly mechanisms. In this context, self-assembling peptide molecules are considered as candidates for catalysis studies due to their dynamic nature, which can be used to closely approximate the flexible and hydrophobic cores found in natural enzymes. In addition, the self-assembled nanofibers are composed of amino acid sequences, which can be derived directly from the peptide chains found in enzyme active sites, thereby preserving the function of enzymes in a simpler system than proteins. The peptides are considered as “scaled-down” proteins that preserve function while eliminating non-

catalytic bulk and their use may allow greater understanding of the structure, activity and active residue preferences of enzymatic site [13, 41, 42, 64].

The self-assembling PA(PA) molecules exhibit several features similar to natural proteins; in particular, their noncovalent interaction-driven assembly (hydrogen bonding, electrostatic interactions, hydrophobic forces etc.), dynamic molecular structure, hydrophobic nanodomains and activity under natural conditions (pH, aqueous medium) are all similar to naturally occurring enzymes [65]. Moreover, their stability, low cost, multivalent presentation on a concise area, ease of synthesis and modification, biocompatibility and ability to present multiple active domains in small areas all enhance their ability to serve as effective enzymatic catalyst [43]. In addition, self-assembled peptide nanofibers can be utilized as “proto-enzymes” that provide minimalist frameworks for catalysis studies, reducing much of the complexity that is inherent to natural enzymes [41, 42, 58, 59]. So far, significant effort has been devoted to the development of peptide designs capable of acting as high-efficiency catalysts or models for understanding the factors involved in the catalytic action [13, 41, 42, 64, 66]. Many of these self-assembled peptide nanostructures focus on the use of single molecules as nucleophilic sites, and utilize variations on a core peptide sequence to investigate the effect of residue identity on enzymatic catalysis.

In this chapter, a multivalent self-assembled nanocatalyst was shown for catalytic action through the synergistic behavior of three separate units; each are naturally present in the catalytic triad of the enzymes. The catalytic triad (CT) containing serine, aspartate and histidine amino acid residues is the model active site for several enzymes [4]. These three residues cooperate to generate a catalytic action and this cooperation mechanism makes CT an attractive catalytic system. To generate an artificial catalytic

triad, each active residue in CT (D, H, S) was incorporated separately to a self-assembling peptide domain (Lauryl-VVAG-) that is able to assemble into β -sheet conformations through noncovalent interactions (Figure 2.1, 2.2). Combinations of the active amino acids were utilized in the PA nanostructure formation. By conjugating a single amino acid to each peptide unit, we provide flexibility to the active site chains, thereby supporting the catalytic pocket formation, which is assembled with the help of physical interactions among amino acids. The system is able to closely mimic the flexibility of the natural enzymatic triads. The catalytic are triads typically present in the hydrophobic pockets of the enzymes, and the peptide nanofiber can create hydrophobic domains intrinsically. The efficiency of the catalytic nanostructures was studied with a model substrate *p*-nitrophenyl phosphate (pNPA) and acetylcholine, which is a natural substrate of acetylcholine esterase. The hydrolysis of the model and natural substrate was achieved with the peptide-based artificial enzyme system in high efficiency.

2.3. Results and Discussion

2.3.1. Self-Assembled Peptide Nanostructures

A functional peptide nanostructure library was designed and screened to understand the role of amino acid conformations for a catalytic triad formation as an artificial enzyme mimetic system. The PA molecules functionalized with chemically active amino acid residues (aspartic acid, histidine and serine) were used to form catalytic nanofibers (Figure 2.1, 2.2). TEM analysis was performed to observe the morphology

of the self-assembled peptide nanostructures. For all peptide combinations (D/H/S, D/H, H/S and D/S), nanostructures were observed in aqueous conditions. They were found to be stable over the course of one week or more (Figure 2.1-2.5) The high surface area containing nanostructures were formed through growth of short peptide nanostructures in one dimension, resulting in a compact packing arrangement [67]. Strong driving forces, such as salt inclusion, high peptide concentrations, and pH [68] are utilized to produce nanostructures. The concentration is an important factor for self-assembly pathways as well [69].

Peptide length is also important contributor during one dimensional structure formation and short peptides have shown higher tendency to display one directional growth [70]. In our study, the use of short peptides in sufficient concentration to form gel at pH 8, was observed to cause the loss of all structural curvature and result in formation of high surface area nanostructures through lateral growth. The width of the individual nanostructures was determined to be approximately 100-200 nm by TEM and by atomic force microscopy (AFM) (Figure 2.3-2.5, 2.7). AFM demonstrated flat morphology of the nanostructures and reveal the height of the nanostructures was around ~6 nm [71, 72] (Figure 2.8). Amide I peak at $1,633\text{ cm}^{-1}$, accompanied by a shallow broad band around $1,681\text{ cm}^{-1}$, indicated amyloid-like characteristics of nanostructures (Figure 2.8) [73-76]. This observation is in agreement with the imaging results, as flat nanostructures are typically formed by amyloid-like β -sheets, which are slightly more stable than fibrillar counterparts due to their thermodynamically favorable arrangement of hydrogen bonds [75-77].

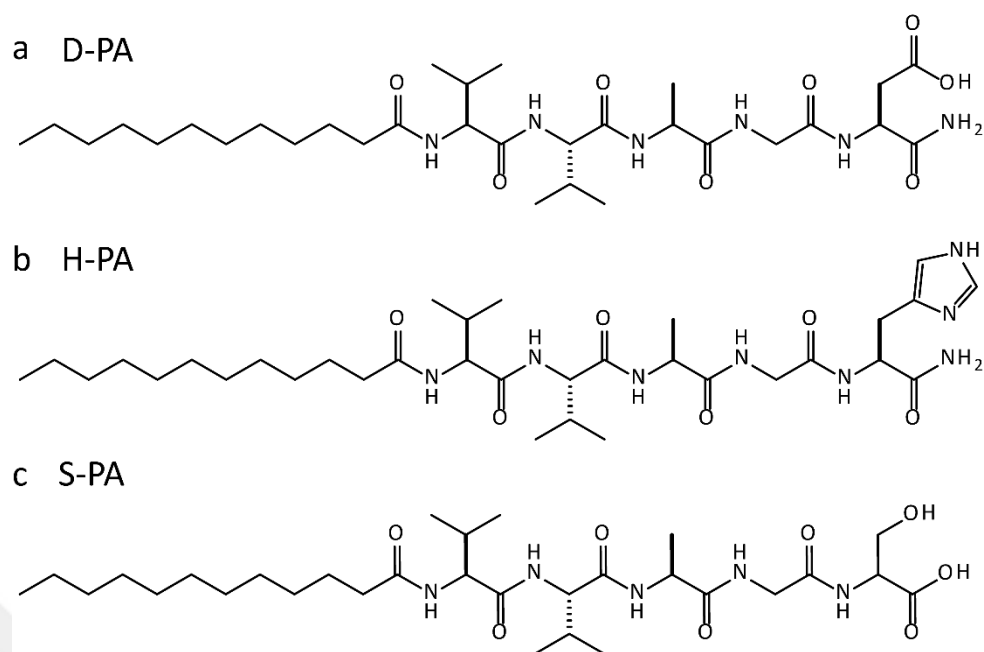


Figure 2.1 Chemical structures of the peptides used to form catalytic nanostructures. a) Lauryl-VVAGD-Am (D-PA), b) Lauryl-VVAGH-Am (H-PA) and c) Lauryl-VVAGS (S-PA).

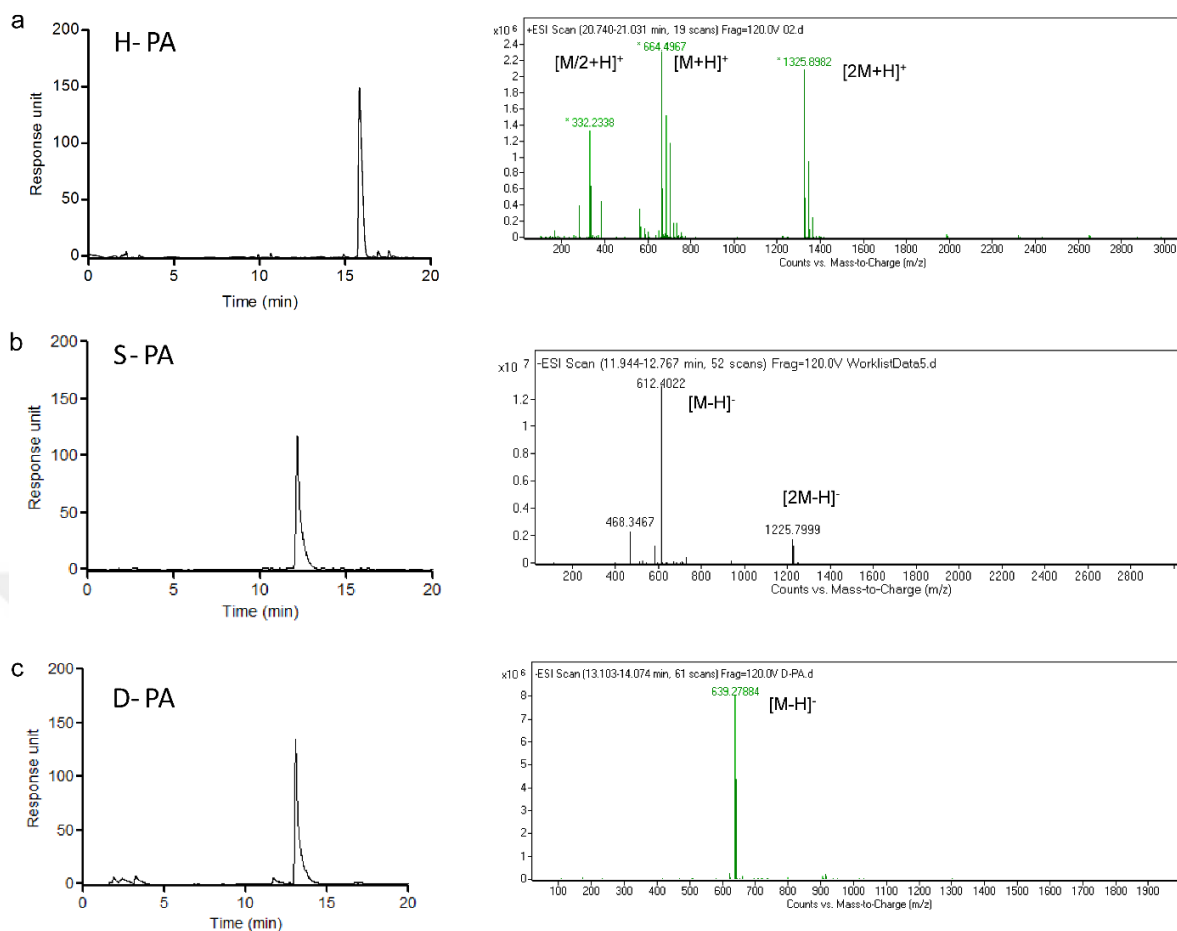


Figure 2.2 Mass spectra of catalytic triad peptides. Following the subtraction of water readings, results found as a) For H-PA, $[M+H]^+$ (calculated) = 664.86, $[M+H]^+$ (observed) = 664.4967, $[M/2+H]^+$ (calculated) = 332.43, $[M/2+H]^+$ (observed) = 332.2308, $[2M+H]^+$ (calculated) = 1326.82, $[2M+H]^+$ (observed) = 1325.8982 . b) For S-PA, $[M-H]^-$ (calculated) = 612.79, $[M-H]^-$ (observed) = 612.4022, $[2M-H]^-$ (calculated) = 1226.58 $[2M-H]^-$ (observed) = 1225.7999 . c) For D-PA, $[M-H]^-$ (calculated) = 639.81 $[M-H]^-$ (observed) = 639.2788.

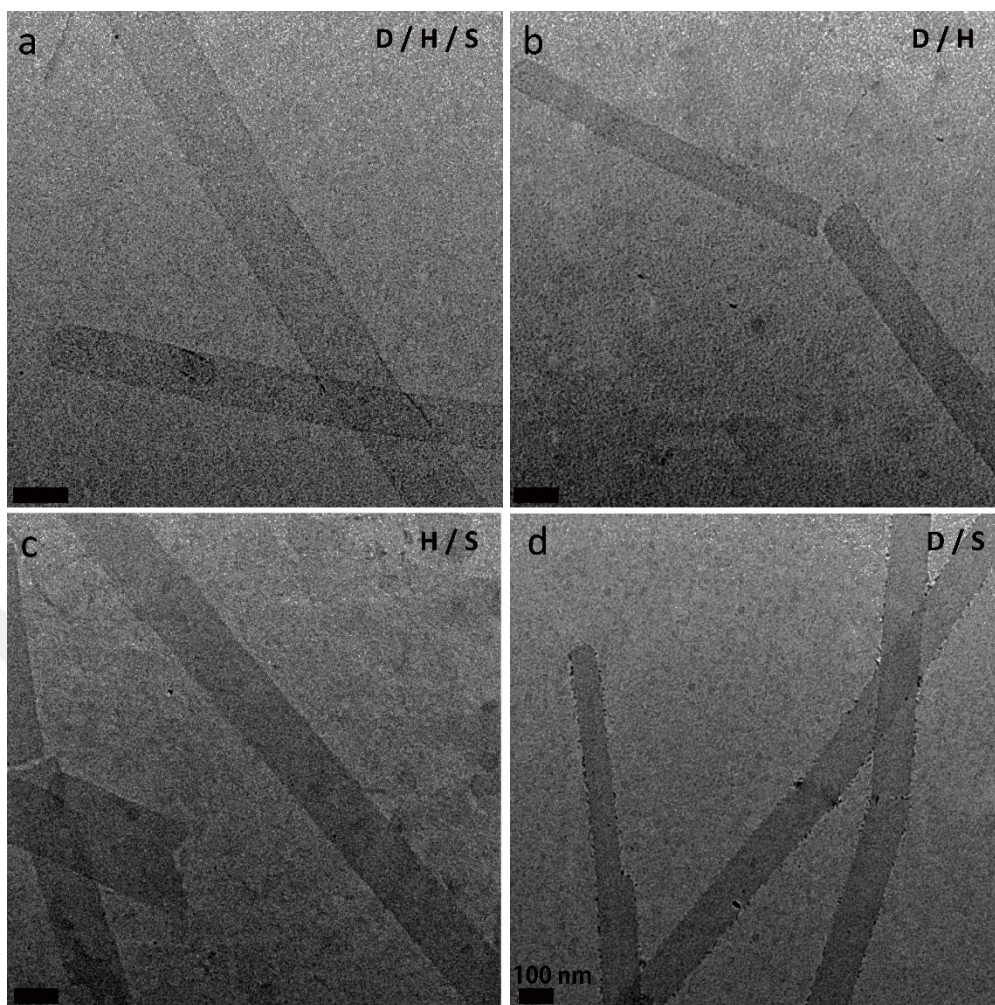


Figure 2.3 TEM images of the peptide nanostructures. a) D/H/S (D-PA+H-PA+S-PA), b) D/H (D-PA + H-PA), c) H/S (H-PA + S-PA) and d) D/S (D-PA+S-PA). Scale bar 100 nm.

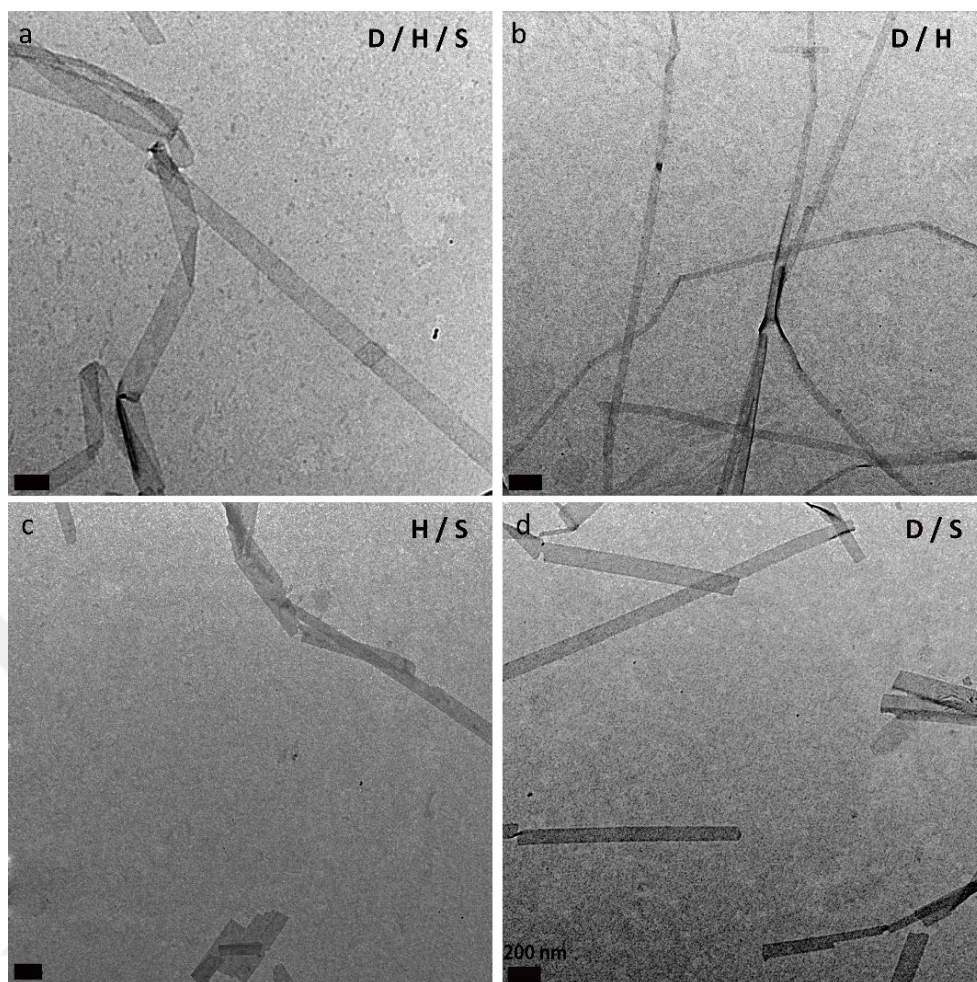


Figure 2.4 Transmission electron microscopy (TEM) images of the nanostructures of the catalytic peptide complexes. TEM images of peptide nanostructures with higher magnification.



Figure 2.5 Transmission electron microscopy (TEM) images of the nanostructures exhibited by peptide molecules. TEM visualization of nanostructures formed by single peptide assembly.

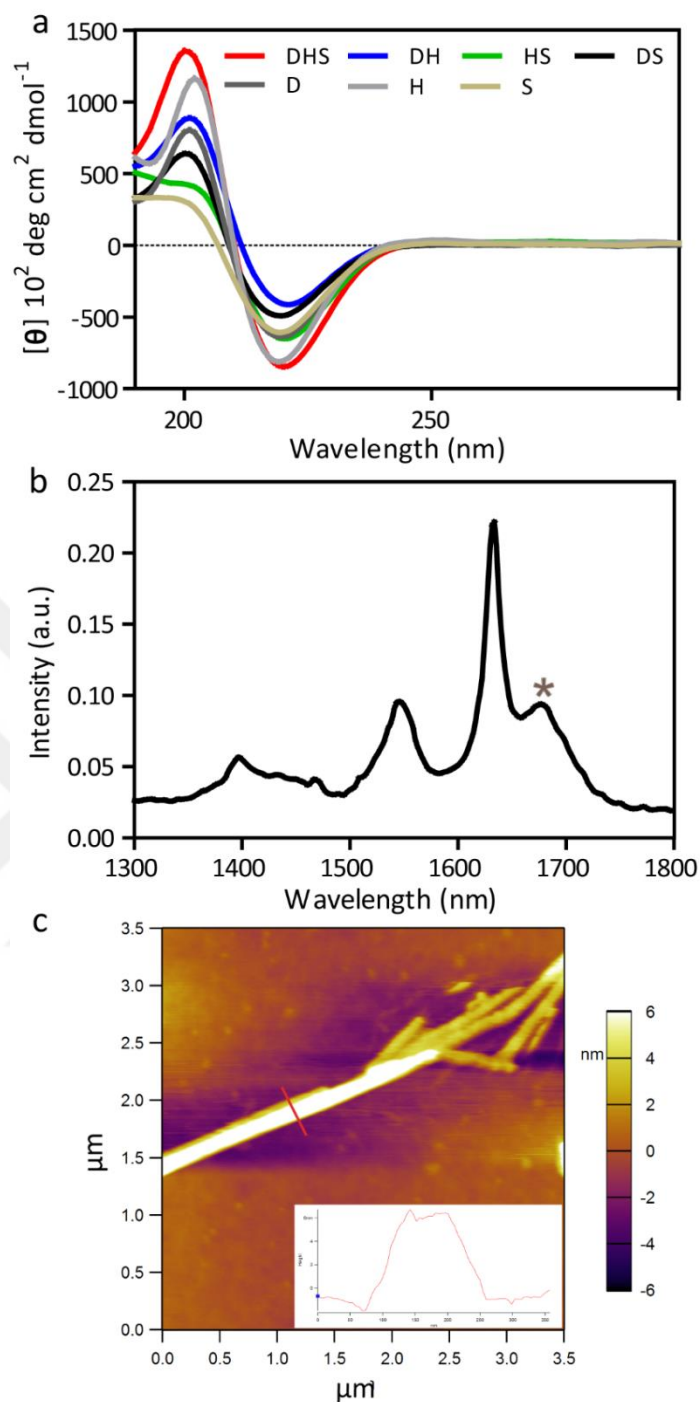


Figure 2.6 Characterization of the secondary structures of nanofibers. a) Circular dichroism analysis of the self-assembled peptide nanostructures. b) FTIR spectrum of the D/H/S peptide nanostructures, and c) AFM image of co-assembled D/H/S nanostructures.

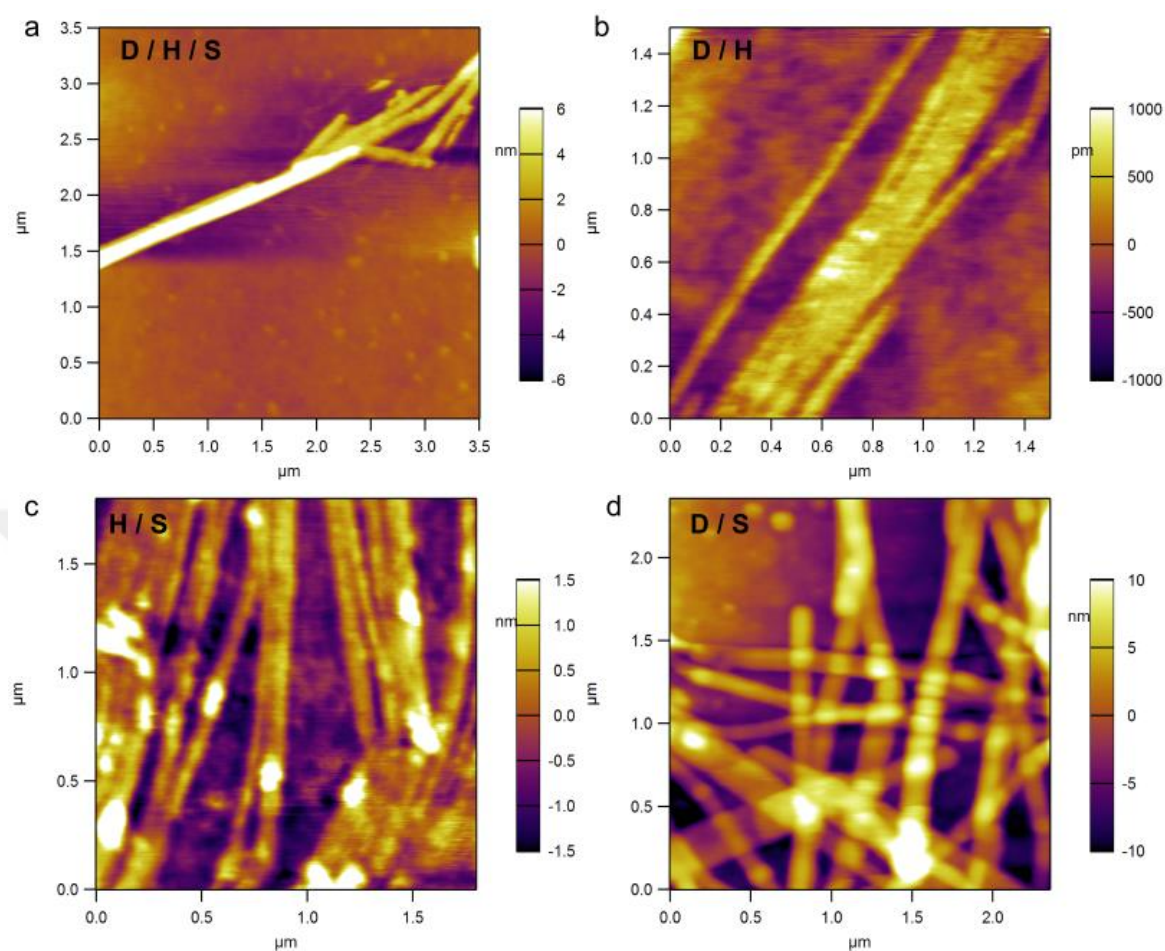


Figure 2.7 Atomic force microscopy images of the nanostructures formed by the peptide complexes. AFM analysis revealed wide and flat structures formed by peptides after assembly.

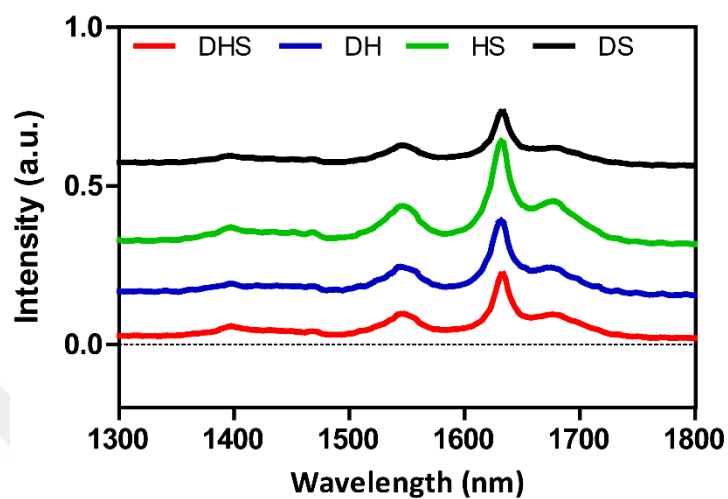


Figure 2.8 FT-IR spectra of the peptide complexes for secondary structure investigation. Hydrogen bonding indicator amide peaks investigated by using FT-IR spectroscopy and all peptide combinations revealed specific amide peaks indicating secondary structure formation.

2.3.2. Catalytic Activity of Peptide Nanostructures

The structures and mechanisms of action of catalytic peptide nanostructures were studied as a dynamic enzyme system. Peptidic catalysts are easy to produce and modify, which makes them ideal materials for understanding the individual and cooperative functions of amino acids in the catalytic pockets of enzymes. PA nanocatalysts which utilized histidine as a catalytic domain were reported in the literature [42, 64, 70]. Here, we showed an artificial catalyst with multiple reactive units, which can emulate the function of a triple action catalyst, catalytic triad. Accordingly, each active residue is presented on the peptide nanostructure, and hydrolytic sites are formed through peptide self-assembly, a process that resembles the natural enzyme folding mechanism. The mixtures of peptides were investigated to determine the function of each reactive residue during ester hydrolysis reaction. Catalytic properties of the peptide nanostructures were analyzed by using *p*-nitrophenylacetate (pNPA), a common model substrate for esterase activities. The reaction rates associated with enzyme catalysis for single, binary and triple peptide combinations were screened (Figure 2.9). The D/H/S PA nanostructure was found to be the most active system for pNPA hydrolysis, followed by the dual combinations H/S, D/H and D/S PAs. Single peptide nanostructures were generally ineffective because these systems are unable to establish the intermolecular associations required for molecular coordination; however, H-PA was observed to exhibit some catalytic activity, possibly due to its nucleophilic nature at neutral pH. This result was found compatible with previous reports, in which covalently conjugated double histidine moieties instead of single histidine, were displayed on periphery of the fiber surface,

and collective interaction among decorated residues have shown upgraded hydrolysis activity [42, 70].

Assembly of active residues and cooperation among residues those units displayed on small area were found significant for enhancement of catalytic action [42, 64, 66, 70]. Significance of assembly on catalytic action was tested by using soluble amino acids (L-aspartic acid, L-histidine and L-serine) and these residues were mixed by using as controls, same preparation method that was used to prepare peptide nanostructures (Figure 2.9). The soluble amino acid mixture (D/H/S) did not shown significant activity for pNPA hydrolysis and this result indicated the importance of cooperation of the reactive groups on a stable support to generate catalytic action.

When, the catalytic activity of D/H/S, H/S, D/H and D/S residues on the PA nanostructures was characterized, all of the nanostructures were found to display measurable activity (PBS buffer pH 7) and they exhibited substrate concentration-initial rate of reaction dependencies consistent with the Michaelis-Menten model (Table 2, Figure 2.10). The catalytic activity was mainly caused by all three residues. The D/H/S supramolecular nanostructures were found to be the most active, exhibiting the highest catalytic efficiency at $k_{cat}/K_M = 126.62 \pm 14.58 \text{ mM}^{-1} \text{ s}^{-1}$. The catalytic efficiency of D/H/S nanofibers was at least 4-fold higher than those of dual systems, suggesting that all three amino acids are required for complete catalytic action. The D/H/S also exhibited a stronger binding affinity to the substrate, because its binding constant, K_M was the lowest among all groups ($K_M=0.035 \text{ mM}$).

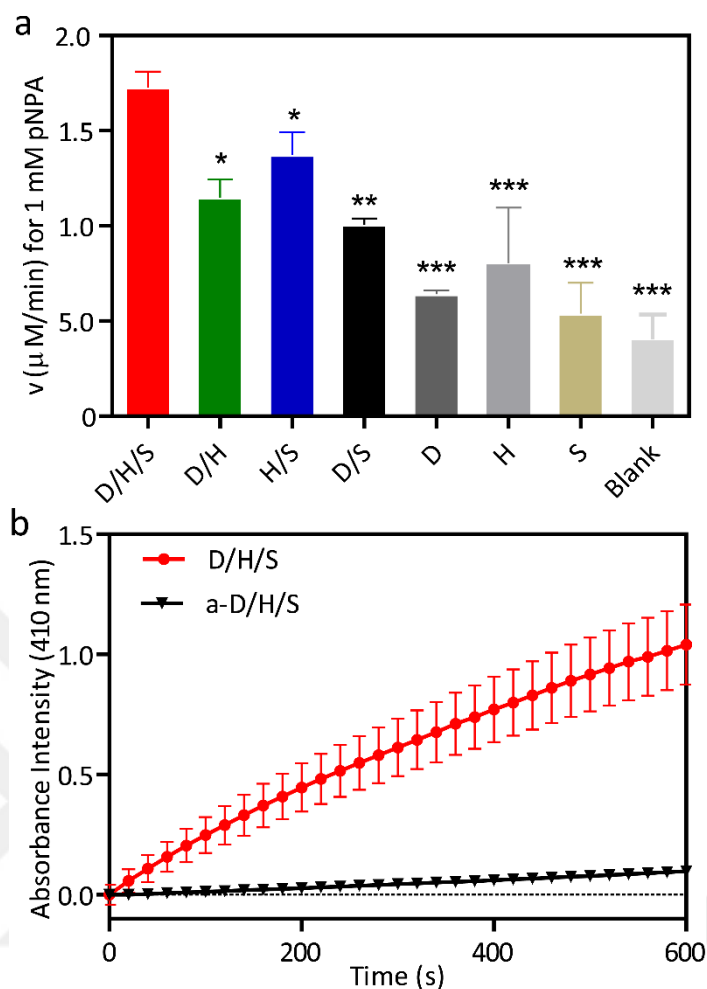


Figure 2.9 Comparison of catalytic reaction rates of peptide combinations for pNPA hydrolysis. a) and b) D/H/S peptide nanostructure vs. soluble amino acids (a-D/H/S) (L-aspartic acid, L-histidine and L-serine) mixture. (Mean $\pm$ SEM, $n \geq 3$ measurements per sample, one-way analysis of variance (ANOVA) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.)

Table 2 Catalytic activity of designed triad and dual peptide nanostructure combinations at pH 7.4 in 1x PBS buffer. Catalytic efficiency, turnover rate and substrate binding constants of peptide nanostructures were showed in this figure.

	k_{cat}/K_M ($M^{-1} s^{-1}$)	$k_{cat} \times 10^{-3}$ (s^{-1})	K_M (mM)
D/H/S	126.62 ± 14.58	4.41 ± 0.51	0.035
H/S	35.90 ± 4.61	2.60 ± 0.33	0.072
D/H	8.97 ± 1.43	2.59 ± 0.42	0.289
D/S	7.63 ± 1.69	1.12 ± 0.25	0.148

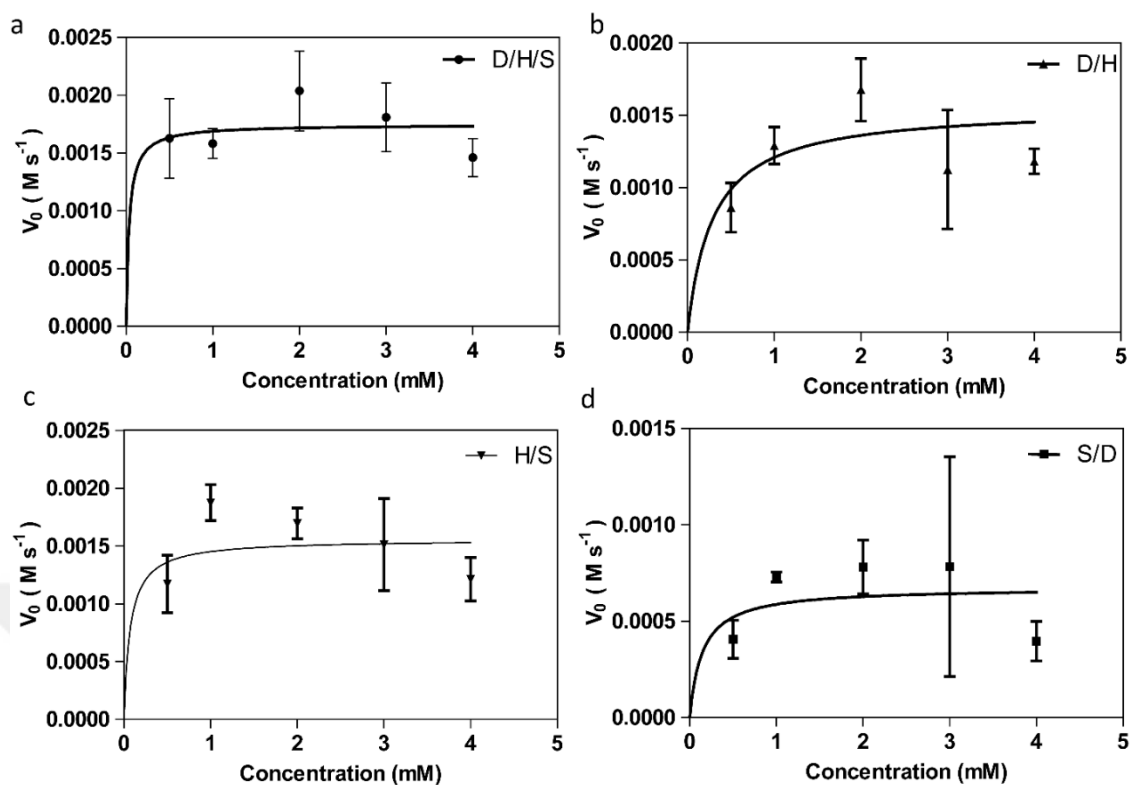


Figure 2.10 The Michaelis–Menten diagram of the catalytic activity of CT-PAs for pNPA. Substrate concentration dependent catalytic rates were measured in 1x PBS and calculated by using Michaelis-Menten enzyme kinetics fitting (error bars in all groups represent standard deviations of at least three independent measurements).

Knowledge of chemical features of active residues could provide better understanding to explain action mechanism. During the enzymatic action, aspartate group serves to facilitate histidine group's capacity as a proton acceptor. Deprotonated imidazole moiety recruits a proton from the hydroxyl side chain of the serine, creating a highly nucleophilic alkoxide group on the serine residue [4]. The catalytic triad and nucleophile generation by histidine and serine residues is important for the enzyme activity [71, 72]. Our observations are in agreement with this common mechanism, as the H/S nanofiber system was observed to have the second-best reaction efficiency ($k_{\text{cat}}/K_{\text{M}} = 35.90 \text{ mM}^{-1} \text{ s}^{-1}$). Previous studies have also indicated the significance of cooperation of each residue. Especially the histidine-serine pair should act together to generate nucleophilic action [71, 73]. The absence of aspartate in the H/S binary system probably decreased the proton accepting capability of histidine, leading to impairments in the formation of alkoxide action. However, the system nevertheless retained its capacity to catalyze pNPA, and exhibited only a minor decrease in binding affinity ($K_{\text{M}} = 0.072 \text{ mM}$).

Serine is responsible for both recruiting the enzyme substrate and performing the hydrolytic attack during catalytic action [4], and forms tetrahedral intermediates that are involved in both functions. The kinetic data of D/H suggests that the exclusion of serine results in greatly reduced catalytic action ($k_{\text{cat}}/K_{\text{M}} = 8.97 \pm 1.43 \text{ mM}^{-1} \text{ s}^{-1}$), and the binding affinity of D/H was likewise lower compared to serine-containing groups. These results are in accordance with the previous findings about the catalytic triad. In addition, comparisons between the catalytic activities of D/S and H/S systems suggest that the function of serine is highly dependent on histidine. The serine-mediated catalysis is greatly inhibited in the absence of the histidine residue. The reaction

efficiency of D/S was $k_{cat}/K_M = 7.63 \pm 1.69 \text{ mM}^{-1} \text{ s}^{-1}$, which is lower than D/H/S and H/S systems. The catalytic efficiency of D/S is also lower than the D/H system, possibly because of the ability of aspartate to enhance the nucleophilic character of histidine but not serine. In addition, histidine itself is a good nucleophile in basic conditions [42, 70] allowing it to perform enzymatic catalysis without support from other residues.

2.3.3. *Acetylcholine Esterase-like Activity*

Acetylcholinesterase is a hydrolase that liberates choline from the neurotransmitter acetylcholine during neurotransmission, and possesses an esterase unit that acts like a serine protease and contains a catalytic triad. Intrigued by the possibility of replacing the function of a natural enzymatic system with an artificial catalytic peptide nanofiber, we utilized acetylcholine as a model ester for studying the catalytic effect of the dual and triple functionalized nanofiber combinations. We analyzed hydrolysis of acetylcholine by mass spectrometry (Figure 2.11).

Acetylthiocholine was exploited as an acetylcholine analogue for spectrometric tracking, and it is chemically similar to acetylcholine in terms of hydrolysis reaction except for the presence of an additional thiol group. After hydrolysis, released thiol groups remain on the choline residues and they can be monitored by Ellman's reagent [74], which is a commonly used method as a colorimetric enzyme assay. This technique is based on the reaction between 5,5-dithiobis(2-nitrobenzoic) acid (DTNB) and thiol groups on thiocholine (TCh), which is released after the cleavage (Figure 2.12). Consistent with our previous results, serine protease-like peptide nanostructures were found to be applicable for the catalysis of natural ester containing substrates. The D/H/S peptide nanocatalyst exhibited the highest catalytic rate. Catalytic activities of

each combination were also in agreement with our previous results (Figure 2.11, 2.12), as D/H and H/S displayed less activity for acetylcholine and D/S was the least active catalyst combination, reflecting the importance of serine and histidine residues during hydrolysis.



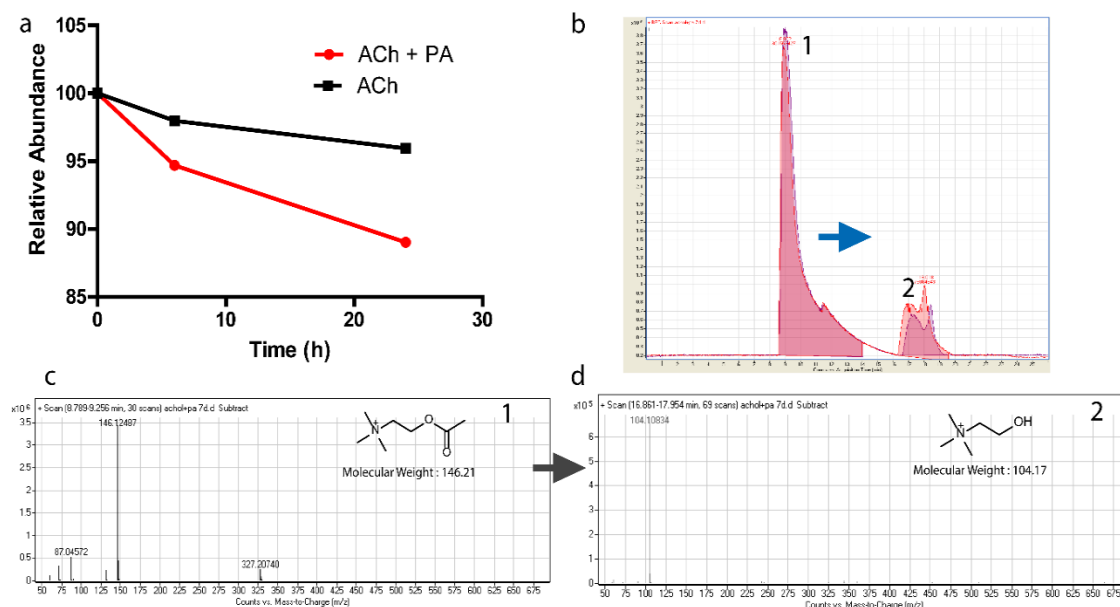


Figure 2.11 Degradation of acetylcholine in the presence of D/H/S CT-PAs, as analyzed with LC-MS. a) Relative abundance calculations used to track PA-dependent hydrolysis. b) Abundance diagram showing choline formation. c) Mass spectrum of the peptide following the subtraction of water readings. $[M]^+$ (calculated) = 146.21, $[M]^+$ (observed) = 146.1267, $[M]^+$ (calculated) = 104.17, $[M]^+$ (observed) = 104.1083.

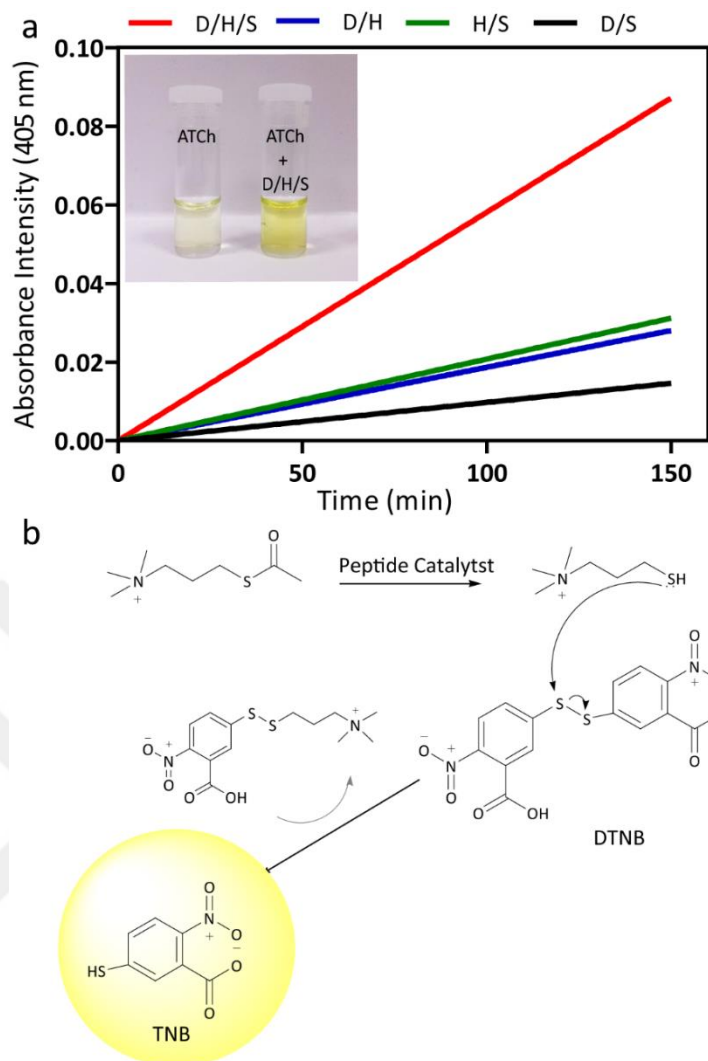


Figure 2.12 Comparison of catalytic reaction rates of all peptide combinations for acetylthiocholine a) Hydrolytic rate comparison among peptide groups in the presence of acetylthiocholine substrate. b) Reaction mechanism of Ellman's reagent following catalysis of substrate. Substrate release quantified by this method.

2.4. Conclusion

In this chapter, self-assembled peptide nanostructures containing chemically active groups on a high-surface were utilized for hydrolysis of model esters. A noncovalent self-assembly process was utilized to form the nanostructures capable of mimicking the structure and function of the catalytic triad present in the esterase active site. The roles of individual amino acid residues were elucidated on the catalytic nanostructure system. We demonstrated the mechanisms of complex enzymatic reactions by using a peptide nanostructure system. The catalytic triad model created on the nanostructures was investigated through the assembly of combinations of reactive amino acid residues. Both natural and synthetic ester containing substrates were used to demonstrate the synergy between the catalytic units. The multivalent nanostructures shown here are promising for the investigation of enzyme functionality, developing artificial enzymes and also for designing multifunctional systems. Potentially, by altering the chemical content of the self-assembling molecules, multifunctional bioactive enzyme mimetic nanostructures can be produced.

2.5. Experimental Section

2.5.1. Synthesis and Characterization of Peptide Molecules

The peptides were synthesized using standard Fmoc (9-Fluorenylmethoxycarbonyl) solid phase synthesis. Protected amino acids, MBHA Rink Amide Resin, Wang Resin and HBTU (O-Benzotriazole-N,N,N,N'-tetramethyl-uronium-hexafluoro-phosphate) were purchased from Novabiochem, lauric acid was purchased from Merck. Serine-

loaded Wang resin (Fmoc-Ser-(OtBu)) or Rink amide MBHA resin was used for peptide synthesis. For each coupling reaction, Fmoc groups were removed by shaking resins in 20% piperidine in N,N-dimethylformamide (DMF) for 20 min. All amino acids were activated by adding 2 molar equivalents of amino acid to 1.95 equivalents of O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and dissolving the mixture in 10 mL of DMF. After the complete dissolution of the amino acid and HBTU in DMF, 3 molar equivalents of N-ethyl-diisopropylamine (DIEA) were added into the solution. Each coupling reaction was performed for 2.5 h. Lauric acid was coupled to the peptide's N-terminal. Peptide cleavage and the removal of the protecting groups were performed with 95:2.5:2.5 trifluoroacetic acid (TFA): triisopropylsilane (TIS): water for 2.5 h at room temperature. TFA was then rotary-evaporated, ice-cold diethyl ether was added to the residue, and the resulting solution was left at -20 °C overnight. The pellet was collected, dissolved in deionized H₂O and freeze-dried. After freeze-drying, peptides were dissolved in 1 mM HCl solution in order to get rid of excess TFA, freeze dried again and characterized by LC-MS. An Agilent Technologies 6530 Accurate-Mass Q-TOF LC-MS equipped with a Zorbax SB-C8 column was used for LC-MS analysis. The concentration of the sample was 0.5 mg/mL. The mobile phase was water (containing 0.1% formic acid for positively charged peptides, 0.1% ammonium hydroxide for negatively charged peptides) and acetonitrile (containing 0.1% formic acid for positively charged peptides, 0.1% ammonium hydroxide for negatively charged peptides). LC-MS was run for 20 min for each sample. Each run was started with 2% acetonitrile and 98% H₂O for 5 min, and the acetonitrile gradient was gradually increased to 100% over 20 min. The solvent flow rate was 0.65 mL/min. 5 µL of sample was injected for each run.

2.5.2. Transmission Electron Microscopy

2 mM peptide solutions were prepared and their pH was adjusted prior to TEM analysis. The peptide gel was diluted in water and a small amount of solution was dropped onto a carbon-covered copper grid. 2% (w/v) uranyl acetate solution was used to visualize the peptide nanostructures. The carbon grid was air dried following uranyl acetate staining. A FEI Tecnai G2 F30 transmission electron microscope was used to image the peptide nanostructures.

2.5.3. Circular Dichroism

Secondary structures of peptide nanofibers (pPA) were analyzed with a JascoJ-815 circular dichroism (CD) spectrometer. 2 mM peptide solutions were prepared in ddH₂O, coassembled by mixing and kept at room temperature in order to achieve complete assembly formation. Peptide nanostructures were then diluted with water and the CD spectra of 1×10^{-4} M peptide solutions were measured from 300 nm to 190 nm with a data pitch of 0.1, scanning speed of 100 nm/min, band width of 1 nm and D.I.T. of 4 s. An average of three measurements was used for each sample, and water was used as baseline. Peaks indicating β -sheet formation were observed in the CD spectra analysis.

2.5.4. Atomic Force Microscopy Experiments

AFM sample preparation was performed by using the same samples prepared for CD measurement. Samples were diluted with water to drop cast onto a cover glass and kept at room temperature for drying. Aggregates were measured via tapping mode in air. A commercial microscope (MFP3D, Asylum Research) was used in the measurements.

2.5.5. Fourier Transform Infrared Spectroscopy Measurements

2 mM of solutions were prepared separately at pH 8 on glass surfaces placed in a 24 well-plate. The plate was immersed in liquid nitrogen to obtain flash-frozen gels, which were then lyophilized for dehydration and to preserve their architectural integrity. Dried aggregates were weighed and mixed with KBr powder at a ratio of 1:100 (w/w). KBr/peptide mixtures were homogenized and KBr pellets were formed by pressure. Fourier transform infrared (FTIR) analysis of the samples was carried out using a Bruker VERTEX 70 with a Hyperion scanning microscope at a range of 300-4000 cm^{-1} .

2.5.6. Determination of the Catalytic Activity of the Peptides

2 mM peptide solutions (or amino acid solutions) were prepared and diluted to 0.2 mM by using PBS. A 100 mM *p*-nitrophenyl acetate substrate stock solution was prepared in DMSO and mixed with the peptide solutions in concentrations ranging from 0.5 mM to 4 mM. For single concentration rate comparison experiments with 1 mM

substrate and 0.1 mM peptide catalysts – triple, dual or single combinations- were used. Immediately following mixing, kinetic measurements were performed by collecting the absorbance spectrum of the resulting *p*-nitrophenyl acetate solution at 410 nm by microplate reader. Reaction rates were calculated for each concentration with an extinction coefficient of $10.166 \text{ M}^{-1} \text{ cm}^{-1}$ and fitted into the Michaelis-Menten equation ($v_0 = k_{\text{cat}}[E]_0[S]_0/(K_M + [S]_0)$).

2.5.7. Tracking of Acetylcholine Hydrolysis by Mass Spectroscopy

An Agilent Technologies 6224 Accurate-Mass TOF LC-MS equipped with an Intersil Diol 4.6 x 250 mm column was used for LC-MS analysis. Initial acetylcholine and peptide concentrations were 5 mM and 0.02 mM, respectively. The mobile phase was ammonium formate (15 mM, pH 3.5) and acetonitrile (10:90 v/v) LC-MS was run for 25 min for each sample. Each run was performed under a stable gradient and with a flow rate of 0.06 mL/min.

2.5.8. Acetylthiocholine Hydrolysis Experiments

100 mM stock solutions of acetylthiocholine and dinitrothiocyanobenzene (DNTB) were prepared in DMSO, and peptide solutions were diluted to 0.1 mM in PBS. 2 μl each of DTNB and acetylthiocholine were then added in sequential order to the reaction mixture. Immediately after substrate addition, absorbance data were collected at 405 nm and converted to reaction rates using a nominal extinction coefficient of $13.600 \text{ M}^{-1} \text{ cm}^{-1}$.

CHAPTER 3

Alkaline Phosphatase -Mimicking Peptide Nanofibers for Osteogenic Differentiation

This work is partially described in the following publication:

Gulseren, G., Garip, C., Ustahuseyin, O., Tekin, E. D., Tekinay, A. B., Guler, M. O.,
Biomacromolecules 16(7), 2198–2208, 2015.

3.1. Objective

Recognition of molecules and regulation of extracellular matrix synthesis are some of the functions of enzymes in addition to their catalytic activity. While a diverse array of enzyme-like materials have been developed, these efforts have largely been confined to the imitation of the chemical structure and catalytic activity of the enzymes, and it is unclear whether enzyme mimetic molecules can also be used to replicate the matrix-regulatory roles ordinarily performed by natural enzymes. Self-assembled peptide nanofibers can provide multifunctional enzyme-mimetic properties, as the active sequences of the target enzymes can be directly incorporated into the peptides. In this chapter, an enhanced bone regeneration efficiency was observed through peptide nanofibers carrying both catalytic and matrix-regulatory functions of ALP, a versatile enzyme that plays a critical role in bone formation by regulating phosphate homeostasis and calcifiable bone matrix formation. Histidine presenting

peptide nanostructures were developed to function as phosphatases. These molecules are able to catalyze phosphate hydrolysis and serve as bone-like nodule inducing scaffolds. ALP-like peptide nanofibers enabled osteogenesis for both osteoblast-like and mesenchymal cell lines.

3.2. Introduction

Bone formation is a challenging process since it contains multiple levels of hierarchical organization [75] and ALP has multiple roles during each level of this process. It increases inorganic phosphate to pyrophosphate ratio (P_i / PP_i) through nonspecific cleavage of phosphate esters, and elevates the local concentration of inorganic phosphate, thereby promoting the formation of hydroxyapatite, which is an essential building block of bone tissue [6, 8]. In preosseous cellular metabolism, ALP shows osteoconductive and osteoinductive characteristics, which stem from its ability to induce osteoblast differentiation and to form a calcifiable bone matrix [76]. Especially, regulation of bone matrix for mineral deposition is a critical process to have well-structured biomineralization. These significant catalytic and metabolic properties of ALP make it a remarkable target for bone regeneration studies.

However, until now ALP has not been considered as a model for peptide catalysis studies or even for artificial mimics other than peptide except macrocyclic tetramine complex used to study ALP activity [77]. ALP based studies mostly depend on the native enzyme that requires elaborate isolation steps and careful handling. For regeneration studies, immobilized native enzyme is commonly used to generate bone formation inducing regenerative matrix [78-80]. In one of these studies, peptide nanostructures were used as enzyme immobilizing agent and this scaffold was able to

show catalytic and mineral inducing activities that accelerates osteoblast differentiation. However, difficult purification and handling procedures require much effort and cost to reach desired bioactivity and affect efficiency of the native enzyme. Therefore, investigation of artificial structures designed by considering active sites and roles of model enzyme is crucial to provide regeneration without nontrivial preparation and application steps.

In catalytic site of ALP, imidazole-containing histidine amino acid was found to be a fundamental moiety for its catalytic action. Around physiological pH, imidazolyl moieties can perform a diverse set of functions (metal aided nucleophile, general base and general acid) in order to generate the desired catalytic action. In ALP, histidine residues that are coordinated with Zn^{II} ions attain organophosphate complexation and deprotonation of the water molecule, which is converted into an intermolecular nucleophile. This nucleophile subsequently attacks on the ester bond of the phosphate monoester initiating the hydroxyapatite mineralization process [6, 81]. In addition to its role in metalloenzymes, the histidine residues that are in catalytic sites of non-metalloenzymes provide catalytic capability by themselves without the requirement of a metal ion due to general acid/base characteristics of imidazole moiety [10, 82].

Here, by taking both catalytic and mineral matrix regulating functions of ALP into consideration, we designed self-assembling artificial phosphatase like peptide nanostructures (pPA) by introducing catalysis centers as multifunctional modules to attain bioactive catalytic and calcifiable synthetic bone matrix constructs (Scheme 1). PA are an important class of molecules for bioinspired catalysis owing to their ease of design, handling, presentation of multiple active epitopes and potential for catalytic applications at physiological pH [70, 83-86]. In this chapter, we exploited peptide

nanostructures as a scaffold like support for biomineralization process enhanced with catalytic activity by providing synergistic effect among amino acid residues. Active site of the individual PA molecules was decorated to present multiple histidine residues at the periphery of the nanofibrous system (Figure 3.1- 3.3, 3.5) where residues organized to achieve optimal cooperation and coordination of multiple zinc ions to mimic the activity of natural ALP. Furthermore, the synthetic ALP mimetic nanostructures presented a calcifiable synthetic extracellular matrix like system for bone-like nodule formation through hydrolysis of the organophosphate molecules for inducing efficient osteogenic differentiation of multiple cell types including stem cells.

3.3. Results and Discussion

3.3.1. Phosphatase-Like Nanostructure Formation and Catalytic Activity

Due to self-assembly properties of the pPA molecule, it is possible to produce multivalent nanostructures with high epitope density. The pPA molecules were designed to self-assemble into nanofibers with active sites presented at the periphery. As a control group, imidazole free E-PA and K-PA combination was used to have active site free fibers. (Figure 3.5). After synthesis and characterization of molecules, the effect of secondary structure formation and metal addition on the self-assembled peptide nanofibers was investigated by circular dichroism (CD) spectroscopy (Figure 3.1-3.4). The nanofibers were mainly formed by β -sheet conformations of the peptides at neutral pH and metal addition strengthened secondary structure signal as a sign of coordination. TEM imaging revealed that the pPA molecules self-assemble into nanofibers with diameters of about 5-10 nm Figure 3.5. Zinc coordinated peptide

nanostructures demonstrated sufficient electron density to provide a contrast to be visible under TEM even without staining of the organic nanostructures (Figure 3.5).

High-density presentation of active sites on the nanofiber surface and metal binding ability of the peptide nanofibers are attractive for designing efficient hydrolytic nanocatalysts due to enhanced reaction surface area. The pPA molecules bind to Zn^{II} ions revealing an exothermic reaction determined by isothermal titration calorimetry (ITC). Thermograms obtained following addition of Zn^{II} to pPA solution are shown in (Figure 3.5). Zn^{II} binding isotherm results showed that pPA interacts with zinc ions at a 2:1 ratio with a binding constant of $4.35 \pm 1.16 \times 10^5 \text{ M}^{-1}$.

Hydrolytic capacity of the ALP mimetic pPA nanofiber systems with and without Zn^{II} was studied by measuring catalytic production of chromogenic *p*-nitrophenol from *p*-nitrophenyl phosphate (pNPP) as a model organophosphate substrate. This process relies on the reaction initiated by the metal-coordinated histidine which deprotonates the water molecule and converts it into a nucleophile to cleave phosphate from monoester. Enzymatic saturation profiles of pPA and pPA + Zn^{II} were analyzed by using the Michaelis-Menten kinetics to study their hydrolytic behaviors. The k_{cat} value of pPA with Zn^{II} was found to be $3.76 \times 10^{-5} \text{ s}^{-1}$, which indicates efficiency of Zn-OH^- nucleophile catalyzing the monophosphate. Interestingly, the pPA nanofibers without zinc ions also exhibited catalytic activity, albeit to a lesser extent than Zn^{II} -containing peptide nanofibers, with a k_{cat} value of $1.83 \times 10^{-5} \text{ s}^{-1}$ (Figure 3.6, 3.7).

This inherent catalytic activity of pPA without zinc ions could be because of positioning two or more imidazoles in efficient proximity to generate effective cooperation among imidazoles to form general bases tuning the microenvironment and optimizing the medium effects. It is well-known that imidazole groups can be included

in catalytic mechanism of many enzymes like Ribonuclease A and can be used as chemical catalyst which plays essential role in transesterification process through deprotonation of nucleophile as the nucleophile attacks the carbonyl [11].

Explaining cleavage process is a bit challenging; in this system, nucleophile might be water, which is deprotonated by concentrated general base activity of imidazoles. Another possible mechanism is that, hydroxyl groups on β -glycerophosphate substrate are deprotonated by imidazole and generated nucleophile subsequently attacks phosphoryl group of substrate to cleave the phosphate ion. This mechanism is similar to histidine-serine portion of catalytic triad mechanism in serine protease [11].

On the other hand, free imidazole- Zn^{II} showed a much less catalytic activity compared to both Zn^{II} -containing peptide nanofibers and Zn^{II} -free peptide nanofibers which proves the importance of histidine residue organization in nanofiber system. These results indicated that the activity of peptide nanofibers is not strictly Zn^{II} dependent and these nanofibers themselves have considerable catalytic activity against organophosphate substrate. However, the imidazole groups need to be presented on a special conformation on the nanofibers in order to achieve this catalytic activity in agreement with previous catalysis studies [10, 82]. In order to develop understanding for Zn^{II} free activity and the effect of Zn^{II} ion (presence/absence) on the catalytic mechanism, active sites of the nanostructures (Ac-GHH-Am and Ac-GHH-Am with Zn^{II}) were investigated by using quantum-chemical calculations. The geometry, stability and metal-imidazole interactions were studied to develop further insight (Figure 3.8).

Chemical reactivity and intrinsic stability of active sites of molecule and metal complex were computationally predicted by Density Functional Theory (DFT)

calculations of highest occupied molecular orbital (HOMO)–lowest unoccupied molecular orbital (LUMO) energy gap. The HOMO–LUMO energy gaps were found to be 5.9 eV for pPA active site and 2.9 eV for Zn^{II} bound pPA active site. Zinc coordination dropped HOMO-LUMO energy by half which stands for approximately one fold increase of reactivity in a similar manner with enzyme kinetics experimental results. In other words, the peptide molecules without Zn^{II} ions are potentially kinetically more stable and less reactive unlike the metal complex. The metal addition enables increase in turnover capacity of pPA system with respect to experimental and theoretical findings. These calculations also imply that pPA itself also has considerable activity due to favorable active site localizations supporting general base activity of imidazole chains.

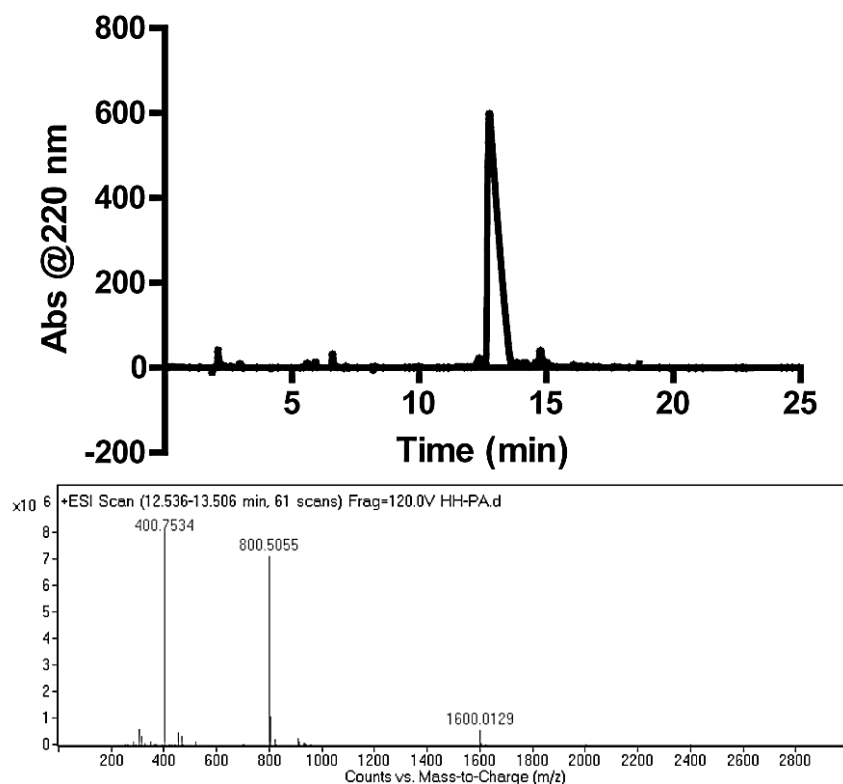


Figure 3.1 Liquid chromatography and mass spectrometry results of the pPA molecule. Mass spectrum of peptide after subtracting mass spectrum of water sample. $[M+H]^+$ (calculated) = 800.00, $[M+H]^+$ (observed) = 800.50, $[M/2+H]^+$ (calculated) = 400.00, $[M/2+H]^+$ (observed) = 400.75, $[2M+H]^+$ (calculated) = 1600.00, $[2M+H]^+$ (observed) = 1600.01.

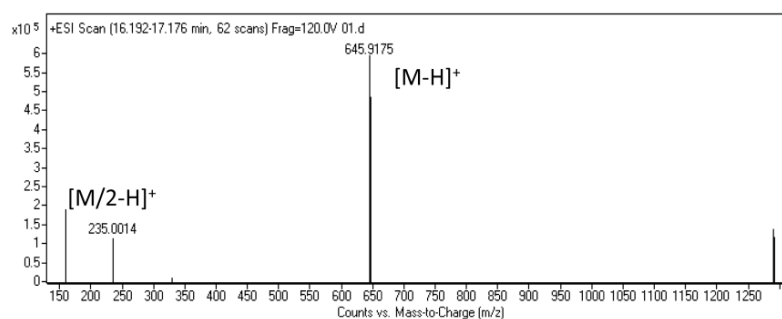
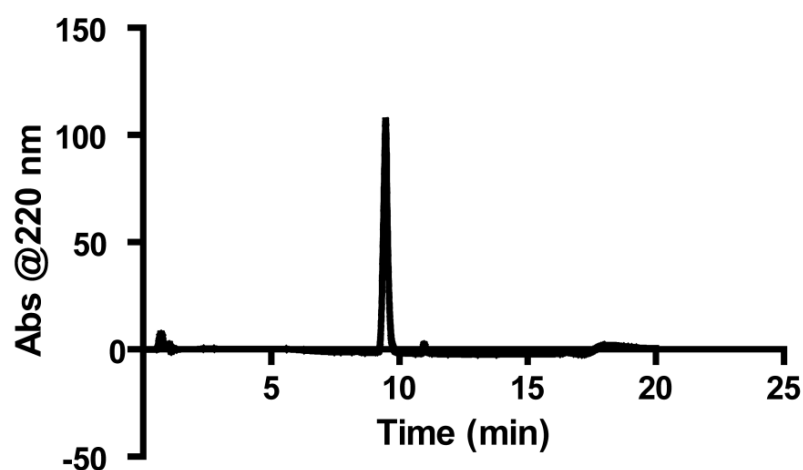


Figure 3.2 Liquid chromatography and mass spectrometry results of the E-PA molecule. Mass spectra of peptides after subtracting mass spectra of water samples. For E-PA $[M-H]^-$ (calculated) = 654.82, $[M-H]^-$ (observed) = 654.40, $[2M-2H]^-$ (calculated) = 1309.64, $[2M-2H]^-$ (observed) = 1309.8064, $[M/2-H]^-$ (calculated) = 326.70, $[M/2-H]^-$ (observed) = 326.70.

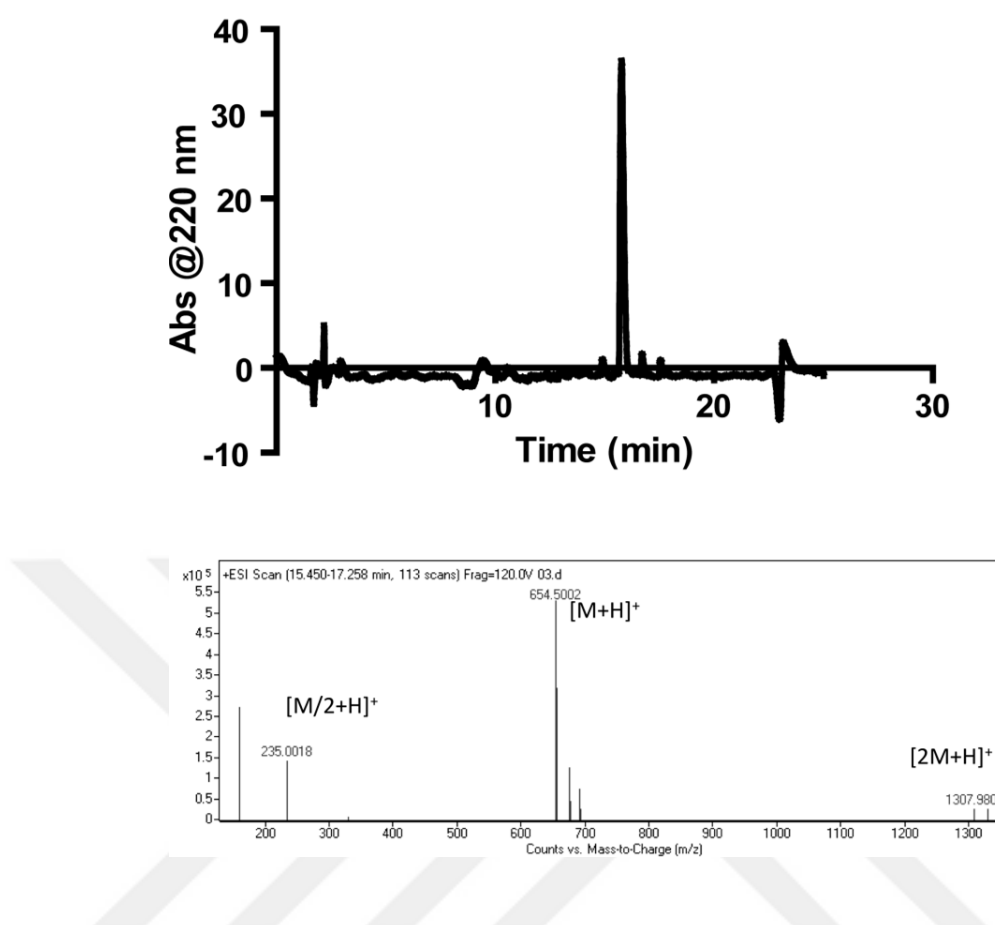


Figure 3.3 Liquid chromatography and mass spectrometry results of the K-PA molecule. Mass spectra of peptides after subtracting mass spectra of water samples. For K-PA $[M+H]^+$ (calculated) = 654.90, $[M+H]^+$ (observed) = 654.50, $[2M+H]^+$ (calculated) = 1308.80, $[2M+H]^+$ (observed) = 1307.98.

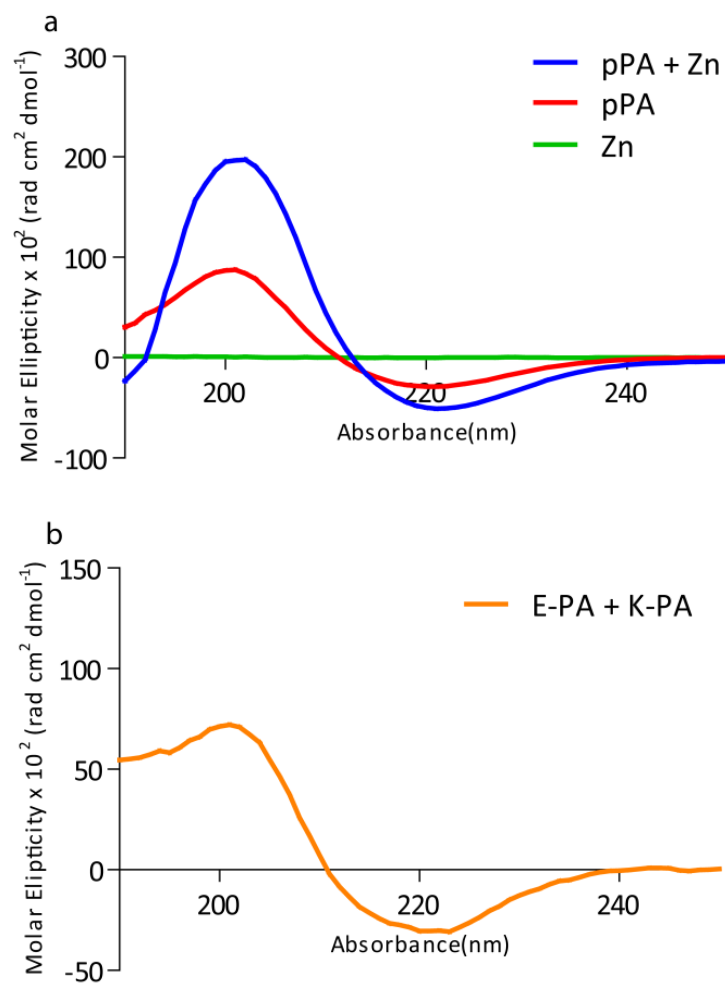


Figure 3.4 Circular dichroism spectroscopy results of pPA. pPA with Zn^{II} addition and only Zn^{II} solution, collected after three readings (3 runs).

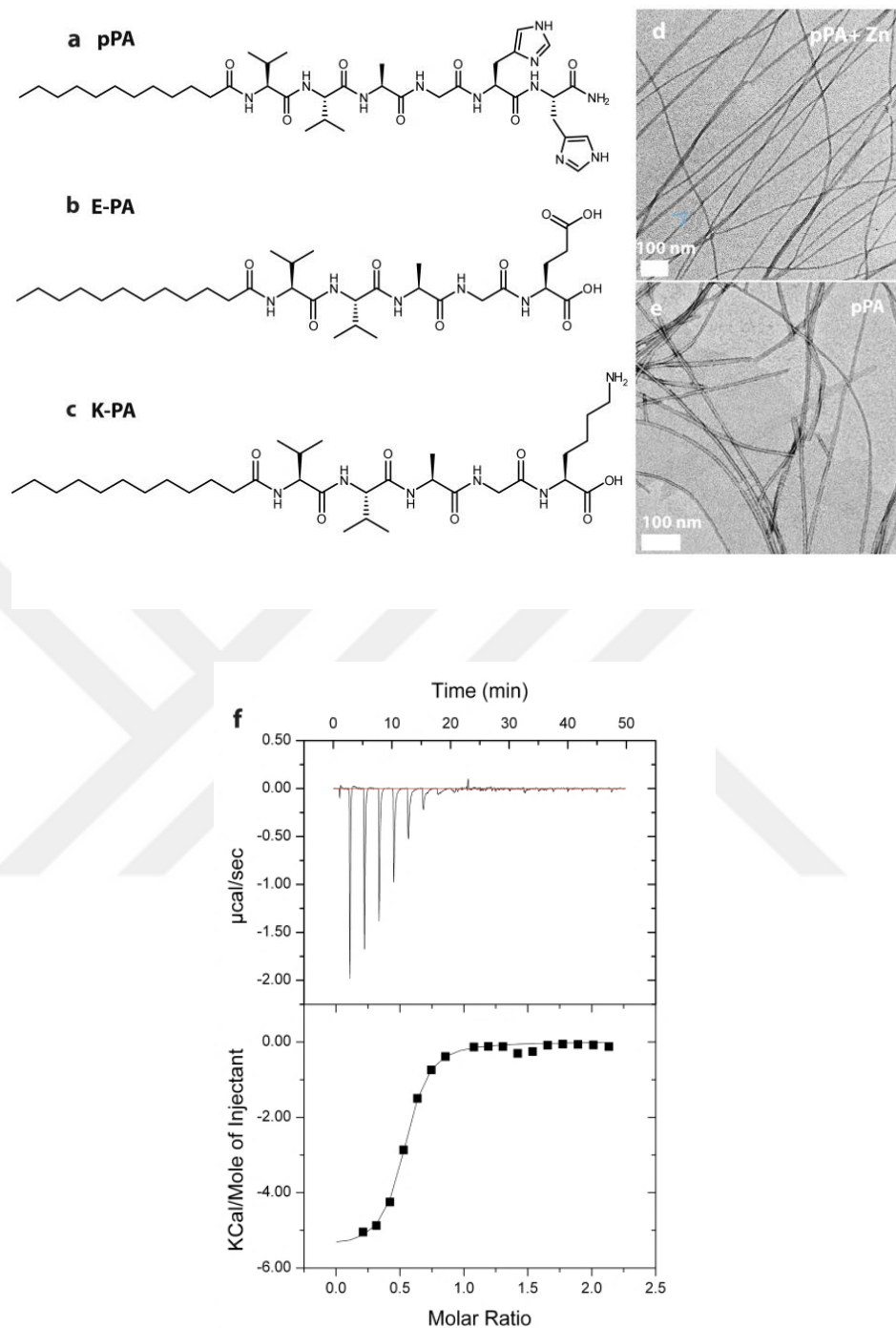


Figure 3.5 Chemical structures and characterization of PAmolecules. a) Chemical structure of the pPA, and control groups b) E-PA, c) K-PA. TEM images of d) pPA, e) pPA + Zn^{II} , f) Isothermal titration thermograms following addition of Zn^{II} to pPA solution.

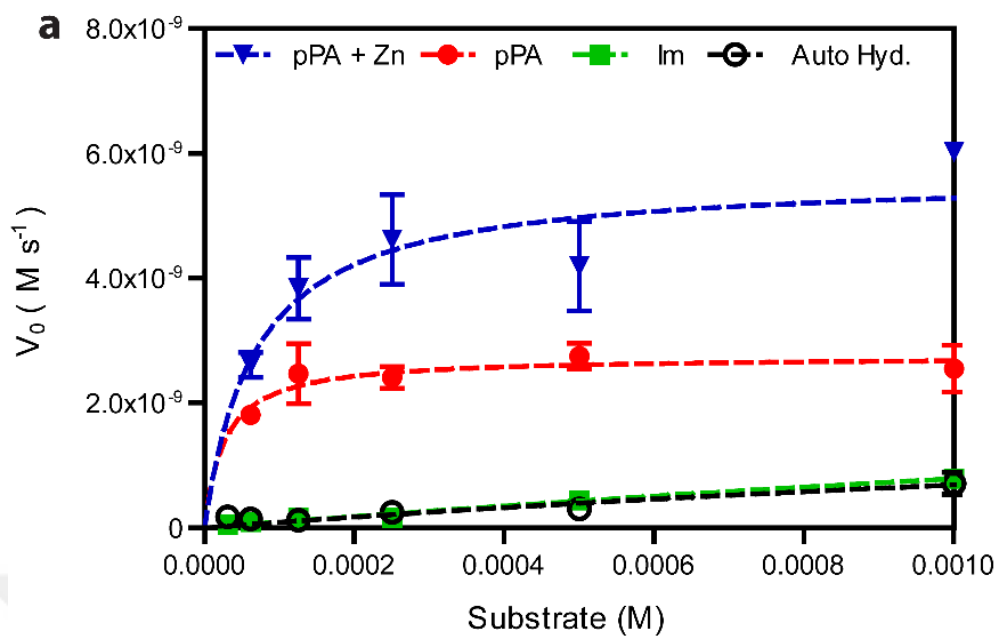


Figure 3.6 Michaelis-Menten fittings of phosphatase activity of peptide nanofibers. Catalytic activity of pPA and control groups in the presence and absence of Zn^{II} . Only imidazole and autohydrolysis control also measured to prove activity of nanostructure.

Table 3. Michaelis-Menten fittings calculations of phosphatase activity of peptide nanofibers at pH 8 in the presence and absence of Zn^{II}.

	$k_{cat} \times 10^{-5} \text{ (s}^{-1}\text{)}$	$K_m \times 10^{-2} \text{ (mM)}$	$k_{cat}/K_m \text{ (M}^{-1} \text{s}^{-1}\text{)}$
pPA	1.83 ± 0.13	2.62 ± 1.55	0.69 ± 0.09
pPA + Zn	3.76 ± 0.40	6.76 ± 2.97	0.56 ± 0.14

	$k_{obs} \times 10^{-7} \text{ (s}^{-1}\text{)}$
Imidazole	7.54 ± 0.36
E-PA\K-PA	6.87 ± 1.31
Auto Hydrolysis	5.75 ± 0.95

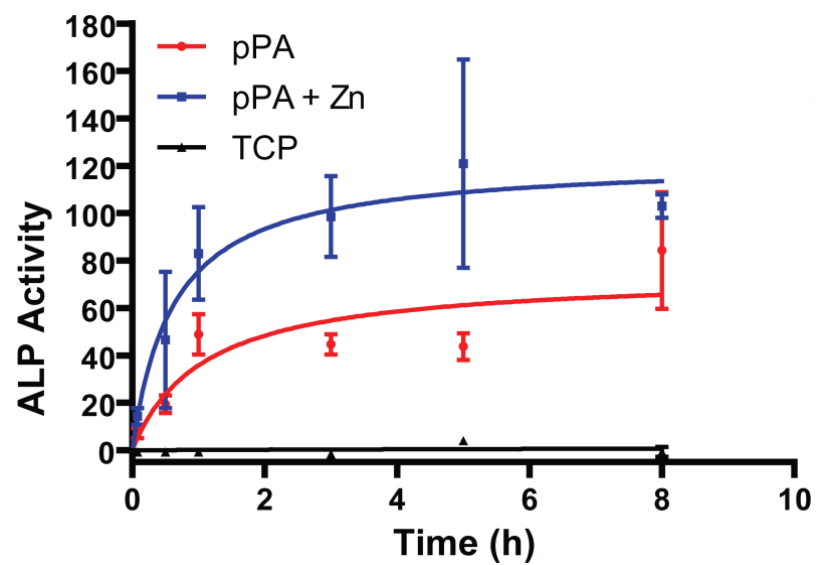


Figure 3.7 pNPA hydrolysis activity of pPA coated surfaces. Released pNP after cleavage monitored by absorbance spectra at 410 nm.

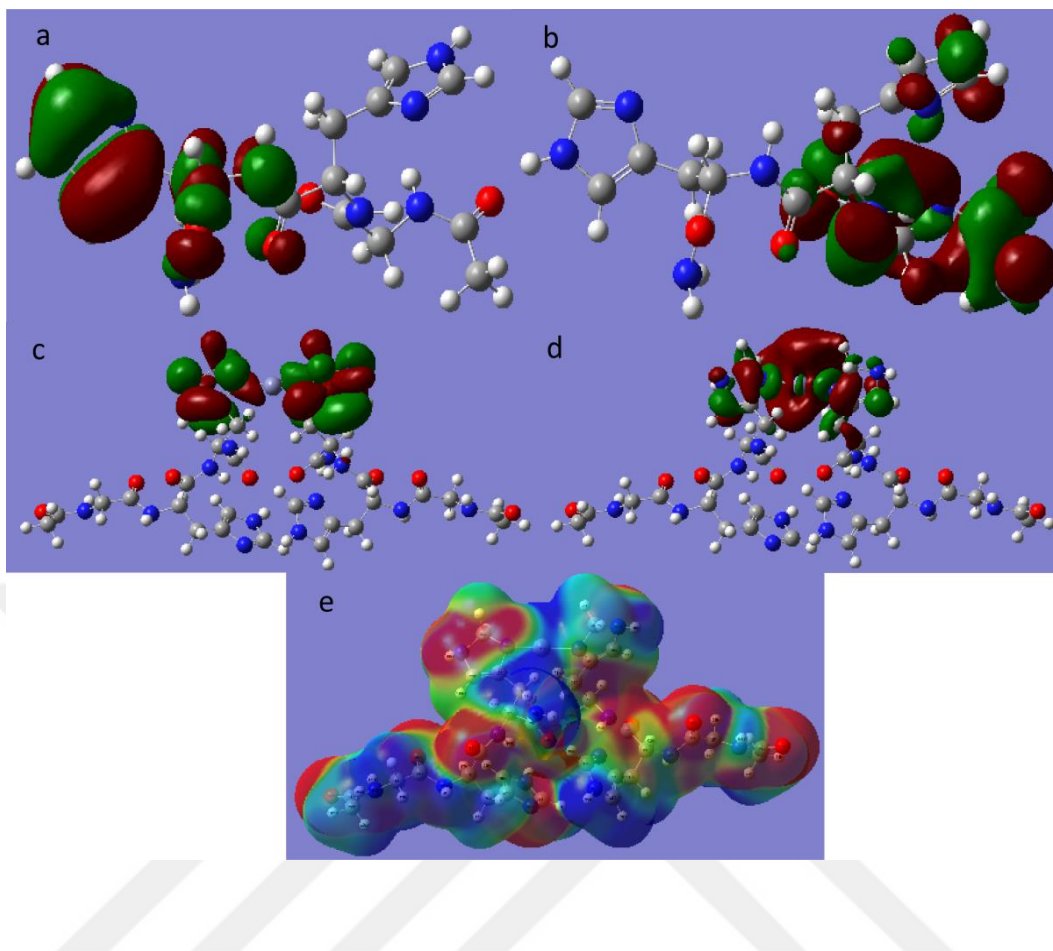


Figure 3.8 HOMO-LUMO active sites of pPAs. HOMO plots of active sites of a) pPA c) pPA + Zn^{II}. LUMO plots of active sites of b) pPA d) pPA + Zn^{II}. ESP plots of active sites of e) pPA + Zn^{II} at the DFT\B3LYP\6-31G (d)\SP level of calculation.

3.3.2 Mineralization on the Catalytic Nanostructures

The ALP enzyme has multiple functions during osteogenesis. It does not only provide a catalytic support for an initial inorganic phosphate (Pi) formation, but also regulates calcifiable bone template construction [12, 13]. In order to mimic ALP activity, it is important to perform both of these functions. In a previous study, native ALP was immobilized on microporous fibrin scaffolds to promote biomineralization and bioactivity on surfaces [79]. Similarly, as a calcifiable bone matrix, we coated surfaces with phosphatase mimicking peptide (pPA) to induce mineral formation. β -sheet containing nanostructures have previously been utilized as templates for CaP mineralization, but for the first time ALP mimicking peptides were used for mineral induction. Thus, β -sheet containing nanofibers formed by self-assembly of pPA molecules with catalytic activity provide the perfect platform to mimic both functions of ALP enzyme. When biomineralization on the ALP-mimetic peptide nanofibers was analyzed, we observed that pPA peptide nanofibers does not only react with substrate but they also serve as suitable templates for CaP crystal formation, similar to natural ALP (Figure 3.9-3.12). Peptide coated surfaces were supplied with calcium and organic phosphate, and CaP deposition was examined by scanning electron microscopy (SEM). Formation of CaP crystals was observable on catalytic pPA nanofibrous surfaces in 24 h, whereas no mineralization was observed on the imidazole free peptide nanofibers (epitope free E-PA and K-PA combination, Figure 3.5), or in the absence of the peptides (Figure 3.9, 3.11). This result confirms the significance of imidazole groups presented on the peptide nanofibers for CaP deposition. Quantification of the calcium content mineralized nodules on the surfaces by Alizarin Red S assay, revealed that calcium mineralization was similar between pPA and pPA

+ Zn^{II} , which were both significantly higher than nanofiber control and uncoated samples on day 1 (Figure 3.9). On the other hand, calcium content on pPA + Zn^{II} surfaces on day 3 was significantly higher than pPA only surfaces (Figure 3.9).

Energy dispersive X-ray (EDX) spectra analysis confirmed the presence of calcium (Ca) and phosphate (P) in the mineralized nodules on the pPA peptide nanofiber-coated surfaces (Figure 3.9, 3.12). X-ray diffraction (XRD) patterns of the CaP crystalline phases on peptide coatings correlated to the miller (hkl) indices (200) and (211) of hydroxyapatite (JSPDS card no 73-0293) (Figure 3.13). Raman spectral mapping was also utilized to visualize the CaP crystal formations, which revealed internal PO_4^{3-} modes. In addition, bands at 1430 and 1465 cm^{-1} are characteristic stretching modes of the CO_3^{2-} groups in hydroxyapatite (Figure 3.14).

When hydrolytic capacity of the bulk PA nanofiber network was assessed by using a nanofiber-coated surface, the bulk system was shown to have better catalytic action due to excess active sites presented on a more concentrated network (Figure 3.7). These results showed that the bulk peptide network allows regulation of free phosphate concentration, providing controlled mineralization in two-dimensional environments. The ALP-mimetic PA nanofibers also serve as catalytic-nucleation surfaces along with the catalytic centers. These multifunctional nanofibers resulted in eventual saturation of the surface and thereby limiting Pi hydrolysis and CaP formation. Accumulation of CaP crystals on the surface hinders the active sites present within the peptide network and gradually limits hydrolytic activity in a manner similar to the gradual decrease in activity observed in the natural enzyme. Catalysis trend acquired from our bulk fiber model is similar to the results associated with soluble enzyme like fiber system.

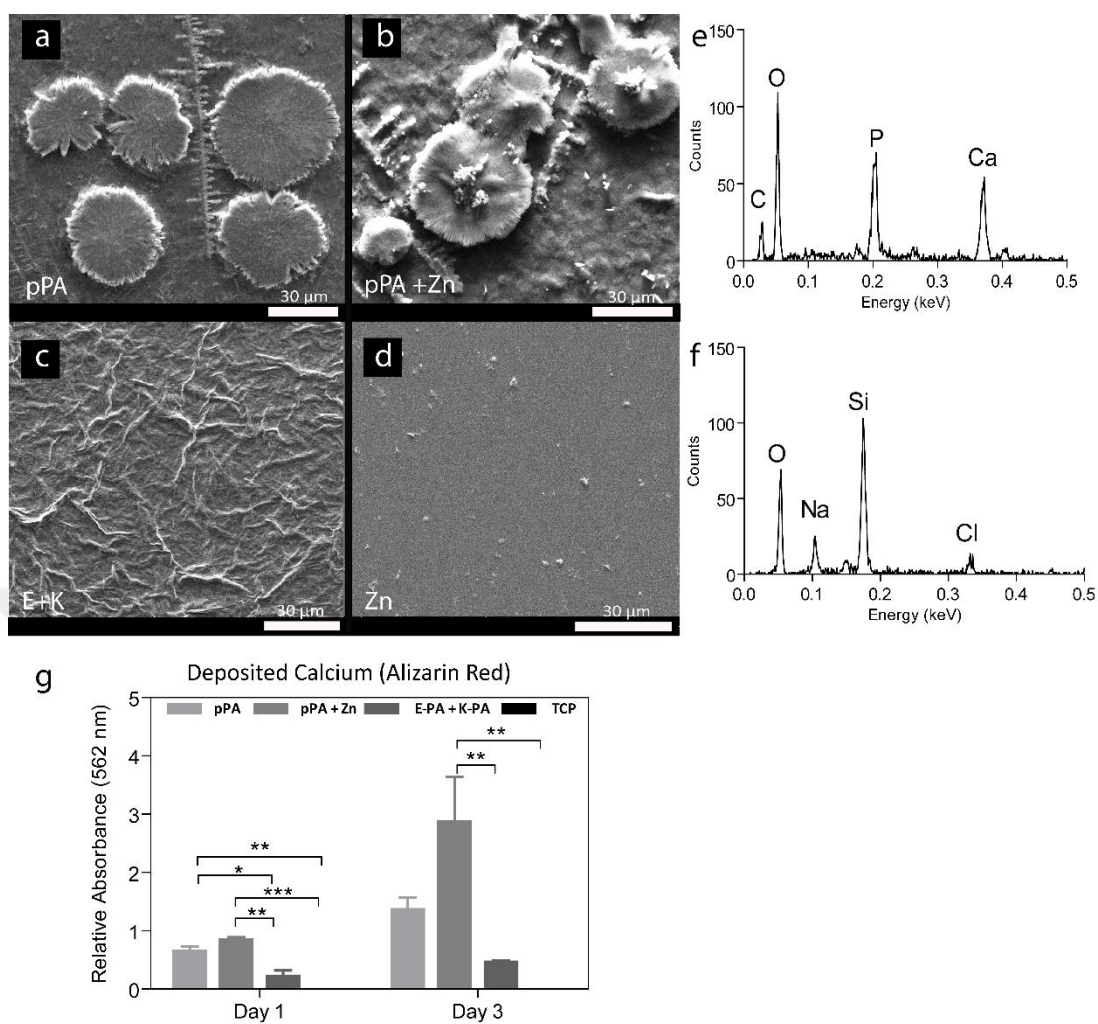


Figure 3.9 CaP deposition on pPA and E-PA + K-PA coated surfaces. SEM images of the CaP nodules at day 1 on a) pPA b) pPA + Zn^{II} c) E-PA + K-PA and d) Zn^{II} coated surfaces. EDX analysis of mineral content on e) pPA + Zn^{II} and for f) Zn^{II} surfaces. g) Alizarin Red S of surfaces at Day 1 for quantification of deposited Ca⁺² on coatings. Statistical significance was determined by Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.0001, n $\geq$ 3 assays).

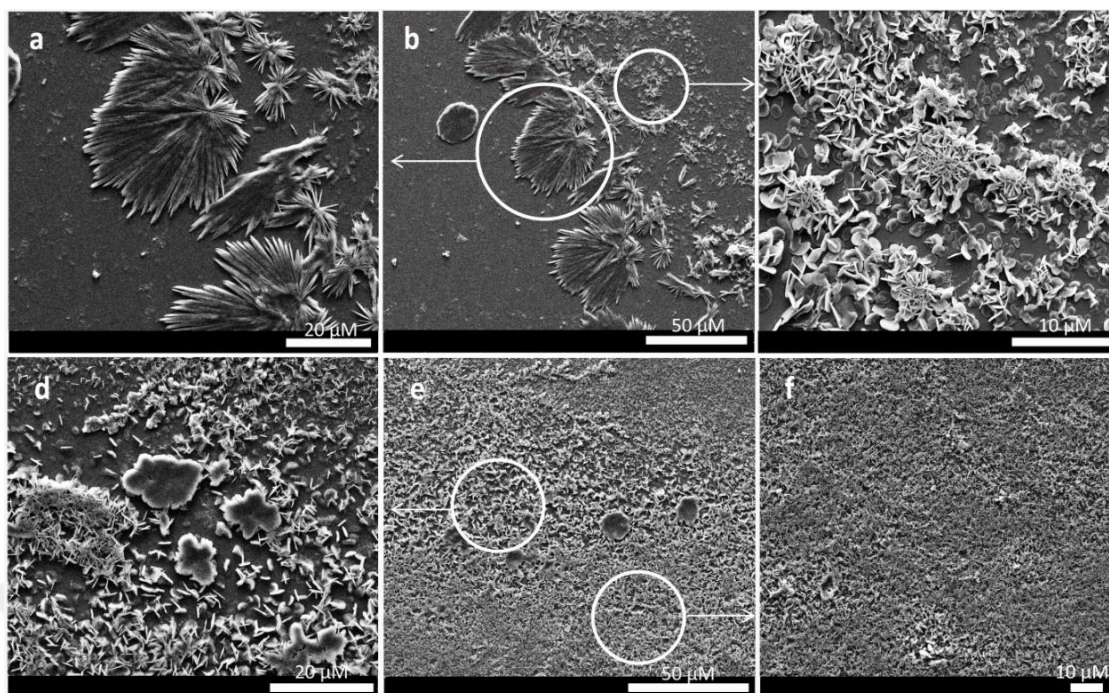


Figure 3.10 SEM images of mineralization on ALP-like peptides. Mineral deposition on a, b, c) pPA; d, e, f) pPA + Zn^{II} surfaces at day 3, monitored by SEM.

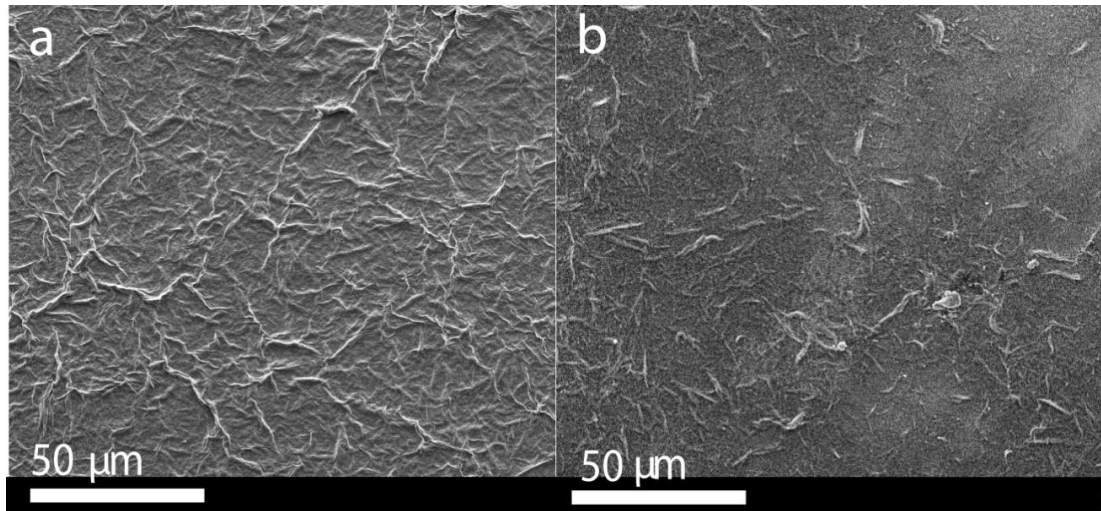


Figure 3.11 SEM images of CaP deposition on E-PA + K-PA coated surface. Only peptide surface was observed at a) day 3, b) day 6, control groups did not induced mineral formation.

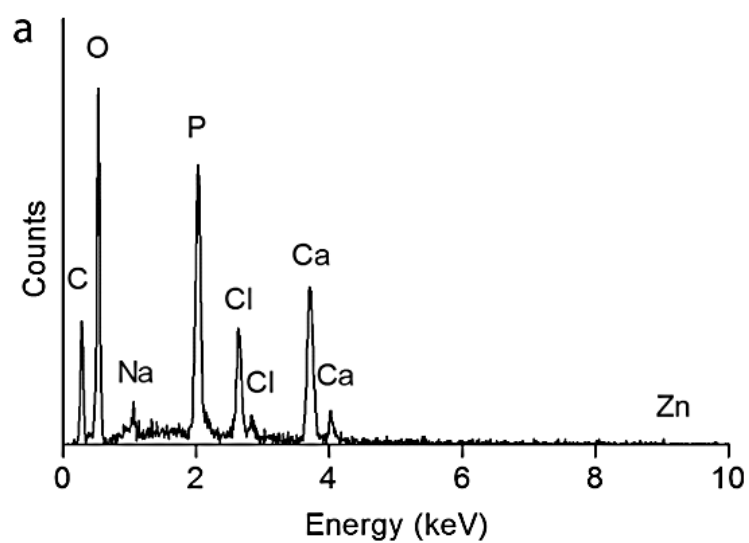


Figure 3.12 EDX spectra of CaP nodules on pPA surface. Elemental characterizations of mineral nodules were analyzed with EDX spectra and CaP deposition was confirmed by this method.

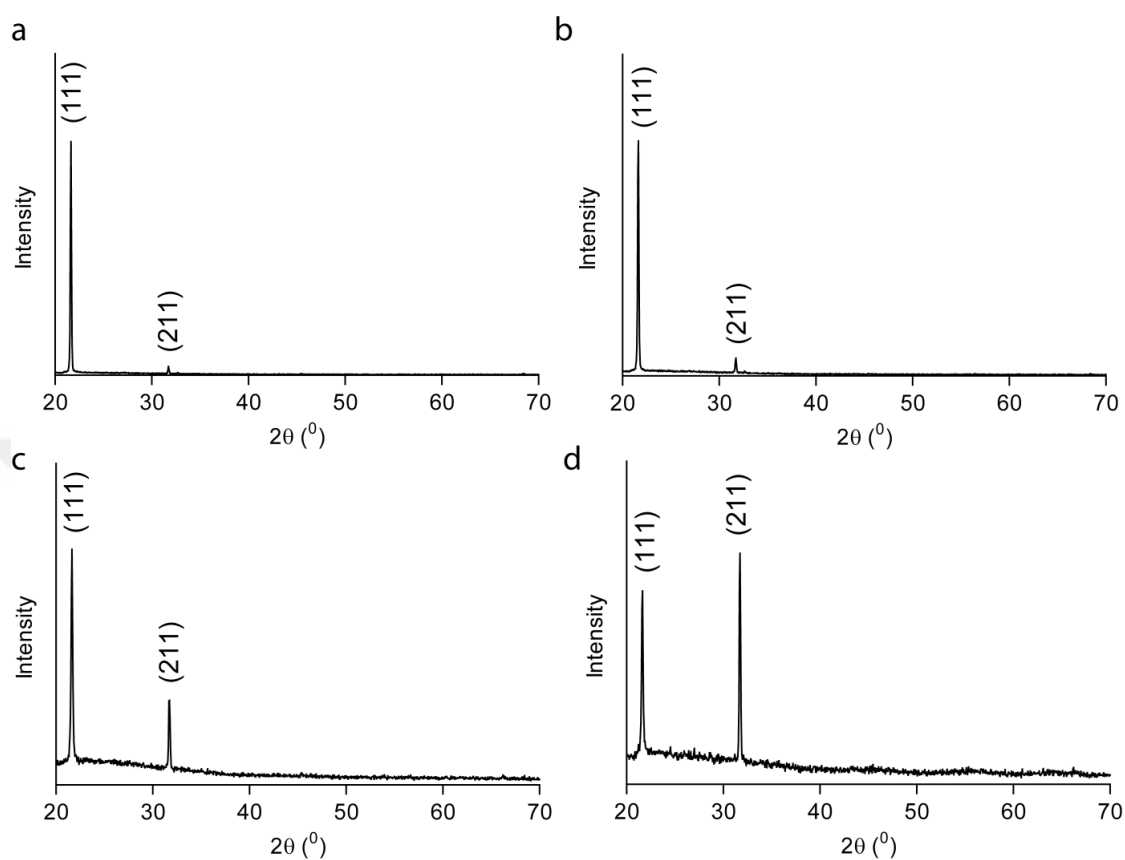


Figure 3.13 XRD patterns of ALP-like peptides a) pPA, b) pPA with Zn^{II} at day 1 and c) pPA and d) pPA with Zn^{II} at day 3.

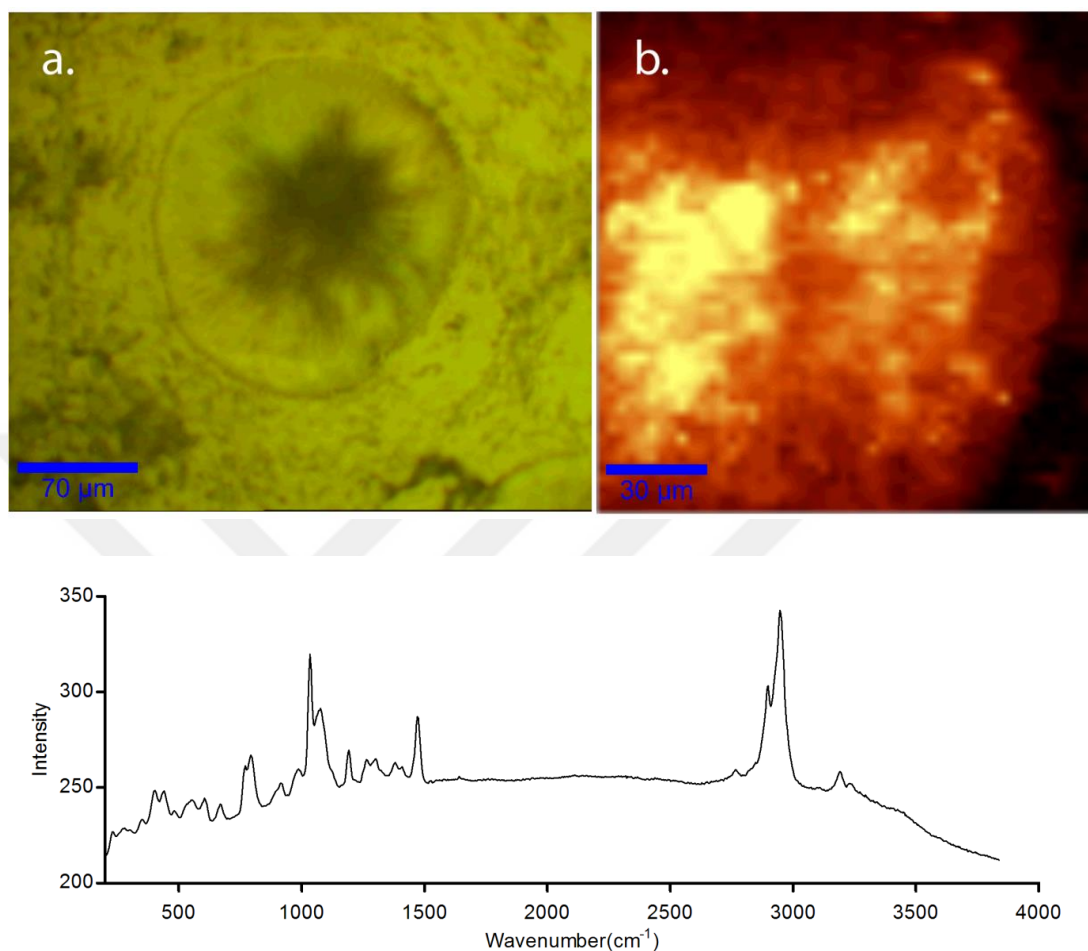


Figure 3.14 Confocal Raman images of calcium phosphate crystals on pPA + Zn^{II} coated surfaces a) Optical (20X) and b) Average Raman spectrum of calcium phosphate crystals formed on the 150 x 150 μm² surface of pPA + Zn^{II}. ν_2 = Doubly degenerate bending mode of the PO₄ group (O-P-O bond) (403,442,484 cm⁻¹) ν_3 = Triply degenerate asymmetric stretching mode of the PO₄ group (P-O bond) (1035,1076 cm⁻¹) ν_4 = Triply degenerate bending mode of the PO₄ group (O-P-O bond) (607 cm⁻¹).

3.3.3. Differentiation of Osteoprogenitor Cells on Catalytic Peptide Nanostructures

Formation of bone tissue is an elaborate process since it contains multiple levels of hierarchical organization. Although there have been studies that utilized different extracellular matrix (ECM) mimicking peptide designs for bone regeneration, these studies focus on mimicking the specific signals or physical properties of ECM [87-90]. However, mimicry of enzymatic components of the bone formation is also a crucial requirement in order to develop a suitable template with multiple functionalities to simulate bone mineralization properly.

ALP-inspired supramolecular nanostructures enhanced biomineralization and osteodifferentiation of osteoprogenitor SaOS-2 cells, while supporting their viability (Figure 3.15). We performed Alizarin Red S staining which chelates Ca^{2+} , and is a commonly used method to evaluate mineralization to determine the mineral deposition ability of osteoblast-like cells. After as early as 3 and 6 days of culturing on the Zn^{II} containing ALP mimetic peptide nanofiber coated surfaces, cells were shown to form bone-like nodules. Both microscopic images and quantitative analysis by cetylpyridinium chloride extraction of calcium, showed that calcium deposition by SaOS-2 cells was significantly enhanced compared to cells on tissue culture plate (TCP), only zinc supplemented surfaces, and peptide nanofibers without histidine residues (E-PA and K-PA), as evidenced by Alizarin Red S staining for both osteogenic and normal culture media (Figure 3.16, 3.17). Osteogenic media (OM) was composed of 10 mM β -glycerophosphate, 0.2 mM ascorbic acid and 100 nM dexamethasone while normal media is similar to maintenance medium with only 10 mM β -glycerophosphate addition. In bone regeneration experiments phosphate source is commonly used and is a necessary factor to direct cells into osteogenic

differentiation. Strikingly, these experiments were performed in normal media without osteogenic supplements, and inclusion of an organic phosphate source was sufficient for bone nodule formation (Figure 3.16, 3.17). To further investigate the osteogenic differentiation, gene expression profiles of osteogenic differentiation markers were analyzed by qRT-PCR. Statistically significant enhancement in expression of both Runt-related transcription factor-2 (RUNX2), an early osteogenic marker, and collagen type I (Col I) on ALP mimetic peptide nanofiber coated surfaces compared to control groups on both days 3 and 6 of culture were shown even in the absence of osteogenic culture medium (Figure 3.17). RUNX2 is an essential transcription factor that regulates expression of specific genes such as collagen I (COL I), osteopontin (OP) and bone sialoprotein (BSP), which are important for bone development [91-93]. Therefore, early upregulation of RUNX2 expression in the early stages is an indicator that the progenitor cells are undergoing or will undergo differentiation [94]. In parallel to Alizarin Red S staining results, cells cultured on the pPA nanofibers exhibited significantly higher expression of RUNX2 during 6 days of incubation compared to control groups both with and without Zn^{II} . Strikingly, the expression of late osteogenic differentiation marker, COL-I, was also up regulated in cells cultured on pPA nanofibers on both day 3 and day 6, whereas osteogenic medium supplemented control group on TCP only started to activate gene expression on day 6, similar to previous studies [43, 95]. Collagen I is the main scaffold of bone matrix, and expression of this protein is regulated by ALP during osteoblast formation and differentiation. These results show that the pPA nanostructures accelerate commitment of osteoblast-like cells into osteogenic lineage by inducing expression of osteogenic genes without the

need of osteogenic medium as evidenced by both gene expression profiles and Alizarin Red S staining.

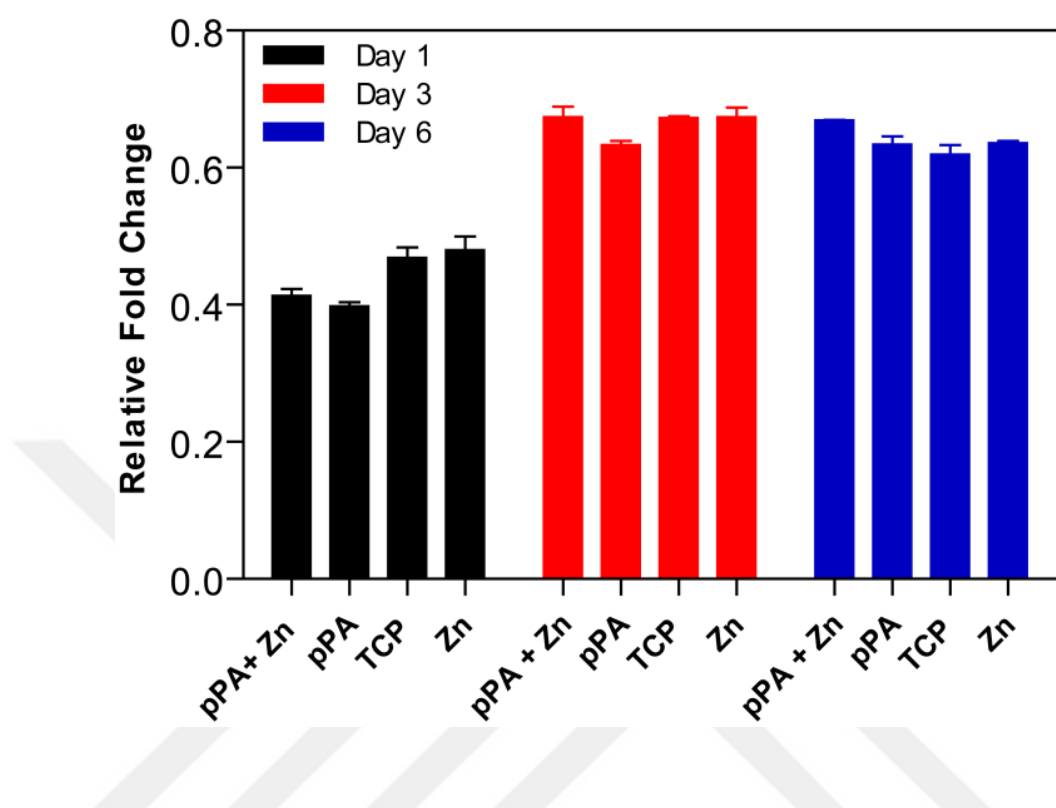


Figure 3.15 Alamar blue assay for analyzing viability of SaOS-2 cells on pPA coating. Biocompatibility of peptides and metal complexes was analyzed with Alamar blue assay (Mean $\pm$ SEM, n = 3).

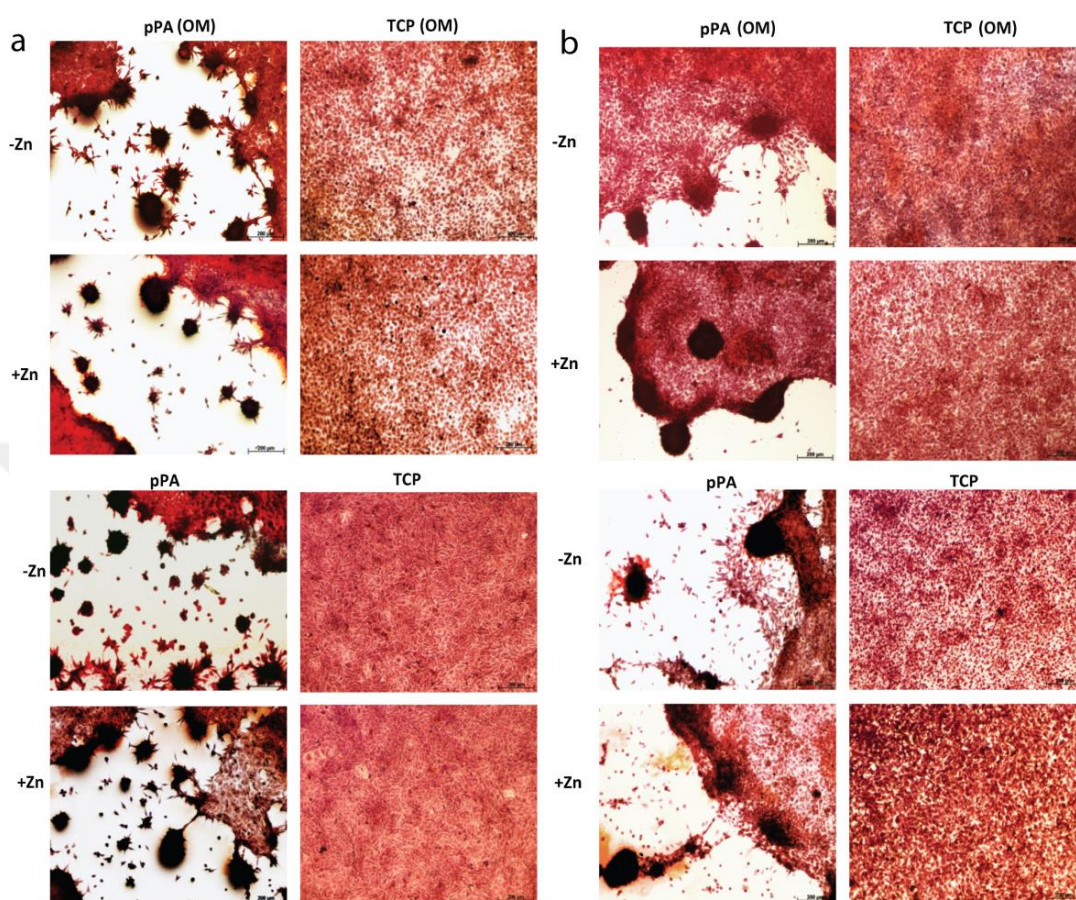


Figure 3.16 Microscopic images of Alizarin Red S staining results of SaOS-2 cells. Deposited minerals were stained and imaged at a) day 3 and b) day 6, with (OM) and without osteogenic medium in the presence and absence of 25 μM Zn^{II} .

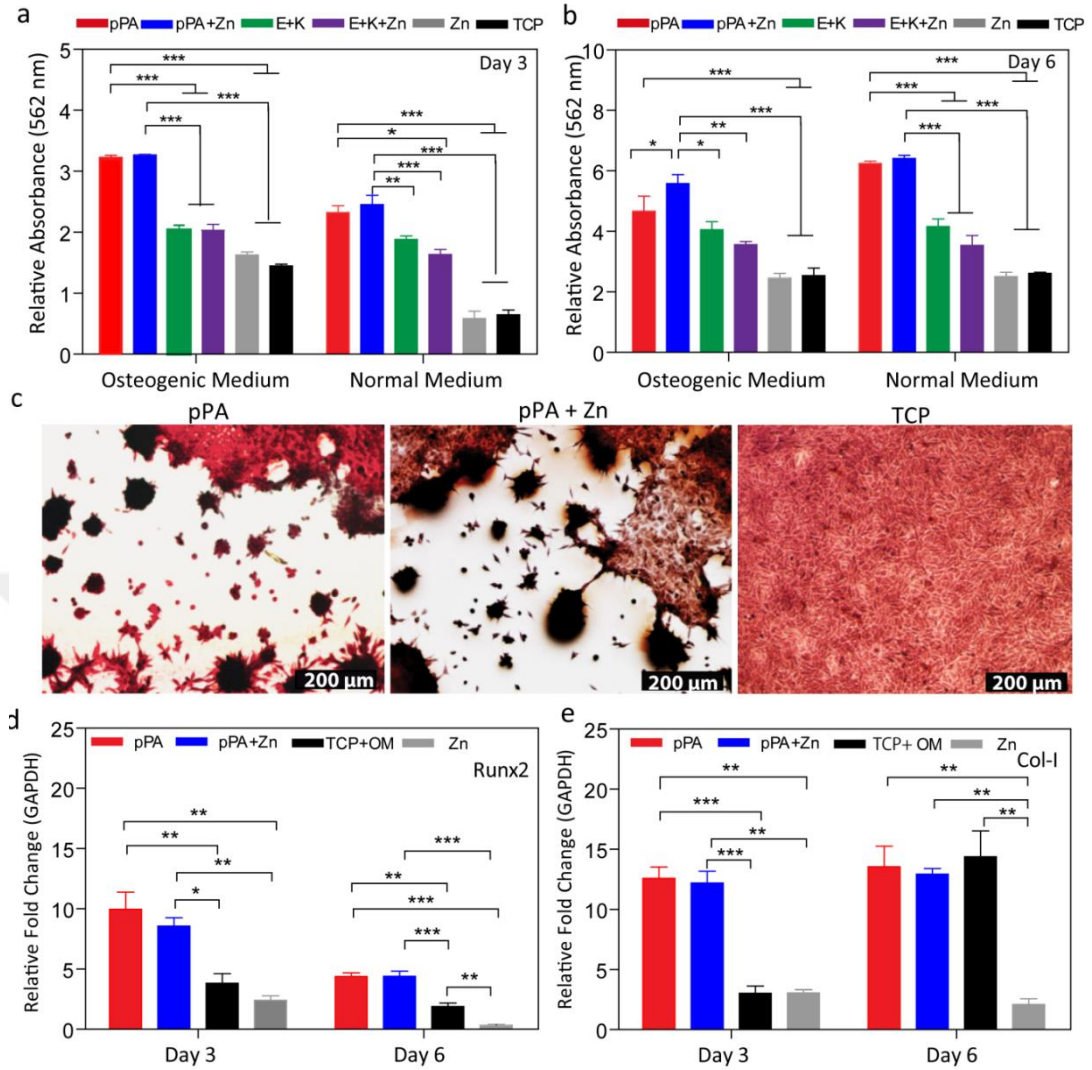


Figure 3.17 Quantitative analysis of Alizarin Red S staining and gene expression of SaOS-2 cells. Alizarin Red S staining at a) day 3, b) day 6 in the presence and absence of osteogenic medium with 25 μM Zn^{II} . c) Optical images of day 6 samples in osteogenic medium with 25 μM Zn^{II} , after Alizarin Red S staining. Gene expression analysis of d) RUNX2, e) COL-I at both day 3 and day 6. Expressions were normalized to GAPDH and TCP + β -gly group. (E+K means E-PA mixed with K-PA, , TCP+OM indicates osteogenic medium added sample) Mean $\pm$ SEM, n=4 coatings per sample, (ANOVA) *P < 0.05, **P < 0.01, ***P < 0.0001.

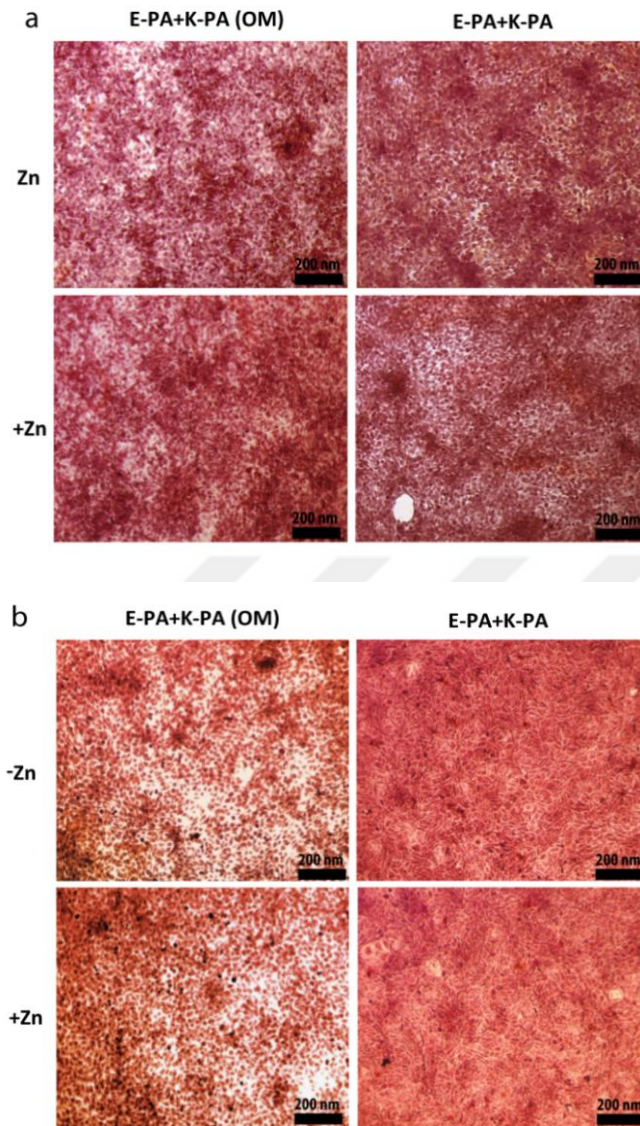


Figure 3.18 Alizarin Red S staining results of cell on control groups E-PA and K-PA. Alizarin Red S staining microscopy images at a) day 3, b) day 6, with (OM) and without osteogenic medium.

3.3.4. Osteogenic Differentiation of Mesenchymal Stem Cells on Catalytic Peptide Nanostructures

The effect of the pPA nanostructures on the osteogenic differentiation of the mesenchymal stem cells (MSCs) were also studied because stem cells are better candidates for regenerative therapy for successful reconstruction of bone tissue and osteogenesis. However, multi-lineage capacity of MSCs makes their use more challenging compared to osteoprogenitor cells. Osteogenic medium has crucial content for osteogenic differentiation of MSCs and for preventing differentiation into other cell fates. Similar to SaOS-2 results, the ALP-mimetic peptide nanostructures with and without Zn^{II} were found to be biocompatible and induced biomineralization of MSCs significantly compared to bare surface as evidenced by Alizarin Red S staining (Figure 3.20, 3.21). In this part of study, activity of pPA nanofibers was verified by only comparison to TCP, for simplicity and ease of analysis. Active site free control group was not included since its effect have already been investigated with osteoprogenitor cells and was not found to be not much enhancing osteogenic differentiation significantly. Here, it was shown that, the pPA nanofibers accelerate differentiation with only β -gly supplemented medium as effectively as with osteogenic medium. It is a significant improvement, because compared to previous reports, differentiation of rMSCs to osteoblasts was shown only after long incubation periods (after two weeks) and in the presence of osteogenic supplements [96]. On the other hand, the pPA remarkably initiated differentiation within three days without any osteogenic support and with significant mineral deposition on day 12 (Figure 3.20).

Expression levels of osteoblast specific genes (Runx2, Col-I and Opn) were also studied, where osteopontin (Opn) was also analyzed as a late osteoblast marker to

verify stem cell differentiation (Figure 3.22). On day 7, statistically significant increase in the expression levels of Runx2, Col-I and Opn were observed in rMSC samples cultured on ALP mimetic peptide nanofiber scaffolds compared to control groups both with and without addition of osteogenic supplements. Osteogenic medium (OM) containing samples exhibited earlier differentiation for rMSCs as shown in Figure 3.22, and all differentiation factors were upregulated with OM at day 7. Non osteogenic medium supplied samples showed lower expression levels compared to OM supported cells, however, cells cultured on ALP mimetic peptide nanofibers showed higher expression compared to control groups. These results indicate that absence of osteogenic medium cause differentiation delay but still peptide itself is still enough to lead stem cells into osteogenic fate. In addition, at day 7, metal addition upregulated Runx2 expression, the fundamental factor for differentiation process, for both OM and normal medium samples. Initial studies showed that zinc addition could increase native ALP specific activity [97] in addition to pPA activity which was shown to be enhanced by Zn^{II} , which might be the reason of initial upregulation of differentiation factor.

In addition to gene expression analysis, differentiation of rMSCs into osteogenic lineage was also analyzed with immunocytochemistry staining of Dentin matrix protein-1 (DMP-1) (Figure 3.22). DMP-1 is a non-collagenous acidic protein present in the mineralized matrix and has a dual functional role as a transcription factor, a matrix protein and important factor [98] to detect maturation level of mineralization, since it can be found in nucleus, cytoplasm and ECM depending on level of differentiation. DMP-1 is mainly localized in the nucleus of undifferentiated osteoblasts, however, mineralization and release of intracellular calcium stimulates its

export to the out of matrix during maturation [98-102]. This property of DMP-1 compared to other differentiation markers, helps us to understand and detect the stage of osteogenic differentiation. Therefore, we carried out protein level DMP-1 analysis and results of DMP-1 protein localization showed that rMSCs undergo faster differentiation and maturation when exposed to pPA system because DMP-1 staining was observed more on the periphery of the cells (mainly in the matrix) on pPAs. Since degree of matrix mineralization is important for differentiation and maturation of differentiated cells, differences in the differentiation stage of committed rMSCs cultured on pPA system might be caused by efficient and rapid catalytic behavior of pPA mineralization. Cells that were cultured on uncoated TPCs showed no staining on day 7, which indicates that the ALP-mimetic peptide nanofibers induce commitment of cells into osteogenic lineage much faster than control system.

Besides efficient activity of phosphatase like nanofibers, according to *in vitro* findings we did not observe a remarkable difference among groups pPA and pPA with Zn^{II} ion. Since cell culture media contains different ions it might have been hard to observe one-fold efficiency difference between groups. For example, when we carried out the same experiment without cells and cell culture media, we observed difference between groups in terms of calcium deposition at day 3.

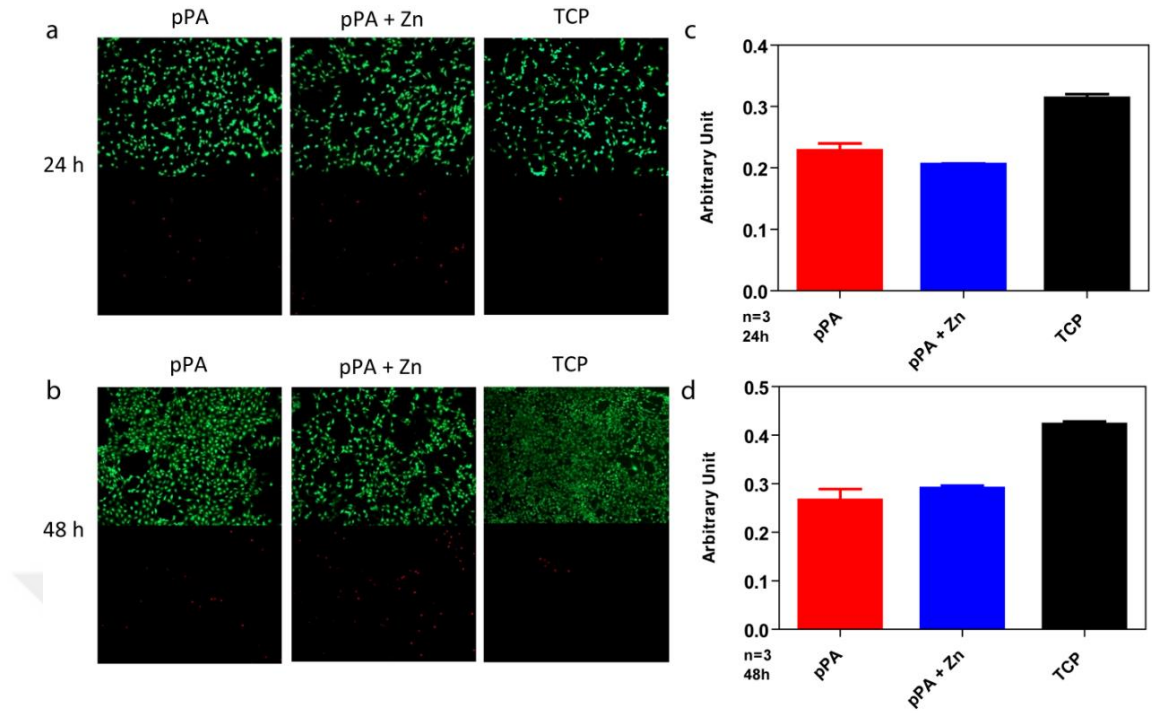


Figure 3.19 Viability analysis of rMSCs cultured on pPA coatings and tissue culture plate. a, b) Representative Calcein Am (Live=green) and ethidium homodimer (Red=dead) staining and c, d) Alamar Blue assay results (Mean $\pm$ SEM, n = 3).

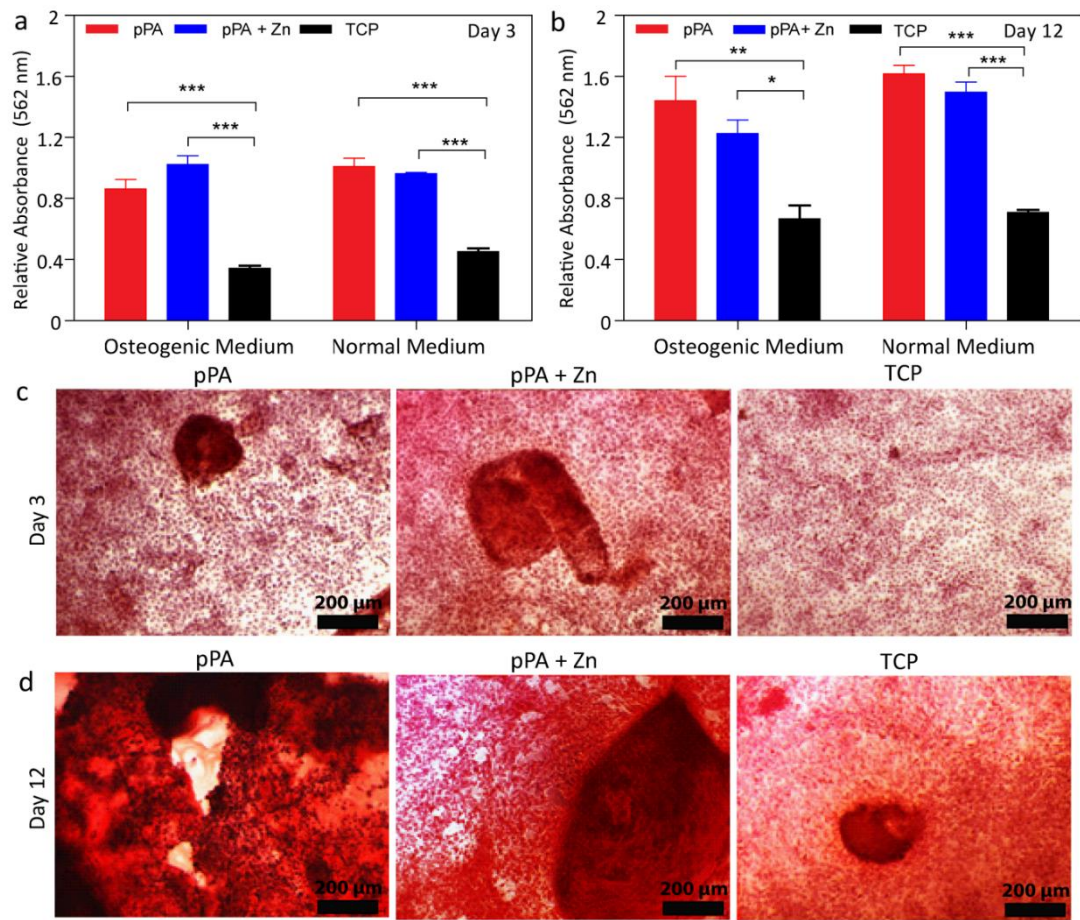


Figure 3.20 Quantitative and qualitative analysis of Alizarin Red S staining of rMSCs. Quantitative analysis of Alizarin Red S at a) day 3 b) day 12. Optical microscopy images of stained cellular CaP deposition in the absence of osteogenic supplement at c) day 3 and d) day 12, demonstrated by Alizarin Red S staining. (Mean $\pm$ SEM, n=4 coatings per sample, one-way analysis of variance (ANOVA) *P < 0.05, **P < 0.01, ***P < 0.0001).

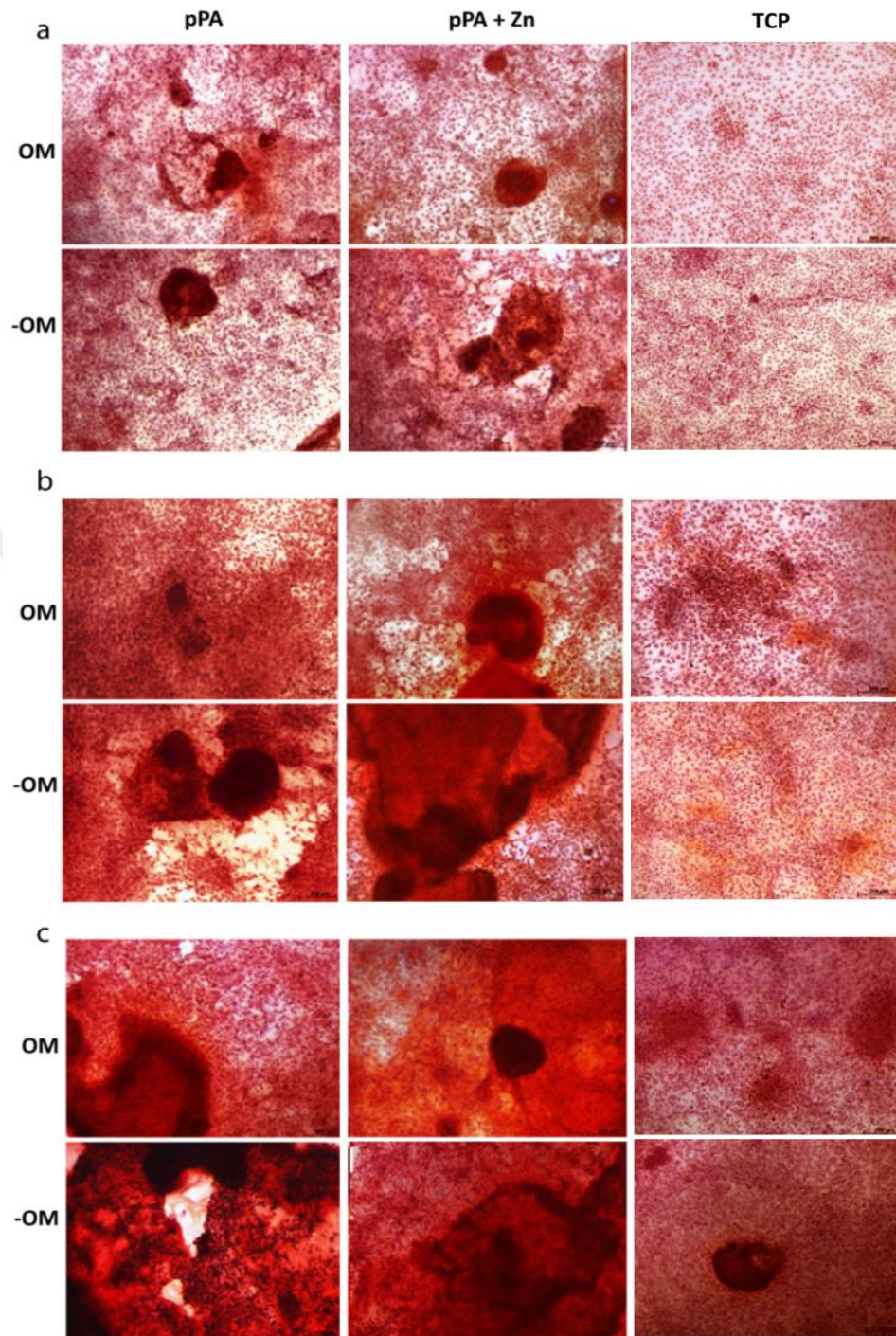


Figure 3.21 Microscopic images of Alizarin Red S staining results of rMSCs. Microscopy images of stained calcium deposition at a) day 3, b) day 7 and c) day 12, with (OM) and without (-OM) osteogenic medium and in the presence and absence of $25 \mu\text{M Zn}^{\text{II}}$.

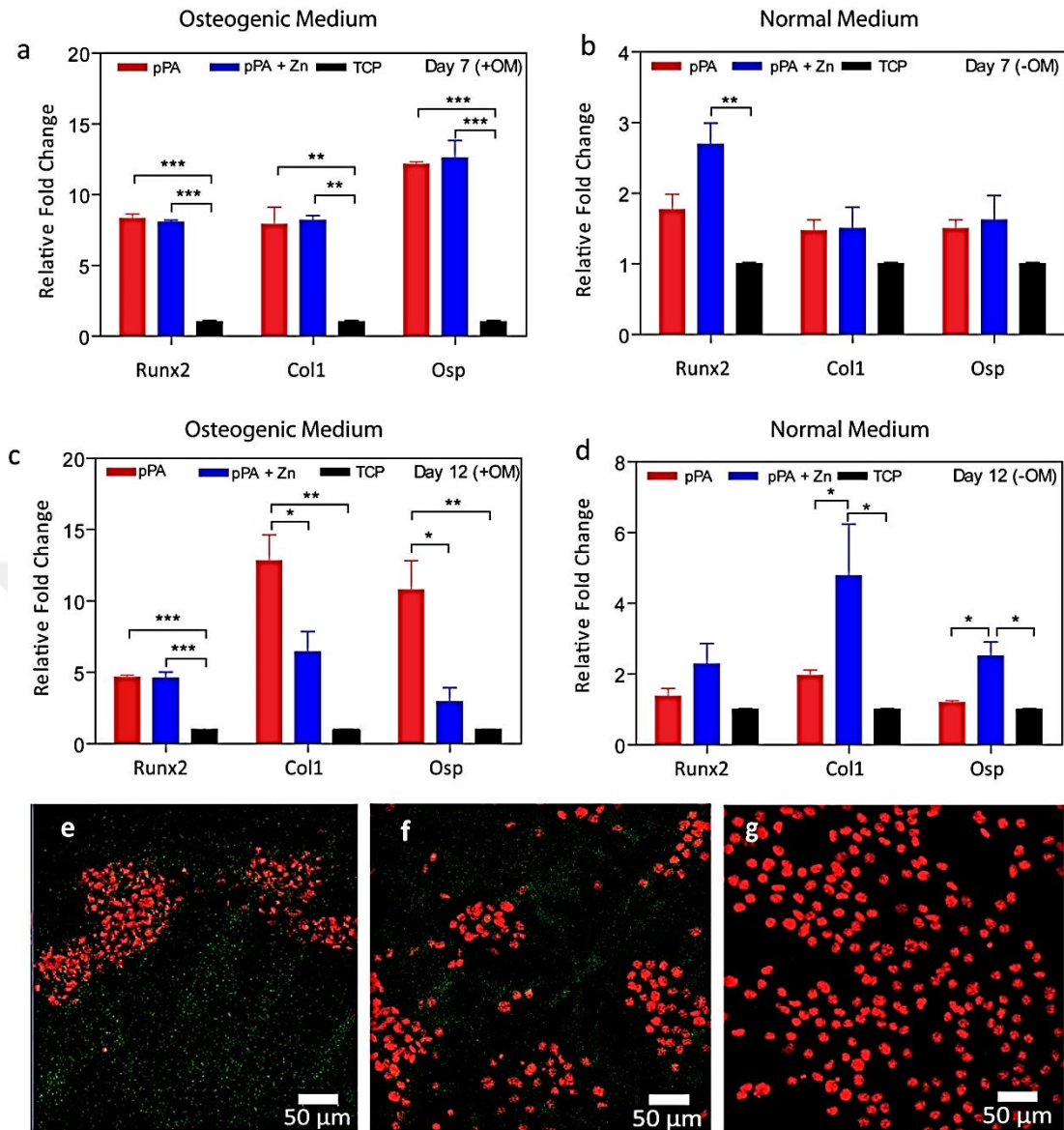


Figure 3.22 Gene expression analysis of Runx2, Col1 and Osp of rMSC with qRT-PCR. Osteogenic marker analysis of seeded cells on pPAs at a) day 7 with OM, b) day 7 without OM (-OM), Expressions were normalized to GAPDH and nonosteogenic medium containing TCP control group. Confocal images of DMP-1 staining at day 7 c) pPA, d) pPA + Zn^{II}, e) bare surface. (Scale bar 50 μm) (Red fluorescence belongs to TO-PRO nucleus staining and green emission is DMP-1 staining).

3.3.5. Osteogenic Differentiation in 3D Culture Model

3D culture elicits a more similar state to physiological conditions compared to 2D culture. As a proof of concept, in order to better mimic physiological conditions, cells were also encapsulated within the pPA gel to obtain a 3D culture model instead of seeding on thin gels. The pPA system offers a facile way to create 3D cell culture, since pPA solution turns into gel only with addition of culture media containing cells at physiological pH. Through this method, cells are homogeneously distributed in the gel system by diffusion and this encapsulation does not interrupt cell to cell interaction or diffusion of soluble factors. Viability of the cells in the pPA gels were tested in 3D cultures through 14 days and pPA culturing method significantly supported viability both in short and long term. Similar to 2D culture, osteogenic differentiation of SaOS-2 cells was also enhanced within 3D method (Figure 3.23, 3.24). SaOS-2 was used in this study as a model cell line since they are easier to handle. As a control gel environment E-PA and K-PA group were used, TCP control is not a proper control for 3D study. Our investigations again showed that phosphatase peptide is more efficient in inducing osteogenic differentiation than control groups. Another interesting finding of this study was the observation of slightly better equilibrium storage modulus of cell embedded gels compared to only gel counterparts. This result implies that cell inclusion supports gel flexibility perhaps through secretion of extracellular matrix components by encapsulated cells (Figure 3.24) since 6 days is enough for secretion of native extracellular components. Overall, 3D cell culture studies demonstrated that ALP like pPA nanofiber system has potential as a catalytic, mineralizing and versatile bone graft, which can also be used for growth factor encapsulation and diffusion for further purposes.

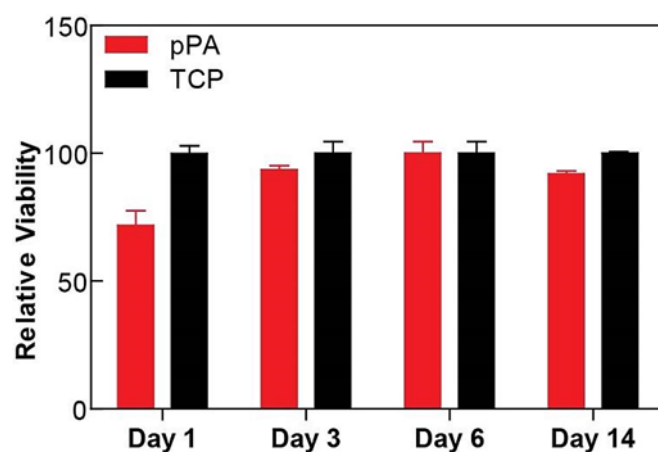


Figure 3.23 Alamar blue assay for analyzing viability of 3D cell culture of SaOS-2 cells. Viability of cells encapsulated in peptide gel was investigated at different time point for understanding of short and long term viability of cells in 3D environment (Mean $\pm$ SEM, n = 3). Data normalized to TCP.

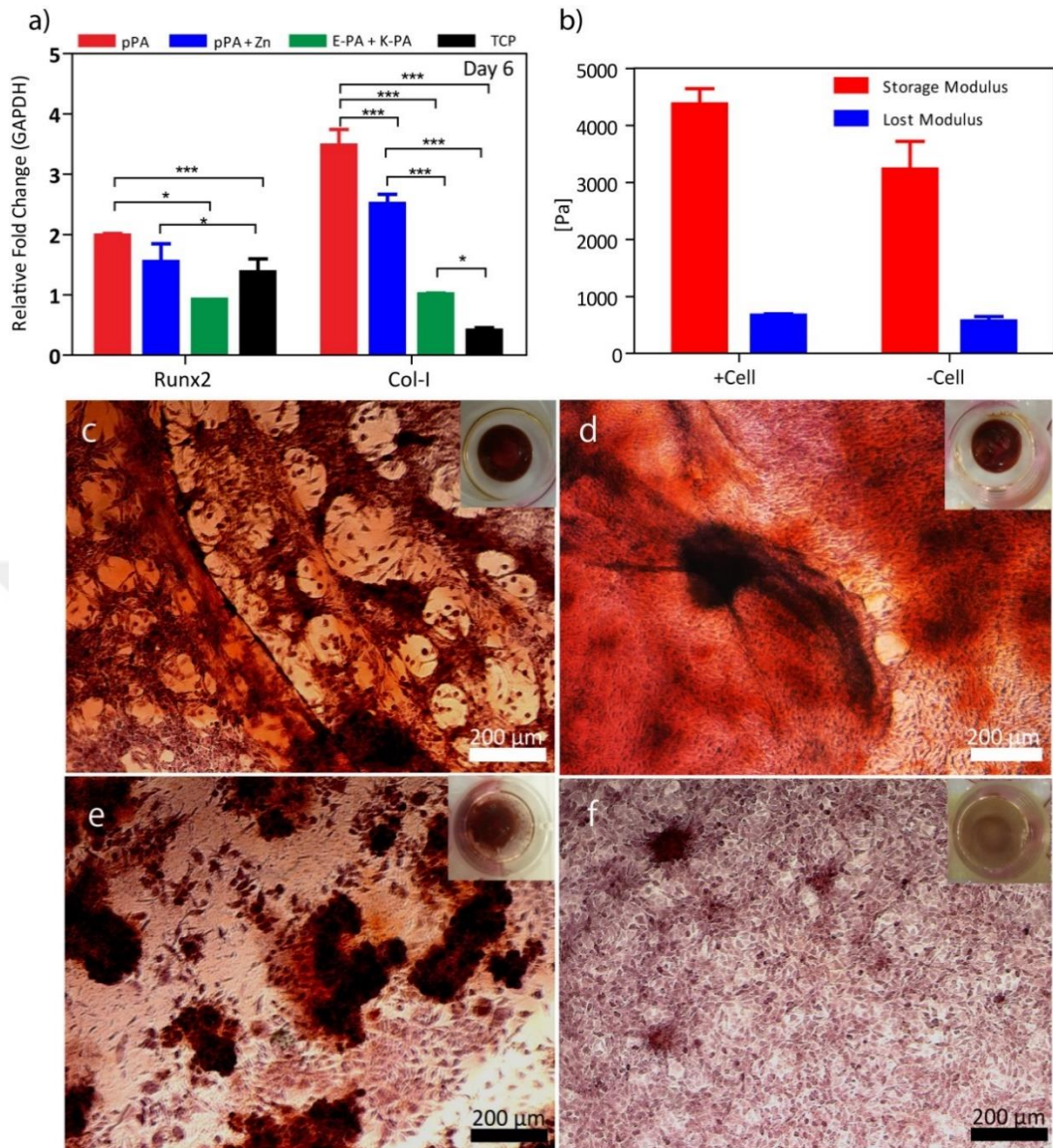


Figure 3.24 3D study gene expression analysis of Runx2 and ColI with qRT-PCR. Osteogenic differentiation specific gene markers was analyzed at a) day 6 w/o OM, expressions were normalized to GAPDH. b) Rheology results showing effect of cell inclusion on gel properties. Alizarin Red S staining of 3D cell culture within c) pPA in d) TCP. (Mean $\pm$ SEM, n=4 gels per sample, one-way analysis of variance (ANOVA) *p <0.05).

3.4. Conclusion

Based on a molecular self-assembly and artificial hydrolysis strategy, multivalent and functional peptide nanofibers were developed as an enzyme activity containing scaffold support for osteogenesis. This approach presents remarkable features with dual hydrolytic and structurally supportive environment to simulate enzymatic level of hierarchical organization of bone formation process. The catalytic sites of the supramolecular nanostructures were also studied by theoretical modeling. In addition, the pPA nanofibers were exploited for inducing *in vitro* osteogenic differentiation without addition of any osteogenic support. The pPA nanofibers facilitated rapid maturation of osteoprogenitor cells and mesenchymal cells into osteoblasts, which is an important challenge for the currently available bone grafts. These robust, versatile and biocompatible peptide platforms can be used to develop new multifunctional biomaterials for biomedical applications.

3.5. Experimental Section

3.5.1 Peptide Synthesis

PA molecules (Figure 3.5) were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Fmoc-protected amino acids, MBHA Rink Amide Resin, and HBTU (O-benzotriazole-N,N,N,N'-tetramethyl-uroniumhexafluoro-phosphate) were purchased from Novabiochem, and lauric acid was purchased from Merck. Fmoc-Glu-(OtBu)-Wang resin or Rink amide MBHA resin was used for peptide synthesis. For each coupling reaction, Fmoc groups were removed by shaking resins in 20% piperidine in N,N-dimethylformamide (DMF) for 20 min. All amino acids were

activated by adding 2 mol equiv of amino acid to 1.95 equiv of O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and dissolved in 10 mL of DMF. After complete dissolution of the amino acid and HBTU in DMF, 3 mol equiv of N-ethyl-diisopropylamine (DIEA) was added into the solution. Each coupling reaction was performed for 2.5 h and washed with 3 time DMF and 3 times dichloromethane (DCM). Lauric acid was coupled to the peptide's N-terminal. Peptide cleavage and the removal of the protecting groups were performed with 95:2.5:2.5 trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water for 2.5 h at room temperature. TFA was rotary-evaporated. After evaporation, icecold diethyl ether was added and the solution was left at $-20\text{ }^{\circ}\text{C}$ overnight. The pellet was collected and dissolved in deionized H_2O and freeze-dried. After freeze-drying, peptides were dissolved in 1 mM HCl solution in order to get rid of excess TFA, freeze-dried again, and characterized by Liquid Chromatography–Mass Spectrometry (LC-MS). An Agilent Technologies 6530 Accurate-Mass Q-TOF LC-MS equipped with a Zorbax SB-C8 column was used for LC-MS analysis. The concentration of the sample was 0.5 mg/mL. The mobile phase was water (0.1% formic acid for positive charged peptides, 0.1% ammonium hydroxide for negative charged peptides) and acetonitrile (0.1% formic acid for positive charged peptides, 0.1% ammonium hydroxide for negative charged peptides). LC-MS was run for 25 min for each sample. Each run was started with 2% acetonitrile and 98% H_2O for 5 min, followed by the increase of the acetonitrile gradient to 100% over 20 min. Finally, acetonitrile concentration was decreased to 2% and the run was continued for a further 5 min. The solvent flow rate was 0.65 mL/min. A total of 5 μL of sample was injected for each run.

3.5.2 Transmission Electron Microscopy

A 1% (w/v) peptide solution was prepared and alkalified with 0.1 M NaOH solution. The peptide gel was diluted with 1 mM NaOH and a small amount of solution was dropped to a carbon-covered copper grid. The 2% (w/v) uranyl acetate solution was used to visualize the organic peptide fibers. The carbon grid was air-dried following staining. A FEI Tecnai G2 F30 transmission electron microscope was used to image the PAnanofibers.

3.5.3. Circular Dichroism.

The secondary structures of peptide nanofibers (pPA) were analyzed with a JascoJ-815 circular dichroism spectrometer. A 1 wt % peptide solution was prepared in ddH₂O and jellified with 1 mM NaOH. The peptide gel was then diluted with water and the circular dichroism (CD) spectrum of 1×10^{-4} M peptide solution was measured from 300 to 190 nm with 0.1 data pitch, 100 nm/min scanning speed, 1 nm bandwidth, and 4 s D.I.T. The average of three measurements was used and sensitivity was selected as standard. Peaks indicating β -sheet presence were observed in the CD spectrum. The pPA molecules were mixed with 2: equivalent of Zn^{II}. The effect of metal binding on secondary structure was tested with CD analysis. Metal binding increased β -sheet formation.

3.5.4. Isothermal Titration Calorimetry

The pPA solutions were titrated with ZnCl_2 solution at pH 8. Cell temperature were 25 °C, reference power was 5 $\mu\text{cal/s}$, and stirring speed was 500 rpm. In Figure 3.5, titration of 0.2 mM pPA at pH 8.0 with 2 mM ZnCl_2 solutions were demonstrated, respectively. The pPA molecule exhibited moderate binding to Zn^{II} . One set of site fitting model was applied to determine values of the binding affinity (K), and enthalpy affinity pattern, according to association constants of affinity sites.

3.5.5. Hydrolysis Kinetics Experiments

1.5×10^{-4} M pPA-only solution and 1×10^{-4} M Zn^{II} containing peptide solution were prepared in 25 mM pH 8.0. $\frac{1}{4}$ excess zinc ion was included to have complete complexation since pPA- Zn^{II} ratio was found to be 2:1 by ITC experiments. Tris buffer was heated to 37 °C. Peptide solutions were kept at 37 °C for 30 min for Zn-peptide complexation. Six different pNPP substrate concentrations (0.3 mM-10 mM) were prepared in 25 mM Tris buffer at pH 8.0. 50 μL of substrate solutions were aliquoted in each well of a 96 well plate. 50 μL of peptide solution was added into each well. Kinetic measurements were started immediately, and absorbance of resulting pNP solution was collected at 405 nm. Results were collected and calculated for each concentration and fitted into the Michaelis-Menten equation.

3.5.6. Theoretical Calculations

The pPA and pPA + Zn^{II} molecules were theoretically studied by a semi-empirical molecular orbital method (called Parameterized Model-3 (PM3) [103] with the

Restricted Hartree-Fock (RHF) [104] formulation. After geometry optimization was carried out, Single Point Energy (SPE) calculation using Density Functional Theory (DFT) [105] with B3LYP (Becke, three-parameter [105], Lee-Yang-Parr [106, 107]) exchange-correlation potential at the basis set 6-31G(d) [108] was performed. These calculations gave us the frontier molecular orbitals HOMO and LUMO, and electrostatic potentials. Gaussian 0939 program package was used for calculations and GaussView 40 was used, respectively for input and visualization. To determine the chemical activity and electrostatic interactions with other molecules, we calculated the electrostatic potential maps of all molecules (Figure 3.8). According to these maps, electron-accepting regions, plotted in blue, are on C atoms, which are double-bonded with the O atoms. The electrostatic charges on these C=O bonds are similar. For 2 pPA + Zn^{II} , the charges take values between +0.57. For pPA, the charges take values between +0.57 and +0.64 a.u. Electron-donor regions (plotted in red) are on the N atoms. For all molecules, the electrostatic charges in these regions range from -0.49 to -0.73 a.u. . For 2 pPA + Zn^{II} , we also calculated the charges on the N and Zn atoms and found N to have a charge of -0.69 a.u. and Zn^{II} to have a charge of +0.64 a.u for 2pPA + Zn^{II} .

3.5.7. *CaP Mineralization*

Glass coverslips were coated with 600 μM peptide with and without 400 μM ZnCl_2 , solution and placed in 24-well plates. A total of 25 mM Tris buffer at pH 7.4, supplemented with 24 mM CaCl_2 and 14.4 mM β -glycerophosphate (β -gly), was added to each well to initiate the mineralization process. The solution was removed from the surface and coverslips were dried at ambient conditions prior to analysis.

3.5.8. Ca Mineralization Quantification

The pPA and glass bare surfaces were supplied with 24 mM CaCl₂ and 14.4 mM β-gly solution. After incubation, the solution was discarded and the plate was washed with water and stained with Alizarin Red S for 20 min. After staining, coatings were washed again with water. Alizarin Red-bound calcium was extracted using 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7) for 20 min at room temperature and the concentration of Alizarin-Red S was determined by microplate reader by measuring the absorbance at 562 nm.

3.5.9. Scanning Electron Microscopy

FEI Quanta 200 FEG environmental scanning electron microscope was used for the acquisition of SEM images. Coverslips were coated with 3 nm Au–Pd prior to imaging. Image acquisition was performed under 10–15 kV voltage. Mineralized surfaces were prepared with the method explained in surface preparation section.

3.5.10. Raman Spectroscopy

A WITec Alpha300S Scanning Near-field Optical Microscope equipped with a Raman module was used to characterize calcium phosphate crystals on pPA surfaces. A Nd:YAG 532 nm laser source was used in the experiments. The integration time was 0.53492 s, and the number of accumulation was 50. For Raman spectral images, a 150

$\mu\text{m} \times 150 \mu\text{m}$ area was scanned with 50 points per line and 50 lines per image. The scan speed was 10.610 s/line and the integration time was 0.2122 s.

3.5.11. X-ray Diffractometry

The crystallographic structure of calcium phosphate crystals was evaluated with a PAN analytical X'Pert X-ray diffractometer using Cu K α radiation. Peptide-coated glass surfaces were prepared as described and measured without further modification. The rotation time was 16 s, scan range was from 20° to 70°, and step size was 0.0525°. The planes observed were (111) and (211) for all of the crystals formed on surfaces.

3.5.12. Hydrolysis Experiments on the Surface

Hydrolysis experiments on peptide-coated surfaces were performed in 25 mM TRIS buffer at pH 7.4. The 20 mM pNPP solutions were prepared in diethanolamine solution from pNPP kit and used for hydrolysis (Sigma). Reactions were catalyzed with 1 mM PA coatings.

3.5.13. Cell Culture and Maintenance

SaOS-2 human osteosarcoma (ATCC HTB-85) cells and rat mesenchymal stem cells (rMSCs) (Invitrogen) were used for *in vitro* cell culture experiments on pPA coated culture surfaces. All cells were cultured and propagated in 75 cm² cell culture flasks using Dulbecco's Modified Eagle Serum (DMEM) supplemented with 10% Fetal

Bovine Serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine. Cells were grown at 37 °C in a humidified chamber supplied with 5% CO₂. All experiments were carried out after 80–90% confluency was reached. rMSCs were cultured in 75 cm² cell culture flasks at a seeding density of 3×10^3 cells/cm² using DMEM supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine for all experiments. rMSCs were used at passage number 7.

Table 3 Contents of Cell Culture Mediums

Maintenance Media	Normal Media	Osteogenic Media
1. Dulbecco's Modified Eagle Serum (DMEM) 2. 10% Fetal Bovine Serum (FBS), 3. 1% penicillin/streptomycin 4. 2 mM L-glutamine.	1. DMEM 2. 10% FBS 3. 1% P/S 4. 2 mM L-glutamine 5. 10 mM β -glycerophosphate	1. DMEM 2. 10% FBS 3. 1% P/S 4. 2 mM L-glutamine 5. 10 mM β -glycerophosphate 6. 0.2 mM Ascorbic Acid 7. 100 nM Dexamethasone

3.5.14. Cell Viability Assays on Peptide-Coated Surfaces

Viability of SaOS-2 cells was analyzed on pPA-coated culture plate surfaces at 24, 72, and 144 h. Cells were seeded onto pPA-coated TCP surfaces in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin at a density of 2×10^4 cells/cm². After the required incubation times, the viability of the cells on pPA coated and control surfaces was quantified by Alamar Blue assay (Invitrogen) by measuring absorbance values at 570 nm (background absorbance at 600 nm). The viability of cells on pPA coatings was normalized to that of cells on bare surfaces. The viability of the 3D cell culture with SaOS-2 cells was evaluated using the same

procedure as the 2D experiments detailed above. The only difference was that 5×10^5 cells/cm² were encapsulated within 10 mM pPA gels. Viability of rMSCs was analyzed with both Alamar Blue assay (Invitrogen) and Live–Dead assay (Molecular Probes, Invitrogen) to observe live and dead cells. Cells were seeded on 96-well plates, incubated for 24 and 48 h, and centrifuged at 2500 rpm for 5 min. Cells were then stained with 1 μ M calcein AM ($n = 4$) and 2 μ M ethidium homodimer for 40 min in dark. Washing steps during the staining protocol were performed at 2500 rpm for 2 min to prevent dead cells from being washed away. Stained cells were observed under fluorescence microscope.

3.5.15. *In vitro* Biomineralization Assay

Biomineralization and calcium phosphate tests with SaOS-2 cells were carried out on 1 mM pPA coated well-plates. pPA was gelated with DMEM medium at pH 7.4. pPA nanofibers were formed on culture plate surfaces as described above and coated surfaces were first incubated at 37 °C to strengthen the gels. Coatings were then allowed to dry in a chemical hood overnight. Calcium deposition on the surfaces was measured on days 3 and 6 using Alizarin Red S as a quantitative colorimetric staining method. Briefly, cells were fixed with ice-cold ethanol for 1 h and stained with 40 mM Alizarin Red S for 15 min. After washing 4–5 times with double-distilled water to remove nonspecific Alizarin Red binding, cells were imaged with optical microscope. Alizarin Red bound calcium extraction was performed by using 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7) for 20 min at room temperature and the concentration of Alizarin Red S was determined by measuring the

absorbance at 562 nm. For Alizarin Red S staining assay, SaOS-2 cells were seeded on 24-well plates at a density of 2×10^4 cells/cm² in DMEM with 10% FBS. Cells were cultured in 10% FBS/DMEM until they reached 90–100% confluence, after which the medium was replaced with fresh osteogenic medium containing 10 mM β -gly, 0.2 mM ascorbic acid, and 100 nM dexamethasone in 10% FBS/DMEM. This medium was replenished every 3–4 days during the course of experiments. The DNA content of each set was controlled with DNA quantification assay to confirm cell number equality for each sample. The procedure of 3D culture calcium deposition was similar, except that 5×10^5 cells/cm² were encapsulated within 10 mM pPA gel, and the cells were fixed with ethanol and stained with Alizarin Red S for 5 min after the incubation period.

3.5.16. Gene Expression Analysis

The gene expression profiles of cells during osteogenic differentiation were assessed by quantitative RTPCR (qRT-PCR) analysis. The expression profiles of RUNX2 and Col I were analyzed for SaOS-2 cells, while these of RUNX2, COL I and Osp were analyzed for rMSCs. Reaction conditions were briefly as follows: 58 °C for 5 min, 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 40 °C for 1 min, followed by a melting curve to confirm product specificity. The reaction efficiencies for each primer set were evaluated with a standard curve using 5-fold serial dilutions of total RNA. SaOS-2 cells were seeded at a density of 4×10^4 cells/cm² on peptide nanofibers and total RNA was isolated using TRIzol (Invitrogen) according to manufacturer's instructions. Yield and purity of extracted RNA were assessed by Nanodrop 2000

(Thermoscientific). Primer sequences were designed using Primer 3 software. cDNA synthesis from RNA and qRT-PCR were performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) according to manufacturer's instructions. Primary gene expression results were normalized to the expression level of GAPDH for the analysis of the expression data. A comparative cycle threshold method was used to analyze the results.

3.5.17. Immunocytochemistry Staining and Imaging

In order to analyze DMP-1 protein localization, rMSCs were seeded onto pPA coated surfaces and glass surface at a density of 1×10^4 cells/cm². After reaching confluence, cells were incubated for 7 days in maintenance medium supplemented with only β -gly. Medium was changed every 4 days. Cells were then fixed in 4% paraformaldehyde/PBS for 10 min and permeabilized in 0.1% Triton X-100 for 15 min. To reduce nonspecific binding, samples were incubated with 10% (w/ v) bovine serum albumin/PBS blocking reagent for 30 min and treated with 6.7 μ g/mL DMP-1 primary antibody (Abcam) overnight at 4 °C. Samples were then incubated with goat anti-rabbit secondary antibody (DyLight 488) at 1:300 dilution for 1 h at room temperature. Extensive washing with PBS was performed between each step. All samples were counterstained with 1 μ M TO-PRO-3 (Invitrogen) in PBS for 15 min at room temperature and mounted with Prolong Gold Antifade Reagent (Invitrogen). Negative controls were obtained by omitting primary antibody and incubating with 1% normal goat serum/PBS. Samples were imaged using confocal microscope (Zeiss LSM510).

3.5.18. Rheology Analysis of 3D Culture

The formation of network structure at pH 7.4 with and without cells was studied by oscillatory rheology. Anton Paar Physica RM301 Rheometer operating with a 12 mm parallel plate configuration was used to probe the structural gel properties of the cell cultured pPA networks. Samples were prepared by mixing pPA solution with DMEM and DMEM containing cells, incubated for 6 days and moved on the lower stage of the rheometer. A gap distance of 0.5 mm was used with 10 rad/s angular frequency and 0.1% shear strain.

3.5.19. Statistical Analysis

All experiments were independently repeated at least twice, with at least four replicas for each experimental or control group in each independent assay. All quantitative results are expressed as mean $\pm$ standard error of means (SEM). Statistical analyses were performed by one-way analysis of variance (ANOVA) or Student's test, whichever applicable. Significant differences were evaluated by the P-value; differences were considered statistically significant at $P < 0.05$.

CHAPTER 4

Serine Phosphorylation and Matrix Formation Alter The Bio-mineralization Efficiency of Dentin Sialophosphoprotein (DSPP)-Mimetic PA

4.1. Objective

Cleavage products of dentin sialophosphoprotein (DSPP) are major components of the dentin matrix, and play crucial roles in the deposition of hydroxyapatite (HA). They have also been shown to be effective in facilitating bone mineralization when incorporated into a peptide structure, making them prime candidates for the design of novel materials for bone and tooth repair. However, the bioactivity of DSPP-derived proteins is altered by the phosphorylation and dephosphorylation of their serine residues, and this regulatory mechanism is difficult to fully replicate in an artificial system. In this chapter, we describe a new PA-based system that closely emulates the structural and functional properties of DSPP, and facilitates the deposition of HA in a phosphorylation-dependent manner. In particular, the mechanical and biochemical properties of PA molecules could be altered through dephosphorylation by ALP, an enzyme that plays a crucial role in the regulation of tooth and bone mineralization. In addition, hydrogel formation could be induced either by dephosphorylation or through the addition of calcium to form biomineralizable surfaces. Our findings suggest that both scaffolding and (free or bound) phosphate presence are required for biomineralization to occur, and that serine-rich, DSPP-mimetic PA networks can be

used for the in-depth investigation of the mineral deposition process that occurs in teeth during early development and reactive dentin formation.

4.2. Introduction

Dentin is a compact but highly specialized tissue that performs a diverse set of functions through a small population of dental pulp stem cells (DPSCs), odontoblasts, fibroblasts, neural cells and capillary and immune system elements [109]. Mature cells of the dental pulp typically differentiate from DPSCs, which are derived from neural crest cells during embryonic development and may subsequently develop into odontoblasts, chondrocytes, adipocytes, myocytes and neural cells [110]. Among these, the maintenance of the odontoblast population is especially crucial for the production of dentin, as these cells are responsible for secreting and facilitating the mineralization of the dentin matrix during both embryonic development and post-injury repair [111, 112]. However, the native odontoblast population may be damaged during dental trauma and remain inoperative for the redeposition of the dentin matrix, necessitating the differentiation of latent DPSCs into odontoblasts for the adequate repair of tooth injuries. Consequently, the formation of new odontoblasts from native or externally introduced DPSC populations is a promising area of research for tooth regeneration, and peptide-based materials incorporating sequences from dentin matrix elements are an effective means of achieving this goal.

Dentin sialophosphoprotein (DSPP) is a non-collagenous SIBLING protein that is expressed strongly by odontoblasts and plays an important role in the mineralization of dentin [112]. As a pro-protein, DSPP is activated through its cleavage into three distinct proteins; dental phosphoprotein (DPP), dentin sialoprotein (DSP) and dentin glycoprotein (DGP) [113]. While DGP has been described relatively recently and its

precise function remains unclear, both DSP and DPP are abundantly present in the dentin matrix and appear to play crucial roles in the initiation and maturation phases of dentin formation, respectively [114]. In addition, DPP is notable for its exceptionally high ratio of phosphorylation, containing numerous Ser-Asp-Asp, Asp-Ser-Ser and Asp-Ser repeats with phosphorylated serine units [114, 115]. This unique structure allows DPP to facilitate mineral deposition by attracting calcium ions and promoting the formation of hydroxyapatite crystals that form the bulk of the dentin structure [116]. However, while the hydroxyapatite-binding and mineralization-inducing capacities of DPP have been replicated in a short, serine-rich, non-phosphorylated peptide sequence [116], the potential effects of serine phosphorylation and dephosphorylation on these properties have not fully been demonstrated in PA systems.

Mass spectrometry (MS) analyses have identified a 185-kDa protein band as the full-length form of DSPP, and the whole amino acid sequence of rat DSPP has been deduced using cDNA libraries [117]. Using this sequence as a reference, we designed a dentin-mimetic PA (SpDSp-PA) molecule that is capable of emulating the structure and function of DSPP in general and DPP in particular, and evaluated its capacity to support the deposition of hydroxyapatite and survival of osteoprogenitor cells. As with DPP, SpDSp-PA hydrogels contain large numbers of Ser(phos)-Asp-Ser(phos) (SpDSp) repeats in order to effectively induce dental mineralization and subsequent differentiation. In addition, the peptide hydrogel system was designed to reversibly alter its mechanical properties through enzymatic dephosphorylation, allowing greater control over the mineralization and differentiation processes through the enzymatic modulation of hydrogel structure. As DPP exhibits the strongest negative charge

among known proteins, we also intended to utilize the selective dephosphorylation capacity of SpDSp-PA to demonstrate the importance of phosphorylated serine residues on the function of this protein. As such, self-assembling SpDSp-PA hydrogels were produced through both calcium ion-mediated charge neutralization and ALP-mediated enzymatic dephosphorylation. A non-phosphorylated control (SDS-PA) was used to evaluate the influence of free phosphoryl groups created by the dephosphorylation process on biomineralization.

SpDSp-PA hydrogels were observed to exhibit a strong capacity for biomineralization, especially after dephosphorylation by ALP, while soluble, non-hydrogel peptides were altogether ineffective for this purpose. In contrast to dephosphorylated SpDSp-PA, non-phosphorylated SDS-PA networks also failed to facilitate mineral deposition despite their nanofibrous structure, suggesting that phosphoryl residues and scaffold formation are both critical for the formation of hydroxyapatite. While phosphorylated DPP has been demonstrated to display a greater binding affinity to HA (though the reverse has also been reported [118]), greater resistance to enzymatic degradation and a greater capacity for calcium deposition [119-121], we conclude that the scaffolding capacity and free phosphoryl groups of ALP-treated SpDSp-PA are effective in facilitating biomineralization. As all three hydrogels also supported the survival of DPSCs, we also suggest that the hydrogel system described here can be used for the further investigations of the dental biomineralization process involving a combination of cellular, mineral and protein hydrogel components.

4.3. Results and Discussion

4.3.1. Design and characterization of DSPP-mimetic PAmolecules

The DSPP-mimetic PA was designed with the lauryl-VVAGSDSG-Am sequence to emulate the Ser-Asp-Ser motifs that occur in large numbers in DSPP and DPP. In this sequence, the hydrophobic lauryl tail acts in conjunction with the β -sheet forming VVAG motif to enhance the self-assembly of the peptide, while the SDSG sequence presents phosphorylated or non-phosphorylated serine residues to facilitate the deposition of hydroxyapatite onto the nanofiber matrix. This sequence was synthesized with and without phosphorylated serine residues through the inclusion of the appropriate amino acid during the synthesis protocol; these variants have been named SDS-PA (for non-phosphorylated PA) and SpDSP-PA (for phosphorylated PA). Following the purification of peptide products by LC-MS (Figure 4.1), the physicochemical characteristics of SDS-PA, SpDSP-PA and their hydrogels were characterized by circular dichroism, oscillatory rheology and SEM and TEM imaging. Two methods were used for the formation of hydrogels from SpDSP-PA solutions: ALP-mediated dephosphorylation and Ca-mediated co-assembly. For the former, SpDSP-PA was treated with a ten-fold excess concentration of ALP to remove the negatively-charged phosphoryl groups that normally prevent the self-assembly of the soluble peptide, while the latter was accomplished by the addition of a ten-fold excess of calcium to neutralize the overall charge of SpDSP-PA. Both ALP- and Ca-treated solutions were observed to rapidly form hydrogels under room temperature and exhibited CD spectra consistent with a β -sheet structure (Figure 4.2). In contrast, SpDSP-PA did not exhibit any secondary structure formation in water, likely due to its

strong negative charge preventing any large-scale assemblies, while SDS-PA had a CD spectrum similar to the ALP-treated SpDSp-PA, due to both peptides being dephosphorylated.

The enzymatic dephosphorylation of SpDSp-PA could be monitored in real time through LC-MS and CD measurements. The unmodified SpDSp-PA elutes at c. 8 min in the water/acetonitrile gradient due to its strongly negative charge, while the ALP-treated group is observed later (at c. 13 min) due to the removal of its phosphoryl groups. The non-phosphorylated S-PA, like dephosphorylated SpDSp-PA, is eluted at c. 13 min, confirming the successful dephosphorylation of the latter (Figure 4.2, 4.3). In addition, time-lapse CD measurements demonstrate that a negative peak appears at 221 nm in the CD spectrum of SpDSp-PA following ALP-treatment, and gradually increases in intensity with time (Figure 4.4). This peak is associated with β -sheet formation and corresponds to the self-assembly of SpDSp-PA, concurrent with its dephosphorylation by ALP. The 221 nm peak appears to reach a plateau at c. 60 min following treatment, suggesting that dephosphorylation (and self-assembly) is complete by that point (Figure 4.4). Concentration-dependent CD measurements also suggest that SpDSp-PA can form nanofibers at concentrations as low as $\sim 5 \mu\text{M}$ (Figure 4.5).

Calcium-induced hydrogel formation was also assayed through TEM, CD and oscillatory rheology analyses (LC-MS measurements were not performed on Ca-treated samples on account that the rapidly-forming hydrogel would occlude the column). While S-PA did not substantially react to the addition of calcium in CD measurements, SpDSp-PA again exhibited a substantial increase in the intensity of β -sheet-associated CD peak at 221 nm, as the negatively-charged phosphoserine residues

in its structure allow it to form self-assembled nanostructures following the addition of calcium (Figure 4.6). In contrast, S-PA does not require a neutralizing agent for its self-assembly, and consequently maintains a β sheet structure with or without the inclusion of a cation. It is, however, notable that the storage modulus of Ca-treated SpDSp-PA was substantially higher than that of S-PA, suggesting that the ion-mediated assemblages may be mechanically more stable than those created through the electrostatic interactions of hydrophobic tail groups and β sheet-forming peptide sequences alone (Figure 4.4). Nevertheless, both hydrogel forms exhibited a fibrous structure common to many PA assemblies.

4.3.2. Mineral deposition capacity of DSPP-mimetic PAs under enzymatic and ionic self-assembly

Following the materials characterization of SpDSp-PA, SDS-PA and their self-assembled forms, an *in vitro* biomineralization assay was performed to determine the effectiveness of each formulation in facilitating the deposition of a mineral matrix. Curiously, the ALP-treated SpDSp-PA group exhibited a very high rate of biomineralization, heavily coating the glass coverslip with calcium depositions matching the Ca:P ratio of hydroxyapatite, while Ca-treated SpDSp-PA supported biomineralization only to a lesser extent and non-treated SpDSp-PA and SDS-PA were both largely ineffective in facilitating the mineralization of the glass surface (Figure 4.7). This is in contrast to prior studies suggesting that dephosphorylated DPP loses a portion of its affinity for hydroxyapatite, resulting in lower rates of biomineralization (or even the inhibition of mineral deposition) compared to its phosphorylated form

[122, 123]. However, it should be noted that free SpDSp-PA is in soluble form while the ALP-treated peptide self-assembles into a nanofibrous matrix that may assist in the deposition of hydroxyapatite by presenting a well-ordered series of amino acid residues. We therefore suggest that the presence of a well-organized nanofiber scaffold can support or substitute for the strongly negative charge of naturally occurring DPP, allowing for efficient mineralization even in the absence of bound phosphoryl groups. In addition, the phosphoryl residues removed by ALP may be available for the formation of hydroxyapatite or even remain in association with the peptide matrix to better facilitate the mineral deposition process.

It should be noted that some of these effects may also apply to the *in vivo* development of dentin and enamel: It is, for example, known that DPP and DSP in teeth are partially dephosphorylated with age [124], resulting in a lowered capacity for biomineralization that may be mitigated through the presence of a well-established scaffold matrix. In addition, casein kinase II and ALP are thought to be responsible for the phosphorylation and dephosphorylation of DPP, respectively, and their activity is necessary for modulating the mineral balance of the tooth by controlling the concentration of calcium and phosphorus in free and HA-bound forms [116]. As SpDSp-PA can also be phosphorylated and dephosphorylated by these two enzymes, and retains enough functionality to act as a bioactive matrix in its dephosphorylated form, we suggest that the present system can serve as a model for further investigation of enzyme activity and its effects on biomineralization. In order to better demonstrate the effectiveness of the SpDSp-PA system as a mimic of the native DPP/DSP matrix, we further performed a set of experiments on the differentiation of dental pulp stem cells on these networks.

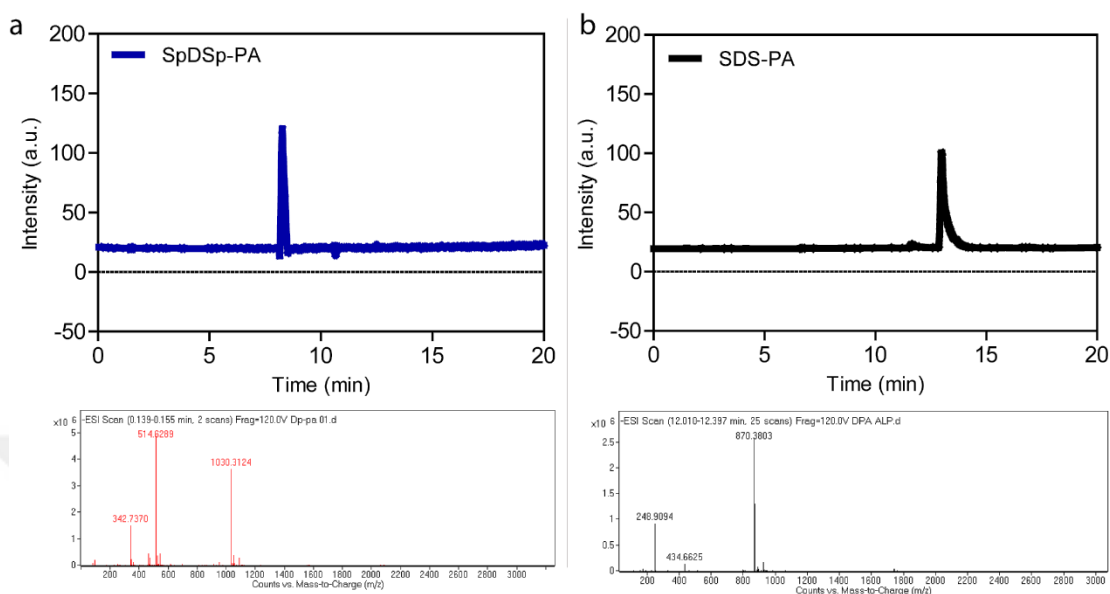


Figure 4.1 Liquid chromatography and mass spectrometry results of the pPA molecule. Mass spectrum of peptide after subtracting mass spectrum of water sample. For SpDSp-PA $[M-H]^-$ (calculated) = 1030.96, $[M-H]^-$ (observed) = 1030.31, $[M/2-H]^-$ (calculated) = 514.49, $[M/2-H]^-$ (observed) = 514.62, $[M/3-H]^-$ (calculated) = 342.65, $[M/3-H]^-$ (observed) = 342.73. For SDS PA $[M-H]^-$ (calculated) = 870.02, $[M-H]^-$ (observed) = 870.38, $[M/2-H]^-$ (calculated) = 434.01, $[M/2-H]^-$ (observed) = 434.66.

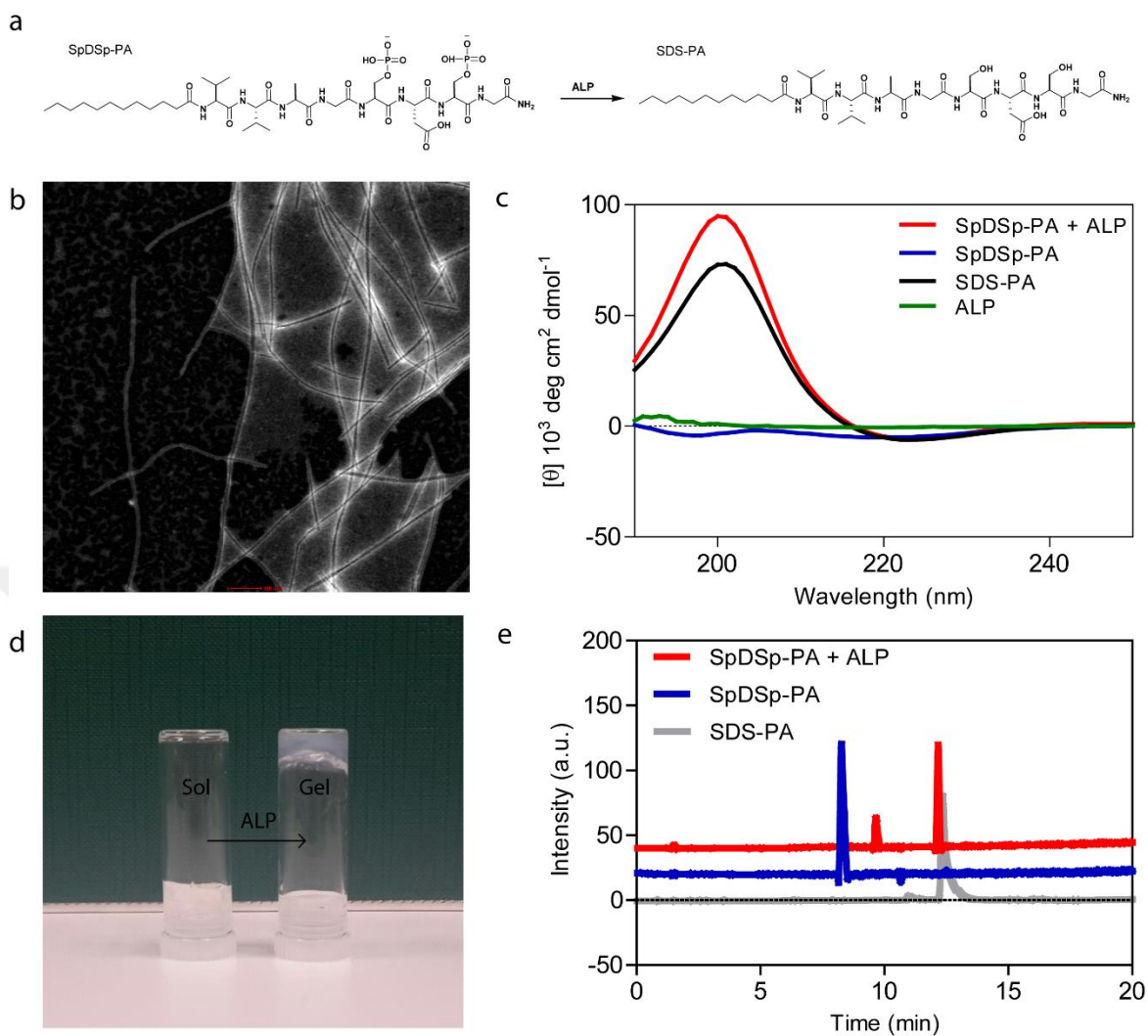


Figure 4.2 Enzymatic cleavage and gel formation analysis for DSPP-like peptides.

a) Chemdraw illustration of enzymatic dephosphorylation process of SpDSp-PA with ALP, b) TEM Images of peptide fibers formed after ALP treatment. c) Circular dichroism data showing β -sheet formation before and after enzymatic treatment, and control D-PA. d) Illustration of enzyme induced gel formation, e) LC-MS analysis of enzymatic cleavage process.

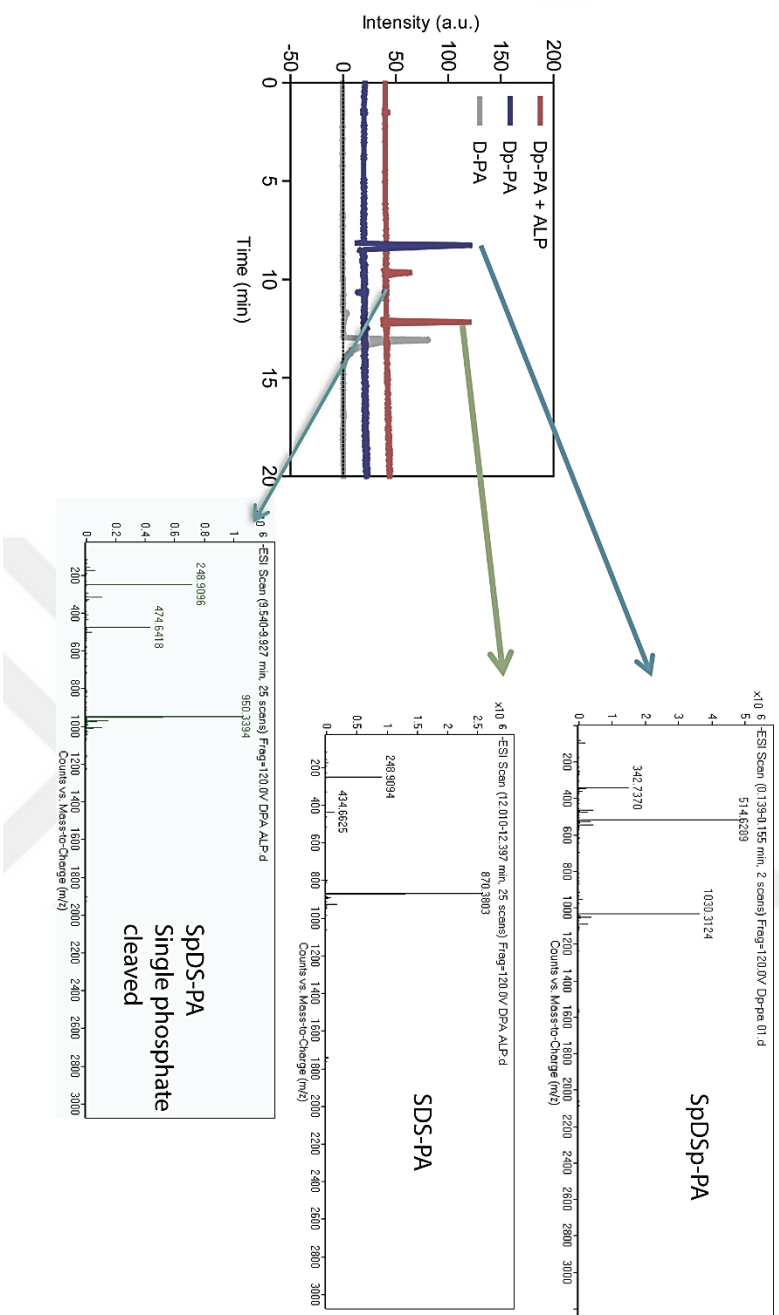


Figure 4.3 Mass analysis of cleavage products after enzymatic treatment. Assessment of dephosphorylation-induced peptide network formation by LC-MS. Single or double phosphate removal of peptide was shown in this figure with retention time and mass analysis.

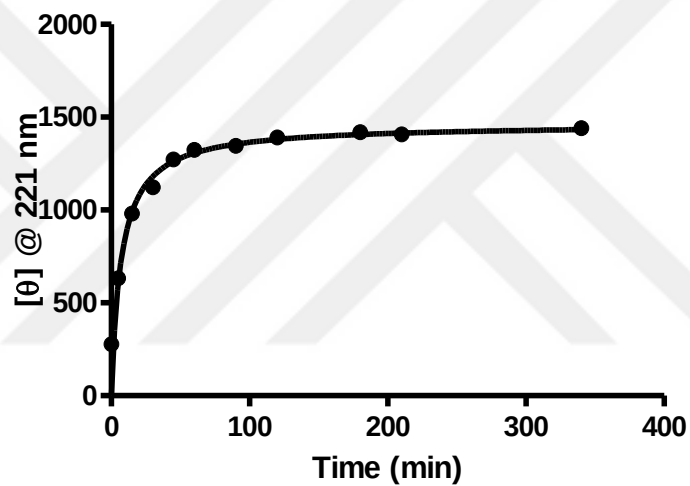
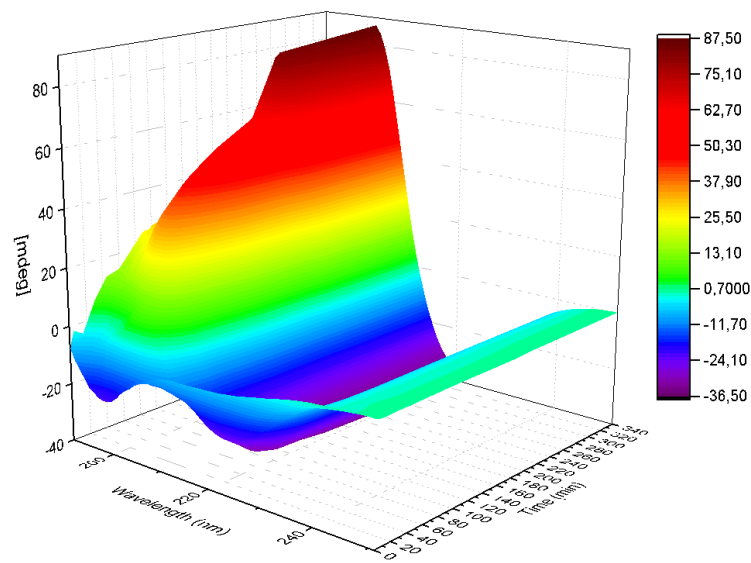


Figure 4.4 Fiber formation rate was tracked with bio-catalytic induction of Dp-PA. In-depth monitoring of network formation by dephosphorylated Sp-PA. (a) Time-course circular dichroism of ALP-treated Sp-PA, showing the gradual formation of a beta-sheet signal. (b) Quantification of the 221 nm beta-sheet peak, showing that dephosphorylation is complete by 60 min.

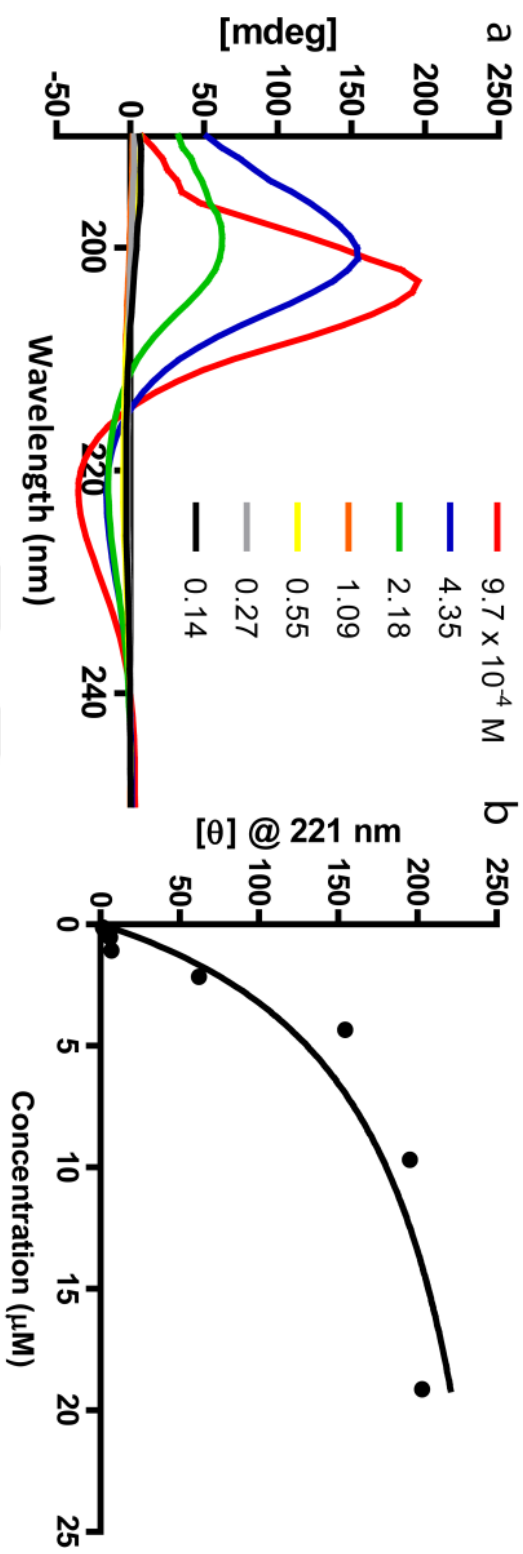


Figure 4.5 Peptide concentration dependent ALP induced fiber formation. Detection of minimum peptide concentration for fiber formation.

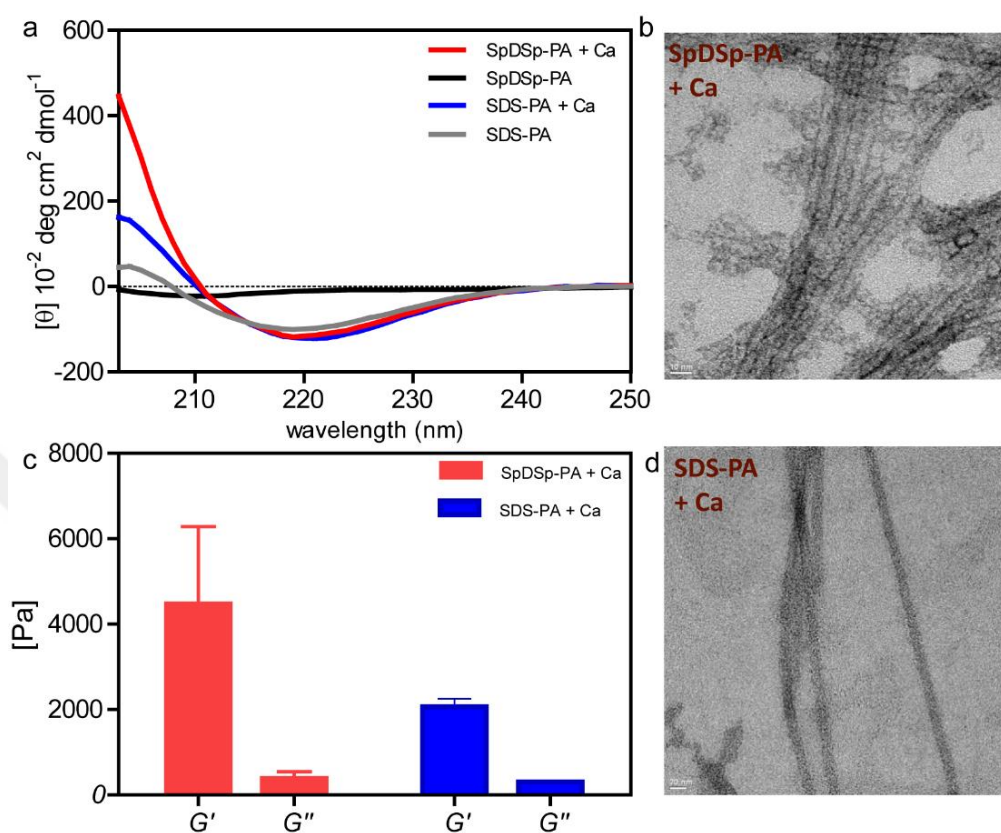


Figure 4.6 Calcium induced gel formation of DSPP-like peptides. In-depth monitoring of network formation by calcium-treated Sp-PA and S-PA. a) Circular dichroism results showing that Ca addition triggers self-assembly in Sp-PA, while S-PA remains self-assembled with and without cation presence. b, d) TEM images of Sp-PA/Ca and S-PA/Ca nanofiber networks. c) Oscillatory rheology of Sp-PA/Ca and S-PA/Ca nanofiber networks.

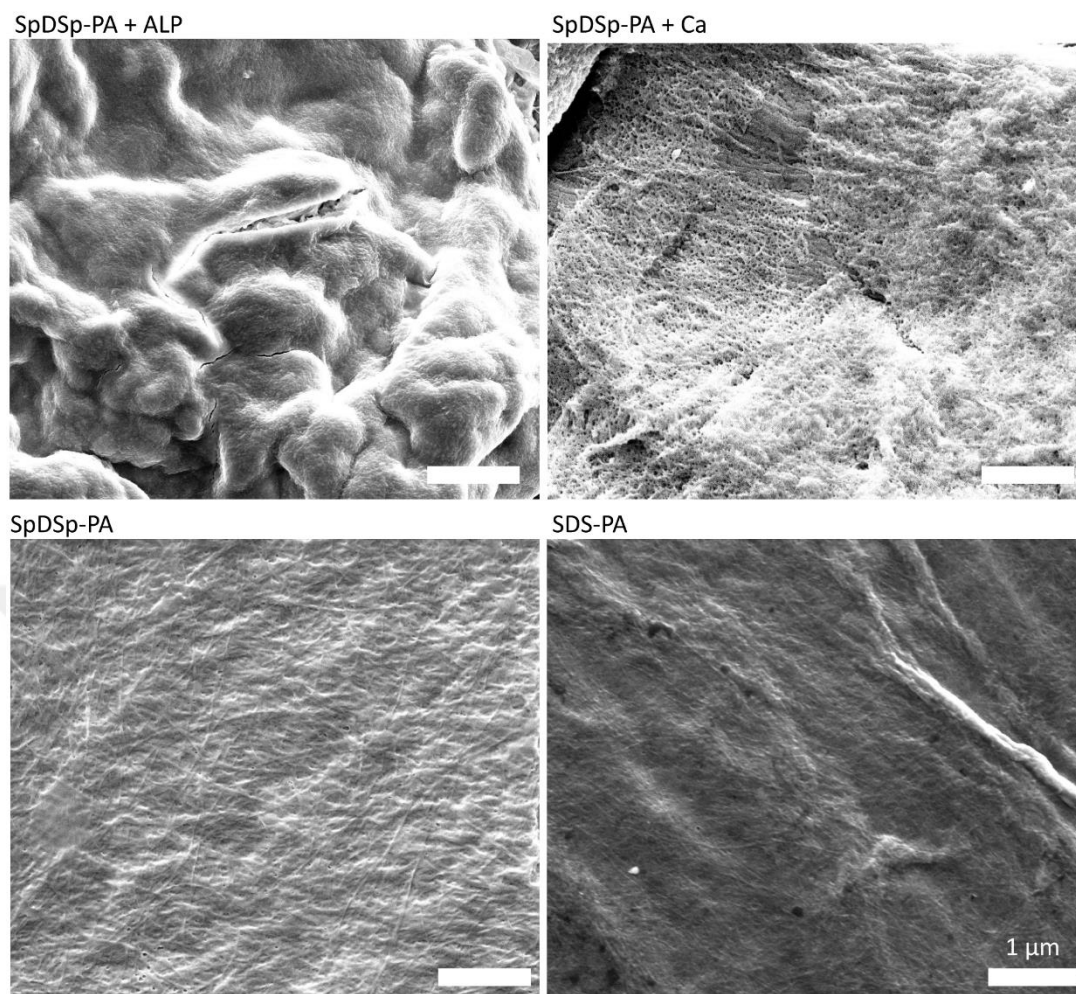


Figure 4.7 Hydroxyapatite deposition on peptide coated surfaces. Mineral deposition on peptide surfaces with and without hydrogel formation. SEM images of (a) Sp-PA/ALP, (b) Sp-PA/Ca, (c) Sp-PA and (d) S-PA are provided.

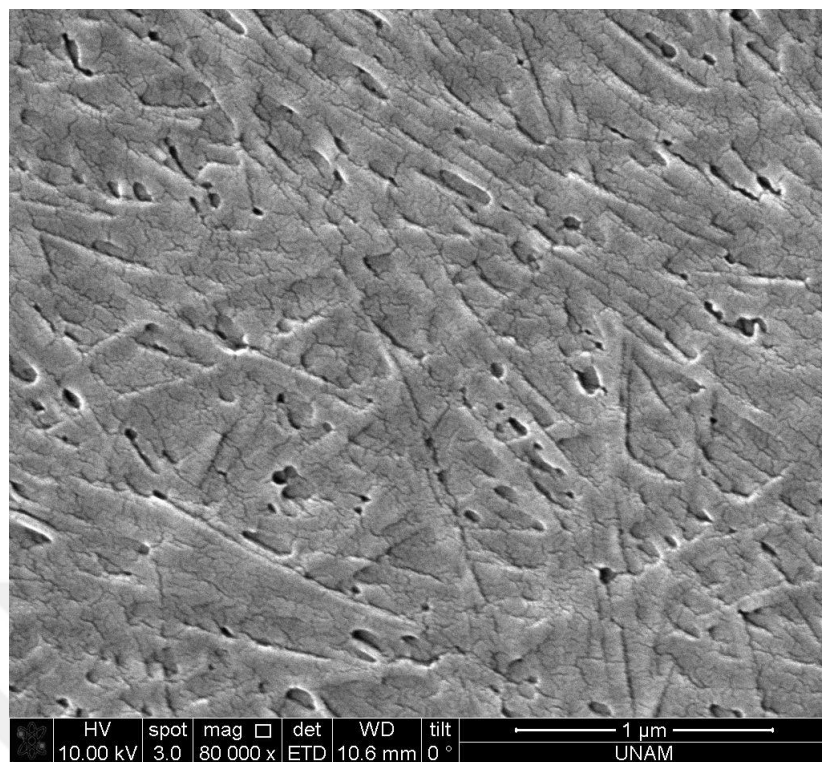


Figure 4.8 SEM image of calcium deposited SpDSp-PA fiber structure. Calcium deposition was confirmed with EDX analysis.

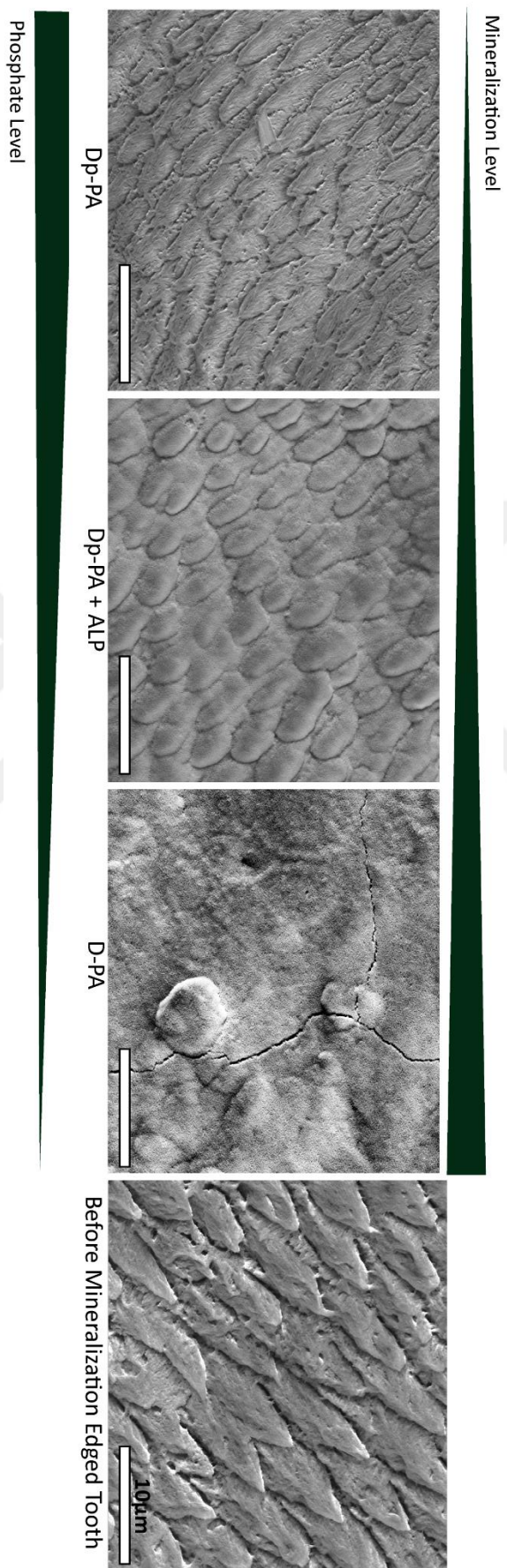


Figure 4.9 Hydroxyapatite formation on demineralized tooth surfaces. Calcium deposition analysis on tooth surfaces treated with 100 μ M DSPP-like peptides in simulated body fluid.

4.3.3. Biocompatibility of DSPP-mimetic PAs

Biocompatibility of DSPP mimetic peptides was tested with live/dead assay for osteoprogenitor cells and dental pulp stem cells. Compatibility of materials with surrounding environment is an important factor to harvest desired function.

Cell supporting environment is required during biomineralization process. In particular, tooth mineralization and interactions between mineralized tissue and cells (odontoblasts, gingivals, dental pulp stems cells) are crucial for formation of tooth segments. Our design was found to be compatible with multiple cell lines and this offers further application for regeneration of dental impairments (Figure 4.9).

This study will be continued with biomineralization studies of osteoblast-like cells osteoblast lineages and effect of peptides on cellular mineralization will be investigated. This study will be helpful to understand complicated tooth mineralization process and regulator factors.

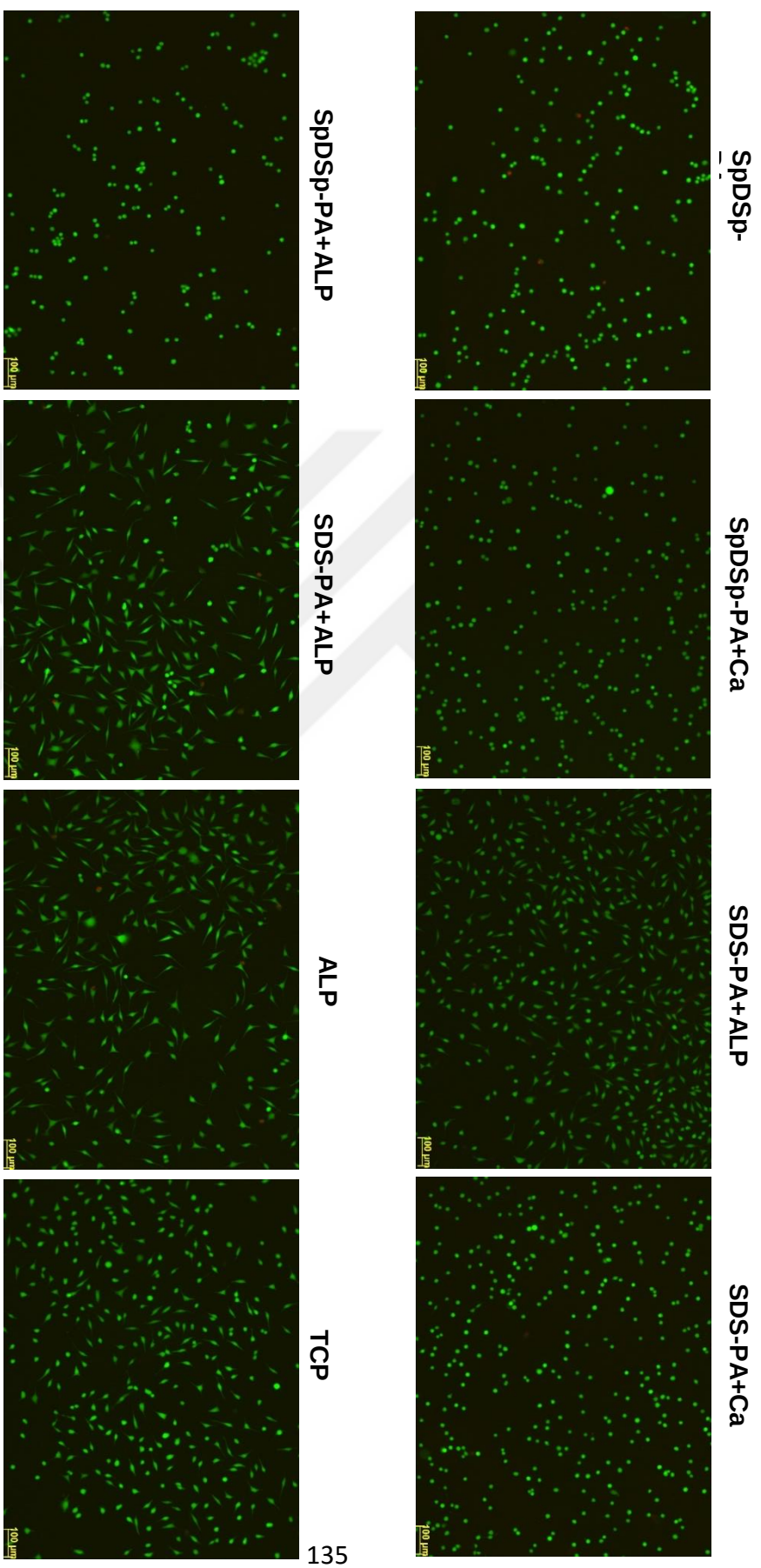


Figure 4.10 Viability of SaOS-2 cells on DSPP-like PA coated peptide nanofibers. a) Representative Calcein Am (Live=green) and ethidium homodimer (Red=dead) staining.

4.4. Conclusion

Here we showed that DSPP-mimetic peptide network is able to mediate its self-assembly and biomineralization capacity through the phosphorylation status of its serine residues. While previous studies have demonstrated that DSPP loses some of its effectiveness following the loss of its phosphoryl groups, both the native protein and its peptide equivalent can nevertheless function in their absence, possibly by retaining a physical scaffold matrix for HA assembly and utilizing free phosphoryl or other negatively charged groups (such as that conferred by aspartic acid) as a substitute for bound phosphoryl residues. In contrast, the soluble SpDSp-PA and gelous-but-not-phosphorylated SDS-PA both failed to facilitate mineral deposition, suggesting that the presence of phosphoryl groups and the nanofibrous composition of the peptide matrix are both vital for this process. In addition, as SpDSp-PA and SDS-PA supported the viability of osteoprogenitor cells in all formulations, the ability of ALP-treated SpDSp-PA to induce biomineralization may be utilized to develop new treatments for the assisted repair of tooth defects, while its structural, biochemical and functional resemblance to the cleavage products of DSPP may allow it to serve as a highly accurate model for the study of the biomineralization process itself.

4.5. Experimental

4.5.1. *Synthesis and purification of PA*

Lauryl-VVAGS(Phos)DS(Phos)G-Am and Lauryl-VVAGSDSG-Am peptides was constructed on glycine-coupled Wang resin at a 0.25 mmol scale. Amino acid couplings were done with 2 equivalents of Fmoc-protected amino acid, 1.95 equivalents of HBTU, and 3 equivalents of DIEA for 2 h. Fmoc removal was

performed with 20% piperidine/dimethylformamide (DMF) solution for 20 min. Peptides were cleaved from the resin with a mixture of TFA:TIS:H₂O in the ratio of 95:2.5:2.5 for 3 h. Excess TFA was removed by rotary evaporation; the remaining viscous peptide solution was triturated with ice-cold ether, and the resulting white product was freeze-dried for use following resolubilization in water. Peptide molecules were characterized by liquid chromatography-mass spectrometry (LC-MS).

4.5.2. TEM imaging of DSSP-mimetic PAs

1 mM peptide solution was prepared and gelified with ALP or 10 mM Ca solution and incubated for 6 h. Peptide gels were then diluted with water and a small amount of solution was dropped to carbon covered copper grids. Peptide fibers were stained with 2% (w/v) uranyl acetate solution for enhanced contrast, and the carbon grids were air-dried prior to TEM measurements. FEI Tecnai G2 F30 transmission electron microscope was used for the imaging of PA nanofibers.

4.5.3. Circular dichroism of DSSP-mimetic PA

Secondary structure analysis was carried out following the synthesis and purification of DSPP-mimetic peptide molecules. All peptides are dissolved in water at a concentration of 1 mM., and ALP or calcium (10-fold excess, 10 mM) was used for gel formation. Peptide/ALP and peptide/Ca mixtures were incubated until gel equilibrium was reached, and subsequently diluted to a final concentration of 5×10^{-5}

M for CD analysis. CD accumulations were repeated 3 times with a scanning speed 100 nm/min.

4.5.4. Enzyme Induced Gel Formation Analysis

2 mM SpDSp-PA and SDS-PA solutions were prepared, and ALP (30 U/ mg) added and only peptide solutions were incubated in humidified incubators with constant 5% CO₂ at 37 °C for 2 h. Resulting solution was tracked with Q-TOF LC-MS equipped with a Zorbax column was used for LC-MS analysis. The concentration of the sample was 0.5 mg/mL. The mobile phase was water containing 0.1% ammonium hydroxide and acetonitrile containing 0.1% ammonium hydroxide due to negative charge of peptide.

4.5.5. Rheology analysis of materials

Oscillatory rheology measurements for were performed determinin viscoelastic properties of DSPP like Ca mixture materials with Anton Paar Physica RM301. 25 mm parallel plate configuration was used for rheometer operating a at 25 °C. 125 µL of each sample (peptide and 10 fold excess calcium chlorirede) and 250 µL total volume with a final peptide concentration of 1 wt% was carefully loaded on the center of the lower plate and incubated for 15 min before measuring. After equilibration, the upper plate was lowered to a gap distance of 0.5 mm. Storage moduli (G') and loss moduli (G'') values were screened from 100 rad/s to 0.1 rad/s of angular frequency, with a 0.5% shear strain.

4.5.6. Surface mineralization assay

Coverslip surfaces were coated with 2 mM peptide solutions and supplied with 1.5x simulated body fluid (SBF). After incubation for 12 h, the solution was discarded and the coverslips were washed repeatedly with water, coated with 3 nm Au–Pd and imaged under scanning electron microscopy (SEM) operating under 10–15 kV.



CHAPTER 5

Biocompatible Supramolecular Catalytic One-Dimensional Nanofibers for Efficient Labeling of Live Cells

This work is partially described in the following publication:

Khalily A., Gulseren, G., Tekinay, A. B., Guler, M. O., *Bioconjugate Chemistry* 26(12), 2371–2375, 2015.

5.1. Objective

The copper-catalyzed azide–alkyne cycloaddition (CuAAC) method attracts great attention for a wide range of applications from chemistry to biology due to its high specificity, orthogonality and feasible reaction mechanism. Therefore, CuAAC has been utilized for biorthogonal click reaction to label and probe various target biomolecules. The cytotoxicity of reaction caused by Cu^{I} , due to reactive oxygen species formation, can be lowered significantly by copper chelating and stabilizing ligands. Besides stabilization, these ligands can also be used to upgrade reaction rates. For this purpose, we used histidine modified PA as a multi-unit ligand for copper to obtain enhanced and biocompatible click reaction mediated cell labeling.

5.2. Introduction

The copper-catalyzed click chemistry method offers a bioorthogonal strategy that can label live cells in a complex environment, allowing the function and dynamics of target biomolecules to be monitored in living organisms. In this chapter, an unnatural sialic

acid precursor bearing a bioorthogonal functional group (alkyne functionalized sugar (Ac₄ManNAI)) was introduced to living cells by using biosynthetic pathways and a chemical tag (biotin-azide) was subsequently attached to the alkyne-bearing sialic acid group by CuAACa. The labeling process was then completed with the addition of a streptavidin–fluorescein (FITC) conjugate [125]. Copper-coordinating ligands used for dynamic imaging studies must meet certain criteria; such as high binding affinity for copper ions, biocompatibility, biodegradability, and the ability to perform their reactions rapidly under physiological conditions. In view of these requirements, we used PA molecules derived from natural amino acids as a ligand to coordinate and stabilize the Cu^I oxidation state and to obtain improved reaction kinetics and minimized cytotoxicity. Cytotoxicity resulting from oxidative damage is one major drawback of copper catalysts for bioorthogonal labeling reactions.

Owing to the stabilization of Cu^I, the peptide ligand can prevent the formation of undesired byproducts and minimize the oxidative damage against target biomolecules and cells. In addition to reduced toxicity, biocompatibility, and biodegradability, another important feature of the supramolecular nanofiber-based catalysis is the availability of a large number of active sites on a small area, which enhances reactivity by better facilitating surface and substrate interactions.

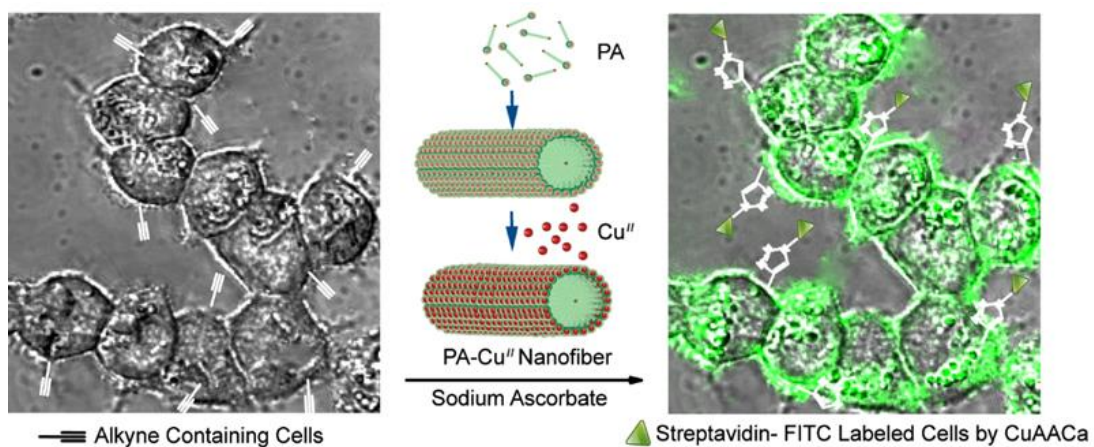


Figure 5.1 Illustration and summary of PA- Cu^{II} catalyzed biorthogonal cell labeling.

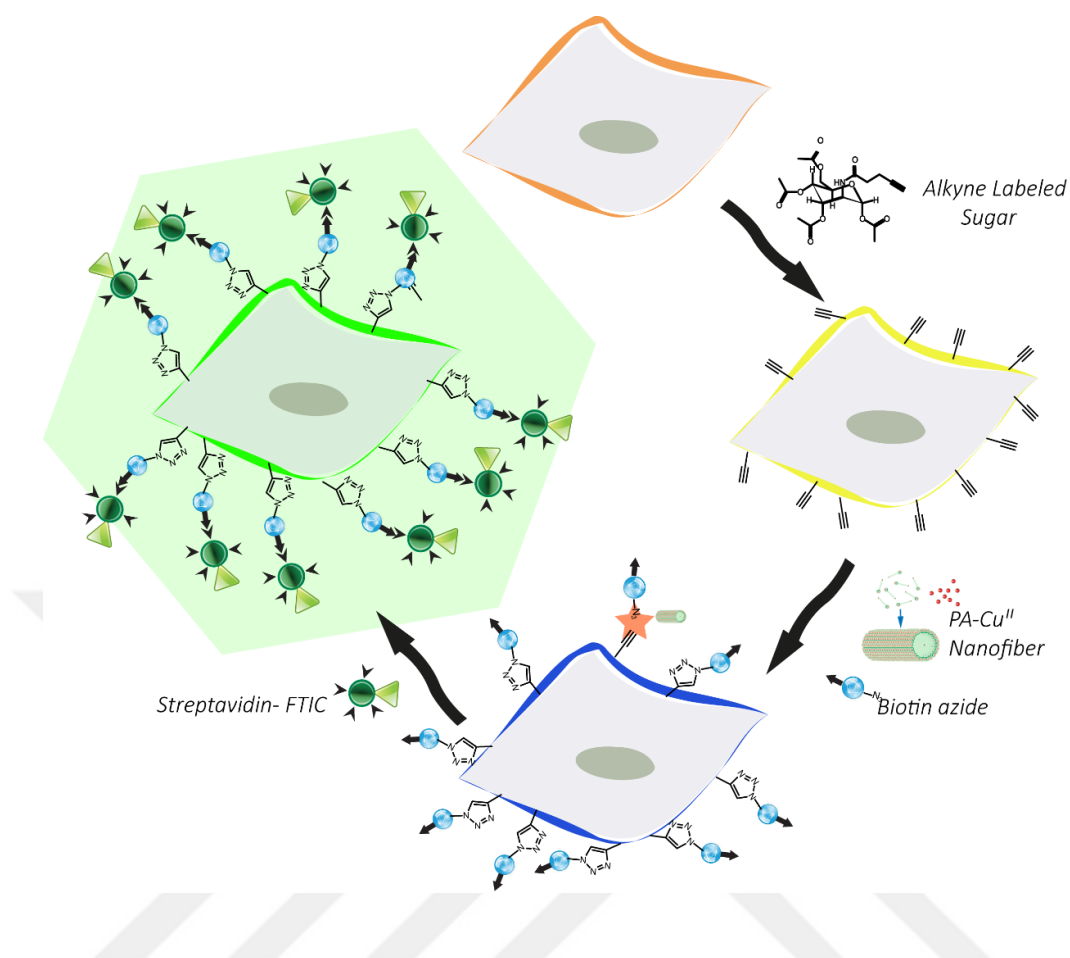


Figure 5.2 Steps of PA-Cu^{II} catalyzed biorthogonal live-cell labeling reaction.

Illustration of cell labeling steps of biorthogonal labeling from cell surface modification to labeling with click reaction.

5.3. Results and Discussion

5.3.1. Bioorthogonal Cell Labeling

To evaluate the potential of PA-Cu^I nanofibers (PA-Cu^{II} + sodium ascorbate) in cell labeling applications, variable concentrations of the PA-Cu^I nanocatalyst (3–0.125 mM) were administered to MCF-7 (breast adenocarcinoma) cells. MCF-7 cells were treated with Cu^I ions (CuSO₄ and sodium ascorbate) in the absence of ligand as positive control, and the effect of ligand incorporation on viability was tested. After 6 h of catalyst exposure, PA-Cu^I nanofibers were found to display significantly lower cytotoxicity compared to copper-alone samples for all copper concentrations (Figure 5.3). Toxicities of PA-Cu^I nanofibers were also found to be lower than Cu^I-only controls over extended exposure times (24 h) (Figure 5.3). In previous bioorthogonal labeling studies with small molecule ligands, toxicity was tested in the presence of Cu^{II}-ligand complexes or lower copper concentrations [126, 127]. However, Cu^{II} is found in the serum as an essential metal and is less toxic than Cu^I, which is the main ingredient of the click reaction mixture administered to cells.

Our PA-Cu^I nanocatalyst approach allows the use of higher Cu^I concentrations for click chemistry, which makes PA nanofiber-based click catalysts promising for effective catalysis in the biological environments. Modification of cell membranes by Ac₄ManNAI was verified and labeling reaction duration was optimized by using fixed MCF-7 cells. Cells were incubated with Ac₄ManNAI for 3 days to introduce alkyne residues to cell surface sialyl glycoconjugates, and fixed with cold acetone. The acetylated form of the alkynyl sugar was administered to MCF7 cells since acetylation enhances cellular uptake compared to free sugars [125, 128]. Following Ac₄ManNAI

incorporation, MCF-7 cells bearing alkyne sialic acid residues were reacted with biotin-azide for 6 h in the presence of PA-Cu^I nanofibers, using a reaction mixture composed of biotin azide, PA-Cu^{II}, and sodium ascorbate.

The reaction mixture treated MCF-7 cells showed a strong labeling signal following the attachment of the biotin residue and staining with a fluorescent streptavidin-FITC probe. Cells treated with Cu^I-only were also labeled, while nontreated control groups were not labeled in the presence of the fluorescent probe (Figure 5.4). This result suggests that alkynyl-tagged mannose moieties were incorporated into cell glycans and could function as chemical tags for PA-Cu^I complex-mediated specific labeling after 6 h of reaction. After establishing optimum reaction times and PA-Cu^I nanofiber concentrations on fixed cells, we extended our study to the labeling of glycans in living cells. A bioorthogonal cell tracking experiment was carried out using the same procedure used for fixed cell labeling experiments, except that the cells were not fixed prior to the experiment. Labeling efficacy was monitored with confocal microscopy imaging following CuAACa reaction and fluorescent probe conjugation. Fluorescence microscopy observations demonstrated that alkyne-tagged glycoconjugates are able to successfully modify MCF-7 cells and that PA-Cu^I nanofiber administered group exhibited fluorescence levels above Cu^I-only group and nontreated cells, suggesting that the labeling of Ac₄ManNAI is specific and PA-Cu^I nanofibers could mediate adequate live-cell labeling reaction (Figure 5.5).

5.3.2. Fiber-Metal Interaction Analysis with Isothermal Titration Calorimetry

To verify metal peptide interaction, we measured the thermodynamic response and stoichiometry of Cu^{II} binding to PA using isothermal titration calorimetry (ITC). To minimize the potential for artifacts (i.e. precipitations and bubbles) in our binding assays arising from alkyl tail of PA, we analyzed only binding site composed of double histidine peptide moiety. Binding properties of single histidine to copper have straight forward interpretation, whereas inclusion of a second histidine introduces the possibility of cooperation in copper binding between the sites present. In our case, two copper binding sites were observed to be similar to previous studies [129]. For the active site-metal interaction, ITC binding isotherms were best fitted by a two-sets of site mode with K values; $K_1 = 5.11 \times 10^4 \pm 3.36 \times 10^4 \text{ M}^{-1}$ and $K_2 = 2.51 \times 10^3 \pm 2.11 \times 10^3 \text{ M}^{-1}$ (Figure 5.6). Binding affinity (c values) of each site was calculated as following; $c_1=19.44$ and $c_2=7.75$, which could be considered as moderate metal binding. According to this model, actual metal to PA stoichiometry remain controversial ($N_1=0.102$ and $N_2=1.03$), histidine rich protein and copper binding studies also did not indicate binding ratio due to the unique binding nature of histidine[129].

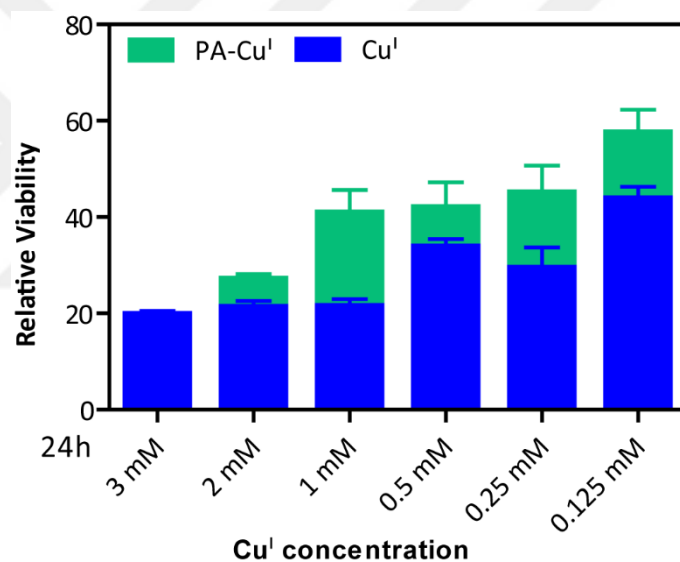
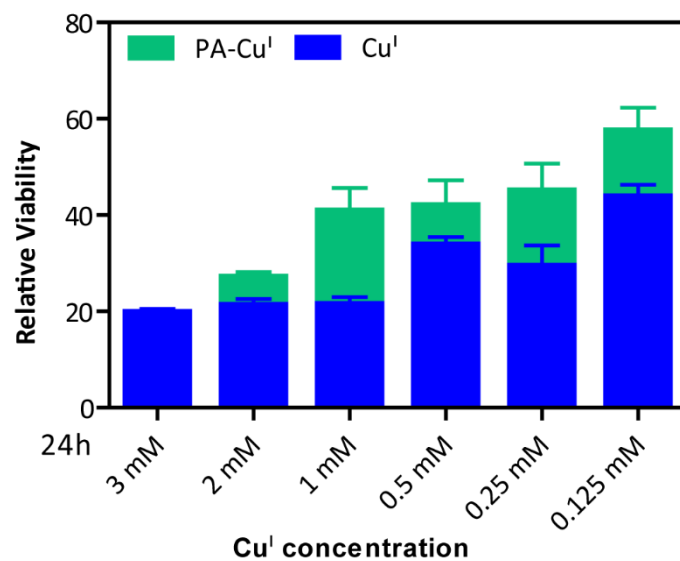


Figure 5.3 Viability of MCF-7 cells in the presence of PA- Cu^{I} and Cu^{I} . Alamar blue viability analysis of peptide-copper complexes, test was carried out after 6 h and after 24 h reaction.

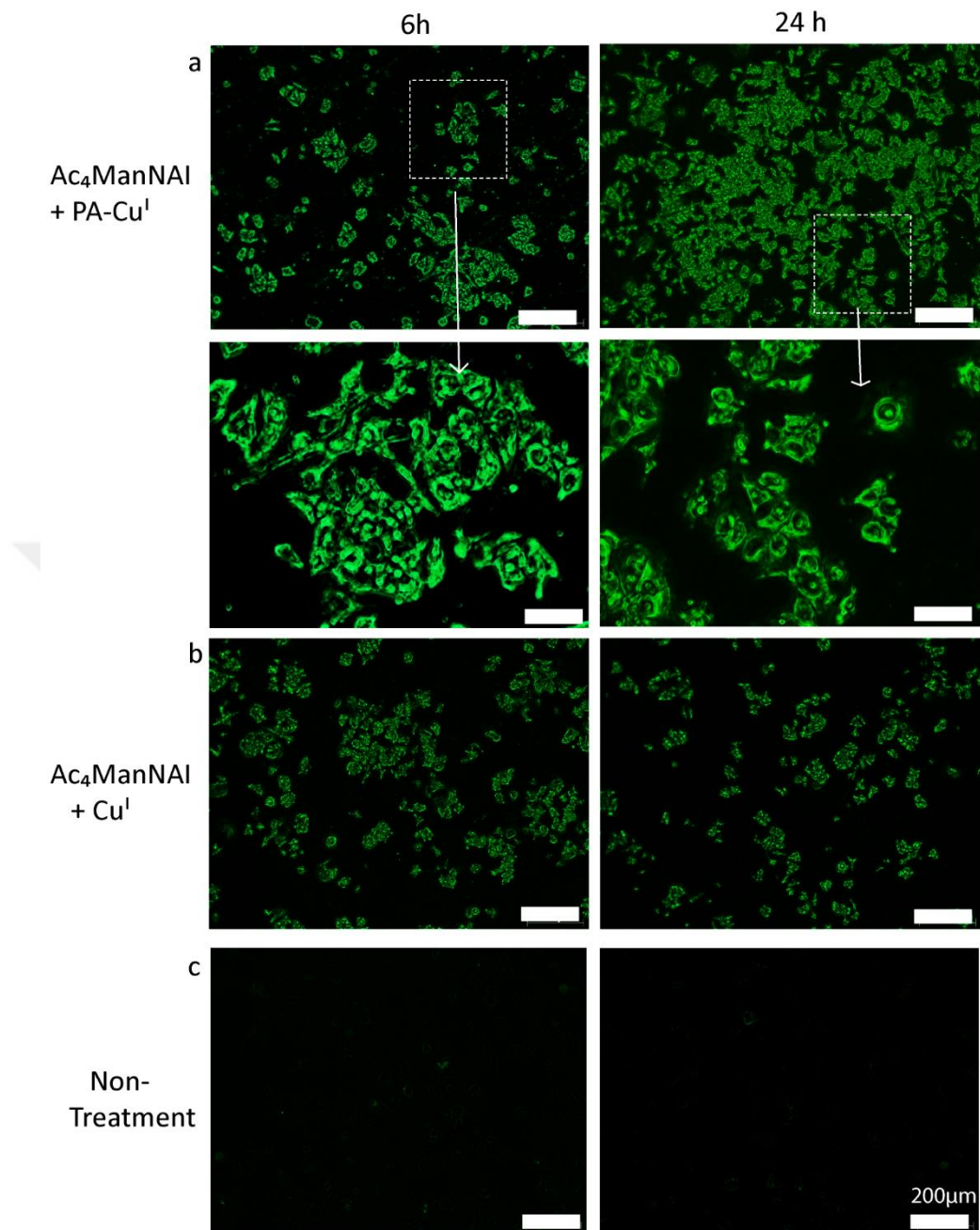


Figure 5.4 Microscopic analysis of fixed cells labeled with peptide nanofibers.

Fixed and sugar tagged cells were labeled nanofibers and control groups (a) PA-Cu^{I} , (b) Cu^{I} and (c) non-treatment group after 24 h reaction. (Scale bars = 200 μm)

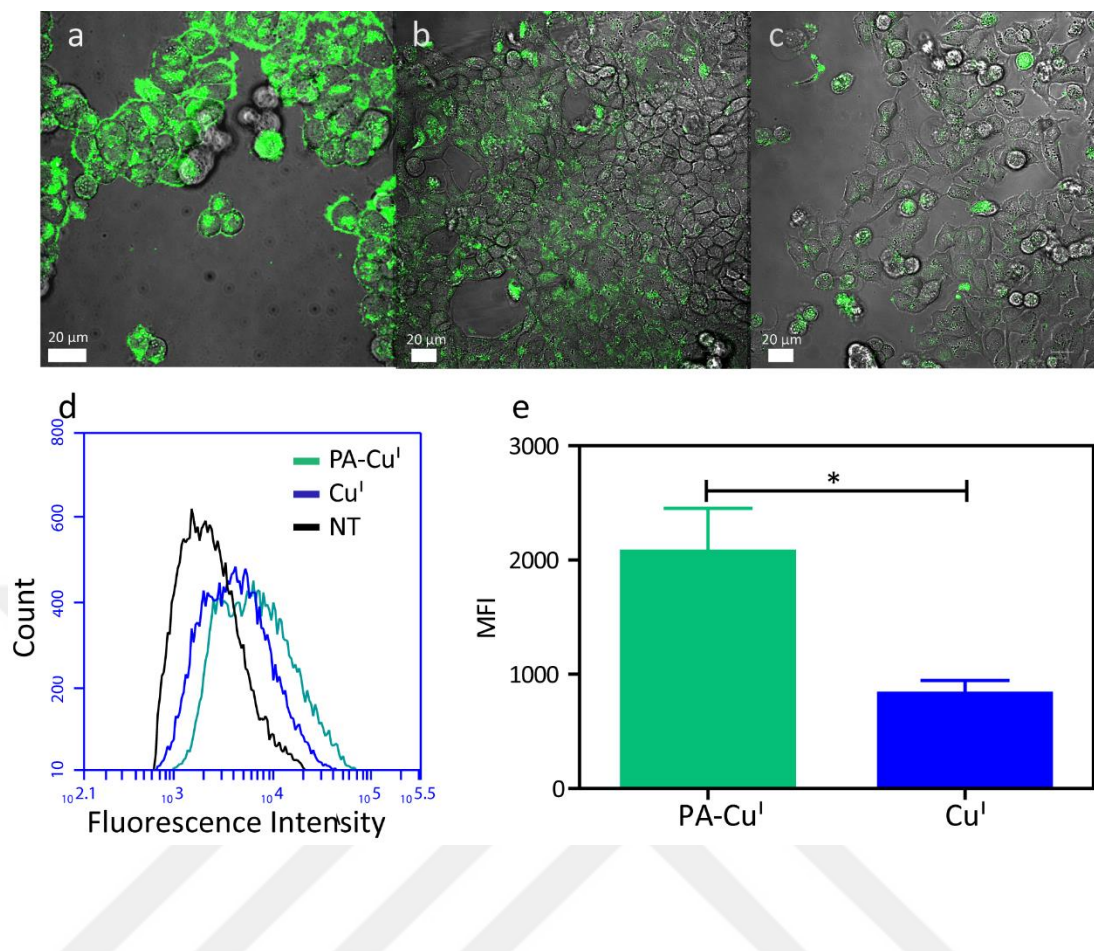


Figure 5.5 Analysis of cells labeled with a) PA-Cu^I complex b) Cu^I and c) non-treatment sample after 6 h reaction. Flow cytometry analysis graph of d) PA-Cu^I, Cu^I, non-treatment groups and e) quantitative analysis of labeling control groups was subtracted (only PA added sample for PA-Cu^I complex group and non-treatment sample for Cu^I group) in order to exhibit emission as a consequence of labeling.

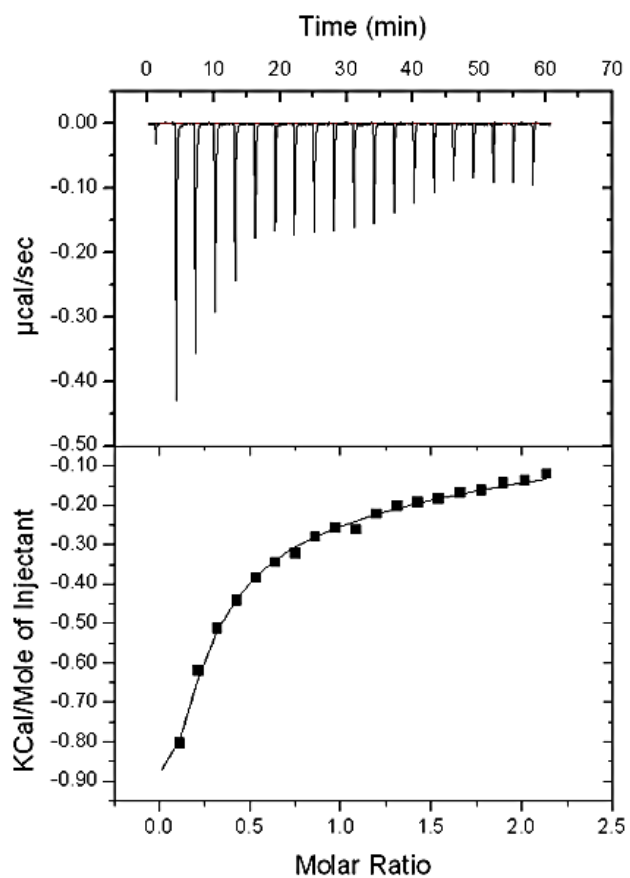


Figure 5.6 Isothermal titration calorimetry of Ac-HH-Am. CuSO_4 solution titrated to Ac-HH-Am peptide to evaluate metal binding kinetics of double histidine decorated peptide.

5.4. Conclusion

In conclusion, a supramolecular nanocatalyst was constructed for bioorthogonal click reaction mediated live-cell labeling. Designed biocompatible and biodegradable supramolecular PA nanofibers structure was administered as nanocatalysts for bioorthogonal reactions. Peptide nanocatalyst enabled reduced cytotoxicity caused by Cu^I ions and improved the reaction yields under physiological conditions. This *de novo* catalyst approach offers a promising and compatible method for investigating biomolecular dynamics and functions in cells and subsequently in living organisms due to its simple dynamics.

5.5. Experimental

5.5.1. Cell Culture and Maintenance

MCF-7 human breast adenocarcinoma cell line was used for *in vitro* cell culture experiments. All cells were cultured and propagated in 75 cm² cell culture flasks using RPMI medium 1640 supplemented with 10% Fetal Bovine Serum (FBS), and 1% penicillin/streptomycin. MCF-7 cells were grown at 37 °C in a humidified chamber supplied with 5% CO₂.

5.5.2. Cell Viability Assay

The cytotoxicity of PA-Cu^I was evaluated by using Alamar BlueTM assay (Invitrogen). MCF-7 cells were cultured in a 96-well plate with a density of 1×10^4 cells/well for 24 h. PA-Cu^I and only Cu^I control group were administered with copper concentrations of 3 mM, 2 mM, 1 mM, 0.5 mM, 0.25 mM and 0.125 mM for 6 h and 24 h (with n=4 per sample). After 6 h and 24 h of treatment, cells were washed with PBS and culture medium was replaced with serum free RPMI supplemented with 1% penicillin/streptomycin and 10% Alamar Blue solutions, after which the samples were incubated for an additional 4 h. Cell viability was quantified by measuring the fluorescence at 560/590 nm excitation/emission with a microplate reader (Molecular Devices Spectramax M5).

5.5.3. Microscopic Analysis of Fluorescent Labeling in Fixed Cells

Breast adenocarcinoma cells (MCF-7) were seeded onto 24-well plates (4×10^4 /500 μ L per well) containing glass coverslips, and were cultivated in 10% FBS/RPMI medium 1640. Growth medium was supplemented with 100 μ M Ac₄ManNA₆. Following incubation for 3 days, cells on coverslips were fixed and permeabilized with cold acetone for 10 min, then subjected to the probe labeling reaction: CuSO₄·5H₂O (0.125 mM), (+) sodium-L-ascorbate (1.25 mM), PA (0.125 mM), and biotin-azide (0.125 mM) at room temperature for 6 h. Subsequently, the fixed and labeled cells were washed with PBS and stained with fluorescein-conjugated streptavidin (5 μ g/mL in PBS) at room temperature for 30 min. All samples were counterstained with 1 μ M TO-PRO-3 (Invitrogen) in PBS for 15 min at room temperature and mounted with

Prolong Gold Antifade Reagent (Invitrogen). Fluorescent images were captured by Zeiss AxioCam™ fluorescence microscope.

5.5.4. Microscopic Analysis of Fluorescent Labeling of Living Cells

MCF-7 cells were seeded onto 6-well glass bottom plates (2×10^5 cells/well), and were cultivated in 10% FBS/RPMI medium 1640. After 24 h, appropriate groups were administered with 100 μ M Ac₄ManNAI and incubated for 3 days. Following 3 day Ac₄ManNAI treatment, the cells were washed with PBS and following reagents were applied for 6 h at 37 °C. PA containing labeling group was composed of CuSO₄ · 5H₂O (0.125 mM), (+) sodium-L-ascorbate (1.25 mM), PA (0.125 mM), and biotin-azide (0.125 mM). For only copper samples, the same reagents were used except PA. After labeling, cells were washed three times with PBS, blocked with 1% BSA (in PBS) for 20 min at room temperature, and streptavidin-FITC conjugate (5 μ g/mL in PBS /0.1 % FBS) was used for staining for 30 min at room temperature. Cells were washed two times with PBS and phenol-red free DMEM. Fluorescent images of cells were captured in 1 mL of phenol-red free DMEM by using confocal (Zeiss LSM510) microscopy system.

5.5.5 Flow Cytometry Analysis of Fluorescent Labeling

MCF-7 cells were cultivated in six-well plates (2×10^5 cells/well) in RPMI medium 1640 supplemented with 10% FBS and 100 μ M Ac₄ManNAI for 3 days at 37 °C. Cells were then harvested, washed with 0.1% FBS/PBS, and suspended (2×10^6 cells) in

200 μ L of click reaction solution ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.125 mM), (+)-sodium-L-ascorbate (1.25 mM), PA (0.125 mM), and biotin-azide (0.125 mM)). The reaction was incubated at 37 °C for 6 h. Following incubation, cells were washed twice with 0.1% FBS/PBS. Cells treated with reaction solution and control groups were stained with fluorescein-conjugated streptavidin (5 μ g/mL in PBS/0.1 % FBS) for 30 min at 4 °C, and washed three times with 1% FBS/PBS. Data were acquired by using BD Accuri™ C6 flow cytometer, and were analyzed by BD Acuri C6 software.

5.5.6. Isothermal Titration Calorimetry Analysis

0.3 mM of Ac-HH-Am was titrated by 3 mM of CuSO_4 solution using iTC200 system (MicroCal, GE Healthcare). The titration was performed at 25 °C with 800 rpm stirring speed. Twenty injections were done, where the injection period was 4 s and the space between injections was 180 s. ITC binding isotherms were best fitted by a two-sets of site mode.

CHAPTER 6

Collagen Mimetic Peptides

6.1. Objective

The aim of the project is to enhance our understanding of collagen mimetic structures and their function to modulate cellular processes and to improve its biological function for biomedical applications. For this purpose, a collagen mimetic short PA was designed from tandem repeat of Pro-Hyp-Gly artificial collagen sequence. Interaction among monomeric units on peptide fiber surfaces was used to create *de novo* collagen mimetic structures for biological applications. Collagen monomer unit-mediated microenvironment is promising for regeneration studies, especially chondrogenic differentiation due to the collagenous structure of cartilage and collagen is important source both as a scaffold and as a bioactive material. Strikingly, our collagen mimetic PA enhanced the chondrogenic differentiation of mesenchymal stem cells without any chondrogenic supplement. The monomeric collagen sequence on peptide fibers lead to multifibrillar nanostructures and induce chondrogenic differentiation by using physical and biochemical cues. This new method offers simple preparation and effective regeneration without the need of external stimuli, therefore this study is promising for future *in vivo* cartilage regeneration studies.

6.2. Introduction

Collagen has fibrous triple-helix structure which is the most abundant component in extracellular matrix and plays an essential role in maintaining the structural integrity

of tissues and in cell development. The triple helix motif found in collagen is unique, highly specific and commonly occurs in biological activities. In addition, the application range of native collagen is limited due to immunologic, pathologic and physical characteristics (weak scaffold with low mechanical strength, shrinkage, large porosity) draw-backs. Hence, the requirement of collagen like structures which can be applied noninvasively, is gaining interest in the field of biomaterial engineering, to obtain enhanced bio functional uses. Collagen mimetic materials are also recognized as promising biomaterials in self-assembled tissue engineering and regenerative medicine. A large portion of studies have been done to develop artificial collagen yet, these researches are still at early stage to construct collagen mimetic fiber to mimic its high-level structure and bio functions.

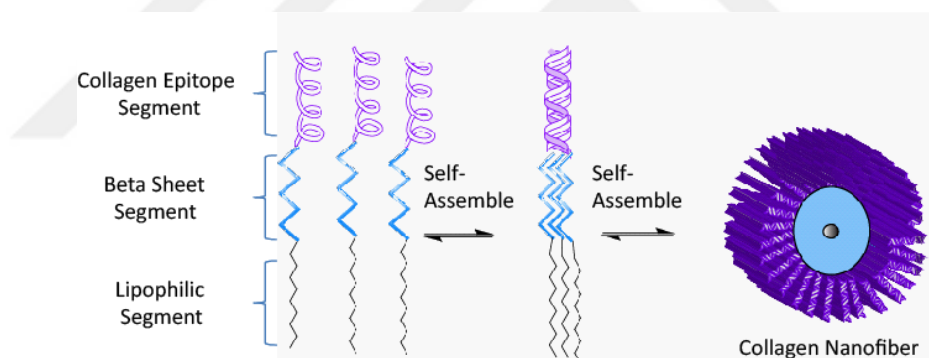


Figure 6.1 Self-assembly of collagen mimetic peptide nanostructure. Schematic illustration of collagen mimetic peptide assembly and fiber formation.

It is known that collagen is not only an ECM structural protein but also induces many vital activities such as it is one of the main component of cartilage. Collagen like cell fate regulator components based bio scaffold or biofilms designed from bio-active peptide sequences, could mimic ECM in its chemical compositions comprising a wide range of bio inductive materials. In addition, collagen can provide proper

microenvironment for stem cell differentiation studies and cartilage matrix production since it contains various significant domains like; cell binding, growth factor binding, and enzymatic cleavage sites. Such designs, mimicking porous and fibrous ECM will mediate signaling and support cell growth. These structures can also be used for induction of tissue production. Therefore, we seek to use this novel construct for cartilage regeneration, in particular MSC chondrogenesis.

Thus, development of ECM analogues and cartilage regenerative which can be used in clinical support needing effective tissue repair back up. Starting from these information, ECM mimicking PA scaffolds is might be useful resolution of the above-mentioned issue. A designed new artificial bio scaffold which are based on highly helical collagen (-Pro-Hyp-Gly-) structure to provide cartilage healing with or to obtain enhanced healing. This system will be supportive for quick regenerative healing therapy by using collagen like (structural or signaling) components. Cost effective and reusable materials are in demand as they provide better chances of infrequent applications though damaged area. Easy synthesis and regenerative active sequence attached and fast and cost-effective PA molecules are expected to be an effective remedy in chronic cases.

6.3. Results and Discussion

6.3.1. Collagen Mimetic Peptide Synthesis and Regeneration

Firstly, monomeric CPA (Figure 6-2) was synthesized by standard solid phase Fmoc peptide synthesis chemistry. In this study, a collagen-mimetic CPA was prepared by conjugating a PA with a collagen-mimetic peptide monomer derived from tandem repeat of Pro-Hyp-Gly sequence. Lauryl-VVAGK-Am was utilized sequence of PA

self-assembly segment for self-assembly and ionic lysine for solubility- and (Pro-Hyp-Gly or POG) was utilized as the specific amino acid sequence which generates a triple helix conformation that resembles the native protein structure of natural collagens. Whole sequence is Lauryl-VVAGKPOG-Am (MCPA). Lauryl-VVAGE (E-PA) peptide will be used as gelator as a counter ion with same beta-sheet forming segment. After gel formation with counter peptide or pH adjustment, secondary structure formation was characterized by circular dichroism (CD), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and rheology to reveal main properties of structure (Figure 6.4, 6.8 – 6.10). Nanofiber formation was obtained by using both spectroscopic and microscopic methods as explained in previous chapters. Rheology results also showed regular peptide hydrogel mechanical characteristics (storage modulus and loss modulus) (Figure 6.5). Additionally, thixotropic test was carried out to understand collagen like properties of designed structure (Figure 6.6). Interestingly, gel recovery was found to be less compared to collagen monomer excluded control group gel. After structural destruction, gel recovery was lowered due to broken molecular interaction among monomer groups. In previous studies, covalently cross-linked gels or collagen itself showed similar low recovery trend and these results indicated, that crosslinking-like ability of POG monomer units along fibers form collagen like structure with better mechanic stiffness and availability manipulation of peptide hydrogel.

The collagen specific amino acid sequence was elongated with five repeat Lauryl-VVAGK(POG)₅-Am (QCPA) for upgrading collagen formation and signal. This elongated construct was characterized with the same methods applied for monomeric CPA (CD, TEM etc.). However, due to the presence of two different secondary

structures (beta-sheet and triple helix) forming sequence inclusion in peptide sequence caused instability and thereby lowered gel stiffness. Thus, this molecule was not considered for further applications since it did not exhibit any superior characteristics compared to monomeric construct.

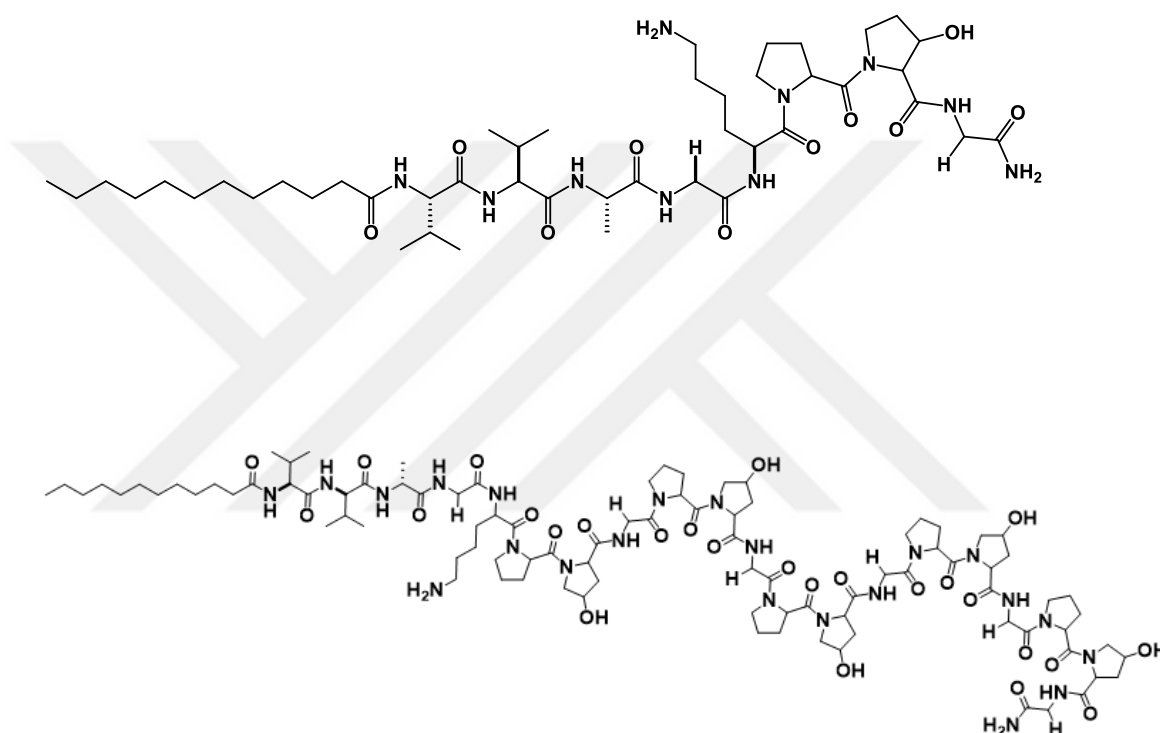


Figure 6.2 Chemical structures of Lauryl-VVAGKPOG-Am (MCPA) and Lauryl-VVAG(POG)₅-Am (QCPA) peptide nanostructures. Molecular formula of designed peptides and depiction of their molecular structures.

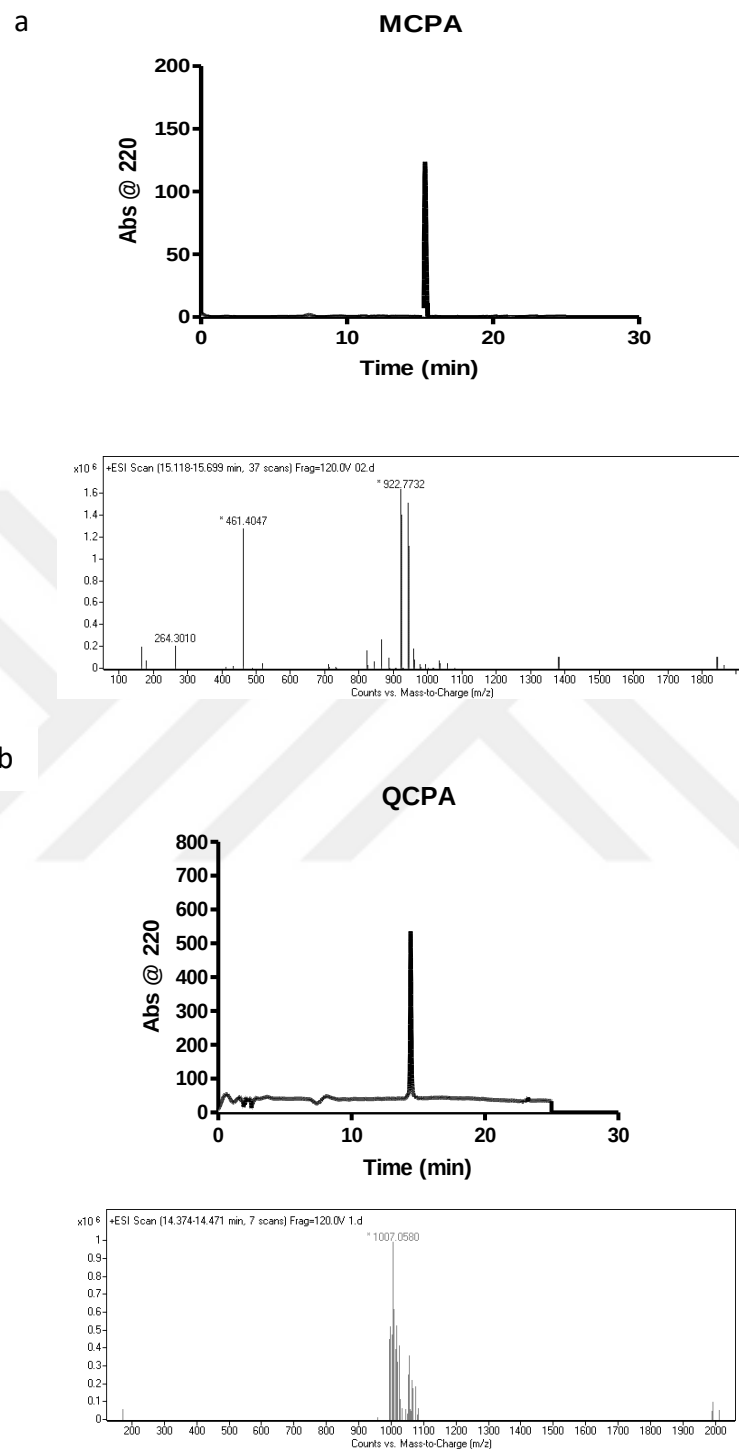


Figure 6.3 Lc-MS results of collagen mimetic peptides. Liquid chromatography and mass analyses of designed peptide molecules.

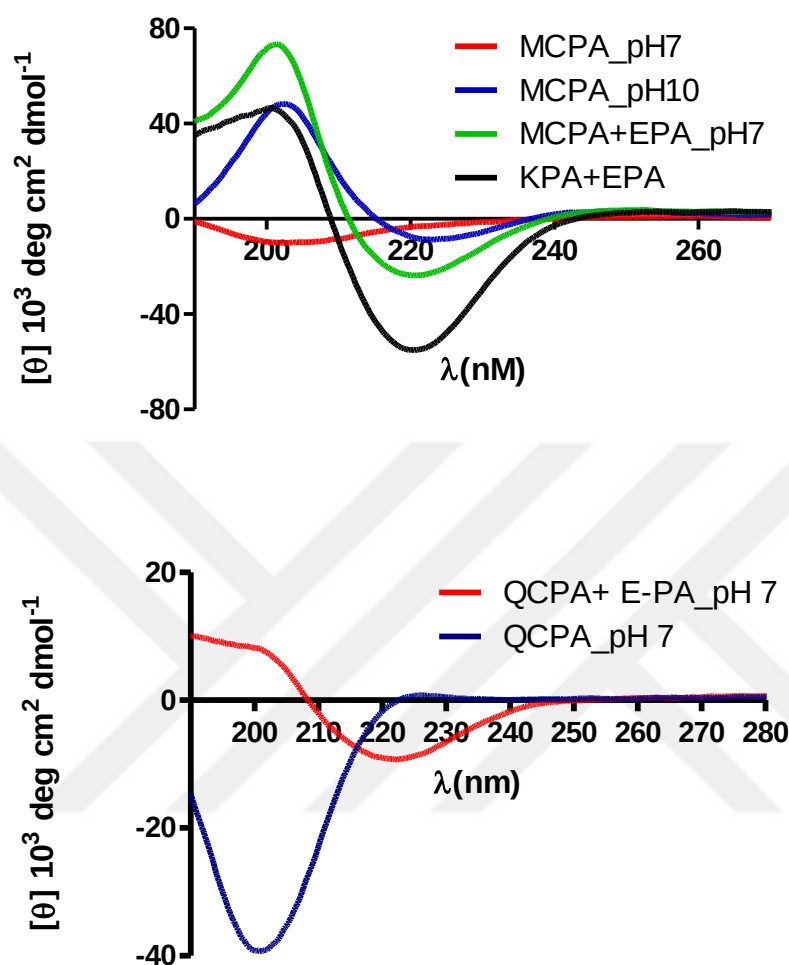


Figure 6.4 Circular dichroism spectra of collagen mimetic peptides. Investigation of secondary structures by the addition of counter peptides and pH adjustment.

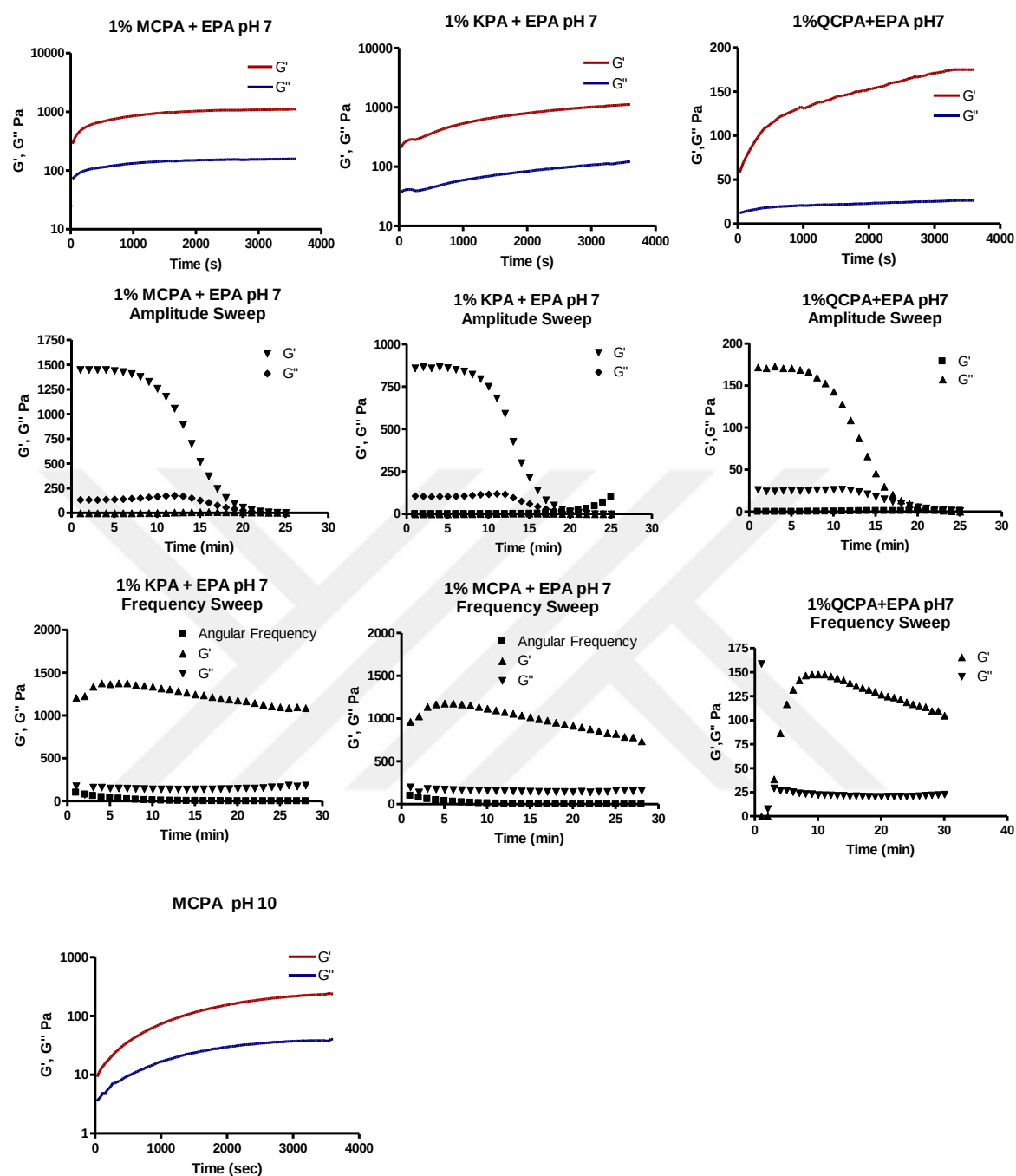


Figure 6.5 Viscoelastic Characterization of collagen mimetic gels with rheometer.

Time, amplitude and frequency sweep analysis of peptide gels with oscillatory rheometer analysis.

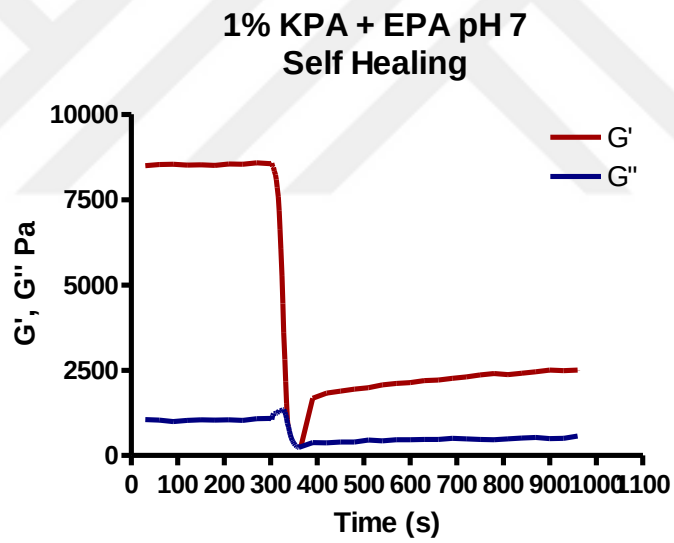
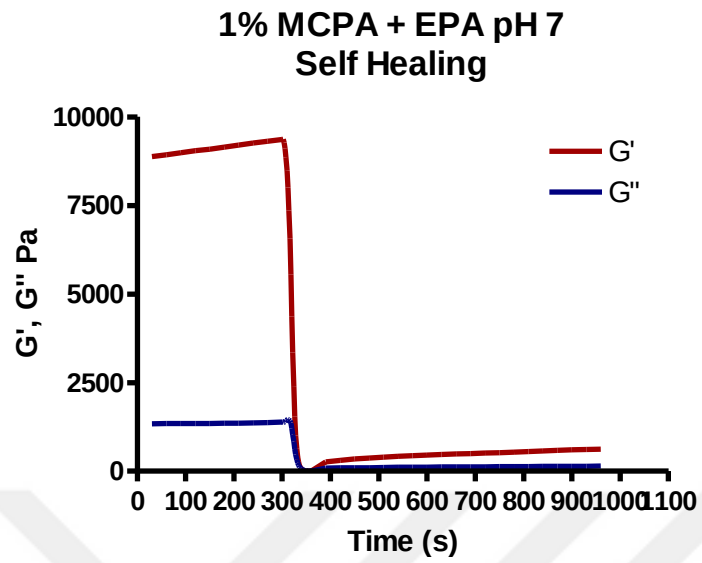


Figure 6.6 Thixotropic rheology analysis for testing recovery capacity of MCPA.

After gel denaturation recovery capacity of gels were tested by thixotropic test of oscillatory rheology.

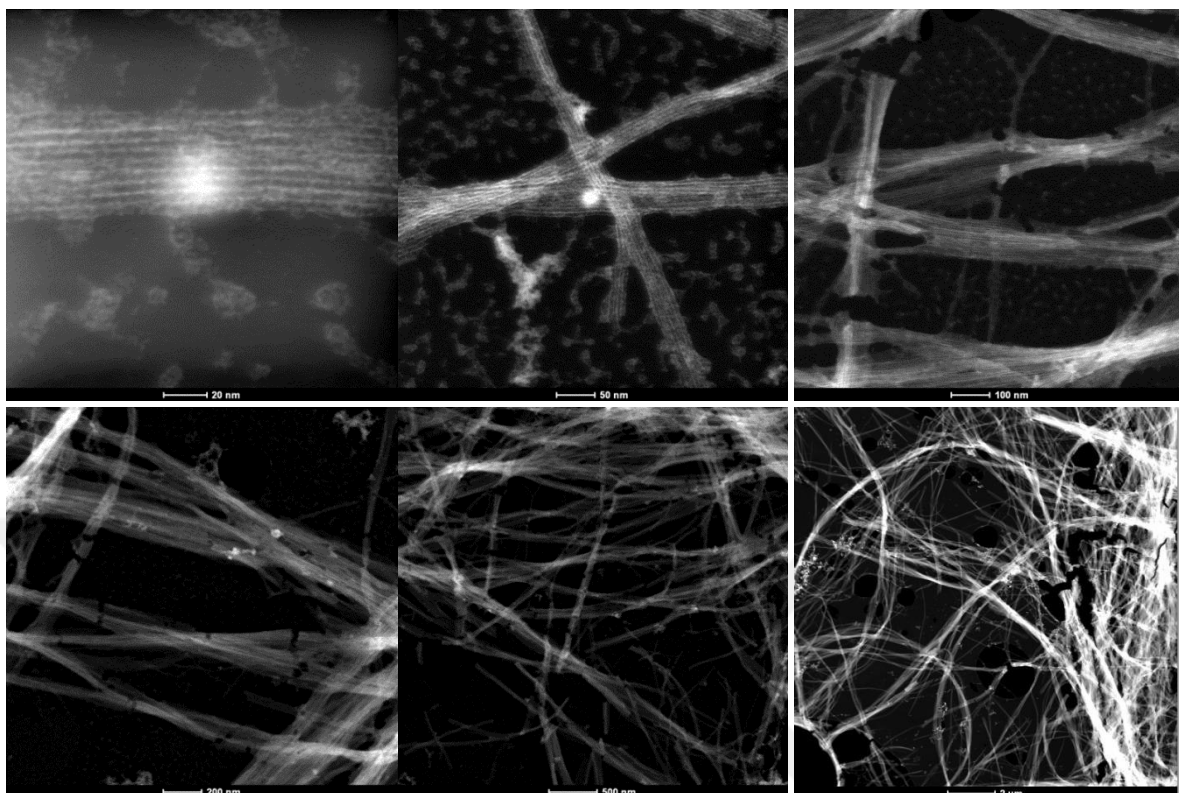


Figure 6.7 TEM image of MCPA bundled fibers. Peptide nanofibers was formed with pH adjustments (pH=10) and collagen like fibers were imaged through TEM.

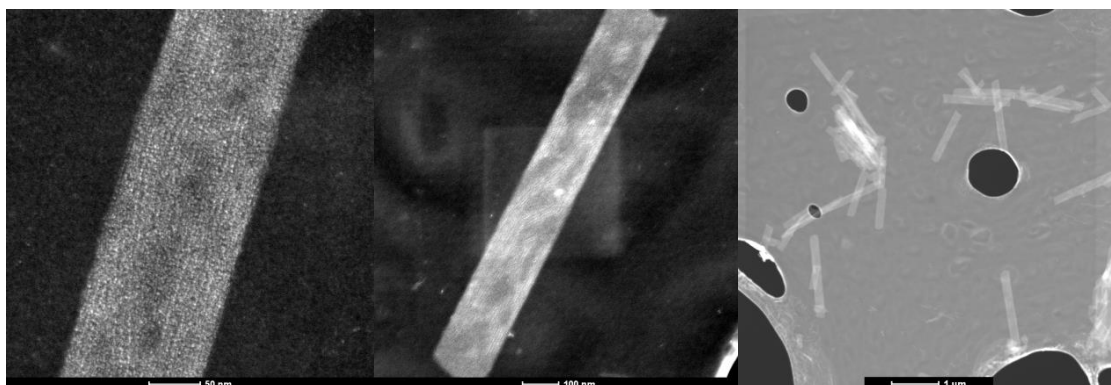


Figure 6.8 TEM image of MCPA bundled fibers formed with E-PA addition.
Peptide nanofibers were formed with addition E-PA and collagen-like fibers were imaged through TEM.

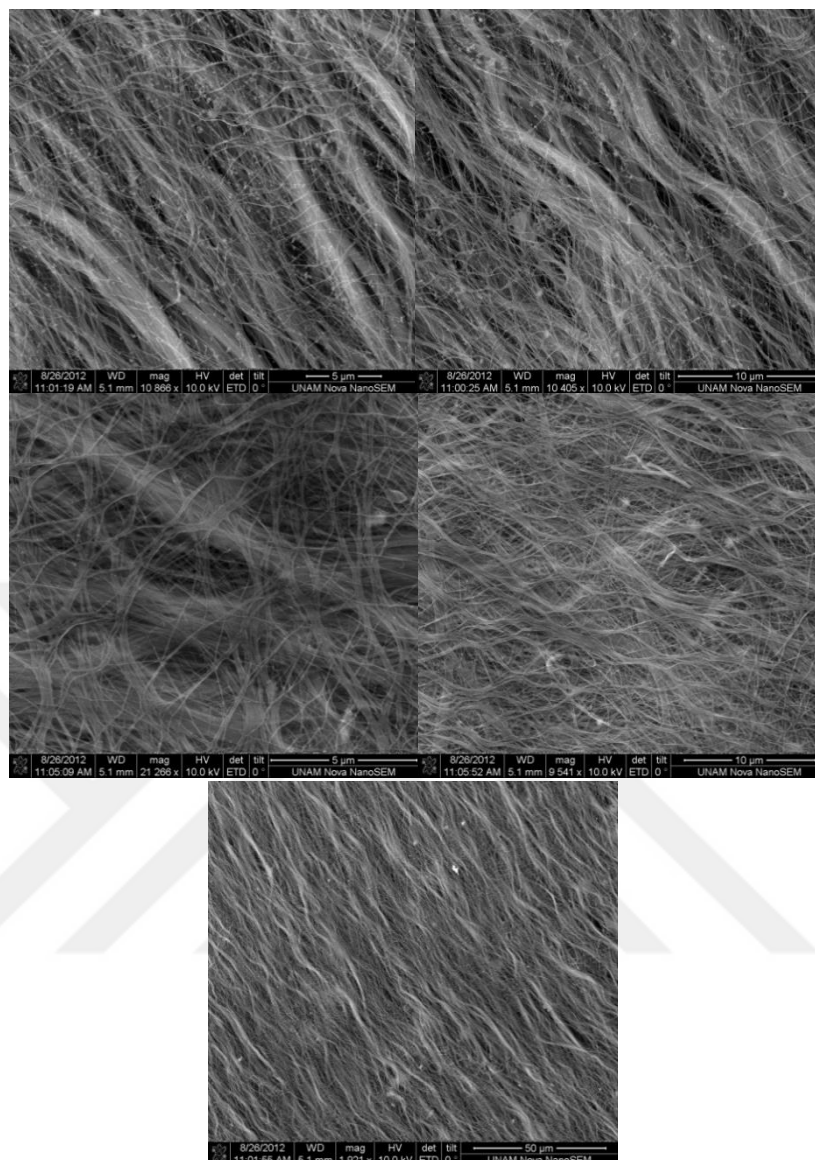


Figure 6.9 SEM image of MCPA bundled fibers formed with pH adjustments (pH=10). Peptide nanofibers was formed with pH adjustments and collagen like fibers were imaged through SEM.

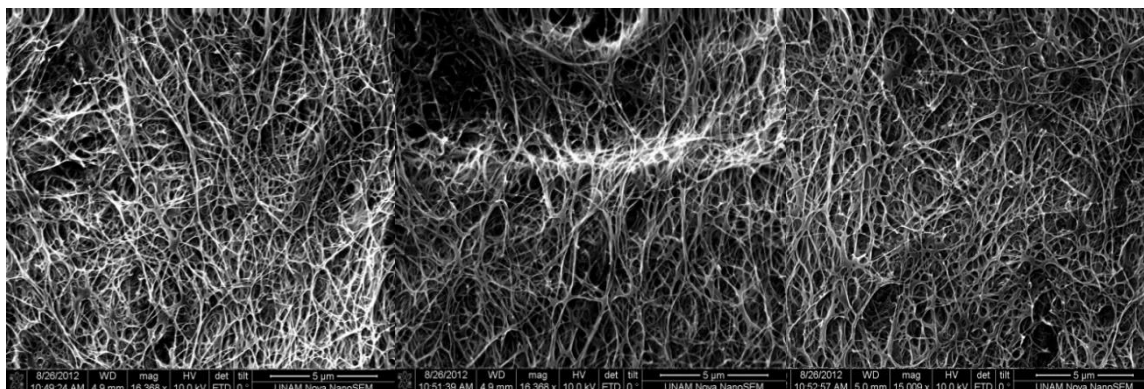


Figure 6.10 SEM image of MCPA bundled fibers formed with pH adjustments.

Peptide nanofibers was formed with pH adjustments (pH=10) and collagen like fibers were imaged through SEM.



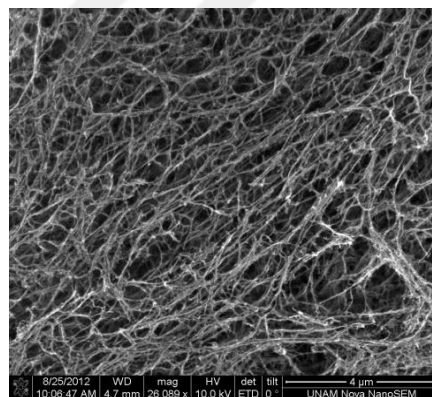
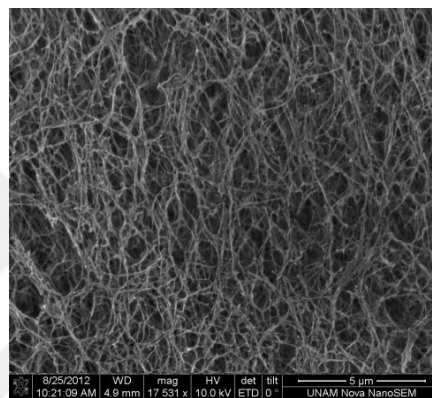


Figure 6.11 SEM image of MCPA bundled fibers formed with E-PA addition.

Peptide nanofibers was formed with addition E-PA and collagen like fibers were imaged through SEM.

6.3.2. Chondrogenic Differentiation Induction with Collagen Mimetic Peptide

Nanostructures

As application part of this project, after full characterization of the material, we carried out biocompatibility and regeneration capability of our peptides by using mesenchymal stem cells (Figure 6.12). Collagen has broad a range of medical applications, such as artificial skin, articular cartilage, and bone due to their biodegradation, cell scaffold characteristics and strong gel formation. In particular, we tested cartilage regeneration potential of the artificial collagen system.

Remarkably, we observed cartilage like cell accumulation and Safranin-O staining was applied qualify direction of stem cells into cartilage (Figure 6.13-6.15). Investigations could be extended to multicomponent containing smart design, for example drug loading and enzyme responsive. Strikingly, the collagen mimetic PA enhanced the chondrogenic differentiation of mesenchymal stem cells without any chondrogenic supplement.

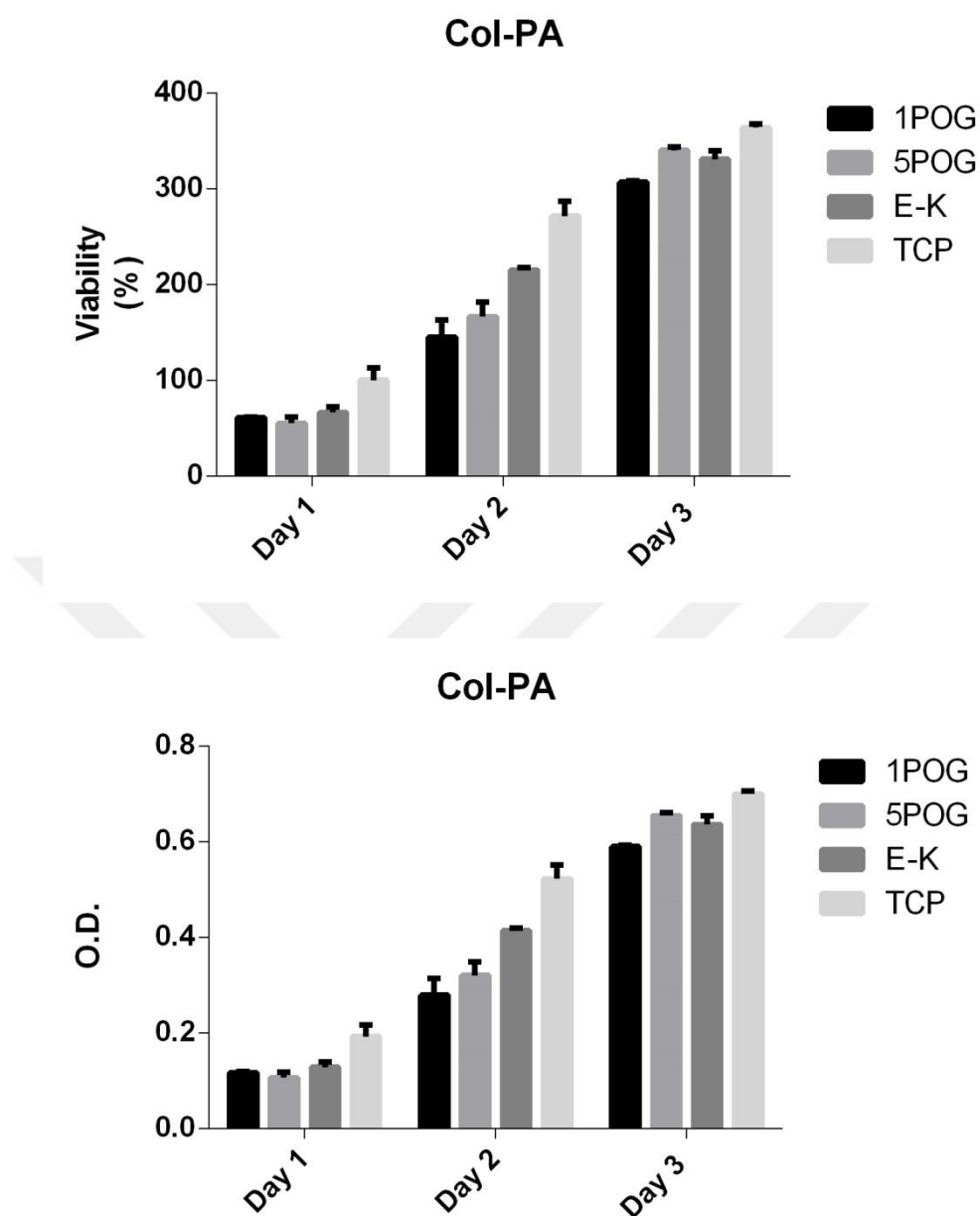


Figure 6.12 Viability analysis of rMSCs cultured on collagen mimetic peptide.

Representative Calcein Am (Live=green) and ethidium homodimer (Red=dead) staining of cells seeded on collagen mimetic peptide coatings, control peptide coatings and tissue culture plate.

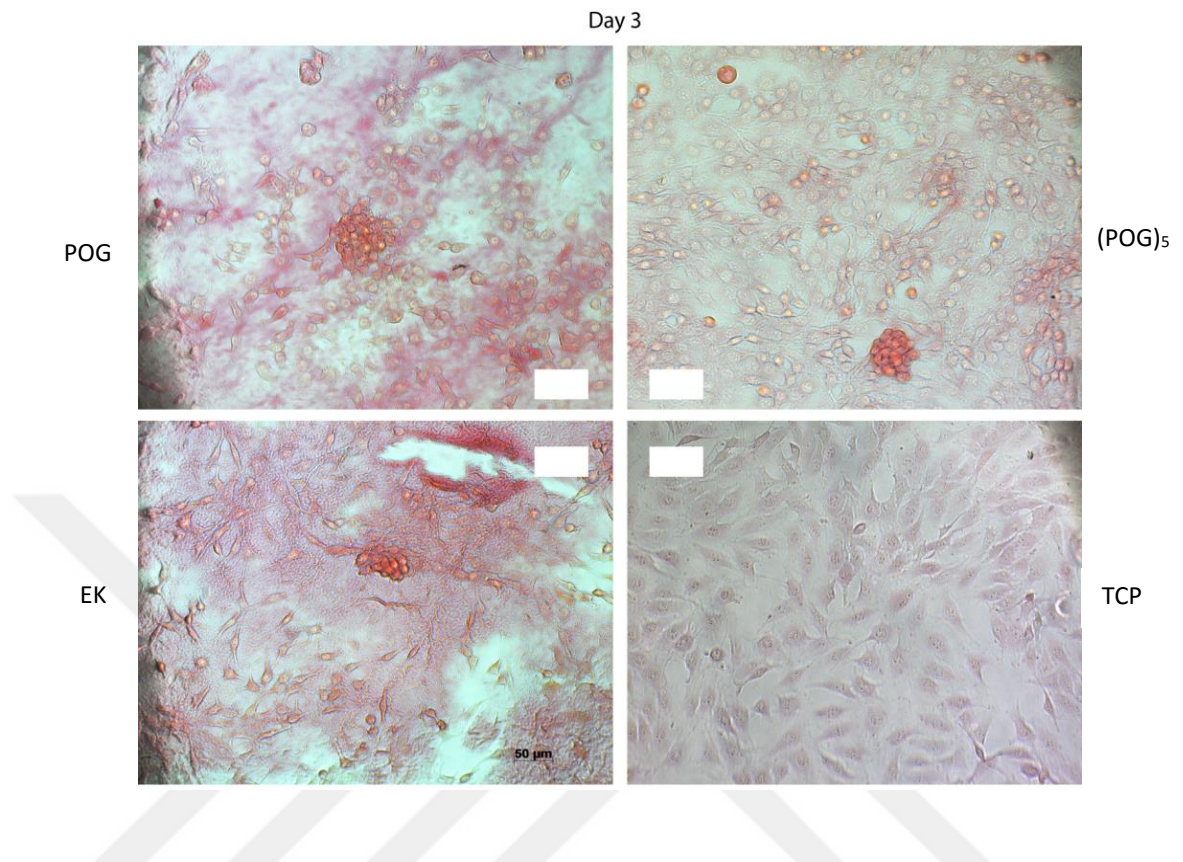


Figure 6 .13 Day 3 Safranin-O staining of rMSCs cells cultured on pPA coatings. Cartilage specific Safranin-O staining was carried out to observe linages of rMSCs on collagen mimetic peptide coatings, control peptide coatings and tissue culture plate (n = 3, scale 50 µm).

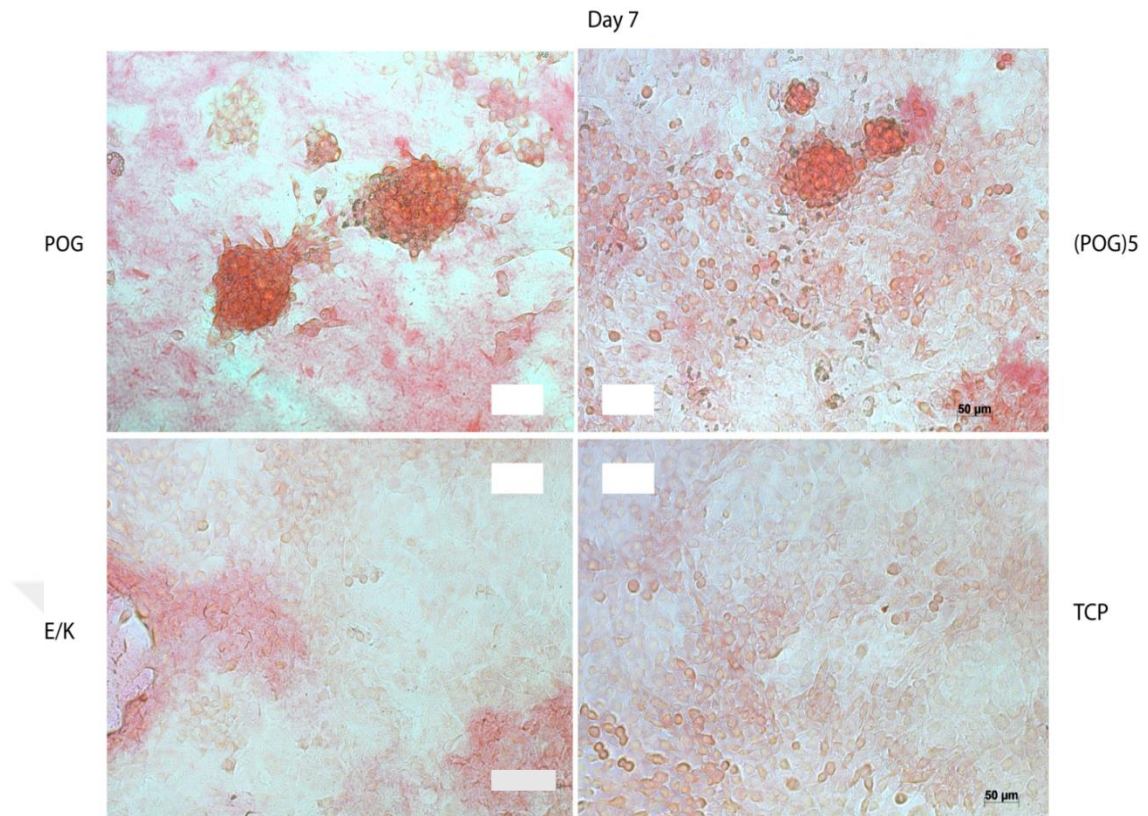


Figure 6.14 Day 7 Safranin-O staining of rMSCs cultured on collagen PA coatings. Cartilage specific Safranin-O staining was carried out to observe linages of rMSCs on collagen mimetic peptide coatings, control peptide coatings and TCP (n = 3, scale 50 μ m).

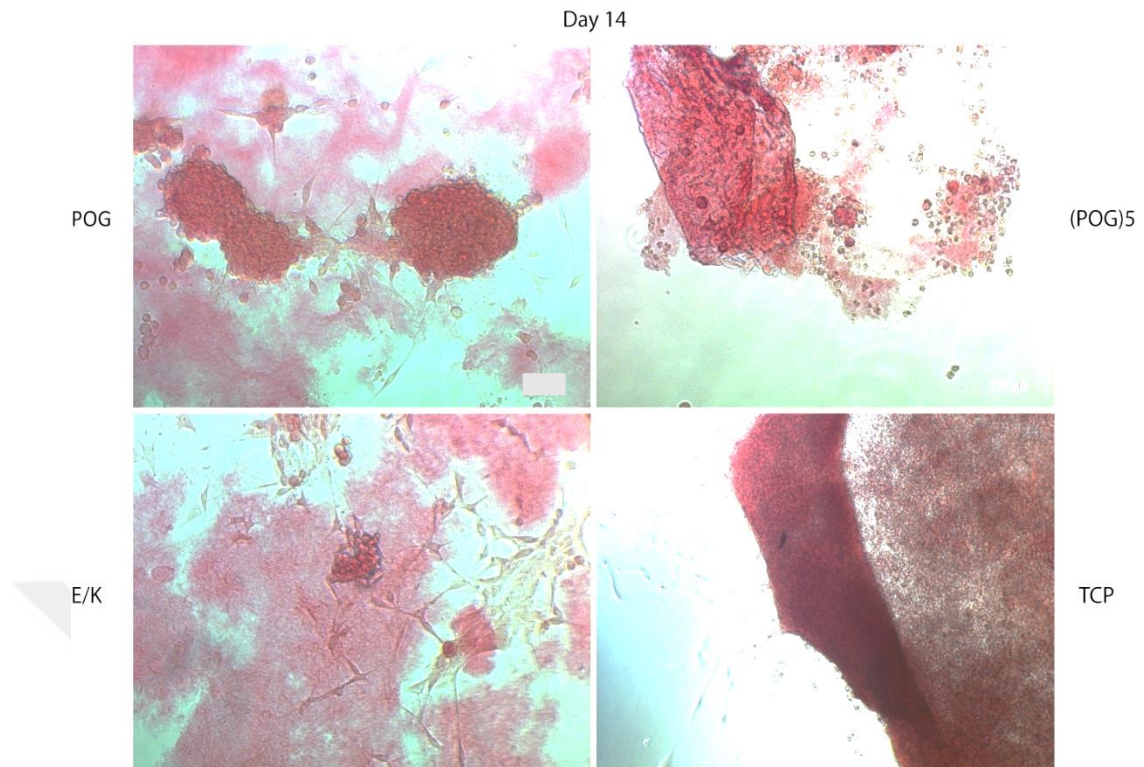


Figure 6.15 Day 14 Safranin-O staining of rMSCs cultured on collagen PA coatings. Cartilage specific Safranin-O staining was carried out to observe linages of rMSCs on collagen mimetic peptide coatings, control peptide coatings and TCP (n = 3, scale 50 μ m).

6.4. Conclusion

In this chapter, we designed and characterized a new type of collagen mimetic material by combining two different approaches in one method. Collagen monomeric unit conjugated β -sheet forming peptides self-assembled into nanofibers resembling the fibrous structure of native collagen and characterizations have elucidated structural properties of artificial nanostructures. Besides unique characteristics of this novel molecule, collagen mimetic peptide nanostructures induced differentiation of rMSCs into chondrogenic lineage. According to cartilage specific Safranin-O staining collagen mimetic peptide have exhibited better differentiation compared to active group free control group. This study could be extended with in vivo cartilage defect studies and also hydrogel structure of material could be considered for multicomponent studies like drug or bioactive materials loading for further applications.

6.5. Experimental Section

6.5.1. Peptide Synthesis and Characterization

Peptide synthesis and characterization was carried out with the same procedure explained in previous chapters. Collagen peptides was constructed on MBHA Rink Amide resin at 0.25 mmol scale. Amino acid couplings were done with 2 equiv of Fmoc-protected amino acid, 1.95 equiv of HBTU, and 3 equiv of DIEA for 2 h. Fmoc removal was performed with 20% piperidine/dimethylformamide (DMF) solution for 20 min. Cleavage of the peptides from the resin was carried out with a mixture of TFA:TIS:H₂O in the ratio of 95:2.5:2.5 for 3 h. Excess TFA was removed by rotary

evaporation. The remaining viscous peptide solution was triturated with ice-cold ether and the resulting white product was freeze-dried.

6.5.2. *CD Spectroscopy*

Peptide solutions were dissolved in water. Each peptide sample was prepared by adjusting pH or mixing with E-PA and was incubated for 15 min to observe nanofiber formation. The samples were diluted with water to final concentration of 1×10^{-4} M. Used parameters; DIT 4 sec, Band width 1.00 nm, Data pitch 0.2 nm, Start mode 1 mm, Scanning mode continuous.

6.5.3. *Rheology Analysis of Materials*

Oscillatory rheology measurement was performed with Anton Paar Physica RM301 s for determining the viscoelastic properties of collagen materials. 25 mm parallel plate configuration was used for Rheometer operating at 25 °C. 125 μ L of each sample was used, and 250 μ L of total volume with a final peptide concentration of 1 wt % was carefully loaded on the center of the lower plate and incubated for 15 min before measuring. After equilibration, the upper plate was lowered to a gap distance of 0.5 mm. Storage moduli (G') and loss moduli (G'') values were screened from 100 rad/s to 0.1 rad/s of angular frequency, with a 0.5% shear strain.

6.5.4. Cell Culture and Maintenance

The rMSCs were seeded in passage numbers between 3 and 7. rMSCs were maintained in 225 cm² flasks in Dulbecco's Modified Eagle's Medium (DMEM) included 20% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultivated at standard humidified incubators with constant 5% CO₂ at 37 °C. Trypsin/EDTA chemistry was used for cell detachment at about 75% confluency.

6.5.5 Safranin-O Staining

After cell seeding on peptides and incubation, cells were washed with PBS and fixed with 10% formalin for 15 min at room temperature. Following fixation cells were washed with PBS again, 1% BSA /PBS blocking reagent was added and blocking was proceeded for 30 min. Cells were treated with 100 µL solution of 0.1% (w/ v) Safranin-O in 1% (v/v) acetic acid for 5 min at room temperature. Unbound dye was removed by excessive washing with 1% (v/v) acetic acid.

CHAPTER 7

Conclusions and Future Perspectives

Peptide based materials provide a remarkable toolbox for designing nature inspired smart materials. As in the usage of toolboxes, synthetic peptide materials could be manipulated with expedient tools allowing wide range of applicability in medicine and industry. In these new days of technology, there has been an ever growing need to use practical synthetic materials. Increasing demand of complex materials with facile preparation methods, bioinspired smart materials gain more importance for achieving multifunctional hybrid materials.

Bioinspired designs can be developed by using combination of functional moieties from multiple sources. In particular, biological cues can be integrated with supramolecular designs to generate bioactive materials. Sometimes multiple functions from a single source can also be tagged to supramolecular constructs. For example, we here proposed that functions of ALP on catalysis and biomineralization could be combined in one design in order to form the closest mimic for ALP . Regarding bioactivity of ALP, designed biomaterial was proven to be practical approach for obtaining phosphatase like activity including, phosphate ester catalysis, ability of biomineralization and cell differentiation induction. Ease of the use of designed material enabled us to apply them for 2D and 3D cell culturing techniques with multiple cell lines.

Nature is an open source for new material research, and assembly driven dynamic structure of enzymes gave us another clue for the next design. By taking advantage of dynamic features of self-assembling peptide nanostructures, catalytic nanofibers were

constructed from separate action units which forms active catalytic pocket after self-assembly. Instead of covalently linked active residues, we applied self-assembly approach on artificial catalysis in order to generate assembly-driven catalytic domain for efficient catalysis. Catalytic triad was used as the model substrate for this study and three residues found in triad activity were individually incorporated to assembling precursors. After assembly, significant activity was observed against model and natural substrate.

Besides enzyme-like constructs, enzyme driven scaffolds are also remarkable for establishing adaptive materials. Inspired from dental sialophospho protein (DSPP), phosphoserine decorated peptides were designed. Great repulsion among phosphate group blocked self-assembly and mineralizable scaffold was obtained by the addition ALP. In addition, assembly could be induced by multiple triggers, and calcium ion was also another important factor to initiate self-assembly. Mineralization on dentin surface was supported with multi trigger initiated mineralizable fibrous mesh. Additionally, these structures supported odontoblast differentiation regarding the DSPP-like characteristics.

As mentioned above peptide nanostructures can be manipulated according to desired function, and besides bioactivity alterations, peptide nanofibers can be used as ligand for metal stabilization during metal mediated chemical reaction. Click is one of the metal (copper) mediated reaction and oxidative stress caused by copper has limited its use in biological applications. Copper ligands were used to solve this problem and cell labeling technique was created by using copper mediated click chemistry, called native chemical ligation. Chemical group functionalized on living cell surface can be tagged with fluorogenic units by using copper stabilizing nanofiber ligand. Copper chelating

groups were decorated on peptidic framework to form assembly driven ligand for copper. Nanofibrous structure lowered intrinsic toxicity and also multiunit recruiting architecture of peptide nanofibers enhanced reaction rate.

Not only functional also structural properties should be considered for establishing next generation materials. In this context, collagen has unique architecture and is very promising for new application areas. Small monomeric units of collagen were integrated to peptide units to create inter-fibrillar interaction after self-assembly, it might be called as non-covalent crosslinking. This novel collagen-like design was characterized and applied to cartilage regeneration studies. This new material is open to further development and applications.

In conclusion, there is always room for improvement in material studies to reach desired performance. Using functions from different sources or multiple functions of single source, using only interactions or only structure can open us new paths to understand complex and dynamic nature and to achieve better envisagement.

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