İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

GOLD BINDING PEPTIDE FUSED ALKALINE PHOSPHATASE FOR REAL TIME MONITORING OF CALCIUM PHOSPHATE BIOMINERALIZATION

M.Sc. Thesis by
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Department : **Molecular Biology and Genetics**

Programme : **Molecular Biology-Genetics & Biotechnology**

JANUARY 2012

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JANUARY 2012

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ALTINA ÖZGÜL BAĞLANAN PEPTİT İÇEREN ALKALİN FOSFATAZ ENZİMİ İLE KALSİYUM FOSFAT BİYOMİNERALİZASYONUNUN GERÇEK ZAMANLI İZLENMESİ

YÜKSEK LİSANS TEZİ

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FOREWORD

The divine aim for human being should be involving in the contribution for the improvement of civilization. It may be sounds naive since this notion have already fell out of favor thus consequently lead to being perceived as a deprecating attitude to zeitgeist. Although it is obvious that mediocrity have infected all of the world without discriminating intellectual occupations, there are still some people who resist to the devastating result of extinction. This thesis is a product that could be accrued with the help of these people.

I feel indebted unless I acknowledge my supervisor Prof. Candan Tamerler who inspired me with a never-ending energy. I am also grateful to Prof Mustafa Ürgen who is one of the most generous and patient advisors in academic world. I should acknowledge to Prof İlhan A. Aksay with tremendous reverence who advised me to study biomimetics at ITU. I want to thank my mentors Urartu Özgür Şafak Şeker as an example of serious and profound professor candidate and to Sibel Çetinel as an interesting design of nature. I should also thank to all faculty of ITU Molecular Biology and Genetics department, my colleagues, labmates and friends in ITU MOBGAM for their support and fruitful discussions which were without doubt meritous intellectual pleasures.

At last, if there are someone to be acknowledged, those are my family not only leading to me to study in academic world but also more importantly, by enduring to a curious mind but never held this against me throughout these years. In today's philistine world their contribution is admittedly invaluable.

February 2012 H. Burak Çalışkan

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ABBREVIATIONS

AFM : Atomic Force Microscopy
AMP : Amorphous Calcium Phosphate
AP : Alkaline Phosphatase
AuBP : Gold Binding Peptide
DPN : Dip Pen Nanolithography
EDX : Energy Dispersive X-ray
ELISA : Enzyme Linked Immunosorbent Assay
FPLC : Fast Protein Liquid Chromatography
GBP : Gold Binding Peptide
GEPI : Genetically Engineered Peptides for Inorganics
GFP : Green Fluorescence Protein
HA : Hydroxyapatite
HABP : Hydroxyapatite Binding Peptide
IEX : Ion Exchange
OCP : Octa Calcium Phosphate
PDMS : Polydimethylsiloxane
QBP : Quartz Binding Peptide
QCM : Quartz Crystal Microbalance
QD : Quantum Dot
RIU : Refractive Index Unit
SAM : Self Assembled Monolayer
SDS PAGE : Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
SPR : Surface Plasmon Resonance
TEM : Transmission Electron Microscopy
WT AP : Wild Type Alkaline Phosphatase
3GBP : Three repeat Gold Binding Peptide
5GBP : Five repeat Gold Binding Peptide

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GOLD BINDING PEPTIDE FUSED ALKALINE PHOSPHATASE FOR REAL RIME MONITORING OF CALCIUM PHOSPHATE BIOMINERALIZATION

SUMMARY

Genetically engineered peptides for inorganics (GEPIs) that have the ability to bind specifically to an inorganic surface provide important advantageous for constructing multifunctional systems which inspired from natue. These short amino acid sequences serve as linker molecules in order to realize striking nanobiotechnological applications. GEPIs fused proteins serve as multifunctional biomolecules which overcome limitations of traditional immobilization techniques.

In this dissertation, alkaline phosphatase enzyme fused to a genetically enginneered gold binding peptide with five tandem repeat (5GBP1-AP) was used for real time monitoring of calcium phosphate biomineralization. Calcium phosphate as being the main component of hard tissues such as bone and teeth is a crucial biomineral which is found in different crystal structures in both vertebrates and invertebrates. Calcium phosphate is widely studied in order to shed light of its formation and crystal phase transitions that are important for theoretically and practically for medical applications. Alkaline phosphatase has the ability to catalyze the hydrolizing reaction of phosphate containing molecules result to release inorganic phospahate group to the environment. For this reason, it is used in mineralization studies to mimic this function for biomineral formation.

Surface plasmon resonance (SPR) spectroscopy is a very sensitive characterization method to analyze molecular interactions in real-time. In addition SPR serve as valuable technique to study protein interactions with solid surface that is commonly gold substrate. GEPI fused alkaline phosphate which have high affinity to gold surafec was analyzed by SPR in order to progress biomineral formation in real-time. The advantage of this method is the ability to provide both monitoring of calcium phosphate formation in real-time and analyzing gold binding property of bifunctional enzyme. In addition GEPI fused AP was found to have higher enzymatic activity according to wild type enzyme. Results indicated that 5GBP1-AP induced biomineral formation upon adsorption onto gold substate and allow to progress biomineral formation by surface plasmon resonance spectroscopy. SPR results are thought to be promising with the possible indications about the crystal phase transsition of calcium phosphate which is a long debated issue about biomineralization.

ALTINA ÖZGÜL BAĞLANAN PEPTİT İÇEREN ALKALİN FOSFATAZ ENZİMİ İLE KALSİYUM FOSFAT BİYOMİNERALİZASYONUNUN GERÇEK ZAMANLI İZLENMESİ

ÖZET

Anorganik yüzeylere özgül olarak bağlanan genetik yöntemlerle tasarlanmış peptitler, doğadan esinlenen çok işlevli sistemlerin oluşturulması için bir önemli üstünlükler sağlamaktadır. Kısa amino asit zincirlerinden oluşan bu peptitler moleküler bağlayıcılar olarak geleneksel yöntemlerin karşılaştığı güçlüklere çözüm olarak kullanılabilecek işlevsel biyomoleküllerdir.

Bu çalışmada beş sıralı tekrar biçiminde altına özgü bağlanan peptitin alkalin fosfataz enzimine moleküler biyoloji yöntemleriyle eklenmiş yapısı, kalsiyum fosfat mineralizasyonunun gerçek zamanlı takip edilmesi amacıyla kullanılmıştır. Kalsiyum fosfat kemik ve diş gibi sert dokuların ana maddesi olan önemli bir biyomineral olarak hem omurgalı hem de omurgasız canlılarda farklı kristal yapılarda bulunmaktadır. Kuramsal açıdan olduğu kadar tedavi alanında pratik bakımından da önemli olmasından dolayı kalsiyum fosfatın hem oluşum hem de kristal faz geçişleri hakkında birçok çalışma yürütülmektedir. Alkalin fosfataz enzimi fosfat grubu içeren kimyasal yapılarndan hidroliz tepkimesi ile anorganik fosfat grubu açığa çıkmasını sağlama özelliğine sahiptir. Bu nedenle mineral oluşumu çalışmalarında biyominerallerin üretilmesi için söz konusu enzimin bu tür tepkimelerinden esinlenilmektedir.

Yüzey plazmonik rezonans spektroskopisi moleküler etkileşimlerin gerçek zamanlı incelenmesini sağlayan çok hassas bir karakterizasyon yöntemidir. Ayrıca SPR proteinlerin katı yüzeylerle özellikle altın yüzeyle etkileşimini incelemek açısında yararlı bir tekniktir. Bu çalışmada GEPI molekülleriyle güçlendirilmiş altına özgü bağlanma özelliği olan alkalin fosfataz enzimi SPR yöntemi kullanılarak mineral oluşumunun gerçek zamanlı incelenmesi amaçlanmıştır. Bu deneysel çalışmalarda izlenen yöntemler hem biyomineral oluşumunun gerçek zamanlı takibine olanak sağlamış hem de genetik yöntemlerle tasarlanan çok işlevli alkalin fosfataz enziminin altına özgü bağlanma özellikleri incelenme imkânı sunmuştur. Ayrıca doğal enzime kıyasla GEPI molekülleriyle işlevselleştirilmiş alkalin fosfatazın daha yüksek aktivite gösterdiği bulunmuştur. Elde edilen sonuçlara göre GEPI bağlı alkalin fosfataz altın yüzey üzerinde biyomineral oluşumunu tetikleme özelliğine ek olarak kalsiyum fosfat oluşumunun yüzey plazmonik rezonans yöntemi ile takibine olanak sağlamıştır. Yüzey plazmonik resonans spektroskopisinin biyomineral oluşumu hakkında uzun süredir tartışmalı bir konu olan kalsiyum fosfatın olası kristal faz geçişlerini destekleyen veriler sağladığı düşünülmektedir.

1. INTRODUCTION

Nature is designing exquisite structures with using a toolbox that contains building blocks which are both organic and inorganic molecules. Starting from molecular level to come up with macroscopic scale structures nature provides highly sophisticated examples of functional materials and systems. The strategy of biological systems for building elegant multifunctional structures is using bottom up approach with harnessing self-assembly and molecular recognition properties of organic molecules. Self-assembly is an organization system in nature which do not require human intervention (Whitesides and Grzybowski 2002). Self-assembly is the common way of nature's processing strategy to build multifunctional materials and sytems. It organizes diverse natural systems including bacterial colonies even galaxies (Whitesides and Grzybowski 2002). Molecular recognition as a partner of self-assembly is another crucial mechanism which as its name indicates, defines the recogniton of two or more different molecules via coded information in them like shape, geometry, charge, surface properties etc. Achieving to produce materials using organic and inorganic structures with a methodology that nature uses, have amazed scientists for a long time.

In the last few decades with the advent of technology, it is now possible to analyze natural processes at molecular scale which is crucial to understand how nature setting up its structures. Since stable structures that can form materials and systems begin with single molecules, it is obvious to analyze natural phenomenons at molecular level which is about a few nanometer. This obligation unveiled a new field of research which is now called nanotechology. Nanoscience includes the study of objects and systems in which at least one dimension is 1-100 nm (Love et al. 2005).

In this new era of science, researchers are able to understand natural processes begining from nanometer scale and with gaining inspiration from these perfect structures, rational design of new systems and materials are now en route. Although today it is difficult to say that theoretical background of nano world is fully deciphered, it is day-by-day advancing both in theory and experimental systems to

shed light onto this fascinating phenomenon. It is now commonly recognized that at nanometer-scale dimensions materials have unique functional properties that can lead to novel engineering systems with highly useful characteristics (Tamerler and Sarikaya 2007). Nature is in fact a guide for nanotechnology. Organization and formation of nanoscale structures can be found in the vast area of biological systems. These biological systems carry a great wealth of engineering principles for the design, synthesis, and manufacturing of materials for practical uses (Tamerler et al. 2003). Animal cells are about 10 μm in size and plant cells can have sizes of up to about 100 μm, so they are both of a suitable scale for nanostructure production (Parker and Townley 2007). The examples in nature trigger a new dicipline for scientists which was a dream for thousand of years that comes to reality in recent years with advanced technology. Biomimetics as a term of not only for biology but also for chemistry, physics and also engineering now using to define synthesizing new materials with the procedures that nature have used for millions of years.

Traditionally, biomimeticists, inspired by the biological structures and their functions, focused on emulating or duplicating biosystems using mostly synthetic components and by following traditional approaches (Tamerler et al. 2003). Nature for gaining inspiration to come up with highly functional and also low cost materials, systems and synthesizing strategies, is an excellent source for scientists (Tamerler et al. 2010). Today for nanotechnology and for nanoscience it is obvious that man-made structures are far away to compete with nature according to the nature's methodology about synthesizing sophisticated structures especially at microscopic dimensions. Increased requirements of highly sensitive devices and systems for human all around world not only for crucial health associated problems but also for better understanding of biological processing of nature lead a subset of nanotechnology.

Nanobiotechnology as term of biology, chemistry, physics, medicine and electronics includes biological processes that harness nanotechnology to understand and improve highly sophisticated systems, materials and devices. It is an opportunity to use nanotechnology that comes from physical sciences and implement it to biological sciences. Since today most of arduous problems of human especially in medicine, root from genetically and cell based disorders and both systems can be defined as nanomachines, it can be said nanobiotechnology is includes the right size for

studying on (Whitesides 2003). Nanobiotechnological systems uses nature's main bulding blocks like proteins, DNA, RNA etc. These biomaterials that are found as a core in biological organisms studied extensively but in few decades molecular biology has an increased key role to understand and manipulate genetic material of life which is called DNA. This magical molecule and its complex product proteins are the most important molecules for biological structures. Proteins, through their unique and specific interactions with other macromolecules and inorganics, control structures and functions of all biological hard and soft tissues in organisms (Sarikaya et al. 2003). Hard tissue formation studies which focus on the main principles of mineral formation by organisms called biomineralization.

Biominerals that produced in mild conditions in nature have great importance both for pratical and theoretical purposes (Sanchez, Arribart and Guille 2005). Along with learning new functional material synthesis researchers take lessons from nature's hard tissues for medical applications because these hard tissues include bones and teeth which are crucial fields of medicine. These structures are formed through template-assisted self-assembly, in which self-assembled organic material (such as proteins or lipids or both) form the structural scaffolding for the deposition of inorganic material (Aksay et al. 1996). Not only as a scaffold material and also with its magical and incredible functionality proteins play the most important role in biological processes. For biomineralization deposited mineral must formed on the scaffold protein which triggers enhanced mineral formation. Also this scaffold materials must have the ability to recognize inorganic material to assist it for tissue formation. Inorganic binding proteins to achieve this tasks were shown to induce biomineralization (Falini et al. 1996). However it is difficult to extract and purify these proteins from bioloigical tissues to harness them for technologically and medically important problems.

As a new strategy for using organic molecules that have affinity to a target inorganic material were proposed by Sarikaya et.al. This strategy uses short amino acid sequences, that is peptides, selected with combinatorial biology approaches like phage and cell surface display and have high affinity to an inorganic material which give an oppurtinity to use them as technologically important compounds (Sarikaya et al. 2003). These peptides called Genetically Engineered Peptides for Inorganics (GEPIs) lead a new bioinspired field which is called molecular biomimetics.

In the scope of this study our overarching goal is to use one of these GEPIs namely Gold Binding Peptide (GBP) as a tandem five repeat structure fused with alkaline phosphatase enzyme (5GBP1-AP) to study real time monitoring of calcium phosphate biomieralization by surface plasmon resonance (SPR) spectroscopy. Calcium phosphate formation as the main component of bone tissue is a controversial issue in literature about crystal phase transitions occur or not during bone maturation. SPR apart from immobilization, binding kinetics and thermodynamic properties of these proteins provided us two other important advantages. Since SPR uses a gold sensor chip we could studied quantitatively 5GBP-AP affinity on bare gold surface directly and after immobilization of proteins, it served us a platform for studying of biomineralization without the need for another intervention. This provided us a different vision to understand about the nature of calcium phosphate mineral formation by real-time monitoring using a biomimetic strategy.

2. BACKGROUND INFORMATION

2.1 Biomimetics and Bioinspiration

Biomimetics is the field of inspiration from nature to come up with perfect design. When compared with synthetic materials and systems, nature's strategy to build multifunctional structures is far away not only about design principles but also from using chemicals and process conditions (Parker and Townley 2007). It can be said that mimicking nature trace back very far from today however, this inspiration was usually very simple and lack of scientific character. Traditional chemical pathways to produce materials that have used for a long time has a nearly opposite protocol according to nature's approach of synthesizing materials especially when it comes to microscopic scale systems. Production of synthetic materials involve a combination of approaches like melting and solidification processes which are controlled by the kinetics and thermodynamics of the system that is known as 'heat-and-beat' approaches. By contrast, in biological systems, biomaterials are highly organized starting from the molecular level to the nano- micro- and the macro-scales often in a hierarchical manner (Sanchez et al. 2005). By using a bottom up approach nature come up with interesting architectures that ultimately make up functional units in a large spectrum including both soft and hard tissues (Sarikaya 1999). With the advent of technology today biological structures can analyze in detail to understand better the underlying principles of nature's way of setting up structures.

Recent developments in nanoscale engineering provide us to extract information about how nature design and build materials and systems. Structural control of materials at the molecular scale is a key to the production of structures with improved properties used in a wide range of nanotechnological systems. Biological hard tissues which are biocomposites provide conceptual models for future biomimetic engineered materials and are striking examples for bioinspired pathways. These tissues are hybrid materials containing biomacromolecules such as both proteins that are building blocks of nature and also bioinorganics like calcite, magnetite, and silica. The hierarchical architecture of this systems resulting in highly

functional magnetic, mechanical, and photonic properties (Frankel, Blakemore and Wolfe 1979, Parker and Townley 2007). It is crucial to inspire from from nature to build highly sophisticated, cheap, durable and environment respecting materials, systems and devices and this is only possible by a colloboration of different scientific fields. Taking lessons from biology, with the recent advances in genetic engineering and molecular biology tools when merge with nanotechnology give rise to an exciting field to control and manipulate intricate nano- and microarchitectures at the molecular level assisting by proteins via self-assembly and molecular recognition.

2.1.1 Self-Assembly Process in Nature

Self-assembly is the organization of molecules spontaneously under equilibrium conditions without any intervention (Whitesides and Grzybowski 2002). Self-assembly can be defined as a process in which molecules or parts of molecules autonomously form ordered aggregates usually by non-covalent interactions (Boncheva and Whitesides 2005). Molecular self-assembly is a common phenomenon in nature between associated molecules and it is the main reason of the spontaneous formation of complex biological structures. Self-assembling processes are nearly everywhere throughout nature. Examples range from formation of crystals and micelles to bacterial colonies and involves a myriad of biological systems like ants, fish, solar system and also galaxies with different kinds of interactions in a large spectrum (Whitesides and Grzybowski 2002). Self assembly as a fabrication strategy for the formation of highly developed structures with biomolecules can be applied to components ranging in size from nano scale microscopic dimensions. Particularly in making structures that are too large to be prepared by chemical synthesis, but too small to be made by traditional methods, self-assembly can give a rise to build materials and systems which can not be made otherwise (Boncheva and Whitesides 2005).

2.1.2 Synthetic Surface Functionalization: Self-Assembled Monolayers

Among the biological self-assembled molecules there are several types of synthetic self-assembled chemicals that have affinity to metals or metal oxides. Known as self-assembled monolayers (SAMs) are now being intensively studied in chemistry, biology, and materials engineering, in systems ranging in size from molecular to

macroscopic (Love et al. 2005). Self-assembled monolayers (SAMs) serve as a simple method with which a target surface or interfacial properties of metals, metal oxides, and semiconductors can be tailored (Love et al. 2005). SAMs are organic molecules formed by the adsorption of molecules from solution allowing appropriate surfactants to assemble on surfaces. Also SAMs can adsorbed from gas phase onto the surface of solids or on the surface of liquids in an autonomous way into crystalline structures. The molecules of SAMs have three parts that serve different functions. A typical SAM have a headgroup, a spacer and a terminal group as shown in Figure 2.1.

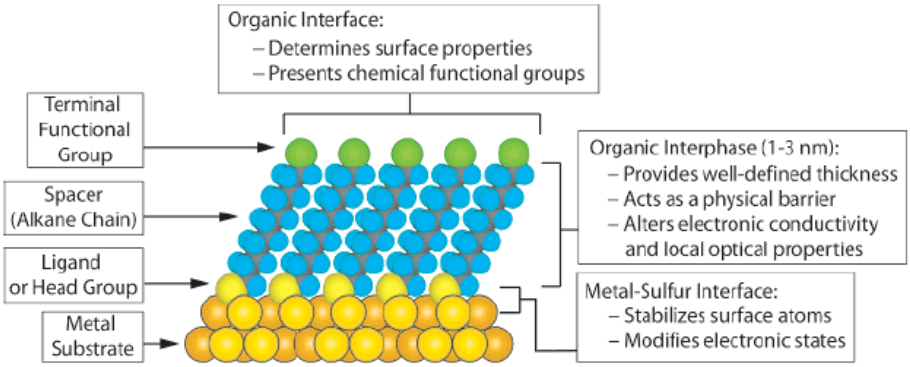

Figure 2.1: Schematic diagram of an SAM of alkanethiolates on a (111) gold surface. Explanation are given about structure, chemical and physical properties and interaction with its substrate of SAM molecule (Love et al. 2005).

Headgroup of a SAM can be defined to have a chemical functionality with a specific affinity for the target material of surface. There are several headgroups that have high affinity to specific metals, metal oxides, and semiconductors (Love et al. 2005). Spacer group of an SAM consists of an alkane chain which can involve different number of carbon atoms. The third part of an SAM molecule is the terminal functional group. With the head group of an SAM, terminal functional group is crucial for self assemble process since the modification of SAM surface to achieve an organized layer or pattern is manipulated from functional groups on the surface. Terminal groups can consist of different functional molecules like carboxyl or amino and can be further reacted with appropriate chemical to come with other functionalties. The most extensively used modification system of alkanethiols based on SAMs for further surface activation is EDC/NHS chemistry. EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimidehydrochloride) and NHS (N-Hydroxy

succinimide) are used to form active amino group on terminal functional part of SAM to react with target surfaces or chemicals like gold. The most studied and well known class of SAMs is the adsorption of alkanethiols on gold surfaces. Also it is known that SAMs adsorped on their target substrate form highly ordered layers onto this surface (Whitesides 2005). Organization of SAM after adsorption of alkanethiols on gold surfaces are shown to be highly organize on its substrate as is shown in Figure 2.2.

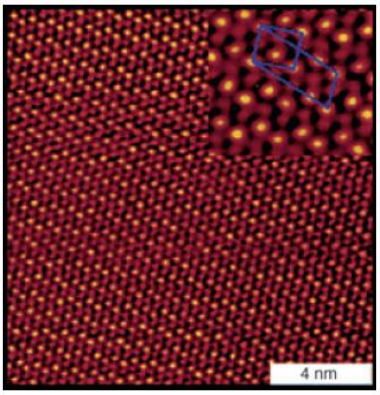

Figure 2.2 : Scanning tunneling microscope image of a self-assembled monolayer (SAM) of decanethiol on gold (Whitesides 2005).

In summary, as a synthetic pathway for surface functionalization, SAMs are capable of modifying surfaces with various functional groups with high efficiency. SAM coating on surfaces generally is not a time-consuming and laborious way which provides another important advantage. On the other hand, upon surface functionalization, non specific attachment of target molecules onto surface such as adsorption of biomolecules from their active site is a serious drawback of SAMs for using in immobilized systems.

2.1.3 Self-Assembly Properties of Proteins

From countless examples of self-assembled molecules in nature maybe the most importants are proteins. As being the crucial building block of nature, proteins through their specific interactions, unique molecular recognition properties, intricate coded informations for diverse functionality and capability to interact with other biomacromolecules and also inorganics, control structures, functions and serve scaffolds of all biological hard and soft tissues in organisms (Sarikaya et al. 2003).
Formation of protein molecules itself is an example of self-assembly process. They

are synthesized as chains autonomously fold into specific 3D structures with complex functionality and surface topology. Biological tissues are synthesized with genetically controlled pathways in aqueous environments under mild physiological conditions. Proteins in this processes provide and serve as the main component of the system in which they both collect and transport raw materials and ultimately self- and co-assemble subunits in a hierarchical manner (Tamerler et al. 2003). Proteins among their diverse functions provide both multifunctionality to the structure and also serve as a scaffold to organize biological tissue formation. For this reason, with their countless properties of proteins, their biological functions, catalyzing important chemical reactions as being enzymes or providing physical performance it can be said that proteins are an indispensable part of biological structures. Therefore, cutting-edge technological systems should include proteins in synthesis, assembly or function (Sarikaya et al. 2003).

2.1.4 Molecular Recognition

There are two associated ways in nanotechnology related fields to be used in the formation of functional materials. One of them is the top-down approach in which targeted product is generated by a means of miniaturization. Especially in electronics this is the preferred way of constructing components of interest. The other way which is also nature harnesses is the bottom-up approach in which materials are formed through self-assembly property with molecule by molecule also in some cases even atom by atom construction (Zhang 2003). Using the second way requires a deep understanding of molecular building blocks that is proteins and peptides and their structures, self-assembly properties, chemical and structural complementarities. Nature's choice of design strategy that is bottom-up approach involves both organic and inorganic material using simultaneously with improved properties which is not fully realized by synthetic patways. To use organic and inorganic molecules in the same structure, in most cases coupled with each other, these two different molecular building blocks must recognized by each one to combine with other. This is achieved by the property of molecular recognition. Biological molecules with their coded molecular recoginition information *know* each other in order to associate and organize by the help of self-assembly. This information comes mostly from geometrical and chemical properties of molecules (Castner and Ratner 2002). The

shape complementarity plays an important role in molecular recognition but it can't explain the whole picture as well. Charge interactions and themodynamic properties of molecules are crucial to dictate the recogniton process (Gitlin, Carbeck and Whitesides 2006). The most important biological molecules such as proteins, nucleic acids and as a subclass of these ones enzymes and DNA or RNA also the ribosome have very specific molecular recognition property (Castner and Ratner 2002). It is vital for the interaction of related molecules to come up crucial metabolic reactions. For this reason, specific molecules should recognize each other and prefer to associate with it but not with another. Although they are harnessed widely for diverse applications and chemical pathways the most two important property of biological molecules, self-assembly and molecular recoginition still are not well understand and explained to be used with their high potential.

2.2 Molecular Biomimetics

Nature as a school for scientists can not be ignored easily by different disciplines such as chemistry, biology, physics or engineering provide inspiration for solving technological challenges. Although there is a rich history of taking lessons from nature for the design of materials and systems only with recent developments biological structures are started to understand in detail. A very high sophistication degree is the key in all living organisms starting from single cell organisms to very complex systems such as vertebrates (Sanchez et al. 2005). In order to harness nature's way of building materials and systems it must be well understood its protocols like selecting the right material for the right function at the right moment from sources available at that environment (Parker and Townley 2007). Comparing synthetic materials that man have used for a long time with nature's structures can give an obvious idea about the long divergence between two approaches.

Nature can achieve to set up a system in which it uses the simplest materials but with a genious strategy to come up with desired product that is both at a high level of sophistication and miniaturization also recyclable, reliable and consume less energy (Parker and Townley 2007). For this reason, scientists started to look deeper to learn more about this impressive mechanism. This obligation lead a new field of research which mimics nature at the molecular level where all the things start. Molecular biomimetics is a promising field of research in which hybrid technologies, like

molecular biology and nanotechnology, are using to overcome challenges that is not possible by traditional methods (Sarikaya et al. 2003). Molecular biomimetics investigate abundant examples of multifunctional materials, devices and systems in nature to understand the bases of synthesis, formation and function in order to emulate their practical utility in everyday applications (Tamerler et al. 2003). Formation of this exquisite structures involves using both organic and inorganic molecules for a desired functionality. Organic molecules that nature harnesses are the building blocks that is proteins whereas inorganic ones are minerals by which biological organisms form their hard tissues. These biomaterials formed by associating organic and inorganic molecules in which organic phase both serve as a scaffold and provide high degree of functionality like toughness, hardness and strongness to the systems (Aksay et al. 1996). Inorganic phase deposit in a highly organized way to build overall structure from the molecular level starting from nano- and macroscales to end with macroscopic dimesions. This strategy results in elegant structures that are shown in Figure 2.3.

Biomaterials when analyzed in microscopic dimensions give excellent examples of how a common material which has a poor mechanical property like calcium carbonate can transform to one of the strongest materials found in nature that is to nacre. As is shown at top left in Figure 2.3. mother-of-pearl, the natural armor of mollusks' shells, is constituted of a layered and segmented hybrid composite of aragonite (orthorhombic $CaCO_3$) and nanostructurally integrated proteins and polysaccharides.

Nanoparticle synthesizing which is very crucial for nanotechnological applications have been using by biological organisms for thousand of years with precise structures that is even very difficult today for material scientists. Magnetotactic bacteria that shown at top right in Figure 2.3 is another striking example for this type of producing. Magnetite (Fe_3O_4) nanoparticles formed with magnetosomes by magnetotactic bacterium Aquaspirillum magnetotacticum are used as an impressive biocompass (Sarikaya et al. 2003, Frankel et al. 1979). Mouse enamel shown in bottom left in Figure 2.3. Consist of hydroxyapatite crystallites assemble into woven rod structure that provide both hard and strong tissue due to the well organized micro and nano architecture. This special structure of mouse enamel provides the essential network resistance to the mixed stresses during mastication, thereby preventing

premature fracture or failure (Tamerler and Sarikaya 2008). Sponge spicule of Rosella racotvitzea shown in bottom right in Figure 2.3 can synthezise a biological optical fibre under 200 m in ocean which consist of layered silica with excellent optical and mechanical properties (Tamerler and Sarikaya 2007). Both the spicular tip (a lens) and the shaft (optical fiber) are molecular composites of silica and bound proteins that provide the structural, architectural and functional properties to the spicular system serve to collect and transmit light effectively across the outer wall of the sponge.

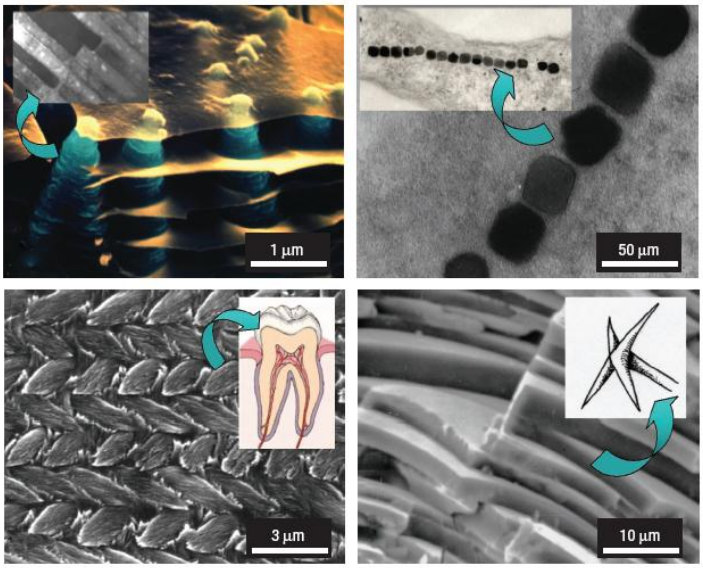

Figure 2.3 : (Top left) SEM image of a growth edge of abalone Haliotis rufescens. (Inset: TEM image of cross section of abalone) (Top right) Magnetite (Fe3O4) nanoparticles. (Inset: TEM image of Fe3O4 nanoparticles. (Bottom left) Unique woven architecture mouse enamel. (Inset schematic representation of human tooth) (Bottom rigth) Sponge spicule (with a cross-shaped apex shown in inset), a biological optical fibre (Sarikaya et al. 2003).

2.2.1 Genetically Engineered Peptides for Inorganics (GEPIs)

Nature exhibit countless examples to form multifunctional systems that have high efficiency and cheaper than man made structures however, it is difficult to build such systems found in biological environment. The reason for that, is the difficulty of using specific proteins for a desired material since biological organisms know what they require and use them by their coded information via molecular recognition and self assembly. Complex biological molecules such as proteins are abundant in the same structure in a system and understanding which protein functions for a specific

task is hard to say with our knowledge. Although it is advantegous to mimic or inspire from nature the question how this can achieved is an ardous problem. It can be promising to use same biological molecules with nature like using proteins that involve in mineral formation for tissue engineering but this not as simple as defining that protein. A possibility would be to extract for example biomineralizing proteins from hard tissues. This procedure should involve their isolation and purification so it comes with its own difficulties since in a hard tissue there are usually many proteins, not just one, all differently active in biomineralization. For example only in enamel there are 40 different protein each has probably specific functions. Another way is to design peptides or proteins using a theoretical molecular approach. This is application that is used for pharmaceutical drugs however can be impractical because of being time consuming and expensive.

Among the major limitations of traditional approaches a new route is the selection of peptides using combinatorial biology techniques such as phage display to mine sequences that have affinity to a target inorganic material (Sarikaya et al. 2003). These special peptides called genetically engineered peptides for inorganics (GEPIs) are short amino acid sequences (7-14 amino acids) that have high binding affinity with physiochemical non-covalent forces to an inorganic compound. Offering three unique advantages, molecular recognition, self-assembly and genetic manipulation these genetically engineered peptides for inorganics provide some promising challenges for nanotechnological applications (Tamerler and Sarikaya 2007). Gaining inspiration from biology peptides can now be genetically engineered to specifically bind to a target inorganic material to be used in applications in nano- and biotechnology (Sarikaya et al. 2003).

Detailed information about the surface-binding characteristics, structure-function relationship and folding mechanism of proteins can not obtained with current knowledge to design proteins for a desired function. For this reason, starting from the molecular level using the recognition properties, inorganic surface-specific polypeptides could be used as material binding molecules to control the organization and specific functions of materials. Using polypeptides also provide different solutions to the improvement of new multifunctional nanostructures. The first is the chance of design at molecular level using genetic engineering techniques. The second is the advantage of harnessing molecular and nanoscale recognition of

peptides to use them as linkers to bind diverse synthetic entities, like nanoparticles, functional polymers. The third one is the ability of biological molecules to self- and co-assemble into highly organized nanostructures which gives a powerful assembly process for to build structures in complex architecture such as those found in nature.

2.2.2 Selection of GEPIs

Selection and isolation of polypeptide sequences for desired function that is for preferentially binding to a surfaces of inorganic substrate can be done with combinatorial biology methods such as phage or cell surface display (Smith and Petrenko 1997, Boder and Wittrup 1997). These protocols have been used for over two decades for the selection of peptides. The two approach are the same in application. Simply a peptide genetically fused into a chimeric protein which is located on the outer surface of bacteriophage or on to the flagella of cell is expressed and exposed to a specific material of interest (Sarikaya et al. 2003).

In phage display usually pIII coat protein of M13 phage is used while for cell surface display it can be an outer membrane or flagellar protein. For the selection of peptides within thousands of possible sequences a library is generated by using random oligonucleotides. The ultimate result is a protein that reside on outer surface of phage or cell each exhibit a different affinity to the target substrate. Then a mixture of cells or phages are exposed to the inorganic surfaec of interest to find out specific binding peptides. After several washing steps weak binders of the phages or the cells are eliminated to recover only strong binders. Then bound phages or cells eluted from the surfaces and after the extraction of genetic material amino acid sequence is determined. These selected peptides are further characterized. An overall flow of both phage and cell surface display methods are given in Figure 2.4.

For an inorganic binding activity cell surface display method was first used to select iron oxide binding peptide (Brown 1992). After that peptides for binding affinity to semiconductor materials were selected by phage display protocol (Whaley et al. 2000). So far there are several inorganic binding peptides defined with either phage or cell surface display to select high binding affinity sequnences to various inorganic materials. Up to date, selection of inorganic binding peptides for gold (Brown 1997), platinum (Sarikaya et al. 2004), palladium (Sarikaya et al. 2004), silver (Naik et al. 2004), SiO_2 (Naik et al. 2002), ZnO and TiO_2 (Thai et al. 2004), $CaCO_3$ (Gaskin,

Starck and Vulfson 2000), Cu_2O (Thai et al. 2004)), GaAs (Whaley et al. 2000), ZnS and CdS (Lee et al. 2002), minerals such as hydroxyapatite (Gungormus et al. 2008), mica (Donatan et al. 2009), sapphire (Krauland et al. 2007), zeolites (Nygaard, Wendelbo and Brown 2002), and carbon nanotubes (Wang et al. 2003) were achieved.

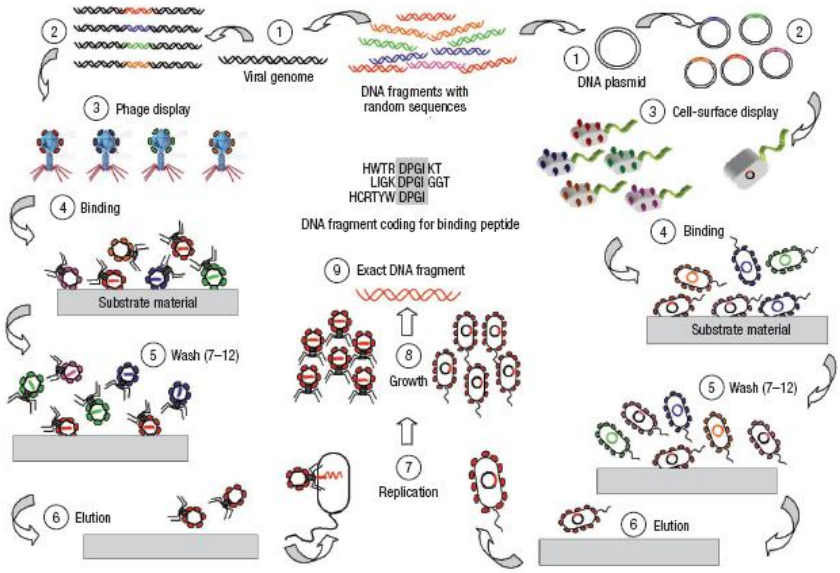

Figure 2.4 : Phage display and cell-surface display protocols used for selecting polypeptide sequences that have binding affinity to given inorganic substrates (Sarikaya et al. 2003).

After the first inorganic binding peptide selection for iron oxide it is thought to be an inappropriate choice to assess binding affinity since the surface character of Fe_2O_3 is not suitable enough for peptide binding. For this reason, selection of gold binding peptide was studied as one of the first material binding biomolecule that have high affinity for gold substrate (Brown 1997).

2.2.3 Currents Applications of GEPIs

GEPIs are promising smart molecules that provide high material specificity for nanobiotechnological applications. In the literature there are striking examples which use GEPIs or GEPI fused proteins. For example assembly of semiconductor quantum dots (QDs) on silica surface was achieved using silica binding peptide QBP1 (Seker et. al., 2011, Oren et al. 2010). Semiconductor quantum dots which have interesting optical and electronic properties however specific immobilization of these molecules on solid surface for their applications are not sufficiently succesful. Using QBP1 it was shown

that QDs can assembly onto silica surface solely with GEPIs as molecular linker. As shown in Figure 2.5, using quartz crystal microbalance and fluorescence microscopy GEPI fused QDs was assembled onto silica surface with a high affinity according to non-functionalized QDs. In this study non-functionalized QDs was used as a control for binding affinity to silica surface. QBP1 on the other hand was used in two different case. In one case QBP1 was immobilized to silica surface and QDs was applied onto this surface for assembly. In the second case QDs were functionalized with QBP1 and then analyzed for their binding affinity to bare silica surface. Functionalization of QDs with QBP1 was resulted in 79.3 fold enhancement according to binding on QBP1 immobilized surface which is only 3.3 fold increased when compared to bare QDs on the silica surface (Seker et. al., 2011).

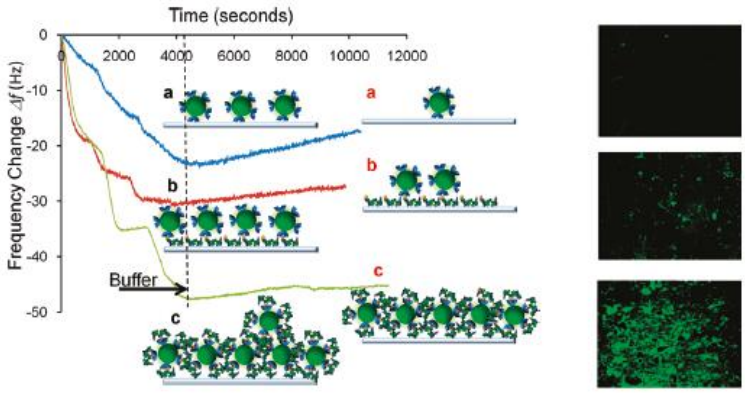

Figure 2.5 : Quartz crystal microbalance results of QDs binding to bare silica, binding of QDs to QBP1 immobilized surface and QBP1 fused QDs binding to silica. Fluorescence microscopy images for these three cases (Seker et. al., 2011).

GEPIs were also used for pattering inorganic surfaces with proteins to fabricate protein microarrays. Immobilization of biomolecules onto inorganic surface require SAMs which have limitations due to random orientation of molecules on surfaces and requering multistep chemical reactions. Using two types of QBP1 (Notman et al. 2010), fused with fluorescein (QBP1-F) and fluorescent quantum-dot nanocrystals with streptavidin (SA-QD), reserchers was achieved to pattern a silica surface with PDMS stamping of GEPI fused constrcuts to fabricate self-assembled protein microarray as is shown in Figure 2.6 (Kacar et al. 2009a).

A striking example of inorganic binding peptides for future technological applications is the fabrication of high power lithium ion batteries using carbon nanotube binding and and a genetically engineered peptide capable of nucleating amorphous iron phosphate. These peptides were expressed on the major coat protein

of M13 virus to harness it for assembling nanomaterials to enable a high power performance battery (Lee et al. 2009).

GEPIs were also used as a *ink* to pattern a surface by using dip pen nanolithography (DPN) tip (Wei et al. 2009). Site specific assembly of bifunctional linkers labeled with fluorescent streptavidin was shown with patterning silica and gold surfaces. 3GBP1 patterned via microcontact printing and QBP1 on silica patterned via DPN were resulted in targeted immobilization and surface bifunctionalization with high-spatial-resolution as shown in Figure 2.8. This approach can be used in nanometer- and micrometer-sized peptide patterns that provide nanobiotechnological applications in future experiments.

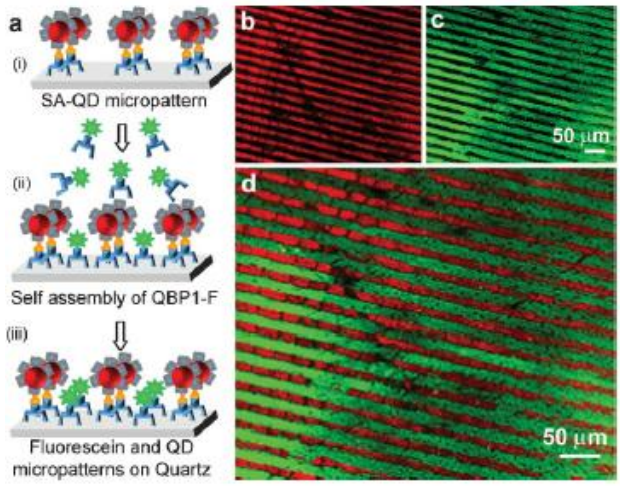

Figure 2.6 : Schematic representation of SA-QD-QBP1 and QBP1-F assembly on silica surface (a). Fluorescence microscopy image of a patterned surface with SA-QD-QBP1-biotin (b) QBP1-F (c) and both SA-QD-QBP1-biotin and QBP1-F (d) (Kacar et al. 2009a)

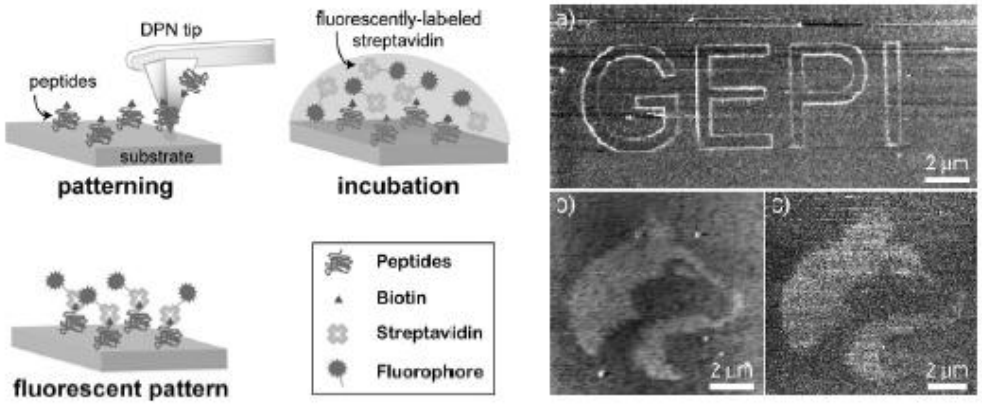

Figure 2.7 : Schematic representation of peptide patterning and lateral force microscopy images of (a) QBP1 on silica (b) QBP3 another silica binding peptide on silica (c) GBP1 on gold surface (Wei et al. 2009).

Biomineral formation studies has a major focus in hard tissue engineering (Khatayevich et al. 2010) including calcium phosphate mineralization which is crucial for biomedical applications. It is important to monitor calcium phosphate mineralization to understand the process of bone and teeth tissue formation. Using combinatorially selected hydroxyapatite-binding peptides (HABP) that genetically linked to the green fluorescence protein (GFPuv) researchers were shown that it is possible to track mineral formation in vitro (Yuca et al. 2011). As is shown in Figure 2.7 alkaline phosphatase based mineralization was monitored under fluorescence microscopy which provide monitoring of calcium phosphate formation either on HA powder and human incisor.

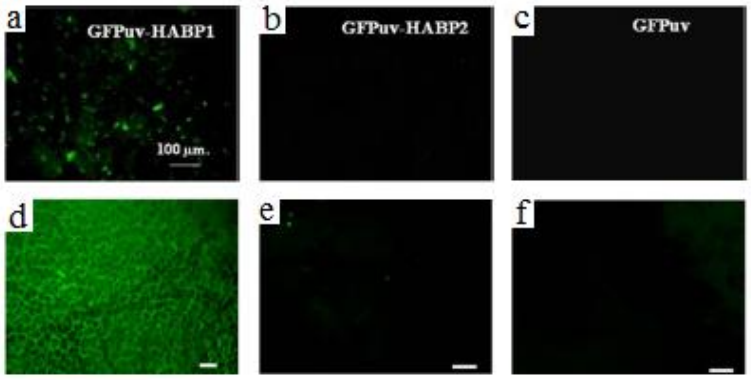

Figure 2.8 : Fluorescence microscopy images of GEPI fused strong binder HABP1 (a), weak binder HABP2 (b) and GBPuv (c). In vitro labeling of human teeth with GFPuv-HABP1 (d), GFPuv-HABP2 (e) and GFPuv (f) (Yuca et al. 2011).

Engineered solid-binding peptides that have high affinity to their target inorganic surfaces serve as a breakthrough for a wide range of area due to potential and promising properties which yet have not utilized sufficiently.

2.3 Biominerals and Biomineralization

Living organisms produce biomaterials that consist of inorganic molecules for the formation of hard tissues. These biocomposite materials includes more than 60 different kind of minerals such as hydroxyapatite, calcium carbonate, and silica (Sarikaya 1999). Using this inorganic materials to form hard tissues like bones, teeth, shells, skeletal units, and spicules biological organisms prefer an elegant way that is to incorporate both organic biomacromolecules (lipids, proteins, and polysaccharides) and minerals simultaneously. The function of organic molecules are

providing a scaffold for the deposition of inorganic material and to control the formation of highly ordered mineral layer. In order to produce an specifically textured hard tissue, depositing of inorganic molecule is crucial. All mineralization processes in nature includes the precipitation of inorganic material from solution (Aksay et al. 1996). During this precipitation formation of inorganic crystals occur which is called biomineralization. In other words biomineralization is the formation of inorganic materials in biological organisms. A large number of biological organisms produce inorganic materials either intracellularly or extracellularly. These hard tissues provide both mechanical and physical functions such as skeletal or piezoelectic properties. Starting from single-celled organisms like bacteria and algae to complex examples like vertebrates biological systems produce inorganic materials such as synthesizing siliceous templates of diatoms or well known example of mammalian teeth bone as shown in Figure 2.9.

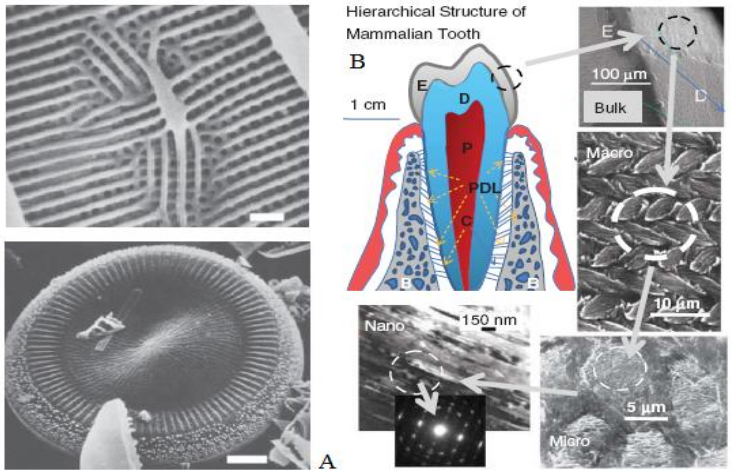

Figure 2.9 : Silicic skeletons of diatoms (A) (Sanchez et al. 2005), and hierarchical architecture of the mammalian enamel at the crown of the tooth (B) (Tamerler and Sarikaya 2008).

Using materials commonly available in the environment, nature design striking examples which normally exhibit poor macro-scale mechanical properties but come up with orders-of-magnitude increased strength and toughness (Ortiz and Boyce 2008). For this reason, scientists are gaining inspiration to form materials based on the design principles found in biological materials. The key role in nature's approach for biomineralization is controlling the formation of highly organized textured and laminated organic–inorganic molecule assemblies by direct or synergistic templating (Parker and Townley 2007). Biologically produced organic-inorganic composites in nature such as bone, teeth, diatoms, and sea shells are formed through highly coupled

and often concurrent synthesis and assembly (Aksay et al. 1996). The structures are fabricated via template assisted self-assembly in such a way that organic molecules such as proteins or lipids or both serve as a structural scaffold for the deposition of inorganic material. Nacre of abalone shell as a classic example for biomimeticists consist of thin films both in organic and inorganic phases. Due to its laminated structure as is shown in Figure 2.10 in which organic and inrganic molecules coupled together nacre has an impressive mechanical strength. Because of their special architecture, biocomposites such as nacre are simultaneously hard, strong and tough (Aksay et al. 1996).

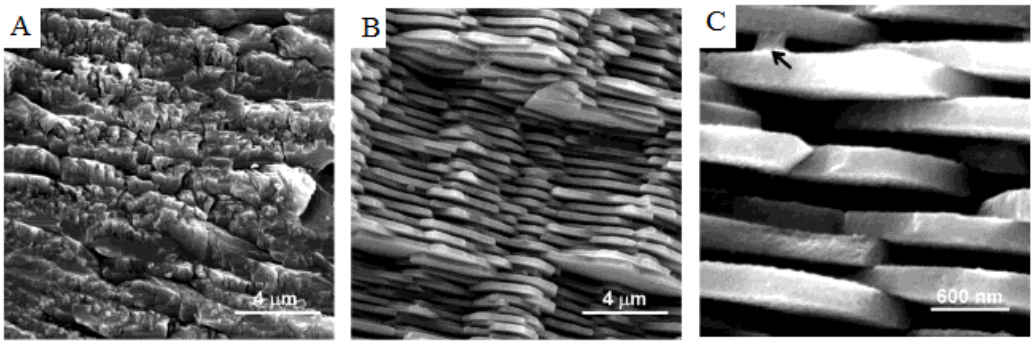

Figure 2.10 : SEM images of the cross section of abalone Haliotis rufescens. (A) Image of the fracture surface of the prismatic section (B) Nacreous section taken at increasing magnification (C) Organic phase serve both as scaffold and adhesive to hold aragonite platelets as indicated by black arrow (Li et al. 2004).

The structural analysis shown that organic template of nacre consist silk like glycine and alanine rich proteins in its core that is also involves a layer of β-chitin between this organic scaffold (Aksay et al. 1996). Organic template has also aspartic and glutamic acids for coating its outer layer. It is known that these biomacromolecules provide a scaffold which serve as control mechanism for the formation of its speficic $CaCO_3$ morphology. Biomaterials that are formed in mild conditions using commonly available chemicals with an environment respectful and high efficient way are hierachially structured composites contains soft organic materials highly organized on lenght scales of 1 to 100 nm.

Inorganic materials synthesized by biological organisms that is biomineralization is a source for engineering of producing functional biomimetic materials (Xu et al. 1998).

It is crucial to express that biological organisms forms laminated nanocomposites harnessing self-assembled organic scaffolds using directed nucleation and deposition

of inorganics (Xu et al. 1998). Using organic template reaction are conducted under mild conditions such as in aqeous solutions below 100 ºC (Xu et al. 1998). This process conditions are nearly opposite for techniques that is used for the formation of synthetic materials. The efforts for the undestanding of natural mineralization proceses can lead new material synthesis approaches. Hierarchical organization of organic-inorganic molecules from nano to macro dimensions for building macroscopic scale structure is an important lesson for not only biomedical but also for innovative engineering applications.

2.3.1 Calcium Phosphate

Calcium and phosphorus are widely distributed elements as being found in a quantity of about 3.4 wt % of calcium and 0.10 wt % of phosphorus on the surface layer of the Earth. When compared with other biominerals calcium phosphates are not found as much as $CaCO_3$ or SiO_2 however they are very important for being the main component of vertebrates skeletal systems (Dorozhkin and Epple 2002). Among their function for the formation of hard tissues of all complex biological systems calcium phosphtases are also an important class of biominerals found in not only in vertebrates but also in microscopic organisms (Mann 1988). Biomineralization is a biological process by which living organisms use organic molecules such as peptides and proteins to control the formation of inorganic hard tissues. In nature there are several types of minerals that synthesized by organisms like calcium carbonate ($CaCO_3$) or silicon dioxide (SiO_2). Also another crucial class of biominerals are iron oxides which is found for example, in magnetotactic bacteria (Blakemore 1982).

The importance of biominerals comes from their function which is the formation of biological hard tissues. Among lots of biominerals there is another very important one exist in nature namely calcium phosphate. Calcium phosphates are the most important inorganic components of biological hard tissues (Dorozhkin and Epple 2002). In vertebrates such as humans bone and teeth tissue are formed by calcium phosphates (Olszta et al. 2007). There are several different types of calcium phosphates according to their Ca/P molar ratio some of which are monocalcium phosphate monohydrate, monocalcium phosphate anhydrate, dicalcium phosphate dihydrate, dicalcium phosphate anhydrate, octacalcium phosphate, tricalcium phosphate, amorphous calcium phosphate, calcium-deficient hydroxyapatite,

hydroxyapatite and tetracalcium phosphate (Dorozhkin and Epple 2002). Being the main components bone and teeth calcium phosphate were studied widely due to their role in biomedicine. Since it formed skeletal and dental system of vertebrates formation and organization of calcium phosphates should understand properly (Dey et al. 2010). Human tooth enamel is composed of 96 % of apatite structure related crystals, whereas the remainder of the tissue is formed by an organic matrix consisting mainly of two classes of proteins: amelogenins and enamelins (Smith 1998). In a same manner human bone is composed of mainly calcium phosphate. Synthetic methods were developed to produce calcium phosphate to mimic hard tissues in order to understand biomineral formation (Bradt et al. 1999, Gungormus et al. 2008).

2.3.2 Calcium Phosphate Mineralization

Biomineralization process is of vital importance from the perspective of hard tissue formation. One of the core research fields in biomedical sciences is the formation and regeneration of hard tissues that bone and teeth. The term bone is in fact should be used to identify a class of materials which are built up of mineralized collagen fibrils (Dorozhkin and Epple 2002). On the other hand teeth are the second major hard tissue of vertebrates which can be defined as a calcification present in mammals. The structure of teeth is in fact more complex than that of bone. Teeth consist of at least 40 different proteins which serve as organic template to control tissue formation and two different biominerals namely enamel at the outside and dentin which is located interior (Dorozhkin and Epple 2002).

Calcium phosphate is the main chemical component that display its function in the building of hard tissues in vertebrates so the mechanism of calcium phosphate deposition during bone formation has some controversials. There are two debates about bone formation one of which is whether it is an active or a passive process. Active process means the self-assembly of calcium phosphate crystals in a matrix with a spatially organized way which was shown by transmission electron microscopy for bone and tooth formation. Passive process on the other hand means that blood serum is supersaturated with respect to calcium phosphate precipitation therefore mineralization should occur spontaneously at a suitable nucleus that is on a collagen fibril (Dorozhkin and Epple 2002). However the second controversial

question which is about the crystal phase transitions during bone maturation trace far back from the other (Weiner and Addadi 2010). For over a hundred years ago it was observed that the mineral phase present in mineralized tissues has a less stable phase during the first stages of mineralization which is known as Ostwald rule. Comparing with mature tissues it was regarded as an expected result since famous chemist, Ostwald, had shown that precipitation process from supersaturated solutions involves a precursor phase which precipitate, is usually not the thermodynamically stable one.

This mechanism that is the most stable phase ultimately formes after tranformation of first formed metastable one is known as Ostwald's Rule of Stages. For a long after this period researchers ignored the phase transitions although being a crucial step in tissue formation. After it was reported that there is a precursor mineral phase that is amorphous iron oxide called ferrihydrite in a mollusk called chitons which then formed magnetite as a more stable form (Kirschvink and Lowenstam 1979). Analyzing sample with TEM researchers identified highly disordered ferrihydrite which subsequently tranforms to thermodynamically stable form to magnetite. In 1990s phase transformation was proved for another organism that is Sea urchin larval spicules (Berman et al. 1993). Spicules that is made of calcite is known to diffract X-rays however researches reveal that intensities of the reflections were weaker since it consist an amorphous phase. Further investigations of larval spicules with X-ray photoemission electron microscopy proved that there are three diffrent phase during spicule maturation (Politi et al. 2008). First one is the amorphus calcium carbonate which is highly disordered (ACC1) transforms to an intermediate phase (ACC 2) and finally calcite (Weiner and Addadi 2010).

In vertebrates the mature bone and teeth has a mineral phase of carbonated hydroxyapatite. The controversial question of precursor phases was a long debate in the past. It is known that in vitro at around pH 8, first precipitated phase from a saturated solution is amorphous calcium phosphate which thermodynamically unstable so transforms into the crystalline mineral phase called octacalcium phosphate (Suzuki 2010). It has a structure that nearly same with hydroxyapatite except an added hydrated layer between apatite layers. Octacalcium phosphate (OCP) when exposed to hydrolysis form hydroxyapatite. The reason for the name of carbonated hydroxyapatite comes from the substution of phosphate molecules to carbonate which is found in a significant amount in the in vivo environment. So

among several types of calcium phosphates three of them are crucial from a perspective of scientific research. Amorphous calcium phosphate (AMP) first member of the most important calcium phosphate family has a Ca/P ratio of nearly 1.33 and is believed to be first deposited phase during bone formation (Mahamid et al. 2008). In vitro and also in vivo studies were revealed that there are some significant evidence that ACP is in fact a precursor phase before bone tissue maturation (Mahamid et al. 2010).

As mentioned above a fundamental question in biomineralization of calcium phosphate for the formation of hard tisues is the nature of the first-formed mineral phase. To address this question several attempts were made in past however recently with the advent of highly resolving analytical techniques for both imaging tissues under hydrated conditions it was found a solution to this long standing debate. Researchers were used a model organism Tuebingen long-fin zebrafish is a wonderful candidate to study mineralization because its fins grow continuosuly for the life span of the fish and new segments are added to the distal end while proximal segments grow and thicken (Mahamid et al. 2008). Since continuously growing fin bony rays of zebra fish provide analysis of three phases during bone maturation results can be accepted to be a prove for the precursor phase that is amorphous calcum phosphate (Mahamid et al. 2008). Using high-resolution scanning, transmission electron microscopy imaging and electron diffraction it can be at least for zebra fish the first deposite mineral is in amorph form which subsequently transform to hydroxyapatite (Mahamid et al. 2008). It is well studied in some model vertebrates but actually the transition of ACP to another calcium phosphate that is octa calcium phosphate in vivo in human could not proved so far.

The second very important member of caicium phosphate family, octacalcium phosphate (OCP) $Ca_8H_2(PO_4)_6.5H_2O$, is a transient phase of calcium phosphate which is thought to be formed after ACP deposition in bone formation. Bone mineral formation has been studied extensively to investigate actual mechanisms however key questions, as yet unanswered, that are mineral delivery to crystallization site and if it is first-formed mineral phase or not. The continuously forming fin bony rays of zebrafish provide researchers a very appropriate model to study mineralization in such a way that whole process is temporally and spatially resolved (Mahamid et al. 2010). There is another advantage comes when fin bones are examined since the

mineralized collagen fibrils are nearly same in structure to that is found all known bone materials. With using recent imaging techniques like synchrotron microbeam x-ray diffraction and small-angle scattering also used with cryo-scanning electron microscopy a lot of information about mineral phase, particles size and shape, and how mineral is deposited as amorphous calcium phosphate nanospheres, which transform into platelets of crystalline apatite within the collagen matrix (Mahamid et al. 2010). It can be said with respect to mineralization studies both in vitro and in vivo hydroxyapatite is the last and the crucial stage of mineral formation. Hydroxyapatite (HA) $Ca_{10}(PO_4)_6.(OH)_2$ may be most important one as being the third member of calcium phosphate family is of great scientific interest in the field of biomaterials due to their function in mineralized tissues. Teeth contain large amount of hydroxyapatite crystals which consist of nearly % 96 of this hard tissue (Bres and Hutchison 2002). Specifically, HA is the main constituent of bone, tooth enamel, and dental calculi, while OCP plays an important role in its formation with ACP which is proved to be a precursor phase during bone maturation at least in model organisms *in vivo*.

2.3.3 Mineralization Pathways

Understanding biomineralization processes in nature is crucial both for fundamental studies about biomineral formation and also for medical reasons to provide a solution of hard tissue related diseases. One of the main problems about this subject is the clarification of the processes involved in bone mineralization. Biomacromolecules proteins and polysaccharides are known to control biomineralization with different mechanisms to induce crystal growth (Belcher et al. 1996).

In biological organism all biomineral formation processes involve precipitation of mineral phase from a solution (Aksay et al. 1996). Inorganic material that already found in these organisms precipitate to form hard tissue from a solution with the help of several proteins. These protein serve not only a scaffold and control the formation of mineralization but also provide the catalytic activity that is sometimes necessary to induce crystal formation. Also essential step for mineralization is the heterogeneous nucleation that is to dictate the formation of inorganic phase directly on the substrate (Aksay et al. 1996). On the other hand homogenous nucleation involves a kinetically controlled process which is not preferable according to

heterogeneous nucleation in which process is controlled by thermodynamically (Aksay et al. 1996). At high level of supersaturation homogenous nucleation is the dominant way that can vary the mineralization time from seconds to months. Apart from fundemental studies of inorganic material formation it is crucial to extract information about biologic hard tissue formation. For this point of view natural organisms were studied widely to understand their mineral producing protocols which contains organic and inorganic phase collaboration.

Mineralizing proteins for the formation of hard tissues by depositing mineral on the scaffold were identified as the organic part of the process. These proteins for example soluble shell proteins of abalone shell can control crystal morphology (Belcher et al. 1996) or in algae it is known there some proteins that provide calcium carbonate formation from solution. On the other hand in vertebrates it is known that osteoblasts are responsible for bone formation. Osteoblasts are a type of cell which secreted osteocalcin which is an essential non-collegenous protein for bone formation (Hoang et al. 2003). It is known that mineralization in vivo is controlled by some proteins which are found in bone, the so-called noncollagenous proteins (Hoang et al. 2003). It was shown that through in vitro studies using chicken osteoblast cultures that bone formation is not a one step process rather can be identified as a multistage mechanism.

The mechanism of bone formation involve the growth of a collagen template (Dorozhkin and Epple 2002). Then non-collagenous proteins are produced that serve as controlling the crystallization of the calcium phosphate. It is believed that mineralization proteins that trigger inorganic phase formation contain generally acidic amino acids (Addadi and Weiner 1985). For example osteocalcin the protein that responsible for vertebrate bone tissue formation contains three tandem repeats of Gla residues that is serve for formation and growth of hydroxyapatite in bone tissue (Hoang et al. 2003). The most abundant noncollagenous protein in bone osteocalcin, whose precise mechanism of action is unclear, influences bone mineralization since its high binding affinity to the mineral component of bone hydroxyapatite (Hoang et al. 2003). Also osteocalcin is found to have a negatively charged protein surface that coordinates five calcium ions in a spatial orientation that is complementary to calcium ions in a hydroxyapatite crystal lattice (Hoang et al. 2003). Although osteoblasts are the main component for the formation of bone tissue in vertebrates

there is some other biological molecules are involved for the production of hard tissues. An important biomacromolecule that is associated with inorganic material production in organism is alkaline phosphatase. Alkaline phosphatase is a hydrolase enzyme that is found commonly in all organisms which has a function for removing phosphate so trigger the formation of inorganic phosphate. Alkaline phosphatase with the ability to form calcium phosphate is believed to play an important role in the formation of the main component of bone of the vertebrates.

There are some evidence about the role of bone formation of AP in organisms (Mornet et al. 2001) since mutations in the genes associated with alkaline phosphatase are related with hypophosphatasia, a rare inherited disorder, characterized by defective bone mineralization (Ritchie 1964). So formation of calcium phosphate with using AP is a common method to study biomineralization. In vitro studies for understanding biomineralization mechanism involves using both organic and inorganic molecules which mimics the formation of hard tissues (Gungormus et al. 2008). In order to achieve biomineralization researchers harnessed natural mineralizing proteins that extracted from tissues which can be impractical to extract and purify these proteins among hundreds of molecules. In order to overcome this problem there are different approaches to induce mineral formation in vitro (Wang and Nancollas 2009). One of these approaches is to use alkaline phosphatase to trigger inorganic material production. This way of mineralization is proved to be a robust method to form calcium phoshate in vitro. In this dissertation mechanism to form calcium phosphate in vitro by using alkaline phosphatase which genetically fused with gold binding peptide is explained in detail.

2.4 GEPI Fused Bifunctional Protein: 5GBP1-AP

2.4.1 Alkaline Phosphatase

Alkaline phosphatases are homodimeric enzymes probably common to all organisms that functions to catalyze the hydrolysis of phosphomonoesters (Stec, Holtz and Kantrowitz 2000).They are responsible for removing phosphate groups with release of inoganic phosphate. The active site of alkaline phosphatase have three metal-binding sites that is called M1 and M2 which both contain zinc ions and M3 occupied by magnesium a ion (Stec et al. 2000). The catalytic mechanism of AP

starts with activation of the catalytic serine by a zinc atom (Stec et al. 2000). The product of this activation is a covalent phosphoseryl intermediate which hydrolysis by a water molecule activated by a second zinc atom (Stec et al. 2000). Finally release of phosphate product occur or it is transfered to a phosphate acceptor. As its name indicates the optimum active range for AP is alkaline environment. The schematic representation of tissue non-specific alkaline phosphatase is shown in Figure 2.11.

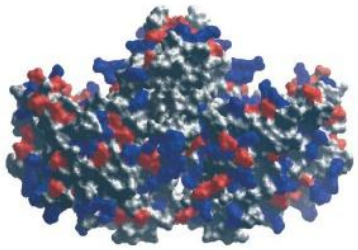

Figure 2.11 : Schematic representation of tissue non-specific alkaline phosphatase surface (Mornet et al. 2001).

Bacterial and eukaryotic AP nearly have same structure with respect to their conserved residues in the active site and ligands coordinating the two zinc atoms and the magnesium ion however mammalian and bacterial AP sequences is only % 25 - 30 identical (Mornet et al. 2001). In humans there are four types of AP are present. The first one is placental (PLAP), the second appears in germ cells (GCAP), the third in the intestine (IAP) and the last one is tissue non-specific (TNAP) alkaline phosphatase which is found in bone, liver, and kidney (Mornet et al. 2001).

Alkaline phosphatase are widely used in molecular biology for removing phosphate groups from 5' ends of linear vectors to prevent recircularization during cloning and dephosphorylation of DNA prior to kinase labeling reactions. It also used commonly with *p*-nitrophenyl phosphate (pNPP) which is a substrate for detecting alkaline phosphatase in ELISA applications. When alkaline phosphatase and pNPP are reacted, a yellow water-soluble reaction product is formed thus provide a chromogenic agent.

An important usage area of AP is biomineralization reactions. As mentioned in previous sections alkaline phosphatase is a crucial enzyme for studying biomineralization for the ability to form inorganic phosphate which then react with calcium that is already exist in medium so trigger mineral formation that precipitated from solution.

2.4.2 Gold Binding Peptides (GBPs)

Gold is a technologically important material as being biologically inert and has diverse applications in material science. Recent developments in nanotechnology provided different application areas for especially gold nanoparticles for their interesting properties. Gold nanoparticles play key role in a wide cutting-edge research area like localized surface plasmon resonance spectroscopy (Willets and Van Duyne 2007). In order to harness metal-protein asseblies in technological applications, the mechanism of the interaction between these materials should be elucidate. Although there are several methods to modify a metal surface with organic molecules (Love et al. 2005) inorganic binding peptides offer some promising solutions to build biomimetic functional systems (Sarikaya et al. 2003). Genetically engineered peptides for inorganics in last nearly ten years were proved to serve as a breakthrough for scientific fields including not only biology, chemistry, material science but also physics, engineering and medicine. As being an important member of GEPI family gold binding peptides were characterized for their high binding affinity to gold and proved to be a promising candidate for future applications (Hnilova et al. 2008, Tamerler et al. 2006a).

Gold binding peptide is one of the first selected inorganic binding peptides through cell surface display technique. GBP was displayed on the surface of bacterium Eschericia coli as part of the maltodextrin porin LamB, fused with alkaline phosphatase (Brown 1997). GBP possessing high binding affinity to gold that has 14 amino acids with a sequence of MHGKTQATSGTIQS (Hnilova et al. 2008). Also tandem repeats of GBP from one up to nine were constructed (Brown 1997). They e-were analyzed to understand the adsorption behaviour of the effect of increasing number of repeating units. It is important to express that GBP has not involve cystein group in its sequence which is known for high affinity to gold due its mercapto group. This offers an advantage since it means GBP has a different mechanism to bind gold rather than interacting of sulphur group with gold which is well-known for example in self assembled monolayers of alkanethiolates (Love et al. 2005). Sulpfur is known to have very high binding affinity to gold which can be said to form a semi covalent bond. GEPIs on the other hand form non covalent bonds which offers advantageous for dissociating mechanisms. The morphological basis of molecular recognition and self assembly of a genetically engineered peptide on (111) gold surface was analyzed

recently by atomic force microscopy (So, Tamerler and Sarikaya 2009b). It was observed a dynamic adsorption behavior proceed in two stages as schematically shown in Figure 2.12.

In first regime peptides form island-like structures when they approach to the surface. In second regime, these islands coalescence a network is formed by structural changes of

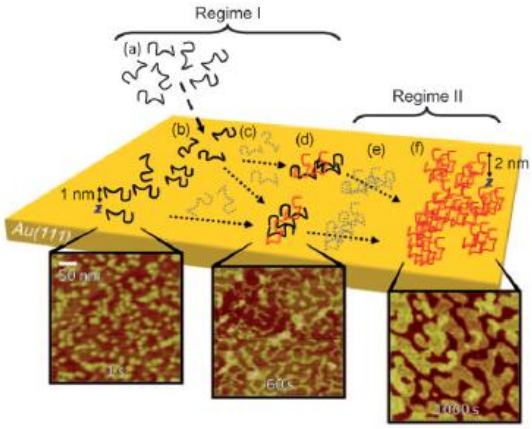

Figure 2.12 : Schematic model showing the proposed mechanism of the binding, diffusion, and assembly of 3rGBP1 on Au(111) (So et al. 2009b).

peptide molecules. Also binding kinetics of 3GBP extracted previously with SPR proved to be valid by fitting data to two-domain Langmuir model (Tamerler et al. 2006). On the other hand catalytic acvitiy of GBP fused with AP was shown by its ability to induce crystal formation (Brown, Sarikaya and Johnson 2000). At first stages of GBP research AP-GBP1 construct was shown that to trigger formation of gold crystals in the absence of any other reducing agent under ambient conditions as is given TEM images in Figure 2.13.

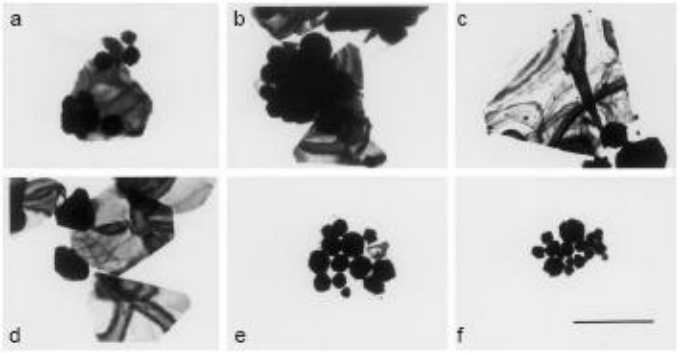

Figure 2.13 : Gold crystals formed by the reduction of $AuCl_3$ with using (a) GBP1-AP, (b) GBP2-AP, (c) 200 uM $AuCl_3$, 200 uM ascorbic acid (d) 200 uM $AuCl_3$ 500 uM citric acid (e) wild type alkaline phosphatase (f) 200 uM $AuCl_3$, 200 uM potassium ascorbate pH 7, 10 mM potassium phosphate pH 7 (Brown et al. 2000).

There are different motifs of gold binding peptides in the literature however the most used one is GBP1 whose sequence is given above. Other gold binding peptides for example AuBP1 and AuBP2 also shown that possess high binding affnity to gold. There are two conformations, linear (l) and cyclic (c) forms AuBP1 and AuBP2 which were constructed with 18 amino acid Cystein-Cystein loops for cyclic forms and open dodecapeptide version for linear one (Hnilova et al. 2008). They contains twelve amino acid with a sequence of WAGAKRLVLRRE for AuBP1 and WALRRSIRRQSY for AuBP2 (Hnilova et al. 2008). There are three additional amino acid in the case of constrained loops so there are overall eighteen amino acid with a sequence of CGPWAGAKRLVLRREGPC for AuBP1 and CGPWALRRSIRRQSYGPC for AuBP2 (Hnilova et al. 2008).

Although there are different constructs of GBP1 fused AP such as explained above, for the mineralizaiton studies explained here, five repeat gold binding peptide fused alkaline phosphatase were used as the main catalyzer for the formation calcium phosphate for some reasons which are the subject of next section.

2.4.3 Gold Binding Peptide Fused Alkaline Phosphatase

Five repeat gold binding peptide (5GBP1) as mentioned above is the one of the first constructed GBP1 motifs, which was fused originally with alkaline phosphatase. 5GBP1-AP as an inorganic binding fusion protein selected through cell surface display technique that displayed on the surface of bacterium Eschericia coli as part of the maltodextrin porin LamB (Brown 1997). Bacteria that strongly adsorped on to gold were exposed to enrichment procedures. In addition, another enrichment was conducted for chromium surface binders (Brown 1997). Nine cycles of enrichment was done for gold and six cycles was done for chromium. Then the inserts from the selected population cloned into pSB2991 that is alkaline phosphatase expression vector (Brown 1997). This procedure result with a metal binding polypeptide from insoluble LamB protein, which transforms into a soluble inorganic binding protein that resides in the periplasmic space of bacteria (Brown 1997). The increased repeats of GBP1 motif in was not shown higher binding activity on gold however up to nine repeat including three- five- seven-repeats were constructed (Brown 1997). Like other GBP1-AP constructs. 5GBP1-AP is well-studied about its both immobilized and catalytic activity after binding to surface as a bifunctional metal binding protein

(Kacar et al. 2009b). Wild type AP and GBP1-AP fusion proteins expressed in the periplasmic space of E. coli cultures. After two step purification by using liquid chromatography technique AP and GBP-AP were purified and differenrt tandem repeats of the GBP1 were analyzed according to their differences on gold binding and enzymatic activities (Kacar et al. 2009b). The characterization of this properties was conducted by using various spectroscopic and imaging methods. Molecular adsorption behaviour on to gold substarates was quantified using quartz crystal microbalance (QCM) and surface plasmon resonance spectroscopy. Using micro-contact printing directed self-assembly of the metal binding bifunctional GBP1-AP enzyme on patterend surface was also studied (Kacar et al. 2009b). The binding activity for 3GBP1 to gold is proved to be better than of single GBP1. For this reason, multiple tandem repeats of cell surface display-selected gold binding peptide were analyzed in detail (Kacar et al. 2009b). It found that 5GBP1-AP, result a high gold binding activity according to other constructs. In Figure 2.14 non contact mode AFM results for the binding of GEPI fused AP and wild type AP is shown.

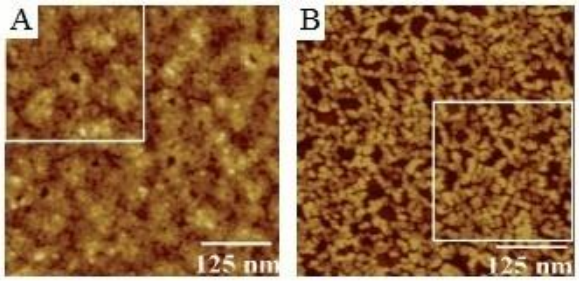

Figure 2.14 : The AFM images of surface topography of the 5GBP1-AP and AP (B) Discrete enzyme molecules are observed in (A) while the molecular distinction is lost and clusters appear (B) (Kacar et al. 2009b).

The particle sizes of the immobilized enzymes are very close with the molecular dimensions of the bacterial AP according AFM measurements. It can be recognized from the images that the GEPI fused AP units are obviously discrete and well ordered on the surface while wild type not. Activitiy assays for the wild-type and GEPI fused alkaline phosphatase constructs was found spectrophotometrically using pNPP as a substrate. Enyzmatic activity was calculated from Beer-Lamber Law due to release of p-nitrophenol. In Figure 2.15 enyzmatic activity of GEPI fused AP for four tandem repeats at different concentrations was given.

In order to monitor the gold binding affinity of GBP1 fused AP QCM measurements were conducted on gold electrode (Kacar et al. 2009b). The 5GBP1-AP and 6GBP1-

AP were identified as strong binders according to 7GBP1- AP or 9GBP1-AP. The 5GBP1-AP had also the highest phosphatase activity; this fusion construct, therefore, was chosen to carry out the subsequent directed immobilization studies (Kacar et al. 2009b). QCM is method to quantify adsorbed mass onto the quartz crystal. In this system a quartz crystal which is known to possess piezoelectric effect is used as a sensor surface (Nicu and Leichle 2008).

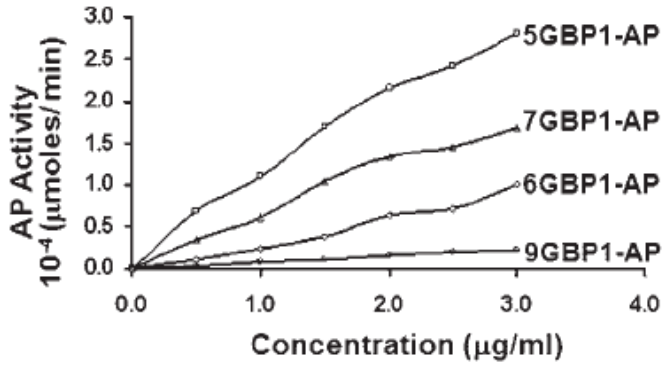

Figure 2.15 : Enyzmatic activity of GEPI fused AP for 5, 6, 7 and 9 tandem repeat constructs at different concentrations (Kacar et al. 2009b)

Piezoelectricity is the property of formation of a mechanical stress across the material when an electrical potential applied on the surface (Buttry and Ward 1992). Also QCM harnesses the piezoelectric effect of quartz crystal. When voltage is applied the quartz crystal of QCM a reorientation of the dipoles is trigger a shear deformation of the crystal (Viitala 2008). This shear deformation generates an acoustic wave travelling across the crystal in resonance (Viitala 2008). Resonant frequency can be used as an indication of adsorbed mass at constant temperature the change in resonance frequency of crystal has a linear relationship with the mass of deposited film onto the crystal (Viitala 2008). For this reason, QCM is widely used to measure the adsorption kinetics of proteins onto crystal surface that is quartz or coated with a thin film of a metal or polymer. When analyzed with QCM, it was found that 5GBP1-AP has the highest binding on to gold surface that is used as sensor surface in QCM as shown in Figure 2.16.

It was found due to the QCM measurements that other bifunctional constructs that is 6, 7, and 9GBP1-APs have low affinity on gold substrate according to 5GBP1-AP motif (Kacar et al. 2009b). After analyzing binding affinity of GEPI fused AP on gold surface, immobilization of protein by micro contact printing onto substrate surface was conducted so as to quantify immobilized enyme activity.

It is crucial in the process of immobilization to keep activity of an enzyme after immobilization to a surface since active sites of molecules should be available to function its catalytic reaction. For quantifying immobilization activity of 5GBP1-AP and wild type were achieved to adsorbed on gold srface by micro contact printing.

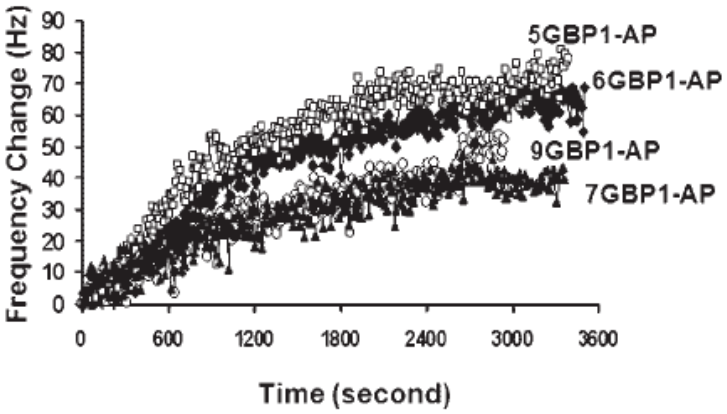

Figure 2.16 : QCM results of GEPI fused AP for different tandem repeat constructs at a concentration of 2.5 mg/mL (Kacar et al. 2009b)

Microcontact printing (μCP) is a flexible method to form patterns of biological molecules without using expensive traditional lithographic equipments (Jackman, Wilbur and Whitesides 1995). The patterning produce requires a poly(dimethylsiloxane) (PDMS) stamp which functions for transfering molecules to the surface of substrate (Xia and Whitesides 1998). PDMS in this procedure is coated with protein solution and simply applied to the surface allowing to contact with it to form protein patterns on the substrate. For wild type AP patterning on gols surface a linker molecules that is oligo (ethylene glycol)-terminated alkanethiols (OH-$(OCH_2)_3$-SH) was used. Thiol group of this molecule was binded to gold surace and functional group is free for the binding of WT AP. Although μCP is a versatile method to pattern surface with biological molecules there is a drawback here to immobilize enzymes on surface. The disadvantage of this procedure is the binding of anyzme to surface non-specifically since it can adsorped to substrate from its active sites however this can decrease activity. So immobilization of 5GBP1-AP is a robust way since it is known that GBP is located at the N-terminus of AP which is the opposite direction according to active site (Kacar et al. 2009b). The schematic represantation of μCP and AFM images of patterned surface with both 5GBP1-AP and WT AP is shown in Figure 2.17.

Quantifying immobilized enzyme activity was conducted both for patterned and non-

patterned surfaces. In the case of non patterned surface it was calculated for wild type AP that immobilization of protein on the surface at a amount of only % 2 could achieved while this amount is % 66 for 5GBP1-AP (Kacar et al. 2009b).

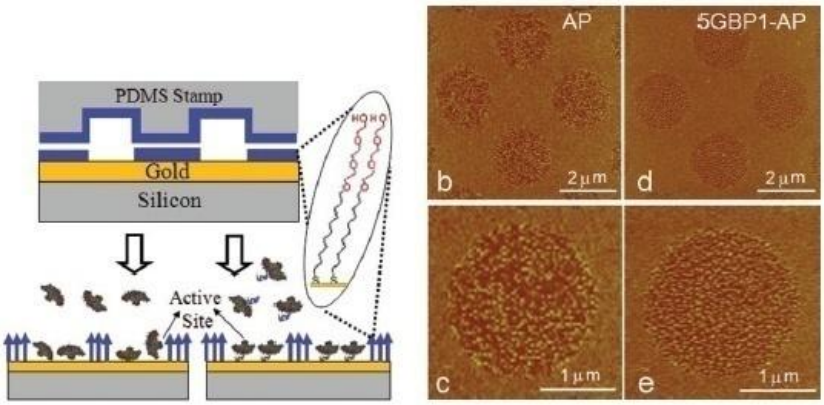

Figure 2.17 : Schematic representation of PDMS stamping of proteins on patterned surface and thier tapping mode AFM images (Kacar et al. 2009b).

For the case of patterned surfaces activity is importantly differs when compared with free enzymes in solution as is shown graphically in Figure 2.18.

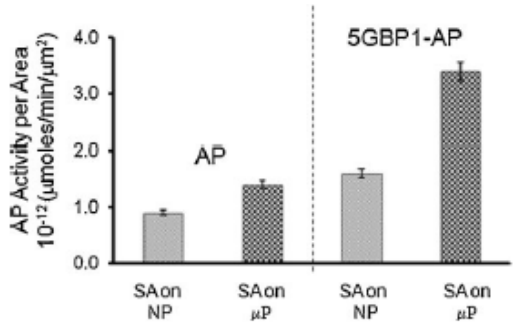

Figure 2.18 : Activities of wild type and 5GBP1-AP for both micro-patterned and non-patterned surfaces (Kacar et al. 2009b).

There is an increase in activity for patterned surfaces for both Wt and 5GBP1-AP however, the activity of GEPI fused AP is higher than that of wild type (Kacar et al. 2009b). Quantitatively the difference between AP with 5GBP1 linker and wild type is nearly three times according to enzymatic activity (Kacar et al. 2009b). So GEPIs offer more advantage than only providing high binding affinity to inorganic surfaces since they also serve as efficient molecular linkers especially for protein immobilization.

Monitoring of calcium phosphate mineralization by using 5GBP1-AP construct is the subject of this dissertation. In order to harness this inorganic biniding bifunctional protein it was first expressed in E.coli cells and extracted from periplasmic space of

cells. In the next step 5GBP1-AP was purified with a two stage purfication protocol using liquid chromatography. The subject of the next section is the procedures that is used for protein purification using liquid chromatography.

2.4.4 Protein Purification Using Liquid Chromatograpy

Protein purification is one the most important topics in biotechnology. There are several techniques and methods to purify the desired protein however it can be difficult to obtain product with desired purity. Purification schemes generally contains the use of chromatography. Chromatography is simply involves the binding of target product to a media that is generally a type of resin loaded on a column and ultimately elution of product with washing steps by different solution at a flow rate which is controlled by a pump. There are some different techniques to purify a protein according to their specific properties. Desired product can be found in several solutions that includes different size of contaminants or proteins at different net surface charges. Selecting one of the appropriate techniques as schematically shown in Figure 2.19 according to product properties results an intented purification.

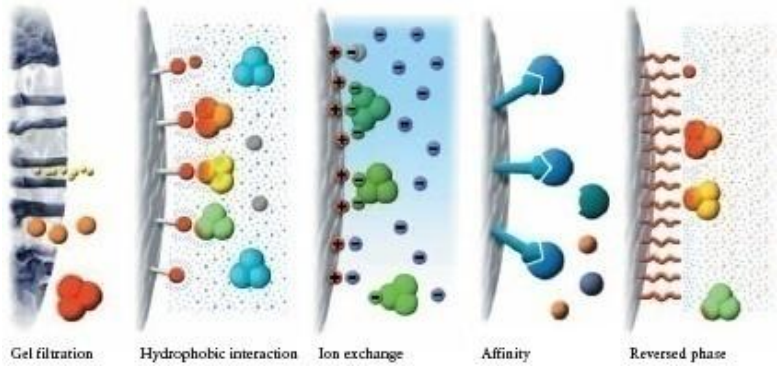

Figure 2.19 : Purification techniques based on the differences of desired product properties (Image taken from Ion Exchange Chromatography & Chromatofocusing Principle & Methods, Amersham Biosciences 2004,http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/3E56FFCAFE43BCADC1257628001D0EC6/$file/11000421AB.pdf Accessed 19 December 2011).

In addition analytical assays during pruification can be an effective way to follow the progress of the process. This analytical ways consist of monitoring UV absorption spectra of sample while passing through a UV flow cell or can be conductivity measurements.

For the purification of 5GBP1-AP two step purification procedure was used that

includes an ion exchange and a size exclusion chromatography. For this reason, these two techniques are explained in detail in this section.

As mentioned above, biomolecules can be purified using one of the chromatographic methods due to their specific properties. Ion exchange chromatography (IEX) is such a technique that harnesses net surface charge of a protein. Proteins have many different amino acids containing acidic and basic groups. A protein's net surface charge is determined by its amino acid sequences and can be change with changing the pH of the environment. Overall net charge of a protein can be determined by its net charge versus pH relationship curve. Since proteins are amphoteric it is possible to charge a protein either positive or negative. Each protein at a specific pH value have no net charge. This is called isoelectric point (pI). Above this pH value protein has a net negative and below this pH it has a net postive charge. IEX chromatography uses this property to bind or elute of desired protein reversibly onto an IEX media.

In common ion exchange purification, depending on the net charge of the target protein at a specific pH value, negatively or positively charged medium should selected to use as IEX media. A protein can bind to a positively charged medium, an anion exchanger, above its pI value that is when it charged negatively. In contrast, same protein binds to a negatively charged medium, a cation exchanger, when below its pI value that is when it charged positively. IEX purification involves four main steps to seperate target product. In the first stage media is equilibrated with buffer solution. The sample is then load to the column and allow to pass through at a fixed flow rate. During this step contaminants that have opposite charged with target protein or uncharged molecules elute directly. In the third stage increasing ionic strength that is making gradient replace ions with proteins. The high ionic strength elute strongly bind proteins from column. In the last stage washing column with high concentration salt or ethanol elutes all binded molecules before re-equilibration.

Another technique for purification of protein is gel filtration or size exclusion. As its name indicates this method harness the size differences of proteins as is shown in Figure 2.21. In contrast with ion exchange chromatography, molecules do not bind to the chromatography medium in gel filtration. Small molecules can diffuse into pores of the medium according to bigger ones so they stay in column longer than others. As a result high molecular weight proteins elute first from column and the smallest

one arrive at last. As mentioned above, the purification of 5GBP1-AP was achieved with using first an ion exchange and then a size exclusion chromatography. The details of purification protocol can be found in Materials and Methods section. High purity wild type and GEPI fused AP, in further experiments used for characterizing their binding affinity to gold by using surface plasmon resonance spectroscopy. In the next section details of SPR spectroscopy can be found.

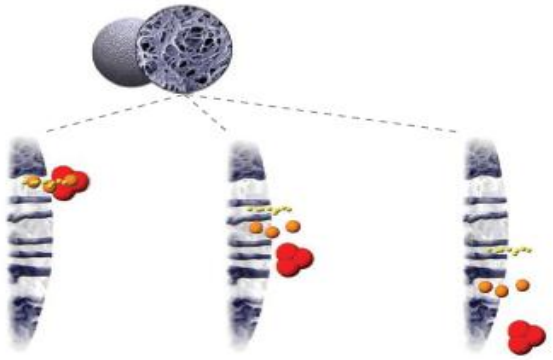

Figure 2.20 : Schematic representation of size exclusion chromatography. Large molecules leave the column first followed by smaller molecules in order of their size (Image taken from Gel Filtration Principles & Methods GE Healthcare 2007, http://www.gelifesciences.com /aptrix/upp00919.nsf/Content/226C564DBB7D5229C1257628001C D36F/$file/18102218AK.pdf Accessed 19 December 2011).

2.4.5 Surface Plasmon Resonance Spectroscopy

Surface plasmon resonance (SPR) spectroscopy is a characterization technique for biomolecular interactions. The fundemental principle of SPR is the resonance of surface plasmons which provide a sensitive method to extract information about interaction mechanisms of molecules of interest (Tudos and Schasfoort 2008). Surface plasmons also known as surface plasmon polaritons are electromagnetic waves, which can be defined as propagating electron density waves occurring at the interface between metal and dielectric (Kooyman 2008). Surface plasmons are essentially light waves that are interact with the free electrons of the metal film and can be defined as surface plasmon polaritons which reflect their hybrid nature (Barnes, Dereux and Ebbesen 2003). Surface plasmon polariton (SPP) is a photon-plasmon surface electromagnetic wave that excite on a conductor surface (Englebienne, Van Hoonacker and Verhas 2003). The wave propagates along the planar interface between a metal and a dielectric medium. The interaction of free electrons with electromagnetic wave result an oscillation in resonance with the light

wave (Barnes et al. 2003). The formation of surface plasmons occur with the excitation of free electrons on the surface of metal commonly gold or silver (Willets and Van Duyne 2007). This excitation is formed through an electromagnetic radiation that hits the surface of a thin metal film and totaly reflected (Tudos and Schasfoort 2008).. Photons, energy carrying particles of light, transfer their energy to surface electrons which cause them to oscillate since they are excited (Kooyman 2008). At a specific angle between the light and the metal, surface electrons are excited in such a way that they oscillate at their resonance frequency (Tudos and Schasfoort 2008). Excitation of surface plasmons at a specific angle results a minimum or a dip in the intensity of the reflected light (Tudos and Schasfoort 2008).. This angle is called resonance angle or SPR angle. This angle is related with optical properties of the system for example the refractive index of the media at both sides of the metal. The propagating of electrons on the metal surface decay evanescently in the z-direction (Willets and Van Duyne 2007). So-called evanescent field is related with the penetration depth of the electromagnetic field decaying exponentially from the metal surface (Willets and Van Duyne 2007). A schematic representation of a SPR spectroscopy is shown in Figure 2.22.

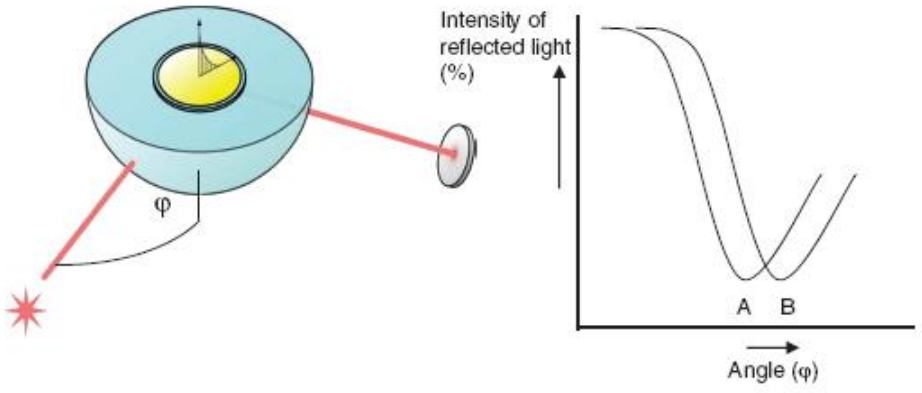

Figure 2.21 : A representation of an SPR system based on a gold sensor surface on a prism. The dip position of the reflected light (Tudos and Schasfoort 2008).

In addition to gold or silver materials with a negative real and small positive imaginary dielectric constant have the ability to possess a surface plasmon resonance (Willets and Van Duyne 2007). The resonance angle is related with the optical properties of the system for example refractive index of the media at both sides of metal. The refractive index of the bottom side of metal that is at prism side is not

change however if a change occur on the metal surface refractive index change due to for example adsorption of a molecules hence cuase a shift in dip position (Tudos and Schasfoort 2008). The change in dip position is related with adsorped mass on the sensor surface so SPR is used obtain kinetic and thermodynamic properties of the binding process (Kooyman 2008). During the adsorption of any molecule onto metal surface or in the case of biomolecular interactions binding of one molecule to the other, the change in dip position versus time is monitored in real-time on a sensogram as shown in Figure 2.23.

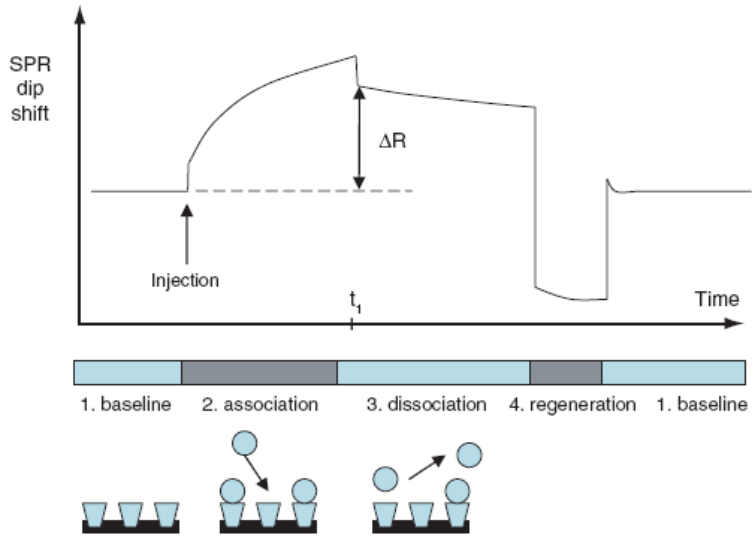

Figure 2.22 : The change in SPR dip position versus time due to the adsorption of molecules onto sensor surface (Tudos and Schasfoort 2008).

Plasmonics as a promising field of research related with particular light-matter interactions uses the shifts in the plasmon resonance which can be monitored in three modes: (a) angle resolved, (b) wavelength shift, and (c) imaging (Willets and Van Duyne 2007). In the angle resolved mode, the reflectivity of light from the metal surface is measured as function of angle of incidence at constant wavelength (Willets and Van Duyne 2007). In the wavelength shift mode the reflectivity of light from the metal surface is measured as function of wavelength at a constant angle of incidence (Willets and Van Duyne 2007). In the imaging mode both constant wavelength and incident angle is used for mapping the reflectivity of the surface as a function of position (Willets and Van Duyne 2007). Generally angle resolved SPR system are used in sensing applications. A widely used setup for SPR systems is the Kreschtmann configuration in which a thin gold film with a 50 nm thickness is

coated on a glass substrate used a sensor surface. A white light source directed to gold surface under the metal passing through a prism. The reflected light is detecting by a detector thus progress the change in dip position can be monitored in real-time. Since the adsorped mass is associated with the change in dip position adsorption and desorption curves provided by SPR can be fitted to a mathematical model to obtain kinetic and thermodynamic constants about the binding process. For kinetic studies there are several models to calculate association (k_a), dissociation (k_d) constants and equilibrium constant (K_{eq}) (Luthgens and Janshoff 2005, Jung and Campbell 2000, Karpovich and Blanchard 1994). In all these models experimental data is fitted to adsorption isotherm however, the most studied one is the Langmuir adsorption model as given in Equation 1. This model explain the rate of surface reaction where Θ is the fraction of surface coverage, c is the concentration of molecule of interest, k_a and k_d defines association and dissociation constants respectively (Karpovich and Blanchard 1994).

$$\frac{d\theta}{dt} = ka\,.(1-\theta\,).c - kd\,.t \qquad \textbf{(2.1)}$$

Integration of Eq. (1) give rise to come up with time course of binding event as given in Equation 2.

$$\theta\,(t) = \frac{C}{C+(\frac{kd}{ka})}[\,1-\exp(-(ka.C+kd).t)] \qquad \textbf{(2.2)}$$

This equation can be simplified as in following Equation 3:

$$\theta\,(t) = K'[1-\exp(-kobs\,.t)] \qquad \textbf{(2.3)}$$

Equation 4 can calculate equilibrium contant using association and dissociation constants.

$$Keq = \frac{ka}{kd} \qquad \textbf{(2.4)}$$

The free energy of the adsorption upon binding also found by Equation 5 as follows:

$$\Delta G = -R.T.\ln(\,Keq) \qquad \textbf{(2.5)}$$

Thermodynamic properties of an interaction between biomolecules or the intearction between a molecule and a surface such as a thin metal film like gold, silver or platinum can be calculated using SPR spectroscopy. After obtaining kinetic constants mathematical models such as Van't Hoff equation (John and Weeks 2000) can be

used to calculate enthalpy, entropy and Gibbs free energy of the process. Equilibrium association and dissociation contants provide the free energy ΔG of interaction with which enthalpy and entropy values can found by using Equation 6.

$$\ln(Keq) = \frac{\Delta H}{R.T} - \frac{\Delta S}{R} \quad \textbf{(2.6)}$$

Plotting K_D versus 1/T values it is possible to calculate enthalpy and entropy values which provide information about binding mechanism of interacting molecules.

2.4.6 Raman Spectroscopy

Raman spectroscopy is a characterization technique to extract information about the properties and structures of target molecules. It is possible with this method to characterize a sample using Raman scattering phenomenon (Nafie 2001). In Raman scattering electromagnetic radiaton scatters as a result of oscillating electrons due to oscillating electric field (Nafie 2001). This scattering is unelastic contrast to Rayleigh elastic scattering. Unelastic scattering means the frequency of the photons coming from generally a laser source has a different energy level according to other scattered incident light. In other words when photons scatter upon interaction with the sample loose or gain some portion of their energy which correspond to the energy of a transition for target molecule. The interaction cause a shift in the frequency of photons which provide vibrational information about the molecules of interest (Nafie 2001).

Raman spectroscopy can serve a robust approach to obtain detailed information about the molecular structure of the sample. It is also possible to exploit full information using improved Raman spectroscopic techniques about chemical structure and composition of the molecule of interest (Kneipp et al. 1999).

Characterization of biominerals were also studied with Raman spectroscopy as a reliable method to analyse mineral samples (Stewart et al. 2002). Calcium phosphate minerals which can be amorphous or different crystal morphologies are analyzed by Raman spectroscopy in order to extract information about their structure including crystal phase transitions (Politi et al. 2006). Transient mineral phases during bone maturation was shown by Raman spectroscopy which provided information for the presence of octacalcium phosphate like structures in vivo (Crane et al. 2006).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The E. coli S2157 cells harboring either the plasmid pSB2991 that encodes AP and, pSB3057 encoding 5-repeat gold binding peptide fused to AP, were provided by S. Brown (University of Copenhagen, Denmark). For growing cells yeast extract tryptone medium was used. Peptone, yeast extract granulated sodium chloride, ampicillin, Tris (Hydroxymethyl) aminomethane, magnesium chloride, sucrose, acrylamide, ammonium peroxodisulfate and sodiumdodecylsulfate were purchased from Merck (Dermstadt, Germany). N,N Methylene bis acrylamide was purchased form Sigma-Aldrich (Stenheim, Germany). Isopropylthiogalactopyranoside (IPTG), p-nitrophenyl phosphate (pNPP), phenyl methane sulphony fluoride (PMSF) were purchased from Biochemica AppliChem (Dermstadt, Germany). Unstained protein molecular weight marker and Bradford reagent and Bradford standarts were purchased from Fermantas (Maryland, USA). Sodium β-glycerophosphate pentahydrate was purchesde from Alaf Aesar (Karlsruhe, Germany) Diethyl amino ethyl Sephacel and Sephacryl S200 resins were purchased from GE Healthcare (Uppsala, Sweden). Amicon Ultra centrifugal concentration tubes were purchased from Millipore (Carrigtwohill, Ireland). Costar 96 well ELISA plates were purchased from LP Italiana (Milano Italy).

3.2 Cell Growth and Periplasmic Protein Extraction

Cell broth sterilized for 5 minutes at 120 ºC using Tomy SX 700 High Pressure Sterilizer. Ampicillin was added as a final concentration of 100 mg mL^{-1} during cell growth. Cells were growth at 37 ºC with shaking at 200 rpm using Thermo Orbital Shaker. Optical density of the cells was measured by Shimadzu UV 1700 spectrophometer until they reached at a absorbance of 0.5 – 0.7 at 600 nm. Then

1mM IPTG added as final concentration and growth conducted for 6 hours with shaking at 37 ºC.

3.2.1 Osmotic Shock Protocol

In order to extract periplasmic protein from cells in which APs were synthesized osmotic shock protocol applied as follows: Cells harvested by centrifugation at 5000 rpm for 15 minutes using Beckman Coulter Avanti JI 30 centrifuge. After centrifugation, cells resuspended by 30 mM Tris buffer pH 7.5. Then centrifuged again at 5000 rpm for 15 minutes and resuspended with % 20 sucrose in 30 mM Tris pH 7.5 buffer, incubated at room temperature for 15 minutes then centrifuged again at 5000 rpm for 15 minutes. Lastly, cell were resuspended by 1 mM MgCl2 in cold 30 mM Tris pH 7.5 buffer, incubated on ice for 10 minutes and centrifuged at 4 ºC for 15 minutes. Supernatant collected and 1mM PMSF added as protease inhibitor. After osmotic shock procedure, supernatant filtered through a filter that has 0.45 uM pore size. Periplasmic fraction was concentrated with ultrafiltration tube using Beckman Coulter Allegra 25 centrifuge to a final volume of 5 mL.

3.3 Purification of Alkaline Phosphatase

3.3.1 Ion Exchange Chromatography

Fast protein liquid chromatography (FPLC) technique used for the two-step purification of APs. For this two-step purification procedure, first an ion exchange chromatography that is specifically an anion exchanger for APs and secondly a gel filtration also known as size exclusion chromatography conducted. BIO-RAD Biologic Duo Flow FPLC system used to purify target proteins. DEAE Sephacel™ resin loaded to a glass column and equilibrated with 20 mM Tris pH 8.0 buffer at a flow rate of 2 $mL.min^{-1}$ until stable UV and conductivity baseline achieved. Buffer preperation is extremely important to conduct an efficient purification. All buffers used during purification should thoroughly filtered with 0.45 μm pore size filters and degassed using ultrasonication for at least 15 minutes or by vacum filtration before used in the system. Sample loaded to column and washed with equilibration buffer. After elution of positively charged and uncharged molecules, AP eluted with making a salt gradient from 0 to 0.1 M NaCl. Fractions of eluted protein collected with a

fraction collector and analyzed with pNPP at a final concentration of 5.5 mM in 10 mM Tris pH 7.5 10 mM $MgCl_2$ that is AP reaction buffer at 405 nm using BIO RAD Benchmark Microplate Reader. Active fractions, that is resulted to give a yellow color to reaction mixture upon reacted with pNPP, then collected and analyzed with sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). AP containing fractions then pooled and concentrated to a final volume of 5 mL.

3.3.2 Size Exclusion Chromatography

The second step of AP purification conducted by using Sephacryl S200™ resin which loaded to a glass column and equilibrated with 30 mM Tris pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl at a flow rate of 0.3 $mL.min^{-1}$. After stable baseline, sample loaded to column and washed with same buffer. Collected fractions analyzed with again 5.5 mM pNPP and analyzed with SDS-PAGE that has a running voltage of 95 V. SDS-PAGE gel stained with Coomassie blue for 10 minutes at 37 ºC with mild shaking and destained with % 20 methanol, % 20 acetic acid solution at least 3 hours. AP containing fraction then pooled and concentrated. Exchange of protein buffer from size exclusion to AP reaction buffer conducted with Amicon 10K MWCO ultrafiltration tubes with centrifugation at 3500 rpm. Protein concentration determined by using Bradford method with using Bovine Serume Albumin protein with a concentration between 0.125 – 2 $mg.mL^{-1}$ as Bradford standarts.

3.4 Instruments and Methods

3.4.1 Surface Plasmon Resonance Experiments

A Reichert SR 700 single channel SPR spectrometer used to characterize binding properties and real-time monitoring of calcium phosphate mineralization of both wild type and 5GBP1 AP proteins. SR 700 uses Kretchman configuration in which a high refractive index prism, sapphire for SR 700 having a refractive index of 1.76, constructed for coupling of thin metal surface as sensor. A light source that is light emitting diode in SR 700 placed under the prism. At a specific angle at, light beam is totally internally reflected causing plasmon resonance on metal film at about 66 º. SPR gold slides are thin gold films with a thickness of about 50 nm coated onto BK7 glass, which has a thin chromium layer for the attachment of gold. Slides treated with

Novascan PSD UV/Ozone cleaner for 10 minutes before used in experiments. UV/Ozone cleaner is a robust technique for removal of molecular organic contaminants by using intense ultraviolet light emitted at wavelengths of 185 nm and 254 nm. UV emission results production of O_3 by decomposing ambient oxygen. Cleaned gold surfaces placed on the sapphire flow cell upon dropping immersion oil for matching refractive index. AP reaction buffer pumped to system with using a peristaltic pump at a flow rate of 0.1 $mL.min^{-1}$. All buffers usef during experiments prepared with deionized water, filtered through 0.2 μm pore size filter and degassed using ultrasonication at leat for 10 minutes. It is very important to prepare fresh buffer solutions for each experiment since a stable baseline can only be achieved with degassed and thoroughly filtered buffers. In addition, the temperature of the system should match with the environment and solutions that used during experiments.

SPR is very sensitive to temperature changes and refractive index is highly dependent on temperature of the sensor area. All buffers and samples should be at same temperature to obtain reliable results form SPR experiments. After a stable baseline achieved with AP reaction buffer, pump immediately switched to protein solution at final concentrations between 2–6 μM. Upon binding curve stabilization, reaction buffer immediately pumped again to system for washing loosely bound proteins from gold surface. Buffer was allowed to pumped to system for at least 10 minutes and then pump switched to mineralization solution. For real-time mineralization experiments, a mixture of Ca^{+2} and β-glycerophosphate was prepared at a final concentration of 24 mM and 14.4 mM respectively. Reaction mixture pumped to system to saturate alkaline phosphatase that assembled onto gold surface with mineralization solution in order to induce mineral formation. Upon saturation of surface finished and stabilization reached, pump stopped and refractive index continued to monitor for 24 hours.

3.4.2 Raman Spectroscopy Experiments

Raman spectroscopic measurements were conducted by Horiba Jobin Yvon Labram 800 micro Raman spectroscope. A He-Ne laser source with a 632.8 nm line was used as the excitation source. Focusing laser beam was achieved in Labram 800 by 50X objective. The diameter of the beam is approximately 1 μm. The power of laser on

the mineral samples was 3 mW. All experiments were conducted at room temperature and mineral samples were analyzed without further sample preparation. All samples were used after collected from SPR experiments and stored at room temperature.

4. RESULTS AND DISCUSSION

4.1 Purification of Alkaline Phosphatases

Alkaline phosphatases used in this study were extracted from periplasmic space of E.Coli cells by osmotic shock procedure and purified in two-step via FPLC with using first an anion exchanger and a gel filtration chromatography. Partially purified protein solution by ion exchange column was analysed with SDS-PAGE technique and further purified to obtain highly pure alkaline phosphatases with size exclusion chromatography. After all purification steps protein fractions were analyzed with SDS-PAGE in order to identify APs according to molecular weight marker.

4.1.1 Anion Exchange Chromatography

Periplasmic fraction of E.coli cells involving either 5GBP1- or WT AP were harvested with an osmotic shock procedure by destruction of outer cell wall and partially purified after concentrated to 5 mL using 10K MWCO ultrafiltration tubes by centrifugation at 3500 rpm. Partial purification conducted with ion exchange chromatography in which DEAE Sephacel™ resin was used as anion exchanger equilibrated with 20 mM Tris pH 8.0 buffer at a flow rate of 2 $mL.min^{-1}$. Since alkaline phosphatase has an isoelectric point at about 4.5, at pH 8.0, it has a net negative charge which enabling the elution of positively charged contaminants, neutral proteins or other cellular components directly by washing with equilibration buffer. In order to elute target protein a linear salt gradient was applied using NaCl with a final concentration of 0.1 M. During salt gradient ions exist in the medium change their locations due to their relative binding capacity to oppositely charge components. Analysis of AP elution with using substrate pNPP was yielded reaction mixture to turn yellow and provide immediate characterization of target protein according to analysis of fractions by SDS-PAGE. For this reason, eluted fractions analysed by pNPP during purification and active fractions then loaded to % 5 stacking % 12 separating SDS gel in order to prove AP purification. Chromatogram

given in Figure 4.1 shows the first purification step of AP using an anion exchanger. The change in UV absorbance and conductivity due to elution of contaminants and target protein is indicated on the graph.

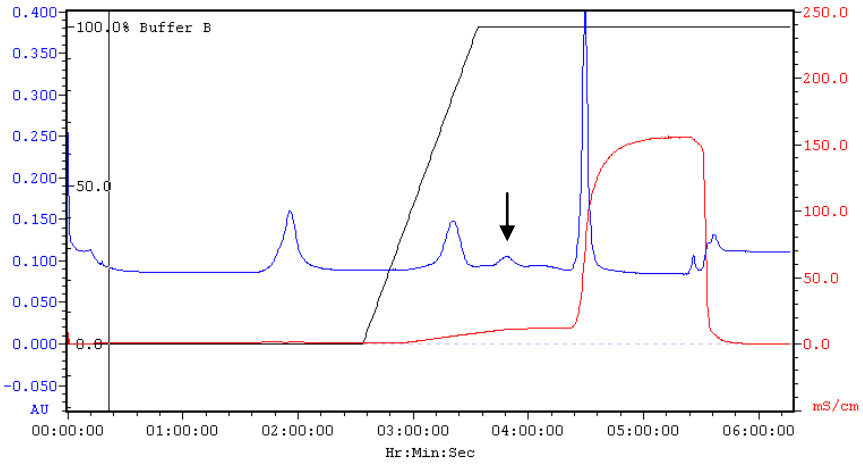

Figure 4.1 : Chromatogram of AP purification by anion exchange chromatography. Blue and red lines represent UV absorption and conductivity of samples passing through the column respectively. Arrow indicates AP elution peak.

4.1.2 Gel Filtration Chromatography

Partially purified 5GBP1- or WT AP by ion exchange chromatography purified further with gel filtration also known as size exclusion chromatography in order to eliminate contaminants to come with highly pure alkaline phosphatase. Ion exchange fractions were analyzed with AP substrate pNPP for a fast chromogenic assay with which it is possible to assess if fractions consist AP or not. Active fraction were then analyzed by SDS-PAGE technique to determine both the existence of AP at about 52 kDA for WT and 57 kDa for 5GBP1-AP. Then AP containing fractions were pooled and concentrated to 5 mL using 10K MWCO ultrafiltration tubes by centrifugation at 3500 rpm. Sephacryl S200™ resin used as size exclusion resin was equilibrated with 10 mM Tris pH, 7.5 1 mM $MgCl_2$ and 150 mM NaCl buffer at a flow rate of 0.3 $mL.min^{-1}$. In size exclusion chromatography, all components loaded to column were eluted directly according to their molecular weight. In other words, contaminants that have high molecular weight were eluted first and low molecular weight molecules were eluted at the end of purification. As in ion exchange step, AP containing fractions were analyzed during purification with AP substrate pNPP, which yielded reaction mixture to turn yellow and provide immediate characterization of AP

existence. Further analysis of active fractions was conducted using SDS-PAGE with %5 stacking %12 separating gel to analyze AP purification. Chromatogram given in Figure 4.2 shows size exclusion purification steps of AP in which change in UV absorbance and conductivity implying the elution of contaminants and target protein.

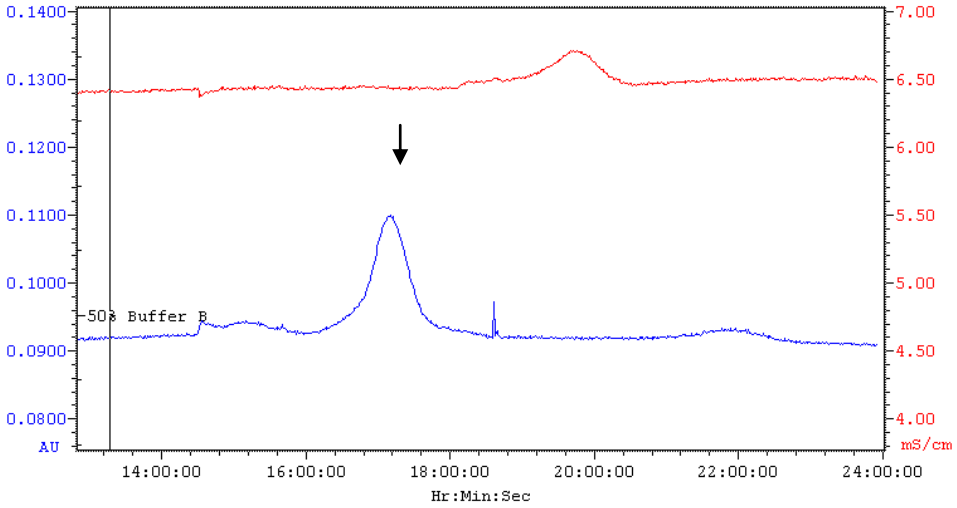

Figure 4.2 : Chromatogram of AP purification by gel filtration chromatography. Blue and red lines represent UV absorption and conductivity of samples respectively. Arrow indicates AP elution peak.

Pure fractions that contain AP were then pooled and concentrated again with using 10 K MWCO ultrafiltration tubes by centrifugation at 3500 rpm. AP reaction buffer 10 mM Tris pH 7.5 and 10 mM $MgCl_2$ is exchanged with size exclusion buffer in order to use AP at its optimum environment.

After concentrated with ultrafiltration tubes at 3500 rpm to 1.5 mL, enzyme concentration was measured by Bradford method. Overall purification products analyzed by SDS-PAGE as given in Figure 4.3.

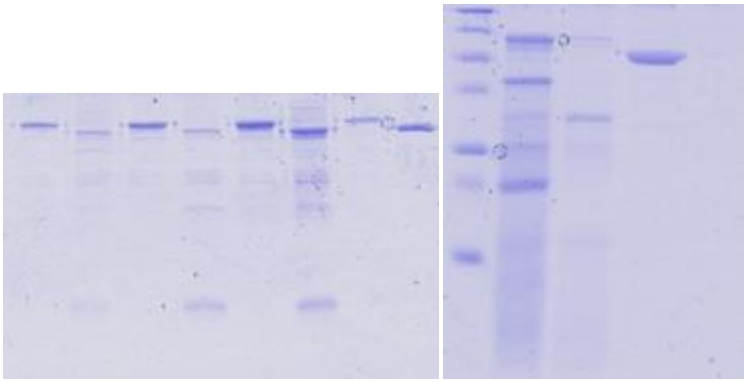

Figure 4.3 : SDS-PAGE results of APs (left) the difference of WT vs 5rGBP-AP (Right) marker, periplasmic fraction, ion exchange and gel filtration samples

4.2 Enzymatic Activity of 5GBP1- and WT Alkaline Phosphatases

Enyzmatic activity is curicial for studying catalytic reactions since progressing of chemical reaction catalyzed by enzyme is important step of overall reaction pathway. It is important to determine optimum ranges for target enzyme to achieve high enyzmatic activity. Alkaline phosphatase is relatively stable at alkaline environment and known to be catalyze specific reactions at 37 ºC in vivo. The most studied activity assays for AP based on chromogenic reaction of pNPP in alkaline medium. Release of phosphate group from substrate by AP can qauntitatively analyzed by spectroscopic methods in which decomposition of pNPP yielding yellow color in turn change reaction mixture's absorbance at a specific wavelenght that is 405 nm.

4.2.1 Activity Assay of Alkaline Phosphatase

Quantitative enzyme activity assays are based on detection of a specific change of the reaction which yield a product upon catalytic reaction that triggered with target enzyme. The interaction between the enzyme and its substrate results with a product which can be spectroscopically monitored in order to determine the progress of reaction in terms of a change at a specific wavelenght. Experimental data can be fitted to one of the mathematical models that are capable of analyzing the acvitivity of an enzyme quantitatively. Michealis-Menten equation given in Equation (4.1) is commonly used for enyzmatic activity assays in which the rate of reaction for different substrate concentrations measured in time intervals to assess the progress of reaction (Fogler 2006).

$$-rs = \frac{kcat\,.(Ce).(Cs)}{(Cs)+KM} \qquad \textbf{(4.1)}$$

The net rate of disappereance of the substrate –rs, can be measured by means of a change that monitored with spectroscopic techniques and results with turnover number, kcat. Turnover number is the number of substrate molecules that is converted to product in a given time on single molecule enzyme (Fogler 2006). In this condition it is important to saturate enzyme with substrate which means $C_S >> K_M$. The K_M constant is a measure of attraction of the enzyme for its substrate so its called affinity constant also known as Michaelis constant (Fogler 2006).

The correlation of product formation in various substrate concentrations results Michealis-Menten plot, where maximum rate of reaction at assay conditions and

affinity constant K_M can be calculated by using a mathematical modification. Exponential plot of Michealis-Menten can simply modified to yield V_{max} and K_M by plotting reciprocal of absorbance and substrate concentrations to come up with Lineweaver-Burk plot (Fogler 2006). Identifying maximum rate of reaction for a given enzyme concentration as given in Equation (4.2).

$$Vmax = kcat\,.\,Ce \tag{4.2}$$

V_{max} and K_M are calculated from the intercept and slope of Lineweaver-Burk plot by fitting of experimental data to Equation (4.3). The intercept of the new graph yield V_{max} and the slope can give rise to calculate K_M by using calculated V_{max} value.

$$-\frac{1}{rs} = \frac{1}{Vmax} + \frac{KM}{Vmax}\left(\frac{1}{Cs}\right) \tag{4.3}$$

Enyzmatic activity of APs in this study were quantitatively analyzed with 0.3 µmol enzyme at 37 ºC and calculated by Michealis-Menten equation with using different concentrations between 0.5-6.5 mM substrate, pNPP. Calculation of 5GBP1- and WT AP activities were conducted in an ELISA plate by taking 15 absorbance data at 405 nm for 15 min. Mean velocity values were calculated from raw data and plotted as mean velocity versus time as given in Figure 4.4 and Figure 4.5 for 5GBP1- and WT AP respectively.

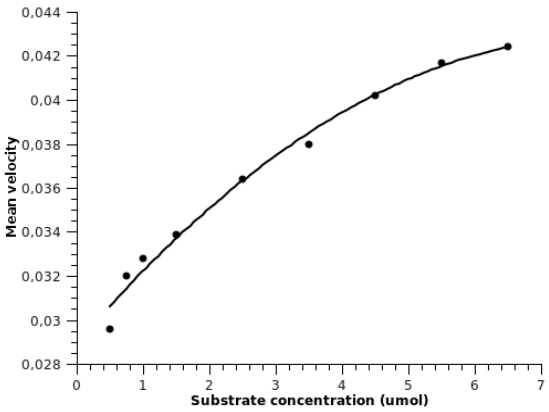

Figure 4.4 : Michealis-Menten plot for 5GBP1-AP where enzyme concentration is 0.3 µmol. Assay conducted at 37 ºC for 15 min.

In order to calculate reliable activity values of enzymes it is important to optimize substrate concentrations and reaction time. Substrate concentrations should consist a minimum concentration value that possess catalytic activity and a maximum concentration value that can possess the end of enzymatic reaction.

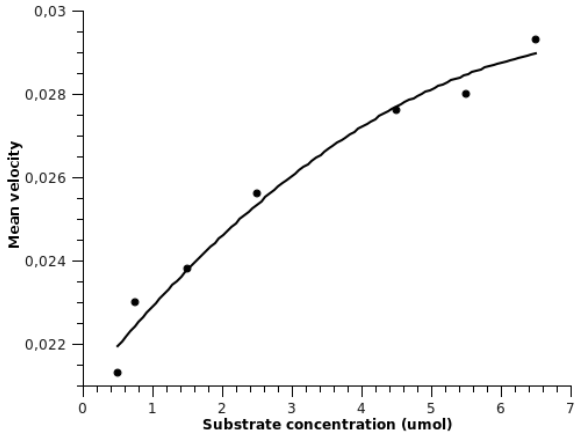

Figure 4.5 : Michealis-Menten plot for WT AP. Assay conducted at 37 ºC for 15 min. Enzyme concentration is 0.3 µmol

For this reason, during enzymatic activity assays high concentrations should represent a saturation which can be shown in Michealis-Menten graphs. The activity assays for both 5GBP1- and WT AP were conducted with ten concentrations of substrate. Velocity measurements were taken every minute for 15 minutes in duplicate essay. Mean velocity values were calculated as the average value of these readings. Plotting reciprocal of mean velocity and substrate concentration values yielded Lineweaver-Burk graph as shown in Figure 4.6 for WT-AP which V_{max} and K_M calculated.

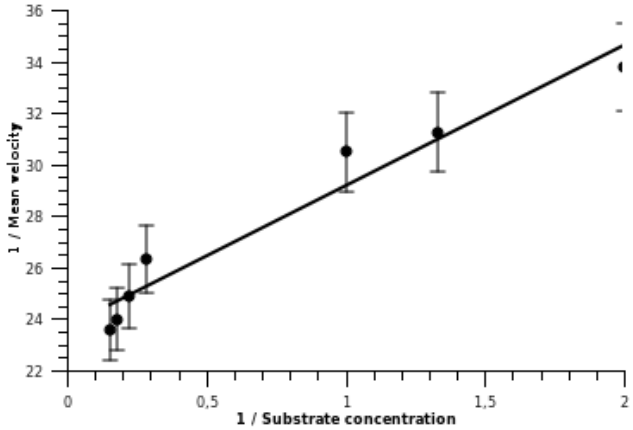

Figure 4.6 : Lineweaver-Burk plot for 5GBP1-AP. Assay conducted at 37 ºC for 15 min. Enzyme concentration is 0.3 µmol

Same graph is shown in Figure 4.7 for 5GBP1 with which V_{max} and K_M calculated. Then it can be possible with these graphs to come up with quantitative information about the activity of the enzyme. These graphs for these reasons is crucial for providing insights to determine how the enzyme will function in experimental conditions.

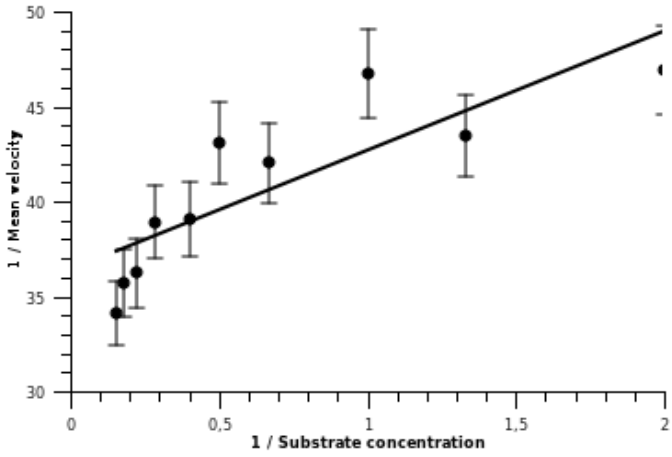

Figure 4.7 : Lineweaver-Burk plot for WT AP. Assay conducted at 37 ºC for 15 min. Enzyme concentration is 0.3 µmol.

The intercept and slope values of Lineweaver-Burk plot were used for calculation of activity constants. Quantitative enzymatic activity values of 5GBP1- and WT AP are given in Table 4.1.

Table 4.1 : Calculated activity constants for 5GBP1- and WT AP

	V_{max} (Abs.min^{-1})	K_M (µM)	$k_{cat}.10^{-6}$ (min)$^{-1}$
5GBP1-AP	0.0416	0.2207	0.0277
WT AP	0.0275	0.1726	0.0183

5GBP1-AP activity was found higher than WT AP for all activity constants that measured at the same conditions. Both V_{max} and K_M are about 1.5 times higher then WT which means the maximum velocity of reaction and affinity of substrate enzyme complex are superior when GEPI fused AP. Also turnover number kcat is about 1.27 times higher than WT AP. Commonly modified enzymes possess low activity according to wild types ones. The main reason for this activity loss stem from the occupied active site of enzyme due to randomly adsorp to the surface of interest. On the other hand, there are lots of application in biotechnological processes in whch enzymes must be immobilized to solid surfaces in order perform a task. In this case 5GBP1-AP was thought to perform lower enzymatic activity however, it was found higher than WT which is an acceptable result. 5GBP-AP has also gold binding feature which can open routes for different applications. In summary gold binding peptide fused AP provide important opportunites as being a bi-functional protein with material selectivity and performing enzymatic activity with its modified form.

4.2.2 Characterization of SPR Spectroscopy Sensor Surface

Real-time monitoring of calcium phosphate biomineralization in situ by harnessing either 5GBP1- or WT alkaline phosphatase in order to induce mineral growth on solid surface, surface plsmon resonance spectroscopy was used in this study. SPR uses a thin gold film with a thickness of about 50 nm on a glass substrate. SPR as mentioned in previous sections, based on plasmon oscillations, which are surface free electrons that excited on gold film. In order to conduct an analysis by using these oscillations sensitivity, first metal film that possess plasmon oscillation should be characterized by using spectroscopic behaviour of SPR device. If SPR sensor, usually gold or silver, can induce plasmon polariton oscillation, biomolecular interactions can be analyzed either as an adsorption on metal surface or molecular interactions between different associated chemical structures.
In summary before conducting an experiment by SPR, sensor surface, here gold, should be characterized properly by means of plasmon oscillation occurance.

4.2.3 Array Scan Function

SPR dip position, the decrease of the intensity of reflected light is the characteristic behaviour of plasmon oscillation due to energy transfer from photons to the surface free electrons. In other words, it is cruical to harness surface plasmon resonance to analyze a binding or molecular interaction when plasmon oscillation is proved to occur on sensor chip. The method to assess plasmon oscillation is the monitoring of reflection spectrum of metal surface, which was exposed to a light source in order to excite free electrons.

Reichert SR 700 system used in this study, provides an array scan property, which enables user to measure reflection spectrum. This process is a spectroscopic analyze of the gold layer in order to monitor energy absorbance behaviour of metal surface. Using array scan function of SR 700 it is possible to analyze dip position of SPR before and after conducting an experiment. A typical array scan of the reflection spectrum of gold surface is shown in Figure 4.8. Real time monitoring of binding, adsorption or biomolecular interactions is the change of dip position of SPR. This change in SPR is monitored in a graph that is time versus an arbitrary unit, which defines the change of dip position.

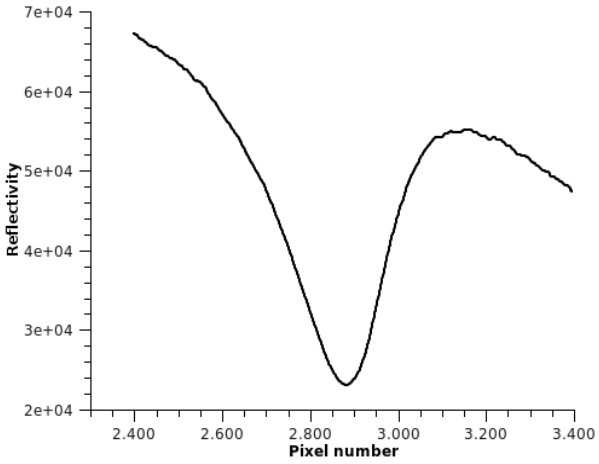

Figure 4.8 : Reflection spectrum of thin gold film coated on glass substrate used a SPR spectroscopy sensor sensor. Dip position in the spectrum is a direct indication of absorbance capability of electromagnetic wave of surface electrons.

4.3 Binding Analysis from SPR Data

In this study, all binding and mineralization experiments were conducted with Reichert SR 700 SPR. In this system, binding event that is the change of SPR dip position monitored as an adsorption isotherm on a graphical interface with time versus μ Refractive Index Unit (RIU). RIU is an arbitrary unit selected for binding analysis of molecules on gold surface, which trigger refractive index change of the medium. The angle of incidence difference due to adsorption and change in the optical properties of the system in turn shifts the dip position of SPR. Detecting of the change of dip position give quantitative data about the interaction that occur on sensor surface. For this reason, analyses of SPR signal for extracting information about the adsorption and desorption events related to target biomolecules is crucial.

4.3.1 Interpretation of Refractive Index Change

Obtaining reliable kinetic and thermodynamic consants by using SPR spectroscopy can only be achieved through a detailed analysis of refractive index change. In SR 700 system change in SPR dip position can be monitored in four different modes: Resonance angle, μRIU, pixel number and ΔμRIU. In this study, all experiments performed by monitoring time versus μRIU since adsorption isotherm that obtained upon binding event fitted to Langmuir adsorption model and it is appropriate to take SPR data as an arbitrary unit to follow binding event.

4.4 Mathematical Modelling of SPR Signal

In this study, all adsorption curves related to binding of both 5GBP1-AP and WT AP on bare gold surface or WT AP on modified gold surface were quantitatively characterized by fitting experimental data to a Langmuir adsorption isotherm. Langmuir adsorption originally gives information about the adsorption of gas molecules onto a solid surface are commonly used to extract information of solid binding behaviour of molecules. Not only for SPR but also other characterization techniques like QCM, Langmuir adsorption is widely used for calculating kinetic and thermodynamic constants related to adsorption event. Although there are several methods to understand kinetic and thermodynamic properties of solid-biomolecule or molecule-molecule interactions such as Monte Carlo simulation, Langmuir model is generally, prefer for interpretation of SPR experimental results.

4.4.1 Langmuir Adsorption

In this model, adsorption and desorption curves are fitted either to a mathematical model that involves both adsorption and desorption event or to a single model explain adsorption behaviour by fitting only binding curve however in turn provide extra information about dissociation that comes from model inself. As given in Equation 4.1, Langmuir model provides both association (k_a) and dissociation constant (k_d) for 1:1 molecular binding mechanism.

$$\frac{d\theta}{dt} = ka\,.(1-\theta).c - kd.\theta \qquad \textbf{(4.1)}$$

In Equation 4.1 (1-Ɵ) is the fraction of surface coverage and c is the concentration of the molecule that is adsorped to solid surface. Integration of Equation (4.1) results the adsorption of molecules onto metal surface as in the form of monolayer in ideal cases as given in Equation (4.2):

$$\theta\,(t) = \frac{c}{c+\frac{kd}{ka}}\,.[1-\exp(-(ka\,.c+kd)\,.t))] \qquad \textbf{(4.2)}$$

Equation (4.2) can be written in form of Equation (4.3) expressing an observable k constant as follows:

$$\theta\,(t) = K'[1-\exp(-kobs.t)] \qquad \textbf{(4.3)}$$

Since it may take time of solution pumped to system to reach an initial adsorption rate Equation (4.3) can further be modified as follows:

$$\theta\ (t - t0) = \ K^{'}[1 - \exp(-\text{kobs.}\ (\text{t} - t0)] \quad \textbf{(4.4)}$$

In addition to 1:1 molecular binding mechanism, which is commonly dominant in adsorption events, modified Langmuir equations for other mechanism can be obtained by simply adding association (k_a) and dissociation constant (k_d) to the model as given in Equation (4.5).

$$\theta\ (t) = K1^{'}[1 - \exp(-k1obs.t)] + K2'\ [1 - \exp(-k2obs.t)] \quad \textbf{(4.5)}$$

Observable kinetic constant provides a concentration dependent association rate which in turn result to calculate association (k_a), dissociation (k_d) and equilibrium (K_{eq}) constants. Following, using K_{eq} it is possible to quantitatively express the surface coverage of gold surface as given in Equation (4.6):

$$\theta\ (\infty) = \frac{c}{c + \frac{1}{Keq}} \quad \textbf{(4.6)}$$

Thermodynamic properties of interaction occur onto gold surface upon adsorption and desorption can be calculated using K_{eq} such as free energy of adsorption or enthalpy and entropy change of the system as given in Equation (4.7) and (4.8) respectively.

$$\Delta G = \ -R.T.\ln\ (Keq) \quad \textbf{(4.7)}$$

$$\ln\ (Keq) = \frac{\Delta H}{R.T} - \frac{\Delta S}{R} \quad \textbf{(4.8)}$$

Calculation of enthalpy and entropy values then can be simply done by plotting K_D versus 1/T graph that provide information about the thermodynamic properties of the interaction.

4.5 Alkaline Phosphatase Binding on Metal Surface

GEPIs that have high material selectivity to inorganic surfaces are used in addition to other biotechnological applications to immobilize protein complexes on different metal surfaces to come with up high performance bi-functional systems. The interaction of protein with metal surfaces is a complex subject which is widely studied to understand the mechanism of binding of protein to inorganic surface. It is not only important to illimunate this interaction theoretically but also to provide

information for construction of diverse multifunctional systems for nanobiotechnological applications.

In this study alkaline phosphate was used as a bi-funcitonal protein which immobilized on gold surface through five tandem repeat gold binding peptide. Also WT AP was used in order to correlate the differences with 5GBP1 property on metal binding. One of the purposes of using GEPI is to prove gold binding actvity of 5GBP1 fused AP with high affinity to gold surface which is also known has higher enzymatic activity then WT AP. In addition the overarching goal of this study is to monitor calcium phosphate biomineralization with immobilized AP by using surface plsmon resonance spectroscopy. Also it is important to assess if 5GBP1-AP has a robust mineralization potential or not according to its high affinity and enymatic activity.

4.5.1 5GBP1-AP Binding Analysis onto Gold Surface

The interaction of 5GBP1 AP with gold surface was studied with SPR spectroscopy. In SPR spectroscopy a thin gold film is used to propagate plasmons in order to analyse interactions on the surface harnessing high sensitivity of plasmon oscillation. Purified 5GBP1-AP was assessed in order to understand its binding to gold surface using different concentrations. SPR as being a very sensitive spectroscopic method provide to study with relatively low concentrations. The experiments for binding of 5GBP1-AP onto gold were conducted with concentration 2, 3 and 4 μMolar. As mentioned in previous sections, SPR experiments were conducted at room temperature and AP reaction buffer was used in all binding analysis as a referance solution. After achieving a stable baseline that is monitored real-time, 5GBP1-AP consisting solution was pumped to system with a volume of about 500 μL. The binding process was allowed to progress until saturation of gold surface that is coverage of metal with protein monolayer was completed.

The binding curve of a protein either 5GBP1 or WT AP can be defined at the first stage of SPR experiments is a qualitative information which provide only the relative measure of binding mass onto metal surface. If the refractive index change is higher than a reference protein here WT AP, it can be deduced that the target protein that is 5GBP1-AP can bind to gold surface more than reference WT AP. Since the refractive index change is sensitive to the change on adsorped mass on sensor

surface, the monitoring of binding in real-time result to analyze directly without calculating affinity constants quantitatively. So during SPR experiments it is possbile to assess qualitative affinity of 5GBP1-AP on gold surface. However it is not sufficient to analyze a binding process only qualitatively since it can not provide kinetic constants. It is crucial to calculate kinetic and thermodynamic constans quantitatively to provide actual information about the nature of binding event. For 5GBP1 and WT AP kinetic and thermodynamic constans were calculated by using Langmuir model after conducting binding experiments with SPR. The binding curves of 5GBP1-AP with different concentrations are shown in Figure 4.9.

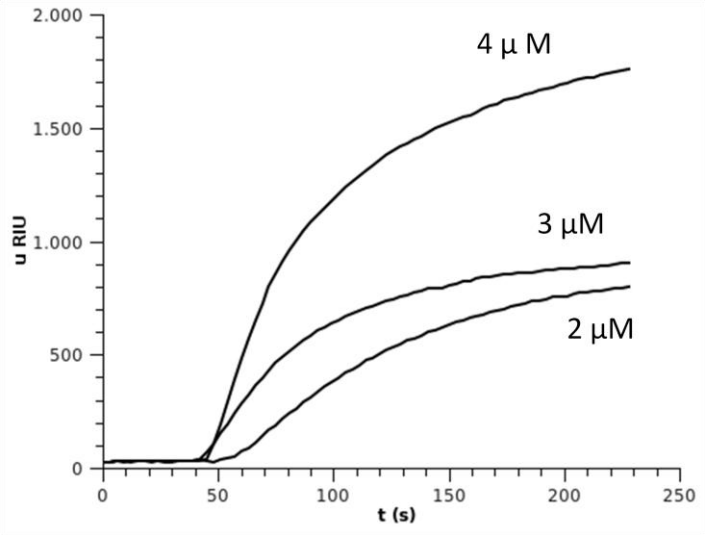

Figure 4.9 : SPR sensogram for binding of 2, 3 and 4 µM 5GBP1-AP onto SPR gold surface.

The binding process of 5GBP1-AP monitored time versus an arbitrary unit µRIU which provided to monitor the progress of interaction of protein with gold surface. After binding process AP reaction buffer was pumped to system in order to analyse dissociation event that is desorption of some AP molecules from surface and also to achieve a stable baseline again for the next step of experiment. The difference between 5GBP1 and WT AP at the same concentration was analysed in order to correlate GBP performance which will be discussed in the next section.

The analysis of gold binding of 5GBP1-AP was conducted with three different concentrations, 2, 3 and 4 µM. It is sufficient to analyze three different concentration in order to calculate kinetic and thermodynamic constants by using Langmuir 1:1 adsorption isotherm. The more concentration means more accurate calculation of

affinity constants. During the optimization of SPR experiments both higher and lower concentrations of AP were used and it was found that concentration between 2-4 μM are optimum. The binding analysis of 3 μM 5GBP1-AP on gold surface was conducted at the same conditions with 2 μM. The adsorption process of 3 μM 5GBP1-AP is shown in Figure 19.

The total adsorped mass on sensor surface upon binding of 3 μM 5GBP1-AP is higher than 2μM 5GBP1-AP as expected since the adsorption of mass on gold surface is related with the concentration of the protein. In theory total refractive index change should change linearly with the increase of protein concentration. However there must be a limit value for concentration at which metal surface is saturated with protein and surface covareage is near to % 100. It is ideal for a protein to bind on metal surface as a monolayer. In addition after binding process, AP buffer was again pumped to system in order to desorp loosely bound proteins either on gold surface or upon monolayer.

At least three concentrations should be analyzed in order to calculate kinetic and thermodynamic constants quantitatively. For this reason, as the highest concentration 4 μM 5GBP1-AP was used in SPR experiments. As an expected result in ideal conditions 4 μM should bind to gold surface more than both 2 and 3 μM protein. This means adsorped mass on sensor surface is higher than the case in lower concentrations. The adsorption isotherm of 4 μM 5GBP1-AP is shown in Figure 19.

As is shown in graphical representaion of experimental data, 4 μM 5GBP1-AP induced high refractive index change according to other concentrations. Qualitatively it is obvious that more AP molecules bind to sensor surface upon interaction of gold binding peptide with gold film. Similar to other SPR experments AP reaction buffer pumped to system upon adsorption process in order to progress desorption event which will be discussed later.

Comparison of the three different concentration of 5GBP1-AP adsorption onto gold surface provide information about the concentration dependent binding of AP onto sensor surface. When analyzed according to increased concentrations, it can be deduced for 5GBP1-AP at these concentration SPR analysis

was reliable since there is a nearly linear change of refractive index which is the ideal case for an SPR analysis.

4.5.2 Wild Type AP Binding Analysis onto Gold Surface

All biomolecules possess relative affinity to metal surfaces due to their amino acid sequnce. For gold, specifically cysteine, histidine and methionine containing proteins can interact with gold surface through sulfur-gold interaction since it is known that sulfur has high affinity (about 45 kcal/mol binding energy) to gold. For this reason, some protein molecules can easily adsorped to metal and can either desorp or not upon washing with buffer solution.

In this study WT AP affinity to gold was analyzed as a reference adsorption event to 5GBP1-AP. In order to compare 5GBP1 binding to gold surface with WT AP, two proteins were used at same conditions. Concentration of both proteins were found optimum at 2, 3 and 4 μM. Same buffer composition and SPR conditions were used for WT AP in SPR experiments to analyze affinity to bare gold substrate. Adsorption isotherm of 2, 3 and 4 μM WT AP onto gold sensor surface of SPR is given in Figure 4.10.

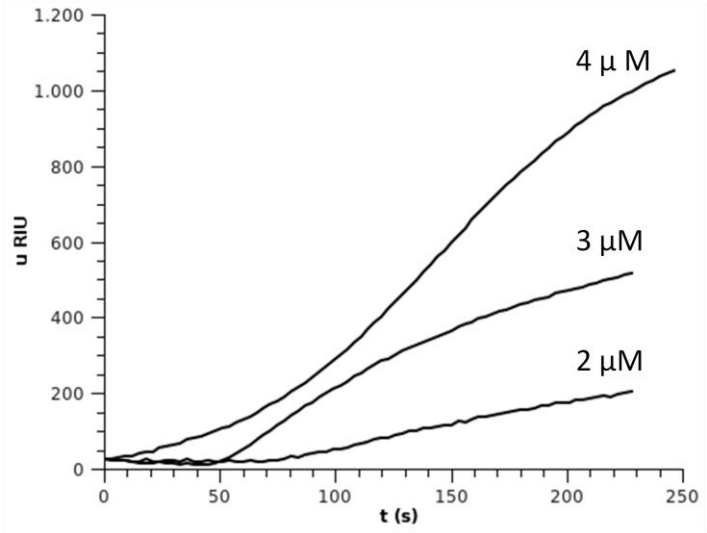

Figure 4.10 : SPR sensoram for binding of 2, 3 and 4 μM WT AP onto SPR gold surface.

WT AP adsorption on gold surface at a concentration of 2 μM lead a change in refractive index unit that is lower than 5GBP1-AP in the same concentration. It is a direct proof for GBP activity, which provides higher adsorption through its material selectivity. The difference between WT and 5GBP1 AP is only the modification of enzyme with 5 tandem repeat gold binding peptide. For this reason, it can be deduced that GEPI fused AP has robust metal binding property due to its GBP content.

The same concentrations of 5GBP1 AP also used for SPR experiments of WT AP in order to compare all three concentrations and to calculate kinetic and thermodynamic constants quantitatively. In Figure 20, adsorption isotherm of 3 μM WT AP onto SPR sensor surface is given. As in the case of 2 μM WT AP, at the concentration of 3 μM, refractive index change of SPR is lower than 5GBP1 AP at same conditions. This observation can be explained as the adsorped mass of 3 μM 5GBP1 AP on gold substrate is higher than WT AP. It is obviously due to high gold affinity of 5GBP1 AP according to gold binding peptide existence. In addition, adsorped mass of 3 μM WT AP onto gold is higher than 2 μM WT AP which is an expected result since at low concentrations the number of interacting molecules with gold substrate is lower than high concentration.

Gold binding analysis of WT AP was also conducted with 4 μM protein. It is sufficient to calculate affinity constants with three concentrations that assessed with SPR spectroscopy. In addition comparing all three concentrations is crucial to come up with results for analysing metarial selective binding of 5GBP1 AP.

Gold binding property of 5GBP1 was again observed at the concentration of 4 μM. As is shown in Figure 20, 4 μM WT AP adsorped onto gold substrate and triggered a change in the refractive index which is lower than 5GBP1 AP at same concentration. This result serve as an additional prove for binding affinity of GBP to gold according to WT.

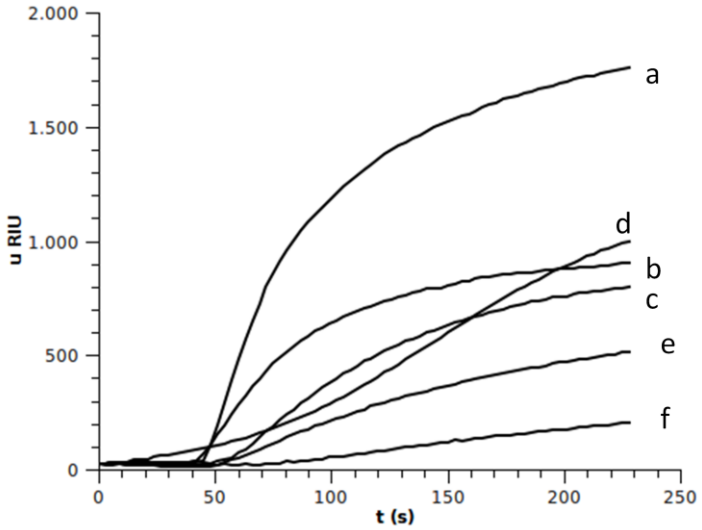

Figure 4.11 : Adsorption of 5GBP1- and WT AP for all three concentrations onto SPR gold surface. (a) 2, (b) 3, (c) 4 μM 5GBP1- AP, (d) 2, (e) 3, (f) 4 μM WT AP respectively.

The difference of adsorption behaviour between 5GBP1-AP and WT AP is obvious when same concentrations compared on a single graph as shown in Figure 4.11. In all three concentrations, 5GBP1-AP adsorption onto gold sensor surface is higher than that of WT AP. These observations serve as a direct proof of GBP's metal binding property. Immobilization of AP onto gold surface was proved to be achieved by using gold binding peptide fused AP.

5GBP1-AP provide advantages as being a bi-functional protein with which there is no need to modify metal surfaces to immobilize enzymes by organic molecules and further functional group modificiations. low enzymatic activity due to binding of enzyme through its active site. Since 5GBP1 fused from the N terminus of AP, active site of the enzyme is thought not occupied and results high catalytic activity.

4.6 Adsorption Kinetics and Thermodynamics of 5GBP1-AP and WT AP

Protein immobilization studies are based on extraction of kinetic properties of biomolecules that adsorped onto surfaces since the constant realted with this binding event provide information about the nature of immobilization. It is important to study immobilization quantitatively to understand reaction mechanisms between the molecules and the surface.

Qualitative results are not sufficient in immobilization studies especially for protein adsorption which are further used at surface for its catalytic activity. If kinetic and thermodynamic constants are not reliable or appropriate to achieve an ideal interaction, conditions should be changed to come with up an efficient immobilization. Otherwise adsorption process can be impractical for that surface with target protein.

4.6.1 Quantitative Analysis of 5GBP1-AP Adsorption

Langmuir adsorption model provides both kinetic and thermodynamic constants about the adsorption process and is commonly used to fit experimental SPR data. In this study both 5GBP1- and WT AP immobilization experiments were conducted with SPR spectroscopy and further analyzed for extracting kinetic and thermodynamic properties.

Raw data for 5GBP1-AP was fitted to 1:1 Langmuir adsorption model and

observable kinetic constant k_{obs} was calculated. Experiments were completed in three different concentration for the calculation of association (k_a) and dissociation constants (k_d). Using k_a and k_d it is possible to calculate equilibrium constant K_{eq}. Experimetal data that fitted to Lagmuir adsorption model is given for all three concentartion in Figure 4.12.

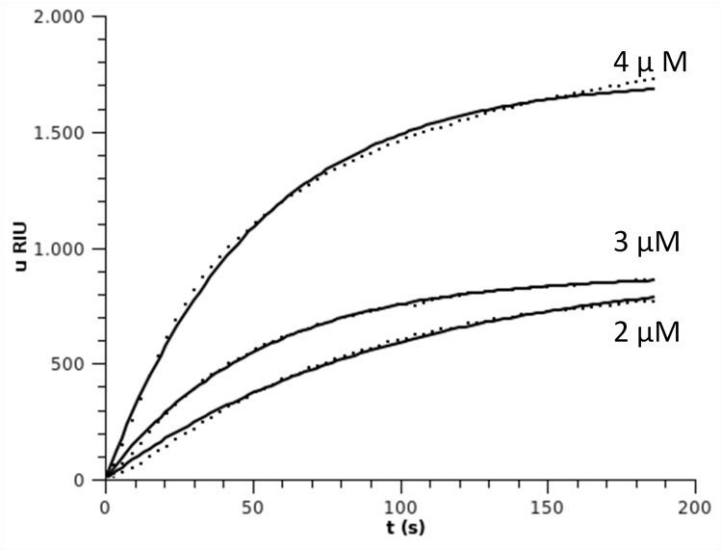

Figure 4.12 : Fit of experimental binding data for all concentrations to Langmuir adsorption isotherm for 5GBP1-AP. Dots represent experimental data.

Quantitative information about the thermodynamic properties of the adsorption process then can be calculated by using K_{eq} value. For this reason, using ideal gas constant (R) and reaction temperature (K) give rise to come up with Gibbs free energy (ΔG) of the system during immobilization. Gibbs free energy is the measure of free enthalpy of the process. It can be defined as the spontaneity of the system at specific conditions. If free energy of a system is negative then this means process can be progress spontenaously without making any intervention.

In summary, for the case of protein immobilization studies free energy of the process possess a negative value that explain the self adsorption of biomolecules under constant temperature and pressure. In other words, the process can be defined as a spontaneous. Calculated values of kinetic and thermodynamic constants for 5GBP1-AP is given in Table 4.2. Both kinetic and thermodynamic constants calculated from Langmuir model for the adsorption of 5GBP1-AP onto bare gold surface were found to be acceptable.

Table 4.2 : Kinetic and thermodynamic constants for 5GBP1-AP

	$k_a.10^3$ ($M^{-1}.s^{-1}$)	$k_d.10^3$ (s^{-1})	K_{eq} (M^{-1})	ΔG_{ads} ($kcal.mol^{-1}$)
5GBP1 AP	4.38 ± 2.55	3.27 ±7.95	1.3394 ± 3.34	-0.1728 ± 1.97

In addition more accurate and reliable values for the interaction of AP with gold surface can be calculated with different mathematical models using several concentration values also with changing expeirmantal conditions.

In this study the overarching goal is to study the mineralization of calcum phosphate through immobilized 5GBP1-AP on gold surface to monitor biomineral formation process. For this reason, kinetic and thermodynamic constants were calculated in order to prove acceptable adsorption process and not for kinetic study of 5GBP1-AP for its material specificity which was previously studied in detail.

4.6.2 Quantitative Analysis of WT AP Adsorption

As in the case of 5GBP1-AP affinity and thermodynamic constant calculations, SPR experimental data were fitted for three differenet concentration of WT APs and k_{obs} values calculated with same mathematical model. After calculation of k_{obs} values kinetic constants simply extracted from the graph of concentration versus k_{obs} which has linear relationship that result to calculate association and dissociastion constants about the adsorption process.

For thermodynamic properties of WT AP same caluculation were done as in 5GBP1-AP. Thereby, equilibrium constant and with using it, free energy of the adsorption of WT AP onto bare gold surface were quantitatively calculated and explained.

Experimetal data that fitted to Lagmuir adsorption model is given for all three concentartion in Figure 4.13. The fits are reliable enough to extract quantitative information from the Langmuir adsorption isotherm of AP binding onto SPR spectroscopy bare gold surface. The fits of 2 μM and 3 μM of AP of the experimental SPR spectrum have a higher value of R^2 however the fit of 4 μM is still in reliable range. Calculated of kinetic and thermodynamic constants for WT AP are given in Table 4.3.

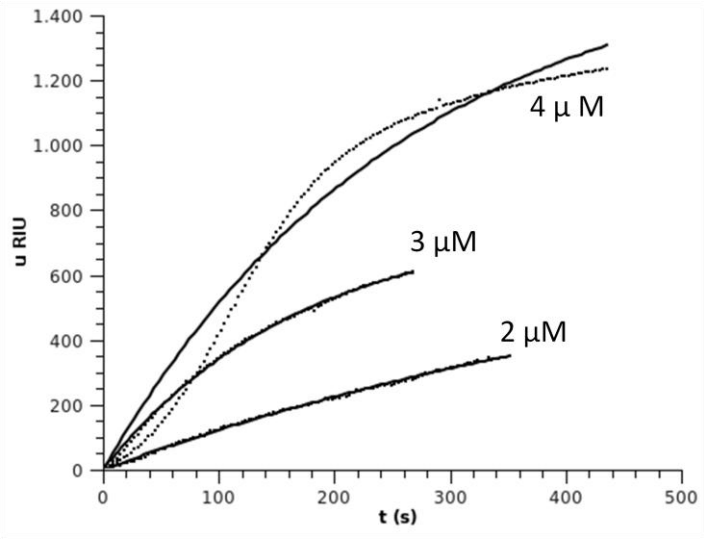

Figure 4.13 : Fit of experimental binding data for all concentrations to Langmuir adsorption isotherm for WT AP. Dots represent experimental data.

Table 4.3 : Kinetic and thermodynamic constants for WT AP

	$k_a.10^3$ ($M^{-1}.s^{-1}$)	$k_d.10^3$ (s^{-1})	K_{eq} (M^{-1})	ΔG_{ads} ($kcal.mol^{-1}$)
WT AP	1.065 ± 1.76	0.561 ± 5.50	1.89 ± 18.87	-0.3791 ± 11.06

As can be deduced from calculated values for 5GBP1-AP and WT AP, kinetic behaviours of these enzyme for the adsorption onto bare gold surface from solution are drastically different. The high association constant of 5GBP1-AP compared to WT AP was dedicated to gold binding affinity due to GBP1. Although higher k_a value for 5GBP1-AP was an expected result dissociation constant is also higher than WT AP. High dissociation constant means the irreversible binding of enzyme onto gold surface which was not observed in SPR studies.

This result maybe originate from the surface properties of alkaline phosphatase. Protein such as Aps through their high amino acid contents and complex surface architechures can adsorp onto solid surface with high affinity through specific interactions such as electrostatic, hydrophilic or hydrophobic etc. AP was shown to possess low desorption behaviour from gold substrate.

4.7 Calcium Phosphate Biomineralization via Immobilized APs

Calcium phosphate as being the main component of hard tissue in several organisms was widely studied due to its importance in medical application. Mineralization of calcium phosphate can be conducted by different methods as mentioned in previous section however one of the practical and efficient way is to use alkaline phosphatase enzyme for this process. In this study GEPI fused AP was analysed in order to induce mineral formation in vitro by SPR spectroscopy. The differences of this work according to other calcium phospahate mineralization researches, is using a modified enzyme with gold binding peptide which serve as a bi-functional protein.

On the other hand calcium phosphate formation is crucial in a theoretical perspective since this biomineral formation mechanism is still not very clear. The most important question about mineral formation for hard tissues in several organisms is a long debate for the reason that biomineral formation is thought to include crystal phase transitions. It was shown in vitro and also in vivo in some organisms that first formed product is in an amorphous phase which progress to come up with octacalcium phosphate and finally hydroxyapatite is formed. All studies related with biomineral formation provide information about the mineral after product formation. However it can be very useful and can provide interesting informations to analyse biomineral while it is forming.

In this study the purpose was to monitor overall mineral formation process in real-time which can result for additional knowledge about the nature of calcium phosphate biomineralization including crystal phase transition.

4.7.1 Real-time Monitoring of Calcium Phosphate Mineralization

The overarching goal of this study is to monitor calcium phosphate biomineralization in real-time by using SPR spectroscopy. For this reason, after immobilization of either 5GBP1- or WT AP onto SPR gold sensor surface, mineralization was induced by pumping β-glycerophosphate calcium chloride solution to the system. Immobilization of APs onto gold was proved to trigger mineral formation by hydrolyzing β-glycerophosphate that result with inorganic phosphate group remove from molecule and react with calcium molecules to form calcium phosphate. Precipitation of formed mineral molecules is the bases of all biomineral formation

process. Similarly, on gold sensor surface of SPR, calcium phosphate mineral was shown to precipitated until reaction on the surface is completed. Plasmon oscillation that is the sensing mechanism of SPR spectroscopy is harness from the refractive index change of the environment on the sensor surface, change of the molecule component of metal surface could progressed in real-time during mineral formation. Adsorption of proteins in solution leaded relatively low refractive index change due to their low concentration however in the case of solid precipitation a drastic change that is very high value of μRIU was observed in mineralization studies. This result originate from the calcium phosphate molecules that formed and precipitate after AP triggering of mineral formation. Upon solid calcium phosphate molecules adsorped onto gold sensor surface from solution, refractive index increased for the whole process of biomineralization. Reaction mixture for inducing biomineral formation was used excess according to immobilized protein molecules on gold surface. The reason for using high concentrations of substrate is to saturate most of enzyme molecules on the surface.

In addition it is important to use specific molar ratios of substrate to come up with minerals that have crystal morphology rather than amorphous. In nature, APs are known to induce the formation calcium phosphate biomineral in vivo so optimum conditions can be defined for the formation of biomineral crystals as 37 ºC. In addition AP is known to has catalytic actvity even at temperatures about 60 ºC. For this reason, after immobilization of enzyme onto gold surface and saturated enzyme immobilized surface with excess substrate, all SPR experiments were conducted by increasing surface temperature to 37 ºC. The expected result with this reaction conditions was to mimic natural biomineral formation such as occur in mammalian bone tissue which consist of calcium phosphate.

All mineralization experiments were allowed to progress about 24 hours which was accepted as an optimum reaction time. The overall mineralization process conducted by immobilized 5GBP1-AP is given in Figure 4.14. For the mineral formation β-glycerophosphate and calcium chloride were used as a molar ratio of 1.66 which is known as the ratio of hydroxyapatite mineral in other words optimum for crystal morphology. At the initial stages of the reaction the velocity of the formation of product is maximum as expected and near the saturation point refractive index increase was not further observed.

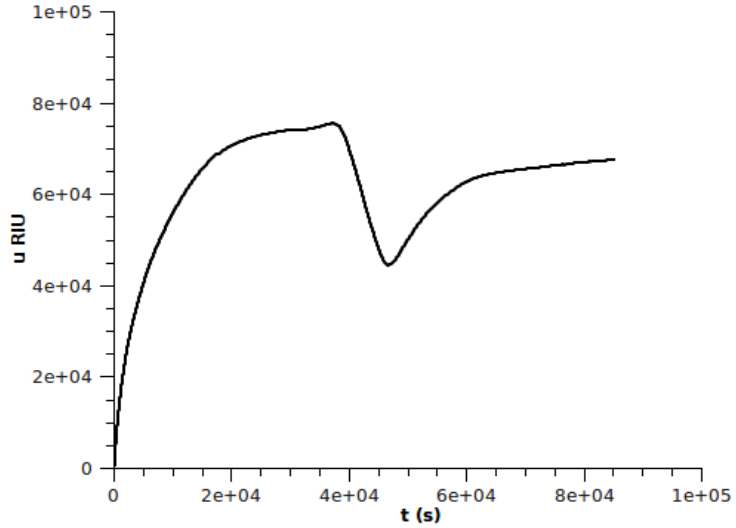

Figure 4.14 : SPR sensogram for the mineralization of calcium phosphate via immobilized 5GBP1-AP.

Mineralization solution that is β-glycerophosphate and calcium chloride was allowed to saturate surface with progressing refractive index change as in the case of adsorption studies. When the surface where immobilized enzyme was saturated with substrate, pumping mineralization solution was stopped and allow SPR to take refractive index data for about 24 hours. For this reason, any change especially after mineral formation is completed can be dedicated to the change of one of the properties of molecules on the sensor surface. The refractive index change that is shown in the sensogram at about 16 hours is thought to be a conformational reorientation of calcium phosphate molecules which will explained in detail in the next section.

The mineralization of calcium phosphate via WT AP was also analyzed under the same reaction conditions. Overall mineralization progress of an immobilized WT-AP **i**s shown in Figure 4.15. WT AP was observed to achieve mineral formation when immobilized onto gold surface of SPR. Although the adsorped mass of the WT AP is lower according to 5GBP1-AP, biomineral precipitation was led to refractive index change taht can be compared with 5GBP1-AP. Although mineral formation could occur with both enzymes, due to its high material selectivity that is gold binding affinity of 5GBP1-AP the mineral formation is thought to be more efficient in tha cese GEPI fused enzyme. This result can be dedicated to self-assembly property of gold binding peptide and may be yield from the immobilization differences.

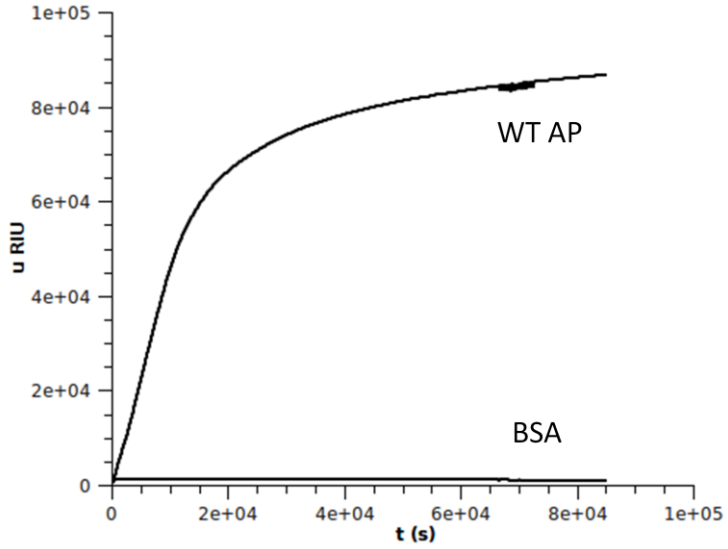

Figure 4.15 : SPR sensogram for the mineralization of calcium phosphate via immobilized WT AP and control experiment conducted with BSA.

Not only 5GBP1-AP is adsorped at a high amount according to WT AP but also probably 5GBP1-AP is oriented on the surface with a form which expose active site of enzyme free and result in high enzymatic activity. The scaffold for the construction of mineral layer is crucial for the efficient formation of biominerals on the surface. As in the case of natural biomineral formation, proteins provide special architecture as a scaffold to come up with highly oriented inorganic phases. 5GBP1-AP could serve as a scaffold with its oriented patterning onto the gold surface and result the construction of calcium phosphate mineral in an highly organized manner. WT AP on the other hand immobilized onto gold surface randomly and yield low ordered protein pattern at which active sites could be occupied due to orientation on gold surface. The inorganic phase for this reason, could be less organized in the case of WT AP.

As a negative control experiment, Bovine Serum Albumin (BSA) at the same protein concentration was used to analyze mineral formation property of AP in order to prove that the refractive index change is a result of precipitated calcium phosphate formed by immobilized AP. For this reason, BSA was immobilized on gold surface of SPR under the same experimental conditions and upon adsorption of protein onto gold surface mineralization solution was pumped to system with same molar ratio with 5GBP1-or WT AP experiments. Refractive index change again monitored via SPR for 24 hours. The SPR sensogram for BSA induced mineralization is given in

Figure 26. As an expected result mineral formation was not observed in the case of immobilized BSA onto gold surface since it could not hydrolyze inorganic phosphate group from β-glycerophosphate and biomineral could not formed despite there are calcium molecules exist at the environment.

4.7.2 Crystal Phase Transition of Calcium Phosphate Mineral

The question of crystal phase transitions of biominerals in both vertebrates and invertebrates have been stand as a controversial issue for nearly 50 years. Although researchers realize that the first formed product in biomineralization is not a thermodynamically stable phase and transform to a more stable phase spontaneously. In the last decades several proof for this mechanism was shown both for in vivo and in vitro mineralization.

As in the case of all biomineral formation processes, calcium phosphate mineralization is also believed to involve two step crystal phase tranisiton. The first formed phase for calcium phosphate is an amorphous structure which is thermodynamically unstable and transform to a crystal phase that is octacalcium phosphate. Although octacalcium phosphate has a crystal morphology and more stable than amorphous phase, it exposed another transformation to yield hydroxyapatite crystal.

Hydroxyapatite is the main component of bone and teeth tissue which is calcium phosphate crystal with a size of about 30 nm. Understanding the mechanism of biomineralization is crucial for both theoretically and practically for medical application such as bone or teeth regeneration.

In addition, enabling to make intervention to the mineral formation can give rise diverse important studies for diseases related with mineral defects. Monitoring mineral formation in real-time can provide useful information about calcium phosphate formation. Another very important issue for biomineral formation is the molar ratio of calcium and phosphate molecules. Very precise ratios in the final product of minerals are observed in natural structures. This molar ratio is define the overall morphology of the mineral and crucial for the property of mineral that is used in a specific tissue. If the ratio is not balanced properly hard tissue formation resulted with important defects which lead some important diseases. In this study calcium

phosphate biomineralization was studied with different molar ratios of mineralization solution in order to analyze the differences between final products. The probable crystal phase transition of calcium phosphate is observed by SPR as is shown in Figure 4.16.

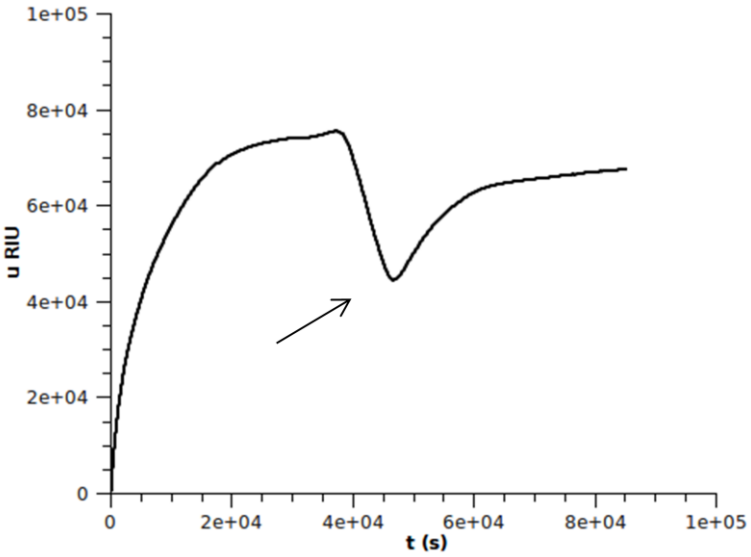

Figure 4.16 : SPR sensogram of the overall mineralization process with a possible crystal phase transition indicated with arrow.

Mineral formation with a molar ratio of 1.66 for mineralization solution was completed after 11 hours. Upon saturation a change in refractive index was occured despite any intervention to the system. This result is thought to happen with the reorientation of calcium phosphate molecules on the surface that is transform to crystal shape from an amorphous phase. It is acceptable to occur a transition process since thermodynamic properties of the system could undergo a phase transformation in experimental conditions. It can be deduced from this observation that when molar ratio of mineralization solution is changed the conformation of the mineral should be different. For this reason, a molar ratio of 1.33 was used as mineralization substrate at the same conditions to conduct same experimental procedure. The result of the overall calcium phosphate mineralization monitoring process as a SPR sensogram is given in Figure 4.17.

It can be said from SPR spectroscopy results that crystal phase transitions could be progressed in real-time with SPR spectroscopy since there is no change in refractive index when the molar ratio is 1.33 that is optimum for amorphous mineral. On the

other hand this hypothesis should be tested by other characterization techniques in order to verify if there is a crystal phase transition in the biomineral.

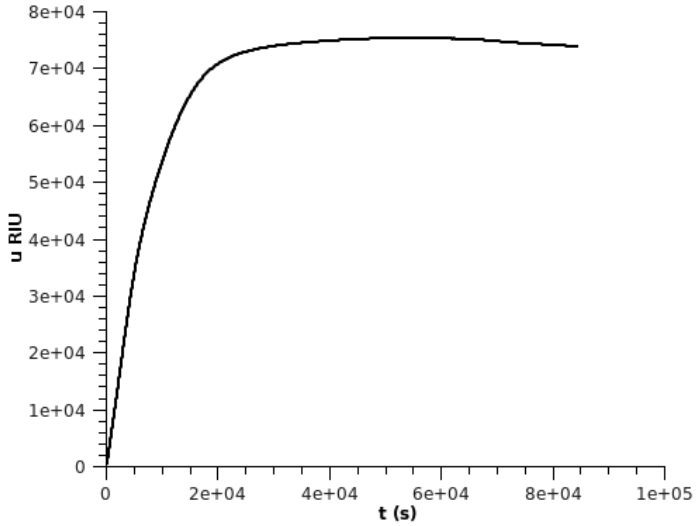

Figure 4.17 : SPR sensogram of the overall mineralization process with a molar ratio of 1.33 for mineralization solution.

4.8 Characterization of Calcium Phosphate Mineral

Although SPR provide original and acceptable results for biomineralization of calcium phosphate, mineral sample that precipitate onto gold surface were analyzed with other charaterization techniques to further analyze the morphology of the product and to provide supportive information about the crystal transition.

4.8.1 Raman Spectroscopy Results

Calcium phosphate minerals were analyzed with Raman spectroscopy technique without any sample preperation and used as they formed onto the gold sensor surface of SPR spectroscopy. Raman spectroscopy is an accepted and reliable method to characterize mineral sample including calcium phosphate. In this methods it is possible to extract information about the morphology of the mineral since crystal phase calcium phosphate according to amorphous or low ordered crystal sample are known to induce red shift in the spectrum of Raman experiments. In addition a direct proof of the existence of crystal like structures in bone tissue in vivo was shown by using Raman spectroscopy.

For these reasons, mineral samples that formed through immobilized APs and different molar ratios of mineralization solutions were analyzed by Raman

spectroscopy. The Raman spectrum for the three samples with different mineralization substrate ratios are shown in Figure 4.18.

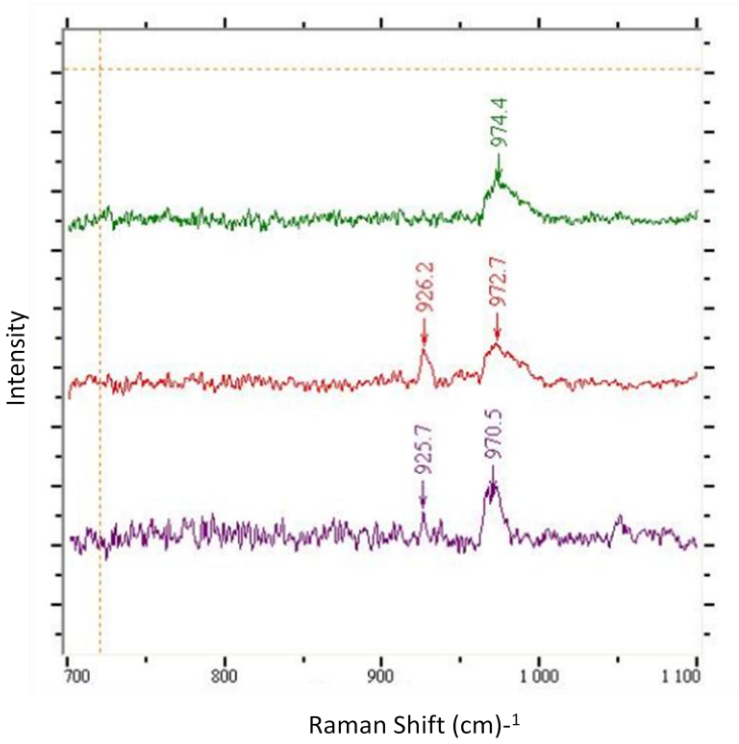

Figure 4.18 : Raman spectrum of calcium phosphate samples formed via immobilized AP on SPR Au sensor surface.

The red shift from 970.5 to 974.4 cm^{-1} was observed which was the mineral that formed onto the gold surface of SPR with mineralization solution optitmum for amorphous and crystal calcium phosphate formation respectively. The peaks in the spectrum of amorphous and low crystalline mineral samples which have low intensity according to main peaks at 970.5 and 972.7 cm^{-1} were thought to be resulted from the low crystalline morphology of the biomineral. The Raman spectroscopy results indicate that samples which are thought to be candidates for crystal phase minerals induce a red shift in the spectrum which means the diffrence crystallinity between minerals that were formed under substrate conditions optimum for amorphous.

Complementary results obtained from SPR and Raman spectroscopy in this study are thought to be acceptable for the characterization of calcium phosphate minerals and from all of these observations it can be deduced at least for experimental conditions used in this study, calcium phosphate possess a crystal phase transition from

amorphous to a thermodynamically more stable crystal form. In summary GEPI fused AP was used to trigger calcium phosphate formation for real-time monitoring of biomineralization and analysis of final product yield promising results for the use of GEPIs enzyme immobilization and further use of their catalytic activity for diverse applications.

5. CONCLUSIONS

In this study, gold binding peptide (GBP) as a genetically engineered peptide for inorganics (GEPIs) with its five tandem repeat construct fused to alkaline phosphatase enzyme was used for monitoring of calcium phosphatae biomineralization in real-time via surface plasmon resonance spectroscopy.

Surface plasmon resonance spectroscopy was harnessed as a method for both quantitatevely analyze binding affinity of 5GBP1-AP onto gold substrate and also for progressing calcium phosphate mineralization in situ. 5GBP1-AP was found to have high binding affinity to gold substrate according to wild type enzyme. Gold binding experiments were conducted with at least three different concentrations for both 5GBP1- and WT AP. Experimental data for binding of APs onto gold substrate were then fitted to Langmuir adsorption isotherms which provide quantitative kinetic and thermodynamic constants of the adsorption event. 5GBP1-AP adsorption was calculated to possess an association constant k_a of $4.38.10^{-3}$ $M^{-1}.s^{-1}$. Wild type AP on the other hand has an association constant k_a of $1.06.10^{-3}$ $M^{-1}.s^{-1}$. These results indicated that gold binding peptide enhanced metal binding property of 5GBP1-AP which in turn provide high efficiency for protein immobilization due to its high adsorped mass on gold surface of SPR. In addition GEPI enhanced AP has higher enzymatic activity which was calculated by varying substrate concentrations. It was found that 5GBP1-AP has a V_{max} value of 0.0416 that is nearly two fold according to WT AP which possess a V_{max} value of 0.0275. K_M that is affinity of enzyme to its substrate for 5GBP1-AP was calculated as 0.2207. Under same conditions WT AP has a K_M value of 0.1726. The activity to catalyze hydrolizing reaction of pNPP was shown more efficient whne using GEPI modified AP according to WT.

As the second part of the study, mineralization of calcium phosphate was studied upon adsorption og APs on gold surface. APs were shown to induce biomineral formation in situ using SPR spectroscopy which provide an promising approach to monitor mineralization in real-time. 5GBP1-AP is thought to possess efficient biomineralization as being a scaffold for mineral growth. Due to its self-assembled property onto gold surface, mineralization of calcium phosphate by using

immobilized 5GBP1-AP can be more efficient according to wild type. The reason for this efficiency is thought to be the self-assembly character of GEPI. Since WT AP was binded to gold surface randomly, the active site of the enzyme could be occupied however GEPI was fused to AP from N terminus which result highly organized adsorption on gold surface. Another important result for biomineralization experiments is the possible crystal phase transitions that monitored by SPR in real-time. Calcium phosphate crystal phase transitions are controversial although it was studied widely according to its health associated importance. SPR results that monitor mineralization in this study were thought to be promising for real-time progressing amorphous to crystal phase transition. It was shown to occur the phase transition when using a mineralization solution with a molar ratio of 1.66 for calcium and phosphate which is known to be optimum for hard tissue formation in vivo. On the other hand there was no transition observed in using a molar ratio of 1.33 that is known to possess amorphous calcium phosphate in organisms. Characterization of mineral samples were conducted by Raman spectroscopy. This method was used widely to analyze biomineral samples to assess morphological properties of minerals. In Raman experiments mineral samples with molar ratio of 1.33 and 1.66 were used for analyzing possible morphological differences. Results indicated that crystal phase transitions could occur in the sample with a molar ratio of 1.66 which is optimum for phase transition since it is known in Raman studies that a red shift is observed when crystallinity of the sample is increased. To sum up in all adsorption and biomineralization experiments it was thought that 5GBP1-AP is a promising candidate for both immobilization and mineral formation due to its high self-assembly property which provide an organized scaffold such as found in natural organisms and possess higher enzymatic activity according to wild type.

GEPI fused protein immobilization and further studies harnessing immobilized protein functionality can realize cutting-edge nanobiotechnological applications in a diverse area. It is possible by using GEPI fused bifunctional biomolecules to come up with molecular constructs which have superior efficiency for the construction of multifunctional biomimetic systems.

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