

ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**SPECIFIC RECOGNITION OF A GENETICALLY ENGINEERED GOLD
BINDING PEPTIDE ON GOLD SUBSTRATE:
INTERFACIAL CHARGE MECHANISMS**

**Ph.D. Thesis by
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Department : Advanced Technologies

Programme : Material Science & Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**GENETİK MÜHENDİSLİĞİYLE ALTINA BAĞLANAN BİR PEPTİTİN
ALTIN YÜZEYİNİ ÖZGÜL OLARAK TANIMASINDA ARAYÜZEY YÜK
MEKANİZMALARININ İNCELENMESİ**

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FOREWORD

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Ayşe Senem Donatan

Materials Scientist

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ABBREVIATIONS

AFM	: Atomic Force Microscopy
AP	: Alkaline Phosphatase
GEPI	: Genetically Engineered Peptides for Inorganics
GBP	: Gold Binding Peptide
OCF	: Open Circuit Potential
PZC	: Potential of Point of Zero Charge
QCM-D	: Quartz Crystal Microbalance with Dissipation Monitoring
QCM-Z	: Quartz Crystal Microbalance with Impedance Monitoring
SAM	: Self Assembled Monolayers
SPR	: Surface Plasmon Resonance

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SPECIFIC RECOGNITION OF A GENETICALLY ENGINEERED GOLD BINDING PEPTIDE ON GOLD SUBSTRATE: INTERFACIAL CHARGE MECHANISMS

SUMMARY

Genetically engineered peptides for inorganics (GEPI) were increasingly utilized as molecular building blocks in bionanotechnological applications in the last decades due to their material-selective properties. Despite considerable work has been accomplished in this area, it is still not well understood how GEPI recognize their counterpart inorganics. Understanding recognition mechanisms will enable the realization of peptide based hybrid molecular technologies and will be a breakthrough for materials science and engineering.

In this dissertation, we investigated the influence of interfacial charge on the molecular recognition of a gold binding peptide on the gold surface in order to gain fundamental understanding about the specific relationship between a GEPI and its associated solid in aqueous environments. Our model peptide was 3rGBP₁ possessing an amino acid sequence MHGKTQATSGTIQS in triply tandem repeat form. In this study, we, intentionally, disturbed the equilibrium interface between peptide, buffer solution and the solid surface by applying external voltage to the solid surface and variation of solution charge through its pH and ionic strength. Subsequently, we observed the differences at several charged environments quantitatively.

All experiments were performed in solution using an electrochemical quartz crystal microbalance with impedance monitoring (EQCM-Z). Acquired data from EQCM-Z was fitted to the two-state conformational change adsorption model. The adsorbed hydrodynamic mass, the effective thickness and other viscoelastic properties of the peptide layer on gold surface were estimated from equivalent circuit analysis model as a function of applied potential, pH and ionic strength.

Results revealed that 3rGBP₁ adapted itself to different charged environments at the peptide-solution-solid interface. Under various applied potentials, pH's and ionic strengths, considerable differences were obtained in adsorbed peptide amount, effective film thicknesses and film viscosities indicating different assemblies of 3rGBP₁ on the gold surface taking place at the peptide-solution-solid interface depending on the interfacial charge. Hence, self-assembly properties of 3rGBP₁ were significantly influenced by interfacial charge alterations.

The adaptation flexibility of GEPI, shown in this study using a model protein 3rGBP₁, will certainly expand their utility in bionanotechnological applications.

GENETİK MÜHENDİSLİĞİYLE ALTINA BAĞLANAN BİR PEPTİTİN ALTIN YÜZEYİNİ ÖZGÜL OLARAK TANIMASINDA ARAYÜZEY YÜK MEKANİZMALARININ İNCELENMESİ

ÖZET

Malzemeleri özgül olarak tanıma özelliklerinden dolayı, genetik mühendisliği kullanılarak oluşturulmuş anorganiklere özgül bağlanan peptitlerin (GEPI) biyonanoteknoloji uygulamalarında moleküler yapı taşı olarak kullanımı giderek artmaktadır. Bu alanda yapılan birçok çalışma olmasına rağmen, GEPI'lerin anorganik yüzeyleri özgül olarak nasıl tanıdıkları henüz anlaşılamamıştır. Moleküler tanıma mekanizmalarının anlaşılması peptit bazlı hibrid teknolojilerin önünü açacak ve malzeme bilimi ve mühendisliği alanında çığır açacak bir adım olacaktır.

Bu tez çalışmasında, altına özgül olarak bağlanan bir peptitin altın yüzeyini moleküler tanınması esnasında arayüzey yüklerinin etkisi araştırılmıştır. Buradaki esas amaç, GEPI ve bağlandığı anorganik yüzey arasındaki özgül ilişki hakkında temel bir kavrayışa vakıf olmaktır. Kullandığımız model peptit 3rGBP₁ ardarda üç kere tekrarlanan MHGKTQATSGTIQS amino asit dizisine sahiptir. Biz bu çalışmada peptit, çözelti ve metal yüzey arasında oluşan arayüzey dengesini metal yüzeyine voltaj uygulayarak ve çözeltinin pH ve ion konsantrasyonu değiştirerek özellikle bozduk. Daha sonra da, farklı yüklere sahip arayüzeylerde ortaya çıkan değişimleri kantitatif olarak gözlemledik.

Tüm deneyler sıvı ortamında impedans görüntüleyecili elektrokimyasal kuvars kristal mikrotarazi (EQCM-Z) sisteminde gerçekleştirilmiştir. Elde edilen veriler iki aşamalı ve konformasyonel değişimi ön gören adsorpsiyon modeliyle eşleştirilmiştir. Yüzeye adsorbe olan hidrodinamik kütle, efektif kalınlık ve altın yüzeyindeki peptit filminin diğer viskoelastik özellikleri metal yüzeyine uygulanan voltaja ve çözelti ortamının pH ve ion konsantrasyonuna bağlı olarak hesaplanmıştır. Viskoelastik özelliklerin hesaplanmasında eşdeğer elektrik devresi modeli kullanılmıştır.

Sonuçlar göstermektedir ki; 3rGBP₁ farklı yüklere sahip peptit-çözelti-metal arayüzeylerin her birine adapte olmuş ve her koşulda bağlanmayı başarmıştır. Uygulanan değişik voltajlar, pH değerleri ve ion konsantrasyonlarında yüzeye bağlanan peptit miktarı, oluşan peptit filminin efektif kalınlığı ve viskozitesinde meydana gelen dikkate değer değişimler, ara yüzeydeki yük miktarı ve kaynağı değiştiğinde 3rGBP₁ moleküllerinin altın yüzeyinde farklı düzenlendiklerine işaret etmektedir. Buradan yola çıkarak, 3rGBP₁ moleküllerinin kendiliğinde dizilme özelliklerinin ara yüzey yüklerinden önemli ölçüde etkilendiği sonucuna varılmıştır.

Bu çalışmada GEPI'lerin adaptasyon esnekliği 3rGBP₁ model peptiti kullanılarak gösterilmiştir. Bu özellik, GEPI'lerin biyonanoteknoloji uygulamalarındaki kullanımını mutlaka arttıracaktır.

1. INTRODUCTION

With advances in micromachining and micro- and nanoelectronics industry, materials science and engineering achieved to process nano-size objects using techniques like lithography. However, it is still a challenge to manipulate nano objects directly. On the other hand, nature fabricates diverse range of nano-scale objects with the help of molecular recognition, self-assembly and precise immobilization strategies. Nature's molecular machines do not have only high precision, flexibility and error correction capacity but they are also self-sustaining and evolving (Sarikaya et. al., 2003, Zhang, 2003, Gray, 2004).

The most important building strategy of nature is molecular recognition, which is the preferred coupling of two or more associated molecules based on geometrical and physicochemical complementarity (Castner and Ratner, 2002, Whitesides and Lipomi, 2009). In molecular recognition processes, a kind of specific interaction occurs between combining regions of associated molecules in aqueous solutions, which is ensured by weak intermolecular forces such as Van der Waals attraction and repulsion, electrostatic interactions and hydrogen bonds etc. (Gitlin et al., 2006, Mallouk and Gavin, 1998). Biological processes like protein folding, nucleic acid hybridization, enzyme-substrate or cofactor interactions and signaling pathways of receptor molecules all require molecular recognition (Zhang, 2003, Whitesides and Lipomi, 2009). In nature, molecular recognition mechanisms can be encountered not only between organic-organic interactions but also between organic-inorganic hybrid interactions. The natural materials such as bone, dentin, mollusk shell, silk, sponge spicules, or magnetotactic bacteria owe their hierarchical organization and their unique functions to the molecular control of their biomacromolecules (Mann, 1996, Sarikaya, 1999). Macromolecules, mainly proteins, in these hybrid structures control nucleation and growth of the inorganic mineral phase and also shaping and adapting mechanical properties from the mineral phase to function (Weiner and Addadi, 1997, Tamerler et. al, 2010).

Taking lessons from nature, scientists started to utilize nature's building strategies for fabrication of novel materials. "Biomimetic thinking" at the nano-scale opened

interesting possibilities for synthesis of new structures at the interface of biology and materials science. At first, Inorganic Binding Proteins (IBPs), which recognize an inorganic surface in molecular scale and direct its' crystallization process, were utilized in the construction of synthetic materials by extracting them from biological hard tissues (Falini et. al., 1996, Shimuzu et. al., 1998, Sudo et. al., 1997, Lang, 2007). However, this approach was restricted with materials found in natural hard tissues and it also involves cumbersome and time-consuming procedures (Tamerler and Sarikaya, 2009). Fortunately, biocombinatorial selection procedures have permitted the isolation of peptides with short amino acid sequences (7-14 amino acids) that associate specifically with inorganic surfaces through physiochemical non-covalent forces. These short peptides, named later as Genetically Engineered Peptides for Inorganics (GEPI), and their observed selective properties expanded the vision of scientists into the technologically relevant materials of choice (Tamerler and Sarikaya, 2009). The idea of utilizing GEPI as molecular building blocks in materials science and engineering was pioneered by Sarikaya and his co-workers (Sarikaya et. al., 2003). They paved the way of emerging the novel research area; Molecular Biomimetics. Using GEPI and nature's building strategies; the challenge of Molecular Biomimetics is to construct multifunctional hybrid materials in nano-micro- and macro- scale which are unfeasible to be constructed by conventional methods of materials science (Tamerler et. al., 2010).

GEPI differentiate from other proteins and polypeptides with their specific molecular recognition properties onto targeted inorganic surfaces (Sarikaya et. al., 2003, Wei and Latour, 2009, Weiger et. al., 2010). These short peptides have great potential for various applications in different areas such as nanobiotechnology, medicine, nanoelectronics due to their material specific properties. Since their discovery, GEPIs' have been successfully utilized in myriad practical applications such as synthesizers and growth modifiers in nanocrystal synthesis or biomineralization processes, as molecular linkers and assemblers in targeted organization of functional hybrid nanostructures (Tamerler et. al., 2010, Whyburn et. al., 2008). Despite to the considerable work that has been accomplished in this area, it is still not well understood how solid-binding peptides recognize their counterpart inorganics (So et. al., 2009, Evans, 2008, Diamanti et. al., 2009). Elucidating the recognition mechanisms will enable the realization of peptide based hybrid molecular technologies (Tamerler and Sarikaya, 2009). Controlled binding and assembly of

GEPI onto their counterpart solids may open new routes in the development of advanced biosensors for pathogen detection, drug screening, bioseparation systems and diagnostics in medicine (e.g. cancer therapeutics). Hybrid GEPI-inorganic nanostructures as molecular building blocks can also be utilized in molecular electronics and photonics (Tamerler and Sarikaya, 2008).

In this dissertation, our overarching goal was to gain fundamental understanding about the specific relationship, namely molecular recognition, between GEPI and their associated inorganics in aqueous environments. In the past, molecular recognition was defined in terms of “lock and key”, referring only to adsorbent and adsorbate. However, this definition excludes the presence of “near-surface” water since most of the biological processes occur in aqueous media. Nowadays, understanding the remarkable contribution of structured water within the first few nanometers of a molecular surface became an important issue in “soft nanoscience” (Whitesides and Lipomi, 2009). Moreover, the existence of charged or polar moieties of amino acids in certain GEPIs was proposed as the most possible sites for strong binding of GEPI to their relevant inorganics (Baneyx and Schwartz, 2007, Hayashi et. al., 2009, Oren et. al., 2010). We, therefore, investigated the influence of interfacial charge on the molecular recognition between a GEPI and its counterpart solid in aqueous media. We, intentionally, disturbed the equilibrium interface between peptide, buffer solution and the solid surface by applying external voltage to the solid surface and variation of solution charge through its pH and ionic strength. Subsequently, we observed the differences at several charged environments quantitatively.

In our experiments, a gold binding peptide, 3rGBP1 possessing an amino acid sequence MHGKTQATSGTIQS in triply tandem repeat form, was utilized as model protein. The binding and assembly characteristics of 3rGBP1 have been very well studied in literature (see section 2.3.3 and following subsections) and therefore it has been an ideal system to study the interfacial charge effect.

All experiments were performed in solution using an electrochemical quartz crystal microbalance with impedance monitoring (EQCM-Z). This system provided us the opportunity of (1) carrying out time-dependent in-situ adsorption experiments concurrently during flow and under the influence of external electrical field (2) observing both the changes in adsorption kinetics and also the alterations in viscoelastic properties of the adsorbed peptide film with the help of impedance

monitoring, (3) obtaining more reliable data about molecular recognition process by driving the overtone frequencies of QCM. During our experiments, QCM-Z system was connected to a potentiostat with the intention of performing electrochemical measurements. Acquired data from EQCM-Z was fitted to the two-state conformational change adsorption model (Yowler and Schengrund, 2004). The adsorbed hydrodynamic mass, the effective thickness and other viscoelastic properties of the peptide layer on gold surface were estimated from equivalent circuit analysis model (Martin et. al., 2000) as a function of applied potential, pH and ionic strength.

2. BACKGROUND INFORMATION

2.1 Molecular Recognition

Nature utilizes molecular recognition mechanisms for structuring biological entities at the macro-, micro- and nano-scale. Multifunctional macromolecular assemblies come together one atom or one molecule by one at a time through molecular recognition. They are ordered and templated via self-assembly and then fabricated into hierarchical structures using again molecular recognition and self-assembly mechanisms (Castner and Ratner, 2002). Biological processes such as protein folding, nucleic acid hybridization, enzyme-substrate or cofactor interactions, signaling pathways of receptor molecules and also molecular biomachines such as hemoglobin, polymerases, ATP synthases, membrane channels, ribosomes all require molecular recognition (Whitesides et al., 1991, Zhang, 2003).

Molecular recognition relies on self associations of biomacromolecules that prefer to combine specifically with each other rather than to interact with other molecules. This intriguing relationship has caught researcher's interest in the beginning of 20th century. Since then, molecular recognition has been observed and investigated in many biological processes such as antigen-antibody interactions, enzyme catalysis or specific actions of drugs and bactericidal substances (Castner and Ratner, 2002).

In 1938, Paul Jordan stated that specific interactions occur between identical or nearly identical molecules because of the quantum mechanical stabilizing interactions operating in between those molecules (Jordan, 1938). In 1940 Max Delbrück and Linus Pauling proposed that biological specificity results from the interaction of complementary molecular structures rather than identical ones (Pauling, 1974).

After the discovery of DNA structure (Watson and Crick, 1953) it became obvious that molecular recognition is based on shape-specific and non-covalent interactions of complementary molecules (Lehn, 1993, Philip and Stoddart, 1996). DNA structure revealed two important features of molecular recognition and self-assembly

mechanisms: (1) The complementary strands have a strong affinity for each other (bonded via hydrogen bonds) (2) They form a predictable structure (double helix) when they associate (Seeman and Belcher, 2002).

Another remarkable example for molecular recognition is the streptavidin-biotin pair, known as one of the strongest receptor-ligand interactions found in nature. The structural origins of high affinity binding between avidin and biotin were clarified in 1989 by Weber et. al. Streptavidin is a tetrameric protein which has the capacity of binding four biotin molecules simultaneously with a very high binding constant ($K_d : 10^{15} \text{ M}^{-1}$). The highly specific interactions between streptavidin and biotin are ensured by the shape symmetry of biotin-binding pockets which are positioned in pairs at opposite faces of streptavidin (Weber et. al., 1989, Chilkoti and Stayton, 1995, Perez-Luna et. al, 1999).

Investigating many other examples in nature, researchers suggest that molecular recognition occurs when numerous shape-complementary atoms from each of the associating molecules come in close contact simultaneously resulting in a specific interaction. This specific interaction is ensured by weak intermolecular forces (Van der Waals attraction and repulsion, electrostatic interactions and hydrogen bond formation etc.) acting between complementary combining regions (Gitlin et al., 2006, Mallouk and Gavin, 1998). Overall, molecular recognition is stated as the geometrical and physiochemical match of associated molecules in aqueous environments (Castner and Ratner, 2002). Nevertheless, the exact pathways of molecular recognition mechanisms for many biological materials and processes are still waiting to be elucidated (Tamerler and Sarikaya, 2009).

2.2 Inspiration from Nature: Inorganic Binding Proteins

In nature, there are many elegant hybrid architectures such as bone, dentin, mollusk shell, coral, pearl, wood, silk, horn, collagen, muscle fibers, extracellular matrices, sponge spicules, siliceous skeletons of single celled organisms, magnetotactic bacteria etc. These natural materials with unique properties owe their hierarchical organization to the molecular control of their proteins or peptides (Mann, 1996, Sarikaya, 1999). Molecular recognition between proteins and inorganic substances in these hybrid structures resembles to those observed in specific organic-organic interactions (Sicheri and Yang, 1995, Makrodimitris et al., 2007).

These hard tissues are biocomposites that include both organic macromolecules like lipids, proteins, and polysaccharides and also different kind of inorganic minerals such as hydroxyapatite, calcium carbonate, and silica. In many of these hybrid organisms, organic macromolecules, mainly proteins, control the formation of precise arrays of inorganic crystals. The crystallization process is called *biomineralization* (Lowenstrom and Weiner, 1989, Mann, 1996).

The main constructional processes in biomineralization are supramolecular pre-organization, interfacial molecular recognition (templating) and cellular processing. First, organic matrices (extended networks of protein, polysaccharide or lipids) were constructed via self assembly of these biomacromolecules. The second step involves the controlled nucleation of inorganic clusters from aqueous solution via interfacial molecular recognition. In this process, organic supramolecular systems provide a framework for the assembly of the inorganic phase (Mann, 1993). During nucleation, a molecular complementary occurs between functional groups of organic molecules and the inorganic crystal nucleus surface at the organic-inorganic interface. This complementary phenomenon is the key for specificity in nucleation process, which is induced by polarity, charge and stereochemical relationships at sites and restricted in size and topology. It is postulated that potential fields surrounding the inorganic and organic surfaces match depending on electrostatic, structural and stereochemical requirements of the functional groups at the organic-inorganic interface and the activation energy for the nucleation is lowered specifically as a function of this matching. Consequently, interfacial molecular recognition causes the construction of inorganic nuclei with a preferred crystallographic orientation and morphology as shown in Figure 2.1 (b&c) (Mann, 1988).

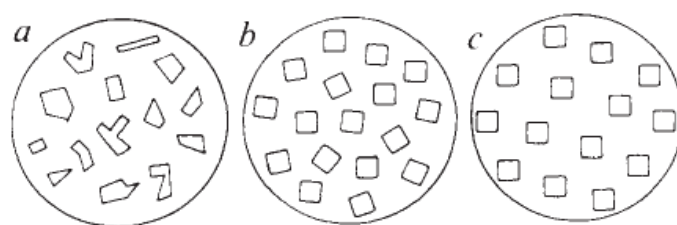


Figure 2.1: Nucleation of biominerals on organic polymeric substrates. (a) Non-specific nucleation in which crystal nuclei of varying orientation are randomly formed across the organic surface (b) and (c) Site-directed nucleation involving molecular specificity (preferred orientation and morphology) at the organic/crystal interface. The crystals are organized randomly in (b) periodically in (c) (adapted from Mann, 1988).

At the final step of biomineralization, genetic control involving larger scale cellular activity steps in the process in order to shape the crystals for their appropriate functions. The cellular processing of biomineral shapes is determined by the compromise between the constraints of crystal physics and the genetic manipulation of morphology (Mann, 1993). The shaping of individual biominerals is often accompanied by their assembly into organized intracellular structures (Figure 2.2 (A)) and also further into extended extracellular matrices (Figure 2.2 (B&C)).

Products of the biomineralization processes are mainly nanomaterials, which are constructed under genetic control, resulting in specific morphology, size and crystallinity of the structures. Remarkably, synthesis and assembly of these biological nanomaterials occurs mainly under physiological aqueous conditions. In the industry, the same materials are trying to be synthesized using caustic reagents, high temperatures and pressures. As well, industrial processing methods often cannot achieve the nanoscale size features that nature produces routinely (Sarıkaya et. al., 2003, Crookes-Goodson et. al., 2008). Hence, owing to their higher order, complex architectures and concurrently simple construction conditions, mineralized biological hard tissues represent a source of inspiration for materials scientists. A closer inspection into the materials properties of some functional examples (Figure 2.2) will certainly lead to the idea that there are lessons, which have to be taken from nature (Tamerler et. al, 2010).

The first example is a single-celled organism, which acts as a biologically made nano-compass (Figure 2.2 (A)). A type of bacteria, *Aquaspirillum magnetotacticum*, produces magnetosomes; a protein and lipid-based closed membrane structure that include a magnetic nanoparticle. These nanoparticles are magnetite (Fe_3O_4) formed as perfect cubo-octahedral single crystals, their size are about 50 nm in diameter. Each nanoparticle has a single superparamagnetic domain, which is stationary within the magnetosome membrane. The particles are crystallographically and morphologically aligned with respect to each other for improved detection of magnetic field. In the presence of a magnetic field, magnetosomes align themselves according to the field, rotating and orienting the cell in the direction of the magnetic field. If 25 magnetosomes align in the same direction, a magnetic moment of 0.5 Gauss is created, which is large enough to sense the Earth's magnetic field. Both the

formation of nano-compass and magnetic sensing are achieved via a network of proteins (Blakemore, 1975, Crookes-Goodson et. al., 2008, Tamerler et. al., 2010).

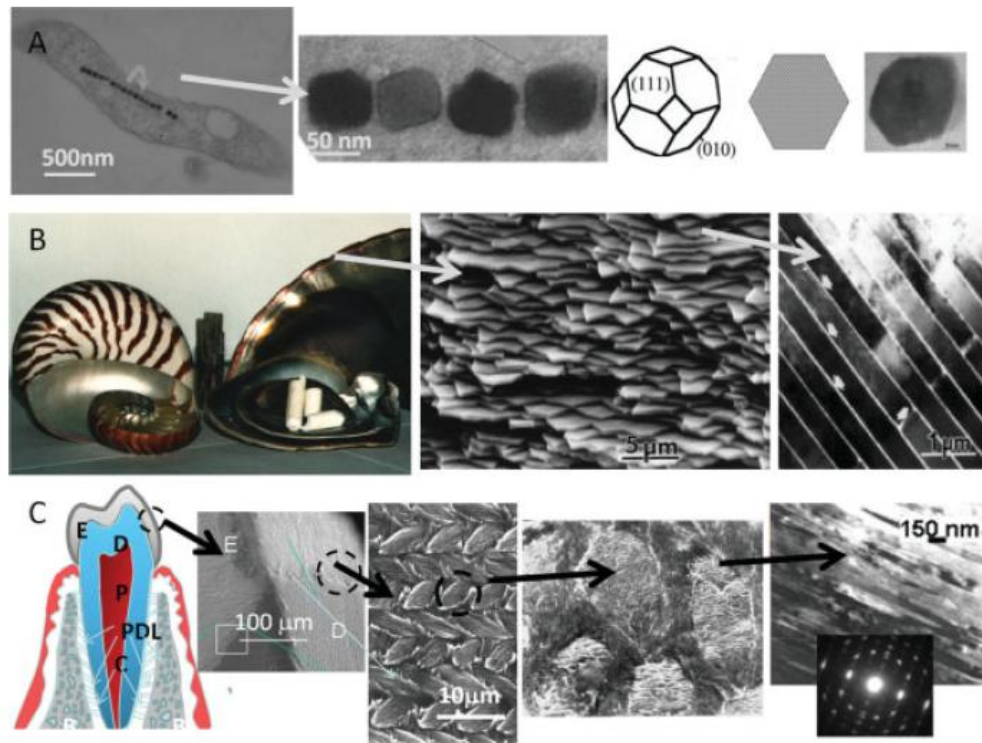


Figure 2.2: Examples of functional natural hard tissues. (A) Magnetotactic bacteria, e.g. *Aquaspirillum magnetotacticum* (B) Nacre (C) Mammalian, which is composed of enamel (E), dentin (D), pulp (P), cementum (C) and periodontal ligaments (PDL) (adapted from Tamerler et. al., 2010).

Another fascinating example is the nacre (Figure 2.2 (B)), which is the natural protective sheathing of mollusks such as red abalone, pearl oyster and nautilus. Long time, it was not understood how mollusk shells can be so tough although they were made up of calcium carbonate, a considerably soft material. Several decades ago, researchers found out that nacre has a layered and segmented hybrid composite structure containing a mixture of aragonite (orthorhombic CaCO_3) and biopolymers (Sarikaya and Aksay, 1992). Proteins and polysaccharides (e.g., chitin) are integrated into the shell structure with a 95/5 inorganic/organic volume ratio. Although the contribution of biopolymers is few in amount, their assistance in biomineralization leads to construction of a material, which has one of the highest specific toughness and fracture strength among the all known ceramic-based materials (Mayer and Sarikaya, 2002).

As the third example, the hierarchical structure of the mammalian tooth (Figure 2.2 (C)) is also coordinated by many proteins (e.g. ameloblastin and amelogenin etc.). The crown of the tooth in mammals is the enamel, which is the hardest material in the body that protects the tooth. The nanostructure of the dental enamel contains ordered woven-like fibers, each consisting of thousands of crystallographically-aligned elongated hydroxyapatite (HA) crystallites. This unique architecture, which is formed under the genetic control of proteins, resists against mechanical stresses occurring because of fracture and failure. Under the enamel lamina, there is dentin, the softer and, therefore, tougher bone-like tissue. This tissue absorbs the minor mechanical stresses arising during cutting and chewing (Paine et al., 1997, Fong et al., 2003).

Aforementioned examples highlight that macromolecules, mainly proteins, are intimately involved in controlling nucleation, growth, shaping and adapting mechanical properties of the mineral phase to function (Weiner and Addadi, 1997, Tamerler et. al, 2010). By extracting them from biological hard tissues, scientists identified many proteins which bind to an inorganic substrate with high affinity and direct its' crystallization process (Falini et. al., 1996, Shimuzu et. al., 1998, Sudo et. al., 1997, Lang, 2007). These *inorganic binding proteins (IBPs)* were utilized in the construction of synthetic materials.

As a biomimetic approach, extraction of IBPs from hard tissues became a popular trend during 1990s (Mann, 1993, Fritz et. al., 1994, Belcher et. al, 1997, Kroger et. al., 1999). However, this approach involves cumbersome and time-consuming procedures such as isolation, purification, sequence and structure analysis of proteins (Tamerler and Sarikaya, 2009). This method has also limited applicability as it covers only IBPs which bind to mineral substrates. Moreover, extracted proteins demonstrated some limitations in applicability when they are utilized as molecular tools in hybrid systems because of their huge size and also their undesirable response to the alterations of working conditions (Castner and Ratner, 2002, Tamerler and Sarikaya, 2009).

Design of IBPs using strategies of biomineralization was suggested as an alternative approach (Mann et. al, 1993, Barth et. al., 2000) The major drawback of this method is that present knowledge about molecular mechanisms of IBPs and their counterpart inorganics is inadequate for designing precise solid surface topography and proper

protein conformation (Tamerler et. al., 2010). On the other hand, design of inorganic binding proteins or peptides after selection via combinatorial techniques for further enhancement of their binding properties became a very popular approach in the last decade (Peelle, 2005, Oren et. al, 2007, Evans et. al., 2008, Shiba, 2010), which will be discussed later in detail.

2.3 Genetically Engineered Peptides for Inorganics (GEPI)

As a breakthrough in research, biocombinatorial selection procedures have permitted the isolation of peptides with short amino acid sequences (7-14 amino acids) that associate specifically with inorganic surfaces through physiochemical non-covalent forces. As peptide-inorganic interactions are not longer restricted with those found in natural hard tissues, these short peptides, referred as *Genetically Engineered Peptides for Inorganics* (GEPI), and their observed selective properties expanded the vision of scientists into the technologically relevant materials of choice (Tamerler and Sarikaya, 2009).

The idea of utilizing GEPI as molecular building blocks in nanoscale science and engineering, pioneered by Sarikaya and his co-workers, paved the way for emerging a novel research area, *Molecular Biomimetics* (Sarikaya et. al., 2003). Using GEPI that contain the information obtained from molecular evolution, the challenge of Molecular Biomimetics was to construct biomimetic hierarchical structures and highly functional materials which are unfeasible to be constructed by conventional methods of materials science (Tamerler et. al., 2010).

Recent studies have demonstrated that GEPI have great potential for various applications in different areas such as nanobiotechnology, medicine, nanoelectronics due to their specific molecular recognition properties onto targeted solid surfaces (Sarikaya et. al, 2003, Baneyx and Schwartz, 2007, Whyburn et. al, 2008). Following successful isolation and characterization of these peptides, GEPI have being exerted successfully in myriad practical applications as synthesizers and growth modifiers in nanoparticle synthesis (Li et. al., 2009, Zhou et. al, 2010) or biomineralization processes (Gungormus et. al., 2008, Crookes-Goodson et. al., 2008), as molecular linkers and assemblers (Matsui et. al, 2007, Kacar et. al., 2009) in targeted organization of functional hybrid nanostructures.

2.3.1 Selection and characterization

For the selection and isolation of GEPI, biocombinatorial approaches have been adapted to organic-inorganic hybrid systems (Sarıkaya et. al., 2004, Kriplani and Kay, 2005). More than two decades ago, combinatorial selection techniques have begun to be utilized in the selection of antigen-antibody, receptor-ligand pairs for applications in biology, biotechnology, and medicine, such as drug and vaccine development. These combinatorial libraries based mostly on phage display (Smith and Petrevko, 1997) or cell-surface display (Wittrup, 2001) enabled the screening and selection of high affinity peptides towards a target from a large population by employing the tools of the molecular evolution.

In 1992, Stanley Brown attempted for the first time to select peptides that have specific affinity to an inorganic surface, namely Fe_2O_3 substrate, using cell surface display technique (Brown, 1992). This achievement was followed by Belcher and coworkers, who utilized the phage display technique for the first time in the selection of semiconductor materials (Whaley et. al, 2000). Thereafter, both cell surface display and phage display, later also yeast surface display have been utilized systematically to select and identify peptide sequences exposing high affinity to various inorganic materials including noble metals (*e.g.*, gold (Brown, 1997, Slocik et al., 2005, Hnilova et. al., 2008), platinum (Sarıkaya et. al, 2004, Seker, 2007), palladium (Sarıkaya et. al, 2004), cobalt (Naik et. al, 2004) and silver (Naik et. al, 2002)), metal oxides (*e.g.*, SiO_2 (Chen et. al., 2006, Naik et al., 2002, Oren et. al., 2007), ZnO and Cu_2O (Thai et al., 2004; Umetsu et al., 2005), TiO_2 (Sano et. al, 2005, Chen et. al., 2006)) semiconductors (*e.g.*, GaAs (Whaley et. al., 2000), ZnS and CdS (Lee et. al., 2002)), minerals (*e.g.*, hydroxyapatite (Gungormus et al., 2008), sapphire (Krauland et. al., 2007), mica (Donatan et. al, 2009), zeolite (Nygaard et. al., 2002)), carbonanotubes (Wang et al., 2003) and even polymers (Adey et. al., 1995, Kenan et. al., 2006).

In biocombinatorial techniques, peptide libraries are generated by insertion of random peptide sequences into the genetic code of chimeric proteins. Hence, a peptide population has been created for screening towards a target material. The chimeric proteins containing a genetically fused peptide sequence are displayed on the surface of a bacteriophage or cellular organism. In phage display technique, the pIII coat protein of a M13 phage is utilized generally as the chimeric protein. In cell

surface display, the desired sequence of the peptide is usually displayed by the flagellin (Sarıkaya et al., 2004; Tamerler and Sarıkaya, 2007, Tamerler et. al, 2010). As shown in Figure 2.3, peptide libraries are exposed to a solid material of interest. Peptides that have high affinity towards the target material binds onto its' surface. Binding step is followed by removal of non-specific interactions through washing. Thereafter, the specifically bound peptides displayed on cells or phage particles were recovered by harsh washing. This cycle is repeated 4-5 times. After selection, the genetic material of the specifically bound peptides is extracted from the organism and their DNA is sequenced (Sarıkaya et al., 2004, Baneyx and Schwartz, 2007).

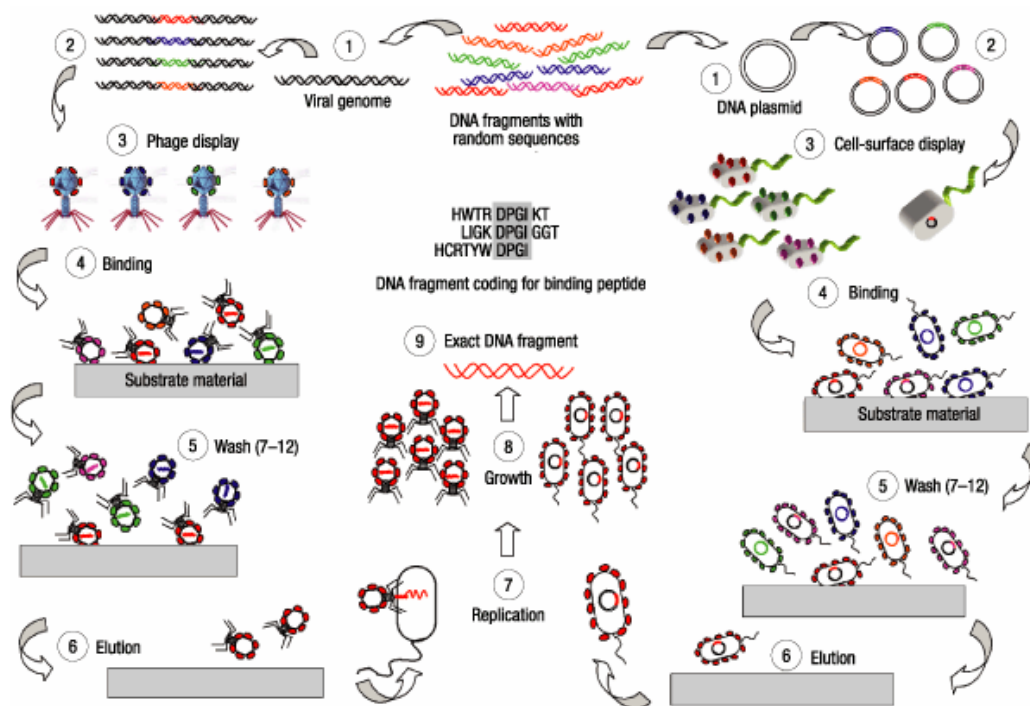


Figure 2.3: General protocols for the selection of the GEPI using phage display and cell surface display technologies (Sarıkaya et. al., 2003).

Although biocombinatorial approaches are relatively straightforward, there are some important considerations that have to be taken into account when they are adapted for selection of GEPI. The size of the peptide libraries is limited. Libraries may not be enough to cover all possibilities to reach the best sequence through directed evolution. In phage display, enrichment of weakly bound clones may occur by the amplification of host organisms (Evans et. al., 2008, Tamerler and Sarıkaya, 2009).

Another problem is the lack of a consensus sequence. In traditional application of display technologies, a consensus sequence is obtained after several cycles of biopanning. However, in the case of GEPI selection only sequence similarities are

encountered generally, rather than a strict consensus amino acid pattern. Several reasons can be stated for this observation. First of all, most solids are compositionally and structurally inhomogeneous at the atomic, crystallographic and morphological levels, and are often oxidized in aqueous environments. Hence, special care must be given when working with inorganic materials in biological buffers. The chemical and physical states of the inorganic surface may affect the efficiency of polypeptide binding during panning procedures. Therefore, solid surfaces must be characterized prior to and after panning procedures in order to determine if any alterations have taken place. Moreover, wash and elution buffers should be checked whether they cause a surface modification, etching or other forms of surface deterioration. On the other hand, forms and shapes of the inorganic solids used in the biopanning processes have a broad variation (e.g. polydisperse powders, single crystals, polycrystals etc.). Different particle sizes and various surface orientations may result in a different binding sequence for each distinct surface topology (Baneyx and Schwartz, 2007, Evans et. al., 2008).

As GEPI obtained through combinatorial biology protocols have a broad range of amino acid patterns, further characterization is required to distinguish among selected GEPI; which one has higher affinity towards others. For this purpose, fluorescence microscopy (FM) and ELISA immunoassays can be conducted as semi-quantitative characterization methods. In FM characterization, each GEPI clone is exposed to the target material separately. Following washing steps, bound clones are fluorescently labeled with either M13-antibody-FITC conjugate or DNA dye in case of phage or cell, respectively. In phage display surface coverage of bound clones is calculated, in cell surface display bound clones are counted under observation of FM. Using the number of bound cells or the surface coverage of bound phages on the target material, selected GEPI can be classified as “strong”, “moderate” or “weak”. (Figure 2.4 (upper)) (Oren et al., 2008, Donatan et. al., 2009). ELISA technique is suggested for materials of interest which are not convenient to work with fluorescent dyes due to overenhancing or quenching of the fluorescence signal. For phage display, ELISA technique can be applied by labeling the mixture of bound phages and powdered material with M13-antibody-HRP conjugate and measuring the shift in absorbance. Sorting of the selecting GEPI due to their affinity depends on the

duration of the enzyme reaction. Surely, higher number of bound phage will result in quicker enzyme reaction (Gungormus et. al, 2008).

Experimentally selected and categorized GEPI can be further “tuned” through application of various molecular-tailoring strategies to improve their affinity and selectivity. Site directed mutations of amino acids within the GEPI sequence (Peelle et. al, 2005, Chen et. al., 2008), the use of multiple sequence repeats (i.e., multimerization) (Seker et. al., 2009), application of conformational restrictions on GEPI structure through genetic control (Seker et. al., 2007, Choe et. al, 2007, Hnilova et. al., 2008) are some examples of molecular-tailoring strategies, which achieved to enhance binding properties of GEPI. Ultimately, the initial sequences serve as a starting point for further improvement or modification.

Besides genetic manipulation, computational studies may introduce novel perspectives for the design of new peptides bearing improved affinity as well as material-specificity. At this point, a successful strategy for the design of GEPI was developed by Sarikaya and his co-workers. In nature, functionally similar proteins usually exhibit some degree of sequence similarity (Attwood, 2000). Based on this finding, Sarikaya group proposed that peptides selected for the same material should have a certain sequence similarity. They developed a methodology using bioinformatic tools. With the combination of different sequence alignment techniques, they produce material-specific scoring matrices using the experimentally observed sequence similarities of quartz binders (Figure 2.4 (lower)). From these matrices, they randomly generated new quartz-binding sequences. Thereafter, they have chosen six strong (high-scored) and four weak (low-scored) predicted quartz binders and tested their binding affinities to quartz. Experimental results were in consistency with the predicted results. As a consequence, second generation GEPI sequences were acquired by using the existing knowledge of the first generation GEPI sequences (Oren et. al., 2008).

Despite all the above stated accomplishments in selection and further improvement of GEPI, the intriguing relationship between solid binding peptides and their associated inorganics has not been revealed clearly. Quantitative characterization is required to elucidate the recognition and assembly mechanisms of these peptides.

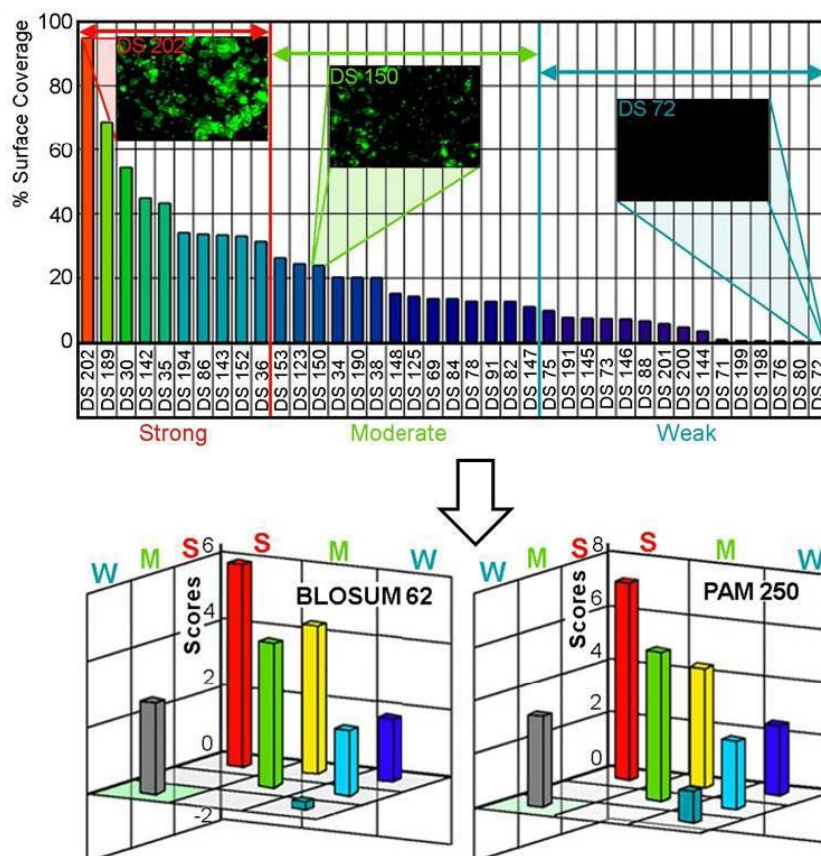


Figure 2.4: Design of second generation GEPI using bioinformatic approach. Experimentally selected and categorized peptide sequences (upper) are used to calculate similarity scoring matrices (lower) (adapted from Oren et. al., 2008).

For this purpose, extensive work has been done in the last decade using a combination of several techniques. Surface plasmon resonance spectroscopy (SPR) and quartz crystal microbalance (QCM) provide in-situ monitoring of peptides' adsorption and desorption kinetics for a certain solid under various buffering conditions (Tamerler et. al., 2006, Seker et. al., 2007, Seker et. al., 2009, Dai et. al., 2005, Sano et. al., 2005, Chen et. al., 2008). Amino acid fingerprint of the peptides on the adsorbed inorganic surface may be obtained via X-ray photoelectron spectroscopy (XPS), time-of-flight secondary ion mass spectroscopy (TOF-SIMS) (Suzuki et. al., 2007 (a&b)), Fourier transform infrared spectroscopy (FTIR), surface enhanced Raman spectroscopy (SERS) (Tullman et. al., 2007, Sengupta et. al., 2008). Secondary structures of GEPI in solution and on the solid surface can be assessed using circular dichroism (CD) spectroscopy technique (Seker et. al., 2007, Kulp et. al., 2007, Hnilova et. al., 2008). Combining CD data with solid- and liquid-state nuclear magnetic resonance (NMR) spectroscopy can provide quantitative information on structural conformations of GEPI (Kulp et. al, 2004, Kulp et. al.,

2007). However, the contribution of solid-NMR method is limited due to the interference coming from the solid surface (Tamerler and Sarikaya, 2009). Another powerful technique is atomic force microscopy (AFM) in order to gain fundamental insight into assembly and diffusion of the peptide on the solid surface (So et. al., 2009). Collective utilization of these techniques will pave the way for a better understanding of GEPI-inorganic associations. A gold binding peptide, GBP₁, which will be discussed in detail in the following sections, is an excellent example as a proof of concept demonstration; how useful data can be obtained with the combination of several techniques for enlightening the molecular mechanisms between GEPI and their counterpart solids.

To obtain a complete picture of GEPI-inorganic interactions, the knowledge obtained from experimental studies should be supported with theoretical models. Thus, the number of molecular simulation studies concerning GEPI-solid surface associations is ever increasing. Molecular dynamics (MD) simulations reveal information about structural behavior of GEPI on the solid surface, and give quantitative data such as binding energy (Braun et. al, 2002, Kantarci et. al., 2005, Tomasio and Walsh, 2009). A noteworthy study in this area was conducted by Oren et. al. Using conformational analysis technique, they found out that platinum binding septapeptides conform into multiple protrusions, referred as *polypods*, which spatially match with the crystallographic metal surface (Oren et. al, 2005). Recently, similar match of surface and GEPI orientation was observed by So et. al. for another GEPI, namely a gold binding peptide 3rGBP₁. In that work, molecular simulation and geometrical docking studies were coupled with experimental AFM observations resulting in integrated information (So et. al., 2009).

As the knowledge on the peptide recognition and assembly processes increases, novel peptides with tailored binding and higher material-specificity will be designed for the control of solid-peptide interface, leading to fabrication of new hybrid materials (Tamerler and Sarikaya, 2009).

2.3.2 Current applications

GEPI have great potential in bionanotechnological applications due to their material specificity and self-assembly properties (Tamerler and Sarikaya, 2008, Baneyx and Schwartz, 2007). They can be utilized as molecular linkers, assemblers and erectors

in targeted immobilization (Zin et. al., 2005&2007, Wei et. al, 2009, Nochomovitz et. al., 2010), as synthesizers and nano-organizers in fabrication of inorganic materials (Naik et. al., 2002&2004, Kramer et. al., 2004, Gungormus et. al., 2008, Grosh et. al., 2009). GEPI have also the advantage to enhance their applicability with desired functionality in nanoscale when inserted into permissive sites of several biomacromolecules (proteins, enzymes, viruses etc.) using genetic engineering techniques (Mao et. al, 2003, Huang et. al., 2005, Sano et. al, 2005, Matsui et. al., 2007, Sengupta et. al., 2008, Kacar et. al., 2009). Another priority of GEPI is their applicability under mild conditions of pH and temperature in aqueous solutions. In this section, only two examples will be demonstrated to give a basic perspective for promising potential of GEPI. In the following sections, the utility capacity of GEPI will be elaborated in a particular model peptide, namely gold binding motifs.

A remarkable proof-of-concept demonstration of 3D heterogeneous multilayered nanostructures, which can lead to the formation of the future batteries, memory elements or biosensors, was achieved using a titanium binding peptide, min-TBP1. This motif (called also T1) has the ability to adhere titanium surface with high affinity and also to control silica mineralization. In this study, min-TBP1 was inserted in ferritin molecule and min-TBP1- ferritin construct (T1-LF) was generated. Ferritin has a hollow structure; this hole can be filled up with Fe, Co and CdSe nanoparticles.

The schematic representation of the bionanofabrication process is given in Figure 2.5(c). With the help of lithographic techniques, Ti patterns were obtained on Pt surface. T1-LF constructs were exposed to the patterned surface and only Ti stripes were occupied because of the specific recognition between min-TBP1 and the Ti surface. Thereafter, ferritin molecules were filled with Fe. Later tetramethyl ortosilicate (TMOS) was added on top of the min-TBP1 decorated ferritin molecules and silification is catalyzed by min-TBP1. After silification another layer of T1-LF molecules is assembled in aqueous solution. This time, ferritin molecules were filled with CdSe. The fourth layer is formed again via silification. The following layer was decorated with T1-LF constructs filled with Co. As shown in Figure 2.5(d), Sano and his coworkers have successfully created controlled arrays of 3D nanostructures via direct patterning of layer by layer self assembly (DP-BioLBL) (Sano and Shiba, 2008).

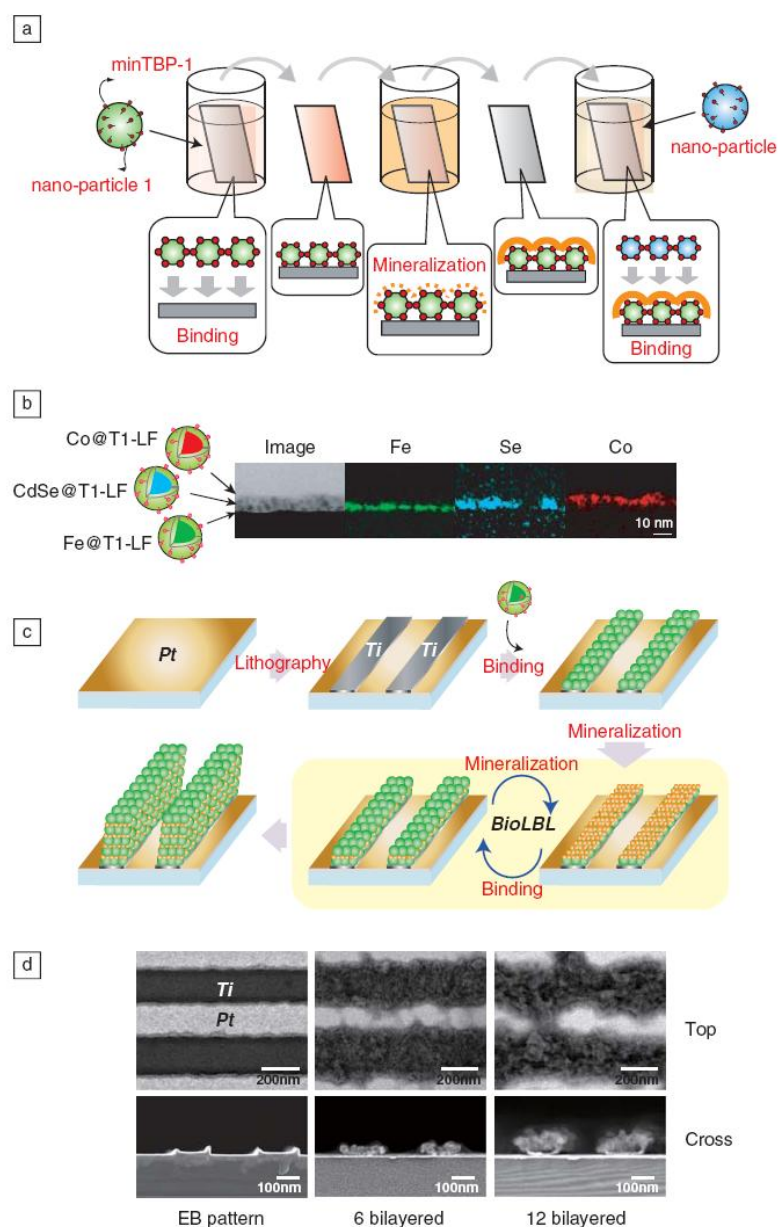


Figure 2.5: In aqua assembly of 3D nanoarchitecture fabricated via biomimetic layer-by-layer (BioLBL) approach. (a) Schematic illustration of BioLBL (b) Energy dispersive spectrometry mapping of cross sections containing Fe-Ferritin-TBP, CdSe- Ferritin-TBP and Co- Ferritin-TBP layers (c) Schematic illustration of direct patterning BioLBL method (DP-BioLBL) (d) Top and cross sectional SEM images of fabricated structures via DP-BioLBL (adapted from Sano and Shiba, 2008).

Another noteworthy application was performed by Belcher and her co-workers. They succeeded to demonstrate the assembly of various material systems with nanometric precision (Figure 2.6).

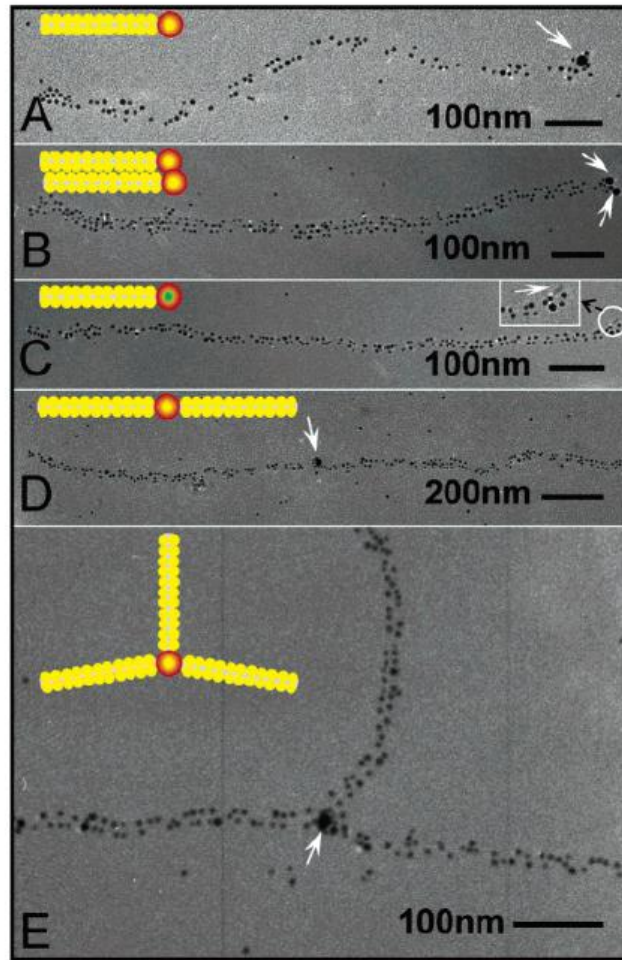


Figure 2.6: (A-E) TEM images of various nanoarchitectures templated by GEPI-virus constructs. The insets demonstrate the assembly routes of observed structures. White represent the virus, yellow dots refer to gold nanoparticles, green dot represents a CdSe quantum dot and red represents the streptavidin coated gold or CdSe particles (adapted from Huang et. al., 2005).

They engineered M13 phage (virus); gold binding motifs were inserted into the permissive sites of p8 proteins, which surround the exterior of the phage. The CdSe binding motif was inserted into the p3 protein, which is on the end of the phage. These engineered viruses were used as templates for nanoparticle assembly. When the form of the biotemplate was changed via genetic engineering tools, the nanoparticle assembly was also changed corresponding to the alteration of the biotemplate. This work highlights the potential promise of bottom-up assembly of GEPI constructs for sophisticated electronic and optoelectronic systems (Huang et. al., 2005).

2.3.3 Gold binding peptides (GBPs)

Gold has great importance in bionanotechnology, not only because it is a biologically inert material but also owing its' high conductivity, plasmonic features, and easy treatability. Hence, just in the beginning of GEPI research, gold binding peptides (GBPs) were selected and identified by Stanley Brown using cell surface display technique (Brown, 1997). Later on, new gold binding motifs were also introduced by other researchers, selected and designed using phage display (Hnilova et. al., 2008) and yeast surface display (Peelle et. al., 2005), respectively. However, the most studied GEPI in literature within the gold binding motifs is GBP₁, selected by Brown. As GBP₁ is the main subject of this dissertation, research about this motif will be reviewed in this section and following subsections.

Gold-binding peptides were originally selected by cell surface display as a fusion partner in the structure of the alkaline phosphatase. They were further isolated as extracellular loops of the maltodextrin porin, LamB protein. GBP₁, possessing an amino acid sequence MHGKTQATSGTIQS, was one of the strongest binders identified and it exposed further enhanced binding affinity when triply repeated in tandem (Figure 2.7). Increasing the repeats from three up to nine did not cause a detectable enhancement in binding. On the other hand, HF treatment of the gold surface further enhanced the adhesion of GBP₁ motif on gold surface indicating that this motif recognizes the gold metal not the impurities or contamination on the surface (Brown, 1997).

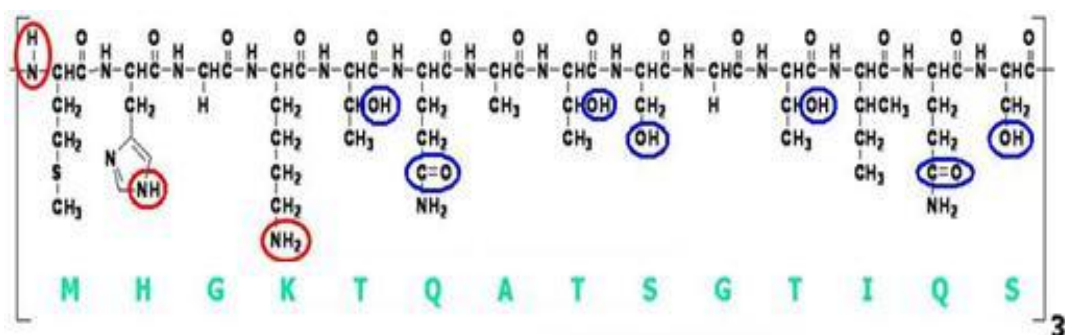


Figure 2.7: Primary structure of 3rGBP₁ (Brown, 1997).

The cysteine-free character of this binding motif and its' removal capability in the presence of detergents suggested the existence of alternative binding mechanisms, different from conventional thiol–gold systems based on chemisorption. The other

interesting feature of GBP₁ binding motif was the adherence to gold at a very high salt concentration, 0.1 M KCl (10 times more than in the absence of salt). This property made the GBP₁ binding motif distinguishable from other gold binding polypeptides, which preferably bind to gold at low salt concentrations. Binding of the proteins was inhibited by salt, which is a commonly encountered case in non-specific protein adsorption studies (Brown, 1997).

Many attempts have been made both experimentally and also theoretically to gain fundamental understanding about intriguing relationship between GBP₁ motif and the gold surface. The first structure prediction study was made by Braun et al.; they tried to model the possible orientation of 3rGBP₁ motif on the gold surface. Results of the model predicted that 3rGBP₁ motif has an anti-parallel beta sheet structure having repetitive random coils. Researchers inferred that a periodic structure of hydroxyl groups on the 3rGBP₁ surface is responsible for 3rGBP₁ binding onto the Au lattice. The proposed model was found to be similar to the electrostatic binding of organic matrix proteins on CaCO₃ surfaces or the binding of small antifreeze proteins with tandem repeats onto ice crystals. Besides, researchers found out that 3rGBP₁ motif demonstrated face specificity; 3rGBP₁ binds onto {211} lattice much less favorable than onto the {111} lattice. The reason for this preference was explained as the difference in atomic density of two planes and also the presence of more water in the {211} surface corrugations that might hinder the direct contact of 3rGBP₁ and gold lattice (Braun et al., 2002).

The second and more detailed study about structural conformation of GBP motifs was performed with the cooperation of Sarikaya and Evans groups using MALDI/TOF and ion-trap mass spectrometry, far UV circular dichroism spectroscopy and solid state NMR techniques. Alterations in secondary structure of the original sequence, GBP₁, and 3rGBP₁ were investigated in the presence of the Au⁺³ ions in solution. They found out that GBP₁ adopts a random-coil extended structure conformation represented by (MHGKTQA) random coil (TSGTIQS) extended, whereas 3rGBP₁ adopts an unfolded, unstable conformational state (probably a combination of random-coil and extended structures) in the presence of the Au⁺³ ions in solution. NMR data implied that the -KTQATS- motif in 3rGBP₁ and the -QAT- motif in GBP₁ play significant roles in interaction with Au⁺³ containing solution (Kulp et al, 2004).

The first detailed report on the adsorption kinetics of a genetically engineered gold binding peptide was achieved by Tamerler *et. al.* They utilized both SPR and QCM techniques to extract quantitative kinetic and thermodynamic data for 3rGBP₁ binding on gold surface. They fitted the obtained data to 1:1 and also biexponential Langmuir adsorption models. The best fit for the SPR data was acquired from biexponential Langmuir model. Hence, a bimodal adsorption was proposed; two simultaneously occurring binding reactions take place on the gold surface. It was found out that 3rGBP₁ motif adheres to gold surface with adsorption equilibrium constants (K_{eq}) $2.54 \times 10^6 \text{ M}^{-1}$ and $4.51 \times 10^5 \text{ M}^{-1}$ respectively. On the other hand, K_{eq} obtained from 1:1 Langmuir fit was $1.12 \times 10^7 \text{ M}^{-1}$ and the binding energy was -9.68 kcal/mol. Results indicated a fairly tight binding with low desorption (comparable to thiol-based systems) and a fast binding process, where the whole surface was covered up to 90% within 20 minutes. Researchers also analyzed the effect of pH on 3rGBP₁ recognition onto gold surface and suggested that the peptide retains its structural conformation over a large pH range. The proposed two stage adsorption model was later confirmed via high magnification atomic force microscopy by the same group (So *et. al.*, 2009).

In parallel with research about molecular structure and adsorption behavior of GBP motifs functional features of this peptide motif have been clarified due to structure-activity relationship of biomolecules. In the next subsections the unique properties of GBPs, particularly 3rGBP₁ motif, will be reviewed.

2.3.3.1 Catalytic activity

In the early stages of GEPI research, Brown and Sarikaya demonstrated the catalytic activity of chimeric AP-3rGBP1 construct that induced the formation of the large flat gold crystals in the absence of any other reducing agent under ambient conditions.

TEM images shown in Figure 2.8 revealed that shapes of the crystals obtained in the presence of GBP motifs resemble to the ones assessed under acidic conditions whereas alkaline phosphatase alone could not induce crystallization. Authors deduced that GBP motifs act catalytically by controlling the local pH. The repeating polypeptides emulate the acidic conditions but probably do not follow a general acid

catalysis mechanism. It was also shown that the -KTQATS- sequence of the GBP motif particularly participates to the nucleation process of gold crystals from Au(III) ions in solution by predominantly adhering onto {111} lattice. Moreover, researchers proposed that GBP motifs accelerated the crystal appearance under kinetically favorable and thermodynamically unfavorable conditions which resembles to the nucleation and growth mechanisms of many natural hard tissues (Brown et al., 2000).

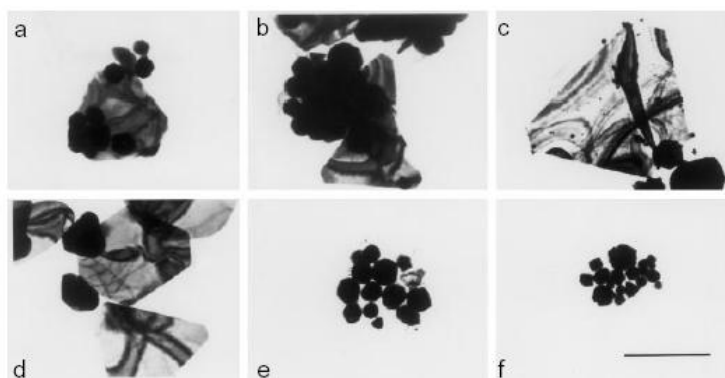


Figure 2.8: TEM images of gold crystals formed by reduction of AuCl₃ in the presence of (a) AP-GBP1 construct (b) AP-GBP2 construct (c) ascorbic acid (d) citric acid (e) only AP (f) potassium ascorbate and potassium phosphate at pH 7 solutions (Brown et. al., 2000).

This study opened a novel route for inorganic material synthesis where GEPI could act as synthesizer and nano-organizer. Thereafter, GEPIs have been utilized for fabrication of various inorganic structures including silver nanoparticles or silver crystals in different shapes (Naik et. al., 2002, Kramer et. al., 2004, Grosh et. al., 2009), Cu₂O nanoparticles (Dai et. al., 2005) silica and cobalt nanoparticles (Naik et. al., 2004), hydroxyapatite crystals (Gungormus et. al., 2008), platinum nanocrystals (Li et. al., 2009). Recently, Wang *et. al.* utilized the one repeat GBP₁ motif alone (without any fusion protein) to catalyze and regulate crystallization of gold nanoparticles (Wang et. al., 2007).

2.3.3.2 Material specificity

Aforementioned studies revealed that 3rGBP₁ motif adheres to gold surface with high affinity. The study conducted by Tamerler et al. clarified that this motif shows also high specificity to gold surface. In this study, a multi-material (Pt/SiO₂/Au) patterned surface was first exposed to biotinylated 3rGBP₁ (bio-GBP1). Following the self assembly of bioGBP1 streptavidin coated Q-dots (SAQD) were immobilized

on patterned surface through biotin streptavidin coupling. After removal of non-specifically interacting molecules, patterned surface was detected under fluorescence (Figure 2.9). Observations indicated that 3rGBP₁ motif preferentially assembles onto gold surface in the presence of another, similar materials. It distinguishes not only between three materials but also between two similar noble metals, namely Pt and Au (Tamerler et al., 2006). Consequently, this study posed that 3rGBP₁ motif can be utilized as a highly specific molecular linker.

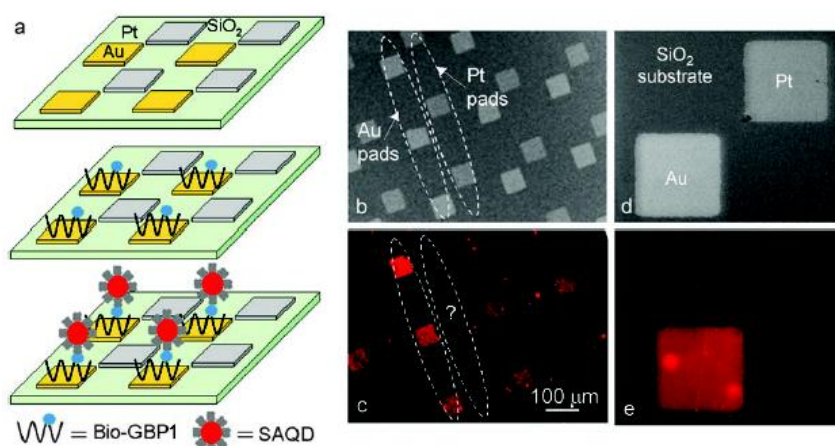


Figure 2.9: (a) Schematic illustration of the directed self-assembly of SAQDs on bio-GBP1 decorated gold pads. Low and high magnification optical microscopy images of the Pt/SiO₂/Au patterned surface in (b) and (d) respectively. Low and high magnification fluorescence images of the SAQD immobilization on self-assembled bio-GBP1 surface in (c) and (e) respectively, demonstrating the selective binding of 3rGBP₁ to the gold surface only (Tamerler et al., 2006).

Another specificity study was conducted by Suzuki et. al. The aim of the study was to unravel the amino acid fingerprints contributing to noble metal binding of GBP₁ motifs. Adsorption of GBP₁ and 3rGBP₁ motifs on noble metals (Au, Ag and Pd) was examined using TOF-SIMS technique. Two different multivariate statistical methods were utilized to interpret the variations in TOF-SIMS spectra for several adsorption conditions. Results revealed that GBP₁ has substrate specificity toward Au compared to Pd and Ag confirming the previous findings. Similar spectral results were obtained for the interactions between three substrates and the ATSGT motif, located in the middle of GBP₁. This motif is therefore considered as the binding site for noble metals. Authors also suggested that the difference in binding specificity of GBP₁ between Au and Ag may arise from the strong binding of histidine to Au and glutamine to Ag, respectively (Suzuki et al., 2007 (a) & (b)).

2.3.3.3 Self assembly

Owing to their self-assembly properties, GBP₁ motifs have been utilized successfully in many applications as molecular linkers and assemblers for targeted organization of functional hybrid nanostructures (Zin et. al, 2007, Park et. al., 2006, Kacar et. al., 2009, Nochomovitz et. al., 2010).

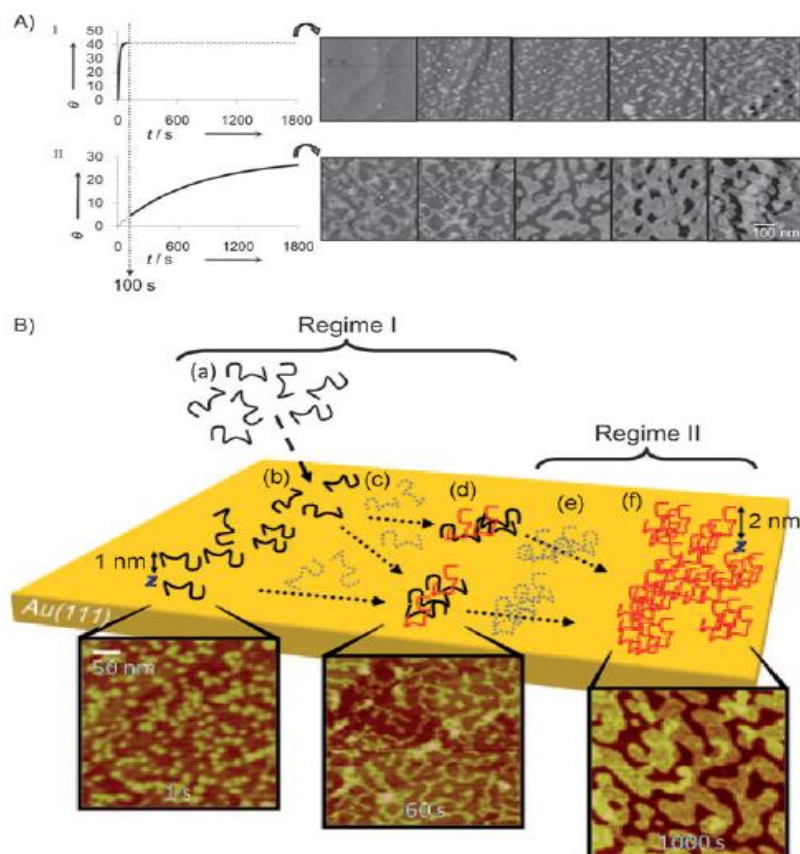


Figure 2.10: A) Deconstruction of the two-domain Langmuir curve into regime I and regime II, B) Schematic illustration for proposed mechanism of the binding, diffusion and assembly of 3rGBP1 on Au (111), in relation with the corresponding AFM images (adapted from So et al., 2009).

Ever growing applicability of these peptides augments the need of improved control. Hence, more efforts have to be given to unravel the complex molecular mechanisms between GEPI and their counterpart solids; how peptides recognize the solid surface specifically.

Recently, a noteworthy study for elucidating the recognition and self assembly mechanisms of GEPI has been performed by Sarikaya group. For the first time, they demonstrated the morphological basis of molecular recognition and self assembly of a genetically engineered peptide on a solid surface. They analyzed concentration-

and time-dependent assembly of 3rGBP₁ on Au (111) surfaces using high magnification atomic force microscopy (AFM). They observed a dynamic adsorption behavior involving multiple stages with different diffusivities for 3rGBP₁ molecules. For simplification they divided the adsorption process into two regimes; peptides first form island-like structures as soon as they approach to the surface, following, these islands coalesce and a peptide network is constituted through a number of structural changes of peptide molecules. Researchers also compared their kinetic data with former SPR results and fit the data to two-domain Langmuir model (Figure 2.10). Observed structures of the adsorbed peptides were interpreted by well-established theories of submonolayer nucleation and island growth developed for metallic and semiconductor thin film deposition systems (So et. al., 2009).

2.3.3.4 Molecular recognition and nanostructuring

Using high magnification AFM, So *et. al.* observed ordered supramolecular 3rGBP₁ structures matching the gold lattice using (Figure 2.11). Further, they investigated the molecular basis for 3rGBP₁ self-association and supramolecular assembly on gold by performing structure refinement (SA/MD) simulations for 3rGBP₁ under implicit solvent conditions. Results confirmed the previous findings obtained from NMR studies indicating that 3rGBP₁ possesses an alternating coil – extended -strand structure and appears to be intrinsically disordered due to its labile unfolded confirmation (So et. al, 2009).

Researchers found out that 3rGBP₁ forms elongated nano-domain assemblies that are crystallographically oriented with the same six-fold symmetry as the Au {111} surface lattice. They suggested the unfolded conformation of 3rGBP₁ leads to simultaneously acting features; (1) driving the gold surface recognition and binding process, (2) formation of the supramolecular assembly. According to the geometrical docking studies shown in Figure 2.12, the unfolded state of 3rGBP₁ first creates an effective surface display of polar (green), non-polar (white) and cationic (blue) residues, which enhances both interfacial solid-peptide and intermolecular peptide-peptide associations. Moreover, the complementarities between the Au docking surface of 3rGBP₁ and the Au {111} interface may induce peptide binding and assembly following the six-fold symmetry of the Au{111} surface (Figure 2.11&2.12).

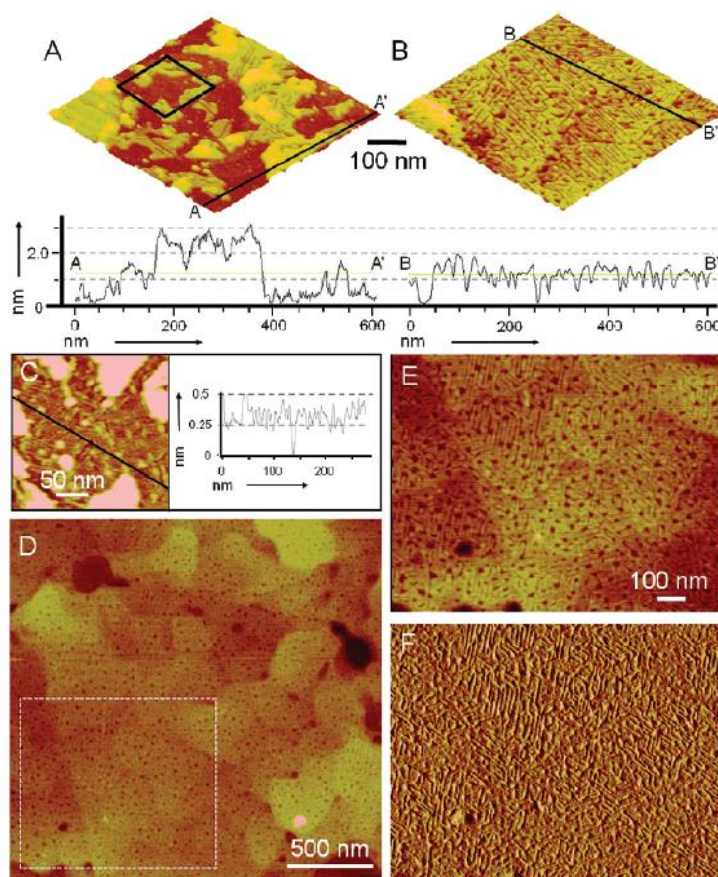


Figure 2.11: AFM observation of ordered supramolecular assembly of 3rGBP₁ on Au {111}. Pseudo 3D AFM images and line profiles of a harshly rinsed surface (A) and gently rinsed surface (B). (C) High resolution AFM image of the area highlighted by the black square in A. AFM height images (D, E) AFM amplitude image (F) that demonstrate the long-range ordering of the assembled peptide film on <111> oriented gold grains (adapted from So et. al., 2009).

Results of this study imply that the tight and concurrently flexible interactions between 3rGBP₁ binding motif and the gold surface may originate from the close contact of the polar (e.g., glutamine, serine, or threonine) and cationic (e.g., lysine or histidine) amino acid side chains with the underlying gold lattice. This study emphasizes also the important role of peptide's unfolded labile conformation and the six glycine residues, which enable conformational flexibility, in specific recognition.

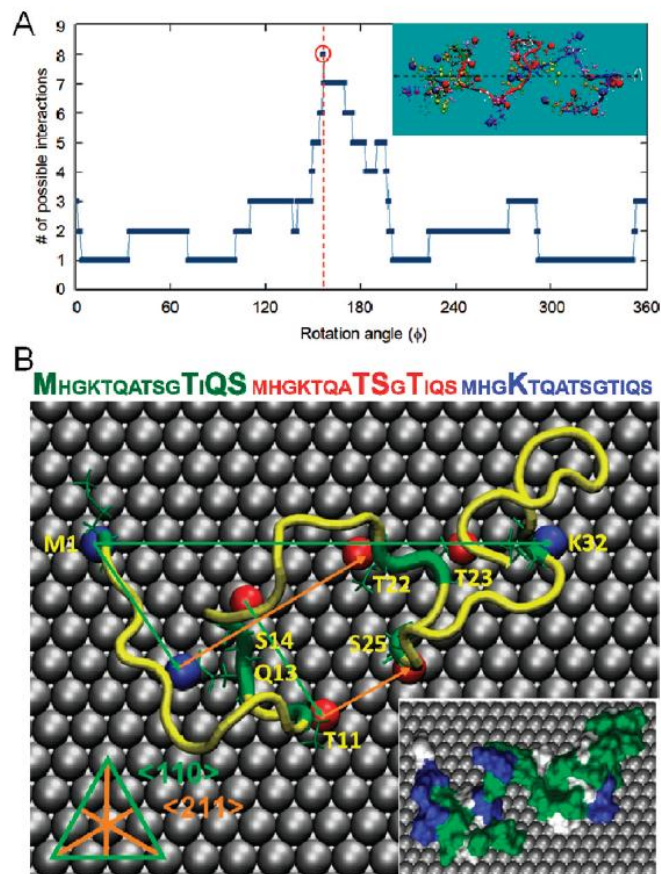


Figure 2.12: Predicted geometric orientation of 3rGBP₁ on the Au{111} surface. (A) Plot of number of possible planar interactions versus rotational angle (ϕ) between the peptide and the Au{111} surface. The rotation angle of the most interactive surface is shown by a red circle. (B) Representation of the circled orientation from (A) and its geometric correlation with the {111} gold surface (adapted from So et. al., 2009).

2.3.3.5 Intrinsically disorder

3rGBP₁ motif has an unfolded and labile conformation in solution and it undergoes conformational changes when it binds to the gold surface. Its internal stabilization depends on external conditions. Moreover, it contains disorder promoting amino acids such as lysine and serine (Kulp et. al., 2004, So et. al., 2009). Because of these properties, 3rGBP₁ behaves very similar to intrinsically disordered proteins (IDPs), which do not lose their functionality in a partially or even fully unfolded state and change their conformation upon adhering to their targets (Delak, 2009).

Intrinsically disorder could be a key feature in molecular recognition of GEPI onto their counterpart inorganics since many identified naturally occurring peptides,

which take place in biomineralization processes, are intrinsically disordered (Evans, 2003).

2.3.3.6 Nano- and bionanotechnological applications of GBPs

Since their identification GBP motifs have been utilized in many applications as synthesizers in materials synthesis or as molecular linkers and constructs in targeted immobilization. Some examples have been already mentioned in the previous subsections. Here, a couple of other important applications, where GBP motifs are used, are summarized to point out that utilization of GEPI, particularly GBP, has a promising potential for the future in nanoscience and nanotechnology.

One of the approaches was the improvement of biosensor efficiency using 3rGBP₁ motif. In a study, the gold surface of SPR biosensor system was modified with 3rGBP₁, and the limit of detection of the biosensor system was increased by a factor of five (Soh et. al., 2003). In another study, 3rGBP₁ was fused into several model proteins; enhanced green fluorescent protein (EGFP), severe acute respiratory syndrome coronavirus (SARS-CoV) envelope protein (SCVme), and core streptavidin (cSA) of *Streptomyces avidinii*. GBP-fusion proteins were immobilized onto the gold nanoparticles and protein nanopatterns were generated on the bare gold surface. Microchannels were fabricated based on these protein patterned platforms. The interactions between fusion proteins and their antibodies were monitored in microchannels using SPR imaging. High throughput detection of biomolecular interactions in microfluidic channels have been demonstrated (Park et. al, 2006).

Another approach was the utilization of GBPs as molecular linkers and assemblers. Ordered arrays of gold nanoparticles were created with the help of 3rGBP₁. The gold binding peptide was assembled on gold surface via micro contact printing (1) directly through physical interactions (2) through covalent bonding between a thiol molecule and N-terminus of 3rGBP₁. Gold nanoparticles were exposed to the 3rGBP₁ modified surface and they were captured by 3rGBP₁. Results revealed that the chemical conjugation increases peptide's ability of capturing gold nanoparticles, whereas the physical binding decreases this ability (Zin et. al., 2005). Immobilization of 3rGBP₁ for capturing gold nanoparticles was also applied to control the emission of Q dots depending on their distance from the gold surface, where 3rGBP₁ binding motif was utilized as molecular linker and also molecular erector (Zin et al., 2007).

In another study, 5rGBP₁ motif was genetically fused to alkaline phosphatase (AP) and immobilized on gold substrate. Higher enzymatic activity per area was observed on micro-patterned gold surfaces where 5rGBP₁-AP constructs were immobilized compared to only AP assembled surfaces (Figure 2.13). Results demonstrate the promising applicability of GEPI as sitespecific molecular linkers for oriented enzyme immobilization with retained activity (Kacar et. al., 2009).

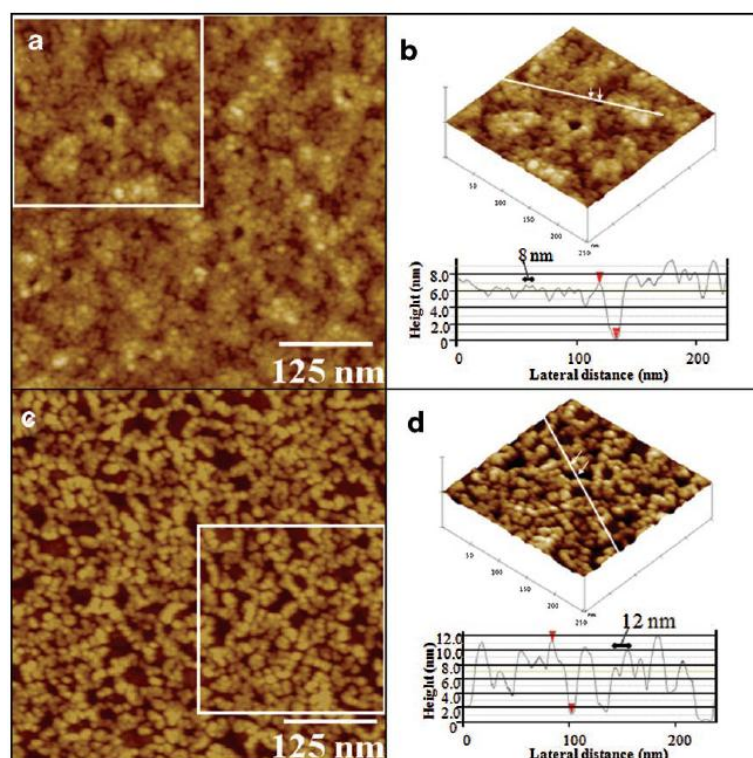


Figure 2.13: 2D and magnified pseudo 3D AFM images with corresponding line profiles of self assembled 5GBP1-AP constructs (a&b) and AP alone (c&d) respectively (adapted from Kacar et. al., 2009).

2.4 Protein Adsorption at Solid-Liquid Interfaces

Protein adsorption can be defined as the thermodynamically and/or kinetically favorable *movement of protein molecules towards a surface*. Since molecular mechanisms about movement and recognition of peptides towards a solid surface were investigated in this dissertation, particular attention has given to adsorption phenomenon at solid-liquid interfaces.

From a materials scientist's point of view, protein adsorption can be assumed as the spontaneous deposition of soft thin films on solid surfaces in a liquid environment. As soft and flexible materials, structural properties of proteins and polypeptides in

aqueous environments will be introduced in the first subsection. Thereafter, dynamics of protein adsorption in aqueous environments will be outlined. In the following subsections characterization techniques, used in protein adsorption studies will be explained by emphasizing quartz crystal microbalance, which was the main experimental technique used in this thesis work.

2.4.1 Proteins in aqueous environments: Structure and properties

Proteins or polypeptides are co-polymers which have unique structural and functional properties depending on their size and sequence. Random variation of 20 different amino acids in different sequences and lengths determines the enormous diversity of proteins. Each amino acid has a carboxylate (COOH) and an amino (NH_2) group which can be deprotonated (COO^-) and protonated (NH_3^+) in aqueous solutions. Each amino acid structure contains also a functional side group, which can be non-polar, polar or charged. A polymer chain, referred as *polypeptide*, is formed when amino acids are linked covalently to each other through their carboxylate and amino groups. Although COOH and NH_2 groups lose their (de)protonation capabilities during polycondensation, the C- and N-terminus placed at the both ends of the chain give a zwitterionic character to polypeptide chains at certain pH values (Berg et. al., 2006). Polypeptides are also amphiphilic molecules because they usually contain non-polar and polar amino acid residues along the same polypeptide chain. In aqueous solutions, non-polar residues are highly hydrophobic, whereas polar and charged side chains are hydrophilic (Israelachvili, 1992). As a matter of fact, zwitterionic and amphiphilic characteristics make polypeptides and proteins intrinsically surface active (Norde, 1986).

Charged and polar groups in amino acid residues play an important role for the structural stability of polypeptide chains in aqueous environments. The charged amino acid side chains exchange protons with water and become ionized and they remain in dynamic equilibrium with their uncharged states under physiological conditions. In globular proteins, more than 20% of all aminoacids are charged and polar groups are found in another 25% of side chains. The effects arising from the electrical charges and dipoles in these very large structures give rise to Coulombic attraction and repulsion and dispersive attractions; the larger the entities become, the more they behave as colloidal particles (Creighton, 1993, Bockris and Reddy, 2000).

The location of charged groups along the protein plays a critical role in determining the nature of the inter- and intra-molecular electrostatic interactions. Charges of the protein may be placed in the active site, on the surface, or in the interior of the protein. Charges may exist in pairs or groups, or may be isolated. If two charged groups are sufficiently close that a water molecule cannot accommodate between them, interactions between those charges are referred to as *local*. Hydrogen bonding, ascribed as salt bridge, occurs between oppositely charged groups which have local interactions. If water or other species can fit between two charges, then their interaction is called as *long-range* (Figure 2.14). One ionizable group of the protein influences the charges of other ionizable groups. This interdependent character of charged groups causes a network between charges of the protein if the charged groups are located in the vicinity of each other. Hence, a *cooperative* effect within the protein charge interactions occurs. When the ionizable groups are located in distance, then the interdependent effect of charges weakens and the charge interactions can be treated as *pair-wise additive*.

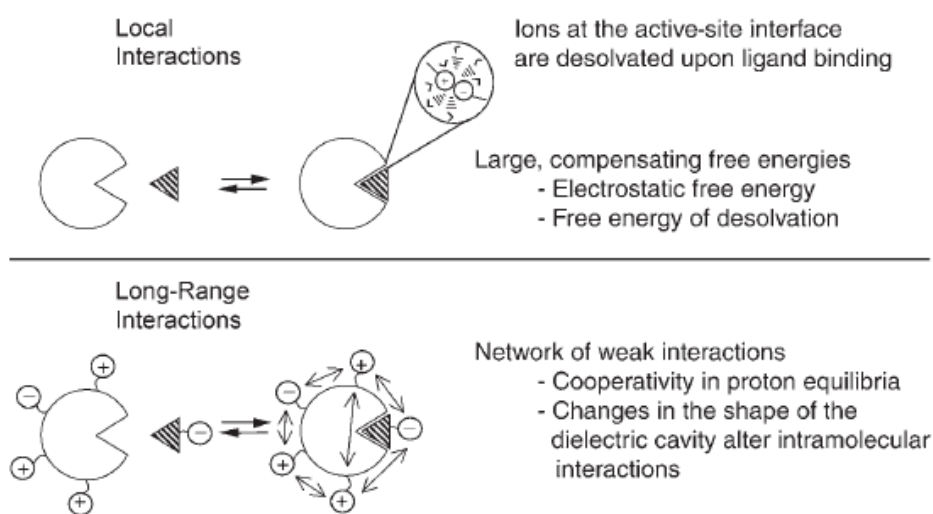


Figure 2.14: Illustration of the difference between local and long-range interactions among charged groups of proteins (Gitlin et. al, 2006).

A fundamental physical property of a protein is its net charge, which is defined as the sum of all charged groups that are tightly associated with a protein (Berg et. al, 2006). Net charge of a protein can be estimated roughly by proton titration or through summation of charges on each ionizable group along the protein sequence. The charges are approximated using a standard value of their ionization constants (pKa values) and the pH value of the solution. There are also other techniques such

as the combination of charge ladders and capillary electrophoresis for obtaining more accurate information about protein charge (Gitlin et. al, 2006). The net charge directly influences the solubility, aggregation and crystallization of the protein (Winzor, 2004, Gitlin, 2006).

Besides charged and polar residues, the contribution of non-polar residues to the structural stability of a protein is significant. Spontaneous dehydration and subsequent aggregation of non-polar amino acid residues in aqueous solutions is called as *hydrophobic interaction* which is characterized by a large entropy increase and a relatively small enthalpy gain. Hydrophobic interactions are the main driving force for protein folding. In aqueous protein solutions, non-polar amino acids will tend to be buried in the interior of the molecule, whereas most of the amino acid residues carrying an electric charge are located at the aqueous periphery of the protein molecule. This induces the formation of secondary structure as helices and pleated-sheet structures and possibly folding of the polypeptide chain to form a compact molecule. Folded proteins have less rotational freedom compared to random coil structures. The degree of rotational freedom depends on steric hindrance by amino acid side chains. Directional forces such as dipole-dipole and charge-charge interactions or hydrogen bonds determine the degree of rotational flexibility along the polypeptide chain and the side chains. Increase in rotational freedom causes entropy and enthalpy gain, whereas decrease of rotational flexibility results in a more stable protein structure (Norde, 1986, Gitlin et. al., 2006).

Another important criterion, which designates protein structure, is the length of polypeptide chain. Shorter chains are more flexible and usually less stable. They only bear a secondary structure. Longer polypeptide chains form three dimensional (3D) *folded* structures under physiological conditions. Formation of 3D structure occurs by intermolecular forces and multiple physical bonds through-out the protein leading to a metastable state. Proteins with 3D structure are generally less flexible and therefore more stable. The distinct and single 3D structure, which is specific for each protein, distinguishes proteins from synthetic polymers (Horbett, 1996). However, the structural singularity of a particular protein generally applies only under physiological conditions. Beyond these conditions proteins usually denature, which means they lose their *native* structure. The protein tend to become much more like a random coil, that is characteristic for synthetic polymers. Denaturated proteins

usually lose their stability and solubility, and lose also biological functions such as enzyme activity (Horbett, 1996). On the other hand, recent studies revealed that some proteins, named *intrinsically disordered proteins* (IDPs), do not lose their functionality in a partially or even fully unfolded state (Delak, 2009).

As stated above, the structure and the physicochemical state of proteins determine their function and activity. Hence, a detailed understanding is necessary about possible inter- and intramolecular associations of proteins and other molecules present in aqueous environments. Understanding the physicochemical origins of protein-water interactions requires a closer look to the thermodynamic behavior of these entities.

Interactions between charged residues during protein folding, ligand binding, and protein-protein interaction often involve changes in their solvation states. *Solvation* occurs when solute molecules are surrounded by solvent, referred as *hydration* in water. When polar and charged groups in proteins are hydrated, they alter and disrupt the local water structure. Hydration of these ionized structures causes a favorable increase in entropy. Yet, in some cases such as ligand binding, charges on the active site of the protein may be transferred from a medium with larger dielectric constant (bulk water) to the one with smaller dielectric constant (interfacial water) because binding of a ligand requires an expulsion of solvent from protein-ligand interface. This phenomenon is referred as *desolvation*, which is thermodynamically unfavorable. During protein-ligand binding in aqueous environments, a competition occurs between the unfavorable energy for desolvation and the favorable energy due to the formation of electrostatic contact between active sites (Gitlin et. al., 2006, Israelachvili, 1992).

Increasing the ionic strength of protein solutions may diminish the free energy of the electrostatic interactions by shielding charged groups in proteins due to the Debye layer. However, in proteins the distances between many charged groups (especially those involved in salt bridges) are significantly smaller than the Debye screening lengths. Therefore, the shielding effect is less than expected.

Hydration of non-polar residues in proteins is a more complicated task. Tetrahedral coordination of water molecules enables a three dimensional network of hydrogen bonds in aqueous environments. When water molecules come in contact with non-polar side chains of proteins, they reoriented themselves in the vicinity of those

molecules in such a way that they do not lose any of their hydrogen bonds (i.e. without necessitating any breakage of hydrogen bonds). Hence, water molecules around a non-polar solute are even more ordered than in the bulk liquid, which is usually called as *hydrophobic solvation* or *hydrophobic hydration*. Both, experimental and theoretical studies suggest that reorientation or restructuring of water around non-polar solutes or surfaces is entropically very unfavorable since it disrupts the existing water structure and imposes a new and more ordered structure on the surrounding water molecules (Israelachvili, 1992). If a protein has more hydrophobic surface exposed to water, then it has less intramolecular hydrophobic interaction within protein molecules (Norde, 1986, Gitlin et. al., 2006). Such a protein may not prefer aggregation.

Overall, protein-protein, protein-water, protein-ion interactions are directed by intermolecular forces (e.g. Coulombic attraction and repulsion, dispersive attractions, hydrophobic interactions or hydrogen bonding) leading to a change in protein structure or intramolecular associations within proteins (Norde, 1986). Since intermolecular forces operating in protein solutions are physical bonds in origin, they are very convenient for holding molecules together in aqueous environments. Hence, the molecules can move and rotate while still remaining physically bonded to each other. In physical bonds, the electron charge distribution of the atoms or molecules are only perturbed and do not change completely such as in the case of chemical bonds. Nevertheless, physical bonds can be as strong as the chemical bonds. They also do not have a certain stoichiometry, strong directionality and usually not high affinity and specificity. On the other hand, short-range physical forces can also be coupled with the long-range forces, which give them the power to regulate all phenomena that do not involve chemical reactions. Thus, molecular recognition processes occurring between biological materials mentioned in the previous sections are mainly directed by intermolecular physical bonds (Israelachvili, 1992).

2.4.2 Dynamics of protein adsorption

Protein adsorption is a complex process, involving many successive steps. The degree of adsorption depends on both protein and solid surface properties and also the type of interfacial solvent. As highly surface active materials, proteins have an inherent tendency to deposit on surfaces as a tightly bound adsorbate (Horbett,

1996). Hence, protein adsorption is mainly an irreversible process, unless removal procedures such as dilution of the solution, adjusting pH or ionic strength or adding a displacer were applied. (Norde,1986).

The physicochemical factors that determine protein adsorption and desorption are demonstrated in Figure 2.15. According to the Figure 2.14, adsorption and desorption processes are summarized in three distinct steps; approach of the proteins towards the solid surface, attachment of proteins involving stabilization of the adhesion interface and detachment of proteins requiring destabilization of the adhesion interface. Yet, without additional work proteins usually do not desorb from solid surfaces (Kidoaki, 2002). During approach, adsorption kinetics is determined by diffusion of proteins towards the solid surface. Then protein molecules adhere to the solid surface by overcoming several energy barriers. Thereafter, protein-solid surface interactions and structural rearrangements become rate-determining. Important parameters for adsorption kinetics are the protein concentration and the flow rate of the protein solution (Xia, 2003).

The thermodynamic aspects for protein adsorption are mainly determined by the type, charge and lyophobicity of both, protein and solid surfaces and also the temperature, pH and ionic strength of the solution environment (Norde, 1986). The charge on proteins and solid surfaces in aqueous solutions is usually neutralized by counterions, leading to the formation of the electrical double layer (EDL). Prior to adsorption as the proteins approach to the surface, the EDLs overlap, the resulting change in the free energy for the electrostatic interactions can be attributed mostly to the enthalpy (Norde, 1992, Norde, 1994). Nearby, rearrangement of water molecules and ions occur causing a change in entropy. During attachment, water molecules adsorbed on the surface are substituted by protein molecules leading to a change in the hydration states of solid and also protein surfaces. Once proteins are adsorbed onto solid surfaces, they may undergo some conformational changes because of their relatively low structural stability which allow them to unfold and form additional contacts with the solid surface (Brash and Horbett, 1987). Depending on the type of intermolecular interactions at protein-solvent-solid interface, conformational changes in the protein structure containing ion pairing, H-bonding, hydrophobic interactions, rotational freedom, bending of the covalent bonds may occur during and after adsorption (Xia, 2003).

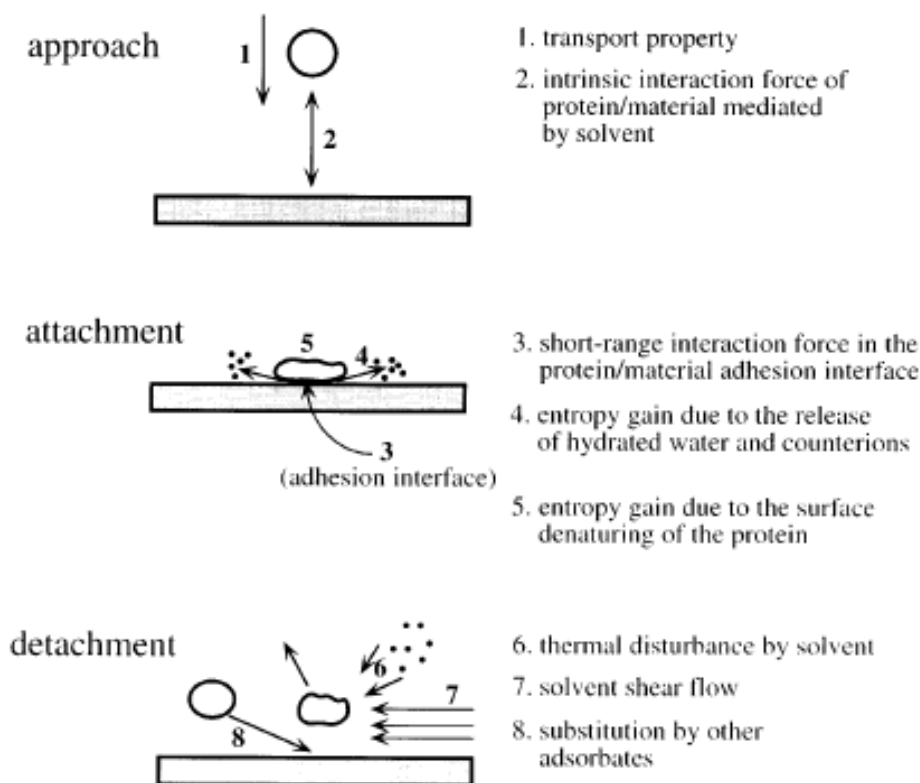


Figure 2.15: Illustration of protein adsorption and desorption processes and the physicochemical forces inducing each process (Kidoaki, 2002).

Hydrophobic interactions are the main driving force in protein adsorption. At hydrophobic surfaces, hydrophobic side chains in the interior of the protein have a high affinity to interact with the solid surface while hydrophilic side chains remaining in contact with water. The reduction of free energy provides a spontaneous change in protein conformation at solid-water interface because the high energy water-hydrophobic protein residues interface is replaced with the low energy hydrophobic surface-hydrophobic protein residues and water-hydrophilic protein residues interfaces (Xia, 2003). For example, in a study of Tilton *et. al.* an enhancement in adsorption was observed by adding non-polar solvents into the aqueous buffers, since the number of high energy water-hydrophobic interfaces are augmented. On the other hand, addition of polar solvents into the same system diminished adsorption because the amount of high energy water-hydrophobic interfaces is decreased (Tilton *et. al.*, 1991).

Besides hydrophobic interactions, the polyelectrolyte behavior of proteins has also a primary role in protein adsorption. Owing to a large variety of polar and charged

amino acid side chains, proteins have both positive and negative excess charges which are distributed around the exterior of the protein. Therefore, depending on the pH and ionic strength of the solvent, a large range of charge interactions can be expected between the protein and a surface. For many proteins, highest adsorption on neutral or slightly charged surfaces is obtained near the isoelectric pH, where the net charge on the protein surface is minimal because the electrostatic repulsion between protein and the solid surface is minimized under this condition. On the other hand, on surfaces carrying a large net charge, protein-solid interactions are dominated by the degree of opposition of protein and solid surface charges, so that negatively charged proteins adsorb preferentially to positively charged surfaces and positively charged proteins to negatively charged surfaces. When a protein approaches a like-charged surface, electrical double layers overlap. Hence, retardation in the adsorption process may occur (Horbett, 1996, Xia, 2003).

The effect of ionic strength in protein adsorption depends on the type of ions present and also sometimes on the type of the adsorbed surface. Increase in ionic strength may shield the electrostatic interactions causing enhancement in adsorbed protein amounts. On the other hand, the role of small ions, especially ions with higher valency, on the protein adsorption is more intricate than non-specifically screening between interacting charges (Kunz et. al., 2004).

The degree of structural stability of proteins is another essential phenomenon for the dynamics of protein adsorption. Proteins with high structural stability, like lysozyme and ribonuclease, bear strong internal coherence and therefore behave as “hard” particles in solution and on the solid surface. Hence, these proteins adsorb on hydrophobic surfaces under all conditions of charge interaction and on hydrophilic surfaces only if electrostatically attracted. Proteins having a relatively low structure stability, such as myoglobin and α -lactalbumin, contain an extra driving force for adsorption, related to structural rearrangements in the molecule. This driving force outweighs the unfavorable contributions from hydrophilic dehydration and electrostatic repulsion. Therefore, these “soft” proteins adsorb on hydrophobic and hydrophilic surfaces under attractive and repulsive electrostatic conditions (Arai and Norde, 1990).

In conclusion, the affinity between a protein and a solid surface increases, if (1) the solid surface and the protein exterior are more hydrophobic, (2) the protein have a

larger tendency to unfold upon adsorption, (3) the electrostatic repulsion between charged groups of the protein and the solid surface is minimum, (4) ions in the medium are less chaotropic and have a higher valency (Xia, 2003).

2.4.3 Characterization techniques for protein adsorption

In spite of enormous efforts given in this area, protein adsorption is a process that is still only rudimentarily understood at the molecular level. Further progress for a fundamental understanding about protein adsorption is highly dependent on reliable measurement techniques that can produce rich and accurate information (Bockris and Reddy, 2000).

Analysis of protein adsorption at solid-liquid interfaces is challenging because adsorption is affected not only from interactions between adsorbed molecules and the solid surface but also from associations within the adsorbed molecules and solution. Another problem is the strong attenuation of the probing signal by the liquid phase for most techniques (Fang et. al., 2009).

In protein adsorption studies, the application of spectroscopic techniques (NMR, CD, infrared or fluorescence spectroscopy), which are generally used for structural analysis of proteins (Mollmann et. al., 2006, Collino and Evans, 2007, Wangoo et. al., 2008), is limited because signals coming from the solid surface, protein surface and the aqueous solvent can hardly be differentiated (Bockris and Reddy, 2000). Nowadays, attenuated total reflectance FTIR (ATR-FTIR), TOF-SIMS and SERS are the promising spectroscopic techniques, which give compositional and structural information about protein-solid interface at molecular level (Xia, 2003, Tullman et. al., 2007, Boujday et. al., 2009, Cerf et. al., 2009, Podstawka-Proniewicz et. al., 2010).

High resolution imaging of adsorbed protein layers are achieved via scanning probe microscopy (SPM) techniques such as STM, AFM, and SFM, which became very popular in the last decades (Veisheh et. al., 2002, Andolfi et. al., 2006). For example, using AFM, quantitative information about protein adsorption kinetics and thermodynamics can be assessed (Gettens et. al., 2005, Mulheran et. al., 2008). Moreover, protein conformation and assembly orientation can be observed both in-

situ and ex-situ with the help of SPM techniques (Bonanni et. al., 2003, Goede et. al., 2006, Andolfi et. al., 2006).

Time dependent kinetic measurements of adsorbed proteins in solutions can be performed using a variety of techniques including SPR, QCM, ellipsometry, reflectometry, optical waveguide lightmode spectroscopy (OWLS), and total internal reflectance fluorescence spectroscopy (TIRF) (Hook et. al., 2002, Brusatori and Van Tassel, 2003, Bremer et. al., 2004, Edvardsson et. al., 2009). To obtain thermodynamic data such as enthalpy, entropy and Gibbs free energy of a protein during equilibrium states of adsorption steps, differential scanning and isothermal microcalorimetry techniques are very convenient (Haynes and Norde, 1995).

Electrochemical methods such as cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to measure adsorption kinetics of proteins in aqueous solutions for a long time. These systems allow reproducible, continuous in situ measurements, besides they are also very sensitive to protein conformational changes in the bulk solution during adsorption. However, they require electrokinetically active molecules to probe adsorption processes (Nakata et. al., 1996, Omanovic and Roscoe, 1999, Jackson et. al., 2000, Cosman and Roscoe, 2004). Nowadays, QCM and SPR techniques are much more preferred compared to these electrochemical studies since they directly probe protein-solid interface in-situ without necessitating any other molecule (Boujday et. al., 2009, Fang et. al., 2009).

Efforts are continuing to develop label-free techniques, which obtain quantitative information from solid-liquid interface. There are some emerging techniques such as SPR Imaging, nanohole arrays, photonic crystals, high Q-microcavities, microcantilevers, reflective interferometry, imaging elipsometry etc. Each of these techniques has its own advantages and limitations (Qavi et. al., 2009). Indeed, SPR and QCM are still the most commonly used techniques for in-situ adsorption kinetic measurements of proteins due to their well-established setups and enormous literature background (Fang et. al., 2009).

2.4.4 Quartz Crystal Microbalance (QCM)

In this dissertation, all experiments were performed in solution using a quartz crystal microbalance with impedance monitoring (QCM-Z). As analysis of QCM data is one

of the main focuses of this study, a comprehensive physical and mathematical background about QCM technique is given in the following subsections.

2.4.4.1 Quartz and the piezoelectric effect

In 1880, Pierre and Jacques Curie discovered that an electrical potential on the surface of quartz could be generated when a mechanical stress across the material is applied (Curie and Curie, 1880). The potential vanished by removal of the stress resulting in elastically deformation of the quartz material. This effect is called as the *piezoelectric effect*. On the molecular scale, this effect is caused by the presence of ionic dipoles that exactly cancel out in solid, crystalline materials whose structures do not possess a center of inversion. Mechanical deformation of the crystal lattice causes an unbalancing of the dipoles resulting in a measurable electrical potential (Nicu and Leichle, 2008, Viitala, 2008).

Since the beginning of the 19th century, quartz crystals have been utilized in a wide range of industrial applications such as in watches, microphones, electronic circuit boards etc. due to its piezoelectric properties (Patel, 2007). In this work, quartz is used in an electrochemical quartz crystal microbalance (EQCM) system to follow the adsorption kinetics of peptides and proteins in-situ.

2.4.4.2 Physical principles of QCM

In a QCM system, quartz crystal replies to the inverse piezoelectric effect. Hence, when voltage is applied across the electrodes of the QCM crystal, a reorientation of the dipoles inside the quartz is induced leading to shear deformation of the crystal due to its AT-cut crystal orientation and piezoelectric character (Tsionsky et. al., 2004). Shear deformation generates an acoustic wave travelling across the crystal in resonance. This resonant frequency depends on several factors including crystal mass, orientation relative to the direction of the electrical field as well as the temperature (Patel, 2007).

Sauerbrey's pioneering work (Sauerbrey, 1959) showed that at constant temperature the change in resonance frequency of AT-cut crystal (the angle between the planes of two electrodes and the crystallographic axis is 35°15') (Figure 2.16) has a linear relationship with the mass of deposited film onto the crystal, if the film is (1)

uniform, (2) thin compared to the thickness of the crystal, (3) rigid and (4) elastically coupled to the crystal such as it can be treated as an extension of the quartz crystal. Thus, the acoustic wave travels a longer distance, a decrease in frequency occurs where the other properties of the acoustic wave remain unchanged because it is assumed that the deposited film material has the same acoustic properties as the quartz (Patel, 2007, Viitala, 2008).

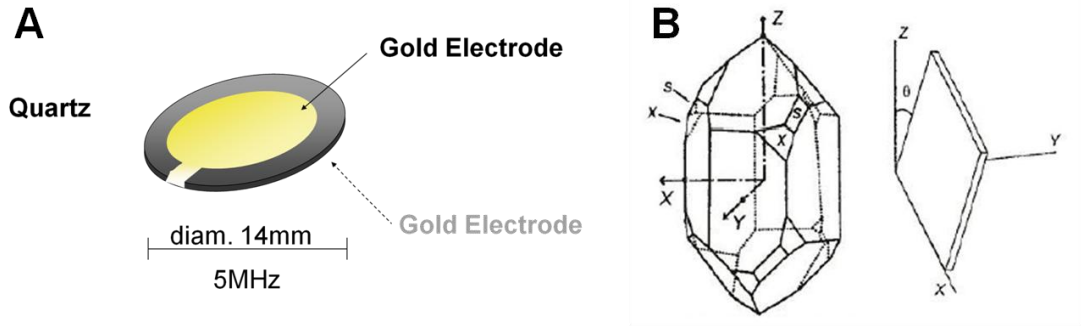


Figure 2.16: (A) Schematic illustration of a quartz crystal with its gold electrodes (B) Crystallographic representation of quartz and the specific AT-cut (Viitala, 2008).

The added mass of the deposited film can be calculated from the well-known Sauerbrey equation:

$$\Delta f = -C_f \Delta m = -C_f \rho_f t_f \quad (2.1)$$

where Δf is the resonant frequency change between unloaded crystal and loaded crystal, Δm is the added mass of the deposited film with density ρ_f and thickness t_f , C_f is the mass sensitivity constant which can be expressed as:

$$C_f = \frac{2 f_0}{v_q \rho_q} \quad (2.2)$$

in which f_0 is the fundamental resonant frequency of the quartz crystal, ρ_q is the density of quartz (2648 kg/m^3) and v_q is the shear wave propagation velocity of sound in quartz (3340 m/s). This value is equal to $17.7 \text{ ng/cm}^2\text{Hz}$ at $f_0 = 5 \text{ MHz}$ (Viitala, 2008). At the fundamental frequency, a standing wave is established when the acoustic wavelength is equal to exactly on-half the thickness of the quartz crystal:

$$t_q = \frac{\lambda_q}{2} \quad (2.3)$$

where t_q is thickness of the crystal and λ_q is the wavelength of the shear wave. In addition to the fundamental resonance, the crystal can be excited to its overtone frequencies. Under these conditions, the resonant frequency of the acoustic wave, f_q , in standing wave condition is given by:

$$f_q = \frac{Nv_q}{t_q} = N f_0 \quad (2.4)$$

where N is the overtone number (1, 3, 5, 7, 9, ...) of the resonant frequency.

Significant efforts have been given by Miller and Bolef (Miller and Bolef, 1968) and thereafter Lu and Lewis (Lu and Lewis, 1972) to express the shear velocity of the quartz and the deposited film in terms of respective material's shear moduli, μ , and mass densities, ρ :

$$v_q = \sqrt{\frac{\mu}{\rho}} \quad (2.5)$$

Overall, these contributions paid the way for a better correlation between the mechanical and acoustic properties of the deposited films in QCM systems when the system is operated in air or vacuum. However, in liquid systems the frequency is not only affected by the mass loading, but also by the viscous coupling with the liquid. Besides, surface stresses at the solid-liquid interface contribute to the frequency shift in a more complicated way (Kucukkaraca, 2005). Since this dissertation have focused on analysis of solid-liquid interfaces via QCM, the influence of viscous loading and viscoelasticity on QCM signal will be asserted in the following sections.

2.4.4.3 Viscous loading

When one phase of the crystal is immersed into a Newtonian liquid, a purely viscous coupling occurs at the interface. Shear wave of the crystal propagating in the solution dissipates energy. In this case, Sauerbrey equation fails. Kanazawa and Gordon demonstrated in 1985 that the frequency change of the crystal in liquid systems is proportional to the square root of the liquid viscosity, η_1 , and density ρ_1 , product (Kanazawa and Gordon, 1985):

$$\Delta f = -N^{1/2} f_0^{3/2} \left(\frac{n_l \rho_l}{\pi \rho_q \mu_q} \right)^{1/2} \quad (2.6)$$

For thin, rigid and uniform layers deposited on the quartz crystal in a Newtonian like liquid the frequency change due to the liquid and the deposited film are additive. Hence, the mass of the deposited layer can be obtained by a Sauerbrey-type analysis. For thicker and less rigid films, a more complex analysis should be performed and several viscoelastic models are available, which will be discussed later.

As mentioned before, the quartz crystal experiences a loss in energy as it oscillates in solution. This viscous loss results in a decrease of the quality factor, Q , of the crystal, which is the ratio of energy stored to that lost per oscillation. Energy loss can also expressed as dissipation factor, D , which is the inverse of Q . For simple viscous loading, the change in dissipation can be related to the viscosity and density of the fluid as follows (Patel, 2007):

$$\Delta D = -2N^{-1/2} \left(\frac{f_0 n_l \rho_l}{\pi \rho_q \mu_q} \right)^{1/2} \quad (2.7)$$

Here it is necessary to mention that changes in frequency and dissipation do not have a linear relationship with harmonic numbers for viscous loading, which was the case in air and vacuum environments. Another concern is the nature of viscous loading. At the fundamental frequency, the equations (2.6) and (2.7) are valid when the deposited layer has a purely viscous character.

2.4.4.4 Viscoelastic loading

When the adsorbed layer is neither purely elastic nor purely viscous, in other words *viscoelastic*, then vibrational motion of the quartz crystal causes a slippage at the adsorbate-quartz electrode interface as a result of the force of inertia acting on the adsorbate (Figure 2.17). Thus, frequency and dissipation shifts depend not only on the viscosity and density of the liquid but also on the viscosity and density of the adsorbed viscoelastic film.

Driving several overtones of the quartz crystal, frequency and dissipation shifts can be obtained for each overtone, so multidimensional information of the quartz crystal and its adlayers can be assessed. By fitting the multiple frequency and dissipation data to a viscoelastic model, mechanical properties of adsorbed viscoelastic films can be correlated with the acoustic outcomes of the QCM system. Hence, it is possible to

calculate the mass, thickness, viscosity and elasticity of adsorbed viscoelastic layers using this models (Viitala, 2008, Bandey et. al, 1999, Voinova et. al., 1999).

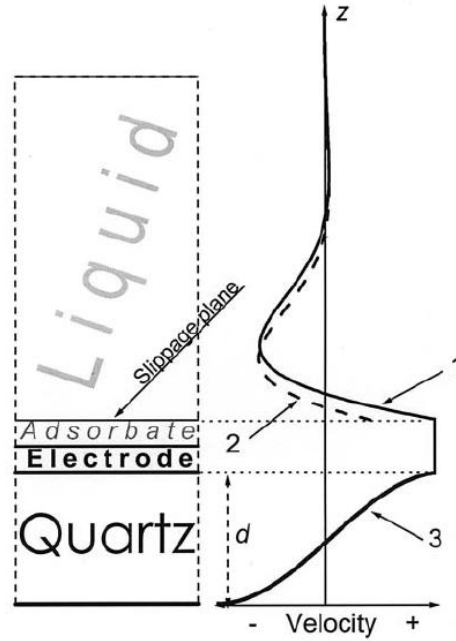


Figure 2.17: Geometry of a quartz crystal electrode covered by a viscoelastic layer oscillating in liquid and the corresponding shear velocity distribution of this system. Curve 1 and Curve 2 represents the velocity distributions at the liquid-adsorbate interface without and with slippage, respectively. Curve 3 is the velocity distribution in the quartz. The thickness of various layers does not match to scale (Tsionsky et. al., 2004).

Among theoretical models, Voight-based viscoelastic model as a continuum mechanics approach is often used together with QCM-D technology. The principle of the Voight-based viscoelastic model relies on the balance of applied stress and ensuing relaxation processes (Rodahl et. al., 1995). When the driving power to a quartz oscillator is switched off at $t = 0$, the voltage over the crystal, U , decays as an exponentially damped sinusoidal:

$$U(t) = U_0 e^{-t/\tau} \sin(2\pi f t + \phi), t \geq 0 \quad (2.8)$$

Where τ is the decay time constant and ϕ is the phase. The dissipation factor is inversely proportional to τ :

$$D = 1/\pi f \tau \quad (2.9)$$

By periodically connecting and disconnecting to/from the driving power of the QCM oscillator, the exponential decay of the signal is recorded (Figure 2.18) and fitted to equation (2.8). From the fit, both the resonant frequency and the dissipation factor

are extracted simultaneously (Rodahl et. al., 1996, Voinova et. al., 1999, Nicu and Leichle, 2008).

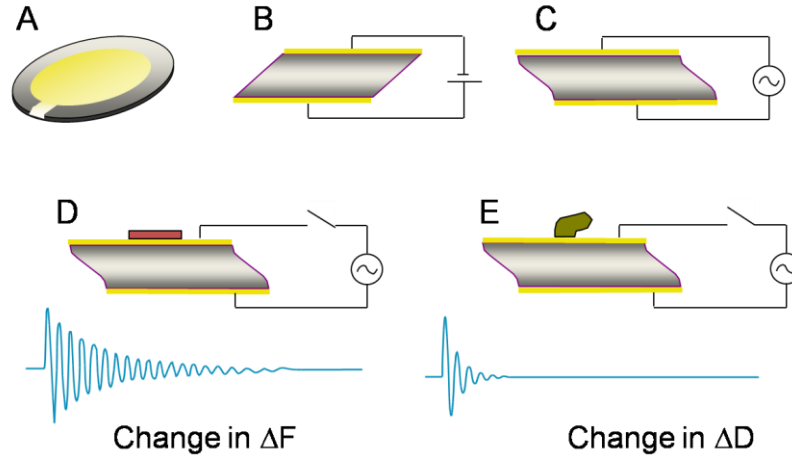


Figure 2.18: Schematic illustration of the working principle of QCM-D technique (A) The quartz crystal used in the QCM-D. (B) Applying of the direct current to the crystal. (C) Applying alternative current to the quartz crystal. (D) The change in the frequency upon adsorption of a layer onto the quartz crystal. (E) The change in the dissipation upon adsorption of a viscoelastic layer on to the quartz crystal.

The relaxation rates are assumed to be the intrinsic properties of the viscoelastic adsorbed layer, whereas the amount of applied stress is determined experimentally (Voinova et. al., 1999). In this model, ΔF and ΔD of an adsorbed layer can be expressed as following:

$$\Delta F \approx -\frac{1}{2\pi \rho_0 h_0} \left\{ \frac{\eta_3}{\rho_3} + h_1 \omega - 2h_1 \left(\frac{\eta_3}{\rho_3} \right)^2 \frac{\eta_1 \omega^2}{\mu_1^2 + \omega^2 \eta_1^2} \right\} \quad (2.10)$$

$$\Delta D \approx -\frac{1}{\pi f \rho_0 h_0} \left\{ \frac{\eta_3}{\rho_3} + 2h_1 \left(\frac{\eta_3}{\rho_3} \right)^2 \frac{\eta_1 \omega^2}{\mu_1^2 + \omega^2 \eta_1^2} \right\} \quad (2.11)$$

where h_0 and ρ_0 are the thickness and the density of the quartz crystal, respectively; η_3 is the viscosity of the bulk solution, $\delta_3 [= (2\eta_3 / \rho_3 \omega)]$ is the viscous penetration depth of the shear wave in the bulk solution and ω is the angular frequency of the oscillation; h_1 , ρ_1 , η_1 , μ_1 refer to the thickness, density, viscosity and elastic shear modulus of the adsorbed viscoelastic layer. Unknown parameters are obtained by fitting the simultaneously measured ΔF and ΔD data at the fundamental resonant

frequency and its overtones to the equations (2.10) and (2.11) (Rodahl et. al., 1996, Voinova et. al., 1999, Fang et. al., 2009).

Another strategy is to represent the QCM system as an equivalent electrical circuit and correlate the measured electrical characteristics of the viscoelastic film deposited on quartz with the mechanical properties of the film. This model is called as *equivalent circuit model*. The electrical properties of the quartz crystal and its adlayers are determined by impedance analysis method.

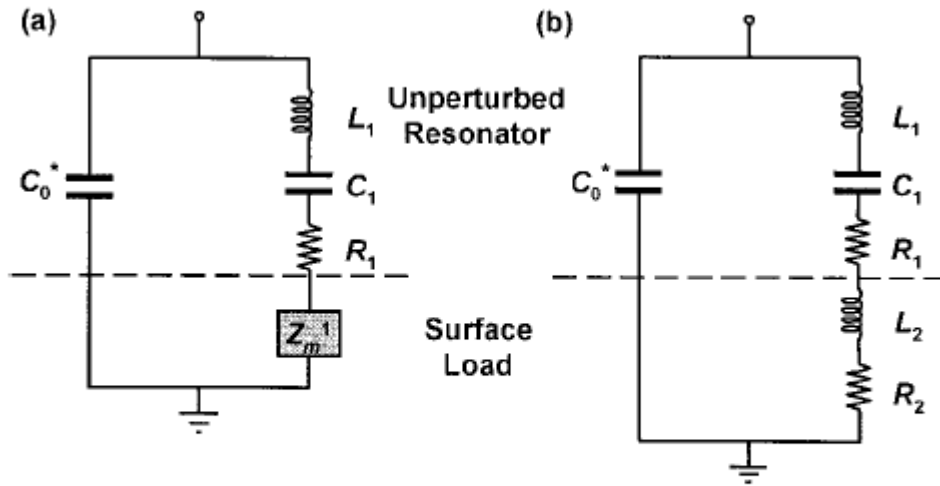


Figure 2.19: Modified Butterworth – Van Dyke equivalent circuit with (a) a complex surface impedance element Z_m and (b) a motional inductance, L_2 , and resistance, R_2 (adapted from Bandey et. al, 1999).

In this method, the quartz crystal is connected to an impedance analyzer, which applies an alternating voltage at various frequencies across the crystal. Measuring the current as a function of applied potential, the magnitude and phase of the impedance are obtained. The impedance spectrum, which consists of impedance-frequency and phase-frequency curves, can be fitted to the unmodified and modified Butterworth – Van Dyke (BVD) equivalent circuits shown in Figure 2.19, where the unloaded and loaded quartz crystal near resonance are modeled, respectively.

In unmodified BVD equivalent circuit (Figure 2.19(a)), there are two parallel *arms*. At one arm, a static capacitance C_0 represents the insulating properties of quartz material between the two electrodes. On the other arm, namely *motional arm*, a capacitor, C_1 , a resistor, R_1 and an inductor L_1 are present in series. Motional arm represents the coupling between electrical and mechanical properties of the piezoelectric quartz crystal; C_1 is referred as the elastic energy stored when the crystal oscillates, R_1 represents the loss of energy to the environment or through

thermal dissipation and L_1 is ascribed for the inertial energy of the oscillating crystal. Near resonance, the motional arm dominates the crystal's admittance, which is the reciprocal of impedance. If the crystal oscillates away from resonance, then the static capacitance dominates the admittance. Martin *et. al.*, modified the BVD equivalent circuit by the addition of electrical elements in the motional arm as shown in Figure 2.19(b) in order to represent the load on the crystal. L_2 and R_2 are representing the viscous load, where L_3 describes the mass load (Martin *et. al.*, 2000). Each of electrical elements in the modified circuit is related to the physical characteristics of the quartz, and the liquid through the following expressions:

$$C_0 = \frac{\varepsilon_{22}A}{t_q} \quad (2.12)$$

$$C_1 = \frac{8K_0^2 C_0}{n^2 \pi^2} \quad (2.13)$$

$$L_1 = \frac{1}{\omega_s^2 C_1} \quad (2.14)$$

$$R_1 = \frac{\eta_q}{\mu_q C_1} \left(\frac{\omega}{\omega_s} \right)^2 \quad (2.15)$$

$$L_2 = \frac{\omega_s L_1}{n\pi} \sqrt{\frac{2\rho_l \eta_l}{\omega \mu_q \rho_q}} \quad (2.16)$$

$$R_2 = \frac{\omega_s L_1}{n\pi} \sqrt{\frac{2\omega \rho_l \eta_l}{\mu_q \rho_q}} \quad (2.17)$$

$$L_3 = \frac{2\omega_s L_1 \Delta m}{n\pi \sqrt{(\mu_q \rho_q)}} \quad (2.18)$$

$$K_0 = \frac{e_{26}}{\sqrt{(\mu_q \varepsilon_{22})}} \quad (2.19)$$

In the above expressions A refers to the active area of the quartz crystal, ω_s and ω represent the angular series resonant frequencies of the motional arm from the unmodified and modified (loaded) BVD-equivalent circuits at electrical resonance, respectively, e_{26} is the piezoelectric stress constant and ε_{22} ascribes the permittivity of quartz. Additionally, an extra parallel capacitance is added to the BVD model for

parasitic capacitance, C_p , which arises from the geometry of the electrodes and the oscillator circuit (Patel, 2007).

The measured admittance is fitted to the electrical elements in the modified BVD model through the following expression:

$$Y = j\omega(C_0 + C_p) + \frac{1}{Z_m} \quad (2.20)$$

Where Y is the admittance of the equivalent circuit, Z_m is the impedance of the motional arm and j is the imaginary number. Admittance curve is a complex quantity; the real part is correlated with the magnitude and the imaginary part is correlated with the phase of the measured admittance. By the way, the expression of the motional arm is as follows:

$$Z_m = (R_1 + R_2) + j\omega(L_1 + L_2 + L_3) + \frac{1}{j\omega C_1} \quad (2.21)$$

So far, measured admittance and impedance curves were converted to their electrical counterparts according to the modified BDV model. Collecting a series of data from admittance and impedance curves and fitting those to the equation 2.21 enables to extract C_1 , R_2 and L_3 parameters. These parameters can be further used to calculate frequency and dissipation values according to the following equations:

$$f = \frac{1}{2\pi\sqrt{L_3 C_1}} \quad (2.22)$$

$$Q = \frac{1}{D} = \frac{2\pi f L_3}{R_2} \quad (2.23)$$

Generally, information from the frequency and dissipation shifts is sufficient to get a qualitative understanding about the processes happening at the quartz crystal surface. However, if more quantitative information is required such as the thickness, viscosity, density and elastic modulus of adsorbed viscoelastic films on the quartz crystal, then the measured electrical parameters have to be converted to mechanical motional impedances originating from the load on a quartz crystal (Viitala, 2008).

The mechanical load on a quartz resonator is represented by surface mechanical impedance, Z_s , which equals the ratio of surface stress to particle velocity at the quartz surface:

$$Z_s = \frac{T_{xy}}{v_x} \Big|_{y=0} \quad (2.24)$$

where T_{xy} is the sinusoidal steady-state shear stress imposed on the contacting medium by the quartz crystal, and v_x is the resulting x-directed surface shear particle velocity. The nature of loading will directly change the surface mechanical impedance. Following expressions represent liquid and viscoelastic loadings:

$$Z_s^{liquid} = \left(\frac{\omega \rho_l \eta_l}{2} \right)^{1/2} (1 + j) \quad (2.25)$$

$$Z_s = Z_s^{film} \left[\frac{Z_s^{liquid} \cosh(\gamma h_f) + Z_s^{film} \sinh(\gamma h_f)}{Z_s^{film} \cosh(\gamma h_f) + Z_s^{liquid} \sinh(\gamma h_f)} \right] \quad (2.26)$$

where $Z_s^{film} = (\rho_f G)^{1/2}$ is the surface mechanical impedance of the viscoelastic film, Z_s^{liquid} is the surface mechanical impedance of a Newtonian liquid described by the equation (2.25), $\gamma = j\omega(\rho_f G)^{1/2}$ is the complex wave propagation constant, where h_f , ρ_f and G refer to the film thickness, film density and elastic modulus of the viscoelastic layer. Using multidimensional information obtained from overtone frequencies, mechanical properties of the viscoelastic film can be extracted by fitting the measured impedance data to the equation (2.26) (Fang et. al., 2009, Viitala, 2008).

2.4.4.5 Probing protein adsorption with QCM

QCM technique is capable to directly probe the adsorption kinetics of proteins in-situ and during flow, without necessitating any labeling. One of the major advantages of the QCM system over other adsorption kinetic probing techniques is that the surface material can be fairly freely chosen without having to consider special properties such as optical transparency or reflectivity, as long as the preferred material can be deposited as a thin film onto the sensor crystals (Hook et. al., 1998, Hook et. al., 2002). However, the utility of QCM technique in protein adsorption studies was limited because QCM data interpretation in liquid environments is difficult. Complex interactions such as viscoelastic effects of the adsorbed protein layer, the trapped and hydrated water in the adsorbed molecular layer, complex dielectric response of the buffer electrolyte, variations in the acoustic coupling of the protein molecules to the

liquid should be taken into account (Hook et. al., 1998, Hook et. al, 2001). Fortunately, with the invention of QCM-D technology and the development of viscoelastic models some of these limitations were converted into advantages. For example, an apparent complication of QCM was that this technique senses the hydrodynamic mass (mass coupled with water) of the adsorbed proteins. Recently, combination of QCM with water-insensitive techniques such as ellipsometry, reflectometry and SPR provided insight into the hydration properties of proteins (capacity of water-holding, amount of trapped liquid etc.) when adsorbed on solid surfaces (Hook et. al, 2002, Zhou et. al., 2004, Bingen et. al., 2008, Edvardsson et. al. 2009). Besides, mechanical properties of adsorbed protein films such as thickness, viscosity, density and elasticity, acquired via viscoelastic models, enabled information about dynamic conformational changes in the protein structure during adsorption (Hook et. al., 2002, Wang et. al., 2007). These improvements enhanced the application of QCM technique for protein adsorption studies in the last decade.

2.5 Interfacial Electrochemistry

Protein adsorption on metal surfaces occurs at electrically charged interfaces since proteins, ionic solutions and metal surface are all charged species. In this dissertation, interfacial charge mechanisms between three distinct interfaces, namely peptide-buffer electrolyte, peptide-gold surface and gold-buffer electrolyte were investigated. Indeed, none of the present interfaces can be treated as isolated regions since they interfere with each other in both aqueous and also charged environments. Movement of solvent, ions, electrons and peptides all create new structural conditions at interfaces. For better insight, fundamental aspects of metal-electrolyte interface under non-polarized and polarized conditions are reviewed in the following subsections.

2.5.1 Metal-electrolyte interface: Electrical double layer

When an ionic conductor, e.g. an electrolyte, is in contact with an electronic conductor, e.g. a metal electrode, a phase boundary occurs. The forces operating on particles behave different in a direction toward the boundary compared with the direction toward the bulk electrolyte. In the bulk, forces are acting the same in all directions (perfect isotropy and homogeneity) whereas at a phase boundary forces

have an anisotropic behavior. Hence, the electroneutrality, a common feature of the bulk, break down at the interphase region causing to the formation of new structures between a metal electrode and electrolyte solution. At a phase boundary polar solvent molecules (usually water) form an oriented network because of their dipole character. Ions in the electrolyte and electrons in the metal adopt comprise positions demanded by the structure and charge density of both phases. New arrangements of solvent dipoles and charged species result in electrification of the interphase, so electrical field begin to operate on the particles at the boundary. This field enables the separation of charges across the electrode-electrolyte interface and formation of the so called *electrical double layer* or just *double layer* (Bockris and Reddy, 1970, Parsons, 1990).

The double layer formed at a boundary between two phases has two fundamental aspects:

- Electrical aspects (e.g. charge densities on each phase, variation of the potential with distance from the interface)
- Structural aspects (e.g. distribution of particles in the interphase region)

Generally, the metal surface carries an excess negative or positive charge which is balanced by the equal amount of unlike charges on the solution side. However, there is also a potential value for each metal surface where the excess charge is zero, which is defined as *potential of zero charge (PZC)* (Bard and Falkner, 2001). PZC of the metal surface is a fundamental aspect used to elucidate the nature of the double layer.

Another important concept for a better understanding of the double layer structure is the type of electrochemical processes occurring in the double layer. Electrochemical processes are categorized depending on the nature of current flow across the interface. When the passage of electrons leads to oxidation or reduction processes according to the Faraday's law (the amount of chemical reaction caused by the flow is proportional to the amount of electricity passed), then those processes are called as *faradaic processes*. In *non-faradaic processes* current flows only due to the variation of electrode potential and solution composition. Adsorption and desorption are usually known as non-faradaic processes. Unfortunately, it is difficult to find an electrode where only non-faradaic processes occur. Usually, both faradaic and non-faradaic processes occur concurrently at electrode-electrolyte interface. If the

faradaic process is not a determining component in the system, its contribution can be eliminated. An interface where only non-faradaic processes occurring with the application of potential can be considered as a capacitor composed of two main plates of equal surface area. One side of this capacitor is the metal surface with the charge density σ_M and the other side is the solution with the charge density σ_S , which is composed of charged species, usually ions, and oriented solvent molecules located at specific distances from the metal surface to balance the electrode charge (Israelachvili, 1992).

Since the aforementioned processes occur at the molecular scale, it is very difficult to observe and understand what is happening inside the double layer region. Thus, several models have been developed for better insight.

2.5.2 Models for double layer structure

The first structural model for double layer arose from the work of Helmholtz and Perrin. They thought that any excess charge on a metallic phase resides strictly at the surface, and therefore, proposed that the counter-charge in solution also resides at the surface. According to their model, the structure of the double layer resembles a *parallel-plate condenser*; two sheets of rigidly fixed charges bearing opposite polarity, separated by a distance of molecular order. The relationship between the stored charge density, σ , and the potential drop, V , between two plates was considered as follows ((Bockris and Reddy, 1970, Bard and Falkner, 2001):

$$\sigma = \frac{\varepsilon_0}{d} V \quad (2.27)$$

where ε is the dielectric constant of the medium, ε_0 is the permittivity of free space and d is the interplate spacing. The differential capacitance is therefore:

$$\frac{\partial \sigma}{\partial V} = C_d = \frac{\varepsilon_0}{d} \quad (2.28)$$

This model predicted a constant capacity which does not change with the variation of potential. However, experimental results contradicted with this model. Surface tension measurements for mercury electrodes have already demonstrated that the differential capacitance of the double layer has a potential dependent nature obtained by double differentiation of electrocapillary curves (Bard and Falkner, 2001).

The second model was proposed by Gouy and Chapman. The idea was that charged species at the solution side of the double layer should not be fixed, whereas the charge in the metallic phase is confined to the surface. Gouy and Chapman independently predicted a diffuse layer model and offered a statistical mechanical approach for its description. According to the theory, the thickness of the double layer was extended in a series of laminae and the ions in the solution behave as point charges. The charge density in each lamina was expressed in terms of Poisson-Boltzmann where potential drop varies with distance from the interface (Figure 2.20):

$$\frac{\partial \Psi}{\partial x} = - \left(\frac{32 \pi k T n^0}{\epsilon} \right)^{1/2} \sinh \frac{ze_0 \Psi_x}{2kT} \quad (2.29)$$

where Ψ is the electrostatic potential, n_0 is the concentration of species in the bulk solution, k is the Boltzmann constant, z is the valence of the species, e_0 is the electronic charge and T is the temperature in Kelvin (K).

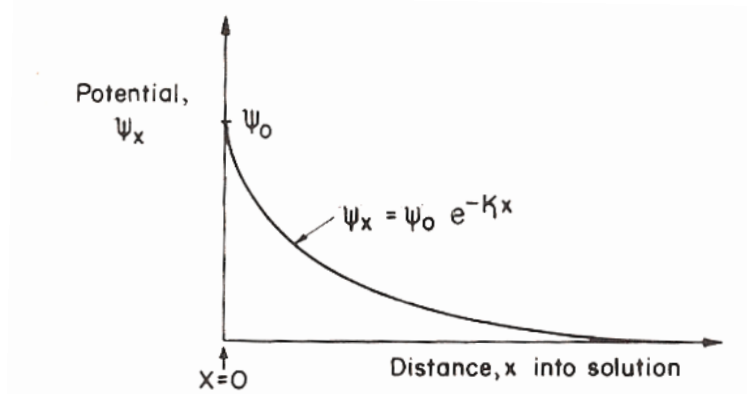


Figure 2.20: The distance variation of the potential in the diffuse charge region (Bockris and Reddy, 1970).

In this model, the total diffuse charge density, q_d , was thought to be scattered under the interplay of thermal and electrical forces, and therefore, assumed to be contained in a Gaussian box:

$$q_d = \frac{\epsilon}{4\pi} \frac{\partial \Psi}{\partial x} \quad (2.30)$$

Combining equations (2.29) and (2.30), q_d can be expressed as follows:

$$q_d = -2 \left(\frac{\epsilon k T n^0}{2\pi} \right)^{1/2} \sinh \frac{ze_0 \Psi_x}{2kT} \quad (2.31)$$

As the metal surface charge density, q_M , is equal to the opposite charge of q_d at $x=0$, q_M can be defined as follows:

$$q_M = -q_d = 2 \left(\frac{\epsilon k T n^0}{2\pi} \right)^{1/2} \sinh \frac{ze_0 \Psi_0}{2kT} \quad (2.32)$$

The differential capacity can be calculated easily assuming that $\Psi_M = \Psi_0$ at $x=0$:

$$C = \frac{\partial q_M}{\partial \Psi_M} = \left(\frac{\epsilon z^2 e_0^2 n^0}{2\pi k T} \right)^{1/2} \cosh \frac{ze_0 \Psi_M}{kT} \quad (2.33)$$

As the cosh function gives inverted parabolas, the capacitance should demonstrate an inverted parabola dependence on the potential across the interface according to Gouy and Chapman theory. The predicted V-shaped capacitance curve resembles to the observed behavior of experimentally obtained differential capacitance functions at low electrolyte concentrations and at potentials in the vicinity of PZC. However, experimental findings show a flattening in capacitance at more extreme potentials and the valley at the PZC disappears at high electrolyte concentrations (Figure 2.21).

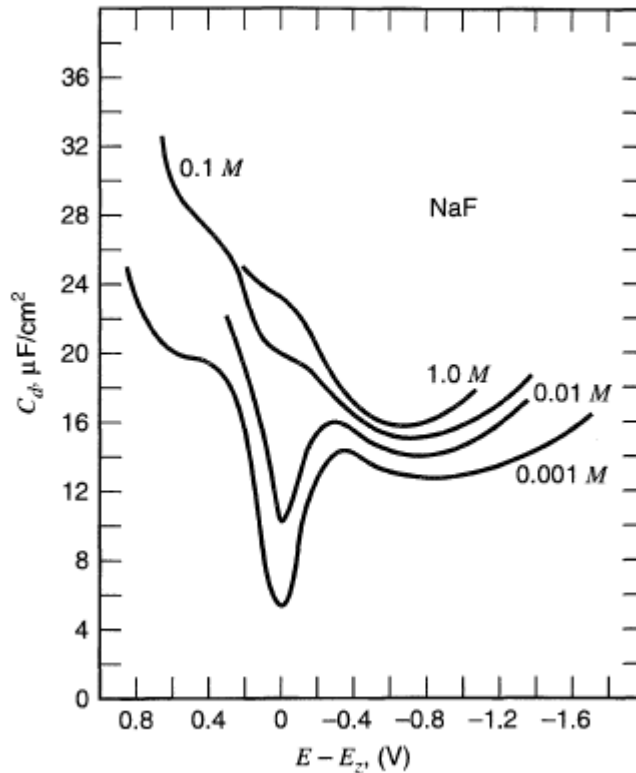


Figure 2.21: Experimental plots of differential capacitance versus potential at different solution concentrations (Bard and Falkner, 2001).

Moreover, the actual capacitance is usually much lower than the predicted value. Besides its failures, this theory has historical importance. It was one of the first treatments of properties of condensed systems by statistical mechanics and paved the way for Debye and Hückel's theory of electrolytes (Bockris and Reddy, 1970, Bard and Falkner, 2001).

The *diffuse charge* model was an antithesis of *parallel-plate* model; the particles were scattered so much that only scattered particles took place in the interphase region, and not any fixed charges within a distance from the metal electrode. However, in reality, there is interplay between the tendency of electrostatic attraction and repulsion forces to fix the positions of the charge carriers and the tendency of thermal processes to randomize them. Because of this trade-off, a finite thickness has to be defined for the double layer.

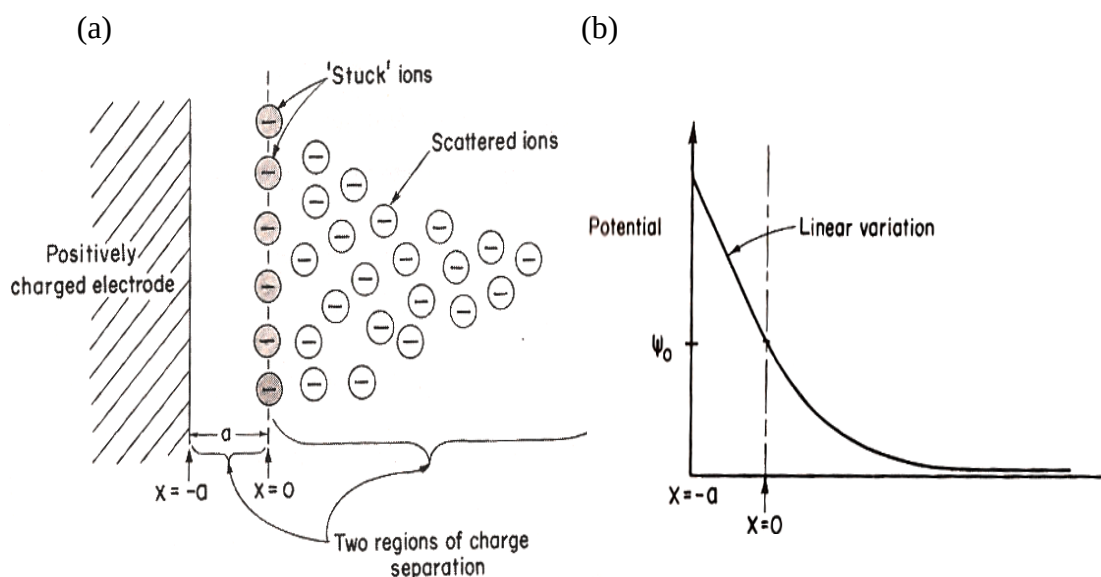


Figure 2.22: (a) Illustration of the Stern model with a layer of 'stuck' ions and the remaining ions in scattered form (b) The potential variation according to the Stern model (adapted from Bockris and Reddy, 1970).

Stern achieved to combine the *parallel-plate* model and the *diffuse charge* model and proposed a more realistic model, which showed a high consistency with experimental data. Stern postulated that ions could not come closer than a certain critical distance, a , from the metal electrode because of their ion-centers. Therefore, some ions are stuck to the electrode within a distance, a , whereas the remaining amount of ions are spread out in the solution. Hence, two regions of charge separation exist in the solution phase as shown in Figure 2.22(a) (Bard and Falkner, 2001).

Two distinct regions of charge separation lead to two different potential drops, which are occurring at the interface (Figure 2.22(b)); a linear region corresponding to the ions stuck in the interface and an exponential region corresponding to the ions which are under the combined influence of the ordering electrical and the disordering thermal forces. The concept of two potential drops emerges an interesting result from the Stern model: The total capacitance of the double layer can be given by the Helmholtz-Perrin and Gouy-Chapman capacities in series:

$$\frac{1}{C} = \frac{1}{C_H} + \frac{1}{C_G} \quad (2.34)$$

According to the equation (2.34), at high electrolyte concentrations C_G becomes large, while C_H does not change, which means that $1/C_G$ becomes smaller compared to $1/C_H$. Thus, in sufficiently concentrated electrolytes, the capacity of the interface is equal to the capacity of the Helmholtz-Perrin parallel plate model. At high electrolyte concentrations, most of the solution charge moves into the Helmholtz plane, few charge is scattered in the Gouy-Chapman diffuse layer. At low concentrations, C_G is low and therefore $1/C_G$ is larger than $1/C_H$, meaning that electrified interface behaves like Gouy-Chapman diffuse charge model. Less charge moves into the Helmholtz plane, while most of the solution charge is scattered in the diffuse layer.

2.5.3 Polarization of the double layer

In systems, in which one of the phases, e.g. metal electrode, is connected to an external source of charge, the electrification of the double layer can be conceived as follows:

- 1) Flow of charge from outside source into one phase (in this case metal electrode)
- 2) Charging of one phase
- 3) Redistribution of the electrolyte particles at the interface
- 4) Charge separation across the interface
- 5) Development of potential difference across the interface

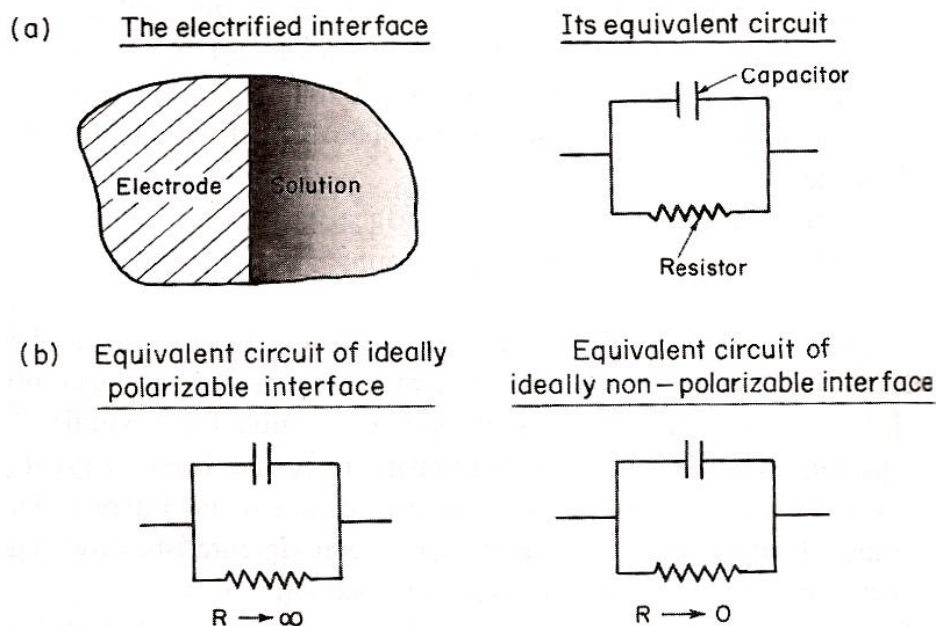


Figure 2.23: (a) Schematic illustration of electrified interface and its corresponding equivalent circuit (b) The difference between polarizable and non-polarizable interfaces in the equivalent circuit model.

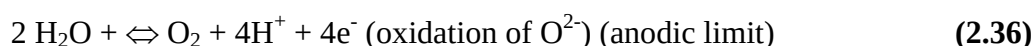
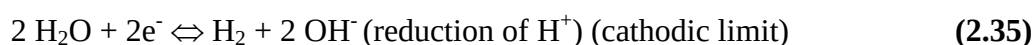
In fact, these processes occur almost simultaneously. Here, one needs to be distinguishing between polarizable and nonpolarizable electrodes. If the electrical resistance of the electrode against current flow is very high, then the capacitor charges up to the value of the potential difference assessed by the source. In this case, the electrode behaves as a *polarizable* electrode. If the resistance is low, then any attempt to charge the capacitor is compensated by leakage of charge through the path of low resistance, which is the behavior of a *nonpolarizable* electrode (Figure 2.23).

Electrochemical processes, which are under investigation, take usually on polarizable electrodes. However, nonpolarizable electrodes play an important role while measuring the potential differences across the polarizable interface. Hence, changes in the potential difference across a polarizable interface can only be measured when it is connected to a nonpolarizable interface that cannot be affected from charging processes. Since reference electrodes bear nonpolarizable interfaces, they are used to measure the potential difference of an electrochemical cell. The requirement of another electrode for potential difference measurements stems from the impossibility of measuring the absolute potential difference at a single electrified interface. Only,

the potential difference between two electrode-electrolyte *interfaces* is measurable, which is a relative quantity (Bockris and Reddy, 1970, Schmickler, 1996)

In most of the electrochemical studies, the conventional three-electrode configuration is used in order to polarize the double layer. In this setup, the working electrode is the metallic surface where the electrochemical processes are observed, the counter electrode takes up the current and thus, allows the passage of current across the cell and the reference electrode, which bears a constant potential, serves to measure the potential difference of the cell (Schmickler, 1996).

The potential range within which the working electrode is polarizable is called as *potential window*. The interval of potential window depends on the type of electrolyte and electrode material and the decomposition characteristics of the electrolyte. For example, in pure water the potential window is limited by reductive and oxidative decompositions of water:



$$E(\text{H}^+/\text{H}_2) = -0.059 \text{ pH V vs SHE} \quad (2.37)$$

$$E(\text{OH}^-/\text{O}_2) = 1.23 - 0.059 \text{ pH V vs SHE} \quad (2.38)$$

Before carrying out voltammetric experiments, determination of available potential window at the present solid-liquid interface is indispensable since no peaks of undesirable impurities should be observed in this potential range. Working at potentials, where some undesired reduction/oxidation processes occur, may cause that unwanted products generated at the potential limits may interfere the system under investigation (Scholz, 2002, Schmickler, 1996).

2.5.4 Structure of an electrified interface in aqueous solutions

If an electrode is immersed in an aqueous solution, then the electrode surface is largely (at least 70%) covered with adsorbed water molecules. Since water molecules have dipole character, they induce charges on metal surfaces leading to dipole-dipole interaction between the metal surface and the water molecules. Dispersion forces and other forces, which induce chemical bonding, operate also in between the metal and water molecules. Consequently, even an uncharged electrode has affinity for water

molecules. Hence, adsorbed water molecules may form ordered structures on metal electrodes (Lipkowski and Ross, 1993).

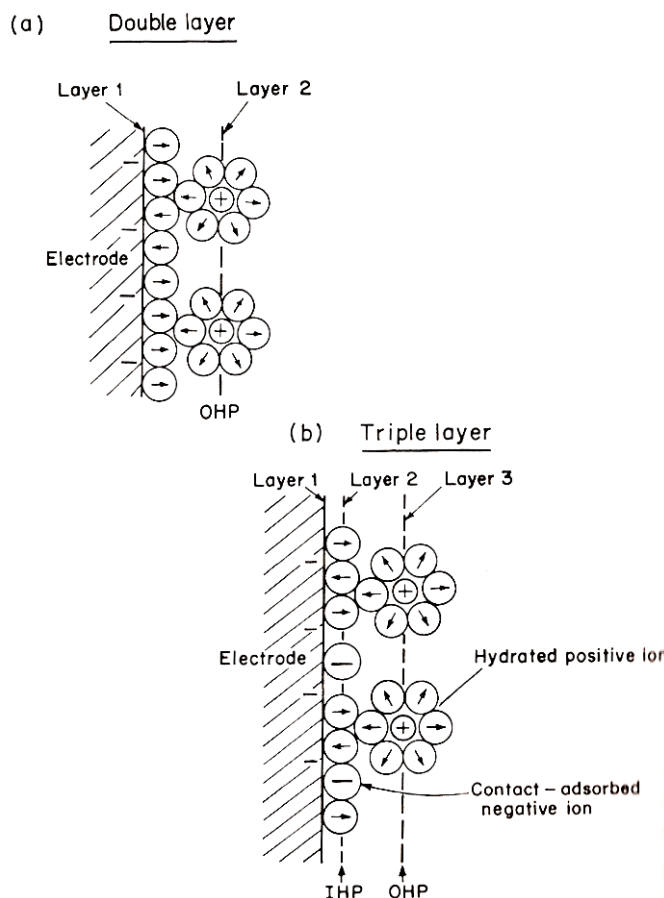


Figure 2.24: A schematic representation of the structure of an electrified interface. Formation of (a) double layer (b) triple layer (adapted from Bockris and Reddy, 1970).

If the metal surface is charged, this charge will stimulate the orientation of the water molecules since dipoles tend to align according to the direction of the electrical field. Ions may also approach to the electrode surface upon electrical field. Here, it is important to mention that ions in aqueous solutions are surrounded by water dipoles. Hydration ratio depends on the radius of the ions. Smaller ions like Na^+ , K^+ , F^- are more tightly hydrated than the larger ions such as Cl^- , Br^- , I^- , so they cannot lose their primary hydration sheath easily. Water molecules in the hydration layer (very close to the metal surface) and in the primary hydration sheath of the ions (surrounding the ion) keep the hydrated ions in a certain distance away from the metal surface. The theoretical plane drawn through the centers of hydrated ions is called as *outer Helmholtz plane (OHP)*, which defines the boundary of the double layer (Figure 2.24(a)). However, in some cases, the double layer (electrified interface) behaves like

a triple layer. It is energetically favorable that larger ions release their hydration sheaths and contact adsorb to the charged metal surface through replacement with a number of water molecules. The theoretical plane drawn through the centers of contact adsorbed ions is called as *inner Helmholtz plane (IHP)*, which defines the first boundary, whereas the OHP refers to the second boundary in the triple layer (Figure 2.24(b)) (Bockris and Reddy, 1970, Kolb, 1986, Parsons, 1990).

It can be seen from (Figure 2.24(b)), that the negatively charged ions are contact adsorbed to the negatively charged metal surface. At first, this phenomenon is called as *specific* adsorption. The reason for naming it *specific* lies on the unexpected behavior of ions; they do not obey to Coulombic rules and adsorb even to like charged metal surfaces. Now, it is known, that not only Coulombic forces but also other forces like dispersion forces or attraction of π - π orbital overlaps could operate in the double layer region. Therefore, the phenomenon is ascribed as *contact adsorption*, which is still debated with the terminology *specific adsorption* (Bockris and Reddy, 1970, Parsons, 1990, Scholz, 2002).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Solid state peptide synthesis

3rGBP₁ was synthesized using an automated solid-phase peptide synthesizer (CS336X, CSBio Inc., Menlo Park, CA). Standard Fmoc solid phase peptide synthesis technique was employed. The -N or -C termini of the peptide was not blocked during synthesis. After synthesis, the peptide was purified by C-18 reverse phase liquid chromatography (RPLC) to a level >95%.

3.1.1 Preparation of peptide and protein buffers

BSA was purchased from Sigma with % 99 purity. For potential dependent experiments, BSA solutions were prepared in 10 mM phosphate buffer (3:1 K₂HPO₄-KH₂PO₄, containing 100 mM KCl) under 100, 300, 500, 1000 nM concentrations, respectively. 3rGBP₁ solutions were prepared either in 10 mM phosphate buffer (3:1 K₂HPO₄-KH₂PO₄, containing 100 mM KCl) or in ultrapure water under 200, 500, 800, 1200 nM concentrations, respectively. The pH of the solutions was adjusted to 7.4 in all potential dependent experiments. All solutions were prepared using ultrapure water (Merck & Co, Inc., USA) and degassed under vacuum before use.

Experiments concerning the variation of solution charge were only performed for 3rGBP₁ solutions. The studies on pH were conducted at 4.5, 7.4, 10.28, and 12. 1M HCl and NaOH were used to equilibrate the desired pH of the 3rGBP₁ solution. KCl content in 10 mM phosphate buffer was varied as 0, 10 and 100 mM for ionic strength dependent experiments. All solutions were prepared using ultrapure water (Merck & Co, Inc., USA) and degassed under vacuum before use.

3.1.2 Quartz crystals

AT cut quartz crystals coated with a polycrystalline gold layer of 10 nm were utilized (KSV Instruments, Norway). They have a fundamental frequency of 5 MHz, a constant factor of $17.7 \text{ ng} \cdot \text{cm}^{-2} \cdot \text{Hz}^{-1}$ and a geometric area of 0.78 cm^2 gold working surface. Before the first use, crystals were annealed using hydrogen flame annealing technique. Thereafter, they were rinsed with ultrapure water, dried under a stream of nitrogen and treated in a UV-ozone cleaner system (Novascan Inc., IA, USA) for 10 min. For removal of organic films after peptide adsorption, crystals were introduced into UV-ozone for 20 min and then exposed into a NH_4OH (28%): H_2O_2 (30%): H_2O (1:1:5 v/v/v) mixture at 75°C for 5 min, and further cleaned in a UV-Ozone chamber for 20 min.

3.1.2.1 Surface characterization

The surface morphology of annealed bare QCM slides was characterized with atomic force microscopy (Nanomagnetics Instruments) using frequency modulation non-contact mode. AFM Probes from Veeco Instruments were used for analysis. The resonance frequency interval of the probes was between 150-250 kHz. $1 \mu\text{m}^2$ images were scanned at 1 Hz at a resolution of 256x256 data points.

The crystallinity of the gold coated quartz crystal resonators used in the experiments was examined by grazing angle X-Ray diffraction method with Philips PW 3710 X2Pert $\text{CuK}\alpha$. Diffractometer including ICDD database.

3.2 Instruments and Methods

3.2.1 Electrochemical quartz crystal microbalance with impedance monitoring

A QCM-Z instrument (KSV Instruments, Norway) having a specially designed, temperature-controlled electrochemical flow cell module was used for this purpose. The cell was made of Teflon and was sealed using O-rings. The quartz crystal resonator was mounted into the cell, where only one side of the crystal was in contact with liquid. The inner volume of the electrochemical flow cell was approximately 1.5 ml. A peristaltic pump (Ismatec SA, Switzerland) was utilized to introduce the desired solutions into the cell. The flow rate was $100 \mu\text{l} / \text{min}$ in all experiments.

In potential dependent experiments, first, buffer solution was supplied into the system. After frequency stabilization, the desired potential was applied in the interval of $[-0.4, 0.4 \text{ V}]$ vs NHE to the gold surface. As polarization of the surface cause drifts at the interface, the resonance frequency might deviate; therefore frequency stabilization was required each time. Following the second frequency stabilization, 1.5 ml peptide or protein solution with desired concentration and then sufficient amount of buffer was fed into the system in order to maintain the flow and exclude all non specific binding contributions.

In pH and ionic strength dependent experiments, buffer solutions under desired conditions were supplied into the system at first. Following frequency stabilization, solutions containing desired 3rGBP₁ concentration were fed into the system under the supplied conditions. Thereafter, sufficient amount of buffer was given into the system in order to maintain the flow and exclude all non specific binding contributions.

In all experiments, electrochemical cell was entirely filled with a degassed buffer electrolyte because solution height and bubbles influence the EQCM response in the magnitude of 1-2 Hz (Tsionsky et. al., 2004), which may lead to erratic results especially when handled with small proteins like 3rGBP₁.

Changes in frequency and dissipation were recorded simultaneously associated with four overtone frequencies (15, 25, 35, and 45 MHz). These frequencies correlate with the third, fifth, seventh, and ninth harmonics ($n=3, 5, 7, 9$). The frequency shifts were normalized to the fundamental frequency by division with the overtone number n whereas the dissipation shifts were normalized by multiplication with the overtone number n . In this paper, normalized frequency and dissipation shifts of the 3rd overtone frequency were reported. Overtone frequency data from 3 to 9 were used for the viscoelastic model.

The schematic illustration of the electrochemical quartz crystal microbalance system with impedance monitoring (EQCM-Z) is shown in Figure 3.1. The QCM-Z system was connected to a potentiostat (Compactstat, Ivium Technologies, Netherlands). Measurements were conducted using a conventional three-electrode configuration. The gold coated quartz crystals were used as the working electrode, a platinum disc was utilized as the counter electrode (positioned parallel to the working electrode) and a small Dri-ref-2SH electrode (World Precision Instruments, UK) containing

KCl gel (3M KCl saturated Ag/AgCl pellets) was served as the reference electrode, which has a redox potential of -225 mV versus NHE at 25 °C. All potentials reported in this dissertation were expressed with respect to NHE.

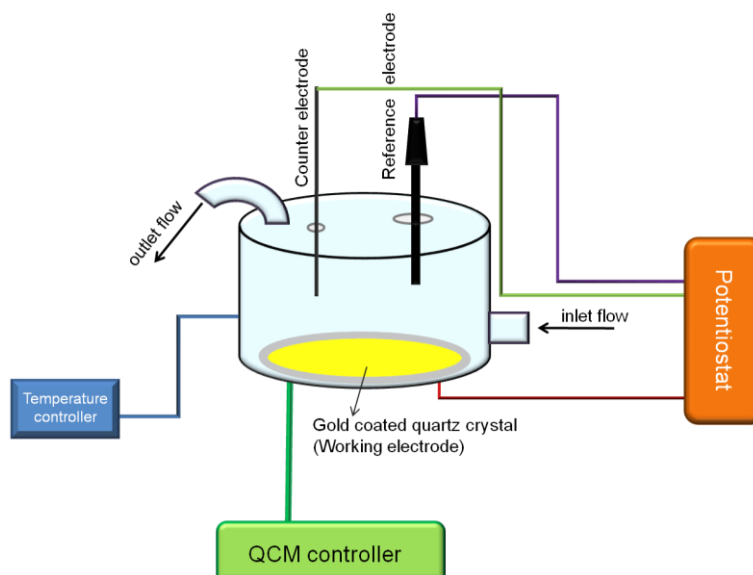


Figure 3.1: Schematic illustration of the EQCM-Z System.

Acquired data from EQCM-Z (Figure 3.2) was fitted to the two-state conformational change model (Yowler and Schengrund, 2004). The adsorbed hydrodynamic mass, the thickness and the viscoelastic properties of the peptide layer on Au {111} surface was estimated from equivalent circuit analysis model (Martin et. al., 2000) as a function of applied potential, pH and ionic strength.

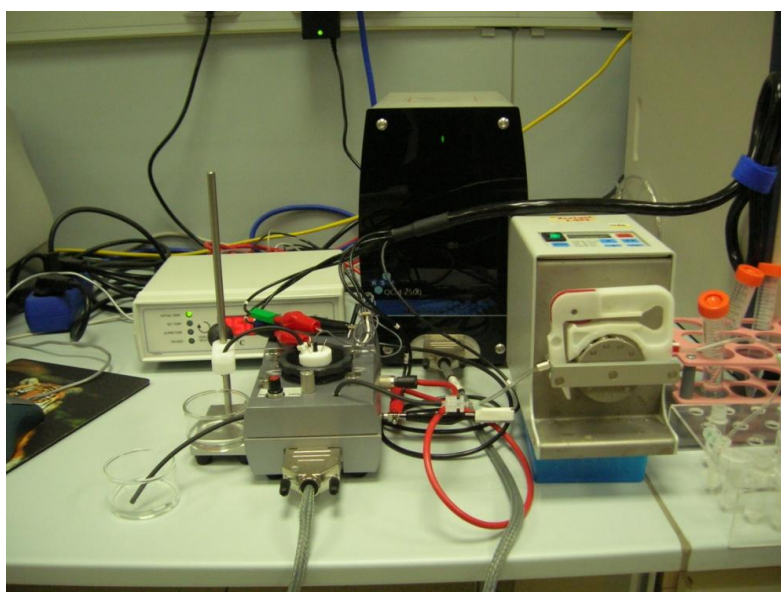


Figure 3.2: Image of EQCM-Z system used in the laboratory.

3.2.1.1 Chronoamperometry

In this thesis work, chronoamperometry technique was employed during potential dependent adsorption experiments. This electrochemical technique measures the current as a function of time at constant potential. The working electrode potential is stepped from an initial potential, which is usually the open circuit potential (OCP) of the system, to a final potential and the potentiostat system tries to keep the potential constant at this desired value (Bard and Falkner, 2001). Control experiments were performed under open circuit potential (OCP) conditions without the application of an external electric field.

3.2.1.2 Cyclic voltammetry

Cyclic voltammetry technique was performed to check the electrochemical stability interval of the metal-liquid interface under desired conditions. Usually, CV is a very powerful method for determination of the redox mechanisms, evaluation of electron transfer kinetics and explaining the surface conditions when dealing with Faradaic processes. In this technique, the potential is applied to the working electrode at a constant scan rate as a function of time. The potential is swept through a specified potential range and reverses the direction of the sweep ending at the starting potential value. The resulting current (I) is plotted as a function of applied potential (I - E curve), which is called as *cyclic voltammogram* (Bard and Falkner, 2001). In all cyclic voltammograms reported in this dissertation, the potential scan rate was 50 mV / s, where the potential scan was applied at first to the positive direction.

3.2.1.3 AC Impedance

AC impedance technique was utilized to measure the differential capacitance values in the double layer. In this technique, AC current with constant frequency and amplitude is applied to the system under a constant rate of potential change. At each potential step, differential capacitance data is extracted from the response signal, namely the AC impedance (Bard and Falkner, 2001). In all differential capacitance measurements, AC current was applied with 60 Hz frequency and 3 mV amplitude. The scan rate of applied potential was 10 mV / s.

Potential of point of zero charge (PZC) of the gold surface was obtained by measuring the differential capacitance values in buffer electrolytes containing 1 mM, 10 mM and 100 mM KCl. The differential capacitance measurements before and after 3rGBP₁ adsorption onto the gold surface were conducted in 10 mM phosphate buffer containing 100 mM KCl with a pH value of 7.4.

4. RESULTS AND DISCUSSIONS

In this dissertation, we probed the molecular recognition mechanisms of a gold binding peptide, 3rGBP₁ onto the gold surface through application of external voltage, hence variation of solution charge at metal/electrolyte interface. The differences at several charged environments were investigated quantitatively in order to elucidate the influence of interfacial charge on the specific relationship between 3rGBP₁ and the gold surface.

4.1 Characterization of the Gold Surface

At the beginning of the study, we annealed the gold coated QCM slides to obtain flat gold surface area. After annealing, we characterized the gold surface using atomic force microscopy. The line profile shown in green line on the AFM image in Figure 4.1 indicate that the roughness of the annealed gold coated QCM slide is 0.5 ± 0.3 nm meaning that the gold surface is sufficiently smooth for peptide adsorption studies.

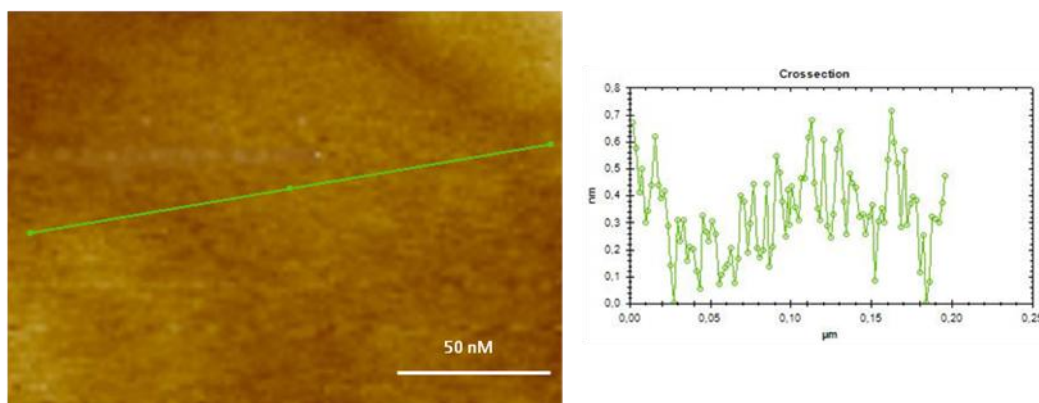


Figure 4.1: AFM image of the annealed gold coated QCM slide.

4.2 Electrochemical Characterization of Gold/Phosphate Buffer Interface

When external voltage is considered to be applied at a solid-liquid interface, at first electrochemical stability interval of the interface should be checked to eliminate any undesired chemical reactions during experiments. Thereafter, potential of point of

zero charge (PZC) of the metal surface should be determined. Once PZC is known, the sign of the metal surface charge is ensured depending on the potential value that is applied to the metal surface. Before adding the molecules of interest into the interfacial region, the contribution of ions to the solid-liquid interface should also be analyzed under positively and negatively polarized surfaces since ion migration into the double layer due to applied external voltage might lead to confusing results. Consequently, a proper characterization of the solid-liquid interface prior to desired experimental studies is a necessity for reliable results in electrochemical experiments.

4.2.1 Working window

According to potential-pH equilibrium diagram for the gold-water system at 25 °C and pH 7.4, hydrogen evolution occurs at -400 mV vs NHE whereas oxygen evolution occurs at +800 mV vs NHE (Pourbaix, 1974). Within this potential interval, gold-water system is stable for given conditions. Here, it is important to emphasize that the gold electrodes employed in the EQCM measurements may have quite different surface properties than electrodes prepared from bulk metal (e.g., wires, foils) (Tsionsky et. al., 2004). Moreover, the ions in the buffer electrolyte may induce undesirable chemical reactions. Thus, cyclic voltammetry measurements were conducted to assess the working window of gold-phosphate buffer interface used in our experimental studies.

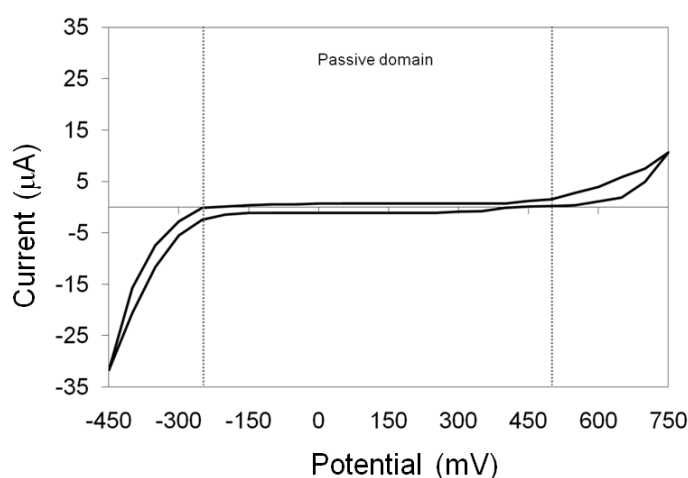


Figure 4.2: CV plot of the gold surface immersed in 100 mM KCl containing phosphate buffer with pH 7.4.

CV data were recorded in phosphate buffer solution having a pH of 7.4 after degassing the electrolyte under vacuum. As shown in Figure 4.2, the region between -280 mV and 500 mV shows no evidence of Faradaic processes occurring and is referred as the double-layer charging region or *passive domain*. At potentials greater than 500 mV the sharp increase in anodic current is due to the oxidation of oxygen and the sharp decrease in cathodic current at potentials lower than -280 mV is because of the hydrogen evolution in the system. From -280 mV up to 500 mV, the system is electrochemically stable. In other words, polarization of the gold surface within this potential interval will prevent both the gold surface and the adsorbates from being reduced or oxidized.

4.2.2 Structure of the double layer

Differential capacitance data from buffer electrolytes with decreasing salt concentration were measured to obtain the potential of point of zero charge (PZC) of the gold surface. PZC was determined from the position of the diffuse layer minimum (Lipkowski and Stolberg, 1992), which corresponds to 143 ± 10 mV vs NHE in the differential capacity curve of 1 mM KCl containing electrolyte solution. According to Figure 4.3, the minima of the differential capacity curves shift to the negative direction with an increase of the electrolyte concentration. This phenomenon is usually observed if the anions of the buffer electrolyte adsorb specifically onto the metal surface (Bockris and Reddy, 1970, Parsons, 1990). Since the buffer concentration of interest for adsorption experiments is 100 mM KCl, under this condition we should take the contact adsorbing Cl^- ions into consideration, which are probably placed in the inner Helmholtz plane (IHP) of the double layer. The hump in differential capacitance curves of 10 mM and 100 mM KCl containing solutions is also an indication for contact adsorption of anions (Bockris and Reddy, 1970).

Following the assessment of PZC, the effect of ions under negative and positive polarizations was tested in the EQCM-Z system. Constant external voltage was applied 200 seconds for each desired potential value. Applied potential intervals are indicated with dotted lines in Figure 4.4.

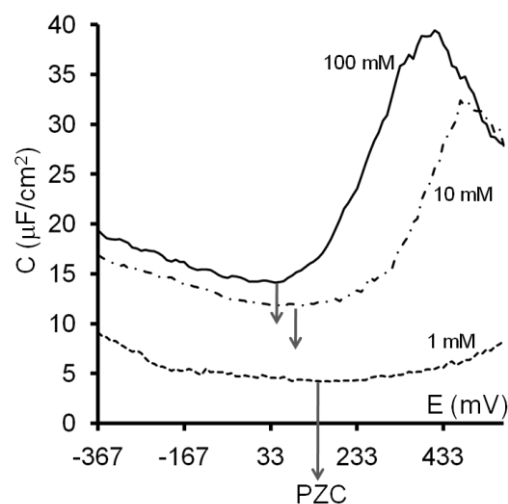


Figure 4.3: Differential capacitance curves of the gold surface in phosphate solutions containing different KCl concentrations. The gray arrows indicate the minima of differential capacity curves. PZC is referred to the potential of point of zero charge.

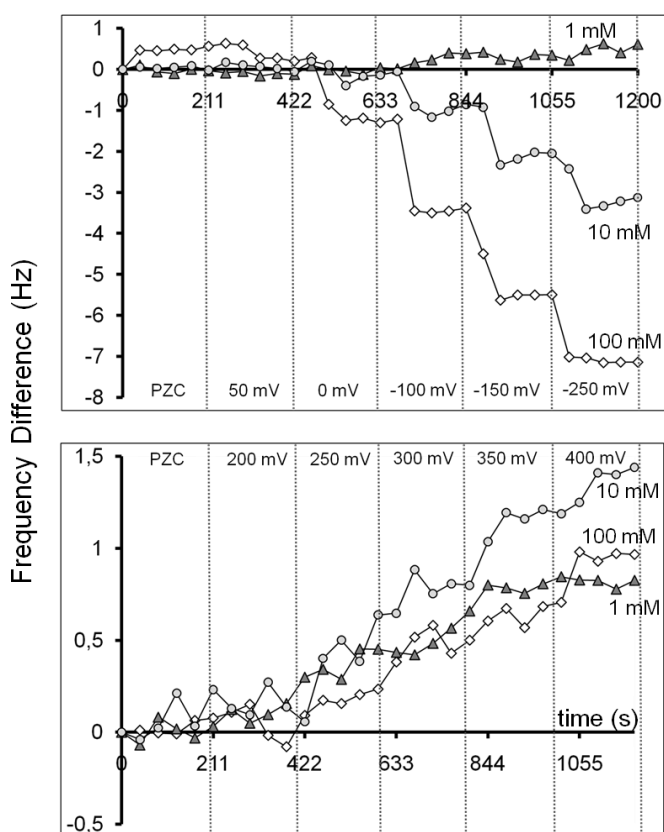


Figure 4.4: Time dependent frequency shifts in phosphate solutions containing different KCl concentrations under negative (top) and positive (bottom) polarizations.

In the top graph of Figure 4.4, the potential shifted to the negative direction away from PZC. It is expected that the K^+ ions migrate to the double layer with the

increase of cation concentration and gold surface charge density due to the electrostatic attraction. Indeed, the low concentration electrolyte (1 mM KCl) is almost not influenced from potential shifts but a strong potential dependency can be observed in solution containing 100 mM KCl; indicating ion migration to the surface and remain there during polarization since a stable frequency data can be observed. When the potential is shifted more to the negative direction, frequency shift increases indicating a higher amount of migrated ions in the double layer. In 10 mM KCl containing solution, the amount of migrated K^+ ions are less compared to the 100 mM KCl solution, and they are also not as stable as the ones in the 100 mM KCl solution.

In the lower graph of Figure 4.4, beginning from 250 mV, further potential shifts to the positive direction causes positive frequency shifts. The frequency increase in this case is very small compared to the frequency shift under negative polarization. However, an increasing trend can be observed in all differently concentrated electrolytes, even 1 mM KCl containing solution is affected from applied potentials. Actually, a decrease in frequency due to electrostatic attraction between Cl^- ions and the positively charged gold surface was expected since the molecular weights of K^+ and Cl^- ions are similar. In contrast, an increase in frequency data is apparent when the potential is shifted to 250 mV and further, which seems like a desorption process away from the gold surface. If Cl^- ions are specifically adsorbed to the metal surface as predicted from differential capacitance measurements, they will lose their charge and therefore do not contribute to the Coulombic interactions in the double layer (Parsons, 1990). Thus, they could not adsorb on or be desorbed from the gold surface upon polarization. Remaining candidates responsible for observed desorption signal may be the K^+ ions. However, since the frequency shifts are in the magnitude of 1-2 Hz, resonance frequency could have been perturbed due to increase in electrical energy upon polarization, which leads to deviation of frequency signal. To eliminate such undesired contributions, potential dependent adsorption experiments were all conducted following frequency stabilization thereafter the surface was polarized.

The conjunction of QCM and potentiostat systems may affect not only electronic but also electrochemical responses. Therefore, it is recommended to repeat at least some of the electrochemical experiments of the gold electrodes separated from the EQCM system (Tsionsky et. al., 2004). We, therefore, constitute another electrochemical cell

in the lab and repeated cyclic voltammetry and differential capacitance measurements by using saturated calomel electrode (SCE) as reference electrode. The results were in consistency with the results obtained from the electrochemical cell of the EQCM system.

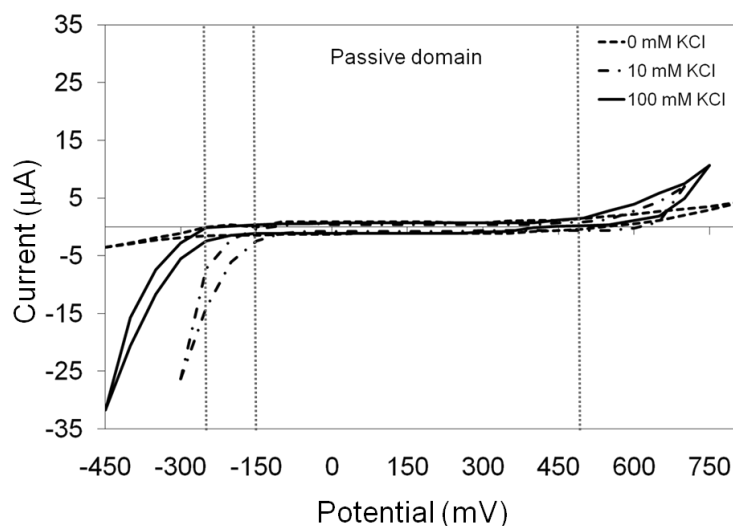


Figure 4.5: CV plots of the gold surface in phosphate solutions containing different KCl concentrations.

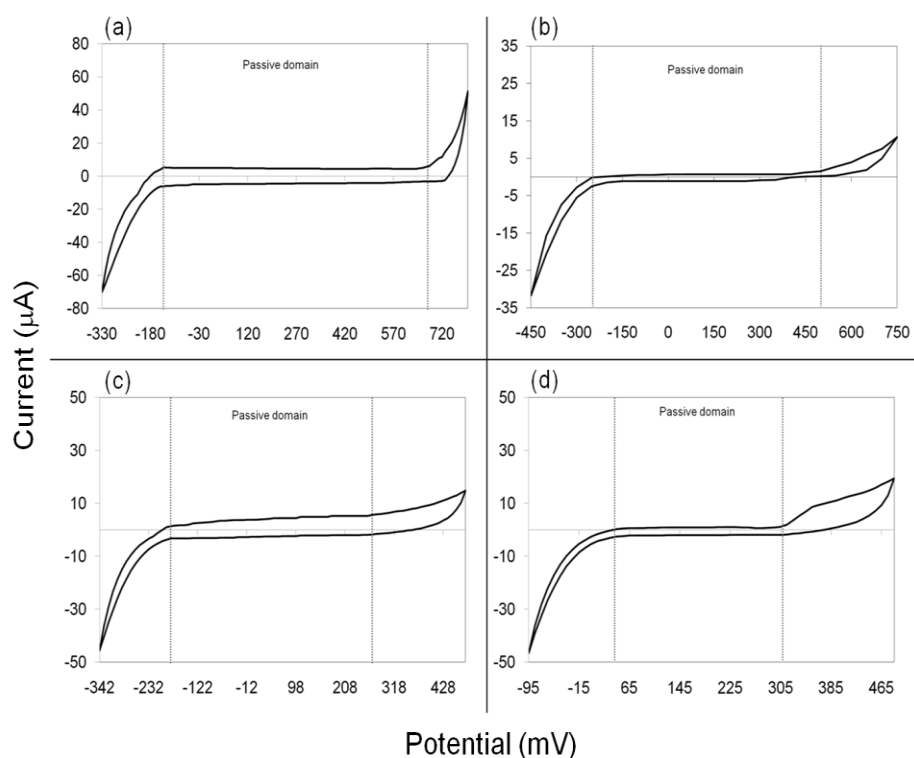


Figure 4.6: CV plots of the gold surface in phosphate solutions having a pH of (a) 4.5 (b) 7.4 (c) 10.28 (d) 12.

Further, the influence of ionic strength and pH on the electrochemical response of the gold-phosphate buffer interface was examined. Figure 4.5 indicates that the working window is contracted from [-280; 500] mV to [-150; 500] mV when the KCl concentration is 10 mM in the buffer electrolyte. On the other hand, working window is expanded up to [-450; 750] mV in solutions without KCl. Alterations in pH affected the working window tremendously compared to the influence of ionic strength on electrochemical stability.

Cyclic voltammetry measurements shown in Figure 4.6 assign that no oxidation or reduction occurs in the range between [-140; 670] mV when the solution pH is 4.5. Lowering pH from 7.4 down to 4.5 caused to extension of the double layer region from 780 mV up to 810 mV and a shift in the working window to the positive direction. At high pH values (Figure 4.6(c) & (d)), the double layer regions are observed in the following intervals [-192; 308] mV and [-55; 305] mV, respectively, indicating a contraction of the working window. CV results in different pH's demonstrate that the gold-buffer interface could compensate the increase of hydronium (H_3O^+) ions upon charging the double layer while the high amount of hydroxyl (OH^-) ions are not easily tolerated.

Open circuit potential (OCP), which is the potential of the electrochemical cell under non-polarized conditions, is another important concept for signifying the double layer properties. In electrochemical cells, open circuit potential (OCP) measures usually a mixture of metal dissolution and hydrogen evolution reactions occurring at the metal-liquid interface (Harrington and Conway, 1987). Since gold does not dissolve easily in aqueous solutions it cannot be oxidized or reduced within the double layer charging region, measured OCP values at gold-electrolyte interfaces corresponds mainly to hydrogen evolution reaction, which occurs through adsorption of H_3O^+ ions onto the metal surface and release of hydrogen from the metal surface after reduction (Bockris and Reddy, 1970).

The pH-potential diagram demonstrated in Figure 4.7 is a strong evidence for the hydrogen evolution reaction occurring on the metal surface, which is measured as OCP in our system. If the amount of H_3O^+ ions at the interface is augmented, the reaction will be favored to the hydrogen reduction causing an increase in the potential. If there are more OH^- ions than H_3O^+ ions at the interface, the reaction will tend to the opposite direction leading to a decrease in the measured potential. Indeed,

the measured OCP values were increased when the pH was lowered and decreased when the pH was raised up.

In the electrochemical cell of our QCM system, the generally measured OCP value in solutions of pH 7.4 was 73 mV vs NHE for gold-phosphate electrolyte interface. Indeed, observed OCP values was varying from one to another unused QCM slide within an interval of 10 mV above or below 73 mV because the response of the EQCM strongly depends on the morphology and roughness of the electrode surface and the fabricated slides do not have always the same surface properties. EQCM response is also highly affected from the history of the QCM electrodes (how many times they are used, how they are cleaned etc.) (Tsionsky et. al., 2004). Hence, significant differences in OCP values (shifts from 50-100 mV from 73 mV) were obtained when experiments were conducted with used slides, although they were cleaned sufficiently. To compensate the effect of these shifts, cyclic voltammetry measurements were conducted before each experiment to ensure the working window interval. Moreover, for reliable results each of the new try was performed with unused QCM slides at least once.

Since OCP of our system (73 mV) is below PZC (143 mV) of the gold surface, the surface of the gold electrode is negatively charged under non-polarized equilibrium conditions, indicating that mostly positively charged ions (mainly K^+) are accumulated in the outer Helmholtz plane (OHP) of the double layer.

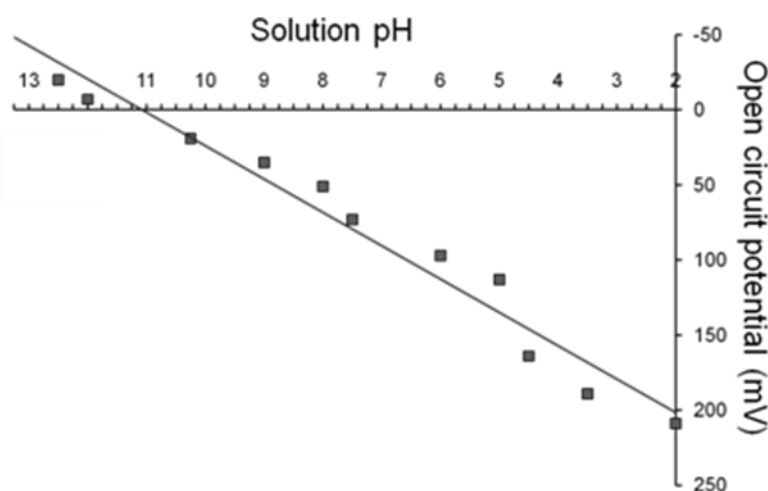


Figure 4.7: pH-potential diagram for the gold coated QCM electrode in phosphate buffer containing 100 mM KCl. The solid line is the linear fit with slope of 5.6 mV per pH unit ($R^2=0.96$).

Overall, we found out that the working window of gold-phosphate buffer interface is from [-280; 500] mV, while PZC and of the gold surface is determined as 143 ± 10 mV and OCP of the gold-phosphate buffer interface at pH 7.4 is acquired as 73 ± 10 mV. Under non-polarized conditions, the gold surface is negatively charged and Cl^- ions are specifically adsorbed on the gold surface and behave as neutral molecules in the IHP, while K^+ ions most probably oriented in the OHP of the double layer. In concentrated solutions, high amount of K^+ ions migrate onto the gold surface under negative polarization. Upon polarization, H_3O^+ ions are more welcome into the double layer region than OH^- ions.

4.2.3 Effect of 3rGBP₁ addition into gold-phosphate buffer interface

Evolution of OCP is dependent on the relative rates of the charging and discharging processes at the metal-liquid interface. If organic molecules are added to the interface, they might accelerate or inhibit these charging and discharging processes. Molecules, which cause to Faradaic processes, are called as *electroactive* molecules. Electroactive molecules alter the interfacial electrochemical properties significantly when they are added to the system. Therefore, prior to adsorption experiments under external voltage, the electrocatalytic activity of 3rGBP₁ was examined. First, OCP values before and after 3rGBP₁ adsorption were compared (Figure 4.8).

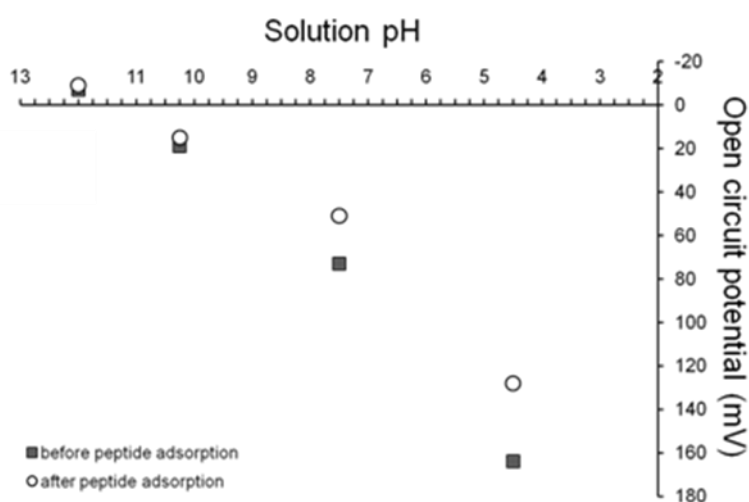


Figure 4.8: Measured OCP values before and after 3rGBP₁ adsorption on gold.

The decrease in measured OCP after peptide adsorption in Figure 4.8 signifies that the adsorbed protein layer hinders the hydrogen adsorption onto the gold surface. This result is consistent with previous studies claiming that physisorbed organic

monolayers decrease the work function of the organic-gold interface (Braun et. al., 2009).

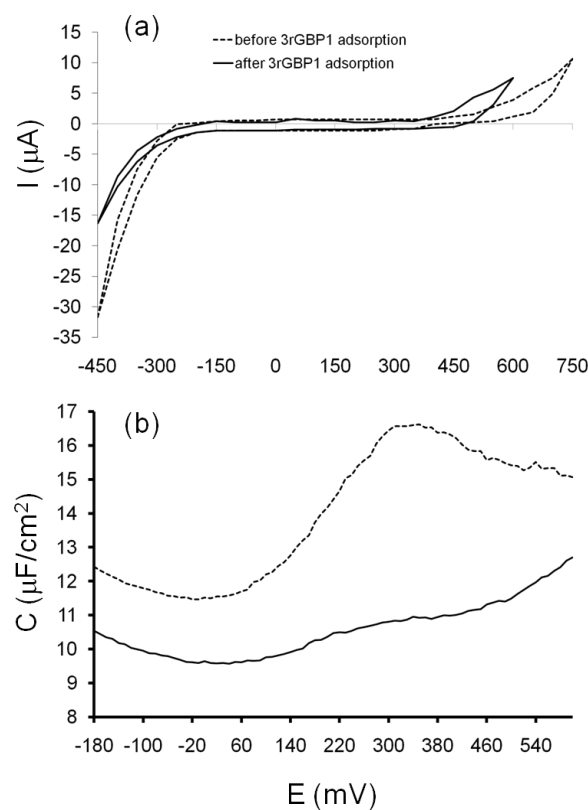


Figure 4.9: (a) CV plots (b) Differential capacity curves of the gold-phosphate buffer interface in solutions with pH 7.4 before (dashed line) and after (solid line) 3rGBP₁ adsorption on gold.

CV measurements indicate that adsorption of 3rGBP₁ does not induce any Faradaic processes at the interface (Figure 4.9(a)). According to the differential capacitance results shown in Figure 4.9(b), the charging capacity of the double layer decreases significantly after peptide adsorption. Results imply that 3rGBP₁ does not act as an electroactive molecule at the metal-electrolyte interface, on the contrary adsorbed peptide layer behaves like a blocking agent.

4.3 Probing Molecular Adsorption through Applied Potential

Following the electrochemical characterization of the gold-phosphate buffer interface, we, first investigated the interfacial charge effect between 3rGBP₁ molecules and the gold surface under applied external voltage. Hence, the gold surface was polarized in both positive and negative direction and alterations during adsorption were observed. Since previous high magnification AFM studies indicated

that the adsorption process of 3rGBP₁ on gold follows two distinct adsorption regimes (So et. al., 2009), acquired data from EQCM-Z was fitted to the modified Langmuir adsorption model, which is the combination of 1:1 Langmuir model (Karpovich and Blanchard, 1994) and two-state conformational change model (Yowler and Schengrund, 2004). Using equivalent circuit analysis model, viscoelastic properties of adsorbed 3rGBP₁ films were obtained for various surface charge densities. Next, alterations in assembly behavior of 3rGBP₁ as a function of surface charge density were discussed considering assembly rate, peptide population and hydration state of adsorbed peptide films.

Further, binding affinities of 3rGBP₁ and BSA to gold surface were compared under the same polarization conditions. BSA is chosen intentionally because it usually binds to gold substrate non-specifically and mainly through electrostatic interactions (Brusatori et. al., 2003). The idea was to distinguish between specific and non-specific effects when proteins are adsorbed on charged metal surfaces.

4.3.1 Specific recognition of 3rGBP₁ at metal/electrolyte interface

Time-dependent adsorption studies of 3rGBP₁ on gold surface under different potentials were performed at pH 7.4 and 25°C. From previous studies, it was known that 1200 nM peptide concentration enables the formation of one 3rGBP₁ layer on gold surface in approximately 20 minutes (So et. al., 2009). Hence, maximum peptide concentration was chosen as 1200 nM in order to ensure working in the monolayer range.

As mentioned above, OCP of the buffer-gold interface was determined as 73±10 mV. First, gold surface was polarized to the negative direction. Substantial changes in adsorption behavior were observed after 150-200 mV shifts from OCP where the maximum frequency shift was assessed at -167 mV. Thereafter, applied potential was shifted to positive direction and maximum frequency shift was then determined under 233 mV. Adsorption of 3rGBP₁ at -167 mV and 233 mV on gold surfaces was analyzed in detail. Actually, we expected alterations in adsorption by smaller shifts of the applied potential. However, the strong hydrophobic nature of the gold surface usually induces an ordered structure of water molecules at gold-water interface, which is different from that of bulk water. When the interaction between the metal surface and the solvent becomes stronger, the influence of the electrical field on the

structure of the surface layers becomes weaker (Tsionsky et. al., 2004). Thus, the required electrical energy, which induces changes in peptide recognition process, may have been achieved by larger potential shifts.

Next, applied potential was shifted to the limits of the passive domain in order to find out the potential E_f where the total desorption of organic molecules from metal surfaces takes place (Lipkowski and Stolberg, 1992). However, stabilizing the frequency was not achieved until shifting the potential values from both limits down to -267 mV and 333 mV, respectively. Thus, alterations in 3rGBP₁ adsorption were observed under shifted conditions. Using E-QCM system, an E_f value could not be determined meaning that 3rGBP₁ molecules did not totally desorb from the gold surface through application of external voltage within the conducted potential interval. We may mention that low desorption, which was confirmed here for 3rGBP₁, is a common feature of inorganic binding peptides (Seker et. al., 2009).

4.3.1.1 Binding kinetics

Adsorption isotherms and binding parameters at applied potentials were obtained by fitting the measured frequency shifts via QCM to the modified Langmuir adsorption model. 1:1 Langmuir adsorption model assumes that the adsorbate molecules adhere to the available sites of the given surface and form a monolayer, meantime the adsorbed molecules on the available adjacent sites are not interacting with each other (Schessler et. al., 1996). Monolayer surface coverage can be considered as a site filling procedure, where a dynamic process (adsorption is followed by desorption) takes place. At equilibrium conditions, the amount of adsorbed molecules is equal to the amount of desorbed molecules. The adsorption rate depends on the concentration of adsorbate (Karpovich and Blanchard, 1994). According to the stated assumptions, the adsorption model is given in the following expression:

$$\frac{\partial \theta}{\partial t} = k_a(1 - \theta)C - k_d\theta \quad (4.1)$$

where θ is the fraction of surface covered by the adsorbate molecules, C is the concentration of the adsorbate, k_a and k_d are the association and dissociation constants, respectively. By integrating of (4.1), we can attain the surface coverage as a function of time:

$$\theta(t) = \frac{C}{C + k_d/k_a} [1 - \exp(-|k_a C + k_d|t)] \quad (4.2)$$

As time approaches infinity at equilibrium conditions, the limit of (4.2) can be taken to calculate the surface coverage:

$$\theta(\infty) = \theta_{\max} \frac{k_a}{k_a C + k_d} = \theta_{\max} \frac{1}{C + k_d/k_a} \quad (4.3)$$

The surface coverage θ can be replaced by the signal of the applied technique, in this case the frequency shift obtained from QCM:

$$\Delta F = \Delta F_{\max} \frac{1}{C + k_d/k_a} \quad (4.4)$$

(4.4) describes the simple 1:1 Langmuir model, where k_d/k_a refers to the dissociation constant K_d . Although Langmuir adsorption model requires desorption, this model fits well to the irreversible protein adsorption data (Schessler et. al., 1996). Therefore, Langmuir model is commonly preferred in protein adsorption studies due to its simplicity. On the other hand, 3rGBP₁ molecules do not adsorb onto the gold surface in one step. The recognition process is more complex following two distinct regimes (So et. al., 2009). Therefore, we combined the simple Langmuir model with the two-state conformational change adsorption model and fitted our data to this modified Langmuir model. Conformational change adsorption model is usually applied to ligand (A) - analyte (B) interactions, where the molecules first form an initial complex (AB) and then a more stable complex (AB*), usually through conformational changes at the interface (Yowler and Schengrund, 2004):



The adsorption equilibrium constant (K_{eq}) of this model is defined as follows:

$$K_{eq} = (k_{a1} / k_{d1})(1 + k_{a2} / k_{d2}) \quad (4.6)$$

By combining both models, binding parameters can be extracted from (4.6) and (4.7):

$$\Delta F = \Delta F_{\max} \frac{1}{C + \frac{1}{(k_{d1}/k_{d1})(1+k_{d2}/k_{d2})}} = \Delta F_{\max} \frac{1}{C + \frac{1}{(K_{eq1})(1+K_{eq2})}} \quad (4.7)$$

The surface coverage; in which percentage the adsorbate covered the entire surface can also be calculated using the following expression:

$$\theta(\infty) \approx \frac{\Delta F}{\Delta F_{\max}} \approx \frac{1}{C + \frac{1}{(K_{eq1})(1+K_{eq2})}} \quad (4.8)$$

Free energies were calculated using the following expression:

$$\Delta G = -RT \ln((K_{eq1})(1 + K_{eq2})) \quad (4.9)$$

where ΔG is the binding energy (kcal/mol), R is the ideal gas constant and T is the temperature in Kelvin.

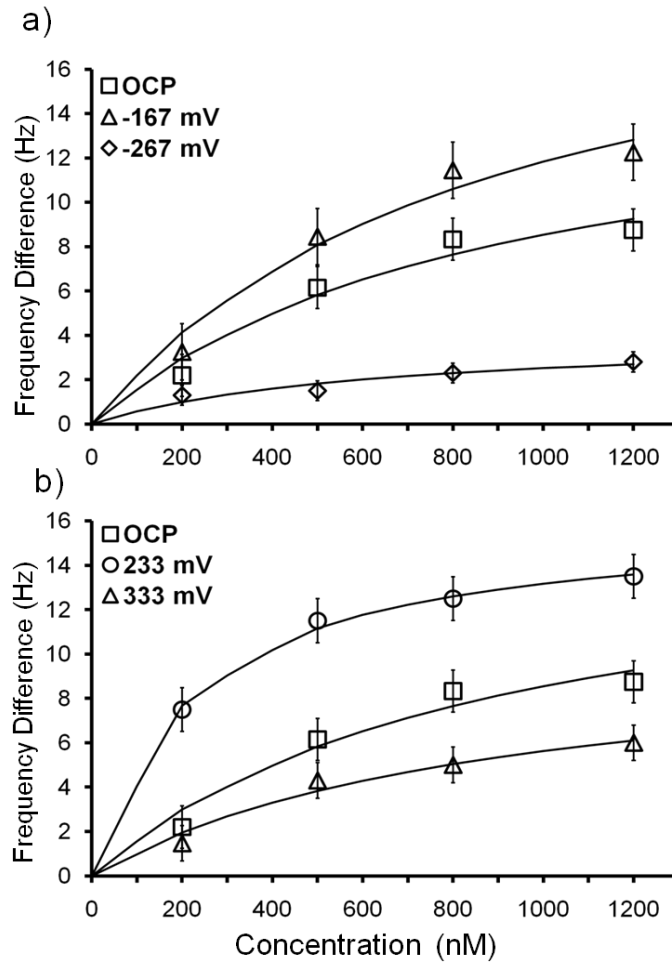


Figure 4.10: Adsorption isotherms for 3rGBP₁, obtained by EQCM-Z, at (a) negatively polarized gold surface (b) positively polarized gold surface in comparison with non-polarized gold surface (OCP), respectively.

Adsorption isotherms for 3rGBP₁ on the gold surface under applied potentials were demonstrated in Figure 4.10. Detailed output from the fitting process is listed in Table 4.1 including theoretical maximum adsorption signal shift under equilibrium conditions (ΔF_{max}), adsorption equilibrium coefficients (stepwise; K_{eq1} , K_{eq2} and total; K_{eq}) and adsorption free Energies (ΔG_{ads}) for 3rGBP₁ depending applied potentials and their corresponding surface charge densities (σ). Surface charge densities were calculated by integration of differential capacitance curve of the gold surface measured in 100 mM KCl containing phosphate solution.

Results in Figure 4.10 indicate that the adsorption behavior of 3rGBP₁ changed significantly by polarization of the gold surface; each isotherm implying to a distinct adsorption process although the same peptide concentration values were used for each experiment. Enhancement in binding was observed at surfaces polarized to -167 mV and 233 mV compared to the adsorption process under OCP. A decrease in frequency data was assessed by further shift of the applied potential to -267 mV and 333 mV respectively. Since -267 mV and 333 mV surface conditions have rather high surface charge densities (Table 4.1), the decrease in adsorption data was not surprising. On polarized metal surfaces with high positive charge density, conduction electrons are expected to recede into the metal by forming a hard wall of metal ions inhibiting the adsorbate-solid surface interactions at the interface (Schmickler, 1993). If the metal surface is highly polarized with excess negative charge, solvated cations are attracted to the surface accumulating in high amounts within the double layer, which restricts also adsorbate-solid surface interplay (Bockris and Reddy, 1970). Adsorption isotherms regarding to -267 mV and 333 mV surface conditions reveal that, in some degree, 3rGBP₁ molecules were able to overcome the stated structural limitations at the interface occurring under high surface charge densities.

Recently, concentration- and time-dependent assembly of 3rGBP₁ on gold surface were analyzed by our group using high magnification AFM (So et. al., 2009). A dynamic adsorption behavior involving multiple stages with different diffusivities was observed for 3rGBP₁ molecules. For simplification, the adsorption process was divided into two distinct regimes; peptides first form island-like structures as soon as they approach to the surface, following, these islands coalesce and a peptide network is constituted through a number of structural changes of peptide molecules. Regarding to this AFM investigation, K_{eq1} and K_{eq2} values in Table 4.1 refer to the

adsorption equilibrium constants for the formation of island-like peptide structures and the peptide network onto the gold surface, respectively.

Table 4.1: Surface Charge Density (σ), Theoretical Maximum Adsorption Signal Shift under equilibrium ($\Delta F_{\max}$), Adsorption Equilibrium Coefficients (stepwise; K_{eq1} , K_{eq2} and total; K_{eq}) and Free Energies (ΔG_{ads}) for 3rGBP₁ at applied potentials^a, respectively.

V_{applied} (mV)	σ ($\mu\text{C}/\text{cm}^2$)	$\Delta F_{\max}$ (Hz)	$K_{\text{eq1}} \times 10^4$ (M^{-1})	$K_{\text{eq2}} \times 10^1$ (M^{-1})	$K_{\text{eq}} \times 10^6$ (M^{-1})	ΔG_{ads} (kcal/mol)
333	13.60 $\pm$ 1.27	10.64 $\pm$ 1.78	6.13 $\pm$ 0.12	1.73 $\pm$ 0.18	1.12 $\pm$ 0.09	-8.21 $\pm$ 0.06
233	9.55 $\pm$ 0.73	16.05 $\pm$ 1.39	6.13 $\pm$ 0.01	7.36 $\pm$ 2.45	4.57 $\pm$ 1.50	-9.06 $\pm$ 0.30
OCP ^b	-3.03 $\pm$ 0.05	15.96 $\pm$ 1.75	6.13 $\pm$ 0.04	1.78 $\pm$ 0.45	1.15 $\pm$ 0.28	-8.22 $\pm$ 0.22
-167	-6.96 $\pm$ 0.32	16.50 $\pm$ 3.14	6.13 $\pm$ 0.19	3.58 $\pm$ 1.12	2.25 $\pm$ 0.76	-8.64 $\pm$ 0.24
-267	-	4.08 $\pm$ 2.42	6.13 $\pm$ 0.47	2.53 $\pm$ 0.51	1.61 $\pm$ 0.36	-8.43 $\pm$ 0.21

a The errors represent standard deviations.

b Open circuit potential is the potential at non-polarized condition (73 $\pm$ 10 mV).

A closer inspection of K_{eq1} and K_{eq2} values reveals that K_{eq1} data do not show dependence on the potential applied to the gold surface. The independent behavior of K_{eq1} data at various applied potentials indicates that polarization of the gold surface do not induce remarkable changes during the first adsorption regime, which was proposed as the faster diffusion controlled adsorption step in above stated AFM study (So et. al., 2009). Here, we can speculate that intrinsic diffusion rates of 3rGBP₁ molecules may be faster than the migration rates generated via applied electrical field in the system. However, additional data is required to prove this prediction.

On the other hand, alterations in K_{eq2} values under polarization affirm that the slower second adsorption step, demonstrate a potential dependent behavior. K_{eq2} under 233 mV polarization is approximately 4 times, K_{eq2} under -167 mV polarization is exactly twice and K_{eq2} under -267 mV polarization is nearly 1.5 times higher than K_{eq2} under non-polarized condition. According to the mentioned AFM study, during the second adsorption regime, 3rGBP₁ molecules coalesce and self-assembly on the gold surface forming finally a monolayer peptide film (So et. al., 2009). Obtained differences in

K_{eq2} values upon applied potential reveal that peptide network formation rate can be tuned by changing the charge density of the gold surface.

4.3.1.2 Assembly behavior of adsorbed peptide films

QCM measures the adsorbed peptide film coupled with water. Changes in dissipation value measured by QCM are generally interpreted in terms of alteration in peptide conformation or water content of the peptide layer. From the shifts in dissipation per frequency shift ($\Delta D/\Delta F$), structural changes during peptide film formation or hydrated state of the peptide film can be inferred (Hook et. al., 1998, Hook et. al., 2001, Van de Keere et. al., 2009). Thus, in this section, structural alterations during peptide assembly at various surface charge densities were investigated first by analyzing $\Delta D/\Delta F$ ratios independent from time. Next, time-dependent frequency and dissipation shifts of various peptide insertions under applied potentials were examined for additional insight. Here, adsorption data of potential of zero charge (PZC) condition was additionally tested to understand how peptide assembly change in the absence and presence of excess surface charge.

Table 4.2: Frequency and dissipation shifts (ΔF , ΔD , respectively), increase in dissipation by a unit frequency change ($\Delta D/\Delta F$) of adsorbed 3rGBP₁ molecules from 1200 nM peptide insertion under applied potentials.

V_{applied} (mV)	ΔF (Hz)	ΔD (10^{-6})	$\Delta D/\Delta F$ (10^{-6})
333	6.20±0.51	5.13±0.91	0.855±0.017
233	13.50±2.43	10.50±1.33	0.777±0.029
PZC	9.70±1.58	2.41±0.40	0.248±0.016
OCP	8.75±0.21	2.50±0.29	0.285±0.076
-167	12.25±1.30	20.00±2.24	1.632±0.091
-267	2.80±0.65	6.00±1.40	2.143±0.052

Conformational change as a result of exposure of hydrophobic regions within the protein is thought to be one of the main driving forces for protein binding to hydrophobic surfaces like gold. Thus, it is expected that at PZC, where the net surface charge of the gold substrate is zero, the main driving force in binding should be the hydrophobic interactions between 3rGBP₁ molecules and the gold surface. In aqueous solutions, proteins usually adhere to surfaces with their hydrophobic

residues because protein-surface interactions are thermodynamically favorable than protein-solution interactions. Consequently, proteins films formed through hydrophobic interactions have low water content. The least $\Delta D/\Delta F$ ratio under PZC condition (Table 4.2) renders that 3rGBP₁ film contains least water at PZC implying a more rigid peptide film. Shifting gold surface charge to the both positive and negative directions caused an increase in $\Delta D/\Delta F$ ratios indicating that excess surface charge induces conformational changes and probably attracts water molecules into the peptide film.

The values shown in Table 4.2 were assessed at saturation stages. According to the given results, $\Delta D/\Delta F$ ratios of PZC and OCP potentials seem to be close to each other. However, the $\Delta D/\Delta F$ ratios for 200 nM 3rGBP₁ insertion under OCP and PZC conditions are 0.6 and 0.19, respectively. At OCP, a decrease in the dissipation per unit frequency shift can be observed when increasing the inserted peptide amount. Exact opposite appears to be occurred under PZC surface condition. As known from other studies (Hook et. al., 1998), the reason for the decrease of $\Delta D/\Delta F$ ratios upon increased concentration is the reorientation of the peptide molecules to a more ordered structure during the adsorption process, so we can deduce that a more compact assembly of peptides occurs under non-polarized condition compared to the peptide assembly at PZC.

Trapped water is a possible cause of the large increase both in dissipation and frequency shifts, compared to that expected for bare protein mass (Hook et. al., 1998). Remarkable increase both in dissipation and frequency shifts were obtained when the gold surface was polarized to the 233 mV and -167 mV implying an increase in the hydrated state of the peptide films. By further shifting the potentials to the 333 mV and -267 mV values, still high dissipation values were observed despite the low frequency shifts. The high increase in dissipation can be attributed to the conformational changes under these conditions. Moreover, the surface charge densities at 233 mV and -267 mV are rather high since these potential values are closer to the limits of the passive domain. Therefore, high charge density under these conditions might have caused an increase in dissipation due to molecular crowding.

The time dependent shifts of frequency and dissipation values at three different applied potentials in Figure 4.11 elaborate the film formation properties from a

kinetic point of view. When the gold surface was polarized to 233 mV and thereafter 200 nM peptide was inserted into the system, peptide molecules achieved equilibrium in 500 seconds. For the same inserted peptide amount, the time-dependent frequency shift pathways under OCP and -167 mV can not be differentiated from each other. They both come to the equilibrium in approximately 700 seconds. Considering all peptide insertions, within the three surface conditions the fastest peptide film is formed at 233 mV.

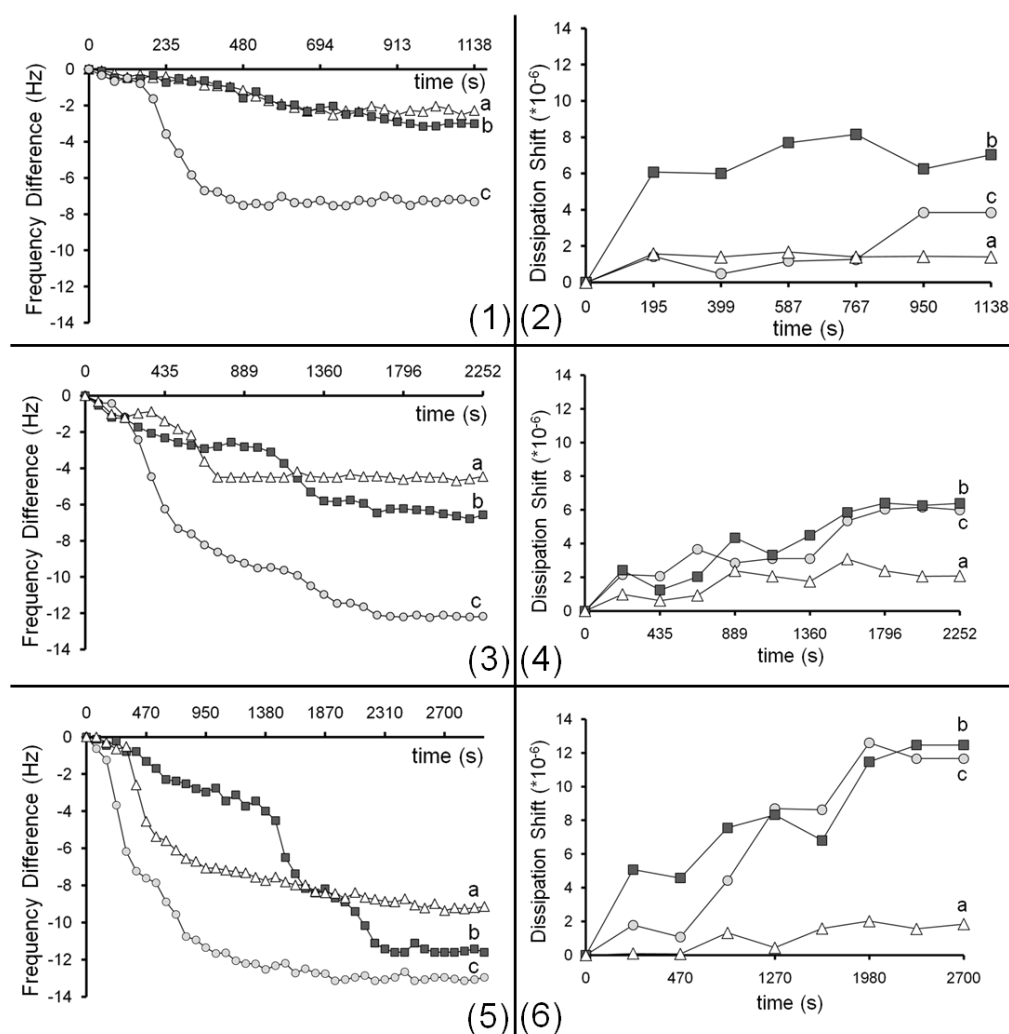


Figure 4.11: Plots of time-dependent frequency and dissipation shifts at different amount of 3rGBP₁ insertions; (1)&(2) 200 nM, (3)&(4) 500 nM and (5)&(6) 800 nM concentrations (a) under non-polarized conditions, when gold was polarized to (b) -167 mV (c) 233 mV, respectively.

With an increase in inserted peptide amounts, two equilibrium stages during adsorption can be observed in the frequency graphs (Figure 4.11 (1),(3),(5)). The frequency pathways for OCP and -167 mV conditions started to be differentiated. Unlike frequency graphs, time-dependent dissipation shift pathways for OCP and -

167 mV conditions look like very differently from the beginning. Under -167 mV, high dissipation shift was assessed despite the low frequency shift meaning that thermodynamically unfavorable conformational changes occur during the early steps of peptide adsorption. This was followed by more ordered configurations of 3rGBP₁ molecules on the gold surface resulting in a soft and compact film at -167 mV.

4.3.1.3 Viscoelastic properties of adsorbed peptide films

Acquired frequency and dissipation data from several harmonics for adsorbed peptide molecules at each applied potential were fitted to a viscoelastic model named as equivalent circuit analysis, which was interpreted in detail in Section 2.4.4.4. Hence, quantitative data about viscoelastic properties of adsorbed 3rGBP₁ films were obtained as a function of applied potential and their corresponding surface coverage ratios. Results of the model including effective thickness, hydrodynamic mass and dynamic viscosity of adsorbed 3rGBP₁ films for 1200 nM peptide insertions are given in Table 4.3. Here, calculated values at -267 mV and 333 mV polarizations were intentionally disregarded, because low signal-to-noise ratio at higher harmonics caused to uncertainty in the model. Viscoelastic properties of adsorbed peptide films for all peptide concentrations can be found in Appendix B.1 with their corresponding applied potentials.

Table 4.3: Effective film thicknesses ($\Delta\delta_{\text{eff}}$), dynamic density (ρ_{eff}) and hydrodynamic mass (Δm_{eff}) of adsorbed 3rGBP1 molecules from 1200 nM peptide insertion under applied potentials.

V_{applied} (mV)	$\Delta\delta_{\text{eff}}$ (nm)	ρ_{eff} (g/cm ³)	Δm_{eff} (ng/cm ²)
233	2.18±0.17	0.954±0.05	194±10
PZC ^a	1.82±0.20	0.944±0.02	162±13
OCP ^b	2.15±0.34	0.925±0.03	191±24
-167	2.43±0.24	0.941±0.06	216±18

^a PZC (potential of point of zero charge) of the gold surface is 143±10 mV.

^b OCP (open circuit potential) of the gold surface referring to non-polarized condition is 73±10 mV.

Results in Table 4.3 indicate that, within error, film thickness obtained for each surface condition did not show significant difference when compared to each other. However, significant distinction can be observed in surface coverage ratios, which

were calculated using kinetic data. Maximum surface coverage was obtained at 233 mV surface condition. If 3rGBP₁ would be a rigid substrate, then we would assume that highest peptide amount should be adsorbed when the surface was polarized to 233 mV. However, since peptides are soft molecules, they take water within themselves in solutions and QCM measures the adsorbed peptide film coupled with water (Hook et. al, 2002). Therefore, the adsorbed film was assumed as a mixture of buffer solution and peptide molecules and hydration ratios were estimated from dynamic density results. Hence, dynamic densities were treated as mixture densities. Figure 4.12 shows the hydration ratios calculated for adsorbed films at applied potentials. Results indicate that polarization caused an enhancement in the hydrated state of peptides. Minimum hydration was obtained under non-polarized condition and maximum hydration was assessed under 233 mV polarization.

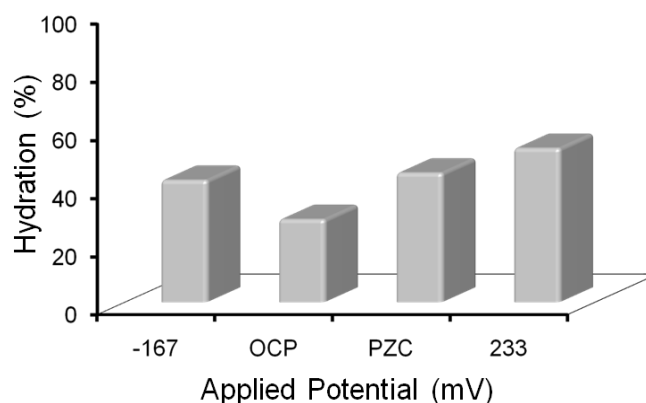


Figure 4.12: Hydration ratios of adsorbed 3rGBP₁ molecules from 1200 nM peptide insertion under applied potentials (OCP and PZC conditions are 73 mV and 143 mV, respectively).

Using hydration ratios, we calculated the mass of dehydrated adsorbed peptides and then the number of adsorbed peptide molecules per covered area (Figure 4.13(a)) for each charged surface condition. According to the results in Figure 4.13(a), minimum adsorption was attained when the excess surface charge was zero (PZC condition). There is an increase in the number of adsorbed peptides when the gold surface potential was shifted from PZC to both negative and positive directions. Hence, maximum adsorbed amount was attained when the surface was polarized to -167 mV. Results reveal that excess surface charge independent from the type of the sign led to an enhancement in the adsorbed peptide population.

In Figure 4.13(b), surface coverage ratios, calculated using kinetic data, are given. There is no linear correlation between the number of adsorbed peptides (Figure

4.13(a)) and their surface coverage ratios (Figure 4.13(b)) due to applied potential. Maximum surface coverage was obtained when the surface was polarized to 233 mV. If 3rGBP₁ would be a rigid substrate, it was expected that highest peptide amount should be adsorbed under this condition. However, maximum adsorbed peptide amount was assessed under -167 mV polarization. The reason of this peculiar behaviour of the peptides may lie on their flexibility. Since 3rGBP₁ molecules are flexible, their conformation and their hydration state on the surface change probably due to alteration of the gold surface charge density.

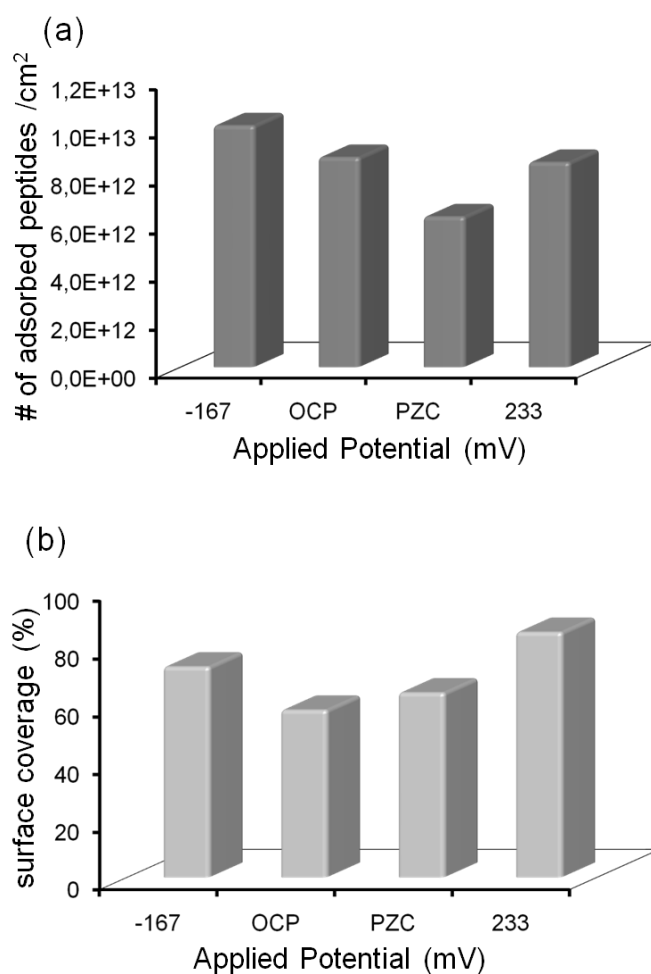


Figure 4.13: (a) Number (b) Surface coverage of adsorbed 3rGBP₁ molecules from 1200 nM peptide insertion under applied potentials, respectively.

As we know the number of adsorbed peptides and covered peptide area for each applied potential, we also calculated the maximum area covered by one peptide molecule on the gold surface under applied potentials. Maximum interaction area of a single 3rGBP₁ molecule on the gold give physical insight for the assembly character of adsorbed peptides; whether the peptides are assembled in a stretched or

condensed structure on the surface. Surely, it does not mean that the all peptide molecules have the same structure on the surface but this finding gives us insight about the structure of the average peptide population adsorbed on gold.

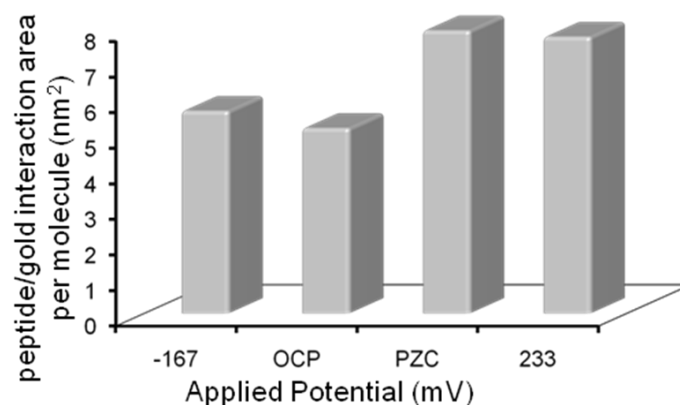


Figure 4.14: Maximum interaction area between a single 3rGBP₁ molecule and the gold surface.

Previous NMR studies revealed that the dimensions of one 3rGBP₁ molecule are 1x2x4 nm (Kulp et. al, 2004, So et. al., 2009). Based on the theoretical dimensions, the largest possible interaction area for one 3rGBP₁ molecule on the gold surface is approximately 8 nm². As demonstrated in Figure 4.14, results obtained when the applied potential was 143 mV (no excess surface charge) and 233 mV (excess positive charge) correspond to the theoretical maximum peptide/gold interaction area for one molecule. Thus, under these conditions peptides are most likely assembled on the gold surface by making the largest possible contact with the underlying gold lattice. It is like as they are stretched on the surface. At applied potentials of 73 mV and -167 mV, namely on negatively charged gold surfaces, peptide/gold interaction area for a single molecule reduces in the amount of 2 ± 0.5 nm. In these cases, peptides probably changed their conformation, making fewer contacts with the surface but enhancement in adsorbed peptide population under these conditions indicates a denser assembly of peptides on the gold surface. For non-polarized surface condition (OCP), results are inconsistency with previous studies (So et. al., 2009), which claim that 3rGBP₁ molecules form polypod structures and a dense assembly. Here, we may predict that excess negative charge induces proper conformation for the dense assembly of peptides on the gold surface. Indeed, to find out what is really happening at the molecular scale, our data should be supported with molecular modeling studies under charged aqueous conditions.

4.3.1.4 Proposed assembly of 3rGBP₁ at several metal surface charge densities

In aqueous solutions, proteins generally adhere to hydrophobic surfaces like gold with their hydrophobic residues because protein-surface interactions are thermodynamically favorable than protein-solution interactions (Norde, 1986). Thus, maximum adsorption of proteins on gold substrate in aqueous solutions is usually observed in the vicinity of PZC of the gold surface (Lipkowski and Stolberg, 1992). Since, in our case, the least adsorbed peptide amount was assessed at PZC surface condition, the small peptide, 3rGBP₁, seems not to follow the common rules of protein adsorption to gold surfaces in aqueous solutions. We also cannot explain the propensity between 3rGBP₁ and the gold surface using simple electrostatics because in solutions with a pH of 7.4, the surface of 3rGBP₁ is positively charged. Hence, it is not common that positively charged peptide surface recognize the positively charged gold surface as in the case of 233 mV and 333 mV surface conditions. Our results revealed that 3rGBP₁ adapted itself to each charged environment whether the excess surface charge was zero, negative or positive. Here, we may state the conformational flexibility of 3rGBP₁ because of peptide's unfolded labile conformation and the existence of six small and flexible glycine residues in peptide's backbone. Moreover, conformational flexibility can be correlated with having intrinsic disorder of 3rGBP₁ molecules. Intrinsic disorder, which is a common feature of naturally occurring inorganic material interacting proteins, may provide adaptation ability to 3rGBP₁ molecules (So et. al., 2009, Evans et. al., 2008, Delak et. al., 2009). The whole results obtained in this study reveal that variation in charge densities of the metal surface caused to alteration of adsorbed peptide population, peptide coverage ratios and hydrated state of adsorbed peptide films leading to a different assembly of 3rGBP₁ under each applied potential.

- **Assembly at potential of point of zero charge:**

When the excess surface charge was zero, peptides covered the surface with their largest surface area, but there were not many available adsorption sites leading to a low population of adsorbed peptides, as illustrated in Figure 4.15. The lack of excess metal surface charge decreased certainly the number of available adsorption sites indicating that electrostatic interactions play an important role in 3rGBP₁ adsorption.

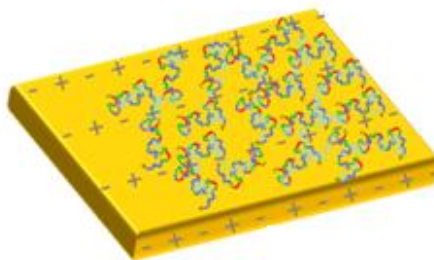


Figure 4.15: Proposed assembly behavior for 3rGBP₁ adsorbed on gold surface when the surface was polarized to 143 mV (PZC). Colors in the molecular structure of the peptide represent corresponding amino acid residues as red refers to anionic, dark blue refers to cationic and green refers to polar residues. Light blue represent water molecules held by peptides.

- **Assembly at negative surface charge densities:**

In section 4.1.2, we already remarked that OCP value was measured as 73 ± 10 mV. An OCP value below PZC indicates that the gold surface is negatively charged under non-polarized condition. In solutions bearing a pH of 7.4, the net surface charge of one 3rGBP₁ peptide is +3 (Berg et. al., 2006). The cationic motifs (N-terminus, lysine residues and the partially positively charged histidines on the 3rGBP₁ surface) are attracted by the negatively charged gold surface, so more peptides move to the gold/buffer interface and stick onto the surface. Previous studies proposed that the tight and concurrently flexible interactions between 3rGBP₁ binding motif and the gold surface may originate from the close contact of the polar (e.g., glutamine, serine, or threonine) and cationic (e.g., lysine or histidine) amino acid side chains of 3rGBP₁ with the underlying gold lattice (So et. al., 2009, Verde et. al., 2009, Suzuki et. al., 2007). Our findings are inconsistency with previous results. Moreover, lysine and histidine amino acids have been previously shown to participate in metal binding either through electrostatic interaction when they exist in their charged states or through the coordination of gold by amine functional groups in their neutral states (Slocik et. al., 2002). Thus, we can confirm that cationic motifs in 3rGBP₁ play an important role for the specific binding of 3rGBP₁ on gold. The excess negative charge on the gold surface, observed in this study under non-polarized condition, explains the high affinity between cationic residues of 3rGBP₁ and the gold surface.

The high viscosity obtained at OCP (see Appendix B.1) supports the predicted “compact and ordered” structure for the peptide assembly under non-polarized condition. It is known from CD and NMR studies that 3rGBP₁ molecule has a labile

conformation (Kulp et. al., 2004). Additionally, molecular docking studies proposed that 3rGBP₁ molecules form a polypod structure on the gold surface (So et. al., 2009). With its periodically repeating cationic motifs and its labile conformation, 3rGBP₁ may form repeating polypods on the negatively charged gold/buffer interface resulting in densely woven network of peptides with low water content as proposed in Figure 4.16.

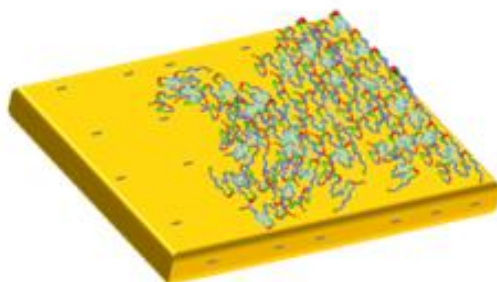


Figure 4.16: Proposed assembly behavior for 3rGBP₁ adsorbed on gold surface at non-polarized condition.

If the gold surface is polarized to the negative direction away from OCP, solvated cations (in our case K⁺ ions) are attracted to the surface and some water molecules in the network change either their place or their orientation within the double layer (Bockris and Reddy, 1970). This new condition probably induces a change in the intermolecular interactions between peptides and the gold surface. Hence, under negative polarization, adsorption rates were higher when compared to the rates under OCP showing that electrostatic stimulation accelerates the adsorption process. Unfortunately, excess negative charge higher than $\pm 10 \mu\text{C}/\text{cm}^2$ led to a decrease in adsorbed peptide population probably because of structural limitations within the double layer occurring at high surface charge densities.

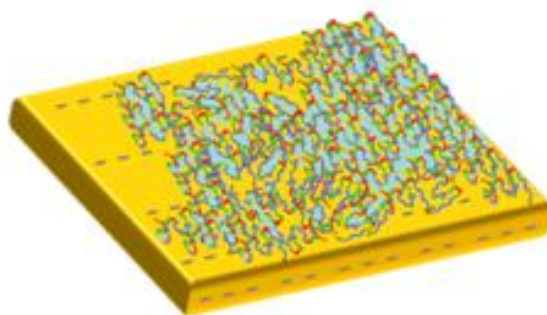


Figure 4.17: Proposed assembly behavior for 3rGBP₁ adsorbed on gold surface when the surface was polarized to -167 mV.

Excess negative charge in the range of $-7 \mu\text{C}/\text{cm}^2$ causes to increment of the available adsorption sites, therefore also the adsorbed peptide amount compared to non-polarized condition. Water holding capacity of assembled peptides increases also at this condition as proposed in Figure 4.17.

Finally, it is noteworthy to mention that we obtained similar peptide/gold interaction areas at two negatively charged surface states (OCP and -167 mV), independent from charge amount (see Figure 4.14). Hence, we may predict that negative charge induces the proper confirmation for the dense assembly of peptides onto gold surface. Indeed, to find out what is really happening at the molecular scale, our data should be supported with molecular modeling studies under charged aqueous conditions.

- **Assembly at positive surface charge densities:**

Since increase in excess negative surface charge cause an increment in the number of adsorbed 3rGBP₁ molecules onto the gold surface, it is expected that the reverse case, namely increase in excess positive surface charge, result in a reduction on the number of adsorbed peptide molecules. At this point, 3rGBP₁ adsorption data observed under 233 mV polarization (Table 4.2&4.3) demonstrates a peculiar behavior. Our results revealed that 3rGBP₁ molecules prefer to interact with negatively charged gold surface through their cationic motifs. As cationic motifs tend to repel from the positively charged gold surface, there should be another recognition pathway for 3rGBP₁ molecules on positively charged gold substrate. When the gold surface was polarized to 233 mV , the lowest viscosity value is estimated for the adsorbed peptide film compared to the other conditions. Low viscosity of the peptide film means that there are many water molecules inside the adsorbed film. Under positive polarization, more water molecules could be migrated to the gold/buffer interfacial region. Presence of many water molecules at the interface reduce generally the hydrophobic interactions between protein molecules and solid surfaces and cause to high energy losses. Besides, hydrogen bonding and dipole-dipole interactions are enhanced at the interfacial region (Norde, 1986). At this condition, the single negatively charged anionic motif, namely the C-terminus, on the peptide surface could be attracted by the positively charged gold surface. Several partially negatively charged polar residues at serine, threonine, glutamine and glycine amino acids on the 3rGBP₁ surface could approach to the interface possibly through

hydrogen bond interactions with water. All these predicted alterations at the interface result in high energy losses. We already remarked that high frequency and dissipation shifts were obtained under 233 mV polarization implying high energy losses and significant conformational changes of peptide molecules at the gold/buffer interface. Besides, according to the results in Figure 4.14, peptides cover the surface with their largest surface area under 233 mV polarization. On the other hand, excess positive charge in the range of $10 \mu\text{C}/\text{cm}^2$ increased both the available adsorption sites and also the hydrated state of the peptides. These findings support the proposed peptide assembly shown in Figure 4.18 for 3rGBP₁ molecules at 233 mV applied potential. When the excess positive charge was shifted above $10 \mu\text{C}/\text{cm}^2$, a decrease in adsorption was observed probably because of structural limitations at high surface charge densities.

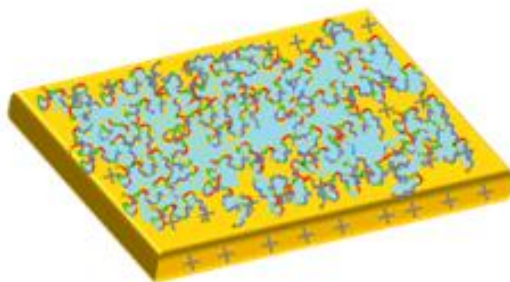


Figure 4.18: Proposed assembly behavior for 3rGBP₁ adsorbed on gold surface when the surface was polarized to 233 mV.

In literature there are some examples such as HSA and IgG proteins where the proteins adsorb onto the gold surface although the metal and the protein surfaces have like charges (Norde et. al., 1986). Such observations were interpreted as adsorption processes are dominated by Van der Waals forces. However, in case of small and soft proteins characterized by lower structural stability, the mechanism cannot be attributed easily to the commonly encountered hydrophobic nature of protein adsorption since the local environment of the electrode/solution interface and structural rearrangement capability of the adsorbing protein molecule play also a vital role in protein adsorption process (Roscoe et. al., 2000).

4.3.2 Non-specific recognition of BSA at metal/electrolyte interface

Bovine serum albumin (BSA) is a globular protein, which usually adsorbs to metal surfaces non-specifically and irreversibly. It is known that BSA molecules adhere on

gold substrates mainly through electrostatic interactions (Brusatori et. al., 2003). BSA has a molecular weight of 66.43 kDa and an isoelectric point of about 4.7–4.9 in aqueous solutions at 25 °C (Ithurbide et. al., 2007). Thus, the surface of BSA at pH 7.4, the conducted pH in up to now presented 3rGBP1 adsorption experiments, is negatively charged. Based on these features, BSA was a proper model protein to analyze the differences between specific and non-specific effects when proteins are adsorbed on charged metal surfaces.

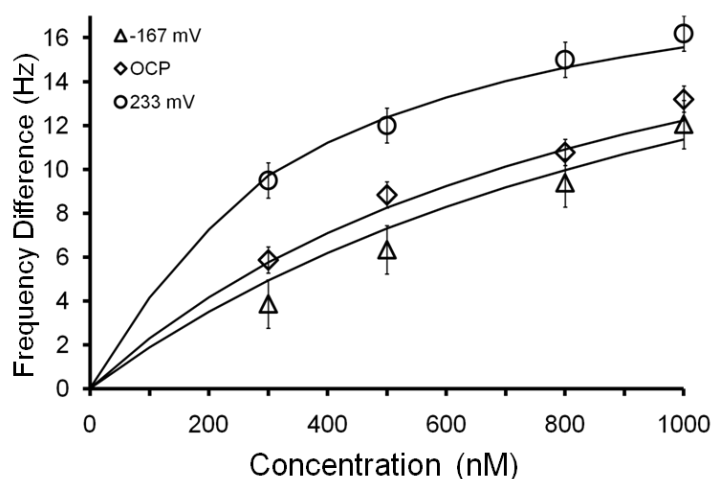


Figure 4.19: Adsorption isotherms for BSA, obtained by EQCM-Z, under -167 mV (negatively polarized gold surface) and 233 mV (positively polarized gold surface) polarizations in comparison with non-polarized conditions (OCP).

Adsorption isotherms for adsorbed BSA on the gold surface at three different potentials are presented in Figure 4.19. An enhancement in measured frequency shifts was observed when the surface was polarized to more positive potentials. This result indicates an increase in electrostatic attraction between oppositely charged metal and protein surfaces. The shift of the potential to the negative direction caused to a slight decrease in the measured frequency shifts probably due to the electrostatic repulsion between like charges of the gold and BSA surfaces.

The binding constants shown in Table 4.4 were calculated by fitting the QCM data to the 1:1 Langmuir adsorption model, because fits obtained from modified Langmuir model displayed higher errors than the single step model. Since BSA is a larger molecule with higher conformational stability compared to 3rGBP₁, two-stage adsorption process may not be prevailing for the case of BSA.

Table 4.4: Adsorption Equilibrium Constants (K_{eq}) and Free Energies for BSA at applied potentials^a.

$V_{applied}$ (mV)	ΔF_{max} (Hz)	$K_{eq} \times 10^6$ (M^{-1})	ΔG_{ads} (kcal/mol)
233	21.00±2.75	2.87±0.67	-8.79±0.20
OCP ^b	23.63±0.71	1.07±0.04	-8.22±0.03
-167	25.69±0.15	0.793±0.12	-8.04±0.08

a The errors represent standard deviations.

b Open circuit potential is the potential at non-polarized condition (73±10 mV).

Since the molecular weight of BSA is circa 15 times larger than the molecular weight of 3rGBP₁, higher shift in frequency and dissipation were expected for BSA molecules when they adsorbed to the gold surface. Especially the changes in dissipation are very few indicating that Sauerbrey relation holds in this case even for a viscoelastic layer. Thus, Δm values in Table 4.5 were calculated using equation (2.1).

Table 4.5: Measured frequency, dissipation shifts and calculated adsorbed mass of adsorbed BSA at three different applied potentials.

$V_{applied}$ (mV)	ΔF (Hz)	Δm (ng/cm ²)	ΔD (10 ⁻⁶)	$\Delta D/\Delta F$ (10 ⁻⁶)
233	16.2±0.21	286±4	2.5±0.29	0.154±0.017
OCP	13.2±0.43	234±8	3.13±0.24	0.237±0.009
-167	12.05±0.55	213±10	2.52±0.40	0.209±0.026

The net surface charge of BSA is negative at pH 7.4 Under the same pH, 3rGBP₁ has a positive net charge. According to the surface coverage ratios of BSA and 3rGBP₁ at corresponding potentials shown in Figure 4.20, enhancement in surface coverage was observed under positive polarization and a decrease was obtained under negative polarization in BSA case. This means that the negatively charged BSA obeys to the simple electrostatic rules. However, for 3rGBP₁ case enhancement in surface coverage occurs whether the polarization positive or negative, which means that 3rGBP₁ does not obey to simple electrostatic rules. The reason of this peculiar behavior of 3rGBP₁ may lie on its flexibility. As indicated in Figure 4.21(a), 3rGBP₁ has a more flexible structure than BSA. If a material is flexible and undergoes many conformational changes, it dissipates more energy at the interface. According to the

dissipation shifts in Figure 4.21(b), we can state that 3rGBP₁ has a more dynamic behavior than BSA under polarization.

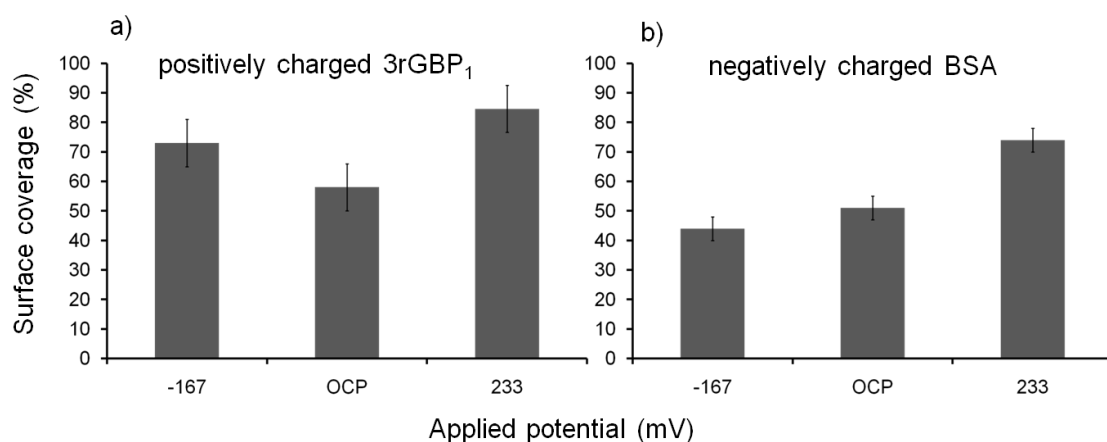


Figure 4.20: Surface coverage ratio of adsorbed a) 3rGBP₁ molecules when 1200 nM peptide concentration b) BSA molecules when 1000 nM protein concentration was inserted to the system at applied potentials.

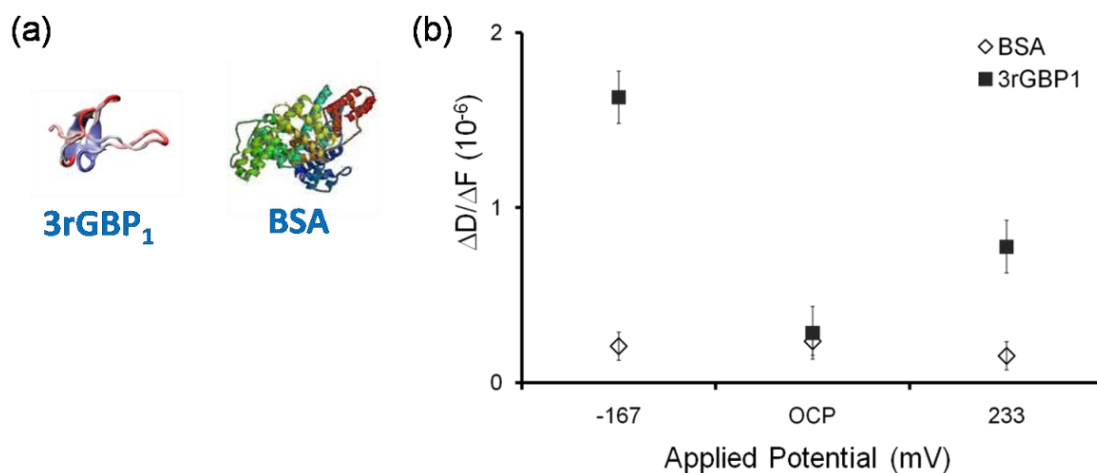


Figure 4.21: (a) Molecular dynamic structure of 3rGBP₁ and BSA (So et. al., 2009, Ithurbide et. al., 2007) (b) Comparison of $\Delta D/\Delta F$ changes of 1000 nM BSA and 1200 nM 3rGBP₁ concentrations as a function applied potential.

4.4 Probing Molecular Recognition of 3rGBP₁ through Solution Charge

In potential dependent adsorption experiments of 3rGBP₁, we observed that the external electrostatic contributions strongly influence the interactions between peptide molecules and the gold surface. Now, alterations in solution charge will be probed.

4.4.1 Variation of peptide's net charge at metal/electrolyte interface

In this part of the study, we changed peptides' overall net charge by altering solution pH and investigated the adsorption behavior of 3rGBP₁ on the gold surface at different pH's. We already know from the results shown in Figure 4.7, that OCP value at the interface decreases with the increase of pH. On this pH-potential diagram PZC condition, where the excess charge is zero, was measured at pH 5.2, meaning that in solutions having a pH value below 5.2, excess surface charge of gold is positive, while the gold surface is negatively charged when solution pH is above 5.2. Thus, we can use this information to determine the sign of excess interfacial charge at desired pH values.

Next, we calculated the overall net charge of 3rGBP₁ as a function of solution pH by using the program Protein Calculator 3.3v. This program assumes that all amino acid residues in the polypeptide chain are equivalent to isolated residues. This estimation is not valid for a folded protein. Since previous CD and NMR studies (Kulp et. al., 2004) already revealed that 3rGBP₁ has an unfolded and labile structure, the program gives, therefore, reliable results for 3rGBP₁, which are presented in Table 4.6 for 4 different pH values.

Table 4.6 contains also OCP values of the gold surface in solutions with their corresponding pH's and the sign of excess interfacial charge under these pH conditions. We arranged pH values so that the surface of 3rGBP₁ first becomes highly positively charged. At pH 4.5, the net charge surrounding the exterior of the peptide arises from protonated 3 lysine and 3 histidine residues, while the positively charged N-terminus compensates the negatively charged C-terminus (Berg et. al., 2006). Since at pH 4.5 the interface is also positively charged, we expected strong repulsion under this condition. Thereafter, we arranged the solution pH so that the peptide surface becomes negatively charged. At pH 12, all cationic motifs, even N-terminus are deprotonated and the only negative charge originates from the C-terminus. Here, again a strong repulsion was expected between negatively charged peptide surface and the negatively charged gold surface. Additionally, we analyzed peptide adsorption at pI, where the net charge of 3rGBP₁ is zero, and also at pH 7.4, where previous studies were generally conducted. When the solution pH is 7.4, N-terminus and 3 lysine amino acids on 3rGBP₁ surface are deprotonated in aqueous environment. As the positive charge at the N-terminus is cancelled out by the

negative charge at the C-terminus, the net charge of 3rGBP₁ equals to +3 (Berg et. al, 2006). Under this condition, the gold-solution interface is negatively charged. Hence, positively charged 3rGBP₁ molecules are probably electrostatically attracted by the negatively charged gold surface, which explains the strong affinity of 3rGBP₁ onto the gold surface claimed by previous studies (So et. al, 2009).

Table 4.6: Overall net surface charge of 3rGBP₁, measured OCP of the gold surface and the sign of excess interfacial charge at chosen pH values.

Solution pH	3rGBP ₁ net charge	OCP ^a (mV)	sign of excess interfacial charge
4.5	+6	164±4	+
7.4	+3	73±10	-
pI ^b	0	19±6	-
12	-1	-7±3	-

a OCP is the open circuit potential of the gold-buffer interface.

b pI (isoelectric point) of the 3rGBP₁ is at pH 10.28.

4.4.1.1 Binding kinetics

Adsorption isotherms for 3rGBP₁ on gold surface at stated pH values are given in Figure 4.22. At pH 12, low frequency shifts indicate low adsorption as expected, probably due to electrostatic repulsion between peptide and the gold surface. However, an unexpected increase in frequency data was observed at pH 4.5. Adsorption at pH 7.4 and isoelectric point conditions cannot be differentiated from each other clearly.

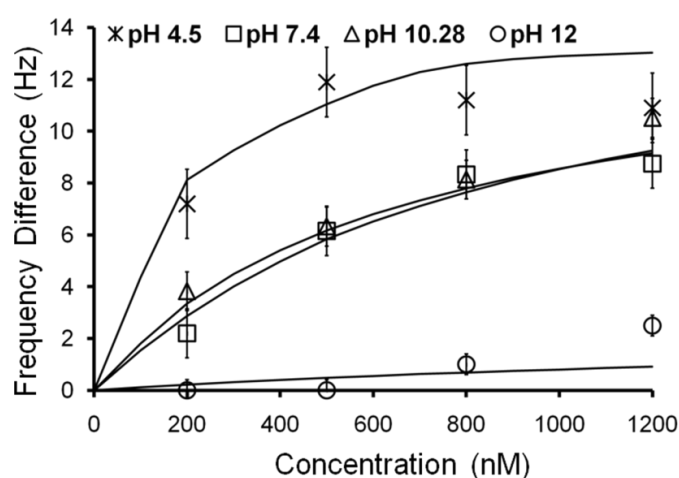


Figure 4.22: Adsorption isotherms for 3rGBP₁ on gold surface at several pH's.

We, then calculated the binding parameters by fitting frequency shifts to the modified Langmuir adsorption model. Here, pH alteration did not change the first diffusion controlled adsorption step (see K_{eq1} results in Table 4.7) but peptide network formation rate (see K_{eq2} results in Table 4.7) is strongly influenced from pH alterations, similar to the results obtained in potential dependent experiments.

Table 4.7: Adsorption Equilibrium Coefficients (stepwise; K_{eq1} , K_{eq2} and total; K_{eq}) and Free Energies for 3rGBP₁ at different pH values.

pH	ΔF_{max} (Hz)	$K_{eq1} \times 10^4$ (M ⁻¹)	$K_{eq2} \times 10^1$ (M ⁻¹)	$K_{eq} \times 10^6$ (M ⁻¹)	ΔG_{ads} (kcal/mol)
4.5	12.00±0.99	6.13±0.05	18.3±0.16	11.3±0.95	-9.61±0.06
7.4	15.96±1.75	6.13±0.04	1.78±0.45	1.15±0.28	-8.22±0.22
pI ^a	14.00±1.25	6.13±0.05	2.50±0.66	1.60±0.26	-8.42±0.12
12	6.00±1.43	6.13±0.01	0.097±0.05	0.12±0.19	-6.50±0.03

a pI is the isoelectric point of 3rGBP₁, which equals to pH 10.28.

An adsorption equilibrium coefficient (K_{eq}), which was 10 times higher than K_{eq} at pH 7.4, was observed at pH 4.5. This result reveals that the peptide film is formed very quickly under this condition despite strong repulsion at the interface. On the other hand, at pH 12, K_{eq} was almost 10 fold lower than K_{eq} at pH 7.4 indicating a very slow adsorption process.

4.4.1.2 Viscoelastic properties of adsorbed peptide films

By further analyzing the viscoelastic properties, we realized that even at low peptide concentrations 3rGBP₁ molecules cover nearly the whole surface at pH 4.5. The effective hydrodynamic thickness, density and mass, together with their hydration ratios of adsorbed peptide films for 500 nM and 1200 nM 3rGBP₁ concentrations were given in Table 4.8 as a function of conducted pH values.

As shown in Table 4.8, there is no significant difference in effective thicknesses between adsorbed 3rGBP₁ at low concentration (500 nM) and high concentration (1200 nM) when the solution pH is 4.5. Under this condition, peptides may have formed a closely packed monolayer over the whole surface, since the maximum theoretical mass for monolayer peptide coverage, which equals to 280 ng/cm², is in

good correlation with the adsorbed hydrodynamic mass results obtained at both 3rGBP₁ concentrations. Besides, low hydration ratios indicate that a very dense monolayer film with low water content is formed at pH 4.5.

Table 4.8: Effective film thickness ($\Delta\delta_{\text{eff}}$), dynamic density (ρ_{eff}), hydrodynamic mass (Δm_{eff}) and hydration ratio (H) of adsorbed 3rGBP₁ molecules from 500 and 1200 nM peptide concentrations at conducted pH values.

V_{applied} (mV)	$C_{(3\text{rGBP}_1)}$ (nM)	$\Delta\delta_{\text{eff}}$ (nm)	ρ_{eff} (g/cm ³)	Δm_{eff} (ng/cm ²)	H (%)
4.5	500	3	0.903	270	9
4.5	1200	2.98	0.902	268	9
7.4	500	1.5	0.875	131	-
7.4	1200	2.15	0.925	185	28
pI ^a	500	0.9	0.934	84	36
pI	1200	1.93	0.946	182	47

a pI is the isoelectric point of 3rGBP₁, which equals to pH 10.28.

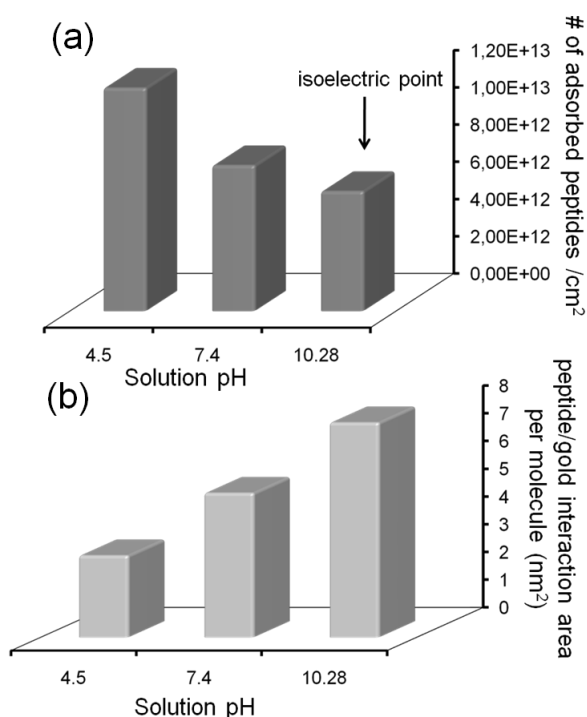


Figure 4.23: (a) Number of adsorbed 3rGBP₁ molecules from 1200 nM peptide concentration (b) Maximum interaction area between a single 3rGBP₁ molecule and the gold surface at conducted pH values, respectively.

If we further analyze the adsorbed peptide amounts from dehydrated thicknesses (Figure 4.23(a)) we can clearly observe that adsorbed peptide amount increases with the decrease of pH, whereas the peptide-gold interaction area decreases with the decrease of pH (Figure 4.23(b)) which means that excess interfacial charge induces conformational changes. As reported in Figure 4.23(b), peptide's conformation is close to the maximum theoretical area at isoelectric point, where the excess peptide's surface charge is zero. Similar results were obtained when excess gold surface was zero under PZC condition.

4.4.1.3 Proposed binding mechanisms at conducted pH values

Proposed binding mechanisms at conducted pH values were illustrated in Figure 24. At isoelectric point (pH 10.28), peptides were probably aligning on the surface horizontally using their maximum surface area for interaction with the gold surface, since the peptide-gold interaction area is close to the maximum theoretical area per one peptide molecule (Figure 4.23(b)). Under this condition, the number of adsorbed peptides were fewest as indicated in Figure 4.23(a). The lack of excess peptide's surface charge decreased certainly the number of available adsorption sites indicating that electrostatic interactions play an important role in 3rGBP₁ adsorption. On the other hand, adsorption occurs even the net excess peptide's surface charge or metal surface charge is zero meaning that electrostatic interactions are not the solely driving forces in recognition mechanism of 3rGBP₁ on gold. In cases where there is a lack of net electrostatic charge at the interface, hydrophobic interactions might become dominated or local charge equilibriums formed could have been contributed to the surface binding. When the solution pH is 4.5, the only negatively charged motif on 3rGBP₁ is the C-terminus. We suggest that a strong interaction between positively charged interface and the C-terminus occurs causing to very dense assembly (a nearly fully covered close packed monolayer peptide film with very low water content) in short time. At pH 7.4, which is the commonly used pH value for 3rGBP₁ adsorption studies, results are the same as assessed under non-polarized conditions; a densely woven peptide network with low water content is suggested.

At pH 12, the adsorbed amount was so few that viscoelastic properties cannot be modeled under these circumstances. Therefore, we were not able to propose an assembly behavior when the solution pH was 12. We speculate that the lack of cationic motifs and the presence of many negatively charged hydroxyl ions may have

changed the nature of peptide-gold interactions at the interface leading to low affinity at this pH value.

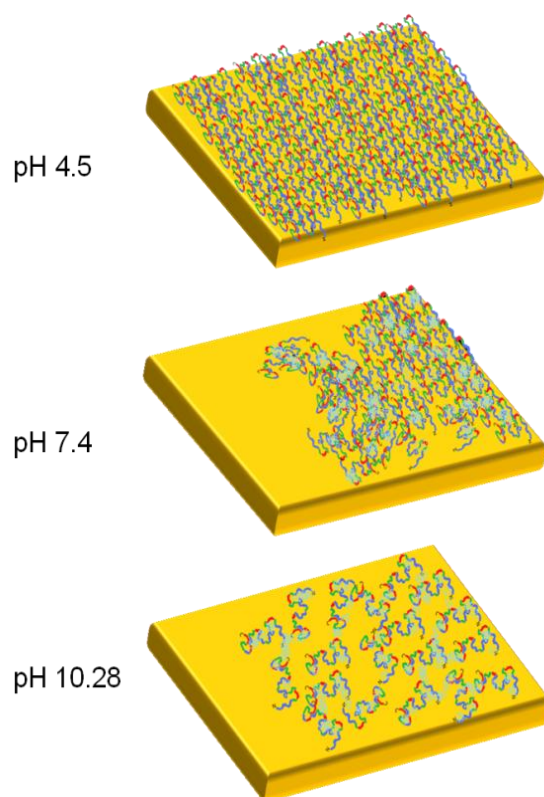


Figure 4.24: Proposed assembly behavior for 3rGBP₁ adsorbed on gold surface at conducted pH's.

4.4.2 Variation of solution's ionic strength at metal/electrolyte interface

In this part of the study, we altered KCl concentration of the solution and investigated the adsorption behavior of 3rGBP₁ on the gold surface at different ionic strengths. Alterations in adsorption behavior were observed depending on the amount of solution charge.

4.4.2.1 Binding kinetics

Adsorption isotherms for 3rGBP₁ on gold surface at stated ionic strength values are given in Figure 4.25. Results in Table 4.9 indicate that the adsorption processes occurring in solutions without KCl and with 10 mM KCl are slower than the recognition observed in 100 mM KCl containing solution.

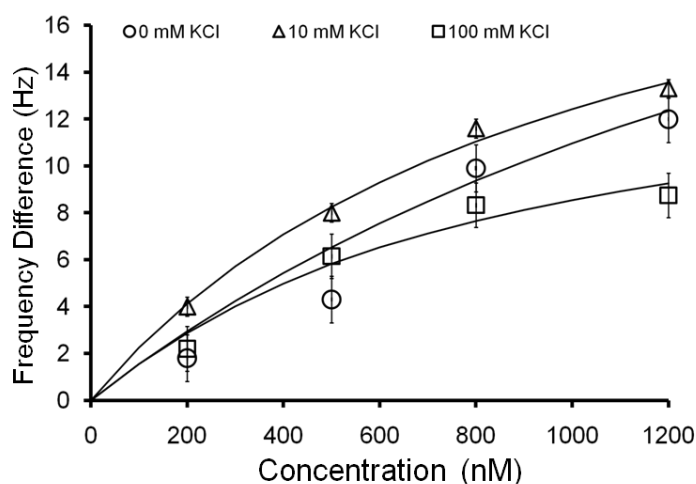


Figure 4.25: Adsorption isotherms for 3rGBP₁ on gold surface in solutions of conducted ionic strengths.

Table 4.9: Adsorption Equilibrium Constants (K_{eq}) and Free Energies for 3rGBP1 at conducted ionic strengths^a.

C (mM KCl)	OCP (mV)	ΔF_{max} (Hz)	$K_{eq} \times 10^6$ (M^{-1})	ΔG_{ads} (kcal/mol)
0 ^b	10±8	34.09±2.83	0.47±0.03	-7.73±0.09
10	15±5	25.08±0.33	0.98±0.05	-8.13±0.03
100	OCP ^c	15.96±1.75	1.15±0.28	-8.22±0.22

a The errors represent standard deviations.

b Under this condition, kinetic data was fitted to 1:1 Langmuir model because fits obtained from modified two step Langmuir model displayed higher errors than the single step model.

c Open circuit potential is 73±10 mV.

The high values of frequency shifts for 0 mM and 10 mM KCl concentrated buffers did imply an enhancement in adsorption when 800 nM 3rGBP₁ was inserted into the system (Figure 4.26 (5)) However, their estimated effective thicknesses exceed the theoretical monolayer thickness, meaning that 3rGBP₁ could lose its native polypod structure when ionic strength is decreased in the medium leading to interlaced peptide structures.

It is known that solvated ions enhance molecular flexibility since they are in random motion. However just dipole-dipole interactions between water and peptide molecules or ion-dipole interactions between solvated ions and peptide molecules could not induce molecular orientation and alignment of macromolecules because

those interaction energies are generally smaller than their thermal energy kT (Israelachvili, 1992). Under these conditions, if there are enough ions in the medium, an increase in the long-range electrostatic interactions and these interactions can induce oriented assembly of molecules on a solid surface. If there are not enough ions in the medium, Van der Waals forces become dominant leading an enhancement in peptide-peptide associations and a possible multilayer formation (Israelachvili, 1992).

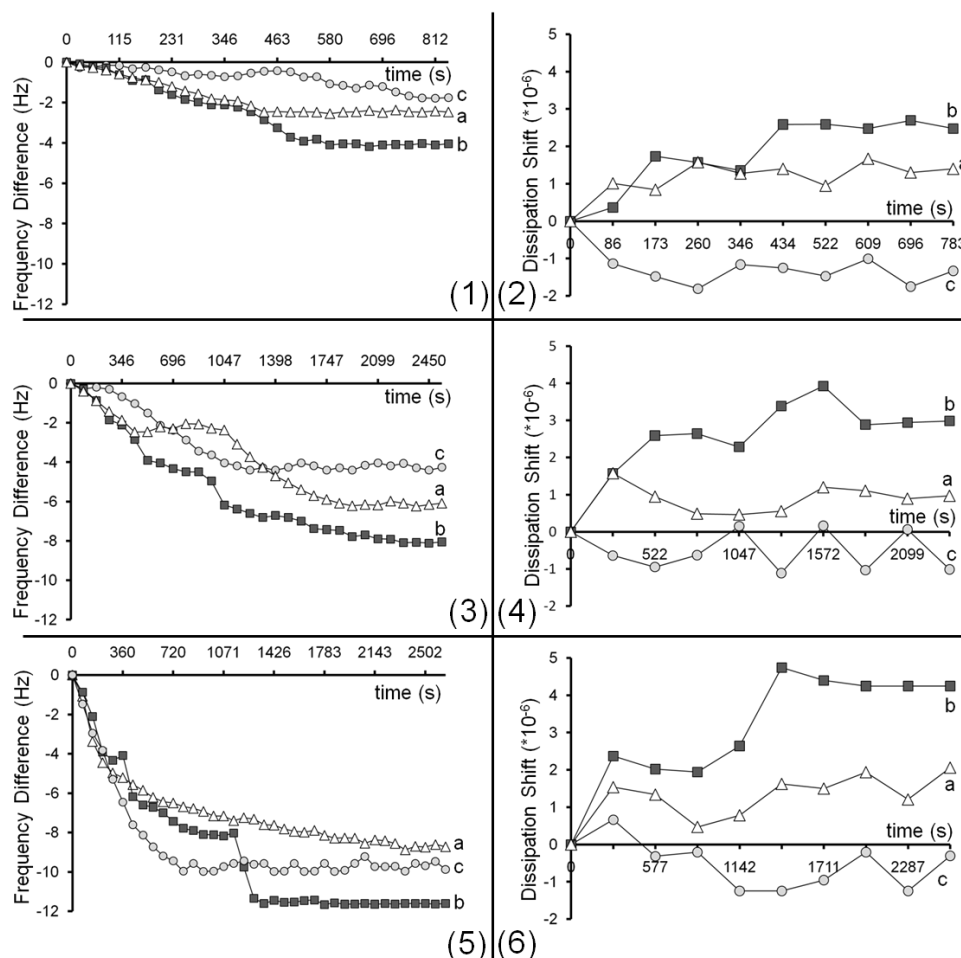


Figure 4.26: Plots of time-dependent frequency and dissipation shifts at different amount of 3rGBP₁ insertions; (1)&(2) 200 nM, (3)&(4) 500 nM and (5)&(6) 800 nM concentrations in solutions containing (a) 100 mM KCl (b) 10 mM KCl (c) 0 mM KCl (ultrapure water), respectively.

The most likely explanation for the smaller dD/dF for 3rGBP₁ adsorbed in solutions containing 100 mM KCl than 10 mM KCl (Figure 4.27), is a more pronounced surface-induced conformational change in the former case and, as a consequence an increased spreading on the surface, which can be seen from the higher surface coverage of 100 mM KCl containing solution results (Figure 4.28(a)).

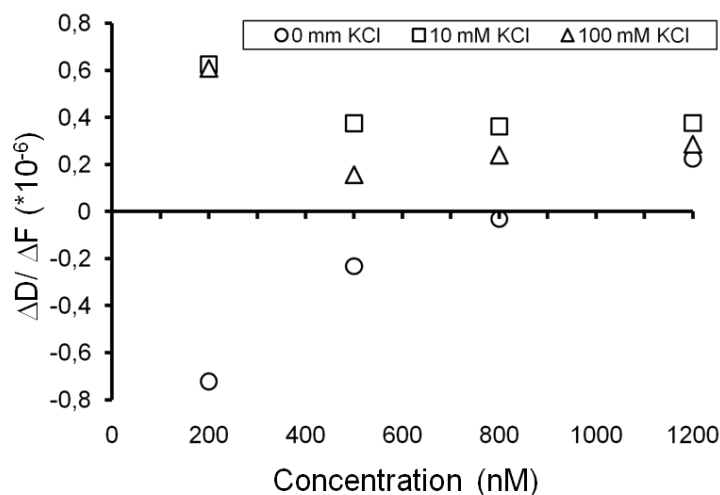


Figure 4.27: $\Delta D/\Delta F$ versus concentration graph at three different ionic strengths.

According to the results in Figure 4.28, if we decrease the ionic strength concentration, a decrease in covered area by peptides can be observed. However, calculated effective film thicknesses did not show the same trend. In solutions containing 0 mM and 10 mM KCl effective thicknesses exceed the theoretical monolayer thickness for corresponding surface coverage ratios indicating multilayer formation under these conditions.

Results are inconsistency with the knowledge from literature. It is already mentioned that solvated ions enhance molecular flexibility when they are in random motion (Israelachvili, 1992). When there are less ions in the media, then the flexibility of molecules will decrease, the native structure of 3rGBP₁ can be disturbed. When molecular flexibility decreases, Van der Waals forces become dominant leading to an enhancement in peptide-peptide interactions and these both factors may result in interlaced peptide structures which ends up with multilayers of peptides.

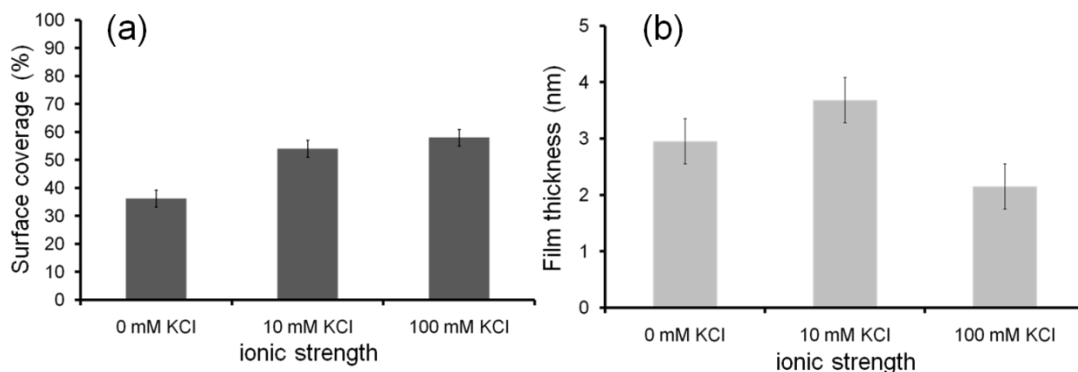


Figure 4.28: (a) Surface coverage (b) Effective film thickness of adsorbed 3rGBP₁ molecules from 1200 nM peptide concentration in solutions of conducted ionic strengths.

4.5 Possible Control of 3rGBP₁ Adsorption on gold via applied potential

4.5.1 Tuning self assembly of 3rGBP₁ on gold

Under non-polarized conditions, a film thickness of 1.31 nm was assessed in solutions with a pH value of 12. When a potential of 233 mV (positive polarization) was applied to the surface at this pH (Figure 4.29), a 0.23 nm increase in the film thickness was assessed. This result shows that the increasing number of positive charges at the interface causes an enhancement in binding most likely by creating additional adsorption sites for the C-terminus of 3rGBP₁. In another experiment conducted in the same solution, a film thickness of 1.56 nm was obtained by first polarizing the gold surface to 233 mV and then sending 3rGBP₁ molecules to the system. When the applied potential was cut off then a desorption process took place leading to a decrease of peptide film thickness to 0.39 nm. This observation also supports that peptide's affinity to the gold surface showed dependence on the amount of positively charged adsorption sites. These results reveal the possibility of tuning the 3rGBP₁ film thickness on gold surfaces by polarization of the surfaces (Table 4.10).

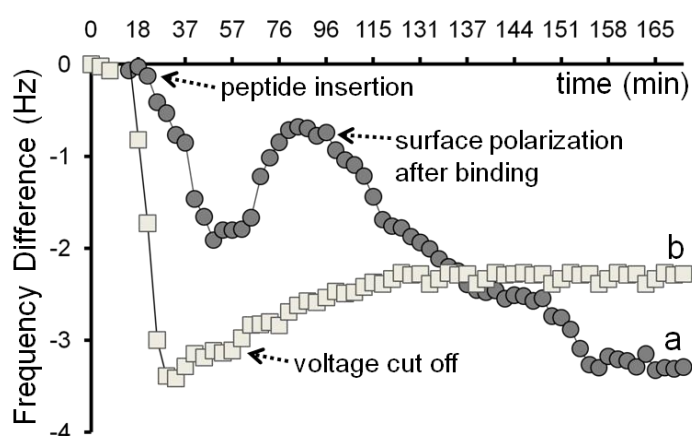


Figure 4.29: Time dependent 3rGBP₁ adsorption at pH 12 (a) under OCP (b) under 233 mV.

Table 4.10: Estimated equilibrium thicknesses for given conditions at pH 12.

3rGBP ₁ binding under OCP		3rGBP ₁ binding under 233 mV	
without polarization	during polarization	during polarization	after voltage cut off
δ_{eq} 1.31 nm	1.54 nm	1.56 nm	1.39 nm

Overall our results showed that the presence of cationic motifs at the interface caused an enhancement in binding. Additionally, alterations in polarization enabled to tuning of the adsorbed peptide amount on the gold surface. These results can be a model for controlled binding of the inorganic materials specific peptides on the desired surface.

CONCLUSIONS

GEPI, genetically engineered peptides for inorganics are a new class of biological molecules, which specifically adhere to their counterpart inorganics. GEPI's potential ability to form hierarchical molecular self assembled structures on the inorganic surfaces, as demonstrated earlier for the case of 3rGBP₁ (So et. al., 2009) makes them alternative eco-friendly biological molecular linkers compared to thiol-based systems.

Controlled binding and assembly of GEPI will be an important breakthrough in science and technology since GEPI serve us many prospects: Targeted binding of GEPI onto their counterpart solids may open new routes in the development of advanced biosensors for pathogen detection, drug screening, bioseparations systems and diagnostics in medicine (e.g. cancer therapeutics). Hybrid GEPI-inorganic nanostructures as molecular building blocks can be utilized in molecular electronics and photonics. Major challenges of these fields are the control of spacing and orientation of molecules, which may be defeated using genetic engineering approaches for desired effects. Novel hybrid platforms made by the utilization of GEPI will enable the synthesis of biology-based materials (Tamerler and Sarikaya, 2008).

In this study, for the first time, the contribution of charge effects at GEPI-solution-gold interface was analyzed quantitatively using electrochemical QCM-Z technique. Remarkable findings were reported about charge mechanisms in specific recognition:

- The adaptation flexibility of a well known GEPI, namely the gold binding peptide 3rGBP₁, was demonstrated as a function of applied potential, pH and ionic strength. For each charged environment, quantitative differences in terms of assembly rate, hydration state, surface coverage ratio and adsorbed peptide population were obtained. Results revealed that 3rGBP₁ adapted itself

to different charged environments at the peptide-solution-solid interface, whether the excess interfacial charge is zero, positive or negative.

- Self-assembly properties of 3rGBP₁ were significantly influenced by interfacial charge alterations. Thus, under various applied potentials, pH's and ionic strengths, considerable differences were obtained in adsorbed peptide amount and effective film thicknesses indicating different assemblies of 3rGBP₁ on the gold surface taking place at the peptide-solution-solid interface depending on the interfacial charge.
- Applied electrical field and arrangements in solution charge did not change the first step of the diffusion controlled adsorption mechanism of 3rGBP₁ molecules. However, the second step, referring peptide coalescence and film formation, was influenced significantly by interfacial charge effects. Results reveal that peptide coalescence and film formation rate can be tuned via variation of interfacial charge.
- Excess negative charge of the metal surface (less than $-10 \mu\text{C}/\text{cm}^2$) and presence of KCl ions in high amounts in the solution are significant parameters for specific recognition of 3rGBP₁ on the gold surface. On the other hand, polarization of the metal surface above $-10 \mu\text{C}/\text{cm}^2$ and high pH (pH 12) causes to a decrease in peptide recognition.
- Ionic strength dependent adsorption studies highlighted the importance of ions for specific recognition. The decrease of ion concentration in aqueous media disturbed the native structure of 3rGBP₁ leading possibly to multilayered peptide islands on the gold substrate.
- Based on the applied potential and pH dependent adsorption studies, we could deduce that particularly positively charged species accelerated the adsorption rates in significant amounts at the peptide-solution-solid interface. The deprotonation of cationic motifs by changing the pH caused a decrease in 3rGBP₁ recognition. However, the introduction of new positively charged adsorption sites by changing the applied potential created a possibility of tuning and enhancing peptide adsorption. These results can be a model for controlled binding of the inorganic materials specific peptides on the desired surface.

In one of his papers, John Spencer Evans pointed out that “Nature’s choice of unfolded, unstable conformations is neither accidental, sloppy, nor boring, but purposeful because an unfolded, undefined conformation would have a better capacity to ‘adjust’ to variations in topology, more so than a folded, stable conformation” (Evans, 2003). Evans’s words summarize the importance of adaptation flexibility in biological processes. Inspired from Nature’s material formation strategies, the adaptation flexibility of GEPI, shown in this study using a model protein 3rGBP₁, will certainly expand their utility in bionanotechnological applications.

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APPENDICES

APPENDIX A : Surface coverage ratios of adsorbed BSA and 3rGBP₁ films on gold surface

APPENDIX B : Viscoelastic properties of adsorbed 3rGBP₁ films on gold surface

APPENDIX A : Surface coverage ratios of adsorbed BSA and 3rGBP₁ films on gold surface

Table A.1: Surface coverage ratios of adsorbed BSA films on gold surface at applied potentials.

BSA concentration (nM)	Applied potential (mV)		
	-167	OCP	233
300	19.2±2.3	24.3±0.8	46.3±7.8
500	58.9±2.9	34.8±1.0	59.0±8.4
800	38.8±3.2	46.1±1.1	69.7±8.1
1000	44.2±3.3	51.7±1.2	74.2±7.6

Table A.2: Surface coverage ratios of adsorbed 3rGBP₁ films on gold surface at applied potentials.

3rGBP ₁ concentration (nM)	Applied potential (mV)					
	-267	-167	OCP	PZC	233	333
200	19.2±2.3	18.7±4.1	18.7±4.1	22.5±5.2	46.3±7.8	46.3±7.8
500	58.9±2.9	36.5±7.0	36.5±7.0	42.0±8.5	59.0±8.4	59.0±8.4
800	38.8±3.2	47.9±8.1	47.9±8.1	53.7±9.7	69.7±8.1	69.7±8.1
1200	44.2±3.3	58.0±8.5	58.0±8.5	63.5±9.9	74.2±7.6	74.2±7.6

Table A.3: Surface coverage ratios of adsorbed 3rGBP₁ films on gold surface at different pH's.

3rGBP ₁ concentration (nM)	pH			
	4.5	7.4	10.28	12
200	8.6±0.5	18.7±4.1	16.4±0.7	18.7±4.1
500	19.2±0.9	36.5±7.0	32.9±1.2	36.5±7.0
800	27.5±1.3	47.9±8.1	44.0±1.4	47.9±8.1
1200	36.2±1.5	58.0±8.5	54.1±1.4	58.0±8.5

APPENDIX B : Viscoelastic properties of adsorbed 3rGBP₁ films on gold surface

Table B.1: Viscoelastic properties of 3rGBP₁ adsorbed films on gold surface under applied potentials.

Vs (mV)	C (nM)	t(nm)	m (ng/cm ²)	G' (MPa)	η (mPas)
233	200	1.14	102	0.018	0.07
233	500	2.15	192	-	-
233	800	2.17	194	-	-
233	1200	2.18	195	-	-
PZC	200	-	-	-	-
PZC	500	1.30	116	1.05	3.25
PZC	800	1.74	155	-	1.35
PZC	1200	1.82	162	-	1.45
OCP	200	-	-	-	-
OCP	500	1.41	126	0.40	7.30
OCP	800	2.00	178	0.30	4.20
OCP	1200	2.15	192	0.42	3.90
-167	200	-	-	-	-
-167	500	1.73	154	-	0.36
-167	800	2.35	210	-	0.30
-167	1200	2.43	217	-	0.32

Table B.2: Viscoelastic properties of 3rGBP₁ adsorbed films on gold surface under several pH's.

pH	C (nM)	t(nm)	m (ng/cm ²)	G' (MPa)	η (mPas)
4.5	200	2.25	201	0.15	2.7
4.5	500	3.64	325	0.25	4.6
4.5	800	3.35	299	0.13	4.2
4.5	1200	3.39	302	0.14	4.2
7.4	200	-	-	-	-
7.4	500	1.41	126	0.40	7.30
7.4	800	2.00	178	0.30	4.20
7.4	1200	2.15	192	0.42	3.90
Ip	200	0.67	68	-	-
Ip	500	0.74	75	-	-
Ip	800	1.42	143	-	-
Ip	1200	0.65	66	0.21	1.5

Table B.3: Viscoelastic properties of 3rGBP₁ adsorbed films on gold surface at different ionic strengths.

C (mM KCl)	C (nM)	t(nm)	m (ng/cm²)	G' (MPa)	η (mPas)
0	200	-	-	-	-
0	500	1.04	105	0.87	1.88
0	800	2.16	218	1.3	3
0	1200	2.61	263	1.33	3.56
10	200	1.3	131	0.17	2.2
10	500	1.82	183	0.325	3.55
10	800	3.22	325	0.21	3.15
10	1200	3.25	328	0.21	3.35
100	200	-	-	-	-
100	500	1.41	126	0.40	7.30
100	800	2.00	178	0.30	4.20
100	1200	2.15	192	0.42	3.90

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