

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

NANO-BIOTECHNOLOGICAL APPLICATION OF
INORGANIC BINDING PROTEINS

Ph.D. THESIS

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Department of Advanced Technologies

Molecular Biology, Genetics and Biotechnology Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**İNORGANİKLERE ÖZGÜN BAĞLANAN PEPTİDLERİN
NANO-BİYOTEKNOLOJİK UYGULAMALARI**

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ABBREVIATIONS

AFM	: Atomic Force Microscopy
AuBP2	: Gold Binding Peptide 2
bsLDH	: Bacillus stearothermophilus lactate dehydrogenase
CV	: Cyclic Voltammetry
E. coli	: Escherichia coli
GEPI	: Genetically Engineered Peptides for Inorganics
GBP1	: Gold Binding Peptide 1
HD	: Huntington's Disease
Htt	: Huntingtin
Htt53Q	: Huntingtin containing 53 glutamine residues
Htt20Q	: Huntingtin containing 20 glutamine residues
IPTG	: Isopropyl- β -D-thiogalactopyranosid
LB	: Lysogeny Broth
LDH	: Lactate Dehydrogenase
NAD ⁺	: Nicotinamide Adenine Dinucleotide
NADH	: Reduced Nicotinamide Adenine Dinucleotide
PCR	: Polymerase Chain Reaction
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPR	: Surface Plasmon Resonance
3GBP	: 3 repeat Gold Binding Peptid 1
5GBP	: 3 repeat Gold Binding Peptid 1

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NANO-BIOTECHNOLOGICAL APPLICATION OF INORGANIC BINDING PROTEINS

SUMMARY

Nature provides enormous structures exhibiting great functions such as strength, optical and magnetic properties. The molecular hierarchy of these structures arises from the hybrid organic-inorganic interfaces. The organic compounds are mainly proteins, which are mediating the assembly of inorganic molecules for ordered structures. The protein moieties interacting with the inorganic molecules could be purified and utilized for biotechnological applications in order to obtain new generation biomaterials. However, the purification of the proteins from hard tissues is time consuming as well as the natural proteins may not even exist for some of the technologically relevant materials. Therefore, combinatorial biology techniques have been adapted and applied in the last decade for the selection of inorganic binding peptides for desired inorganic materials. The inorganic binding peptides selected and further characterized by Sarikaya and Tamerler group was named as GEPIs (Genetically Engineered Peptides for Inorganics) and this term is widely used in the literature. In the last decade, inorganic binding peptides have been selected for various inorganic materials by our group and others. In the last years GEPIs were demonstrated to exhibit a number of functions such as linking inorganic-inorganic particles, synthesizing inorganic materials and immobilizing biomolecules on inorganic surfaces through their controlled interactions at the biological-material interface based on molecular recognition. In this study, our purpose to develop fusion proteins incorporating inorganic binding domain and demonstrate their versatile use in a wide range of area from biomedical to biotechnology. We utilized two of the inorganic gold binding peptides, with different length and binding characteristics for directed immobilization of Huntingtin protein, which is responsible for Huntington disease, and Lactate dehydrogenase, which is an important enzyme in chemical and pharmaceutical industries

In the biomedical studies related part of our study, one of the gold binding peptides (GBP1: MHGKTQATSGTIQS) was used as fusion partner with Huntingtin protein (Htt) to mediate its directed immobilization onto gold surface. Htt is a eukaryotic protein, which is causing to Huntington's disease in mutated form. The main pathology of the disease is the inclusion bodies found in cytoplasm and nucleus, which are composed of aggregated Htt proteins. Therefore, investigating the aggregation profile of the protein is critical and expected to have an impact on the progress of the disease. In order to investigate aggregation kinetics, we utilized SPR, which is a surface based system. Preformed fibrils composed of mutant Htt proteins were immobilized on gold sensor surface with the gold binding ability of the fusion partner GBP1. Two different stages of fibrillization were monitored which are fibril and proto-fibril elongations. The data obtained for fibril elongation was suggested a two-stage model indicating the interaction of monomer to fibril in the first stage and

the conformational change of newly added monomer in the second stage. In the proto-fibril elongation, a one-stage model was proposed indicating the addition of monomer to the fibril. Difference between the first stage KD values of two individual steps showed that fibril elongation is slower in the early times of fibrillization which increases while the fibril is formed. The obtained fibrillization kinetics lead for the detection of the effect of new drug targets either for preventing or inducing aggregation. Our studies demonstrate that the GBP1 mediated immobilization could be utilized for the investigation of aggregation kinetics for other neurodegenerative diseases as well through the attaching the proteins to the electrode surfaces with an orientation control and following the interaction with the counterparts dynamically.

In the second part of our study, we used another gold binding peptide AuBP2, which is much smaller than GBP, as the fusion partner for *Bacillus stearothermophilus* LDH (bsLDH). The AuBP2 link was utilized to provide to direct LDH's immobilization onto gold surface with an orientation control and result in self-organization of the fusion product. bsLDH is a consumable enzyme in food, chemical and pharmaceutical industries and it is therefore industrially an important enzyme. Additionally, enzyme based biosensors for lactate detection is required LDH utilization. Finally, as being a redox enzyme, LDH has application area in biofuel cell systems, which has getting increasing interest in the recent years. However, the enzyme has to be immobilized without catalytic activity loss which requires orientation control at the close proximity to increase the efficiency for both biosensor and biofuel cell applications. In consequence of reduced retained activities and efficiencies in electron circulation due to conventional surface immobilization techniques, we utilized GEPI mediated immobilization for LDH attachment onto gold surfaces. AuBP2 peptide region was genetically fused to the N-terminal of the enzyme. Enzyme activity analysis of the fusion construct indicated that AuBP2 insertion doesn't effect the enzyme activity. On the other hand, gold binding kinetics of fusion construct concluded that AuBP2 insertion resulted in gold binding ability to LDH enzyme. As expected, the immobilized enzyme was still keeping its activity. Even, at the end of the 30th day, the relative activity of the immobilized enzyme was about %100 compared to free enzyme. Finally, redox catalysis efficiency of the enzyme was investigated by using cyclic voltammetry. The data enabled the detection of reducing reaction catalysed by LDH, in correlation with increasing substrate concentration. These data together provides that LDH immobilized by AuBP2 could be used for biosensor and biofuel cell applications.

Overall, our results demonstrate that inorganic binding peptides are new type of surface functionalization linkers that provide functional biomolecules, ie. proteins and enzymes to be immobilized on the desired surfaces with orientation control at a close proximity through self assembly of the fusion molecules. This self-organization has potential in diverse technological areas from monitoring to energy conversion.

İNORGANIKLERE ÖZGÜN BAĞLANAN PEPTİDLERİN NANO-BİYOTEKNOLOJİK UYGULAMALARI

ÖZET

Bir çok organizma protein anorganik etkileşimlerinden meydana gelen organik-anorganik hibrid sistemler içerir. Bu hibrid sistemlerin koruyucu tabakalar ve destekleyici dokular oluşturmak, yük ve iyon transferi yapmak, bazı optik ve mekanik özellikler geliştirmek gibi mükemmel fonksiyonları mevcuttur. Çeşitlendirilmiş fonksiyonlara sahip bu sistemler, biyoteknoloji ve nano-biyoteknoloji alanında kullanılabilecek yeni sistemlerin tasarımında örnek teşkil eder (biyobenzetim). Anorganik yüzeyleri tanıma ve kuvvetli bir şekilde tutunma özelliğine sahip olan peptitlerin (GEPI) kullanılmasını esas alan bir yaklaşım ile nanoparçacık ve biyomoleküllerin istenen anorganik yüzeye immobilize edilmesi ya da yeni özellikte nanoyapıların sentezlenmesi mümkündür.

Biyomoleküllerin anorganik yüzeylere immobilizasyonları aktivite ve stabilite artışı sağlaması amacıyla önemlidir. Kimyasal immobilizasyon yöntemleri ile elde edilen sistemler biyomolekülün aktivitesinde azalmaya ya da kayba neden olabilmektedir. Bu nedenle GEPI'ler aracılığı ile immobilizasyon denenmiş ve başarılı örnekler elde edilmiştir. GEPI aracılığı ile immobilizasyonda peptidler, biyolojik moleküle birlikte üretilerek çift fonksiyonlu bir molekül elde edilir. Molekül peptidten gelen yüzey tanıma özelliği ile matrikse tutunurken, biyomolekülü oluşturan bölge istenen biyolojik fonksiyonu göstermeye devam eder. Bu tür bir tutuklanma sisteminde protein yapısının kimyasal reaksiyonlarla bozunması ya da yüzeye kontrolsüz olarak bağlanması engellenmiş olur. Bu çalışmada altın yüzeye özgün bağlanan iki farklı peptid sırasıyla Huntingtin proteinini ve Lactate dehidrogenaz enziminin altın yüzeylere tutuklamak amacıyla kullanılmıştır.

Çalışmanın ilk kısmında altın yüzeye özgün bağlanan GBP1 peptidi, Huntingtin proteininin N-terminal ucuna eklenerek proteinin altın sensör yüzeyine bağlanmasına aracılık etmek amacıyla kullanılmıştır. Huntingtin proteini mutant formunda Huntington hastalığına neden olan ökaryotik bir proteindir. Proteinin mutant formu N-terminal bölgesindeki glutamin tekrarlarının sayısında artış meydana gelmiş halidir. Normal protein 35 ve daha az glutamin tekrarı içerirken, mutant proteinde bu sayı 110 a kadar ulaşabilmektedir. Glutamin tekrarlarındaki artış miktarı hastalığın seyri ile doğru orantılı bulunmuştur. Hastalığın en belirgin hücre patolojisi sitoplazma ve nukleusta inklüzyon cisimciklerinin gözlenmesidir. Inklüzyon cisimcikleri mutant proteinin agregatlar oluşturmaları sonucu meydana gelir. Bir grup araştırmacı hastalık patolojisinde etkin mekanizmanın protein agregasyonu sonucu oluşan fibril yapıların varlığı olduğunu düşünmektedir. Ancak fibril yapıların sayısında azalma olmaksızın hastalık patolojisinin engellenebildiği durumların varlığı, bazı araştırmacıların fibrilasyonun aslında hücrenin korunma mekanizması olduğunu düşünmelerine yol açmıştır. Hastalık oluşumunun daha iyi anlaşılabilmesi ve etkin tedavi yöntemlerinin geliştirilmesi için Huntingtin fibrilasyon mekanizması

çalışılmaktadır. Ancak şu ana kadar farklı fibrillasyon basamaklarında meydana gelen agregasyon kinetiği nicel olarak araştırılmamıştır. Biz yüzey plazmon rezonans spektroskopisini kullanarak (SPR) Htt fibrilasyon kinetiğini incelemeyi hedefledik.

Yüzey plasmon rezonans spektroskopisi çalışmalarında proteinlerin altın sensor yüzeyine immobilize edilmeleri gerekmektedir. Altın yüzeyine protein immobilizasyonunu gerçekleştirmek için GBP1 kullanılmıştır. GBP1-Htt füzyon proteinleri hem fibril hem de proto-fibril formlarda SPR yüzeyine tutuklanmışlardır. Ardından monomer haldeki Htt proteinleri yüzeye tutuklanmış fibril yapıların üzerine gönderilerek bağlanma kinetikleri incelenmiştir. Kontrol olarak Htt fibril ve proto-fibril yapıları altın yüzeyine kovalent immobilizasyonla tutuklanarak da deneyler tekrar edilmiştir. Kontrol çalışmaları GBP1 aracılıklı immobilizasyon ile en az kovalent tutuklanma kadar etkin bir immobilizasyon gerçekleştirildiğini göstermiştir. Ayrıca fibril ve proto-fibril uzamasının kinetiğinin incelenmesine olanak sağlamıştır.

Elde edilen veriler, fibril uzaması için iki basamaklı bir fibrillasyon modeli önerilmesine sebep olmuştur. Model, ilk basamakta monomerin fibril ucuna eklenmesini, ikinci basamakta ise konformasyonel değişimini içermektedir. Bu konformasyonel değişim sonucu monomer artık fibrilin bir parçası haline gelmiş ve yeni eklenecek olan monomer proteinlerle etkileşmeye hazır hale gelmiştir. Proto-fibril uzamasında ise tek basamaklı bir fibrilasyon kinetiği modeli önerilebilmektedir. Bu modele göre monomer proto-fibril ucuna eklenmiş ve uzamaya neden olmuştur. Fibril ve proto-fibril uzamaları için elde edilen denge sabitleri, fibrilasyonun başlangıç aşamalarında daha yavaş olduğunu göstermiştir. Bu veri nükleasyona dayalı polimerizasyon olarak değerlendirilen Huntingtin fibril uzamasını destekler niteliktedir. Bu modelde fibrilasyonun başlaması için öncelikle bir nükleus oluşumu gerekliliği ve bu basamağın da hız kısıtlayıcı basamak olduğu düşünülmektedir. Proto-fibril uzamasının da bu nedenle fibril uzamasından daha yavaş olması anlamlı bulunmuştur. Çünkü proto-fibriller aslında nükleus dan daha büyük ancak henüz fibril büyüklüğüne ulaşmamış ara yapılardır. Elde edilen fibrilasyon kinetiği verileri ile hastalık tedavisi için geliştirilen olası ilaç prokürsörlerinin agregasyona etkileri araştırılabilir. Ayrıca GBP1 aracılıklı immobilizasyon diğer agregasyon profili gösteren proteinlerin kinetiklerinin incelenmesinde de kullanılabilir bir yöntem olarak sunulabilir. Bu sayede Huntington benzeri nörodejeneratif hastalıkların da araştırılması mümkün görülmektedir.

Çalışmanın ikinci kısmında diğer bir altına özgün bağlanan peptid olan AuBP2 (WALRRSIRRQSY), LDH enziminin N-terminaline eklenmiş ve enzimin altın yüzeyine immobilizasyonunda kullanılmıştır. Laktat dehidrogenaz enzimi birçok organizma tarafından üretilmektedir. Ancak bu çalışmada aktitesi ve üç boyutlu yapısı aydınlatılmış olanı termostabil organizmalardan *Bacillus stearothermophilus* tarafından üretilen enzim (bsLDH) kullanılmıştır. bsLDH NADH/NAD⁺ kofaktör çiftini kullanarak pürivat (oxo asid) ve laktat (hidroksi asid)'ın dönüşümünü katalizlemektedirler. Reaksiyon ürünleri yarı-sentetik penisilin ve antihipertensifler gibi farmasötiklerin yapımı ve fenilketonüriinin tıbbi tanısında kullanılmaktadırlar. Ayrıca diyagnostik alanında laktat miktarının tayininde yararlanılan bir enzimdir. Son zamanlarda ise nano boyutlu bio-enerji sistemlerinin geliştirilmesinde bir redoks enzimi olan LDH'in direk elektron transferini sağlama özelliğinden yararlanılması üzerine çalışılmaktadır. Hem biyosensör hem de biyo-enerji sistemlerinde LDH'den yararlanabilmek için uygun bir matrikse immobilize edilmesine ihtiyaç vardır.

Bu çalışmada, kimyasal tutuklama yöntemlerine alternatif olarak LDH immobilizasyonunu AuBP2 peptidi aracılığı ile gerçekleştirilmiştir. AuBP2 peptid dizisi, rekombinant DNA teknoloji kullanılarak LDH proteinin N-terminal ucuna eklenmiştir. Oluşturulan füzyon proteinin LDH aktivesi göstermeye devam edip etmediği enzim aktivite tahlilleri ile test edilmiştir. Sonuçlar AuBP2 eklenmesinin LDH aktivitesinde ciddi bir azalmaya sebep olmadığını göstermiştir. Ardından yüzey plazmon resonans spektroskopisi kullanılarak füzyon proteinin altın yüzeye bağlanma ilgisi araştırılmıştır. SPR sonuçları AuBP2 varlığında enzimin altın yüzeye ilgisinin on kat kadar arttırıldığını ve yüzeye tutuklandıktan sonra uzaklaştırılmadığını göstermiştir. Bu çalışmalar ile AuBP2-LDH füzyon proteinin hem altın yüzeye bağlanabildiği hem de LDH aktivitesi göstermeye devam ettiği bulunmuştur.

Füzyon proteinin immobilize halde iken ne kadar aktif olduğu da enzimler altın nano parçacıklara tutuklanarak incelenmiştir. Enzimlerin immobilizasyon sonrası serbest enzime kıyasla %80 civarında aktivite gösterdikleri bulunmuştur. Enzim immobilizasyonlarında %50 üzerinde immobilize aktivite elde etmenin iyi bir veri olduğu düşünüldüğünde AuBP2 ile immobilizasyonun etkinliği görülmektedir. Ayrıca immobilize enzimlerin 30 güne kadar aktivitelerini korudukları da izlenmiştir. Serbest enzimler ise 30 gün sonunda immobilize enzime kıyasla daha fazla aktivite kaybına uğradıkları gözlenmiştir.

Son olarak, LDH enziminin redoks reaksiyonunu incelemek amacıyla siklik voltametri çalışması yapılmıştır. Bu çalışmada AuBP2-LDH altın elektrod yüzeyine immobilize edilmiştir. Sisteme substrate olarak laktat ve kofaktor olarak NAD beslenmiştir. Elektrod yüzeyine potansiyel farkı uygulayarak, redüklenme ve oksitlenme reaksiyonları izlenmiştir. Siklik voltametre analizlerinde enzimin NAD redüksiyonu gözlenmiştir. Bu gözlem, hem biyosensör hem de biyo-enerji sistemlerinde elde edilen redüklenme miktarından daha yüksek bulunmuştur. Bu sonuç, AuBP2 gibi çok küçük bir molekül ile yüzeye tutturulan enzimin, elektroda yakınlığı sonucunda elektrod ile enzim arasındaki elektron transferine olanak sağlandığını düşündürmüştür.

Tez çalışması genel olarak incelendiğinde altına özgün bağlanan peptidlerin hem protein hem de enzim immobilizasyonunda etkin olarak kullanılabileceği görülmüştür. GEPI aracılıklı immobilizasyon ile huntington hastalığı gibi protein agregasyonuna dayalı diğer nörodejeneratif hastalıkların da çalışılması mümkündür. Ayrıca sensör yüzeyine yaklaştırılmak istenen enzimlerin GEPI aracılıklı immobilizasyon ile yüzeye tutuklanmaları olanağı vardır.

1. INTRODUCTION

1.1 Inorganic Binding Peptides

Biological hard tissues are hybrid systems, composed of organic phase together with inorganic materials. These biocomposites employ proteins, lipids or carbohydrates as organic phase in combination with inorganic materials such as hydroxyapatite, silica and magnetite. Because of their molecular recognition ability, proteins, specifically inorganic binding proteins, interact with and organize inorganic materials to perform protective layer formation, ion transferring and developing some optical and mechanical properties. Inspired by nature, hybrid materials can be developed combining molecular biology tools with nanotechnology under the name “Molecular Biomimetics” to overcome difficulties encountered in nano- and nanobio-technological applications.

1.1.1 Importance of inorganic binding peptides in nature

Nature provides great examples of materials and systems with enormous functions. These various functions are evolutionarily improved and accounted for elegant properties of biological systems for a given condition. These amazing formations could be seen even in the single cell organisms; such as magnetotactic bacteria, *Aquaspirillum magnetotacticum* [1, 2]. One of the organelles of this bacterium contains magnetite (Fe_3O_4) nanoparticles that are aligned to sense Earth's magnetic field and allows the organism's motility by magnetotaxis. These single crystal nanoparticles are formed and held together with protein and lipid-based closed membrane (Figure 1.A).

Mother-of-pearl of seashells is another example for inorganic-organic hybrid systems. The shell is a layered structure composed of aragonite (orthorhombic CaCO_3) and mixture of proteins and polysaccharides (Figure 1.B). This unique organization results with higher toughness and fracture strength [3].

Mammalian tooth is another unique architecture as providing the hardest tissue of the body, dental enamel. Enamel, covering the tooth and integrating with the soft tissue

(dentin), adsorbs the stress during cutting and chewing. In the structure, proteins are seen to be controlling the formation of enamel rods, elongated hydroxyapatite (HA) crystals (Figure 1.C). In case of abnormal protein production, this unique architecture is deformed resulting with premature failure or fracture [4].

Magnetotactic bacteria, seashell and enamel are best but not the least examples of high hierarchical organizations of organic-inorganic systems. Examples could be extended to magnetite (Fe_3O_4) particles in teeth of chiton [1]; silica (SiO_2) as tiny light-gathering lenses and optical waveguides in sponges [5] and hydroxyapatite ($\text{Ca}_2\text{C}(\text{OH})_3$) in bones [6].

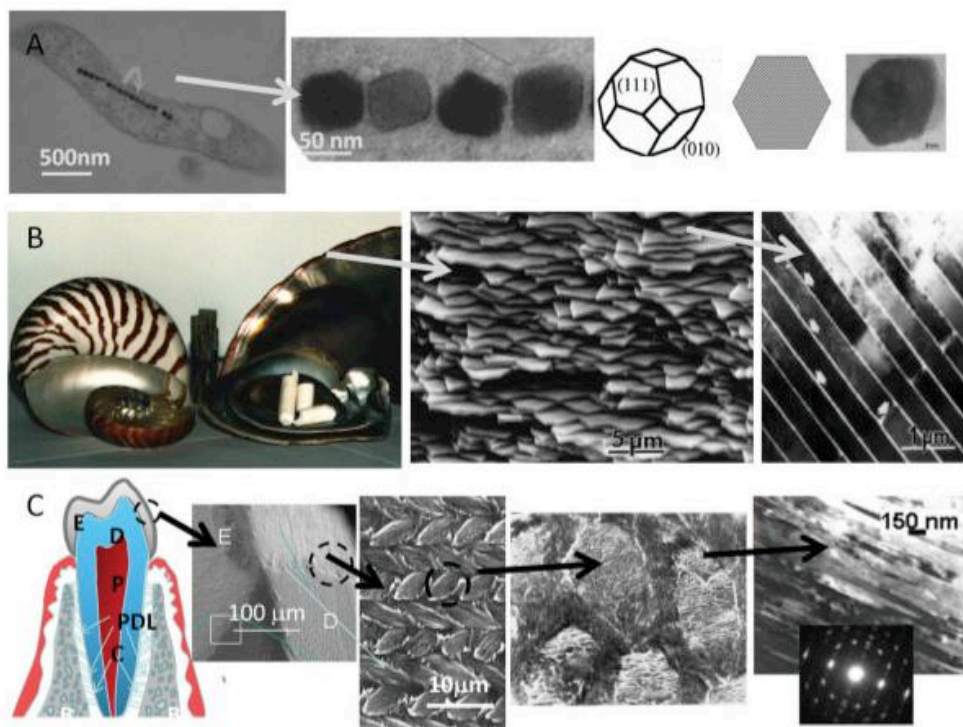


Figure 1.1 : Examples of functional biological materials systems. (A) Magnetotactic bacteria, *Aquaspirillum magnetotacticum*, have aligned cubo-octahedral-shaped superparamagnetic particles. (B) Nacre, mother-of-pearl is a segmented laminated composite of calcium carbonate and biomolecules having mechanical properties that provide armor. (C) Mammalian tooth is a hierarchical multimaterial system composed of enamel (E), dentin (D), pulp (P), cementum (C) and periodontal ligaments (PDL). Enamel, such as the one shown here from mouse, is *100% hydroxyapatite crystals that have a hierarchically ordered woven structure providing stability against complex mastication mechanical stresses [7].

In the long run, wherever is looked in hard tissue organizations, biological molecules, especially proteins are playing the critical role. Exhibiting enormous

diversity of functions, they consequently perform most of the biological processes such as cellular structure formation, catalyzing biological reactions and controlling physical conditions of biomaterials. In fact, proteins constitute the most valuable part of tissues by serving as transporters and self- and co-assembly into short and long range ordered substrates [8-11]. Variable properties of proteins rely on the fact that they are highly specific and selective towards their ligands. Proteins interact with other macromolecules and inorganics to control the structures and the functions of biological systems [8, 9]. While macromolecular interactions are observed in soft tissues such as muscle, skin and etc. [12], macromolecular-inorganic hybrids form the basis of hard tissues [7, 8, 13].

1.1.2 Importance of inorganic binding peptides in biotechnology

Technological improvements and life facilitating ideas are mainly originated from Nature. Performed more observations on nature and detailed research on it's solutions to problems, resulted in nature imitating technologies such as velcro, imitating the attachment mechanism of plant burrs and paints mimicing the superhydrophobic and self-cleaning nature of Lotus leaf.

In materials science and engineering field, traditional material synthesis requires melting and solidification processes, followed by thermo-mechanical treatments, or solution/vacuum deposition and growth processes. These processes are accomplished in ambient conditions such as high temperature and pressure. Moreover, toxic byproducts are possibly produced during the synthesis [9]. On the other hand, nature produces ordered and complex nanostructured materials in aquaous environment under mild conditions such as ambient temperature, pressure and neutral pH.

As exemplified in Figure 1.1 and detailed in section 1.2.1, inorganic material synthesis in nature is under the control of proteins, namely inorganic binding proteins. Inspiring from nature, an emerging field, Molecular Biomimetics is utilizing the molecular recognition ability of inorganic binding proteins for the assembly of hybrid materials in molecular scale. This approach enables the selection, biological expression and further modification of inorganic binding proteins/peptides and combines them with solid surface science to result in new nanotechnological materials and devices [7, 9, 13-15]. Recent studies of Sarikaya and co-workers

indicated the great potential of these peptides for various applications in different areas such as nanobiotechnology, medicine and nanoelectronics [7, 9, 13-15]

1.1.3 Selection and characterization of inorganic binding peptides

There are naturally occurring inorganic binding proteins as mentioned in previous sections. The most known example of these is ice-binding protein, which is identified, extracted and characterized [16]. There are possible ways to identify and obtain natural inorganic binding proteins. They could be extracted and purified from hard tissues, then the indicating genes could be cloned for further applications [17, 18]. However this approach is limited with the known natural hybrid systems besides being time consuming among the long purification processes. For unidentified proteins, theoretical molecular approaches could be applied using computer technologies, which is an impractical and expensive way [19].

Besides the complications of inorganic binding protein identifications by previously explained ways, utilizing the whole protein for material science applications is not applicable for nanotechnology. Therefore, as a following step, small regions of those proteins, which are the responsible sides for inorganic recognition, growth or nucleation has also to be determined. By using combinatorial biology approaches, peptide sequences could be selected through their inorganic surface recognition and binding abilities [20]. Since peptides are shorter sequences, amino acid motifs for inorganic binding function could be explored and help to clarify molecular binding mechanisms. Moreover, they could be genetically engineered for improved functions or fused with other molecules to gain multifunctional molecular constructs.

Combinatorial biology approach depends on the selection of peptides against a specific inorganic material from combinatorial biology libraries [9, 14]. These libraries composed of random peptides with the same number of amino acids but different in sequence compositions. “Phage Display” and “Cell Surface Display” are well-known *in vitro* combinatorial biology techniques [21, 22]. These display techniques mainly were carried out to perform the characterization of receptor and antibody binding sites, the study of protein-ligand interactions and the isolation and evolution of proteins or enzymes exhibiting improved or altered binding characteristics for their ligands [23] are also adapted for the selection of inorganic binding peptides [9, 14, 23] (Figure 1.2). Sarikaya and co-workers called the peptides

they selected towards various inorganic surfaces as “Genetically Engineered Peptides for Inorganics” –GEPI-. Both this group and the others selected many peptides for solid surfaces up to date. Among them; gallium arsenide, silica, silver, zinc sulfide, calcite, cadmium sulfide, mica and noble metals such as platinum and palladium, iron oxide, gold, zinc oxide, zeolites and cuprous oxide specific binders could be enumerated [24-36].

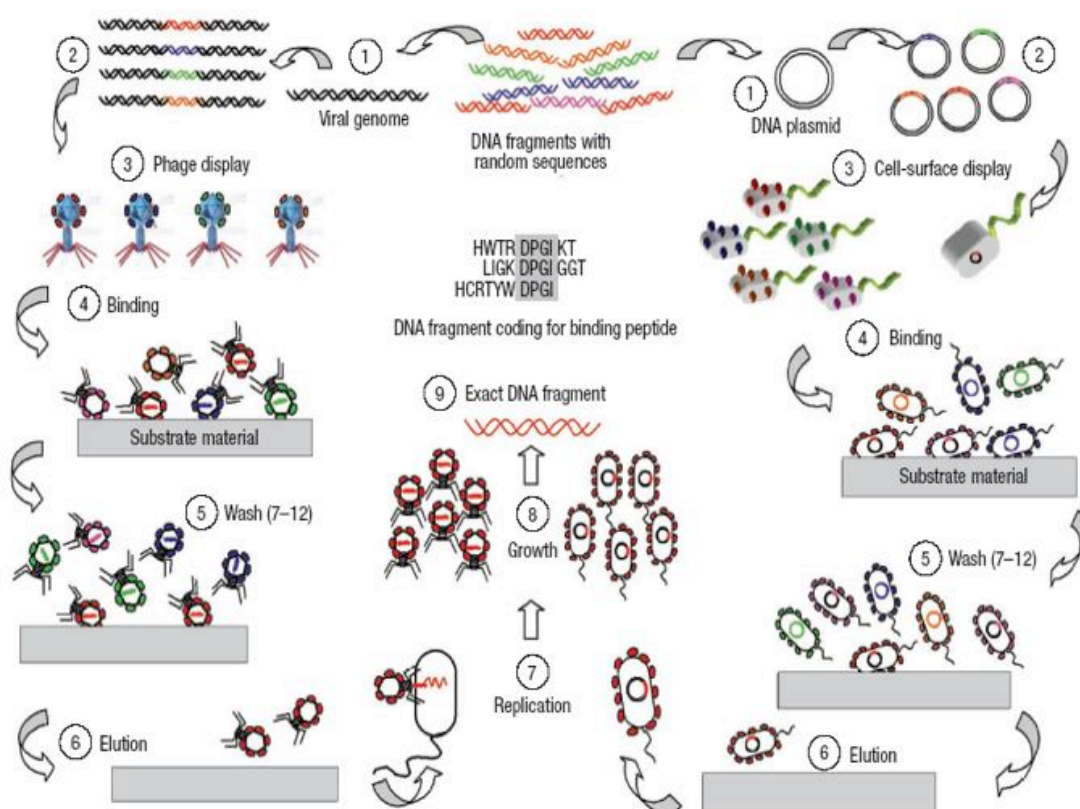


Figure 1.2 : Phage display and cell-surface display. Principles of the protocols used for selecting polypeptide sequences that have binding affinity to given inorganic substrates [14].

While discussing GEPI selection, it should be kept in mind that combinatorial selection ends up with 30-50 peptide sequences for a given inorganic. These peptides could be used for any application without any further characterization and/or improvement applied via mutations. However, characterization of the peptides could lead to obtain much selective and strong binders and insight about molecular recognition of inorganic materials. Sarikaya’s group optimized various techniques for the molecular binding characterization of GEPIs [37]. The first step they apply is a semi-quantitative binding assay using Fluorescent Microscopy (FM) imaging. In this technique, affinities of selected phage or cell clone’s surface coverages are

estimated via immunolabelling with anti-M13 antibodies or DNA-binding fluorescent dyes, respectively. FM chracterization lead researchers to classify experimentally selected peptides into three groups according to their binding affinities; strong, moderate and weak [7, 9, 14]. These first generation peptides, than, are used in sequence alignment methods and standart scoring matrices to generate a new “material-specific sequence scoring matrix” that can determine specific sequence patterns responsible for strong and weak binding. When a scoring matrix for an individual inorganic is applied to millions of amino acid sequences, peptides with highest and lowest similarity scores are defined as strongest and weakest binders and named as *de novo* peptides for a given inorganic [38, 39].

Although selecting and designing inorganic binding peptides could give specific and strong binders, their solid surface recognition affinities should also be determined quantitatively. Surface Plasmon Resonance Spectroscopy (SPR) and Quartz Crystal Microbalance (QCM) are the applied techniques for real time monitoring of peptide binding on solid surfaces and enable to calculate adsorption and desorption kinetics together with thermodynamics under various buffer and temperature conditions (Figure 1.3.a) [40-43].

Since the conformation of the peptide is the key factor for understanding the binding mechanism, Nuclear Magnetic Resonans (NMR) Spectroscopy can be applied for the determination of molecular conformation of GEPIs (Figure 1.3.b). While peptides are short sequences, they exhibit different secondary structures in solution. Using Circular Dichroism (CD), the structure of GEPI in solution can be identified (Figure 1.3.b). [34, 44-46]

A complementary method to gain conformational information is the computational modeling. Computational modeling tools enable researches to predict the molecular conformation, find out the amino acids close to the solid surface and estimate the mechanism of crystallographic surface recognition (Figure 1.3.c). [35, 47]

Atomic Force Microscopy (AFM) is another characterization method practiced by resaerchers for monitoring the peptides surface interaction. Moreover, AFM imaging provides insight for supramolecular arrengment and self-assembly functions of GEPI (Figure 1.3.d) [48, 49].

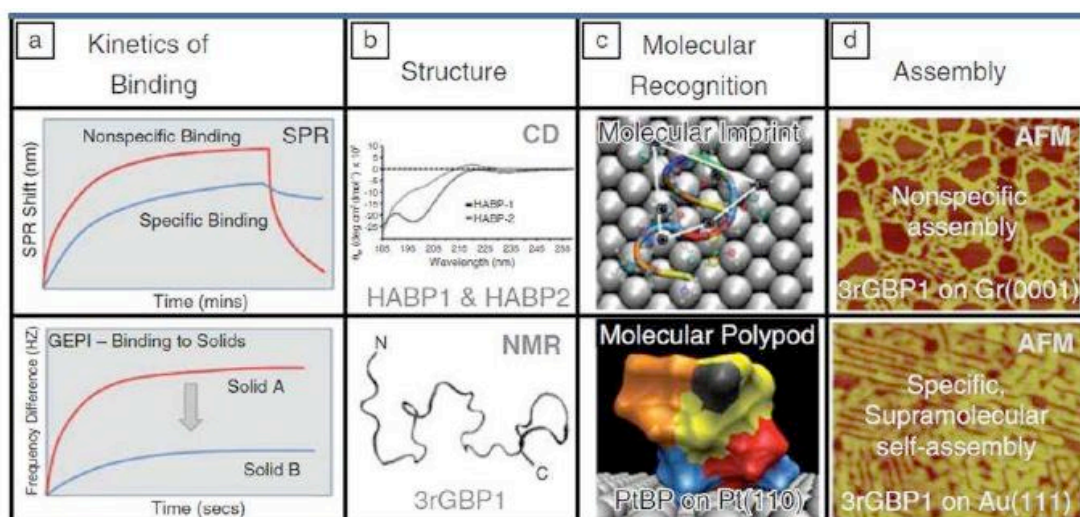


Figure 1.3 : Binding and Structural Characterization of GEPIs. a) SPR and QCM determination of the specificity and kinetics of peptide binding on solid surfaces. b) The detailed molecular structure characterization of GEPI by (CD) and (NMR) spectroscopy. c) Computational modelling of GEPIs for understanding the molecular conformation and mechanism of crystallographic surface recognition. d) Supramolecular self assembly of gold binding peptide on graphite (0001) and Au [37].

All characterization techniques explained above and applied for determination of molecular recognition mechanisms of GEPI are more appropriate when utilized in combination for each individual inorganic binding peptide. As our knowledge on an individual peptide's characteristics increase, further tailoring of this peptide would be possible for designing new hybrid materials.

1.1.4 Current and potential applications of GEPIs (Genetically Engineered Peptides for Inorganics)

Genetically engineered peptides have the function of controlled binding and assembly on inorganic surfaces. As biological molecules, GEPI can be genetically modified to serve as a biolinker and assembler for biological molecules. Subsequent to the chemical modifications, they can also be used for the attachment of chemical molecules on a desired surface. Besides these, GEPI has the ability for controlled synthesis of nano-inorganics. [7, 37]

Acting as linkers, GEPI can be an alternative way for conventional immobilization techniques for the assembly of nano-components on material surfaces [50, 51]. In a study, biotinylated peptides; gold binding peptide (AuBP2), three repeat of platinum binding peptide (3R-PtBP) and silica binding peptide (QBP1) were used to

immobilize streptavidin conjugated quantum dots (SAQ) on a micropatterned surface [7, 34, 52]. Immobilization was controlled with the red color that emitted by SAQ under fluorescent microscope (Figure 1.4). Following this proof-of-principle experiment, researchers demonstrated the self-assembly of quantum dot emitters at a material specific location in a color-conversion LED containing several material components including a metal, a dielectric and a semiconductor. Material specific location of SAQ was achieved by QBP1 peptide in this study. And showed that, photoluminescence magnitude could be increased by peptide-decorated quantum dot immobilization [53]. The same peptide, in another study, inserted at the semiconductor-dielectric interface to precisely control the threshold voltage of pentacene-based organic thin film transistor [54]. By this way, it's demonstrated that GEPI modification can adjust the threshold voltage, which is a crucial requirement for integration of transistor into electronic circuits.

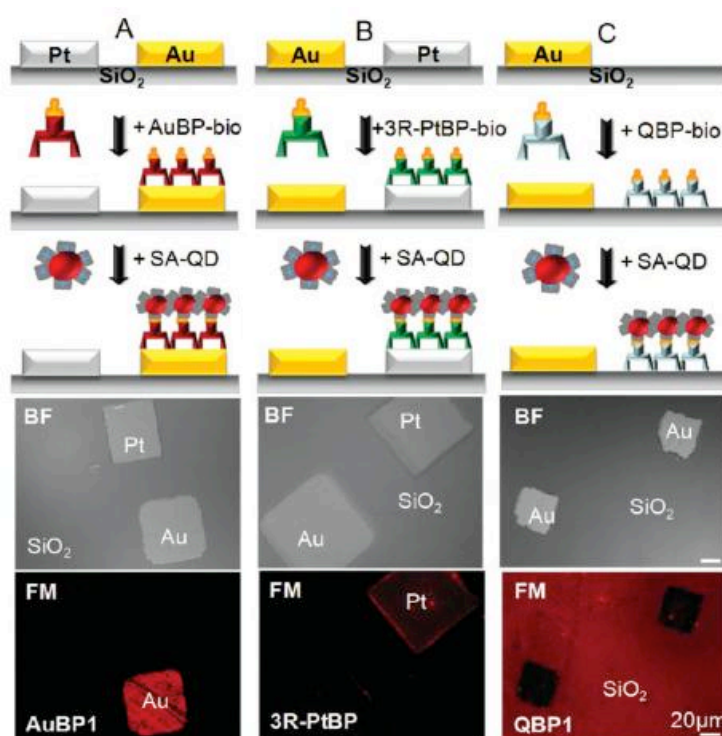


Figure 1.4 : Preferential assembly of SA-QD through GEPI biotin conjugates on different inorganic surfaces. The substrates composed of either Pt and Au or only micropads on silica were incubated with either AuBP-bio (A), or 3R PtBP-bio (B) or QBP-bio (C). After the procedure schematically shown for each peptide was carried out the substrates were examined under FM [7].

GEPI could also be conjugated with small molecules such as organic dyes and thus, used as bio-inks for different lithography techniques. Figure 1.5 shows an example

for Dip-pen-nanolithography (DPN) patterned surface, modified with three different biotinylated peptides (QBP1 and QBP3; silica binding peptides. 3R-GBP1; three repeat of gold binding peptide), which were labeled with streptavidin-fluorophore conjugate. [55]

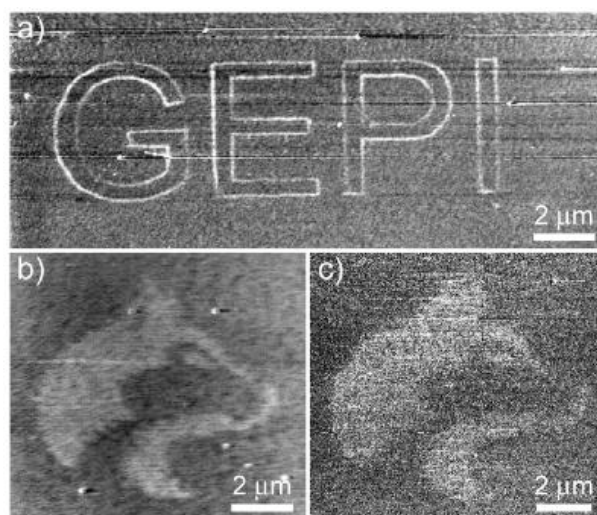


Figure 1.5 : LFM image of DPN-patterned a) silica-binding peptide (QBP1) on SiO₂, b) another silica-binding peptide (QBP3) on SiO₂, and c) gold-binding peptide (3R-GBP1) on gold [55].

Instead of using an affinity conjunction between the GEPI and the nano-material, as exemplified in figure 1.4 and previous studies, two inorganic binding peptides can be fused to obtain a bifunctional molecule. In this way fused QBP1-AuBP2 peptide conjugate was used for the immobilization of gold nanoparticles on silica surface without requiring any surface modifications [56]. This bifunctional peptide not only immobilizes gold nanoparticles, but also could synthesize them on the surface. Gold nanoparticle synthesis by surface attached peptides is the next development follow-up the nanoparticle growth acceleration and morphology altering in solution, achieved by another gold binding peptide GBP1 [57].

As implicated in previous example, GEPI can synthesis and control the growth and morphology of nanoparticles. However, they are not only acting on metal materials but also on bioceramics. Gungormus *et al.* explained how hydroxyapatite-binding peptides (HABP1; strong binder, HABP2; weak binder) could control the formation and morphology of HA (hydroxyapatite) crystals. Their *in vitro* biomineralization studies indicated that structurally different peptides affect the mineralization kinetics and size of crystals formed [44]. In a further study, *in vitro* biomineralization

mediated by HABP1 was triggered both with biochemically and cementoblast cells, in a hydrogel matrix [58]. The peptide-based hydrogel system, they developed, showed the potential of GEPI utilization in medical area such as tissue engineering application.

The main concern for implant materials and tissue engineering applications is the uncontrolled interactions between the material and human tissue. Therefore, synthetic material surface is under modification either for improved cell adhesion or antibacterial properties. GEPI has the potential of surface modification under ambient conditions and without producing any toxic byproducts, which could be lethal for medical applications. Khatayevich *et al.* demonstrated the applicability of this propotion in their study [59]. They successfully imparted cell-resistance properties to gold and platinum surfaces by using the gold- and platinum binding peptides conjugated with polyethyleneglycol (PEG). Also induced cell adhesion and spreading of fibroblast cells on glass and titanium surfaces, which were modified with silica- and titanium binding peptides fused with integrin ligand RGD (Figure 1.6).

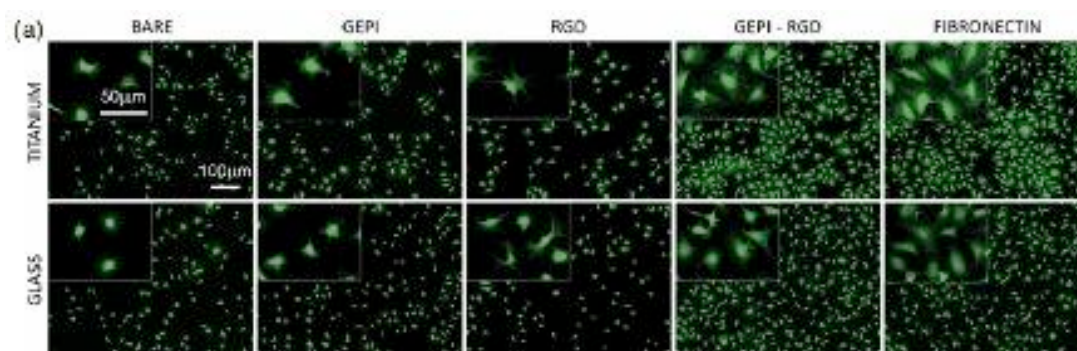


Figure 1.6 : FM micrographs of phalloidin stained NIH 3T3 cells on glass and titanium surfaces [59].

As being biological molecules, GEPIs are suitable for DNA level modification such as site-directed mutations, gene level multimerizations and fusions with other biomolecules. Using this opportunity, GEPI could be utilized as a fusion partner of proteins and enzymes, thus direct their immobilization onto the desired surface and/or nano-material. Using peptides for protein immobilization instead of conventional methods help to overcome the disadvantages of these processes such as uncontrolled assembly, activity loss and denaturation. These possible advantages of GEPI-mediated immobilization were demonstrated in three different studies. In the first study, Alkaline Phosphatase enzyme was fused with GBP1-gold binding

peptide- for direct immobilization of the enzyme onto gold surface [60]. Here, they demonstrated the potential of GEPI as a site-specific molecular linker for oriented enzyme immobilization with retained activity. In another study, Sedlak *et al.* inserted 5R-GBP1, 7R-GBP and QBP1 peptides into a permissive site of tral gene coding a DNA binding protein, respectively. Fusion constructs were both able to bind DNA with the activity of the Tral protein and interact with gold/silica nanoparticles using the inorganic binding ability of GEPIs [61]. In a recent study, Yuca et al. constructed GFPuv-HABP1 (Green Fluorescent Protein-HA binding peptide) fusion protein and reported that this multifunctional protein can targets calcium phosphate minerals and allows monitoring the biomineralization under fluorescent light [62].

The literature and examples reported in this section clearly explicate the potential applications of GEPIs as molecular assemblers, erectors and signalers [7]. The studies accomplished till the date should be accounted as baby steps. Towards the developments in molecular biology tools for tailoring GEPIs and open-minded applications on hybrid material and device production will speed up the construction of bio-nanotechnological systems. These systems will potentially developed in many areas including, not only materials science and engineering but also nano-photonics, nano-optics, nano-electronics and medicine.

1.2 Protein Immobilization Methods

For decades DNA and RNA microchips were in the center of array systems. Recently proteins came forward in sensing technologies, because of their diverse functions and specific interactions with other micromolecules. In time, protein biosensors became one of the core biotechnological systems not only for sensing applications but also for the detection of receptor ligand interactions, drug discovery, enzyme activity analysis, and antigen-antibody recognition and even in fuel cell constructions [63-67].

In order to build biosensors, ligand molecule (DNA, RNA or Protein) has to be attached to a solid surface. Thus, small amount of ligand is used to obtain a reuseable system with increased stability of biomolecule. However, the main consideration is choosing the right immobilization strategy that doesn't effect the conformation and function of the molecule for retained biological activity. DNA immobilization is easier in comperative with proteins, due to their uniform structure, stability, high

resistance to activity loss and containing one interaction site with its target. While proteins have various different structures, include hydrophobic and charged regions, are sensitive to any changes in structure causing activity loss and contain different interaction sites. Therefore, random orientation causing closure of interaction sites and structural deformations resulting with activity loss should be avoided during protein immobilization.

Generally, the choice of the right immobilization technique depends on the physicochemical and chemical properties of both the surface and the protein. There are many immobilization strategies developed for protein attachment onto different solid surfaces. These techniques can be categorized under three main mechanisms: physical, chemical and bioaffinity immobilization. Finally, a new strategy will be proposed based on GEPI-mediated immobilization.

1.2.1 Physical immobilization

Proteins can be adsorbed onto inorganic surfaces via intermolecular forces such as ionic bonds, hydrophobic and polar interactions [68]. Surface properties of the inorganic material directly affect the physical attachment of protein. Therefore, surface modification using self-assemble monolayers (SAM) could be applied to increase protein adsorption.

Hydrophobic interaction between the surface and the protein is more favorable than hydrophilic interactions [69, 70]. As the hydrophobicity of the surface increased, protein adsorption increases [70]. Moreover, insertion of hydrophobic regions into a protein structure could alter its adsorption behavior in terms of increasing the attraction more hydrophobic one [71].

In case of hydrophobic and charged surfaces both hydrophobic and electrostatic interactions take part. However, for proteins undergoing limited or no interfacial conformational changes, hydrophobic interaction is favored instead of electrostatic interaction [69]. Also, for proteins undergoing large conformational changes, during adsorption, hydrophobic interactions enter into instead of electrostatic interactions. Moreover, van der Waals interactions seem to be important between these proteins and the surface [69, 72]. On the other hand, at a hydrophilic and charged surface, adsorption mainly proceeds through electrostatic interactions as an attraction between the surface and the protein with opposite charge [69].

The adsorption capacity of flat surfaces is limited due to the geometric size of the protein to be immobilized. High density loading can block the active sites of the protein. Mainly, porous surfaces are preferred for physical immobilization applications due to their large surface area, pores able to adsorb protein and high loading capacity [68, 73].

Random orientation and weak attachment of the proteins are the main drawbacks of physical absorption [68]. Random orientation causes to activity loss and/or unreproducible activity in repeated trails. Whereas, weakly attached proteins could be washed away with buffers or mild detergents used during assays. Thus could originate to obtain false assay results.

1.2.2 Chemical immobilization

Mostly, proteins are covalently attached to an inorganic surface through the functional groups of exposed amino acids. Covalent bond is formed between these groups of the protein and the suitably modified functional groups on the immobilization support [68]. Thus, irreversible binding and high surface coverage is established. However, protein immobilization is mostly random oriented resulting with loss in biological activity [68, 74]. Ideally, only the proteins with one active amino acid for chemical attachment could be orientedly immobilized. In order to establish oriented (site specific) immobilization for a variety of proteins, modification of the protein or the surface or both is required [68, 75, 76]. Commonly used functional groups of proteins and surface functionalities required for proper chemical immobilization could be followed from Table 1.1.

Table 1.1 : Commonly used functional groups of amino acids in proteins and functionalities required for surfaces [68].

side groups	amino acids	surfaces
–NH ₂	Lys, hydroxyl-Lys	carboxylic acid active ester (NHS) epoxy
–SH	Cys	aldehyde maleimide pyridyl disulfide vinyl sulfone
–COOH	Asp, Glu	amine
–OH	Ser, Thr	epoxy

In order to produce functional groups on solid supports, chemical treatments are required. Self-assembled monolayers (SAMs) provide a convenient, flexible, and

simple system to tailor the interfacial properties of inorganic surfaces [77]. SAMs are organic assemblies that could be chemically attached onto solid surfaces and organize spontaneously into crystalline/semicrystalline structures [78]. The molecular structures of SAMs contain a chemical functionality provided by the headgroup, which presents the affinity for an inorganic surface (Figure 1.7). There are various headgroups that bind to specific metals, metal oxides, and semiconductors. Among them, the most extensively used SAMs are alkanethiols for metal surfaces (gold, silver, copper, palladium) and alkyl- and alkenesiloxanes for glass and aluminum oxide [78, 79]. While headgroup of a SAM provides surface binding, terminal functional group determines the properties of modified surface. A number of terminal groups could be generated in a SAM molecule as summarized in table 1.1, which will be a part of covalent bond formation between the protein and the surface during immobilization.

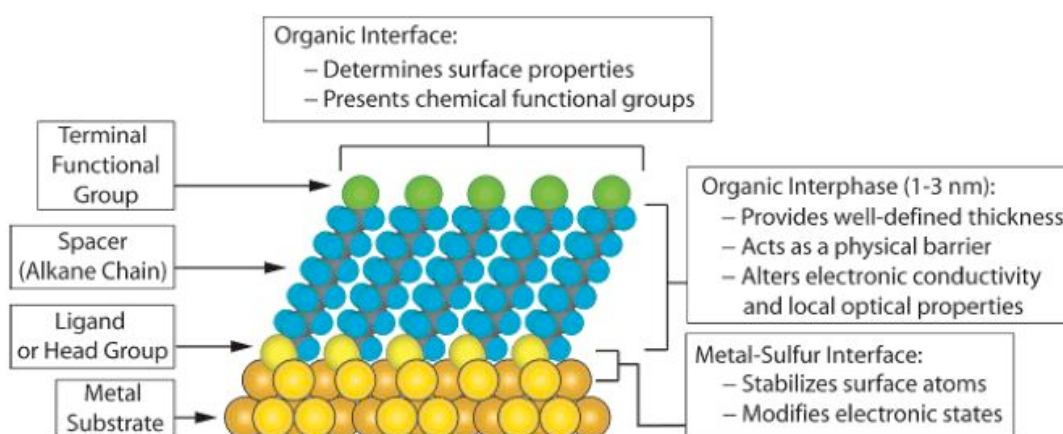


Figure 1.7 : Schematic diagram of an alkanethiolane SAM on a gold surface with a (111) texture [78].

As seen in Table 1.1, both proteins and SAMs contain various functional groups. Thus, different chemical immobilization strategies were developed for different functionalities. Above all, chemical immobilization could be discussed in two main categories depending on the orientation of the protein on solid surface; Non-specific immobilization and Site-specific immobilization.

Non-Specific Immobilization: In principle, all the functional groups given in Table 1.1. can react with a SAM coated surface. When amino group on the side chain of a lysine residue is reacted with the SAM to form the covalent bond, the strategy is called as amine chemistry. N-Hydrozysuccinimide (NHS) derivatized SAM can be utilized to form stable amide bond between the NHS ester and the amino group of

Lysine (Figure 1.8.a). Also, an aldehyde group containing SAM could react with amino group of Lysine residue. Here, a labile Schiff's base is formed that can be stabilized by reduction creating a stable amide bond (Figure 1.8.b). Although amine chemistry is widely used, the abundance of amino groups on protein surface creates the risk of multipoint attachments yielding heterogeneity and restricting conformation flexibility.

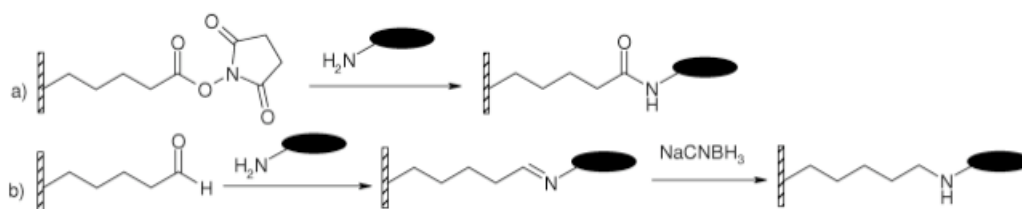


Figure 1.8 : Amine Chemistry on (a) NHS-Derivatized and (b) Aldehyde-Derivatized Surfaces [68].

Cysteine is another amino acid utilized for immobilization. The functional site used in attachment is a unique group, thiol group, in its side chain. Thiol chemistry utilizes different coupling approaches for cysteine residues to be immobilized on functionalized surfaces (71-73). Maleimide, disulfide and vinyl sulfone derivatized surfaces are effectively used in order to produce thioether or disulfide bonds between the protein and the surface (Figure 1.9). Since cysteines are not as abundant as lysines, random orientation is less likely to occur. On the other hand, reversibility of disulfide linkage in exposure to reducing agents is a problem in stable protein immobilization. Also, immobilization has to be achieved in aqueous/organic environment due to the insolubility of disulphide reagents in water [68].

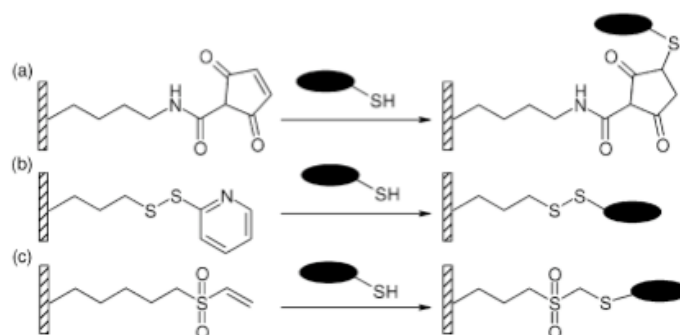


Figure 1.9 : Thiol Chemistry on (a) Maleimide-Derivatized, (b) Disulfide-Derivatized, and (c) Vinyl Sulfone-Derivatized Surfaces [68].

Carboxyl chemistry, on the other hand, depends on the immobilization via carboxyl groups found in side chains of aspartic and glutamic acid residues. Following the

activation of carboxyl groups of these molecules, chemical coupling of the protein with the amino groups on the surface is occurred (Figure 1.10). The main advantage of this strategy is the employment of mild conditions for protein activation.

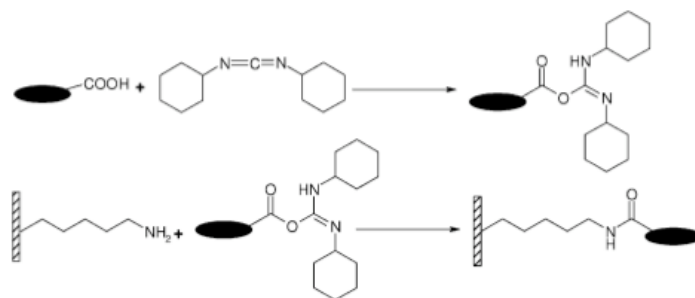


Figure 1.10 : Carboxyl Chemistry Using Carbodiimide Activation [68].

Photoactive chemistry is an alternative strategy for chemical immobilization. Photoimmobilization is based on the utilization of photosensitive reagents activated by UV light [68]. After activation, reagents undergo chemical processes leading to the formation of covalent bonds between the photogenerated surface and the protein [80]. Advantages of this strategy are being a single-step reaction, efficiency, fastness and applicability for biomolecules lacking active functional groups. Also, site-directed immobilization is applicable prior to the formation of patterned surfaces using masks during irradiation (Figure 1.11) [81, 82].

Site-Specific Immobilization: In order to orient proteins on surfaces to expose their active sites to the sample solution, site-specific immobilization has to be accomplished [68]. Therefore, different techniques have been developed. “Click” chemistry is one them, where an azide and alkyne are cycloadded to form 1,2,3-triazole [83, 84]. The effortlessness of introducing azides and alkynes into biomolecules such as carbohydrates and proteins and effectiveness on their stability make click chemistry a useful strategy [85]. Among these, triazole formation is irreversible, yielding a stable immobilization.

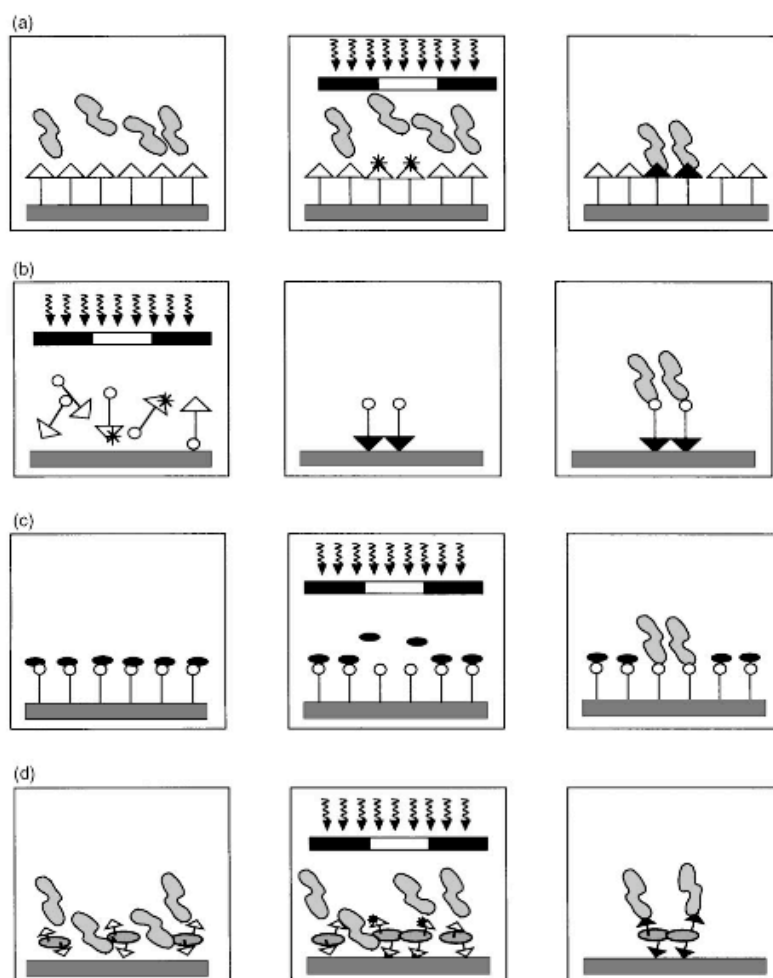


Figure 1.11 : Photochemical protein patterning. (a) A substrate derivitized with a photochemical molecule is incubated with a protein solution. Following the irradiation, activated regions bind protein. (b) A substrate incubated with a photochemical molecule is irradiated in localized regions. Activated molecules are localized at that regions and bind to the protein. (c) A substrate derivitized with a 'caged' molecule is irradiated, to remove caging group. Protein binds within the localized active regions. (d) A substrate incubated with a cross-linking polymer containing several photochemical species and a protein solution is irradiated, activating polymer within localized regions. The activated polymer binds to the substrate and the protein within these regions [82].

Similar to “click” chemistry, *Diels-Alder Cycloaddition* takes place between an electronically matched pair of a dienophile and a conjugate diene to form an unsaturated hexa-ring [68]. Diels-Alder-mediated immobilization has been used as a straightforward method for the preparation of peptide chips. Here, the surface, modified with a quinone group, is reacted with cyclopentadiene group conjugated to the peptide [86]. The main advantage of this technique is processing the reactions in water at room temperature.

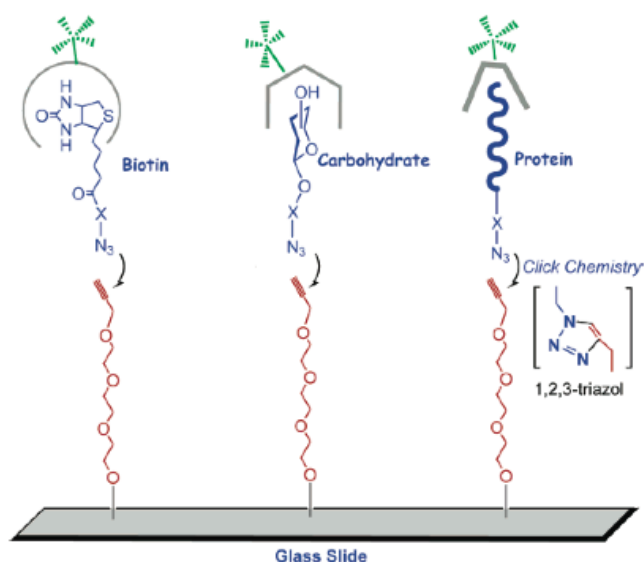


Figure 1.12 : Illustration of protein immobilization via “click” chemistry [85].

In a special technique, *Peptide Ligation*, peptides or proteins can be coupled using a variable chemiselective capture steps followed by an intramolecular acyl transfer reaction [68, 87]. The chemoselective capture requires a nucleophile localized at the N-terminus while a compatible electrophile at the C-terminus or vice versa. The nucleophile and electrophile pair brings two termini into such a close proximity to permit an intramolecular acyl transfer reaction forming an amide bond [88]. While weak-base nucleophile containing amino acids (cysteine, serine, histidine, threonine) have been found to be the most suitable in N-terminus, an ester or a thioester is favoured for the C-terminus. Peptide ligation technique is also applied for protein and peptide immobilization onto modified surfaces (Figure 1.13) [68].

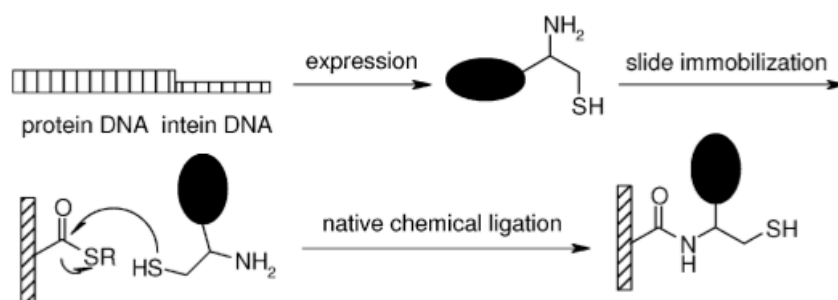


Figure 1.13 : Protein Immobilization Using a Native Chemical Ligation Reaction of N-Terminal Cysteine-Containing Proteins Expressed by Intein-Mediated Protein Splicing [68].

Straudinger ligation, can also be considered as peptide ligation, depends on the reaction between an azide group and a functionalized phosphine-containing thioester [68, 87, 89]. The reaction forms an amide via aminophosphorane intermediate. The

main advantages are being rapid and high yielding, also favorable at room temperature. Besides peptide/protein coupling, protein immobilization to solid supports is also achieved by Straudinger ligation [90].

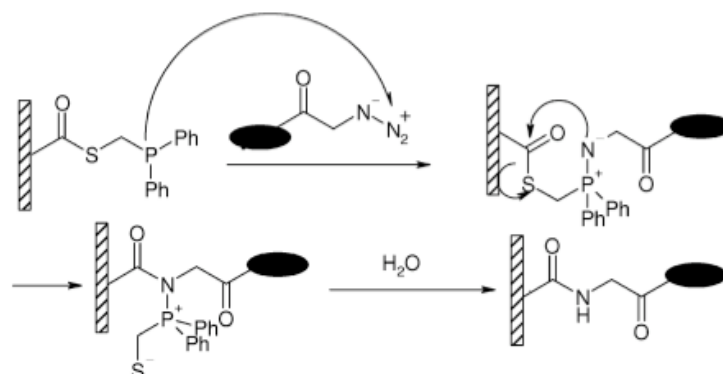


Figure 1.14 : Straudinger ligation [68].

1.2.3 Bioaffinity immobilization

Genetic engineering not only enables proteins to be modified via mutations but also permits two of them to be fused in DNA level leading the expression of bifunctional proteins. Fusions can be applied to either or both sides of the target gene depending on the application and ensuring the continuity of target gene products activity. Fusion partners have been utilized to construct reporter region, display proteins on surfaces of cells or phages and increase the circulation half-life of protein therapeutics. Even if the fusion partner is a protein or specific amino acid having bioaffinity for another molecule, than could be renamed as affinity tag. Affinity tag fusions allow the affinity purification of recombinant proteins [91]. Besides purification, in recent years, affinity tags have been applied for immobilization of proteins taking the advantage of gentle immobilization, homogeneous attachment and allowment of surface regeneration.

One of the examples of affinity tags used for immobilization is polyhistidine and polyarginine tag having affinity to IMAC and ionexchange resins respectively [92, 93]. The most utilized affinity tag is His-tag system, due to its small size, compatibility with organic solvents and low immunogenicity. Proteins fused with His-tag on C- or N-terminus can be immobilized on a nickel-chelating complex such as Ni-nitriloacetic acid (NTA). NTA is covalently bounded to the surface and loaded with a metal cation, usually Ni^{+2} [68]. His-tagged protein interacts with the Nickel ion via two imidazole groups of His-tag (Figure 1.15). In order to obtain proper

surface for His-tagged protein immobilization, NTA could be covalently bound to silanized glass or carboxy-dextran surfaces [94]. Besides the easy preparation of the immobilization system, there are also some drawbacks. The main drawback is the additional interactions between the surface and the protein with high Histidine content exposed to surface of the protein. Also, reversible interaction and the low strength of the affinity could be counted as disadvantages depending on the application requirements.

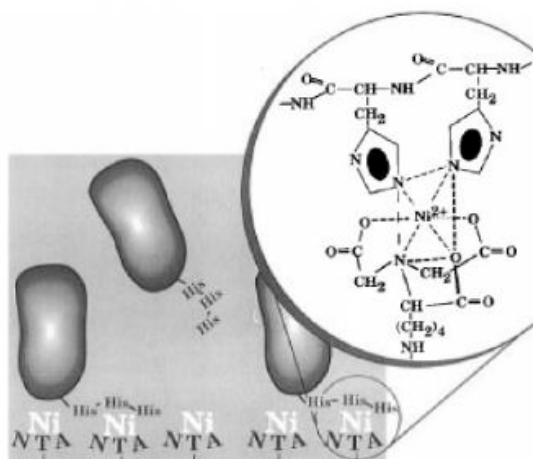


Figure 1.15 : The binding of His-tag proteins on NTA surface [95].

An alternative is the Biotin-Avidin system depending on the strong affinity of biotin to avidin or bacterial protein streptavidin (K_d is 10^{15} M^{-1}) [96, 97]. Due to its small size, biotin can be attached to proteins in order to obtain biotinylated molecules. Following the surface coating with avidin/streptavidin, biotinylated proteins can be immobilized effectively [98]. On the other way, surface could be coated with biotin. Avidin/streptavidin fused proteins can be immobilized onto biotinylated surface. However, avidin found to be binding to the closely packed biotinylated-SAMs in low affinity whereas, showing significantly higher binding affinity for loosely packed biotinylated surfaces [99]. For this reason, biotinylation should be controlled by using mixed SAMs, such as a mix of thiol and silane molecules, one biotinylated and one not [68].

Single-stranded DNA (ssDNA) shows a strong affinity to its complements and turns to a stable and robust double-stranded form via hybridization. Taking the advantage of DNA hybridization, DNA-directed immobilization is applicable for protein immobilization. Once ssDNA had to be covalently immobilized from one end, onto the surface for proper coating. On the other side, protein to be immobilized has to be

conjugated to the same end of the complementary ssDNA. When DNA conjugated with protein hybridized with the complementary DNA attached to the surface, the oriented protein immobilization has been achieved [68]. DNA-protein conjugates can be obtained in different strategies such as direct covalent attachment, bifunctional linkers, interaction via streptavidin bridge and expressed protein ligation [100, 101].

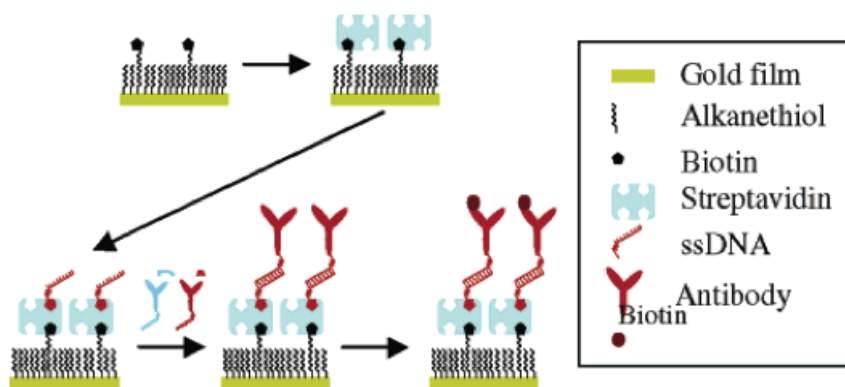


Figure 1.16 : The general construction of the DNA-directed antibody immobilization using a streptavidin bridge [102].

DNA-directed immobilization proceeds with high efficiency and site specificity due to the formation of a rigid, double-stranded DNA spacer arm between the surface and the protein. Since the immobilized proteins can be removed by alkaline denaturation of the DNA double strand, a complete regeneration of the surface can be achieved. While this is taken as an advantage for reusable chips, it could also be a problem for some applications. Also, DNA-based systems are not suitable for analytes containing nucleases, which may degrade DNA. However, the main drawback of the system is the difficulties in nucleotide incorporation into large proteins [68].

1.2.4 Biological (GEPI mediated) immobilization

Genetically engineered peptides for inorganics are short amino acid sequences selected from combinatorial libraries among their specific affinities for a given inorganic [7, 9, 13, 14, 37]. Their selective and specific affinity for solid surfaces make them good candidates for immobilization of proteins when used as a fusion partner with the target protein. As being biological molecules, GEPIs are suitable to be fused with proteins at the DNA level. GEPI can be fused at either end of the protein and also in any permissive site if it exists. DNA level modifications carry the advantage of not using chemical reactions for conjugation, which may cause damage to the protein.

structure. Because of their small size, they usually don't disrupt the 3D structure of the protein resulting with the retained biological activity.

On the other hand, utilization of GEPIs for protein immobilization brings the advantage of directed immobilization and self-assembly of the protein on the surface. As being fused to a specific region, all the proteins will be immobilized in the same way resulting with an oriented immobilization, which is important to establish retained protein activity. Moreover, GEPI mediated immobilization does not require any surface modifications such as SAM coatings due to their natural affinity for the surface and self-assemble function. The possible advantages of GEPI-mediated immobilization have already been demonstrated in different studies, performed by Sarikaya group [60-62]

1.3 Gold Binding Peptides

A number of inorganic binding peptides have been selected up to date and some of them were utilized in various applications in biotechnology. Among the gold binding peptides GBP1 (MHGKTQATSGTIQS) and ϵ -AuBP2 (cyclic-AuBP2) (CGPWALRRSIRRQSYGPC) have been utilized in this thesis project as a fusion partner for directed protein immobilization [25, 34]. Detailed characterization and applications of these peptides are explained in following subsections.

1.3.1 Selection and characterization of GBP1

The first gold binding peptide series were selected by bacterial cell surface display technique. Repeated tandem oligonucleotides were displayed on the surface of *Escherichia coli* as part of the maltodextrin porin protein, LamB [25]. Following a series of selection experiments, peptides showing affinity for gold were cloned to Alkaline Phosphatase expression vector for further analysis. Among them, a 14 amino acid long peptide possessing high binding affinity to gold was named as GBP1. GBP1 with the sequence of MHGKTQATSGTIQS had been produced in 3, 5, 7 and 9 repeated forms. Their binding characterizations implied that increasing the number of repeating units can change binding affinity to its target, gold [25].

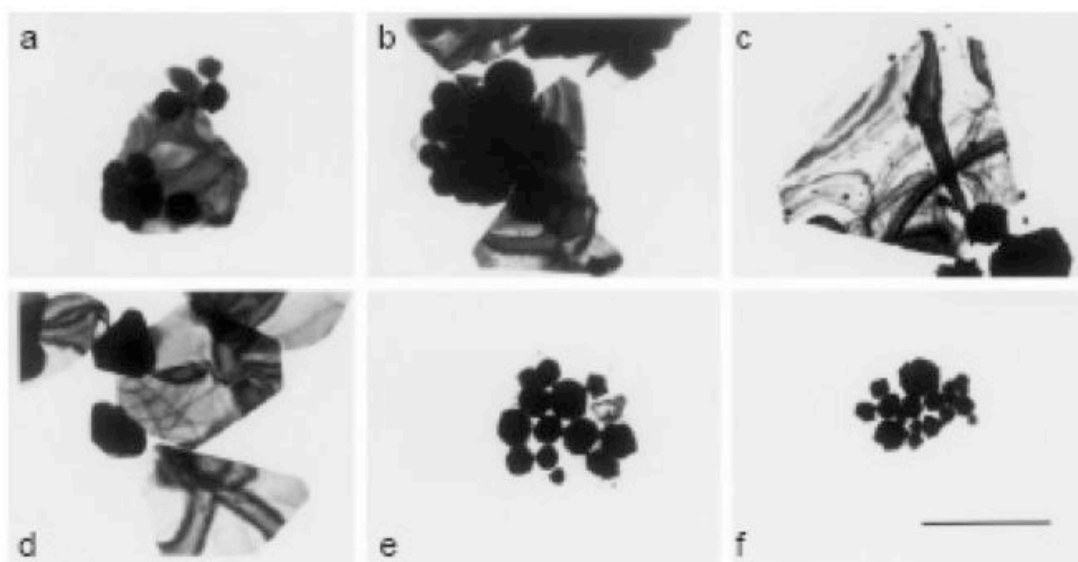


Figure 1.17 : TEM images of gold crystals formed by the reduction of AuCl_3 using (a) GBP1-AP, (b) GBP2-AP, (c) 200uM AuCl_3 , 200uM ascorbic acid (d) 200uM AuCl_3 , 500uM citric acid (e) wild type AP (f) 200uM AuCl_3 , 200uM potassium ascorbate, 10mM potassium phosphate, pH 7 [57].

Besides recognizing gold surface, GBP1-AP fusion protein also has the ability to induce crystal formation [57]. The formation of gold crystals by GBP1-AP does not required any other reducing agents and crystal growth was performed under ambient conditions (Figure 1.17). Moreover, like the nucleation mechanism of biological hard tissues, GBP1 enhanced the crystal formation under kinetically favorable and thermodynamically unfavorable conditions [57].

On the other hand, GBP1 was shown to have specific-binding and recognition ability for gold. Even its exposed to silica surface patterned with Pt and Au squares, peptides directly recognize the gold regions (Figure 1.18). Also, QCM measurements quantitatively showed that GBP1 has a significantly higher binding affinity to gold than platinum. Results indicated the impact of specific recognition ability of GBP1 not only between two materials but also between two nobel metals (Au and Pt) [50].

Surface binding characterizations of 3R-GBP1 was also monitored and calculated by using SPR spectroscopy. Binding kinetics extracted from SPR experiments resulted with the proposal of a biexponential kinetics [42]. This two-domain Langmuir model was explaining the surface heterogeneity resulting from two different adsorption processes taking place simultaneously, each with its own energy (Figure 1.19).

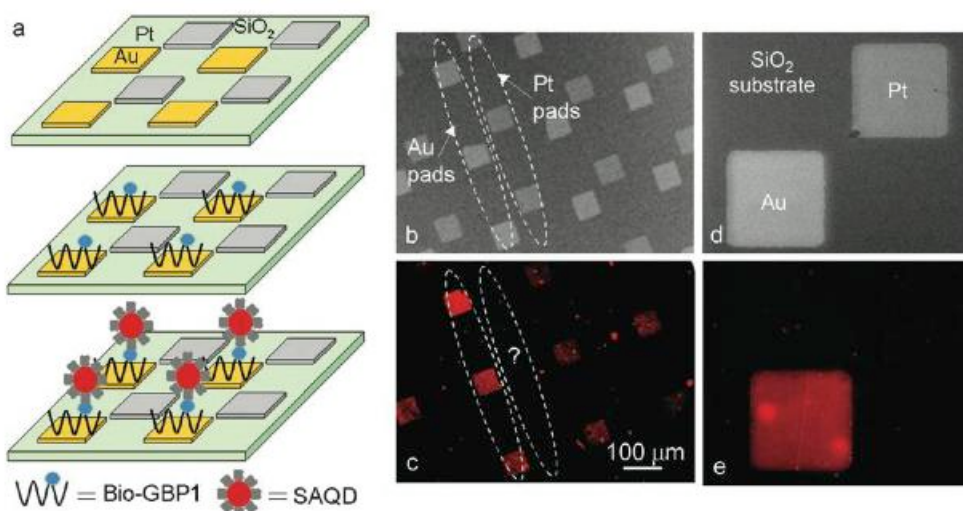


Figure 1.18 : a) Schematic illustration of the directed self-assembly of SAQDs on bio-GBP1 only on the gold regions of a silicon dioxide substrate containing both gold and platinum pads. The micrographs in (b) and (d) are optical microscopy images of the microfabricated template with gold and platinum pads. The fluorescent images in (c) and (e) (low and high magnification, respectively) reveal SAQD immobilization on the self-assembled bio-GBP1 [50].

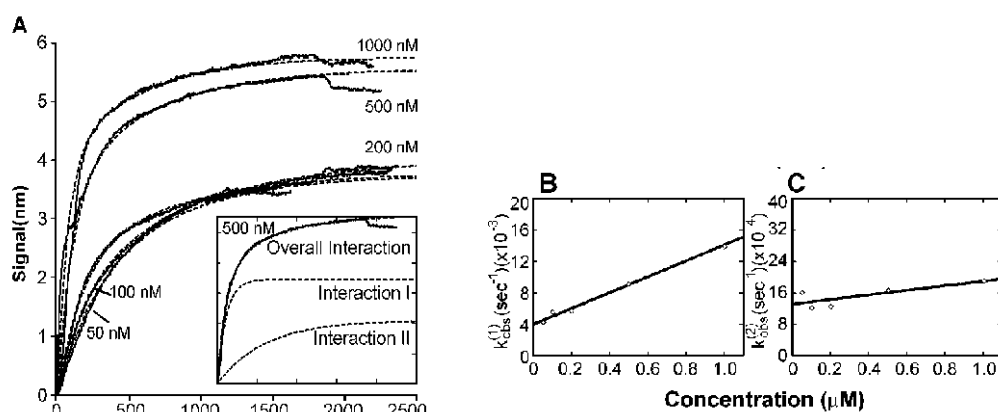


Figure 1.19 : (A) SPR analysis of 3R-GBP1 and the biexponential fits of Langmuir adsorption isotherms to the experimental data. The inset shows the model of the two types of adsorption of the molecule. The concentration dependence of k_{obs} for the two simultaneous reactions are in (B) for $k_{obs}(1)$ and (C) for $k_{obs}(2)$ [42].

Recently, the morphology of molecular recognition and self assembly of GBP1 on (111) gold surface was analyzed by atomic force microscopy [49]. According to the results a two staged dynamic adsorption behavior was proposed (Figure 1.20). Where, in first regime, peptides form island-like structures when they approach to the surface. In second regime, these islands coalesce a network formed by structural changes of peptide molecules. This two-staged model was also consistent

with the binding kinetics of 3GBP extracted previously with SPR, which was fitting to a two-domain Langmuir model [42].

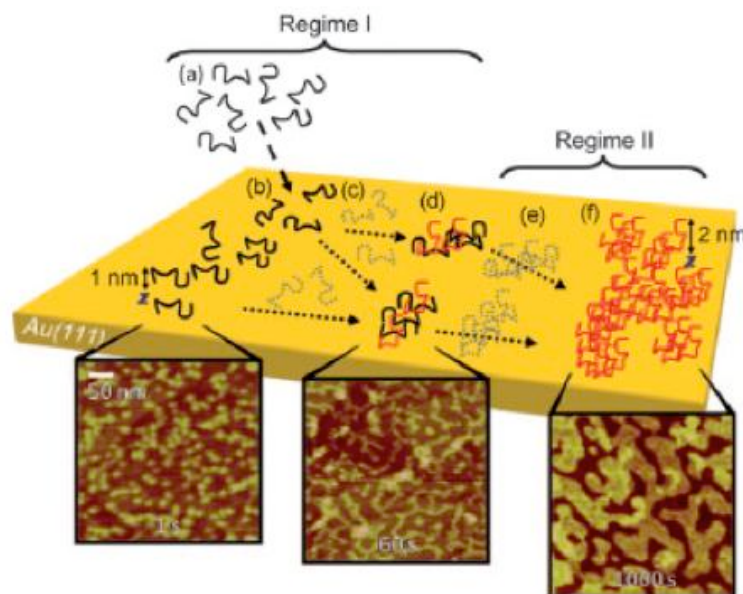


Figure 1.20 : Schematic model showing the proposed mechanism of the binding, diffusion, and assembly of 3rGBP1 on Au(111) [49].

As well as experimental studies, structure prediction, molecular dynamics and geometrical docking studies have been applied to detail the formation of an ordered supramolecular self-assembly of 3R-GBP1 [48, 49, 103]. Simulated annealing molecular dynamic (SA/MD) studies based on nuclear magnetic resonans (NMR) were applied. Results confirmed the intrinsic disorder of 3R-GBP1 and identified putative Au docking sites where surface-exposed side chains align with gold surface (Figure 1.21) [48].

Besides the fundamental studies achieved to understand binding strength and mechanism of GBP1, some applications have also been demonstrated [104, 105]. In the first study, 3R-GBP1-AP fusion construct was used for direct immobilization of the enzyme onto gold surface [60]. In another example, 5R- and 7R-GBP1 were ligated into a permissive site of Tral protein. Fusion constructs were both able to bind DNA with the activity of the Tral protein and interact with gold nanoparticles using the inorganic binding ability of GBP1 [61].

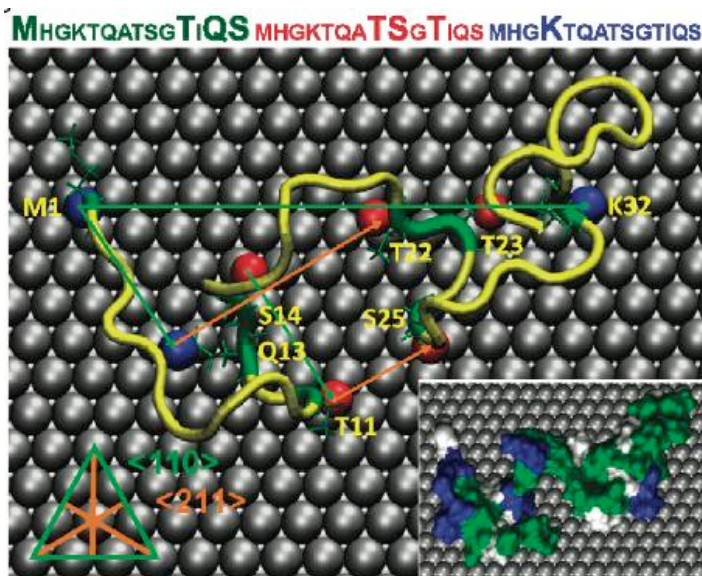


Figure 1.21 : Representation of the circled orientation of 3R-GBP1 and its geometric correlation with the {111} gold surface. The polypeptide backbone is represented in ribbon format (yellow) with docking residues (M1, T11, Q13, S14, T22, S23, T25, and K32) highlighted (green) along with their respective heteroatoms involved in surface interactions represented as space-filling atoms in either blue (N) or red (O). The crystallographic directions for specific docking surface atoms are presented in green (<110>) or orange (<211>), depending on their correspondence with the appropriate Miller indices of the Au surface lattice. Inset shows surface representation of potential inter-molecular interactions with 3R-GBP1 while bound to Au{111}, highlighting free polar (green), nonpolar (white), and cationic (blue) surfaces [48].

1.3.2 Selection and characterization of AuBP2

The second gold binding peptide series were selected from a commercially available bacterial cell surface display library, FliTrx™ (Invitrogen). The library was constructed with random dodecapeptides positioned in the active site loop of the thioredoxin protein (trxA) inside the dispensable region of the bacterial flagellin gene (fliC). The resultant recombinant fusion protein (FLITRX) is exported and assembled into partially functional flagella on the bacterial cell surface.

Withal the selected gold binding peptides, first characterization experiments were carried out with immuno-fluorescent labelling of the cells bound to Au. Depending on the results, two of the strong binders were further examined; AuBP1 (WAGAKRLVLRRE) and AuBP2 (WALRRSIRRQSY) [34]. These 12 amino acid peptides were synthesized both in linear form (*l*-AuBP1, *l*-AuBP2) and a constrained form (*c*-AuBP1, *c*-AuBP2) to mimic their localization on thioredoxin protein. In order to constraint the peptides, a Cys-Cys loop was introduced with CGP-

AuBP1/AuBP2-GPC amino acid sequence. The difference in binding strenght of the peptides in different conformations was examined using SPR (Figure 1.22). While all the peptides were showing similer affinities, *c*-AuBP2 was possessing a significantly lower desorption rate. Thus, this lower desorption rate leads to higher K_{eq} and ΔG values than other AuBPs (Table 1.2) [34].

Table 1.2 : Adsorption Rate (k_a), Desorption Rate (k_d), Equilibrium Coefficient (K_{eq}), and Free Energy for Linear (*l*) and Constrained (*c*) Gold-Binding Peptides. (Remaded from the Table 2 of reference [34]).

Peptide	$K_{eq} \times 10^6 (M^{-1})$	$k_a \times 10^5 (M^{-1} s^{-1})$	$k_d \times 10^{-4} (s^{-1})$	$\Delta G_{ads} (kcal/mol)$
<i>l</i> -AuBP1	3.24 ± 1.31	1.32 ± 0.21	41 ± 8	-8.9 ± 0.2
<i>c</i> -AuBP1	2.51 ± 0.50	1.10 ± 0.22	44 ± 2	-8.7 ± 0.1
<i>l</i> -AuBP2	2.34 ± 0.34	1.00 ± 0.03	43 ± 5	-8.7 ± 0.1
<i>c</i> -AuBP2	13.50 ± 3.00	1.35 ± 0.12	10 ± 2	-9.7 ± 0.2

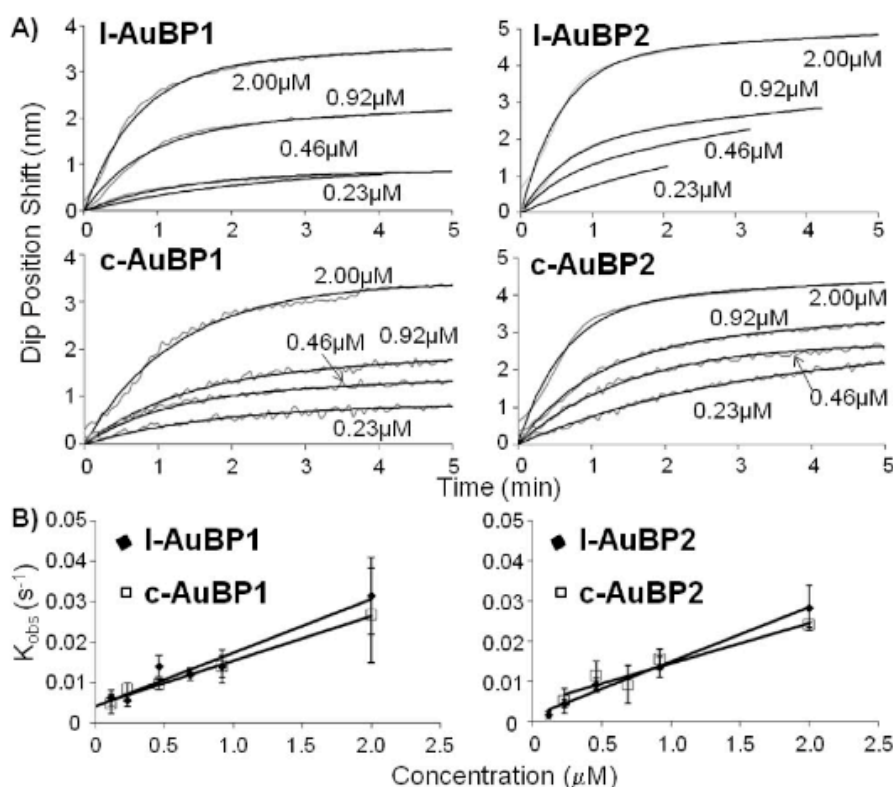


Figure 1.22 : (A) Adsorption isotherms for linear and constraint forms of AuBP1 and AuBP2, obtained by SPR, at different concentrations. The gray lines represent experimental data points, and the black lines are Langmuir model fits. (B) Observed rate coefficients (K_{obs}) plotted as a function of the peptide concentration. Linear regression was used to calculate the adsorption (k_a) and desorption (k_d) rates [34].

Also, the conformational and molecular dynamic properties of the peptides have been investigated using CD analysis and MD simulations, respectively. Results indicated that the random coil (RC) structure is the main conformation in cyclic peptides. However, linear versions have some degree of polyproline type II (PPII) rigid structures in addition to the random coil structure (Figure 1.23). The percentage of PPII structure in *l*-AuBP2 is greater than in *l*-AuBP1. Thus, leads to the higher difference between *c*- and *l*- forms of AuBP2 rather than AuBP1 [34]. This significant difference in adapted secondary structures between cyclic and linear AuBP2 peptides may explain the binding affinity decrease in linear form. On the other hand, the structure similarity between cyclic and linear AuBP1 explains the similar binding energies obtained in SPR experiments.

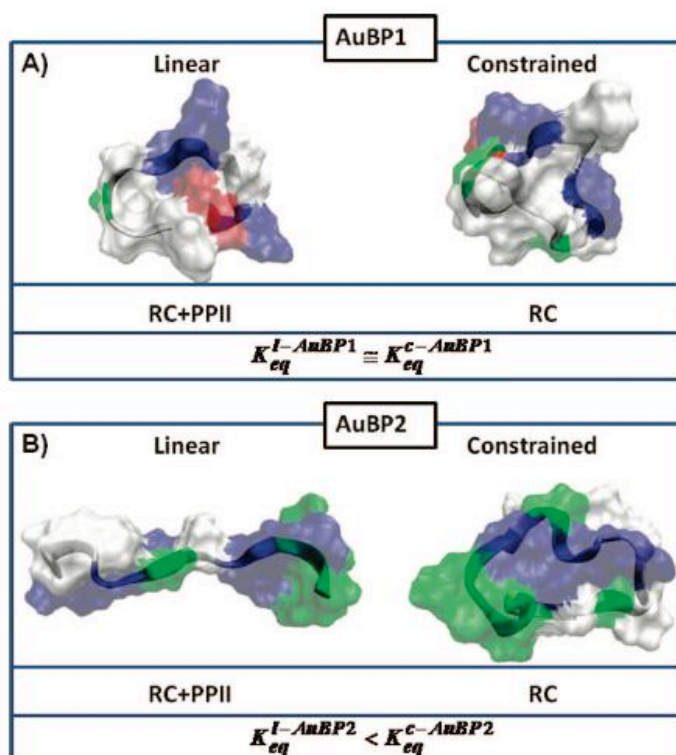


Figure 1.23 : Overlapped ribbon and transparent surface models of the predicted structures of the *l*- and *c*-versions of AuBP1 (A) and AuBP2 (B) [34].

As in GBP1 case, AuBP2 was also utilized in different applications. The first examples were depending on the gold surface immobilization of nanoparticles and facilitating the assembly of DNA. In these studies, biotinylated AuBP2 was used to prepare a platform for streptavidin binding, which serves as a second platform for the assembly of bio-QD or bio-DNA [106, 107]. In a following study, QBP1-AuBP2 peptide conjugate was used for the immobilization of gold nanoparticles on silica

surface without requiring any surface modifications. This bifunctional peptide was able to not only immobilize gold nanoparticles, but also synthesize them on the surface [56].

1.4 Huntingtin Protein

Huntingtin (HTT) gene was first mapped on the chromosome in 1983 [108]. The gene was found to be located in proximal 4p16-3 and 180 kb long consist of 67 exons. *IT15* gene has two alternatively polyadenylated transcripts of 10.5 kb and 13.5 kb which are expressed at varying ratios in almost all tissues. Huntingtin (Htt), the predicted 348 kDa protein product of the gene, comprises 3144 amino acids. Htt is a soluble protein and ubiquitously expressed in human and rodents [109]. Intracellularly, huntingtin is found to be associated with many organelles such as nucleus, endoplasmic reticulum and golgi complex and also found in neurites, synapses, vesicular structures, endosomal compartments and microtubules [110-113]. The primary amino acid sequence of huntingtin doesn't exhibit structural domains or homology to any known proteins (Figure 1.24). The main characteristic of the protein is the polyQ region, which begins at 18th amino acid and contains up to 34 glutamine residues. The importance of this region is the variability in glutamine repeats, which lead to Huntington's disease in increased repeat number [114]. Also, was shown to form a polar zipper structure and thought to be important to bind transcription factors containing a polyQ domain [115, 116]. The following region of the Htt protein is a polyproline (polyP) stretch, which could be effective in the solubility via binding with intrabodies and WW domain proteins [117-120]. Downstream of these regions are HEAT repeats, which are ~40 amino acid long sequences occurring multiple times and are involved in protein-protein interactions [115, 121]. The repeats are highly conserved in a wide variety of large eukaryotic proteins involved in cytoplasmic and nuclear processes, microtubule dynamics and chromosome segregation, suggesting that Htt might also be involved in such processes [121]. C-terminal of the protein contains a nuclear export signal (NES) sequence and a less active nuclear localization signal (NLS) indicating that Htt protein might be involved in transport of molecules from nucleus to cytoplasm [122]. The evidence for this hypothesis is the interaction of first 17 amino acids of Htt with

the nuclear pore protein TPR (translocated promoter region), which exports proteins from nucleus [123].

Huntingtin is subjected to proteolysis. There are three well-defined cleavage sites for caspases (a family of aspartyl protease), which are involved in programmed cell death. Huntingtin is cleaved in vitro by *caspase 3* at amino acids 513 and 552, by *caspase 6* at amino acid 586 and by *caspase 2* at amino acid 552 (Figure 1.24) [124]. Caspase generated Htt fragments are found both in nucleus and cytoplasm. Proteolytic cleavage of mutant Htt is suggested to lead to the development of aggregates compromising cell viability [125]. There is also evidence that calpain (a calcium dependent protease) is involved in the cleavage of Htt [126]. Active calpain cleavage sites have been described for mouse and human huntingtin [126, 127]. While contribution of Htt proteolysis to cell function is unclear, calpain inhibitors are found to prevent cell death whereas caspase inhibitors do not [128]. Also, it's demonstrated that modifications in the activity of caspase and calpain reduce the proteolysis and toxicity of mutant protein and delay disease progress [125, 127].

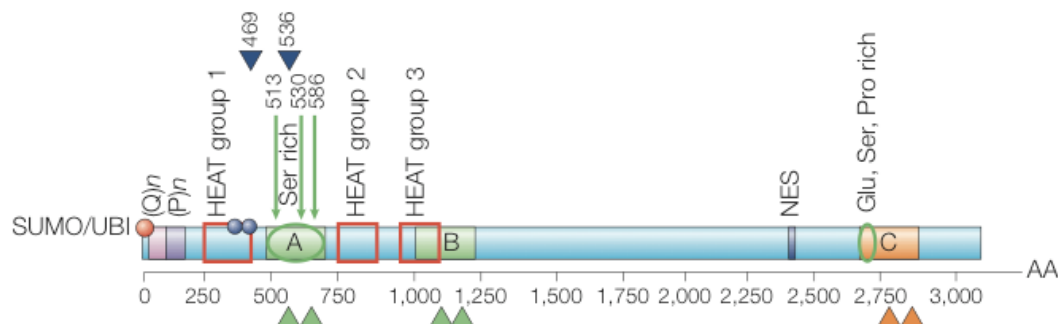


Figure 1.24 : Schematic diagram of huntingtin amino acid sequence. (Q)*n*: the polyglutamine tract, (P)*n*: the polyproline sequence, Red squares: The three main clusters of HEAT repeats, Arrows: the caspase cleavage sites, Blue arrowheads: the calpain cleavage sites, B: the regions cleaved preferentially in the cerebral cortex, C: the regions cleaved mainly in the striatum, A: the regions cleaved in both. Green and orange arrowheads: Protease cleavage regions, NES: the nuclear export signal, Red dot: ubiquitination (UBI) and/or sumoylation (SUMO) site, Blue dots: phosphorylation sites (blue). The glutamic acid (Glu)-, serine (Ser)- and proline (Pro)-rich regions are indicated (serine-rich regions encircled in green) [129].

Huntingtin is post-translationally modified either by sumoylation, ubiquitination, phosphorylation and palmitoylation. Sumoylation and ubiquitination are occurred at the N-terminal lysines K6, K9 and K15 [118, 130], whereas phosphorylation at serines

S421 and S434 [131, 132]. Both modifications are effective in cleavage and toxicity, which is reduced in diseased form [118, 123, 130, 132]. Palmitoylation is achieved by Htt co-partner; huntingtin-interacting protein 14 (HIP14, a palmitoyl transferase), but the precise amino acid position involved is unknown. The palmitoylation of Htt provides to propose its role in regulating vesicular trafficking, because palmitoylated proteins are often involved in the dynamic assembly of components that control vesicle trafficking and synaptic vesicle function [133].

1.4.1 Functions of Huntingtin protein

Huntingtin, intracellularly, is found to be associated with many organelles and demonstrated to be interacting with various proteins. Among these knowledge obtained by in vitro, cell culture and animal studies, diverse functions of huntingtin have been proposed.

Huntingtin is found to be essential for embryonic development. Mice model of huntingtin-knockout, couldn't reach to the 8.5th day of embryonic development [134, 135]. On the other hand, pathological polyQ expansions don't cause embryonic lethality in huntingtin-null mice [136, 137]. Even for the homozygous mutant individuals born without any apparent defects suggesting that glutamine tract doesn't effect the physiological development. The only requirement for embryogenesis is the full dose expression of Htt even in normal or mutant form. In fact, in case of reduced huntingtin levels more than 50%, leads defects in epiblast (the structure that will give rise to the neural tube) in mice [138]. Thus, neurogenesis is reduced and malformations of cortex and striatum are profound. These findings indicate that huntingtin is essential for the development of central nervous system (CNS) [139].

In vitro studies of conditionally immortalized striatum- derived cells over-expressing huntingtin were provided its neuroprotective role in brain cells exposed to various apoptotic stimuli. Also, this *in vitro* study demonstrated that the 548 N-terminal amino acids are exhibiting anti-apoptotic activity [140]. The enhancement of neuroprotection with increased level of protein indicates a gene-dosage effect. The molecular mechanisms are shown that huntingtin maintains cell survival by inhibiting the processing of pro-caspase 9 and the complex formation between huntingtin interacting protein 1 (HIP1) and HIP1 pro-apoptotic interactor (HIPPI) [140-142].

Among anti-apoptotic role, huntingtin has also been considered as a cell survival factor. Htt is found to be a substrate for Akt (a serin/threonin kinase) that activates pro-survival pathways [143]. Also, has been increasing the production of brain derived neurotropic factor (BDNF), which is important for the survival of striatal neurons and the activity of cortico-striatal synapsis [144, 145]. BDNF is mostly produced in cerebral cortex, transported to axon terminals and released from there to be captured by striatal neurons. Thus give rise to intracellular signal [146]. On the other hand, BDNF controls the release of glutamate at cortico-striatal synapse and allows striatal neurons to survive excitotoxin-induced neurodegeneration [147, 148]. On molecular level, huntingtin controls the expression of BDNF by regulating the activity of BDNF promoter [140, 149].

Htt also regulates BDNF transport along the cortico-striatal afferent and thereby affect BDNF to reach to its striatal targets. Huntingtin facilitates the vesicular transport of BDNF by interacting with the p150 subunit of dyactin (an essential component of molecular motors) via Huntingtin associated protein 1 (HAP1) (Figure 1.25) [150]. In the case of reduced or interrupted Htt production, fast axonal transport has been defected as shown in *D. melanogaster* neurons [151]. The main function of Htt and HAP1 in neuronal transport might be serving as scaffold proteins to stabilize the assembly of the dynein-dynactin complex to selected cargo vesicles, thereby enabling the transport of cargo along microtubules.

Huntingtin has been shown to interact many transcription factor and activators, suggesting its role in transcriptional regulation in neurons. Huntingtin is found to interact with RE1 silencing transcription factor (REST or NRSF), which is the regulator of neuron-restrictive silencer element (RE1 or NRSE). REST represses transcription via binding to NRSE. Huntingtin sequesters cytoplasmic REST and prevent co-repression of NRSE nuclear site, thereby allow the gene transcription [152]. The other transcriptional factor that Htt interacts with are cAMP response-element binding protein (CREB binding protein: CBP) [153, 154], p53 [153, 154], co-activator CA150 [155] and co-repressor C-terminal binding protein (CtBP) [113]. However, the mechanism of Htt influence on transcription as a consequence of the interaction with these factors is still unclear. Huntingtin was also reported to bind both with transcriptional activator Sp1 and co-activator TAFII130 [156]. TAFII130 is known to interact directly with Sp1 and stimulate transcription. Therefore, Htt

function was suggested to act as a scaffold that links transcriptional activator Sp1 to transcriptional machinery. Consequently, Htt might be stimulating the expression by strengthening the interaction between DNA-bound Sp1 and TFIID multiprotein complex including TAFII130.

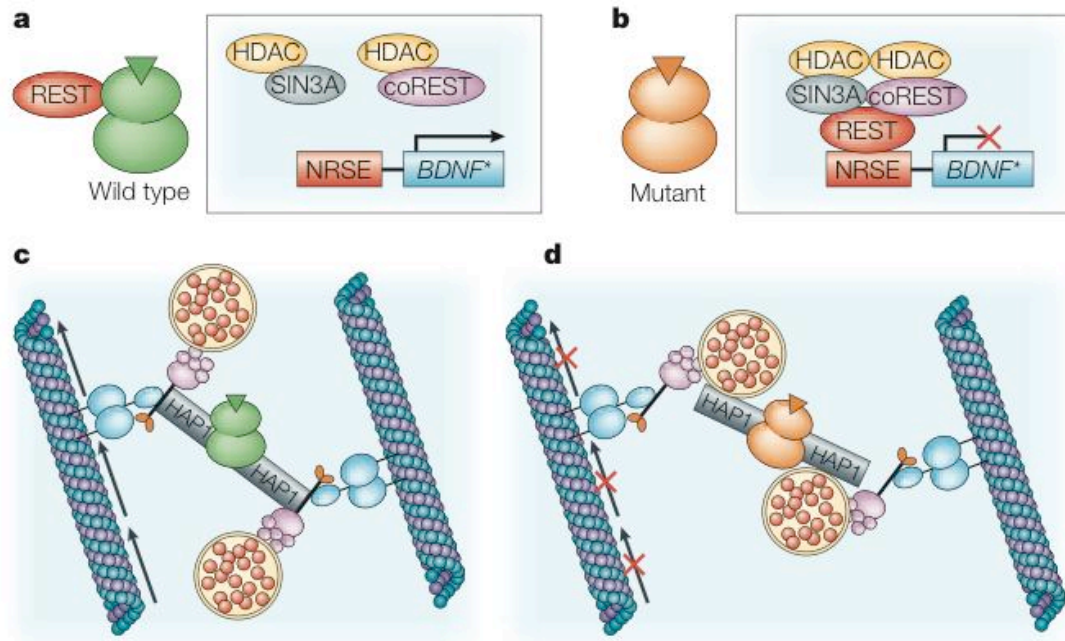


Figure 1.25 : Molecular mechanism of wild-type huntingtin function. (a) Wild-type and (b) mutant Huntingtin activity on the transcription of brain-derived neurotrophic factor (BDNF). BDNF transport (c) with wild-type huntingtin, where it binds to huntingtin-associated protein 1 (HAP1) and indirectly regulates the assembly of p150 (Glued) (grey) with the dynein (blue) and dynactin complexes (pink) that ultimately control the microtubule transport of BDNF vesicles (yellow), (d) with mutant huntingtin, binding more tightly to HAP1, reduces the transport of BDNF vesicles along microtubules. coREST; REST co-repressor, HDAC; histone deacetylase, SIN3A; SIN3 homologue A, a transcription regulator [129].

Recent experiments implicated that huntingtin and its interacting proteins HIP1, HIP12, PACSIN1, SH3GL3 and HIP-14 could play role in *clathrin-mediated endocytosis* in neurons [133, 157-159]. It's possible for Htt to enhance the assembly of HIP1 with AP-2 (adaptor protein 2 acting in clathrin polymerization for membrane budding) and clathrin for the formation of clathrin-coated pits. Moreover, Htt could be effective in the association of vesicles with actin filaments through interacting with HIP1 and HIP12 [160]. While interacting with PACSIN1, huntingtin is thought to influence actin dynamic and coated pit formation [158]. Huntingtin also interacts with SH3GL3 (endophilin 3), a Grb2-like protein that mediates vesicle invagination

and fission [161, 162]. Although the effect of htt-SH3GL3 binding on this process is unclear, htt-SH3GL3 interaction is considered to be regulating dynamin and synaptojanin function. Nonetheless, different functions of huntingtin proposed here are demonstrating the hierarchy of protein-protein interactions. Thereby, general function of huntingtin in clathrin-mediated endocytosis is possibly to mediate a multiprotein scaffold.

Huntingtin is found to interact with a number of cytoskeletal and synaptic vesicle proteins, suggesting its role in *synaptic transmission* and *dendritic morphology* [163, 164]. Huntingtin binds to Src homology 3 (SH3) domains of postsynaptic density protein 95 (PSD95), which interacts with glutamate receptors such as NMDA (N-methyl-d-aspartate) and kainate receptors at postsynaptic density, and cytoplasmic signalling proteins such as GTPase activating protein (SynGAP) [164]. PSD95 is found to act in a protein-protein complex and considered to be a key molecule in synaptic transmission and plasticity. Thus, Htt is thought to influence post-synaptic transmission by effecting the organization of PSD95 with its binding partners and hence, receptor clustering and downstream signalling [115].

1.4.2 Huntington's Disease

Mutation on huntingtin gene characterized by the expansion of poly glutamine region of Htt, results with a neurodegenerative disorder called Huntington's disease (HD). HD was first described by George Huntington in 1897 [165]. While wild-type huntingtin is produced from *HTT* gene containing a 6-35 CAG (glutamine coding codon) repeats, disease-causing allele contains more than 36 repeats. The length of the expansion is inversely correlated with the age of disease onset, as juvenile onset is associated with the alleles more than 70 CAG repeats. Autosomal dominant inheritance of the disease shows an intergenerational instability [114]. About 80% of juvenile patients inherit the disease from their father [166]. This augmented paternal repeat instability is possibly because of the greater number of cellular divisions that take place during spermatogenesis.

The disease symptoms typically begin in mid-life and duration of patients life is about 15-18 years after onset. In adult patients, the most common motor symptom is chorea existed with memory deficits, affective disturbance and changes in personality.

However, bradykinesia, rigidity, epilepsy, severe dementia and progressing disease are usually seen in juvenile-onset patients [167].

Clinicopathology of the disease is the diffuse and severe atrophy of neostriatum. Because of the loss of striatopallidal projection fibers, the globus pallidus can show a volume loss compared to that seen in the striatum. In early onset cases, cerebellar Purkinje cell loss is also seen along with generalized atrophy of the brain [168].

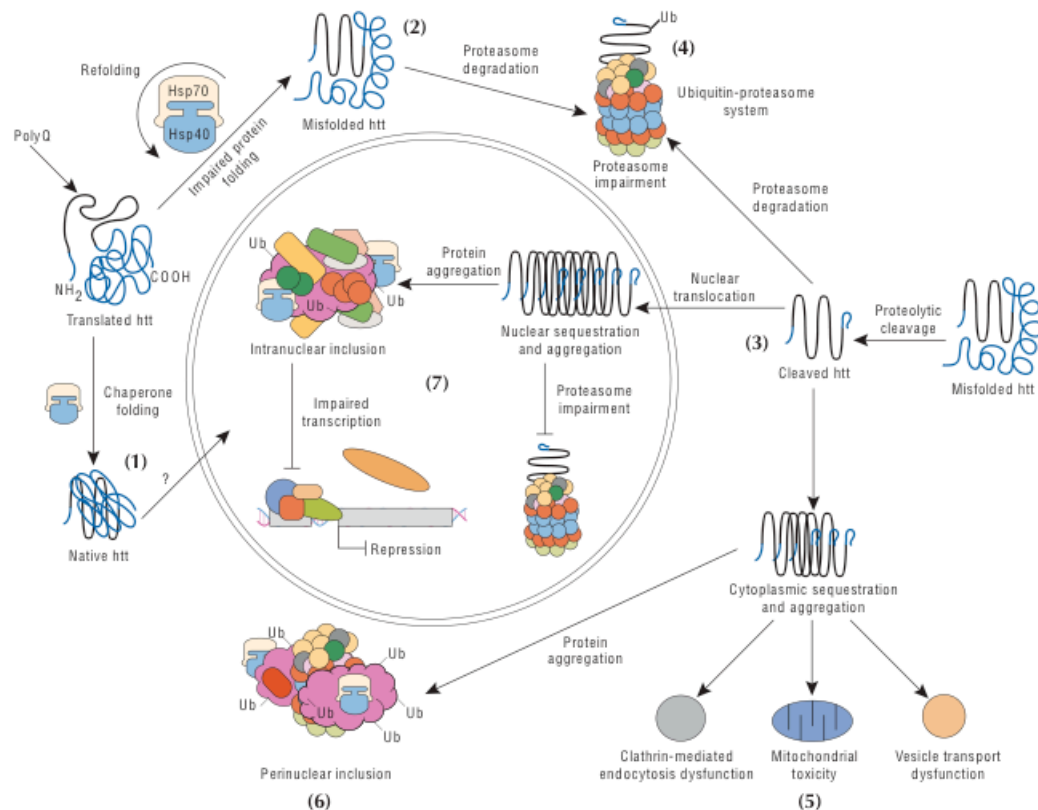


Figure 1.26 : The cellular pathogenesis in Huntington's disease. (1) Hsp70 and Hsp40 promote the folding of htt into a native structure. (2) The abnormal proteins are subjected to refolding or ubiquitination (Ub). If not corrected by chaperones, is accumulated in the cytoplasm. (3) Mutant htt might also be proteolytically cleaved and translocated into nucleus. (4) In the cytoplasm, mutant htt impairs UPS, leading to the accumulation of more misfolded proteins. (5) It also impairs normal vesicle transport and clathrin-mediated endocytosis and activates proapoptotic proteins directly or indirectly by mitochondrial damage. (6) The cell accumulates toxic fragments into ubiquitinated cytoplasmic perinuclear aggregates to protect itself. (7) Nuclear inclusions disrupt transcription and the UPS [169].

Electron microscopy examinations of patient tissues are exhibited large nuclear and cytoplasmic inclusions or aggregates [170]. Intracellular aggregates are composed of N-terminal Htt fragments, suggesting that mutant protein undergoes proteolytic

cleavage for nuclear localization [171]. The accumulation of aggregates is thought to be critical for progressive neuronal degeneration and dysfunction. While increased expression of normal huntingtin reducing neurotoxicity, the normal htt can also fold in huntingtin aggregates. Therefore, neurodegeneration in HD is suggested to be a combined effect of gain-of-function in mutated Htt and a loss-of-function in wild type protein [134, 136, 169, 172].

The huntingtin aggregates also, sequester chaperones, proteasomal proteins and transcriptional factors. Mutant huntingtin is suggested to induce disease, because of this interference with normal function of cellular proteins (Figure 1.26). Numerous studies shown that transcriptional factors such as p53, CREB-binding protein (CBP), specificity protein 1 (SP1) and TATA-binding protein (TBP) are recruited to intranuclear aggregates suggesting the inducement of transcriptional dysregulation in HD [152, 155, 156, 173]. Besides, Hsp70 and Hsp40 family of chaperones have been shown to colocalized with aggregates implying that impairment of protein folding gives rise to HD pathology [174]. The sequestration of chaperones into aggregates decreases the amount of soluble chaperones in the cell and results with enhanced abnormal protein folding. This hypothesis is strengthening by showing that overexpression of Hsp70 and Hsp40 chaperones effect to rescue neurodegeneration and alter fibrillar aggregates to detergent-soluble amorphous structures [175, 176]. Another feature of HD is that inclusions are ubiquitinated and contain several proteasome subunits. Thus, a failure in the degradative machinery and impairment of the ubiquitin-proteasome system (UPS) is suggested to be central in HD pathogenesis. The eukaryotic proteasome is known to be incapable of digesting peptides with 9-29Q residues [177]. Therefore, attempt to digest mutant Htt might result in release of aggregation-prone polyQ-containing fragments from proteasome. Either, these fragments couldn't exit the proteasome and result with the impairment of UPS function [178]. Mutant huntingtin also impairs normal vesicle transport and clathrin-mediated endocytosis. Besides, leads to mitochondrial damage, probably depending on transcriptional dysregulation [179]. Eventually, a serin-threonin protein kinase called mammalian target of rapamycin (mTOR) is detected in htt aggregates. mTOR sequestering into aggregates decreases its kinase activity and thereby leads to the induction of autophagy, which is a key clearance pathway for

toxic htt fragments [180]. Therefore, in HD, aggregates are suggested to have a key role in inducing autophagy by suppressing mTOR activity.

Together with the accelerating knowledge about the molecular processes on cellular pathology of the disease, investigation of treatment strategies have begun. However, the only FDA approved drug available for Huntington's disease is tetrabenazine and the pharmacologic strategy is to improve the quality of patient's lives by mitigating the chorea symptoms [181]. There have been many attempts to develop appropriate therapeutics for HD [165]. Therapeutic target developmental research is focused on cellular pathology processes of HD (Figure 1.27). Among the targets, caspase and calpain inhibitors are under development to inhibit htt proteolysis and therefore inhibit nuclear inclusion formation. In order to modulate gene transcription, histone deacetylase (HDAC) inhibitors have been tested. HDAC inhibitors were effective in increasing transcription and reducing toxicity, however didn't affect the number of aggregates [182]. Creatine, on the other hand, is found effective to reduce mitochondrial dysfunction and attenuate brain atrophy and formation of htt aggregates [183]. Another drug target, riluzole, is developed to decrease glutamate release and thus inhibit excitotoxicity [184]. It's also found to inhibit htt aggregation. While htt aggregates are toxic when directed to nucleus, preventing the aggregation is considered as another therapeutic strategy. Trehalose treatment is found to reduce striatal atrophy and number of aggregates [185].

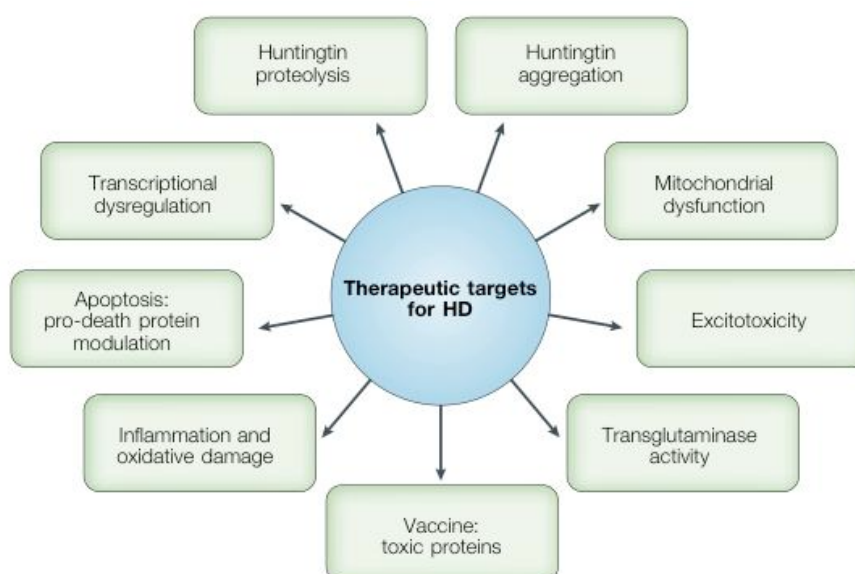


Figure 1.27 : Downstream targets for therapeutics for Huntington's disease [165].

The entire therapeutic targets have been tested in animal models and show a number of improvements in the disease pathology. However, their effectivity on human is still unclear and each of them is effective on an individual process. While continuing research on appropriate drug targets, combinational therapy is suggested for a combined effect on different disease mechanisms [165].

1.4.3 Aggregation profile of Huntingtin (Htt) protein

The main cellular pathology of Huntington's disease is the observation of neuronal and/or cytoplasmic inclusion bodies composed of aggregated Htt proteins together with its binding partners [171, 186]. The huntingtin protein aggregates found in inclusion bodies are granular or fibrillar structures [187]. The presence of these detergent insoluble protein aggregates are highly correlated with the disease pathology [188]. However, there are animal and cell culture studies indicating strongly that aggregation is neither necessary nor sufficient for neuronal dysfunction [189]. In this case, neuronal toxicity might come from the freely roaming mutant htt proteins and/or prefibrillar amorphous structures or oligomers [190]. Thereby, aggregation might be the protection way of the cell from insoluble toxic oligomers. To better understand the molecular basis of the glutamine repeat disorder, identifying the aggregation mechanism could be crucial. Also, this knowledge might allow for the development of new therapeutic strategies. Therefore, a number of studies focused on the aggregation mechanism of huntingtin protein both *in vivo* and *in vitro*.

N-terminus of huntingtin protein was found to be subjected to proteolytic cleavage *in vitro*. Following the cleavage, these N-terminal fragments (coded by exon 1 of Htt) containing the polyQ expansions could form into amyloid-like aggregates [170]. While observing such aggregates also in cell nucleus, researchers suggest that proteolytic cleavage product of Htt is transported into the nucleus and cause neuronal death by the formation of insoluble amyloid-like fibrils [170]. The formation of the fibrils was proposed to be nucleation-dependent polymerization in which, a nuclear structure has to be formed before fibril formation. This nucleus formation might be the rate limiting step of fibrillization [191]. On the other hand, fibrillization rate was higher for the longer polyQ containing proteins and in increased protein concentrations, *in vitro*. These data suggests that htt fibrillization is length and concentration dependent [191]. The length dependency of fibrillization could also be

correlated with the age-of-onset of the disease pathogenesis [192]. On the other hand, normal length htt protein could successfully be added to the preforming polyQ aggregates. Presence of normal length htt, enhances nucleation kinetics also in a concentration and polyQ repeat length dependent, *in vitro*. In fact, accelerates aggregation formation and cytotoxicity *in vivo* [172].

In an *in vitro* study, globular assemblies (oligomers) and protofibrillar intermediates have been observed in early times of fibrillization [193]. These β -structured assemblies were suggested to associate to form mature fibrils, over time (Figure 1.28). On the other hand, annular and amorphous aggregates were also observed and their acceleration was interrupted by molecular chaperones, Hsp40 and Hsp70, which are protective in neurodegeneration [194]. Taking these data together it's also possible to suggest that amorphous aggregates might be the byproducts of fibrillization rather than intermediates [195].

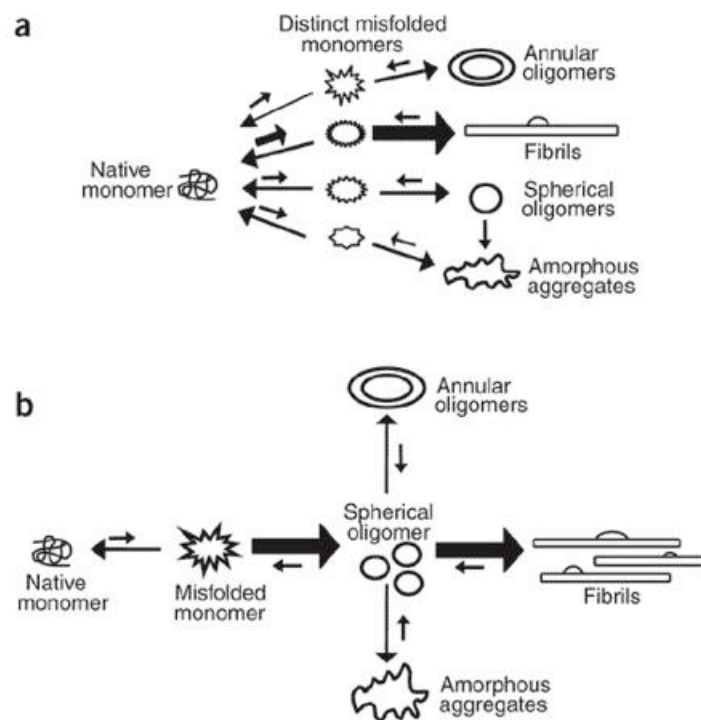


Figure 1.28 : Two models for the assembly of expanded polyQ proteins into amyloid-like fibrils.(a) Monomers may exist in a number of distinct misfolded conformations. Only one altered conformer is on-pathway for fibril formation (bold arrows) (b) Spherical oligomeric structures are obligate on-pathway intermediates for fibril assembly (bold arrows), whereas annular and amorphous structures are metastable off-pathway assemblies [194].

Both normal and expanded polyQ containing huntingtin monomers were found to be unstructured (random coil) suggesting that fibrillation is not correlated with the monomer conformation [188]. However, fibrils were showing high degree of β -structured conformation [193]. Therefore, many structural models for aggregated polyQ peptides have been proposed up to date including antiparallel, parallel, hairpin, compact random coil and compact β -sheets and β -helix [196]. Thakur and Wetzel have induced β -turns with proline-glycine (PG) insertion into polyQ peptides and determined their aggregation. They observed aggregation in peptides composed of multiple repeats of ≥ 7 glutamine residues interrupted with PG regions. These data were suggestive for a model comprised of alternating β -strand and β -turn elements, with an optimum of 7 constitutive glutamine residues within each β -strand (Figure 1.29) [197]. This compact β -sheet model is supported and confirmed in various studies [116, 192, 193, 198, 199]. In addition, β -strand forming polyQ peptides are suggested to be toxic both in monomeric and oligomeric structures [200].

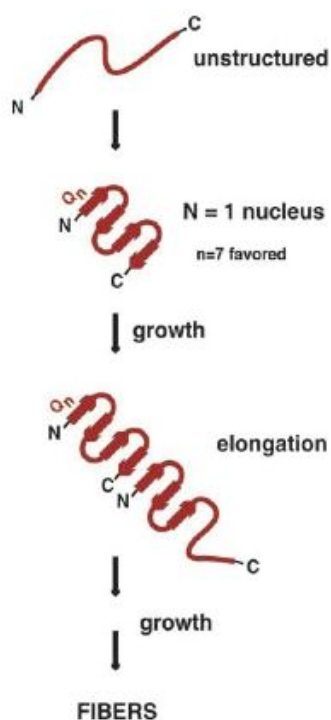


Figure 1.29 : Model of polyGln aggregate initiation and elongation as proposed by Thakur and Wetzel [196].

While polyQ region is found the most sustainable for htt aggregation, the effect of flanking sequences have also been investigated. The insertion of additional proline residues into polyP region of htt resulted with decreased aggregation and toxicity [201]. In another study, flanking polyP region was found to induce the polyproline

type II-like helix structure, which is opposite the aggregation-prone β -sheet [120]. A more recent study indicated the effect of N-terminal insertions on aggregation. The presence of c-myc epitope region on N-terminal of mutant htt proteins slowed down the fibrillization. This result suggested that c-myc sequence interferes with the intermolecular interactions mediated by N-terminal region of htt, thus altering fibrillization behaviour [202]. Proceeding from these data, N-terminal 17 amino-acid sequence of htt (htt^{NT}) has been analyzed, recently. Findings indicated that htt^{NT} shows an helical structure [203]. Also fibrils formed by htt^{NT} alone and together with mutant htt-exon1 displayed a degree of α -helix structured regions. The N-terminal helical segment is suggested to bring polyQ domains together with intermolecular helix-helix interactions, thus increasing the local concentration and leading to fibrillization initiation (Figure 1.30) [204].

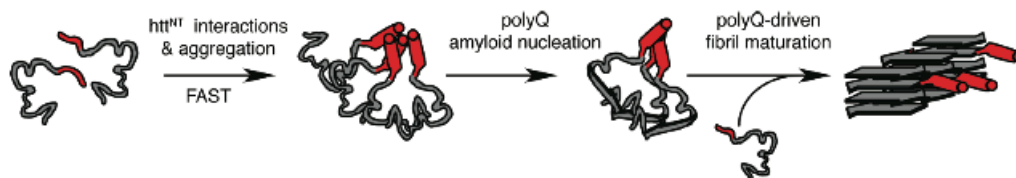


Figure 1.30 : Schematic model of the htt^{NT}-initiated aggregation process; The htt^{NT} segment initiates the aggregation process, mediated by htt^{NT}-htt^{NT} interactions resulting in oligomeric species featuring substantial helicity. This permits the formation of the amyloid core, involving the generation of β -sheet secondary structure within the polyQ stretch. Upon maturation, a highly rigid and dehydrated amyloid core (consisting of polyQ) is decorated with N-terminal residues that are relatively mobile and solvent exposed [204].

1.4.4 Detection of fibrillization kinetics using Surface Plasmon Resonance Spectroscopy (SPR)

Huntingtin fibrillization was investigated by using various techniques. Among them fluorescent microscopy, circular dichroism (CD), quasielastic light scattering, electron microscopy, mass spectroscopy, fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), high pressure liquid chromatography (HPLC) and atomic force microscopy (AFM) could be encountered [192, 193, 198, 205, 206]. These approaches provide information about fibrillogenesis over relatively long periods of time (hours). To track the fibrillogenesis relatively in shorter times (from 3 sec to 30 min), surface based detection methods such as surface plasmon resonance (SPR) spectroscopy and quartz

crystal microbalance (QCM-D) could be used. These methods not only decrease the time required for analysis of fibrillogenesis but also, allow direct measurement of the kinetic rate constants at each step of the fibrillogenesis as a function of time. Nonetheless, effects of additional compound on the fibrillogenesis such as ions, chaperons and antibodies could be studied with these methods [207-210].

The first attempt to utilize SPR for fibrillization studies was to track quantitative kinetic data for fibril elongation of A β protein [208, 211, 212]. Meanwhile, the technique was being applied to study the interactions of recombinant PrP^c with various molecules. Then, also successfully utilized to monitor the fibrillization kinetics of PrP^c protein and allowed researcher to elaborate the previously proposed polymerization model by using quantitative data [213-216]. The first study on huntingtin that takes advantage of SPR was focused on the molecular interactions of the protein [209, 217]. The interaction of an antibody (MW1) Fab fragment with huntingtin protein were examined in this study. Results entail researchers to propose a lattice model where expanded polyQ proteins bind to ligand (MW1) with higher affinity than the normal length protein. Recently, molecular interactions of huntingtin with two of its partners, SH3GL3 and 2B4 antibody was investigated in a similar manner [218]. SPR was also successfully applied to identify inhibitors for huntingtin aggregation [219].

However, the benefit of SPR on the detection of fibrillization was not engaged with huntingtin aggregation. In this thesis project we applied SPR technique to monitor real time fibrillization kinetics of huntingtin. Due to the requirement of a proper immobilization technique for SPR applications, we aimed to use GEPIs. One of the gold binding peptides, GBP1 [25, 49], was utilized as a fusion partner for Htt proteins and lead their immobilization to SPR sensor chip, gold surface. Then fibril elongation kinetics in different fibrillization stages were monitored and results were discussed to propose a fibrillization model.

1.4.4.1 Surface Plasmon Resonance Spectroscopy

SPR is a characterization technique for biomolecular interactions [220]. The fundamental principle of SPR is the resonance of surface plasmons, which provide a sensitive method to extract information about interaction mechanisms of molecules of interest [221, 222]. Surface plasmons can be defined as propagating electron

density waves occurring at the interface between metal and dielectric. The formation of surface plasmons, occur with the excitation of free electrons on the surface of metal, which are commonly gold or silver [223]. This excitation is formed through an electromagnetic radiation that hits the surface of a thin metal film and totally reflected. Photons, energy carrying particles of light, transfer their energy to surface electrons, which cause them to oscillate since they are excited. At a specific angle between the light and the metal, surface electrons are excited in such a way that they oscillate at their resonance frequency [224]. Excitation of surface plasmons at a specific angle results a minimum or a dip in the intensity of the reflected light (Figure 1. 31). This angle is called resonance or SPR angle. This angle is related with optical properties of the system for example the refractive index of the media at both sides of the metal [223].

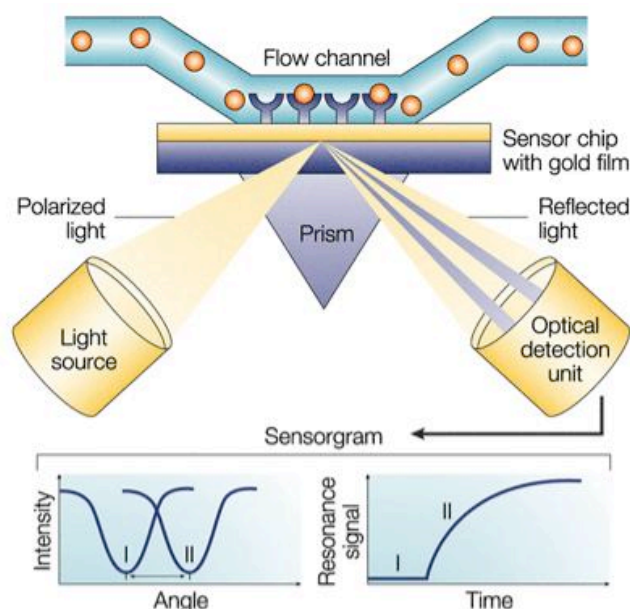


Figure 1.31 : Working principle of Surface Plasmon Resonance Spectroscopy. The SPR angle shifts (from I to II in the lower diagram) when biomolecules bind to the surface and change the mass of the surface layer. This change in resonant angle can be monitored in real time as a plot of resonance signal versus time [65].

By monitoring this dip position versus time, SPR spectroscopy provides a powerful kinetic technique for the detection of the changes on the surface. These surface changes might arise from the differences in the liquid solutions to which it's exposed or molecular interactions take place on the surface. When the gold surface is functionalized with biomolecular receptors, it is a very effective tool for measuring the kinetics and equilibrium constants of bioaffinity interactions [225].

1.5 Lactate Dehydrogenase (LDH) Enzyme

Lactate dehydrogenases (LDH) (EC: 1.1.1.27, L-lactate NAD⁺ oxidoreductase) are members of the family NAD-dependent 2-hydroxycarboxylate dehydrogenases [226]. LDH is a ubiquitous enzyme among vertebrates and catalysis the interconversion of pyruvate to lactate by utilizing the NADH/NAD⁺ reduction couple [227]. The conversion of pyruvate to lactate constitutes the last step of glycolysis, which provides ATP as the source of energy. Therefore, the major biological function of LDH is considered as maintaining glycolytic flux during prolonged periods of exposure to anoxic or hypoxic conditions.

LDH is a tetrameric enzyme, which is existed in different isozyme forms [228]. There are five isozymes numareted as LD1-LD5 and are consisted of two different subunits designated as B or M (muscle) and A or H (hearth) [229]. These five isozymes are either homo or heterotetramers; H₄, H₃M₂, H₂M₂, HM₃ and M₄. Besides then, one more isozyme (LD-X) is found in testicular postmeiotic, haploid cells and spermatozoa. LD-X is a homotetramer formed by C₄ subunits. Although, subunits A, B and C are translated form different genes, all display a molecular weight around 35 kDa. The homotetramers H₄, M₄ and X₄ show different catalytic activities with regard to difference in their amino acid contributions [230]. These characteristics have generally been used to explain the physiological role and tissue distribution of the isoenzymes. While LDH is common for all tissues, the quantitative distribution of isozymes differs for each tissue. Therefore, when LDH is released to the serum in case of an injury, following the LDH isozyme distribution change in serum is helpfull to track the injured tissue.

Lactate dehydrogenase is also present in invertebrates, plants and microbes [231-233]. Many bacteria produce some lactate and fermentations of a mixed type are frequent. The bacteria forming lactate are generally named as lactic acid bacteria. Some lactic acid bacteria convert 85% of glyucose into lactate, where some converts only half while the rest of the glyucose is converted into CO₂, acetate and/or ethanol [234]. Bacterial LDHs could be divided into two group; NAD dependent, nLDH and NAD independent, iLDH. iLDHs are mainly found in catalase-positive organisms, where they enable bacteria to use lactose as a carbon source. Among nLDH, some of them found to produce L-lactate (L-LDH) and some D-lactate (D-LDH). D-LDHs

are seen in lower organisms [235, 236]. Also in these organisms, some results indicated that the active form of the enzyme is a dimer. Although vertebrate LDHs are non-allosteric, some bacterial LDHs are activated by an allosteric effector, fructose-1,6-bisphosphate (FBP) [237-239].

1.5.1 Structure of *Bacillus stearothermophilus* LDH (bsLDH)

The investigation of the structure and enzymic mechanism of bacterial lactate dehydrogenases rather than eukaryotic ones offers several advantages. The first advantage comes from their susceptibility to genetic manipulation, thus providing an opportunity for discerning the structural and mechanistic roles of individual amino acids. Also many bacterial enzymes are stable to heat and may yield information on the structural factors, which give rise to their thermostability. Besides, their activation by fructose-1,6-bisphosphate renders them suitable vehicles for observing the precise molecular events in mediate effector control. Lastly, they can provide information on the forces and amino acid residues involved in dimer-to-tetramer assembly [240, 241].

The *Bacillus stearothermophilus* LDH (bsLDH) enzyme is a typical prokaryotic enzyme, which exhibits allosteric activation (by FBP). Also it lacks the N-terminal arm, common for eukaryotic enzyme, which is the part responsible for tetramerization. The main reason to study bsLDH is its close structural relativity with vertebrate enzymes. The amino acid sequence of thermophilic LDH from *Bacillus stearothermophilus* was obtained by cloning the gene in *E.coli* and sequencing thereafter [242-244]. The gene consists of a 951bp long open reading frame (ORF) starting with ATG codon and stopped with TGA. There is a shine-dalgarno sequence upstream of the start codon, which indicates the gram-positive ribosome binding [242]. Thermophilic bsLDH enzyme consists of 317 amino acids which are not forming disulphide bridges [244]. Among the primary structure, 3D structure and substrate, cofactor, allosteric activator and ligand binding sites of the enzyme are characterized by x-ray crystallography studies (Figure 1.32) [245-249].

Although bsLDH is structurally similar to vertebrate counterpart, it's lack of the first 14 amino acids of the vertebrate enzyme [250]. This lacking region is suggested to be important in tetramer formation, thus tetrameric form of bsLDH is unstable in low concentrations. While dimeric enzyme can exhibit activity, an increase in the

catalytic activity is observed in the presence of fructose 1,6 biphosphate, FBP. Therefore, allosteric activator is found to be important in the conversion of dimer to tetramer forms. Each dimeric form of the enzyme binds to a single FBP and two of these intermediates combine to form a liganded tetramer [251]. X-ray crystallography studies revealed a 3D structure of the tetrameric enzyme, which is available from Protein Database (PDB) with the PDB ID of 2LDB (Figure 1.33).

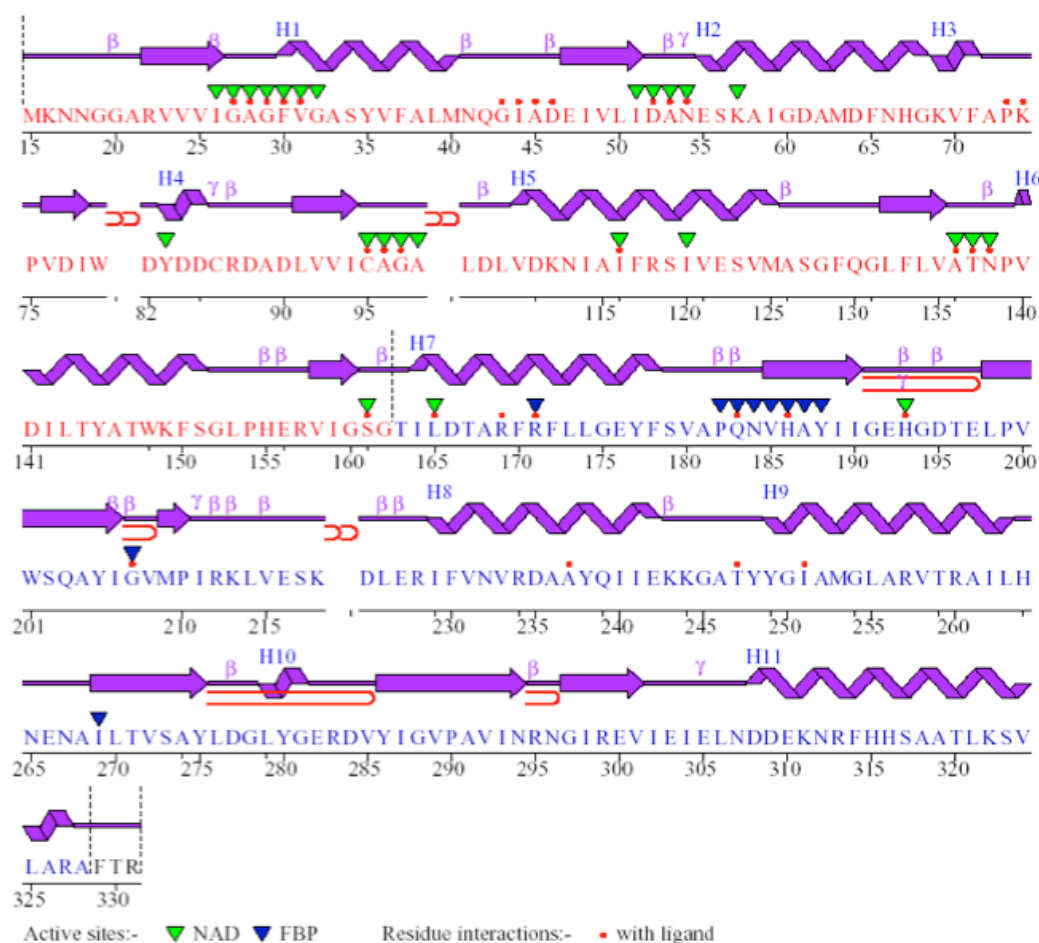


Figure 1.32 : Primary amino acid sequence of bsLDH. Structural domains are shown in purple. Cofactor (NAD), allosteric activator (FBP) and ligand interaction sites are labelled with green triangle, blue triangle and red dots, respectively (MMDB ID: 53831, PDB ID: 2LDB).

The active site of the enzyme is a loop occurred by the residues 98 to 110. The conformation of the loop determines the substrate specificity. The Glu-105 of eukaryotic enzyme is replaced with a proline residue in bsLDH providing thightness to the loop. Thus, restrains it in a more open conformation [249].

The interaction of coenzyme NAD with bsLDH is required to produce the correct conformation needed for catalysis. NAD mediates hydrogen bonding with the enzyme as well as with a number of water molecules. The major difference between the binary and ternary structures concerns the proposed hydrogen bond between the nitrogen atoms of the carboxamide side-chain of the coenzyme with the main-chain carbonyl group of residue 138.

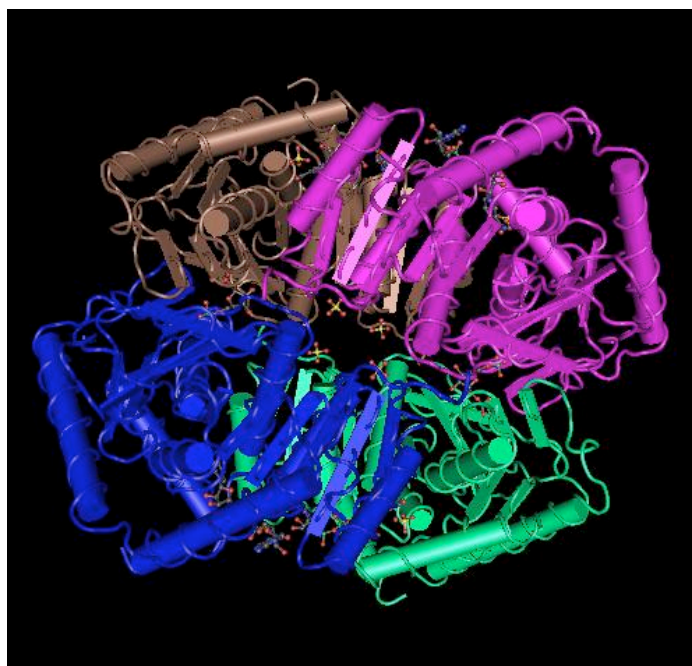


Figure 1.33 : 3D structure of tetrameric bsLDH. Each subunit is indicated in different colors (blue, brown, green and purple) (PDB ID: 2LDB) [247].

The substrate, pyruvate, binds to bsLDH-NAD ternary complex. The carboxylate of the substrate forms a salt bridge with side chain of Arg-171 and oriented by this interaction [252]. In the active complex, pyruvate's methyl side chain is then postulated to sit under the 98-110 loop [253]. The carbonyl group forms a hydrogen bond with the unprotonated imidazole ring of His-195. During catalytic activity, His-195 donates a proton and is stabilized by Asp168. The substrates with larger side chains than pyruvate would extend into region occupied by Gln-102, Arg-109 and Thr-246 (Figure 1.34) [247, 249, 254, 255]. Two arginine residues 109 and 171 as well as His-195 are available to provide a partner for an ion-pair. Arg-109 that is involved in catalysis and polarizes the carbonyl bond of pyruvate is the epsilon-nitrogen [256, 257]. The terminal nitrogen is linked to a network at the break between helices α -1G and α -2G (Try-237, main chain nitrogen of 236 and carbonyls of 232 and 233) at the β -H/ β -H turn [242].

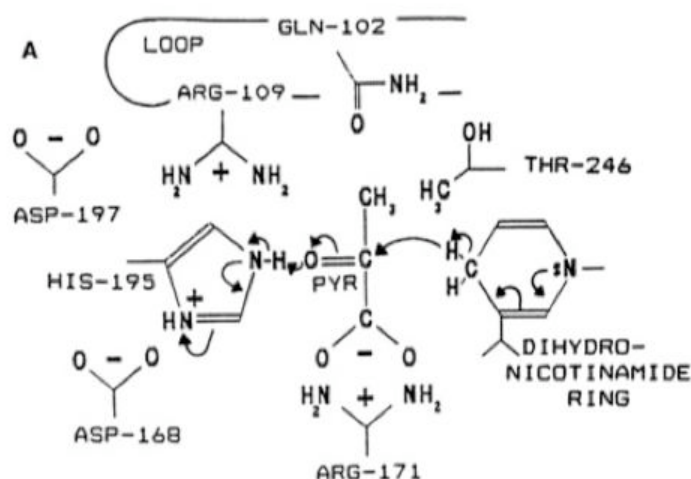


Figure 1.34 : The residues responsible for catalytic efficiency of LDH are shown; Arg109 polarizes the carbonyl bond, His195 donates a proton, Arg171 binds and orients the substrate and Asp168 stabilizes the protonated His195 [258].

1.5.2 Kinetic mechanism of bsLDH

Lactate dehydrogenase enzyme could bind to its substrate lactate or pyruvate only in the presence of coenzyme NAD^+ or NADH . Therefore the first step in the kinetic mechanism is considered as coenzyme binding [259]. Binding of coenzyme, induces the conformational change of the enzyme, in which residues 98 to 110 of the chain move through a relatively large distance to close over the active site in the ternary complex [250]. The dissociation of enzyme- NADH binary complex is a rate limiting step [241].

When lactate is added to the haloenzyme at neutral pH 7, NAD^+ is reduced rapidly leading to attain equilibrium between E-NAD^+ -lactate and E-NADH -pyruvate. The main reason for the equilibrium of two ternary complexes is the rapid hydride transfer as well as the slow dissociation of pyruvate. When the pyruvate dissociates, the equilibrium is displaced toward products. Then a slower dissociation of NADH occurs, which is the rate-limiting step in the steady state [241].

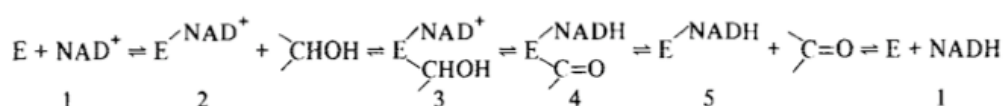


Figure 1.35 : The ordered binding of substrates to lactate dehydrogenases. The point at which the proton is taken up is not shown. >CHOH represents lactate and C=O represents pyruvate [241].

Although the binding of coenzyme is independent; there is still an allosteric regulation on bsLDH [241, 260]. The binding of allosteric regulator, FBP, stabilizes the tetrameric form of bsLDH. The stabilized tetrameric form binds pyruvate 50 times more tightly than the dimeric form does. The turnover numbers of tetramers and dimer are the same, and so they form a V-system.

Both mammalian and bacterial LDHs are inhibited by high concentrations of substrate, pyruvate. At high substrate concentrations, before the release of oxidized cofactor NAD^+ from the enzyme, pyruvate binds to the E-NAD^+ binary complex and form the inhibitory abortive adduct, $\text{E-NAD}^+\text{-Pyr}$ [261, 262]. This inhibition is a consequence of the formation of a covalent adduct between pyruvate and the oxidized cofactor [263]. The pyruvate inhibition takes place not only in high concentrations but also in low or high pH conditions [264]. Substrate inhibition is due to an abortive NAD^+ -pyruvate complex decreasing the steady state concentration of functional LDH [265, 266]. Researches are under progress for reducing substrate inhibition by focusing on reducing the affinity of the LDH-NAD^+ complex for pyruvate or reducing the affinity of the enzyme for NAD^+ [264, 267-269].

1.5.3 Importance of LDH in biotechnology

Enzymes play a crucial role in chemical transformations important to the pharmaceutical, chemical, agriculture and food processing industries because of their ability to produce chirally pure and complex molecules with interesting chemical and biological properties. They offer a number of advantages over conventional chemical catalysts [270-272].

Dehydrogenases are important in the production of chiral compounds [273]. One of the dehydrogenases, LDH catalyses the interconversion of pyruvate (oxo acid) and lactate (hydroxy acid) using the NADH/NAD^+ pair as a redox cofactor. The chirally pure α -hydroxy acids are used to produce various pharmaceuticals such as semi-synthetic penicillin (using $\text{S-}\alpha$ -hydroxyisocaproic acid) and antihypertensives (using $\text{S-}\alpha$ -hydroxyl-4-phenyl butanoate) [274]. The most important α -hydroxy acid produced by bsLDH is lactic acid (2-hydroxypropionic acid). Lactic acid consumption is high due to its wide utilization in cosmetic, pharmacy, food and chemical industry. This consumption arises from the role of lactic acid as complexion agent and sequester, solvent, cleaning agent, and raw material for

chemical synthesis [275].

The optical purity of lactic acid is also crucial to the physical properties of poly (lactic acid) (PLA). Only an optically pure L(+)- or D(-)-lactic acid, rather than racemic DL-lactic acid, can be polymerized to a high crystalline PLA. The production of pure L(+) or D(-) lactic acid might be achieved by using biocatalyst, LDH, rather than chemical synthesis. Thus, leads to the synthesis of the biodegradable polymer, PLA, which is currently used in packaging as films, thermoformed containers and short-shelf life bottles [276-278].

On the other hand, LDHs have an application area in medicine, mainly in diagnosis. They are used to detect S-ketoisohexanoic acid, which is an indicator of phenylketonuria [279]. LDH is also able to diagnose maple syrup urine diseases by detecting S-ketoisocaproic acid [280].

Since lactate is produced during normal metabolism and exercise, detection of lactate concentration plays an important role in clinical diagnostics, medicine validations and food analysis [281-284]. Biosensors based on the lactate detection facilitated by LDH provide an accurate and faster method over traditional chromatographic and spectrophotometric systems [285, 286]. The main consideration for biosensor design is the immobilization of LDH on the sensor surface with less reduction in its biologic activity. A number of methods have been utilized for LDH immobilization such as, adsorption, cross-linking, covalent attachment, conducting polymer entrapment and confinement in sol-gel matrixes [287-291].

LDH is utilized in one more area of biotechnology, which is enzymatic biofuel cell. Biofuel cells are an alternative energy source, which are capable of transforming chemical energy into electrical energy by biocatalysts [292]. LDH is one of the redox enzymes used in biofuel cells due to its direct electron transfer capability [293, 294]. However, direct electron transfer between enzymes and electrode is usually prohibitively slow due to the burying of electroactive prosthetic groups of the enzyme during immobilization. In order to promote direct electron transfer in fuel cell applications, various nanomaterials are applied such as carbon nanotubes, gold, titania and clay nanoparticles [295-298]. Besides these mediators such as pyrroloquinoline quinone, PQQ, are also applied to promote electron transfer [293].

In this thesis project, we aimed to use GEPIs as a proper immobilization mediator for

bsLDH. One of the gold binding peptides, AuBP2, was utilized as the fusion partner of bsLDH. The efficiency of immobilization was determined by using SPR monitoring. Then the activity of immobilized enzyme was analyzed and the retained activities due to immobilization was calculated. While immobilized enzymes provide many opportunities such as low enzyme consumption and reusability, LDH also takes attention due to its redox potential. The ability of direct electron transfer makes LDH, a candidate for biofuel application. In order to detect bsLDH's electron transfer capability when immobilized via AuBP2, cyclic voltammetry analysis were applied.

2. MATERIALS AND METHODS

2.1 GEPI-Htt Fusion Proteins

Gold binding peptide in two different repeats (3GBP1 and 5GBP1) were inserted to the N-terminus of Htt proteins containing different length polyQ tracks, in order to modulate their immobilization onto gold. Fusion constructs were transformed into *E.coli* cells and protein expressions were accomplished. Cytoplasmic fusion proteins were purified from total protein using affinity chromatography. The first analysis on the fusion proteins were AFM to visualize whether they were forming fibrils. Then, fibril elongation kinetics was monitored using SPR analysis. Experimental procedures applied are detailed in following subsections.

2.1.1 Construction of 3GBP-Htt and 5GBP-Htt proteins

We already had an expression vector coding 5GBP (5 repeat GBP) in pQE2 plasmid (Figure 2.1). Both three and five repeat forms of GBP, 3GBP and 5GBP, were amplified from pQE2 vector via PCR using site-specific primers. Primers were designed to insert BamHI and EcoRI restriction enzyme recognition sites to 3' and 5' ends of the GBP sequence respectively (Figure 2.2). The forward primer sequence was 5'-TAGGTTATTGGATCCATGCATGGAAAACTCAGGCAAC-3', while reverse primer was 5'-TTTAATATAGAATTCGCTCTGGATAGTCCCCGCTG-3'. Polymerase chain reaction was performed in a 50 μ L total volume containing 2 μ L template DNA (pQE2-5GBP plasmid), 1 μ L of 50 μ M primer for each forward and reverse and 25 μ L PCR-2X-master mix (The concentration of Taq polymerase, dNTPs and buffer from Fermentas). The optimized PCR conditions were 2 min. 94°C initial denaturation, 35 cycles of 20 sec. 94°C, 20 sec. 52°C, 1 min. 72°C and 10 min. final extension at 72°C.



Figure 2.1 : pQE2-5GBP plasmid vector map.

PCR product and DNA marker (Appendix A) was loaded on 2% agarose gel and electrophoresis was performed in 1XTBE (89 mM Tris-borate and 2 mM EDTA, pH 8.3) running buffer. 3GBP and 5GBP fragments of PCR product were cut from agarose gel. Then, purified from gel by using Qiagen Gel Extraction Kit according to manufacturers instructions.

pQE2-5GBP																					
CAT ATG ACC CGC CAA [ATG CAT GGA AAA ACT CAG GCA ACC AGC GGG ACT ATC CAG AGC], TTA TTA																					
PGEX-6P-1_Htt(n)Q																					
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCG GAA TTC ATT ATG TGT GAA CAA																					
L E V L F Q G P L G S P E F I M C E Q																					
PGEX-6P-1_5GBP-Htt(n)Q																					
GGG CCC CTG GGA TCC ATG CAT GGA AAA ACT CAG GCA ACC AGC GGG ACT ATC CAG AGC GAA TTC ATT ATG																					
CCC GGG GAC CCT AGG TAC GTA CCT TTT TGA GTC CGT TGG TCG CCC TGA TAG GTC TCG CTT AAG TAA TAC																					
G P L G S E F I M																					

Figure 2.2 : General strategy for GEPI-Htt fusion construction. 3GBP and 5GBP sequences were amplified from pQE-5GBP plasmid by inserting BamHI and EcoRI restriction enzyme sites to each end. pGEX-6P-1-HDnQ plasmid was digested with the same restriction enzymes and GBP sequences were ligated into.

The gel extracted, blunt-ended PCR fragments were ligated into pDrive TA cloning vector (Qiagen PCR cloning kit). Ligation was completed with 1-hour incubation at 37°C. Ligation mixture was used for bacterial transformation. *E. Coli* DH5α chemical competent cells were used as host strain. 5 µL ligation mixture was used to transformed into 50 µL competent cell. Following the ice incubation of 15 min., DNA uptake was promoted by 45 sec heat exposure of 42°C. Transformation mixture was than incubated for 1-hour at 37°C. At the end of incubation cells were plated on LB agar plates containing ampicillin as the selective marker of pDrive plasmid. The next day, cell colonies on plate were grown in 5 mL liquid LB medium for overnight at 37°C in variuos shaking. These cultured cells were subjected to plasmid isolation by

3GBP and 5GBP coding regions were digested from pDrive plasmids with BamHI and EcoRI (fast digest enzymes from Fermentas Inc.) restriction enzymes. DNA digestion was performed by the incubation at 37°C for 10 minutes. Digestion reaction and DNA marker (Appendix A) was run on 1% agarose gel and 3GBP and 5GBP fragments were gel extracted.

pGEX-Htt20Q and pGEX-Htt53Q vectors were also digested with the same restriction enzymes to obtain a linear plasmid DNA. Linearized plasmids were run on 1% agarose gel with Lambda DNA marker (Appendix B) and cut from gel for further extraction from gel. Gel purified 3GBP/5GBP fragments, which are sticky-ended were ligated with linearized pGEX-Htt vectors. Ligation was performed using Roche T4 fast ligase enzyme (in rapid DNA ligation kit) with an incubation of 15 min. at room temperature. A 3:1 molar excess of DNA fragment to plasmid DNA was used. Each ligation reaction was transformed into *E. Coli* DH5α chemically competent cells. Transformant colonies were selected from LB agar plate containing ampicillin as the selection marker for pGEX vector. Plasmids isolated from various positive clones were analyzed via DNA cycle sequencing reaction using 5' and 3' sequencing primers.

Based on DNA sequencing results, plasmid DNAs of fusion constructs with no mutations were transformed into *E. Coli* SURE cell that is the proper expression host. Competent cells were purchased from Stratagen and instructions of the manufacturer were followed for transformation. Finally, clones containing the fusion constructs were stocked in 20% glycerole and stored at 4°C till used for protein expression.

2.1.2 Expression of Htt fusion proteins in *E.coli*

The recombinant *E. coli* SURE strains harboring pGEX-Htt20Q, pGEX-3GBP-Htt20Q, pGEX-5GBP-Htt20Q, pGEX-Htt53Q, pGEX-3GBP-Htt53Q and pGEX-5GBP-Htt53Q expression constructs were grown in 50 ml LB containing ampicillin (100 µg/mL). The culture growth was performed overnight at 37°C by shaking at 200 rpm. The overnight culture was used to inoculate 1 L LB medium. Cells were grown at 37°C with shaking till O.D₆₀₀ reaches to 0.5. At that point, expression was induced by IPTG addition to a final concentration of 1mM. The culture growth was continued at 30°C for 4 hours, following the induction.

2.1.3 Purification of GST-tag Htt fusion proteins

The grown culture was transferred into centrifuge tubes. Cells were harvested by centrifugation for 20 min. at 4000 rpm, and the supernatant was discarded. Harvested cells were resuspended in wash buffer (50 mM NaPO₄, 150 mM KCl, 1 mM EDTA, pH; 8.0). As protease inhibitor, PMSF was added to a final concentration of 1mM. Finally lysozyme was added to 1 mg/mL, and the sample was incubated on ice for 30 minutes. Then, the lysate was sonicated three times for 10 sec with 10 sec pauses at 200 W. Following the sonication, bacterial lysate was centrifuged at 10.000xg at 4°C for 30 minutes and the supernatant composed of total protein was used for column purification.

Since fusion constructs are containing GST-tag, affinity chromatography was used for purification. Chromatography column was prepared with Glutathione Sepharose resin and equilibrated with wash buffer. Total protein was loaded to the column and flow through was collected. Then unbound traces were removed by washing the column with 5-column volume of wash buffer. GST-tagged proteins were eluted with elution buffer (wash buffer containing 15 mM reduced glutathione) in 3 mL fractions.

Protein samples collected during purification were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous buffer system. SDS-PAGE gels were prepared according to Molecular Cloning: a laboratory manual from J. Sambrook [299] and 4% stacking gel was used with 12% separating gel. 10 µL of each protein fraction was mixed with 10 µL of 2X-protein loading dye (Fermentas) and denatured at 95°C for 5 minutes. Protein samples and the molecular weight marker were run at 80 volt for stacking gel and 150 volt for separating gel.

2.1.4 Removal of GST-tag from proteins

Pure elution fractions were pulled and buffer exchanged to 1X cleavage buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDT, 1 mM DTT pH; 7.0) using 10000 MWCO ultra-filtration centrifugal devices. GST-tag was removed from purified proteins by 45 min cleavage with PreScission Protease performed in 1X cleavage buffer on ice. To get rid of GST and protease, protein solution was incubated with Glutathione Sepharose for 45 min at 200 rpm shaking and pure protein recovered in supernatant following a 5 min. centrifugation at 1000xg.

2.1.5 Atomic Force Microscopy imaging of fibrils

Fibrillization of Htt53Q was visualized under Atomic force microscopy. 6 μ M, 20 μ M and 40 μ M Htt53Q samples were prepared in Cleavage buffer. GST-tag was removed by the addition of PreScission protease (4 units/100 μ g of fusion protein) at time zero to initiate aggregation. Samples were incubated at 37 °C with shaking at 1400 rpm for the duration of the experiment. At time 1, 3 and 6 h after cleavage of the GST, 5 μ L of sample was taken from each concentration and deposited onto freshly cleaved muscovite mica (Ted Pella Inc., Redding, CA or Structure Probe, Inc., West Chester, PA) and template stripped gold. Template stripped gold samples were prepared using the protocol established by Hegner *et al*; briefly (111) gold evaporated on mica are bonded to silicon via Epo-Tek 377 epoxy (Epoxy Technology, Inc, Billerica, MA) and soaked in tetrahydrofuran for 10 minutes. Mica backing is then removed exposing the first deposited layer of gold [300].

Protein samples on mica and gold were waited till completely evaporated. Then, surfaces were pipetted with 200 μ L of DI H₂O for 10 minutes to dissociate salts and non-specific proteins from the interface. Images were generated in air at room temperature via a Veeco Multimode IIIa AFM system (Veeco, Plainview, NY) under acoustic/mechanical isolation using high resonance frequency non-contact tips (Nanosensors, Neuchatel, Switzerland). A Large 150x150 μ m scanner (Digital Instruments, Santa Barbara, CA) was used for imaging of surfaces above 10 μ m. For large area scanning, a resonance offset (~10% total amplitude) was used to allow for higher tip velocities.

2.1.6 Surface Plasmon Resonance Spectroscopy of fibrillization

Fibril elongation was monitored by using Surface Plasmon Resonance (SPR) spectroscopy from Reichert Instruments. The instrument has a flow cell placed on top of the prism coupler of the device. SPR sensor chips coated with 50 nm gold with 5 nm chromium underneath were used as sensor surfaces. SPR experiments were carried out in two ways in terms of fibril immobilization that are chemically and GBP mediated immobilization.

2.1.6.1 SPR monitoring due to chemical immobilization

Gold-coated SPR slides were embedded in the 1 μ M MUA (11-mercaptoundecanoic acid) solution and the formation of the self-assembled monolayer proceeded for 10 hours. Following the incubation, SAM modified surfaces were washed with ethanol and dried under N_2 . Prepared slides were placed in SPR chamber. A baseline was established by flowing DI water through the flow cell and the surface bound SAM –COOH ends were activated using a (1:1) mixture of 100 mM NHS-EDC solution. Following the activation of the –COOH groups on the surface, preformed fibrils were immobilized through covalent bonding. Following the washing step with DI water, the freely available carboxyl sites were blocked using ethanolamine. Access amount of ethanolamine was washed with DI water and a baseline was established with pumping 1X cleavage buffer. After the baseline was established, the Htt proteins in monomer form (just after GST removal) were sent onto immobilized fibrils on sensor surface. Soon after the interaction of the Htt with surface bound fibrils was seen (almost 2 minutes), buffer solution was pumped to observe desorption of the non-interacting monomers and unbound monomers to the fibril surface.

2.1.6.2 SPR monitoring due to biological immobilization

Gold-coated SPR slides were cleaned with a basic piranha solution (1:1:3 NH_3 , H_2O_2 and H_2O). The elongation experiments were carried out at 37°C to mimic the body temperature. Buffer solution (Cleavage buffer as wash buffer in SPR experiments) was pumped with a peristaltic pump and it was flown through the flow cell to establish a baseline. After a baseline was established, the fibrils of GBP-Htt were sent on sensor chip at a flow rate of 100 μ L/min. Fibrils prior to immobilization, were sonicated in a water bath for 5 minutes to succeed a more homogenous and controlled assembly of the fibrils [205]. The fibrils were sent on the surface of the gold sensor until an equilibrium state was reached, this nearly refers to an amount of 2000 μ RIU (Refractive Index Unit) of 3GBP-Htt. Following the equilibrium state, to remove the loosely and non-specifically bound fibrils, buffer solution was pumped to sensor surface. To track the fibril elongation of the Htt, proteins in monomer form (just after GST removal) were sent onto immobilized fibrils on sensor surface. Soon after the interaction of the Htt with surface bound fibrils was seen (almost 2

minutes), buffer solution was pumped to observe desorption of the non-interacting monomers and unbound monomers to the fibril surface.

2.1.7 Calculation of fibrillization kinetics

A two step model for fibril elongation and a one step model for proto-fibril elongation were used to fit the data. Using the kinetic model, the response for the fibril elongation was calculated. The calculated signal was compared with the measured response, and the values of the rate constants were found by minimizing the sum of square errors. To achieve this, the Solver function of Microsoft Excel was used, which performs a constrained nonlinear fitting based on the generalized reduced gradient algorithm.

SPR response for the adsorption step is represented with equation (a) and desorption step with equation (b) in the two-step model. For the one step model, SPR response for the adsorption step is represented with equation (c) and desorption step with equation (d). Whereas, the R_s is the calculated response, as the k_{a1} and k_{a2} are the rates for the adsorption and conformational change rates, k_d is the desorption rates from the monomers adsorbed on the fibril surface.

$$R_s = (K_1 * (1 - \text{EXP}(-k_{a1}t)) + (K_2 * (1 - \text{EXP}(-k_{a2}t))) \quad (a)$$

$$R_s = (R_1 * (\text{EXP}(-k_{d1}t)) + (R_2 * (\text{EXP}(-k_{d2}t))) \quad (b)$$

$$R_s = (K_1 * (1 - \text{EXP}(-k_{a1}t))) \quad (c)$$

$$R_s = (R_1 * (\text{EXP}(-k_{d1}t))) \quad (d)$$

2.2 GEPI-LDH Fusion Proteins

Gold binding peptide, AuBP2 was inserted to the N-terminus of LDH protein, in order to modulate their immobilization onto gold surface. Fusion construct was transformed into *E.coli* cells and protein expressions were accomplished. Cytoplasmic fusion proteins were purified from total protein using affinity chromatography. Following the activity analysis of the fusion protein, gold recognition ability and binding kinetics were determined using SPR technique. Then, immobilized enzyme activities were tested using gold nanoparticles as the immobilization support. Finally, direct electron transfer capability of the enzyme

was analyzed using cyclic voltammeter device. Experimental procedures applied are detailed in bellowing subsections.

2.2.1 Construction of AuBP2-LDH fusion proteins

wtLDH gene in pEQ2-6His vector was provided by Assos. Prof. Nevin Gül Karagüler (ITU). AuBP2 sequence was inserted into N-terminal of the protein using PCR. For AuBP2 fusion protein, two double step PCR reactions were performed. In the first step PCR, pQE2-LDH template was amplified using Primer 1 as the forward primer. This 15-cycle reaction was used as the template of the second step PCR reaction, where Primer 1 short is used as forward primer. Primers were given in Table 2.1. and PCR mixture and reaction conditions are summerized in Table 2.2.

In order to insert full lenght AuBP2 and linker region, a second PCR set had to be applied by using first PCR set as the template. Here, primers namely; primer 2 and primer 2 short were used for 1st and the 2nd steps of PCR reaction, respectively.

Table 2.1 : PCR primers for AuBP2 insertion.

AuBP2-LDH	Primer 1	5' AGATCCATCAGGAGACAGTCCTACG GTCCTTGCGGTGGTGGTTCCATGAAAA ACAACGGTGGAGC 3'
	Primer 1 short	5' AGATCCATCAGGAGACAG 3'
	Primer 2	5' TAGAGAGCTCAATGCGGCCCTTGGGC CCTGAGGAGA TCCATCAGGAGACAG 3'
	Primer 2 short	5' TAGAGAGCTCAATGCGGCCCT 3'
	Reverse Primer	5' GCACAAGCTTTCATCGCGTAAAAG 3'

Table 2.2 : 1st and 2nd step PCR conditions for AuBP2 insertion.

1 st step PCR		Conditions	
Expand High Fidelity PCR System 5X Buffer	4 µl	94°C 2 min	15 cycle
dNTP (10 µM)	0.3 µl	94°C 20 sec	
Primer 1/Primer 2 (10 µM)	1 µl	50°C 30 sec	
Template (pQE2-LDH or pQE2-FDH)	1 µl	72°C 4 min	
Enzyme Mix	0.2 µl	4°C α	
H ₂ O	13.4 µl		
2 nd step PCR		Conditions	
1st step PCR	20 µl	94°C 2 min	30 cycle
Expand High Fidelity PCR System 5X Buffer	6 µl	94°C 20 sec	
dNTP (10 µM)	0.3 µl	55°C 30 sec	
Primer 1 short / Primer 2 short (10 µM)	1 µl	72°C 4 min	
Reverse Primer	1 µl	4°C α	
Enzyme Mix	0.5 µl		
H ₂ O	21 µl		

PCR amplified fusion protein sequence (AuBP2-LDH) was ligated into a TA cloning vector pDrive (Qiagen PCR cloning kit). Ligation was completed with 1-hour incubation at 37°C and ligation mixture was transformed into *E. Coli* DH5α chemical competent cells. Transformation mixture was then incubated for 1-hour at 37°C. At the end of incubation cells were plated on LB agar plates containing ampicillin as the selective marker of pDrive plasmid. The next day, cell colonies on plate were grown in 5 mL liquid LB medium for overnight at 37°C in various shaking. Plasmid isolation was performed to these cultured cells by using Qiagen Plasmid Isolation kit according to manufacturer's instructions. Isolated plasmids from various cell colonies were subjected to DNA sequencing analysis in order to catch un-mutated clone containing pDrive-AuBP2-LDH. *E. Coli* DH5α strain harbouring pDrive-AuBP2-LDH plasmid was stocked in 20% glycerol and stored at -80°C.

AuBP2-LDH coding region was digested from pDrive cloning vector using SacI and HindIII restriction enzymes. Restriction reaction was performed with double enzyme in 37°C incubation for 10 min. Restriction mixture was run on 2% agarose gel and sticky-ended DNA fragment was removed. AuBP2-LDH coding fragment was extracted from agarose gel using Qiagen Gel Extraction kit. On the other hand pQE2 expression vector was also digested with the same enzymes to obtain an open plasmid for proper ligation, and ligated into pQE2 vector (Figure 2). AuBP2-LDH coding fragment was with linearized pQE2 vector in the presence of Roche T4 fast ligase enzyme (in rapid DNA ligation kit) with an incubation of 15 min. at room temperature. A 3:1 molar excess of DNA fragment to plasmid DNA was used. Each ligation reaction was transformed into *E. Coli* DH5α chemically competent cells. Transformant colonies were selected from LB agar plate containing ampicillin. Plasmids isolated from various positive clones were analyzed via DNA cycle sequencing analysis to find proper construct.

Final construct was obtained after the insertion of PreScission Protease Cleavage site between His-tag and the fusion protein. Thus, expressed protein would be removed from His-tag, which would interfere with gold surface. For this purpose, site-directed mutagenesis was applied to the fusion constructs. One set primer; forward primer (containing the protease cleavage site) and reverse primer were used in combination with Gene-Tailor site directed mutagenesis kit (primer sequences are given in table 2.3). First, 100 ng of templates (pQE2-LDH and pQE2-AuBP2-LDH) were methylated in

the presence of 10X-SAM and 4U DNA Methylase enzyme. Methylation reaction was performed in 1-hour at 37°C. Methylated DNA was used as template in site-directed mutagenesis PCR. 2µL methylated template was amplified using 10 µM from each primer and 1U Platinum *Taq* High Fidelity polymerase (Invitrogen). The cycling conditions applied were 2 min initial denaturation at 94°C, 20 cycles of 30 sec. 94°C, 30 sec. 55°C and 7 min. 68°C, followed by 10 min. final extension at 68°C.

Table 2.3 : Site-directed mutagenesis PCR primers for PreScission Protease insertion.

For AuBP2-LDH	Forward primer SDM-AuBP-LDH	5'CATGCGGGAGCTCAACTGGAAGTTCTGTTCC AGGGGCCCTGCGGCCCTT 3'
	Reverse primer SDM-AuBP-LDH	5 TTGAGCTCCCGCATGCATATGGTGATG 3'
For wtLDH	Forward primer SDM-LDH	5'CATGCGGGAGCTCAACTGGAAGTTCTGTTCC AGGGGCCCCATGAAAAACAACG 3'
	Reverse primer SDM-AuBP-LDH	5' TTGAGCTCCCGCATGCATATGGTGATG 3'

2-5µl of PCR reaction was utilized for transformation into *E. coli* DH5α chemical competent cells. Positive clones were analyzed through DNA sequencing and stocked in 20% glycerol. The schematic diagram of final AuBP2-LDH fusion construct is given in Figure 2.5.



Figure 2.5 : Schematic of AuBP2-LDH Fusion Construct.

2.2.2 Expression of LDH and AuBP2-LDH proteins in *E. coli*

The *E. coli* DH5α cells containing pQE2-LDH and pQE2-AuBP2-LDH fusion constructs were grown in 50 mL LB containing ampicillin (100 µg/mL). The culture growth was performed overnight at 37°C by shaking at 200 rpm. and used to inoculate 1 L LB medium. Cells were grown at 37°C with shaking till O.D₆₀₀ = 0.5. Then, expression was induced by IPTG addition to a final concentration of 0.5 mM. The culture growth was continued at 37°C for an additional 4 hours.

2.2.3 Purification of LDH fusion proteins

The grown cells were harvested by centrifugation for 10 minute at 4000 rpm, and the supernatant was discarded. Harvested cells were resuspended in lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH: 8.0). Lysozyme was added to 1 mg/ml, and the sample was incubated on ice for 30 minutes. Then, the lysate was sonicated three times for 10 sec with 10 sec pauses at 200 W. Following the sonication, lysate was centrifuged at 10.000xg at 4°C for 30 minutes and the supernatant containing total protein used for further purification.

50% slurry of Ni-NTA resin (Qiagen) was packed into a syringe in order to built a column. Ni-NTA column was equilibrated with more than 5 column volume of wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole pH 8.0). Then, total protein is loaded to the column and flow through was collected. To remove excess protein stacked in the column was eliminated with the washing steps carried out with wash buffers containing different imidazole concentration. Column was washed first with wash buffer containing 1% triton X-100 and 20 mM imidazole, than with wash buffer containing 1% triton X-100 and 50 mM imidazole. Proteins were eluted in elution buffer (wash buffer containing 250 mM imidazole). Elution was collected as 3 mL fractions.

Protein fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous buffer system. SDS-PAGE gels were prepared according to Molecular Cloning: a laboratory manual from J. Sambrook [299] and 4% stacking gel was used with 12% separating gel.

10 μL of each protein fraction was mixed with 10 μL of 2X-protein loading dye (Fermentas) and denaturated at 95°C for 5 minutes. Protein samples and the molecular weight marker were run at 80 volt for stacking gel and 150 volt for separating gel.

2.2.4 Affinity tag (His-tag) removal from LDH proteins

Pure elution fractions were pulled and buffer exchanged to 1X cleavage buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDT, 1 mM DTT pH; 7.0) using 30000 MWCO ultra-filtration centrifugal devices. His-tag was removed from purified proteins by 45 min cleavage with PreScission Protease performed in 1X cleavage buffer on ice. To get rid of His-tag protein solution was incubated with Ni-NTA resin for 45 min at

200 rpm shaking and protein recovered in supernatant following a 5 min. centrifugation at 1000xg. Then protein solution was incubated with Glutathione Sepharose resin for 45 min at 200 rpm shaking to remove protease, which is GST-tagged. Pure protein was obtained following a 5 min. centrifugation at 1000xg. Tag removed LDH and AuBP2-LDH enzymes were exchanged to 50 mM Tris buffer, pH 7.5 for further applications using 30000 MWCO ultra-filtration centrifugal devices.

2.2.5 LDH enzyme activity assay

Enzyme activities were measured at 25°C in 50 mM Tris buffer at pH 7.5 containing 0.2 mM NADH, 5 nM enzyme and 0–16 mM pyruvate. Activities were determined by observing the oxidation of NADH at 340 nm in 96 well elisa plates. The data were calculated using Enzyme Kinetics tool.

2.2.6 Surface binding studies using Surface Plasmon Resonance Spectroscopy

Binding kinetics of LDH and AuBP2-LDH enzymes were analyzed using SPR spectroscopy from Reichert Instruments. The instrument has a flow cell placed on top of the prism coupler of the device. SPR sensor chips coated with 50 nm gold with 5 nm chromium underneath were used as sensor surfaces.

The baseline was established by flowing 50 mM Tris Buffer pH 7.5 at a rate of 100 μ L/min. When the baseline obtained, enzyme was pumped to surface for ~10 minutes to follow the surface binding. Desorption of the enzymes were monitored while washing with Tris buffer. At least three different enzyme concentrations were tested for each enzyme, LDH and AuBP2-LDH, in order to extract kinetic and thermodynamic properties of the system with using Langmuir adsorption model. The raw data of each enzyme concentration was fitted to Langmuir isotherm and binding constants were calculated.

2.2.7 Determination of immobilized enzyme activities

In order to investigate immobilized enzyme activities, fusion proteins were immobilized onto 15 nm gold nanoparticles (AuNP from Ted Pella Inc.). 0.5 μ M enzyme (in final concentration) was incubated with 40 μ L AuNP (15 nm) in 100 μ L final volume. Incubation was established in room temperature for 10 min. with 350 rpm shaking. Then, AuNPs were settled with a 10 min. centrifugation at 13000 rpm and re-suspended in 50 mM Tris buffer, pH 7.5. Unbound enzyme concentration was

determined with Bradford reagent and used to calculate the bound enzyme concentration. Enzyme immobilized AuNP solution was used for activity assay. For comparison, the same amount of free enzyme was also assayed. Activity assays were performed in 50 mM Tris Buffer pH 7.5, 2.5 mM sodium pyruvate and 0.2 mM NADH. Activities were determined by observing the oxidation of NADH at 340 nm in 96 well elisa plates.

2.2.8 Determination of electron transfer capacity of immobilized LDH

A QCM-Z instrument (KSV Instruments, Norway) with temperature-controlled electrochemical flow cell module was used for determination of electron transfer between the enzyme and electrode. QCM-Z was connected to a potentiostat (Compactstat, Ivium Technologies, Netherlands). Measurements were conducted using a conventional three-electrode configuration. The gold coated quartz crystals (KSV Instruments, Norway) 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. 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 $\mu\text{L}/\text{min}$ for all solutions.

First, 50mM Tris buffer pH 7.5 was supplied into the system. After frequency stabilization, 2 mL of 0.5 μM enzyme was injected. When a new stabilization of the frequency was established 2 ml of NAD-lactate mixture was supplied. Cyclic voltammetry was performed to check the electrochemical reaction on working electrode. Cyclic voltammograms were recorded at gold working electrode modified with AuBP2-LDH or LDH from 0.2 to 1.7 V versus Ag/AgCl reference electrode in 50 mM tris buffer pH 7.0. Scan rate was set for 100 mV/s^{-1} in all experiments.

3. RESULTS AND DISCUSSIONS

3.1 Detection of Huntingtin Fibrillization Kinetics

Huntington's disease is characterized with intranuclear and/or cytoplasmic inclusion bodies caused by the mutant huntingtin aggregation [171, 186]. The aggregation of the mutant huntingtin is correlated with the poly-glutamine expansion in N-terminus of the protein. Since, disease pathology is highly related with the protein aggregates [187-190], it's crucial to identify aggregation kinetics. Thus, new therapeutic strategies could be developed.

In this study, fibrillization kinetics of mutant Huntingtin protein, Htt53Q, containing 53 glutamine residues were investigated. As a control protein, Htt20Q was selected which is found in healthy people, in other words the protein not causing the disease. In order to investigate fibrillization kinetics, Surface Plasmon Resonance (SPR) Spectroscopy was utilized. During the SPR experiments proteins had to be immobilized on gold sensor chip of the device. Therefore Htt proteins were expressed as the fusions with Gold Binding Peptide (GBP1) [25]. Repeated forms of GBP1 were inserted to the N-terminus of the Htt proteins. The reason for using multiple repeat forms of GBP1 was to increase gold binding affinity of the peptide. Previous studies have showed that 3 repeat GBP1 (3GBP1) has a better binding affinity for gold than single repeat one [25, 41, 42]. Initially, 3 and 5 repeat forms of GBP1 were inserted to Htt proteins in order to follow their effect on fibril formation and gold binding properties. According to the results, one of the repeated peptides was selected for further investigation of fibrillation kinetics. The following subsections will represent the detailed information about the experimental results and final discussions.

3.1.1 Construction of 3GBP-Htt and 5GBP-Htt proteins

Huntingtin proteins with expanded glutamine regions are tend to aggregate and form fibrils [170, 188, 193]. Aggregation of the protein would start even they have been translated in the living cell. In order to prevent protein aggregation and to facilitate

purification, Htt proteins have been expressed with different affinity tags. Among them, Flag-tag, GFP-tag and GST-tag can be mentioned [170, 301, 302].

In our experiments, Htt proteins were expressed with an N-terminal GST (Glutathione-S-Transferase)-tag. The expression vector utilized was pGEX-6P-1 (GE-Healthcare) and constructs for GST-tagged Htt proteins were kindly provided by Prof. Dr. Paul Muchowski (UCSF). We have inserted GBP peptides between GST-tag and Htt protein coding sequences in pGEX-6P-1 expression vector containing Htt20Q and Htt53Q proteins.

3GBP and 5GBP coding sequences were amplified from pQE2 vector -previously constructed for 5GBP expression and purification using 6His-tag system- via PCR using site-specific primers (Figure 3.1). Primers were designed to insert BamHI and EcoRI restriction enzyme recognition sites to 3' and 5' ends of the GBP1 sequence respectively. When PCR product was run on agarose gel, five different DNA fragments were observed. Each PCR fragment was belonging to different repeated forms of GBP1.

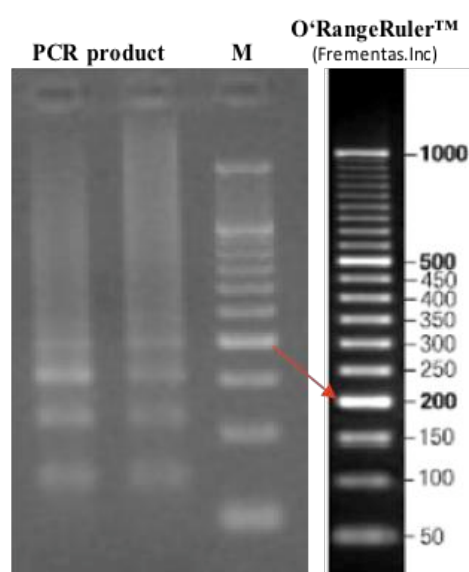


Figure 3.1 : Agarose gel electrophoresis image of 1-5 repeat GBP coding DNA fragments as the PCR products of 5GBP-pQE2.

Each DNA fragment of PCR reaction was cuttened and extracted from agarose gel. Then, gel extracted PCR fragments for each different repeats of GBP1 were ligated into pDrive cloning vector resulting with pDrive vectors containing 1, 2, 3, 4 and 5 repeat forms of GBP1. Each cloning vector was tranformed into *E.coli* DH5α cells

and obtained in multiple copies. The pDrive vectors positive for coding 3GBP and 5GBP were selected with restriction enzyme digestion using BamHI and EcoRI (Figure 3.2). Their sequences were verified by DNA sequencing analysis and the clones without mutations were stocked with glycerol. According to Figure 3.2, first pDrive clone was found to be coding 5GBP and named as pDrive-5GBP, whereas clones 4, 5 and 6 were found to be coding 3GBP and named as pDrive-3GBP.

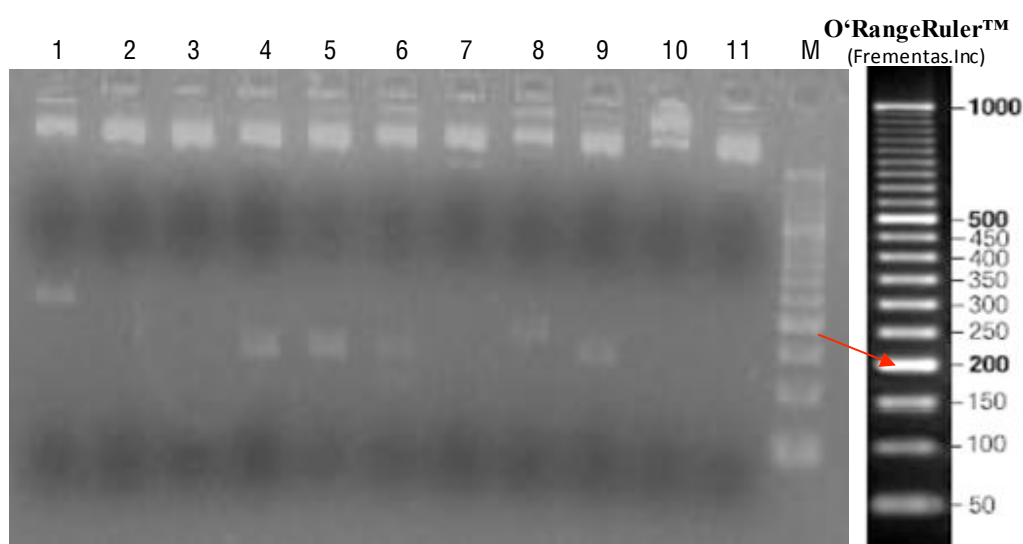


Figure 3.2 : Agarose gel electrophoresis image of Restriction enzyme digestion of pDrive vectors containing 5GBP (lanes 1-3) and 3GBP (lanes 4-11).

Following the cloning vector amplification, 3GBP and 5GBP coding sequences were digested from pDrive by using BamHI and EcoRI restriction enzymes. Meanwhile pGEX-6P-1 vectors containing Htt-53Q and Htt-20Q were also digested with the same enzymes (Figure 3.3). GBP1 sequences were ligated with pGEX-6P-1 vectors in the presence of T4 ligase (Quick ligase from NEB) and transformed into *E.coli* DH5 α -T1 CaCl₂ competent cells. These cells were used because of their high transformation efficiencies. After obtaining the GBP-Htt constructs, their sequences were verified with DNA cycle sequencing. GBP-Htt constructs without any mutations were transformed into *E. Coli* SURE cells for protein expression. The general schematic view of GBP-Htt fusion proteins is given in Figure 3.4.

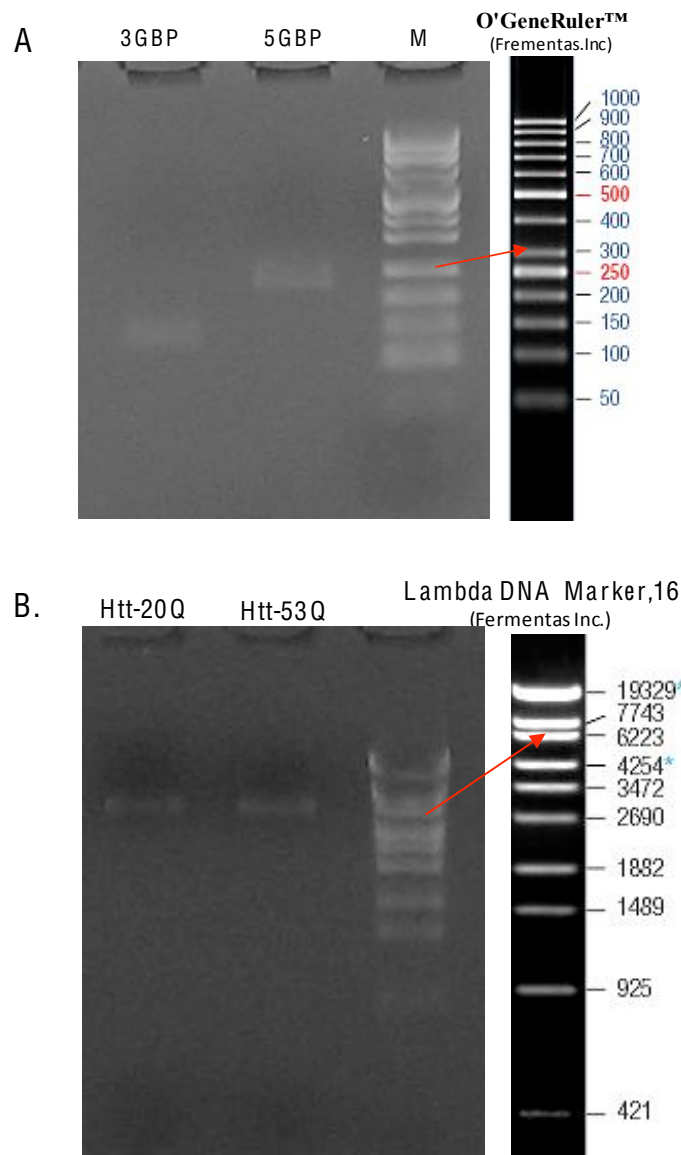


Figure 3.3 : Agarose gel electrophoresis images of A. 3GBP and 5GBP coding DNA fragments following the restriction enzyme digestion, B. pGEX-Htt53Q and pGEX-Htt20Q vectors following restriction enzyme digestion.

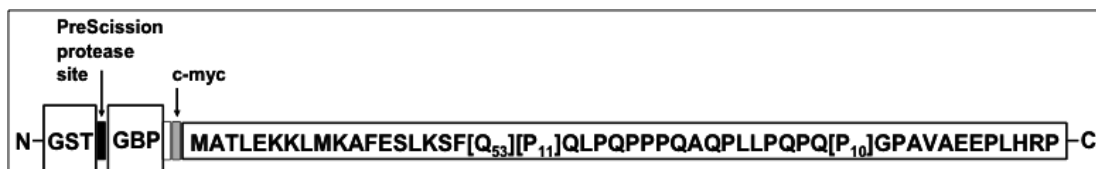


Figure 3.4 : Schematic of GBP-Htt fusion constructs.

3.1.2 Expression of Htt fusion proteins in *E.coli*

For the expression of Htt and GBP-Htt proteins, *E. coli* SURE cells were used as the bacterial host. This strain was chosen because it lacks the components of the pathways that catalyze the rearrangement and deletion of nonstandard secondary and tertiary structures, including cruciforms (caused by inverted repeats) and Z-DNA, that occur frequently in eukaryotic DNA [303, 304]. In the case of Htt proteins, there are large Glutamine repeated regions, which are coded with CAG codon making the RNA transcripts very sustainable for deletion. The usage of SURE cells as the expression host resulted with full-length protein products. Both Htt and GBP-Htt protein expressions were achieved by the induction of the culture for 4 hours at 30°C in the presence of 1 mM IPTG.

3.1.3 Purification of GST-tag Htt fusion proteins

Expressed proteins containing a GST-tag were purified from Glutathione Sepharose under non-denaturing conditions (wash and elution steps were performed in phosphate buffer). The expression level, purity and molecular weights of eluted fusion proteins were analyzed by SDS-PAGE (Figures 3.5 and 3.6).

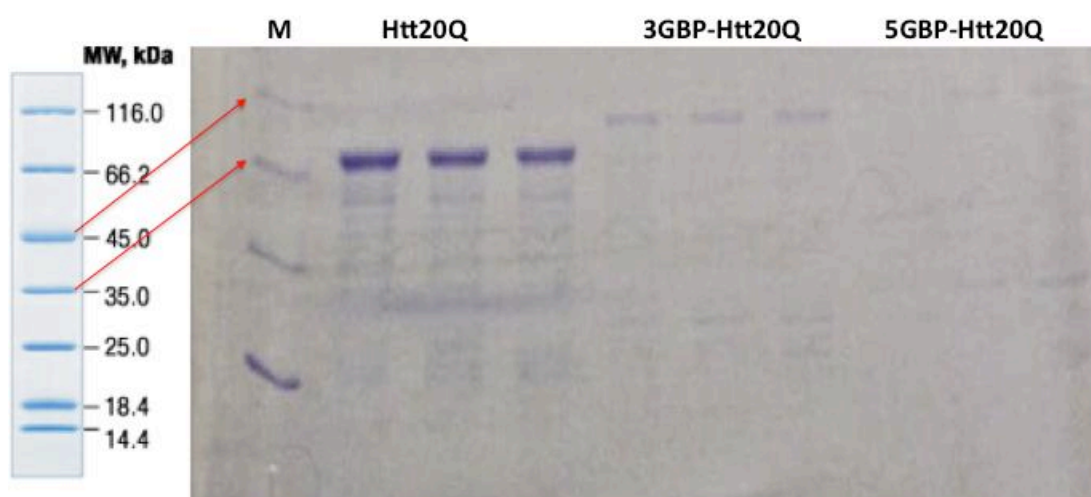


Figure 3.5 : SDS-PAGE analysis for elution fractions of Htt53Q and GBP-Htt53Q proteins. M; Protein Weight Marker (Appendix C), Three elution fractions of Htt53Q, 3GBP-Htt53Q, 5GBP-Htt53Q, respectively.

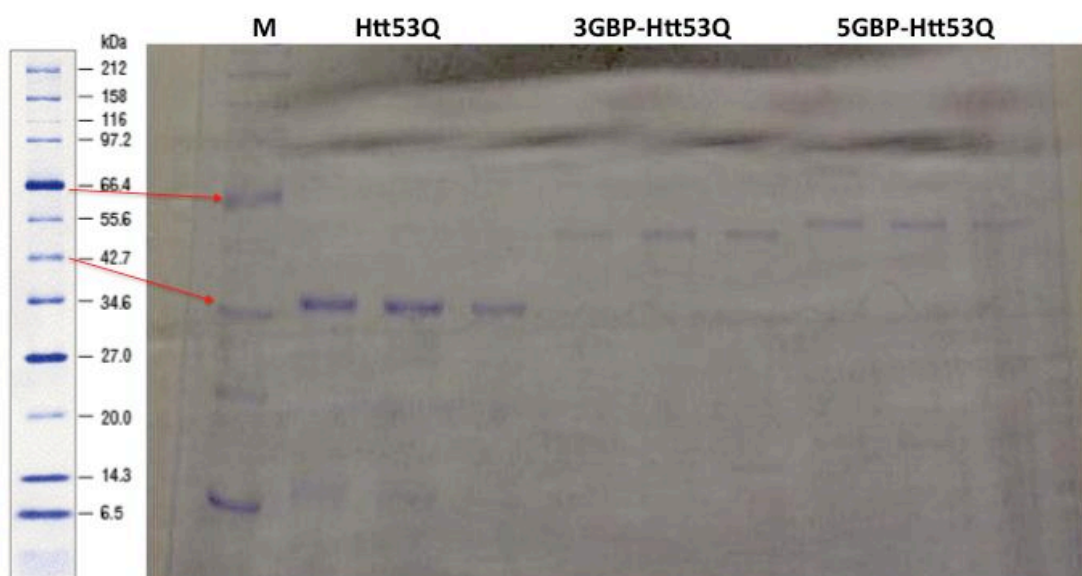


Figure 3.6 : SDS-PAGE analysis for elution fractions of Htt53Q and GBP-Htt53Q proteins. M; Protein Weight Marker (Appendix D), Three elution fractions of Htt53Q, 3GBP-Htt53Q, 5GBP-Htt53Q, respectively.

Molecular weights of fusion proteins were calculated (Table 3.1) and compared with the proteins bands on SDS-PAGE images. GST-Htt20Q protein was observed more than 35 kDa whereas GST-5GBP-Htt20Q was around 45kDa. As expected GST-3GBP-Htt20Q protein band was between 35 kDa - 45 kDa. On the other hand, GST-Htt53Q was observed more than 42.7 kDa, which is close to calculated weight. However, GST-3GBP-Htt53Q was around 50 kDa and GST-5GBP-Htt53Q was slightly bigger than that.

Table 3.1 : Fusion Proteins Constructs and Calculated Molecular Weight.

GBP-Htt construct	Calculated MW	MW with GST-tag
Htt20Q	12768.62 Da	38768.62 Da
3GBP-Htt20Q	17054.37 Da	43054.37 Da
5GBP-Htt20Q	19824.46 Da	45824.46 Da
Htt53Q	17094.05 Da	43094.05 Da
3GBP-Htt53Q	21195.61 Da	47195.61 Da
5GBP-Htt53Q	24052.77 Da	50052.77 Da

3.1.4 Removal of GST-tag from Proteins

Following the protein purification, GST-tag was removed from proteins during 45 minutes cleavage with PreScission Protease in Cleavage buffer. When cleavage is completed, GST and protease (which is GST-tagged also) had to be removed in order to obtain pure protein. Therefore, protein solution was incubated with Glutathione Sepharose 4B for 45 minutes and pure protein recovered in supernatant following a 5 minutes centrifugation at 1000xg. Pure protein was eluted in the flow through fraction (Supernatant after the centrifugation step). Then an elution step was applied to recover GST-tagged PreScission Protease, GST-tag and uncleaved GST-GBP-Htt proteins. Fractions were immediately denatured for preventing Htt proteins from aggregation. Therefore proteins would be analysed on SDS-PAGE (Figure 3.7).

As mentioned before, Htt proteins are forming aggregates when they lost GST tag and the aggregates formed are SDS insoluble [170, 188, 305]. SDS-PAGE analysis of GST-3GBP-Htt53Q cleavage indicated that, despite the aggregation tendency of Htt proteins, GST tag removal would be observed in SDS-PAGE at the 75th minute after cleavage had started. Even if some of the protein turned into fibrils, they wouldn't be able to enter the gel and not be visualized. Despite this, our observation is indicating that most of the Htt-53Q protein is still in monomeric form and therefore could be utilized as the monomer protein during SPR experiments.

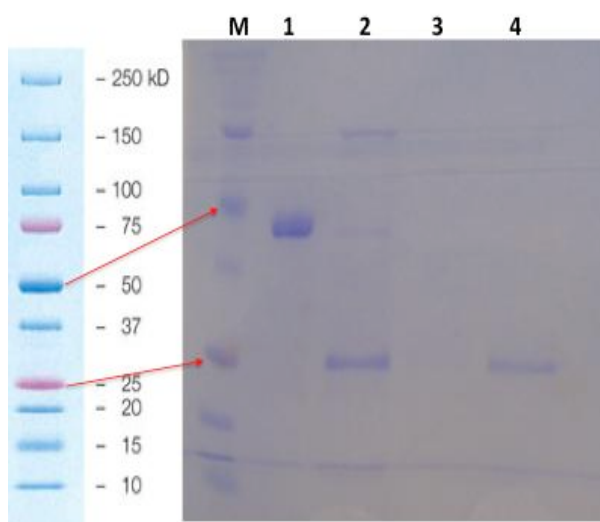


Figure 3.7 : SDS-PAGE analysis of GST-3GBP-Htt53Q cleavage reaction. M; Protein Weight Marker, Lane 1; GST-3GBP-Htt53 protein before cleavage, Lane 2; Elution fraction, Lane 3; Wash fraction, Lane 4; Flow through fraction.

3.1.5 Atomic Force Microscopy imaging of 5GBP and 3GBP-Htt fibrils

Atomic force microscopy has been utilized to perform aggregation profile analysis of Htt proteins with regard to GBP fusion. Individual 5GBP-Htt53Q and 3GBP-Htt53Q monomers and fibrils were scanned on mica surfaces, firstly, to determine the time points of monomer, proto-fibril and fibril structure formations. Fibrillization was initiated by the removal of GST-tag. The tags were cleaved with PreScission protease and during the cleavage reactions; samples were collected (before cleavage, 1st, 3rd, 6th and 18th hour of the cleavage) and analyzed under AFM.

AFM experiments showed that proteins fused with GBP were still forming aggregates, which is a requested result. According to the results, time points for monomers to be converted into proto-fibrils was 15-20 hours for 5GBP-Htt53Q protein whereas 6-8 hours for 3GBP-Htt53Q proteins (Figure 3.8). It was already known from previous experiments that first visuable Htt53Q fibrils are formed in 3-5 hours [194, 306]. These observations indicated that presence of GBP as fusion partner with Htt, increases the fibrillation time. Moreover, this increase is depending on the GBP repeat number. 3GBP slows down the fibrillation of Htt53Q by almost two fold while the decrease in fibril formation with 5GBP is more than five-fold.

There are two main considerations on following the fibrillization kinetics with GBP based immobilization. The first one is, to attach Htt proto-fibrils/fibrils on the gold surface and the second one is protecting the wtHtt protein properties such as fibrillization profile. GBP in any repeated forms have a great affinity for gold surface therefore utilizing even 5GBP or 3GBP would not make big difference on surface binding [41, 42, 60]. However the second consideration restricts the GBP repeat according to the results obtained from AFM experiments. Fibrillization time elapse in the case of 5GBP indicates a great difference in aggregation profile and would give a deviation on the fibrillization kinetic constants. However, close prefibrillization time of 3GBP-Htt to wtHtt, makes it a better candidate for following the fibrillization kinetics. Since this, 3GBP-Htt constructs were used for fibril elongation experiments carried out with SPR.

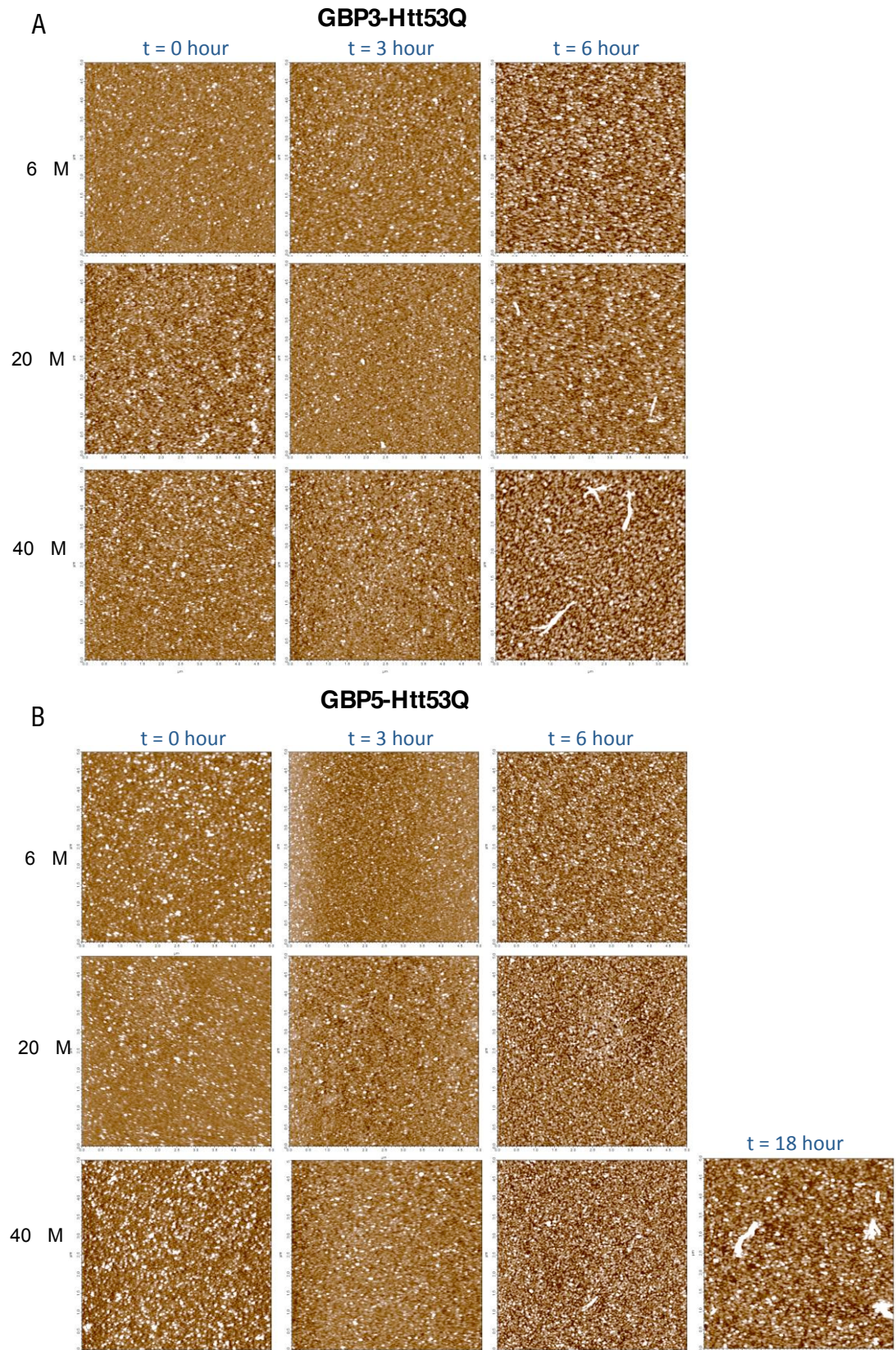


Figure 3.8 : AFM analysis of fibril formation. Time 0 indicates the initiation of GST cleavage reaction. a. Fibrillation of 3GBP-Htt53Q proteins with different concentrations b. Fibrillation of 5GBP-Htt53Q proteins with different concentrations (All the images are in 5μm x 5μm scale).

According to the AFM results (Figure 3.8), time points for monomers to be converted into fibrils and proto-fibrils were determined. Thereafter, monomer, proto-fibril and fibril proteins used in SPR experiments were performed according to these observations. Where, both Htt53Q, 3GBP-Htt53Q and 5GBP-Htt53Q proteins were found in monomer forms following the 2.5-hour cleavage and purification period. During fibrillization experiments, Htt53Q cleaved and purified in less than 2 hours was used as monomer protein. On the other hand, 3GBP-Htt53Q and 5GBP-Htt53Q were accepted to form into proto-fibrils at 6 hr and 15 hr, respectively. Therefore, 3GBP-Htt53Q proto-fibrils used in fibrillization experiments were prepared with 5-hour incubation following the GST cleavage. Besides, proto-fibrils of 5GBP-Htt53Q were prepared with 15-hour incubation following the GST cleavage. Mature fibril formation of both proteins was performed with an overnight incubation.

3.1.6 Gold surface binding profiles of 5GBP and 3GBP-Htt fibrils

AFM was also used to visualize GBP1 effect on immobilization. The immobilization results of the proteins alone and in the construct form, genetically fused to GBP, show interesting results (AFM images in Figure 3.9). Both Htt53Q and 5GBP-Htt53Q fibrils were visualized on mica and gold surfaces. Htt53Q proteins were formed huge fibrils after an overnight cleavage reaction (Figure 3.9 a and b). These fibril structures could be identified as bundles, which are formed by the fibril-fibril interactions. In the case of Htt53Q, although the fibril sizes are constant when they immobilized on mica and gold surfaces, the surface coverage was higher on gold surface. This might be the gold binding tendency of Htt53Q protein.

On the other hand, in the presence of GBP1 in the construct (Figure 3.9 c and d), formation of fibril bundles has decreased. This might be due to the GBP content, which is preventing fibrils to interact with each other. Therefore, small fibrillar structures have been obtained in the same protein concentration with Htt53Q. Thus leads a more accurate surface coverage. When Htt53Q coverage on gold surface is compared with 5GBP-Htt53Q, it's obvious that GBP containing construct shows a much higher surface coverage. Since, obtaining higher surface coverage is preferred ability for SPR experiments, GBP fused Htt53Q proteins were considered as good candidates for surface based fibrillization detections.

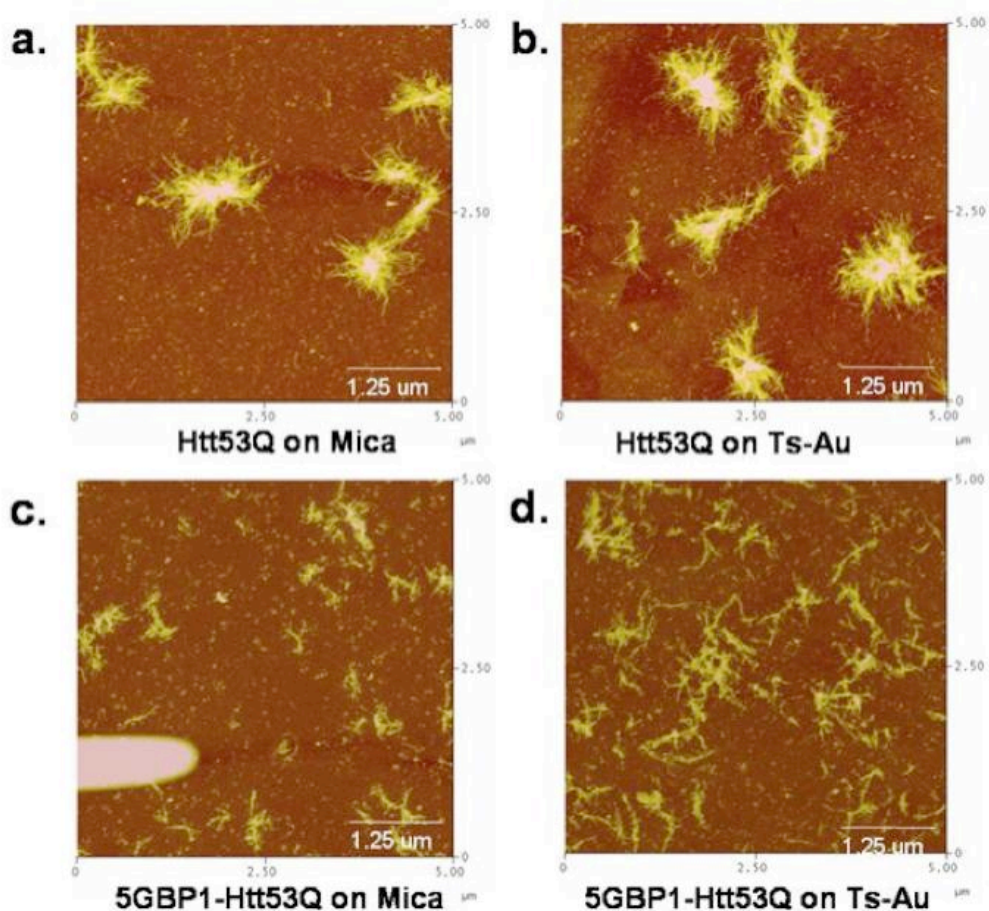


Figure 3.9 : Htt53Q and 5GBP1-Htt53Q fibrils are seen on gold and mica surfaces to illustrate the increase of nucleation and decrease of growth rate of the fibrillation in the presence of GBP1 in the constructs. The fibrils were formed after an overnight incubation of the purified monomers.

3.1.7 Surface Plasmon Resonance Spectroscopy monitoring of fibrillizations

Huntingtin fibrillization was investigated by using various techniques such as CD, quasielastic light scattering, FTIR, TEM and AFM [192, 193, 198, 205, 206]. These approaches provide valuable information about fibrillogenesis, which focuses on relatively long periods of fibrillization (hours). In order to measure the rate kinetic constants at each step of fibrilligenesis, a surface based detection method such as surface plasmon resonance (SPR) spectroscopy could be used. SPR could also be applied for the determination of additional compounds effect on the fibrillogenesis such as ions, chaperons and antibodies [207-210].

In this study, fibrillization was monitored with SPR experiments. The setup of these experiments involved the attachment of proto-fibril/fibril on the gold sensor of the

device and recording the fibrillization kinetics while pumping the monomer proteins onto the preseeded protofibril/fibrils. In the light of AFM observations, 6th hr incubated proteins after GST cleavage was utilized as 3GBP-Htt53Q proto-fibrils and 15 hr incubated proteins as 3GBP-Htt53Q fibrils. Htt53Q proteins without GBP fusion were used as monomer proteins and used right after cleavage process is completed (approximately in 2.5 hours).

Initially, fibrillation experiments were carried out with both 5GBP and 3GBP tagged Htt proteins. However we were not able to monitor the fibril elongation while using the 5GBP inserted proteins. Insertion of the 5GBP may affect the structure of the Htt and thus detrimentally affected the fibril formation of Htt. This result was also consistent with AFM analysis shown in Figure 3.8 as 5GBP insertion drastically changes the fibrillation period of Htt. These observations made us keep working with 3GBP as fusion partner to minimize the effect of GBP insertion on the structure and so on fibril formation.

As seen in Figure 3.9 (a) and (b), fibrils are forming bundles in the absence of GBP fusion. This bundle formation can decrease the surface coverage and therefore fibrillation sites of protein. Therefore, in the literature in most cases for the monitoring of the fibril formation the fibrils were sonicated to increase efficiency of immobilization with maximum sites for fibrils elongation available [307]. In our approach we also tested the effect of the sonication on the assembly of 3GBP-Htt53Q fibrils. As can be seen on Figure 3.10, sonication can slightly change the adsorption kinetics of the fibrils depending on the time of sonication. However the final amount of immobilized fibril did not deviate too much, which is around 2000 mRIU. When we consider that 5GBP-Htt53Q proteins are not forming such as many bundles as Htt53Q proteins form, 3GBP should have a similar affect. This observation together with sonication experiments were indicated that 3GBP insertion increases the surface coverage by decreasing the bundle formation and sonication is not necessary for fibrils to be immobilized on SPR surface.

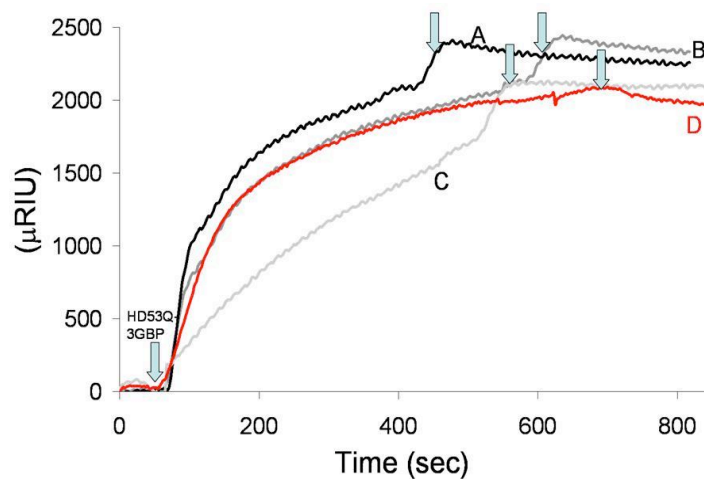


Figure 3.10 : Comparison of the sonicated and non-sonicated fibrils for their adsorption rates. The arrows indicate where the buffers were sent to remove non-specifically and weakly bound fibrils from gold surface. A. 2 mins sonicated fibril B. 5 mins sonicated fibril C. 10 mins sonicated fibril and D. Unsonicated fibril.

3.1.7.1 Control experiments

Fibrillization models suggest that monomer proteins form the nuclei, which serve as the seed for fibril elongation and preformed fibrils come together to form bundles resulting with the inclusion formations [193, 196]. We assume that Htt monomers are specifically binding to the available sites on the surface of the bound fibrils. Therefore, we expect Htt monomers not to be washed away as they become a part of the bound fibril and cause a further elongation via this specific interaction. Fibril bundles were observed in Htt53Q protein aggregation, where these structures might be interacting fibrils with each other. However, in the case of GBP-Htt53Q, bundle structures were not observed. In the first experimental set, the answer for whether fibrils also interact with each other and form recent inclusions was investigated.

Therefore, 3GBP-Htt53Q fibrils were monitored whether they cause any elongation on the bound fibril. As seen on Figure 3.11, an increase in the refractive index upon the interaction of the Htt fibrils with bound fibril was noticed. However, this causes no significant elongation, as it was removed from the bound fibril surface by buffer injection. This experiment showed that fibril-fibril rearrangement is not one of the ways of fibril elongation in the case of 3GBP-Htt53Q proteins.

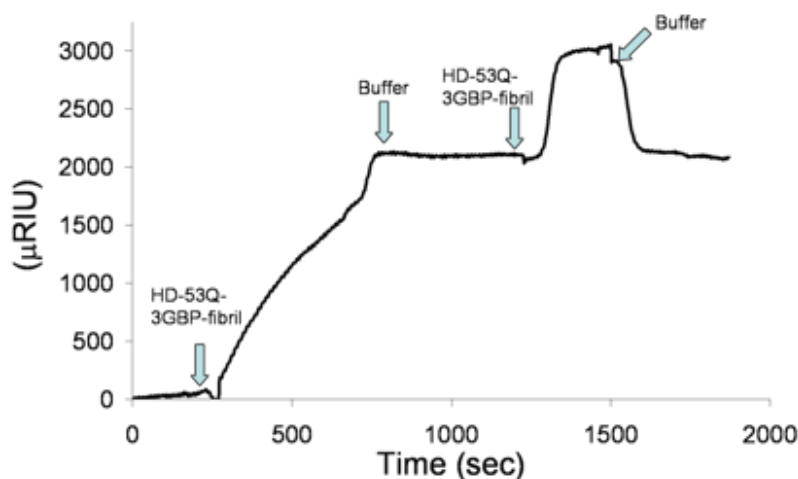


Figure 3.11 : The control experiments where Htt53Q fibril was pumped over surface bound 3GBP-Htt53Q fibrils. Htt53Q fibrils were dissociated upon buffer wash.

As the negative control, BSA solution was sent on the immobilized fibrils on sensor surface (Figure 3.12). BSA would either interact with the fibrils and/or with the gold surface. BSA injection caused to an increase in refractive index, indicating its adsorption to either fibrils or gold surface. However, when wash buffer was injected to the system, all bound BSA was washed away and the SPR signal was reduced to baseline. This desorption was an indication of the non-specific binding of BSA. The non-specific binding is believed to be between the BSA and the fibril but not with the surface because BSA could attach on gold surface [308]. Here, it's not standing on the surface possibly because of the full-filled surface with the fibrils. This finding also verifies that any molecule pumped through the surface bound fibrils will be interacting only with the fibrils but not with the surface.

In order to test the monomer addition to the fibrils, Htt53Q monomer was send to the surface of the immobilized fibril. This positive control experiment was resulted with an increased adsorption signal first. When buffer wash is applied to remove unbound monomers, a desorption signal was establihes. However, this desorption doesn't result with a baseline formation. Thus, addition of monomers onto surface bound fibrils resulted with an increase amount of fibril on the sensor surface through fibril elongation (Figure 3.12).

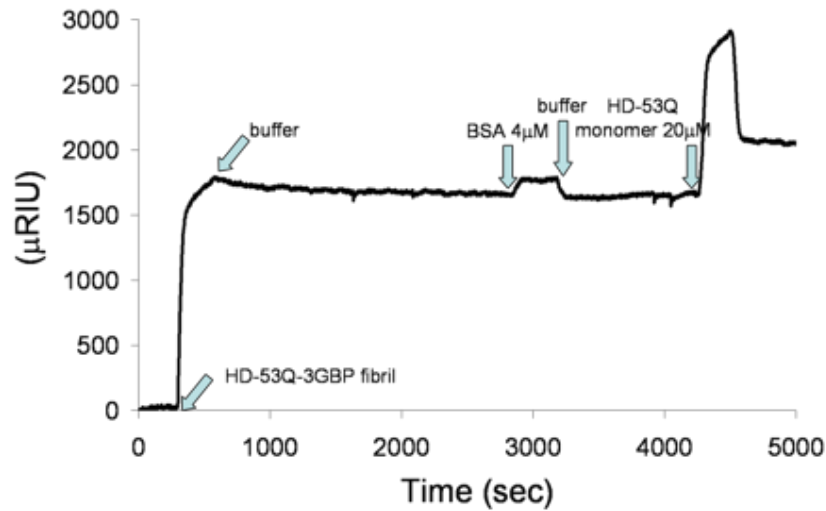


Figure 3.12 : A positive and negative control to test the Htt53Q monomers are interacting with the surface bound fibrils. BSA was sent as a negative control and show that only the monomers are interacting with the surface bound seed fibrils.

Htt is known to form fibrils when the number of glutamine repeats in the structure of the Htt reaches over 35 [170, 193, 309]. In addition to the experiment carried out above, another control experiment for non-fibril forming protein, Htt-20Q was tested. 3GBP-Htt20Q was incubated overnight for fibrillization, than immobilized onto the sensor surface. When fresh monomers of Htt20Q were sent on the pseudo fibrils of 3GBP-Htt20Q, both two different concentrations of monomers were removed without interacting with the surface bound protein (Figure 3.13).

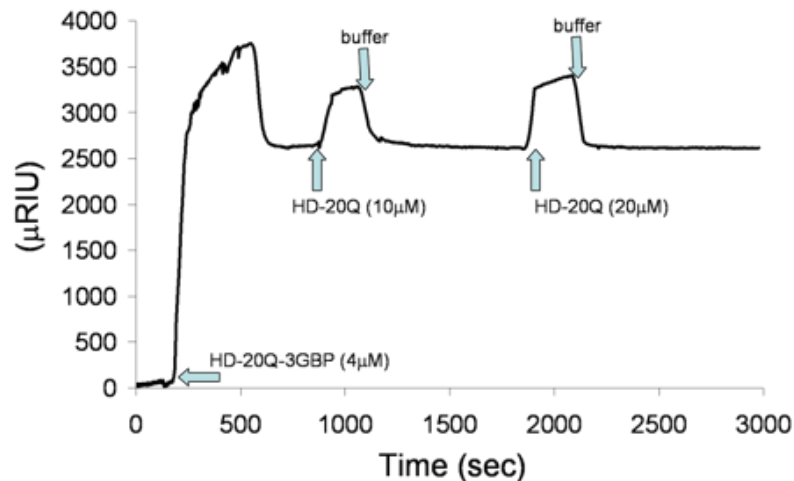


Figure 3.13 : The fibril elongation test for the Htt20Q. 3GBP-Htt20Q proteins were incubated overnight for fibril formation. Then, attached on the SPR surface. Monomer Htt20Q protein pumped on syface bound protein and totally dissociated following the buffer wash.

3.1.7.2 Detection of fibril elongation with chemical immobilization

Although the binding efficiency of GBP content of the fusion protein have been observed with AFM experiments and initial SPR studies, GBP mediated immobilization has to be compared with chemical immobilization. Therefore, fibrillization was monitored on Htt53Q fibrils immobilized onto gold surface by chemical attachment.

Htt53Q pre-fibrils were immobilized on gold sensor surface by using NHS-EDC chemistry. Following the fibril immobilization, Htt53Q monomers at concentrations of 4 μM , 10 μM and 20 μM were sent on the surface of immobilized fibrils. Obtained sensograms (Figure 3.14) were analyzed using the two-stage binding model. The rate constants were calculated using this two-step model is given in Table 3.2.

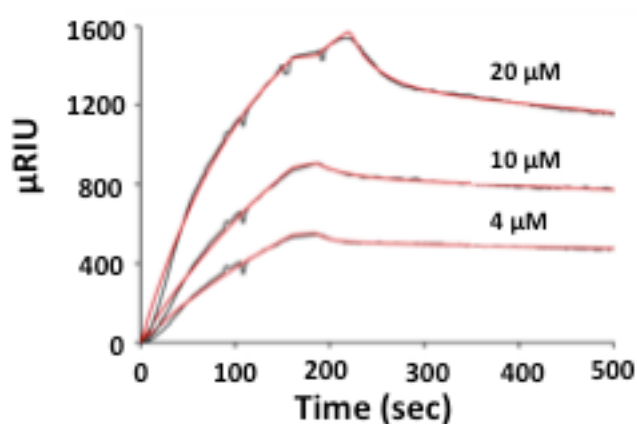
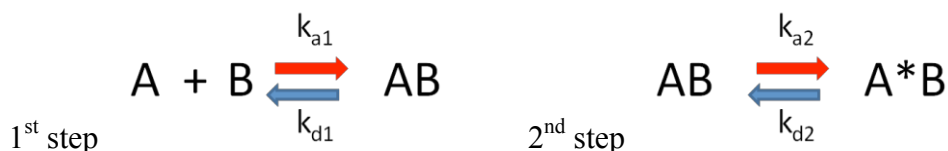


Figure 3.14 : SPR sensograms for Htt53Q fibril elongation. The lines represent the data obtained for fibril elongation in Htt53Q. The red line represents the fitted lines to the experimental data.

Table 3.2 : Kinetic rate constants for Htt53Q fibril elongation

Rate	Fibril Elongation (Chemical imm.)
$k_{a1} (\text{M}^{-1}\text{s}^{-1})$	2.77×10^2
$k_{d1} (\text{s}^{-1})$	$3.3 \pm 0.11 \times 10^{-2}$
$k_{a2} (\text{M}^{-1}\text{s}^{-1})$	0.1002×10^2
$k_{d2} (\text{s}^{-1})$	$2.98 \pm 1 \times 10^{-4}$

The two-stage model could explain the interaction of the monomers with the fibrils, which is followed by a conformational change of the monomer upon sticking to the fibril.



The first (docking) step of the model includes the interaction of the monomers (A) with preformed fibrils (B) immobilized on SPR gold sensor surface. The second (locking) step, is the step where the adsorbed monomer (AB) changes its conformation (A*B) and irreversibly binds to the fibrils on the surface and causes the fibril elongation.

Depending on the Circular dichroism experiments and computational simulations, the monomers in the bulk phase taught to remain in the random coil conformation until they aggregate [192, 310, 311]. This second step involves the conformational change, which can be called as the denaturation of the monomer into the sheet form, which ends up with the elongation of the fibril.

3.1.7.3 Detection of fibril elongation with GEPI mediated immobilization

In order to obtain fibril eleongation kinetics on immobilized fibrils with GBP content of the protein, fibril elongation was monitored on 3GBP-Htt53Q fibrils immobilized on gold sensor chip. The pre-formed 3GBP-Htt53Q fibrils were immobilized on gold-coated surface while they flown trough the SPR sensor. 3GBP region of the protein served as the immobilization mediator and lead fibril adsorption on gold. Fibrillization was monitored while Htt53Q monomers at concentrations of 4 μ M, 10 μ M and 20 μ M were sent on the surface of immobilized fibrils. The monomer binding to seeded fibrils was observed as an adsorption signal. Then, wasf buffer was pumped to remove excess amount or un-bound monomer proteins. Thus, lead an desorption signal in SPR. Obtained sensograms (Figure 3.15) were analyzed using the two-stage binding model and the rate constants were calculated (Table 3.3). The result of equilibrium analysis, the K_D values of 31 ± 9 and 1.81 ± 75 mM were calculated for dock and lock steps, respectively.

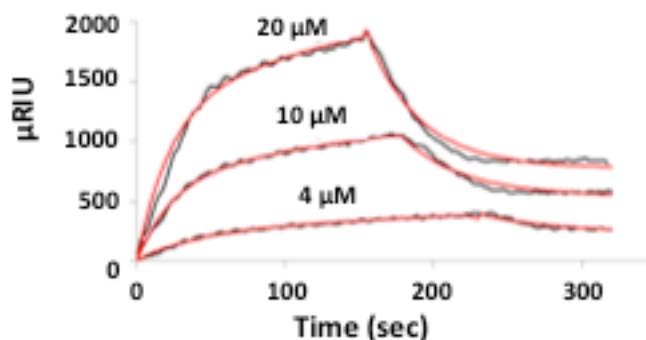


Figure 3.15 : SPR sensograms for 3GBP-Htt53Q fibril elongation. The lines represent the experimental data. The red line represents the fitted lines to the experimental data.

Table 3.3 : Kinetic rate constants for 3GBP-Htt53Q fibril elongation.

Rate	Fibril Elongation (3GBP mediated imm.)
$k_{a1} (M^{-1}s^{-1})$	$9.17 \pm 1.41 \times 10^2$
$k_{d1} (s^{-1})$	$2.80 \pm 0.43 \times 10^{-2}$
$k_{a2} (M^{-1}s^{-1})$	$1.25 \pm 0.51 \times 10^2$
$k_{d2} (s^{-1})$	$2.01 \pm 0.1 \times 10^{-4}$

Obtained SPR data and calculated kinetic values (Table 3.3) were found to be comparable with the results of chemical immobilization (Table 3.2). This result explained that GBP is not disrupting the structure of fibrils and do not occupy the free ends available for monomer addition. Thiol linkage could lead to uncontrolled immobilization and limit the structure of the macromolecule due to the covalent bonding between the surface and the protein [312, 313]. Therefore, the accessibility of newly added monomers to the fibril ends could be limited in the chemically attached fibrils. On the other hand, N-terminal insertion of GBP tag lead Htt to assemble on the gold surface with non-covalent interactions and therefore do not constraint the structure of the fibril on the surface, even gave the flexibility for monomer interaction. These could be the reasons for the higher adsorption values obtained for the elongation of 3GBP-Htt53Q fibrils compared to Htt53Q fibrils.

Despite the difference between the adsorption rates, desorption rates were the same for both chemical and GBP mediated systems, which indicates the monomer stability on the fibril end independently from monomer concentration. Therefore GBP based

system was thought to be as efficient as chemical attachment and could be an alternative way for protein immobilization in fibrillization studies.

3.1.7.4 Detection of proto-fibril elongation with GEPI mediated immobilization

Here, in order to follow up the initial fibrillation steps proto-fibril elongation was monitored. The proto-fibrils of 3GBP-Htt53Q were formed by incubation of the monomers for 5 hours. Formed proto-fibrils were sonicated for 5 min. to obtain a more homogenous suspension and then were immobilized on the surface of the SPR sensor. Following the assembly of the proto-fibrils in SPR flow cell, 4 μM , 10 μM and 20 μM of Htt53Q monomers were flown on the immobilized proto-fibrils. The monomer binding to seeded fibrils was observed as an adsorption signal. Then, wasf buffer was pumped to remove excess amount or un-bound monomer proteins. Thus, lead an desorption signal in SPR.

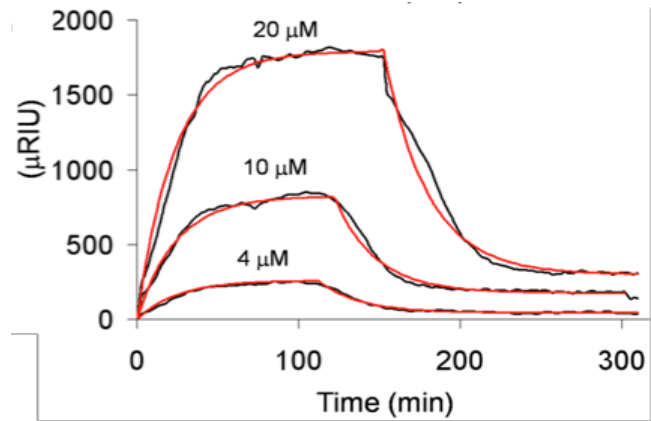
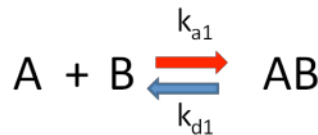


Figure 3.16 : SPR sensograms for 3GBP-Htt53Q proto-fibril elongation. The lines represent the experimental data. The red line represents the fitted lines to the experimental data.

The data collected (Figure 3.16) was fitted to a first order kinetic model, which indicates the adsorption of monomers (B) to the proto-fibril surface (A) resulting with the fibril elongation.



The calculated signal was compared with the measured response, and the values of the rate constants (Table 3.4) were found by minimizing the sum of square errors. To

achieve this, the Solver function of Microsoft Excel was used, which performs a constrained nonlinear fitting based on the generalized reduced gradient algorithm.

The K_D value for proto-fibril elongation was calculated as $203 \pm 58 \text{ mM}$, which was almost seven times higher than the first stage of the fibril elongation ($31 \pm 9 \text{ mM}$). The data indicated that monomers adsorbed on fibril surface desorb slower which represents a more stable structure. However, in the case of the proto-fibrils, they desorb from the fibril surface faster because of the less favored interaction between the proto-fibril and the monomer.

Table 3.4 : Kinetic rate constants calculated for proto-fibril elongation using a one-stage model.

Rate	Proto-fibril Elongation
$k_{a1} (\text{M}^{-1} \text{s}^{-1})$	$1.99 \pm 0.47 \times 10^2$
$k_{d1} (\text{s}^{-1})$	$3.86 \pm 0.14 \times 10^{-2}$

3.1.8 General discussion on Huntingtin fibrillization

The quantitative analysis of the fibril elongation has been studied with different models such as the single Langmuir adsorption model, two and three stage models [192, 208, 216, 314]. The main difference in these models was the protein used and the characteristics of the fibril elongation. In the case of Huntingtin fibril elongation we proposed a two-stage model, which was well fitted to our data. This model represented adsorption onto the seed fibrils (immobilized on SPR chip surface) in the first stage and the conformational change in the monomer attached to fibril in the second stage. However, on the proto-fibril elongation, only seed grow was observed which made us use a one-stage model. According to the proposed fibril elongation model, the conformation of monomer rearranges while binding to unique site on the end of the fibril. This, results with a more stable complex and further addition of bulk monomer to the fibril prevents the release of previously bound peptides [208, 314].

The K_D value of proto-fibril elongation was calculated as $203 \pm 58 \text{ mM}$, which is almost seven times higher than the first stage of the fibril elongation. This indicated that proto-fibril elongation is slower than the fibril elongation. This observation is correlated with the nucleation dependent fibrillization, where nucleation is the rate-limiting step [191, 192]. Despite the fact that, proto-fibrils are not the seeds (nucleus)

but they are the elongated seeds which will form the fibrils. This could be the reason of a survival process where fibrils can absorb monomers in a faster way to compensate the time required for new seed formations.

During the SPR experiments, both proto-fibrils and fibrils were immobilized till 2000 RIU shift was obtained. Even proto-fibrils are shorter than fibrils, there has to be more free ends than fibrils has, at the same concentrations. In this assumption more monomer could find a free fibril end to interact and yield a higher adsorption rate, which is lower according to the data. This made us concludes that the rate-limiting step of fibrillogenesis is not the fibril concentration but the conformation of the fibril ends. Even fibrils get elongated, the more stable the backbone get and therefore rate of fibrillization increases [314, 315].

The comparison of adsorption rates for the fibril and proto-fibril elongation steps shows that monomer addition is faster on the fibril ends compared to proto-fibril ends. Moreover, higher desorption rate for proto-fibril elongation indicates that monomers less favorable bind to the proto-fibril ends and tend to release. The more monomer release from the oligomer surface and/or less monomer addition to the ends, leads to increase free monomer concentration in the cellular compartments. Since the monomers and the small seeds can travel through nucleus and mitochondria and cause toxicity [115, 156, 316, 317], proto-fibrils might be leading toxicity by increasing the monomer concentration. Therefore some compounds could be investigated which are increasing the proto-fibril elongation rate can help to lower the toxicity.

3.2 LDH Immobilization on Gold Surfaces

LDH catalyzes the interconversion of pyruvate and lactate. Thus, produces lactic acid, which is highly consumable in cosmetic, pharmacy, food and chemical industry [275]. LDH also leads to the synthesis of the biodegradable polymer, PLA (poly lactic acid), which is currently used in packaging as films, thermoformed containers and short-shelf life bottles [276-278]. On the other hand, LDHs have an application area in medicine, mainly in diagnosis. Biosensors based on the lactate detection facilitated by LDH provide an accurate and faster method over traditional chromatographic and spectrophotometric systems [285, 286]. As being a redox enzyme, LDH is also used in biofuel cells as an anodic enzyme due to its direct

electron transfer capability [293, 294].

The enzyme has to be immobilized for both biosensor and biofuel cell applications. The main consideration of enzyme immobilization is to obtain retained catalytic activity for biosensors. In the case of fuel cell applications, exposure of electroactive prosthetic groups of the enzyme during immobilization is crucial for direct electron transfer between the enzyme and the electrode.

In this thesis project, we aimed to use GEPIs as a proper immobilization mediator for directed attachment of bsLDH onto gold surfaces. One of the gold binding peptides, AuBP2 [34], was utilized as the fusion partner of bsLDH. The efficiency of immobilization was determined by using Surface Plasmon Resonance (SPR) spectroscopy monitoring. Then the activity of immobilized enzyme was analyzed and the retained activities due to immobilization was calculated. While immobilized enzymes provide many opportunities such as low enzyme consumption and reusability, LDH also takes attention due to its redox potential. In order to detect bsLDH's electron transfer capability when immobilized via AuBP2, cyclic voltammetry analysis were applied.

3.2.1 Construction of AuBP2-LDH fusion proteins

bsLDH gene in pEQ2-6His vector was kindly provided by Assoc. Prof. Nevin Gul Karaguler (ITU). AuBP2 sequence was inserted into N-terminus of the protein using 5' extension PCR. In order to gain flexibility to the AuBP2 fusion peptide, a linker region has also been inserted between the peptide and the bsLDH gene. This was 4 amino acid long linker composed of GGGS (Glycine-Glycine-Glycine-Serine). The reason of using 3 glycine residues was to produce a flexible region and thereby perform the exposure of AuBP2 for proper gold binding affinity [318, 319]. Peptide and linker regions were added to N-terminus of bsLDH gene by using two sets of double step PCR reactions. In final PCR step, primers used were also added SacI and HindIII restriction enzyme recognition sites to 3' and 5' ends of AuBP2-LDH fusion construct, respectively. Following the PCR reaction was completed; PCR products were run on 2% agarose gel (Figure 3.17).

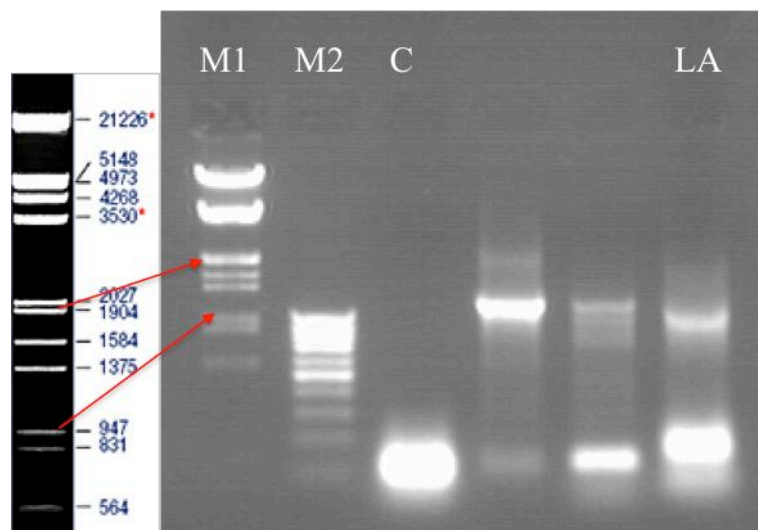


Figure 3.17 : PCR products for LA; AuBP2-bsLDH M1; Lambda Marker 3 (Appendix E), M2; 1000bp mass ruler (Appendix F), C; Control PCR

PCR product was containing a high amount of primer dimers as seen in the Figure 3.17. In order to clean the amplified gene region from excess amount of primers, DNA fragment was cut from the gel and purified with Qiagen gel extraction kit. Then, PCR amplified fusion protein sequence (AuBP2-LDH) was ligated into a TA cloning vector pDrive (Qiagen PCR cloning kit) and ligates were transformed into *E. Coli*. DH5 α -T1 chemical competent cells. Plasmids were isolated from positive transformants and screened with restriction enzyme digestion, where SacI and HindII restriction enzymes were used.

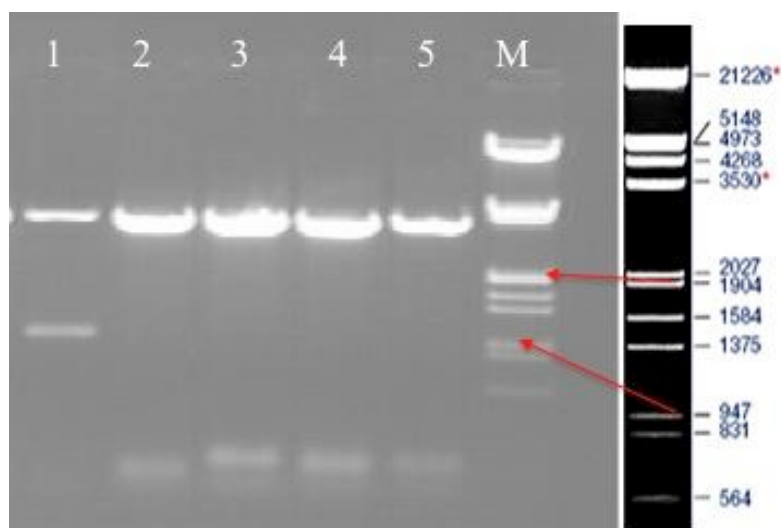


Figure 3.18 : PCR products for LA; AuBP2-bsLDH M1; Lambda Marker 3 (Appendix E), M2; 1000bp mass ruler (Appendix F), C; Control PCR.

Digestion reactions were run on 2% agarose gel to observe the plasmids containing insertions (Figure 3.18). Then, the plasmids containing ~1026 bp. long insertions were subjected to DNA sequencing in order to catch the clones containing unmutated pDrive-AuBP2-LDH.

In the next step pQE2 plasmid containing AuBP2-LDH was constructed. For this purpose, AuBP2-LDH coding region was digested from pDrive cloning vector using SacI and HindIII restriction enzymes and purified from agarose gel. On the other hand pQE2 expression vector was also digested with the same enzymes to obtain an open plasmid for proper ligation (Figure 3.19). AuBP2-LDH coding fragment was ligated with linearized pQE2 vector and transformed into *E. Coli*. DH5 α -T1 chemically competent cells. Transformant colonies were selected from LB agar plate containing ampicillin. Plasmids isolated from various positive clones were analyzed via DNA cycle sequencing and clones containing pQE2-AuBP2-LDH construct without any mutations have been stocked in glycerol.

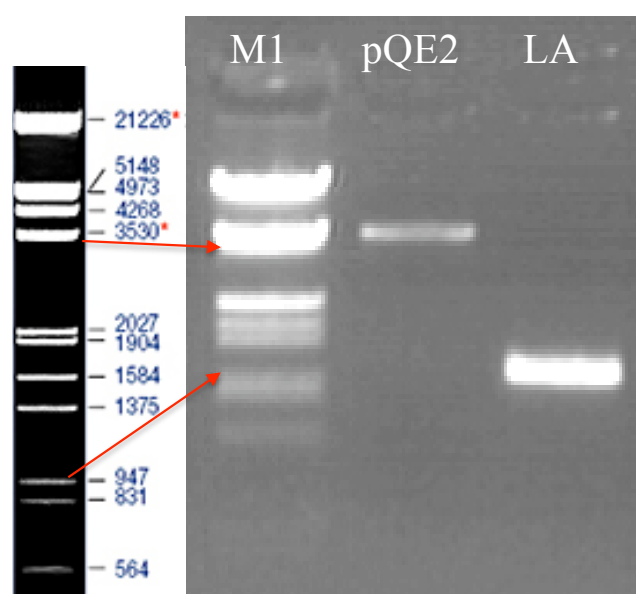


Figure 3.19 : Restriction enzyme digested pQE2 and AuBP2-LDH before ligation M1; Lambda Marker 3 (Appendix E).

In order to obtain a pure protein, the fusion tag region has to be removed. The removal of affinity tags is mainly required when large proteins are utilized as tags like Glutathione-S Transferase, Maltose Binding Protein or Streptavidin [320]. Histidine has a great affinity for metal chelators and only 6 histidine residues is enough to obtain a proper affinity tag [321]. Because of its small size, his-tag is not

removed in many applications [322, 323]. However, the histidine residues might attach to gold surface and interfere with the affinity of AuBP2. Therefore we aimed to construct a tag removal region for efficient his-tag cleavage. Although pQE2 vector was designed for His-tag removal by using two different cleavage enzymes; the activities of these proteases are relatively low compared to PreScission protease [320, 324, 325]. Therefore, final construct was obtained after the insertion of PreScission Protease Cleavage site between His-tag and the fusion protein. Thus, expressed protein would be removed from His-tag, which might attach to gold surface [326, 327]. For this purpose, site-directed mutagenesis was applied to the fusion constructs. One set primer; forward primer (containing the protease cleavage site) and reverse primer were used in combine with Gene-Tailor site directed mutagenesis kit. Site-directed mutagenesis applied for PreScission protease site insertion was also performed on pQE2-LDH construct. Site-directed mutagenesis PCR amplified whole plasmid (Figure 3.20), which is directly transformed into expression host following the gel purification. The expression host used for AuBP2-LDH and LDH enzymes was *E. Coli*. DH5 α -T1. DNA sequence of final constructs was verified with DNA cycle sequencing analysis and clones were stocked in glycerol.

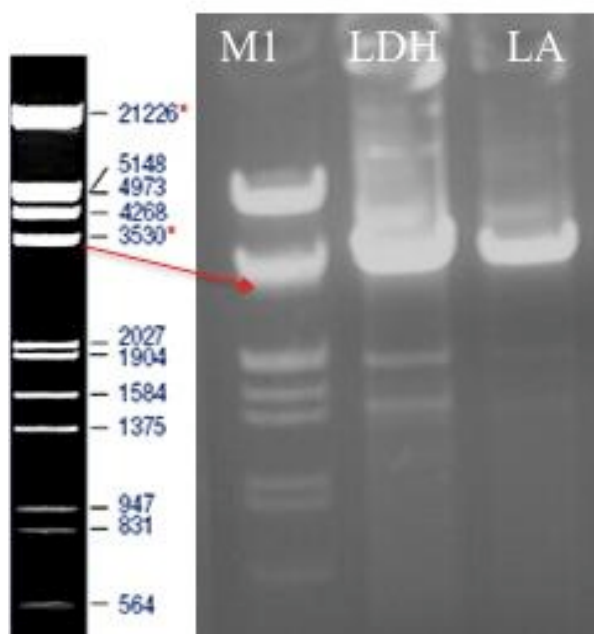


Figure 3.20 : Site-directed mutagenesis PCR of LDH; pQE2-LDH and LA; pQE2-AuBP2-LDH M1; Lambda DNA Marker, 3 (Appendix E).

3.2.2 Expression and purification of LDH and AuBP2-LDH proteins

For the expression of LDH and AuBP2-LDH proteins, *E. coli* DH5 α -T1 cells were used as the bacterial host. The expression of proteins was achieved by the induction of the culture for 4 hours at 37°C in the presence of 0.5 mM IPTG.

Expressed proteins containing His-tag were purified from Ni-NTA resin under native conditions according to QIAexpressionist manual from Qiagen. Two individual washing steps were applied by using 20 mM imidazole and 50 mM imidazole containing wash buffers (Up to 1% Triton X-100 was added to wash buffers). The expression level, purity and molecular weights of eluted fusion proteins were analyzed by SDS-PAGE (Figures 3.21 and 3.22).

Molecular weights of fusion proteins were calculated (Table 3.5) and compared with the proteins bands on SDS-PAGE images. LDH enzyme was observed around 35 kDa whereas AuBP2-LDH was bigger than 35kDa.

Table 3.5 : Fusion Proteins Constructs and Calculated Molecular Weight.

Protein construct	Calculated MW	MW with His-tag
LDH	35500 Da	37376 Da
AuBP2-LDH	38000 Da	39723 Da

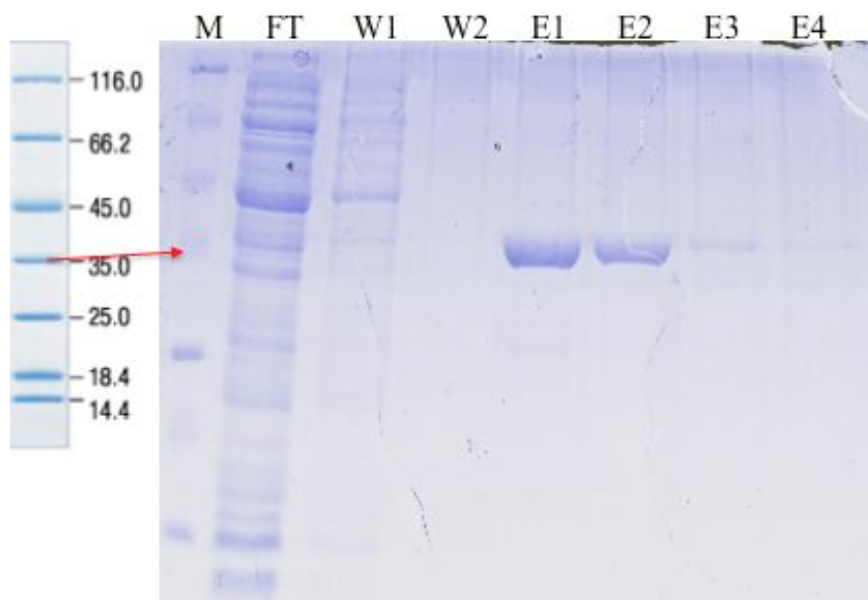


Figure 3.21 : SDS-PAGE image of LDH purification. M: Protein weight marker, FT: Flow through, W1: 1st wash fraction (20 mM imidazole), W2: 2nd wash fraction (50 mM imidazole), E1-4: Elution fractions.

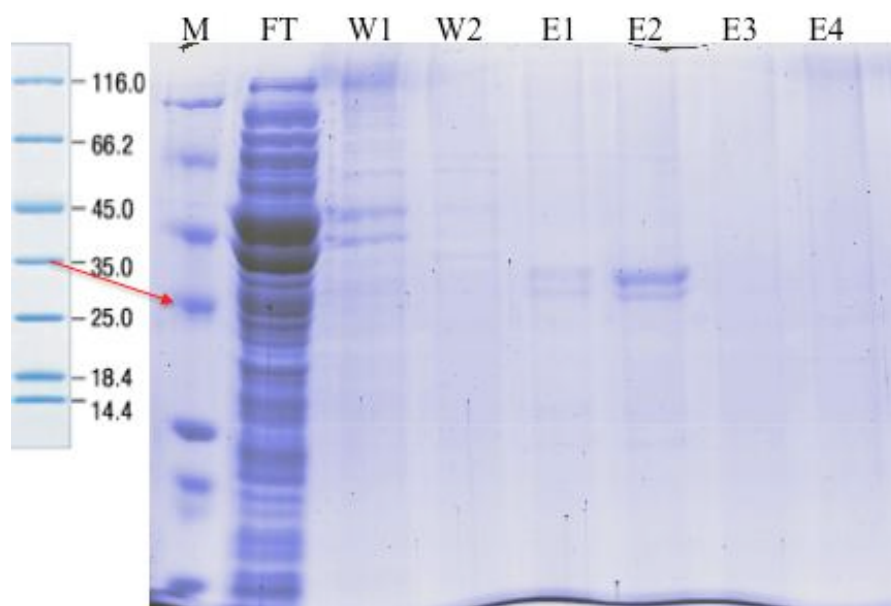


Figure 3.22 : SDS-PAGE image of AuBP2-LDH purification. M: Protein weight marker, FT: Flow through, W1: 1st wash fraction (20 mM imidazole), W2: 2nd wash fraction (50 mM imidazole), E1-4: Elution fractions.

3.2.3 Affinity tag (His-tag) removal from LDH proteins

Histidine tail of the purified proteins was removed using PreScission Protease. Cleavage reaction was performed in protease buffer under shaking at 4°C. Following the cleavage, reaction mixture was incubated with Ni-NTA and GST resins for removing His-tag and protease, respectively. His-tag free fusion proteins don't bind to either Ni-NTA or GST resins therefore collected as supernatant following a brief centrifugation.

His-tag free AuBP2-LDH and LDH enzymes were run on 12% SDS-PAGE together with His-tagged AuBP2-LDH enzyme (Figure 3.23). Since removed tag region is smaller than 1 kDa, observing the cut and uncut enzymes on gel was not accurate. Even, there is a slight difference between the protein bands of AuBP2-LDH and his-tag removed protein, it wasn't possible to make a statement for tag cleavage from SDS-PAGE analysis. On the other hand, initial SPR experiments verified that his-tagged protein displaying an unreproducible binding affinity while tag-free enzyme binding was highly reproducible. Therefore, we could assume that Figure 3.23 was showing the his-tag removal from AuBP2-LDH enzyme.

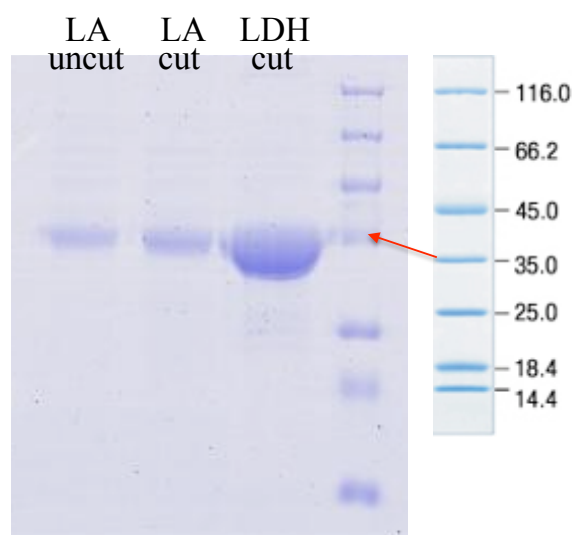


Figure 3.23 : SDS-PAGE image of His-tag removed proteins LA uncut; AuBP2-LDH with His-tag, LA cut; AuBP2-LDH after his-tag removal, LDH cut; LDH after his-tag removal, M; Protein weight marker.

Following the cleavage and purification steps, tag free LDH and AuBP2-LDH enzymes were exchanged to 50 mM Tris buffer, pH 7.5 for further applications, such as enzyme activity assays, surface binding characterization and cyclic voltammetry experiments.

3.2.4 LDH enzyme activity assay

Enzyme activities were measured at 25°C in 50 mM Tris buffer at pH 7.5, 0.2 mM NADH, 5 nM enzyme, 0–16 mM pyruvate. Activities were determined by observing the oxidation of NADH at 340 nm in 96 well elisa plates. The data were calculated using Enzyme Kinetics tool.

Table 3.6 : Enzyme activity kinetics.

	LDH	AuBP2-LDH
$V_{\max}$ (abs/min)	0.22 ± 0.019	0.12 ± 0.006
K_m (mM)	3.98 ± 0.51	4.59 ± 1.05
k_{cat} (s^{-1})	118.54 ± 10.55	66.8 ± 3.74
K_{cat}/K_m	29.78	14.55

Native bsLDH shows a catalytic activity of 240 s^{-1} , when purified with ion-exchange chromatography [249]. Karaguler *et. al.*, expressed the enzyme with His-tag for rapid purification and achieved an active LDH with a k_{cat} value of 116 s^{-1} [322]. We

utilized this his-tagged enzyme in our experiments with the difference of his-tag removal. The data in Table 3.6 was indicated that removing His-tag region from the enzyme didn't affect the catalytic activity. However, k_m increased from 1.02 to 3.98 when tag region was removed. On the other hand, catalytic kinetics indicated that AuBP2 addition was slightly decreased the catalytic activity (k_{cat}) and enzyme efficiency (K_{cat}/K_m). The decreased efficiency of the enzyme was partly because of the higher K_m value. This data was implied that AuBP2 insertion slightly decreasing the enzyme activity both in terms of catalytic activity and enzyme efficiency. Although, AuBP2-LDH is still an active enzyme that could be utilized for biosensor and biofuel cell applications even if gold surface binding ability was also gained. In order to follow gold binding ability of the enzyme, SPR spectroscopy experiments were performed.

3.2.5 Surface binding kinetics via AuBP2

Binding kinetics of LDH and AuBP2-LDH enzymes were analyzed using SPR spectroscopy. Gold coated SPR slides were used and a baseline was obtained by flowing 50mM Tris Buffer pH7.5 with 100 μ l/min. Then enzyme was flown through the surface for ~10 minutes to follow the surface binding. Desorption of the enzymes were monitored while washing with Tris buffer. Binding kinetics of AuBP2-LDH was monitored in three different enzyme concentrations; 0.25 μ M, 0.5 μ M and 1 μ M. On the other hand, in LDH case the SPR signal couldn't be established in the same enzyme concentrations. However, higher concentrations of LDH enzyme were required in order to obtain reliable SPR signal, which were 2 μ M, 4 μ M, 6 μ M and 8 μ M.

In order to extract quantitative kinetic information on the binding regime of either wt or AuBP2 enhanced LDH enzyme, SPR spectroscopy results were interpreted in terms of association and dissociation constants. wtLDH enzymes could be identified as a reference for binding since the effect of AuBP2 was investigated throughout these measurements. As seen qualitatively in the sensograms (Figure 3.24 and 3.25), AuBP2 content allow the LDH to better bind onto bare gold surface compared to wtLDH. It should be noted that even at the same concentrations wtLDH could not trigger proper amount of refractive index change upon binding to the surface. In other words, AuBP2-LDH has the ability to adsorb onto gold surface with relatively

low concentrations, which could be interpreted as the effectiveness of the AuBP2 fusion. As expected from the qualitative information for binding, calculated kinetic constants represented the difference between wt and AuBP2-LDH constructs. As given in Table 3.7, in case of AuBP2, there is about 10 times difference in binding constants that is k_a , which is the direct evidence about the mechanism of binding affinity. This result was achieved with using AuBP2-LDH concentration of 1 μM and below which are lower than wtLDH. That is crucial when making comparisons about binding regimes, since the refractive index change in the lowest concentration of AuBP2-LDH, was only established with 8 μM concentration of wtLDH. On the other hand, dissociation constants were represented a similar desorption pattern which should be analyzed in terms of complex surface-enzyme interactions. Once adsorbed onto the surface, it's difficult for complex molecules such as enzyme to desorb from surface due to molecular interaction with gold. In addition, desorption from the surface with buffer solution is a different process from binding which incorporate distinct mechanisms and for this reason reflected similar dissociation constants both for wt and AuBP2-LDH. Another crucial information extracted from SPR spectroscopy analysis was Gibbs free energy calculations. As a measure of the spontaneous nature of the system, Gibbs free energies for either wt or AuBP2-LDH were found lower than zero as expected. This means the whole process for two enzymes are self-driven systems and the difference in free energy values as well as equilibrium constants stem mainly from the distinct binding rate constants due to the AuBP2 content.

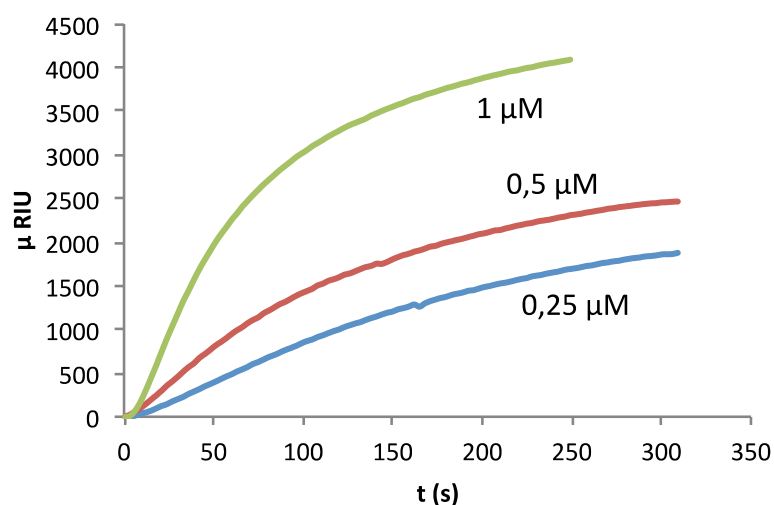


Figure 3.24 : Adsorption of AuBP2-LDH binding on gold surface. Three different enzyme concentrations were monitored.

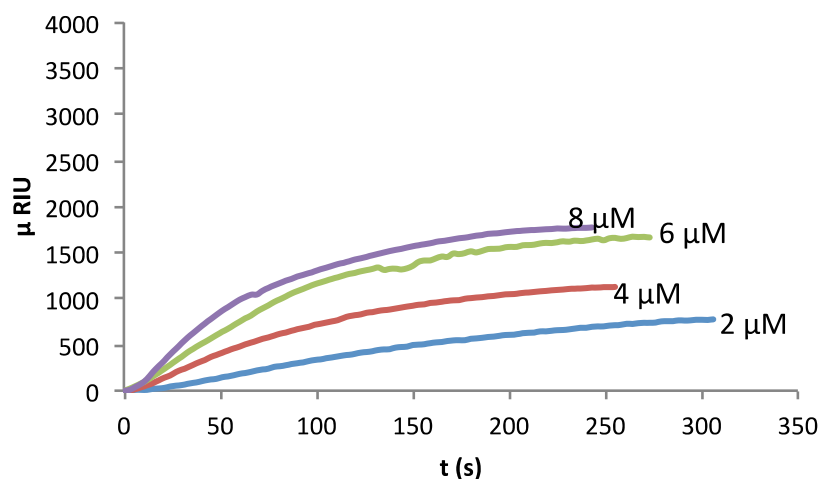


Figure 3.25 : Adsorption of LDH binding on gold surface. Four different enzyme concentrations were monitored.

Table 3.7 : Gold binding constants for AuBP2-LDH and LDH.

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_{eq} (M^{-1})	ΔG ($kcal.mol^{-1}$)
AuBP2-LDH	$1,1277.10^{-2}$	$7,35.10^{-4}$	15,34	- 1,6151
wtLDH	$1,4050.10^{-3}$	$5,00.10^{-4}$	2,81	- 0,611

3.2.6 Determination of immobilized enzyme activities

Lactate dehydrogenase has broad application areas in biotechnology. Among them, utilization of LDH for lactate detection and catodic enzyme in biofuel cell applications are the most attractive ones. For both biosensor and biofuel cell applications, enzyme has to be immobilized on a sensing surface without destruction in catalytic activity. There have been many attempts for enzyme attachment on sensor surfaces including adsorption, cross-linking, covalent attachment, conducting polymer entrapment and confinement in sol-gel matrix [287-291]. However, none of these methods have ability to result with oriented enzyme immobilization. Therefore, immobilized enzyme ends up with decreased catalytic activity and/or buried redox center. In this study, we proposed inorganic binding peptide mediated immobilization in order to obtain oriented enzyme attachments. The SPR results indicated that AuBP2-LDH enzyme has a great affinity for gold surface leading enzyme immobilization on gold. Although enzyme could be immobilized using the fusion partner AuBP2, it should also have a retained activity prior to immobilization

for further utilization. Therefore we tested the activities of the enzymes, which were immobilized by using AuBP2 fusion partner.

In order to investigate immobilized enzyme activities, 0.5 μM fusion proteins were immobilized onto 15 nm gold nanoparticles. Following the enzyme immobilization, nanoparticles were settled down with centrifugation and unbound enzyme was recovered in supernatant. Unbound enzyme concentration was determined with Bradford reagent and used to calculate the bound enzyme concentration. Enzyme immobilized AuNP solution was subjected to enzyme activity assays. For comparison, the activity of free enzyme in same concentration was also measured. Activity assays were performed in 50 mM Tris Buffer pH 7.5, 2.5 mM sodium pyruvate as substrate and 0.2 mM NADH as cofactor. Activities were determined by observing the oxidation of NADH to NAD^+ at 340 nm in 96 well elisa plates.

In Figure 3.26, free (un-immobilized) enzyme activities were given. The activities were measured more than 30 days and as a last measurement, 30th day activities were given in the graph. It could be seen from the figure that AuBP2-LDH has a decreased catalytic activity compared to LDH, in solution. The activity loss by time was similar in both enzymes till 10th day. However, after 10th day, the reduction in activity of AuBP2-LDH was slightly higher than the activity loss in LDH.

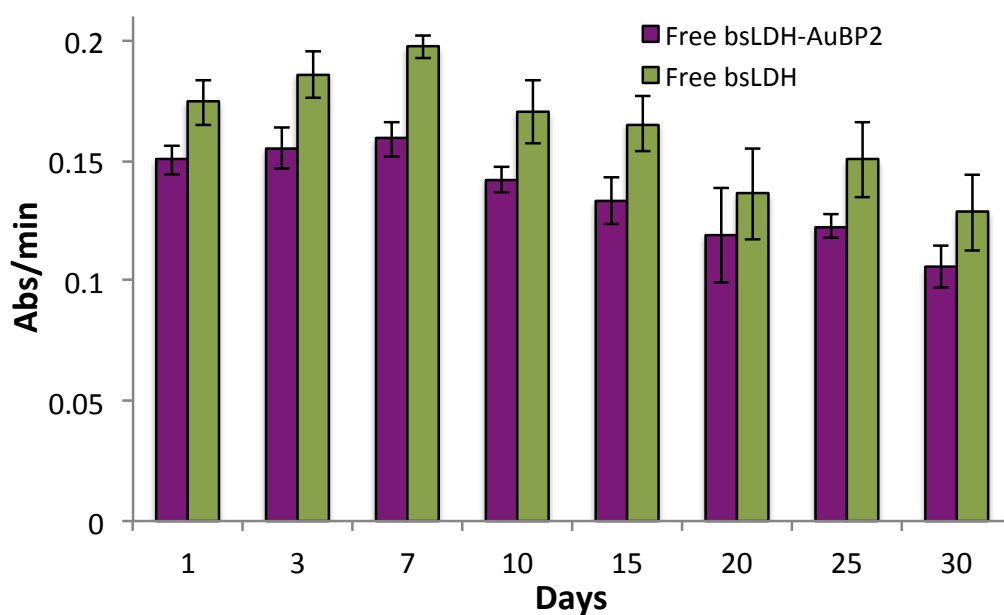


Figure 3.26 : Enzyme activities of free LDH and AuBP2-LDH measured for 30 days.

On the other hand, AuNP immobilized AuBP2-LDH and LDH activities were also compared (Figure 3.27). The activities were measured for more than 30 days and as a last measurement, 30th day activities were given in the graph. Although LDH had a higher activity than AuBP2-LDH in terms of activity kinetic constants (Table 3.6), immobilized LDH displays a lower activity. This might be arising from the un-oriented immobilization, which inhibits the exposure of the catalytic site.

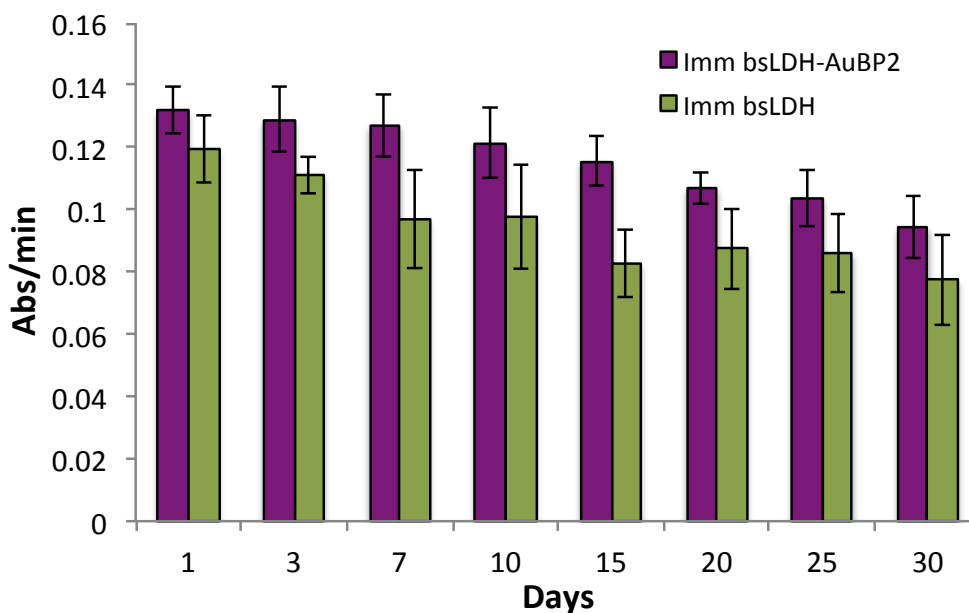


Figure 3.27 : Enzyme activities of LDH and AuBP2-LDH enzymes immobilized on gold nanoparticles measured for 30 days.

Since the difference between the activities of immobilized AuBP2-LDH and LDH indicate the site specific orientation and higher catalytic activity of AuBP2 mediated immobilization. The relative activities between the immobilized and free enzymes were also calculated, in order to gain information about retained activity prior to immobilization. Relative activities were defined as the specific activity ratio of immobilized enzyme to free (un-immobilized) enzyme. In case of LDH, relative activity was found around %70 the day of immobilization (Figure 3.28). The experiment was continued till 30th day. The data obtained allow to conclude retained activity was not lower than %60 among 30 days long experiment, however any half life for enzyme activity couldn't be calculated. The incorporated relative activity results of repeated days might be explained by the enzyme release from the immobilized nanoparticle.

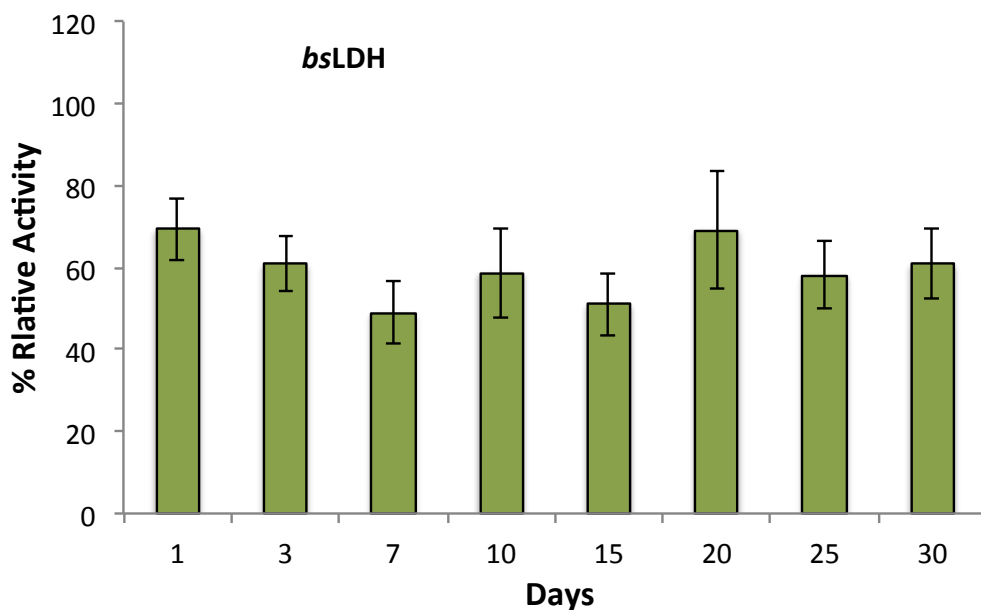


Figure 3.28 : % Relative enzyme activity of immobilized LDH / free LDH. Enzyme activities were measured for 30 days.

On the other hand, relative activities of AuBP2-LDH were showing an increasing pattern day by day. This is because of the activity loss in free enzyme, in contrast to stable activity in immobilized enzyme. Therefore, the relative activity of immobilized to free AuBP2-LDH increases up to 30th day of the experiments. This result indicates that AuBP2 mediated immobilization is efficient not only for retained activity but also for enzyme stability.

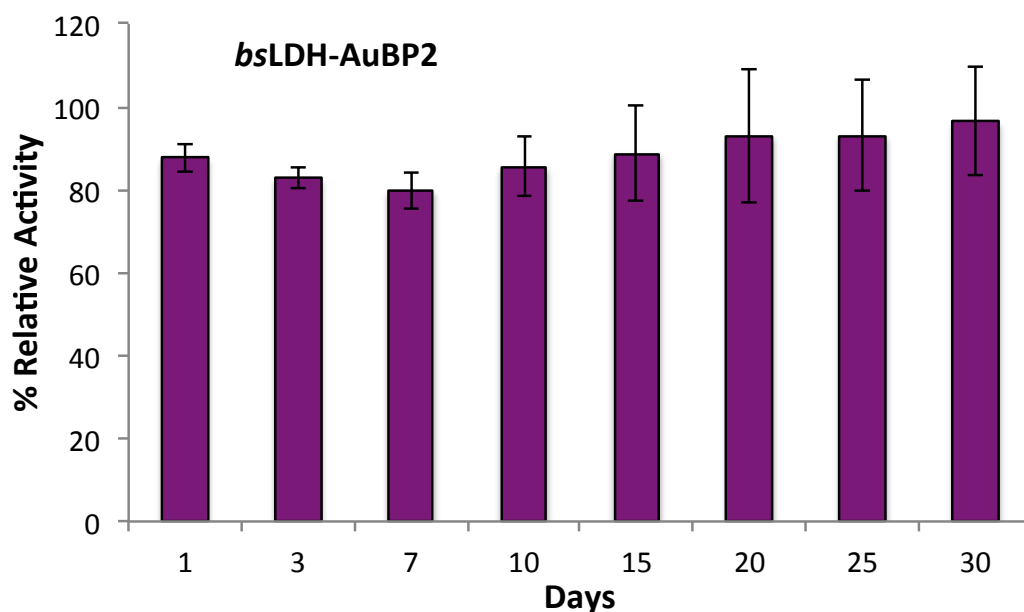


Figure 3.29 : % Relative enzyme activity of immobilized AuBP2-LDH / free AuBP2-LDH. Enzyme activities were measured for 30 days.

3.2.7 Determination of electron transfer capability of immobilized LDH

Since lactate could be produced during natural metabolism and exercise processes, detection of lactate concentration in blood or in commercial samples is important for diagnostics and medicine validations. The main detection method for lactate concentration is high-performance liquid chromatography (HPLC) conducted with spectrophotometry or refractive index detection [328]. However, this method is time consuming due to the complicated procedure. In order to generate fast and accurate lactate detections, enzyme based biosensor systems have investigated for last decades.

The amperometric detection systems depend on the catalysis of lactate to pyruvate in the presence of oxidized nicotinamide adenine dinucleotide (NAD^+) [270, 281, 283, 329, 330]. NAD^+ is reduced to NADH in this reaction and is detected amperometrically with the sensing layer. However, biocatalysis is hard to be followed because redox center of the enzyme is generally buried in the enzyme or enzyme could not be localized close enough to the sensing surface to enable the detection of electron transfer [331]. Therefore, mainly the reoxidation of NADH is detected in amperometric systems. This approach also has a drawback that the NADH oxidation occurs at high potentials [286]. Yet, applying high potential might result in the interference with other electroactive molecules mainly present in real samples. Therefore, strong oxidizing agents called mediators are utilized for electrocatalysis of NADH [332]. These oxidizing agents transfer charge from the substrate (NADH in this case) to the electrode surface. Mediators mainly used can be encountered as PQQ, metal nanoparticles and carbon nanotubes [295-298].

In this study, we propose an alternative immobilization method for LDH, based on the utilization of a gold binding peptide AuBP2. The SPR spectroscopy and immobilized enzyme activity results have showed that AuBP2 was an effective linker for immobilization of LDH on gold surface (Section 3.2.5 and 3.2.6). Finally, we aimed to investigate the electron transfer detection of LDH based system on the gold surface. Therefore, AuBP2-LDH was immobilized onto gold working electrode of the electrochemical cell. Then 10 mM NAD^+ and various concentrations of lactate were added and the redox reaction was investigated by using cyclic voltammetry.

Since, all the experiments were performed in 50 mM Tris buffer pH 7.0, the possibility of interference of buffer solution was investigated first (Figure 3.30). There was a typical redox reaction in Tris buffer, which is due to the electrochemical oxidation and reduction on the electrode surface. Then, 10 mM NADH and 10 mM NAD^+ was pumped separately to the system and cyclic voltammograms were recorded. In the case of NADH, a slight oxidation was observed. On the other hand, there was also a re-reduction (oxidation) for NAD^+ .

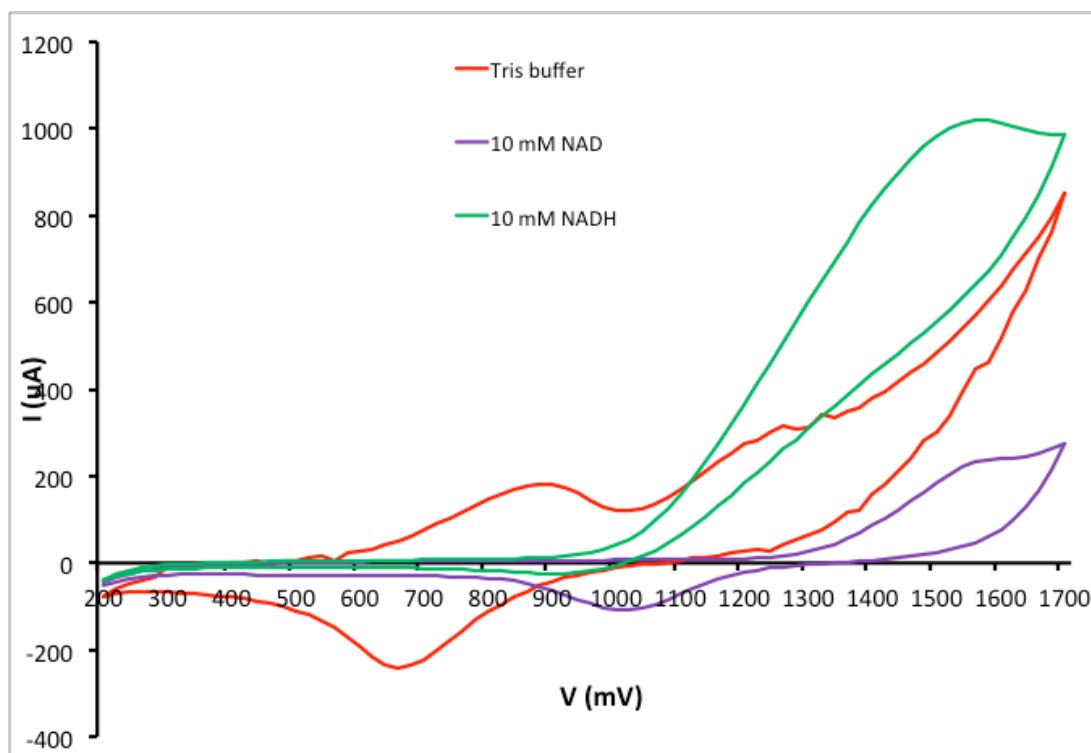


Figure 3.30 : Cyclic voltammetry of 50 mM Tris Buffer pH 7.0, 10 mM NAD^+ and 10 mM NADH. 2-LDH. Experiments were performed at 25°C and a scan rate of 100 mV/s^{-1} .

Then, AuBP2-LDH was immobilized on gold working electrode. When the potential was applied, there were mix reduction and oxidation peaks observed (Figure 3.31). However, when 10 mM NAD and 2 mM lactate were pumped to the system, cyclic voltammetry was recorded only a reduction signal. This reduction signal was thought to be the reduction of NAD^+ in the redox center of the enzyme. When cyclic voltammetry was repeated with increased lactate concentration, an increased in the reduction signal was recorded. Since the reduction signal was linearly correlated to the substrate concentration, the signal observed was suggested to arise from the catalysis of NAD^+ to NADH.

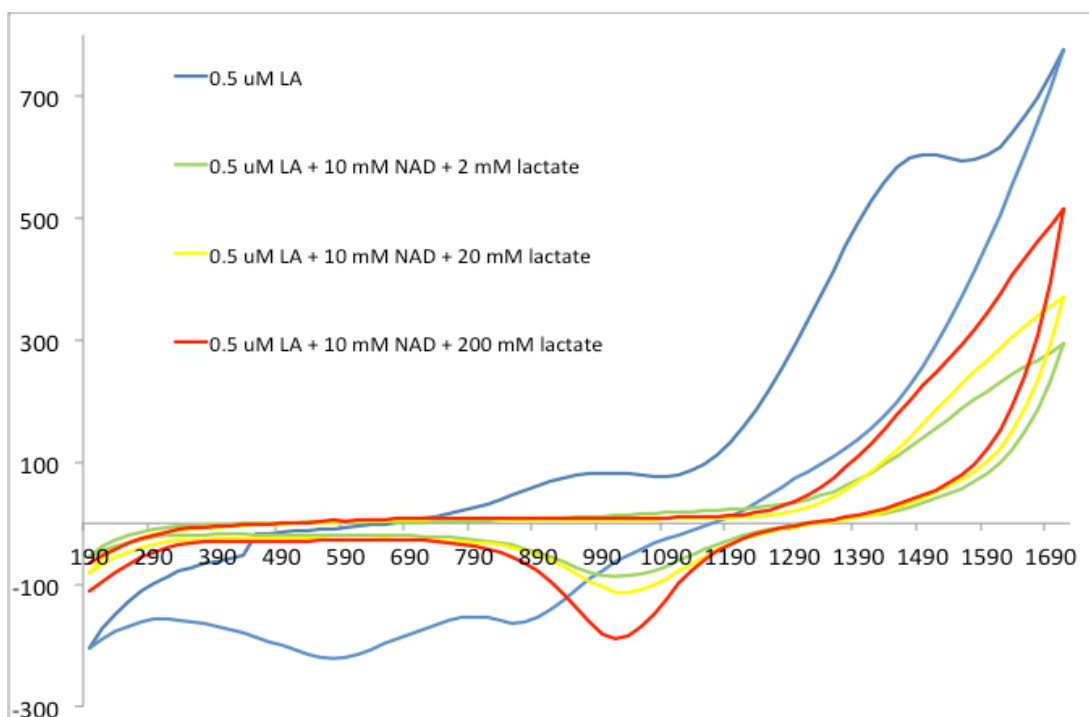


Figure 3.31 : Cyclic voltammetry of AuBP2-LDH immobilized on gold working electrode and AuBP2-LDH following the addition of 10 mM NAD^+ together with 2 mM 20 mM or 200 mM lactate. Experiments were performed in 50 mM Tris buffer pH 7.0, at 25°C and a scan rate of 100 mV/s^{-1} .

In order to analyze whether AuBP2 mediated immobilization is as effective as chemical immobilization for the detection of catalytic reaction on gold surface, LDH was immobilized with EDC-NHS chemistry on working electrode. Under the same experimental conditions (10 mM NAD^+ and 2 M lactate), cyclic voltammetry of chemically immobilized LDH was resulted with both oxidation and reduction peaks (Figure 3.32). The observed redox signals were very similar to that observed when only NAD^+ was added to the electrochemical cell in the absence of enzyme and lactate (Figure 3.30). The only difference between the cyclic voltammograms of NAD^+ and LDH- NAD^+ -lactate systems is the left shift in redox peaks. This left shift might be due to the pH increase arising from the lactate concentration. When lactate concentration is reached to 2 M, the pH of the solution rises to 8. This increase leads to a negative shift both in anodic and cathodic peaks [333, 334]. This result suggests that catalytic signal for NAD^+ to NADH reduction could not be observed when LDH is chemically immobilized on working electrode. On the other hand, it's possible to follow the catalytic signal in case of AuBP2 mediated LDH on working electrode

(Figure 3.31 and 3.32). This might be due to the small size of AuBP2, which could overcome the tunnelling effect [335-337] and allow electron transfer between the enzyme and the electrode. However in this system re-oxidation of NAD^+ could be detected. The reason might be either stem from the difficulty of reaching to the required potential for NADH oxidation or from the very rapid reduction of NAD^+ compared to oxidation of NADH [286]. Therefore, the oxidized NADH signal could be missed in cyclic voltammetry detections.

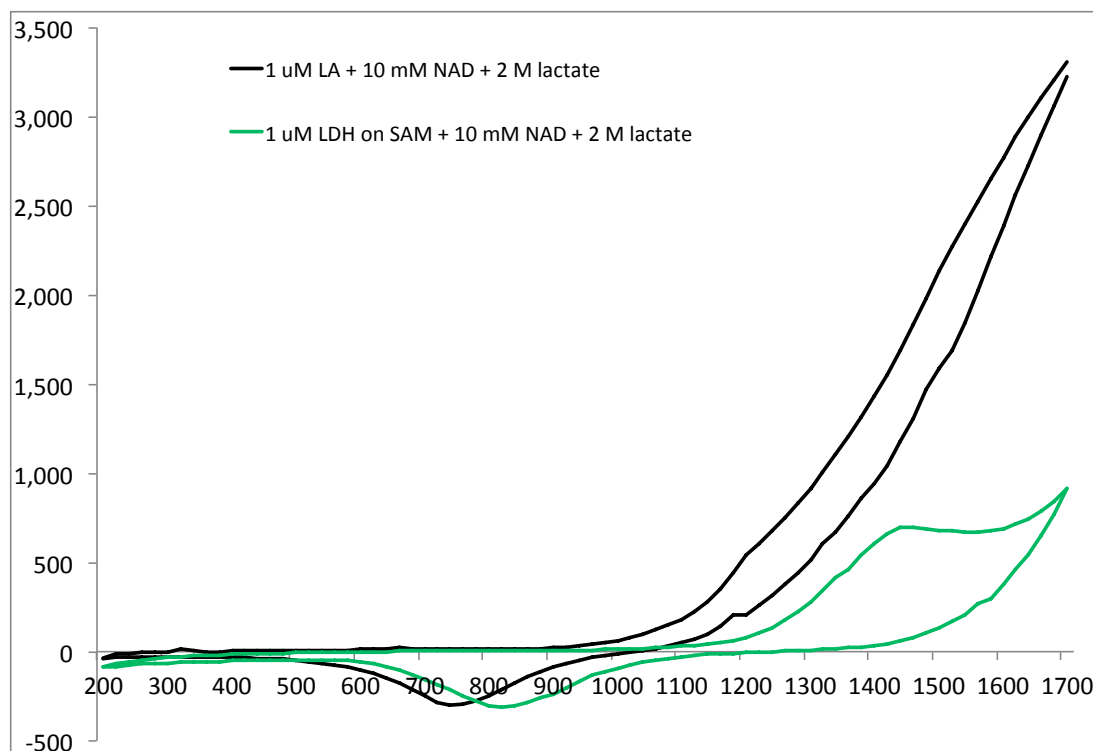


Figure 3.32 : Cyclic voltammetry of AuBP2-LDH and LDH immobilized on gold working electrode following the addition of 10 mM NAD^+ and 2M lactate. Experiments were performed in 50 mM Tris buffer pH 7.0, at 25°C and a scan rate of 100 mV/s⁻¹.

In final experimental set, the response of the system to various lactate concentrations was examined. AuBP2-LDH immobilized working electrode was scanned between 0.2-1.7 V in the presence of 10 mM NAD^+ and 20 mM- 2 M lactate. The reduction signal was increased with the increasing concentrations of lactate (Figure 3.33). This catalytic signal obtained was higher than many systems in the literature [293]. Since amperometric lactate sensors are based on the detection of NADH oxidation, the linear range and detection limits they provide are lower than our system. However, they also obtain the reduction signal which is much smaller than the signal obtained with AuBP2 mediated LDH immobilization system [293]. Therefore, we could

conclude that AuBP2 provides a new and efficient immobilization method for LDH leading to enzyme location close to the sensing surface and therefore allow efficient electron transfer between the enzyme and the electrode.

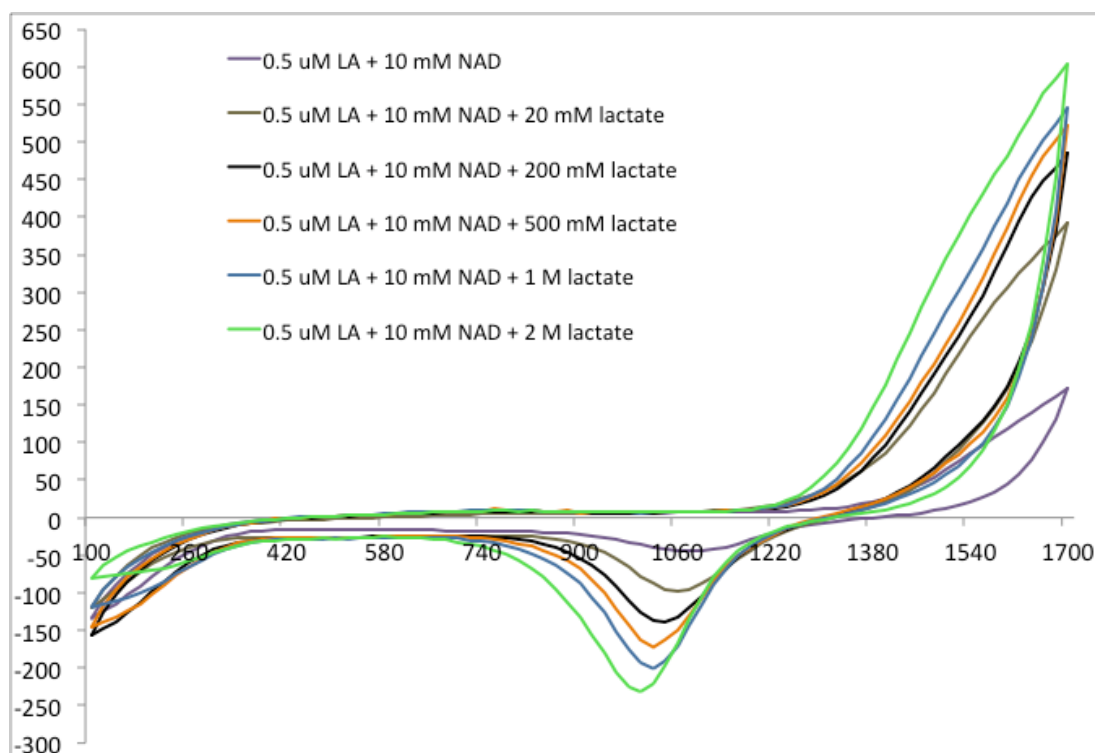


Figure 3.33 : Cyclic voltammetry of AuBP2-LDH immobilized on gold working electrode following the addition of 10 mM NAD⁺ together with 20 mM, 200 mM, 500 mM, 1 M or 2 M lactate. Experiments were performed in 50 mM Tris buffer pH 7.0, at 25°C and a scan rate of 100 mV/s⁻¹.

In order to detect NADH oxidation in lactate biosensors, oxidation mediators are in use. There is the question whether AuBP2 could be utilized as an electron acceptor, thus oxidation of NADH could also be observed. The possibility of changing the pH to change the peptides net charge could be considered for converting AuBP2 to an appropriate electron carrier. On the other hand, dehydrogenases are in use for biofuel cell applications. AuBP2-LDH could provide the cathodic enzyme for such biofuel cell applications, combined with an anodic counterpart.

4. CONCLUSIONS

Nanotechnology, dealing with systems on atomic and molecular scale, has diverse applications on surface science, organic chemistry, molecular biology, semiconductor physics, microfabrication and energy production. Advances in nanotechnology arise from the combined utility of conventional physics, chemistry and materials science with the approaches based on molecular self-assembly in order to develop new materials and devices. At that point, as a branch of nanotechnology, bionanotechnology exhibits enormous attraction. Bionanotechnology depends on the use of biomolecules together with nanomaterials in order to develop new approaches. Since biomolecules, especially proteins, have the ability of inorganic recognition and self-assembly, they have a potential in development of new generation nanomaterials. Also, because of the working conditions of proteins such as neutral pH and ambient temperature, their utilization in nanotechnology gives the opportunity of easy manipulation. With better understanding of biomolecule-material interactions, which is the underlying mechanism of nature-made inorganic-organic structures, it's possible to generate new hybrid systems in nano scale. In order to gain more information about interactions between biomolecules and nanomaterials, biomimetics is an important area to take advantage of.

Molecular biomimetics offers the utilization of the recognition and self-assembly abilities of biomolecules for the assembly and fabrication of materials [7-9, 13-15]. Taking the lesson of Biomimetics, Sarikaya and Tamerler have proposed the utilization of peptides with inorganic binding affinities for the development of new hybrid systems. Many peptides have been selected by their group and the others, for various inorganic materials. Sarikaya et al. called these peptides as "Genetically Engineered Inorganic Binding Peptides", GEPIs in short.

In this thesis project, two different GEPIs were used for the preparation of hybrid functional platforms based on GEPI-protein constructs. Taking advantage of inorganic recognition and binding abilities of GEPIs, we were able to attach functional proteins on solid surfaces. This GEPI mediated protein immobilization was yielded as efficient as chemical covalent immobilization strategies in terms of

protein stability but more efficient in terms of the exhibited functional activities of proteins.

In the first part of the thesis, one of the gold binding peptides (GBP1) was fused with huntingtin proteins. Huntingtin (Htt) is an eukaryotic protein exhibiting a number of functions in neuronal cells and is the responsible protein in Huntington's disease. The disease is formed, when a mutation is generated in the gene of protein, resulting with the expansion of N-terminal poly-glutamine region of the protein. The mutant proteins tend to form aggregates and fibrils in both cytoplasm and nucleus. In fact, the pathology of the disease is relevant with the protein aggregation. To better understand the disease molecular pathology, aggregation mechanism of Htt proteins has to be identified. The more information on aggregation could result with more development on new therapeutic strategies.

In order to investigate Huntingtin fibrillogenesis we aimed to apply a surface based technology, Surface Plasmon Resonance Spectroscopy. The reason beyond this was to measure the kinetic rate constants at individual steps of the fibrillogenesis as a function of time. With this purpose, we monitored the elongation kinetics of both fibril and proto-fibril stages. The pre-fibrillar/fibrillar proteins were attached onto SPR surface by the gold recognition ability of GBP1, fused to huntingtin proteins. GBP1 insertion is shown not to inhibit fibril formation and lead fibrils to be assembled on gold surfaces. Hence, GBP mediated Huntingtin fibril immobilization was successfully performed to obtain a suitable surface for fibril elongation analysis.

Surface based detection of the fibrillogenesis is given preferences because of its convenience and high repeatability compared to that of in solution methods. The main limitation here is to find out a suitable immobilization method to stick the fibrils of interest on to the desired surface. In conventional methods thiols chemistry was utilized to stick the fibrils on a surface to monitor fibril elongation [207-209]. However, chemical immobilization with thiols can have an aftermath of enclosing the fibril elongation sites on the surface of the fibril. Therefore, GBP is genetically fused to Htt proteins and utilized as an alternative immobilization agent by using its gold recognition ability. Bound fibrils were successfully utilized as seed to monitor fibril elongation and a two state kinetic model was used to determine the kinetic constants for the fibril formation in Htt53Q. This method resulted with success in terms of monitoring of fibril elongation indicating that GEPI (GBP in this case)

mediated immobilization would be utilized for the investigation of different disease related proteins. Moreover, this method could be utilized as an *in vitro* assay for screening drug targets together [338]. And the fibrillogenesis model proposed, would give an opportunity to further investigate proteins and compounds effective on fibrillogenesis to identify the initial step they may be directly taking part.

In the second part of the thesis, another gold binding peptide, AuBP2, was fused with Lactate dehydrogenase enzyme (LDH). LDH is an industrial enzyme used for medicine, lactat detection and recently in fuel cell applications with its redox potential. In order to develop biosensors and biofuel cells based on LDH utilization requires the immobilization of enzyme. In this study, we aimed to immobilize LDH onto gol surfaces by using the gold recognition ability of AuBP2. Surface binding kinetics of the fusion consturct, AuBP2-LDH was monitored with surface plasmon resonance spectroscopy (SPR). Our results showed that AuBP2-LDH have the ability to adsorp onto gold surface in relatively low concentrations which could be interpreted as the effectiveness of the AuBP2 fusion. When calculated kinetic constants for wt enzyme and AuBP2-LDH were compared, a 10 times higer k_a was obtained in case of AuBP2 presence, which indicates the increased surface binding affinity.

Besides, AuBP2 insertion is shown to decrease enzyme activity not seriously, making the fusion construct a preferable enzyme in applications. On the other hand, the activities of immobilized enzyme have lasted more then the free enzyme in a 30-day experiment. Therefore, the relative activity of immobilized to free AuBP2-LDH increases up to 30th day of the experiments. This result indicates that AuBP2 mediated immobilization is efficient not only for retained activity but also for enzyme stability.

The GEPI mediated immobilization of LDH was also investigated in terms of electrone transfer capabilities of the enzyme. Cyclic voltammetry was used to detect redox reaction performed by the enzyme, in the presence of NAD^+ and lactate. There was a reduction signal in cyclic voltammetry, which is tought to come from the NAD^+ reduction since signal was increased with the increasing concentrations of lactate. This catalytic signal obtained was higher than many systems in the literature [293]. Since amperometric lactate sensors are based on the detection of NADH oxidation, the linear range and detection limits they provide are lower than our

system. However, they also obtain the reduction signal which is much smaller than the signal obtained with AuBP2 mediated LDH immobilization system [293]. Therefore, we could conclude that AuBP2 provides a new and efficient immobilization method for LDH leading to enzyme location close to the sensing surface and therefore allow efficient electron transfer between the enzyme and the electrode.

In conclusion, GEPIs, the small peptide sequences exhibiting specific inorganic binding affinities have the potential to be genetically engineered and used as fusion partners with functional proteins. Taking the advantage of being biomolecules themselves, GEPIs don't destroy the structure of proteins they are fused with. Moreover, GEPI fusion results with the formation of bi-functional molecular structures having functional properties of the protein together with inorganic binding ability coming from the peptide region. Therefore, GEPI serves as an immobilization-mediating partner for the protein of fused. In GEPI mediated immobilization strategies, it's obvious to obtain oriented protein assembly, which results with the less biological activity in protein.

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APPENDICES

APPENDIX A : O'Range Ruler™ DNA Ladder (Fermentas Inc.)

APPENDIX B : Lambda DNA/Eco130I (StyI) Marker, 16 (Fermentas Inc.)

APPENDIX C : Unstained Protein Weight Marker (Fermentas Inc.)

APPENDIX D : Protein Marker Broad Range 2-212 (NEB)

APPENDIX E : Lambda DNA/EcoRI+HindIII Marker, 3 (Fermentas)

APPENDIX F : GeneRuler™ 100 bp DNA Ladder, 100-1000 bp (Fermentas)

Appendix A : O'Range Ruler™ DNA Ladder (Fermentas Inc.)

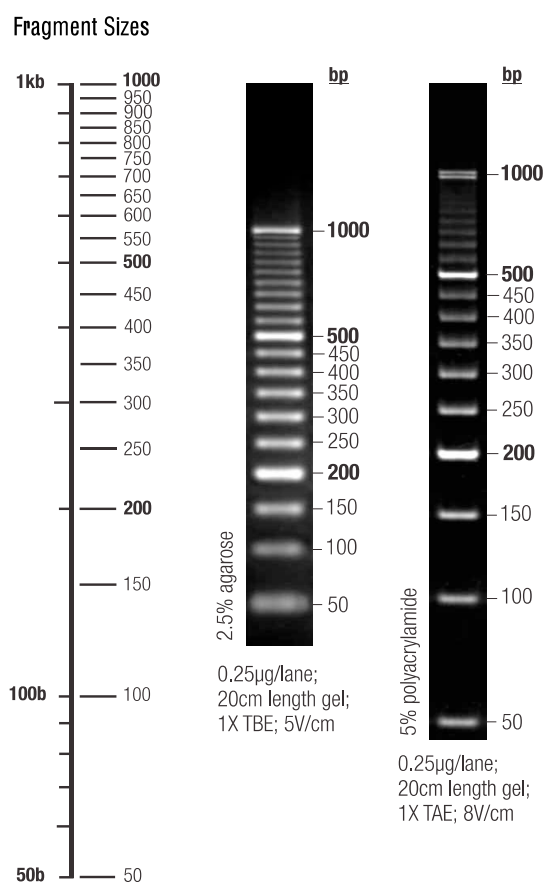


Figure A : O'Range Ruler™ DNA Ladder run on 2.5% agarose and 5% polyacrylamide gels.

Appendix B : Lambda DNA/Eco130I (Styl) Marker, 16 (Fermentas Inc.)

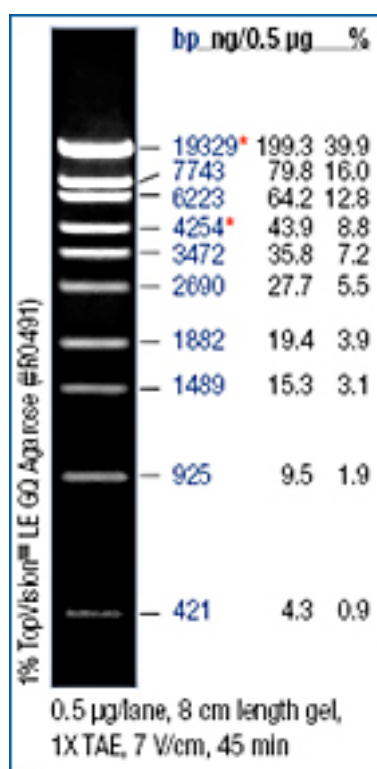


Figure B : Lambda DNA/Eco130I (Styl) Marker, run on 2.5% agarose and 5% polyacrylamide gels.

Appendix C : Unstained Protein Weight Marker (Fermentas Inc.)

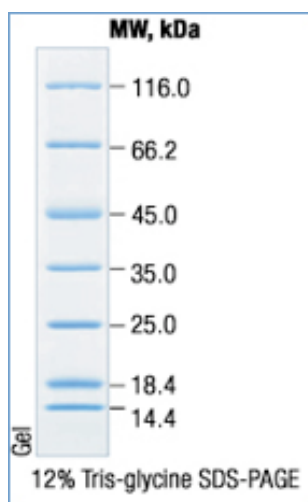


Figure C : Unstained Protein Weight Marker run on 12% polyacrylamide gel.

Appendix D : Protein Marker Broad Range 2-212 (NEB)

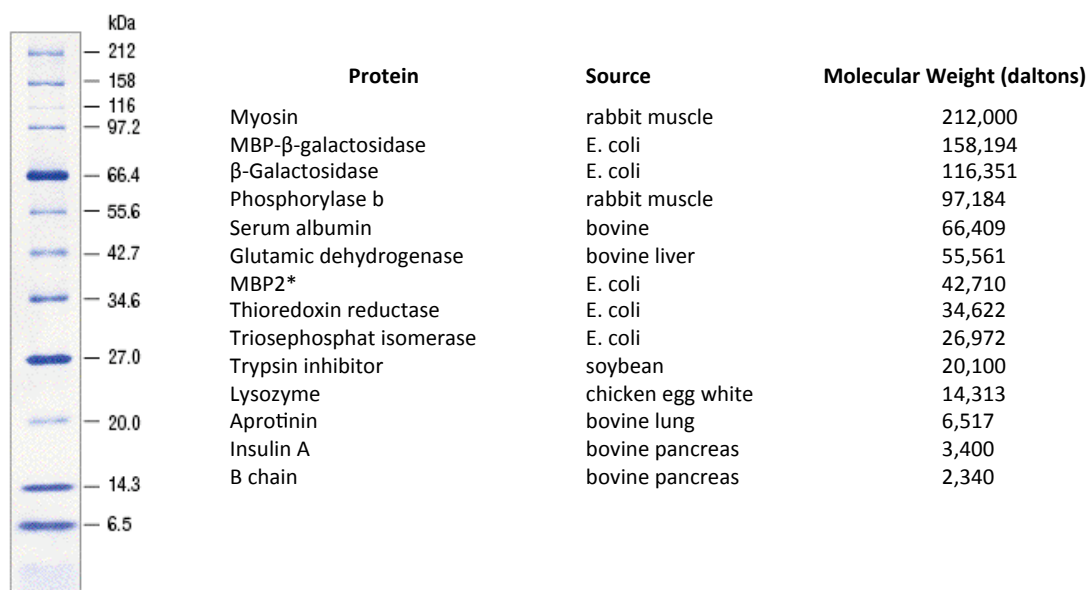


Figure D : Protein Marker Broad Range 2-212 run on 12% polyacrylamide gel.

Appendix E : Lambda DNA/EcoRI+HindIII Marker, 3 (Fermentas)

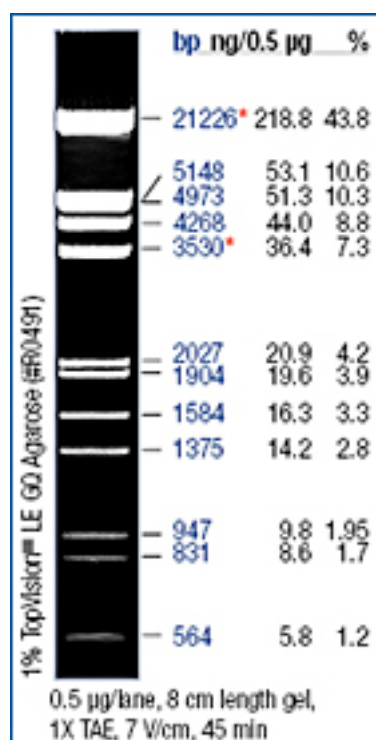


Figure E : Lambda DNA/EcoRI+HindII Marker run on 11% agarose gel.

Appendix F : GeneRuler™ 100 bp DNA Ladder, 100-1000 bp (Fermentas)

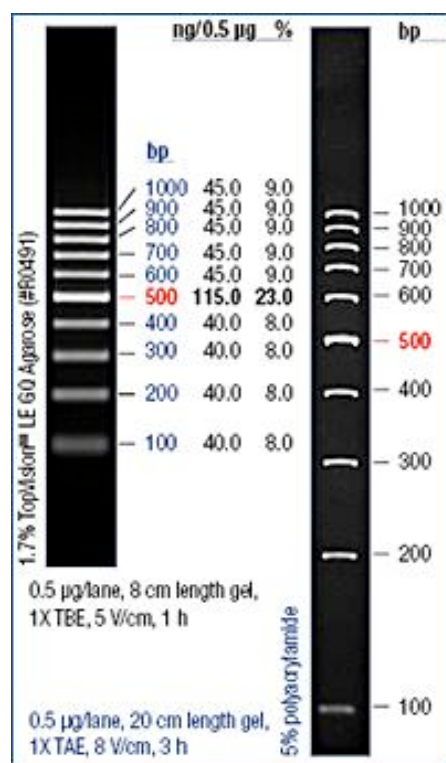


Figure F : GeneRuler™ 100 bp DNA Ladder run on 1.7% agarose and 5% polyacrylamide gels.

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PUBLICATIONS/PRESENTATIONS ON THE THESIS

Cetinel S., Seker U. O. S., Legleiter J., Arslan E., Muchowski P., Sarikaya M. and Tamerler C., “Real Time Monitoring of Huntingtin Fibrillization Kinetics Using GBP-Htt Constructs” (submitted to *Langmuir*)

Cetinel S., Yucesoy D., Caliskan H. B., Yuca E., Karaguler N.G., Sarikaya M., Tamerler C., “GEPI mediated Lactate Dehydrogenase Immobilization on Gold and Graphite Surfaces” *Eleventh International Symposium on Biomimetic Materials Processing*, February 25-28 2011, Nagoya, Japan

Yucesoy D., Cetinel S., Caliskan H. B., Yuca E., Karaguler N.G., Sarikaya M., Tamerler C., “The Utilization of GEPI-FDH Fusion Proteins in the Enzymatic Fuel Cells” *Eleventh International Symposium on Biomimetic Materials Processing*, February 25-28 2011, Nagoya, Japan

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