

**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE**  
**ENGINEERING AND TECHNOLOGY**

**MOLECULAR RECOGNITION BASED SELF ASSEMBLY OF ENGINEERED  
PROTEINS ON NANOSCALED GOLD SURFACES**

**Ph.D. THESIS**

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**Molecular Biology-Genetics and Biotechnology Department**  
**Molecular Biology-Genetics and Biotechnology Programme**

**Thesis Advisor: Prof. Dr. Candan TAMERLER**  
**Co-Advisor: Assist. Prof. Bülent BALTA**

**MARCH 2015**



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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

**NANO-ÖLCEK METAL YÜZEYLERDE MOLEKÜLER TANIMA ESASLI  
KENDİLİĞİNDEN MONTAJ OLABİLEN PROTEİN TASARIMI**

**DOKTORA TEZİ**

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**MART 2015**



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*To my spouse and parents,*



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Banu TAKTAK KARACA  
(M.Sc.)

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## ABBREVIATIONS

|                      |                                                                   |
|----------------------|-------------------------------------------------------------------|
| <b>aa</b>            | : Amino acid                                                      |
| <b>A (Ala)</b>       | : Alanine                                                         |
| <b>R (Arg)</b>       | : Arginine                                                        |
| <b>N (Asn)</b>       | : Asparagine                                                      |
| <b>D (Asp)</b>       | : Aspartic acid                                                   |
| <b>C (Cys)</b>       | : Cysteine                                                        |
| <b>E (Glu)</b>       | : Glutamic acid                                                   |
| <b>Q (Gln)</b>       | : Glutamine                                                       |
| <b>G (Gly)</b>       | : Glycine                                                         |
| <b>H (His)</b>       | : Histidine                                                       |
| <b>I (Ile)</b>       | : Isoleucine                                                      |
| <b>L (Leu)</b>       | : Leucine                                                         |
| <b>K (Lys)</b>       | : Lysine                                                          |
| <b>M (Met)</b>       | : Methionine                                                      |
| <b>F (Phe)</b>       | : Phenylalanine                                                   |
| <b>P (Pro)</b>       | : Proline                                                         |
| <b>S (Ser)</b>       | : Serine                                                          |
| <b>T (Thr)</b>       | : Threonine                                                       |
| <b>W (Trp)</b>       | : Tryptophan                                                      |
| <b>Y (Tyr)</b>       | : Tyrosine                                                        |
| <b>V (Val)</b>       | : Valine                                                          |
| <b>AFM</b>           | : Atomic force microscopy                                         |
| <b>AgBP</b>          | : Silver binding peptide                                          |
| <b>AP</b>            | : Alkaline phosphatase                                            |
| <b>Anti-AP</b>       | : Antibody against AP                                             |
| <b>AuBP</b>          | : Gold binding peptide selected by cell surface display technique |
| <b>AuBP1</b>         | : Gold binding peptide containing 'WAGAKRLVLRRE' aa residues      |
| <b>AuBP2</b>         | : Gold binding peptide containing 'WALRRSIRRQSY' aa residues      |
| <b>Au NP</b>         | : Gold nanoparticle                                               |
| <b>Bi-GEPI</b>       | : Bifunctional peptide                                            |
| <b>cm</b>            | : <i>Candida methylica</i>                                        |
| <b>CSD</b>           | : Cell surface display                                            |
| <b>DF</b>            | : Dark-field                                                      |
| <b>DsRed</b>         | : Red fluorescence protein                                        |
| <b>DsRed-AuBP</b>    | : Genetic fusion of AuBP to DsRed                                 |
| <b>ELISA</b>         | : Enzyme-linked immunosorbent assay                               |
| <b>FDH</b>           | : Formate dehydrogenase                                           |
| <b>FDH-AuBP2</b>     | : Genetic fusion of AuBP2 to FDH                                  |
| <b>F</b>             | : Maximum fluorescence intensity in the presence of quencher      |
| <b>F<sub>0</sub></b> | : Maximum fluorescence intensity in the absence of quencher       |
| <b>FITC</b>          | : Fluorescein isothiocyanate                                      |
| <b>FM</b>            | : Fluorescence microscopy                                         |
| <b>FRET</b>          | : Fluorescence resonance energy transfer                          |

|                                        |                                                              |
|----------------------------------------|--------------------------------------------------------------|
| <b>GBP</b>                             | : Gold binding peptide selected by phage display technique   |
| <b>GEPI</b>                            | : Genetically engineered polypeptides for inorganic surfaces |
| <b>IPTG</b>                            | : Isopropyl $\beta$ -D-1-thiogalactopyranoside               |
| <b><math>k_{cat}</math></b>            | : turnover number of enzyme                                  |
| <b><math>K_m</math></b>                | : Michaelis-Menten constant                                  |
| <b><math>K_{sv}</math></b>             | : Stern-Volmer constant                                      |
| <b>LSP</b>                             | : Localized surface plasmon                                  |
| <b>LSPR</b>                            | : Localized surface plasmon resonance                        |
| <b>LSPR <math>\lambda_{max}</math></b> | : Maximum wavelength of LSPR                                 |
| <b>MBP</b>                             | : Maltose binding protein                                    |
| <b>MBP-AuBP1</b>                       | : Genetic fusion of AuBP1 to MBP                             |
| <b>NP</b>                              | : Nanoparticle                                               |
| <b>PEO</b>                             | : Polyethylene oxide                                         |
| <b>PBS</b>                             | : Phosphate buffered saline                                  |
| <b>PDMS</b>                            | : Polydimethylsiloxane                                       |
| <b>PMSF</b>                            | : Phenylmethanesulphonyl fluoride                            |
| <b>Q</b>                               | : Quencher                                                   |
| <b>QBP</b>                             | : Quartz binding peptide                                     |
| <b>QCM</b>                             | : Quartz crystalline microbalance                            |
| <b>SAM</b>                             | : Self-assembled monolayer                                   |
| <b>SEM</b>                             | : Scanning electron microscopy                               |
| <b>SPP</b>                             | : Surface plasmon polariton                                  |
| <b>SPR</b>                             | : Surface plasmon resonance                                  |
| <b>TEM</b>                             | : Transmission electron microscopy                           |
| <b><math>V_{max}</math></b>            | : Maximum velocity of enzyme                                 |
| <b><math>\mu</math>CP</b>              | : Microcontact printing                                      |
| <b><math>\lambda</math></b>            | : Wavelength                                                 |

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# **MOLECULAR RECOGNITION BASED SELF ASSEMBLY OF ENGINEERED PROTEINS ON NANOSCALED GOLD SURFACES**

## **SUMMARY**

In recent years, a wealth of novel, nature-inspired materials have been explored across a wide range application in medicine and technology. Hierarchical organization is one of the key components that are reflected in the diverse structure and function in biological materials. In mimicking these functions in our engineering approaches have been challenging due to the complexity of these organization. Biological self-assembly principle based upon precise molecular-scale recognition is the key in Nature's design. Biological machinery facilitates self-organization and self-regulation that we observe in biological materials. Proteins are perceived as crucial interaction components in this machinery owing to their versatile characteristics. The protein assemblies may be the key in making next generation materials mimicking Nature's design.

Peptides as smaller domains of proteins become crucial components in constructing new types of materials having the versatility that we observe in Nature. Inorganic material specific peptides have attracted increasing interest in the recent years as novel bio-surface functionalization agents. Such functionalization can recruit biological cues to the material's surface and promote the otherwise challenging coupling of distinct materials. Their modularity also offers a unique opportunity to link them to numerous other functional proteins. Herein, we provide the details for their design and recombinant incorporation into functional proteins and enzymes to achieve addressable self-organization.

In the scope of this dissertation, we first constructed an AuBP1 peptide genetically fused to the C-terminus of maltose-binding protein (MBP) using two different linkers to produce MBP-AuBP1 hetero-functional constructs in order to accomplish the biological self-assembly on gold surfaces. Using various spectroscopic techniques, surface plasmon resonance (SPR), and localized surface plasmon resonance (LSPR), we verified the binding of MBP-AuBP1 fusion protein on gold substrates. The AuBP1 peptide tag were demonstrated to direct the organization of recombinant MBP protein onto various gold surfaces via efficient control of the organic-inorganic interface at the molecular level. Furthermore, using a combination of soft-lithography and self-assembly techniques, AuBP1 peptide tag controlled the formation of protein assemblies onto gold nanoparticle arrays with high molecular packing density and patterning efficiency following a series of simple, reproducible steps. This model system offers layer-by-layer assembly capability upon specific AuBP1.

In our second design, we developed multifunctional monitoring molecule through genetic fusion of gold binding peptide (AuBP2) to a red fluorescence protein,

DsRed-Monomer. AuBP2 was utilized as a material specific linker to construct novel bio-imaging, drug delivery, and targeting bio-nano hybrid systems. The AuBP2c peptide tag was enabled the self-immobilization of the DsRed protein onto a variety of nanoscale gold surfaces. Functional DsRed monomer generated by protein engineering methods using AuBP2 allowed variety of properties for biological sensing as well as analytical applications. The red emission of DsRed protein offered new opportunities at the nano- to micro-scale of a material surface such as multicolor labeling and fluorescence resonance energy transfer (FRET) applications. Our designed fusion protein with a fluorescent tag provided quick and accurate visualization of the self-immobilized proteins on the gold surface. Additionally, DsRed constructs have a selective and reversible binding affinity to copper. This unique binding property of DsRed protein to copper ions and gold nanoparticles results in tunable quenching of its fluorescence activity. Given the importance of gold nanoparticles and copper ions in several application areas, DsRed-AuBP2 was proposed as a bio-sensing modality through its bi-functional properties, fluorescence and self-assembly. We also tested the efficiency of the DsRed-AuBP2 protein as an integrated component in nanofiber system. The fibers were formed in the presence of the protein provided the integration of gold nanoparticles along the fiber length through Au-binding peptide tag. These nanofibers that feature gold nanoparticles with proteins did not affect the red fluorescence property of the protein, rather protein allowed the visualization of the protein construct along the fibers. Engineering proteins that can decorate gold nanoparticles well integrated into PEO polymer fibers offers several opportunities to create versatile structural templates for metallization in formation of conducting nanofibers.

Our final set of design included a fusion enzyme that is designed to have gold binding activity. Formate dehydrogenase, FDH, was fused to an AuBP2 fusion tag to construct FDH-AuBP2. This fusion enzyme was utilized to develop a circuit-based electrode system that provided verification of the redox catalytic ability of the self-immobilized enzyme on gold electrode by subsequent addition of formate. We achieved this validation following the self-immobilization of FDH-AuBP2 onto a gold electrode surface and monitored the conversion of formate to CO<sub>2</sub> electrochemically. The circuit-based system we constructed consisted of two gold electrodes submerged in a buffer solution. Prolonged catalytic activity of the enzyme was observed by subsequent addition of format into solution.

The engineered multi-functional biomolecular constructs developed were demonstrated to self-organize, forming functional hybrid hierarchical entities. However, no all-encompassing solution exists to control the orientation of the enzymes on surfaces using inorganic binding peptide tags. Each peptide and enzyme system requires detailed evaluation following genetic conjugation to investigate the performance of the chimeric functions.

Our results demonstrate the engineered inorganic binding peptides as a promising platform technology by their role in producing bi-hybrid nanostructured systems including fluorescent biosensors for metals, bioelectrodes for biobatteries, and nanofibers for conductive nanomaterial. Based upon an in-depth investigation of the limited research currently published in this growing field, we have developed

a strategy for the design of new protein constructs as well as their over-expression as recombinant products. Moreover, we provide a valuable approach to generate bioactive nanostructures for the engineering protein complexes by co-expression, purification, and characterization, coupled with bionanotechnological applications. Our engineering approach to conjugate proteins with inorganic binding peptides to create nanodevices propels this technology forward, one step closer to becoming a reality. Our fabrication process is based upon a bio-enabled self-assembly technique and is, herein, proven capable for engineering multilayered protein and nanometallic assemblies that utilize modular gold-binding peptides, such as AuBP1 and AuBP2.

Overall, the reported findings in this dissertation demonstrate a great potential to design novel nanostructured materials that incorporate the engineered proteins that are designed in the scope of this thesis. Our multi-functional engineered proteins display high binding affinity and specificity to various gold surfaces at the nano- to micro scale under ambient conditions. These novel platforms can be used for a variety of purposes such as preparing protein micro/nano-arrays, biosensors, biobatteries, and reagents for molecular imaging and targeting. Our established model constitutes biological routes for biofabrication of various protein arrays, plasmon-active nanometallic assemblies, and devices that feature controlled organization, packing density, and architecture.



## **NANO-ÖLÇEK METAL YÜZEYLERDE MOLEKÜLER TANIMA ESASLI KENDİLİĞİNDEN MONTAJ OLABİLEN PROTEİN TASARIMI**

### **ÖZET**

Son zamanlarda, tıp ve teknolojiye yönelik geniş bir uygulama alanında, doğadan esinlenerek geliştirilen yeni malzemeler yoğun bir şekilde araştırılmaktadır. Bu biyolojik malzemelerin çeşitli yapı ve işleve sahip olmalarında rol oynayan anahtar unsurlardan bir tanesi hiyerarşik organizasyondur. Bu organizasyonun çok karmaşık olması nedeniyle söz konusu işlevlerin bire bir aynısının üretilmesi bizim mühendislik yaklaşımlarımızla bile zordur. Moleküler-ölçek tanıma esasına dayalı biyolojik olarak kendiliğinden montaj olabilme ilkesi ise Doğa'nın tasarımındaki anahtardır. Bu mekanizma, biyolojik malzemelerde gördüğümüz kendiliğinden organizasyon ve kendiliğinden düzenlemeyi kolaylaştırmaktadır. Söz konusu mekanizmada rol alan en önemli etkileşim moleküllerinin ise çok yönlü olmalarından dolayı proteinler olduğu anlaşılmıştır. Proteinlerin kendiliğinden montaj olabilme yeteneği gelecekte Doğa'daki tasarımların bire bir benzerlerinin yapılmasında rol oynayabilir.

Proteinlerin daha küçük yapıları olan peptidler de Doğa'daki gibi çok yönlülüğe sahip yeni malzemelerin üretilmesinde önemli bileşenlerdir. Son zamanlarda anorganik malzemelere özgün olan peptidler yeni biyo-yüzey fonksiyonlaştırıcı ajan olarak cazip hale gelmişlerdir. Yüzeyleri peptidlerle fonksiyonlaştırma, biyolojik moleküllerin malzeme yüzeyine tutunmasını artırabilmekte ve diğer farklı malzemeler ile de kaplanmasını sağlamaktadır. Peptidlerin bu denli modülerite olmaları onların aynı zamanda çok farklı işlevsel proteinlerle de birleştirilme imkanını sağlamaktadır. Bu çalışmada, güdümlü bir şekilde kendiliğinden organizasyonu gerçekleştirme yeteneğine sahip işlevsel protein ve enzimlerin, peptidlerle olan tasarımı ve rekombinant birleştirilmesine ilişkin detayları sunmaktayız.

Bu tez kapsamında, ilk önce, altın yüzeylere biyolojik olarak kendiliğinden montajı başarabilecek MBP-AuBP1 hetero-işlevsel yapı üretebilmek amacıyla, AuBP1 peptidini genetik yollarla iki farklı bağlayıcı kullanarak maltoz bağlayan protein (MBP) ile birleştirdik. Yüzey plazmon rezonans (SPR), lokalize yüzey plazmon rezonans (LSPR) gibi spektroskopik tekniklerle MBP-AuBP1 füzyon proteinin çeşitli altın yüzeylere bağlanma ve kendi kendine tutunma özelliklerini karakterize ederek kanıtladık. Burada AuBP1 peptidi moleküler seviyede anorganik-organik ara yüzeyini etkili bir şekilde kontrol ederek, MBP'nin altın yüzeye uygun bir konformasyonda organizasyonunu sağlamıştır. Dahası soft-litografi ve kendiliğinden montaj tekniklerinin kombinasyonu ile AuBP1 peptidi, altın nanoparçacık dizilişlerin (array) üzerine yüksek moleküler yoğunlukta ve bir takım kolay ve tekrarlanabilir adımlardan sonra etkili bir motif oluşturarak proteinin montajını kontrol etmiştir. Bu model sistem spesifik AuBP1 aracılığıyla kat-kat montajlama (layer-by-layer assembly) yeteneğini göstermektedir.

İkinci tasarımıımızda, altın bağlayan ikinci peptidimiz (AuBP2) ile kırmızı flüoresan protein olan DsRed-monomeri genetik füzyon tekniğıyle birleştirerek yeni, çok fonksiyonlu denetleme molekülü geliřtirdik. Biyolojik görüntüleme, ilaç salınımı ve hedefe yönelik tanı mekanizmalarında kullanılmak üzere kendi kendine montajlanan altyapı birimlerinin oluşturulması için AuBP2 özgün bir bağlayıcı malzeme olarak nedeniyle kullanıldı. Ayrıca, AuBP2 peptidi çeşitli nano yüzeylerde fonksiyonel biyo-prob olarak kullanılacak olan altının kullanıldığı malzemelerin tasarımında DsRed'in immobilize olabilmesi için kullanıldı. AuBP2 aracılığıyla çok daha fonksiyonel DsRed-monomer üretilerek hem biyolojik algılama uygulamaları hem de analitik uygulamaları gösterilmiştir. DsRed'in kırmızı fluoresan özelliğinden dolayı fluoresan enerji transfer (FRET) uygulamalarında çok tercih edilmektedir. Çünkü nano ve mikro yüzeylerde çok avantajlı ve güvenilir sonuçlar vermektedir. Bu nedenle bu çalışmamızda kırmızı flüoresan özellikli ve altın yüzeylere immobilize olabilen füzyon protein ürettik. Bununla birlikte DsRed füzyon proteininin bakır metaline bağlanma potansiyelini arařtırdık. DsRed'in bakır iyonuna karşı olan bu ilgisi altın nanoparçacıklarla da incelenmiştir. DsRed-AuBP2 bakır iyonu ve altın nanoparçacıklarıyla flüoresan şiddeti azalmaktadır. Bu da bağlanma kinetiğı hakkında bilgi vermektedir. Bakır iyonu ve altın nanoparçacıklarının teknolojik önemlerinden dolayı DsRed-AuBP2'li biyosensor üretilmiştir. Bununla birlikte DsRed-AuBP2'nin nanofiber oluşumunda da yer alabileceğini gösterdik. Oluşturulan nanofiberler altın nanoparçacığı ve kırmızı flüoresan protein içermektedir. Burada DsRed-AuBP2'nin görevi altın nanoparçacıklarının PEO (polietilenoksit) polimeriyle derinlemesine etkileşimini sağlayarak fiber oluşumunun organizasyonunu sağlamaktadır. Böylece çok yönlü ve iletken nanofiberler üretilmiştir.

Son olarak altın bağlama aktivesi de olan bir füzyon enzim tasarladık. Format dehidrogenaz (FDH), AuBP2 peptidiyle birleştirildi. Buradaki amaçla elektro katalitik aktiviteyi ölçen devre tasarımında kullanılan bir biyoelektrot üretildi. FDH-AuBP2'nin altın yüzeye immobilize olmasıyla biyoelektrot oluşturuldu ve bu elektrotu enzimin redoks aktivitesinin ölçülebileceğı bir elektrik devre tasarımında kullandık. Enzimin format substratını CO<sub>2</sub>' e dönüřtürmesi, tasarlanan elektrik devresinde gözlenmiştir.

Geliştirilen çok fonksiyonlu biyomoleküler yapıların kendiliğinden organizasyonu gerçekleřtirdiğı ve işlevsel hiyerarşik hibrit birimlerin meydana geldiğı kanıtlanmıştır. Ayrıca, anorganiklere bağlanan peptidler, enzimlerin yüzeylere oryantasyonunun kontrolünü yapmaktadır. Her bir peptid ve enzim sistemlerinin genetik birleřtirmenin ardından kimerik işlevlerin performansını arařtırmak için detaylı bir şekilde deęerlendirilmesi gerekmektedir. Bu tezde sunulan tüm bulgular ve sonuçlarla ürettiğimiz proteinleri kullanarak yeni nanoyapılı malzemelerin oluşturulabileceğı gösterilmiştir. Tasarladığımız çok fonksiyonlu proteinler altın yüzeylere yüksek affinite ve seçicilikle bağlanabilmektedir. Bundan dolayı protein tabanlı mikro/nano diziler, biyosensörler, biyopiller ve moleküler görüntüleme ve tanımlama sistemlerinde kullanılmıştır. Anorganiklere özgün bağlanan peptidlerin umut verici olan altyapı teknolojisi ve nano yapıları sistemlerdeki rolleri gösterildi. Bu amaçla metallere özgü flüoresan biyosensor, biyolojik pil üretimi için biyolojik elektrot ve iletken nanomat için nanofiber gibi nanoyapılar ürettik. Nano cihazların söz konusu peptid ve proteinlerden tasarlanması gelecekteki yöntemlere umut vadetmesine

rağmen hala bir takım araştırılması gereken önemli hususlar vardır. Bu konu üzerinde sınırlı çalışmaların yayınlandığını gördük ve bu amaca yönelik rekombinant olarak, oldukça yüksek oranda üretilabilen, yeni protein yapıların (constructs) tasarlanmasına ilişkin sağlam bir strateji geliştirdik. Dahası, biyoaktif nanoyapılar oluşturmak için protein bileşiklerini kullanarak biyo-nanoteknolojik uygulamalara yönelik bir yaklaşım ileri sürdük. Bu yaklaşım yeni nano cihaz üretimi için anorganiklere özgün bağlanan peptidlerin proteinlerle birleştirilmesidir. Bu üretim sürecinde kendi kendine tutunma tekniğini kullanan ve altına özgün bağlanan (AuBP1 ve AuBP2) peptidleri kullandık. Daha önceki çalışmalarımızda gösterdik ki bu peptidler altın yüzeylere oldukça yüksek bir affinite ve seçicilikle bağlanmaktadır.

Sonuçta, bu tez kapsamında geliştirilen proteinlerle yeni nano yapıları malzemelerin tasarlanabildiğini bu çalışmada yer alan bulgular göstermektedir. Geliştirdiğimiz çok işlevli proteinler uygun koşullar altında, nano ölçekten mikro ölçeğe kadar çeşitli altın yüzeylere karşı yüksek bir bağlanma ilgisine ve özgünlüğüne sahiptir. Bu yeni platformlar pek çok amaç için kullanılabilir. Örneğin, protein mikro/nano dizilişler, biyosensörler, biyobateriler ve moleküler görüntüleme ve hedefleme amacıyla kullanılacak belirteçler hazırlanabilir. Belirlediğimiz bu model, organizasyon, kaplama ve mimariyi kontrol edebilen çeşitli protein dizilişleri, plazmon-aktif nanometalik montajlar ve cihazların üretilmesinde tamamen biyolojik bir yol izlemektedir.



## **1. INTRODUCTION**

### **1.1 Bioinspired Nanotechnology**

Nanotechnology defined as the application of materials and devices with characteristic length scales between 1 and 100 nm to the development of new products and processes [1-3]. Bionanotechnology is the interface of nanotechnology with biological systems. Biology has various examples of materials and structures that share a common length scale with nanotechnology [4-8]. The combination of biology and nanotechnology creates the opportunity to create and apply novel materials, hybrid structures and devices that exploit the distinctive features of both. This combination spans the use of nanomaterials as tools in biological research, the development of novel approaches to diagnose and treat disease as well as new ways to generate energy or clean up the environment [9-16]. Additionally, the link between biology and nanotechnology plays an important role in common processes for both domains such as self-assembly of the importance of kinetic rather than thermodynamic control in creating and maintaining structures. Because of having the marginal stability of many biological structures at ambient temperatures regarding non-functional states, they can have important implications for building hybrid bionano constructs. Therefore, they are favorable in the design and fabrication of such "hard-soft" interfaces as biological materials that bionanotechnology's distinctive flavor side.

The diverse properties of biological materials have been studied by many groups over a long period of time with at least one early example going back to Sir Thompson's book, i.e., "On Growth and Form" published in 1917 [17]. Thompson pointed out the correlations between biological forms and mechanical properties. Today's accumulated knowledge on biological materials are continuing to attract the interest of materials more than ever due to their exceptional properties resulting from Nature's inherent approach concealed in molecular scale design [2, 7, 14].

The remarkable difference in the design strategies used by Nature compared to today's engineering generates an important barrier in translating the lessons learned from biological materials to the design of new materials. Many biomaterials are structured in a highly organized-, hierarchical-manner at the nano-, micro-, meso-, and macro-scales over a long period of evolutionary adaptations [18-20]. The way Nature evolves the biological systems serves to bring multiple distinguishing functions that are built upon biomolecular machinery. One of the unique features is probably their inherent ability to self-organize over both multiple times and multiple length scales. By using basic biological building blocks such as proteins, lipids, nucleic acids and polysaccharides; living organisms synthesize a vast variety of tissues, sensors, and organisms using bottom-up approaches. Hard tissues, such as bones, teeth, spicules, shells, and bacterial nanoparticles, are examples that all have a high percentage of minerals that is integrated within a protein-rich matrix. This matrix can possess one or more protein-based components that control structural formation. [21-23]. Examples shown in Figure 1.1 include silaffins and silicateins in silica-based structures, amelogenin in enamel, collagen in bone, calcite- or aragonite-forming proteins in mollusk shells and magnetite-forming proteins in magnetotactic bacteria [24-27]. Each of these functional biological systems has self-organization and self-regulation characteristics. These kinds of characteristics are difficult to achieve in purely synthetic systems, even with the recently developed bottom-up processes that rely on the use molecules and nanocomponents. Moreover, the quality of these biological systems can extremely be astounding when compared to their man-made counterparts. They often are much tougher than synthetic ones formed at high degrees owing to the fact that they are synthesized at ambient temperature and chemical concentrations.

Research over the years has identified specific proteins found in living organisms that promote nucleation, promote growth, restrict growth, and even dissolve hard tissue. Cells in the organism control the expression of these proteins in order to grow and control hard tissues of the appropriate shape, size, and microstructure. Nature also has an amazing ability to recognize and communicate on the molecular level. The ability to even partially mimic these processes would represent a major technological advance for science.

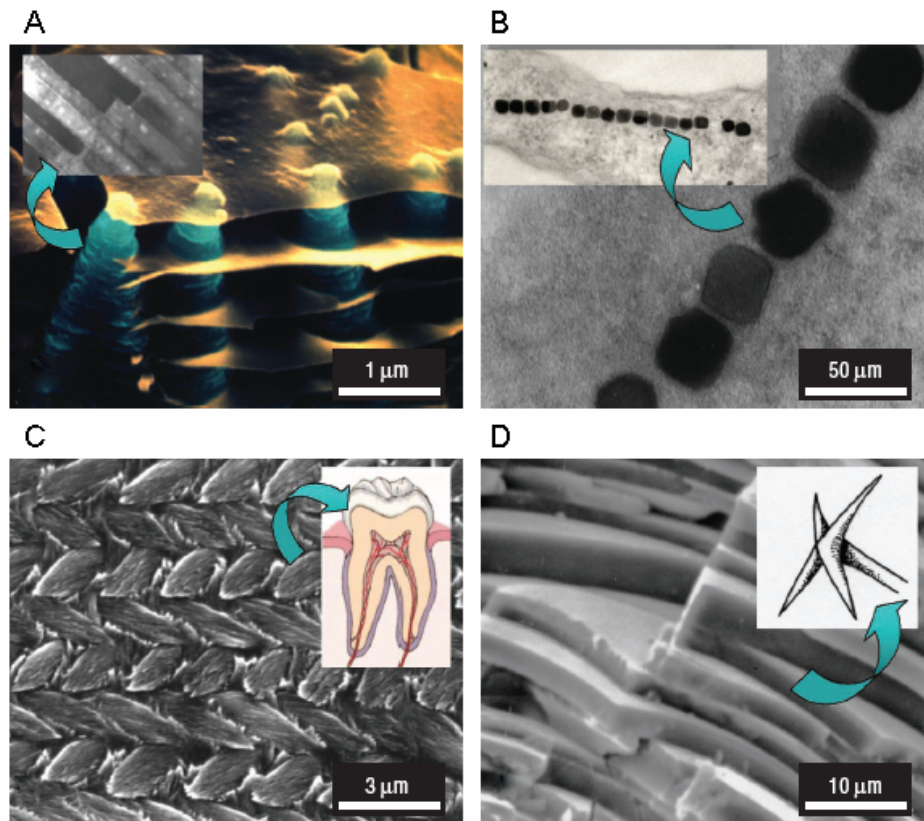


**Figure 1.1** : Example of biologically synthesized microstructures: A) SEM image of abalone shell cross-section (TEM image inset), B) TEM image magnetic nanoparticles in magnetotactic bacterium, C) SEM image of mouse enamel (inset: schematic of tooth), D) SEM image of sponge spicule of Rosella [2].

### 1.1.1 Protein nanotechnology

Protein nanotechnology is an emerging field and comprises the intersection of protein science, which exists naturally at the nanoscale, and the burgeoning field of nanotechnology. Proteins exist in nanoscale and they are a very versatile family of molecules with diverse functions, so they are finding utility in a host of nanotechnological applications. The diversity originates from the twenty different amino acid residues that are combined in different permutations to form all the hundreds of thousands of proteins found in nature. The amino acid sequences form specific secondary structures leading to higher order structure. The specific structure of the resulting protein is what allows specific function. This functionality is the result of millions of years of evolution. Proteins offer a diverse platform that can be harnessed using a controlled process of evolution in order to create controlled systems.

Proteins have been recognized as the most versatile of the building blocks, however this versatility comes with the cost of complexity. Moreover, proteins are also hard to control in a predictive manner. In contrast peptides are increasingly investigated as building blocks for nanostructures [28-37]. Most exciting applications are being explored for their use in creating functional biological materials [38-45]. Successes include the generation of biomaterials from peptides that bridge the nano- to mesoscales [33, 43, 46-49]. Structures assembled from peptides synthesized chemically are often much more stable than proteins. However, peptides lack the functionality of proteins, and are expensive to synthesize at large scale [50]. This, coupled with routine techniques to create proteins with designer properties, provides to explore proteins as building blocks to design nanostructures and nanodevices.

Mimicking molecular scale interactions of biomolecules at the material interfaces was initiated with the search for control over directing molecular evolutions toward technologically relevant materials [7, 20, 23, 51]. Peptides, with specific materials recognition have been a major biocomponent in this approach. In the last decade many groups, including the authors, used combinatorial biology-based approaches to generate peptides with a specific affinity and selectivity for a material of technological interest. These new set of peptides have revealed that novel ways of addressing the biomolecular interfacial interactions can be generated by mimicking the natural evolution process. There is a continued interest to identify additional peptides and understand their lexicon for molecular level interactions with different materials such as metal, metal oxides, and ceramics. As the fundamental level of peptide-based interfacial interactions continues, peptides are making their ways into bio-enabled materials and systems assembly and synthesis processes.

Already there appears to be a path to follow in order to design peptide-based biomimetic systems: (1) The inorganic-specific peptides are identified by initiating a fast evolution toward the materials of interest, using combinatorial biology-based libraries; (2) These peptide building blocks can be further engineered to tailor their recognition and assembly properties using rational design or computational biology approaches, including bioinformatics and structural biology. This step is similar to natural evolution, where successive cycles of mutation and selection potentially leads to improved progeny; (3) The engineered peptides can be used to self- or co-assemble into an ordered functional biomolecular layer, building upon the controlled

biomolecular-inorganic materials interface; and (4) Building upon the modularity of the peptide domains, a domain can be used as molecular building blocks to combine: (a) two-different materials binding functions to couple different materials within a close proximity, and (b) a biological cue to any type of materials with size, shape, and composition leading to the design of multifunctional hybrid materials [9, 10, 12, 13]. The outcome of this process will continue to have great impact on medical and technological applications, including self-assembled-, multi-functional-, molecular-structure constructs, from nanophotonics to nano- medicine, as well as to devise systems that can be built upon the multitude of self-regulatory operational modes intrinsic to protein.

Proteins offer enormous diversity in three-dimensional architecture and endless scope for modification and functionalization. For the last decade this opportunity has been suggested [26, 52-61] but the challenge of designing and controlling the interactions in vitro to produce useful functionality is a large one [62-65]. In contrast to the simpler peptide building blocks, designed self-assembly of proteins has been hampered by chemical heterogeneity and the large size of molecular surfaces required for biological interactions [66]. Since proteins composed of diverse amino acid residues can non-specifically interact with any surfaces through their side chains, material selectivity becomes an important parameter toward the integration of the desired molecular recognition properties into peptide-based materials and systems [6, 32, 67-71] .

### **1.1.2 Peptides in protein nanotechnology**

Progress in the development of combinatorial selection techniques created a major tool for a myriad of biotechnological applications including discovery antigen–antibody interactions, studying peptide–ligand interactions, and drug and vaccine development [72-74]. In the last decade, combinatorial biology-based molecular library protocols have been adapted from molecular biology to materials science and engineering, with the intention to select peptides that recognize and bind to an inorganic surface.

Peptide self-assembly is a burgeoning research field finding increasing scientific and application focus. To date various activities have centered on the design of three dimensional structures, such as scaffolds and gels for cell organization, and the

design of nanostructures in bulk, for example, fibrils or modification of the two dimensional inorganic-organic interface, i.e. in biosensing applications [75, 76]. Inorganic binding peptides are a class of peptides that have been biocombinatorial selected to bind to an inorganic surface. They have presented the effort of scientist to understand and mimic the process found in nature of synthesizing inorganic materials and provided a new approach to self-assembly and formation in nanotechnology. The shorter sequence of peptides compared to natural proteins allow for better control and easier study of their function [9, 37, 77, 78]. Interest in these peptides has been fueled by such applications as nanomedicine, biosensors [79], and biocompatible materials. Specific studies have shown the ability of peptides as synthesize nanoparticles [8, 15, 80], molecular linkers[9, 32], immobilization platforms [81-83], and assemble nanostructures [2, 32, 45, 84, 85]. Inorganic binding peptides have been suggested for these applications because of their long-term stability, compatibility with living systems, and their ability to recognize different surfaces. Several studies conducted in recent years have shown that inorganic binding peptides do indeed possess these qualities when compared to synthetic chemical approaches. Also, several proof of principle studies have been published or are underway to show the usefulness of such peptides in a variety of applications in both engineering and medical systems [13, 86-93].

### **1.1.3 Inorganic binding peptides in protein nanotechnology**

Inorganic binding peptides sequences have been selected using several methods. Some peptides have been chosen by imitating sequences found in natural proteins [94]. Among the combinatorial biology methods, phage display (PD) and cell surface display (CSD) are two well-adapted techniques used to screen peptides against an inorganic target surface [72-74, 95]. These methods allow a library of  $10^9$  -  $10^{11}$  randomly generated peptide sequences to be screened for their binding ability to an inorganic surface. The basic principle of each technique relies on the link between phenotype and genotype. DNA sequences corresponding to random peptides are inserted into the genome of microorganisms where they will express, within the context of proteins localized on the surface of phage or the cell. The random peptide sequences can be displayed on the target surface of the phage or bacterial cells within fimbria, lipoprotein, and flagellar protein of the cell or on the major or minor coat protein of phage [72, 96]. Among cell surface and phage display combinatorial

approaches, the phage display system has risen to become more dominant owing to better efficiency than the cell surface technique.

The typical biopanning round that is applied in selecting inorganic-binding peptides is composed of multiple steps. The randomized peptide library displayed on phage or bacterial surfaces first interacts with the inorganic target having a specific material with a defined composition, shape, or size. Next, the nonbinding phage or cell clones are washed off the surface, followed by an elution protocol to recover the bound clones from the inorganic surface. Depending on the materials and solution properties, the elution process might involve physical and/or chemical methods. The recovered pooled clones are amplified for a subsequent panning round. Generally, a biopanning cycle is repeated 3–5 times (Figure 1.2).

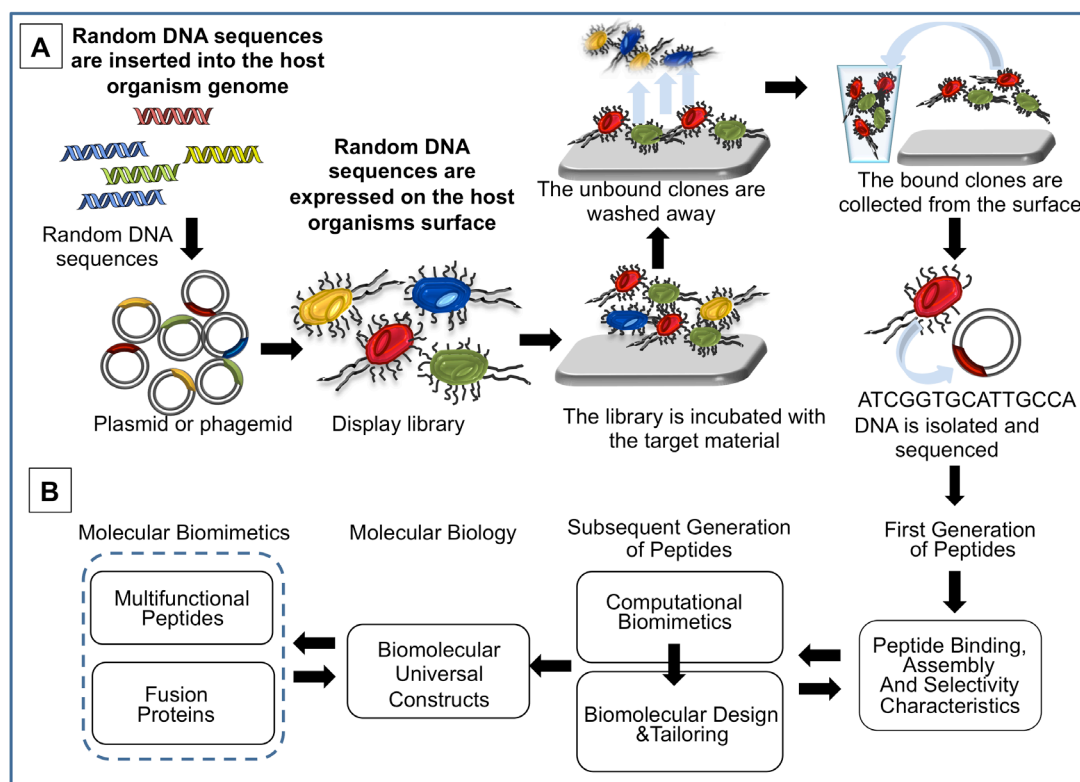


**Figure 1.2 :** Selection and Engineering of Inorganic-binding Peptides and Fusion Proteins: A) Schematic of experimental steps in biopanning process; B) Schematic of engineering steps in materials specific fusion protein design.

In early rounds, low affinity binders can be retrieved if the selection is performed under mild conditions. In later rounds, as the elution conditions get harsher (more stringent), high-affinity binders are also recovered. Because the chimera is encoded

within the phage genome or in a plasmid carried by the cell, the amino acid sequence information can be readily deduced by DNA sequencing (Figure 1.2).

The screening process generally yields several dozen peptide sequences. These sequences are generally narrowed down to several sequences that are used for further study and applications. The final sequences can be selected by relative number of bound phages [97] or through experimental binding characterization such as fluorescence microscopy [69, 98]. These methods have been used to identify peptide sequences that bind to gold [73], silver [15], platinum [2], zinc oxide [99], gallium arsenide [100], and titania [101] just to name a few with published sequences in Table 1.1. Beyond the ability to bind to a wide range of inorganic surfaces, studies have shown that in some case inorganic binding peptides can form ordered structures on surfaces [6]. So far, various peptide sequences for different inorganic materials, e.g., metals, Au [69, 73], Ag [15, 102], Pt [87, 103], SiO<sub>2</sub> [34, 104], Cu<sub>2</sub>O [99], ZnO [105-107], TiO<sub>2</sub> [13, 89, 97] [13, 89, 101], and ZrO<sub>2</sub> [108], minerals including hydroxyapatite, calcite, sapphire [30, 109, 110], semiconductors including GaN, ZnS, and CdS [54, 100, 111], graphite [84, 112], and diamond-like carbon [113] have been isolated using combinatorial molecular biology procedures, e.g., phage and cell surface displays. The experimentally selected peptides that show promising affinity in preliminary binding experiments can be used directly or can be further engineered by either bioinformatics tools or rational engineering approaches to produce the next generation of engineered peptides.

Once the peptide sequences have been selected, their binding characteristics are determined using a variety of methods. The quantitative characterization of peptide binding is important because it confirms the usefulness of the peptide while not connected to a phage or flagella. Some important traits are: the binding affinity of the peptide to the inorganic surface, the kinetic parameters of peptide binding, the selectivity of the peptide for the surface on which it was selected, the surface coverage of the peptide, the density of the self-assembled monolayer (SAM), the stability of the SAM, and the thermodynamics of binding. The importance of each of these traits depends on the mode of implementation. For example, in linking applications, affinity and stability are of utmost importance, while many biosensor applications require dense monolayers and high surface

coverage. In self-assembly applications, organization is likely kinetically driven, making binding kinetics important [69, 103, 114, 115].

**Table 1.1 :** Examples of Inorganic Surfaces and Peptide Sequences that Show Binding Affinity [2].

| Material                       | Sequence       |
|--------------------------------|----------------|
| Au                             | MHGKTQATSGTIQS |
|                                | WAGAKRLVLRRE   |
|                                | WALRRSIRRQSY   |
| Pt                             | DRTSTWR        |
|                                | QSVTSTK        |
|                                | SSSHLNK        |
| Pd                             | SVTQNKY        |
|                                | SPHPGPY        |
| Ag                             | TGIFKSARAMRN   |
|                                | EQLGVRKELRGV   |
| SiO <sub>2</sub>               | RLNPPSQMDPPF   |
|                                | QTWPPPLWFSTS   |
| Cr <sub>2</sub> O <sub>3</sub> | VVRPKAATN      |
|                                | RIRHRLVGQ      |
| Fe <sub>2</sub> O <sub>3</sub> | RRTVKHHVN      |
| GaAs                           | AQNPSDNNTHTH   |
|                                | TPPRPIQYNHTS   |
| ZnS                            | NNPMHQN        |

The “know-how” in biological systems has been built throughout billions years of evolution. The laboratory imposed cycle of generations and mutations have been an important part of the improved progeny found in these screening exercises. However, some groups introduced bioinformatics tools and computational approaches in order to reach the next level of control [34, 78, 116, 117]. They are combined with experimental characterization to generate peptides with improved properties. The application area was able to dictate the desired properties. For example, depending on the multi-material environments, peptides can be constructed with specific material selectivity properties or they can be tuned toward catalytic ability enabling control over nucleation and growth of a desired material with a specific morphology.

#### 1.1.4 Nanoparticles in protein nanotechnology

Nanoparticles comprised of noble metals, metal oxides, semiconductors are ideal candidates for single-molecule biophysics applications. They can also be synthesized readily in large quantities from various materials by simple chemical methods with a range of one to several hundred nanometers in size [118, 119]. Generally,

nanoparticles possess strong optical, electronic, magnetic, and catalytic properties which are not displayed by their corresponding bulk forms and which are often size-dependent [118, 119]. The synthesis of commonly used nanoparticles will be briefly mentioned as follows.

Due to the unique properties at the nanoscale, nanoparticles (NPs) have been developed as analytical probes, drug carriers and imaging agent for cancer [120-124]. Nanoparticles have both positive and negative properties in more applications of nanotechnology. Both sides of NPs are related to their dramatic biological membrane permeability and their strong interactions with biomacromolecules, such as proteins. Therefore, analytical methods and strategies are required to research NP-protein interactions to understand the mechanism of NP's biological activity.

## **1.2 Nanoparticle-based Detection Methods for Biosensing**

### **1.2.1 Fluorescence-based detection**

Proteins are polymeric complexes of amino acids and have fluorphores, such as aromatic amino acids, which are tyrosine, tryptophan and phenylalanine. Among all existing analytical techniques to investigate NP-protein interaction, fluorescence spectroscopy is most sensitive one to detect intermolecular interactions [125-131]. Moreover, this method is sensitive to protein dynamics because the excited fluorescent state persists for nanoseconds. This fluorescence lifetime is exactly the time scale of many important biological processes such as molecular binding, and conformational changes [125, 128, 132, 133]. In sensing applications, detection is based on a change in fluorescence properties of molecular probe that happens when it interacts with the target molecule. Therefore, this probe displays both biological recognition as well as fluorescence activity. As a simple example, fluorescent recognition element is a dye that also exhibits binding affinity towards a certain target (chemical sensor). For most of the applications, integration of one or two dyes at appropriate positions in the molecular recognition element that is specific binder to the target is needed [133-135].

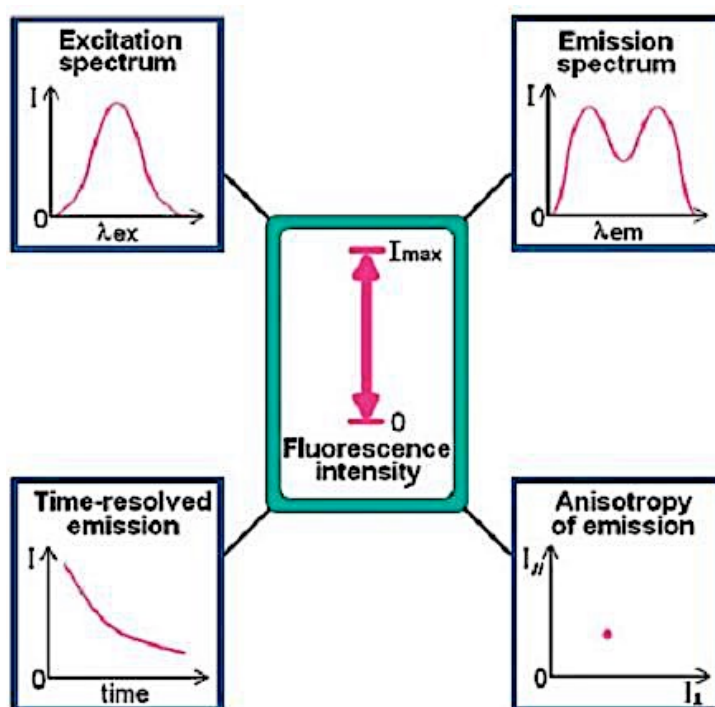


**Figure 1.3 :** Fluorescence parameters used for obtaining the sensor response [133].

Figure 1.3 summarizes the fluorescence parameters to monitor the sensor response. Fluorescence intensity ( $I$ ) is the simplest parameter that can be measured in the steady-state spectrum at particular excitation and emission wavelengths. Intensity ( $I$ ) measured in relative units is sensitive to all quenching effects. Its dependence on excitation wavelength  $I_{ex}$  and on emission wavelength  $I_{em}$  gives the excitation and the emission spectra, respectively. Positions of their maxima may be sensitive to intermolecular interactions of reporting dye. The rate of emission decay gives the fluorescence lifetime. The emission anisotropy is obtained by recording fluorescence emission at two perpendicular polarizations, vertical and horizontal. All of these parameters can be measured as a function of excitation and emission wavelengths [134].

When NPs are labelled with fluorescence probes, fluorescence emission can be detected from NPs. NP-protein binding can be monitored by steady state and time resolved fluorescence spectroscopy, fluorescence energy transfer, fluorescence polarization and fluorescence quenching methods [136-141].

Upon binding to target molecule, a conformational change in the receptor unit changes: (a) the distance and therefore the interaction between two fluorophores or between a fluorophore and a quencher, (b) the interaction of the fluorophore with the

receptor and/or its exposure to the solvent, (c) the environment of the fluorophore, leading to a direct interaction between the dye and the bound target.

### 1.2.1.1 Quenching of fluorescence

Fluorescence lifetime and quantum yield are informative characteristics for a fluorophore molecule. Fluorescence lifetime determines the information available from emission of the fluorophore. The fluorophore interacts with or diffuses in the environment in time. The fluorescence quantum yield is measured as ratio of number of the emitted photons to the number of absorbed photons. Higher quantum yield increases the intensity [142, 143]

Quenching is the decrease in the intensity of the fluorescence by a variety of mechanisms. One experimentally useful type of quenching is collisional or dynamic due to collisions between quenching agents and fluorophores. The dynamic quenching agent provides a non-radiative route for loss of the excited state energy. The excited state fluorophore is deactivated upon contact with the quencher molecule. Molecules such as oxygen, halogens, amines, and electron deficient molecules can act as collisional quenchers. Formation of non-fluorescent complex by the fluorophore and the quencher molecule is another quenching process. This second type of quenching is static quenching, which is sometimes confused with dynamic quenching [139-141]. Both quenching need direct contact between the fluorophore and the quencher molecule. During dynamic quenching, the result of the interaction between quencher and fluorophore is loss of the fluorescence pathway for return to the ground state.

Dynamic quenching exhibits a concentration-dependence that is described by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q] \quad (1.1)$$

where  $\tau_0$  is the lifetime of the fluorescent state in the absence of the quenching agent. If the quenching is not known to be due to dynamic quenching,  $K_D$  is replaced by  $K_{SV}$ .

For dynamic quenching,

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} \quad (1.2)$$

because the quenching agent decreases the lifetime of the excited state. Dynamic quenching increases with temperature, because temperature increases diffusion rates.

The term  $k_q$  is the second order rate constant that describes the quenching process. It is proportional to the effectiveness of the quencher and the accessibility of the fluorophore to collisions with the quencher. The quenching rate constant is actually comprised of two terms:

$$k_q = f_Q k_0 \quad (1.3)$$

where  $f_Q$  is the fraction of collisions that result in quenching, and  $k_0$  is the diffusion-controlled bimolecular rate constant:

$$k_0 = \frac{4\pi N_0}{1000 \frac{cm^3}{L}} (r_f + r_q)(D_f + D_q) \quad (1.4)$$

where  $N_0$  is Avogadro's number,  $r_f$  and  $r_q$  are the radii of the fluorophore and quencher, and  $D_f$  and  $D_q$  are the diffusion coefficients of the fluorophore and quencher.

Typical values of  $k_0$  for free fluorophores and free quenchers are  $\sim 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ . If the fluorophore is bound to the surface of a protein, the  $k_0$  will be roughly half of this value due available surface occupied by the protein. If the fluorophore is buried within the protein, the value of  $k_0$  will be even smaller, depending on the accessibility of the fluorophore.

Static quenching is the result of the formation of a non-fluorescent complex between the fluorophore and the quencher. The association constant for the quencher-fluorophore complex describes the effectiveness of a static quencher:

$$K_s = \frac{[FQ]}{[F][Q]} \quad (1.5)$$

where [FQ] is the complex concentration, and [F] and [Q] are the concentrations of free quencher and free fluorophore. Because the total fluorophore concentration,  $[F]_0 = [F] + [FQ]$ ,

$$K_s = \frac{[F]_0 - [F]}{[F][Q]} \quad (1.6)$$

which rearranges to:

$$K_s = \frac{[F]_0}{[F][Q]} - \frac{1}{[Q]} \quad (1.7)$$

and therefore

$$\frac{[F]_0}{[F]} = 1 + K_s[Q] \quad (1.8)$$

If all of the decrease in observed fluorescence is due to complex formation, the equation becomes:

$$\frac{F_0}{F} = 1 + K_s[Q] \quad (1.9)$$

which is identical in form to the Stern-Volmer equation for dynamic quenching. Static and dynamic quenching can be distinguished by lifetime measurements (which require instruments capable of time resolutions in the nanosecond range), because dynamic quenching reduces the apparent fluorescent lifetime, while static quenching merely reduces the apparent concentration of the fluorophore. Alternatively, temperature effects can be used to distinguish the two forms of quenching. Diffusion rates, and therefore dynamic quenching rates, increase with higher temperature. In contrast, complex formation strength tends to be inversely proportional to temperature, and therefore static quenching tends to be higher at lower temperatures.

In some cases, the effect of the quencher is due to a combination of static and dynamic quenching. This results in a modified equation:

$$\frac{F_0}{F} = (1 + K_D[Q])(1 + K_s[Q]) = 1 + (K_D + K_s)[Q] + K_D K_s [Q]^2 \quad (1.10)$$

Quenching agents are used to probe the environment around fluorophores. Differential quenching by positively and negatively charged quenchers suggests a

charged environment. Small  $K_D$  values typically reflect steric hindrance of quencher-fluorophore collisions.

Quenching studies may allow isolation of signals from different fluorophores. They may also allow characterization of conformational changes that alter the accessibility of the fluorophore to the quenching agent [132, 144, 145].

Fluorophore can be quenched by metallic nanoparticles. In the quenching mechanism by metallic nanoparticles the distance is larger than the mechanism for molecular quenchers and FRET [131, 146]. Dulkeith et al. have shown that fluorescent dyes quenched at distance as large as 16 nm [147, 148]. In addition, it has been found that QDs quenched at 21 nm distance [149].

It has been suggested that the efficiency of quenching by gold nanoparticles depends on the size and shape of the nanoparticle, distance from the fluorophore, spectral overlap and dipole orientation [147, 148]. On the other hand, the exact mechanism for the quenching has not been exhibited yet [150]. Au NPs provide the additional nonradiative decay pathway like energy transfer for quenching of QDs [Pons, 2007 #96]. However, a change in the radiative rate of the fluorophore induces more significant quenching than energy transfer [148]. It has been also shown that surface bound dye have a 10 fold decrease in radiative rate while there is a 10 fold increase in non-radiative rates [147]. This study also suggested that 5-10 nm diameter gold NPs are optimal quenchers [150]. Gold nanoparticles mostly used as quencher for molecular beacons exploiting fluorophores or QDs in nucleic acid analysis.

### **1.2.1.2 Fluorescence resonance energy transfer (FRET)**

In fluorescence resonance energy transfer (FRET) sensor, the efficiency depends highly on the distance between the donor and acceptor dyes. The energy transfer from the one molecule (donor) being initially excited to other (acceptor) being able to receive the energy and to convert it to its own emission is possible at distances of 10–60 Å and sometimes up to 100 Å [134]. The formation of the target-the fluorescent sensor complex results in a change in the proximity range between the two dyes, leading to an increase in the intensity of the acceptor fluorescence, and a decrease in the donor fluorescence. FRET can provide an easy and convenient way for determining the target concentration. During the sensing event, the switching between donor and acceptor emissions can be recorded as the ratio of the

corresponding emission intensities, providing compensation for various instrumental factors as well as the concentration of sensor molecules [134, 138, 146].

### 1.2.2 Localized surface plasmon resonance spectroscopy

Localized surface plasmon resonance (LSPR) spectroscopy of metallic nanostructures is a robust technique for chemical and biological sensing applications [139-147]. Basically, a surface plasmon resonance, SPR, is occurred by the materials that posses a negative real and small positive imaginary dielectric constant. This resonance is caused by a coherent oscillation of the surface conduction electrons excited by electromagnetic radiation [151]. There are two types of surface plasmons; the propagating and the localized [152]. Localized surface plasmons (LSPs) that oscillate around the nanoparticle with a frequency, called as the LSPR, is generated when the light interacts with the particles much smaller than the incident wavelength (Figure 1.4), [154]. The LSPR occurs in metallic nanoparticles, mostly silver and gold, in the 10-200 nm size range and results in enhancement at the electric field ( $E$ ) near the particle surfaces such that  $|E|^2$  can be 100–10,000 times stronger in intensity than the incident field [155]. The enhanced electric field nearby the particles has a spatial range on the order of 10-50 nm and is highly dependent on the nanoparticle size, shape and local environment [152, 153]. On the other hand, propagating plasmons, generally known as surface plasmon polaritons (SPPs), (will be discussed in the following subsection) require a planar platform containing a thin film of silver and gold with thicknesses within the range of 10-200 nm. Comparing to LSP, propagating surface plasmons lead to smaller field enhancements (10–100 times) and larger spatial range ( $\sim 1000$  nm) [155].

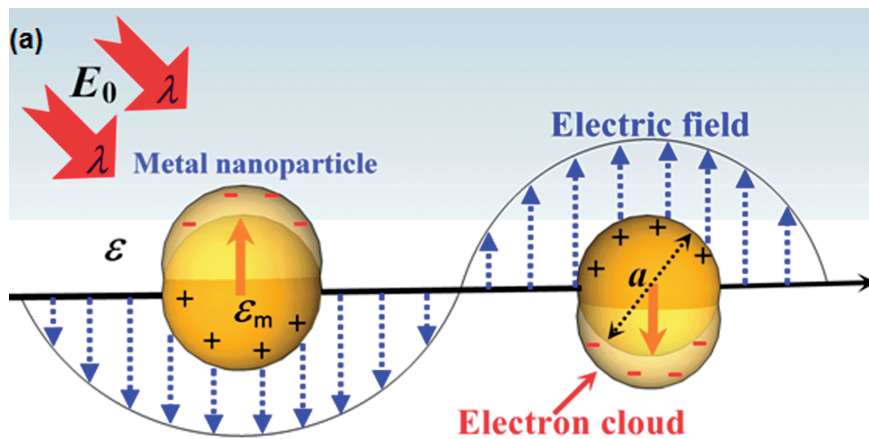


**Figure 1.4 :** Schematic representation of a localized surface plasmon [86].

Both the SPR and the LSPR are very sensitive to changes in the local dielectric environment [154, 155]. Based on this fact, the plasmonic materials have been already used in instruments which provide monitoring of the thermodynamics and kinetics of biological interactions using SPR spectroscopy [156, 157]. In SPR spectroscopy, researchers use thin (~50 nm) metal films, mostly gold, as the sensing platform. These platforms can generate SPPs. Besides, with recent development of synthetic techniques, such as colloid chemistry based nanoparticle synthesis, and NSL, researchers have started to achieve controlled fabrication and manipulation of metallic nanostructures and to use them in new applications that take advantage of the LSPR [155, 158].

For LSPR based sensing, the changes in the local environment are detectable through an LSPR wavelength-shift measurement, although a variant of angle-resolved sensing for the LSPR is also possible [159]. The sensor converts the changes in local refractive index caused by the changes in local environment, to wavelength shifts of the LSPR extinction band maximum (LSPR  $\lambda_{\text{max}}$ ). As mentioned above, upon the interaction between metal nanoparticles and the light, a collective oscillation of the conduction electrons around the particles occurs and this leads to LSPR extinction which results in wavelength-selective absorption with extremely large molar extinction coefficients ( $\sim 3 \times 10^{11} \text{ M}^{-1} \text{ cm}^{-1}$ ), resonant Rayleigh scattering with efficiency equivalent to that of 106 fluorophores [156]. LSPR  $\lambda_{\text{max}}$  is also dependent on the size, shape, composition, and dielectric environment of the nanoparticles [155].

The tuning of optical properties of nanoparticles can also affect the sensing capability of LSPR sensors [86, 156]. LSPR sensors can sense the binding of target molecules at very low concentration. Also, the LSPR sensors are potentially applicable for in vivo cell detection because the sensing reagents are at nano-scale [156].

### **1.2.3 Surface plasmon resonance spectroscopy**

Surface plasmon resonance (SPR) spectroscopy is, perhaps, the most extensively utilized optical technique for various biological and chemical sensing applications such as detection of glucose and urea, DNA binding assays, immunosensing for immunoassays (for proteins, hormones, drugs, viruses whole bacteria, etc.) [160,

161]. SPR spectroscopy is associated with the SPPs that propagate in the x- and y- directions along the metal-dielectric interface, for a distance range from tens to hundreds of microns, and diminish evanescently in the z- direction with  $1/e$  decay lengths on the order of 200 nm (Figure 1.5) [154]. When the light hits the metal film, the interaction between the metal surface and the electromagnetic wave produce SPPs along the metal-dielectric interfaces, which are so sensitive to any change in dielectric environment. For the SPR sensors, the binding of target molecule to the probe molecule at the gold surface alters the surrounding environment of thin metal film, leading shifts in the plasmon resonance condition which can be monitored in three modes: 1) wavelength shift, 2) angle resolved, and 3) imaging. In modes 1 and 2, sensor records the reflectivity of light from the metal surface as function of either wavelength (at constant angle of incidence), or angle of incidence (at constant wavelength). However, the mode utilizes light of both incident angle and constant wavelength to scan a certain two-dimensional region of the metal film, mapping the reflectivity of the surface as a function of position [154].

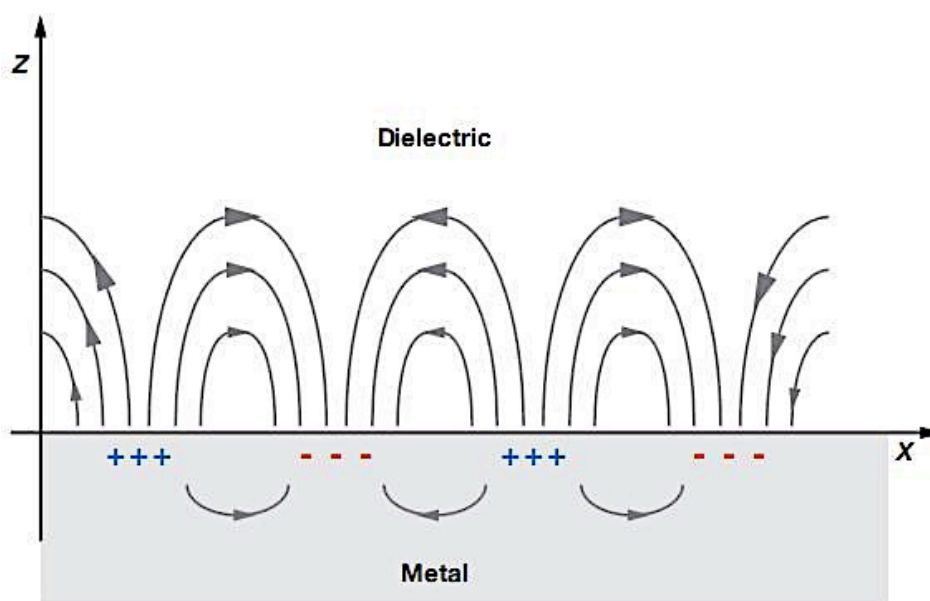


**Figure 1.5 :** Schematic representation of a surface plasmon polariton [154].

In SPR sensors, the Kretschmann configuration is mostly used setup to excite plasmons [153]. The configuration is shown in Figure 1.6. A glass slide is coated with a thick gold or silver film by vacuum deposition. Then, the biosensing layer containing probe molecule adsorbs to the metal surface by immobilization techniques including, mostly, SAMs of thiol molecules. The slide is then coupled to a prism through an index-matching fluid followed by getting the light incident at the prism.

The reflection of the light is monitored. At a certain qsp, coupling of the electromagnetic wave to the interface generates a surface plasmon while an evanescent field propagates away from the interface that moves away up to 100 nm above and below the metal surface. At this angle, the reflectivity signal decreases, resulting in a dip. In the case of bare metal film, this dip in reflectivity occurs at one angle. However, for the metal film coated with sensing layer, this angle shifts (mode 2). Upon the binding of target molecules to the sensing layer, a further red shift in the SPR coupling angle occurs. Simply, the sensor converts the target binding to the angular shift (change in qsp) [163, 164]. Alternatively, this can be also applied to the mode 1 where the reflectivity of the light is recorded as a function of wavelength at constant [103].

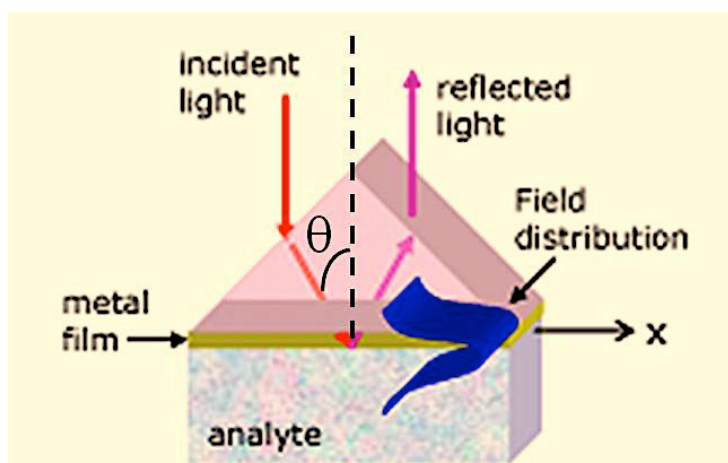


**Figure 1.6 :** Schematic diagram for the Kretschmann configuration [162].

Both propagating SPR and LSPR sensors have an intrinsic advantage over other optical biosensors such as fluorescence-based systems that necessitate a fluorophore or other label to sense the binding event [153, 163]. The response of LSPR and SPR sensors is related with the amount of bound molecule, thin layers and refractive index [155]. These sensors also allow researchers to monitor real-time information for various binding events being used in biotechnology and medicine [153, 155]. In terms of the changes in bulk refractive index, SPR spectroscopy gives higher sensitivity comparing to LSPR spectroscopy [114, 164]. However, the responses of two techniques are comparable when sensing the short-range changes in the refractive index due to a molecular adsorption layer [164]. In other words, SPR sensors provide larger sensing volume since the electromagnetic field decay length is 40-50 times longer than that of LSPR sensors [151].

### 1.3 Gold Binding Peptide Selected by Cell Surface Display Method

A great number of inorganic binding peptides have been selected up to date and the limited numbers of them were well characterized and utilized in various biotechnological applications. Recently, the identification and characterization of two 12 aa cationic peptide sequences that interact with Au surfaces with high affinity were reported and  $K_{eq}$  values of gold binding peptides were determined between  $2.5 \times 10^6$  to  $13.5 \times 10^6 \text{ M}^{-1}$  [165]. These two high-affinity Au binding peptide sequences, AuBP1 and AuBP2, both have aromatic Trp (W) residues and other amino acids in charge of hydrogen bond formation such as Arg, Lys, Tyr and Ser. The AuBP sequences were tested in both the linear (l-AuBPs) and cyclic forms (c-AuBPs) in order to determine the effect of molecular constraint on peptide functionality on gold. In this dissertation, we specifically used *l*-AuBP1 (MW: 1454.7, WAGAKRLVLRRE) and *c*-AuBP2 (MW: 2106.4, CGPWALRRSIRRQSYGPC) to design multifunctional fusion proteins resulting in formation of stable and water dispersible peptide-capped Au nanostructures (Table 1.2).

**Table 1.2 :** Synthesized gold-binding peptide sequences. MW, pI and net charge were computed by ProtParam (expasy.com); introduced tripeptide linkers were highlighted in red [69].

| Name    | Sequence           | MW     | pI    | Charge |
|---------|--------------------|--------|-------|--------|
| l-AuBP1 | WAGAKRLVLRRE       | 1454.7 | 11.71 | +3     |
| c-AuBP1 | CGPWAGAKRLVLRREGPC | 1967.3 | 9.67  | +3     |
| l-AuBP2 | WALRRSIRRQSY       | 1591.8 | 12.00 | +4     |
| c-AuBP2 | CGPWALRRSIRRQSYGPC | 2106.4 | 10.72 | +4     |

AuBPs were selected by bacterial cell surface display (CSD) method, which has FliTrx library (Invitrogen). This FliTrx bacterial expression system was used to select peptide sequences directed against gold substrates and as a scaffold for various size of Au nanoparticle self-assemblies and formations [69]. The system uses the engineered extracellular flagellar protein (flagellin) to interact with external substrates. The main feature of FliTrx peptide libraries is that random peptides are inserted as fusions within the thioredoxin active-site loop, which is itself inserted into dispensable region of the flagellin gene, as opposed to the N-terminal location of fusions made to coat proteins in phage display techniques. Thus random peptides are displayed on the surface of bacteria within the structural context of the thioredoxin

active-site loop, having both their N- and C- termini anchored by the rigid and stable tertiary structure of thioredoxin itself.

After selection of gold binding peptides, first characterization experiments were carried out with fluorescent labeling of cells bound to gold. The binding characterization study allowed us to sort selected clones into three groups: strong, moderate and weak binders depending on their gold-binding affinity [69]. Depending on the results, two of the strong binders, AuBP1 and AuBP2, were further tested.

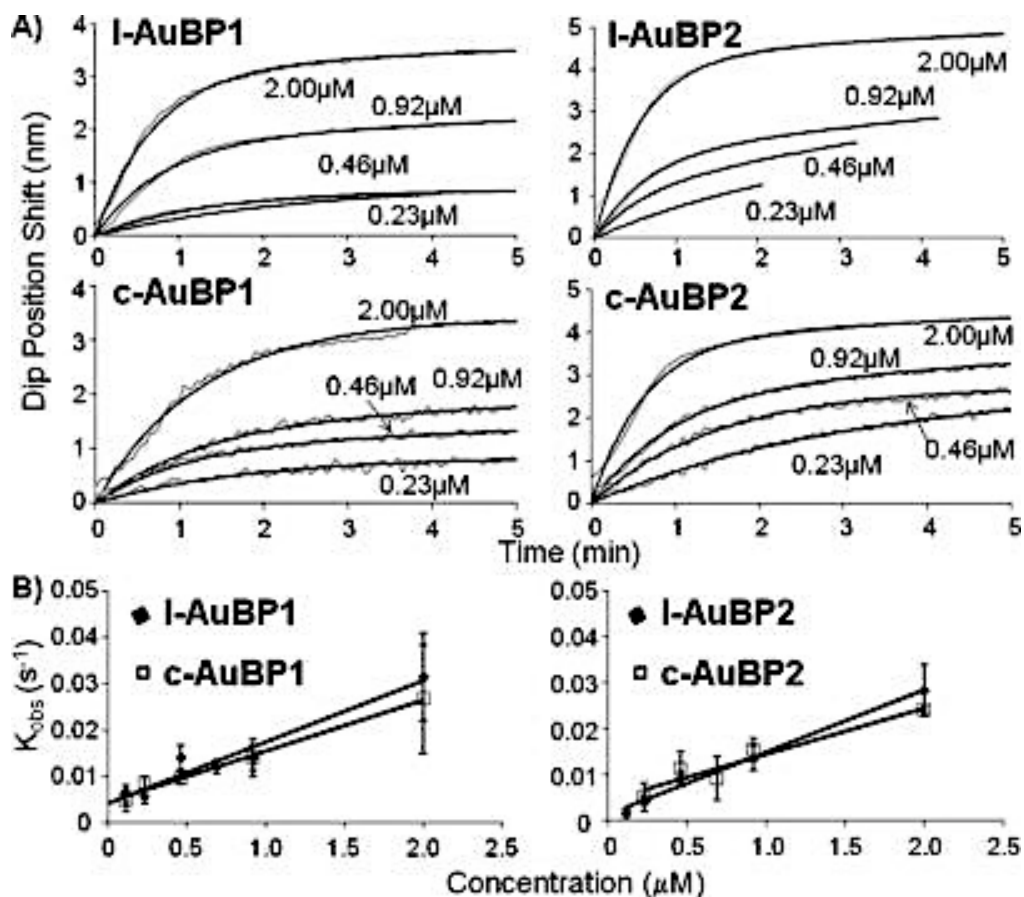


**Figure 1.7 :** (A) Adsorption isotherms for linear and cyclic forms of AuBP1 and AuBP2, obtained by SPR, at different concentrations. The gray lines represent experimental data points, and the black lines are Langmuir model fits. (B) Observed rate coefficients ( $K_{obs}$ ) plotted as a function of the peptide concentration. Linear regression was used to calculate the adsorption ( $k_a$ ) and desorption rates,  $k_d$ . [69].

These AuBPs were produced both in linear form (l-AuBP1, l-AuBP2) and cyclic form (c-AuBP1, c-AuBP2). Cyclic peptides have a Cys-Cys loop, which is introduced as CGP tripeptide at both N- and C-terminus of peptide, for example, CGP-AuBP-GPC in Table 1.2. Binding affinity of these peptides in different conformations was investigated by SPR (Figure 1.7). Although all peptides showed

the similar binding characteristics, c-AuBP2 possess a significantly lower desorption rate, which is leakage rate as seen in Table 1.3. Because of lower desorption rate, values  $K_{eq}$  and  $\Delta G_{ads}$  would be higher than other AuBP conformations.

**Table 1.3 :** Adsorption Rate ( $k_a$ ), Desorption Rate, Equilibrium Coefficient ( $K_{eq}$ ), and Free Energy for Linear (*l*) and Cyclic (*c*) of gold binding peptide [69]

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

#### 1.4 Monomeric Red Fluorescent Protein (DsRed-monomer)

The latest explosion in the diversity of existing fluorescent proteins (FPs) promises a wide variety of new biomaterials for biological imaging and biosensors [127, 130, 137, 166-171]. There are general requirements for the successful use of a fluorescence protein in an imaging or sensing experiments. First, the fluorescence protein should be expressed efficiently and non-toxic in the chosen system. Also, it should provide enough signals above auto fluorescence to obtain reliable and accurate detection. Second, if the fluorescence protein is incorporated with another protein of interest, it should not oligomerize. Third, it should be useful for quantitative interpretation of experimental results because of their sensitivities to environmental conditions. Finally, the number of fluorescence proteins used in multiple-labeling methods should have negligible crosstalk in their excitation and emission wavelengths.

The newly released DsRed-Monomer (Clontech) is described as the bright monomeric red fluorescent protein (RFP) presently available. As the discovery of DsRed, red fluorescence protein, various works have been performed to address the limitations seen for the native tetrameric protein; much of this work has focused the need to create a monomeric mutant [172]. Yanushevich et al. [173] developed the first commercially available non-aggregating mutant of DsRed, in 2002. The optimized monomeric DsRed, DsRed M1 was described by Strongin et al. [174, 175]. DsRed monomer contains 45 amino acid substitutions in native DsRed; it overcomes the oligomerization seen for the native protein. Its molecular weight is ~28 kDa, consistent with a single DsRed unit. The DsRed monomers generated by both

directed evolution and site-specific mutagenesis of amino acid residues have an almost ideal set of properties for biological sensing and analytical applications.

As the spectral properties of DsRed-monomer are less ideal than those seen for the tetrameric DsRed, DsRed-Express (Clontech), the maturation mechanism is much faster. The chromophore of tetrameric DsRed is extremely slow to mature, taking several days at room temperature to reach full red expression. This red chromophore forms through a green intermediate, which is seen at about 7 hours post induction. After 48 hours this green emission completely disappears and red fluorescence reaches 90% maximal intensity [176]. However, the chromophore of DsRed-monomer fully matures within hours. Crystallographic studies of DsRed expose that it is tetramer even at nanomolar concentration. Since it requires more time for all four chromophores within the tetramer structure to fully mature, the slow maturation was observed in DsRed resulting in oligomerization. It has also been suggested that this tetrameric form may be responsible for DsRed's resistance to photo bleaching, which is four to five fold greater than that of GFP [177, 178].

DsRed tetramer presents four individual units, each contains 11-stranded  $\beta$  barrel with a central helix. This central helix consists of the chromophore and  $\alpha$  helical cap on each of the ends of the barrel [178, 179]. Although DsRed shares only 26% amino acid sequence identity with GFP, their three dimensional structures are nearly identical. The superimposed structures reveal that the loops in DsRed are shorter than those in GFP, due to a reduced number of amino acids in the comparable loop regions and higher hydrophobicity of the residues present. Further, a pronounced bulge is seen centered on Ser146 in DsRed, a similar bulge is not seen for GFP in this region. Finally, residues 97-100 and 140-145 are significantly closer to chromophore in DsRed, compared to GFP, which probably impacts chromophore formation. In comparison to GFP, DsRed is more favorably used because it has higher photo stability and higher quantum yield [180]. With no doubt, DsRed-monomer is a better choice for in vivo studies and biochemical studies as it eliminates the unfavorable characteristics of GFP.

DsRed-Monomer displays a fluorescence excitation maximum at 556 nm and emission at 586 nm, respectively. Interestingly, it has an intrinsic copper-binding site that shows its affinity toward binding copper ion resulting in fluorescence quenching. By having this novel characteristic, DsRed is widely used for biosensor design for

copper detection whereby red fluorescent protein can act as a probe, which can be targeted to specific organelles for detection of copper [181, 182]. Although copper is recognized as a pollutant in the environment and exhibits toxic effect, copper is widely used as anti-fouling agents for commercial shipping vessels in the industrial area [183]. Nevertheless, high copper intake may also cause infant liver damage and devastating effect to human health. Therefore, the ability to bind copper ions of red fluorescence protein can now be applied in determining the copper levels in biological and environmental samples [183]. Moreover, red fluorescent protein may also be used as a probe in the medical field for diagnosis, for example, Menkes and Wilson's diseases that arise from the problem of copper transport system in human. Patients with these diseases have high level of free copper ion concentration in their blood due to inability to metabolize copper effectively [181, 183-185]

Current methods for determining copper levels in biological and environmental samples have lots of limitations. Samples may be destroyed when applying atomic absorption spectroscopy and ICP-MS methods. Also, other fluorescence indicators and electrochemical techniques are rather slow and lengthy [186].

Since DsRed-monomer has some advantages aforementioned above, it can play important roles in many biotechnological fields, including diagnostics and biosensors. Often, DsRed is used in immobilization form as a probe. With conventional immobilization techniques, the protein adheres in random orientation: the protein active site may face the solid phase rather than bulk medium, impeding ligand access and leading to sub-optimal catalytic performance. The ability to immobilize DsRed in a directional manner, such that the active site would always face outwards from the insoluble matrix, would maximize protein activity and could increase DsRed range of actual and potential applications [75, 127, 185, 187, 188]. Because of these reasons, DsRed was designed and expressed with gold binding peptide as a multifunctional fusion protein to use in bionanosensors as the high surface sensitivity of nanomaterials.

## **1.5 Formate Dehydrogenase (FDH) As An Industrial Enzyme**

Among Nature's vast repertoire of biomolecules, a variety of oxidoreductases are industrially important enzymes capable of catalyzing key metabolic reactions. These metabolic processes are classified as redox reactions that involve oxidation or

reduction of substrate molecules as well as electron pair transfer between organic substrates and concomitant cofactor molecules [189, 190]. Dehydrogenases produce pure chiral molecules with their enantioselective oxidative and reductive catalytic properties, promoting these enzymes as highly valuable tools in the pharmaceutical, chemical, agriculture, and food processing industries. Nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent formate dehydrogenase (FDH, EC 1.2.1.2) is an important member of the oxidoreductase family. This enzyme catalyzes the conversion of formate into carbon dioxide coupled with irreversible reduction of NAD<sup>+</sup> to NADH [191]. Its ability to regenerate NADH cofactor via an irreversible reaction using a considerably cheaper substrate makes the enzyme a very promising candidate to be employed in NAD<sup>+</sup>/NADH regeneration processes. Furthermore, FDH enzymes have been used for sensing applications to detect formate quantitatively as an important fermentation product of aerobic and anaerobic bacteria [191, 192]. More importantly, with the capability to transfer electron pairs between specific substrates and NAD<sup>+</sup> molecules, FDH enzymes have the potential to power bioelectronics devices<sup>41</sup>. The industrial importance of NAD<sup>+</sup>-dependent FDH has led researchers to develop different kinds of immobilization strategies through a variety of support materials to efficiently enable the use of the enzyme [193-195]. FDH enzymes have been successfully isolated and produced from many different species, but methanol-consuming yeast species, such as *Candida methylica*, have received the most attention due to the enhanced stability and relatively high activity of their isolated NAD<sup>+</sup>-dependent FDH enzymes [191, 196, 197].

## **1.6 Purpose of Thesis**

Herein, a select research is given some of the exciting nanostructures that can be created using proteins, and the sorts of applications that protein engineers can be reaching towards in the nanotechnology space. This provides inspirational case studies, along with tips and tools to manipulate proteins into new forms and architectures, beyond Nature's original intentions.

The overall goal is to design fusion proteins with inherent inorganic binding abilities that facilitate in the development of hybrid constructs for a diverse range of technological applications. In previous findings by Tamerler and Sarikaya group, it was hypothesized that the specific peptide tag presented in the fusion protein

constructs would increase the affinity, orientation and molecular organization for the self-assembled proteins onto inorganic surfaces. However, there are very limited studies in the literature providing a proof of concept demonstration using bi-functional proteins as surface-specific linkers. Therefore, in this research, the gold binding peptide (AuBP), which is well-characterized one of the inorganic binding peptides has been coupled with other biological molecules such as maltose-binding protein (MBP), red fluorescence protein (DsRed-monomer) and formate dehydrogenase (FDH), and investigated with the emphasis on the potential use of this peptide as a molecular linker in functional nanodevices.

In bionanosensors, biomolecules and nanomaterials can be combined to mutual advantage and produce devices with applications in clinical, environmental and bioprocess monitoring. The enhancement of bionanosensors compared to conventional biosensors arises from many nanomaterials, which have optical, electronic or magnetic properties

Fabrication of hybrid structures, particles, fibers, wires, pores or films with enhanced optical, electronic, magnetic or mechanical characteristics generate a new family of base sensors that lack only the molecular specificity necessary to use them in complex backgrounds. Actually, it is such molecular recognition specificity that is the hallmark of biomolecules and the interface between the two moieties is what provides bionanosensors with their analytical power.

As with conventional biosensors however, the native biomolecular properties are not continuously enough to provide an effective sensing interface when they have evolved to fit a specific biological role, not a sensing one. If the situation is more acute with bionanosensors as the high surface sensitivity of nanomaterials is best exploited when the biomolecule retains a high level of function. Molecular engineering can address this issue as well as others such as enhancing stability or adjusting functionality.

For a long time, the area of biotechnology has invested in using biological principles or approaches and components for medical and technological applications. In this endeavor, biomacromolecules, such as proteins, enzymes, and antibodies have been the major components previously used for a variety of applications in materials science. Here the ultimate aim is to bring more traditional engineered materials and

the components of nanotechnology, toward achieving efficient, addressable, adaptable biologically-controlled, and multifunctional hybrid molecular materials and systems.



## **2. MATERIALS AND METHODS**

### **2.1 Genetically Design and Production of AuBP-based Fusion Proteins**

In this dissertation, we particularly demonstrate the utility of combinatorial selected gold-binding peptide (AuBP) [69] as an excellent alternative linker enabling immobilization of various fusion molecules and nanostructures onto gold interface using combination of self-assembly and soft-lithography processes. By this approach, we genetically fused specific AuBP peptide tag to C-terminus of maltose-binding protein (MBP), red fluorescence protein (DsRed), and formate dehydrogenase (FDH), individually. We could design MBP-AuBP1, DsRed-AuBP2, and FDH-AuBP2.

#### **2.1.1 Bioengineered MBP-AuBP1 fusion proteins**

The two functional domains, which are AuBP1 tag and MBP, were fused each other by cloning procedures. In this design two different spacers were placed between MBP and AuBP1 in order to address the linker effect on fusion protein functionality. These spacers are PGPGPG, rigid linker, and SGGG, flexible linkers. The two different MBP-AuBP1 derivatives, MBP-(PG)<sub>3</sub>-AuBP1 and MBP-S(G)<sub>3</sub>-AuBP1, encoding by constructed pMAL-AuBP1 vectors and wild type MBP were expressed in *Escherichia coli* (*E. coli*) ER 2507 cells.

##### **2.1.1.1 Expression and purification of MBP-AuBP1 fusion proteins**

The glycerol stocks of each recombinant *E. coli* ER 2507 strains harboring pMal-c2X, pMAL-(PG)<sub>3</sub>AuBP1 and pMAL-S(G)<sub>3</sub>AuBP1 expression constructs were used to express fusion proteins. Firstly, the bacteria were streaked on a plate containing LB agar (Luria-Bertani, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 15 g/L agar, pH 7.0) with 100 µg/ml of ampicillin and incubated at 37°C for 16 hours. Then, a single colony was picked by a sterile loop and grown in 3 ml of LB broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl, pH 7.0) containing 100 µg/ml of ampicillin on the incubator shaker at 37°C for 16 hours. 1 ml of overnight cell culture

was added to 50 ml of LB broth with 100 µg/ml of ampicillin and grown at 37°C until an optical density of 0.6 to 1.5 at 600 nm. The starter culture flask was kept in the 4°C overnight. For protein expression, this cell culture was equally divided among 400 ml volumes of sterile LB broth with 100 µg/ml of ampicillin in 1 L flasks. Cells were grown at 37°C until an optical density of 0.5 to 0.65 at 600 nm, then the protein induction was initiated by IPTG addition to a final concentration of 0.3 mM. After addition of IPTG, the culture growth was continued at 37°C for 4 hours.

After expression of protein, cells were harvested by centrifugation at 4,000xg for 30 minutes at 4 °C. The centrifuged cell pellet was re-suspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and disrupted by sonication at 200 W for 1.5 min (puls on 10 s / puls off 10 s) using Branson Digital Sonifier with a double stepped microtip. Cell debris was removed by centrifugation (15,000xg for 30 min at 4 °C). 10 ml supernatant was loaded onto amylose resin column (2.0 x 15 cm) equilibrated with the column buffer. Following the removal of the unbound proteins, MBP-tagged fusion protein was eluted from the column with an elution buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM Maltose, pH 7.4). The pure fractions were analyzed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) on a 4 % stacking gel and 12 % separating gel.

### **2.1.2 Bioengineered DsRed-AuBP2 fusion protein**

In this bioengineered fusion protein, we used the gold binding peptide as a proper immobilization mediator for red fluorescence protein (DsRed). One of the gold binding peptide, AuBP2, was used as a fusion partner with DsRed by SGGG spacer between DsRed and AuBP2. DsRed gene from pDsRed-Monomer N1 vector and cyclic AuBP2 gene were cloned into pMal-c4 vector harboring maltose binding protein gene and transformed into *Escherichia coli* strain ER2507 in order to express tagged DsRed-monomer protein.

| <b>DsRed-AuBP2</b> |            |            |            |            |
|--------------------|------------|------------|------------|------------|
| MDNTEDVIKE         | FMQFKVRMEG | SVNGHYFEIE | GELEGKPYEG | TQTAKLQVTK |
| GGPLPFAWDI         | LSPQFQYGSK | AYVKHPADIP | DYMKLSFPEG | FTWERSMNFE |
| DGGVVEVQQD         | SSLQDGTFIY | KVKFKGVNFP | ADGPVMQKKT | AGWEPSTEKL |
| YPQDGVLKGE         | ISHALKLKDG | GHYTCDFKTV | YKAKKPVQLP | GNHYVDSKLD |
| ITNHNEDYTV         | VEQYEHAEAR | HSGSQSGGGC | GPWALRRSIR | RQSYGPC    |

**Figure 2.1 :** Genetically design of DsRed-AuBP2 fusion protein. Blue shows the linker between DsRed and AuBP2, red shows the cyclic formation and green is the AuBP2 sequence.

### 2.1.2.1 Construction of DsRed-AuBP2 fusion protein

The vector pDsRed-Monomer N1 (Clontech) was used as a template in all polymerase chain reactions (PCR). Oligonucleotides and primers in Table 2.1 were designed for PCR experiments according to the amino acid sequences of AuBP2, DsRed-monomer and the corresponding linker sequence. The recognition sites of EcoRI and HindIII restriction enzymes were inserted to 5' ends oligonucleotides and primers. The PCR experiments were performed using Phusion High-fidelity DNA polymerase (New England Biolabs, Catalog # M0530S). This enzyme generates blunt ends in the amplification products. To obtain A- added fragments, Taq DNA polymerase (New England Biolabs, Catalog # M0530S) was added into the reaction before the final extension (Table 2.2).

**Table 2.1 :** Oligonucleotides and primers.

| <b>Name</b>                                  | <b>Sequence</b>                                                          |
|----------------------------------------------|--------------------------------------------------------------------------|
| DsRed-AuBP-2c reverse-Ia                     | 3'CGGGCGGTGAGGCCGAGGGTCAGCCCA<br>CCTCCAACGCCAGGCACCCGAGACGCAG<br>CAAGC'5 |
| DsRed-AuBP-2c reverse-Ib                     | 3'GGCACCCGAGACGCAGCAAGC'5                                                |
| DsRed-AuBP-2c <i>Hind</i> III<br>reverse-IIa | 5'GCCAAGCTTTTAGCACGGACCATACGA<br>CTGACGACGAATCGAACGACGCAGAGCC<br>CA'3    |
| DsRed-AuBP-2c <i>Hind</i> III<br>reverse-IIb | 5'GCCAAGCTTTTAGCACGGACCATACGA<br>CTG'3                                   |
| Forward <i>Eco</i> RI-DsRed                  | 5'CGGGAATTCATGGACAACACCGAGGAC<br>GTC'3                                   |
| Reverse <i>Hind</i> III DsRed                | 5'GCCAAGCTTTTACTGGGAGCCGGAGTG<br>GCGGGC'3                                |

DsRed encoding sequence was amplified using the forward *EcoRI*-DsRed and the reverse *HindIII*-DsRed primers containing extra residues including a *EcoRI* or *HindIII* site at their 5' ends, respectively. The DNA fragment encoding DsRed-fused gold binding peptide AuBP2c (DsRed-AuBP2c) were obtained in a four step PCR reactions.

The first PCR reaction was kept in 15 cycle and performed by using the forward primer *EcoRI*-DsRed and the primer DsRed-AuBP2c reverse-Ia bearing the first part of gold binding peptide (AuBP2c) coding sequence (Table 2.2 and 2.3).

**Table 2.2 :** Polymerase Chain Reaction (PCR).

| Reagent                   | Volume ( $\mu$ L) |
|---------------------------|-------------------|
| Sterile dH <sub>2</sub> O | 39.3              |
| 10X Taq Buffer            | 5.0               |
| 10mM dNTP (2.5mM each)    | 2.0               |
| 25mM MgCl <sub>2</sub>    | 3.0               |
| Forward Primer (10pmole)  | 1.0               |
| Reverse Primer (10pmole)  | 1.0               |
| Template DNA (1 $\mu$ g)  | 0.5               |
| Taq DNA polymerase        | 0.2               |
| Total Volume              | 50                |

**Table 2.3 :** 1<sup>st</sup> and 3<sup>rd</sup> PCR conditions for DsRed-SGGG-AuBP2 fusion protein.

| Temperatures ( $^{\circ}$ C) | Time   | Cycles |
|------------------------------|--------|--------|
| 98                           | 30 sec | 1      |
| 98                           | 10 sec | 15     |
| 50                           | 10 sec |        |
| 72                           | 2 min  |        |
| 72                           | 5 min  | 1      |

The second PCR reaction was kept in 30 cycle and performed by using the forward primer *EcoRI*-DsRed, the reverse primer DsRed-AuBP2c-Ib, and the first PCR product as a template (Table 2.4). The PCR product was purified by gel extraction and used as a template for the third PCR reaction. The third PCR reaction was kept in 15 cycle and performed by using the forward primer *EcoRI*-DsRed and the reverse

primer DsRed-AuBP2c-IIa bearing the second part of AuBP2c coding sequence with HindIII cleavage site (Table 2.1). The fourth PCR reaction was kept in 30 cycle and performed by using the forward primer *EcoRI*-DsRed and the reverse primer DsRed-AuBP2c-IIb and the third PCR product as a template (Table 2.4).

**Table 2.4 :** 2<sup>nd</sup> and 4<sup>th</sup> PCR conditions for DsRed-SGGG-AuBP2 fusion protein.

| Temperatures (°C) | Time    | Cycles |
|-------------------|---------|--------|
| 98                | 30 sec  | 1      |
| 98                | 10 sec  | 30     |
| 54                | 30 sec  |        |
| 72                | 30 sec  |        |
| 72                | 100 min | 1      |

The amplified PCR fragment and pMal-c4X vector were first digested with *EcoRI* and *HindIII*, and then extracted from agarose gel (1.5 %). The digestion reaction was shown in Table 2.5.

**Table 2.5 :** Digestion Reaction.

| Reagent                            | Volume (µl) |
|------------------------------------|-------------|
| DNA (631 ng/µl) or pMal-c4X vector | 25          |
| 10X NEBuffer                       | 5,0         |
| <i>HindIII</i> (10 U/µl)           | 1,0         |
| <i>EcoRI</i> (20 U/µl)             | 0,5         |
| Sterile distilled water            | 18,5        |
| <b>Total volume</b>                | <b>50</b>   |

Agarose gel electrophoresis was performed for analysis and extraction of digested PCR product and vector. Samples and Lambda DNA/*Eco130I* (StyI) marker (Fermentas (Catalog # SM0161, Appendix A2) were run onto the 2% of agarose gel. Electrophoresis was performed in 1X TBE (89 mM Tris-borate and 2 mM EDTA, pH 8.3) running buffer at 80V for 45 minutes. PCR product and digested vector were extracted from agarose gel using MinElute Extraction Kit (Qiagen, Catalog # 27106). The desired DNA fragments were excised from the agarose gel with a clean scalpel. Gel slices were placed into microfuge tubes, and their weight was measured. Compared to their weight, a 3-gel volume of QG buffer was added to one volume of

the gel slices. (600 µl buffer for 100 mg gel). When gels were excised from a 2 % or more concentrated gel, a 6-gel volume of QG buffer was applied. Sliced gels were incubated at 50°C for 10 min. by vortexing every 2 min to dissolve the gel completely. After the gel dissolved, the color of the mixture was checked. QG buffer contains a pH indicator and gives a yellow color at pH less than 7.5, the optimum pH for DNA adsorption. After that, 1 gel volume of room temperature isopropanol was added and mixed by inverting the tube several times to dissolve the DNA. This mixture was applied to min-elute column to bind the DNA to the column and centrifuged for 1 min. at 13000 rpm to remove the undesired molecules from the DNA. Flow-through was discarded from the collection tube. 500 µl QG buffer was applied to column and centrifuged for 1 min at 13000 rpm to wash the DNA. Again flow-through was discarded from the collection tube and centrifuged for an additional 1 min. Finally, spin column placed in a 1.5 ml microfuge tube and 10 µl Elution Buffer was applied to elude the DNA. After 1 min. incubation, tube was centrifuged for 1min. Eluted DNA was stored at -20 °C for further use.

The agarose gel extracted and double digested products were ligated with T4 DNA ligase (New England Biolabs, Catalog # M2200S), the 21µL reaction was prepared (Table 2.6) and the reaction mixture was incubated overnight at 16°C. Treating the mixture at 72°C for 5 minutes inactivated the ligase. The ligation mix was then used for transformation into *Escherichia Coli* ER2507.

**Table 2.6 :** Ligation reaction (1:6 insert/vector ratio).

| Component                                   | Volume |
|---------------------------------------------|--------|
| pMALc-4X vector digest (6611 bp, 143 ng/µl) | 7,9 µl |
| Insert digest ( 85 ng/µl)                   | 2,1 µl |
| 2X Quick Ligation Buffer*                   | 10 µl  |
| Quick T4 DNA Ligase                         | 1,0 µl |
| Total volume                                | 21 µl  |

\*Quick Ligation™ Buffer is supplied with Quick Ligation™ Kit from New England Biolabs (Catalog # M2200S)

The ligation product was transferred into chemically competent *E.coli* ER2507 cells. The transformed cells were spread on LB agar plates with 100µg/mL of Ampicillin

and incubated for overnight at 37°C. The remaining transformation product was poured in 50 mL of LB containing 100µg/mL of Ampicillin and incubated for overnight at 37°C on shaker at 220 rpm. The presence of the DsRed-AuBP2 encoding gene was controlled by restriction enzymes after plasmid isolation. The reaction was set up as mentioned before. Plasmid isolation was performed by Roche High pure plasmid isolation kit (Roche, Catalog # 11 754 777 001) according the manufacturer instructions. The presence of DsRed-AuBP2 in the pMal-c4x vector was confirmed with DNA sequence analysis by using Big Dye<sup>®</sup> Terminator V 3.1 cycle sequencing kit from Applied Biosystems on ABI PRISM 3100-Avant Genetic Analyzer (PE, Applied Biosystem, CA). The reaction conditions were given in Table 2.7.

**Table 2.7 :** Preparation of cycle sequencing reaction.

| Component                  | Volume |
|----------------------------|--------|
| Template                   | 2,5µl  |
| M13 -40 primer (3.2 pmole) | 1µl    |
| 5X Sequencing Buffer       | 1 µl   |
| Big Dye Terminator         | 2 µl   |
| Distilled water            | 3,5 µl |
| Total Volume               | 10 µl  |

#### **2.1.2.2 Expression and purification of DsRed and DsRed-AuBP2**

The glycerol stocks of each recombinant *E. coli* ER 2507 strains harboring pMal-DsRed and pMAL-DsRed-AuBP2 expression constructs were used to express fusion proteins. Firstly, the bacteria were streaked on a plate containing LB agar (Luria-Bertani, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 15 g/L agar, pH 7.0) with 100 µg/ml of ampicillin and incubated at 37°C for 16 hours. Then, a single colony was picked by a sterile loop and grown in 3 ml of LB broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl, pH 7.0) containing 100 µg/ml of ampicillin on the incubator shaker at 37°C for 16 hours. 1 ml of overnight cell culture was added to 50 ml of LB broth with 100 µg/ml of ampicillin and grown at 37°C until an optical density of 0.6 to 1.5 at 600 nm. The starter culture flask was saved in the 4°C

overnight. For protein expression, this cell culture was equally divided among 400 ml volumes of sterile LB broth with 100 µg/ml of ampicillin in 1 L flasks. Cells were grown at 37°C until an optical density of 0.5 to 0.65 at 600 nm, then the protein induction was initiated by IPTG addition to a final concentration of 0.4 mM. After addition of IPTG, the culture growth was continued at 30°C for 48 hours.

After expression of protein, cells were harvested by centrifugation at 4,000xg for 30 minutes at 4 °C. The centrifuged cell pellet was re-suspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and disrupted by sonication at 200 W for 1.5 min (puls on 10 s / puls off 10 s) using Branson Digital Sonifier with a double stepped microtip. Cell debris was removed by centrifugation (15,000xg for 30 min at 4 °C). 10 ml supernatant was loaded onto amylose resin column (2.0 x 15 cm) equilibrated with the column buffer. Following the removal of the unbound proteins, MBP-tagged fusion protein was eluted from the column with an elution buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM Maltose, pH 7.4). The pure fractions were analyzed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) on a 4 % stacking gel and 12 % separating gel. Separating and stacking gel parts were prepared by mixing the components stated in Tables 2.8 and 2.9.

**Table 2.8 :** Components of 12% of separating gel.

| Component                 | Volume |
|---------------------------|--------|
| dd H <sub>2</sub> O       | 1,6 ml |
| 30% acrylamide mix        | 2.0 ml |
| 1.5 M Tris Buffer, pH 8.3 | 1.3 ml |
| 10% SDS                   | 50 µl  |
| 10% APS (fresh)           | 40 µl  |
| <b>TEMED</b>              | 10 µl  |

10 µl of each protein samples were mixed with 4 µl of 2X protein loading dye and denaturated at 95 °C for 5 minutes. The molecular weight marker (Appendix A1) was run at 80 volt for stacking gel and 200 volt for separating gel.

**Table 2.9 : Components of 4 % Stacking Gel**

| Component               | Volume  |
|-------------------------|---------|
| dd H <sub>2</sub> O     | 1.36 ml |
| 30% acrylamide mix      | 0.34 ml |
| 1 M Tris Buffer, pH 6.8 | 0.26 ml |
| 10% SDS                 | 20 µl   |
| 10% APS (fresh)         | 20 µl   |
| <b>TEMED</b>            | 2 µl    |

### 2.1.2.3 Affinity tag (MBP-tag) removal from DsRed proteins

Pure protein fractions were concentrated to 1 mg/ml of volume by 10000 MWCO of Amicon Ultra-15 protein centrifugal filter (Merck Millipore, Catalog # UFC901008). Then, protein samples were transferred to 1X cleavage buffer containing 20 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl<sub>2</sub> (pH 8.0) by ultrafiltration using the same centrifugal filter tubes. 40 µl of 1 mg/ml Factor Xa (New England Biolabs, P8010S) was added to 2.5 mg/ml of fusion protein in 1X cleavage buffer. The reaction was performed at 16 °C for overnight.

The second affinity chromatography technique was used for further purification of the DsRed and DsRed-AuBP2 proteins. This method depends on copper chelating affinity chromatography. The red fluorescent protein, DsRed, has a binding affinity for copper ions. On the basis of this interaction, purification method for DsRed was developed using copper immobilized column to remove MBP-tag and Factor Xa enzyme from DsRed proteins. A metal chelating ligand attached to resin was used in this study to immobilize copper ions on chelating resin. As the crude protein was passed through the copper immobilized column, DsRed bound to the copper immobilized column whereas other interfering proteins did not bind to the column and hence were removed. DsRed was eluted using a competitive ligand imidazole that binds to copper ions. This is a simpler approach of protein purification based on their inherent metal ions affinity.

HiTrap Chelating HP Column (GE Healthcare, Catalog # 17-0409-01) packed with 5 mL of Sepharose High Performance with an iminodiacetic acid as chelating group

was used for purification of DsRed proteins to remove MBP-tag and Factor Xa enzyme. The column was prepared by washing with 15 mL of distilled water to wash away the 20% ethanol in which the packing material was initially stored. After the wash step, 3 mL of 0.1 M CuSO<sub>4</sub> was loaded onto the column followed by an additional 15 mL wash of distilled water. The column was then washed with 15 mL of acidic buffer (0.02 M sodium acetate, 1.0 M NaCl, pH 4.0) in order to elute loosely bound copper ions. Once all of the unbound copper ions were completely removed, the column was equilibrated with 15 mL of binding buffer (50 mM sodium phosphate buffer, 300 mM NaCl, pH 8.0). 5 ml of the digested protein mixture containing MBP, Factor Xa and DsRed proteins in binding buffer was loaded onto the copper immobilized HiTrap column and incubated for 30 min at 37 °C in the hybridization incubator. The column was then washed with 20 mL of binding buffer followed by 100 mL of wash buffer 1 (50mM sodium phosphate buffer, 1 M NH<sub>4</sub>Cl and 300 mM NaCl, pH 8.0). A final wash of the column was performed using 100 mL of wash buffer 2 (50 mM sodium phosphate buffer, 2 mM imidazole and 300 mM NaCl, pH 8.0). Purified DsRed proteins were then eluted with 30 mL of elution buffer (50 mM sodium phosphate, 300 mM NaCl and 4 mM imidazole, pH 8.0). For the entire purification procedure a flow rate of 1 mL/min was used. Eluted fractions, which displayed DsRed fluorescence emission, were collected and the purity of proteins was analyzed by SDS-PAGE.

### **2.1.3 Bioengineered FDH-AuBP2 fusion enzyme**

In this design, the biocombinatorially selected AuBP2 peptide was genetically fused to the N'-terminus of the cmFDH with a structurally flexible (GGGS) linker sequence to ensure that the final fusion protein's distinct functionalities were not restricted by one another. To avoid any potential non-specific interactions that may arrive due to histidine affinity tag used in the system, both enzymes were introduced new cleavage sites to compare the effectiveness of the peptide tag introduced to the enzyme system. For the production of the fusion enzyme, bacterial expression systems and metal-chelate affinity-based Ni-NTA purification matrices were utilized. The enzyme activity assays revealed that the engineered cmFDH-AuBP2 fusion protein construct delivers its enzymatic function without any significant reduction to the overall catalytic efficiency values in solution.

### 2.1.3.1 Expression and purification of FDH and FDH-AuBP2

The pQE2-cmFDH or pQE2-cmFDH-AuBP2 vectors in *Escherichia coli* DH5 $\alpha$  cells were used for protein expression. Cells were first grown overnight at 37°C in 5 ml of Luria Bertani (LB) media containing 100  $\mu$ g/ml ampicillin. Next, 1 ml of overnight culture was inoculated into fresh LB media and incubated at 37°C for 4 hours until to reach an optical density (OD<sub>600</sub>) 0.65. For protein expression the cells were induced with isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) as 0.5 mM and the culture was incubated on the incubator shaker for 16 hours at 25°C. The cells were harvested by centrifugation at 5000xg for 20 min using a pre-weighed centrifuge tube. The supernatant was decanted and pellet was allowed to drain for removing as much liquid as possible. Then, the wet weight was determined to be 1.53 g. The cell pellet was frozen at -20°C for in order to disrupt the cell for protein purification.

After protein expression, enzymatic and physical cell disruption methods were applied to the frozen cell pellet. Firstly, the cell pellet was completely re-suspended by pipetting or gentle vortexing at room temperature in sodium phosphate buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0) containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF). The cells were lysed by 1 mg/ml lysozyme treatment for 30 minutes on ice and sonicated at 200 W 6 times for 10 seconds. Insoluble cell debris was removed by centrifugation at 10000xg for 30 min at 4°C. Finally, the supernatant, which is called crude extract was transferred into a fresh tube and the soluble crude extract was ready to load directly onto the Ni-NTA column for one step purification.

The affinity purification method, i.e. nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography, was used to purify the cmFDH and cmFDH-AuBP2 enzymes. Pre-packed Ni-NTA column of 5 mL was first equilibrated with sodium phosphate buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole at pH 8.0) and then 5 mL of crude extract containing fusion protein was loaded into the column. Histidine-tagged fusion proteins were specifically bound to the column through interaction of nickel and imidazole ring of histidine. Unbound and non-specifically bound proteins were removed from the column by washing with ten column volumes of sodium phosphate buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 50 mM imidazole at pH 8.0) containing 1% Triton X-100 solution. The fusion proteins were eluted by competitive elution method using the 250 mM imidazole in the sodium phosphate buffer. Finally, the

purification process was confirmed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

#### **2.1.3.2 Affinity tag (His-tag) removal through engineered cleavage site**

For the removal of the histidine-tag, enzymes purified in sodium phosphate buffer were first exchanged into 1X PreScission Protease cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0) using 10000 MWCO ultra-filtration tube. PreScission Protease Cleavage reaction was performed at +4°C for 30 minutes. Proteolytic digestion requires 2 units enzyme/100 µg of HisTag-fusion protein. After cleavage reaction, His-tag and protease were removed from *cmFDH* or *cmFDH*-AuBP2 enzymes in 20 mM Tris-HCl buffer at pH 8.0 through Ni-NTA and Glutathione-Sepharose resins, respectively. Firstly, protein mixture was incubated with Ni-NTA resin for 45 minutes on the shaker at 200 rpm to bind His-tag to the resin. Then, this mixture was spun at 1000xg for 5 minutes to remove tag from protein solution. After that, the supernatant was incubated with Glutathione-Sepharose resin for 45 minutes on shaker at 200 rpm to bind the enzyme, which is GST-tagged, to the resin. Pure proteins, tag-free *cmFDH* and *cmFDH*-AuBP2 were obtained following a 5 minutes centrifugation at 1000xg. In order to remove His-tag, cleavage reaction was. Additionally, Glutathione-Sepharose resin was used to remove restriction enzyme from tag free FDH and FDH-AuBP2. The purity of proteins was analyzed by SDS-PAGE.

#### **2.1.3.3 Enzyme activity measurements**

The enzymatic activity was measured in a total reaction mixture of 200 µl containing 20 mM Tris-HCl buffer at pH 8.0, 1 mM NAD<sup>+</sup>, 0–40 mM formate, and 0.4 µM enzyme at both 25°C and 37°C. Corresponding to the reduction of NAD<sup>+</sup>, reaction was monitored at 340 nm. The increase in absorbance at 340 nm was monitored at 10 seconds interval for 3 minute. In this assay, the enzyme concentration was determined through Bradford protein determination assay, and then catalytic reaction was calculated as a rate change based on the data collected from spectrophotometer (absorbance and time).

Reaction rate was calculated by dividing how much absorbance changed between the first and last measurement with how much time elapsed between the first and last measurement, as in the following equation:

$$\text{Reaction Rate } (\Delta A/\text{min}) = \frac{\Delta_{340\text{nm}}}{\Delta t} \quad (2.1)$$

where  $\Delta A/\text{min}$  is the average change in absorbance per minute, and  $A_{3\text{min}}$  and  $A_{1\text{min}}$  are the absorbance readings at 3 min and 1 min respectively.

Now that a measurement of change in absorbance was obtained per minute, this value was used to calculate enzyme activity. Technically, the rate of absorbance change was converted into rate of product formation by determining the concentration of product needed to generate a particular absorbance (e.g., Lambert Beer's Law). In this experiment, the amount of light absorbed at 340 nm by formation of NADH was very well monitored based on formate concentrations. As a result, a series of standard solutions of formate were prepared to plot a standard curve for translation of absorbance into product concentration. Finally, enzyme activity was directly calculated based on the following equation:

$$\text{Activity (U/L)} = \frac{(\Delta A/\text{min} * \text{total reaction volume} * 10^6)}{\text{Extinction Coefficient} * \text{path length} * \text{enzyme volume}} \quad (2.2)$$

where extinction coefficient of NADH ( $\epsilon_{340}$ ) is  $6220 \text{ M}^{-1}\text{cm}^{-1}$ , the total reaction volume is 200  $\mu\text{l}$ , which is subsequently multiplied by  $10^6$  to convert to  $\mu\text{l}$  to liter measurements. The pathlength (how far light travels through the reaction solution) is 0.48 cm, and the enzyme volume is 5  $\mu\text{l}$ . The activity in different concentration of substrate was calculated using the above equation. The kinetic parameters  $V_{\text{max}}$  and  $K_{\text{m}}$  for the substrate, sodium formate, were determined for both enzymes. Activity assay was repeated three times and all formate concentration assays were performed in triplicates. The mean rates were fitted to the Michaelis-Menten model.

## **2.2 Molecular Structure Analysis of AuBP-based Fusion Proteins**

### **2.2.1 Circular dichroism spectroscopy**

A solution containing 0.4 mg/ml protein and 20 mM Tris-HCl at pH 7.4 was prepared for circular dichroic analysis. Circular dichroism (CD) spectra from 190-260 nm were recorded on Jasco J-815 Circular Dichroism Spectrometer in a 1 mm path cuvette at room temperature with a 0.5 nm/s scan rate. Each spectrum represents an average of 20 scans. The averaged spectrum, which was then subtracted with the appropriate buffer background, was smoothened using the Savitzky-Golay algorithm. A section of the smoothened spectrum (from 190 – 240 nm) was compared to the five component reference spectra [(1) a helix, (2) b sheet, (3) b turn, Type-I, (4) b turn, Type- II, and (5) random coil] compiled by using a constrained least-squares fit. The secondary structure estimates are reported as the fractional weight  $\pm$  the standard deviation. All spectral smoothing and secondary structure estimation were executed using commercial graphing software (IGOR Pro. 6.0). The CD machine was carefully calibrated using (1S)-(+)-10-camphorsulfonic acid (Aldrich, 99%). Ellipticity is reported as mean residue ellipticity,  $\theta_M$  (deg cm<sup>2</sup> dmol<sup>-1</sup>).

### **2.2.2 Molecular structure analysis through ATR-FTIR**

Fourier Transform Infrared (FTIR) Spectroscopy was used to determine the secondary structure of proteins and to show the presence of protein in nanomats. Infrared spectrum is molecular vibrational spectrum. The range of infrared region is 12800 ~ 10 cm<sup>-1</sup> and can be divided into near-infrared region (12800 ~ 4000 cm<sup>-1</sup>), mid-infrared region (4000 ~ 200 cm<sup>-1</sup>) and far-infrared region (50 ~ 1000 cm<sup>-1</sup>). When exposed to infrared radiation, molecules selectively absorb radiation of specific wavelengths, which causes the change of dipole moment of molecules. As a consequence, the vibrational energy levels of molecules transfer from ground state to excited state. The frequency of the absorption peak is determined by the vibrational energy gap. The number of absorption peaks is with relation to the number of vibrational freedom of the molecule. The intensity of absorption peaks is related to the change of dipole moment and the possibility of the transition of energy levels. Therefore, by analyzing the infrared spectrum, the structure information of a molecule can be obtained readily.

The infrared spectra of samples were recorded on Perkin Elmer Spectrum-400 ATR-FTIR spectrometer at room temperature between 4000-650  $\text{cm}^{-1}$  with a resolution of 4  $\text{cm}^{-1}$ . To obtain reasonable signal to noise ratio the average of 20 scans was taken. Samples were analyzed directly on a single bounce diamond (ATR).

## **2.3 Experimental Characterization of Protein-surface Interactions**

### **2.3.1 Surface plasmon resonance spectroscopy**

The SPR measurements were carried out using a four-channel instrument (Kretschmann configuration) developed by the Radio Engineering Institute, Czech Republic. The setup consists of a polychromatic light source (Ocean Optics, USA) coupled with an optical fiber and an Ocean Optics SD 2000 detector. The data were collected using WinSpectral 1.03; this software was supplied along with the instrument. This program normalizes the acquired SPR spectrum using the dip in the wavelength shift at regular intervals that then is fit with a 4th-order polynomial for generating the metric sensogram as a function of time. During the experiments, a baseline was established by pumping the reaction buffer first, and then the 6-port valve (Upchurch Scientific, USA) was switched to protein/peptide solution until a full saturation was achieved. The buffer was then pumped once again (at a rate of 80  $\mu\text{L}/\text{min}$ ) to monitor the desorption behavior.

### **2.3.2 Quartz crystal microbalance with dissipation monitoring (QCM-D)**

For the experimental procedure involving the use of the QCM-D, a Q-Sense E4 QCM-D using a four-channel system was used. The Q-Sense E4 is a real-time analytical instrument for studies of molecular events occurring on surfaces. The E4 measures mass and viscoelastic properties of molecular layers as they build up or change on the sensor surface, which plays a key role in areas such as materials, protein and surfactant research.

The instrument has four flow modules, each holding one sensor enabling four parallel measurements. The flow modules are exchangeable and can be separately cleaned. All wetted parts (titanium, Teflon and Viton) can easily be removed and cleaned using standard cleaning procedures such as ultra sonication. Using the same chamber base, accessory modules are available for different application needs. At present there is an electrochemistry module, a window module, a humidity module

and an open module. 7 frequencies can be measured simultaneously, from the fundamental frequency up to the 13th overtone, enabling improved modeling of a wide range of surfaces. In a routine measurement situation, the kinetic resolution is about one data point per second from all 4 sensors. However, each of these data points is an average of several measurements of  $f$  and  $D$ , thus enhancing the signal to noise ratio. If maximum kinetic resolution is desired, more than 200 data points per second can be measured. QTools enables quantification of viscosity, elasticity, and thickness of any soft film measured with the QCM-D at minimum two frequencies. In addition kinetic constants and reaction kinetics can be extracted. The software simplifies interpretation of data, modeling and different kinds of presentations of performed measurements.

All gold-coated quartz crystal electrodes were purchased commercially (Q-Sense). The diameter of the electrodes was 14 mm with a 0.3 mm thick gold coating. The electrodes used had a resonance frequency of 4.95 MHz  $\pm$  50 kHz with an optically polished surface roughness of less than 3 nm (RMS). After clean electrodes were mounted into the individual modules, 100 ml of 2% Hellmanex solution, followed by 200 ml of DDI water was pumped through to clean the system. To establish a stable baseline, phosphate buffer solution (PBS) pH 7.4 was introduced to the electrodes before adding the protein solutions. The frequency change of the crystal in pure buffer solution was recorded for 30-60 min. After a baseline was established, the desired amount of proteins was allowed to flow at 0.1 ml/min and 22°C through the system and the resulting frequency and dissipation shift was recording simultaneously. The system was then washed with PBS pH 7.4 to monitor the subsequent frequency shift related to the protein washing off the surface. Immediately following the experiment, the electrodes were taken out of the module and sonicated with acetone/ methanol solution (1:1) for 5 min. The electrodes were then washed using ethanol and DDI water, then dried with ultra-pure nitrogen gas and stored in a desiccator for humidity control.

All four QCM-D modules were set up in parallel with each. After a baseline with the buffer was established, each concentration of protein was allowed to flow through the system onto an electrode with no proteins previously adsorbed until saturation was reached. 20 mL of each concentration for proteins was allowed to flow through the system, which translated into the protein running over the surface for a little over

3 hrs at a flow rate of 0.1 mL/min. This step is extremely important for a proper analysis of the protein adsorption using the computational model developed that will be discussed in a subsequent section. Although a large amount of protein is used, this procedure insures that complete saturation of the protein onto the gold surface has taken place compared to other techniques where the pump is stopped after a small amount of protein is added and saturation is assumed.

### **2.3.3 Atomic force microscopy (AFM)**

The samples were scanned using an AFM using a Digital Instruments Multimode scanning probe microscope with a Nanoscope V controller, USA under acoustic and mechanical isolation. High frequency ( $\sim 300$  kHz) silicon nitride cantilevers were purchased from Molecular Imaging, USA and used at a scanning velocity of  $\sim 3$   $\mu\text{m/s}$  in order to reduce feedback artifacts. Topographic feature sizes and cross-sectional analysis were performed using the Nanoscope software (ver 5.3r1) provided with the AFM by Digital Instruments Co., USA.

### **2.3.4 Fluorescence microscopy (FM)**

The dried substrates taped on glass slides were mounted on a Nikon Eclipse TE-2000U Optical Microscope (Nikon, Japan) coupled with a Hamamatsu ORCA-ER cooled charge-coupled device (CCD) camera. FITC (exciter 460–500, dichroic 505, emitter 510–560, Nikon). Dark-field mode was maintained by 50X and 100X DF lenses and DF filter set from Nikon, Japan. The mercury illumination (X-cite 120, EXFO, USA) was utilized as the light source. The images are recorded through Metamorph Software (Universal Imaging, USA).

### **2.3.5 Cytation 3 imaging multi-mode reader**

Cytation 3 is a cell imaging multi-mode microplate reader. This system has automated digital microscopy and conventional microplate detection. Moreover, Cytation 3 contains both high sensitivity filter-based detection and a flexible quadruple monochromatic based system for unmatched versatility and performance. Due to the upgradable fluorescence cellular imaging module of this system, rich cellular visualization analysis without the complexity can be performed successfully. Fluorescence microscopy is a powerful technique for visualizing cellular responses to understand cell proliferation, protein expression, cytotoxicity and other

expressions. It has the ability to perform both conventional quantitative fluorescence measurements and cell imaging with temperature, gas such as CO<sub>2</sub>/O<sub>2</sub>, orbital shaking control systems. Cytation 3 is supported with BioTek's Gen5™ software, specifically designed to make plate reading and image capture easy.

## **2.4 Immobilization of Gold Nanoparticles and Proteins Through Peptide**

### **2.4.1 Micro contact printing of proteins on gold surface**

Gold-coated film (25 nm thick) on silica wafer was evaporated onto precoated chromium layer (~3 nm thick) by electron beam evaporation. The gold surface (1cm X 1cm) was subsequently cleaned by ultra sonication in cleaning solution (methanol/acetone (1:1), isopropyl alcohol, and DI water) for 5 minutes followed by drying with nitrogen gas. Polydimethylsiloxane (PDMS) stamps were cleaned by ultra sonication in ethanol and DI water for 20 minutes followed by nitrogen gas drying. 100 µl of 20 µM MBP-AuBP1 constructs and wt-MBP solutions were incubated on the patterned side of a clean PDMS stamp for 10 minutes. The excess protein solution was gently removed by pipette from the surface of the stamp and the stamp was dried with nitrogen gas following brief washing with DI water. Gold-coated glass substrates were thoroughly rinsed by isopropanol and ethanol and then, dried with inert gas. One of the clean gold surface was placed on the protein-loaded stamp, pressed using a gently force for 10 sec and left on the stamp surface for up to 10 minutes. The substrate was separated from the stamp and the side contacted with the stamp was washed with DI water for 1 minute and dried under a flow of nitrogen gas. The unreacted gold surface was then blocked with 1% BSA in PBS solution for 1 hour followed by a 1 min wash and drying with nitrogen gas. The immobilized MBP fusion proteins were visualized after 30 minutes incubation with fluorescently prelabeled anti-MBP (New England Biolabs, Catalog # E8032S) using Zenon-Alexa-546 or Zenon-Alexa-488 labeling kit (Life Technologies, Catalog # Z-25004 or Z-25102) as follows: 2 µL of anti-MBP solution were mixed with 8 µL PBS and 5 µL of Zenon-Alexa IgG labeling dye and incubated for 30 min on ice at the shaker with slow speed. The mixture was again diluted up to 500 µl with the reaction buffer (PBS) and applied to the gold surface pre-stamped with protein for 1 hour at room temperature. In the final step substrate was gently washed for 1 min with DI water,

dried, and imaged using a fluorescence microscope (Nikon, Yokohama, Japan) and Metamorph Software (Universal Imaging LLC, Burbank, CA).

#### **2.4.2 Layer-by layer protein self-organization**

To create the gold nanoparticle patterned surfaces, the GEPI bi-functional peptide QBP1-AuBP1 (200  $\mu\text{g/ml}$ ) was micro-contact printed on glass cover slide (Thermo Fisher Scientific, Catalog # 22X701) in the same manner as described above (Section 2.5.1) using 15 minutes incubation time. After the stamping and washing steps, the peptide-arrayed substrate was incubated with 15 nm Au NP for 30 min, washed for 1 min, and dried under nitrogen gas. MBP-AuBP1 or wt-MBP proteins were incubated on gold patterned surface for 1h, followed by 1 min washing and nitrogen drying steps. The un-reacted surface was blocked with 1% (w/v) BSA in PBS and the immobilized proteins were immune-stained using the methods described above (Section 2.5.1).

#### **2.4.3 Self-assembly of DsRed-AuBP2 on gold Surface**

100  $\mu\text{l}$  of 50  $\mu\text{M}$  of fusion protein in reaction buffer was applied onto the patterned side of the PDMS stamp and incubated for 1 hour at room temperature. The protein solution was gently removed by pipette from the surface of the stamp and then dried with nitrogen following brief washing with DI water. Gold-coated glass substrates were thoroughly rinsed by isopropanol and ethanol and then, dried with inert gas. The clean substrate was then brought to contact with the surface of the stamp and pressed using a gently force for 10 sec and left on the stamp surface for up to 10 min. The substrate was separated from the stamp and the side contacted with the stamp was washed with DI water for 2 min and dried under a flow of nitrogen. The patterned gold substrate was characterized by using fluorescence microscopy (Nikon, Yokohama, Japan) and Metamorph Software (Universal Imaging LLC, Burbank, CA).

### **2.5 Gold Nanoparticle-based Detection Methods**

#### **2.5.1 Localized surface plasmon resonance (LSPR) for MBP-AuBP1**

The effect of proteins on the plasmon excitation wavelength for Au NP was analyzed by recording the light absorbance of Au NPs and protein-functionalized Au NPs by

spectrophotometer. Citrate-capped  $\sim 200 \mu\text{M}$  of 15 nm gold nanoparticles (Au NP) (Ted Pella Inc., Catalog # 15704-20) were incubated with serial dilutions of MBP-AuBP1 fusion or wild type MBP (wt-MBP) proteins (1.0 to 0.001 mM) for 2 h at room temperature. The replacement of the citrate ions by protein molecules was monitored by measurement of LSPR band shift recorded using Sapphire spectrophotometer (Tecan, San Jose, CA). The absorbance scan was performed between 400-700 nm wavelengths. Each spectrum was an average of three individual samples recorded twice and smoothened using the Savitzky-Golay algorithm.

### **2.5.2 Localized surface plasmon resonance (LSPR) for DsRed-AuBP2**

DsRed-AuBP2 proteins functionalized various sizes of Au NPs (5, 15, 50 nm) for design of biologically active probes. The plasmon resonance change was monitored by recording the light absorbance of Au NPs with and without DsRed and DsRed-AuBP proteins on the microplate reader (Cytation 3 Imaging Multi-Mode Reader, BioTek Instruments, Inc., Vermont, USA). Firstly, different size of Au NPs (5, 15, 50 nm) were incubated with serial dilutions of DsRed proteins (4-0.0001 mM) in 100  $\mu\text{l}$  DI water for 2 hours at room temperature on the shaker with low speed. 100  $\mu\text{M}$ , 50  $\mu\text{M}$  and 25  $\mu\text{M}$  Au NPs were used according to their size, 5 nm, 15 nm and 50 nm, respectively. Then, the absorbance of functionalized-Au NPs was scanned between 400-800 nm wavelengths on the microplate reader. Following the first absorbance scanning this hybrid mixture was centrifuged at 13000 rpm for 10 minutes to remove the unbound proteins. The supernatant was kept for further experiment. The pellet was washed with DI water of 200  $\mu\text{l}$  and then centrifuged at 13000 rpm for 10 minutes. This washing step was repeated three times to generate fusion protein based gold probe. Finally, the last pellet was resuspended with DI water and absorbance scans were recorded. Each spectrum was an average of three individual samples recorded twice and smoothened using the Savitzky-Golay algorithm.

The magnitude of the protein induced plasmon shift on Au NP is given by below equation (Equation 2.3). This equation allows an estimation of the strength for the binding arising from proximity of nanoparticles in the Protein-NP conjugates. Particularly, the plasmon shift of gold nanoparticle in the presence of DsRed proteins provides a measure of the apparent interparticle distance [232];

$$\Delta\lambda/\lambda_0 = 0.18 \exp[-(s/D)/0.23] \quad (2.3)$$

where  $\Delta\lambda/\lambda_0$  is the fractional plasmon shift,  $s$  is the distance between the surfaces of the particle,  $D$  is the particle diameter, and 0.23 is the decay constant for the universal trend of plot  $\Delta\lambda/\lambda_0$  versus  $s/D$  [232].

### 2.5.3 Stabilization of gold nanoparticles via inorganic binding proteins

The stability of the protein-functionalized Au NP was monitored in salt agglomeration assay by sequential addition of 5M NaCl solution to the Au NP to a final concentration of 1 M and 1.5 M. Au NP agglomeration was examined by color change and by the shift of absorption bands recorded using spectrophotometer. In agarose gel experiments, washed Au NP functionalized with serial protein concentrations, were loaded to a 0.5% (w/v) agarose gel. After 45 minutes migration in TAE buffer at 60 V the protein-stabilized nanoparticles were detected on the agarose gel as red bands and compared to non-functionalized particle samples.

To understand the protein orientation onto gold surface, washed Au NP was functionalized with either 0.05 mM fusion MBP-AuBP1 or with wt-MBP proteins. The resulting mixtures were immobilized on a nitrocellulose membrane and the immobilized MBP protein was detected by a monoclonal anti-MBP-HRP conjugate (New England Biolabs, Catalog # E8038S) at 1:1.000 dilution and reacted with the TMS substrate (Pierce, Catalog # 34021) to produce a colored product.

### 2.5.4 Fluorescence quenching studies with DsRed proteins

In quenching experiments with DsRed proteins, the quenching agent, which is Au NPs, can interact with the protein in ways that alter the protein structure or that affect the degree of quenching observed. For examining quenching states of proteins, Au NPs with different sizes were used as a quencher in these studies. The protein concentration was fixed at 0.1  $\mu$ M. Nanoparticle concentration range was 0 to 110  $\mu$ M. Fluorescence scanning was performed for each hybrid mixtures containing Au NP and protein by using Cytation 3 fluorescence spectrophotometer (BioTek). Excitation was applied at 530 nm and emission scan was recorded between 550 and 700 nm wavelengths. Fluorescence emission was measured at 22 °C, 25°C and 28 °C buffer pH 8.0.

#### **2.5.4.1 Stern-Volmer Plots**

The fluorescence intensity ratio,  $[F_0/F]$ , was plotted against the nanoparticle concentration to calculate Stern-Volmer constant ( $K_{sv}$ ) for DsRed proteins. DsRed-Monomer protein was used as the control protein to compare the fluorescence readings with DsRed-AuBP2c. Different concentrations from 0  $\mu\text{M}$  to 100  $\mu\text{M}$  of nanoparticles was added to 100  $\mu\text{l}$  of 0.1  $\mu\text{M}$  protein in 20 mM MOPS, pH 8.0. A fluorescence intensity measurement was performed by measuring the emission at 590 nm when exciting the sample at 558 nm for each protein. Fluorescence measurements were corrected using the same buffer.

#### **2.5.5 Enzymatic kinetics of FDH and FDH-AuBP2 on gold surface**

In order to determine the immobilized enzyme activity, fusion proteins were immobilized onto 15 nm gold Au NP. 0.5 $\mu\text{M}$  enzyme interacted with 50  $\mu\text{M}$  Au NP (40  $\mu\text{l}$  from stock solution) in 100  $\mu\text{l}$  final volume. Wt-FDH was used as control samples to determine the AuBP2 effect on gold surfaces. The hybrid mixtures with Au NP and enzymes were incubated at 22°C for 15 minutes. And the enzyme immobilized Au NP solution was used for activity assay. For comparison, the same amount of free enzyme was also assayed at the same time. Activity assays for FDH-AuBP2 were performed in 50 mM Tris-HCl, pH 7.4, 2.5 mM sodium pyruvate and 0.2 mM  $\text{NAD}^+$  by measuring absorbance change for 3 minutes 340 nm in 96 well microplate.

### **2.6 Application of Fusion Proteins in Bio-nanotechnology**

#### **2.6.1 Anti-MBP detection using MBP-AuBP2 probe**

Following cleaning procedure for the glass slides (see Section 2.4.1), the PDMS stamping of bifunctional peptides was carried out on the cleaned substrates using the procedure described in Section 2.4.2. Gold nanoparticles were then immobilized onto the patterned glass and incubated for 30-40 minutes.

MBP-AuBP2 solution (2.5  $\mu\text{M}$ ) was prepared in 2.5 mM PBS buffer, pH 7.4 and incubated onto the gold immobilized glass surface for 2 hours. Following wash step with DI water and drying under nitrogen, binding of the target molecule was achieved by applying 30 nM Anti-MBP in the reaction buffer.

Absorption spectra of each substrate were recorded using Cytation 3 Multi Mode microplate reader in just after gold nanoparticle immobilization (as bare), protein (as probe) and antibody (as target) assembly. Spectral data were plotted and LSPR  $\lambda_{\text{max}}$  for each step was determined using software Graphpad Prism6.

### **2.6.2 Copper detection on DsRed-AuBP2 probe**

The purified protein was concentrated and its buffer was changed to 20 mM MOPS buffer, pH 7.4, to remove the imidazole. The imidazole-free protein solution was passed through a Chelex-100 column to remove any trace levels of  $\text{Cu}^{2+}$  (copper ions). Also, copper ions were removed from the imidazole free protein solutions by ultrafiltration using buffer containing EDTA. To understand the quenching mechanism of DsRed proteins the binding study was carried out against copper ions. Different concentrations of a copper solution were added to 100  $\mu\text{L}$  of 1  $\mu\text{M}$  DsRed-Monomer and DsRed-AuBP2c protein solutions in an individual microtiter well. The fluorescence readings were measured using microplate reader (Cytation 3 Imaging Multi-Mode Reader, BioTek Instruments, Inc., Vermont, USA) by excitation of the samples at 558 nm and the resulting emission at 590 nm was recorded. The fluorescence intensity ratio,  $[F_0/F]$ , was plotted against the copper concentration to calculate the copper dissociation constant for DsRed proteins. DsRed-Monomer protein was used as the control protein to compare the fluorescence readings with DsRed-AuBP2c.

#### **2.6.2.1 Stern-Volmer plots**

Different concentrations from 1  $\mu\text{M}$  to 100  $\mu\text{M}$  of copper solution was added to 100  $\mu\text{L}$  of 1  $\mu\text{M}$  protein in 20 mM MOPS buffer, pH 7.4 in an individual microtiter well. Exciting the sample and measuring the emission, at the appropriate wavelength for each protein, took a fluorescence reading.

### **2.6.3 Nanofiber formation using DsRed-AuBP2**

The polymer solution was prepared in an air-tight bottle using DI water as solvent to dissolve the PEO powder under magnetic stirring for ~24 hours at ambient temperature (~20°C). The 10 wt % of PEO solution was used to integrate the proteins. Phosphate buffer saline (PBS) solution with a pH~7.4 was prepared at ambient temperature and added to the purified MBP-DsRed-AuBP2 engineered

protein (molecular weight  $\sim 73$  kDa) using a micropipette. Adding 410  $\mu\text{l}$  of PBS solution achieved a working stock solution of 50  $\mu\text{M}$ . The concentrated gold nanoparticle solution was added to the gently shaken protein solution. 40 g of PEO solution was added to 0.4 ml of the gold nanoparticle-protein mixture.

Infused gyration method was used to prepare protein-integrated nanofibers. The experimental set up consists of a rotary aluminum cylindrical vessel containing 20 small round orifices on the face. The dimensions of vessel and orifices are 60 mm in diameter with a height of 25 mm, and 0.5 mm in diameter with the same height, respectively. One end of the vessel is joined to a syringe pump through a rotary joint, which can control flow of the protein-polymer mixture or control polymer solution into the vessel. The bottom end of the vessel is connected to a DC motor, which can produce variable speeds up to 36000 rpm. Because of the centrifugal force and the hydrostatic force, which overcomes of the surface tension force at the orifice, a jet is formed. The flow in the vessel is kept constant to minimize the hydrostatic force effect during gyration. A gradual increase of the rotating speed will elongate the formed jet when it comes out via the orifices and subsequently solvent evaporates occur to form solid nanofibers. This unit is placed in a transparent plastic container to collect the polymer fibers conveniently. The polymer nanofibres were collected using a stationary aluminum foil within the container. In order to investigate the nanofibre size and size distribution under different conditions, the polymer solutions were spun at different flow rates (5000  $\mu\text{l}/\text{min}$ , 4000  $\mu\text{l}/\text{min}$ , 3000  $\mu\text{l}/\text{min}$ , 2000  $\mu\text{l}/\text{min}$ , 1000  $\mu\text{l}/\text{min}$  and 500  $\mu\text{l}/\text{min}$ ) under a constant rotating speed (36000 rpm) at the ambient temperature ( $\sim 20^\circ\text{C}$ ) and relative humidity ( $\sim 42\%$ ).

#### **2.6.4 Biobattery Design using FDH-AuBP2**

To create an enzyme-activated electrode system, gold-coated silicon wafer (Platypus Technologies, Madison, WI) was used. The gold-coated substrates were cut in 1cm x 1cm dimensions to be utilized as gold electrodes. Molecules of cmFDH-AuBP2 enzyme were next adsorbed onto one of the electrodes to activate the gold surface. For adsorption process a 200  $\mu\text{l}$  of 10 $\mu\text{M}$  cmFDH-AuBP2 enzyme in 20 mM Tris-HCl buffer, pH 8.0, was applied onto a gold surface and incubated for 15 minutes in a humidity chamber at room temperature. The enzyme-immobilized electrode was subsequently washed with Milli-Q water to get rid of excess proteins from surface.

The enzyme-activated electrode and bared gold electrode were placed into a beaker containing buffer solution (20 mM Tris HCl and 0.1 M KCl) vertically. The submerged gold electrodes were next connected in parallel to the resistor using alligator. A 22 K $\Omega$  resistor was attached to the lead of the electrodes. Output current was recorded using a HP 974A Multimeter.

In this circuit setup, activated enzyme electrode on gold could act a cathode terminal, the other electrode was bare gold used as anode terminal. The negatively charged electrode, which is cathode, attracts cations. Also, the cathode is the source of electrons. And the cathode can generate electrons that are the electrical species doing actual movement in the circuit. The bare gold electrode would be the anode terminal, which has a positive charge. There is a movement of charged molecules that is current.

After obtaining stable current, 1mM NAD<sup>+</sup> and 0.25 mM formate were added into the solution. Within 15 minutes the current increased and reached a stable output. Each addition of NAD<sup>+</sup> and formate gave rise to change in the current because of the enzymatic reaction. In this reaction, FDH-AuBP2 binds NAD<sup>+</sup> prior to formate on the enzyme. Consequently, a direct transfer of a hydride ion occurs from the formate to the nicotinamide ring of NAD<sup>+</sup>, forming carbon dioxide and NADH. When formate is oxidized, two electrons are transferred from the enzyme to the electrode. These electrons flow through the circuit.



### **3. RESULTS and DISCUSSIONS**

#### **3.1 Bioengineered MBP-AuBP1 Fusion Proteins**

To describe and demonstrate the feasibility of the hypothesis mentioned in the Section 2.1, different fusion protein derivatives with specific AuBP1 tag and MBP were engineered genetically by using two structurally different spacing motifs between peptide and protein. It has been shown that the design of linkers separating two functional domains is crucially important for efficient production and fabrication of hetero-functional protein units [42, 198-200] MBP fusion partner was rationally selected due to the lack of cysteine amino acids preventing the sulfide-induced non-specific protein interactions with gold surface. It is very useful to tolerate C-terminal genetic fusion as well as to enable high protein yield when expressed in bacterial cells. Especially, the MBP-AuBP1 fusion constructs containing either a rigid (PGPGPG)- or flexible (SGGG)-spacer separating the AuBP1 peptide from the MBP-tag were designed. Either of the purified heterofunctional MBP-AuBP1 fusion proteins was then used to investigate the effect of the engineered AuBP1 peptide tag on protein adsorption onto various forms of gold surfaces using biochemical, spectroscopic and molecular imaging techniques, as described in following sections.

##### **3.1.1 Expression and purification of MBP and MBP-AuBP fusion protein**

For the expression of MBP-(PG)<sub>3</sub>-AuBP1 and MBP-S(G)<sub>3</sub>-AuBP1 proteins, *E. coli* ER 2507 cells were used as the bacterial host. This strain was chosen because the *malE* gene, which encodes maltose-binding protein, is deleted as part of the *malB* deletion. The *malE* gene is included in the *malB* deletion, so this strain does not make any MBP from the chromosome. Therefore, it is useful to purify the only fusion proteins. To express and purify wt-MBP protein, *E. Coli* TB1 strain was used as the bacterial host. TB1 strain is a derivative of JM83 that has been made *hsdR* so it will not restrict unmodified DNA. It has *lacZ*ΔM15 allele so it is proper for α-complementation (blue-to-white screen on Xgal).

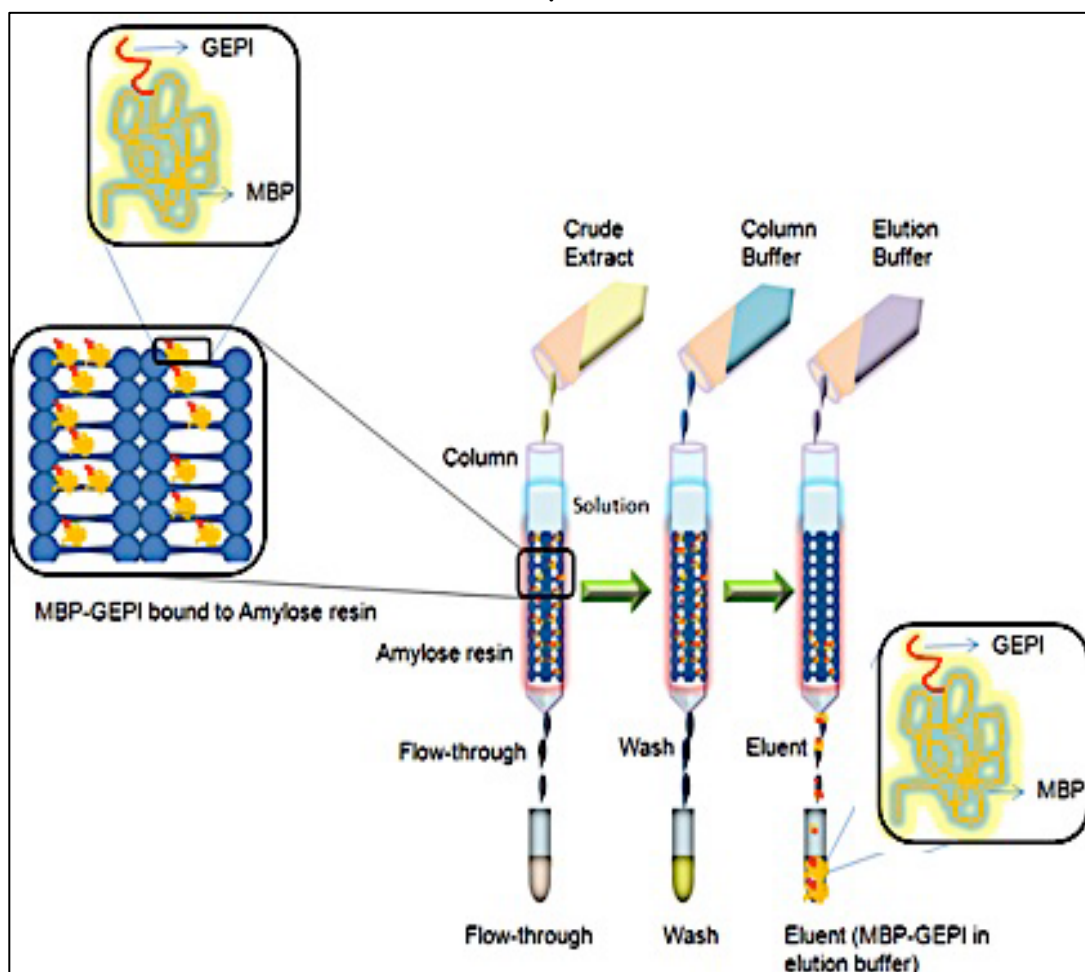


**Figure 3.1 :** Schematic illustration of purification of MBP-tagged fusion proteins.

The expressed proteins were purified by using affinity chromatography method as shown in Figure 3.1. Firstly, the optimization studies were performed to obtain high yield of protein. The efficient amount of amylose resin, size of column and the ingredients of buffers (column and elution buffers) were optimized. 1 ml of bed volume of amylose resin can bind 3 mg of MBP-tagged protein. Since MBP fusion protein might not stick to the amylose column efficiently due to certain factors in the extracts that interfere with binding, or present a low intrinsic affinity. Factors in the crude extract that would interfere with binding and cellular components are released during alternative methods of lysis (lysozyme/sonication). In addition, cells grown in LB have substantial amounts of an amylase that interferes with binding. By adding glucose to the media, expression of this amylase was repressed and the problem was alleviated. Under normal conditions (crude extract from 1 liter of cells grown in LB + 0.2% glucose, 15 ml column), the column loses 1–3% of its initial binding capacity

in each time it was used. If the yield of fusion protein under these conditions is 40mg, this means that after 3 to 5 runs there would be a decrease in the yield

The purification process of both wt-MBP and MBP-AuBP1 was performed using a modified New England Biolabs protocol (Figure 3.1). Amylose column was packed gently and equilibrated through column buffer (Appendix A2) In this purification step, the first modification was addition of protease inhibitors into the column buffer to inhibit the undesired proteases. The second one was the use of  $\beta$ -mercaptoethanol of 1mM in the column buffer, which is 10-fold less than the standard procedure to prevent interchain disulfide bond formation upon lysis. Consequently, proteins were eluted from the cross-linked amylose column with a buffer containing 10 mM maltose without reducing agent.

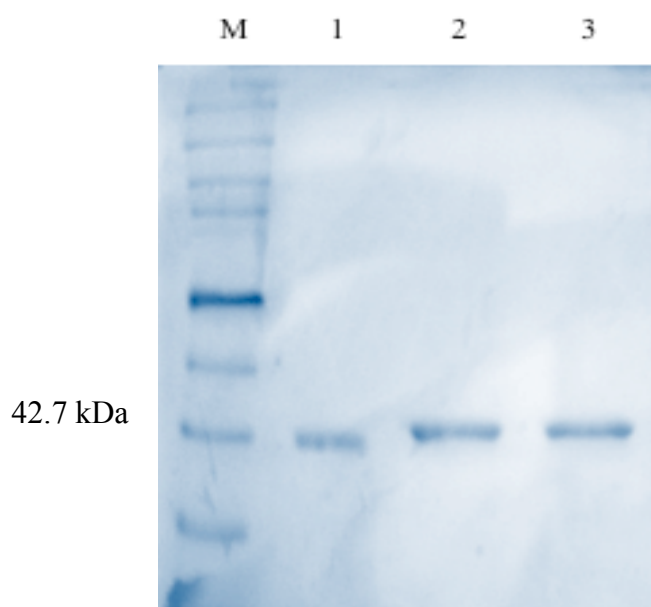


**Figure 3.2 :** Lane (M), broad range protein marker (Fermentas, Appendix A1), lane (1), wt- MBP; lane (2), MBP-S(G)<sub>3</sub>-AuBP1; lane (3), MBP-(PG)<sub>3</sub>-AuBP1.

The purity and molecular weight of eluted fusion proteins were analyzed by SDS-PAGE (Figure 3.2). The apparent molecular mass of the fusion proteins is ~ 45 kDa which corresponds to the theoretical ones. Wt-MBP has an electrophoretic mobility of 42.5 kDa. The molecular mass of individual bi-functional proteins is given in Table 3.1.

**Table 3.1 :** Purification yield and mass spectroscopy (MS-Maldi-TOF) results of expressed and purified fusion proteins with stiff or flexible linkers and wild type MBP

| Proteins                     | Yield of Total Protein (mg/L culture) | Yield of Fusion Protein (mg/L culture) | Predicted Molecular Weight (kDa) | Measured Molecular Weight (kDa) |
|------------------------------|---------------------------------------|----------------------------------------|----------------------------------|---------------------------------|
| Wt-MBP                       | 180                                   | 40                                     | 42.481                           | 42.492                          |
| MBP-(PG) <sub>3</sub> -AuBP1 | 150                                   | 41                                     | 44.381                           | 44.470                          |
| MBP-S(G) <sub>3</sub> -AuBP1 | 85                                    | 37                                     | 44.376                           | 44.395                          |

### 3.1.2 Experimental characterization of protein-surface interactions

Once MBP protein fused to inorganic binding peptide had been purified, their binding characteristics were determined using a variety of methods as will be discussed in the next section. The quantitative characterization of peptide binding is important because it confirms the usefulness of the inorganic binding peptide while connected to a functional protein. Some important traits are: the binding affinity of the fusion protein to the inorganic surface, the kinetic parameters of protein binding, the selectivity of the fusion protein for the surface on which it was designed with inorganic binding peptide, the surface coverage of the protein, the density of the SAM, the stability of the SAM, and the thermodynamics of binding. The importance of each trait depends on the mode of implementation. For example, in linking applications, affinity and stability are of utmost importance, while many biosensor applications require dense monolayers and high surface coverage. In self-assembly applications, the organization of proteins is likely driven kinetically, which makes binding kinetics important.

#### 3.1.2.1 Surface plasmon resonance spectroscopy

Quantitative study of the binding characteristics of fusion protein with inorganic binding peptide is critical. Peptides are often used in the literature for a variety of applications, despite the fact that their binding properties have not been well studied when used in designer proteins. First, quantitative data is necessary for a scientific understanding of the binding process and/or mechanisms. For example, binding

energies can indicate if a certain binding event is due to covalent bonding, ionic interactions or secondary interactions. Second, in order to evaluate the binding of an engineered protein, it needs to have quantitative data to compare it to other binders such as thiol or silane. Third, when peptides are used in a specific application, quantitative information can indicate what peptide or buffer conditions will allow the protein to function properly. Additionally, even a peptide sequence may bind to a specific surface, it may necessarily bind when that same sequence is attached to a different entity, such as a larger protein.

Here the binding characteristics of purified fusion proteins were rigorously tested using SPR. SPR spectroscopy was used to study the effect of engineered AuBP1 peptide tag present in hetero-functional MBP-AuBP1 fusions on protein–solid interactions and binding. Figure 3.3 shows an example of the experimental SPR sensograms recorded for MBP-(PG)<sub>3</sub>-AuBP1 at various protein concentrations fitted with a Langmuir isotherm binding model.

The Langmuir isotherm model was used to deduce the kinetics of the adsorption process using the experimental SPR data. Here, firstly,  $k_{obs}$  was calculated using Eq 3.1; where  $C$  is the protein concentration, and  $k_a$  and  $k_d$  are the association and dissociation constants, respectively. Next, the equilibrium constant ( $K_{eq}$ ) and the equilibrium surface coverage ( $q$ ) data were obtained using Equation 3.2 and Equation 3.3, respectively.

$$k_{obs} = (k_a \times C) + k_d \quad (3.1)$$

$$K_{eq} = k_a / k_d \quad (3.2)$$

$$q = C / [C + (1/K_{eq})] \quad (3.3)$$

The apparent binding rates ( $k_{obs}$ ) were derived from nonlinear curve fitting of the recorded SPR sensograms to the Langmuir binding isotherm as described earlier [9]. The kinetic parameters of the adsorption and desorption process were further calculated from a linear regression (Equation 3.1) (Figure 3.3)

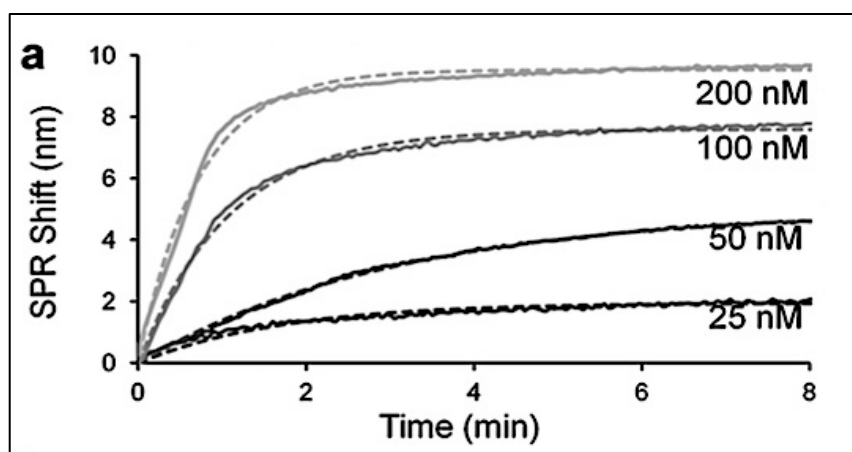


**Figure 3.3 :** SPR binding analysis of expressed fusion proteins. Example of SPR sensograms recorded for MBP-(PG)<sub>3</sub>-AuBP1 at various concentrations.

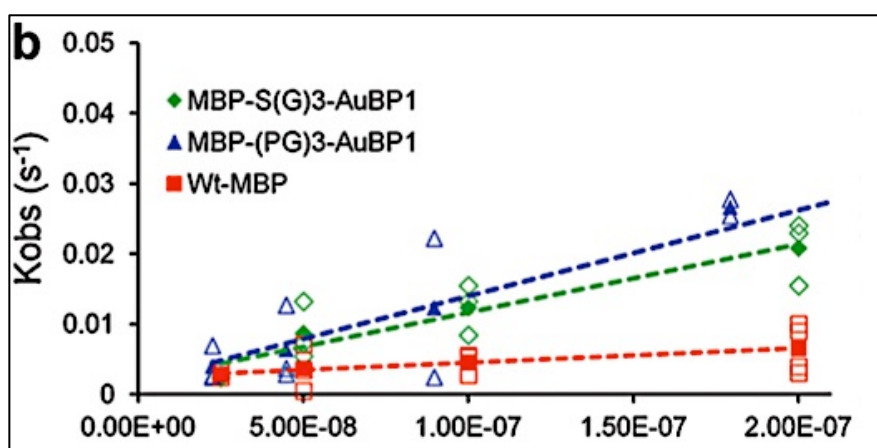


**Figure 3.4 :**  $k_{obs}$  plots for wild type MBP (red), MBP-S(G)<sub>3</sub>-AuBP1 (green), MBP-(PG)<sub>3</sub>-AuBP1 proteins (blue).

The dissociation constants ( $K_D$ ) can be then calculated using Equation 3.2. The observed association rate constant  $k_a$  (mean  $\pm$  SE) values for fusion MBP-AuBP1 proteins ( $8.95 \pm 0.58$ )  $10^4 \text{ M}^{-1} \text{ s}^{-1}$  for MBP- S(G)<sub>3</sub>-AuBP1 and ( $11.65 \pm 0.35$ )  $10^4 \text{ M}^{-1} \text{ s}^{-1}$  for MBP- (PG)<sub>3</sub>-AuBP1 were considerably higher than the  $k_a$  obtained for the wt-MBP protein of ( $2.01 \pm 0.27$ )  $10^4 \text{ M}^{-1} \text{ s}^{-1}$  (Table 3.2).

**Table 3.2 :** Observed association ( $k_a$ ) and dissociation ( $k_d$ ) rate constants for wild type and fusion MBP and respective calculated values of dissociation constant (mean  $\pm$  SE).

| Protein                      | $k_a \times 10^4 (M^{-1}s^{-1})$ | $k_d (s^{-1})$    | $K_D (\mu M)$     |
|------------------------------|----------------------------------|-------------------|-------------------|
| wt MBP                       | $2.01 \pm 0.27$                  | $0.003 \pm 0.000$ | $0.124 \pm 0.024$ |
| MBP-S(G) <sub>3</sub> -AuBP1 | $8.95 \pm 0.58$                  | $0.003 \pm 0.001$ | $0.036 \pm 0.009$ |
| MBP-(PG) <sub>3</sub> -AuBP1 | $11.65 \pm 0.35$                 | $0.004 \pm 0.001$ | $0.035 \pm 0.006$ |

The four- to six-fold enhancement in observed association rate constants for both hetero-functional fusion proteins compared to wt-MBP is statistically significant at a 0.01 in t-test and can be contributed to the enhanced binding affinity of the fusion protein to the gold surface through the incorporated AuBP1 peptide tag. On the other hand the AuBP1 tag did not affect the dissociation rate constants ( $k_d$ ) shown in Table 3.2. Consequently, the corresponding calculated dissociation constant ( $K_D$ ) values of both fusion MBP-AuBP1 proteins were significantly lower than the control wt-MBP (Table 3.2). Overall, these observed differences in binding affinity constants indicate that both fusion MBP-AuBP1 derivatives have significantly higher binding affinity to gold compared to wt-MBP as well as suggest that AuBP1 peptide tag is similarly functional in both fusion proteins (Table 3.2). Furthermore the observed nanomolar  $K_D$  values for these fusion proteins are comparable to our other fusion protein constructs and roughly correspond to adsorption parameters of alkanethiol molecules on gold surface used in conventional chemical approaches [32].

### 3.1.2.2 Self-assembly of proteins to form nanolayers

Recently protein arrays of controlled spatial distribution have been produced using various lithography techniques, including both soft-lithography [212, 213], dip-pen lithography [54, 214], and photolithography [215] or by various immobilization chemistry [216]. By taking advantage of the high- affinity gold-binding capacity of AuBP1 peptide tag and high efficiency of conventional micro-contact printing technology ( $\mu$ CP), we first demonstrate the fabrication of protein arrays on gold surfaces with enhanced molecular density in a single reaction step. As shown in Figure 3.5, fluorescence microscopy results of MBP-AuBP1 fusion protein arrays

immunodetected by a specific anti-MBP-Alexa complex revealed enhanced binding of both MBP-AuBP1 fusion proteins compared to wt-MBP alone.

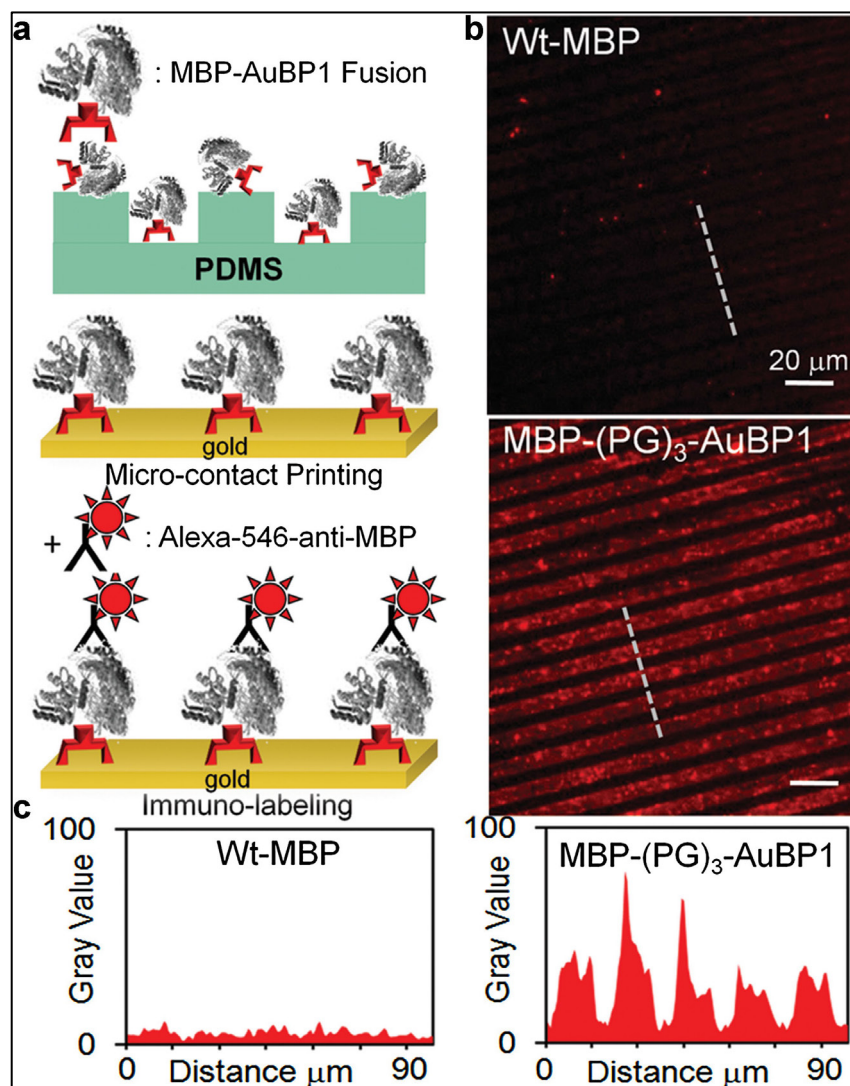


**Figure 3.5 :** Controlled protein patterning on flat gold surface via micro-contact printing technique.

It is apparent that MBP- AuBP1 protein assembles on gold surface with high molecular packing density and patterning efficiency evidenced by bright-, well-defined antibody-to-protein patterns (Figure 3.5 b–c). This result suggests that the specific AuBP1 epitope tags present in MBP-AuBP1 fusion proteins indeed control and direct the immobilization of fusion proteins onto the surface and represents an excellent interface for protein–surface interactions.

Figure 3.5 shows schematics of micro-contact printing and protein labeling (panel a); representative fluorescent images of 20 mM wt-MBP and MBP-(PG)<sub>3</sub>-AuBP1 proteins labeled with anti-MBP-Alexa-546 antibody immobilized on planar gold

surface using micro- contact printing (panel b); respective line scan plots of fluorescence images generated using Image J (NIH, Bethesda, MD) software (panel c). The protein-arrayed gold surfaces were imaged using a fluorescence microscope with corresponding fluorescent filter. Dashed lines indicate the respective positions on fluorescence images used in ImageJ line scan analysis.

### **3.1.2.3 Layer-by layer protein self-organization**

In this section, a multilayered protein-Au NP arrays were fabricated on a solid surface via a strategy using a combination of peptide-enabled layer-by-layer self-assembly and  $\mu$ CP technique. Firstly, a novel bi-functional peptide (QBP1-AuBP1) that includes both gold- and silica-binding motifs previously selected from combinatorial peptide libraries [34, 69] was produced. By taking advantage of the multi-functional modality inherent to the designed QBP1-AuBP1 peptide, Au NP arrays were fabricated on top of the silica surface using a soft-lithography patterning technique as described in the “Materials and Methods” section of this dissertation and in Figure 3.6a. The detailed scans from atomic force microscopy, as well as images from dark field microscopy of Au NP patterns fabricated via bi-functional QBP1- AuBP1 peptide linkage are given in Figure 3.6b–c.

Figure 3.6 shows schematics of preparation of Au NP patterned surface by micro-contact printing and targeted protein immobilization on Au NP patterned surface by specific self-assembly (panel a); detail of atomic force microscopy scan of representative Au NP patterns immobilized on glass surface through QBP-PPP-AuBP bi-functional peptide (panel b); representative fluorescence and dark field images of 20 mM wt-MBP and MBP-(PG)<sub>3</sub>-AuBP1 proteins immobilized on Au NP arrays labeled with anti-MBP-Alexa-488 antibody (panel c); corresponding line scan plots of fluorescence images generated using ImageJ software (NIH) (panel d). The protein molecules immobilized on Au NP arrayed surfaces were imaged using a fluorescence microscope with corresponding fluorescent filter.

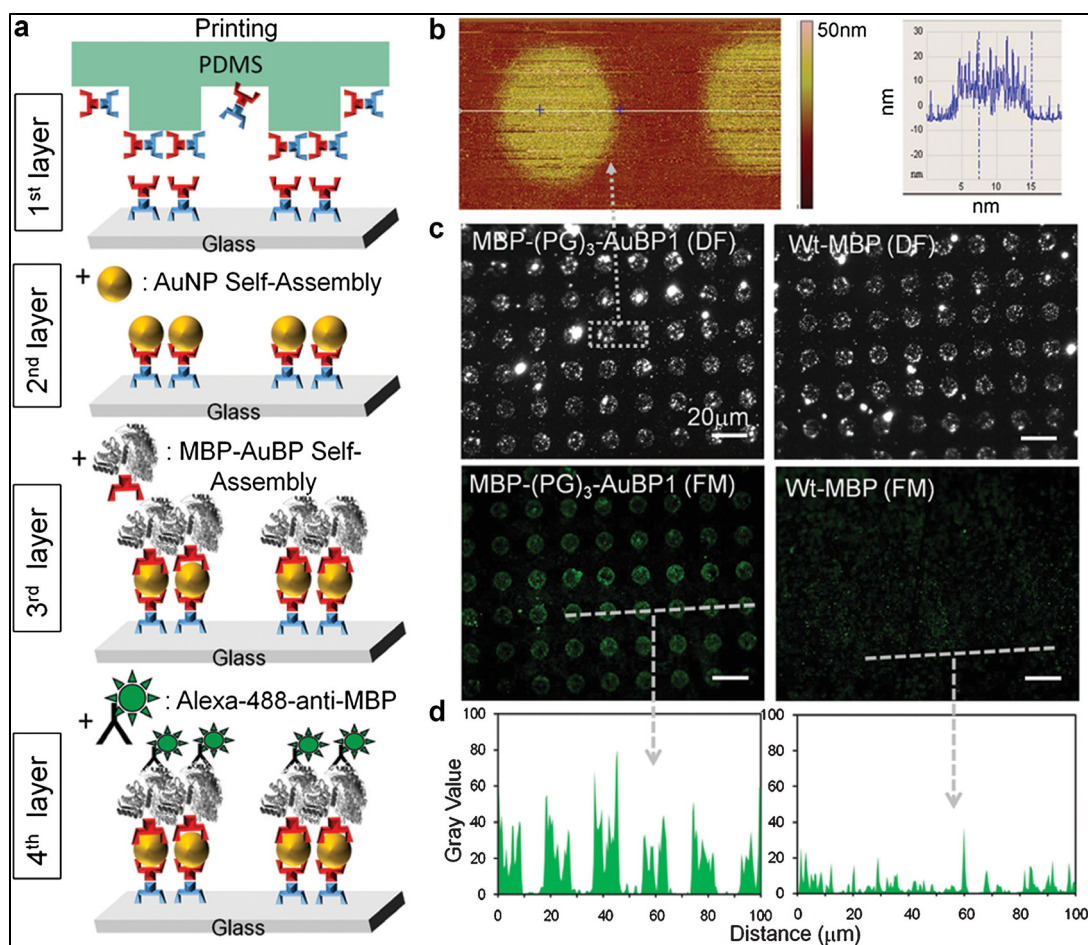


**Figure 3.6 :** Layer-by-layer protein immobilization on Au NP-arrayed surface.

Collectively, AFM and DF data demonstrate that the designed and engineered bifunctional QBP1-AuBP1 peptide can control the assembly of gold nanostructures on silica surfaces and, thus, can be used successfully to fabricate gold nanoparticle arrays via a simple and robust protocol in aqueous solutions. In the next step the hetero-functional MBP-AuBP1 protein selectively immobilized onto Au NP arrays via of AuBP1 peptide tag through its high affinity and specific interactions with gold surface. The immobilized MBP-AuBP1 fusion proteins were immuno-detected by a specific anti-MBP-Alexa complex. The fluorescence microscopy images shown in Figure 3.6c–d confirm the high-affinity and material-selective self-assembly of MBP-AuBP1 fusion proteins onto Au NP, in a process resulting in the fabrication of spatially controllable multilayered protein arrays. In contrast, wt-MBP protein did not reveal production of protein arrays and rather resulted in random non- specific immobilization of MBP onto both solid surfaces (Figure 3.6c–d).

### 3.1.3 Gold nanoparticle based detection methods

#### 3.1.3.1 Localized surface plasmon resonance (LSPR) for MBP-AuBP1

Gold nanoparticles (Au NP) either immobilized on planar surfaces or dispersed in solution are of great interest in developing novel hybrid nanoscale diagnostics tools due to their strong LSPR properties [217-219]. Stable and controlled modifications of Au NP surface with either functional proteins or recognition molecular probes are crucial prerequisite by their successful practical utility for in vivo and in vitro bioassays [32, 82, 153, 163, 218]. Herein, the replacement of citrate ions by protein molecules on gold NP surface was analyzed by the LSPR spectrum shift upon exposure to our fusion proteins (0.001 to 2mM). The premade 15nm citrate-capped Au NPs (Ted Pella) exhibit a characteristic LSPR peak at 520 nm wavelength. Upon protein-surface interaction and binding, absorbance spectrum demonstrates a red-shift. This shift is due to the local change of dielectric permittivity resulting from the formation of a protein layer on the surface of Au NP [218, 220]. Comparison of the observed LSPR shifts recorded at various protein concentrations (Figure 3.7b–c) reveals that MBP-AuBP1 fusion proteins bind to Au NP with higher affinity than either wt-MBP. In fact, observed LSPR shifts recorded for up to 0.1mM MBP-AuBP1 proteins show 5–4 nm greater shift compared to control wt-MBP (Figure 3.7c). Dissociation constants ( $K_D$ ) were directly calculated by fitting Langmuir binding isotherm to the observed LSPR shifts via nonlinear curve fitting (Figure 3.7b).

Corresponding  $K_D$  values (mean  $\pm$  SE) for MBP-AuBP1 fusion proteins ( $0.024 \pm 0.004 \mu\text{M}$  for MBP-S(G)<sub>3</sub>-AuBP1 and  $0.039 \pm 0.013 \mu\text{M}$  for MBP- (PG)<sub>3</sub>-AuBP1) were found to be significantly lower than for wt-MBP protein ( $0.205 \pm 0.056 \mu\text{M}$ ) (Figure 3.7d). The six- to eight fold decrease of observed dissociation constant for hetero-functional fusion proteins compared to wt-MBP is statistically significant at a  $\alpha=0.05$  confirmed by the t-test and show outstanding correlation with calculated affinity constants observed in SPR binding assays. On the other hand, as with SPR results, we observed no statistically significant difference in binding affinity between the tested fusion proteins at a  $\alpha=0.05$  in the t-test suggesting that both fusion proteins are equally functional. The detected enhanced binding affinities of the engineered

MBP- AuBP1 proteins to the gold surfaces can be attributed to the AuBP1 peptide tag.

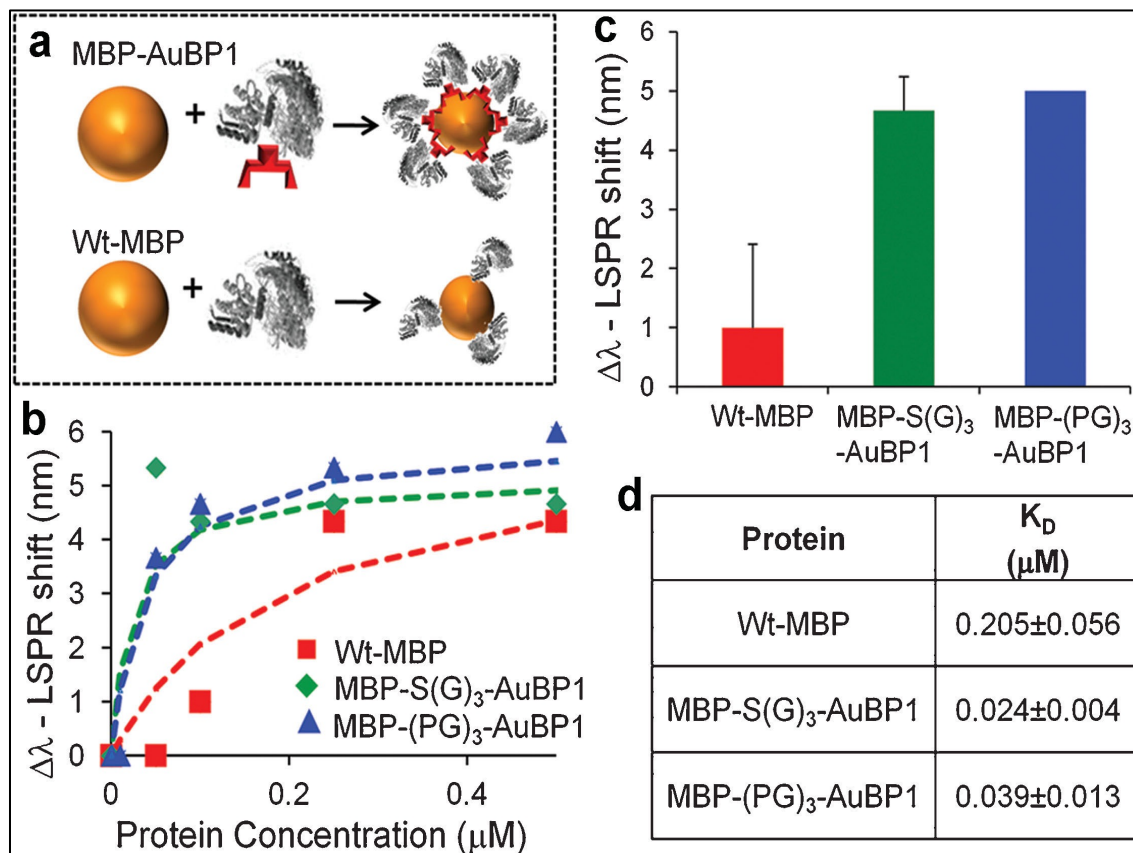


**Figure 3.7 :** LSPR binding analysis and protein-functionalization of gold nanoparticles (Au NP).

Figure 3.7 illustrates the functionalization of Au NP (a), LSPR shifts for fusion and wild type proteins plotted at various concentrations fitted with Langmuir curve (b); LSPR shifts for fusion and wild type proteins at 0.1 mM concentration (c) and respective observed dissociation constants ( $K_D$ ) (d).

Moreover, these findings suggest that the fusion MBP-AuBP1 proteins exhibit similar binding affinity to both flat planar and nanoparticle gold surface and that protein binding and self-assembly processes exhibit no or only negligible steric hindrance effect.

### 3.1.3.2 Stabilization of gold nanoparticles via inorganic binding proteins

Previously it has been reported that Au NPs stabilized with loosely bound surfactants such as citrate, aggregate upon salt addition, for example, NaCl, due to the screening of the electrostatic repulsion between them [220]. In salt-induced agglomeration assays, Au NP agglomeration is evidenced by a significant change in LSPR spectrum, such as decreased intensity for the characteristic LSPR band at 520nm and an increased absorption at longer wavelengths (650–700 nm) conveyed with corresponding color change from pink to blue. Here, the Au NP modified with fusion MBP-AuBP1 proteins exhibited exceptional stability and can withstand up to 0.5 M NaCl solution. In contrast, Au NP modified with wt-MBP protein agglomerated at lower salt concentrations, as evidenced by solution color transition to blue and an appearance of large red-shifted absorption peak (Figure 3.8a). This result suggests that the bound wt- MBP protein is probably adhered to the Au surface with low binding strength as well as more random orientation, thus resulting in exposed surface of Au NP for interaction with salt ions promoting agglomeration.

Images of functionalized Au NP with proteins are shown in Figure 3.8. Au NPs were functionalized with 1mM of either wt-MBP, MBP-AuBP1 derivatives and non-functionalized Au NP control before and after addition of 1 M NaCl (a); schematic cartoon for agarose gel electrophoresis of functionalized Au NP after functionalization reaction with 0.05mM proteins is shown in (b); immunoblotting onto nitrocellulose membrane using wt-MBP and MBP-(PG)<sub>3</sub>-AuBP1 functionalized Au NP labeled with anti-MBP-HRP antibody and TMS substrate is in (c).

These conclusions were further supported by experiments assaying protein-induced Au NP stability in gel electrophoresis (60V) using low percentage of agarose gel of 0.5% (Figure 3.8b). In the agarose gel experiment, a significantly enhanced stability of Au NP functionalized with MBP-AuBP1 proteins was observed. Functionalized Au NP appeared as pink-colored bands compared to agglomerated NP functionalized with wt-MBP and “bare” citrate-capped NP, which appeared as blue-color bands in the agarose gel assay. Also, the enhanced surface modification induced by fusion MBP-AuBP1 proteins was demonstrated in a simple blotting experiment on nitrocellulose membrane (Figure 3.8c).

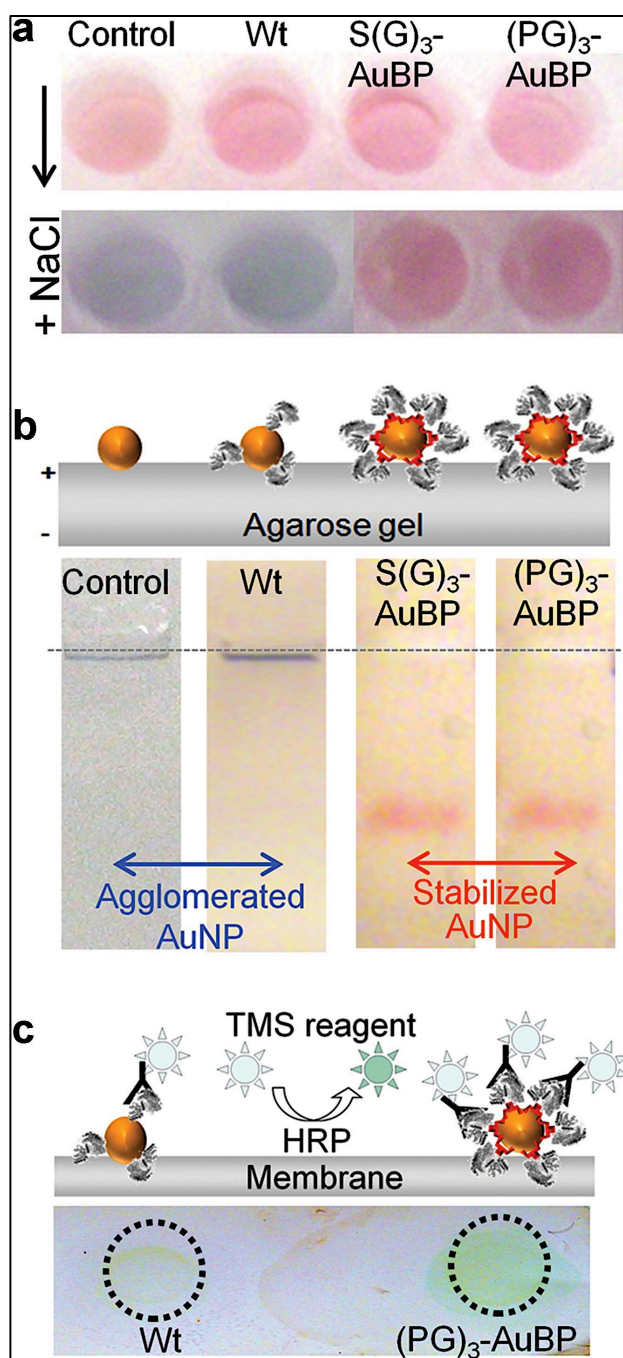


**Figure 3.8 :** Stability of protein-functionalized gold nanoparticles.

The data shown in Figure 3.8c clearly demonstrate enhanced Au NP surface modification with fusion MBP-AuBP1 compared to wt-MBP control ([protein] for both at 0.05mM). In summary, the observed NP characteristics, such as exceptional stability in the absence of excess capping ligand, as well as enhanced surface modifications induced by MBP-AuBP1, suggest that these functional Au NPs would be particularly suited for biological applications or for building nanostructures using biological interactions [220].

### 3.1.4 Application of fusion proteins in bio-nanotechnology

#### 3.1.4.1 Anti-MBP detection using MBP-AuBP2 probe

Noble metal nanoparticles in colloidal solution can be immobilized onto a glass, forming a suitable stage for LSPR based detection [281, 341]. Since bi-functional peptides were successfully utilized for gold nanoparticle immobilization on glass in 2.4.2, the resultant substrates could be proper platforms for biosensing. To explore their potential, another LSPR biosensor was designed, where glass substrate with gold nanoparticles immobilized through bi-functional peptide (see Section 2.4.2) and MBP-AuBP1 were used as platform and probe, respectively. Here, instead of using the alkanethiol-based linkers for the attachment of protein and nanoparticle onto the inorganic surface, the appropriate inorganic binding peptide was used to maintain linkage between the interfaces.

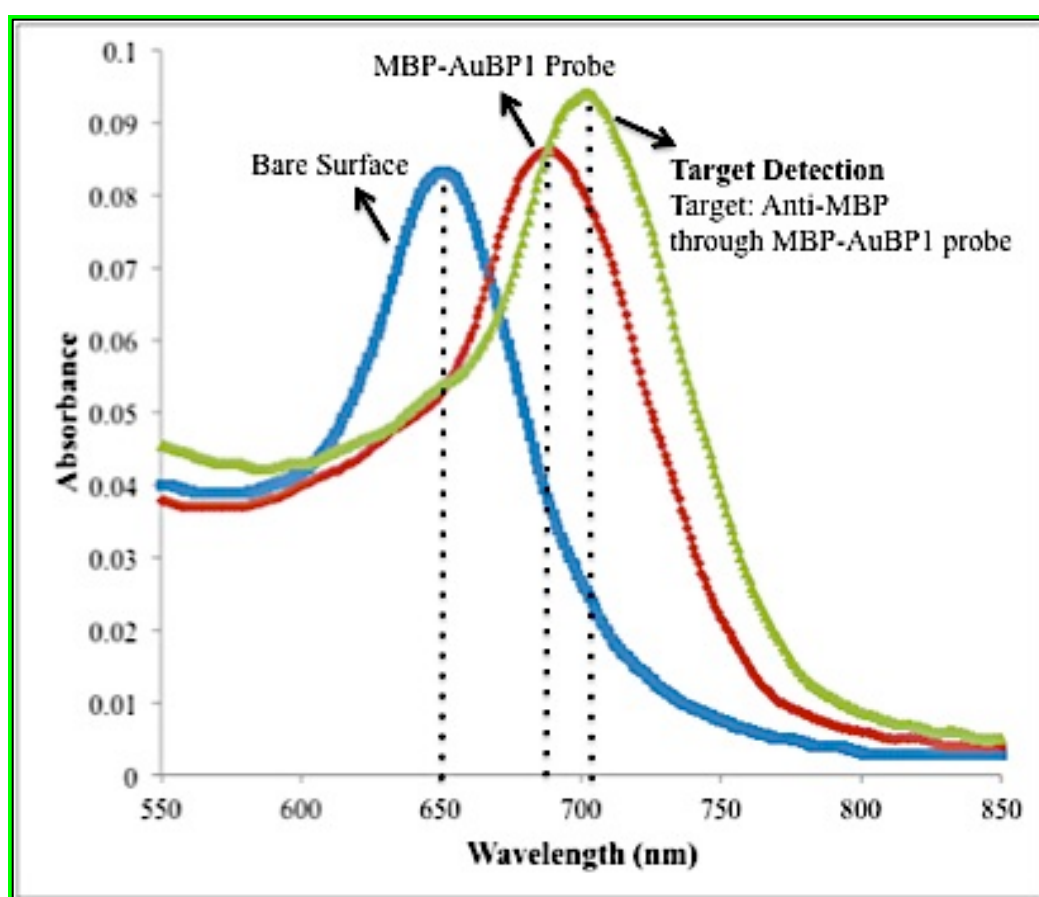


**Figure 3.9 :** Immuno-detection of Anti-MBP (50  $\mu\text{g/ml}$ ) using LSPR based biosensor composed of gold nanoparticles immobilized through bi-functional peptide and a genetically engineered fusion probe, MBP-AuBP1. LSPR Spectra with  $\lambda_{\text{max}}$  values are highlighted in blue for bare, red for probe assembly, and green for Anti-MBP assembly.

Firstly, using LSPR spectroscopy, the assembly performance of the fusion protein was investigated and compared with the control molecule, wt-MBP (Figure 3.9 and Figure 3.10). The red shift at LSPR  $\lambda_{\max}$  corresponding MBP-AuBP1 assembly on gold nanoparticles was more than the red shift recorded for wt-MBP assembly. Even though the sizes of the molecules are closer to each other, higher red shift from MBP-AuBP1 was attributed to higher number of molecules assembled on the gold surface comparing to wt-MBP.

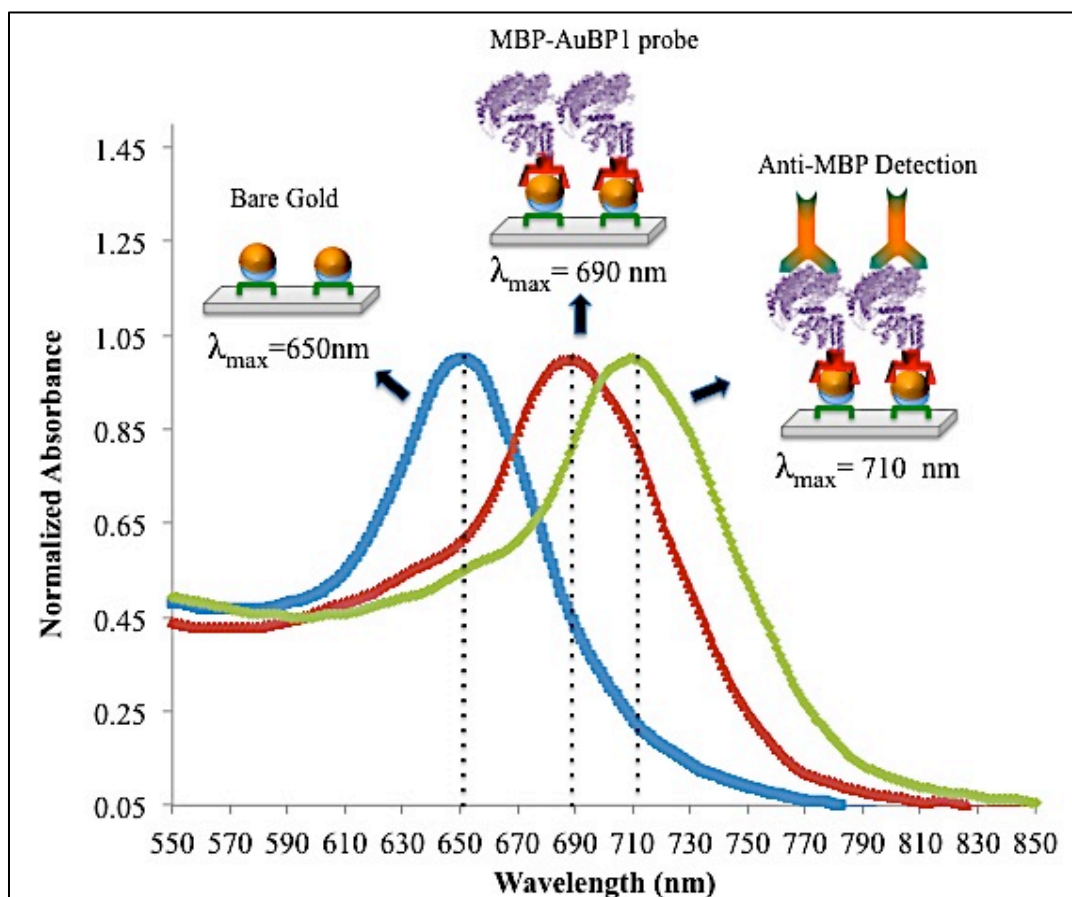


**Figure 3.10 :** Immuno-detection of Anti-MBP (50  $\mu\text{g/ml}$ ) using LSPR based biosensor using MBP-AuBP1 probe. LSPR Spectra with  $\lambda_{\max}$  values are highlighted in blue for bare, red for probe assembly, and green for Anti-MBP assembly. Absorbance data was normalized to observe the red shift.

Next, binding of Anti-MBP (target molecule) to probe immobilized on the chip was studied. Here, the signals coming from LSPR  $\lambda_{\max}$  of the gold nanoparticles attached onto the glass surface were recorded before and after the probe assembly, and then after target assembly (see Materials and Methods Section 2.6.1). Figure 3.9 and Figure 3.11 show the LSPR spectra of the each step in Anti-AMBP detection where MBP-AuBP1 and wt-MBP were used as probe. Due to the local refractive

index change, the probe and subsequent target assembly resulted in red shifts at the LSPR  $\lambda_{\text{max}}$ . Since the number of assembled MBP-AuBP1 molecules on gold surface is higher than MBP, detection of 50  $\mu\text{g/ml}$  Anti-MBP solution was achieved by higher shift (Figure 3.9 vs Figure 3.11).

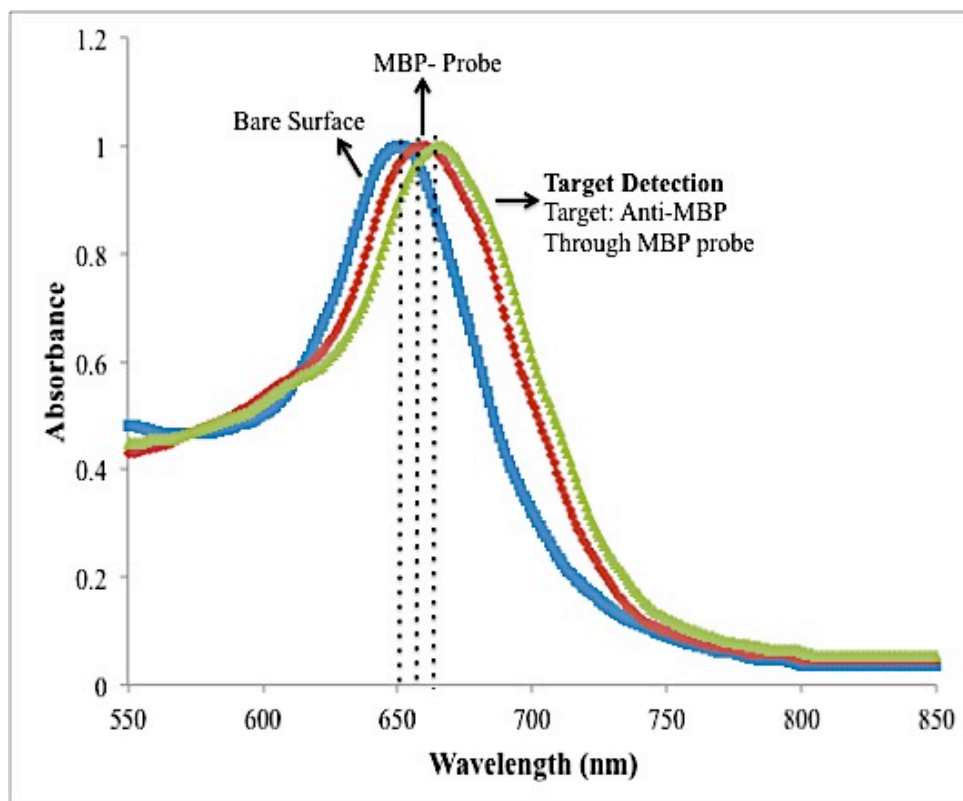


**Figure 3.11 :** Immuno-detection of Anti-MBP (50  $\mu\text{g/ml}$ ) using LSPR based biosensor composed of gold nanoparticles immobilized through bi-functional peptide and a genetically engineered fusion probe, wt-MBP. LSPR Spectra with  $\lambda_{\text{max}}$  values are highlighted in blue for bare, red for probe assembly, and green for Anti-MBP assembly.

Overall results in Section 3.1.4 show that one can prepare optically active platforms for detection of biomolecules where probe molecule and even inorganic nanostructures can be immobilized and organized using inorganic binding peptides with appropriate binding activity. All assembly procedures can be done at ambient conditions such as room temperature, aqueous environment, excluding complex chemical reactions.

### **3.2 Bioengineered DsRed-AuBP2 Fusion Protein**

DsRed and DsRed-AuBP2 genes were inserted pMal-c4X expression vector by Dr. Esra Yuca in our group and kindly obtained from her. Because of the purification problem with wt-DsRed, the constructed vectors were re-transformed into *E.coli* ER 2507 strain and expression conditions were optimized to overcome the purification problem. Finally, DsRed and DsRed-AuBP proteins were successfully expressed and purified by applying two different chromatography techniques. Through MBP-tagged affinity chromatography, MBP-DsRed and MBP-DsRed-AuBP2 were purified. After cleavage of MBP from the DsRed proteins by Factor Xa proteolytic enzyme, the further purification was achieved using copper chelating affinity chromatography to remove the MBP tag and enzyme.

#### **3.2.1 Expression and purification of DsRed and DsRed-AuBP2**

AuBP2 gene was inserted into C-terminus of DsRed-monomer using a flexible linker, SGGG. The reason of using 3 glycine residues (G)<sub>3</sub> was to produce a flexible region and obtain specific gold binding ability of AuBP2

The plasmid encoding fusion proteins were expressed in *E.coli* 2507 cells. MBP fusion method was used for high expression protein yield and easy purification. Firstly, the expression and purification conditions were optimized. The induction time at different temperatures, i.e. at 25°C, 30°C, and 37°C, was tested. (Figure 3.12 and Figure 3.13). For each of the fusion proteins, the optimal expression condition resulting in the highest protein expression was followed after the addition of 0.4 mM IPTG at 30°C for an incubation period of 48h. The purified protein amount was found as ~100 mg/L for MBP-DsRed-AuBP and ~45 mg/L for MBP-DsRed.

The expressed proteins were purified through amylose column. The purified samples were analyzed by SDS-PAGE to verify their purity and molecular weights (Figure 3.14 and Figure 3.15). The single protein bands of MBP-DsRed, MBP-DsRed-AuBP2, were observed at the 71 and 75 kDa values, respectively (Figure 3.14 and Figure 3.15).

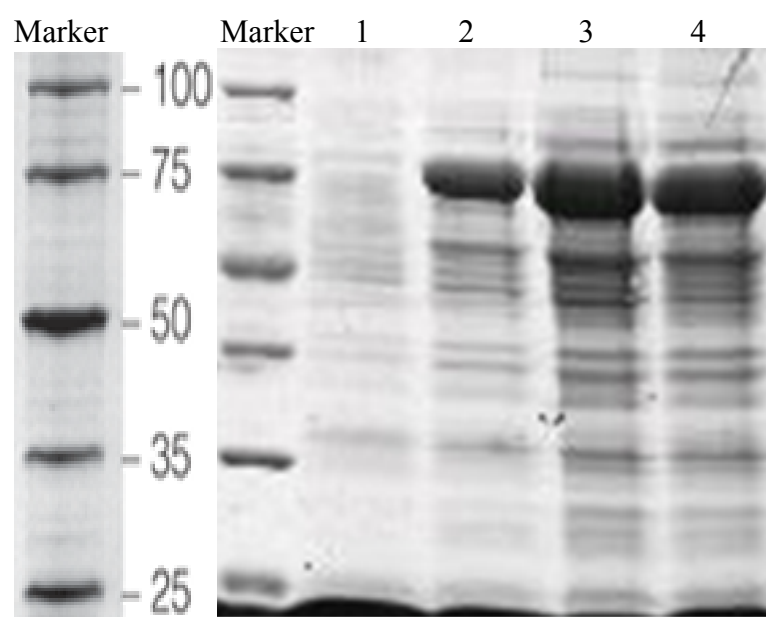


**Figure 3.12 :** The optimum conditions for expression of proteins following different incubation times: SDS-Page analysis of MBP-DsRed (MBP-DsRed; ~71 kDa). Lane1; Unstained protein marker (Fermentas), Lane2; Uninduced cells, Lane3; 24h incubation time after IPTG addition, Lane4: 48h incubation time after IPTG addition, Lane5: 72h incubation time after IPTG addition.

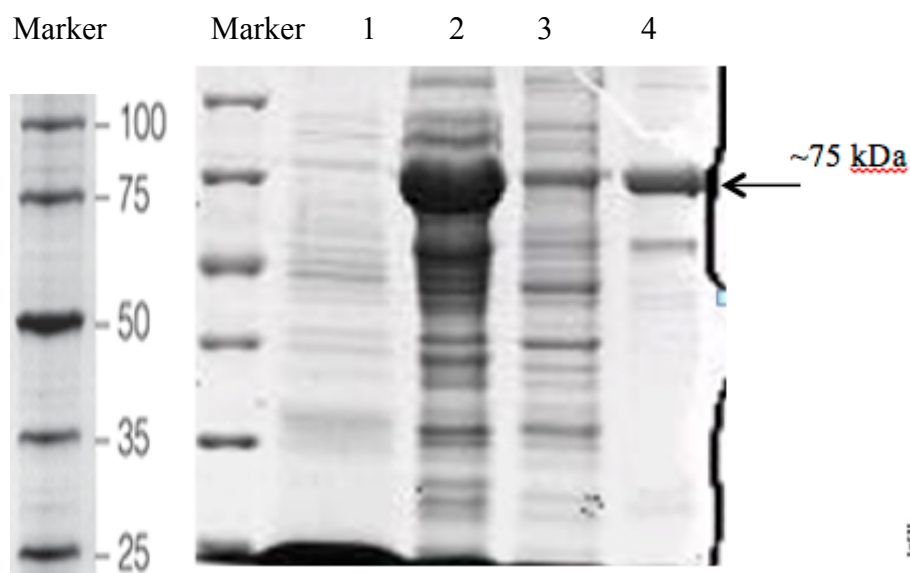


**Figure 3.13 :** The SDS-Page analysis of the protein binding to amylose resin for MBP-DsRed-AuBP2c protein (~75 kDa) Lane1; Unstained protein marker (Fermentas) Lane2; Uninduced MBP-DsRed-AuBP2c, Lane3; Crude Extract of MBP-DsRed-AuBP2c, Lane4; Insoluble cell MBP-DsRed-AuBP2c, Lane5; MBP-DsRed-AuBP2c bound to amylose resin.

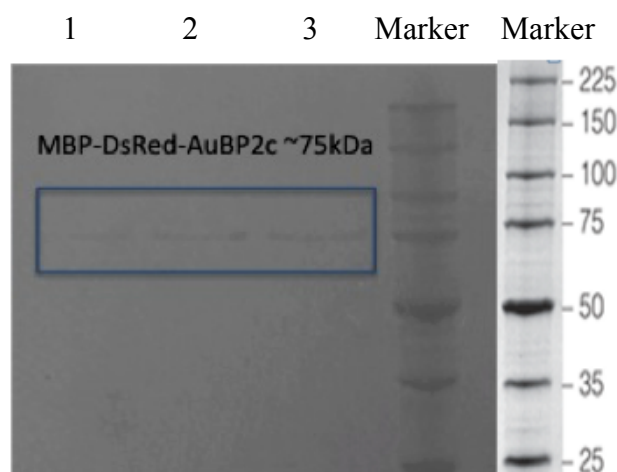


**Figure 3.14 :** SDS-Page analysis of MBP-DsRed-AuBP2c (~75 kDa) after amylose affinity column chromatography. Lane 1-3; pure protein, Lane 4; Unstained protein marker (Fermentas).

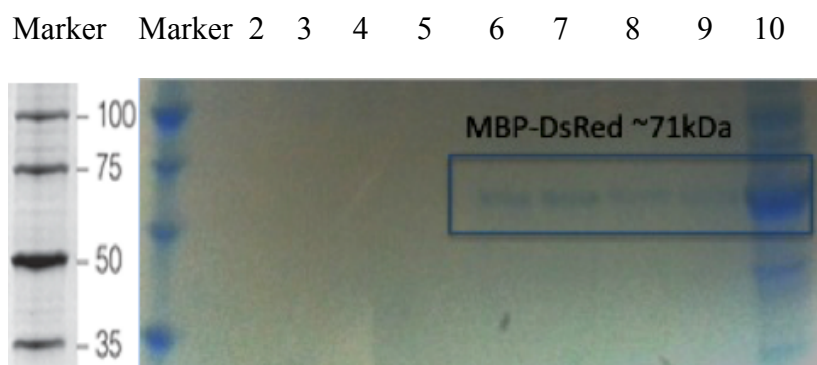


**Figure 3.15 :** SDS-PAGE analysis of MBP-DsRed (~71 kDa) after amylose affinity column chromatography. Lane 1: Unstained protein marker (Fermentas), Lane 6-9; pure protein, Lane 10; crude extract containing MBP-DsRed.

Our results also indicate that the AuBP2 peptide did not cause any burden on the expression of the target protein. The concentration of the purified protein was determined using Bradford assay. The percent recovery of the purified protein was estimated by passing a known quantity of purified fusion protein through the amylose column. The recovered protein was calculated to be ~92% for the MBP-Dsred-AuBP2 fusion.

### 3.2.1.1 Affinity tag (MBP-tag) removal from DsRed proteins

The DsRed-monomer and DsRed-AuBP2 proteins were further purified in this part utilizing their inherent copper binding ability. MBP tag was cleaved by Factor Xa enzyme and removed from DsRed proteins through copper immobilized column. The purity of DsRed and DsRed-AuBP2 were analyzed by SDS-PAGE. Their bands onto

the gel were revealed at the 28 kDa for DsRed and ~35 kDa for DsRed-AuBP2 (Figure 3.16 and Figure 3.17).

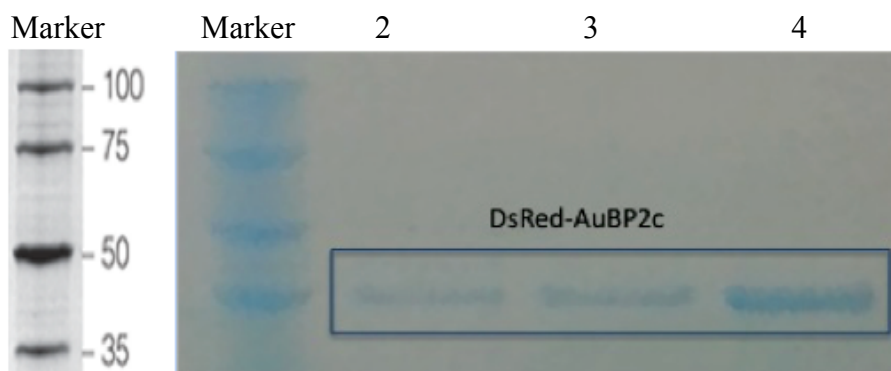


**Figure 3.16 :** SDS-PAGE analysis of DsRed-AuBP2c (~35 kDa) to test the purity of the protein. Lane1; Unstained protein marker (Fermentas), Lane2-4; pure DsRed-AuBP2.

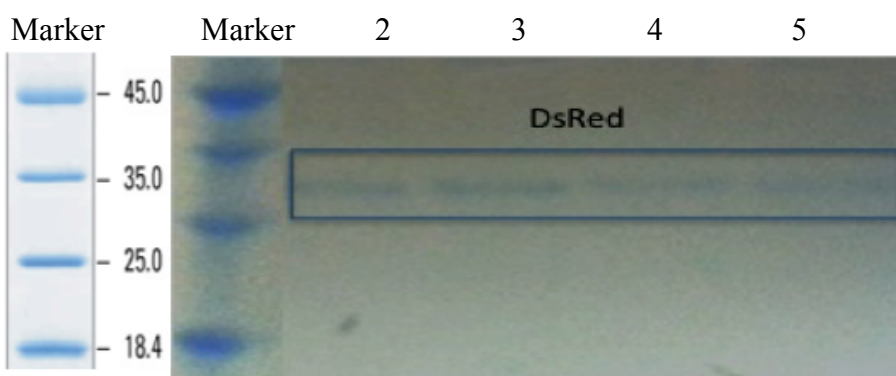


**Figure 3.17 :** SDS-PAGE analysis of DsRed-Monomer (~28 kDa) to test the purity of the protein. Lane1; Unstained protein marker (Fermentas), Lane 2-5; pure DsRed.

### 3.2.2 Molecular structure analysis of DsRed and DsRed-AuBP2

#### 3.2.2.1 Circular dichroism spectroscopy

Fluorescent properties of DsRed-monomer and DsRed-AuBP2 were first characterized by fluorescence spectroscopy. We demonstrated that both of the proteins have equivalent excitation and emission wavelengths, 558 nm and 590 nm, respectively (Figure 3.18a). This implies that fluorescence property of DsRed was conserved upon the insertion of the designed peptide, AuBP2. Next, the CD spectra performed correspond to the dominating  $\beta$ -sheet structure (Figure 3.18b). The presence of the  $\beta$ -barrel core in the DsRed that is formed by embedding hydrophobic residues inside and the exposure of hydrophilic residues to the outside of the barrel structure. There is, however, a change in the amount of molar ellipticity possibly

revealing a loss, in DsRed-AuBP2, of protein structure. This result shows comparison of these proteins with different molecular weight. The change in the molar ellipticity values by the alteration of DsRed structure correlates with the difference in molecular weight of these proteins, since DsRed has higher molar ellipticity value as compared to that of DsRed-AuBP2.

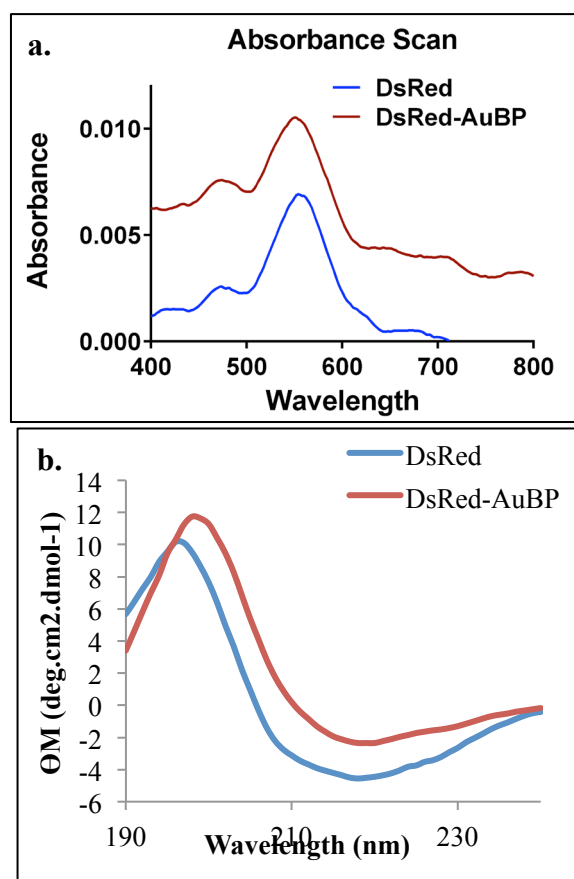


**Figure 3.18 :** a. UV-visible spectra of the purified DsRed and DsRed-AuBP2. The peak at ~558 nm shows the excitation wavelength of proteins. b: Secondary structure analysis of DsRed and DsRed-AuBP2 proteins using CD, far-UV wavelength scans.

### 3.2.3 Experimental characterization of protein-surface interactions

#### 3.2.3.1 Quartz crystal microbalance with dissipation monitoring (QCM-D)

Quartz Crystal Microbalance has found many applications in the liquid-phase sensing of biological molecules [201, 202]. In this method, proteins are introduced to a resonating quartz crystal coated by the gold. As proteins attach to the surface the resonant frequency of the crystal decreases, which is measured in real-time. The change in frequency is directly proportional to the wet mass of attached protein (protein plus bound water). In this way the amount of protein attached to the surface

can be monitored in real-time, as shown in Figure 3.19. This allows the kinetics of binding to be monitored. An example of real-time binding data is shown in Figure 3.19. QCM-D allows the dissipation of energy by the protein to be measured. This property is related to the conformation of the molecule. Thus information about the conformation of the peptide on the surface is obtainable [203, 204]. The main drawback of this method is its inferior sensitivity compared to other methods like SPR [205]. Also, the change in frequency is related to the wet mass of the peptide as opposed to the dry mass, which adds another variable into the system.

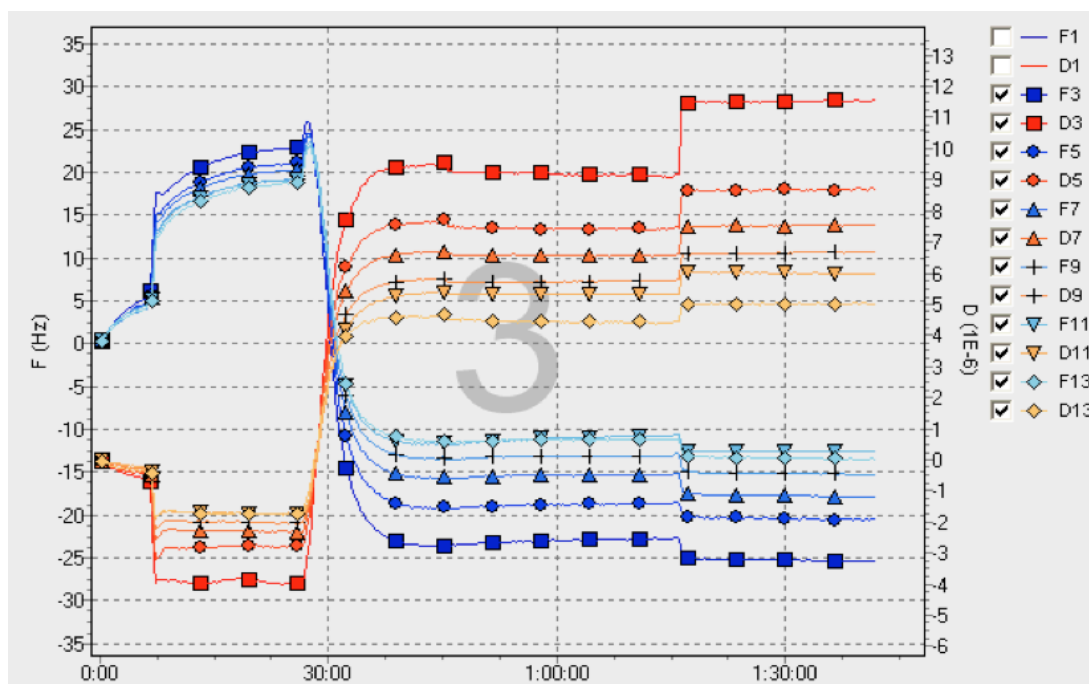


**Figure 3.19 :** Real time measurement by QCM. The change in frequency is shown as blue plots with 3rd (F3), 4th (F4), 5th (F5) and 7th (F7) harmonic, which are decreasing due to the interaction between gold surface and protein. The dissipation of energy by the protein is measured and shown as red plots with 3rd (D3), 4th (D4), 5th (D5) and 7th (D7) harmonic, which are increasing due to the interaction between gold surface and protein.

QCM was utilized to conduct affinity measurements for DsRed and DsRed-AuBP2 onto flat gold surfaces. Due to the poor flow and lower sensitivity of the QCM system comparing to SPR, a modified kinetic model rather than Langmuir isotherm model was used for characterizing binding kinetics of proteins on gold surfaces. In this approach, increasing concentrations of protein (0.1 to 0.4  $\mu\text{M}$ ) were introduced into the flow cell. The frequency and dissipation were given time to stabilize at each concentration. Then, the cumulative shift was plotted versus concentration and fit

using the remodelled Langmuir adsorption model. An example of these plots is shown in Figure 3.20 and Figure 3.21 for DsRed-AuBP2 and DsRed, respectively. This work was done in collaboration with grad student James Edward Meyer from University of Kansas.

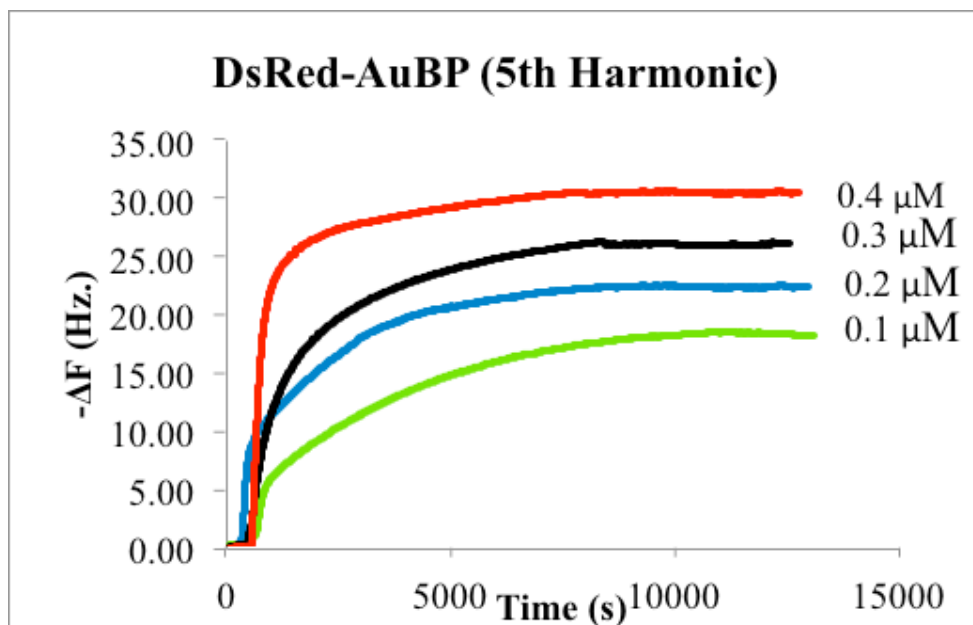


**Figure 3.20 :** QCM results for DsRed-AuBP2 binding onto gold surface.

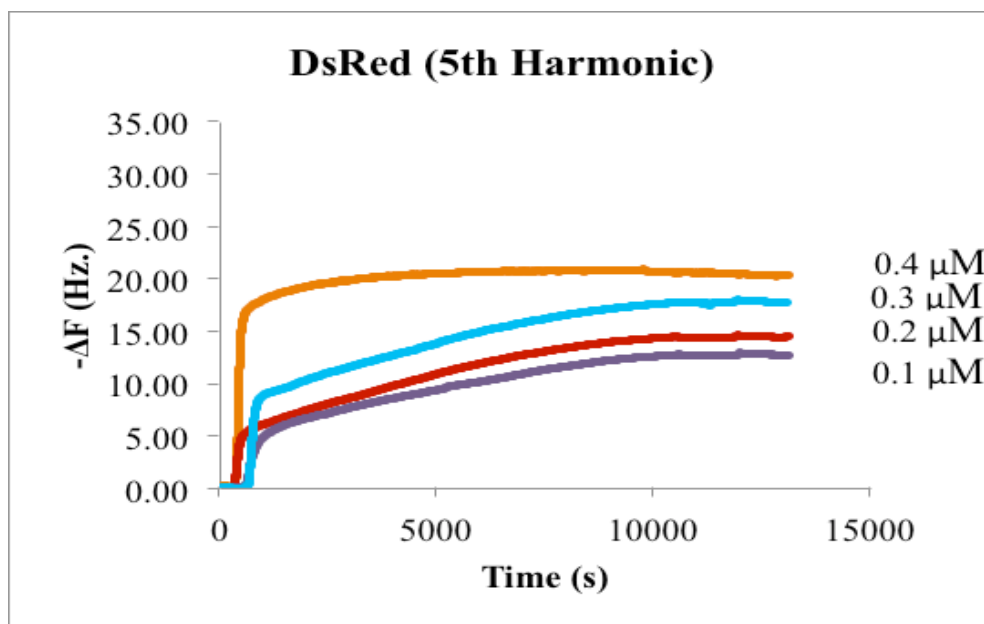


**Figure 3.21 :** QCM results for DsRed binding onto gold surface.

From the raw data, for each concentration of DsRed and DsRed-AuBP2, the DsRed-AuBP2 displayed a higher frequency shift than the DsRed wild type protein. Since the frequency shift directly relates to mass adsorption on the crystal, this result can

be interpreted as the amount of protein that was able to adsorb onto the surface given a specific concentration. It should be noted here however that in this type of measurement technique, the deposited mass that causes the frequency shift includes the water trapped or coupled inside the adsorbed layer of protein and does not actually represent the dry mass of this protein layer.

As can be seen from the results in Figure 3.20, DsRed-AuBP2 was able to adsorb more mass onto the gold surface of the crystal at each concentration. This is judged by the maximum frequency shift, which in this case relates to the saturation frequency seen after stabilization of the signal. While this result can be interpreted as simply the DsRed-AuBP protein was able to adsorb more on the surface compared to DsRed wild type, a more in depth analysis using a specific isotherm is needed to calculate the kinetic and thermodynamic properties of these proteins.

Various concentrations of PBS (1X, 1:2, 1:4, 1:8, 1:16, and 1:32) in succession were allowed to flow across the modules after the protein solution had completed and reached saturation. The step PBS wash procedure revealed that DsRed-AuBP2 largely remained bound to the electrode surface more so than the DsRed, specifically at 1:8 concentration. This would be the concentration used in later experiments for the wash step.

The change in dissipation, which reflects the viscoelastic properties of the layer being formed on the surface of the electrode, shows that while DsRed-AuBP seemed to reach the same max dissipation at each concentration, DsRed had a saturation point in the dissipation that was different for each concentration. Interesting to note that the max dissipation reached for DsRed at the highest concentration tested did not reach any of the maximum dissipations recorded for DsRed-AuBP. The results can be seen in Figure 3.22 and Figure 3.23

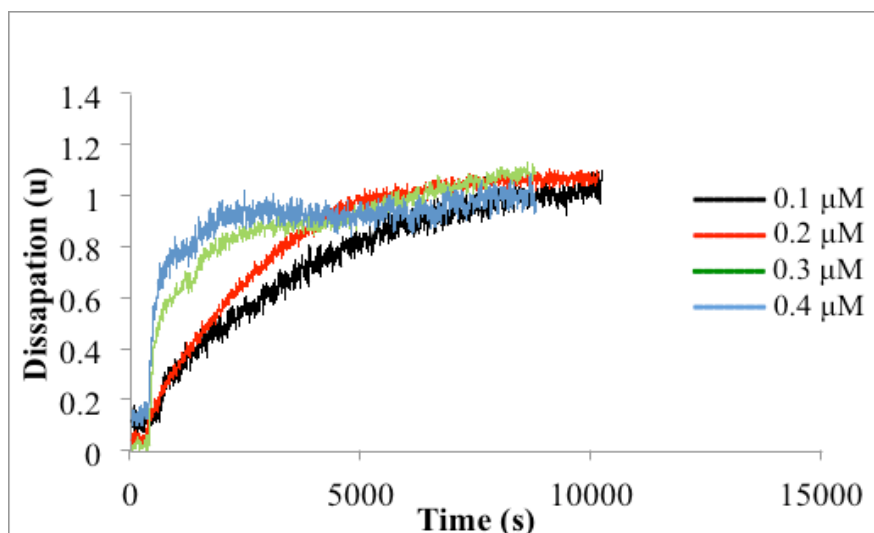


**Figure 3.22** : Dissipation data of DsRed-AuBP.

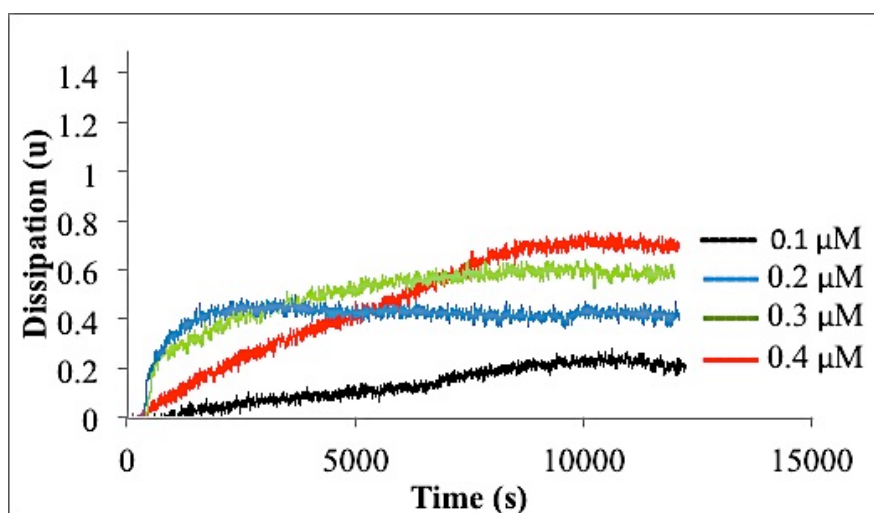


**Figure 3.23** : Dissipation data of DsRed.

### 3.2.3.2 Kinetics Isotherm & Langmuir

The most commonly used isotherm to describe protein adsorption behavior onto solid surfaces, largely due to its simplicity, is the Langmuir Isotherm. Although originally developed to model rigid particle adsorption onto solid surfaces, it has been repeatedly used by many research groups to analyze the much more complex process of protein adsorption. However, proteins do not behave like rigid particles in the presence of a solid interface. While rigid particles attach and detach from a solid with specific adsorption and desorption probabilities, the intricate nature of protein structures cause a multitude of other effects, such as cooperative effects, surface aggregation, size exclusion effects, and structural rearrangements. Due to this significantly more complex process that is slowly being revealed to us through research, several adaptations of the Langmuir Isotherm have been produced. One

such adaptation is what we developed here in an effort to break down a simple adsorption curve into two curves. This kind of separation of a single adsorption curve relating to protein adsorption has been done before, however this was just to show that a bimodal model fit the experimental data better than a single constant exponential model and did not calculate any specific kinetic parameters of the two curves. Here, we took that idea a step further and developed a program that not only has the ability to decompose a single curve into two curves, but also calculates specific characteristics of the curves that we can relate to kinetic properties.

By breaking a single adsorption curve up this way, we can assume the first curve predominately describes just the protein adsorption onto a ‘blank’ surface. We relate this first curve to what we call the ‘primary effect’ of the adsorption process. The primary effects of the protein adsorption process relate to how we would assume the protein would interact with strictly the surface, i.e. none to very minimal adsorbent-adsorbent interactions. The second curve we attribute to any ‘secondary effects’ that begin to happen as adsorption areas fill up on the surface. These secondary effects would include cooperative interactions between the absorbed proteins and the protein attempting to adsorb on the surface such as cooperative effects, diffusion, and even organization of the proteins on the surface. This analysis method would allow us to see the individual contributions of the two different effects to the overall adsorption curve and give us specific curve properties that can be translated into kinetic parameters for each.

We started from the original Langmuir isotherm, where the rate of the surface reaction is illustrated with the rate equation:

$$\frac{d\theta}{dt} = k_a(1 - \theta)C - k_d\theta \quad (3.4)$$

where  $\theta$  is the fraction of surface covered,  $C$  is the protein concentration and  $k_a$  and  $k_d$  are the adsorption and desorption constants, respectively. After the integration of Equation 3.4, the time evolution of the monolayer formation can be displayed as:

$$\theta(t) = \frac{C}{C + \left(\frac{k_d}{k_a}\right)} [1 - \exp(-(k_a C + k_d)t)] \quad (3.5)$$

then substitute  $\theta(\infty) = \frac{C}{C + \left(\frac{k_d}{k_a}\right)}$  and  $k_{obs} = k_a C + k_d$  into Equation 3.5, resulting in:

$$\theta(t) = \theta(\infty)[1 - \exp(-k_{obs}t)] \quad (3.6)$$

Assuming that there is a linear relation between  $\Delta f(t) = \alpha \theta(t)$  (where  $\alpha$  is the proportionality constant, Equation 3.6 can be expressed in terms of the measured frequency different ( $\Delta f$ ):

$$\Delta f(t) = \Delta f(\infty)[1 - \exp(-k_{obs}t)] \quad (3.7)$$

With using a single exponential model to describe the adsorption curve, the parameters of Equation 3.7 ( $\Delta f(\infty)$  &  $k_{obs}$ ) would then be optimized using a nonlinear optimization algorithm. This would allow the  $k_{obs}$  at different concentrations to be plotted with the slope and y-intercept of the best-fit line between these points being our  $k_a$  and  $k_d$  kinetics parameters. However, this is where we altered the traditional single exponential model equation (Equation 3.7) into a bi-exponential model. In order to do this we transformed Equation 3.7 into a Prony Series and set the total frequency shift ( $\Delta f$ ) as a sum of exponentials:

$$\Delta f(t) = \sum_{i=1}^n A_i (1 - e^{-k_i t}) \quad (3.8)$$

where A has been substituted for  $\Delta f(\infty)$  and represents the max frequency shift of the individual curve described by the k constant (Equation 3.8). This series analysis method would potentially allow us to account for more than two effects by breaking the entire curve up into several curves, however here we only explore the kinetic parameters given to us by a two series expansion, giving us a bimodal model:

$$\Delta F(t) = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) \quad (3.9)$$

Where  $k_1$  describes the nature of the primary curve,  $A_1$  is the max frequency shift of the curve described with  $k_1$ , with  $k_2$  and  $A_2$  having the same relationship. Using this mathematical derivation of the Langmuir isotherm as our model, we were able to input our raw data into the Matlab program we developed and effectively fit the raw data with the generated model. The model could then be analyzed using the same technique described with the single exponential model. The result would be a k constant for both the primary and secondary curves that could then be graphed with concentration to obtain adsorption and desorption kinetic parameters of both effects.

For this mathematical model to be an effective analysis method for quantifying our protein adsorption data, a program was needed that could accomplish three goals: accurately model the raw frequency shift data from the QCM-D using Equation 3.9, decompose that model into a primary and secondary curve, and to calculate the characteristics of the individual curves. Those characteristics that describe the nature

of the individual curves could then be translated into kinetic properties of the two proteins being compared. To accomplish these goals, we developed two programs that run in conjunction with each other. The first establishes a base model using Equation 3.9 and a non-linear regression method to locally optimize the model. The second uses the best local optimum found in the first program and further optimizes the model within a stricter set of parameters. The result is a very repeatable set of curve features that describe the primary and secondary curves.

It can be seen from Figure 3.20 and Figure 3.21 that at each concentration tested with the QCM-D, the genetically modified DsRed-AuBP2 protein was able to deposit more mass on the surface than its wild type protein, DsRed. Since mass deposition directly relates to adsorption we can say more DsRed-AuBP protein was able to adsorb onto the gold electrode surface compared to DsRed. This can be simply seen by graphing the raw data. A more in depth analysis was made possible by using the Matlab program described above. The program was successfully able to break the adsorption curves from the raw data up and provide characteristic curve properties for all curves generated. These curve properties could then be translated into kinetic properties used to describe the adsorption phenomena for each protein. The quantification of these parameters could then be compared between the two proteins.

The results of the first step of using our program to build an accurate model to describe the raw QCM-D data can be seen in Figure 3.24 and Figure 3.25.

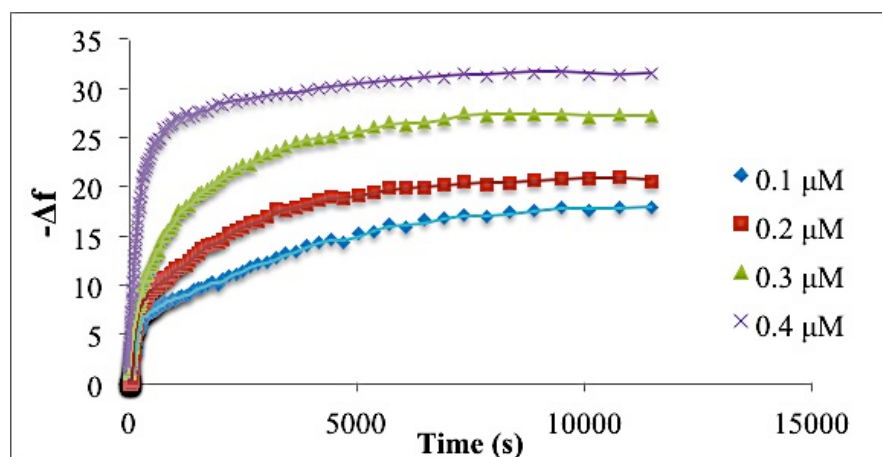


**Figure 3.24 :** Modeling data of DsRed-AuBP.

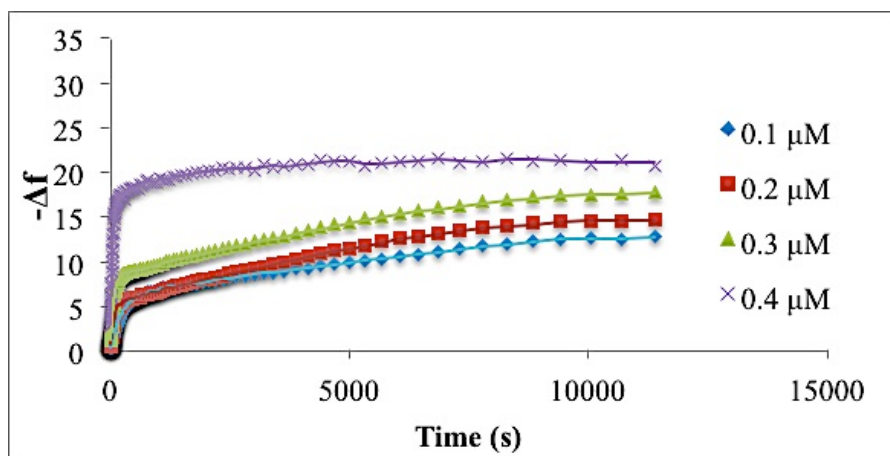


**Figure 3.25 :** Modeling data of DsRed.

As can be seen, the models developed for each individual concentration of both proteins closely follows the raw adsorption data. After modeling the data, the program decomposed each model into two individual curves that represented the primary and secondary effects. Both effects can be quantified by three parameters of the curve: amplitude of the curve or maximum frequency shift ( $A$ ), the curvature of the arc ( $k$ ), and the time that corresponds to  $\frac{1}{k}$  ( $t$ ). An example of this can be seen in Figure 3.26.

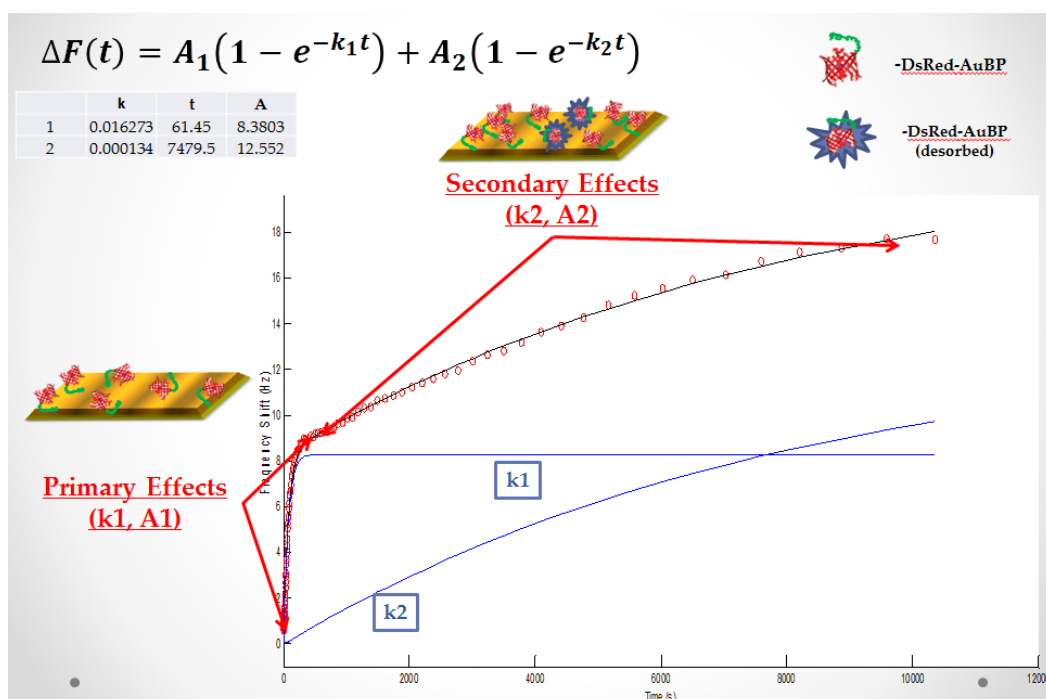


**Figure 3.26 :** Adsorption and desorption of DsRed-AuBP. Each model into two individual curves that represented the primary and secondary effects.

Each of the three parameters can be translated into an individual property of the entire adsorption. The amplitude ( $A_1$ ) describes the maximum amount of protein that was able to adsorb onto the surface during that effect. The curvature ( $k$ ) describes the rate at which the protein reached maximum adsorption for that effect. The time ( $t$ ) describes at what time roughly 60% of the adsorption for that effect has already taken place. Together they help paint a bigger picture of the adsorption process that is occurring during each effect.

While the amplitude and time of each effect can be compared directly for each protein, the curvature ( $k$ ) can be further analyzed to retrieve specific adsorption and desorption properties relating to the protein. This can be done by taking the calculated  $k$  value for each concentration and plotting those vs concentration. According to Equation 3.9, the slope and y-intercept of the best-fit line of these data points relates to the adsorption and desorption characteristic of the protein. Since we break this process up into primary and secondary effects, we can use this method to calculate these characteristics for both effects. The results can be seen in Figure 3.27.

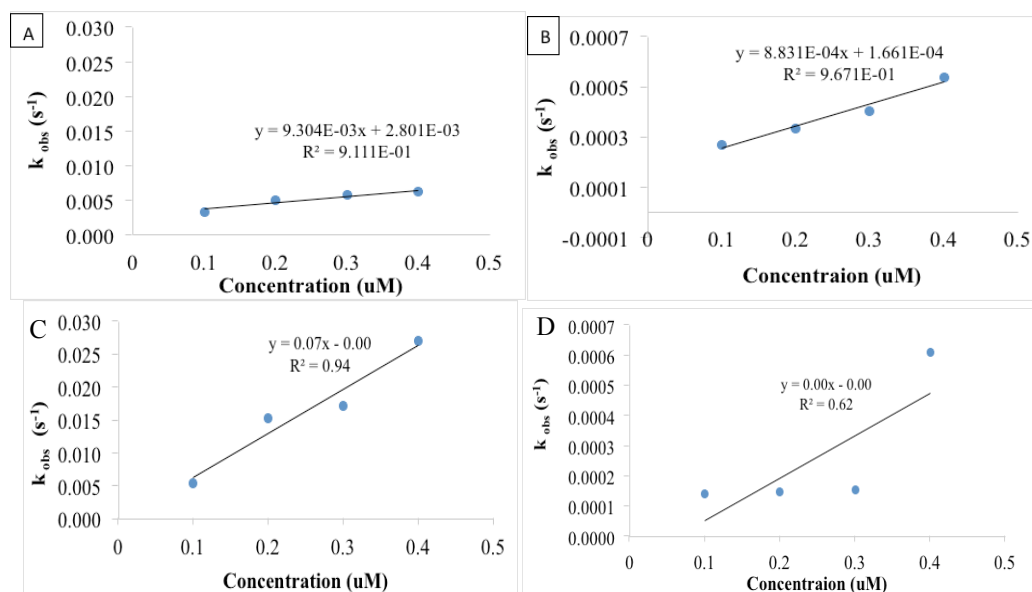


**Figure 3.27 :** Primary effect (A) and secondary effect (B) of binding process on DsRed-AuBP2, primary effect (C) and secondary effect (D) of binding process on DsRed.

DsRed-AuBP reached equilibrium slower but had more mass deposited. DsRed reached equilibrium faster but was unable to deposit as much mass (adsorb as much protein) perhaps DsRed may be agglomerated on the surface and not allow further proteins to be adsorbed while DsRed-AuBP is taking a longer time to adsorb

complete due to the surface organization occurring at the interface due to the AuBP tag.

### 3.2.3.3 Self -assembly of protein to form nanolayer

Due to its parallel nature, micro-contact printing ( $\mu$ CP) has been proven to be a convenient technique to pattern a variety of biological molecules with sub-micron features over a large area ( $> 1 \text{ cm}^2$ ) without the need for expensive lithographic equipment. In our previous work, we studied extensively combinatorial selected and designed peptides' adsorption and demonstrated their role on surface binding [69]. Here, we studied the fusion protein's assembly on gold surface compared with control protein, which lacks the gold binding peptide.

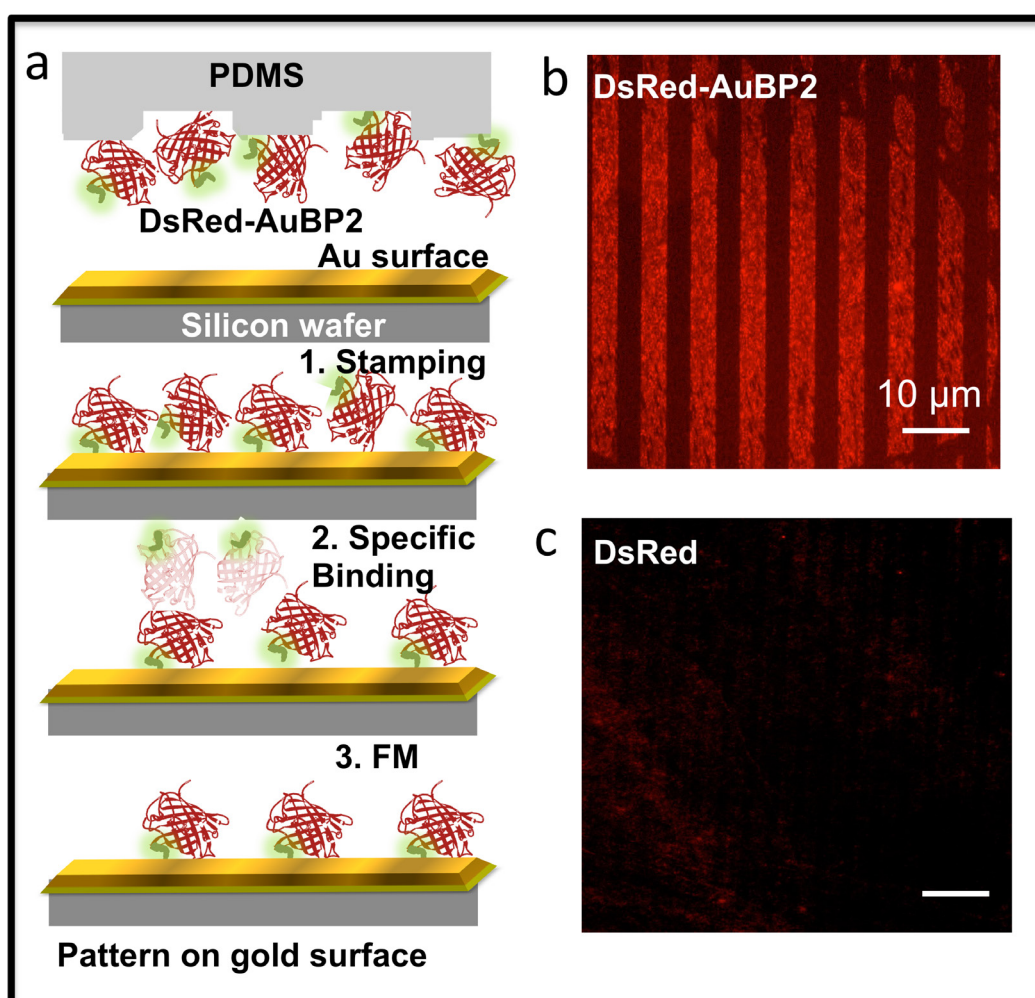


**Figure 3.28 :** Protein Patterning onto a Flat Gold Surface with the Micro-Contact Printing Technique: schematics of micro-contact printing (a), fluorescent images of 50  $\mu\text{M}$  DsRed- AuBP2c protein (b) and wild-type DsRed-monomer protein as control (c).

We first generated the gold surface using the micro-contact printing and applied the proteins onto the surfaces. Figure 3.28 shows the fluorescence microscopy images of the proteins on a patterned gold substrate. These images demonstrate the organization of DsRed-AuBP2c fusion protein onto gold surface is effective compared to control, i.e. DsRed- monomer. The fusion proteins form well-defined, self-organized arrays that feature almost none non-specific binding onto the undesired regions.

The effect of the immobilization protocol on the efficiency of adsorption and the fluorescence intensity was explored by comparing DsRed-Monomer and DsRed-AuBP2c fusion. The AFM image (Figure 3.29 recorded in tapping mode with silicon cantilever at scan rate 1 Hz) shows that DsRed-AuBP2c binds to the gold region of the patterned surface.

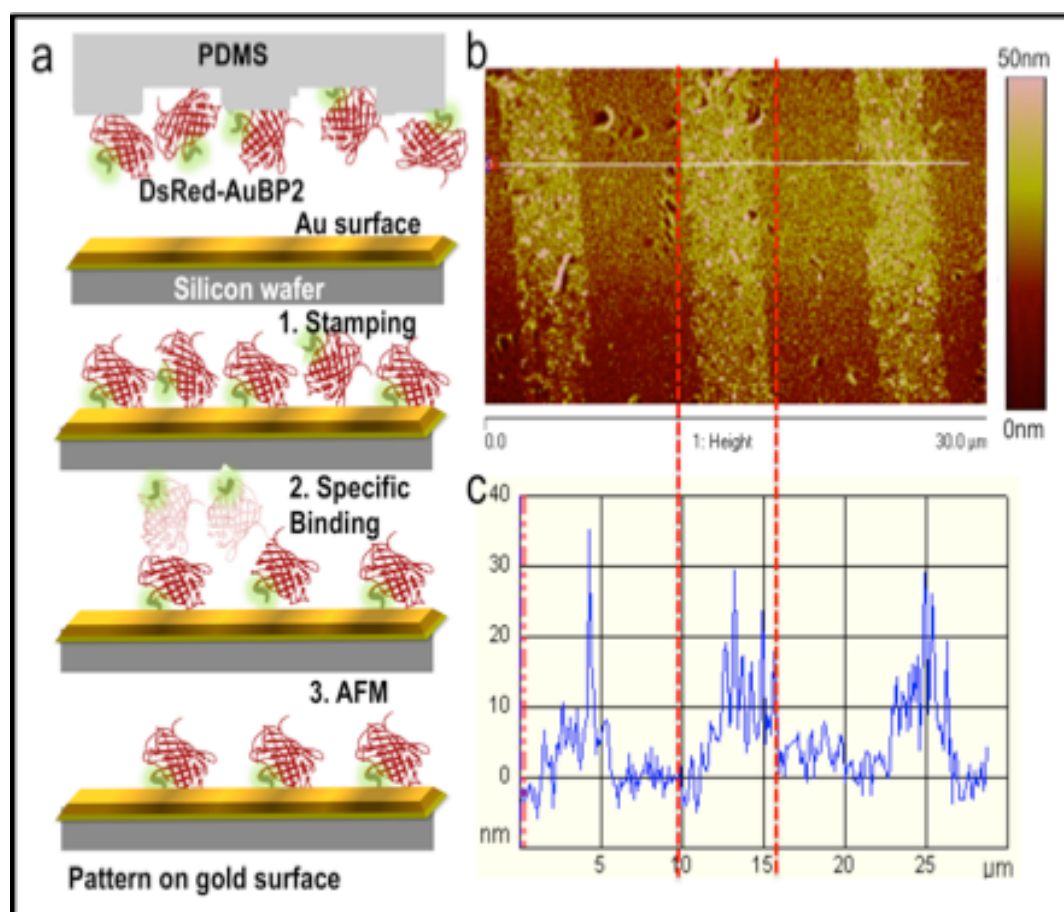


**Figure 3.29 :** Schematic representation of the experimental procedure for the generation of two-dimensional arrays of immobilized proteins on a patterned substrate fabricated through  $\mu$ CP (a) and AFM image of gold substrate following self-assembly of DsRed-AuBP2c (b).

Combining micro-contact printing ( $\mu$ CP) technology and bio-molecular self-assembly, we successfully demonstrated a bio-friendly, one-step surface functionalization process to develop protein arrays on gold surfaces. The AuBP2c peptide tag provided the self-immobilization on the gold surface, consequently directing the engineered fluorescence protein self-assembly to develop an efficient and well-defined protein based pattern.

Interestingly, the immobilization of DsRed-AuBP2c onto the patterned surface resulted in higher fluorescence intensity, when compared to DsRed alone. Using an inorganic binding peptide (GEPI) as the fusion linker offers variety of advantages. For example, the efficient patterning allows for compact assembly in a confined area on the surface. GEPIs feature enhanced control over protein immobilization that may increase the local folding stability. Therefore, the inorganic binding peptide, AuBP2, not only provides specific material selective adsorption onto the gold surface, but also allows for oriented immobilization of proteins while maintaining their fluorescent properties.

### **3.2.4 Gold nanoparticle-based detection methods**

#### **3.2.4.1 Localized surface plasmon resonance (LSPR) for DsRed proteins**

In this study LSPR biosensing measurement was performed based on the resonant interaction induced by the biomolecules binding onto the nanoparticle surface. The experimental schematic is shown in Figure 3.29. The change in the local dielectric owing to molecule recognition and binding was measured by a shift in the LSPR  $\lambda_{\text{max}}$ . An ideal biosensor should be both specific and sensitive to the target analyte.

While the experiments aim to quantify and improve biosensor specificity, sensitivity should be inherent to the specific biosensor system. Therefore, new method was examined to increase the biosensor sensitivity through inorganic binding peptide that increases the  $\lambda_{\text{max}}$  shift from the same amount of analyte.

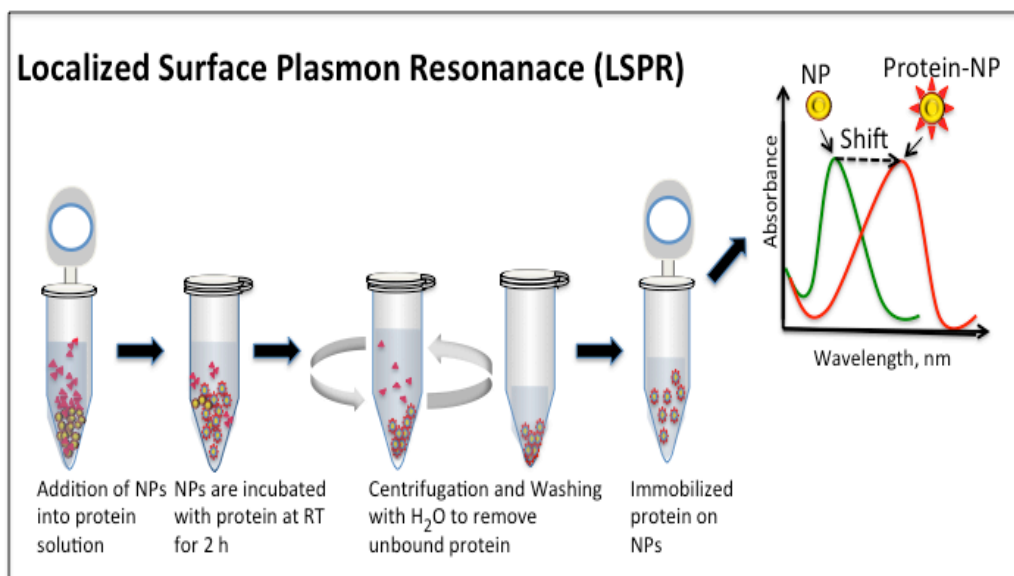


**Figure 3.30 :** Schematic representation of the experiment of Localized Surface Plasmon Resonance (LSPR).

Owing to the short (3-5 nm) decay length of LSPR sensors, the sensitivity losses appear when detection system bases on longer molecular linkers or ligands such as in ELISA. In the sandwich-style ELISA the detecting molecule is placed far from the nanoparticle surface and outside the sensing volume. Therefore, we demonstrated that nanoparticle-labelled sensing material would have a significant impact on the LSPR  $\lambda_{\text{max}}$  shift by the genetically engineered protein. For this, we used our bi-functional fluorescence protein, DsRed-AuBP2. This bi-functional red fluorescence protein was conjugated to various sizes of Au colloids, 5 nm, 15 nm and 50 nm. As expected, the  $\lambda_{\text{max}}$  shift was enhanced, with the degree of enhancement increasing with the concentration of DsRed-AuBP2-AuNP conjugate (Figure 3.31 and Figure 3.33). DsRed-AuBP2-NP conjugate was stable after rinsing with DI water and the enhanced  $\lambda_{\text{max}}$  shift was less change compared with DsRed (Figure 3.32). Here, DsRed-monomer let Au NP agglomerate after rinsing with DI water. Therefore, LSPR shift of DsRed-monomer was not observed. Apart from these results, the inorganic binding peptide tag of DsRed-AuBP2-AuNP conjugate increased not only  $\lambda_{\text{max}}$  shift, but also binding affinity between DsRed and nanoparticle. Based on surface area calculations, each gold colloid was functionalized with between 20-30 protein molecules, depending on AuBP orientation. This means that each interaction between the DsRed and the gold surface is mediated by gold binding peptide (AuBP). The improved affinity induced by nanoparticle-protein conjugate offers an additional source of sensitivity for the plasmonic label-enhanced LSPR sensor

because biosensor sensitivity is inherently based on the strength of the binding interaction between surface and the analyte.

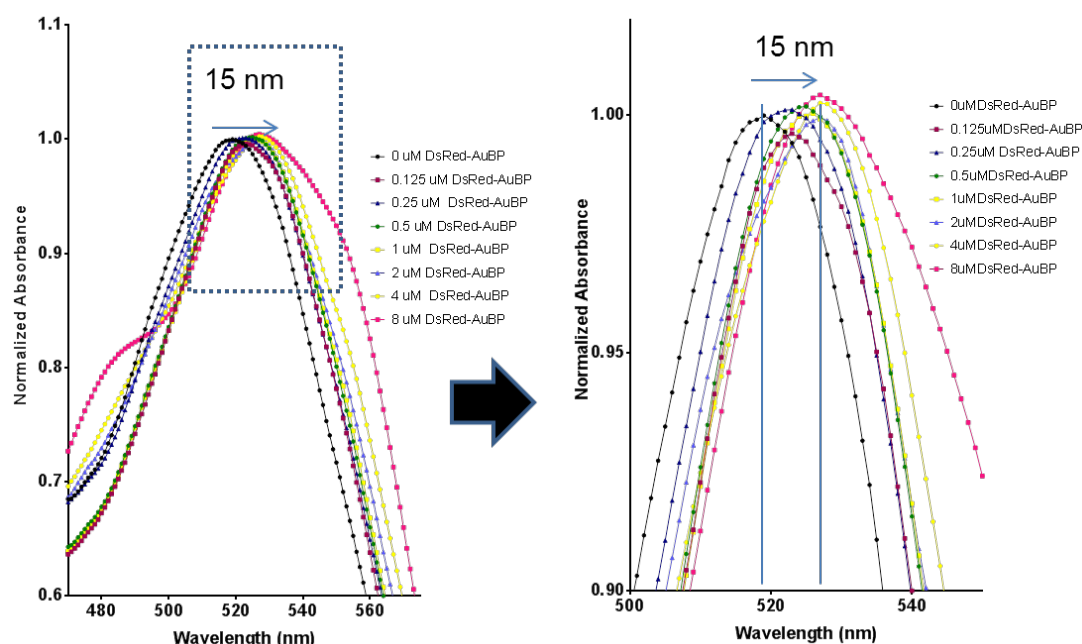


**Figure 3.31 :** Absorbance spectrum of normalized data from LSPR  $\lambda_{\text{max}}$  shift of 0.125-8  $\mu\text{M}$  DsRed-AuBP2 with 100  $\mu\text{M}$  Au NP (15 nm).

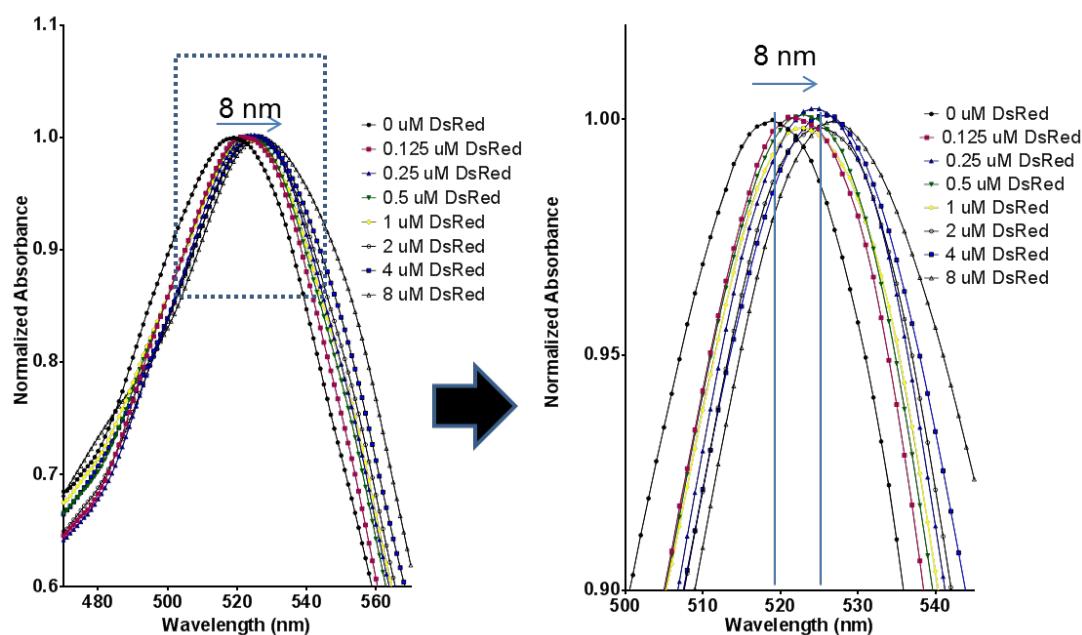


**Figure 3.32 :** Absorbance spectrum of normalized data from LSPR  $\lambda_{\text{max}}$  shift of 0.125-8  $\mu\text{M}$  DsRed-monomer with 100  $\mu\text{M}$  Au NP (15 nm).

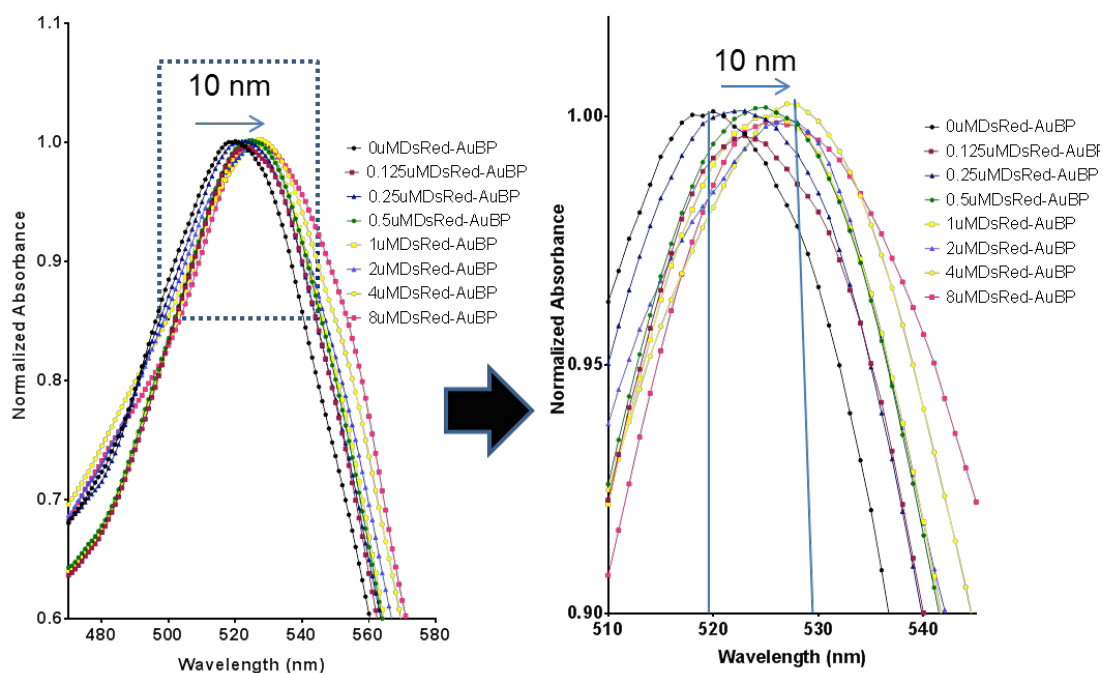


**Figure 3.33 :** Absorbance spectrum of normalized data from LSPR  $\lambda_{\text{max}}$  shift of 0.125-8  $\mu\text{M}$  DsRed-AuBP2 with 100  $\mu\text{M}$  Au NP (15 nm) after washing with water.

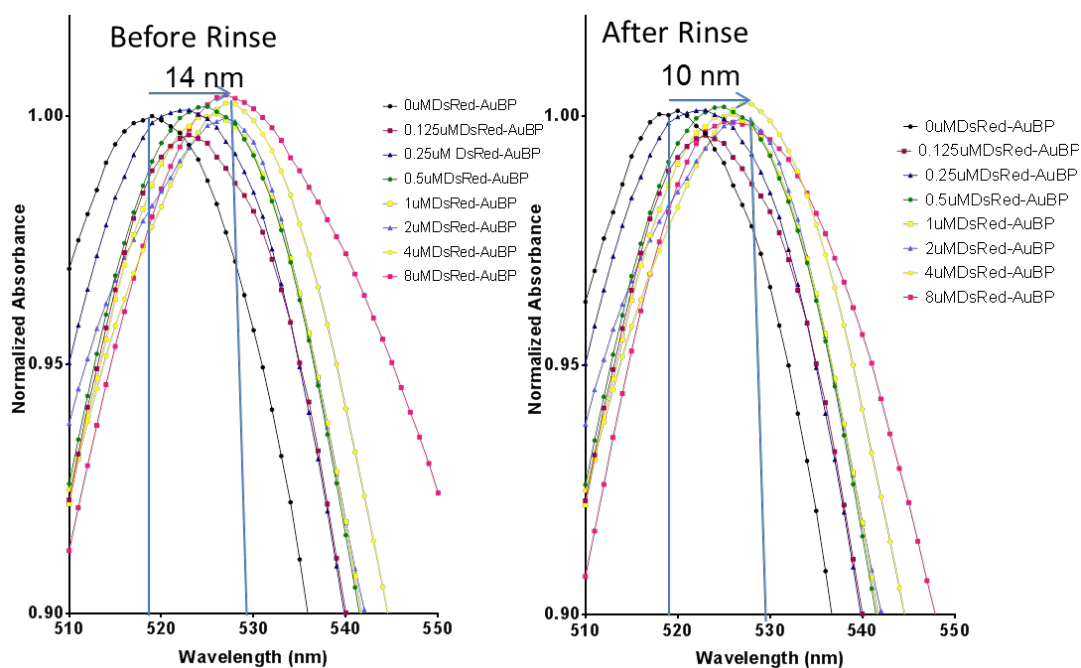


**Figure 3.34 :** **Before Rinse:** Absorbance spectrum of normalized data from LSPR  $\lambda_{\text{max}}$  shift of 0.125-8  $\mu\text{M}$  DsRed-AuBP2 with 100  $\mu\text{M}$  Au NP, **After Rinse:** Absorbance spectrum of normalized data from LSPR  $\lambda_{\text{max}}$  shift of 0.125-8  $\mu\text{M}$  DsRed-AuBP2 with 100  $\mu\text{M}$  Au NP after washing with water.

The effect of Au NPs on LSPR  $\lambda_{\text{max}}$  shift of the DsRed-AuBP and DsRed is illustrated in Figure 3.35 and Figure 3.36. Evidently, Au NPs having a diameter in a range between 5-50 nm efficiently affect the LSPR  $\lambda_{\text{max}}$  of DsRed-AuBP2, as evidenced by the progressive increase in the LSPR  $\lambda_{\text{max}}$  shift with increasing NP size. This result indicates a direct interaction with the Au NP and DsRed-AuBP2.

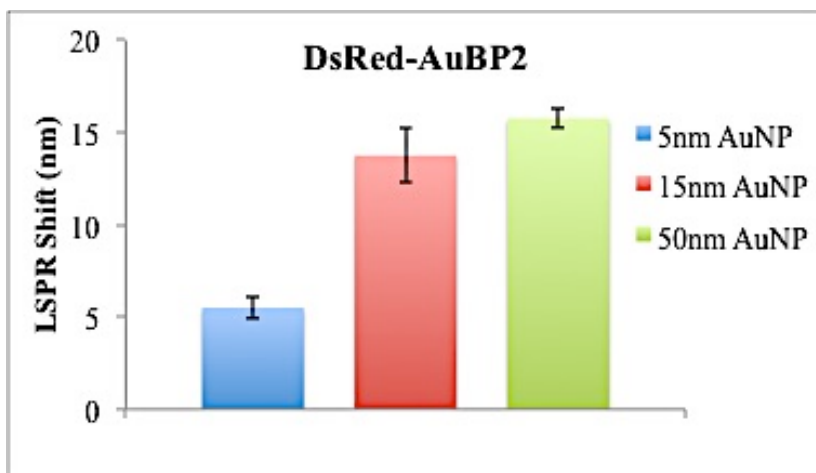


**Figure 3.35 :** LSPR  $\lambda_{\text{max}}$  shift of DsRed-AuBP2 with different size of Au NPs.

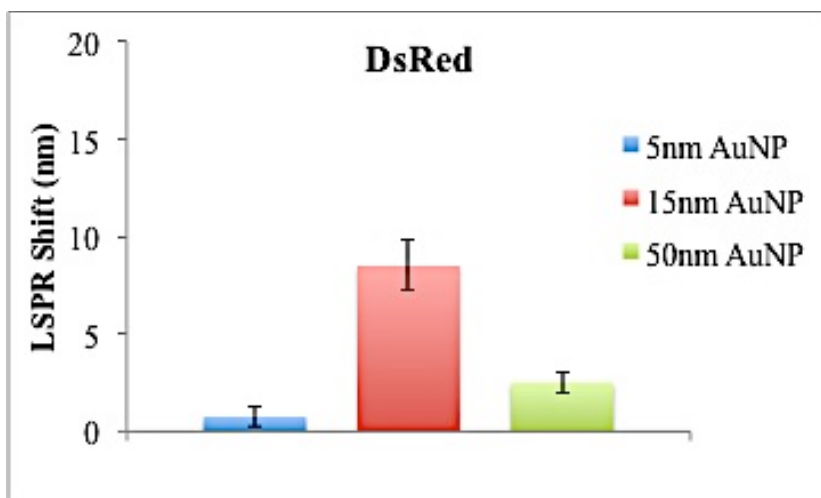


**Figure 3.36 :** LSPR  $\lambda_{\text{max}}$  shift of DsRed with different size of Au NPs.

Stronger binding of the proteins to the NP naturally should also give rise to conformational changes of the protein, where the functional amino acids of the bound protein are in proximity with surface, and more LSPR  $\lambda_{\text{max}}$  shift. Particle size, charge, and other thermodynamic factors affecting the strength of protein-NP should influence the binding equilibrium constant ( $K_D$ ), which quantifies the relative strength of protein-NP binding.

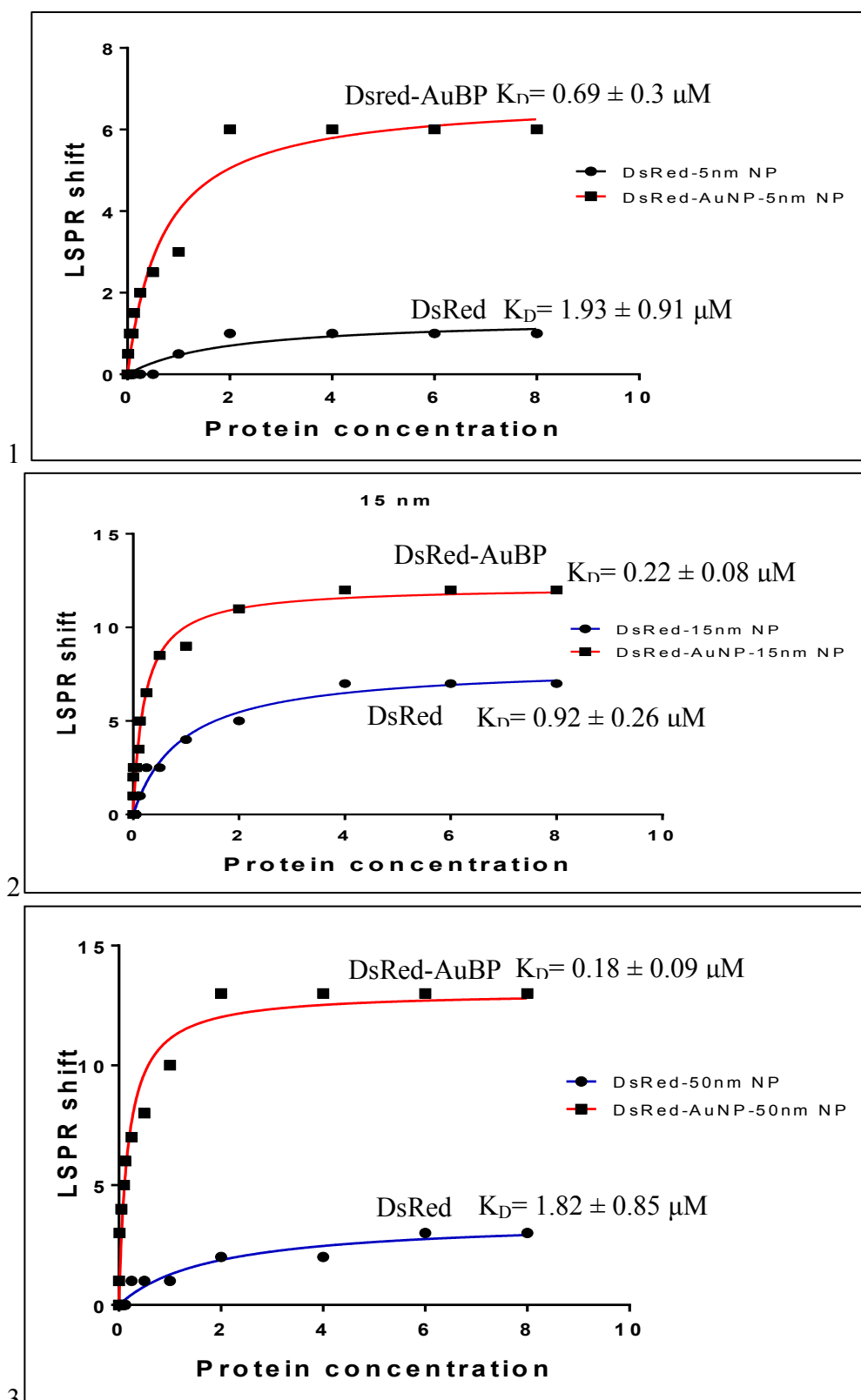


**Figure 3.37 :** Binding equilibrium constant values ( $K_D$ ) of proteins with Au NPs.

Nanoparticles in the diameter range 1-100 nm have physical properties, which are dependent on the size, shape, distance of interparticle, and nature of shell layer about nanoparticle. There are several reasons related to binding of protein to nanoparticles.

Specifically, the orientation of the protein onto the nanoparticle surface and chemical interactions between them were considered in this section. There is the more subtle matter of multifunctional nature of binding interactions of the proteins to the NP, which is function of the inorganic binding peptide. We quantitatively determine a measure of the binding below and explore how NP size affects this specific binding property.

The binding equilibrium constant ( $K_D$ ) for DsRed and DsRed-AuBP onto Au NPs were found to be in the range of  $10^6$  to  $10^7$  mol/L, the same range as that found previously for the binding of MBP-AuBP1 and wt-MBP to 15 nm citrate-coated Au NPs. As shown in Figure 3.37, the binding equilibrium constants for DsRed-AuBP2 onto Au NPs decrease progressively with NP diameter in the range of 5 to 50 nm. This means that DsRed-AuBP2 has almost high binding ability to Au NP. However, DsRed displays lower binding ability because  $K_D$  values are higher than of DsRed-AuBP2. The enhanced binding affinities of the engineered DsRed-AuBP2 to gold surfaces can be attributed to the AuBP2 peptide.

#### **3.2.4.2 Fluorescence quenching studies for DsRed proteins**

The application of fluorescence spectroscopy to the study of the function, structure and conformation of proteins has proven to be successful. Especially, fluorescence quenching has been widely applied in biochemical issues due to its high sensitivity and reproducibility. The emission characteristics of fluorescence proteins can provide convenient handle for researching conformational changes and binding abilities upon association with small molecules such as nanoparticles. This present study takes advantage of the fact that gold capably quenches the emission of the chromophore of the red fluorescence proteins, DsRed-AuBP2 and DsRed. The efficiency of this fluorescence quenching depends on the proximity or specific interaction between the quencher and the chromophore of fluorescence protein. The measurements of fluorescence quenching by DsRed proteins expose information over the accessibility of Au NPs to protein chromophore group. The experimental approach is illustrated in Figure 3.38.

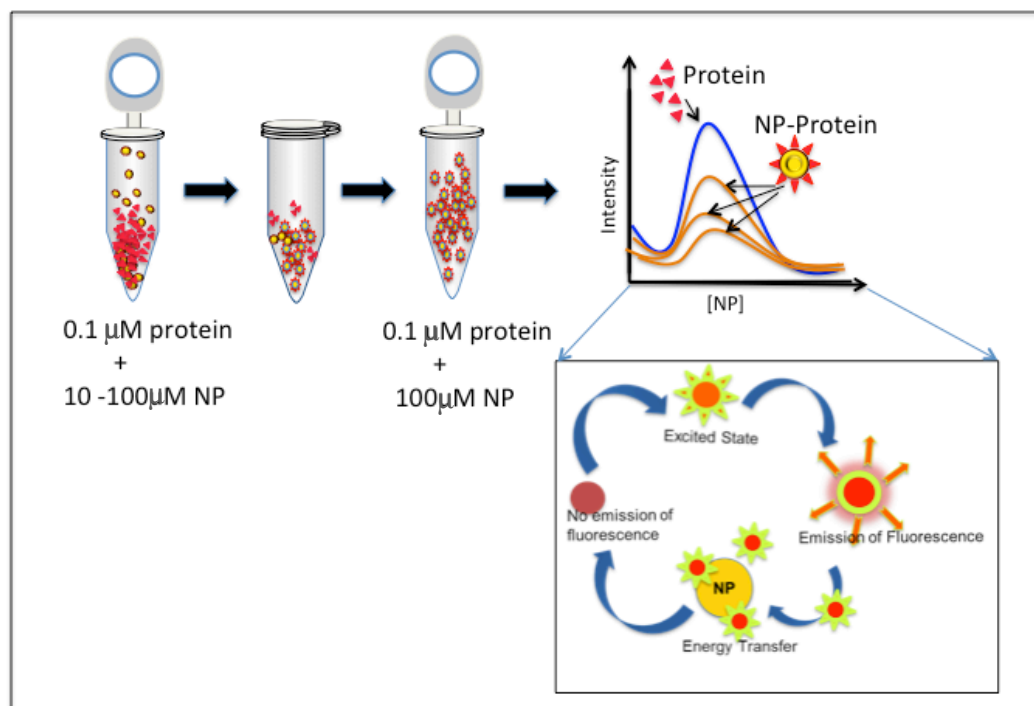


**Figure 3.38 :** Schematic illustration of quenching study of fluorescence proteins by Au NPs.

The effect of Au NP on the fluorescence intensity of our bi-functional protein containing AuBP, DsRed-AuBP2, and DsRed-monomer was illustrated in Figure 3.39 and Figure 3.40.

The emission characteristic of DsRed chromophore tripeptide amino acid residues (Gln-Tyr-Gly) in protein can provide convenient handle for investigating binding and conformation changes upon association with small molecules and nanoparticles. Gold efficiently quenches the emission of many chromophores. The efficiency of the fluorescence quenching depends on the distance between quencher and chromophore.

The effect of Au NPs on the fluorescence intensity of DsRed proteins is illustrated in Figure 3.39 and Figure 3.40, which show the emission spectra of DsRed-AuBP2 and DsRed in the control case where the NPs are absent and in presence of Au NPs. In these figures, Au NPs quenched the proteins as evidenced by the progressive decrease in the emission maximum intensity with increasing NP concentration. This quenching effect indicates significant information about the fluorescence protein layer adsorbed onto Au NP.

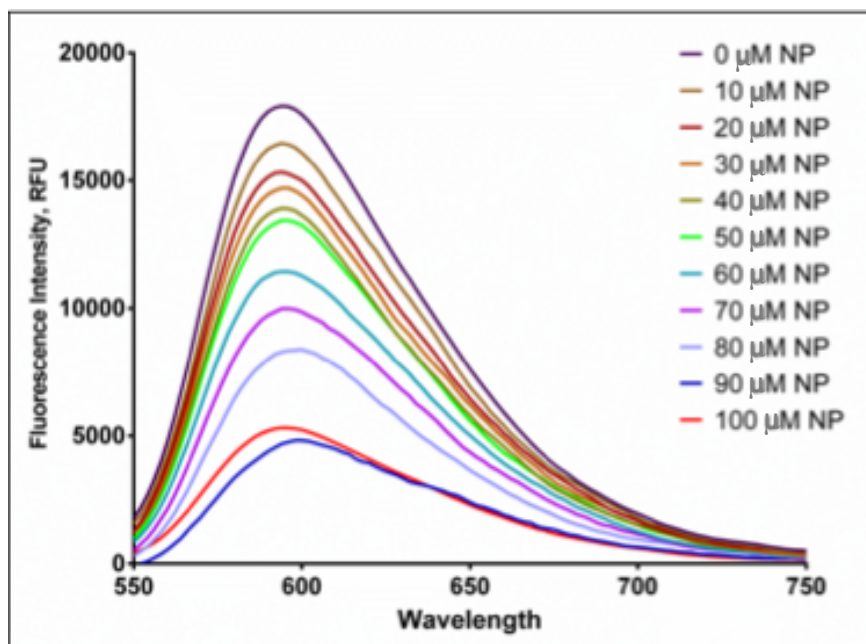


**Figure 3.39** : Fluorescence quenching of DsRed-AuBP2 by Au NP measuring.

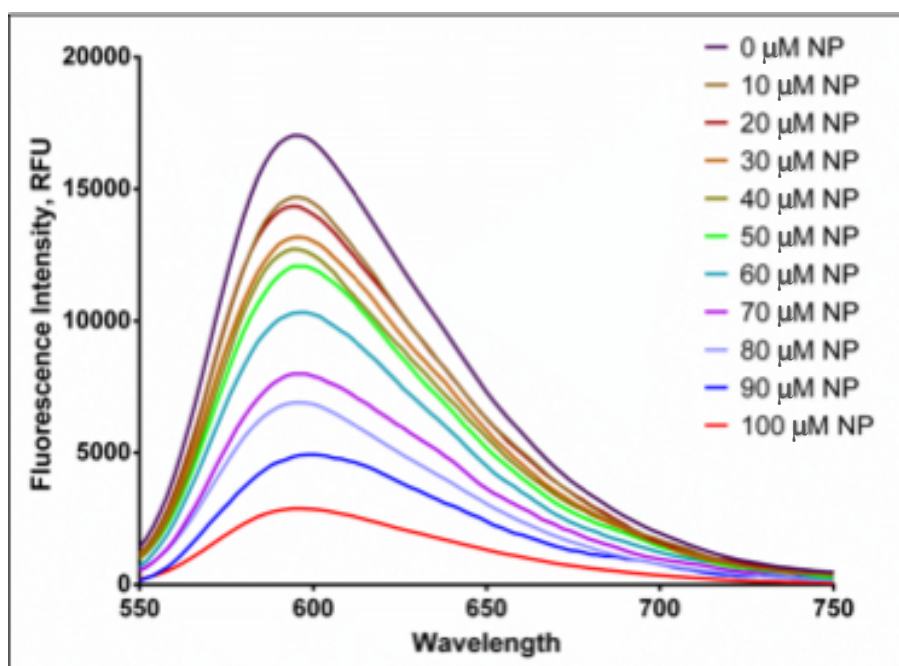


**Figure 3.40** : Fluorescence quenching of DsRed-monomer by Au NP measuring.

The change in maximum fluorescence intensity  $I_{\max}$  arises from molecular contacts, which in the present case is owing to adsorption onto the NPs, and reflects the changes in the proximity between the active fluorescence sample and the quencher (Au NP). The strong decrease in  $I_{\max}$  with NP size indicates that the relative fluorescence quenching is increasing with protein concentration, as shown in Figure 3.31 and Figure 3.32, since the amount of absorbed protein follows this basic trend. The results from quenching measurements showed that DsRed-AuBP2 has better

binding to Au NP than of DsRed-monomer. Also, these findings supported results from LSPR based methods in Section 2.5.2. Stronger binding of the proteins to the Au NP caused conformational changes of the protein, where AuBP2 tag of bound protein in proximity with the surface, and more efficient fluorescence quenching.

Indeed, a variety of molecular interactions, such as excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional mechanism, can result in quenching. Quenching can be from collisional encounters between the fluorophore and quencher, which is called collisional or dynamic quenching. If quenching resulting from binding between the fluorescence sample and the quencher, it is called static quenching, which can give valuable information about binding affinity. Both static and dynamic quenching requires molecular contact between the fluorescence sample and quencher. In collisional quenching, the quencher must diffuse to the fluorophore during lifetime of excited state. In the case of static quenching, a complex, non-fluorescent, is formed between fluorophore and quencher

Quenching measurements can reveal the accessibility of fluorophores to quenchers. Furthermore, it is an important way to investigate the binding of materials to proteins and their transport. The understanding of protein-ligand binding is of prime interest in biochemistry and life sciences.

To investigate whether the mechanism of this quenching is a dynamic or static process we generated a Stern-Volmer plot [43] for DsRed and DsRed-AuBP. This plot was generated by measuring the fluorescence of the protein upon the addition of quencher at a variety of points of temperatures. The relative kinetic efficiency of fluorescence quenching can be estimated by fitting the dependence of  $F_0/F$  on Au NP concentration (Figure 3.41 and Figure 3.42) based on a fitting to the Stern-Volmer equation

In the presence of Au NP, a different type of quenching was observed with DsRed-AuBP2 and DsRed-monomer. We used Stern-Volmer plot to understand the mechanism responsible for quenching. These plots were constructed by plotting the measured fluorescence of each protein after the addition of Au NPs. Linear plot for DsRed-AuBP (Figure 3.41) and upward curve for DsRed-monomer (Figure 3.42) were generated. These results indicated a static process in that only one type of

quenching occurred when DsRed-AuBP2 was conjugated with Au NP (Figure 3.41 and Figure 3.42). Also, DsRed-monomer showed a combined quenching (Figure 3.42 and Figure 3.43).

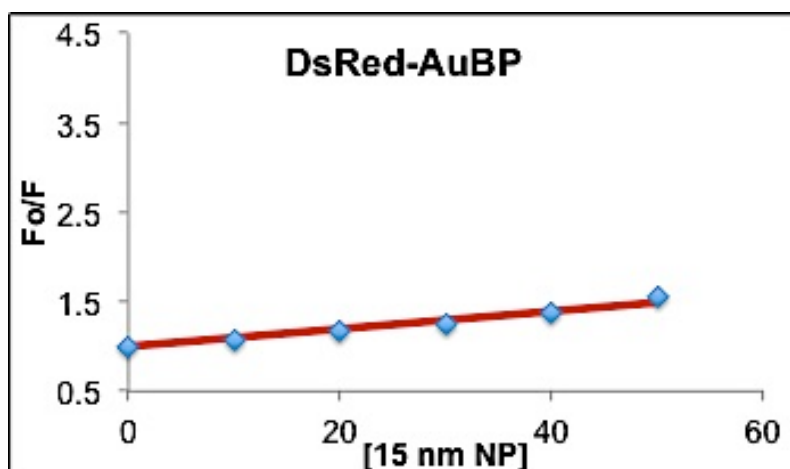


**Figure 3.41 :** Static quenching mechanism of DsRed-AuBP2 for Au NP.

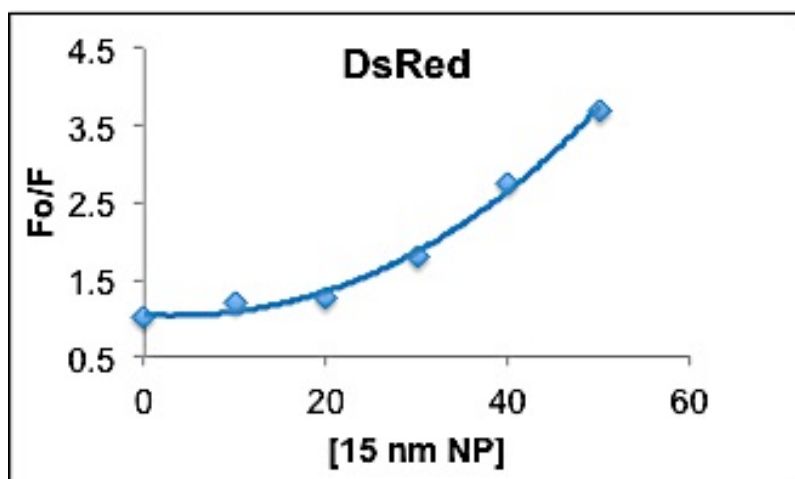


**Figure 3.42 :** Combined quenching (dynamic and static) plot of Au NP effects to DsRed-monomer fluorescence intensity.

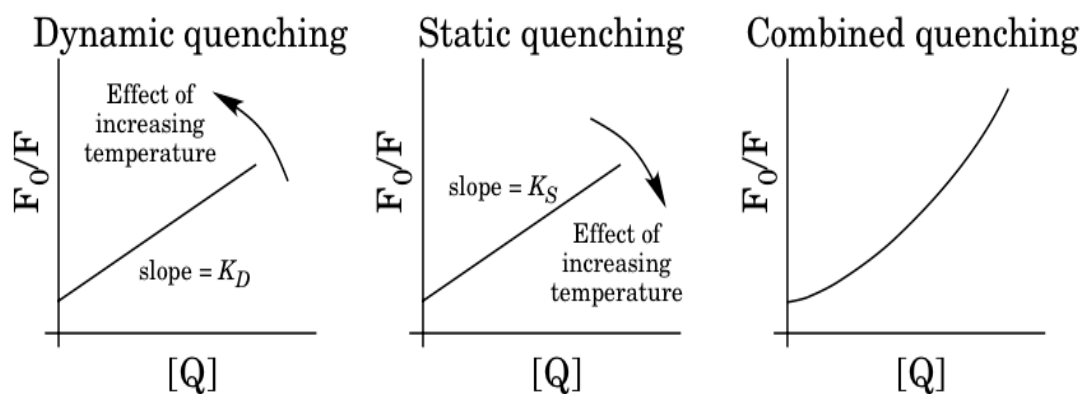


**Figure 3.43 :** Quenching process at different states.

Quenching types exhibit a concentration-dependence that is described by the Stern-Volmer (Equation 3.10). The Stern-Volmer expression is the linear in quencher concentration,  $[Q]$ , and  $K_{sv}$  is obtained as the slope of  $F_0/F$  vs  $[Q]$ , if the assumed mechanism of quenching is effective. Plotting  $F_0/F$  versus  $[Quencher]$  two types of plots may be obtained. A linear Stern-Volmer plot indicates that only one type of quenching, static or dynamic occurs. When the same fluorophore can be quenched both for collisions and by complex formation, an upward curvature is observed (Figure 3.43). The plot obtained with DsRed is shown in Figure 3.42 and it deviated significantly from linearity, which is indicative of interactions occurring between Au NP and DsRed-monomer when the protein is in its ground electronic state (static quenching) and in its first excited single state (dynamic quenching)

In such circumstances it is more convenient to use the modified form of the Stern-Volmer equation:

$$F_0/F = (1 + K_D [Q])(1 + K_{sv} [Q]) \quad (3.10)$$

where  $K_D$  and  $K_{sv}$  are the dynamic and static quenching constants, respectively. This equation is second order in quencher concentration, which accounts for the upward curvature observed when both static and dynamic quenching occurs for the same fluorophore.

Temperature effects can be used to distinguish the two forms of quenching. Diffusion rates, and therefore dynamic quenching rates, increase with higher temperature. In contrast, complex formation strength tends to be inversely proportional to temperature, and therefore static quenching tends to be higher at lower temperatures. The plot obtained with DsRed- AuBP2 is shown in Figure 3.44. It shows that DsRed- AuBP2 binds to Au NP to form a complex hybrid molecule because there is a static quenching between fluorescence protein and nanoparticle.

In quenching experiments with DsRed proteins, the quenching agent may interact with the protein in ways that alter the protein structure or that affect the degree of quenching observed. Here, the relative kinetic efficiency of fluorescence quenching was estimated by fitting dependence of  $F_0/F$  with Au NPs based on a fitting to Stern-Volmer equation. We found that  $K_{sv}$  of Dsred-AuBP2c is higher than of DsRed, and, respectively. This means DsRed-AuBP2c ( $K_{sv} \sim 54000 \text{ M}^{-1}$ ) has higher fluorescence intensity than DsRed-monomer ( $K_{sv} \sim 20000 \text{ M}^{-1}$  and  $K_D \sim 15000 \text{ M}^{-1}$ ). Additionally,

we calculated the quenching rate constants, which are  $6.2 \times 10^{12} \text{ M}^{-1}\text{s}^{-1}$  for DsRed-monomer and  $1.4 \times 10^{13} \text{ M}^{-1} \text{ s}^{-1}$  for DsRed-AuBP2c. It is proportional to the effectiveness of the quencher and the accessibility of the fluorophore to collisions with the quencher.

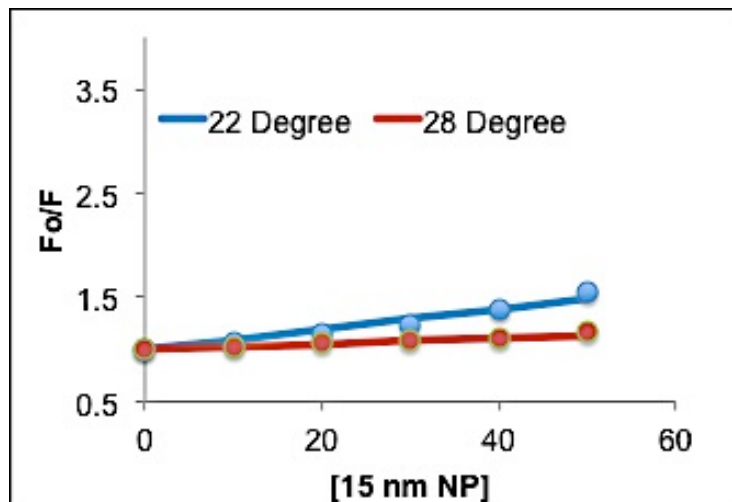


**Figure 3.44 :** Static quenching mechanism of DsRed-AuBP2 for Au NP at different temperatures.

Quenching mechanism of DsRed proteins were also investigated with 5 nm Au NPs as quencher. The same data were obtained and results are shown in Figure 3.45. DsRed displays a combined quenching mechanism and DsRed-AuBP2 has static quenching mechanism (Figure 3.46).

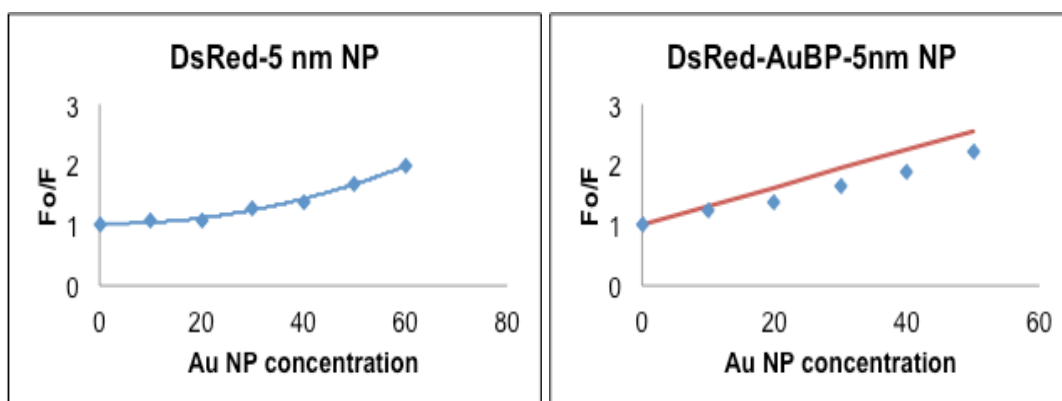


**Figure 3.45 :** Stern-Volmer plots for DsRed and Dsred-AuBP2.

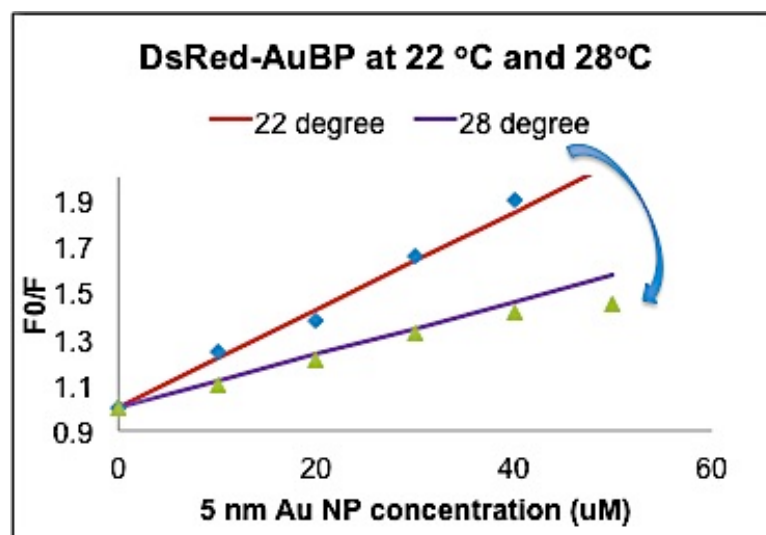


**Figure 3.46 :** Stern-Volmer plots for Dsred-AuBP2 at 22°C and 28°C.

When 50 nm AuNP was used as quencher, Stern-Volmer plots of DsRed and DsRed-AuBP2 display a linear curve (Figure 3.47). In this time, temperature effects were investigated for both proteins. The quenching rate of DsRed increases with higher temperature because there is dynamic quenching, but quenching rate of DsRed-AuBP decreases with higher temperature.

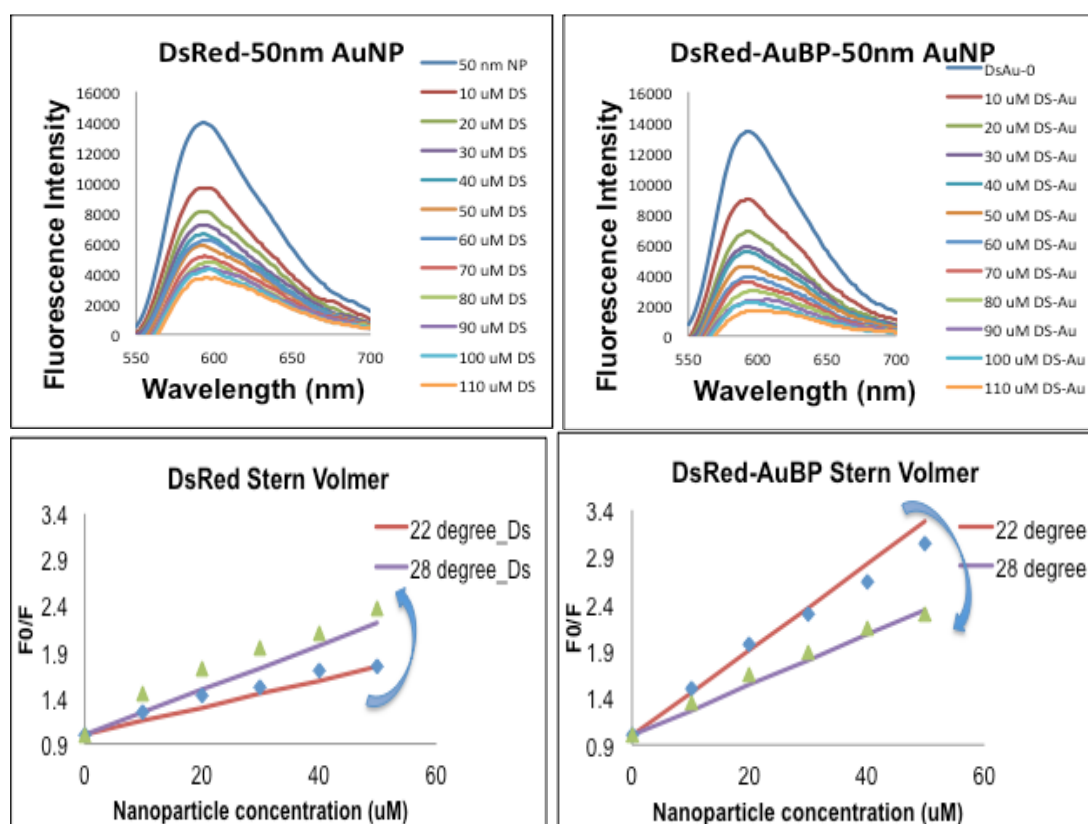


**Figure 3.47 :** Fluorescence quenching of proteins with 50 nm Au NP and Stern-Volmer plots.

We consider that Au NPs bind to a specific amino acid region on the protein, for the static quenching to happen. The consecutive binding of Au NPs to the amino acid residues may bring NP close to the chromophore of the protein to allow donation of an electron from the chromophore to NP, possibly forming a non-fluorescent ground state complex. In this process the inorganic binding protein, AuBP2, may lead the Au NP to reach the chromophore easily and manage the efficient interaction causing the static quenching.

#### **3.2.4.3 Stabilization of gold nanoparticles via inorganic binding proteins**

Owing to the electrostatic repulsion between Au NP and surfactant such as citrate, Au NPs are stabilized with loosely bound surfactants. Initially, we monitored the stability of hybrid Au NP produced after incubation with fusion DsRed-AuBP of various concentrations and compared to DsRed-monomer controls at identical reaction conditions. The Au NP agglomeration is evidenced by a significant change in LSPR spectrum by intensity decrease of characteristic LSPR band at  $\sim 520$  nm and increased absorption at longer wavelengths ( $\sim 650$ - $700$  nm) conveyed with color change from pink to purple. Here we observe that the Au NP modified with fusion DsRed-AuBP2 ( $0.01 \mu\text{M}$  and higher) are showing exceptional stability and can withstand up to  $1.5 \text{ M}$  NaCl solution. In contrast Au NP modified with DsRed-monomer agglomerated as evidenced by solution color transition to purple and a large red- shifted absorption peak (Figure 3.48 and Figure 3.49). These results showed lower binding affinity of DsRed-monomer compared to those of fusion DsRed-AuBP2. It also suggested that the bound DsRed-monomer is non-specifically bound to gold surface with low binding strength. Also, DsRed-monomer may have low orientation control exposing surface of Au NP for interaction with salt ions causing agglomeration.

The absorbance spectrum results from salt-induced agglomeration assays were supported by agarose gel electrophoresis. After addition of salt, protein-bound Au NP stability was confirmed onto low percentage of agarose gel of  $0.5 \%$  at  $40 \text{ V}$  (Figure 3.50 and Figure 3.51). The electrophoresis studies again showed that significantly low concentrations of DsRed-AuBP2 stabilizes Au NP more effectively (Figure 3.50). We found the enhanced stability of DsRed-AuBP2-functionalized Au NP, which has pink-colored bands compared to 'bare' citrate-capped nanoparticles.

Because of agglomeration with NaCl, the citrate-capped nanoparticles in TEA buffer has purple-color onto agarose gel.

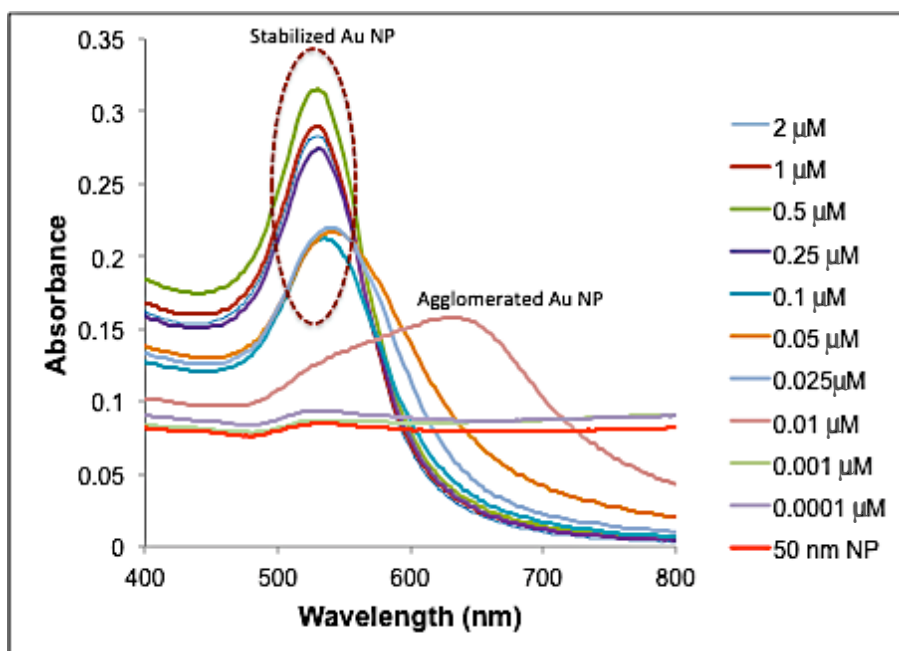


**Figure 3.48 :** Absorbance spectrum of DsRed-AuBP2-Au NP conjugate after addition of 1.5 M of NaCl salt solution.

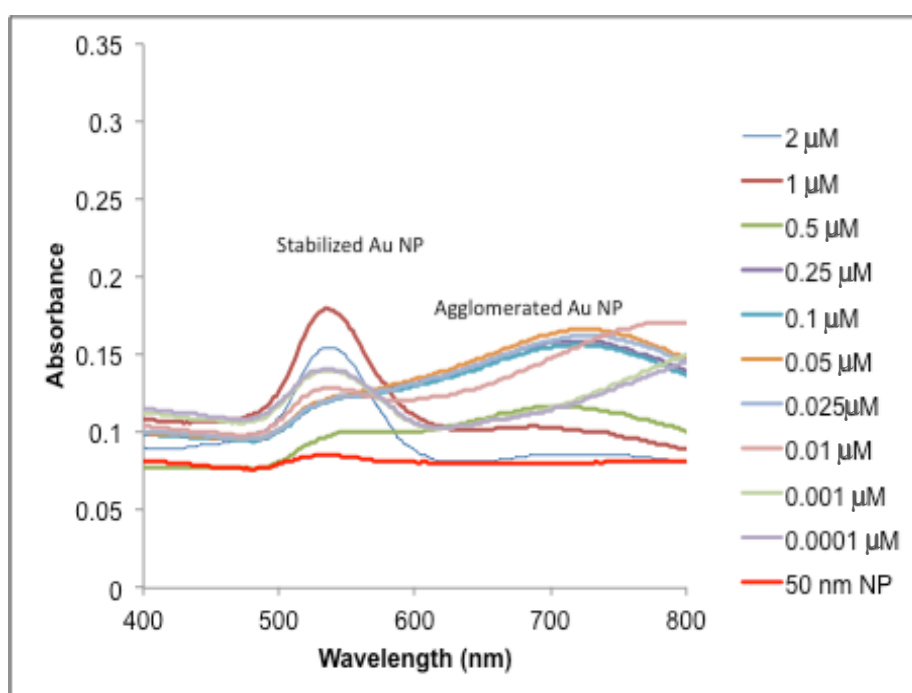


**Figure 3.49 :** Absorbance spectrum of DsRed-Au NP conjugate after addition of 1.5 M of NaCl salt solution.

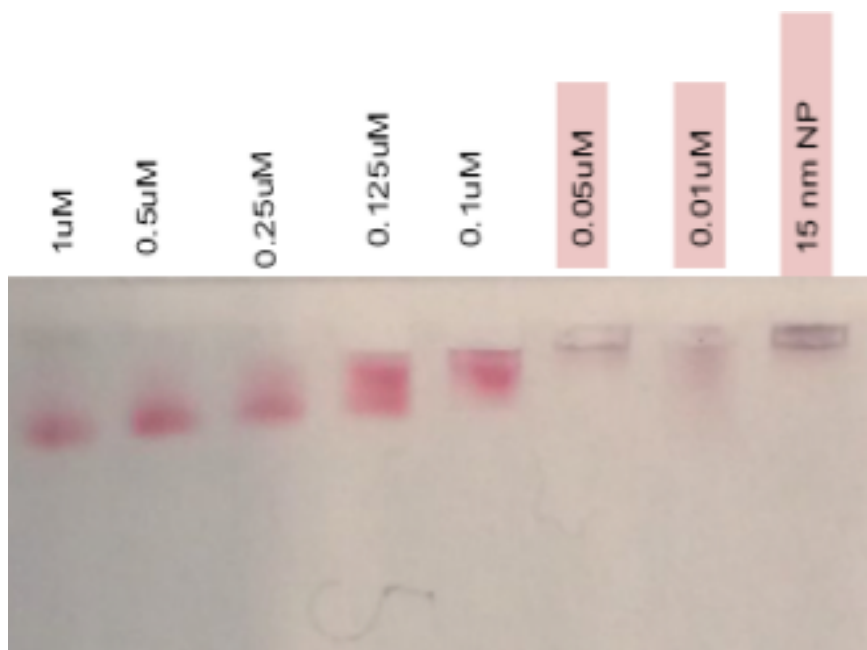


**Figure 3.50** : Agarose gel result of DsRed-AuBP2-Au NP conjugate after addition of 1.5 M of NaCl salt solution.



**Figure 3.51** : Agarose gel result of DsRed-Au NP conjugate after addition of 1.5 M of NaCl salt solution.

Observed protein-induced stability of Au NP in the absence of excess capping ligand suggest that these particles would be particularly suited for biological applications or for building nanostructures using biological interactions.

#### 3.2.4.4 Design of novel purification method using Au NP

In order to further demonstrate the other application of DsRed-AuBP2 fusion protein, we designed a nanoparticle based purification protocol. DsRed-AuBP2 was successfully purified through 15 nm Au NP in 30 minutes. The pure protein was analyzed by SDS-PAGE electrophoresis. Figure 3.52 clearly demonstrated the result of the Au NP-based purification method of DsRed-AuBP2 compared to DsRed-monomer.

In Figure 3.52, the samples were run onto 10 % of SDS polyacrylamide gel at 180 Volt for 45 minutes. The first well is unstained protein marker (Fermentas). Second well is first supernatant, which has various proteins after washing step during purification. However, the pellet has only one protein, which is DsRed-AuBP2. We performed the same purification method for DsRed-monomer, and the pellet of DsRed-monomer could not have the DsRed- monomer after washing step. This result supported that our suggested purification method was performed successfully and we purified the fluorescence protein via Au NP because of the presence of inorganic binding peptide.

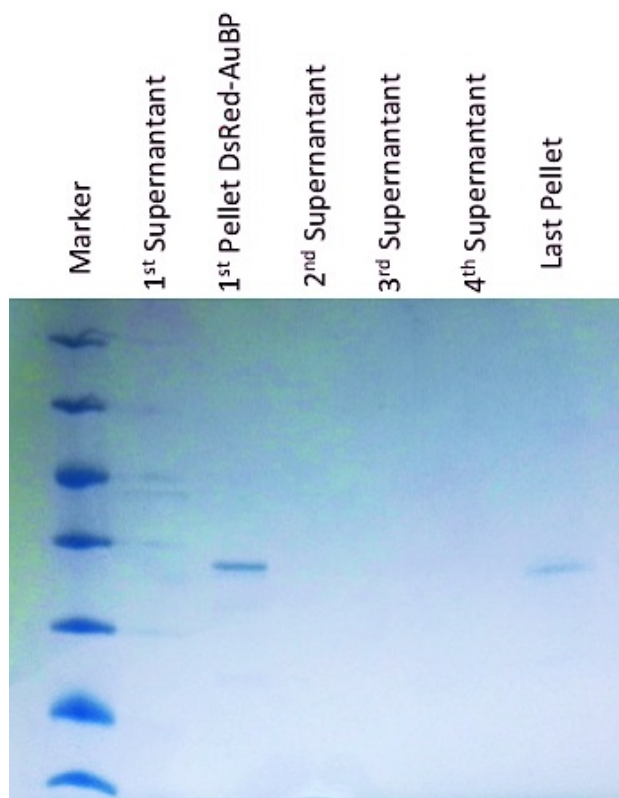


**Figure 3.52 :** The SDS-PAGE result of protein purification through Au NP.

### 3.2.5 Application of DsRed-AuBP1 in bio-nanotechnology

#### 3.2.5.1 Copper sensor design

Recently, the red fluorescence protein has been shown to bind to copper ions. In this part of our study, we investigated if the fluorescence intensity was affected by the addition of metal ions, the fusion protein, which consists of DsRed monomer having AuBP2c insertion and the control, i.e. DsRed monomer. The proteins were titrated with copper ( $\text{Cu}^{2+}$ ) ion at different concentrations. The change in emission intensity at 590 nm (red) was obtained by increasing the copper concentration. The red emission decreased by 50-90% at copper concentrations greater than 10  $\mu\text{M}$ . Following the addition of copper ions, fluorescence intensity of the protein was decreased (Figure 3.53). Consequently, we observed the increase in quenching against total copper concentration (Figure 3.53 and Figure 3.54).

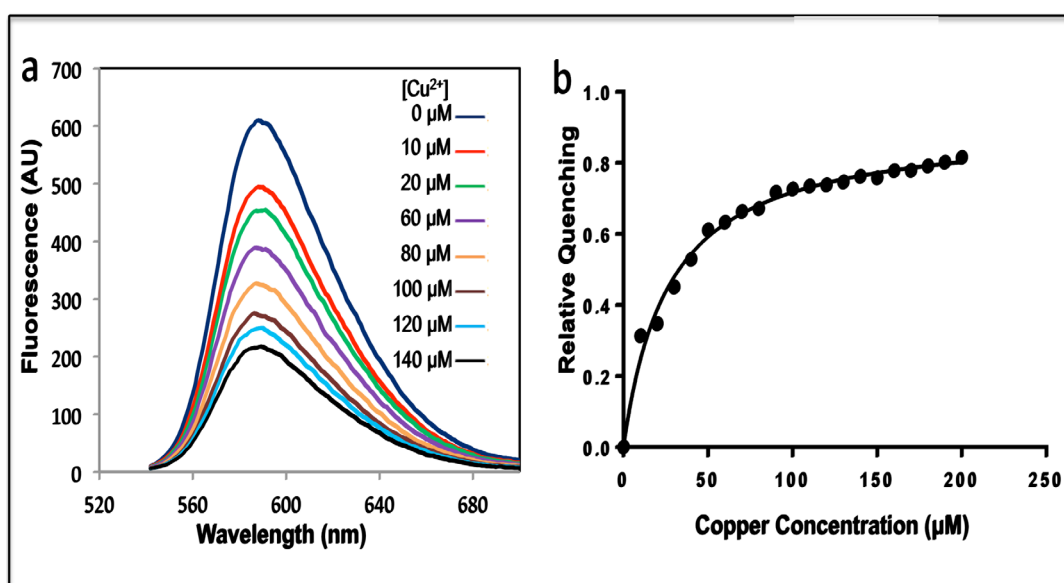


**Figure 3.53 :** Titration of DsRed-AuBP2c with  $\text{Cu}^{2+}$ . Emission spectra of DsRed-AuBP2c (a) was obtained by excitation at 558 nm in the presence of 0, 10, 20, 60, 80, 100, 120, 140  $\mu\text{M}$   $\text{Cu}^{2+}$ . The change in quenching (b) was integrated over a wavelength range of 560-650 nm and was plotted against the copper concentration. The value of 0.8 on the axis indicates a 100% change in observed fluorescence.

Also the excitation and emission properties of DsRed-AuBP2 were checked with metal ions. DsRed-AuBP2 kept their inherent copper binding ability. The emission maxima and excitation maxima of DsRed-AuBP2 is 558 nm and 590 nm, respectively.

Next, a copper binding study was carried out by adding different concentration of copper to a protein solution (20 mM MOPS buffer, pH, 7.4) in microtiter wells. The addition of ions to the fluorescence proteins caused the quenching of fluorescence. Normalized curves were generated for copper by plotting fluorescence intensity against wavelength (Figure 3.54). A copper concentration of 10  $\mu\text{M}$  showed 50 % quenching of DsRed-AuBP fluorescence and 35 % quenching for DsRed-monomer. When the copper concentration was increased to 40  $\mu\text{M}$ , approximately 75 % of the fluorescence was quenched in DsRed-monomer. However, increasing the copper concentration quenched 90% of the fluorescence intensity of DsRed- AuBP. This result showed that the copper sensitivity of DsRed-AuBP is higher than that of the DsRed-monomer.

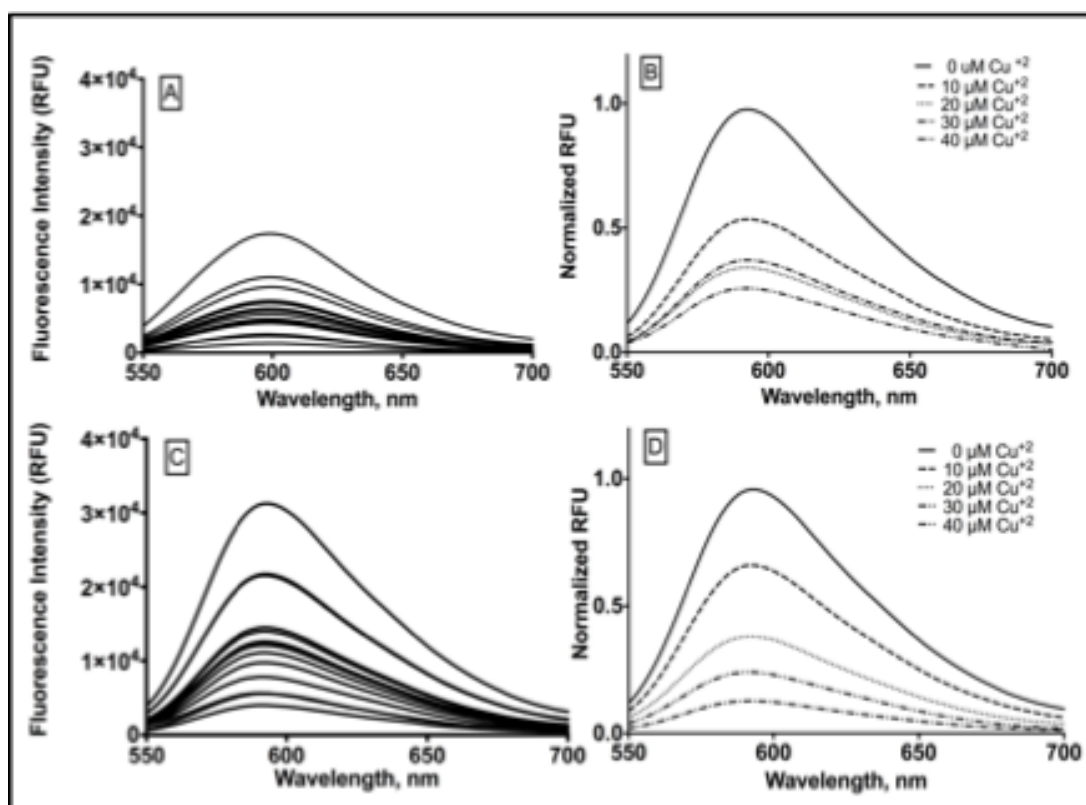


**Figure 3.54 :** Titration of DsRed proteins with  $\text{Cu}^{2+}$ . Emission spectra and normalized fluorescence intensity for DsRed-Monomer (A and B) and for DsRed-AuBP2c (B and C) was obtained by excitation at 558 nm in the presence of 0, 10, 20, 60, 80, 100, 120, 140  $\mu\text{M}$   $\text{Cu}^{2+}$ .

The binding affinity of the fusion protein to copper was quantified by fitting the conventional model using Equation 3.11 and Equation 3.12, below, to the collected fluorescence data.

$$F = F_0 + \Delta F [\text{bound}] / [\text{P}] \quad (3.11)$$

where  $F$  is the measured fluorescence after normalization,  $F_0$  is the fluorescence in the absence of copper, and  $\Delta F$  is the fluorescence change due to copper binding. The concentration of the protein bound to copper, [bound], is shown in Equation 3.12,

$$[\text{bound}] = \{ K_D + [\text{Cu}]_{\text{tot}} + [\text{P}] - \sqrt{([K_D + [\text{Cu}]_{\text{tot}} - [\text{P}])^2 - 4[\text{Cu}]_{\text{tot}} [\text{P}]} \} / 2 \quad (3.12)$$

where  $K_D$  is the dissociation constant of the copper binding sites, total copper concentration is  $[\text{Cu}]_{\text{tot}}$ , and  $[\text{P}]$  is protein concentration. The fitted values of  $K_D$  were calculated to be  $0.77 \pm 0.05 \mu\text{M}$  for DsRed-AuBP2c and  $1.61 \pm 0.2 \mu\text{M}$  for DsRed-AuBP (Figure 3.55a-b). The dissociation constant observed in the case of DsRed-AuBP is much lower, indicating stronger copper binding by DsRed-AuBP than by DsRed-monomer. This result indicates that DsRed-AuBP has a stronger affinity to copper than does DsRed-monomer.

A quenching process is described as one that strives for the spontaneous emission process and thereby shortens the lifetime of emitting molecule. Technically, quenching reactions are energy transfer or electron transfer processes. In some instances transient complex formation in the excited state may occur. These excited state complexes may or may not emit their own characteristic emission that is likely to be different from that of the original molecule. In solution, the possibility of weak complex formation in the ground state is also present. In the limit this may be connection of fluorescent protein and quencher molecules within the solvent cage at the moment of excitation. This may cause immediate loss of electronic energy and is called static quenching. Static quenching requires direct contact between the fluorophore and the quencher. And a result of this contact is loss of the fluorescence intensity for return to the ground state. This quenching type exhibits a concentration-dependence that is described by the Stern-Volmer equation.

The Stern-Volmer expression is the linear in quencher concentration and  $K_{\text{sv}}$  is obtained as the slope of  $F_0/F$  vs  $[\text{Q}]$ , if the assumed mechanism of quenching is effective (Figure 3.55c). In the presence of copper ions, a static quenching was observed with DsRed proteins. We used Stern-Volmer plots to understand the mechanism responsible for this quenching. These plots were constructed by plotting the measured fluorescence of each protein after the addition of copper ions (Figure 3.55c). Linear plots were generated for both DsRed-AuBP2c and DsRed-Monomer. These results indicated a static process in that only one type of quenching occurred.

Using the slopes of these plots, the Stern–Volmer constant ( $K_{SV}$ ) was calculated for each protein (Figure 3.55d). Our findings show static quenching interactions between the proteins and copper ions. Thus,  $Cu^{2+}$  can form a complex at a specific site on the protein. To further investigate the quenching seen between the proteins and  $Cu^{2+}$ , the  $K_{SV}$  (Stern- Volmer) constants, were used to calculate the quenching rate constant,  $k_q$ , by the Equation 3. 13:

$$k_q = K_{SV}/\tau_0 \quad (3.13)$$

where  $\tau_0$  represents the actual lifetime of the protein's fluorophore, which was reported to be 3.3 ns for DsRed in the absence of bimolecular quenching. If the  $\tau_0$  is measured independently of  $K_{SV}$ , the rate constant  $k_q$  for bimolecular quenching step can be determined. For an efficient quencher,  $K_{SV}$  is generally found around  $\sim 10^2$ - $10^3 \text{ M}^{-1}$ . If  $\tau_0$  corresponds to  $10^{-8} \text{ s}$ , then  $k_q$  becomes  $\sim 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  which would be the same order as the counter frequency [86].

The  $k_q$  values calculated in our study were  $10^{13} \text{ M}^{-1} \text{ s}^{-1}$ , for both DsRed proteins in the presence of  $Cu^{2+}$ . These values are 100-fold larger than the maximum scatter collision- quenching constant for quenchers ( $2.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ ) [139]. These  $k_q$  values confirm that the quenching observed for the DsRed protein in the presence of  $Cu^{2+}$  was caused by the formation of complexes between the proteins and  $Cu^{2+}$ . The efficiency of this fluorescence quenching depends on the distance between the quencher and chromophore [156]. The Stern-Volmer plot is linear, which indicates that the only one type of quenching occurs. The proportional decrease in fluorescence lifetime and yield proves that the observed quenching is due to a diffusive process.

In quenching experiments with DsRed proteins, the quenching agent may interact with the protein in ways that alter the protein structure or that affect the degree of quenching observed. Here, the relative kinetic efficiency of fluorescence quenching was estimated by fitting dependence of  $F_0/F$  with copper ions based (Figure 3.55 C) based on a fitting to Stern-Volmer equation. We found that  $K_{SV}$  of Dsred-AuBP2c is higher than of DsRed, and, respectively. This means DsRed-AuBP2c ( $K_{SV} \sim 76000 \text{ M}^{-1}$ ) has higher fluorescence intensity than DsRed-monomer ( $K_{SV} \sim 46000 \text{ M}^{-1}$ ).

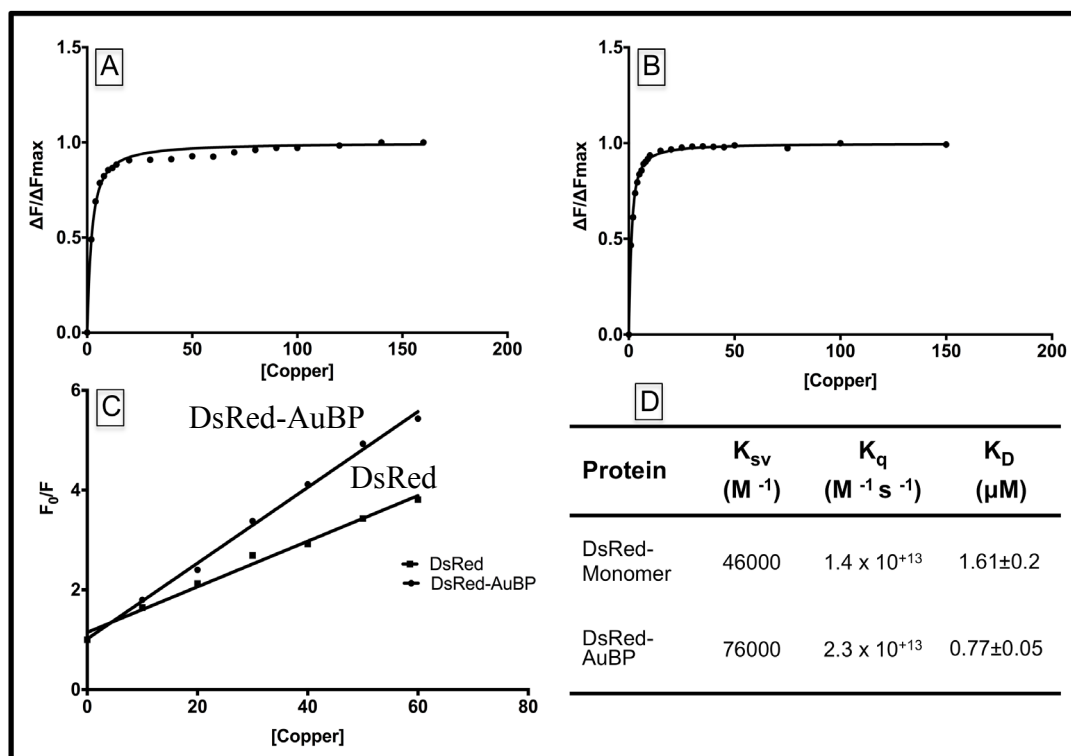


**Figure 3.55 :** Plot of  $\Delta F/\Delta F_{max}$  against copper concentration, where  $\Delta F$  is the change in measured fluorescence and  $\Delta F_{max}$  is the maximum fluorescence change. Plot A corresponds to results obtained using DsRed-Monomer and plot B corresponds to results obtained with DsRed-AuBP2c. Plots C are the Stern–Volmer plots generated by adding  $Cu^{2+}$  to DsRed proteins, DsRed-monomer and DsRed-AuBP2c. Data from DsRed-Monomer is presented as curve (o) and data from DsRed-AuBP2c is presented as curve (■). Stern–Volmer constants ( $K_{SV}$ ) determined from the slope of the Stern–Volmer plot and quenching rate constants determined using the equation  $K_q = K_{SV}/\tau_0$ , where  $K_{SV}$  is Stern–Volmer constant and  $\tau_0$  is the lifetime of the fluorophore (D).

Additionally, we calculated the quenching rate constants, which are  $1.4 \times 10^{13} M^{-1}s^{-1}$  for DsRed-monomer and  $2.3 \times 10^{13} M^{-1} s^{-1}$  for DsRed- AuBP2c. It is proportional to the effectiveness of the quencher and the accessibility of the fluorophore to collisions with the quencher. We consider that copper ions bind to a specific amino acid region on the protein, for the static quenching to happen. The consecutive binding of copper ions to the amino acid residues may bring copper close to the chromophore of the protein to allow donation of an electron from the chromophore to copper, possibly forming a non-fluorescent ground state complex. In this process the inorganic binding protein, AuBP2, may lead the copper ions to reach the chromophore easily and manage the efficient interaction causing the static quenching.

### 3.2.5.2 Role of bioengineered DsRed protein in nanofiber formation

Here, we have invented a new material that consists of polymer (PEO, polyethylene oxide) and protein to generate fibers using infused gyration method. This work was done in collaboration with group of Dr. Mohan Edirisinghe (University College London). Using the combination of infusion and the centrifugal spinning, we developed a new bio-enabled process to generate nanofibers in which the peptides are the integral part of the nanostructure. This process is illustrated in Figure 3.56 and described in Materials and Methods section.

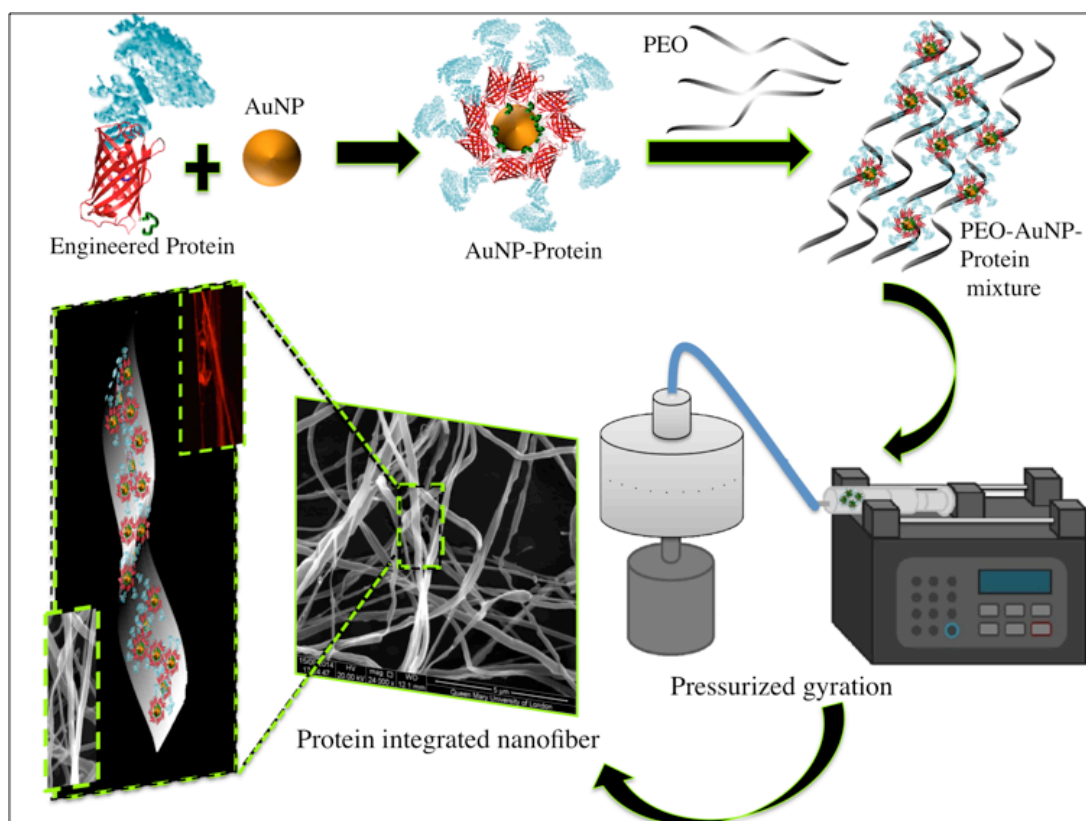


**Figure 3.56 :** Protein-integrated nanofiber formation process.

During the nanofiber formation process, we used one of the gold binding dodecapeptides, namely, AuBP2, i.e. WALRRSIRRQSY, which have been previously shown to assemble gold nanoparticles on surfaces using soft lithography techniques [206]. To easily track the integration of peptide in nanofibers, we genetically conjugated gold binding peptide to a biomarker protein, i.e. red fluorescence (DsRed). The resulting protein with gold binding peptide tag (DsRed-AuBP2) is hypothesized to blend into fiber formation by providing well-defined organic-inorganic surface interactions to decorate gold nanoparticles along the nanofibers. The incorporation of fluorescent protein has enabled multi-analyte and

multicolor detection due to its emission wavelength at a relatively far-red region, 592 nm.

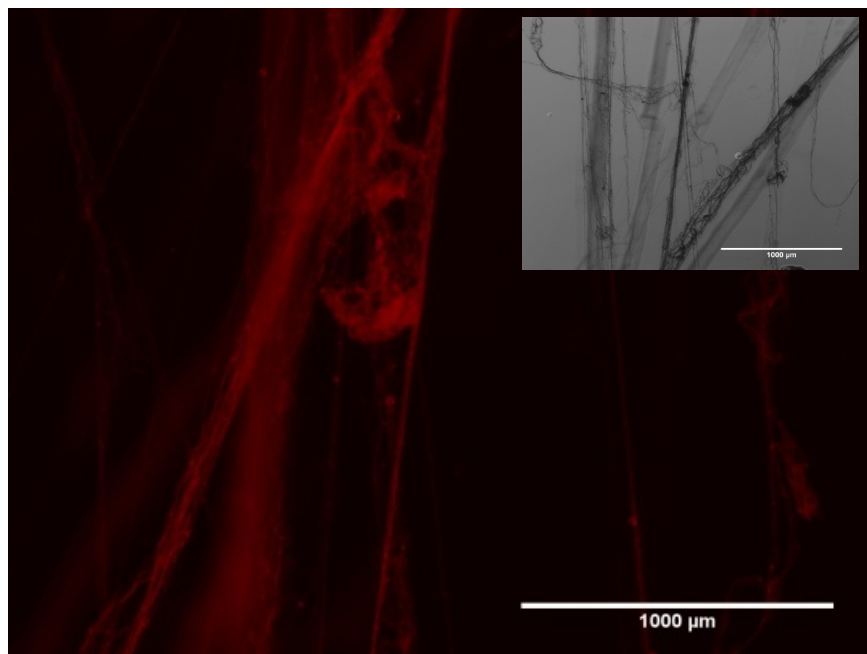


**Figure 3.57 :** Fluorescence microscopy images of the protein integrated nanofibers.

Modification of the fiber ingredients by metallization yields multifunctional 1D nanostructures that extend the range of applications. Semiconductor nanofibers with special optical and electronic properties were prepared. There have been many different efforts to decorate nanofibers with metallic nanoparticles [207]. Here, genetically engineered protein was used to template Au nanoparticles and direct their spatial order into 1D particle assemblies (Figure 3.57). Decorating the hybrid nanofibers with discrete Au nanoparticles change significantly their optical properties. The optical characteristics of both the nanofibers and the Au decorated assemblies were evaluated by using UV-Vis and fluorescence spectrophotometry (Figure 3.58).

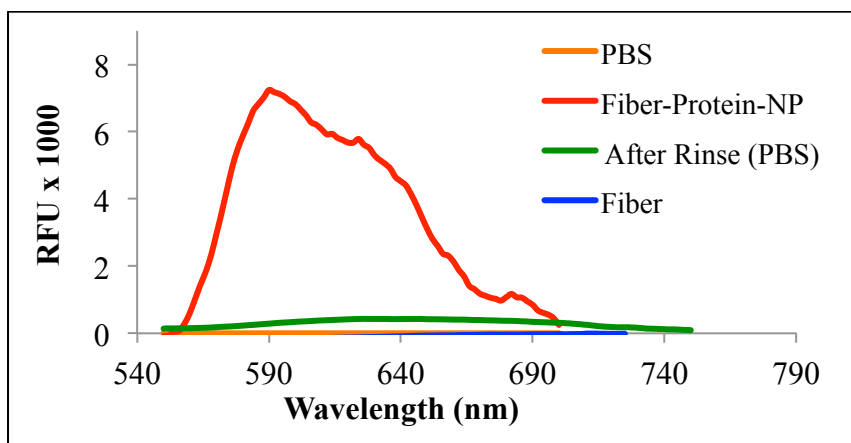


**Figure 3.58 :** Fluorescence intensity of protein-integrated nanofiber.

We have found that integration of Au nanoparticles onto the surface of the fibers has a significant effect on the optical properties. For example, hybrid nanofibers in PBS buffer exhibit a strong fluorescence emission band at  $\lambda_{\text{max}} \sim 590$  nm in Figure 3.58. Interestingly, protein based nanofibers did not dissolve in PBS buffer and show any leakage of protein to the buffer. Protein integrated nanofibers in PBS yielded maximum red fluorescence when samples were excited at 558 nm. As nanofibers were removed from PBS buffer, the fluorescence intensity of PBS buffer decreased completely because of lacking the nanofibers (Figure 3.58). This result shows for fluorescent protein not to be leaked into PBS buffer. Figure 3.59 shows the fluorescence intensity of nanofiber in PBS, which is red column and there is not any fluorescence property of PBS after removal of nanofiber.

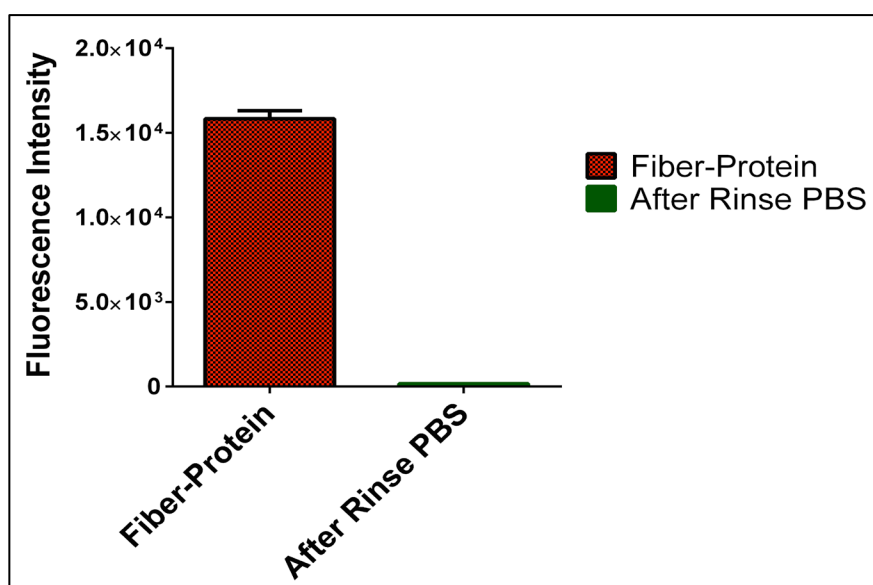


**Figure 3.59 :** Fluorescence intensity of protein-integrated nanofiber in PBS and only PBS after removal of nanofiber.

In this present study, the interaction between proteins and nanoparticles is accompanied by energy or electron, or spectroscopic change, size and shape alterations on both moieties. Therefore, we tested hybrid nanofibers using Localized Surface Plasmon Resonance (LSPR) method. This method is depended on .the ability of noble metal nanoparticles or nanostructures to absorb light in a specific wavelength range. Metallic nanostructures are able to sense alterations at their surfaces by shifting the frequency of the light absorbed or reflected. The shift and broadening of the absorption spectra of the metal nanostructures containing proteins base on the nanoparticle size, shape, and local dielectric environments. For instance, it was shown that the red shift ( $\sim 3$  nm) in the absorption spectra of MBP-DsRed-AuNP integrated nanofibers in solution (Figure 3.60). Firstly, this engineered red fluorescence protein was conjugated to 10 nm Au NP and bio-hybrid nanofibers were formed using infused gyration method. These protein-integrated nanofibers were analyzed in DI water by addition of 50 nm gold nanoparticles in different amounts. Interestingly, bio-hybrid nanofibers was stable in DI water, and 3 nm of  $\lambda_{\text{max}}$  shift was observed because of interaction between 50 nm Au NP and bio-hybrid nanofibers (Figure 3.60A).

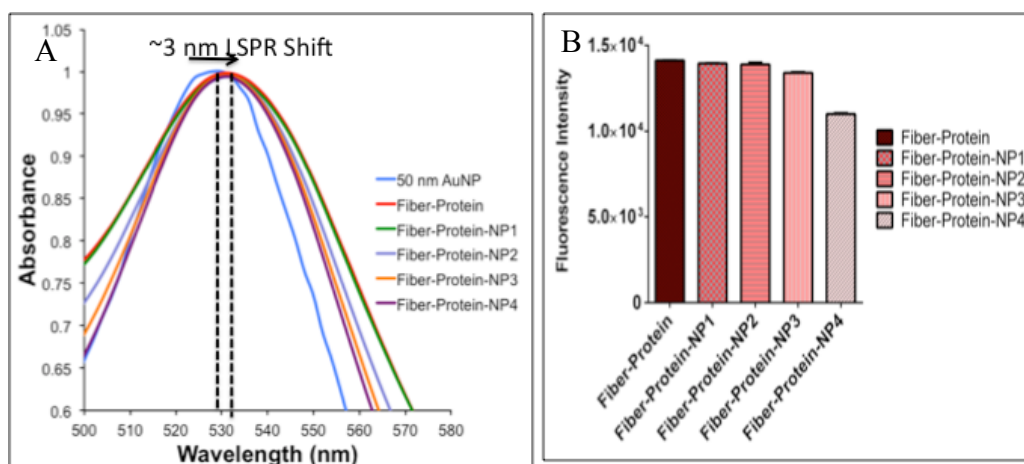


**Figure 3.60 :** Hybrid nanofibers analysis through Localized Surface Plasmon Resonance (LSPR).

Apart from these results, nanofibers affected not only  $\lambda_{\text{max}}$  shift, but also surface interaction between nanofibers and nanoparticle because of the inorganic binding peptide tagged protein. Based on surface area calculations, each gold colloid was functionalized with between 10-20 protein molecules, depending on AuBP2 orientation and size of moieties. The interactions between the proteins and the gold surface may be mediated by gold binding peptide. The improved affinity induced by

nanoparticle-protein conjugate offers an additional source of sensitivity for the plasmonic label-enhanced LSPR sensor because biosensor sensitivity is inherently based on the strength of the binding interaction between surface and the analyte. Finally, we demonstrated that nanoparticle-labelled sensing material would have a significant impact on the LSPR  $\lambda_{\text{max}}$  shift.

Moreover, the fluorescence intensity changes of nanofibers were tested by adding different amounts of gold nanoparticles if there is more binding of nanoparticles to nanofibers fluorescence ability of nanofibers can be decreased. This is the fluorescence quenching process, which decreases the intensity of the fluorescence emission. Herein, the final concentrations of gold nanoparticles were adjusted as and 80, 100, 200, and 400 nM. As a result of this, there were not significant changes in fluorescence intensity of nanofibers (Figure 3.60B). This result supported the LSPR data of nanofibers. If there were more interaction between excess nanoparticles and nanofiber there would be both different  $\lambda_{\text{max}}$  shift than 3 nm and more changes in fluorescence intensity of hybrid- nanofibers.

FTIR analyses of the PEO and bio-hybrid (PEO/Protein) fiber mats were conducted to confirm that both the PEO and protein are present in the fiber nanomat. Figure 3.61 shows the FTIR spectra for bare PEO nanofiber, protein integrated nanofiber and rinsed protein-integrated nanofiber after washing with PBS buffer, respectively. Spectrum A clearly showed that the PEO nanofiber exhibited only the characteristic peaks of PEO. MBP-DsRed protein in PEO absorbs around  $1700\text{ cm}^{-1}$  region as would be expected [209, 210]. Bare PEO nanofiber has characteristic peaks at  $2900\text{ cm}^{-1}$  (methylene group  $\text{CH}_2$  molecular stretching), and at  $1100\text{ cm}^{-1}$  and  $960\text{ cm}^{-1}$  (C-O-C group stretching), which agrees with the PEO fiber peaks seen in our data [211]. A change in bandwidth for the absorption centered around  $2880\text{ cm}^{-1}$  also occurs and changes with protein integration. Our genetically engineered protein in PEO/Protein nanofiber generated FTIR peak at  $1720\text{ cm}^{-1}$  representing amide bonds of protein in spectrum B [210]. After washing of PEO/Protein nanofiber with PBS , the same FTIR peak at  $1720\text{ cm}^{-1}$  was observed in spectrum C. This carbonyl peak suggests that protein has still been integrated into PEO nanofiber after washing step. Similar work in the literature was observed by such and such [208].

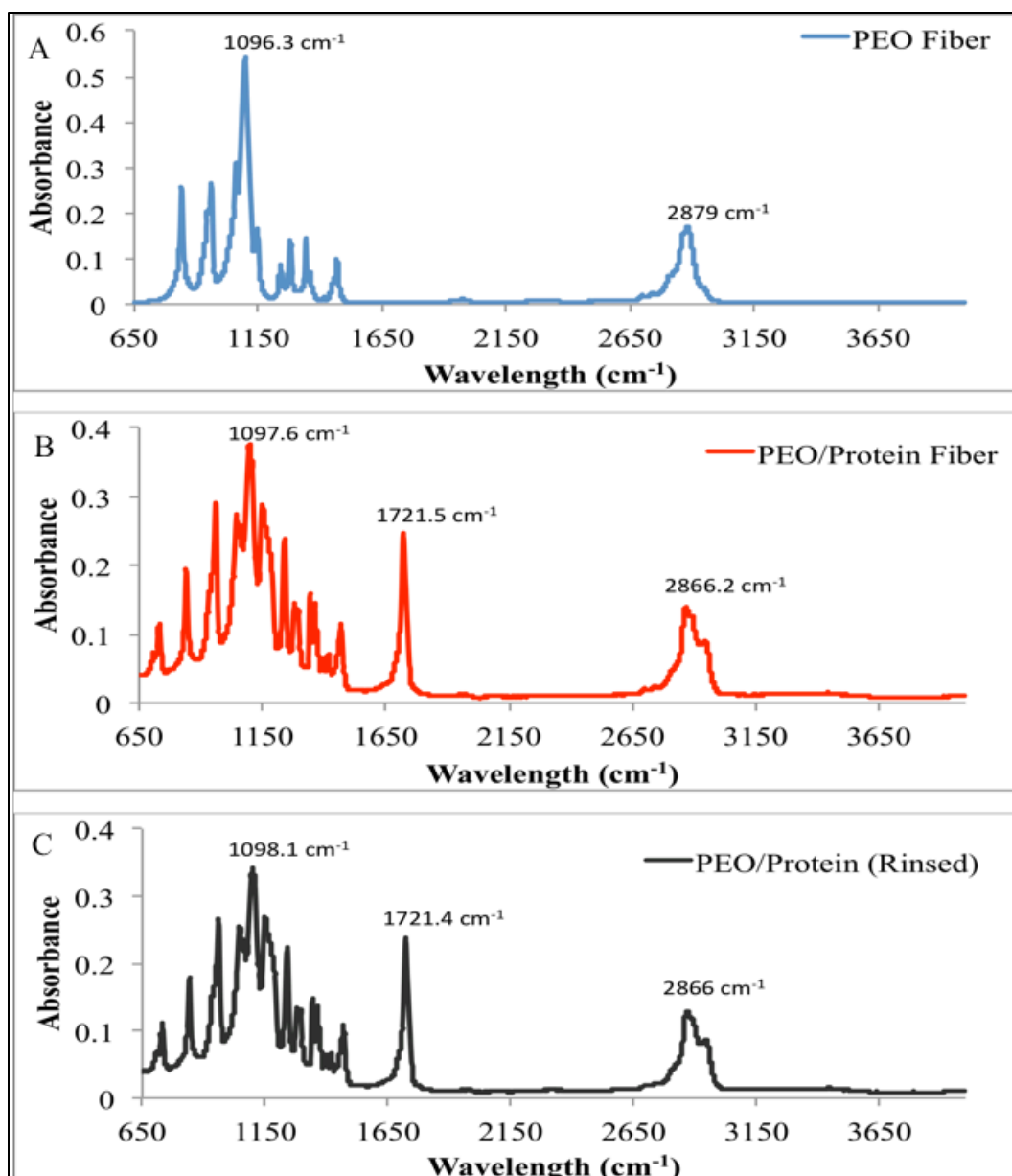


**Figure 3.61 :** FTIR spectra for (a) PEO nanofibre, (b) Protein-integrated PEO nanofibre, (c) After rinse of protein-integrated nanofibre.

Additionally, bio-hybrid nanofibers were analyzed in dry and wet conditions under Cytation 3 cell imaging multi mode reader. Interestingly, protein based nanofibers did not show any leakage of protein to the buffer and performed similarly fluorescent effect both in solution and in dry conditions. Figure 3.62 indicates that protein integrated samples did not dissolve in water and the fluorescent images were obtained under the microscope in dry form and wet form. However, PEO nanofibers without protein dissolved in water, completely. Therefore, any fluorescent images could not be obtained under the same conditions. In the presented method, a fluorescent protein, genetically engineered DsRed-monomer, with the excitation and

emission wavelengths in the red region of the spectrum was utilized because of low auto fluorescence in red region, which yields high signal to noise ratio.

UV-Visible and Fluorescence Spectrophotometry measurements of the nanofibers confirmed the presence of the protein and showed DsRed protein shows the red fluorescence ability on the fiber surfaces. FTIR analysis also confirmed the presence of the protein, and revealed the characteristic bound in the protein structure that is amide bound. The integration of protein to the fibers using infused gyration method enabled the mat to become less soluble in water primarily due to gold nanoparticles functionalization with our genetically engineered protein. This novel fiber formation method and resultant hybrid nanofiber shows promise for various applications and is subject of on-going work because of mat stability.

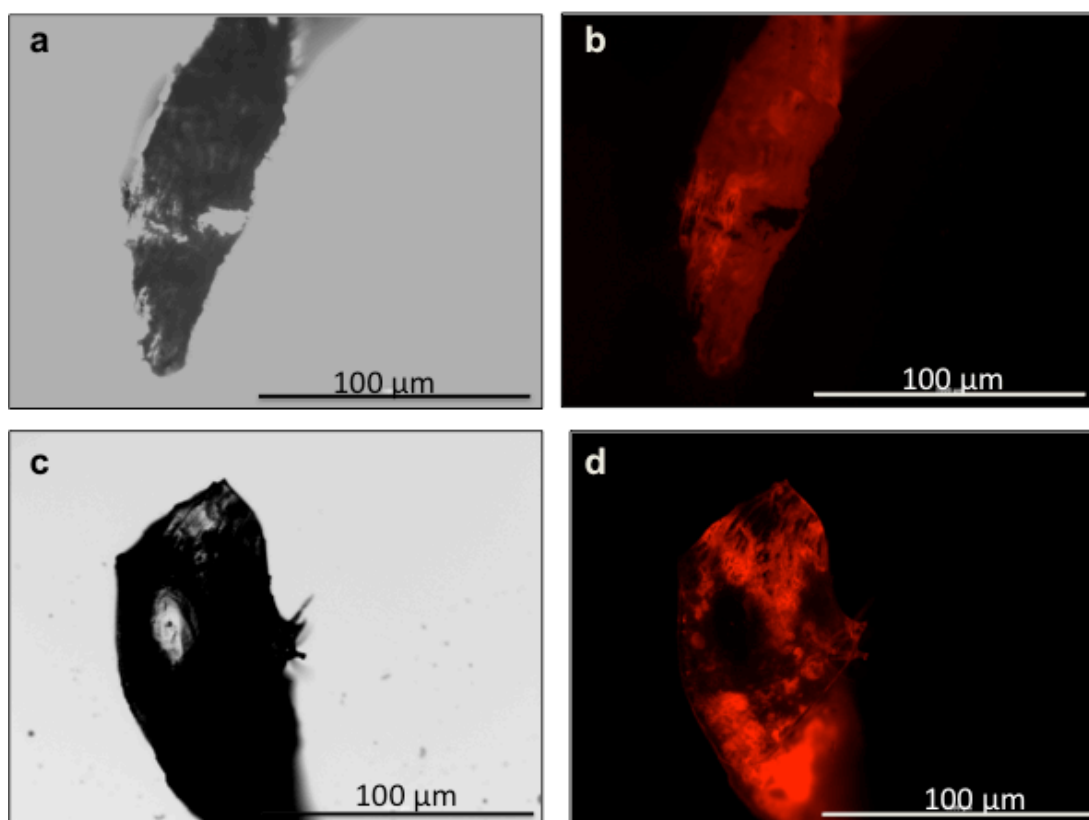


**Figure 3.62 :** Dry versus wet samples.: Fluorescence Microscope image of protein integrated nanofibers (a-b) samples were in PBS buffer, (c-d) samples in dry condition.

The integration of biological building blocks i.e. peptides to nanofiber processing may allow to harness the extraordinary diversity and protein functions to generate smart bio-hybrid materials for imaging, bioelectronics, biosensing and tissue engineering applications.

### 3.3 Bioengineered FDH and FDH-AuBP2 Fusion Enzyme

#### 3.3.1 Expression and purification of FDH and FDH-AuBP2

To purify the fusion enzymes with high yield, we chose a poly (His) affinity tag based approach. It has been previously shown that the N-terminal histidine tag does not influence the activity of cmFDH [189-191]. In order to avoid the unpredictable properties of poly (His) affinity tag on newly introduced specific gold surface recognition ability, both peptide-fused and control constructs were specifically designed by introducing a protease cleavage site at the end of poly (His) region. This extra design step in our cloning approach allowed for complete removal of the poly-histidine tag region from the protein following completion of the purification process. The PreScission protease recognition and cleavage sites were introduced to the final enzyme constructs using a site-directed mutagenesis strategy. The plasmids encoding cmFDH and cmFDH-AuBP2 proteins were successfully expressed in *E. coli* DH5 $\alpha$ -T1 strains and purified using Ni-NTA matrices under native conditions. Purity and molecular weights of the expressed proteins were analyzed by SDS-PAGE (Figure 3.63). The protein bands indicating cmFDH and cmFDH-AuBP2 were observed approximately at 41 and 43.5 kDa, respectively referring to the expected molecular weights for both enzymes following the inclusion of new sequences.

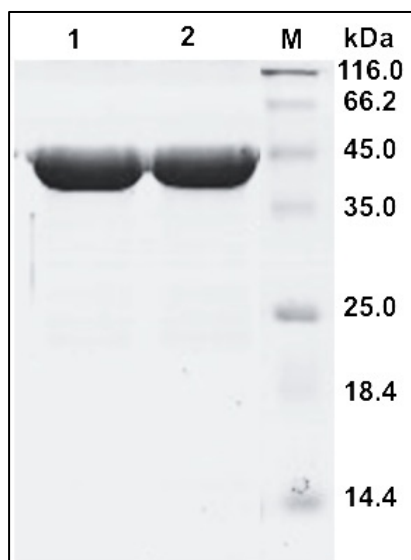


**Figure 3.63 :** SDS-PAGE image of purified enzymes after removal of poly (His) tag; lane 1: *cmFDH* and lane 2: *cmFDH-AuBP2*, M: protein weight marker with corresponding molecular masses.

### 3.3.2 Experimental characterization of FDH and FDH-AuBP2

#### 3.3.2.1 Enzymatic kinetics of FDH and FDH-AuBP2

After the successful expression and purification of the enzymes, the biocatalytic activity of the wild type and engineered enzymes were determined in detail (Table 3.3). The unmodified wild-type *cm*FDH shows a very similar biocatalytic activity ( $0.592 \pm 0.0194 \text{ s}^{-1}$ ) values when compared to other studies in the literature ( $0.5 \pm 0.1 \text{ s}^{-1}$ ). This confirms that the inclusion of the new excision site to remove the poly-histidine tag from the enzyme did not affect the enzymatic activity.

**Table 3.3 :** Apparent kinetic parameters (Data represent mean  $\pm$  SD).

|                                      | <i>cm</i> FDH      | <i>cm</i> FDH-AuBP2 | Pure FDH after digestion of His-tag |
|--------------------------------------|--------------------|---------------------|-------------------------------------|
| $V_{\max}$ (abs/min)                 | $0.083 \pm 0.0029$ | $0.092 \pm 0.0031$  | -                                   |
| $K_m$ (mM)                           | $5.219 \pm 0.6888$ | $5.549 \pm 0.7332$  | $4.49 \pm 0.6$                      |
| $k_{\text{cat}}$ ( $\text{s}^{-1}$ ) | $0.592 \pm 0.0194$ | $0.615 \pm 0.0207$  | $0.5 \pm 0.1$                       |
| $k_{\text{cat}}/K_m$                 | $0.113 \pm 0.0282$ | $0.111 \pm 0.0283$  | 0.1                                 |

A variety of the immobilization techniques result in a significant loss of catalytic activity of enzymes owing to the undesired interaction between active sites of enzymes and the surface. We therefore first determined if the catalytic activity of the enzymes would be preserved within an acceptable range. The calculated enzyme kinetic parameters  $k_{\text{cat}}$ , which indicates the turnover rate of substrate to product, and  $K_m$  (the Michaelis constant), which describes an enzyme's affinity for its substrate, are provided in Table 1. The  $k_{\text{cat}}$  value obtained for the *cm*FDH-AuBP2 ( $0.615 \pm 0.0207 \text{ s}^{-1}$ ) is slightly higher than *cm*FDH ( $0.592 \pm 0.0194 \text{ s}^{-1}$ ) suggesting that the enzymatic breakdown of formate is faster for the fusion enzyme than for the wild type. Interestingly, the  $K_m$  value is almost same for the *cm*FDH-AuBP2 ( $5.549 \pm 0.7332 \text{ mM}$ ) and *cm*FDH ( $5.219 \pm 0.6888 \text{ mM}$ ), which means that the affinity of *cm*FDH-AuBP2 enzyme to formate is same. The ratio of  $k_{\text{cat}}/K_m$  is an indicator of the overall catalytic efficiency of the enzyme; either the turnover rate,  $k_{\text{cat}}$ , is large or  $K_m$  is small and the enzyme has a high affinity for its substrate. In our case, there is less than a 2% difference on the overall  $k_{\text{cat}}/K_m$  values between the wild type and fusion enzymes. This difference is negligible enough to confirm that the apparent kinetic

parameters of the wild-type *cmFDH* were not affected by the incorporation of AuBP2 and more than 98% of the enzyme activity was retained.

### **3.3.3 Application of FDH-AuBP2 in bio-nanotechnology**

#### **3.3.3.1 Biobattery design**

The successful design of any enzyme-based device is dependent upon the efficiency of the immobilization strategy and the catalytic activity of the employed enzyme on its associated surface. With this aim in mind, the *cmFDH*-AuBP2 fusion enzyme was immobilized onto a gold electrode surface and next tested for its catalytic activity. We monitored the conversion of formate to CO<sub>2</sub> electrochemically by designing a circuit-based system consisting of two gold electrodes submerged in solution (20 mM Tris-HCl buffer, pH 8.0, and 0.1 M KCl) and coupled the system with a 22 K $\Omega$  resistor used as a load. Figure 3.64 illustrates our experimental setup for monitoring the conversion of formate by the enzyme-activated gold electrode. To activate the selected gold electrode, 10  $\mu$ M of *cmFDH*-AuBP2 enzyme was applied on the surface. After allowing enough contact time with the surface, electrode was washed with buffer to get rid of excessive amount of any enzyme that may leave on the surface. The enzyme-activated electrode was then placed into a sterile beaker vertically, facing the bare gold electrode to achieve optimal electron transfer. The submerged gold electrodes were next connected in parallel to the resistor. The observed potential difference (V, voltage) between the electrodes was recorded by using a digital multimeter placed in parallel. In this circuit setup, the isoelectric point of *cmFDH*-AuBP2 is 6.5 and this protein has a negative charge at pH 8. The engineered enzyme makes the gold electrode as the cathode terminal in this setup after immobilization on gold electrode in 20 mM Tris-HCl buffer, pH 8.0. The cathode is the negatively charged electrode and attracts cation or positive charge. Additionally, the cathode is the source of electrons. It may accept positive charge. The cathode can generate electrons that are the electrical species doing actual movement. The bare gold electrode behaves as an anode terminal, which has a positive charge.

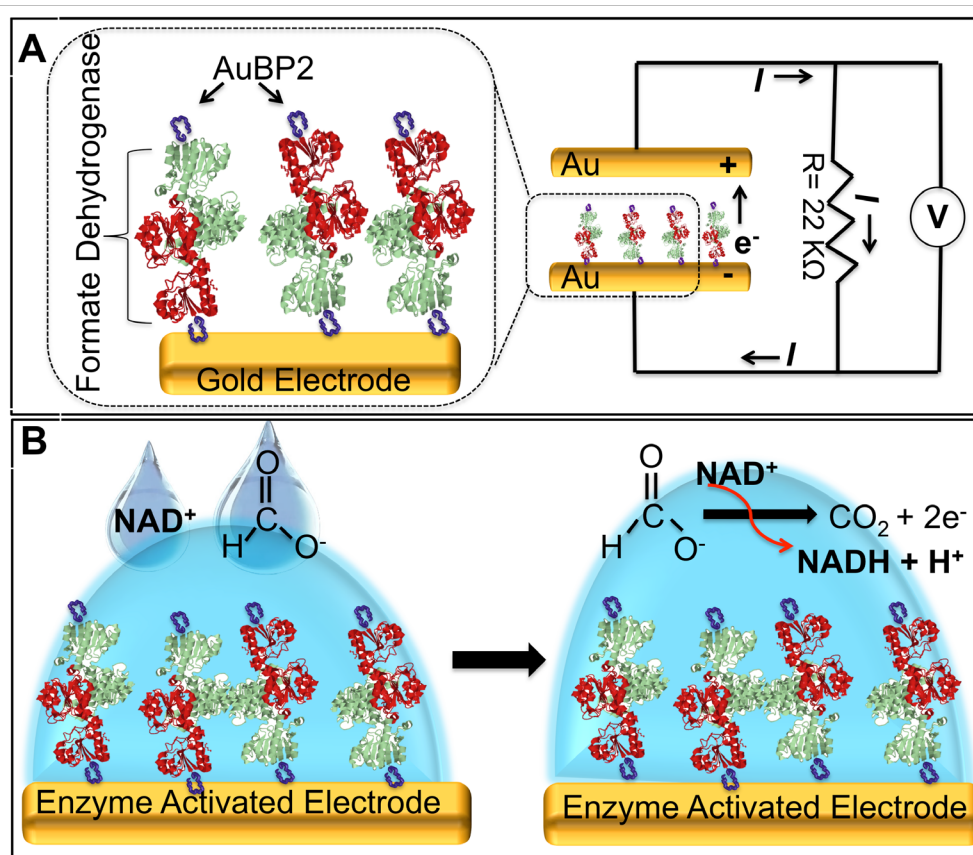


**Figure 3.64 :** Schematic representation of (A) circuit based biosensor and enzyme activated gold electrode and (B) the catalytic conversion of formate by engineered enzyme immobilized onto the electrode surface. When formate is oxidized, the two electrons flow through the circuit.

After establishing a baseline measurement, 0.25 mM formate as substrate and 1mM  $\text{NAD}^+$  as the cofactor were added to the system and output current was monitored continuously. The energy released in the reaction due to the movement of electrons between the enzyme-activated and bare electrodes in the designed circuit gives rise to a potential difference equal to the electromotive force (emf,  $\epsilon$ ) [194]. The resulting change in current across the circuit elements was calculated with Equation 3.14 in Ohm's law:

$$V = I R \quad (3.14)$$

where  $V$  is the potential difference measured across the circuit in units of Volts (V),  $I$  is the current through the resistance in units of amperes (A), and  $R$  is the resistance of the circuit in units of ohms ( $\Omega$ ).

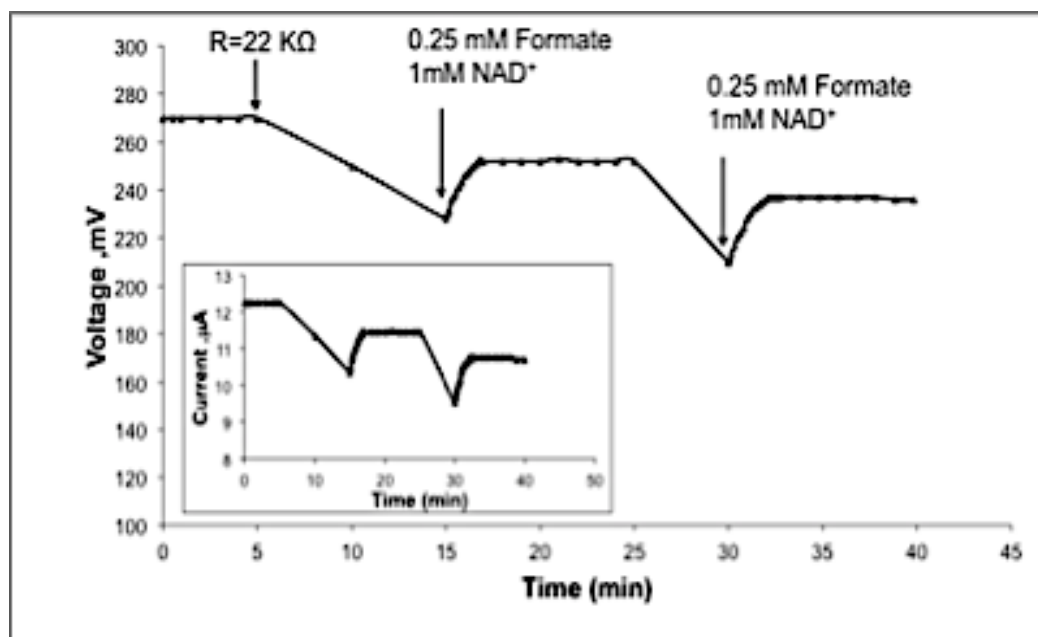


**Figure 3.65 :** Change in total current output in response to time built upon enzyme directed self-organized electrode system.

The initial output voltage was first recorded as 270 mV, then resistor was connected to the system and consequent voltage drop was observed in Figure 3.65. Next, 0.25 mM formate and 1mM NAD<sup>+</sup> were added to the solution (~230mV) to monitor the enzyme based electrochemical conversion. Herein, the enzyme catalyzes the formate oxidation by NAD<sup>+</sup> until all formate is converted into its products of CO<sub>2</sub>, NADH and H<sup>+</sup>. The electrons transferred from the enzyme-coated electrode to the bare gold electrode resulted in a rise in the voltage to about 250 mV. The electron transfer between enzyme and electrode caused an increase in the output voltage. Consecutive additions of formate resulted in approximately 10% increase in the output voltage, which was maintained over a 10 min period. Then, a decrease in the output voltage was observed following the complete conversion of the formate. In this circuit, FDH-AuBP2 binds NAD<sup>+</sup> prior to formate binding on the activated electrode. Consequently, a direct transfer of a hydride ion occurs from the formate to the nicotinamide ring of NAD<sup>+</sup>, forming carbon dioxide and NADH. When formate is oxidized, two electrons are transferred from the enzyme to the electrode. These electrons flow through the circuit. Overall our results demonstrate the catalytic capability of the immobilized enzyme using a bio-engineered circuit design. Our results also indicate the potential of extending the duration of output voltage as well as current by subsequent formate addition over multiple cycles. The circuit design can be further improved by optimizing the physical layout and electrical connections

as well as individual components e.g. enzyme load, formate, internal and external resistances introduced to the system. Potentially, bio-enabled circuit based sensor system can be used for formate detection or NADH regeneration from NAD<sup>+</sup> by pharmaceutical and agrochemical industries.

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#### 4. CONCLUSIONS

Engineering of new protein forms as a biological tool is of great importance for emerging products and applications in medicine, pharmaceutical science, military environmental science, electronics and photonics. The controlling power of genetic engineering to modify or exploit the multi-functional properties of proteins is performed through the relatively straightforward procedures of molecular biology. Rational-based modification of proteins leans an intuitive approach, depend on precise knowledge of structure and function to create desired changes to the molecular system. In the beginning, the viability, functionality and solubility of any recombinant construct needs engineering of a sequence to correctly combine protein with appropriate domain boundaries. The chemistry, size, length, function and position of any extraneous tag can also affect the functionality, solubility and viability of a final expressed construct.

We describe herein a successful engineering and production of a hetero-functional protein unit containing two structural and functional domains; a MBP protein and a highly specific AuBP1 peptide-tag, originally selected from FliTrx peptide combinatorial library. In this design, AuBP1 tag was fused the MBP protein by two structurally different spacers between them. Either a rigid (PGPGPG) or a flexible (SGGG) linker that provided further insights into the potential effect of spacers on the functionality of the fusion protein was used separately. Quantitative binding assays consisting of SPR and LSPR reveal that both engineered MBP-AuBP1 derivatives containing either proline-rich or glycine-rich linker preserved their gold-binding affinities as documented by their similar binding parameters and characteristics. This is probably related to the fusion construction parameters, C-terminal genetic fusion as well as to the considerably smaller size of AuBP1 tag peptide compared to MBP fusion partner.

Furthermore, the results demonstrate the utility of selective AuBP1 peptide tag that enables enhanced immobilization affinity and orientation of MBP-AuBP1 constructs on various gold surfaces using its exceptional molecular recognition and binding

characteristics. Specifically, both of the engineered MBP-AuBP1 proteins demonstrated considerably higher binding affinities to various gold surfaces compared to wt- MBP as evidenced by nearly an order of magnitude enhanced binding parameters for MBP-AuBP1 fusions. Moreover we demonstrate that the novel peptide tag technology is highly modular and versatile by designing a multi-functional QBP1- AuBP1 peptide for an addressed linkage of two different inorganic surfaces and effective bottom-up array fabrication. Collectively, we demonstrate that the effective short AuBP1 peptide tag can be successfully used in various arrangements, such as in hetero-functional peptides and proteins to facilitate effective bottom-up assembly of spatially distributed multi- layer protein microarrays on metallic nanostructures on various solid supports.

This efficient and controlled layer-by-layer strategy for protein immobilization on micro- and nano-patterned surfaces may also be used for preparation of high-throughput LSPR or SERS biosensors with improved outcomes. As shown here, these peptide tags can concurrently direct, control and enhance the protein immobilization onto defined and specific solid surfaces. In contrast to conventional chemical and physical methods for protein adsorption, the robust peptide based approach described here is highly intriguing due to being relatively simple, biologically and environmentally friendly without the requirements of extreme chemical or physical conditions. Overall the results demonstrate that engineered peptides provide a viable alternative to the conventional chemical coupling to produce various protein and hybrid nanostructure assemblies with highly controllable organization and architecture. These layered architectures can be used in a wide range of practical applications, such as controlled bottom-up assembly of hybrid nanostructures, nanobio- photonic platforms and biosensing.

Another work presented in this thesis also focused on the use of DsRed-Monomer for both purification and synthesis of multi-functional fusion protein, DsRed-AuBP2, exploiting the metal-binding affinity, such as gold and copper. Fusion of the gold binding peptide, AuBP2 to the full-length DsRed-Monomer was accomplished by using complete gene synthesis. Maltose Binding Protein (MBP) tag was used to facilitate protein purification. In designing fusion proteins and the expression vector, an exoproteolytic restriction site was localized between malE gene and N-terminus of fusion protein for the Factor Xa protease enzyme. The desired vector was constructed

by designing the oligonucleotides and their primers based upon the AuBP2 peptide sequence, i.e. “WALRRSIRRQSY” and the spacer sequence, i.e. “SGGG” between peptide and DsRed-Monomer on the expression vector. Following protein expression multifunctional protein were purified using an MBP-tag which was later cleaved from the DsRed proteins by Factor Xa proteolytic enzyme. Further purification was accomplished using copper chelating affinity chromatography to remove the cleaved MBP and the DsRed proteins. Purified proteins yielded maximum red fluorescence when protein was excited at 558 nm similar to the expected emission maxima and excitation maxima of DsRed-Monomer, 558 nm and 590 nm, respectively. The gold binding affinity of the fusion protein was also confirmed by AFM, QCM, LSPR and fluorescence based detection methods.

In this work both the natural fluorescence of DsRed-AuBP2 and its unique metal-binding affinity, which results in fluorescence quenching, was exploited. The copper-binding affinity was utilized for the purification of DsRed-AuBP2 and in the development of copper sensing systems onto gold surfaces. We performed studies to identify the mechanism of this fluorescence quenching seen for DsRed-AuBP2 in the presence of copper and gold nanoparticle. Through a series of studies, which include determining the metal dissociation constant, spectroscopic studies in the presence and absence of metal and the effect of gold nanoparticle on this copper-binding affinity. These studies suggested that, upon gold-binding peptide, a sphere of action static quenching mechanism results in the observed fluorescence quenching. These studies indicated the possible role of AuBP2 in this gold-binding feature. By understanding this quenching mechanism and the gold-binding ability of DsRed fusion protein, we believe that improvements can be made in the other metal-binding affinities. We would also like to expand on the use of the fluorescence-based detection methods, of such DsRed-peptide fusion proteins, by demonstrating the viability of developing further methods for studying target protein-ligand pair binding.

Furthermore, bio-hybrid nanofibers containing the genetically engineered protein and gold nanoparticles (AuNPs) were developed following these steps: i) gold nanoparticles (AuNPs) were functionalized with DsRed-AuBP2 in which the DsRed protein was fused with gold binding peptide, ii) polyethylene oxide (PEO) polymer solution was mixed with DsRed AuBP protein functionalized AuNPs, iii) using the

combination of infusion and gyration method, we produced bio-hybrid nanofiber embedded with functional protein and nanoparticles. The integration of biological building blocks, i.e. peptides, to nanofiber processing may provide the ability to harness their extraordinary diversity and functionalities to generate smart, bio-hybrid materials for imaging, bioelectronics, biosensing, and tissue engineering applications. Our results have multiple implications. The presence of the protein in the nanofibers was confirmed through both UV-Visible and Fluorescence Spectrophotometry measurements. As the integral part of the nanofibers, the assembly of the engineered proteins along the fibers was tracked by coupled red fluorescence activity of the proteins. FTIR analysis also confirmed the presence of the protein revealing the characteristic peak of the amide bond in the protein structure within the nanofibers. The integration of protein to the fibers using the new infused gyration method enabled the mat to become less soluble in water potentially due to the incorporation of protein coated gold nanoparticle. The functionalized-gold nanoparticles may provide both electrostatic and necessary steric interactions to stabilize nanofiber formed by polymer, PEO. Reserving biological functionality in the fibers to respond the changing solution conditions offers many implications for wide range of applications. This novel fiber formation method and resultant hybrid nanofiber is subject to on-going work, owing to mat stability. Our studies demonstrate that biological building blocks can contribute to bio-hybrid nanofiber formation by providing control over nanoscale assemblies using infusion gyration process. The integration of biological building blocks, i.e. engineered proteins with inorganic binding peptides, into nanofiber processing may grant the ability to harness the extraordinary diversity and protein functions to be coupled with smart bio-hybrid materials for imaging, bioelectronics, biosensing and tissue engineering applications.

Templating of inorganic materials by proteins is a route that is used extensively in nature, for example in the fabrication of hard tissues such as bones, teeth, spicules, shells and bacterial nanoparticles. Proteins containing specific amino acid residues, which target the nucleation of the inorganic counterpart, often mediate this templating, for example negatively charged amino acids for calcium nucleation. This strong templating feature of inorganic structures by biological materials may be used as fabrication strategy for nanostructured materials destined for novel applications. For instance, templating of metals by proteins and inorganic binding peptides is of

great technological importance, because the facility to produce metal-integrated fiber may be the first step towards their positioning between electrodes and nano-circuit fabrication. Herein, such a proof of concept is demonstrated by introducing the AuBP2 fused to DsRed to self-assembly; the exposed AuBP2 acted as nucleation site for gold nanoparticles. Following DsRed-AuBP2 production, formation of conducting nanofibers was demonstrated. Small building blocks such as short inorganic binding peptide that self-assemble into fibers can also be used as versatile structural templates for metallization. Such engineering protein-integrated fibers are particularly attractive since they can be formed under mild, physiological conditions, and they are biological friendly. Furthermore, the possibility of applying the different type of proteins with gold binding peptide offers the big advantage of tailor-made modifications. Introduction of other inorganic binding peptides, such as silver-, titanium-, platinum-, or graphite-binding peptide, through rational design at the sequence of functional protein is a particularly attractive strategy for templating of metal nanoparticles. We demonstrated a case study of rationally designed gold-binding peptide containing engineered protein and basic protocols for their metallization with gold.

As a last part of this thesis, we describe expression of the other engineered fusion protein, which is enzyme called as *cmFDH*-AuBP2, to form a self-organized enzyme integrated circuit based sensor. Our approach is composed of three steps, i) designing an engineered protein which features an FDH catalytic unit from *Candida methylica* and a highly specific gold-binding peptide (AuBP2) as a genetically conjugated tag that enables direct assembly of the enzyme onto a gold surface, ii) the verification of the individual activities in the system by applying biochemical as well as surface binding assays, iii) designing a circuit based system that is integrated with the self-organized engineered enzyme where the catalytic conversion of the formate can be monitored by the subsequence generation of electrons as an output in the current. In this design, the biocombinatorially selected AuBP2 peptide was genetically fused to the N'-terminus of the *cmFDH* with a structurally flexible (GGGS) linker sequence to ensure that the final fusion protein's distinct functionalities were not restricted by one another. To avoid any potential non-specific interactions that may arrive due to histidine affinity tag used in the system, both enzymes were introduced new cleavage sites to compare the effectiveness of the peptide tag introduced to the enzyme

system. For the production of the fusion enzyme, bacterial expression systems and metal-chelate affinity-based Ni-NTA purification matrices were utilized. The enzyme activity assays revealed that the engineered *cmFDH*-AuBP2 fusion protein construct delivers its enzymatic function without any significant reduction to the overall catalytic efficiency values in solution. The electrocatalytic detection of formate conversion implies that the enzyme is active in its immobilized form. Furthermore, SPR-based quantitative binding assays showed that the gold binding affinity of the engineered fusion protein was 13-fold greater than that of the wild type, *cmFDH*. This proves that the genetic insertion of gold binding peptide into the *cmFDH* enzyme has granted the ability to recognize and bind onto gold surfaces.

Collectively, our results demonstrated the effective use of solid binding peptides as genetically feasible molecular linkers allowing a single step enzyme immobilization purposes. The peptide-based enzymatic platforms can be used in a variety of biosensing applications for both formate detection and NADH regeneration from  $\text{NAD}^+$  by pharmaceutical and agrochemical industries. The enzyme immobilization can be enhanced with an orientation control using solid binding peptides. Solid-binding peptides offer a single-step, bio-friendly alternative to the conventional chemical and physical immobilization methods, without the requirement of undesired surface activation processes. This strategy can be applied to a wide range of practical applications such as biosensing devices, power-on-a-chip platforms, or bioreactors. The proposed immobilization strategy is a step toward achieving self-integrated enzyme harboring platforms and may lead to the improvement of sensing and fuel cell devices in the future. The modular peptide tag-based surface biofunctionalization can inspire novel opportunities in programmable material synthesis and design.

As a summary, the gold binding peptides were investigated as a novel bio-surface functionalization method, i.e., to couple different materials together or to bring proteins to the material surfaces. Their modularity offers a unique opportunity to link them with other functional proteins. Here we provided examples on their design and recombinant incorporation into functional proteins and enzymes to achieve addressable self-immobilization. The engineered multi-functional biomolecular constructs were demonstrated to self-organize to form hybrid hierarchical entities while preserving their original function. However, there is no encompassing solution for orientation control of the proteins on surfaces using inorganic binding peptide

tags. Each peptide and protein set need to be evaluated in detail once they are genetically conjugated; this will help achieve optimum performance for the both existing and additional functional domains.

Consequently, controlling the biological-material interfaces will contribute to the developments of new generation biomolecule mediated devices and systems. These engineered biomolecular systems will serve as dynamic surface functionalization linkers providing functional biomolecules the ability to self-organize at the interfaces within a controllable proximity. This self-organization displays a great deal of potential in creating new materials and systems.



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## **APPENDICES**

**APPENDIX A1:** Protein markers.

**APPENDIX A2:** Materials

**APPENDIX A3:** Computer software



## APPENDICES

### APPENDIX A.1: Protein Markers

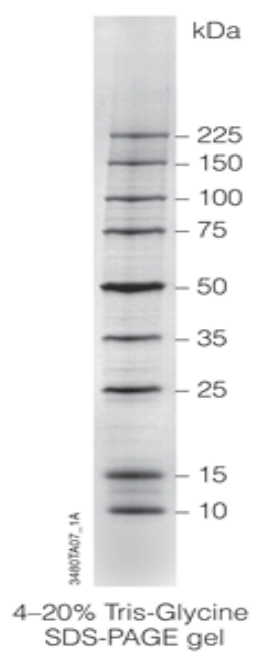


**Figure A.1 :** Broad Range Protein Molecular Weight Markers. 4–20% Tris-Glycine SDS-PAGE gel.

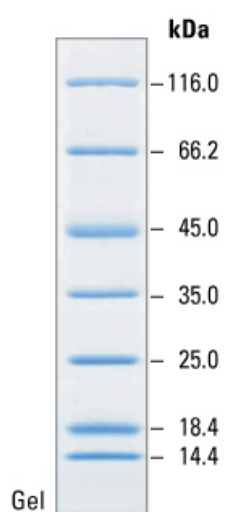


**Figure A.2 :** Unstained Protein Molecular Weight Marker. 12% Tris-glycine gel (SDS-PAGE) stained with coomassie blue gel stain.

## APPENDIX A.2: Materials

### Bacterial Strains

#### **Escherichia coli K12 TB1 strain**

*Escherichia coli* **K12 TB1 strain** is a derivative of JM83 that has been made *hsdR*. The genotype of this strain is  $F^- \text{ ara } \Delta(\text{lac-proAB}) [\Phi80\text{dlac } \Delta(\text{lacZ})\text{M15}] \text{ rpsL(Str}^R) \text{ thi hsdR}$ . (New England Biolabs, Catalog # E4122S).

#### **Escherichia coli K12 ER2507 strain**

*Escherichia coli* **K12 ER2507 strain** is a derivative of RR1. The *malE* gene, which encodes maltose-binding protein, is deleted as part of the *malB* deletion. The genotype of this strain is  $F^- \text{ ara-14 leuB6 fhuA2 } \Delta(\text{argF-lac})\text{U169 lacY1 glnV44 galK2 rpsL20 xyl-5 mtl-5 } \Delta(\text{malB}) \text{ zjc::Tn5(Kan}^R) \Delta(\text{mcrC-mrr})_{\text{HB101}}$  (New England Biolabs, Catalog # E4121S)

#### **Escherichia coli MAX Efficiency DH5 $\alpha$ strain**

*Escherichia coli* **MAX Efficiency DH5 $\alpha$  strain** can be used for blue/white screening of colonies on bacterial plates containing Blueo-gal or X-gal. The genotype of this strain is  $F^- \Phi80\text{lacZ}\Delta\text{M15 } \Delta(\text{lacZYA-argF}) \text{ U169 recA1 endA1 hsdR17 (r}_k^-, \text{m}_k^+) \text{ phoA supE44 } \lambda\text{-thi-1 gyrA96 relA1}$  (Invitrogen, Catalog # K4520-01 20).

### Vectors

The information regarding the vectors pMal-c2X (New England Biolabs), pMal-c4X (New England Biolabs), pDsRed-monomer (Clontech), and pQE2 (Qiagen) is given in Section Materials and Methods

### Primers

The primer list is given in Section Materials and Methods. The design of the primers was performed with the help of the pDRAW software and the San Diego Supercomputer Center (SDSC) Biology Workbench Internet website.

### Enzymes

All the enzymes are recombinant and produced in *E. coli*. The restriction enzymes were purchased from New England Biolabs, the buffers used accordingly as provided and suggested by the supplier. All the restriction enzymes were supplied with the reaction buffer called 10X NEBuffer with each restriction endonuclease to ensure optimal (100%) activity. Most of the enzymes were supplied with one of four standard

### **MscI Restriction Endonuclease**

The source of the restriction enzyme is an *E. coli* strain that carries the MscI gene from *Micrococcus* species. It is supplied with NEBuffer 4 (10X) from New England Biolabs (Catalog # R0534S). Its recognition and cutting site is 5'-TGG<sup>^</sup>CCA-3'.

### **PstI Restriction Endonuclease**

The source of the restriction enzyme is an *E. coli* strain that carries the PstI gene from *Providencia stuartii* 164. It is supplied with NEBuffer 3 (10X) and BSA (100X) from New England Biolabs (Catalog # R0140T). Its recognition and cutting site is 5'-CTGCA<sup>^</sup>G-3'.

### **NlaIV Restriction Endonuclease**

The source of the restriction enzyme is an *E. coli* strain that carries the NlaIV gene from *Neisseria lactamica*. It is supplied with NEBuffer 4 (10X) and BSA (100X) from New England Biolabs (Catalog # R0126S). The recognition and cutting site is 5'-GGN<sup>^</sup>NCC-3'.

### **XmnI Restriction Endonuclease**

The source of the restriction enzyme is an *E. coli* strain that carries the XmnI gene from *Xanthomonas manihotis* 7AS1. It is supplied with NEBuffer 4 (10X) and BSA (100X) from New England Biolabs (Catalog # R0194S). The recognition and cutting site is 5'-GAANN<sup>^</sup>NNTTC-3'.

### **EcoRI Restriction Endonuclease**

EcoRI is an endonuclease enzyme that is isolated from *E. coli* strain along with it is part of the restriction modification system. It is supplied with NEBuffer 3 (10X) and BSA (100X) from New England Biolabs (Catalog # R0101S). The recognition and cutting site is 5'-G<sup>^</sup>AATTC-3'.

### **HindIII Restriction Endonuclease**

*HindIII* is a type II site-specific deoxyribonuclease restriction enzyme. The source of the restriction enzyme is an *E. coli* strain that carries the HindIII gene from *Haemophilus influenza*. It is supplied with NEBuffer 3 (10X) and BSA (100X) from New England Biolabs (Catalog # R0104S). The recognition and cutting site is as follows, 5'-A<sup>^</sup>AGCTT-3'.

### **Factor Xa Protease**

Factor Xa cleaves after the arginine residue in its preferred cleavage site Ile-(Glu or Asp)-Gly-Arg. It will sometimes cleave at other basic residues, depending on the conformation of the protein substrate. The most common secondary site, among those that have been sequenced, is Gly-Arg. There seems to be a correlation between proteins that are unstable in *E.coli* and those that are cleaved by Factor Xa at secondary sites; this may indicate that these proteins are in a partially unfolded state. Factor Xa will not cleave a site followed by proline or arginine. It is supplied with New England Biolabs (Catalog # P8010S).

### **Lysozyme (EC 3.2.1.17)**

50,000 units/mg lyophilized powder containing sodium acetate buffer salts and sodium chloride was purchased from Sigma (Catalog # L7001). It was dissolved in deionized water to get 10-20 mg/ml concentrations. This enzyme breaks down the cell walls of bacteria so it was used for cell lysis.

### **Protein Molecular Weight Markers**

The Protein Molecular Weight Markers are Unstained Protein Molecular Weight Marker from Fermentas (Catalog # SM0431) and Protein Marker, Broad Range (2-212 kDa) from New England Biolabs (Catalog #P7702S)

### **Bacterial Culture Media**

#### **Luria Bertani (LB) Medium**

20 g LB Broth including 10 g tryptone, 5 g NaCl and 5 g yeast extract (Sigma, Catalog # ) was dissolved in 1 L of distilled water at pH 7.0. The dissolved solution was sterilized by autoclaving at 121-124°C for 15 minutes.

#### **LB Agar Medium**

35 g LB Agar including 10 g tryptone, 5 g NaCl, 5 g yeast extract and 15 g agar (Sigma, Catalog # ) was dissolved in 1 L of distilled water by heating at pH 7.0. The dissolved solution was sterilized by autoclaving at 121-124°C for 15 minutes.

### **Stock Solutions**

#### **1000x Ampicillin (Sodium Salt)**

1 g Ampicillin (Sigma) was dissolved in 10 ml deionized water, sterilized by filtration using 0.22 µm filter. This is a 1000x stock solution and stored at -20°C. Its working strength is 100µg/ml.

**X-gal/IPTG Stock Solution**

1.25 g IPTG (Sigma) and 1 g X-gal (Fermentas) were dissolved in 25 ml DMF (Riedel-de-Haen). The solution was stored at -20°C in the dark.

**1 M IPTG Stock Solution**

2.38 g IPTG was dissolved in 10 ml deionized water, sterilized by filtration using 0.22 µm filter and stored at -20°C

**Glycerol Stock Solution**

80 ml glycerol (Riedel-de-Haen) and 20 ml distilled water were mixed to have 80% (w/v) solution. It was sterilized by autoclaving at 121-124°C for 15 minutes.

**Buffers****Sodium Acetate Buffer**

3 M of Na-Ac (Riedel-de-Haen) was dissolved in 65 ml distilled water. pH was adjusted to 5.2 and distilled water was added up to 100 ml.

**10X TBE Buffer (1000ml)**

108 g of Tris base, 55 g boric acid and 20 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 liter deionized water, its pH was titrated to 8.3 and sterilized for 15 minutes under 1.5 atm at 121°C.

**Column Buffer (1000ml)**

20 ml 1.0 M Tris-HCl, pH 7.4, and 2.0 ml 0.5 M EDTA were mixed and 11.7 g NaCl was dissolved this solution. Optionally, 1.0 ml 1M sodium azide and 0.7 ml β-mercaptoethanol were added into this solution

**Elution Buffer (1000ml)**

4 g Maltose was dissolved in 1 liter column buffer without beta-mercaptoethanol

**Chemicals**

Amylose resin in 20% Ethanol was purchased from New England Biolabs (Catalog # E8021). Tris, NaOH, bromophenol blue, SDS, HCl, CBB-R250, CBB-G250, glacial acetic acid, glucose, MgCl<sub>2</sub>, TEMED, NaH<sub>2</sub>PO<sub>4</sub> and EDTA were purchased from Merck., NaCl, NH<sub>4</sub>Cl, CaCl<sub>2</sub>, isopropanol, glycerol, absolute ethanol, absolute methanol and acetic acid (glacial) were purchased from Fluka. Maltose, beta-mercaptoethanol, NaN<sub>3</sub>, DTT, RbCl<sub>2</sub>, acrylamide, APS, N,N'-Methylenebisacrylamide and electrophoresis grade glycine were purchased from Sigma.

### **APPENDIX A.3: Computer Software**

Gel Documentation System: UVIPhotoMW Version 99.05 for Windows 95 & 98, UVIttec Limited.

Homology search: Basic Local Alignment Search Tool, BLAST. Standard protein BLAST (blastp) (Altschul et al. 1990; Nakamura et al., 2000). Available at <http://www.ncbi.nlm.nih.gov/BLAST/>

Nucleotide alignments: Multiple sequence alignment tools, ClustalW (Higgins et al., 1994). Available at <http://www.ebi.ac.uk/clustalw/index.html>

Translator tool: JustBio Translator tool, (Rodriguez P.). Available at [www.justbio.com](http://www.justbio.com).

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- *B.Sc.*, Biochemistry Department, Ege (Agean) University, Izmir, TURKEY, Graduated June 2005.

## AWARDS, FELLOWSHIPS & GRANTS

- NIH Grant, University of Kansas, 2014-2015
- TUBITAK 2214 International Doctoral Reserach Fellowship, 2013-2014
- Graduate Student Award with Honorarium, Materials Science and Engineering Department, University of Washington, Seattle, WA, USA, 2010
- Visiting Graduate Student, National Science Foundation (NSF) MRSEC Program through the University of Washington Genetically Engineered Materials Science and Engineering Center (DMR 0520567), 2007-2008
- Graduate Student, International Research Experience for Students in Molecular Biomimetics TUBITAK/NSF Joint project, Project Number: TBAG 107T250, 2007-2010
- Master Fellowship, Production of enzyme based raw materials for drug industries, Turkish State Planning Organization (SPO) Funded project, 2006

## PROFESSIONAL EXPERIENCE

- 2013-Present            Research Scholar, Bioengineering Research Center, University of Kansas, Lawrence, Kansas, USA
- 2006-Present            Teaching and Research Assistant Molecular Biology and Genetic Department, Istanbul Technical University, Istanbul, TURKEY
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## PUBLICATIONS ON THE THESIS:

- **Karaca, B. T.,** & Tamerler, C. (2015) . Plasmon-Enhanced Nanoprobes from Fluorescence Protein coupled to gold nanoparticles through gold binding peptide, *ACS Nano, Submitted paper*
- Zhang, S., **Karaca, B. T.,** VanOosten, S. K., Yuca, E., Mahalingam, S., Edirisinghe, M., & Tamerler, C. (2015) Nanoscale assembly of functional polymer nanofibers integrated with genetically engineered proteins by coupling flow and gyration, *ACS Nano, Submitted paper*
- Yucesoy, D. T., **Karaca, B. T.,** Cetinel, S., Caliskan, H. B., Adali, E., Gul-Karaguler, N., & Tamerler, C. (2014) Direct Bioelectrocatalysis at the Interfaces by Genetically Engineered Dehydrogenase, *Bioinspired, Biomimetic and Nanobiomaterials*, DOI: 10.1680/bbn.14.00022
- **Karaca, B.T.,** Meyer, J., VanOosten, S. K., Richter, M. & Tamerler, C. (2013) Modular Peptide-Based Hybrid Nanoprobes for Bio-Imaging and Bio-Sensing, *MRS Proceedings*, 2013 Volume 1621 / 2014 DOI: <http://dx.doi.org/10.1557/opl.2014.368>

- **Karaca, B. T.**, Hnilova, M., Park, J., Jia, C., Wilson, B. R., Sarikaya, M., & Tamerler, C. (2012). Fabrication of hierarchical hybrid structures using bio-enabled layer-by-layer self-assembly. *Biotechnology and Bioengineering*, 109(5), 1120-1130. doi: 10.1002/bit.24405

## INTERNATIONAL BOOKS/BOOK CHAPTERS

- **Karaca, B.T.**, Hnilova, M., & Tamerler C. (2014) “Addressable Biological Functionalization of Inorganics: Materials Selective fusion Proteins in Bio-nanotechnology” in “Bio-Inspired Nanotechnology: From Surface Analysis to Applications”, (Editors: Tiffany R. Walsh and Marck R. Knecht) *Springer, Chapter 8*, 221-225 (DOI: 10.1007/978-1-4614-9446-1\_8)

## PATENT APPLICATION

**Banu Taktak Karaca**, & Candan Tamerler, Enhanced metal binding bioprobe:DsRed-AuBP2.

## PRESENTATIONS ON THE THESIS:

- **Karaca, B. T.**, Maloney, R., Deay, D., Tomas, B. Richter, M. & Tamerler, C. (2015) Single step biofabrication of self-organized hybrid molecular assemblies, *144th TMS Annual Meeting and Exhibition*, Poster Presentation, Orlando, USA, Poster
- Meyer, J. E., Singh, V., **Karaca, B. T.**, Spencer, P., Mishra, A., & Tamerler, C. (2015) Dynamic modeling approach to follow adsorption kinetics of engineered proteins that self-assembles through biomolecular recognition, *144th TMS Annual Meeting and Exhibition*, Poster Presentation, Orlando, USA
- Mustafaoglu, N., **Karaca, B. T.**, Meyer, J., Urgan, M., & Tamerler, C. (2014) Bio-enabled Self-organization for Hybrid Thin Films 143<sup>rd</sup> *TMS Annual Meeting and Exhibition*, Biological Materials Science Symposium, Oral Presentation, San Diego, CA, USA
- **Karaca, B. T.**, Karaca, E., Balta, B., Sarikaya, M., & Tamerler, C. (2013). Metal Binding Fluorescent Proteins for Bio-imaging and Bio-sensing Applications. *142th TMS Annual Meeting and Exhibition*, Biological Materials Science Symposium, Poster Presentation, San Antonio, TX, USA  
Poster Awarded

- **Karaca, B. T.**, Yazici, H., Yuca, E., Balta, B., Sarikaya, M., & Tamerler, C. (2012). Genetically Designed Paptide-based Fusion Proteins as Novel Tools. *MRS 2012 Fall Meeting, Symposium Q: Functional and responsive Materials Exploiting Peptide and Protein Self-Assembly*, Poster Presentation, Boston, MA, USA, November 25-30, 2012 Poster
- **Karaca, B.T.**, Hnilova, M., Park, J., Wilson, B. R., Jia, C., Sarikaya, M., & Tamerler, C. (2011) Controlled Site-Directed Protein Immobilization via Genetically Engineered Material Recognition Peptides, *The 11th International Symposium on Biomimetic Materials Processing (BMMP-11)*, Poster Presentation, Nagoya, Japan
- Hnilova, M., **Karaca, B. T.**, Fong, H., Kacar, T., Tamerler, C., & Sarikaya, M. (2010) Peptide-Mediated Formation of Hybrid Metallic Nanostructure, *TMS Annual Meeting and Exhibition, Symposium: Biological Materials Scinece: Bio-inspired Materials Design and Processing I:Macromolecular Concepts and Application*, , On-Site Speaker, Seattle, WA, USA, February 14-18, 2010