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Site-specific immobilization of protein layers on gold surfaces via orthogonal sortases

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ABSTRACT

We report a site-specific, sortase-mediated ligation to immobilize proteins layer-by-layer on a gold surface. Recombinant fluorescent proteins with a Sortase A recognition tag at the C-terminus were immobilized on peptide-modified gold surfaces. We used two sortases with different substrate specificities (*Streptococcus pyogenes* Sortase A and *Staphylococcus aureus* Sortase A) to immobilize layers of GFP and mCherry site-specifically on the gold surface. Surfaces were characterized using fluorescence and atomic force microscopy after immobilizing each layer of protein. Fluorescent micrographs showed that both protein immobilization on the modified gold surface and protein oligomerization are sortase-dependent. AFM images showed that either homogenous protein monolayers or layers of protein oligomers can be generated using appropriately tagged substrate proteins. Using Sortase A variants with orthogonal peptide substrate specificities, site-specific immobilization of appropriately tagged GFP onto a layer of immobilized mCherry was achieved without disruption of the underlying protein layer.

1. Introduction

Immobilization of proteins on surfaces and the generation of protein 3D assemblies have drawn particular attention recently, mainly due to their importance in many nano-biotechnological applications such as proteomics, biosensors, or tissue engineering [1–3]. Site-specific immobilization of protein layers on the surface can be used to generate 3D protein assemblies. Generating protein arrays or 3D assemblies on the surface without negatively impacting protein stability or function requires a benign and controlled protein ligation approach [4]. These well-oriented protein assemblies can be used as scaffolds that mimic the extracellular matrix (ECM) [5,6] or as bioactive hydrogels [7] and biocompatible drug carriers [8]. In addition, generating photoconductive protein layers on surfaces can be used to produce electricity [9].

The conductivity and surface plasmon resonance properties of gold make it an excellent platform for biosensors and biohybrid device electrodes. Many techniques that detect biomolecular interactions, such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and electrochemical biosensors, require protein immobilization on gold surfaces. Binding proteins in a specific orientation to the gold chip has a great impact on the availability of the binding site, and therefore on the apparent kinetics and signal obtained from a binding interaction [10,11]. Having control over protein orientation is also important in immobilizing photoconductive proteins such as photosystem I (PSI) on gold, since these proteins transfer electrons vectorially from the P700 reaction center near one end of the protein complex to the F_A/F_B acceptor sites at the other end [12,13].

Although many different techniques have been used to date to ligate proteins/peptides together and immobilize proteins on the surface, a great need still exists for a robust, gentle, universal method of protein immobilization that can site-specifically ligate proteins to the surface and to other biomolecules. Different techniques such as physisorption [14], chemical conjugation to the functional group [15], click chemistry methods such as cycloaddition [16] or Staudinger ligation [17], trans splicing [18], or mutagenesis to introduce exposed cysteines have all been used to immobilize ligands on surfaces [19,20]. Physical methods have little control over protein orientation and the weak, noncovalent interaction between the protein and the surface lacks inherent stability. Chemical methods such as amine-reactive crosslinking provide more stable protein ligation, but these methods typically suffer from nonspecific orientation of the protein on the surface

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caused by availability of more than one reactive site in the protein structure; additionally, reagents, conditions, and modification sites are not always compatible with optimal protein stability and function. Introducing a cysteine in the amino acid sequence to generate a gold thiolate bond between the engineered proteins and gold surface or disulfide bond between protein layers can likewise affect the structure and function of the protein. Click chemistry can enable site-specific protein immobilization of proteins containing a single unnatural amino acid without using harsh chemical conditions; however, engineering proteins to contain unnatural amino acids is costly and time-consuming and can have a negative impact on protein expression [21]. Alternatively, introducing an azide or an alkyne reagent to the protein structure by reacting with side chain amines, as an alternative click chemistry approach, suffers from a lack of site specificity. Click chemistry also has some other drawbacks that limit its applicability. For instance, in Huisgen 1,3-dipolar cycloaddition, alkynes homocoupling reduces azide–alkyne reaction and cycloaddition efficiency; similarly, air oxidation of phosphine reagents in Staudinger ligation affects reaction kinetics. The limited commercial availability of the general reagents and building blocks and stability of some azide derivatives is another limitation in some click chemistry conjugation methods [22]. Although trans-splicing using intein-based methods has the advantages of traceless protein ligation, intein tags can lead to protein insolubility and expression issues. Moreover, the trans-splicing method is not always applicable for all target proteins, as the inteins are often specific for their natural exteins [23].

An enzymatic method using short, noninterfering peptide tags affords the advantage of immobilizing and oligomerizing proteins covalently using site-specific ligation conditions close to neutral pH and near physiological conditions. In the Gram-positive bacterium *Staphylococcus aureus*, Sortase A, a transpeptidase that recognizes an LPXTG sequence near the C-terminus of a surface protein, cleaves between T and G to yield an acyl–enzyme intermediate. This enzyme–substrate intermediate is resolved by a nucleophilic attack from the amine at the N-terminus of a pentaglycine [24–26]. These short substrate tags can be used in a reaction to immobilize proteins to a surface or ligate them together using Sortase A without altering the protein structure and function. Sortase-mediated protein immobilization has also the advantage of shorter incubation times compared to click chemistry and trans-splicing methods. Sortase A was previously used to immobilize GFP and PEG on agarose or polystyrene beads [27,28] an antibody on a carboxymethylated Biacore sensor chip [29], glycosyltransferase on a sepharose surface [30], recombinant thrombomodulin [31] and influenza virus on modified glass slides [32], recombinant RFP on modified polystyrene microparticles [33], and a fusion protein bilayer composed of recombinant azurin–cytochrome P450 [34].

Sortases inherently recognize 'head' and 'tail' tags, thereby encoding a vectorial component to their reactivity that is invaluable for layer-by-layer assembly. Here, we used sortase-mediated protein ligation to immobilize layers of recombinant fluorescent proteins on a gold surface in a single layer or multiple layers. Two different Sortase A enzymes with different substrate specificities were used. Orthogonal sortase ligation has been previously used to circularize and functionalize interferon [35] and to attach two different moieties onto two capsid proteins in a single phage particle [36]. *S. aureus* Sortase A (Sa-SrtA) is specific toward the LPETG and GGG substrate pair, cleaving between T and G before ligation to tris–glycine; *Streptococcus pyogenes* Sortase A (Sp-SrtA) is more specific toward LPETA and AAA [37], with cleavage between T and A before ligation to tri-alanine. In order to immobilize protein layers on the surface with control over protein orientation, oligomerization site, and homogeneity of each layer, Sp-SrtA and Sa-SrtA were used sequentially. Recombinant fluorescent protein model systems were thereby robustly immobilized to gold in layers.

2. Materials and methods

2.1. Protein expression and purification

A plasmid containing a gene encoding GFP-LPETG was used for expression of soluble GFP as previously described [27]. The mCherry variants were made by PCR using plasmid "pJK148-Pbip1-signal peptide-linker-mCherry-AHDL" as a template. *S. aureus* Sortase A was cloned out of the pHTT27 plasmid. All constructs were inserted into a pET26 vector using NdeI and XhoI restriction sites. A 6xHis-tag sequence is located after the multiple cloning sites in the pET26 vector. In sortase plasmid pMR5, a 6xHis-tag sequence is inserted at the N-terminus and a stop codon is placed before the pET26 XhoI restriction site to prevent expression of the second His-tag at the C-terminus. An engineered Sa-SrtA with higher activity toward LPETG substrate (Sa-SrtAm4) was made using directed mutagenesis [38]. Four mutations (P94S, K137T, D160N, and D165A) were made to Sa-SrtA using the Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and Phusion DNA polymerase (New England Biolabs, Ipswich, MA), following the manufacturers' recommended protocols, to generate plasmid pMR5m4.

Proteins were expressed in BL21(DE3) cells (Life Technologies, Grand Island, NY). A cell culture was grown from a starting OD_{600} of 0.05–0.1 in 250 ml of LB Kanamycin (50 μg/ml) media to OD_{600} of 0.5–0.7 at 37 °C with shaking at 225 rpm, induced by adding IPTG to a final concentration of 1 mM, and pelleted after 3–4 h of induction. Sa-SrtAm4 protein was induced by adding IPTG to a final concentration of 0.5 mM and incubating for 3 h in a 30 °C shaker. The cells were frozen at −20 °C for 16 h. Proteins were extracted after thawing the cells on ice by resuspending cells from a 250 ml culture in 10 ml B-PER reagent (Thermo Fisher Scientific, Waltham, MA) with 10 μl (2 U/μl) DNase I (New England Biolabs, Ipswich, MA), in accordance with the B-PER manual. The target proteins were soluble and found in the supernatant. Proteins were purified using His-tag purification by TALON resin (Clontech Laboratories, Mountain View, CA), following the manufacturer's batch/gravity protocol. The purified proteins were then dialyzed using a Slide-a-lyzer cassette with MW cutoff of 10,000 kD (Thermo Scientific) or buffer exchanged against TBS buffer (50 mM Tris–base, 150 mM NaCl, pH 7.5) using Amicon centrifugal filters (Millipore, Billerica, MA) with MW cutoff of 10,000 kD to remove imidazole. Protein concentration was determined using an A_{280} measurement with a Bio-Rad SmartSpec 3000 spectrophotometer. 2.5–10 mg of the proteins were produced from 250 ml cultures. Proteins were concentrated to 500–1200 μM. The concentrated proteins were stored in aliquots for up to 6 months at −20 °C for future use. For short-term storage, proteins were stored at 4 °C.

2.2. Peptide assembly on the gold surface

Gold-coated microscope slides with 100 Å gold thickness were purchased from Platypus Technologies (Madison, WI). Slides were cut into 10 mm × 10 mm pieces and washed with RCA solution (30% NH_4OH:30% H_2O_2:H_2O; 1:1:5 ratio by volume) at 70 °C for 15 min, then rinsed with a copious amount of molecular biology-grade *dd*H_2O and dried under a nitrogen stream. The GGGC, AGGC and AAAC peptides were purchased from Genscript (Piscataway, NJ) with >95% purity. Dimethylformamide (DMF) was added drop-wise to the peptide powder until the solution was clear, then oxygen-free water (to discourage disulfide formation between Cys) was added to the solution to make 0.1–1 mM solutions. The clean gold slides were submerged in the peptide solution for 16–24 h at room temperature in a glove box flushed with nitrogen gas. The slides were then washed with TBS buffer (pH 7.5) and *dd*H_2O and dried under a nitrogen stream.

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